

# Hormonal Modulation of Citrus Responses to Flooding

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**Abstract** In this work, variations in endogenous levels of several hormones were measured in citrus under conditions of continuous flooding following a time-course design. The use of three genotypes differing in their ability to tolerate waterlogging has allowed the discrimination between common and specific hormonal responses. Data suggest an essential involvement of the aerial part in the regulation of tolerance to flooding, whereas in roots more general responses were detected. The progressive increase in leaf abscisic acid (ABA) correlating with the different tolerance of genotypes confirms the involvement of this hormone in plant responses to stress. The late increase in 1-aminocyclopropane-1-carboxylic acid, concomitant with severe leaf injury, points to ethylene as a promoter of leaf senescence in citrus. Leaf putrescine increased in all flooded genotypes, suggesting a general protective role, whereas a higher protective ability of spermidine and spermine was enforced by their exclusive accumulation in the sensitive genotype. Leaf jasmonic acid (JA) increased rapidly and transiently under flooding, suggesting a role for this hormone in triggering downstream responses. In stressed roots, while indole-3-acetic acid increased, JA and ABA levels rapidly decreased to reach almost complete depletion in all flooded citrus genotypes. This suggests that not only should the increase in the so-called stress hormones be considered a signal but also their reduction. The results contribute to the understanding of the intricate set of connections between plant hormones that regulate physiologic responses to stress.

**Keywords** Abscisic acid · 1-Aminocyclopropane-1-carboxylic acid · Indole-3-acetic acid · Jasmonic acid · Putrescine · Spermidine · Spermine · Waterlogging

## Introduction

Flooding causes several disturbances in plant physiology and biochemistry. This is of special relevance in citrus plants because they lack specific adaptation to this environmental factor such as aerenchyma formation or lenticel hypertrophy (Arbona and Gómez-Cadenas, unpublished results). The genus *Citrus* and related species are classified as flooding-sensitive (Ford 1968), although differences among genotypes regarding their ability to tolerate waterlogging have been reported. Under continuous flooding, Carrizo citrange (CC), a hybrid genotype used as a rootstock, shows relatively high tolerance to flooding whereas Cleopatra mandarin (CM) is very sensitive. Citrumelo CPB4475 (Cit), another hybrid genotype, exhibits an intermediate behavior. This different tolerance has been associated with a distinct ability to scavenge active oxygen species (Arbona and others 2008).

In other plant systems, tolerance to flooding has been linked to morphologic adaptations apparently regulated by specific hormones (Dat and others 2004; Pierik and others 2007). Under flooding, increases in abscisic acid (ABA) content have been reported in leaves of alfalfa (Castonguay and others 1993), tobacco (Hurng and others 1994), pea (Jackson and others 1988), and tomato (Else and others 1996) and in roots of *Gerbera jamesonii* (Olivella and others 2000). These increases have been related to either an increased de novo synthesis (Jackson and others 1988; Castonguay and others 1993) or a diminished export to

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other organs (Else and others 1995, 2001). The ABA increase in response to flooding has been related to several adaptative responses such as stem elongation in deep-water rice (Azuma and others 1995) or stomatal closure in tomato (Else and others 1996) and alfalfa (Castonguay and others 1993). In addition, the exogenous application of ABA increased tolerance to anoxia in *Arabidopsis* (Dat and others 2004).

Ethylene has also been shown to act either alone (Pierik and others 2007) or coordinately with indole-3-acetic acid (IAA) in the stimulation of shoot elongation under flooding (Voeselek and others 2003). In this sense, interaction among different plant hormones has been linked to the induction of several morphologic modifications to cope with waterlogging as shown by extensive research carried out on *Rumex palustris* (Voeselek and others 2003; Cox and others 2004). This is in agreement with the proposal of hormonal networks controlling the responses of plants to environmental changes (Dodd 2003; Ho and others 2003; Devoto and Turner 2005).

Although references to its role as a modulator of plant responses to flooding have not been found in the literature, jasmonic acid (JA) has been extensively studied in response to wounding, herbivore or insect feeding, and fungal infection (Wasternack 2007). This plant hormone has been suggested to act coordinately with ethylene and salicylic acid to induce physiologic responses against pathogen infection in tomato (O'Donnell and others 2003). In addition, increased levels of JA were observed in tomato under salt stress (Pedranzani and others 2003) and in papaya in response to progressive drought and rewatering (Mahouachi and others 2007).

Besides signaling roles, polyamines have been proposed as cell membrane, nucleic acid, and protein stabilizers. A role for these plant growth regulators in detoxification of activated oxygen species (AOS) has also been suggested (Nayyar and Chander 2004). Some polyamines such as putrescine (Put), spermidine (Spd), and spermine (Spm) are ubiquitous in plant systems whereas others are rare or not very abundant (Bouchereau and others 1999). Their involvement in the regulation of plant responses to flooding is unclear. Accumulation of Put seems related to an increased tolerance to hypoxia (Bouchereau and others 1999). In this sense, Put pretreatment of tomato plants prevented root injury induced by hypoxic conditions (Nada and others 2006).

Despite all the information available on plant hormone signaling and action in species such as *Rumex* or pea, it is not always possible to extrapolate conclusions to woody plants. As far as we know, there is a report on ethylene induction of hypertrophied lenticels in mango under flooding (Larson and others 1993), but there is no literature on hormonal regulation of citrus responses to flooding. The

aim of this work is to depict plant hormonal variations in a woody perennial subjected to flooding stress. The metabolic precursor of ethylene, ABA, JA, and polyamines were analyzed due to their involvement in plant responses to abiotic stress together with IAA, which plays an important role in root and shoot elongation under flooding stress.

It was therefore hypothesized that a different type of hormonal regulation could be at the basis of tolerance to flooding stress. To check this hypothesis, three citrus genotypes, well characterized for their different ability to tolerate flooding (Arbona and others 2008), were subjected to continuous soil waterlogging. Root and leaf levels of ABA, JA, IAA, polyamines, and 1-aminocyclopropane-1-carboxylic acid (ACC) were measured throughout a time-course experiment and the results discussed in terms of physiologic relevance.

## Materials and Methods

### Plant Materials, Treatments, and Sample Collection

One-year-old seedlings of Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan., CM), Citrumelo CPB 4475 (*Poncirus trifoliata* L. Raf. × *Citrus paradisi* L. Macf., Cit), and Carrizo citrange (*Poncirus trifoliata* L. Raf. × *Citrus sinensis* L. Osb., CC) were used for these experiments. Plants were purchased from a commercial nursery and immediately transplanted to 2.5-l plastic pots. A mixture of peat moss:perlite:vermiculite in an 8:1:1 ratio was used as a substrate. Plants were watered three times a week as described in Arbona and others (2006) and allowed to acclimate for 2 months before the onset of the experiments. During plant acclimation and experiments, plants were kept in a greenhouse under the following conditions:  $26 \pm 4.0^\circ\text{C}$  day temperature,  $18 \pm 3.0^\circ\text{C}$  night temperature, relative humidity between 50 and 80%, and a 16-h photoperiod. A total of 42 homogeneous seedlings per genotype were chosen: 21 plants were kept as control and watered regularly as described above, whereas the other 21 were continuously flooded by submerging pots in 4-l containers filled with tap water so the water level was 2 cm above the soil surface. Pots were wrapped in opaque plastic sheets to prevent algal proliferation. More water was added as required to maintain the water level. As described previously (Arbona and others 2008), only mature leaves at an intermediate position and young roots were harvested, washed with distilled water, and immediately frozen in liquid nitrogen. At day 32, leaves from Cit and CM were not harvested due to their generalized senescence symptoms. Before storage at  $-80^\circ\text{C}$ , the tissue was ground to a fine powder with a prechilled mortar and pestle. Each plant

was processed as a biological replicate and therefore stored separately. For each of the analyses described below (malondialdehyde and different plant hormones), two independent extractions per plant were performed.

#### Physiologic Parameters of Leaf Damage

Visible symptoms of leaf damage and malondialdehyde (MDA) concentration were used as markers of the negative effects of flooding on plant physiology. The number of leaves showing visible symptoms such as midrib vein yellowing, curling, and wilting was recorded and values encoded in a 0–5 scale, where 0 indicates no visible symptoms and 5 indicates all leaves affected.

MDA content was assayed as in Arbona and others (2008). Briefly, 0.4 g of frozen plant material was extracted in 5 ml of cold 80% ethanol (Panreac S.A., Barcelona, Spain). After centrifugation, different aliquots of the supernatant were mixed with either 20% trichloroacetic acid (Panreac) or a mixture of 20% trichloroacetic acid and 0.5% thiobarbituric acid (TBA, Sigma-Aldrich, St. Louis, MO). After reaction in a boiling bath for 1 h, samples were allowed to cool down in an ice bath and centrifuged. Absorbance at 440, 534, and 600 nm was read on both supernatants against a blank. Calculation of MDA concentration was performed as follows:

1.  $[(\text{Abs } 532_{+\text{TBA}}) - (\text{Abs } 600_{+\text{TBA}}) - (\text{Abs } 532_{-\text{TBA}} - \text{Abs } 600_{-\text{TBA}})] = A$
2.  $[(\text{Abs } 440_{+\text{TBA}} - \text{Abs } 600_{+\text{TBA}}) \times 0.0571] = B$
3.  $\text{MDA equivalents (nmol ml}^{-1}\text{)} = (A - B/157,000) \times 10^6$

#### IAA, ABA, and JA Analysis

Plant hormones were analyzed by HPLC coupled to tandem mass spectrometry as described in Gómez-Cadenas and others (2002) and Durgbanshi and others (2005). Frozen plant tissue was extracted in ultrapure water using a tissue homogenizer (Ultra-Turrax, Ika-Werke, Staufen, Germany). Before extraction, samples were spiked with 5 ng of  $d_2$ -IAA (Isotech, Sigma-Aldrich), 100 ng of  $d_6$ -ABA (prepared as in Gómez-Cadenas and others 2002), and 100 ng of dihydrojasmonic acid (as in Kristl and others 2005) to assess recovery and matrix effects. After extraction and centrifugation, the pH of the supernatant was adjusted to 3.0 and partitioned twice against di-ethyl-ether (Panreac, Barcelona, Spain). The organic layers were combined and evaporated in a centrifuge vacuum evaporator (Jouan, Saint-Herblain, France). The dry residue was thereafter resuspended in a water:methanol (9:1) solution, filtered, and injected in a HPLC system (Alliance 2695, Waters Corp., Milford, MA). Hormones were then

separated in a reversed-phase Kromasil 100 C18 column (100 × 2.1 mm 5- $\mu\text{m}$  particle size) using methanol and 0.01% acetic acid in water as solvents at a flow rate of 300  $\mu\text{l min}^{-1}$ . The mass spectrometer, a triple quadrupole (Quattro LC, Micromass Ltd., Manchester, UK), was operated in negative ionization electrospray mode and the different plant hormones were detected according to their specific transitions using a multiresidue mass spectrometric method. Further details on the determination procedure are given in Durgbanshi and others (2005).

#### Polyamine Analysis

Polyamines were determined as dansyl chloride derivatives according to Hunter (1998). Tissue (0.4 g) was extracted in 10%  $\text{HClO}_4$  (Panreac) using a tissue homogenizer (Ultra-Turrax). After centrifugation at 4°C to pellet debris, 200  $\mu\text{l}$  of the supernatant was combined with 200  $\mu\text{l}$  of a saturated  $\text{NaHCO}_3$  solution and 400  $\mu\text{l}$  of a 5  $\text{mg ml}^{-1}$  dansyl chloride solution (Fluka, Buchs, Switzerland) in ice-cold acetone. Samples were incubated at 70°C in a water bath for 10 min and subsequently allowed to cool down at room temperature. Afterward, 100  $\mu\text{l}$  of a 100  $\text{mg ml}^{-1}$  solution of proline (Panreac) was added as a quencher for dansyl chloride and incubated in the dark at room temperature for 30 min. Then, extracts were partitioned against 500  $\mu\text{l}$  of toluene (Panreac) that was recovered and evaporated in a centrifuge vacuum evaporator (Jouan). The dry residue was resuspended in 800  $\mu\text{l}$  of acetonitrile (Scharlab) and filtered through 0.22  $\mu\text{m}$  cellulose acetate filters prior to injection in a HPLC system (Agilent 1100 Series, Agilent Technologies Ltd., Palo Alto, CA). Samples were separated in a C18 column (Kromasil 100, 5  $\mu\text{m}$ , 146 × 4.6, Scharlab) at a flow rate of 1.5  $\text{ml min}^{-1}$  using an acetonitrile:water gradient. Derivatized Put, Spd, and Spm were detected by fluorescence. A constant amount of 1,5-diaminoheptane (Sigma-Aldrich) was used as an internal standard to normalize peak areas.

#### ACC Determination

ACC was extracted from tissue (0.5 g) in 5 ml of 50% ice-cold ethanol using a tissue homogenizer (Ultra-Turrax). After centrifugation at 4°C, the supernatant was recovered and evaporated in a centrifuge vacuum evaporator (Jouan) at room temperature. The dry residue was then resuspended in ultrapure water and partitioned three times against chloroform to eliminate impurities. ACC in the sample was oxidized to ethylene using  $\text{HgCl}_2$  as a catalyst and then quantified with a gas chromatograph fitted with a packed silica column and a flame ionization detector (Agilent 4890D, Agilent Technologies). Commercial ACC (Sigma-Aldrich) was added as a standard.

Further details on ACC analysis are given in Arbona and others (2003).

### Statistical Analyses

Data mean comparisons were performed with STATGRAPHICS PLUS v.5.1 software (Statistical Graphics Corp., Herndon, VA). One-way analysis of variance (ANOVA) was performed to assess differences between control and flooded plants considering a significance value of 0.05.

## Results

### Leaf Damage

Visible leaf damage increased in all flooded plants, although with differences in intensity among genotypes (Table 1). In CM, visible leaf damage progressed very fast and after 20 days of continuous soil waterlogging, plants exhibited midrib vein yellowing, curling, and wilting in all leaves. In Cit, soil flooding had a less drastic effect on leaf integrity although at day 27 most of the leaves were injured. The least affected genotype was CC, showing injury symptoms in approximately 40% of the leaves after 27 days of treatment.

### MDA

Leaf MDA content in flooded plants of CM remained significantly higher than controls from day 11 until the end of the experimental period. In Cit plants, the leaf MDA increase in response to flooding was observed after 14 days and afterward was always higher than controls. In flooded CC plants, significant increases were recorded only after 20 days of continuous soil waterlogging (Table 2).

**Table 1** Visible leaf damage in citrus plants exposed to continuous soil waterlogging

Days after flooding	Leaf damage		
	Cit	CC	CM
6	0.53	0.00	0.98
11	1.68	0.28	2.24
14	2.74	0.83	2.92
20	3.53	2.08	5.00
27	4.52	2.08	5.00

Leaf damage is expressed from 0 (no leaf visible damage) to 5 (all leaves showing visible symptoms). Data are mean values of three replicate experiments. Control plants always showed no significant visible leaf damage, values above 0 were always significant at  $p \leq 0.05$

### ABA

Flooding increased leaf ABA concentration (Figure 1, upper panel) in all tested genotypes, although with differences in the pattern of accumulation among them. Sharp increases in ABA content were observed in flooded Cit and CM at 14 and 20 days (8.6- and 12.9-fold increase, respectively) and therefore ABA levels in stressed plants remained very high until the end of the experimental period. In flooded CC, despite slight increases from day 14, the strongest ABA accumulation occurred at day 32 (17.8-fold above controls). In addition, ABA level in nonflooded seedlings showed clear differences among genotypes: in Cit it ranged between 171.4 and 671.9 pmol g<sup>-1</sup> fw, whereas in CC and CM it was much lower, between 61.0 and 283.5 pmol g<sup>-1</sup> fw.

On the other hand, waterlogging reduced root ABA below control levels in all genotypes (Figure 2, upper panel). This drop in the endogenous hormone levels occurred early and progressed rapidly, reaching, in some cases, almost complete ABA depletion. Therefore, in all genotypes, flooding significantly reduced root ABA from the first day of sampling and never recovered. The basal levels of ABA in roots varied between 27.5 and 70.7 pmol g<sup>-1</sup> fw without much difference among genotypes. Interestingly, root ABA levels were very much reduced in comparison to leaf ABA levels.

### JA

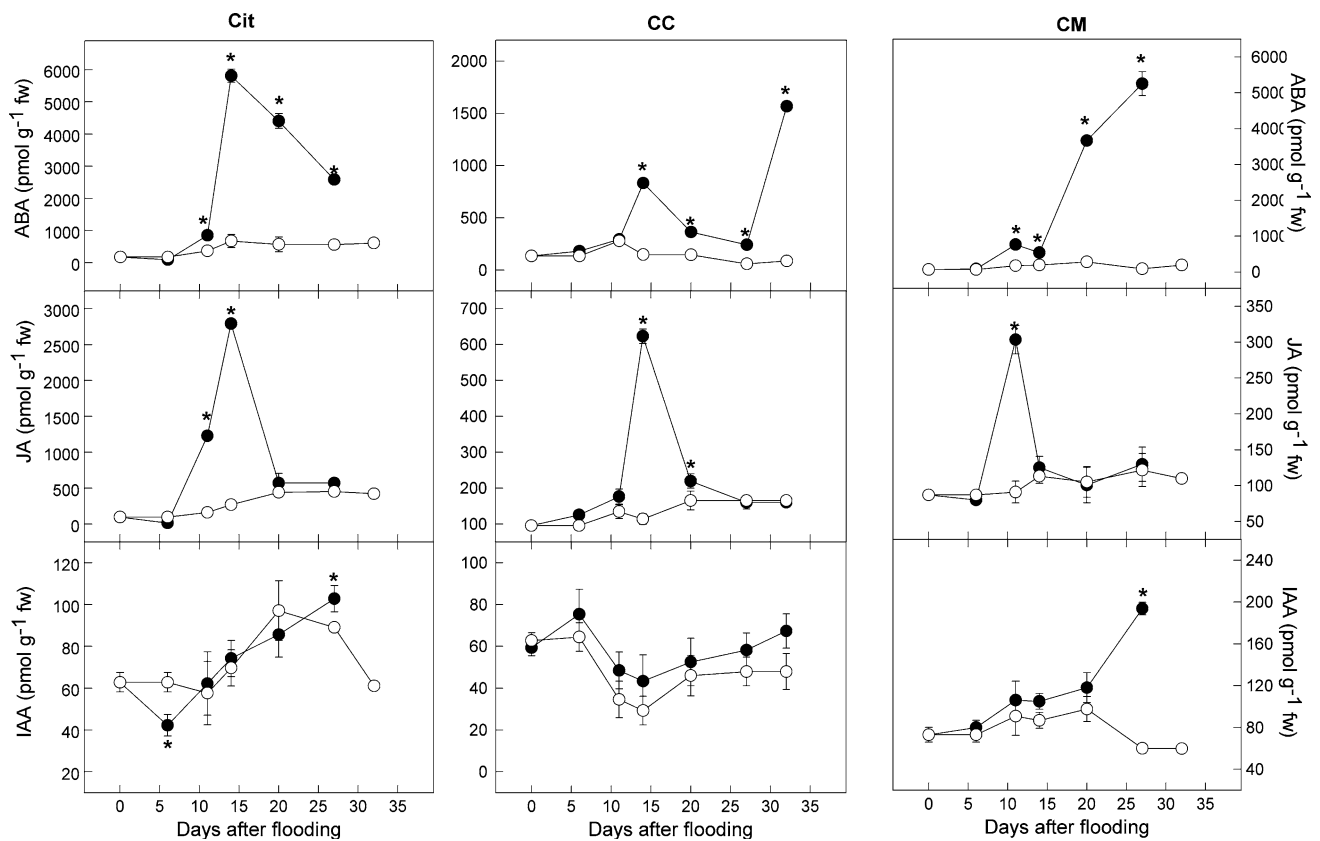
Stress caused transient increases in leaf JA concentration in all genotypes (Figure 1, middle panel), although at different times throughout the period of study. In CM, the most flooding-sensitive genotype, this increase occurred earlier than in the rest, reaching levels 3.3-fold higher than controls at day 11 after flooding. After this date, JA concentration in leaves of flooded CM dropped to control levels. Similarly, both stressed Cit and CC seedlings transiently increased leaf JA concentration (up to 13.4-fold and

**Table 2** Malondialdehyde (MDA) content in citrus leaves

Days after flooding	MDA (nmol g <sup>-1</sup> fw)					
	Cit		CC		CM	
	Control	Flooding	Control	Flooding	Control	Flooding
6	79.21	85.12	104.16	110.64	43.22	42.55
11	76.11	81.15	115.80	111.40	47.64	73.80*
14	78.32	107.22*	100.27	103.10	43.63	73.30*
20	85.11	155.52*	115.11	170.12*	45.19	90.24*
27	59.29	133.64*	88.90	145.92*	48.40	78.80*

Data are mean values of six independent determinations

\* Significant difference with controls at  $p \leq 0.05$



**Fig. 1** Endogenous levels of plant hormones in citrus leaves. From upper to lower: abscisic acid (ABA), jasmonic acid (JA), and indole-3-acetic acid (IAA). In each graph, (○) represents control and (●)

flooded plants. Data are mean values ± standard errors of six independent determinations. Asterisks denote statistical significance at  $p \leq 0.05$  between control and flooded plants

to 5.5-fold above control, respectively) after 14 days. There were important differences among genotypes in leaf JA basal levels, with CM being the genotype with lowest levels and Cit the genotype with the highest.

Root JA concentration (Figure 2, middle panel) drastically and rapidly decreased after flooding in all studied genotypes. Decreases were significant in all groups of plants from the first day of sampling after flooding imposition, reaching reductions close to 100% (for example, after 32 days of stress, levels in Cit were 9.8 pmol g<sup>-1</sup> fw in flooded plants versus 794.2 pmol g<sup>-1</sup> fw in control plants). Differences in root JA basal levels among genotypes were small and not as drastic as in leaves.

**IAA**

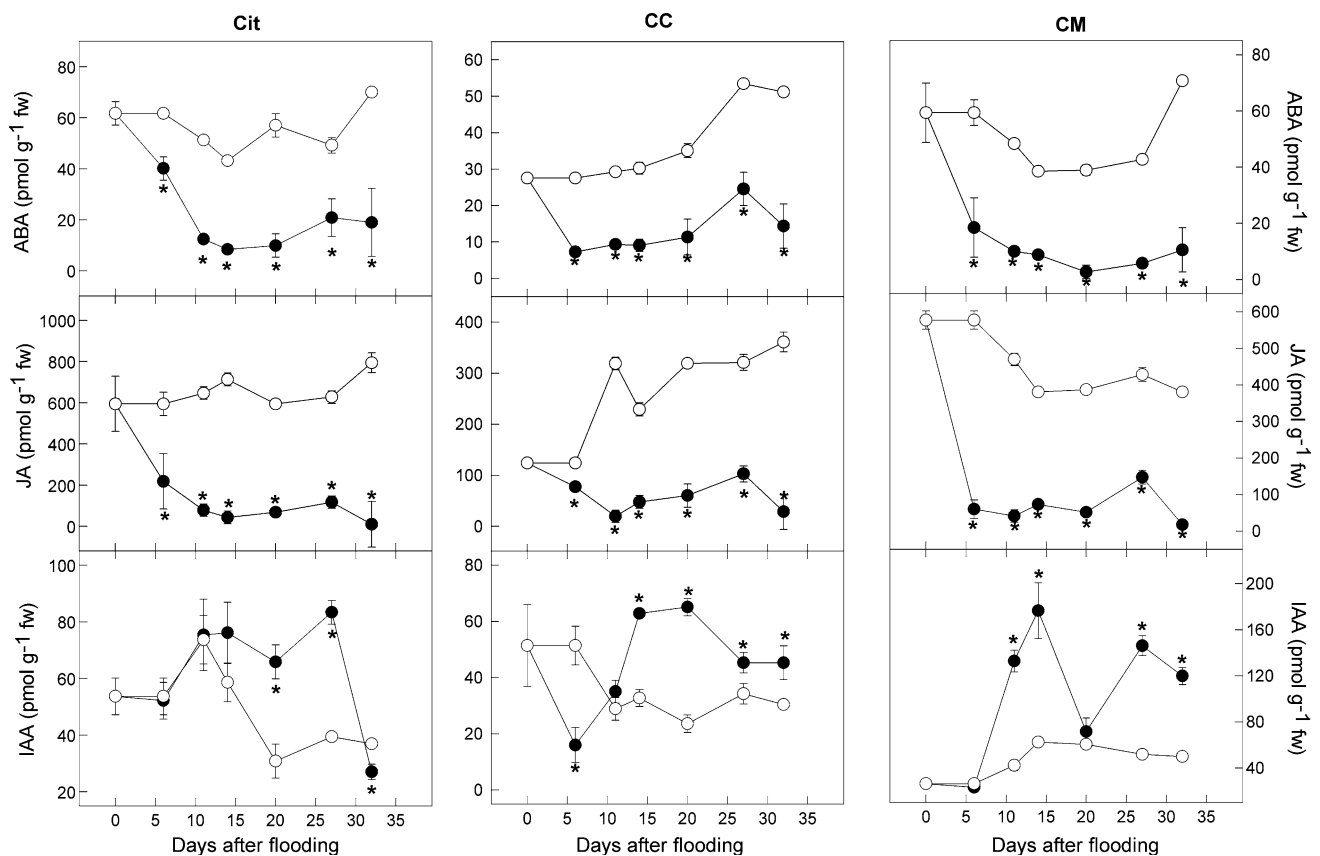
During most of the time-course experiment, flooded and control seedlings of all tested genotypes showed similar leaf IAA levels ranging from 34.6 to 97.6 pmol g<sup>-1</sup> fw (Figure 1, lower panel). Continuous flooding, however, induced a significant increase of this plant hormone in Cit and CM at the end of the experimental period. In flooded CC seedlings, no significant change in leaf IAA content was observed in response to flooding.

Endogenous levels of IAA in flooded roots increased in all tested genotypes (Figure 2, lower panel) showing similar relative accumulations ranging between 2.1 and 2.8 times over control levels. In CM, a biphasic scheme in root IAA accumulation was observed with two transient accumulations that reached maximum levels at days 14 and 27.

**Polyamines**

Leaf Put increased in response to flooding in all tested genotypes although to different extents (Figure 3), CC had the highest accumulation of all three (levels were 16.4-fold higher than controls at the end of the experimental period), whereas Cit and CM had lower increases (6.6- and 8.1-fold, respectively). Flooded Cit seedlings had the maximum accumulation at day 27, whereas in CM maximum levels were observed at day 14, decreasing thereafter to almost control levels. Leaf Spd increased in CM after the imposition of the stress conditions, whereas in Cit and CC levels remained close to control levels most of the time and only increased at the end of the experimental period (1.2- and 1.3-fold of increase, respectively). Similarly, Spm content in leaves of Cit and CC showed no change or even a





**Fig. 2** Endogenous levels of plant hormones in citrus roots. From upper to lower: abscisic acid (ABA), jasmonic acid (JA), and indole-3-acetic acid (IAA). In each graph, (○) represents control and (●)

flooded plants. Data are mean values  $\pm$  standard errors of six independent determinations. Asterisks denote statistical significance at  $p \leq 0.05$  between control and flooded plants

decrease (in some cases reaching a 10% reduction) after stress imposition, whereas Spm concentration in CM increased to reach levels 1.1-fold higher than controls 27 days after flooding. Root polyamine levels exhibited a similar profile with increases in Put in all genotypes after flooding and Spd and Spm accumulation restricted only to flooded CM seedlings.

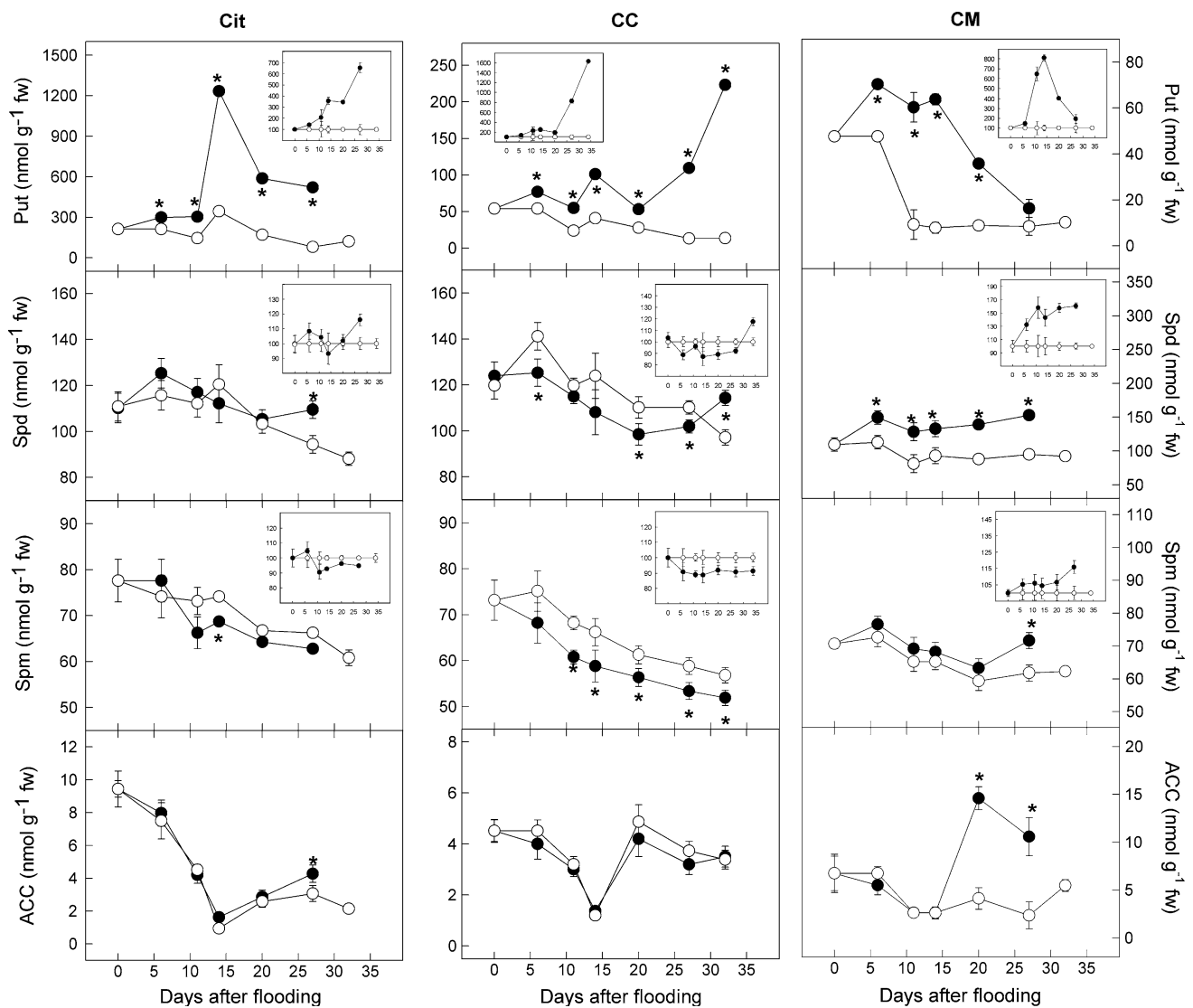
## ACC

Flooding significantly increased leaf ACC concentration in CM seedlings (Figure 3, lower panel): The ethylene metabolic precursor increased up to 3.5-fold above basal levels at day 20. Afterward, ACC levels in flooded CM plants remained higher than in controls. In waterlogged Cit plants, a small significant increase with respect to control values was observed (1.4-fold) after 27 days. In stressed CC seedlings, no significant differences with respect to control values were recorded during the whole experimental period. ACC levels in flooded roots of all citrus genotypes did not show any significant differences compared to controls throughout the experimental period (Table 3).

## Discussion

Hormonal interactions are thought to control different aspects of plant physiology (Gómez-Cadenas and others 1996; Dodd 2003; Ho and others 2003; O'Donnell and others 2003; Cox and others 2004; Devoto and Turner 2005). In this work, the use of three citrus genotypes with a different ability to tolerate flooding has allowed the discrimination between common and specific hormonal responses. Results in Table 1 indicate that Cit and CC plants are more tolerant to soil flooding than CM (see also Arbona and others 2008; Ford 1968). This higher tolerance seems to be associated with an increased ability to delay oxidative damage as indicated by MDA concentration, known to be an indirect marker of oxidative stress, in the different genotypes subjected to soil flooding (see also Arbona and others 2008). However, it is not known which hormonal factors are involved in the regulation of the physiologic responses and how these factors interact.

JA levels transiently increased in leaves of all waterlogged citrus genotypes although not at the same time. The increase in JA occurred earlier in CM, the flooding-sensitive genotype, and later in Cit and CC plants. Moreover,



**Fig. 3** Endogenous levels of plant hormones in citrus leaves. From upper to lower: Putrescine (Put), spermidine (Spd), spermine (Spm), and 1-aminocyclopropane-1-carboxylic acid (ACC). In each graph, (○) represents control and (●) flooded plants. Data are mean values ± standard errors of six independent determinations. Asterisks

denote statistical significance at  $p \leq 0.05$  between control and flooded plants. Data in insets are normalized mean values ± relative standard error. Values for flooded plants were calculated as percentage of control values within each day

**Table 3** 1-Aminocyclopropane-1-carboxylic acid (ACC) concentration in citrus roots

Days after flooding	ACC (nmol g <sup>-1</sup> fw)					
	Cit		CC		CM	
	Control	Flooding	Control	Flooding	Control	Flooding
6	3.32	3.42	3.91	2.70*	5.53	6.02
14	4.91	4.23	3.43	6.01	6.93	8.73
27	6.12	6.05	5.40	9.62	5.61	6.41

Data are mean values of six independent measurements

\* Significant difference at  $p \leq 0.05$

this transient increase always preceded the progressive accumulation of ABA in leaves of stressed citrus seedlings. These data might suggest an involvement of JA in triggering downstream events. In other reports, a similar pattern of JA increase has been described (O'Donnell and others 2003; Pedranzani and others 2003; Mahouachi and others 2007), supporting the role of JA as an early mediator between stress perception and the induction of physiologic responses. On the other hand, leaf ABA increase was detected first in CM, second in Cit, and finally in the most tolerant genotype, CC; therefore, this pattern of accumulation seems likely to be a consequence of the severe and

continuous stress pressure leading to drastic plant responses (Gómez-Cadenas and others 1996, 1998, 2000). Finally, there were no changes in leaf IAA content in CC throughout the studied period and just a slight increase was observed in leaves of flooded Cit plants at the end of the experimental period. Only in the flooding-sensitive CM was an important increase in leaf IAA detected at the last data point. At that moment, severe leaf damage had already occurred in CM which allows us to discard IAA as a signal in this case. On the other hand, the increased levels of this plant hormone seem to be a consequence of the senescence process and might be due to a reuse of IAA in decaying leaves of citrus.

The rapid and strong decrease in ABA and JA observed in roots of all citrus genotypes despite the difference in tolerance points to a common response in this primary sensing organ. The drop in ABA and JA contents in roots can be due to the oxygen requirement of the enzymes responsible for ABA and JA biosynthesis: carotenoid epoxidases and lipoxygenases. However, the involvement of active regulatory processes in the rapid reduction observed cannot be excluded. These data seem to discard the translocation of ABA and JA from roots to shoots in the medium or long term as suggested before in pea (Jackson and others 1988), although early root-to-shoot signaling (Else and others 1996) cannot be ruled out. In contrast to ABA and JA, IAA showed an increase in roots of flooded citrus seedlings irrespective of the relative tolerance of the different genotypes. This observation suggests an essential involvement of IAA in the physiologic responses of roots to flooding, probably related to the induction and growth of new roots that could contribute to coping with stress (Dat and others 2004). It is interesting to note that there is also an oxygen-requiring step in IAA biosynthesis, the conversion from indole-3-acetaldehyde to IAA catalyzed by an oxidase (AAO1). The IAA increase in the anoxic conditions of the flooded root system points to alternative pathways for IAA biosynthesis already described (Pollmann and others 2006) and/or to a reuse of previously conjugated forms (Seidel and others 2006).

The studied genotypes exhibited different leaf polyamine patterns related to their tolerance to flooding. In response to stress, Put accumulated to a higher extent in CC, whereas in Cit and CM Put increases were lower. Spd content increased in CM earlier than in Cit and CC where only moderate increases were observed at the end of the experimental period. In addition, Spm increased only in CM. The observed polyamine variation pattern could be associated with either a protective role against flooding-induced oxidative stress or a signaling role. Data presented here and in a previous work (Arbona and others 2008) show that under flooding conditions, CC and Cit plants delay the onset of oxidative damage and the subsequent

foliar injury with respect to CM plants. Other authors have suggested that polyamines could scavenge AOS and delay senescence (Bouchereau and others 1999). In this context, it is likely that Put accumulation, together with other antioxidant factors in CC (Arbona and others 2008), was enough to cope with flooding-induced oxidative stress. In contrast, an impaired antioxidant response in CM could lead to the activation of subsequent steps in the polyamine biosynthetic pathway and, as a result, accumulation of Spd and Spm occurred. Our results are consistent with the high antioxidant capacity of these tri- and tetracationic amines as previously suggested (Bouchereau and others 1999; Nayyar and Chander 2004).

Previous reports on flooding-induced senescence in tobacco concluded that neither ABA nor polyamines were involved in the induction of leaf senescence (Hurng and Kao 1993; Hurng and others 1994). However, it is known that several genes involved in the polyamine biosynthetic pathway are induced by ABA or JA (Bouchereau and others 1999). Our data are in agreement with these findings although further work is needed to distinguish between the signaling and effector roles of these metabolites and their relationship with leaf senescence in citrus.

In citrus, a close relationship between the amount of ACC and the production of ethylene has been demonstrated (Gómez-Cadenas and others 1996, 1998, 2000). Leaf ACC increased only in flooded CM seedlings at the end of the experimental period concomitantly with severe leaf injury. This points to ethylene as a promoter of senescence in citrus under flooding as it has been shown before under a different stress situation (Gómez-Cadenas and others 2000). Root ACC levels did not vary during the whole experimental period in any of the genotypes subjected to flooding. This observation is consistent with the model that postulates that the accumulation of ABA in the roots and fruits of citrus under different stress conditions is a prerequisite for ACC synthesis (Gómez-Cadenas and others 1996, 1998, 2000).

The use of closely related citrus genotypes, well characterized from an agronomical point of view, has allowed us to differentiate between common and specific responses that could be the basis for a higher tolerance of citrus to flooding. This approach has been previously used to characterize the involvement of the integrity of PSII in tolerance of citrus to high salinity (López-Climent and others 2008). Data presented in this work suggest an essential involvement of the aerial part in the regulation of tolerance to flooding rather than the underground organs. This is supported by the fact that in distinct citrus genotypes with differing abilities to tolerate waterlogging similar root hormonal profiles were found, whereas important differences were observed in leaves. This suggestion is also in agreement with a previous work that



showed higher differences in the ability for AOS scavenging in leaves, whereas only small changes were recorded in roots (Arbona and others 2008). In addition, it is expected that under stress the so-called stress hormones increase in concentration. In fact, ABA increases in roots of *Gerbera jamesonii* under anoxic stress (Olivella and others 2000) and in leaves and roots of citrus plants under salt (Gómez-Cadenas and others 1998) and water stress (Gómez-Cadenas and others 1996) have been reported. In contrast, our data show drastic reductions not only in root ABA levels but also in JA concentrations. As far as we know, this is the first report showing the almost complete depletion of these plant hormones in the sensing organs under stress conditions. This opens new possibilities for the study of hormonal regulation, because not only the increase in a “stress hormone” is considered a signal but its reduction as well. In this sense, this work contributes to the understanding of the complicated set of connections between plant hormones that regulate physiologic responses to stress. These connections may be further explained by using modern integrative approaches.

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