

# The impact of winter flooding with saline water on foliar carbon uptake and the volatile fraction of leaves and fruits of lemon (*Citrus × limon*) trees

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**Abstract.** We investigated the consequences of recurrent winter flooding with saline water on a lemon (*Citrus × limon* (L.) Burm.f.) orchard, focussing on photosynthesis limitations and emission of secondary metabolites (isoprenoids) from leaves and fruits. Measurements were carried out immediately after flooding (December), at the end of winter (April) and after a dry summer in which plants were irrigated with optimal quality water (September). Photosynthesis was negatively affected by flooding. The effect was still visible at the end of winter, whereas the photosynthetic rate was fully recovered after summer, indicating an unexpected resilience capacity of flooded plants. Photosynthesis inhibition by flooding was not due to diffusive limitations to CO<sub>2</sub> entry into the leaf, as indicated by measurements of stomatal conductance and intercellular CO<sub>2</sub> concentration. Biochemical and photochemical limitations seemed to play a more important role in limiting the photosynthesis of flooded plants. In young leaves, characterised by high rates of mitochondrial respiration, respiratory rates were enhanced by flooding. Flooding transiently caused large and rapid emission of several volatile isoprenoids. Emission of limonene, the most abundant compound, was stimulated in the leaves, and in young and mature fruits. Flooding changed the blend of emitted isoprenoids, but only few changes were observed in the stored isoprenoids pool.

**Additional keywords:** *Citrus*, isoprenoids, photosynthesis.

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## Introduction

Salinisation of soils and its negative impact on plant production and quality is an urgent problem worldwide. An increasing amount of soil is subjected to salinity, probably greater today than the 7% of global land area reported by Munns in 2002 on the basis of data collected during the last decade of the past century (Munns 2002). Pitman and Lauchli (2002) reported that the area that is subjected to salinity ranges from 20% to an impressive 50% of the total irrigated lands. Salinisation is generally associated with irrigation with water that has a high salt concentration (Levy and Syvertsen 2004), and therefore salinisation is worse during the dry and warm seasons when

irrigation becomes necessary. Arid and semiarid zones are also characterised by high evapotranspiration rates, which may lead to rising saline water tables, a phenomenon known as ‘dryland salinity’ (Lambers 2003). This may also exacerbate the salinity problem during rainless periods. In the Mediterranean basin, 25% of the total irrigated land is exposed to saline water (Paranychianakis and Chartzoulakis 2005). In rainy months and in well drained soils, salinisation is generally lessened by leaching (Levy and Syvertsen 2004). However, salinisation may also occur because of an increase in saline ground water levels, for example in tropical regions subjected to high rainfalls (Calvert 1982). In this case, the problem of high salinity is

often associated with temporary flooding, which is also known to negatively affect the physiology and productivity of plants by oxygen deprivation in the root system (Vartapetian 1991).

*Citrus* is a high-value horticultural crop that grows preferentially in semiarid areas where irrigation is required to produce maximum yield. *Citrus* species are considered sensitive to salt (Storey and Walker 1999), although there is considerable variation in sensitivity due to rootstock and cultivar selection (Maas 1993). Salt sensitivity is due to the combination of osmotic and ionic stress, the latter probably being more important in altering *Citrus* physiology and productivity (Levy and Syvertsen 2004). Citrus trees are also sensitive to flooding (García-Sánchez *et al.* 2007), as their root system does not withstand long periods of anaerobiosis. Flooding in *Citrus* can reduce photosynthesis and stomatal conductance (García-Sánchez *et al.* 2007); decrease tree growth, yield and fruit size (Reighard *et al.* 2001); and increase susceptibility to pests and root loss (Li *et al.* 2006). Flooding with saline water creates a combination of stresses that can amplify the impact of the single stress (Kozłowski 1997).

In the area of Vendicari, situated in the south-east part of Sicily (36°47'39 "N 15°5'45"E), flooding and high salinity stresses co-occur. The area of Vendicari is largely covered by a natural lagoon system made by three different ponds that were formed in the Hellenistic age (Pirrotta and Barbano 2011). The lagoon was used as salt ponds or for fisheries. It was connected to the sea through canals that were maintained by farmers and allowed a yearly cycle of water management (Ientile *et al.* 2011). In winter, the ponds were refilled by intrusion of marine water and by the limited contribution of freshwater (both from rain and springs). In summer, the ponds dried and the residual water was conveyed to the sea through the canal system. The Vendicari lagoon system is a humid area of international importance according to the Ramsar (1971) convention. After the 1990s, the Vendicari area became a natural reserve to protect its rare ecosystem. The management of the reserve has not regularly maintained the canalisation system since then. Consequently, the water cycle inside the ponds has changed. Nowadays, the ponds rarely dry out in summer, which has important consequences on the microclimate and on the ecology of plant and animal components of the natural ecosystem (Ientile *et al.* 2011). During winter, the water regularly overflows from the ponds onto the surrounding agricultural areas, which are mainly cultivated with typical high-value horticultural crops, namely almond (*Prunus dulcis* Mill.), olive (*Olea europaea* L.) and citrus (lemon; *Citrus × limon* (L.) Burm.f.) fruit trees.

Our research was carried out in a lemon orchard that has been subjected to winter flooding with saline water for the last 10 years. A detailed characterisation of the photosynthetic carbon metabolism of lemon plants was carried out. Lemon trees are known for the fragrance of their leaves, flowers and fruits. Besides having commercial interest for perfumery, and being an important parameter in fruit quality (Moufida and Marzouk 2003), the stored isoprenoids pool and the blend of volatiles emitted by lemon trees also have a fundamental role in ecological interactions with pollinators (Harborne 2001) and herbivores or pathogens (Salvatore *et al.* 2004; Yamasaki

*et al.* 2007). Therefore, the impact of combined flooding and salinity on the volatile emissions from leaves and fruits was also investigated.

## Materials and methods

### Plant material and experimental conditions

Measurements were carried out in a *Citrus* orchard on 50-year-old lemon trees (*Citrus × limon* L. cv. Femminello Siracusano, grafted on sour orange (*Citrus × aurantium* L.) rootstock). The 5-ha orchard was planted with 6 × 6 m spacing between trees on a field gently sloping towards sea level. The part lying just above sea level (~1 ha wide) has been flooded by the water overflowing the surrounding ponds during the last 10 winters. As a consequence, a 5–10 mm thick waterbed covers the soils for around one month every winter during the period of heavy rainfalls (November–December). At the time of our measurements in December, a salt (NaCl) concentration of 1.2% and an electrical conductivity of 1.8 dS m<sup>-1</sup> were measured in the water still residing over the flooded area, thus indicating that the floodwaters had a high salinity level.

Measurements were made in the flooded area and in a control area ~300 m apart and ~1.5 m above sea level, on plants of the same age. The soil was sandy with a 10% limestone skeleton and with very low clay content all over the orchard. The characteristics of the soil are consistent with high drainage soil properties.

Measurements were carried out three times to assess plant physiology (a) during or immediately after the flooding (December 2009), (b) at the end of winter (April 2010), and (c) after the irrigation period in summer (September 2010). Summer irrigation was scheduled periodically (every ~15 days) with travelling sprinklers providing around 300 L of water per tree (~120 m<sup>3</sup> ha<sup>-1</sup>). The irrigation well was 160 m deep and provided water with excellent overall quality and salinity below 0.02%.

For the purpose of this experiment, the orchard was divided into 10 plots (five control and five flooded) each made by two rows of trees. On each plot, one representative tree was selected; thus measurements were done on five trees per treatment. Destructive and nondestructive samples were taken from young leaves (still expanding at the time of measurements and about half the size of a fully expanded leaf), and from mature, fully expanded leaves of each selected tree. In December, mature, ready to market fruits from the two locations (flooded and control) were also collected and examined for their characteristic profile of volatile organic compounds.

### Leaf Na<sup>+</sup> and K<sup>+</sup> and pigment content

Destructive measurements to determine Na<sup>+</sup>, K<sup>+</sup> and pigment content were carried out on young and mature leaves of flooded and control plants. Twenty leaves were randomly collected from all selected trees and from each experimental period. For cation analysis, leaves were dried at 70°C for one week and the dry weight was measured. Dry leaf samples weighing 0.1 g were digested in high-pressure Teflon tubes (THOMSON Instrument Company, San Diego, CA, USA) with a nitric–perchloric acid mixture (3:1, v/v) in a microwave oven (Excel-Shangai EU, Microwave Chemistry Technology Co. Ltd, Hong Kong, China). Digestion consisted

of four steps: (1) 1 min at 100°C and 250 PSI, (2) 1 min at 140°C and 350 PSI, (3) 1 min at 170°C and 450 PSI, and (4) 12 min at 200°C and 550 PSI. Determination of the two cations was made by atomic absorption spectrophotometry (Analyst 300; Perkin Elmer, Rodgau, Germany) in five replicate samples for each treatment.

For pigment content determination, three discs of 0.6 cm diameter were collected from young and mature leaves from the two treatments. The disks were weighed and then immersed in the appropriate solution. Chlorophyll and total carotenoids were extracted with 80% acetone (2.0 mL solvent per 0.05 g leaf tissue), whereas anthocyanins were extracted with 1% HCl in methanol (w/v) (1.5 mL solvent per 0.05 g leaf tissue). All extractions were completed in the dark at 4°C. Spectrophotometric analyses of the pigments were performed using a Perkin-Elmer Lambda Bio20 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). Total chlorophyll (chlorophyll *a* + *b*) and carotenoids were determined following methods and equations by Lichtenthaler and Wellburn (1983).

The total anthocyanin content was estimated from the difference between absorbance at 536 nm and 600 nm (to correct for phaeophytin absorbance) and converted to  $\mu\text{mol g}^{-1}$  FW using the molar extinction coefficient of 33 000 following the method of Hodges and Nozzolillo (1996).

#### *Gas exchange and chlorophyll fluorescence measurements*

CO<sub>2</sub> and H<sub>2</sub>O exchange rates and chlorophyll fluorescence were simultaneously measured over a 2-cm<sup>2</sup> leaf area using an open gas-exchange system (LI-6400, LI-COR, Lincoln, NE, USA) with an integrated fluorescence chamber head (LI-6400-40, LI-COR). Photosynthesis (*A*), stomatal conductance (*g<sub>s</sub>*), transpiration (*T*), intercellular CO<sub>2</sub> concentration (*C<sub>i</sub>*), and the photochemical efficiency in dark-adapted (the maximal quantum efficiency of PSII,  $F_v/F_m$ ) and illuminated leaves ( $\Phi_{\text{PSII}}$ ) were then calculated using LI-6400 software. Water use efficiency (WUE) was calculated as  $WUE = A/T$ . Leaves were maintained in darkness for 20 min before measurement of  $F_v/F_m$ . The  $\Phi_{\text{PSII}}$  was calculated as  $(F_m' - F_s)/F_m'$  where the prime symbol denotes measurements of maximal ( $F_m$ ) and steady-state ( $F_s$ ) fluorescence in illuminated leaves (Genty *et al.* 1989). Nonphotochemical quenching (NPQ), which reflects thermal dissipation of excess light energy, was calculated as  $NPQ = (F_m - F_m')/F_m'$  (Bilger and Björkman 1991). Measurements were repeated on all three experimental dates on 10 leaves per treatment: five young and five mature leaves within each treatment. Leaves were selected in the illuminated part of the crown and in shoots that were not attacked by pathogens or herbivores. Measurements were carried out using a flow rate of 500 mL min<sup>-1</sup> on leaves exposed to 1000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of incident light intensity. The relative humidity was maintained at 50–60%, and the leaf temperature was set at 30°C. The selected conditions allowed normalised measurements of gas exchanges while mirroring, as closely as possible, the ambient conditions that were characterised by clear sky, relative humidity >50%, and temperature ranging between 22°C and 24°C in

December and April, and between 29°C and 32°C in September. CO<sub>2</sub> concentration was set at 400  $\mu\text{mol mol}^{-1}$  except when the CO<sub>2</sub> response was measured. The relationship between photosynthesis and intercellular CO<sub>2</sub> concentration ( $A/C_i$ ) was measured at 1000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light intensity, and external CO<sub>2</sub> concentrations that varied between 50 and 2000  $\mu\text{mol mol}^{-1}$  after removing the diffusive limitations on *A* as described by Centritto *et al.* (2003). The mechanistic model developed by Farquhar *et al.* (1980) was used for  $A/C_i$  curve-fitting and estimation of biochemical parameters.

#### *Measurements of volatile fluxes from intact samples*

The volatile fraction of the isoprenoids contained in lemon leaves and fruits was measured as a flux outside of gas exchange cuvettes. This volatile fraction will hereafter be called the volatile isoprenoids. To measure volatile isoprenoid emissions from the leaf samples, a second LI-6400 system, with a 6 cm<sup>2</sup> window for leaf gas exchange was used. The same conditions as stated for measurements of other gas exchange parameters were set (see above). When photosynthesis and transpiration were steady, the leaf cuvette output was disconnected from the LI-6400 system, and the flow exiting the cuvette was diverted into a silcosteel cartridge packed with 200 mg of Tenax (Markes International Limited, Llantrisant, UK). A volume of 10 L of air was pumped through the trap at a rate of 200 mL min<sup>-1</sup>. The cartridge was analysed by gas chromatography–mass spectrometry (GC-MS, Agilent Technologies, Wilmington, DE, USA). The gas chromatograph (GC) was supplied with a UNITY thermal desorber (Markes International Limited), with a splitless injector and an HP-5MS capillary column 30 m in length, 250  $\mu\text{m}$  internal diameter and 0.25  $\mu\text{m}$  film thickness. The column oven temperature was kept at 40°C for the first 5 min, then increased by 5°C min<sup>-1</sup> to 250°C and maintained at 250°C for 2 min. Helium was used as a carrier gas. The concentration of each volatile compound was calculated by comparison with the peak area of the gaseous standard. The GC was calibrated weekly using cylinders with standards of isoprene, monoterpenes and sesquiterpenes at a concentration of 100 parts per billion (Rivoira, Milan, Italy). The compound identification was made using the National Institute of Standards and Technology library provided with the GC-MS ChemStation software (Agilent Technologies). GC peak retention time was substantiated by analysis of parent ions and main fragments on the spectra.

To measure volatile emissions from fruit samples, whole fruits were placed in a 1.7-L glass cuvette. Fruits were exposed to synthetic air made by mixing O<sub>2</sub>, N<sub>2</sub> and CO<sub>2</sub> from cylinders deprived of contaminants. Atmospheric concentrations of the three gases (20%, 80% and 390 parts per million, respectively) were set with mass flow controllers (BROOKS Instruments, Dresden, Germany). The air flow was set at 2.0 L min<sup>-1</sup>. During sampling, air temperature, light intensity and relative humidity were maintained at constant levels (30°C, 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 56%, respectively). The outlet of the glass cuvette was connected to a silcosteel cartridge and the flow was pumped through the trap at a rate of 150 mL min<sup>-1</sup>. A volume of 1.0 L of air was collected. The analyses of volatile emission from fruits, using trap desorption and GC-MS

determination, were performed as already described above for the leaf measurements.

#### *Measurements of isoprenoid content from stored pools in leaves and fruits*

The composition of the isoprenoid fraction of lemon leaves and fruits was analysed in December 2009. Leaves and fruits were collected in the field and stored at  $-80^{\circ}\text{C}$  until analysed at Consiglio Nazionale delle Ricerche – Florence laboratory. At least five leaves and five fruits were collected from the same trees used for physiological measurements. Young and mature leaves were collected from flooded and control plants, while only mature fruits were analysed, since we were interested in assessing the quality of winter production.

#### *Gas chromatography-flame ionization detection determination*

Foliar tissues (0.2 g) or fruit peel (0.1 g) were ground in liquid nitrogen and extracted with 2.0 mL of *N*-pentane; the sample was filtered and a 0.5  $\mu\text{L}$  volume was injected in the GC in splitter mode (a 20:1 split ratio; see below for details). Analyses were performed with the AutoSystem XL GC (Perkin-Elmer) equipped with an automatic sampler for liquid sample injections and with the TotalChrom™ 6.2.0.0.0:B27 chromatography software.

The separation of the different enantiomeric monoterpenes was performed on an Elite-Betacydex Betacyclodextrin capillary column 30 m long and 0.25 mm in diameter (Perkin-Elmer). Cyclodextrins and their derivatives are useful tools in inducing asymmetric reactions, and have been used extensively as stationary phases in chromatographic enantioselective separations of a wide variety of chiral compounds (Allahverdiev *et al.* 1999). GC analysis was carried out using hydrogen as carrier gas at  $2.0\text{ mL min}^{-1}$  by a flame ionisation detector at  $250^{\circ}\text{C}$  and at an injector temperature of  $230^{\circ}\text{C}$ . The oven temperature programming started at  $40^{\circ}\text{C}$  for 3 min and increased to  $200^{\circ}\text{C}$  at a rate of  $1^{\circ}\text{C min}^{-1}$ ; the final temperature of  $200^{\circ}\text{C}$  was maintained for 10 min. Isoprenoids (mono- and sesquiterpenes) were identified by comparison of retention times with those of standards under the same conditions. High purity components were obtained from Sigma-Aldrich S.r.l., Milan, Italy and Acros, Geel, Belgium. The relative amount (proportion of the profile) of each monoterpene was expressed as a percentage of total monoterpenes, and each sesquiterpene was calculated as a percentage of total monoterpenes plus sesquiterpenes.

#### *Headspace GC-MS determination*

Fruit peel samples (0.3 g each) were ground in liquid nitrogen and placed in a glass vial, sealed with Teflon septum and crimped aluminum cap. The vials were stored at  $-20^{\circ}\text{C}$  until analysed. Headspace GC-MS was then performed on a GC-MS system from Perkin-Elmer Technologies composed of a GC AutoSystem XL coupled to a TurboMass mass spectrometer. The gas chromatograph was equipped with the column described above, operating with helium as a carrier gas at a constant flow of  $3\text{ mL min}^{-1}$ . The oven program was as follows: the initial temperature was  $40^{\circ}\text{C}$  for 3 min, and then an increase of

$1^{\circ}\text{C min}^{-1}$  brought the final temperature to  $200^{\circ}\text{C}$ . The inlet temperature was  $200^{\circ}\text{C}$ . The mass spectrometer was operated with an electron ionisation of 70 eV, scanning the mass range from 35 to 350  $m/z$ . Ion source temperature was  $200^{\circ}\text{C}$ . The GC-MS control and data elaboration were performed using Perkin-Elmer Technologies TurboMass Chemstation ver. 5.4.2.1617 software. The mass spectrometer was calibrated using perfluorotributylamine as the calibration standard, using the Chemstation software.

Samples were analysed by means of headspace gas chromatography with a Perkin-Elmer TurboMatrix 40 automatic headspace sampler and using the following conditions: He (carrier gas) at  $3\text{ mL min}^{-1}$ ; pressurisation time, 5 min; injection time set at 0.05 min to minimise peak broadening; thermostat temperatures,  $80^{\circ}\text{C}$ ; thermostat times, 30 min.

The analysis of pure standards in the same analytical conditions allowed the attribution of some GC-MS signals. When a pure standard was not available, an identification was attempted comparing the recorded mass spectra with a mass spectrometry spectral database (Wiley Library, <http://onlinelibrary.wiley.com>), taking also the chromatographic order of elution into consideration.

#### *Statistical analyses*

Data shown represent the means and s. e. of measurements. Physiological, pigment and mineral data were analysed by a three-factorial ANOVA including the factors treatment (flooded and unflooded), leaf age (young and mature) and season, and their interactions. The results of this factorial analysis are presented in Table 1. The significant difference between treatments (control vs flooded samples) were calculated by Student's *t*-test.

The parameters estimated by  $A/C_i$  response modelling (Table 2) were analysed by one way ANOVA and a *post hoc* Tukey's test was applied.

In the case of isoprenoid emissions and contents, the measurement data were not normally distributed (Kolmogorov–Smirnov one sample test) and were analysed by Kruskal–Wallis and Mann–Whitney nonparametric tests.

Statistical analyses were performed using SYSTAT ver. 12.0 software (Systat Software Inc., San Jose, CA, USA).

## **Results**

### *Salt accumulation in leaf tissues*

In December, after the flooding event,  $\text{Na}^+$  concentration was higher in young leaves than in mature leaves (Fig. 1a). However, no difference in  $\text{Na}^+$  concentration between control and flooded treatments was found in young leaves, whereas the accumulation of  $\text{Na}^+$  was significantly higher in flooded than in control mature leaves. In April, at the end of winter,  $\text{Na}^+$  accumulation in young control and flooded plants decreased with respect to those measured in December, whereas the  $\text{Na}^+$  concentration of mature flooded leaves increased. At our final sampling in September, after summer irrigation, the concentration of  $\text{Na}^+$  decreased in all samples, but was still higher in young leaves than in mature ones.  $\text{Na}^+$  concentration was significantly influenced by the treatment, the leaf age and the season, and



**Table 1. Results of a three-way ANOVA statistical treatment to assess the effects of three independent factors: treatment (flooded and unflooded), leaf age (young and mature) and season (December, April and September) on concentrations of Na<sup>+</sup>, K<sup>+</sup>, total chlorophyll (Chl), carotenoids (Car), anthocyanins (Ant), photosynthesis (*A*), stomatal conductance (*g<sub>s</sub>*), intercellular CO<sub>2</sub> concentration (*C<sub>i</sub>*), and chlorophyll fluorescence parameters ( $\Phi_{PSII}$ , NPQ,  $F_v/F_m$ )**

ANOVA significance level: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; n.s., not significant

	Na	K	Chl	Car	Ant	<i>A</i>	<i>g<sub>s</sub></i>	<i>C<sub>i</sub></i>	WUE	$\Phi_{PSII}$	NPQ	$F_v/F_m$
Treatment	***	n.s.	**	n.s.	***	**	n.s.	***	***	*	***	n.s.
Leaf age	***	***	*	n.s.	***	***	n.s.	***	***	***	**	***
Season	***	*	**	n.s.	***	**	***	***	***	***	***	n.s.
Treatment × leaf age	***	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	*	*	n.s.	n.s.
Treatment × season	n.s.	n.s.	n.s.	n.s.	***	**	***	***	***	n.s.	***	n.s.
Leaf age × season	***	***	n.s.	n.s.	***	***	*	***	***	n.s.	n.s.	n.s.
Treatment × leaf age × season	***	n.s.	n.s.	n.s.	n.s.	n.s.	**	***	**	*	n.s.	n.s.

**Table 2. Maximum Rubisco carboxylase activity ( $V_{cmax}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and maximum rate of electron transport ( $J_{max}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) of young and mature leaves of lemon trees exposed to winter flooding with saline water or grown under control conditions**

The two parameters were estimated from  $A/C_i$  curve fittings using the biochemical model of Farquhar *et al.* (1980) and following the procedure outlined by Centritto *et al.* (2003). Data collected at the end of winter (April) and after the irrigation period in summer (September) are shown as average values and s.e. ( $n = 5$ ). Means were statistically separated by ANOVA and different letters indicate significant differences between means of young and mature leaves of control and flooded trees at  $P < 0.05$  (Tukey's test)

	April 2010				September 2010			
	Young leaves		Mature leaves		Young leaves		Mature leaves	
	Control	Flooded	Control	Flooded	Control	Flooded	Control	Flooded
$V_{cmax}$	11.8 ± 1.1 <sup>d</sup>	12.5 ± 2.5 <sup>d</sup>	50.0 ± 9.9 <sup>b</sup>	36.2 ± 6.8 <sup>b</sup>	25.0 ± 1.4 <sup>c</sup>	20.8 ± 3.1 <sup>c</sup>	90.5 ± 8.7 <sup>a</sup>	91.7 ± 10.2 <sup>a</sup>
$J_{max}$	36.3 ± 2.6 <sup>d</sup>	44.0 ± 2.0 <sup>c</sup>	98.5 ± 10.4 <sup>a</sup>	65.3 ± 6.9 <sup>b</sup>	54.6 ± 1.7 <sup>b</sup>	51.0 ± 3.8 <sup>bc</sup>	128.3 ± 12.1 <sup>a</sup>	113.3 ± 15.5 <sup>a</sup>

by the interaction between leaf age × season and between the three variables (treatment × leaf age × season) (Table 1).

The foliar level of K<sup>+</sup> did not vary significantly due to the treatment; however, in September, a moderate increment of K<sup>+</sup> was observed in flooded leaves. K<sup>+</sup> was always higher in young leaves than in mature leaves (Fig. 1b). A significant interaction between leaf age and season was observed to have an effect on K<sup>+</sup> accumulation, whereas the treatment did not interact with age or season effects (Table 1).

#### Pigment analysis

In December, the total chlorophyll content (chlorophyll *a* + *b*, Fig. 2a) and the carotenoid content (Fig. 2b) were similar for all treatments and leaf ages. The pigment values of mature control leaves were slightly but not significantly higher than those of flooded trees. No anthocyanins accumulated in the young or mature leaves of flooded or control trees at this sampling time (Fig. 2c).

In April, chlorophylls and carotenoids were higher in the controls than in the flooded plants. The difference was especially significant in the case of mature leaves (Fig. 2a, b). A large accumulation of anthocyanins was observed in lemon leaves in April. Anthocyanin content was higher in control leaves than in flooded leaves of the same age, and higher in young leaves than in mature ones (Fig. 2c).

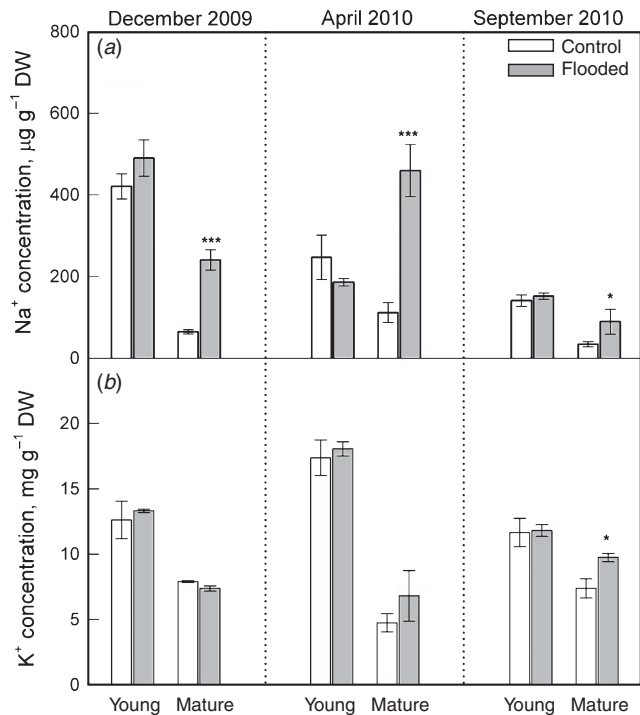
In September, mature leaves of flooded and control trees did not show any difference in pigment concentrations. In young leaves, the concentration of pigments was generally lower than in April and young control leaves still showed a higher pigment

concentration than young flooded leaves (Fig. 2). Factorial ANOVA analysis showed that chlorophyll and anthocyanin contents were significantly influenced by treatment, leaf age and season, whereas carotenoids were unaffected by flooding in both young and mature leaves during the different seasons (Table 1).

#### Photosynthesis and photosynthetic limitations

Photosynthesis (*A*) of both young and mature leaves was low in December, and was significantly lower in flooded plants than in control plants (Fig. 3a). In April, the *A* of young leaves decreased further, and was near to compensation point in flooded plants. Photosynthesis of mature leaves, on the other hand, increased significantly in April compared with December, and was higher in control plants than in flooded trees. In September, *A* was again higher in mature leaves than in young leaves, but no differences were observed between control and flooded plants. Photosynthesis was significantly influenced by treatment, leaf age and season. An interactive effect of leaf age × season on *A* was observed. Differences in *A* between control and flooded trees were also strongly dependent on the season (Table 1).

Although *g<sub>s</sub>* was not significantly affected by flooding and leaf age (Table 1), it followed a similar trend to *A*, being far lower in December than in the other two sampling dates in both leaf types (Fig. 3b). In April, *g<sub>s</sub>* was higher in control than in flooded plants, but this difference was only significant in mature leaves. In September, however, the trend was reversed and the *g<sub>s</sub>* of young and mature leaves of the flooded treatment were higher



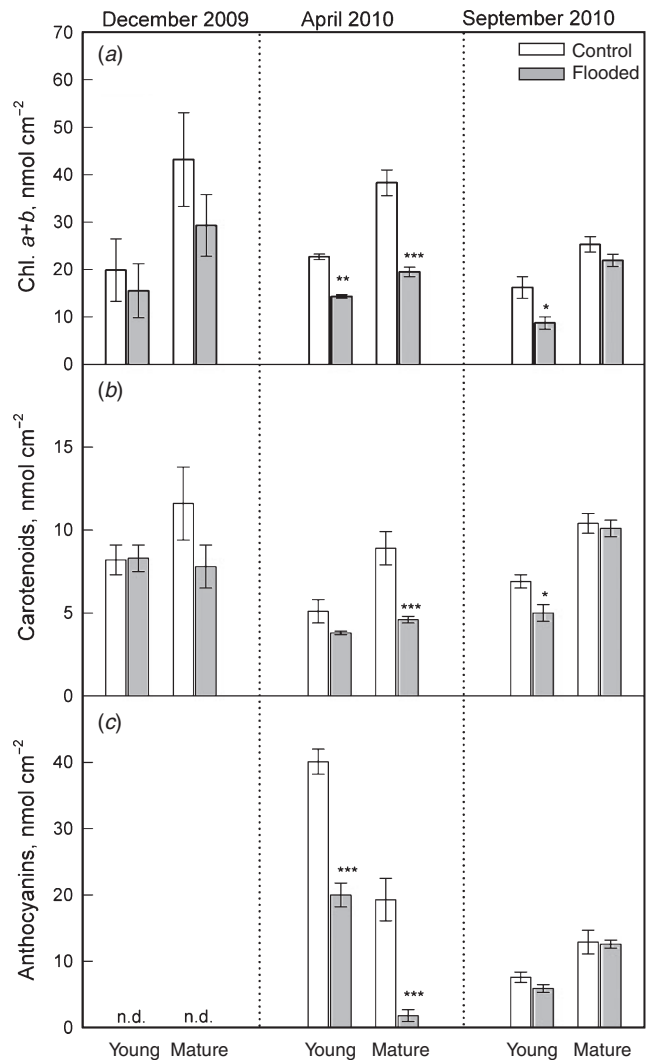
**Fig. 1.** Changes in (a) Na<sup>+</sup> and (b) K<sup>+</sup> concentration in young and mature leaves of lemon trees during flooding with saline water (December), at the end of winter (April) and after the irrigation period in summer (September). Bars represent control (white) and flooded plants (grey). Data are means  $\pm$  s.e. of 20 leaves from different plants in each group. Asterisks indicate significant effects at  $P < 0.05$  (\*) and  $P < 0.001$  (\*\*\*) as assessed statistically by *t*-test on control and flooded sample pairs.

than that of control plants. All variables interacted significantly among them with respect to  $g_s$ .

$C_i$  was significantly higher in flooded than in control leaves in December, whereas in April and September, this difference was less evident or absent (Fig. 3c).  $C_i$  was, however, significantly affected by all variables and by their interactions, with the exception of the treatment  $\times$  leaf age interaction (Table 1).

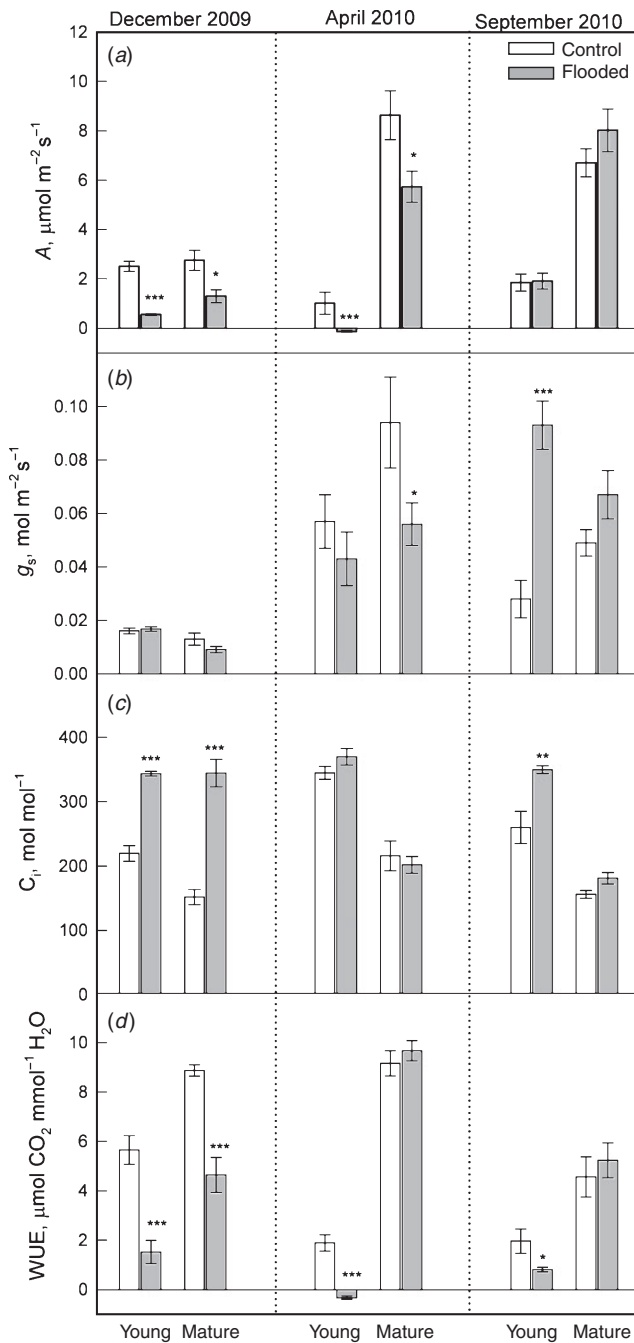
The ratio between photosynthesis and transpiration is a measure of the instantaneous WUE of the leaves. WUE was significantly higher in the controls than in the flooded plants in December (Fig. 3d). A similar trend was observed in the WUE of young leaves in April and September, whereas the WUE of mature leaves was similar in control and flooded plants on these two sampling dates. Factorial analysis revealed significant interactions between the three variables in our experimental conditions (Table 1).

The relationship between  $A$  and  $C_i$  was investigated in April and September (i.e. when photosynthetic rates attained levels that are high enough to make this measurement meaningful in order to assess the biochemical limitations of photosynthesis). In April, the  $A$  of young leaves did not respond to increasing intercellular CO<sub>2</sub> concentration (Fig. 4a). In mature leaves,  $A$  was largely stimulated by increasing CO<sub>2</sub> and the  $A$  of control leaves was higher than in flooded leaves even at lower than ambient CO<sub>2</sub> levels. In September,  $A$  stimulation was again higher in



**Fig. 2.** Changes in (a) total chlorophyll (Chl *a* + *b*), (b) carotenoids and (c) anthocyanins in young and mature leaves of lemon trees during flooding with saline water (December), at the end of winter (April) and after the irrigation period in summer (September). Bars represent control (white) and flooded plants (grey). Data are means  $\pm$  s.e. of five plants from each group. Asterisks indicate significant effects at  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*) as assessed statistically by *t*-test on control and flooded sample pairs.

mature leaves than in young leaves (Fig. 4b), but control and flooded plants did not show significantly different responses to CO<sub>2</sub> (Table 2).  $A/C_i$  responses also showed a considerably higher compensation point (the point at which CO<sub>2</sub> uptake by photosynthesis is compensated by CO<sub>2</sub> release due to photorespiration and mitochondrial respiration) in young leaves of the treated samples compared to mature leaves at both sampling dates. Based on the best-fit curves calculated by the model, the compensation points of young leaves ranged between 301–424  $\mu\text{mol mol}^{-1}$  (April) and 173–178 (September)  $\mu\text{mol mol}^{-1}$ , being higher in flooded plants. In mature leaves, the compensation points were between 69  $\mu\text{mol mol}^{-1}$  and 83  $\mu\text{mol mol}^{-1}$  at the same sampling dates.



**Fig. 3.** Changes in (a) photosynthesis ( $A$ ), (b) stomatal conductance ( $g_s$ ), (c) intracellular  $\text{CO}_2$  concentration ( $C_i$ ) and (d) water use efficiency (WUE) in young and mature leaves of lemon trees during flooding with saline water (December), at the end of winter (April) and after the irrigation period in summer (September). Bars represent control (white) and flooded plants (grey). Data are means  $\pm$  s.e. of five plants from each group. Asterisks indicate significant effects at  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*) as assessed statistically by  $t$ -test on control and flooded sample pairs.

The strong effect of leaf age on the compensation point might be due to higher rates of respiratory activity in young leaves. Indeed, the rates of respiration in the dark (due to mitochondrial respiration only) were found to be significantly higher in young

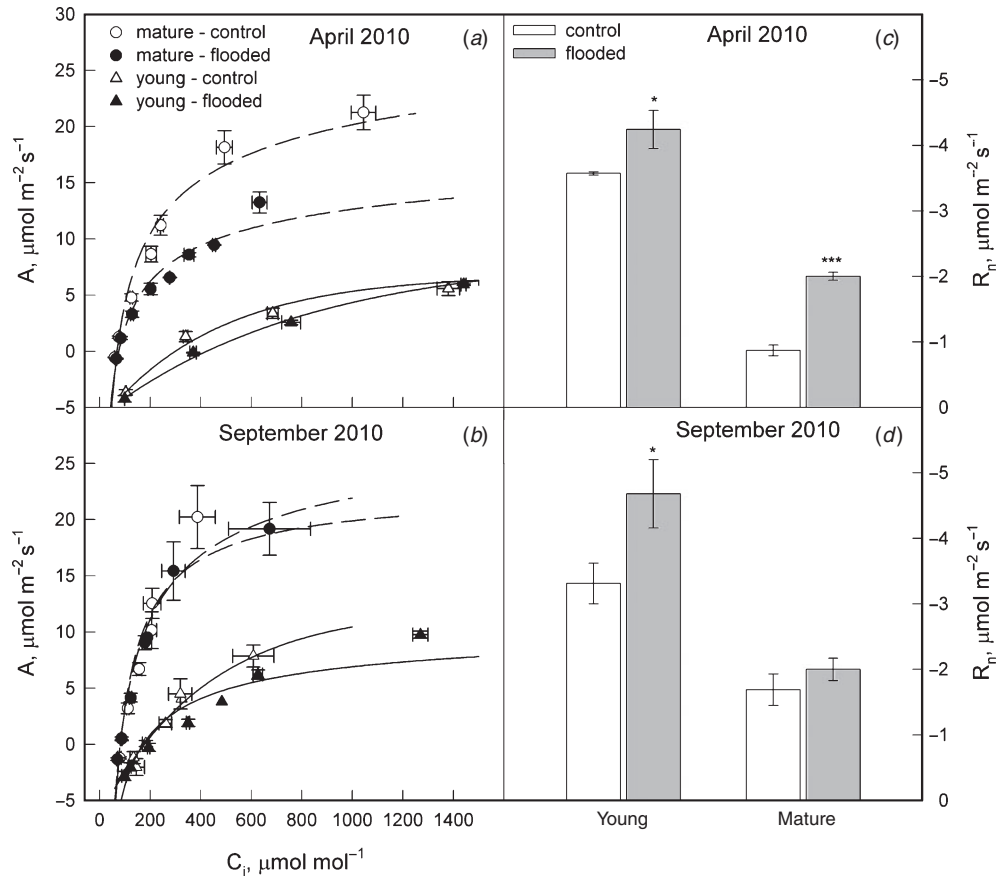
leaves than in mature leaves both in April and September (Fig. 4c, d). Moreover, respiration was significantly increased in response to flooding in both young and mature leaves in April (Fig. 4c), and the effect was still visible, in young leaves only, in September (Fig. 4d). Biochemical modelling of  $A/C_i$  responses suggested that maximum Rubisco carboxylation activity ( $V_{\text{cmax}}$ ) was not affected by flooding in both types of leaves and in both sampling periods (Table 2). On the other hand, mature leaves were characterised by a significantly higher  $V_{\text{cmax}}$  than young leaves in both flooded and control samples. Model estimates of ribulose-1,5-bisphosphate regeneration capacity (which can be estimated by maximum rate of photosynthetic electron transport ( $J_{\text{max}}$ ); see, for example, Wullschleger 1993 and Wohlfahrt *et al.* 1999) were also significantly lower in young leaves.

The efficiency of the photochemistry of photosynthesis was analysed by chlorophyll fluorescence. Flooding significantly reduced the photochemical efficiency of PSII ( $\Phi_{\text{PSII}}$ ) in mature leaves in December (Fig. 5a). However, flooding did not affect  $\Phi_{\text{PSII}}$  in all other sampling periods, irrespective of leaf age. In all sampling dates,  $\Phi_{\text{PSII}}$  was clearly lower in young leaves compared with mature leaves (Fig. 5a and Table 1). The NPQ, an indicator of the capacity to dissipate energy nonradiatively, was similar between treatments and leaf ages in December and September (Fig. 5b). However, in April, a much higher NPQ was observed in control leaves than in the leaves exposed to flooding, which made the treatment  $\times$  season interaction significant regarding this parameter (Table 1).

The maximum quantum yield of PSII photochemistry ( $F_v/F_m$ ) occurs in darkened leaves. This fluorescence parameter is an indicator of permanent photochemical damage, mainly related to photoinhibition (Maxwell and Johnson 2000).  $F_v/F_m$  was lower in young leaves than in mature leaves in December (Fig. 5c). On this sampling date, the efficiency of PSII was also slightly but significantly inhibited in mature flooded leaves compared with mature controls. In April, the  $F_v/F_m$  of control leaves was significantly higher than in flooded leaves of the respective age classes. In September,  $F_v/F_m$  was uniformly high in mature flooded and control leaves, whereas young leaves of flooded plants still presented lower maximal photochemical efficiency than the control trees.

#### Fluxes in leaf and fruit volatiles, and content of isoprenoids from stored pools

In December, flooding caused a large increase in the emission of volatile isoprenoids from leaves. The most abundant isoprenoids (those with emissions above  $3 \text{ nmol m}^{-2} \text{ s}^{-1}$ ) are shown in Fig. 6, whereas the other volatile organic compounds are shown in Table 3. The most abundant isoprenoids emitted were four monoterpenes (limonene,  $\beta$ -phellandrene,  $\alpha$ -pinene and linalool). Among the other isoprenoids, the emission of eight more volatiles (monoterpenes:  $\beta$ -pinene,  $\beta$ -myrcene, terpinene,  $\alpha$ -phellandrene, *cis*- $\beta$ -ocimene, 3-carene and citral, and a sesquiterpene:  $\beta$ -caryophyllene) was observed in response to flooding. For all these compounds, the flood-induced stimulation was higher in mature leaves than in young leaves (Table 3).



**Fig. 4.** Photosynthesis response to intercellular  $\text{CO}_2$  concentration ( $A/C_i$  response curves) in young and mature leaves measured (a) at the end of winter (April) and (b) after the irrigation period in summer (September). The curve fitting of  $A/C_i$  responses is also shown. In the other panels, changes in the dark respiration ( $R_n$ ) of young and mature leaves measured in (c) April and (d) September are reported. Bars represent control (white) and flooded plants (grey). Data are means  $\pm$  s.e. of five plants from each group. Asterisks indicate significant effects at  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*) as assessed statistically by  $t$ -test on control and flooded sample pairs.

Measurements in April showed a dramatic reduction in all emitted volatile isoprenoids, many of which were below the detection limit, across all treatments and leaf types (Fig. 6, Table 3). However, a large peak of another volatile organic compound, acetone, was observed (Table 3). The acetone peak was significantly higher in young flooded leaves than in young control leaves.

In September, the emission of some volatile isoprenoids was observed again, but the pattern was not as clear as that observed in December. The emission of monoterpenes was generally higher in young than in mature leaves, and sesquiterpenes were not emitted in detectable amounts. The emission of acetone was observed, but only in flooded plants, and to a similar extent in young and mature leaves (Fig. 6, Table 3).

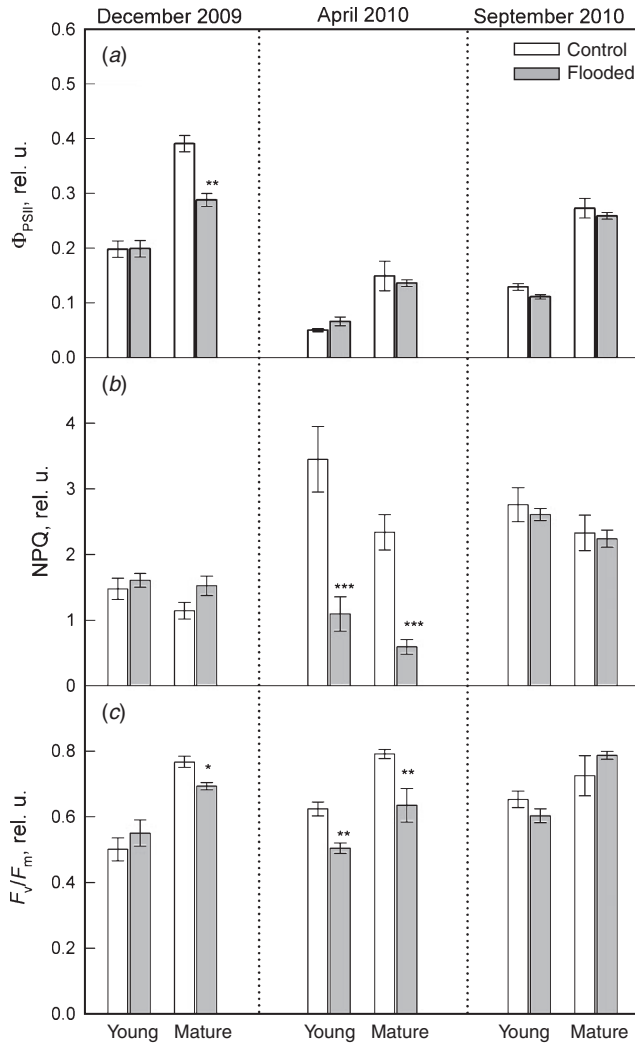
Isoprenoid emission was also measured in young and mature fruits in December. A significant increase of limonene, the main compound in the fruit blend of volatile isoprenoids, was found in young fruits of flooded trees as compared with young fruits of the controls (Fig. 7a). However, the limonene emission by mature fruits was similarly high in control and flooded trees. The other isoprenoids that are minor

components of the blend of fruit emission responded differently to the treatment.  $\alpha$ -Pinene and  $\beta$ -myrcene were only emitted by mature fruits and this pattern was not altered by the flooding (Fig. 7b, d). *Trans*- $\beta$ -ocimene was only emitted in control fruits and, to a larger extent, in mature fruits (Fig. 7e). In young and mature fruits of the flooded plants, the emission of *trans*- $\beta$ -ocimene was suppressed to below the detection limit. On the other hand, a stimulation of  $\beta$ -pinene and terpinene emission was observed in young flooded fruits compared with controls (Fig. 7c, f).

The percent composition of isoprenoids (i.e. the liquid pools of volatile and semivolatile isoprenoids) in the leaves and fruits of lemon trees was also assessed during the December sampling period (Fig. 8). In our analysis, we also discriminated between the enantiomeric forms of several different monoterpenes. This is important because the two enantiomers of chiral monoterpenes might exhibit completely different biological effects (Gershenzon and Dudareva 2007) and can influence the lemon fruit quality (aroma) (Serra 2011).

In both young and mature leaves of the control and flooded plants, (+)-limonene and the oxygenated monoterpenes geranial

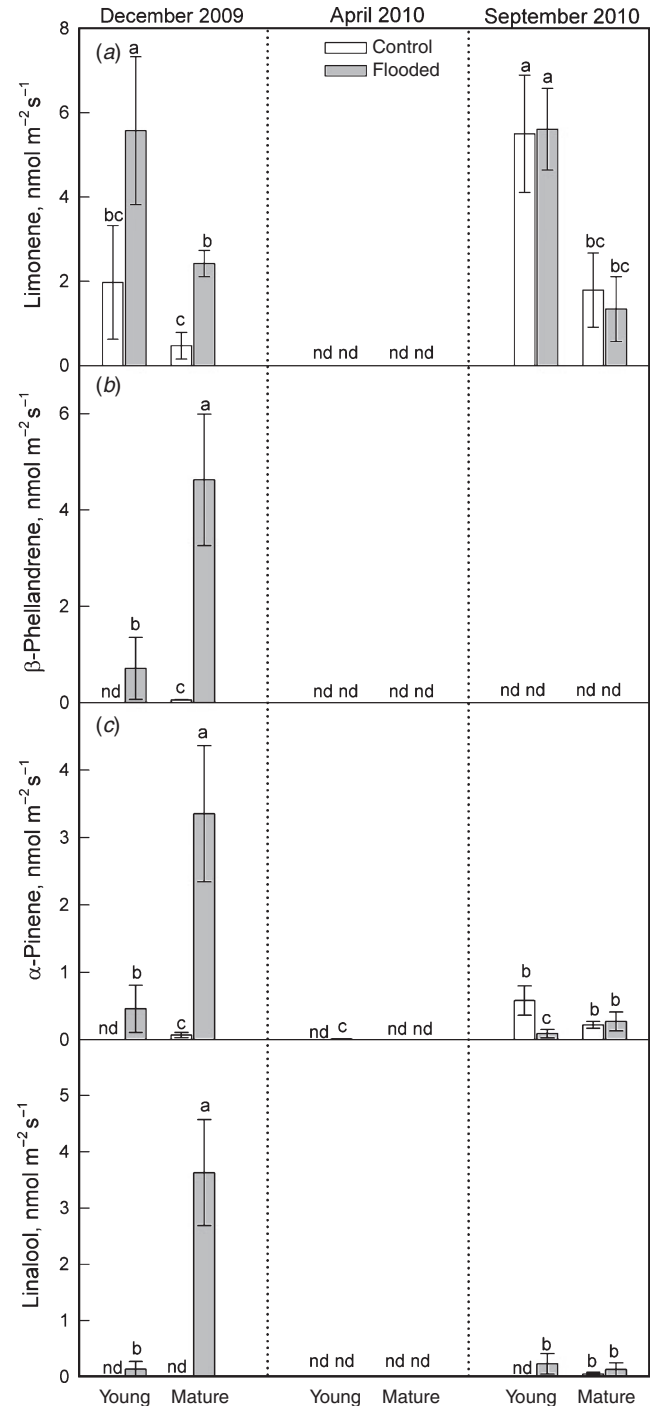




**Fig. 5.** Changes in (a) quantum efficiency of PSII in light adapted leaves ( $\Phi_{PSII}$ ), (b) nonphotochemical quenching (NPQ) and (c) maximum quantum yield of PSII in dark adapted leaves ( $F_v/F_m$ ) in young and mature leaves of lemon trees during flooding with saline water (December), at the end of winter (April) and after the irrigation period in summer (September). Bars represent control (white) and flooded plants (grey). Data are means  $\pm$  s.e. of five plants from each group. Asterisks indicate significant effects at  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*) as assessed statistically by *t*-test on control and flooded sample pairs.

(citral A) and neral (citral B) were the most represented isoprenoids. Differences between young and mature leaves, and between flooded and control plants were only found in a few minor compounds of the foliar isoprenoid blend (Fig. 8). In particular, the percent compositions of 3-carene, (-)- $\alpha$ -phellandrene and terpinolene were significantly reduced in mature leaves of flooded plants compared to mature leaves of control plants.

A few significant changes were observed in the relative content of major compounds of the fruit isoprenoids, which could be related to the flooding (Fig. 9). In particular, the percent content of (+)-limonene, which represents 70–80% of the total isoprenoids in lemon fruits, was reduced significantly in



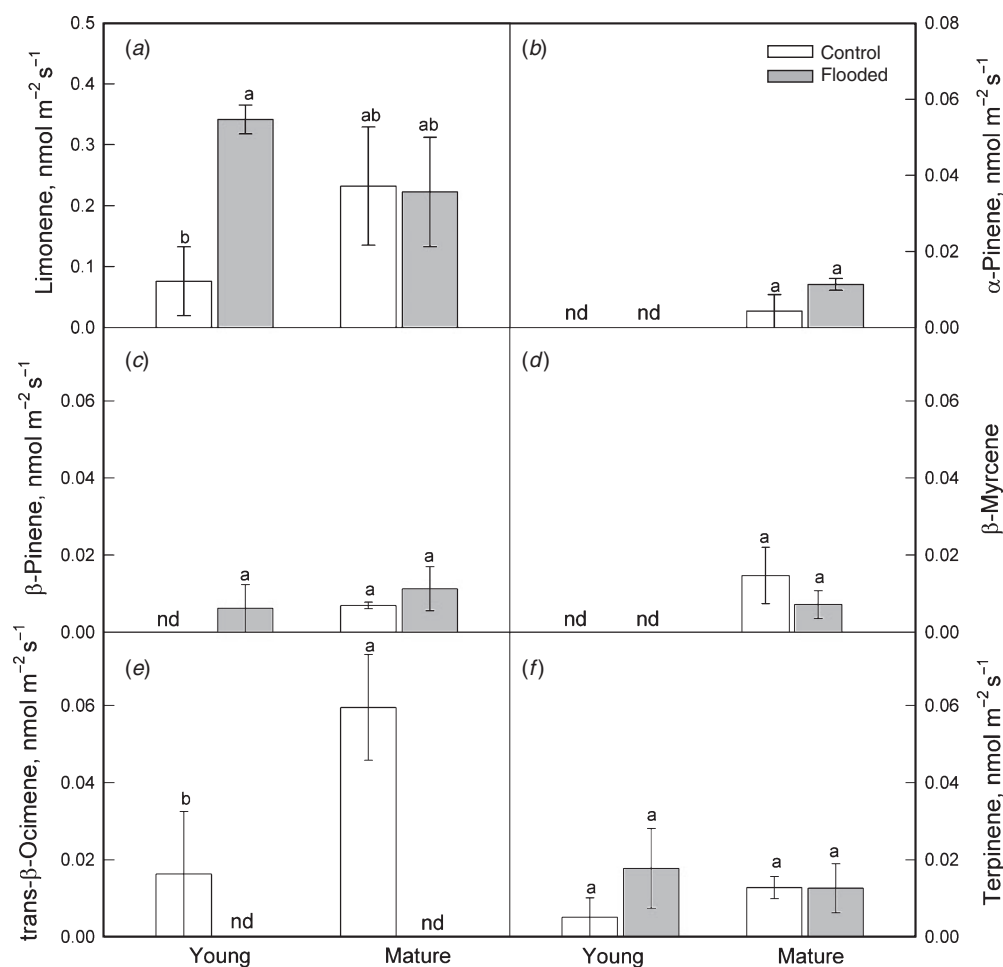
**Fig. 6.** Changes in the most abundant volatile isoprenoids released from young and mature leaves of lemon trees during flooding with saline water (December), at the end of winter (April) and after the irrigation period in summer (September). (a) limonene; (b)  $\beta$ -phellandrene; (c)  $\alpha$ -pinene; (d) linalool. Bars represent control (white) and flooded plants (grey). Data are means  $\pm$  s.e. of five plants from each group. Different letters indicate significant effects at  $P < 0.05$ .

flooded fruits in comparison to the controls. On the other hand, the percent contents of geranial and neral were increased significantly by the flooding treatment.

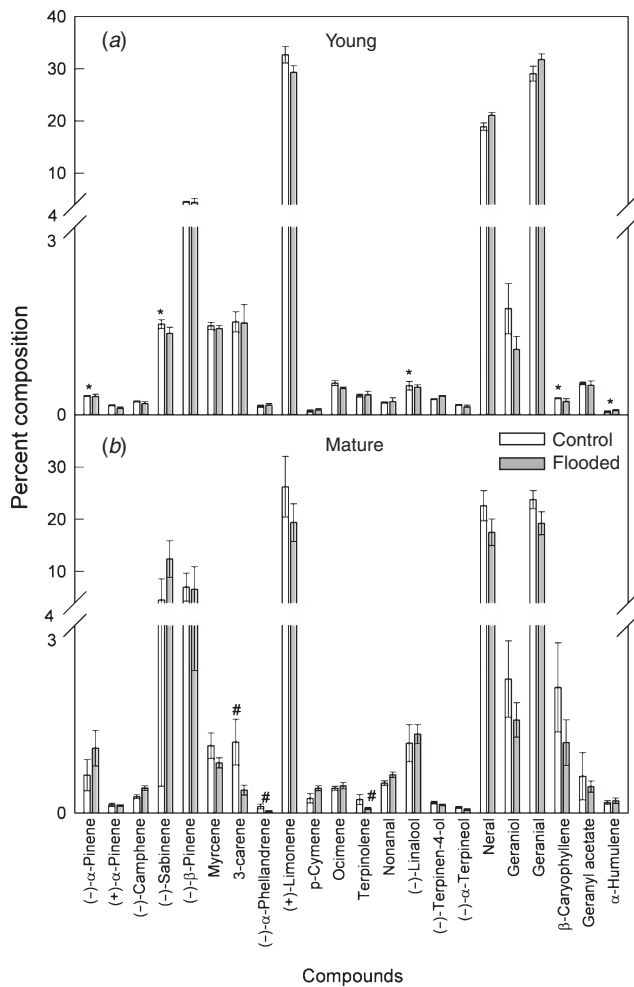
**Table 3. Changes in the volatile organic compounds emitted ( $\text{nmol m}^{-2} \text{s}^{-1}$ ) from young and mature leaves of lemon trees during flooding with saline water (December), at the end of winter (April) and after the irrigation period in summer (September)**

Data are means  $\pm$  s.e. of five plants from each group. Undetectable emissions are labelled as n.d. Different letters indicate statistically significant differences between means of controls and flooded plants ( $P < 0.05$ ).

	December 2009				April 2010				September 2010			
	Young leaves		Mature leaves		Young leaves		Mature leaves		Young leaves		Mature leaves	
	Control	Flooded	Control	Flooded	Control	Flooded	Control	Flooded	Control	Flooded	Control	Flooded
$\beta$ -Pinene	0.1 $\pm$ 0.0 <sup>c</sup>	0.5 $\pm$ 0.2 <sup>b</sup>	0.1 $\pm$ 0.0 <sup>c</sup>	1.3 $\pm$ 0.3 <sup>a</sup>	0.1 $\pm$ 0.0 <sup>c</sup>	0.1 $\pm$ 0.0 <sup>c</sup>	0.1 $\pm$ 0.0 <sup>c</sup>	n.d.	0.4 $\pm$ 0.2 <sup>b</sup>	1.0 $\pm$ 0.4 <sup>a</sup>	0.4 $\pm$ 0.3 <sup>b</sup>	0.2 $\pm$ 0.0 <sup>b</sup>
$\beta$ -Myrcene	n.d.	0.4 $\pm$ 0.1 <sup>a</sup>	n.d.	0.5 $\pm$ 0.0 <sup>a</sup>	n.d.	n.d.	n.d.	n.d.	0.8 $\pm$ 0.3 <sup>a</sup>	0.2 $\pm$ 0.1 <sup>b</sup>	0.5 $\pm$ 0.1 <sup>ab</sup>	0.3 $\pm$ 0.2 <sup>ab</sup>
Terpinene	n.d.	0.1 $\pm$ 0.1 <sup>b</sup>	n.d.	0.5 $\pm$ 0.1 <sup>a</sup>	n.d.	n.d.	n.d.	n.d.	0.3 $\pm$ 0.1 <sup>ab</sup>	0.4 $\pm$ 0.1 <sup>a</sup>	0.2 $\pm$ 0.1 <sup>b</sup>	n.d.
$\alpha$ -Phellandrene	n.d.	0.4 $\pm$ 0.4 <sup>a</sup>	n.d.	0.2 $\pm$ 0.0 <sup>a</sup>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<i>cis</i> - $\beta$ -Ocimene	n.d.	0.4 $\pm$ 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-Carene	n.d.	0.3 $\pm$ 0.0 <sup>a</sup>	n.d.	0.1 $\pm$ 0.0 <sup>b</sup>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caryophyllene	n.d.	n.d.	n.d.	0.1 $\pm$ 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Citral	0.1 $\pm$ 0.0 <sup>b</sup>	0.4 $\pm$ 0.0 <sup>a</sup>	n.d.	0.1 $\pm$ 0.0 <sup>b</sup>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acetone	n.d.	n.d.	n.d.	n.d.	11.3 $\pm$ 4.2 <sup>b</sup>	32.1 $\pm$ 2.4 <sup>a</sup>	1.1 $\pm$ 0.0 <sup>c</sup>	1.2 $\pm$ 0.3 <sup>c</sup>	n.d.	4.4 $\pm$ 4.4 <sup>c</sup>	n.d.	8.5 $\pm$ 4.0 <sup>bc</sup>



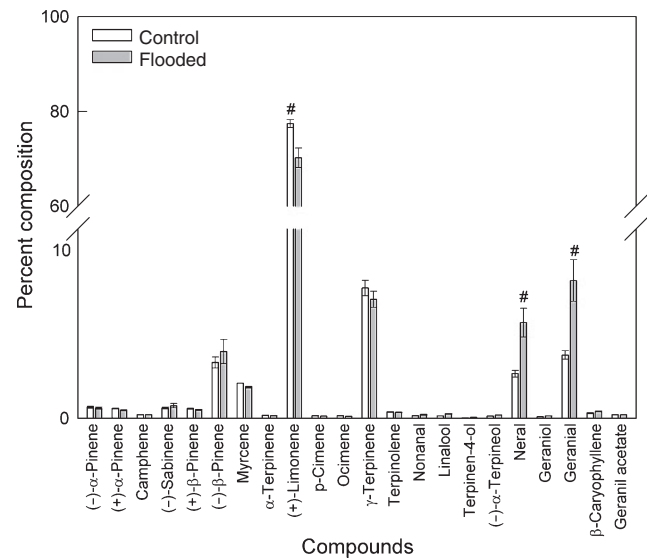
**Fig. 7.** Volatile isoprenoid emission from whole young and mature lemon fruits measured during flooding with saline water (December). (a) limonene; (b)  $\alpha$ -pinene; (c)  $\beta$ -pinene; (d)  $\beta$ -myrcene; (e) *trans*- $\beta$ -ocimene; (f) terpinene. Bars represent control plants (white), and flooded plants (grey). Data are means  $\pm$  s.e. of five plants from each group. Different letters indicate significant effects at  $P < 0.05$ .



**Fig. 8.** Isoprenoid pool composition (%) of (a) young and (b) mature lemon leaves of control (white bars) and flooded plants (grey bars) measured during flooding with saline water (December). Data are means  $\pm$  s.e. of five plants from each group. Data were analysed by the nonparametric Mann–Whitney test. \* indicates significant differences between young mature leaves of control; # indicates significant differences between control and flooded plants.

## Discussion

The combination of flooding and saline water is a rare event in nontropical areas such as the Mediterranean basin. In the lagoon of Venticari, the management of the canal system that traditionally allowed excess water to drain from the ponds to the sea in winter, and to dry out the ponds in summer, has been abandoned in recent years. This causes water to overflow above cultivated areas that surround the ponds during the heavy rainfall season, generally concentrated at the end of autumn and the beginning of winter. This management provides a case study for investigating the impacts and responses of a *Citrus* orchard to such a combined and repeated event. Our study only focusses on physiological traits, and specifically addresses the impacts of flooding on primary metabolism that may in turn affect growth and productivity, and on secondary metabolites that constitute the volatile fractions of leaves and fruits, as these are important



**Fig. 9.** Isoprenoids pool composition (%) of fruit peel of lemons from control (white bars) and flooded plants (grey bars). Fruits were collected during flooding with saline water (December). Data are means  $\pm$  s.e. from five plants from each group. Data were analysed by the nonparametric Mann–Whitney test; # indicates significant differences between control and flooded plants.

features for communication with pollinators and pests, and for the quality of lemon fruits.

## Salt accumulation

An interesting initial observation was that  $\text{Na}^+$  accumulation varied among leaves of different ages over the season. Overall, we expected a much higher foliar  $\text{Na}^+$  accumulation due to the fact that flood water was heavily salinised, and because salt uptake is thought to be linearly related to salt accumulation in the soil (Nieves *et al.* 1992). Sour orange is defined as a rootstock that is susceptible to salinity (Levy and Syvertsen 2004) but is also known to efficiently restrict  $\text{Na}^+$  uptake and transport (Maas 1993). This might have limited salt uptake in our experimental system. Salt accumulation is also known to influence the uptake of other cations (Syvertsen and Yelenosky 1988). However, no effect was found in the accumulation of  $\text{K}^+$  between flooded and control plants, again suggesting that salt accumulation was not so large as to affect ion uptake significantly. Perhaps the most striking observation was that  $\text{Na}^+$  accumulation mainly occurred in the young leaves in December (i.e. immediately after flooding). Sodium and  $\text{Cl}^-$  accumulate preferentially in the older leaves of *Citrus* plants (Syvertsen *et al.* 1988). Unfortunately, we did not measure  $\text{Cl}^-$  accumulation and are unable to say whether this also accumulates in young leaves due to the combination of flooding and salinity. In December, we also observed a very low stomatal conductance ( $g_s$ ), mirroring a low transpiration rate that could have contributed to the reduced salt uptake. Since  $g_s$  was particularly low in mature leaves, the xylematic transport of salt in winter was possibly redirected towards young leaves. Indeed, in April, under higher  $g_s$  and transpiration,  $\text{Na}^+$  accumulation was higher in mature leaves than in young flooded leaves, confirming previous results in

*Citrus* (Syvertsen *et al.* 1988). Finally, the measurements carried out in September revealed that summer irrigation with nonsaline water was sufficient to leach out salinity, as the  $\text{Na}^+$  concentration was similarly low in all samples.

#### Primary metabolism

Flooding with saline water reduced photosynthesis in both young and mature leaves immediately after plants were flooded and until the beginning of spring, when mature leaves had high photosynthetic rates. The inhibition of photosynthesis was never associated with a reduction in  $C_i$ . In fact,  $C_i$  increased significantly in December in flooded leaves, indicating the onset of nonstomatal (i.e. nondiffusive) limitations of photosynthesis. Our results therefore confirm earlier reports that flooding has a very rapid negative effect on photosynthesis (Kozłowski 1997; Arbona *et al.* 2009; Hossain *et al.* 2009) and that biochemical or photochemical factors enhance photosynthesis limitation in flooded (Schaffer *et al.* 1992; Arbona *et al.* 2009; Hossain *et al.* 2009) or salt-stressed (Kozłowski 1997) plants. Photosynthesis inhibition was not observed in September in flooded plants. Clearly, summer irrigation, which reduced salinity to control levels, is sufficient to restore full functionality of the photosynthetic apparatus.

Analysing  $A/C_i$  responses provides insights into biochemical limitations (Sharkey *et al.* 2007) and chlorophyll fluorescence reveals photochemical limitations (Baker *et al.* 2007) to photosynthesis. From  $A/C_i$  responses, it can be inferred (Farquhar and Sharkey 1982; Sharkey *et al.* 2007) that the substantial difference between mature and young leaves during the measurement periods, and in particular in April and September when considerably higher photosynthetic rates can be reached, was caused by the following features of young leaves: (a) a much lower Rubisco activity, as indicated by the lower slope of the  $A/C_i$  response at low  $\text{CO}_2$  concentrations; (b) a lower capacity to regenerate RuBP, in turn due to inefficient photochemistry (see also below); and (c) a significantly higher mitochondrial respiration that increased the compensation point and was also clearly seen when measuring the rates of dark respiration. In April, a comparison between flooded and control trees indicated the occurrence of similarly heavy photosynthetic limitations in young leaves. However, in mature flooded leaves, the RuBP limitation due to inefficient electron transport regeneration in photosynthetic substrates was larger than in mature control leaves, as shown by the lower plateau of the flat part of the  $A/C_i$  response. In September, no significant differences were found in the  $A/C_i$  responses between flooded and control plants, confirming that the effect of flooding was completely alleviated, with no residual photosynthetic limitation at the Rubisco and RuBP level.

It was interesting to observe that flooding significantly increased the rate of mitochondrial respiration in young and mature leaves in April, and still affected the respiration of young leaves in a similar way in September. Perhaps increasing mitochondrial respiration indicates a general increase in catabolic processes under flooding, possibly related to higher enzyme activities in the glycolytic pathway (Kennedy *et al.* 1992) caused by higher demand of carbohydrates for fermentation processes at the root level (Loreti *et al.* 2005).

Young leaves also showed a generally lower maximal photochemical capacity (as indicated by the fluorescence parameter  $F_v/F_m$ ; Maxwell and Johnson 2000) compared to mature leaves at all seasonal stages. This explains the lower capacity of RuBP regeneration indicated by the  $A/C_i$  responses. Flooding significantly affected the maximal photochemical capacity but this was more visible in mature leaves (showing higher photochemical capacity than younger leaves) both immediately after flooding (December) and overwintering (April). Overall, our findings indicate that the young *Citrus* leaves may have experienced photoinhibition and that this was exacerbated by flooding in overwintering leaves (Arbona *et al.* 2009). Photochemical limitations to photosynthesis were completely alleviated in September, again indicating a total resilience to the stressful conditions due to flooding. When photons cannot be used to generate photosynthetic electron transport, the light energy is generally dissipated as heat (Müller *et al.* 2001). Surprisingly, however, the NPQ, which estimates heat-dissipated light energy, did not increase when the photochemistry of photosynthesis was downregulated by flooding. In fact, in April, the NPQ was highest in young and mature control leaves, in which photosynthesis and maximal photochemical efficiency were also higher than in flooded plants. This may indicate that flooding reduced the number of photosynthetic reaction centres or the ability to dissipate excess light. Such a reduction of photochemical capacity is generally observed in plants that also show a strong reduction of photosynthetic pigments in response to abiotic stress. Salinity (Syvertsen *et al.* 1988) and flooding (Arbona *et al.* 2008) often induce leaf chlorophyll degradation. We observed such an effect on the chlorophyll and carotenoid content of leaves of flooded plants that overwintered. The effect was still visible in September, but only in young leaves that had lowest photochemical efficiency. Pigment analysis also revealed that the anthocyanin formation typical of overwintering leaves that experience cold stress (Hughes and Smith 2007) was significantly attenuated in flooded plants in April. Since anthocyanins may also protect the photochemical apparatus by absorbing light, reacting as antioxidants or increasing leaf temperature (Hatier and Gould 2009), increasing photodamage in flooded and overwintered *Citrus* leaves may also be attributed to inhibition of biosynthesis of important pigments, especially in young leaves in which the photosynthetic capacity is still constitutively low.

#### Volatile isoprenoid emission, and foliar and fruit isoprenoid pools

The impact of flooding and salinity on secondary metabolites is important to assess in *Citrus* plants, as these components of foliar and flower scent and fruit flavour drive communication between plants and herbivores or pollinators (Dicke and Baldwin 2010), and also strongly influence fruit quality and have important roles in medicine (Fisher and Phillips 2008). Foliar emission of volatile isoprenoids showed a clear seasonal pattern and was generally enhanced immediately after flooding in young and mature leaves. The blend of emitted volatiles also changed in flooded plants, as some volatiles (e.g.  $\beta$ -myrcene, terpinene,  $\alpha$ -phellandrene,  $\beta$ -ocimene and 3-carene), which were



not detected in control leaves, were emitted in high concentration from flooded plants in December. The overall stimulation of volatile isoprenoids was limited to December, and therefore we believe it was a transient effect of flooding. However, our findings suggest that, firstly, the methylerythritol-phosphate pathway of isoprenoid biosynthesis and emission was transiently stimulated by flooding. As the functions of volatile isoprenoids include an antioxidant role (Loreto and Schnitzler 2010), they possibly help plants cope better with general oxidative stress induced by flooding (Arbona *et al.* 2008). Secondly, isoprenoid emission is not controlled at the genetic level only, and may be altered by flooding and probably other environmental stresses. Thirdly, flood-induced changes in the volatile blend may temporarily alter communication with plant hosts; in the case of lemon trees, emission changes due to winter flooding may interact with mite infestations in particular (Choi *et al.* 2004) or fungi attacks (Yamasaki *et al.* 2007), or in communication with pollinators. None of these interactions were assessed in our experiment but our results may lay the ground work for further investigations in this field.

The emission of acetone was not detected in December, but this oxygenated compound was largely emitted in April, especially from young leaves, and the emission was further stimulated in flooded plants. The emission of acetone is seasonal in plants. A large acetone emission was reported by Fares *et al.* (2011) from orange (*Citrus × sinensis* (L.) Osbeck) and lemon (*Citrus × limon* (L.) Burm.f.) leaves. Acetone emission may indicate active growth (Fares *et al.* 2009) and consequent metabolism of fat reserves (Sharkey 1996). This explains the high emission rates from young lemon leaves observed in April. Acetone emission may also indicate fatty acid destruction during leaf senescence (de Gouw *et al.* 1999) or caused by environmental stresses (Cojocariu *et al.* 2005). An emission of acetaldehyde was also expected after flooding, as this volatile molecule is synthesised from ethanol formed in anoxic roots and then translocated to the canopy of plants (Loreto and Schnitzler 2010). However, acetaldehyde emission was not found in *Citrus* plants, and we speculate that the very low stomatal conductance restrained acetaldehyde emission and possibly also emission of other oxygenated volatiles (Loreto and Schnitzler 2010) immediately after flooding.

In December, the isoprenoid composition inside leaves showed only minor changes in flooded leaves in comparison to the controls. This confirms that flooding caused an overall increase in isoprenoids biosynthesis, as indicated by the general increase of volatile isoprenoid emissions. Salinity is known to affect fruit size and number (Nieves *et al.* 1990). As for the biochemical traits of *Citrus* fruits, only sugar content and total soluble solids appear to be affected by salt (Prior *et al.* 2007), but the effect of salt is controversial (Maas 1993). Levy and Syvertsen (2004) speculated that salinity stress might enhance the concentration of secondary metabolites in the fruit. The impact of flooding on fruit characteristics, to our knowledge, has not been well investigated (Kozłowski 1997). Our results support the idea of Levy and Syvertsen (2004) that flooding with saline water may enhance secondary metabolite production in *Citrus* fruits. However, there was no common pattern in the emission of volatile isoprenoids from lemon fruit. In the case

of limonene, the most characteristic and abundant volatile in lemon juice (Di Vaio *et al.* 2010), emissions significantly increased only in young fruits after flooding. However, of the blend of volatile isoprenoids that are emitted in low amounts, *trans*- $\beta$ -ocimene was totally suppressed after flooding. Changes in emissions may not reflect changes in the overall composition of the internal pool of isoprenoids in lemon fruits. In fact, the internal pool of isoprenoids was remarkably stable. Thus, in agreement with plant chemosystematic studies showing that constitutive terpenes from stored pools are under strong genetic control and are not affected greatly by abiotic stress (Langenheim 1994), the stored isoprenoid profile can be used as a biochemical marker to characterise different *Citrus* species and cultivars (Lota *et al.* 2002). However, as significant differences between flooded and control plants were observed when comparing some of the most abundant fruit monoterpenes, flooding may affect overall fruit quality and defence mechanisms. These possibilities should be assessed in future studies.

## Conclusions

It was shown that recurrent flooding with saline water may have a series of consequences on lemon physiology and fruit quality. From a physiological standpoint, the photosynthetic properties of lemon leaves were heavily reduced by the winter flooding stress, but the plants showed an unexpected resilience, eventually leading to recovery of photosynthetic performances by September of the next year, when salt concentration in the leaves returned to levels similar to those of the controls, probably due to irrigation with good quality water and consequent salt leaching. The biosynthesis and emission of foliar and fruit volatile compounds were also significantly affected by the combined stress (flooding and salinity), but few changes in the internal pools of isoprenoids occurred in flooded plants in comparison to the controls. Variations in the emission of volatile compounds and in the stored isoprenoid pools may lead to qualitative changes of production, and it is an important field for further studies.

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