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Ozone uptake by citrus trees exposed to a range of ozone concentrations

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ABSTRACT

The *Citrus* genus includes a large number of species and varieties widely cultivated in the Central Valley of California and in many other countries having similar Mediterranean climates. In the summer, orchards in California experience high levels of tropospheric ozone, formed by reactions of volatile organic compounds (VOC) with oxides of nitrogen (NO_x). Citrus trees may improve air quality in the orchard environment by taking up ozone through stomatal and non-stomatal mechanisms, but they may ultimately be detrimental to regional air quality by emitting biogenic VOC (BVOC) that oxidize to form ozone and secondary organic aerosol downwind of the site of emission. BVOC also play a key role in removing ozone through gas-phase chemical reactions in the intercellular spaces of the leaves and in ambient air outside the plants. Ozone is known to oxidize leaf tissues after entering stomata, resulting in decreased carbon assimilation and crop yield. To characterize ozone deposition and BVOC emissions for lemon (*Citrus limon*), mandarin (*Citrus reticulata*), and orange (*Citrus sinensis*), we designed branch enclosures that allowed direct measurement of fluxes under different physiological conditions in a controlled greenhouse environment. Average ozone uptake was up to 11 nmol s^{-1} m^{-2} of leaf. At low concentrations of ozone (40 ppb), measured ozone deposition was higher than expected ozone deposition modeled on the basis of stomatal aperture and ozone concentration. Our results were in better agreement with modeled values when we included non-stomatal ozone loss by reaction with gas-phase BVOC emitted from the citrus plants. At high ozone concentrations (160 ppb), the measured ozone deposition was lower than modeled, and we speculate that this indicates ozone accumulation in the leaf mesophyll.

1. Introduction

Ozone is formed through photochemical reactions of volatile organic compounds (VOC) with oxides of nitrogen (NO_x) in the presence of sunlight. Sources of VOC and NO_x can be biogenic (e.g. plants, soils) or anthropogenic (e.g. motor vehicles). Exposure to elevated ozone concentrations produces biochemical and physiological changes in plants (Darrall, 1989; Sandermann et al., 1997; Zheng et al., 2002). Inhibition of carbon assimilation by photosynthesis and decreased plant growth are common effects, often associated with visible injuries (Fares et al., 2006; Vollenweider and Gunthardt-Goerg, 2005; Feng and Kobayashi, 2009). This in turn decreases the benefits (environmental, economic, social) that natural and cultivated plant ecosystems can offer. Ozone is also a greenhouse gas and thus a positive forcing of climate change, which will likely cause further warming in the future owing to its increasing background concentrations in the low troposphere (Shindell et al., 2006; Cooper et al., 2010). Plants act as natural sinks for ozone (Kurpius and Goldstein, 2003; Fares et al., 2008) and have therefore been argued to phytoremediate the atmosphere (Taha, 1996; Nowak and Dwier, 2007).

The uptake of ozone by ecosystems is attributed to stomatal and non-stomatal sinks. At plant level, stomatal absorption is the major contributor to the total uptake of ozone (Loreto and Fares, 2007) and is considered to be the main uptake pathway responsible for plant injuries (UNECE, 2004). Inside the leaves ozone can be

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completely detoxified but can also induce membrane denaturation and the formation of free radicals, with a possible cascade of negative effects on plant physiology and biochemistry (Pell et al., 1997; Oksanen et al., 2004). Conductance to ozone is the inverse of the sum of resistances that ozone meets along the path from outside the leaf to the reaction site inside the apoplast (Fares et al., 2008). The dominant resistances at a leaf level are the boundary layer (the layer of air surrounding the leaf), the stomata, the mesophyll, and possibly the chloroplast. Stomatal conductance regulates the concentration gradient between the outside and inside the leaf. It is usually assumed that ozone reacts rapidly inside the intercellular spaces so its concentration is close to zero (Laisk et al., 1989). However, a non-linear relationship between ozone fluxes and stomatal conductance was shown for some herbaceous plants and tree species, which suggests that at high concentrations ozone may accumulate in the intercellular spaces and also reach the leaf mesophyll (Loreto and Fares, 2007). Environmental variables such as light, temperature and water availability in the plant–soil system affect stomatal conductance and, indirectly, the ozone uptake by leaves (Fares et al., 2008).

Non-stomatal ozone uptake processes include physical deposition to soil, stems, cuticles or any other external surface. Deposition on the cuticles can be limited under dry conditions (Cape et al., 2009), but on wet canopies this process may represent a major sink for ozone (Altimir et al., 2006). Non-stomatal ozone uptake processes also include chemical deposition resulting from gas-phase reactions between ozone and biogenic volatile organic compounds (BVOC) emitted from the ecosystem (e.g. plants or soils) (Kurpius and Goldstein, 2003). Previous work has reported significant non-stomatal ozone fluxes owing to reaction with BVOC (Goldstein et al., 2004; Bouvier-Brown et al., 2009). The emissions of many BVOC increase with light (Niinemets et al., 2004) and exponentially with temperature (Tingey et al., 1991; Monson et al., 1992). High emitters are typically deciduous plants, with higher emissions occurring during spring and summer (Holzinger et al., 2006).

Enclosures (=cuvettes) are useful tools to isolate specific plant elements, control environmental parameters and study their effects on the physiological properties of the plant (for reviews on the enclosure techniques see Tholl et al., 2006; Ortega and Helmig, 2008). Quantitative uptake and emission rates can then be scaled to the ecosystem level to quantify canopy fluxes and the effect on atmospheric concentrations (Helmig et al., 1999; Ortega et al., 2008; Vizuete et al., 2004; Wieser et al., 2008). Measurements of CO_2 and H_2O are relatively easy due to the low reactivity of these gases, while for many reactive BVOC and ozone special materials need to be used that minimize physical or chemical deposition on the walls of the enclosure during measurements. In this study, we designed dynamic Teflon enclosures to expose whole branches to three different levels of ozone concentrations while measuring fluxes of ozone, CO_2 and water.

Citrus species, in particular orange (*Citrus sinensis*) and mandarin (*Citrus reticulata*), are among the most cultivated tree crops in the Central Valley of California; they account for 82,600 ha. Citrus is also widely cultivated in other countries with Mediterranean climates, such as Italy, Spain, Morocco, and Israel, and cultivations are often close to densely populated areas. The warm climates, along with high insolation required for citrus cultivation is often associated to the formation of high ozone levels. Past studies have identified orange as a moderate BVOC emitter (Winer et al., 1992; Ciccioli et al., 1999; Hansen and Seufert, 2003). Significant emissions of ozone precursors and the topography in the agriculturally rich valleys of California routinely lead to high concentrations of ozone (Howard et al., 2010). Ozone concentrations in California's Central Valley often exceed 100 ppb on hot afternoons (California Air Resources Board) which is well above the 40 ppb phytotoxic threshold for vegetation (UNECE, 2004). The objective of this research was to characterize the capacity of three citrus species to remove ozone from the atmosphere and explore the underlying biological mechanisms. Since BVOC react with ozone in the intercellular spaces and outside the leaf (Kurpius and Goldstein, 2003; Loreto and Fares, 2007; Bouvier-Brown et al., 2009), we quantified BVOC emissions from the plants, determined the total ozone flux to the plants at varying ozone concentrations, and separated the flux into stomatal and non-stomatal ozone deposition, the latter mainly attributable to reaction of ozone with BVOC.

2. Material and methods

2.1. Experimental system

Experiments were carried out from June to September in the Oxford greenhouse facility of the University of California, Berkeley. Three citrus species were used: lemon (*Citrus limon* 'Improved Meyer'), mandarin (*C. reticulata* 'W. Murcott' on C-35 rootstock), and orange (*C. sinensis* 'Parent Navel' grafted on Volk rootstock). For each species, sets of 10 individual plants of the same genotype were obtained from a commercial nursery (Willits and Newcomb) and placed in the greenhouse in February to allow adaptation to the greenhouse conditions. All trees were 2–3 years old, planted in individual 19 L pots, irrigated daily, and fertilized weekly to promote favorable growing conditions. Temperatures in the greenhouse were controlled to simulate typical diurnal patterns, with night values around 17 °C and mid-day values up to 28 °C. Light conditions were not controlled and followed natural conditions outside the greenhouse. Relative humidity was controlled within the range of 40–65%.

A pair of dynamic enclosures (Fig. 1) were designed to enclose a portion of a branch (10–500 g of leaf biomass) with an air flow rate allowing adequate signal/noise ratio for all the instrumentation monitoring trace gas concentrations entering and leaving the enclosure (Tholl et al., 2006; Ortega and Helmig, 2008). The cuvettes were built to optimize uniform air circulation (cylinder of 40×64 cm, and conic volume at the base of the cylinder where the branch was wrapped, 40×8 cm, total volume $\sim$84 L). A rigid Teflon frame was sealed within a transparent Teflon layer (Richmond air craft products, Inc.) allowing more than 95% of the incident photosynthetically active radiation (PAR) to reach the leaves. Teflon was used for all surfaces to minimize potential surface deposition of ozone and VOC. Enclosures were supplied with air that had been purified of ozone, CO_2 and hydrocarbons using a Zero Air Generator (Aadco mod. 737) and maintained at a constant 380 ppm CO_2 by diluting gas from a pure CO_2 cylinder into the zero air stream using a mass-flow controller (MKS Instruments, Inc.). Air flow to the cuvette was controlled at 8.5 L min^{-1} using a similar mass-flow controller through ¼″ 0.64 cm Teflon tubing (see Fig. 1) and distributed in the cuvette through a shower-like Teflon ring with multiple holes that promoted well mixed conditions. This method was chosen over the use of a mixing fan inside the cuvette, because it allowed us to exclusively use Teflon material. Residence time of the air in the enclosure was estimated at $\sim$10 min.

When branches were enclosed in the cuvette, the stems were gently wrapped with the Teflon film to minimize damage. In all cases, the measurements started 24 h after the enclosure was installed, to minimize effects of plant handling. Each enclosure was equipped with a PAR sensor (LICOR mod. Li-190), an RH & T sensor (Omega engineering mod. HX93 AV-RP1), and a thermocouple wrapped around the branch and touching the leaves to measure leaf temperature (Omega Engineering, Precision Fine Wire thermocouples).

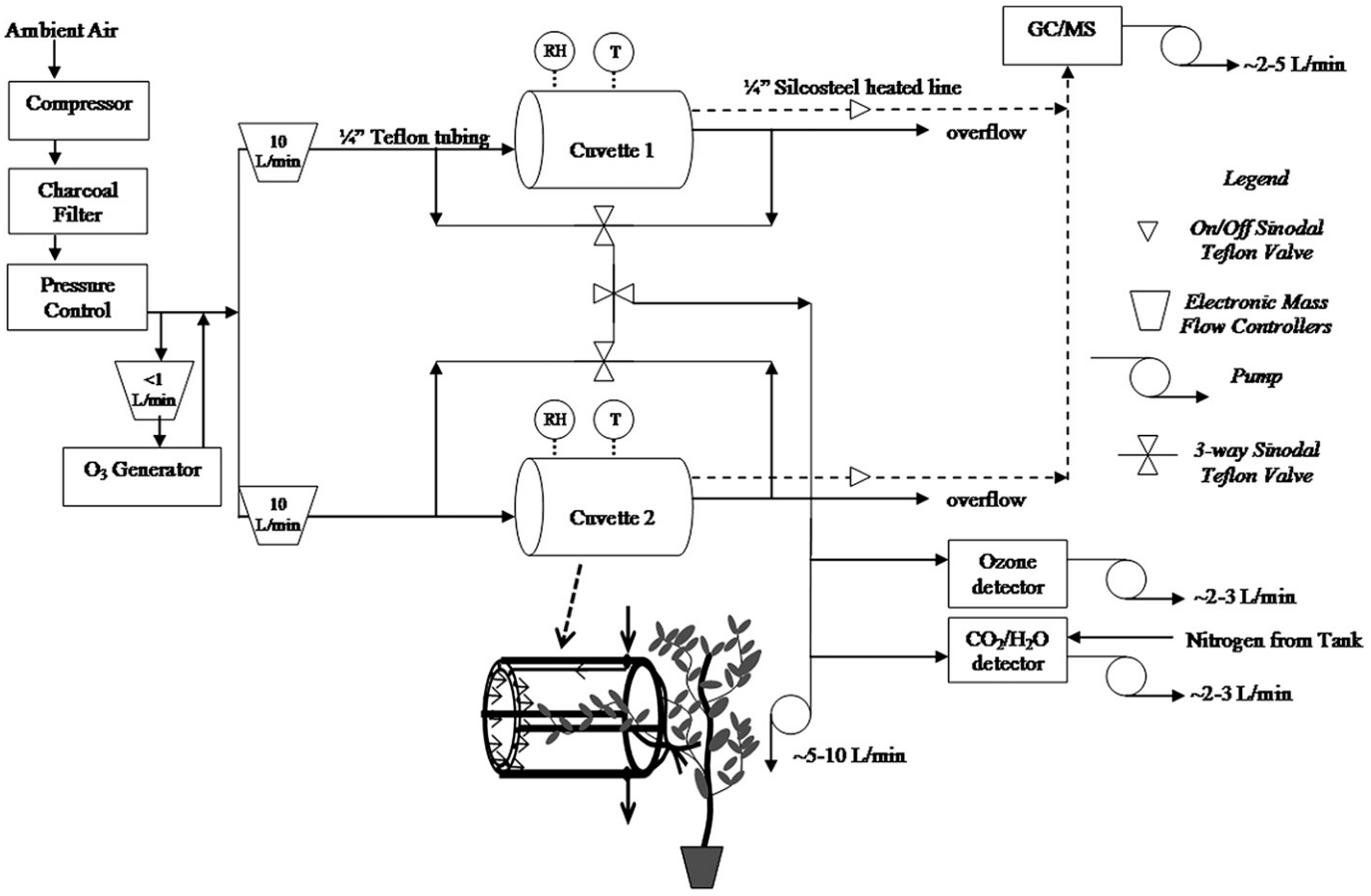


Fig. 1. Simplified experimental design of the dynamic enclosure systems for gas-exchange measurements.

Relative humidity and leaf temperature were used to calculate the vapor pressure deficit (VPD) between the leaves and air.

2.2. Measurement approach

We measured two individuals of the same species simultaneously by enclosing each one in a different cuvette. A single set of instruments was used, switching every 15 min between the outflow of cuvette 1 and 2 using a system of 3-way solenoid valves (TEQCOM Industries, Fig. 1) controlled by a datalogger (Campbell Scientific mod. CR10x and mod. SDM-CD16AC). The first 3 min of each 15-min cycle were dedicated to monitoring the air entering the enclosures. CO_2 and H_2O were measured with a closed-path infra-red gas analyzer (IRGA) (LICOR mod. 6262). During the first three days after enclosure, plants were kept in ozone-free air to acclimate to the enclosure conditions. After this period, ozone-enriched air was introduced in the enclosure. Ozone was produced by a UV ozone generator (DASIBI mod. 1008-RS) and diluted with the zero air entering the enclosures to reach the desired concentrations (40, 100 and 160 ppb). These concentrations simulated zero, low, medium and very high levels of ozone concentrations to approximate levels that may be found in the Central Valley of California and in many other Mediterranean areas. On the same individual, two days of continuous measurements were dedicated to each ozone concentration, starting with 40 ppb, then switching to 100 ppb and finally to 160 ppb. Ozone concentrations were measured at the inlet and outlet of the chamber with a UV analyzer (DASIBI mod. 1008-AH).

Fluxes (Φ) of H_2O, CO_2, ozone, by convention positive if towards the atmosphere and negative if towards the leaf, were calculated using the differential approach described by Fares et al. (2008) summarized in Equation (1), where F is the cuvette air flow, A^L is the leaf area of the plant material enclosed measured with a leaf area meter (LICOR mod. 3100C), $[X]_{in}$ is the gas concentration at the inlet of the enclosure and $[X]$ is the concentration measured at the outlet of the cuvette:

$$\Phi_X = \frac{F}{A^L} \cdot ([X]_{in} - [X]) \quad (1)$$

Equation (2) was used to measure ozone deposition velocity ($O3Vd$) to the cuvette, a term which indicates the rate at which ozone is deposited in the cuvette and represents the ozone flux normalized for the concentration gradient between the inside and outside the leaf:

$$O3Vd = \frac{\Phi_{O_3}}{[O_3]_{ci} - [O_3]} \quad (2)$$

In a first approximation, we assumed that the intercellular ozone concentration ($[O_3]_{ci}$) is zero (Laisk et al., 1989). In this preliminary form $O3Vd$ accounts for stomatal and non-stomatal depositions inside the cuvette; the latter including deposition on the cuvette wall ($O3Vd_{wall}$), on leaf and branch surfaces ($O3Vd_{surf}$), and chemical deposition in the gas-phase through reactions between ozone and BVOC ($O3Vd_{BVOC}$). The stomatal deposition was then calculated subtracting the non-stomatal components:

$$O3Vd_{sto} = O3Vd - O3Vd_{BVOC} - O3Vd_{surf} - O3Vd_{wall} \quad (3)$$

Using non-photosynthesizing plant material inside the cuvette we found that deposition on plant surfaces was negligible after more

than 2 h exposure (less than 2% of total measured deposition). The Teflon enclosure minimized the deposition on the enclosure walls which we measured with the empty enclosure for each different ozone concentration. $O3Vd_{wall}$ accounted for approximately 3% of the total measured deposition, and was subtracted from all measurements. $O3Vd_{BVOC}$ was calculated as:

$$O3Vd_{BVOC} = \frac{V}{A^L} \cdot \sum_i [BVOC_i] \cdot k^i \quad (4)$$

where V is the enclosure volume and k^i is the specific reaction rate constant of each *BVOC* species with ozone assuming a reaction time faster or equal to the air turnover time in the enclosure, as described in Fares et al. (2008).

Stomatal conductance to ozone, $Gsto_{O_3}$, was calculated from stomatal conductance to water vapor $Gsto_{H_2O}$, measured with the gas-exchange system and multiplied by the ratio of the diffusion coefficients of ozone and water vapor in air (Equation (5)):

$$Gsto_{O_3} = Gsto_{H_2O} \cdot \frac{d_{O_3}}{d_{H_2O}} \quad (5)$$

The diffusion coefficients of water vapor (d_{H_2O}) and ozone (d_{O_3}) are 0.25 and 0.167 $cm^2\,s^{-1}$, respectively (Marrero and Mason, 1972; Laisk et al., 1989). Fluxes were reported in $mol\,m^{-2}\,s^{-1}$, while conductances and depositions were reported in $cm\,s^{-1}$. Water use efficiency (WUE) is, by definition, equivalent to CO_2 fixed divided by water transpired on a mass basis, and was averaged over the middle of the day (11:00–15:00). Similarly, we calculated ozone uptake efficiency (OUE) as ozone absorbed divided by carbon fixed on a mass basis.

2.3. GC–MS measurements of BVOC

No BVOC measurements were made for the first 24 h after plant enclosure so that branches could acclimate to the enclosure and minimized possible emissions due to mechanical damage. Measurements of branches exposed to ozone-free air took place on the second and third days after branch enclosure. Hourly-resolved VOC concentrations were measured using an automated in-situ gas chromatograph (HP mod. 5890) equipped with both a mass-selective detector (HP mod. 5971) and a flame ionization detector (adapted from Millet et al., 2005). The instrument pre-concentrated ~600 mL samples of cuvette effluent on adsorbent traps over a 30-min period and thermally desorbed them onto capillary columns; the FID-analyzed sample was collected on a glass bead/Carbopak B/Carboxen 1000 adsorbent mix and injected onto a DB-624 column, while the MSD-analyzed sample was collected on Tenax-TA, then injected onto a Rtx-5 column. Calibrations were performed using gas-phase monoterpene standards and liquid standards for more reactive compounds (e.g. sesquiterpenes and unstable monoterpenes). Interpolation of the 1-h data was performed to obtain 30-min data to be used for calculations.

3. Results and discussion

3.1. Physiology

Average daily PAR, VPD and temperature during the experiments are reported in Fig. 2. PAR never exceeded 900 $\mu mol^{-2}\,s^{-1}$ and temperatures in the greenhouse were controlled to never exceed 28 °C, a level well tolerated by *Citrus* species. Relative humidity was highest during the central hours of the day reaching values up to 65% owing to the higher transpiration rates of the plant in the cuvette, resulting in a drop of VPD during mid-day (Fig. 2) to

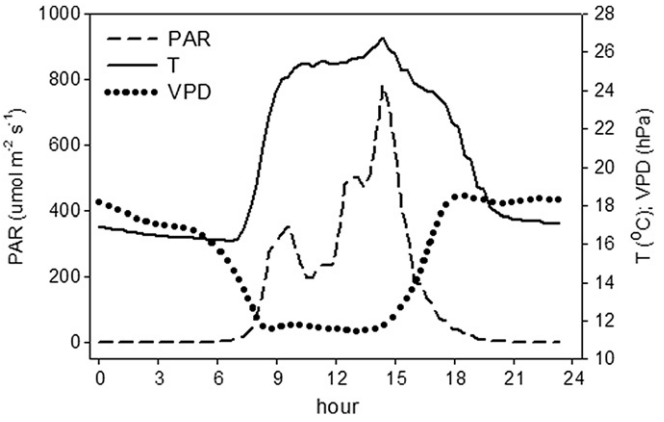


Fig. 2. Photosynthetically active radiation (PAR), leaf temperature and vapor pressure deficit (VPD) between leaf and air averaged every 15 min and for all species during the experiment.

about 11 hPa. During the middle hours of the day, water transpiration and photosynthesis (Fig. 3) reached values up to 2 $mmol\,m^{-2}\,s^{-1}$ and up to −10 $\mu mol\,m^{-2}\,s^{-1}$, respectively, with no statistical differences between controls and ozonated branches. In the case of mandarin, the transpiration values at 100 ppb ozone were statistically higher than those of controls ($P < 0.05$). We explain this result via an accidental increase in greenhouse temperature of 2 °C during the days of measuring at 100 ppb ozone. A statistical analysis of the slope of the linear correlation between stomatal conductance and photosynthesis did not reveal statistical differences caused by ozone exposure with respect to controls (data not shown). We therefore conclude that the *Citrus* species measured in this study tolerate short periods of exposure to high tropospheric ozone levels without apparent damage as determined by photosynthetic values since our 6-d exposure to a range of different ozone levels did not affect primary metabolism or stomatal movement.

The photosynthetic and transpiration rates of our plants were consistent with those reported in other experiments with *Citrus* trees, performed both in the field and under controlled conditions (Manes et al., 1999; Ortuno et al., 2006; Ribeiro et al., 2009), which indicates that the absence of effects due to ozone was not due to previous stress conditions. However, *Citrus* photosynthesis is in the lowest range among trees, because of high resistances to CO_2 diffusion both at stomatal level and in the mesophyll (Loreto et al., 1992). Low stomatal conductance also limits transpiration of *Citrus* leaves, in turn allowing relatively high water use efficiency (WUE). In plants that were not exposed to ozone we calculated WUE of 9.3, 8.2, 8.2 g (CO_2) kg (water transpired)$^{-1}$ for lemon, mandarin and orange, respectively, during the central hours of the day. These values did not statistically differ from data obtained during ozone exposure. We caution that WUE was calculated in absence of drought stress and optimal temperature conditions, and may be higher during drought stress episodes in the field due to the effect of a partial stomatal closure on transpiration (Flexas et al., 2004). Our data may therefore be representative of well irrigated *Citrus* plantations where the stomata do not represent a main limitation to water and carbon exchange.

We did not expect any negative impacts from moderate to high ozone exposure on the physiology of *Citrus* because the high diffusive resistances of *Citrus* plants (Loreto et al., 1992) are believed to dramatically reduce ozone entry, even in presence of high external ozone concentrations. However, exposure to high tropospheric ozone, which typically occurs in most Mediterranean countries, may result in a decrease of productivity of *Citrus* orchards

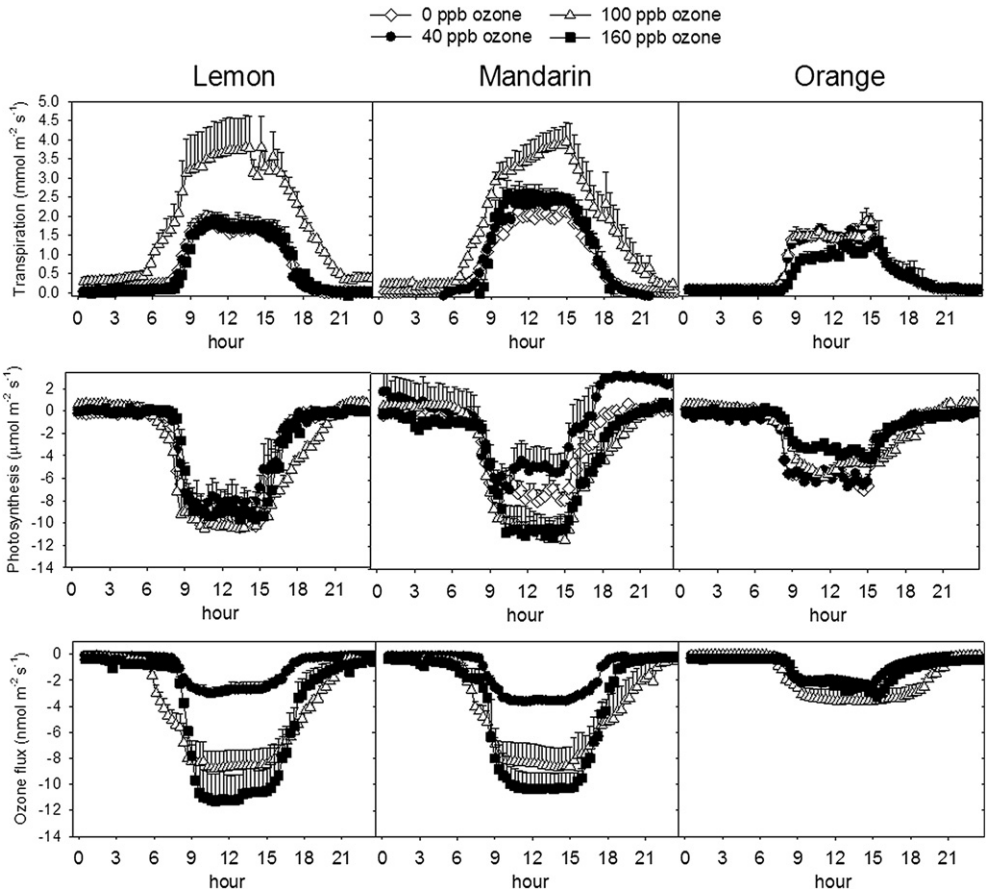


Fig. 3. Transpiration, photosynthesis and ozone uptake of lemon (*Citrus limon*), mandarin (*Citrus reticulata*), and orange (*Citrus sinensis*) measured from branch enclosures in the greenhouse facility at UC Berkeley. Data are reported as an average of samples ($n = 3 \pm$ standard errors) collected during control conditions (ozone-free air inside the enclosures), 40 ppb, 100 ppb and 160 ppb ozone except for ozone fluxes, measured at 40 ppb, 100 ppb and 160 ppb ozone. Each level was measured for two days on the same branch.

up to 15% (Olszyk et al., 1988; Delgado-Saborit and Esteve-Cano, 2008). Additionally, previous studies have shown that several months of exposure to chronic ozone levels produces a decrease in physiological properties of *Citrus* trees (Olszyk et al., 1992; Calatayud et al., 2006). Moreover, long-term ozone exposure can trigger protective mechanisms against oxidative stress (Iglesias et al., 2006). Finally, it should be mentioned that our results may not apply to natural and uncontrolled conditions if other unaccounted factors that accompany high ozone levels impair photosynthesis, stomatal aperture and ozone uptake. For instance, it has been shown that periods of water deficit (Vu and Yelenosky, 1988; Erismann et al., 2008), parasite infections (Sagaram and Burns, 2009; Ribeiro et al., 2004), high temperatures (Guo et al., 2006) and nitrogen deficiencies (Bondada and Syvertsen, 2003) can significantly depress physiological parameters in *Citrus* species. An excessive mechanical harvesting practice may also produce short-term physiological unbalances (Li and Syvertsen, 2005). Environmental pollutants in the soil (Rajaie et al., 2009) and in the air (e.g. ozone) are also important limiting factors for *Citrus* plant growth.

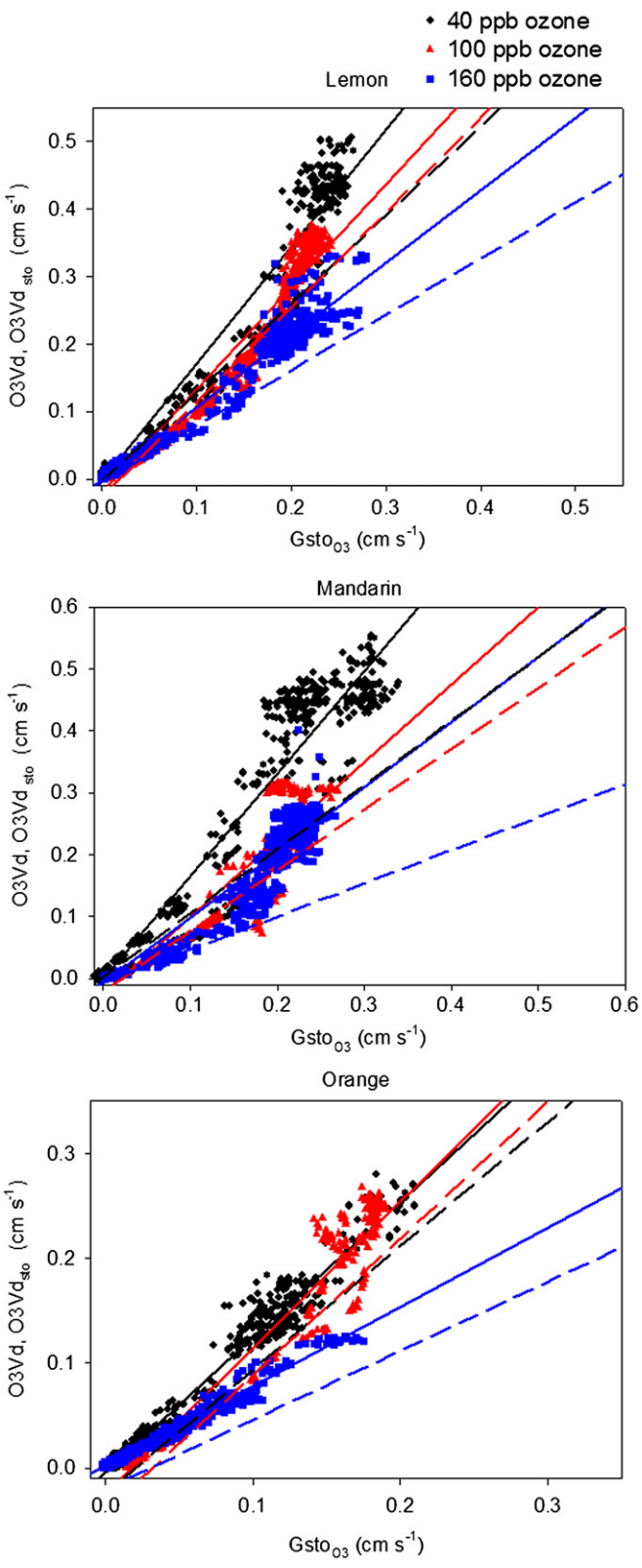
3.2. Ozone flux quantification

Ozone fluxes ranged between −2 and −11 nmol m^{-2} s^{-1} with the highest levels corresponding to the highest levels of ozone exposure during the central hours of the day (Fig. 3). Among all species, navel orange experienced the lowest fluxes due to lowest stomatal conductance. In contrast, for all species the daily peak in ozone fluxes corresponded to the peaks in stomatal conductance and water transpiration (Fig. 3). This association indicates that much of the ozone is removed after entering through stomata and reacting inside the intercellular spaces. Our results are in agreement with studies on direct measurement of leaf ozone fluxes on black poplar and holm oak (Fares et al., 2008), snapbean, California black oak, and blue oak (Grulke et al., 2007), which highlighted dependencies of ozone fluxes on stomatal conductance.

To quantify the ozone fluxes per unit of CO_2 fixed by photosynthesis (including respiration processes), we calculated the ozone uptake efficiency. At 40 ppb ozone during the peak level of stomatal conductance we obtained values of OUE of 0.27, 0.27 and 0.34 mg of ozone per gram of CO_2 fixed for lemon, mandarin and orange, respectively. These values increased almost linearly at 100 ppb of ozone (0.72, 0.57, 0.61 mg (O_3) g $(CO_2)^{-1}$), and slightly increased again at 160 ppb of ozone (0.85, 0.89, 0.69 mg (O_3) g $(CO_2)^{-1}$). We propose the OUE as a suitable indicator to estimate the maximum efficiency of *Citrus* plants to scavenge ozone without suffering immediate physiological damage.

3.3. Ozone flux: stomatal versus non-stomatal components

In Fig. 4 we show the relationship between stomatal conductance to ozone ($Gsto_{O_3}$) calculated via Equation (5) and the measured ozone deposition described in Equation (2) (*O3Vd*), under the assumption that the intercellular ozone concentration was zero (Laisk et al., 1989). Ideally, if all ozone is taken up entirely via stomata with intercellular ozone decreasing quickly to zero, the calculated and measured quantities should return the same values.



However, we observed that at 40 and 100 ppb ozone the slope of the relationship between $Gsto_{O_3}$ and *O3Vd* was often >1, with values up to 1.74 for lemon (Table 1). This preliminary observation suggests that in *Citrus* leaves the high values of measured ozone deposition (*O3Vd*) can be due to unexplained mechanisms that contribute to ozone deposition via non-stomatal pathways.

In addition to stomatal uptake, we investigated ozone deposition due to gas-phase reactions with BVOC inside the enclosure. Studies performed on pine forest ecosystems demonstrate that BVOC participate in non-stomatal ozone uptake through gas-phase reactions (Kurpius and Goldstein, 2003; Goldstein et al., 2004; Bouvier-Brown et al., 2009). This finding has been confirmed in plant enclosures (Fares et al., 2008). In a field study in an orange orchard, Ciccioli et al. (1999) also reported that some undetermined BVOC species are responsible for ozone removal in the atmosphere. *Citrus* is a well known BVOC emitter and is thus expected to have significant non-stomatal ozone fluxes (Lamb et al., 1993; Winer et al., 1992; Ciccioli et al., 1999; Hansen and Seufert, 2003). It should be highlighted that the air residence time in the enclosures (10 min) was long enough to allow gas-phase reactions between ozone and the most reactive chemical species (e.g. sesquiterpenes and some monoterpenes) given the known ozone reaction rate constants for these BVOC (US EPA AOPWIN, 2000; Table 2). A residence time of 10 min is close to natural conditions under moderate turbulence (Martens et al., 2004), but the decrease in VPD values we observed (Fig. 2) are unlikely occurring in warm climates, when heat and turbulent mixing of the air are faster thus reducing the amount of water vapor at the canopy level leading to increases in VPD. Beside BVOC, other compounds like NO_x can contribute to ozone removal, but we considered NO_x emissions from plants negligible under no or limited oxidative stress (Velikova et al., 2008). Yet NO_x emissions from soils in a natural environment may significantly contribute to ozone losses.

To estimate ozone deposition by reaction with BVOC, we measured the BVOC species emitted from the *Citrus* trees focusing on compounds with high and known reactivity towards ozone (e.g. myrcene, limonene, β-caryophyllene, and α-humulene). We observed maximum levels of emission during the middle hours of the day, following the light and temperature dependencies of the emission of these terpenes described elsewhere (Niinemets et al., 2004; Tingey et al., 1991; Monson et al., 1992). A summary with the minimum and maximum values of BVOC concentration in the cuvettes is reported in Table 2. The $O3Vd_{BVOC}$ term reported in Equation (4) was then calculated, and incorporated in the calculation of the net stomatal ozone deposition ($O3Vd_{sto}$, equation (3)). These corrections decrease *O3Vd* and consequently reduce the value of the slope between $Gsto_{O_3}$ and $O3Vd_{sto}$. The new slopes are less steep (Table 1, Fig. 4) although at low ozone concentrations the slope is still above 1. A similar result was obtained in past studies (Fares et al., 2008), which showed that a high BVOC emitter (holm oak) produced an $O3Vd_{sto}$ value much higher than $Gsto_{O_3}$ with a slope > 1. After correcting for BVOC reactivity in the gas-phase, the slope decreased from 1.88 to 1.27, but never reached the 1:1 value. These results suggest that unaccounted BVOC, or other

Fig. 4. Stomatal conductance to ozone ($Gsto_{O_3}$) versus ozone deposition to the cuvette (*O3Vd*) and to the stomata ($O3Vd_{sto}$) for *Citrus* plants exposed to 40 ppb (black circles), 100 ppb (red triangles) and 160 ppb ozone (blue squares). Each point represents a 5-min average and results from three different experiments carried out at 40, 100, and 160 ppb of ozone concentration. The best-fit lines were generated by Sigma plot 2002 software (Systat) and are not constrained through the origin. These lines are reported for each series with the same color of the series. When continuous, the line refers to the data series showed in the graph, when broken, the linear regression was drawn for the correlation between $Gsto_{O_3}$ and $O3Vd_{sto}$. *R*-squared coefficients and slopes for linear regressions are provided in Table 1.

Table 1
R-squared values, slopes (statistically different, $P < 0.05$), intercepts on the *y*-axis of the linear regressions between stomatal conductance to ozone ($Gsto_{O_3}$) and ozone deposition velocities reported in Fig. 4, measured during exposure to three different levels of atmospheric ozone concentrations (40, 100, 160 ppb) for each species. These values were calculated for the correlation between *O3Vd* and $Gsto_{O_3}$ (norm., equation (3)) and the correlation between $O3Vd_{sto}$ and $Gsto_{O_3}$ (corr., equation (3)). The table also shows the percentage of non-stomatal ozone flux (NS-flux) and averaged values of intercellular ozone concentrations ($[O_3]ci$) calculated during the light hours, ±standard errors.

Ozone (ppb)	*R*-square		Slope		Intercept		NS-flux (%)	$[O_3]ci$ (ppb)
	Norm.	Corr.	Norm.	Corr.	Norm.	Corr.		
Lemon								
40	0.97	*0.89*	1.74	*1.43*	−0.04	*−0.03*	0	n.s.
100	0.96	*0.93*	1.52	*1.19*	−0.02	*−0.02*	44 ± 15	19 ± 5
160	0.97	*0.88*	1.07	*0.82*	−0.01	*−0.04*	17 ± 4	23.8 ± 11
Mandarin								
40	0.94	*0.84*	1.69	*1.04*	−0.01	*−0.01*	33 ± 13	n.s.
100	0.9	*0.89*	1.25	*0.98*	−0.02	*−0.02*	33 ± 10	5.8 ± 1
160	0.94	*0.79*	1.05	*0.53*	−0.01	*−0.01*	27 ± 12	46 ± 12
Orange								
40	0.98	*0.95*	1.29	*1.18*	−0.04	*−0.02*	31 ± 1	n.s.
100	0.96	*0.94*	1.39	*1.3*	−0.02	*−0.04*	15 ± 1	20 ± 9
160	0.98	*0.87*	0.76	*0.66*	−0.01	*−0.02*	31 ± 8	63 ± 7.5

biogenic compounds that are able to react with ozone (e.g. leaf antioxidants) may be responsible for the overestimation of the $O3Vd_{sto}$ value. During the light hours (PAR > 100 μmol m^{-2} s^{-1}), we calculated that the percentages of non-stomatal uptake due to BVOC reactivity in the gas-phase at the three levels of ozone exposure were on an average around 30% for lemon, mandarin and orange, respectively (Table 1). These values are slightly lower compared to previous research carried out on a *Pinus ponderosa* forest (Fares et al., 2010a,b), and in a mixed hardwood forest (Hogg et al., 2007) which ranged between 30 and 70%, although these forest ecosystems are known to be higher BVOC emitters. BVOC measurements were done using control branches with no ozone fumigation, because the gas-phase reactions between ozone and BVOC in the cuvette during ozone fumigation would invalidate the measurement. Since ozone did not modify the physiological properties of *Citrus* plants we speculate that BVOC emission was not significantly affected by this short-term ozone exposure, but we cannot exclude an ozone-induced emission of BVOC during the time of ozone exposure, thus leading to some uncertainties in non-stomatal absorption. Some studies showed that ozone fumigation can increase the emission of terpenes by activating the biosynthesis of these defensive compounds, although other studies showed no or a decreased BVOC emission after ozone exposure especially under non-acute ozone exposure (for a detailed review see Loreto and Schnitzler, 2010).

In contrast, at high ozone concentrations (160 ppb) the slope of the $O3Vd/Gsto_{O_3}$ relationship is close to one and even below for orange (0.76, Table 1). Moreover, the $O3Vd_{sto}/Gsto_{O_3}$ value at 160 ppb ozone is lower than 1 in all species after correcting for BVOC reactivity. This indicates that at very high ozone concentrations other factors, which decrease ozone deposition with respect to that estimated from the stomatal conductance to ozone, come into play.

If at increasing ozone concentrations ozone starts to accumulate inside the intercellular spaces, then the gradient between inside and outside the leaf decreases, which produces a decrease in the flux magnitude according to Fick's first law of diffusion in isotropic substances (Nobel, 1974). We attempted to calculate intercellular ozone concentrations using the equation: $O3Vd_{sto} = Gsto_{O_3}$ by considering $[O_3]ci$ reported in Equation (3) as the unknown term. At 40 ppb of ozone exposure during light hours (7 AM–6 PM), we found values of O_3ci close to zero (~7 ppb), suggesting that under those atmospheric concentrations most ozone entering stomata reacts immediately. At 100 ppb, we calculated values averaging 15 ppb and at 160 ppb averaging 44 ppb for lemon, mandarin, and orange, respectively. This indicates that incomplete ozone removal inside the mesophyll plays a role in determining total ozone uptake by *Citrus* leaves and should be considered when calculating $O3Vd_{sto}$ at high external ozone concentrations. However, this hypothesis contradicts the general assumption that ozone intercellular concentration is close to zero (Laisk et al., 1989). Previous work by Moldau and Bikele (2002) and Loreto and Fares (2007) experimentally demonstrated that a certain amount of ozone can accumulate in intercellular spaces. In this controlled study, we cannot exclude that certain non-stomatal sinks were underestimated during ozone exposure. Surface deposition could be an enhanced sink for ozone, although Cape et al. (2009) demonstrated that deposition on wax surface is in the order of few mm s^{-1} and not responding to different concentrations of BVOC in the cuticular waxes and in relative humidity. Surface deposition may be less important in our experimental approach than it would be at the whole canopy level. If surface deposition was a significant loss process for ozone then the amount of ozone accumulated in intercellular spaces would be higher than estimated.

Table 2
Concentration of monoterpenes (myrcene and limonene) and sesquiterpenes (b-caryophyllene and a-humulene) measured in the plant enclosures when lemon, orange and mandarin branches were included one day before ozone fumigation. The rate constant with ozone and the estimated retention time in the cuvette are provided. The latter is reported for 40, 100, 160 ppb ozone in the cuvette, respectively. The range of BVOC concentrations is reported with the minimum and the maximum level reached in mid-day. Rate constants were calculated based on chemical structure using the Environmental Protection Agency's Estimation Program Interface Suite (US EPA AOPWIN, 2000).

BVOC	$k^{O_3} \times 10^{-18}$ (cm^3 s^{-1})	RT (min)	Concentration range (ppb)		
			Lemon	Mandarin	Orange
Myrcene	470	33,13,8	0–0.08	0–0.1	0.01–0.3
Limonene	420	37,15,9	0–0.9	0–1.5	0–0.06
β-Caryophyllene	12,000	1,1,<1	n.d.	0–0.02	0–1.18
α-Humulene	12,000	1,1,<1	n.d.	0–0.04	0–0.05

3.4. Nocturnal ozone deposition

Nocturnal ozone uptake is a process which may occur in all plant ecosystems. Since stomata never completely close during the night (Dawson et al., 2007; Caird et al., 2007; Rannik et al., 2009), a consistent amount of ozone can reach the intercellular spaces (Musselman and Minnick, 2000; Grulke et al., 2004; Vitale et al., 2007). In our study, we quantified the total nocturnal ozone uptake as the integral of the measurements taken during the night (when PAR < 0.5 μmol m^{-2} s^{-1}) and report it as a percentage of the total flux during both day and night. The percentage of nocturnal uptake did not change significantly between the three different ozone exposures. The means (±standard errors) for all ozone exposures were 5 ± 1, 12.5 ± 0.6, 6.9 ± 0.3 percent for lemon, mandarin, and orange, respectively. Despite it being a small fraction compared to the total diurnal stomatal ozone uptake, nocturnal uptake may contribute to ozone damage in the nighttime. This is due to the fact that the antioxidant defenses of plant (e.g. ascorbic acid) are low at night because the biosynthetic pathway of most antioxidants is often light-dependent and associated with photosynthesis (Heath et al., 2009; Fares et al., 2010a,b).

The intercept on the *y*-axis of the correlation between *Gsto* and *O3Vd* is close to zero (Table 1), suggesting that under hypothetical

conditions of total stomatal closure, ozone deposition is negligible. Moreover, a very limited amount of BVOC was measured during the nighttime and the relative humidity during nighttime was lower (~20–30%) than during the day because of the limited leaf transpiration, thus minimizing potential wet deposition. This leads to the conclusion that the nocturnal ozone deposition sinks are mainly the stomata. As previously demonstrated (Fares et al., 2008), non-stomatal deposition due to gas-phase reactions between ozone and BVOC are indeed very low during night. Under atmospheric conditions, nocturnal ozone deposition may occur also on soils and by reactions with NO emitted from soils or from anthropogenic sources (Dorsey et al., 2004; Michou et al., 2005; Wang et al., 2006).

4. Conclusions

We conclude that total ozone deposition on citrus leaves is influenced by two important coexisting processes affecting the magnitude of the deposition in opposite directions. On one hand, emitted BVOC are responsible for an increase in ozone loss in the branch chamber due to their reaction in the gas-phase with ozone. On the other hand, ozone does not instantly disappear in the intercellular spaces as commonly assumed, but rather accumulates and decreases the stomatal ozone flux, especially under high ozone concentrations. This result is particularly relevant considering that citrus in agricultural areas of California often experience atmospheric ozone concentrations above 100 ppb during the warm season and concentrations of atmospheric ozone are expected to increase further in response to increasing temperatures.

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