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ORIGINAL PAPER

Involvement of jasmonates in responses of sunflower (*Helianthus annuus*) seedlings to moderate water stress

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Abstract Jasmonates (JAs), a type of phytohormone, are involved in sensing and signaling of several environmental stresses (biotic and abiotic). Jasmonic acid (JA) has been suggested to function in plant responses to drought, because this type of stress induces expression of several genes that also respond to JA. We investigated the involvement of JA and its precursor (12-oxo-phytodienoic acid; OPDA) on seedling morphological and physiological characteristics of two sunflower (*Helianthus annuus*) inbred lines with contrasting responses (sensitive vs. tolerant) to water stress. Our experimental treatments were based on moderate water stress (simulated by application of mannitol 400 mM) and on blocking of JA biosynthesis (by the chemical inhibitor salicylhydroxamic acid; SHAM). Water stress resulted in reduction of primary root (PR) growth and lateral root (LR) growth, but in increased LR number. SHAM treatment increased PR length, LR number, and LR length, thus strongly affecting root architecture. Water stress had differential effects on various physiological parameters, including relative water content (RWC), stomatal conductance, and content of photosynthetic pigments (chlorophylls, carotenoids). OPDA and JA accumulation in aerial part and roots induced by water stress was reversed by combined water stress plus SHAM treatment at day 14. Our findings suggest that SHAM effectively inhibits de novo JA biosynthesis induced by water stress, and that JAs play a protective role in responses of sunflower seedlings to this stress. JAs, particularly OPDA, are highly effective signaling molecules in mediation of sunflower seedling responses to water stress.

Keywords Jasmonic acid · 12-Oxo-phytodienoic acid · Morphological characteristics · Salicylhydroxamic acid · Sunflower · Water stress

Abbreviations

DAS	Days after sowing
JA	Jasmonic acid
JAs	Jasmonates
JA-Ile	JA-isoleucine
LR	Lateral roots
MPa	Megapascal
OPDA	12-oxo-phytodienoic acid
PR	Primary root
RWC	Relative water content
S + SHAM	Water stress plus SHAM
SHAM	Salicylhydroxamic acid
SD	Stomatal density
SI	Stomatal index

Introduction

Drought is a severe type of abiotic stress that affects growth and development of plants and causes yield loss in crops

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worldwide (Waraich et al. 2011). The global agricultural area used for sunflower (*Helianthus annuus* L.) production has increased steadily during recent decades because this crop has high yield and photosynthetic capacity, and is adaptable to a variety of environmental conditions (Agele et al. 2007). In Argentina, cultivation of sunflower has been displaced by that of soybean from the central region toward marginal agricultural areas characterized by irregular water regimes and lower quality soils, such as the Pampa Central-West region. A major obstacle to high sunflower production yield in these areas is lack of synchronized crop establishment resulting from adverse weather and soil conditions. Water stress imposed by PEG-1000 during V8-growth stage of hybrid sunflower results in morphological changes such as reductions in height, leaves and roots number, and shoot fresh and dry weight (Turhan and Baser 2004). A dynamic physiological change typically observed under water stress is disruption in endogenous concentrations of phytohormones (Farman et al. 2017). Many studies have shown that an intricate hormonal network modulates intensity of physiological responses to stress (Peleg and Blumwald 2011). Jasmonic acid (JA) and its metabolites, collectively known as jasmonates (JAs), are phytohormones that play well-established roles in regulating plant growth and stress responses (Wasternack and Hause 2013).

Involvement of JAs in responses to water stress is well documented, but accumulation of JAs during such stress varies. Several studies show increased levels of JAs under water stress, e.g., in shoots and roots of maize seedlings (Xin et al. 1997), *Carica papaya* seedlings (Mahouachi et al. 2007), *Pinus pinaster* plants (Pedranzani et al. 2007), *Arabidopsis thaliana* plants (Brossa et al. 2011), *Oryza sativa* leaves (Du et al. 2013), and citrumelo CPB 4475 (a commercial citrus rootstock) (de Ollas et al. 2013). De Domenico et al. (2012) found that in stressed and non-stressed roots of two chickpea (*Cicer arietinum*) varieties, levels of 12-oxo-phytodienoic acid (OPDA), JA, and JA-isoleucine (JA-Ile) were higher in stress-tolerant than stress-sensitive varieties. These results demonstrated the JAs play a role in early drought signaling in this species, indicating their involvement in tolerance mechanisms. In a study by Grebner et al. (2013), roots of wild-type and lipoxygenase (LOX) mutant *Arabidopsis* plants treated with sorbitol (osmotic stress) showed differential accumulation of JAs (OPDA, JA, JA-Ile). In roots exposed to air (water stress), JA-Ile was at its detection limit, JA remained low, and there was no notable increase in OPDA. In contrast, roots of osmotic-stressed plants showed moderate accumulation of OPDA and JA, and JA-Ile was at its detection limit. JA and OPDA levels were increased in *lox2* and *lox3*/*lox4* mutants, but remained at basal levels in *lox6* mutants. Thus, production and regulation of JAs appeared to be regulated by different mechanisms in response to osmotic versus water stresses. JA was implicated as a key player in drought responses based on its positive regulatory role in stomatal closure (Munemasa et al. 2007). Accumulation of OPDA without that of JA was observed upon elicitation of cell cultures and during tendril coiling, suggesting the existence of OPDA-specific responses (Wasternack and Strnad 2016, review). In a study of *Arabidopsis*, Savchenko et al. (2014) found that under drought JA remained at basal level, whereas OPDA increased. Plants with higher OPDA levels displayed improved drought tolerance and reduced stomatal aperture size.

Various approaches can be used to determine how an endogenous phytohormone regulates a particular physiological process; e.g., hormone-deficient mutants and biosynthetic inhibitors that block hormone production. Metabolism of stress-associated phytohormones, and the responses they trigger, can be studied using chemical inhibitors that block different steps of hormonal metabolic pathways. Inhibitors of JA biosynthesis include ibuprofen (IBU), propylgallate (PG), indoprofen (INP), and salicylhydroxamic acid (SHAM) (Zhu et al. 2006; Maksymiec and Krupa 2007; Rudús et al. 2009). SHAM is a specific inhibitor of JA biosynthesis and significantly reduces levels of JA and its precursor OPDA. It inhibits biosynthesis of JA by blocking expression and activity of the genes that encode LOX enzyme (Gao et al. 2003). Kong et al. (2005) demonstrated that SHAM reversed the inhibitory effect of theobroxide on potato stem elongation, and that such reversion was associated with endogenous JA reduction.

In the present study, we used chemical inhibition of the JA biosynthetic pathway to elucidate the effect of OPDA and JA on seedling (V2-growth stage) morphological and physiological characteristics of two sunflower inbred lines with differing water stress tolerance, grown under normal (non-stress) conditions and moderate water stress mimicked by mannitol application.

Materials and methods

Plant material

Seeds of sunflower (*Helianthus annuus* L.; family Asteraceae) inbred lines B59 and B71 (respectively sensitive and tolerant to water stress), supplied by Daniel Alvarez, M.Sc., were sown in an experimental field of EEA-INTA Manfredi (31°51′9.00″S, 63°44′55.91″W), Córdoba, Argentina.

Assays and treatments

Germination assay and early growth assay were performed as described previously (Andrade et al. 2013).

Chemicals applied for experimental treatments were SHAM 10 µM (Sigma-Aldrich; Buenos Aires, Argentina)

and mannitol 400 mM (Biopack; Buenos Aires), which was used as a drought simulator and generated a moderate water stress level (−0.989 MPa). Starting at day 4 after planting of seeds in sand, and when seedlings were six days old, they were watered to field capacity (FC) with four solutions (treatments): (1) Hoagland's solution, 50% of full-strength (control); (2) mannitol 400 mM (moderate water stress); (3) SHAM 10 µM (biosynthetic JA inhibitor); (4) mannitol 400 mM + SHAM 10 µM. Irrigation with each of the four solutions was performed every three days until the end of experiments.

Various morphological and physiological seedling characteristics as described below were studied during the course of the experiments and at harvest time. Each sample consisted of 15 seedlings.

Measurement of morphological characteristics

Aerial part length

Aerial part length of 15 selected seedlings was recorded every 2 days.

Stomatal density

Stomatal density (SD) (number of stomata per unit leaf area) on the same 15 selected seedlings was determined by impression technique. The abaxial and adaxial surfaces were carefully coated with clear nail varnish (adhesive) in the mid-area between central vein and leaf edge, and the adhesive dried for 2–3 min. A thin film of epidermal tissue (~5 × 15 mm) was gently peeled away from the leaf lamina. Sections were mounted on microscope slides, covered immediately with cover slips, lightly squeezed with fine point tweezers, and sealed with varnish. Photomicrographs were taken using a Zeiss Axiophot microscope with image capture and digitalization program AxioVision 4.3, and Axio Cam HRc camera. Number of stomata was counted from three randomly selected fields (area 1.03 mm^2) at magnification ×200, and number per field was converted to number per mm^2. The Image-Pro Plus 4.5 software program (Media Cybernetics; Silver Spring, MD, USA) was used for stomatal analysis (http://www.mediacy.com/action.htm).

Stomatal index

Stomatal index (SI) was calculated as the percentage of open stomata out of 100 stomata, per unit area.

Leaf area

Leaf area was determined for the first pair of leaves of the 15 seedlings as above, and estimated as described by Schneiter (1978).

Leaf growth rate

Leaf size was calculated as the product of the length and width each 2 days until the end of experiment.

Root architecture

The two main sunflower root types, primary root (PR) and lateral root (LR), were measured after 14 days of the various treatments. PR and LR lengths were determined using the Image J software program, V. 1.38 (http://rsbweb.nih.gov/ij/), and LR number was visually counted using a magnifying glass. Lateral exploratory roots were measured in the region extending 1 cm from the base of the aerial part. Root length data were averages from three independent experiments, each with n = 5 seedlings.

Measurement of physiological characteristics

Relative water content (RWC)

RWC was estimated as described by Turner (1981), using the equation:

$$\text{RWC}\ (\%) = \left[(\text{FW} - \text{DW})/(\text{TW} - \text{DW})\right] \times 100,$$

where FW = fresh weight, TW = turgid weight (measured after leaf discs were floated in distilled water in a closed Petri dish for 24 h in the dark at room temperature), and DW = dry weight (measured after drying the leaf discs to a constant weight at 70 °C). Leaf discs were collected from the middle section of either seedling to minimize age effects. Experiments were performed in quadruplicate.

Stomatal conductance

Stomatal conductance was measured for healthy, mature leaves using a Leaf Porometer, Model SC-1 (Decagon Devices; Pullman, WA, USA). Measurements were taken near the midline of the leaf, at ~9 AM, with n = 5 leaves for each treatment. Experiments were performed in quadruplicate.

Photosynthetic pigments

Chlorophyll *a* and *b* were extracted from 100 mg FW leaves and estimated as described by Porra (2002). Total carotenoids were then extracted with diethyl ether as

described by Meléndez-Martínez et al. (2007). Data for chlorophylls and carotenoids were quantified as described by MacKinney (1941) and Vernon (1960). Experiments were performed in quadruplicate.

Extraction and purification of endogenous hormones

OPDA and JA were extracted from 200 mg DW seedlings (aerial part and roots) as described by Durgbanshi et al. (2005), with some modifications. Plant material was homogenized in an Ultraturrax T25 basic homogenizer (IKA, Staufen; Germany) with 5 ml deionized water. D_5-OPDA and D_6-JA (Leibniz-Institute of Plant Biochemistry; Halle, Germany) were used as internal standards, and samples were added with 50 ng of each. Samples were centrifuged at 1540×*g* for 15 min, supernatant was adjusted to pH 2.8 with 15% (v/v) acetic acid and extracted twice with diethyl ether, and organic fraction was evaporated under vacuum. Dried extracts were dissolved in 1 ml methanol and filtered on a vacuum manifold at flow rate <1 ml min^{-1}, and eluate was evaporated at 35 °C under vacuum in a SpeedVac SC110 (Savant Instruments; New York, NY, USA). Four biological replicates were used for assays.

Hormone identification and quantification by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS-MS)

OPDA and JA were separated from plant tissues by reversed-phase HPLC, using an Alliance 2695 separation module (Waters; Milford, MA, USA) equipped with a Restek Ultra C18 3 µm column (100 × 3.0 mm). Fractions were separated using a gradient of increasing methanol concentration, constant glacial acetic acid concentration (0.2% in water), and initial flow rate 0.2 ml min^{-1}. The gradient was increased linearly from 40% methanol/60% water-acetic acid at 25 min, to 80% methanol/20% water-acetic acid. Initial conditions were restored after 1 min, and the system was allowed to equilibrate for 7 min. Hormones were identified and quantified using a quadruple tandem mass spectrometer (Quattro Ultima, Micromass; Manchester, UK) fitted with an electrospray ion (ESI) source, in multiple reactions monitoring mode (MRM) using precursor ions and their transitions (*m*/*z*) to OPDA (*m*/*z* 291/225), D_5-OPDA (*m*/*z* 296/ 230), JA (*m*/*z* 209/59), and D_6-JA (*m*/*z* 215/59), with respective retention times 23.44, 23.41, 8.81 and 8.72 min. Collision energies used were 20 eV for JA and 30 eV for OPDA, and cone voltage was 35 V. The MassLynx spectrometry software program, V. 4.1 (Waters) was used for data analysis.

Statistical analysis

Experiments were set up using randomized block design, and each was performed four times, consecutively (two trays per treatment). Data were subjected to one-way analysis of variance (ANOVA), and significant differences between means were determined using Tukey's test at $P \leq 0.05$ level. The Statgraphics Plus software program, V. 3 (Manugistics 1997; Rockville, MD, USA) was used for statistical analysis.

Results

Effects of water stress and JA inhibitor (SHAM) on morphological characteristics

In B59 (water stress-sensitive) seedlings (Fig. A1), aerial part length was significantly reduced by moderate water stress (treatment with 400 mM mannitol solution) (Fig. A1.B). In contrast, SHAM treatment caused an increase of this parameter observed 6 days after sowing (DAS), and a significant difference from control value at 12 DAS. Water stress plus SHAM (S + SHAM) treatment produced a response significantly different from that to water stress alone at 10 and 16 DAS (Fig. 1a). In B71 (water stress-tolerant) seedlings (Fig. A2), mannitol treatment strongly reduced aerial part length (Fig. A2.B) at the beginning of treatment (6 DAS). There was no significant difference between SHAM-treated versus control seedlings at any time point. The effect on aerial part length of S + SHAM treatment was the same as that of water stress alone, including the notable reduction at 6 DAS (Fig. 1b).

Leaf growth rate of B59 was consistently lower than that B71 (Figs. A4, A5). In B59, water stress (mannitol treatment) caused a slight decrease of leaf growth rate at the beginning of treatment (Fig. A4). The rate remained relatively stable until 12 DAS, and was significantly different from that of control at 14 and 16 DAS. SHAM treatment had no significant effect on leaf growth rate at any time point. S + SHAM treatment produced a small (non significant) decrease in this parameter relative to water stress alone (Fig. A4). In B71 seedlings, water stress produced a substantial reduction of leaf growth rate at 10 DAS (Fig. A5). In contrast, the rate was slightly higher in SHAM-treated than control seedlings. The rate was approximately the same for S + SHAM treatment as for water stress alone (Fig. A5).

At harvest time, the morphological parameters described in M&M (SD, SI, leaf area, PR length, LR number, LR length) were measured for each treatment group.

Stomata were 100% anomocytic (characterized by absence of subsidiary cells) and showed dispersed distribution, except on veins where they were arranged in rows (Fig. A6). They were more abundant in abaxial epidermis.

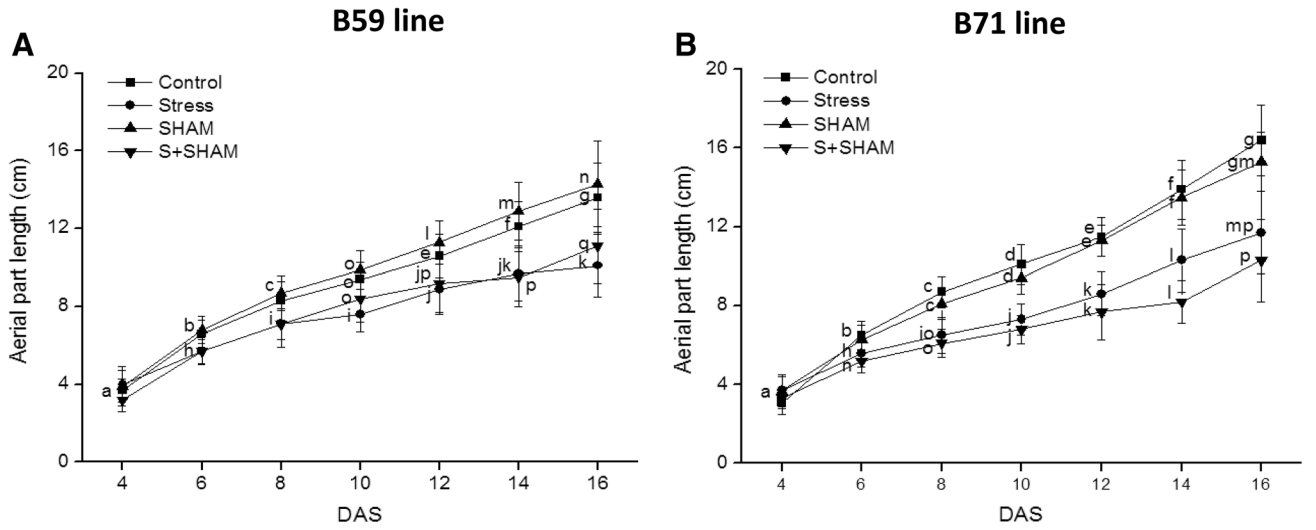

Fig. 1 Aerial part length of B59 (**a**) and B71 (**b**) sunflower seedlings grown under optimal conditions (control), water stress (mannitol 400 mM), SHAM treatment, and combination of water stress plus SHAM treatment (S + SHAM), at various days after sowing (DAS). Data shown are mean ± SE from three replicates. Values with the same letter are not significantly different at $P \leq 0.05$

The leaf micromorphological parameters were differentially affected by the four treatments. In B59, none of the treatments affected SD on the abaxial leaf surface, whereas SHAM treatment decreased SD on the adaxial surface relative to control (Fig. A7). SHAM also significantly reduced SI on the abaxial surface. In B71, water stress significantly reduced SI on the abaxial surface (Fig. A8).

Water stress reduced leaf area relative to controls in seedlings of both lines: by 36% in B59 and by 40% in B71. SHAM decreased leaf area 2.6-fold in B59, but had no significant effect on B71. In both lines, the effect of S + SHAM on leaf area was similar to that of water stress alone (Fig. A3).

In regard to seedling root architecture, water stress reduced PR length in B71, but had no such effect in B59. SHAM treatment significantly increased PR length in B59 (in agreement with previous reports that root growth is inhibited by endogenous JAs), but had no effect on this parameter in B71 line. S + SHAM did not cause reversion of root phenotype in either line (Table 1). Interestingly, water stress increased LR number by 28% in B59 and by 45% in B71 (Table 1). SHAM reduced LR number in B59, but increased this parameter in B71. The effect of S + SHAM on LR number was the same as that of water stress alone for both lines. LR number was higher for B59 than for B71 under control, water stress, and S + SHAM treatments. LR length was reduced by water stress in B59 and slightly increased (12%) by SHAM in B71. The inhibitory effect of water stress on LR length was reversed by S + SHAM in both lines (Table 1).

Table 1 Effects of four experimental treatments on root architecture of B59 and B71

Line	Treatment	Primary root length (cm)	Lateral root number	Lateral root length (cm)
B59	Control	45 ± 0.4 acf	8 ± 1 aef	3 ± 0.1 ac
	Stress	5.3 ± 0.9 bc	11 ± 1 c	2.6 ± 0.1 b
	SHAM	5.3 ± 0.4 b	6 ± 1 bd	3 ± 0.1 ad
	S + SHAM	5 ± 0.6 bf	11 ± 1 c	3 ± 0.1 ad
B71	Control	4.6 ± 0.3 b	5 ± 1 b	2.9 ± 0.2 ab
	Stress	3.5 ± 0.5 d	9 ± 1 ae	2.8 ± 0.2 ab
	SHAM	4.6 ± 0.5 abc	7 ± 1 df	3.3 ± 0.1 cd
	S + SHAM	3.9 ± 0.4 ad	9 ± 1 ae	3.3 ± 0.1 cd

Within each column, values with the same letter are not significantly different at $P \leq 0.05$

Table 2 Effects of four experimental treatments on chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid content (mg g^{-1} FW) of B59 and B71

Pigment	Treatment			
	Control	Stress	SHAM	S + SHAM
Chlorophyll *a*				
B59	117.5 ± 2.7 a	150.6 ± 31.5 c	125.3 ± 9.4 a	158.4 ± 19.2 c
B71	188.3 ± 6.3 b	83 ± 3.7 d	109.3 ± 10.8 a	189.8 ± 2.4 b
Chlorophyll *b*				
B59	58.8 ± 1.7 a	75.5 ± 14.1 bcd	65.5 ± 6.2 a	80 ± 11.7 bcd
B71	96.8 ± 7.9 b	83 ± 3.7 bc	75.6 ± 3.5 d	82.5 ± 3.5 cd
Total chlorophyll				
B59	175.7 ± 3.7 a	226.1 ± 45.6 c	190.8 ± 15.6 a	238.5 ± 30.8 c
B71	282.6 ± 2.3 b	147.2 ± 14.7 d	184.9 ± 14.3 a	272.3 ± 1.1 b
Carotenoid				
B59	81 ± 0 a	93 ± 9.8 c	73.6 ± 1.3 e	113.1 ± 17 c
B71	306.1 ± 11 b	202.7 ± 3.7 d	100.9 ± 1.2 c	202.6 ± 7.5 d

For each type of pigment (paired rows), values with the same letter are not significantly different at $P \leq 0.05$

Effects of water stress and SHAM on physiological characteristics

Relative water content (RWC)

Control seedlings of lines B59 and B71 showed no difference in RWC. RWC was greatly reduced by water stress, and values again did not differ between the two lines (Fig. 2a). The strong reduction in RWC clearly demonstrated that the seedlings were stressed. SHAM treatment resulted in RWC values similar to those of control in both lines. Effects of S + SHAM on RWC were the same as those of water stress alone (Fig. 2a).

Stomatal conductance

Stomatal conductance was higher for B59 than for B71 in controls. Water stress produced a striking 50-fold decrease of stomatal conductance for B59 and a 30-fold decrease for B71. SHAM also reduced this parameter in both lines, but to a lesser degree than did water stress. S + SHAM slightly reversed the reduction caused by water stress (Fig. 2b).

Content of chlorophylls and carotenoids

Chlorophyll and carotenoid contents were lower in leaves of B59 than in B71. Chlorophyll *a* content was higher than that of chlorophyll *b* in both lines. Water stress significantly increased content of all pigments in B59, but reduced chlorophyll *a* (by 56%), total chlorophylls (by 48%), and

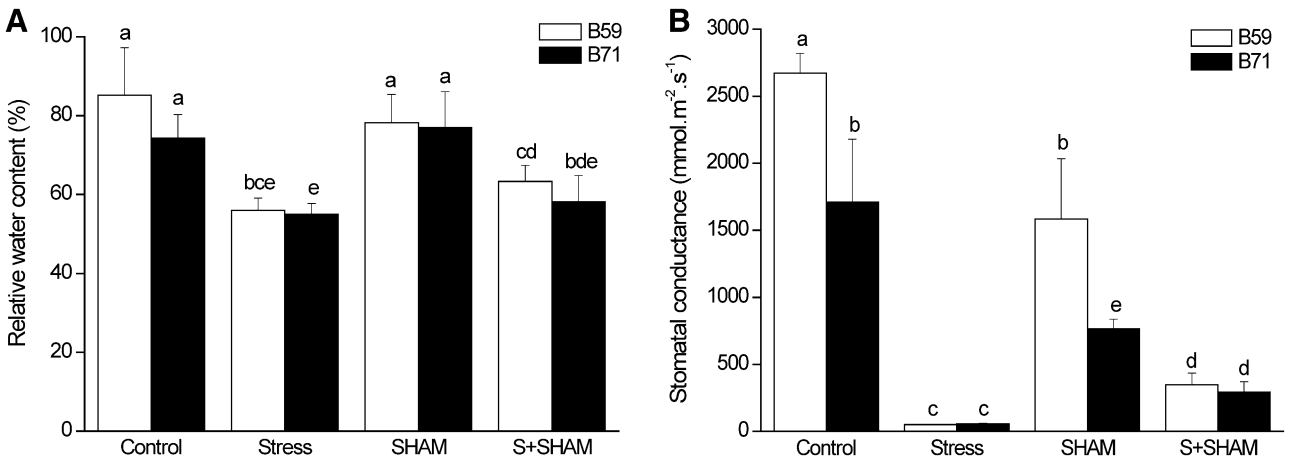

Fig. 2 **a** Relative water content (RWC) of B59 and B71 seedlings under four experimental treatments as in Fig. 1. **b** Stomatal conductance. Data and statistical conventions as in Fig. 1, but with five replicates

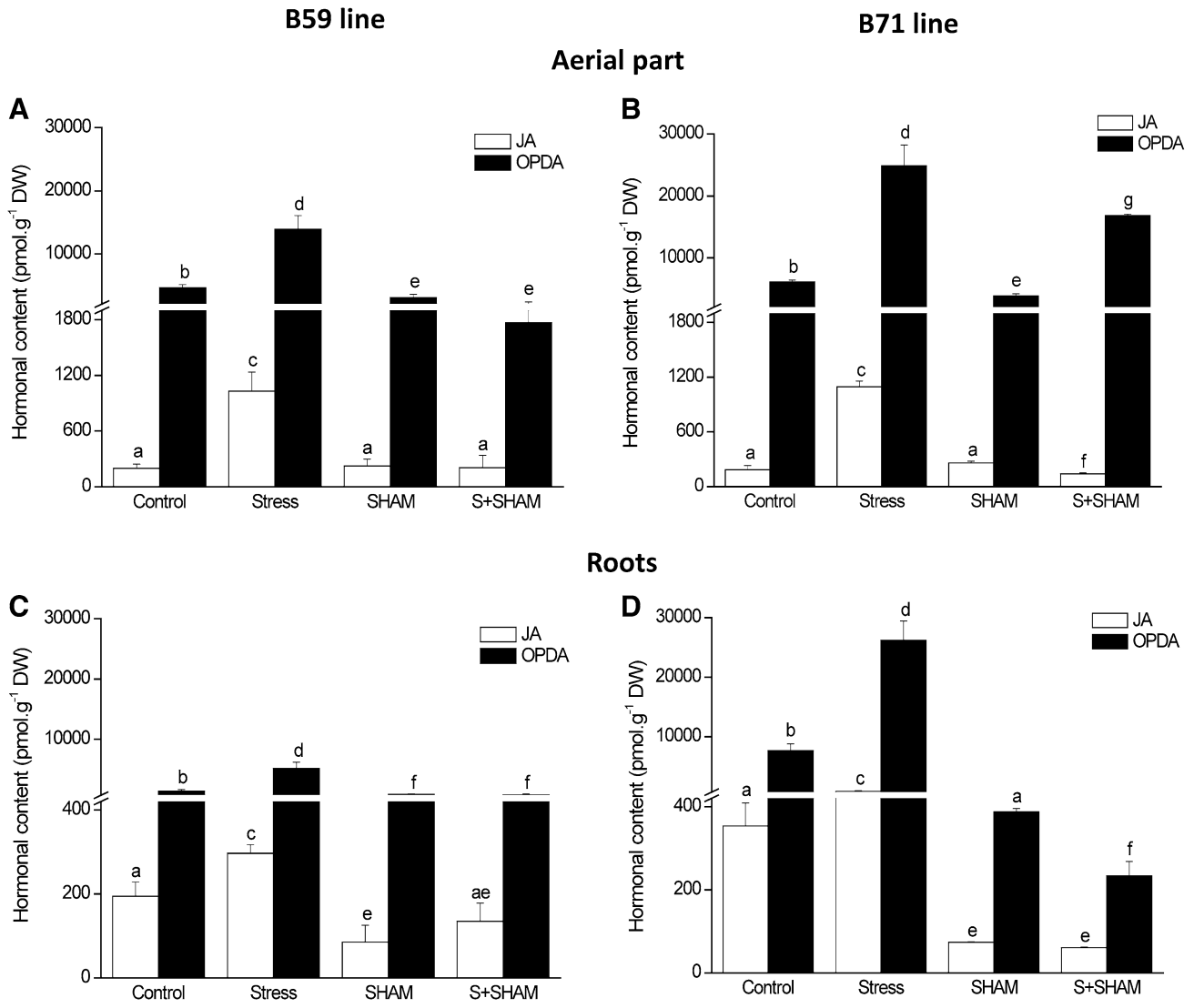

Fig. 3 JA and OPDA content in aerial part (**a, b**) and roots (**c, d**) of B59 and B71 seedlings under four experimental treatments as in Fig. 1. Data and statistical conventions as in Fig. 1, but with four replicates

carotenoids (by 34%) in B71 (Table 2). SHAM reduced chlorophyll content in B71, but had no significant effect on this parameter in B59. Effects of S + SHAM were the same as those of water stress in B59, whereas in B71 S + SHAM reversed the chlorophyll *a* reduction caused by water stress (Table 2).

Effect of water stress on OPDA and JA content

OPDA and JA were detected in aerial part and roots of B59 and B71 seedlings (Fig. 3). OPDA content was higher than that of JA in aerial part and roots of controls. Water stress resulted in differential accumulation patterns of these two phytohormones. OPDA and JA in aerial part were significantly increased by water stress in both lines, but to a greater degree for JA than for OPDA (Fig. 3a, b). OPDA was increased threefold by water stress in B59 (Fig. 3a), and fourfold in B71 (Fig. 3b). JA accumulation in aerial part was similar for the two lines (Fig. 3a, b). Water stress significantly increased OPDA in both lines (Fig. 3c, d), but to a greater degree (3.4-fold) in B71 than in B59. JA accumulation was significantly increased in both lines (1.5-fold in B59; 2.4-fold in B71). Under water stress, content of OPDA was 31-fold higher than that of JA in B71 roots (Fig. 3d), but only 17-fold higher than that of JA in B59 roots (Fig. 3c).

Effect of SHAM on OPDA and JA content

In aerial part of both lines, SHAM reduced OPDA content but had no effect on JA content (Fig. 3a,b). In B59 roots,

SHAM caused a twofold reduction of JA and 1.6-fold reduction of OPDA (Fig. 3c). Levels of the two hormones were also reduced by SHAM in B71 roots, but to a greater degree for OPDA (20-fold reduction relative to control) (Fig. 3d).

Effect of S + SHAM on OPDA and JA content

For aerial part and roots of both B59 and B71, S + SHAM treatment resulted in OPDA and JA levels much lower than those under water stress (Fig. 3). JA content of aerial part under S + SHAM was reduced eightfold in B71 (Fig. 3b) and fivefold in B59 (Fig. 3a). In B71 roots, S + SHAM caused a 33-fold reduction of OPDA (Fig. 3d). In B59 roots, S + SHAM caused a sevenfold reduction of OPDA and twofold reduction of JA (Fig. 3c).

Supplemental Table A1 shows percentage reductions in OPDA and JA content in aerial part and roots of B59 and B71 resulting from S + SHAM treatment, relative to the enhanced content caused by water stress alone. A greater reduction of OPDA was observed in roots (97% for B71; 85% for B59) than in aerial part for both lines. In contrast, a greater reduction of JA was observed in aerial part (87% for B71; 80% for B59).

Discussion

Induction of biosynthetic pathways of various phytohormones is a key physiological mechanism used by plants to enhance stress tolerance (Cutler et al. 2010; Qin et al. 2011). However, our knowledge of the roles of certain phytohormones in responses to water stress is far limited and fragmentary. In the present study, we used the JA biosynthesis inhibitor SHAM to elucidate the roles of OPDA and JA in modulation of sunflower seedling growth under normal and water stress conditions.

Growth is one of the most drought-sensitive physiological processes in plants. Drought (water stress) inhibits mitosis, cell elongation, and cell expansion, resulting in reduced growth. In a previous study of sunflower B59 and B71 seedlings (Andrade et al. 2013), we found that water stress substantially reduced various morphological characteristics, including shoot and root relative fresh weight, shoot dry weight, and the ratio of root dry weight to shoot dry weight.

Water stress alters leaf stomata number and the associated parameters SD and SI, leading to changes in water uptake. In B71 leaves, SD increased in response to mannitol treatment (moderate water stress), indicating the drought adaptation ability of this line. A study of rice showed a similar increase of SD in leaves under moderate water stress (Meng et al. 1999). In contrast, our findings suggest that reduction of SI in B71 under water stress is part of a long-term strategy to reduce water loss under drought condition. In a study of two genotypes of *Populus balsamifera* (balsam poplar), Hamanishi et al. (2012) found that SI was lower in leaves of water-stressed plants as compared with non-stressed plants. Our analysis of leaf micromorphological parameters in SHAM-treated B59 suggests a relationship between a reduced proportion of stomatal cells relative to total epidermal cells and decreased content of endogenous OPDA. We therefore hypothesize that OPDA has a direct or indirect effect on leaf micromorphological parameters such as SI.

PR length and LR production are key parameters affected by water stress. In this study, PR length in B71 was reduced by water stress. A possible explanation is that accumulated JA in roots of stressed plants inhibits cell elongation, cell division, and consequently roots length. On the other hand, water stress in both B59 and B71 led to increases of LR number and root surface area. Thus, PR of both lines maintained its LR formation ability in response to water stress. In B59, LR length was reduced by water stress, in agreement with the observation by Van der Weele et al. (2000) that water stress of −0.5 MPa or less strongly reduced LR length. These findings, taken together, suggest that both B59 and B71 respond to water stress by modification of root architecture, a developmental adaptation important for successful root system formation and seedling establishment. The observed increases of PR length, LR number, and LR length in SHAM-treated plants at day 14 demonstrated that endogenous JA modulates the sunflower root architecture in several ways, as reported previously by Corti-Monzón et al. (2012).

Our finding that SHAM treatment led to increased aerial part length in B59 suggested that this parameter may be affected by OPDA, and that JAs may inhibit shoot growth in a manner similar to their inhibitory effect on root elongation. On the other hand, the observed differences in leaf growth rate between B59 and B71 suggest that genetic variation in this parameter is determined by differential cell wall properties. The reduction in leaf growth rate of both lines under water stress is consistent with the similar finding of Lechner et al. (2008) in *H. annuus* and *A. thaliana*. The strong reduction of individual leaf area in both B59 and B71 under water stress presumably results from limited expansion and development of the leaf surface due to reductions of cell number and cell size. Leaf areas differed notably between SHAM-treated B59 and B71. A possible explanation is that SHAM, in addition to inhibiting JA biosynthesis, also affects crosstalk with other phytohormones such as auxin (Kalve et al. 2014).

Drought conditions are well known to adversely affect RWC of plant tissues. In our study, mannitol treatment caused a moderate water stress, with consequent reduction of RWC. Ünyayar et al. (2004) reported similar RWC reduction in leaves of sunflower plants under water deficit. Leaf water status is always interrelated with stomatal conductance. In our study, stomatal conductance of B59 and B71

under water stress was strongly reduced but never reached zero, indicating that the seedlings were able to maintain some stomatal conductance even at low leaf water potential. This result agrees with Gomes Gonçalves et al. (2017) and Mo et al. (2016) about decline in stomatal conductance in soybean and watermelon plants subjected to drought. Reduction of stomatal conductance can also result from decreased RWC. Interestingly, stomatal conductance values of control B59 and B71 were high, but differences were found between them. Drought-tolerant species often control stomatal function to allow some carbon fixation under stress, thus improving water use efficiency, and open stomata rapidly when water deficit is relieved (Yordanov et al. 2003). Thus, stomatal conductance in drought-tolerant B71, in comparison with B59, may confer greater capacity to maintain biological activity under water stress. Stomatal movements are regulated through integration of environmental signals and endogenous hormonal stimuli. Abscisic acid (ABA), the primary regulator of this process, acts in a cooperative manner with other phytohormones such as JA, OPDA, and salicylic acid (SA) (Poór and Tari 2012; Savchenko et al. 2014). SHAM treatment under our experimental conditions may induce partial stomatal closure through modification of endogenous OPDA levels, resulting in the stomatal conductance values observed in SHAM-treated B59 and B71. ABA is the best-known stress hormone involved in stomatal closure, and stomatal movements are affected by interactions between ABA and JA signaling pathways (Daszkowska-Golec and Szarejko 2013). Still, our findings suggest that OPDA may also help promote stomatal closure in these sunflower inbred lines.

Another plant response to drought is alteration of photosynthetic pigment content in tissues. Water stress resulted in differential modification of pigment content in B59 and B71. The pigment content increase in B59 may be related to reduce leaf area, as part of a defensive strategy against the adverse effects of drought. Similarly, Rahbarian et al. (2011) reported that drought-sensitive chickpea genotype MCC448 showed maximal chlorophyll and carotenoid contents under drought. On the other hand, the reduction of all photosynthetic pigments in leaves of B71 under water stress may be due to the effects of such stress on light-harvesting complexes of thylakoid membranes. Feng et al. (2007) reported that SHAM had no direct effect on photosynthesis because it did not reduce chlorophyll content. In contrast, SHAM reduced chlorophyll content in B71 leaves in our study, possibly through inhibition of alternative oxidase (AOX) pathway. S + SHAM increased pigment content in B71 leaves. A possible explanation is that inhibition of de novo JA biosynthesis by this treatment altered the seedlings' capacity to induce leaf senescence in response to water stress, thus promoting survival.

JAs have been implicated as key factors in drought resistance based on their accumulation during drought (Shan and Liang 2010; de Ollas et al. 2013). Along this line, we observed accumulation of OPDA and JA under water stress, suggesting that JAs may act as triggers of plant responses. The increase of JA content in aerial part of both B59 and B71 is consistent with the fact that shoots are the main site of JA biosynthesis (although it also occurs in roots). OPDA increase was more pronounced than JA increase in B59 and B71 roots. As a signaling molecule, OPDA has both overlapping and distinct functions from those of JA and other JAs (Savchenko et al. 2014). It is possible that OPDA-mediated signaling is best suited for responses to certain abiotic stresses, e.g., drought. The differential accumulation of OPDA in roots of stressed B59 and B71 observed in this study may reflect differing mechanisms of water stress tolerance, mediated by this phytohormone. Many recent studies support the role of OPDA as an important player in plant drought tolerance, in part through expression of stress genes and control of stomatal aperture size (De Domenico et al. 2012; Grebner et al. 2013; Savchenko and Dehesh 2014).

SHAM had no direct effect on JA content of aerial part in either line. In contrast, SHAM had a notable inhibitory effect on JA content of roots, indicating successful modulation of endogenous hormone. It is probable that SHAM had a greater effect in roots than in aerial part because this chemical inhibitor was dissolved in the irrigation solution and preferentially absorbed by roots. Similarly, de Ollas et al. (2013) observed in citrus plants that endogenous JA modulation by SHAM was more effective in roots. In our study, the OPDA and JA accumulation in aerial part and roots induced by water stress was reversed by S + SHAM treatment. This finding suggests that SHAM effectively inhibits de novo JA biosynthesis triggered by water stress, and that JAs play a protective role in responses of sunflower seedlings to this stress. The greatest inhibitory effect of SHAM on OPDA content in roots of both lines was observed for S + SHAM treatment (Table A1). Thus, B59 and B71 roots may synthesize JAs independently of aerial part, in agreement with previous findings by our lab (Abdala et al. 2003) and others. Grebner et al. (2013) demonstrated that production of JAs by roots of *Arabidopsis* in uncoupled from production by shoots, notwithstanding the low expression of various JA biosynthesis enzymes.

In conclusion, endogenous JA content in sunflower modulates root phenotype and leaf development, presumably through crosstalk between the JA pathway and other phytohormone pathways. Induction of increased OPDA content by water stress facilitates adaptation and protection of seedlings against this stress factor because OPDA plays a direct or indirect role in regulation of root architecture, stomatal closure, and content of photosynthetic pigments. Our findings demonstrate that JAs, particularly OPDA, are

highly effective signaling molecules in mediation of sunflower seedling responses to water stress.

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References

Abdala G, Miersch O, Kramell R, Vigliocco A, Agostini E, Forchetti G, Alemano S (2003) Jasmonate and octadecanoid occurrence in tomato hairy roots. Endogenous level changes in response to NaCl. J Plant Growth Regul 40:21–27

Agele SO, Maraiyesa IO, Adeniji IA (2007) Effects of variety and row spacing on radiation interception, partitioning of dry matter and seed set efficiency in late season sunflower (*Helianthus annuus* L.) in a humid zone of Nigeria. Afr J Agric Res 2:080–088

Andrade A, Vigliocco A, Alemano S, Llanes A, Abdala G (2013) Comparative morpho-biochemical responses of sunflower lines sensitive and tolerant to water stress. Am J Plant Sci 4:156–167

Brossa R, López-Carbonell M, Jubany-Marí T, Alegre L (2011) Interplay between abscisic acid and jasmonic acid and its role in water-oxidative stress in wild-type, ABA-deficient, JA-deficient, and ascorbate-deficient plants. J Plant Growth Regul 30:322–333

Corti-Monzón G, Pinedo M, Lamattina L, de la Canal L (2012) Sunflower root growth regulation: the role of jasmonic acid and its relation with auxins. Plant Growth Regul 66:129–136

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol 61:651–679

Daszkowska-Golec A, Szarejko I (2013) Open or close the gate—stomata action under the control of phytohormones in drought stress conditions. Front Plant Sci 4:138

De Domenico S, Bonsegna S, Horres R, Pastor V, Taurino M, Poltronieri P, Imtiaz M, Kahl G, Flors V, Winter P, Santino A (2012) Transcriptomic analysis of oxylipin biosynthesis genes and chemical profiling reveal an early induction of jasmonates in chickpea roots under drought stress. Plant Physiol Biochem 61:115–122

de Ollas C, Hernando B, Arbona V, Gómez-Cadenas A (2013) Jasmonic acid transient accumulation is needed for abscisic acid increase in citrus roots under drought stress conditions. Physiol Plant 147:296–306

Du H, Liu H, Xiong L (2013) Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. Front Plant Sci 4:389–397

Durgbanshi A, Arbona V, Pozo O, Miersch O, Sancho JV, Gómez-Cadenas A (2005) Simultaneous determination of multiple phytohormones in plants extracts by liquid chromatography-electrospray tandem mass spectrometry. J Agric Food Chem 53:8437–8442

Farman A, Asghari B, Fazal A (2017) Recent methods of drought stress tolerance in plants. Plant Growth Regul 82:363–375

Feng H, Li H, Li X, Duan J, Liang H, Zhi D, Ma J (2007) The flexible interrelation between AOX respiratory pathway and photosynthesis in rice leaves. Plant Physiol Biochem 45:228–235

Gao XQ, Yang Q, Minami C, Matsuura H, Kimura A, Yoshihara T (2003) Inhibitory effect of salicylhydroxamic acid on theobroxide-induced potato tuber formation. Plant Sci 165:993–999

Gomes Gonçalves C, da Silva Junior AC, Rocha Pereira MR, Gasparino EC, Martins D (2017) Morphological modifications in soybean in response to soil water management. Plant Growth Regul 83:105–117

Grebner W, Stingl NE, Oenel A, Mueller MJ, Berger S (2013) Lipoxygenase6-dependent oxylipin synthesis in roots is required for abiotic and biotic stress resistance of *Arabidopsis*. Plant Physiol 161:2159–2170

Hamanishi ET, Thomas BR, Campbell MM (2012) Drought induces alterations in the stomatal development program in *Populus*. J Exp Bot 63:695–709

Kalve S, De Vos D, Beemster GTS (2014) Leaf development: a cellular perspective. Front Plant Sci 5:362

Kong F, Gao X, Nam KH, Takahashi K, Matsuura H, Yoshihara T (2005) Theobroxide inhibits stem elongation in *Pharbitis nil* by regulating jasmonic acid and gibberellin biosynthesis. Plant Sci 169:721–725

Lechner L, Pereyra-Irujo GA, Granier C, Aguirrezábal L (2008) Rewatering plants after a long water-deficit treatment reveals that leaf epidermal cells retain their ability to expand after the leaf has apparently reached its final size. Ann Bot 101:1007–1015

MacKinney G (1941) Absorption of light by chlorophyll solutions. J Biol Chem 140:315–322

Mahouachi J, Arbona V, Gómez-Cadenas A (2007) Hormonal changes in papaya seedlings subjected to progressive water stress and rewatering. Plant Growth Regul 53:43–50

Maksymiec W, Krupa Z (2007) Effects of methyl jasmonate and excess copper on root and leaf growth. Biol Plant 51:322–326

Manugistics (1997) Statgraphics plus for Windows 3.0. Manugistics, Rockville, MD

Meléndez-Martínez AJ, Vicario IM, Heredia FJ (2007) Review: analysis of carotenoids in orange juice. J Food Comp Anal 20:638–649

Meng L, Li L, Chen W, Xu Z, Liu L (1999) Effect of water stress on stomatal density, length, width and net photosynthetic rate in rice leaves. J Shenyang Agric Univ 30:477–480

Mo Y, Yang R, Liu L, Gu X, Yang X, Wang Y, Zhang X, Li H (2016) Growth, photosynthesis and adaptive responses of wild and domesticated watermelon genotypes to drought stress and subsequent re-watering. Plant Growth Regul 79:229–241

Munemasa S, Oda K, Watanabe-Sugimoto M, Nakamura Y, Shimoishi Y, Murata Y (2007) The coronatine-insensitive 1 mutation reveals the hormonal signaling interaction between abscisic acid and methyl jasmonate in *Arabidopsis* guard cells. Specific impairment of ion channel activation and second messenger production. Plant Physiol 143:1398–1407

Pedranzani H, Sierra-de-Grado R, Vigliocco A, Miersch O, Abdala G (2007) Cold and water stresses produce changes in endogenous jasmonates in two populations of *Pinus pinaster* Ait. Plant Growth Regul 52:111–116

Peleg Z, Blumwald E (2011) Hormone balance and abiotic stress tolerance in crop plants. Curr Opin Plant Biol 14:290–295

Poór P, Tari I (2012) Regulation of stomatal movement and photosynthetic activity in guard cells of tomato abaxial epidermal peels by salicylic acid. Funct Plant Biol 39:1028–1037

Porra RJ (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth Res 73:149–156

Qin F, Shinozaki K, Yamaguchi-Shinozaki K (2011) Achievements and challenges in understanding plant abiotic stress responses and tolerance. Plant Cell Physiol 52(9):1569–1582

Rahbarian R, Khavari-Nejad R, Ganjeali A, Bagheri A, Najafi F (2011) Drought stress effects on photosynthesis, chlorophyll fluorescence and water relations in tolerant and susceptible chickpea (*Cicer arietinum* L.) genotypes. Acta Biol Cracov Ser Bot 53:47–56

Rudús I, Weiler EW, Kepczyńska E (2009) Do stress-related phytohormones, abscisic acid and jasmonic acid play a role in the regulation of *Medicago sativa* L. somatic embryogenesis? Plant Growth Regul 59:159–169

Savchenko T, Dehesh K (2014) Drought stress modulates oxylipin signature by eliciting 12-OPDA as a potent regulator of stomatal aperture. Plant Signal Behav 9:e28304

Savchenko T, Kolla VA, Wang CQ, Nasafi Z, Hicks DR, Phadungchob B, Chehab WE, Brandizzi F, Froehlich J, Dehesh K (2014) Functional convergence of oxylipin and abscisic acid pathways controls stomatal closure in response to drought. Plant Physiol 164:1151–1160

Schneiter AA (1978) Non-destructive leaf area estimation in sunflower. Agron J 70:141–142

Shan C, Liang Z (2010) Jasmonic acid regulates ascorbate and glutathione metabolism in *Agropyron cristatum* leaves under water stress. Plant Sci 178:130–139

Turhan H, Baser I (2004) In vitro and in vivo water stress in sunflower (*Helianthus annuus* L.). Helia 27:227–236

Turner NC (1981) Techniques and experimental approaches for the measurement of plant water status. Plant Soil 58:339–366

Ünyayar S, Keles Y, Ünal E (2004) Proline and ABA levels in two sunflower genotypes subjected to water stress. Bulg J Physiol 30:34–47

van der Weele CM, Spollen WG, Sharp RE, Baskin TI (2000) Growth of *Arabidopsis thaliana* seedlings under water deficit studied by control of water potential in nutrient-agar media. J Exp Bot 51:1555–1562

Vernon LP (1960) Spectrophotometric determination of chlorophylls phaeophytins in plant extracts. Ann Chem 32:1144–1150

Waraich E, Ahmad R, Ashraf M, Saifullah Y, Ahmad M (2011) Improving agricultural water use efficiency by nutrient management in crop plants. Acta Agric Scand Sect B 61:291–304

Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann Bot 111:1021–1058

Wasternack C, Strnad M (2016) Jasmonate signaling in plant stress responses and development -active and inactive compounds. New Biot 33:604–613

Xin ZY, Zhou X, Pilet PE (1997) Level changes of jasmonic, abscisic, and indole-3yl-acetic acids in maize under desiccation stress. J Plant Physiol 151:120–124

Yordanov I, Velikova V, Tsonev T (2003) Plant responses to drought and stress tolerance. Bulg J Plant Physiol Special Issue 2003:187–206

Zhu C, Gan L, Shen Z, Xia K (2006) Interactions between jasmonates and ethylene in the regulation of root hair development in *Arabidopsis*. J Exp Bot 57:1299–1308

Image-Pro Plus - application notes. Silver Spring: Media Cybernetics (2002). http://www.mediacy.com/action.htm