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BARD

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**Ozone Altered Stomatal/Guard Cell Function:
Whole Plant and Single Cell Analysis**

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Vicia faba, *Nicotiana tabacum*, *Arabidopsis thaliana*, *Pinus brutia*, *Pinus halepensis*, *Commulina communis*

Abbreviations commonly used in the report, in alphabetical order:

H₂O₂: hydrogen peroxide, O₃: ozone

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BARD Final Scientific Report

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Gro Torsethaugen 203 74 8073

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1		1	2
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Description Cooperation:

Dr. Eva Pell visited Israel in May of 1998 where she discussed the progress of the project and especially some different results between the U.S. experiments and those obtained from patch clamp experiments of H₂O₂ exposed guard cells conducted in Israel. She also gave to seminars at The University of Jerusalem in Rehovot and in Jerusalem.

Dr. Nava Moran (Israel) and Dr. Gro Torsethaugen (USA), both involved in research related to this project, met at the annual ASPP meeting in Baltimore, USA in July of 1999.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
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Abstract

Original objectives (revisions from original proposal are highlighted)

1. Elucidate the direct effects O₃ and H₂O₂ on guard cell function, utilizing assays of stomatal response in isolated epidermal peels and whole cell gas exchange.
2. Determine the mechanistic basis of O₃ and H₂O₂ effects on the plasma membrane through application of the electrophysiological technique of patch clamping to isolated guard cells.
3. Determine the relative sensitivity of Israeli cultivars of economically important crops to O₃, and determine whether differential leaf conductance responses to O₃ can explain relative sensitivity to the air pollutant: transfer of technological expertise to Israel.

Background to the topic

For a long time O₃ has been known to reduce gas exchange in plants; it has however been unclear if O₃ can affect the stomatal complex directly. Ion channels are essential in stomatal regulation, but O₃ has never before been shown to affect these directly.

Major conclusions, solutions, achievements

1. Ozone inhibits light-induced stomatal opening in epidermal peels isolated from *Vicia faba*, *Arabidopsis thaliana* and *Nicotiana tabacum*. In *V. faba* plants this leads to reduced assimilation without a direct effect on the photosynthetic apparatus. Stomatal opening is more sensitive to O₃ than stomatal closure.
2. Ozone causes inhibition of inward K⁺ channels (involved in stomatal opening) while no detectable effect is observed on the outward K⁺ channels (stomatal closure).
3. Hydrogen peroxide inhibits stomatal opening and induces stomatal closure in epidermal peels isolated from *Vicia faba*.
4. Hydrogen peroxide enhances stomatal closure by increasing K⁺ efflux from guard cells via outward rectifying K⁺ channels.
5. Based on epidermal peel experiments we have indirectly shown that Ca²⁺ may play a role in the guard cell response to O₃. However, direct measurement of the guard cell [Ca²⁺]_{cyt} did not show a response to O₃.
6. Three Israeli cultivars of zucchini, Clarita, Yarden and Bareqet, were shown to be relatively sensitive to O₃ (0.12 µl l⁻¹).
7. Two environmentally important Israeli pine species are adversely affected by O₃, even at 0.050 µl l⁻¹, a level frequently exceeded under local tropospheric conditions. *P. brutia* may be better equipped than *P. halepensis* to tolerate O₃ stress.
8. Ozone directly affects pigment biosynthesis in pine seedlings, as well as the metabolism of C₅ precursors, thus affecting the allocation of resources among various metabolic pathways.
9. Ozone induces activity of antioxidant enzymes, and of ascorbate content in the mesophyll and epidermis cells of *Commelina communis* L.

Implications, both scientific and agricultural

We have improved the understanding of how O₃ and H₂O₂ do affect guard cell and stomatal function. We have shown that economical important Israeli species like zucchini and pine are relatively sensitive to O₃.

Achievements

Significance

We determined that O_3 inhibits stomatal opening in epidermal peels isolated from *Vicia faba*, *Arabidopsis thaliana* and *Nicotiana tabacum*. This is an important finding because it means that O_3 can affect the stomatal complex directly, without the influence of the photosynthetic apparatus in the mesophyll cells. It has been shown earlier that O_3 can affect the photosynthetic apparatus, leading to increased intercellular CO_2 concentration, thus inducing a decrease in stomatal conductance secondarily. Our findings with epidermal peels were confirmed with non-destructive gas exchange measurements in *V. faba*. Ozone did cause an inhibition of stomatal opening in plants that showed no effect on A/Ci curves, which indicates that the photosynthetic apparatus was not affected by O_3 in these plants.

To our knowledge we have for the first time shown that O_3 can affect ion currents across biological membranes with the sensitive technique of patch clamping. We found that the inward K^+ currents in *V. faba* guard cells are inhibited by O_3 . Since inward K^+ currents are essential in stomatal opening, this could be the mechanism explaining the epidermal peel and gas exchange results. We offer two hypotheses that could explain the effect of O_3 on the inward K^+ channels. First hypothesis: O_3 or its breakdown products, directly oxidize the channel. Second hypothesis: Inhibition of inward K^+ currents is a secondary effect caused by increased cytosolic Ca^{2+} concentrations. We used single channel patch clamp recording to address the first hypothesis and Ca^{2+} imaging to answer the second. Neither approach gave us clear answers, leaving to be resolved the explanation for how O_3 affects the inward K^+ channels. We did achieve our original goal of determining a mechanism by which O_3 might affect plasma membranes.

We did show that H_2O_2 , which is produced in plants during O_3 exposure, enhanced the outward K^+ channels. This mechanism could be involved in O_3 -induced stomatal closure observed in many species. We did for the first time show that H_2O_2 inhibits stomatal opening and promotes stomatal closure in epidermal peels from *Vicia faba*, although similar results have previously been reported in *Commelina* and *Arabidopsis*.

Antioxidant enzyme activity and antioxidant content was studied in epidermal tissue and mesophyll tissue of O₃ exposed *Commelina communis*. Differences were found between tissues and treatment, which might help us better understand defense mechanisms against oxidative stress.

We found that O₃ directly affects pigment biosynthesis in pine seedlings, as well as the metabolism of C₅ precursors, thus affecting the allocation of resources among various metabolic pathways.

All these findings improve our understanding of the cellular mechanisms underlying effects observed on the whole plant level, like reduced gas exchange and potentially even reduced growth. This could be useful in generation of more ozone-resistant plants.

The O₃ sensitivity of different cultivars of zucchini and two different species of pine were investigated. The relative sensitivity was determined, but it was not determined if the differential leaf conductance could explain their relative sensitivity to O₃.

Two pine species commonly grown in Israel were proven to be sensitive to O₃. Effects of the pollutant were observed at doses as low as 0.050 µl l⁻¹ O₃, a concentration commonly exceeded in Israel. *P. brutia* may be better equipped than *P. halepensis* to tolerate O₃ stress. This could become important information for political discussions about air quality standards.

Agricultural and economic impacts of research findings, if known

While these studies were not designed to measure the agricultural and economic impact of O₃ on crop plants the results do have implied effects. The fact that O₃ could impact pine at relatively low concentrations of O₃ suggests the possibility of lower growth rates and productivity. The fact that both O₃ and H₂O₂ can inhibit stomatal opening could have a major impact on plants. Frequently air pollution levels are highest during times of drought when stomata tend to be closed. If, when the drought breaks, stomata opening more slowly or less completely as a result of O₃ or H₂O₂ injury, net photosynthesis will be impaired. The end result could lead to reduced productivity.

Details of cooperation

The Israeli group had developed a phosphate buffer that could be used for patch clamping of *Vicia faba* guard cell protoplasts. The US group used this phosphate buffer for all their patch clamp experiments with O₃ and H₂O₂.

The Israeli group found increased outward K⁺ current and no effect on the inward K⁺ currents after treating *Vicia faba* guard cell protoplasts with 10 mM H₂O₂. The US group did not find a significant effect of 10 mM H₂O₂ on either inward or outward K⁺ channels under apparent similar conditions. Through Eva Pell's visit in Israel in May 1998 and communication via e-mail, potential explanations for our apparent differences in results were discussed. Many of the experiments conducted by the U.S. group were designed based on these discussions.

Foliage of zucchini exhibited symptoms postulated to reflect O₃-injury in the field in Israel. Seeds were sent to the US for controlled O₃- fumigation experiments. Experiments in open top chambers did show that these zucchini cultivars were relatively sensitive to O₃. However, the symptoms induced by O₃ were different from those observed in the field. Thus, while zucchini plants are sensitive to O₃, the symptoms observed in Israel were probably attributable to a different causal agent.

List of publications

Torsethaugen G, Pell EJ, Assmann SM. (1999) Ozone inhibits guard cell K⁺ channels implicated in stomatal opening. *Proc. Natl. Acad. Sci.* 96: 13577-13582.

Shamay Y, Raskin VI, Brandis AS, Steinberger HE, Marder JB, Schwartz A (2001) Ozone treatment affects pigment precursor metabolism in pine seedlings. *Physiologia Plantarum* (in press)

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Torsethaugen G, Pell EJ, Assmann SM. (1999) Ozone inhibits guard cell K⁺ channels implicated in stomatal opening. *Proc. Natl. Acad. Sci.* 96: 13577-13582

2. In Press paper

Shamay Y, Raskin VI, Brandis AS, Steinberger HE, Marder JB, Schwartz A (2000) Ozone treatment affects pigment precursor metabolism in pine seedlings. *Physiologia Plantarum* (in press).

3. Unpublished Data

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3.1 Ozone inhibits light-induced stomatal opening in epidermal peels isolated from *Arabidopsis thaliana* and *Nicotiana tabacum*.

We have shown in epidermal peel experiments with *Arabidopsis thaliana* and *Nicotiana tabacum* that O₃ causes an inhibition of light-induced stomatal opening (Fig. 1). The stomata were closed at the start of exposure and the epidermal peels were exposed to light (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) simultaneously with the start of O₃ exposure. Significant inhibition of stomatal opening was observed after exposure to 0.30 $\mu\text{l l}^{-1}$ O₃ for 3 1/2 hours in *A. thaliana* and *N. tabacum* and 1.0 $\mu\text{l l}^{-1}$ O₃ for 3 hours in *Vicia faba* (Torsethaugen et al. 1999). Epidermal peels from *V. faba* are known to contain high concentrations of phenolics. The phenolics can act as antioxidants protecting the plant tissue against oxidative stress. We speculate that different levels of phenolics can explain the differential O₃ sensitivity amongst the species used for the epidermal peel experiments. The importance of these experiments is that O₃ directly can impact stomatal function without the presence of the photosynthetic mesophyll tissue.

Figure 1. Light-induced stomatal opening in epidermal peels from *Nicotiana tabacum* Bel W3 (A) and *Arabidopsis thaliana* (B) in response to O₃ exposure. Abaxial epidermal peels were floated cuticular side up on 100 mM KCl solution (A) or distilled water (B). Epidermal peels were isolated from plants in the dark and then kept in the dark until exposure started to ensure closed stomata. The peels were exposed to O₃ free air and air containing 0.3 $\mu\text{l l}^{-1}$ O₃ for 3 1/2 hours under a light intensity of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The flow through the chamber during exposure was 400 ml min⁻¹. Stomatal apertures were measured with an ocular micrometer and a light microscope (B n=120, C n=90). Significant difference between treatments is indicated with an asterisk (*).

3.2 Hydrogen peroxide inhibits stomatal opening and induces stomatal closure in epidermal peels isolated from *Vicia faba*.

Epidermal peel experiments were conducted to investigate if H₂O₂ affects stomatal regulation in *Vicia faba*. The epidermal peels were placed floating on a CO₂-free phosphate buffer (0.2 M) containing 35 mM KCl with various concentrations of H₂O₂. The CO₂ free buffer was made from boiled distilled water (15 min) and the buffer was bubbled with argon for 30 minutes before use. Epidermal peels with initially open stomata were floated on 1 mM H₂O₂ in the CO₂ free phosphate buffer under continuous

light for 3 hours. The peels exposed to H_2O_2 showed a significant reduction in stomatal aperture compared to the control peels (Fig. 2A). Stomata in epidermal peels from *Commelina communis* have previously been shown to respond similarly to H_2O_2 (McAnish et al., 1996).

To investigate the effect of H_2O_2 on stomatal opening, epidermal peels with initially closed stomata were exposed to various concentrations of H_2O_2 , in the range from 0.1 to 10 mM, for 3 hours under continuous light. The H_2O_2 treatments inhibited light-induced stomatal opening in a dose dependent matter (Fig. 2B).

The fluorescein diacetate assay (Widholm, 1972) was used to evaluate the viability of the guard cells in the epidermal peels after exposure to H_2O_2 . The green fluorescence in the epidermal peels exposed to 0 or 0.1 mM H_2O_2 for 3 hours was discretely located to the chloroplasts in the majority of the guard cells. Most of the guard cells of the epidermal peels exposed to 1 or 10 mM H_2O_2 also showed a green fluorescence (indicating viable cells). The fluorescence was more diffusely spread throughout the whole cell in the peels treated with the highest concentrations of H_2O_2 . This could indicate that H_2O_2 (1 and 10 mM) irreversibly damaged to the cells. McAnish et al. (1996) also found that exposure of epidermal peels from *Commelina communis* to 1 mM H_2O_2 for 3 hours caused a diffuse fluorescence in the FDA-treated guard cells compared to the control cells. Three hours is a considerably longer exposure time than was used for the protoplast treatments in the patch clamp experiments (see below).

Figure 2: (A) Hydrogen peroxide (1 mM) causes stomatal closure in epidermal peels from *Vicia faba*. The epidermal peels were floated on a CO_2 -free 0.2 M phosphate buffer with 35 mM KCl with and without 1 mM H_2O_2 for 3 hours in the light. The stomata were initially open. (B) Hydrogen peroxide inhibits light-induced stomatal closure in epidermal peels from *Vicia faba*. The epidermal peels with initially closed stomata were made in the dark and transferred to light while floating on a CO_2 -free 0.2 M phosphate buffer containing 35 mM KCl and different concentrations of H_2O_2 ranging from 0 to 10 mM.

3.3 Hydrogen peroxide enhances plant stomata closure by increasing K^+ efflux from guard cells via outward rectifying K^+ channels.

In an attempt to understand the effects of the common pollutant ozone on plant stomatal aperture, we examined the effect of one of the ozone intermediates, H_2O_2 , on stomatal guard cells of *Vicia faba*. Pretreatment with ozone, either for 3 days prior to leaf harvesting (ozone_I), or during the stage of epidermal peels in the process of protoplast isolation on the day of the experiment (ozone_{II}), increased the subsequently measured outward potassium currents via K_D channels (Fig. 3). This was due (a) to increased maximum conductance, G_{max} , and (b) to the shift, by several mV, of the voltage dependence of gating to more hyperpolarized potentials. The effect on outward rectifying K^+ channel (K_D channel) gating was reflected also in the shortening of the activation half-time after the prolonged ozone exposure.

Since H_2O_2 (≤ 10 mM) decreased stomatal aperture in response to opening signals and closing signals, and since stomatal closure results from osmotically decreased volume of guard cells due to the loss of ions, we examined the effect of H_2O_2 on the outwardly-rectifying K^+ channels (K_D channels), which conduct the K^+ efflux via the plasma membrane. In electrophysiological (patch clamp) recordings from whole guard cell protoplasts, bath application of H_2O_2 (8-10 mM final conc.) consistently increased the outward K^+ current through K_D channels, and this effect seemed to peak within 10-20 min of treatment. This was chiefly due to a 20 mV shift of the voltage dependence of K_D channels activity in the direction of hyperpolarization, as determined at the apparent peak of H_2O_2 effect. In contrast, the maximum conductance G_{max} , did not increase significantly with H_2O_2 treatment. Taking into account the gradual spontaneous decline of channel activity during our experiments, the H_2O_2 -elicited effect may become even more dramatic during prolonged exposures *in situ*. H_2O_2 is an early intermediate in various plant responses to environmental challenges such as exposure to ozone, mechanical stimulation, response to pathogens, etc. It appears to be correlated with increased membrane permeability to ions. Enhanced K_D channel recruitment and, perhaps, also, increased unitary conductance, possibly underly the increased ions loss from plant cells during an oxidative burst response.

Figure 3. Typical I_K currents through K_D channels in ozone-pretreated (A-B) and in control (C) guard-cell protoplasts. **Left-top panel:** Pulse protocol; 2.5 s -long depolarizing pulses were applied at 13 s intervals, increasing from -57 mV to 83 mV in steps of 20 mV; between pulses, membrane potential was held at -87 mV. **A-C:** Whole-cell current traces, superimposed, recorded from the following groups (see text): A: "Ozone_I"; B: "Ozone_{II}"; C: Control_I"; **D:** Current-voltage (I-V) relationships of the currents depicted in Figs 1A-1C; for clarity, relations between figures are shown by symbols. **E:** Mean I-V relations ($\pm$ SE) of the 4 groups of protoplasts: "Ozone_I" ($\square$; n=4); Ozone_{II} ($\circ$; n=17); Control_I ($\diamond$; n=27); Control_{II} ($\square$; n=5)

3.4 Ca^{2+} imaging of O_3 exposed epidermal peels of *Nicotiana tabacum*

An O_3 exposure system was developed to perform imaging on submerged abaxial epidermal peels, which was necessary to perform Ca^{2+} imaging. Peels were fixed to a glass cover slip with high-pressure vacuum grease and submerged in an O_3 saturated 100 mM potassium phosphate buffer (pH 5.8) for 15 minutes in the dark. After 15 minutes this buffer was removed and replaced with 100 mM KCl and the peels were exposed to white light. Inhibition of stomatal opening by O_3 was observed consistently in submerged abaxial epidermal peels of *Nicotiana tabacum* cv SR-1 (data not shown). To determine indirectly if Ca^{2+} plays a role in the stomatal response to O_3 we pretreated the exposure buffer with Chelex, to remove Ca^{2+} . The experiments were performed following the same

protocol as the previously mentioned submerged peel experiments except the 100 mM potassium phosphate buffer was pretreated with Chelex (a Ca^{2+} chelating resin) and removed, before O_3 saturation. We consistently found a loss of O_3 induced inhibition of stomatal opening in submerged abaxial epidermal peels of *N. tabacum* cv SR-1 when Ca^{2+} was removed from the exposure buffer (Fig. 4).

Figure 4. Abaxial epidermal peels from leaves were freshly prepared from *Nicotiana tabacum* cv. Petite Havana taken from the dark, to ensure closed stomata. Peels were submerged in a 100 mM potassium phosphate buffer (pH 5.8) +/- O_3 saturation for 15 minutes in the dark. The buffer was then removed and peels were submerged in 100 mM KCl and placed in the light. Stomatal apertures were determined with a light microscope and an ocular at 90, 210 and 350 minutes after end of exposure. (A) < 100 ml/l O_3 exposure of submerged abaxial epidermal peels. Representing the mean of three individual experiments ($n=60 \pm \text{SE}$). (B) < 100 ml/l O_3 exposure of submerged abaxial epidermal peels. Potassium phosphate buffer pretreated with Chelex to remove Ca^{2+} present during the O_3 exposure. The data are presented as the mean of four individual experiments ($n=100 \pm \text{SE}$). (Weightman S.R., E.J.P., S.M.A., unpublished results).

To further investigate the role of Ca^{2+} we used imaging technology. Indo-1 was introduced into the tobacco guard cells of submerged epidermal peels by acid loading according to the recently developed method of Allen et al. (1999). Confocal imaging confirmed retention of the dye in the cytosol. Cytosolic Ca^{2+} concentration was measured by taking a ratio of the fluorescence emission intensity at the Ca^{2+} -dependent wavelength (400 nm) versus that of a Ca^{2+} -independent wavelength (475 nm). Measurements were taken once every minute for a total of 15 minutes after treatment. We were unable to detect any changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ after exposure to the same O_3 dose that resulted in inhibition of stomatal opening (Fig. 5).

Figure 5. Measurement of Guard Cell $[Ca^{2+}]_{cyt}$ in Response to O_3 Treatment. Abaxial epidermal peels of *Nicotiana tabacum* cv. Petite Havana were acid loaded (see Materials and Methods) with Indo-1. Peels were taken from plants after the 8 h dark cycle to ensure closed stomata after acid loading. (A) The zero time point represents resting $[Ca^{2+}]_{cyt}$ before +/- O_3 treatment in a 100 mM potassium phosphate buffer (pH 5.8). Immediately after the initial measurement +/- O_3 saturated potassium phosphate buffer was added; measurements continued every 60 s for 15 min. Open circles (○) represent the addition of O_3 -free potassium phosphate buffer, and closed circles (●) represent the addition of O_3 saturated potassium phosphate buffer. There was no effect on $[Ca^{2+}]_{cyt}$ after the addition of O_3 -free buffer ($n=8$). Additionally, there was no effect of O_3 on guard cell $[Ca^{2+}]_{cyt}$ ($n=13$), showing no significant change in $[Ca^{2+}]_{cyt}$ over time. The results represent the average fluorescence ratio of individual cells, $\pm$ SE. (B) Qualitative representation of $[Ca^{2+}]_{cyt}$ during control and O_3 treatment. Pseudocolor images are from one representative cell's response out of the total treated. The top row represents the control cells and the bottom row represents the O_3 -treated cells. The first column represents time 0, illustrating

resting $[Ca^{2+}]_{cyt}$, with each subsequent column representing 5 min intervals. There was no change in the dye distribution or color intensity, which would indicate a response in either treatment. (Weightman S.R, E.J.P., S.M.A, unpublished results).

The direct measurement of guard cell Ca^{2+} conflicts with the peel experiment data, which suggested Ca^{2+} was involved in the inhibition of light-induced stomatal opening. One possible explanation for the absence of a change in $[Ca^{2+}]_{cyt}$ is that if an increase in $[Ca^{2+}]_{cyt}$ did occur it was below the limits of detection of the system. Based on the statistical analysis of the Ca^{2+} standard calibration using Indo-1, the system requires a ≥ 100 nM change in $[Ca^{2+}]_{cyt}$ to detect a significant change in $[Ca^{2+}]_{cyt}$ (personal communication, Simon Gilroy). The free Ca^{2+} of the potassium phosphate buffer was found to be approximately 10 nM, and based on the imaging data, is lower than that present at resting in the guard cell cytosol. Therefore, there was probably insufficient Ca^{2+} present in the external buffer to observe an increase in $[Ca^{2+}]_{cyt}$.

3.5 Single K^+ channel recording of O_3 -exposed membrane patches isolated from *Vicia faba*.

We tried to apply the single channel patch clamp technique to determine if the effect of O_3 on ion currents is membrane-delimited. We were able to record single channel activity from out-side out patches, but found it difficult to obtain the seal when perfusing the bath dish with a solution that would not react with O_3 (100 mM K^+ , 10 mM $CaCl_2$). Therefore, we can not conclude whether O_3 reacts directly with the K^+ channels or if the observed reduction in K^+ currents at the whole cell levels is caused by an indirect mechanism.

3.6 Evaluation of O_3 sensitivity in three Israeli cultivars of zucchini.

Visible symptoms were observed on zucchini in Israel and it was thought that these symptoms could be caused by O_3 . Seeds of three cultivars, Clarita, Yarden and Bareqet were sent to the US for controlled O_3 -exposure experiments. The plants were exposed to $0.12 \mu l O_3 l^{-1}$ for 8 hours a day in open top chambers. Already after 2 days of exposure the first visible symptoms appeared on two of the O_3 -exposed cultivars, Yarden and Bareqet. All the O_3 -exposed plants showed symptoms after one week of daily exposure (Fig. 6). These results show that these cultivars of zucchini are relatively sensitive to O_3 . However the visible symptoms did not resemble those observed in field grown zucchini. Insect damage may account for that injury (van de Ven et al, 2000).

Figure 6. Ozone symptoms of three cultivars of *Curcubita pepo* L. Plants in open-top chambers were treated with $0.120 \mu\text{l l}^{-1}$ O_3 from 1000-1800 hours each day. **A-C** depict O_3 injury in Yarden (A), Bareqet (B), left Clarita and right Yarden (C). **D** (Bareqet) and **E** (Yarden) illustrate the contrast between O_3 -treated (right) and charcoal-filtered air treated plants (left).

3.7 The influence of O_3 on two environmentally important pine species in Israel

The effects of O_3 on seedlings and saplings of *Pinus halepensis* and *Pinus brutia* were investigated. In short-term exposures to $0.30 \mu\text{l l}^{-1}$ ($=300$ ppb) O_3 , both species showed signs of visible injury, but in *P. halepensis* the extent of seedlings chlorosis was double that in *P. brutia*. The rate of photosynthesis in saplings exposed to unpolluted air was higher in *P. halepensis* than in *P. brutia*, while a decrease in photosynthesis was measured in both species after exposure to O_3 (Fig. 7). In chlorophyll *a* fluorescence measurements, both species showed declines in relative variable fluorescence values, interpreted as increased fluorescence from disorganized chlorophyll (data not shown). This phenomenon appeared in both seedlings and saplings. There was also an increase in the activity of the superoxide dismutase enzyme (data not shown). The results indicate that *P. brutia* is more tolerant to O_3 stress than *P. halepensis*.

Figure 7. Net CO₂ assimilation (A) in pine sapling needles measured after 8 days exposure to 0 ppb (ozone-free air) or 300 ppb ozone. Photosynthesis was measured immediately after the O₃ treatment.

3.8 Ozone-induced Antioxidant enzyme activity in the mesophyll and epidermis of *Commelina communis* L.

Epidermis and mesophyll tissues differed from each other in the antioxidative activity. On the basis of equal protein concentration, the enzymes peroxidase and cytoplasmic superoxide dismutase were more active in epidermis tissue than in mesophyll, under control conditions. While the enzymes catalase, chloroplastic superoxide dismutase and glutathione reductase were significantly more active in mesophyll tissue. Ozone treatment increased the antioxidative activity and ascorbate content in both tissues, only when stomata were open. When abscisic acid was applied, the antioxidative activity was not affected by the ozone treatment.

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Ozone inhibits guard cell K⁺ channels implicated in stomatal opening

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Ozone (O₃) deleteriously affects organisms ranging from humans to crop plants, yet little is understood regarding the underlying mechanisms. In plants, O₃ decreases CO₂ assimilation, but whether this could result from direct O₃ action on guard cells remained unknown. Potassium flux causes osmotically driven changes in guard cell volume that regulate apertures of associated microscopic pores through which CO₂ is supplied to the photosynthetic mesophyll tissue. We show in *Vicia faba* that O₃ inhibits (i) guard cell K⁺ channels that mediate K⁺ uptake that drives stomatal opening; (ii) stomatal opening in isolated epidermes; and (iii) stomatal opening in leaves, such that CO₂ assimilation is reduced without direct effects of O₃ on photosynthetic capacity. Direct O₃ effects on guard cells may have ecological and agronomic implications for plant productivity and for response to other environmental stressors including drought.

Ozone (O₃) in the upper atmosphere is highly beneficial as a UV filter, but O₃ of anthropogenic origin at ground level is a serious pollutant. Elevated ground-level O₃ is associated with impaired human health (1, 2), reduced primary production in forest ecosystems (3), and losses in agricultural yield estimated at \$3 billion annually for the U.S. alone (4). However, the cellular targets of O₃ action remain essentially unknown. In plants, O₃ reduces rates of photosynthetic carbon fixation. Such decreases could reflect (i) direct effects of O₃, and/or the reactive oxygen intermediates generated on O₃ exposure (-OH, H₂O₂ etc.), on the interior, photosynthetic mesophyll cells; or (ii) a decrease in CO₂ availability to the mesophyll cells, as a result of impaired function of guard cells in the plant epidermis. Guard cells control rates of CO₂ entry to the mesophyll by regulating the apertures of the stomatal pores in the leaf epidermis through which gas exchange occurs. Reduced stomatal conductance is commonly observed after O₃ exposure (5, 6), and it is possible that these reductions reflect a primary response of the guard cells to O₃ (7). Alternatively, reduced stomatal conductance could be a secondary response of the guard cells after either O₃ injury of adjacent epidermal cells (8) or alterations in the leaf environment caused by O₃ damage to the mesophyll tissue. In particular, high internal CO₂ concentrations, as result from inhibition of carbon assimilation, are a potent signal for stomatal closure (9) and have been hypothesized to be the proximate cause of the stomatal responses to O₃ (10).

Because the changes in guard cell volume that regulate stomatal apertures are driven in large part by ion uptake and loss, we hypothesized that, if O₃ does directly affect guard cells, it may do so by altering the activity of ion channels in the guard cell plasma membrane. There is indirect evidence that O₃ affects the plant plasma membrane because altered membrane permeability and ion leakage are frequently detected in leaves and in the unicellular alga *Chlorella sorokiniana* after treatment with the gas (11). Little research has been performed on the underlying mechanisms, although two studies have demonstrated that membrane vesicles isolated from leaves of O₃-treated pinto bean (*Phaseolus vulgaris* L. var Pinto) plants display altered Ca²⁺ transport (12) and a decrease in K⁺-stimulated ATPase activity (13). However, all of these studies lack specificity with regard to cell type and involvement of molecularly defined transport

molecules. In mammals, an effect of O₃ on ion transport in airway epithelial cells also has been suggested by indirect measurements (14, 15) but never tested by direct electrophysiological measurements of ion channel activity.

In response to stimuli that elicit stomatal opening, K⁺ influx increases guard cell osmotic content, resulting in water uptake, a bowing out of the two guard cells that define each stomatal pore, and an increase in stomatal aperture. K⁺ influx occurs through inwardly rectifying K⁺ channels and is driven by membrane hyperpolarization caused by H⁺ ATPase activation and anion channel inactivation. Conversely, stimuli that elicit stomatal closure inhibit H⁺ ATPase activity and activate anion channels, resulting in membrane depolarization that both activates outwardly rectifying K⁺ channels and provides the driving force for K⁺ efflux (16, 17).

In the present study, we used the technique of whole cell patch clamping to directly assess the effects of O₃ on plasma membrane K⁺ channels of guard cells of fava bean (*Vicia faba*), the most commonly used species for cellular studies of guard cell function. The influence of O₃ on stomatal regulation was also studied in isolated epidermal peels and whole plants.

Materials and Methods

Growth Conditions. Broad bean (*V. faba*) plants were grown from seeds in 3 parts Metro-mix 360 to 1 part Perlite and were watered 3–4 times per week with ¼ strength Hoagland solution. Plants used for epidermal peel and patch-clamp experiments were grown in environmental growth chambers under a 10-hr light/14-hr dark cycle with a light intensity of 200 μmol·m⁻²·s⁻¹ and a temperature of 25/23°C. Plants used in the gas exchange experiments were grown in a greenhouse under natural illumination with 200 μmol·m⁻²·s⁻¹ supplemental illumination between 8 a.m. and 8 p.m. The youngest fully expanded leaves from 3- to 4-week-old plants were used in all experiments.

Ozone Exposure. Protoplasts were isolated from three fully expanded leaves as described (18, 19). The protoplasts were exposed to O₃ in a 300 mM K⁺-phosphate buffer (pH 5.7) containing 2 mM MgCl₂, 1 mM CaCl₂, and 40 mM K₂SO₄ to a final osmolality of 440 mOsm·kg⁻¹. Ozone was introduced into the buffer, which was determined not to scavenge O₃, by bubbling 930 ml·liter⁻¹ O₃ (in O₂) through the buffer for 30 min. The buffer was treated with O₃ before cell exposure. Ozone concentration in the buffer was determined spectrophotometrically by using the indigo method (20). In the first experiment (Fig. 1 A and B), the protoplasts were pretreated with the O₃-containing buffer (33–354 μl·liter⁻¹) for <5 min and were immediately transferred to bath solution I (see below) for patch clamping. In the second experiment (Fig. 1 C and D), O₃-

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Fig. 1. Ozone exposure of *V. faba* guard cell protoplasts reduces inward K^+ currents. (A) Representative whole cell currents recorded from one O_3 -treated and one nontreated cell using 100 mM K^+ bath and pipette solutions (I). Currents were identified as K^+ currents based on their reversal potentials (data not shown) and their characteristic time-activation and voltage-dependence (24). Seal resistance for the cells in A was 2.0 G Ω for the O_3 -treated cell and 1.2 G Ω for the nontreated cell. (B) Average voltage-activated whole cell K^+ currents, normalized for cell size, measured at membrane potentials of +76 mV and -164 mV. For both A and B, protoplasts were exposed to 33–354 $\mu\text{L}\cdot\text{liter}^{-1}$ O_3 ($n = 21$) for <5 min in a 300 mM K^+ -phosphate buffer (see *Material and Methods*). Controls were protoplasts exposed to O_2 -treated phosphate buffer (O_2 , $n = 14$) or to nontreated phosphate buffer (N, $n = 11$). Protoplasts were transferred to bath solution I for immediate patch clamping after exposure, and whole cell recordings were performed between 8 and 50 min after exposure, depending on the time required to obtain a whole cell seal. Seal resistance ranged from 0.9 to 4 G Ω for the 46 cells of this experiment and did not differ significantly between treatments (data not shown). Ozone induced a significant ($P < 0.01$) reduction in inward currents compared with O_2 - and nontreated protoplasts; there was no significant treatment effect on the outward currents. (C) Voltage-activated whole cell currents measured before and after treatment (treatment at time 0) at membrane potentials of +63 mV for O_3 -treated (●) or nontreated (○) protoplasts and at -177 mV for O_3 -treated (■) or nontreated (□) protoplasts. The effects on inward K^+ current of O_3 , time, and O_3 treatment by time are significant at the $P < 0.05$ level. (D) Current-voltage relationships from whole cell measurements recorded 10 min (indicated by arrow in C) after adding O_3 -treated (■, $n = 6$) or nontreated buffer (□, $n = 8$) directly to the bath solution (phosphate buffer described above). Ozone significantly inhibited inward K^+ current at voltages ≤ -117 mV. For both C and D, the whole cell configuration was obtained with a 10 mM K^+ bath solution (II) and a 100 mM K^+ pipette solution (II). The bath was thereafter perfused with the phosphate buffer described in *Materials and Methods* for 3 min, and 200 μL of O_3 -treated or nontreated phosphate buffer was added to the 1-ml bath dish ≈ 5 min after the end of perfusion. The initial O_3 concentration in the dish ranged from 10 to 30 $\mu\text{L}\cdot\text{liter}^{-1}$ and in the presence of the protoplasts the concentration dropped by 75% within 10 s.

containing buffer was added during the patch clamp experiment to protoplasts bathed in the phosphate buffer described above. The initial concentration after adding O_3 to the dish ranged from 10 to 30 $\mu\text{L}\cdot\text{liter}^{-1}$ O_3 . The concentration dropped by 75% within 10 s. Because O_3 is so unstable and remained in solution for brief periods of time, it was not possible to obtain a repeatable concentration of the gas in each of these experiments. Despite this variation, similar inhibition of the inwardly rectifying K^+ currents ($I_{K_{in}}$) was observed throughout the range of O_3 con-

centrations. It has been observed by others (see ref. 11 for review and references) that relatively high concentrations of O_3 are required to elicit a response when cells or biochemicals are exposed to the gas *in vitro*. This may relate to the solubility of the gas and the scavenging potential of the cells.

The epidermal peels were exposed to air with or without 1.0 $\mu\text{L}\cdot\text{liter}^{-1}$ O_3 for 3 hr in a custom-made Petri dish with a gas inlet and outlet. The flow through the Petri dish during exposure was 400 $\text{mL}\cdot\text{liter}^{-1}$. Whole plants were treated with 0, 0.10, or 0.18

$\mu\text{l-liter}^{-1}$ O_3 in continuous stirred tank reactors (21) located in a charcoal-filtered greenhouse.

Patch Clamp Recording. Two different sets of solutions were used for the patch-clamp recordings presented in Fig. 1 *A* and *B* (bath and pipette solution I) and *C* and *D* (bath and pipette solution II). Bath solution I contained 100 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 5 mM Mes, 5 mM Hepes at pH 5.6 (adjusted with Tris), and sorbitol to a final osmolality of 460 $\text{mOsm}\cdot\text{kg}^{-1}$. Pipette solution I contained 80 mM K^+ glutamate, 20 mM KCl, 2 mM MgCl_2 , 10 mM Hepes, 2 mM EGTA, and 2 mM Mg-ATP (Sigma) (added daily) at pH 7.8 (KOH) and adjusted to 480 $\text{mOsm}\cdot\text{kg}^{-1}$ with sorbitol. Bath solution II contained 10 mM K-glutamate, 1 mM CaCl_2 , 2 mM MgCl_2 , 10 mM Mes, 0.25–0.50 mM *N*-methyl glucamine (pH 5.5), and mannitol to a final osmolality of 480 $\text{mOsm}\cdot\text{kg}^{-1}$. This solution was used initially in experiments presented in Fig. 1 *C* and *D* because it promoted formation of a high resistance ($\text{G}\Omega$) seal. Before O_3 exposure of the cells, this solution was exchanged by bath perfusion and was replaced with the phosphate buffer previously described. Pipette solution II contained 100 mM K-glutamate, 2 mM MgCl_2 , 0.2 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate, 4 mM Mg-ATP, 20 mM Hepes, and mannitol to a final osmolality of 520 $\text{mOsm}\cdot\text{kg}^{-1}$.

Whole-cell currents were measured in response to 1.8-s voltage pulses decremented in -20-mV steps from $+63\text{ mV}$ to -177 mV , using an Axopatch-1B patch-clamp amplifier and a TL-1 DMA Interface (both from Axon Instruments, Foster City, CA). Between measurements, the holding potential was kept for 10.25 s at -54 mV and -67 mV , respectively, in the experiments presented in Fig. 1 *A* and *B* and Fig. 1 *C* and *D*. A schematic of the voltage stimulus is given in Fig. 1*A*. The sampling rate was 1 ms. Currents were filtered at a -3dB frequency of 500 Hz by the lowpass Bessel filter of the patch-clamp amplifier. Data analyses were accomplished by using PCLAMP 6.0.2 software (Axon Instruments). Whole-cell currents were corrected for time-independent or leak currents and were normalized—i.e., expressed in pA/pF —to eliminate differences caused by cell-to-cell variations in surface area (22). Potentials reported have been corrected for liquid junction potentials (23).

Epidermal Peel Assay. In stomatal opening experiments, abaxial epidermal peels were floated on 35 mM KCl during 3 hr of exposure to air with or without $1.0\text{ }\mu\text{l-liter}^{-1}$ O_3 . The plants used for isolation of epidermal peels were kept in the dark overnight before each experiment to ensure that stomata were closed. The isolated epidermal peels were simultaneously exposed to light ($200\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) in the presence or absence of O_3 . In stomatal closure experiments, abaxial epidermal peels were floated on 35 mM KCl/0.5 mM CaCl_2 for 3 hr during exposure to air in the presence or absence of $1.0\text{ }\mu\text{l-liter}^{-1}$ O_3 under continuous light ($200\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Before the isolation of the epidermal peels for the closure experiments, the abaxial side of detached leaves was exposed to light ($200\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 2 hr to ensure open stomata. Widths of stomatal apertures were determined with an ocular micrometer and a light microscope (Leitz Dialux 20).

Gas Exchange Measurements. Stomatal conductance, assimilation, and A/C_i curves (carbon assimilation as a function of the intercellular concentration of CO_2) were measured with a Li-Cor 6400 gas exchange system (Li-Cor, Lincoln, NE). Conditions in the gas exchange cuvette during measurements were 30% relative humidity, $350\text{ }\mu\text{l-liter}^{-1}$ CO_2 and $800\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity provided by a red/blue light source (Li-Cor).

Statistical Analysis. The patch clamp data were analyzed by analysis of covariance (ANCOVA) using O_3 concentration and time between treatment and measurement of whole cell current

Table 1. Change in the extent of opening or closure of stomata in epidermal peels of *V. faba* on O_3 exposure ($1.0\text{ }\mu\text{l-liter}^{-1}$ for up to 3 hr)

Time, hr	Stomatal opening: Mean widths, $\mu\text{m} \pm \text{SE}$		Stomatal closure: Mean widths, $\mu\text{m} \pm \text{SE}$	
	O_3	Air	O_3	Air
0	$0.5 \pm 0.2^*$	$0.5 \pm 0.2^*$	$8.1 \pm 0.5^*$	$8.1 \pm 0.5^*$
1			7.3 ± 0.7	8.1 ± 0.4
1.5	6.4 ± 0.6	7.4 ± 0.7	—	—
2	—	—	6.3 ± 0.6	7.1 ± 0.6
3	$8.4 \pm 0.5^+$	10.6 ± 0.6	6.1 ± 0.6	7.0 ± 0.6

Ten stomatal apertures were measured on three epidermal peels for each treatment/time point in each of the six replicates of both opening and closure experiments (total $n = 180$).

*The 0-hr time point represents a set of peels measured before the initiation of treatments.

⁺Significant difference ($P < 0.01$) between O_3 and air treatment.

as covariates in the first experiment (Fig. 1*B*). In the second experiment (Fig. 1 *C* and *D*), O_3 concentration was used as the covariate in a multiple analysis of covariance (MANCOVA). Multiple analysis of variance (MANOVA) was used to analyze the data from the epidermal peel and gas exchange experiments.

Results and Discussion

Two types of whole-cell patch clamp experiments were performed. In the first set of experiments (Fig. 1 *A* and *B*), protoplasts were pretreated with O_3 and subsequently subjected to whole-cell patch-clamp analysis. In the second set of experiments, protoplasts were exposed to O_3 after attainment of the whole-cell patch-clamp configuration, such that current responses before and after O_3 exposure could be evaluated on the same cell (Fig. 1 *C* and *D*). In both types of experiments, O_3 significantly ($P < 0.05$) reduced the magnitude of inward K^+ currents but had no significant effect on the magnitude of the outward K^+ currents. The reduction in inward K^+ current over time was significantly greater in O_3 -treated than in control cells. The current-voltage relationship of the inward K^+ currents was negatively shifted by O_3 exposure whereas the characteristic voltage dependence of the channels was maintained (Fig. 1*D*). No appreciable effects were observed either on the kinetics of current activation (Fig. 1*A*; data not shown) or on ion selectivity, as ascertained by tail current analyses (data not shown).

These results are noteworthy for three reasons. First, they demonstrate specific inhibition of an ion channel by O_3 , as has not been previously demonstrated for any biological system. Second, they demonstrate that guard cells are affected by O_3 directly. Third, because the inward K^+ channels were inhibited, these results lead to the hypothesis that O_3 acts not, or not exclusively, by promoting stomatal closure, as has been widely assumed (6), but rather that O_3 is a potent inhibitor of stomatal opening. These two different loci of action have significantly different agricultural and ecological consequences (see *General Discussion*).

To test the hypothesis derived from the patch clamp experiments that O_3 significantly inhibits stomatal opening, we measured stomatal apertures in isolated epidermal peels of *V. faba* stripped from the mesophyll tissue and exposed to O_3 . Stomatal opening in response to light was significantly inhibited during a 3-hr O_3 exposure (Table 1). Although stomatal opening was inhibited by only 20%, these results have been confirmed in two other species, *Arabidopsis thaliana* and *Nicotiana tabacum*. Ozone exposure ($0.30\text{ }\mu\text{l-liter}^{-1}$ for 3.5 hr) resulted in 20 and 31% inhibition of light-induced stomatal opening in epidermal peels of *A. thaliana* and *N. tabacum*, respectively, with both

Fig. 2. Stomatal conductance (A and D) and assimilation (B and E) measured on the first fully expanded leaves of *V. faba* plants during exposure to charcoal-filtered air containing 0 (□), 0.10 (▲), or 0.18 (●) $\mu\text{l-liter}^{-1} O_3$ for 4 hr in continuous stirred tank reactors (24). (A–C) The plants were kept in the dark overnight and until the start of exposure to allow observation of the influence of O_3 on light-induced stomatal opening (natural illumination supplemented with $150 \mu\text{mol-m}^{-2}\text{s}^{-1}$). Both O_3 treatments resulted in significant ($P < 0.01$) reductions in stomatal conductance and assimilation. (D–F) The plants were kept in the light (natural illumination supplemented with $150 \mu\text{mol-m}^{-2}\text{s}^{-1}$) for 4 hr before exposure to ensure open stomata at start of exposure. Only the highest O_3 treatment resulted in significant ($P < 0.01$) reduction of stomatal conductance (after 3 and 4 hr) and assimilation (after 4 hr). In each replicate experiment, gas exchange was measured on five individual plants for each treatment/time point. (C and F) Assimilation (A) versus intercellular CO_2 concentration (C_i) measured within 60 min after the end of O_3 exposure, on the first fully expanded leaf of two individual plants for each treatment. The average A/C_i curve for the plants exposed to $0.10 \mu\text{l-liter}^{-1} O_3$ was not significantly different from that of plants exposed to charcoal-filtered air whereas plants exposed to $0.18 \mu\text{l-liter}^{-1} O_3$ had significantly lower A/C_i curves ($P < 0.05$). For all panels, the average of three experiments is shown.

reductions significant at the $P \leq 0.05$ level (S. R. Weightman, E.J.P., and S.M.A., unpublished work). The importance of these results is two-fold. First, the data support the hypothesis derived from the electrophysiological measurements that O_3 inhibits stomatal opening. Second, consistent with the patch clamp results, they demonstrate that intact guard cells are indeed directly affected by O_3 . Although this possibility had been previously inferred (25), no direct evidence had ever been presented to support it. The same concentration of O_3 did not stimulate significant differences in stomatal closure at any of the individual time points measured. Guard cells retained competence to drive stomatal closure under these circumstances, as evidenced by stomatal closure when control peels were transferred to darkness under otherwise identical conditions [final aperture of $1.8 \pm 0.3 \mu\text{m}$ after 3 hr of dark incubation (data not shown)]. Greater stomatal closure of O_3 -treated epidermal peels was statistically significant when data from all individual time points were pooled (not shown), suggesting that there could be a small O_3 effect. Our inability to detect effects of O_3 on outward K^+ channels could have resulted because the impact was minimal and variability in electrophysiological recordings precluded detection. Alternatively, O_3 induction of stomatal closure might have resulted from effects on transporters other than K^+ channels. For example, opening of anion channels has been proposed to play a central role in stomatal closure by both directly mediating anion loss and by depolarizing the membrane, which then drives loss of K^+ (26, 27). Taken together, the results of the patch clamp and epidermal peel experiments led to two hypotheses concerning stomatal function in the intact plant. First, we hypothesized that, if leaves were treated with O_3 under conditions in which stomata initially were closed, subsequent stomatal opening would be impaired. Second, we hypothesized that O_3 -inhibition of stomatal opening and thus of CO_2 influx could lead to reductions in photosynthetic rate even in the absence of any direct effects of O_3 on mesophyll photosynthesis. To test these hypotheses, whole *V. faba* plants were exposed to 0, 0.10, or $0.18 \mu\text{l}\cdot\text{liter}^{-1}$ O_3 for 4 hr. Exposures were initiated under darkness, a condition in which stomata are closed and stomatal conductance is low. Subsequent illumination stimulated stomatal opening, resulting in increases in stomatal conductance, as measured through nondestructive gas exchange analysis. Both 0.10 and $0.18 \mu\text{l}\cdot\text{liter}^{-1}$ O_3 significantly inhibited the increase in stomatal conductance and decreased rates of mesophyll carbon assimilation relative to control plants (Fig. 2A and B). At the end of the exposure, photosynthetic capability of the mesophyll tissue was assessed by A/C_i curves (Fig. 2C). Because C_i is experimentally manipulated in these protocols, the role of stomatal response in controlling photosynthetic rates is "eliminated." The A/C_i curves revealed that, at the lower O_3 concentration, there was no direct effect of O_3 on carbon assimilation; at a given C_i , assimilation rates were identical in O_3 -treated and control plants. Therefore, the reduced rates of carbon assimilation seen under those conditions could only be attributed to a reduced availability of the substrate CO_2 to the mesophyll, as a result of the O_3 -inhibition of stomatal opening. At the higher O_3 concentration, a direct effect of O_3 on mesophyll photosynthesis could be observed as well, detected as a depression in the A/C_i curve; these results agree with previous reports (28–31). The same O_3 treatments then were applied to plants with initially open stomata (Fig. 2D–F). Only when the photosynthetic apparatus was directly affected by the O_3 treatment (at $0.18 \mu\text{l}\cdot\text{liter}^{-1}$ O_3) as indicated by the A/C_i curve (Fig. 2F), did significant stomatal closure and reduction in assimilation occur (Fig. 2D and E). At the lower O_3 dose ($0.10 \mu\text{l}\cdot\text{liter}^{-1}$ O_3), stomata did not close significantly in response to O_3 , despite the fact that this dose sufficed to inhibit stomatal opening (Fig. 2A), and photosynthesis was unaffected. These results further support the conclu-

sion that the process of stomatal opening is more sensitive to O_3 than is the induction of stomatal closure.

General Discussion

The experiments reported here show that O_3 directly affects guard cells themselves, targeting inward K^+ channels, and that O_3 inhibits stomatal opening. These results challenge the prevailing dogma that O_3 , either directly or indirectly, promotes stomatal closure. Moreover, it has been thought that, if stomata are closed at the time of O_3 exposure, there will be minimal adverse effects because the gas will be unable to penetrate to the interior photosynthetic mesophyll (32, 33). However, our results do not support this theory, showing instead that O_3 exposure initiated while stomata are closed can depress subsequent carbon assimilation via O_3 action solely on the guard cells. Our whole plant experiments were conducted with concentrations of O_3 that can be found in polluted atmospheres (34); this is particularly true for the treatment with $0.10 \mu\text{l}\cdot\text{liter}^{-1}$ O_3 . These studies, therefore, have important agronomic and ecological ramifications, as illustrated by the following three examples. First, stomatal closure during a period of drought may be less readily reversed in O_3 -exposed plants. This is particularly relevant because the highest O_3 concentrations are sometimes associated with times of drought: e.g., as was reported in Great Britain during significant droughts in 1976 and 1982 (35). In fact, when ash trees (*Fraxinus excelsior* L.) received long term O_3 exposures during periodic drought exposures, there was some evidence that stomatal conductance might recover more slowly during re-watering in O_3 -treated than control plants (36). Second, in major agricultural regions with high light environments and significant O_3 exposure—e.g., the South Coast Air Basin of California, which has the most extreme O_3 levels in the U.S. (37)—midday stomatal closure ("mid-day depression") often occurs because of the low ambient humidity that results from the high light, high temperature conditions of midday. Because the generation of O_3 in photochemical smog depends on high solar irradiation (34), O_3 inhibition of stomatal opening could significantly retard stomatal reopening in the afternoon after this mid-day depression and consequently reduce crop yield. Third, the presence of nighttime O_3 has been documented in rural locations due to long distant transport from urban sources (34) and at high elevation sites, including sites of ecological importance such as Shenandoah National Park in Virginia (38, 39). Although it has been reported that there can be an adverse impact on biomass accumulation when O_3 exposure of plants occurs exclusively in the dark (39, 40), this impact was previously explained by the modest stomatal conductance that could be detected under darkness and the concomitant O_3 uptake that would be expected. Our results suggest that inability of closed stomata to open completely after a dark exposure could be of equal or greater importance in reducing plant productivity.

One possible mechanism for the observed O_3 effect on guard cell ion currents is direct oxidation of the channel proteins. It is plausible that the exposed amino acids of the inward K^+ channels are more susceptible to oxidation compared with those of the outward K^+ channels. The inwardly rectifying K^+ channel of guard cells, which is shown as the target of O_3 action in this study, has been cloned from the model plant species *A. thaliana* (41, 42). One next step will be to assess whether alterations, either in ion channel amino acids particularly susceptible to oxidation or in levels of cellular reductants (43, 44, 45), may improve rates of guard cell recovery after the termination of an O_3 event. It will be of interest to determine whether other guard cell ion transporters are also targets of O_3 action, although the inhibition by O_3 of stomatal opening, but not induction of stomatal closure under our conditions, implicates O_3 targeting of an "opening-

specific" component, for which the inwardly rectifying K⁺ channels is the best example. Alternatively, elevation of cytosolic Ca²⁺ levels after O₃ exposure is another plausible mechanism to explain the electrophysiological responses documented here. Cytosolic Ca²⁺ has been implicated as a signaling molecule in guard cell responses to other oxidative stresses (46) and has been reported to inhibit the inward K⁺ channel (47). Interestingly, an increase in cytosolic free Ca²⁺ levels also has been reported after exposure of human tracheal epithelial cells to O₃ (48). Our identification of a specific ion channel as a target for O₃ action may prompt comparable studies in mammalian systems, leading to improved understanding of and treatment for the disease

etiologies exacerbated by O₃. Finally, as implied by our whole plant experiments, genetic alterations in channel properties or cellular signaling events that would lead to accelerated rates of recovery of stomatal function after O₃ exposure could improve plant productivity in geographic regions with significant O₃ exposure.

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Ozone treatment affects pigment precursor metabolism in pine seedlings

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Abstract

Five-week-old seedlings of *Pinus halepensis* Mill. and *Pinus brutia* Ten. were exposed to air polluted with ozone (250 nl l⁻¹, 12 h day⁻¹, 4 days) or to ambient air containing ca 10 to 20 nl l⁻¹ ozone, in the light (180 μ mol m⁻² s⁻¹ PPFD, 12 h day⁻¹) and then fed for 24 h in the light (100 μ mol m⁻² s⁻¹ PPFD) with various radioactive precursors of chlorophyll (Chl) and carotene biosynthesis: 5-[4-¹⁴C]-aminolevulinic acid (¹⁴C-ALA), L-[¹⁴C(U)]-glutamic acid (¹⁴C-Glu), or D,L-[2-¹⁴C]-mevalonic acid (¹⁴C-MVA). Pigments were then extracted from cotyledons and fully expanded needles. Chl *a* and carotene were separated by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), and their specific activities (SA) were determined. ¹⁴C-ALA and ¹⁴C-Glu labels were incorporated into Chl *a* and carotene. Exposure to ozone did not inhibit incorporation of ¹⁴C-ALA into Chl *a* molecules, but hydrolysis of Chl *a* showed that ozone inhibited phytol labelling of Chl *a*. Labelling of carotene was also inhibited by ozone, but not when ¹⁴C-MVA was used as the label. These data suggest that ozone treatment inhibits (directly or indirectly) the biosynthesis of isoprenoids from products of ALA and Glu metabolism in the plastid, but not from MVA in the cytosol. This inhibition was more prominent when ¹⁴C-ALA was used as the label than when ¹⁴C-Glu was the labelling precursor. A significant increase in pheophorbide *a* (Pheide) a tetrapyrrole component of Chl *a* labelling and a concomitant decrease in phytol labelling was observed following incubation of ozone-treated pine seedlings with ¹⁴C-ALA and ¹⁴C-Glu. Stronger inhibition of carotene biosynthesis and activation of Chl *a* tetrapyrrole labelling by ¹⁴C-ALA (in comparison with ¹⁴C-Glu) indicated that exposure to ozone inhibits the conversion of ALA to Glu as the first step in ALA catabolism. These results also suggested a more intensive Glu metabolism (in

comparison with ALA) for carotene biosynthesis in the cytosol, as well as cooperation between two pathways of isopentenyl diphosphate (IPP) biosynthesis.

Abbreviations - AOS, active oxygen species; ALA, 5-aminolevulinic acid; ^{14}C -ALA, 5[4- ^{14}C]-aminolevulinic acid; ^{14}C -Glu, L-[^{14}C (U)]-glutamic acid; Fv/Fm, ratio of variable to maximal fluorescence; Φ_{II} , quantum yield of photosystem II electron transfer; IPP, isopentenyl diphosphate; MVA, mevalonic acid; ^{14}C -MVA, D,L-[2- ^{14}C]-mevalonic acid; q_{P} , photochemical quenching coefficient; Pheide, pheophorbide α ; Pheo, pheophytin α ; POR, protochlorophyllide reductase; PPFD, photosynthetic photon flux density; SA, specific activity.

Introduction

Ozone (O_3) is the most important anthropogenic air pollutant of the lower atmosphere (Thompson 1992). Over large rural areas of the industrialised countries its average monthly concentration has increased during the last century to the range of 20 to 80 nl l^{-1} (Lucas et al 1993, Heath 1994). During episodes of severe air pollution, concentrations as high as 400-500 nl l^{-1} have been monitored (Seinfeld 1989, Sakaki et al 1994). The effect of air polluted with O_3 on various aspects of plant growth has been documented in a wide range of species but the mechanism of its phytotoxicity to biosynthesis and metabolism are still only partially understood (Darrall 1989, Heath 1994, Pell et al 1997). Plant responses to O_3 are dependent on the concentration of the pollutant, the length of the exposure, the stage of plant development, and conditions that affect stomatal conductance to gas diffusion into the leaf (Darrall 1989, Heath 1994, Schmieden and Wild 1995). The low sensitivity of younger leaves to O_3 has been suggested to result from the higher activity of free-radical scavenging enzymes (Alonso et al 1999). Long-term, chronic exposure of vegetation to low levels of O_3 (30-70 nl l^{-1}) can cause depression of photosynthesis, increased ion leakage, accelerated senescence and affect sugar allocation (Lucas et al 1993, Mikkelsen et al 1995, Schmieden and Wild 1995, Pell et al 1997). Exposure to higher levels of O_3 (150-400 nl l^{-1}) can cause acute injury which is often characterised by necrotic lesions that develop within a few hours (e.g., Wingsle et al 1992, Sakaki et al 1994, Ciompi et al 1997). Because O_3 is highly reactive, it probably degrades into a number of other active oxygen species (AOS) immediately

after reaching the apoplast of cell adjacent to the stomatal pore (Laisk et al 1989). The formation of AOS in plant tissues following exposure to O_3 was detected using electron spin resonance (Mehlhorn et al 1990). The superoxide EMBEDanion $O_2^{\bullet-}$, hydroxyl radical $HO^\bullet$ and hydrogen peroxide H_2O_2 are reportedly the main degradation products of O_3 in aqueous solutions (Grimes et al 1983, Byvoet et al 1995) and in plant tissues (Kanofsky and Sima 1995, Runeckles and Vaartnou 1997, Schraudner et al 1998). H_2O_2 and free oxygen radicals are also formed in plant cells as a by-product of the mitochondrial and photosynthetic electron transport systems, especially under conditions of environmental stress and during pathogenic infection (Wingsle et al 1992, Ciompi et al 1997, Pell et al 1997). Lipid analysis of leaf tissue following acute or chronic exposure to O_3 revealed clear-cut changes in the lipid composition of the chloroplast membrane (Sakaki et al 1994, Wellburn et al 1994, Pell et al 1997). Disintegration of thylakoid membranes has been suggested to precede the breakdown of photosynthetic pigments (Sakaki et al 1994, Heath 1987, Matile et al 1999). The decrease in Chl in response to ozone could result from its breakdown or from the inhibition of its biosynthesis as an acclimatization strategy in plants in an effort to avoid photoinhibition (Mikkelsen et al 1995).

The objectives of the research reported herein were to determine the effect of ozone on specific steps in the biosynthesis of Chl and carotene in cotyledons and needles of *Pinus halepensis* and *Pinus brutia* seedlings. *P. halepensis* (Aleppo pine) is considered an O_3 -sensitive conifer (Karenlampi 1987). Visible injury and physiological effects of air polluted with ozone have been described in trees of this species grown in Mediterranean areas and in California (Karenlampi 1987, Elvira et al 1998). A significant reduction in Chl and carotenoids was observed following exposure to moderate concentrations of O_3 in older needles of *P. halepensis* (Alonso et al 1999). The pollutant also caused a significant alteration in antioxidant enzyme activity and induced activation of the xanthophyll cycle (Alonso et al 1999). In our preliminary experiment *P. brutia* cotyledons from O_3 -treated seedlings showed significantly less visible injury than *P. halepensis* (data not shown). Therefore, in the present report we compare the responses to O_3 in these two species. Pine seedlings were exposed to a high ozone concentration (250 nl l^{-1} , 12 h day^{-1} for 4 days). First signs of injury were observed at the end of the treatment on cotyledons of both

species. We show that exposure of pine seedlings to ozone essentially changes the distribution of ^{14}C -ALA and ^{14}C -Glu label, from the phytol to the pheophorbide parts of Chl *a*. Labelling of carotene was inhibited except when ^{14}C -MVA was used as the label. No significant difference was found between the effects of O_3 on label redistribution in the two pine species.

Materials and methods

Growth conditions

Pinus halepensis Mill. and *Pinus brutia* Ten. seeds were soaked in tap water for about 12 h, and then planted (1-1.5 cm apart) in pots (20×20×15 cm) containing moist vermiculite. After 2 weeks of germination and growth at 25°C in the dark, the seedlings were transferred to light, 12 h day⁻¹, at a photosynthetic photon flux density (PPFD) of 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ from a bank of Cool-White fluorescent lamps (OSRAM, L40W/20S, USA). PPFD was measured using a Quantum Meter, Li-189 (Li-Cor, USA). The seedlings were grown in the light for 3 weeks (referred to below as 5-week-old seedlings), before being exposed to the ozone treatment.

Ozone exposure

The 5-week-old seedlings were exposed for 4 days (12 h day⁻¹) to air containing 250 nl l⁻¹ O_3 or to ambient air containing ca 10 to 30 nl l⁻¹ ozone in a gas-tight Plexiglas chambers (50×50×100 cm). The chambers were placed in a temperature controlled growth room at temperatures 25/20 ± 2°C 12 h day/12 h night. Relative humidity in the chambers ranged between 65 and 80%. Light was supplied by halogen lamps (OSRAM, HOL 400W, Germany) at a PPFD of 180 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at plant level. Ozone was generated from pure oxygen by ozone generators (Series 1000, McMillan Electronics, USA), and its concentration was continuously monitored with an ozone analyser (Model 49, Thermo Electron, USA). The growth chambers were opened each evening, and the seedlings were watered. The increase in PPFD from 100 to 180 $\mu\text{mol m}^{-2}\text{s}^{-1}$ upon transfer of the pine seedlings to the growth chambers where the ozone treatment was applied did not affect the Chl *a* fluorescence parameters (F_v/F_m , - ratio of variable to maximal fluorescence, Φ_{II} - quantum yield of PSII electron transfer, and q_p - the photochemical quenching coefficient) (Table 1). It is therefore

Table 1 Parameters of Chlorophyll *a* fluorescence as measured in 5-week-old intact *P.*

halepensis seedlings grown at PPFD of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ and then transferred to higher PPFD of $180 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 6 h and 12 h. Results are means of seven replications $\pm$ SD.

PPFD	Fv/Fm	Φ_{II}	1- q_{P}
100	0.84 ± 0.02	0.62 ± 0.02	0.16 ± 0.02
180 (6h)	0.84 ± 0.02	0.62 ± 0.04	0.15 ± 0.03
180 (12h)	0.85 ± 0.02	0.63 ± 0.05	0.14 ± 0.03

not likely that PS II activity was photoinhibited by increasing PPFD. It has also been previously shown that PS II activity is not photoinhibited under growth conditions similar to those used in our experiments (Gilmor and Yamamoto 1991, Ogren 1991).

Chlorophyll fluorescence

Fluorescence transients were measured at room temperature in intact seedlings using a Hansatech MFMS/2S modulated fluorescence system (Hansatech Instrument Ltd, King's Lynn, Norfolk, UK) as described previously (Raskin and Marder 1997). The intensity of the modulated light was $0.3 \mu\text{mol m}^{-2} \text{s}^{-1}$. F_s , steady-state fluorescence, was recorded during continuous irradiation with actinic light at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. The quantum yield of photosystem II electron transfer [$\Phi_{\text{II}} = (F_m' - F_s)/F_m'$, F_m' being the maximum fluorescence in light-adapted seedlings] and photochemical quenching [$q_{\text{P}} = (F_m' - F_s)/(F_m' - F_o')$] were calculated according to Genty et al (1989), and Oxborough and Baker (1997).

Radiolabelling

Three samples, each comprising three or four pine seedlings (whole plants), were selected randomly from the ozone-treated and control plants. The seedlings were uprooted and their roots washed gently with tap water. Paired samples from control and ozone-treated plants were placed in the same flat-bottomed cuvette containing buffer solution (15 mM HEPES, pH 7.3, 40 ml) and 2.96 MBq of one of the radiolabelled precursors: ^{14}C -ALA ($1.76 \text{ TBq mol}^{-1}$), ^{14}C -Glu ($10.95 \text{ TBq mol}^{-1}$) or ^{14}C -MVA (2.1 TBq mol^{-1}). The seedlings were incubated for 24 h under an irradiance of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ (PPFD) at 25°C and were turned over every 6 h. For “pulse-chase” experiments, the radioactive solution was replaced after 24 h with a similar medium containing 0.5 mM non-radioactive ALA and the incubation was continued for 10 h or 24 h, as stated in the Results (Raskin et al 1995).

Pigment extraction and analysis

After labelling, the plants were washed four times with tap water. The cotyledons and needles (0.3-0.5 g) were excised and homogenised in acetone at 4°C using a mortar and pestle. The homogenate was centrifuged at 10 000g for 15 min. The pellet was extracted three times with cold acetone and the extracts were pooled and stored at -20°C in darkness for pigment determination. Overall pigment analysis was performed by HPLC following extraction of pigments with methanol.

The absorption of the extract solution was measured with a spectrophotometer (Model U-3000, Hitachi, Japan). Chl *a*, *b* and total carotenoid contents of the acetone extract were calculated according to Lichtenthaler (1987). Carotenes collected in methanol after HPLC were measured according to the extinction coefficient ($A^{1\%}_{1cm}$) at the absorption maximum for α - and β -carotene (Britton 1985). Carotenes collected from TLC were measured according to the extinction coefficient of 2250 at 470 nm for total carotenoids in methanol (Lichtenthaler 1987). Measurements with α -carotene (Fluca, Switzerland) and β -carotene (Sigma, USA) standard mixtures confirmed the validity of this coefficient; these samples were measured with a spectrophotometer before HPLC analysis.

Isolation of chloroplast fraction

Cotyledons and fully expanded needles (0.3-0.5 g) of control and ozone-treated seedlings were homogenised at 4°C in a buffer containing 0.01 M NaCl, 0.01 M Tricine buffer and 0.4 M sucrose (pH 8). The homogenate was immediately filtered through four layers of 50 μ m nylon mesh and the filtrate was centrifuged at 3000g for 10 min at 4°C. The chloroplast pellet was then resuspended and washed twice in the same buffer.

Purification of Chl *a* and carotene

TLC analysis. After addition of water (to 20%) to the acetone extract and alkalisation with 1 mM NaOH to pH 9, the Chl pigments and carotenes were separated from unesterified pigments by phase-partitioning (three times against the same volume of n-hexane) between n-hexane and acetone. The pooled n-hexane extract was concentrated under a gentle flow of nitrogen. The concentrated n-hexane fraction was used for TLC analysis. For Chl *a* separation we used carbon tetrachloride:acetone:ethanol:water (400:100:4:1 by volume) and for carotene separation n-octane:chloroform (100:3 by volume) as developing solvents (Hager and

Meyer-Bertenrath 1966). TLC was carried out with HPTLC-Platten Kieselgel 60 (Merck, Germany). The bands of Chl *a* and $\alpha+\beta$ -carotene were scraped from the plate and dissolved in methanol for HPLC analysis.

HPLC analysis. Pigment identification was carried out in a Multisolvant Delivery System (Waters 600E, System Controller, USA) equipped with a photodiode array detector (Waters 991). A LiChrospher 100 RP-18 (5 μ m, 250x4 mm) column (Merck, Germany) was used to separate the pigments; the mobile phase was 100% HPLC-grade methanol (Patzlaff and Barry 1996) and the flow rate was 0.8 ml min⁻¹. The column was equilibrated with methanol for 2 h prior to sample injection. These conditions resulted in the reproducible separation of Chl *a*, α -carotene and β -carotene. The retention times and the spectra of α - and β -carotene in the samples were compared with those of α -carotene (Fluca, Switzerland) and β -carotene (Sigma, USA) standards. For separation of α - and β -carotene by HPLC we used the mixture of $\alpha+\beta$ -carotene obtained from TLC and dissolved in methanol.

Liquid scintillation counting

The fractions containing pigments were evaporated, and the residues were dissolved in 10 ml scintillation liquid (Quicksafe A, Zinsser Analytic, GB). Radioactivity was measured with a Tri-Carb 1600TR Liquid Scintillation Analyzer (Packard Instrument Company, CT). After TLC, the activities and SA of $\alpha+\beta$ -carotene and Chl *a* were determined. The Chl *a* and $\alpha+\beta$ -carotene samples were injected separately for HPLC analysis. After HPLC, α - and β -carotene were collected and the activities and SA of these pigments were measured. No significant difference was found between the sum of the α - and β -carotene activities collected after HPLC and the activity of $\alpha+\beta$ -carotene before injection, for the same quantities of pigments. The purity of Chl *a* was monitored by HPLC analysis. The SA of Chl *a* after HPLC and after TLC were not significantly different, and any difference was usually within the error range for the replications of TLC or HPLC measurements.

Pheide radioactivity determination

After Chl *a* purification (TLC and HPLC) and SA determination, the methanol solution was evaporated to dryness under nitrogen and the residues were dissolved in 0.2 ml of diethyl ether. Concentrated HCl (2 ml) was added and the solution was

allowed to stand at room temperature for 60 min, resulting in the removal of Mg and hydrolysis of the phytol-ester linkage, to yield Pheide and phytol, (Weinstein and Beal 1983). Unreacted pheophytin *a* (Pheo) and phytol were removed by adding 2 ml of water and extracting twice with 4 ml of ether. After addition of 4.8 ml water to the aqueous phase, the Pheide was transferred to diethyl ether (two 3-ml aliquots) by vigorous shaking, as described by Weinstein and Beale (1983).

Replication and statistics

The experiments involved two pine species - *Pinus brutia* and *P. halepensis* - and consisted of ozone treatment followed by ^{14}C labelling, and were performed at least twice for each species. The data presented are means of three replications of one experiment. Analysis of variance (ANOVA) followed by the least significant difference (LSD) test was used to compare the means.

Results

Visible signs of chlorosis were observed in cotyledons and needles of 5-week-old seedlings of *P. halepensis* and *P. brutia* after 4 days of ozone fumigation (250 nl l^{-1}). The effect of exposing light-grown *P. halepensis* seedlings to O_3 on the incorporation of ^{14}C into the chloroplast, during a 24-h labelling with ^{14}C -ALA, ^{14}C -Glu and ^{14}C -MVA following the O_3 treatment, is presented in Table 2. Pretreating the

Table 2. Label incorporation into the chloroplast fraction isolated from 5-week-old *P. halepensis* seedlings. The uprooted seedlings were incubated in the light ($100 \mu\text{mol m}^{-2} \text{ s}^{-1}$) in a radioactive precursor for 24h following the ozone treatment. Values are radioactivity incorporated into chloroplast fraction per Chl *a* (GBq mol^{-1} Chl *a*). Results are means of three replications $\pm$ SD.

Treatment	^{14}C -ALA	^{14}C -MVA	^{14}C -Glu
Control	35.5 ± 5.2	53.8 ± 7.5	47.5 ± 6
Ozone	39.7 ± 8.2	52.2 ± 8.5	48.8 ± 6.2

seedlings with O_3 did not affect the amount of ^{14}C incorporated into the chloroplast fraction from the three tested pigment precursors (Table 2). Only ca 5 to 20% of the ^{14}C -ALA that was incorporated into chloroplast fraction during the incubation with ^{14}C -ALA was actually detected in Chl *a* (Chl *a* SA ~ 2.5 - 7.5 GBq mol^{-1}) (Tables 3, 4).

Tab. 3. Labelling of Chl *a* and ($\alpha+\beta$)-carotene in control and ozone-treated seedlings of *P. halepensis* and *P. brutia* with ^{14}C -ALA in pulse-labelling (24 h) and pulse-chase experiments. For ($\alpha+\beta$)-carotene TLC results are shown. The purity of the Chl *a* was monitored by HPLC. Values are pigment SA (GBq mol $^{-1}$). Results are means of three replications $\pm$ SD. Chl *a* and ($\alpha+\beta$)-carotene [($\alpha+\beta$)-Car] in samples were 20-50 nmol and 5-15 nmol, respectively. Δ , the change of SA after ozone treatment (SA for control sample -100%). LSD between treated and control plants, * $P \leq 0.05$, ** $P \leq 0.005$. NS - not significant.

Pre-treatment	24 h pulse-labelling		10 h chase-labelling		24 h chase-labelling	
	Chl <i>a</i>	($\alpha+\beta$)-Car	Chl <i>a</i>	($\alpha+\beta$)-Car	Chl <i>a</i>	($\alpha+\beta$)-Car
<i>P. halepensis</i>						
Control	2.18 $\pm$ 0.37	0.81 $\pm$ 0.21	0.42 $\pm$ 0.14	0.34 $\pm$ 0.06	0.40 $\pm$ 0.11	0.42 $\pm$ 0.11
Ozone	4.24 $\pm$ 1.07	0.15 $\pm$ 0.07	2.77 $\pm$ 0.35	0.06 $\pm$ 0.025	3.54 $\pm$ 0.66	0.08 $\pm$ 0.03
Δ , %	+94 *	-81 *	+560 **	-82 **	+785 *	-81
<i>P. brutia</i>						
Control	4.10 $\pm$ 1.06	1.20 $\pm$ 0.29	2.72 $\pm$ 0.72	0.50 $\pm$ 0.12	2.75 $\pm$ 0.67	1.50 $\pm$ 0.23
Ozone	4.97 $\pm$ 1.11	0.24 $\pm$ 0.11	11.87 $\pm$ 2.50	0.10 $\pm$ 0.06	7.73 $\pm$ 0.85	0.14 $\pm$ 0.06
Δ , %	+21 (NS)	-80 *	+336*	-80*	+181 **	-91 *

Table 4. Incorporation of ^{14}C into Chl *a*, α - and β -carotene in ozone-treated and control pine seedlings. The numbers are SAs (GBq mol $^{-1}$). The seedlings were incubated in light for 24 h with ^{14}C -precursors. Chl *a* and ($\alpha+\beta$)-carotenes were separated by TLC, the purity of Chl *a* was monitored by HPLC, and α - and β -carotene were separated by HPLC. Results are means of three replications $\pm$ SD. LSD between treated and control plants, * $P \leq 0.05$, ** $P \leq 0.005$, NS - not significant.

Precursor	Treatment	Chl <i>a</i>	α - carotene	β -carotene
<i>P. halepensis</i>				
^{14}C -ALA	control	3.62 $\pm$ 1.46	2.25 $\pm$ 0.80	2.08 $\pm$ 0.37
	ozone	6.57 $\pm$ 1.72 *	0.32 $\pm$ 0.17 *	0.35 $\pm$ 0.07 *
^{14}C -Glu	control	0.38 $\pm$ 0.08	2.31 $\pm$ 0.72	2.22 $\pm$ 0.77
	ozone	0.21 $\pm$ 0.06 *	0.93 $\pm$ 0.18 *	0.83 $\pm$ 0.44 *
^{14}C -MVA	control	0.092 $\pm$ 0.062	0.89 $\pm$ 0.15	0.90 $\pm$ 0.21
	ozone	0.088 $\pm$ 0.048	1.06 $\pm$ 0.20	0.95 $\pm$ 0.16
<i>P. brutia</i>				
^{14}C -ALA	control	6.12 $\pm$ 1.22	1.92 $\pm$ 0.34	2.25 $\pm$ 0.44
	ozone	7.42 $\pm$ 1.82 (NS)	0.31 $\pm$ 0.09 *	0.24 $\pm$ 0.08 *
^{14}C -Glu	control	2.03 $\pm$ 0.56	4.65 $\pm$ 0.47	4.25 $\pm$ 0.34
	ozone	1.20 $\pm$ 0.33 (NS)	1.14 $\pm$ 0.24 **	1.07 $\pm$ 0.30 **
^{14}C -MVA	control	0.40 $\pm$ 0.13	1.73 $\pm$ 0.22	1.66 $\pm$ 0.25
	ozone	0.46 $\pm$ 0.17	1.59 $\pm$ 0.16	1.67 $\pm$ 0.34

The effect of light on the incorporation of ^{14}C in photosynthetic pigments was further studied by measuring the SA of the isolated pigments following incubation of seedlings with ^{14}C -ALA in the dark or in the light (Table 5). Following dark

Table 5. Pigments labelling in the pine seedlings following incubation with ^{14}C -ALA in darkness or in the light. The numbers are SA (GBq mol^{-1}). Chl *a* and ($\alpha+\beta$)-carotene were separated by TLC. The purity of Chl *a* was monitored by HPLC. Results are means of three replications $\pm$ SD.

Growth conditions	Labelling conditions	Pine species	Chl <i>a</i> (GBq mol^{-1})	($\alpha+\beta$) carotene (GBq mol^{-1})
2-week-old dark grown	48 h dark	<i>P. halepensis</i>	1.30 ± 0.19	0.01 ± 0.01
		<i>P. brutia</i>	3.76 ± 0.48	0.02 ± 0.01
5-week-old light-grown	48 h dark	<i>P. halepensis</i>	0.05 ± 0.02	0.01 ± 0.006
		<i>P. brutia</i>	0.03 ± 0.01	0.005 ± 0.006
5-week-old light-grown	24 h light	<i>P. halepensis</i>	2.54 ± 0.20	0.76 ± 0.12
		<i>P. brutia</i>	3.74 ± 0.44	1.42 ± 0.13

incubation of seedlings with ^{14}C -ALA, labelling of Chl *a* was clearly detected in the 2-week-old dark-grown seedlings, but not in 5-week-old light-grown seedlings. In contrast to Chl *a*, labelling of carotene could not be detected in the 2-week-old dark grown seedlings following the dark incubation with ^{14}C -ALA. In 5-week-old light-grown seedlings labelling of both Chl *a* and $\alpha+\beta$ -carotene occurred almost entirely when incubation took place in the light (Table 5). These results are in accordance with the well-known fact that greening of conifer seedlings can take place in the dark as well as in the light (Drumm-Herrel and Mohr 1994).

The effect of ozone on pigment labelling with ^{14}C -precursors was quantified by measuring pigment SA after TLC and HPLC (Tables 3, 4). An example of band-labelling on TLC plates with the chromatograms of the hexane fraction was visualised by exposure to phosphor-imaging screen (BAS-IIIIs, Fuji, Japan) followed by scanning with a BAS 1000 Phosphor-Imager (Fuji). Figure 1 shows an

Fig. 1. The "photographic" and autoradiographic image of ^{14}C -labelled pigments extracted from 5-week-old seedlings of *Pinus halepensis* incubated with ^{14}C -Glu for 24 h at PPFD of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$. The hexane fractions of the total pigment extract were separated by TLC using octane-chloroform. The "photographic" image obtained by scanning the photograph of TLC plate on the Hewlett-Packard scanner. The autoradiogram was obtained by a Fuji phosphor imager. Extract of control seedlings on the left and of ozone-treated seedlings on the right. In both cases 80 nmol of Chl *a* were applied on the chromatogram origin. car - $\alpha+\beta$ -carotenes.

autoradiographic image of ^{14}C -Glu-labelled $\alpha+\beta$ -carotene extracted from *P. halepensis* and separated by TLC with *n*-octane-chloroform, which is used as the developing solvent for the isolation of carotene. Quantification of radioactive band density by BAS 1000 image analyser (Fuji) resulted in ratios of carotene to origin of 0.034 and 0.016 for control and ozone treated samples, respectively.

After TLC, the bands of Chl *a* and $\alpha+\beta$ -carotene were extracted with methanol, and HPLC-analysis was carried out separately for Chl *a* and $\alpha+\beta$ -carotene extracts. After liquid scintillation counting the SA of Chl *a*, $\alpha+\beta$ -carotene, α - and β -carotene were determined (Tables 3, 4). The differences in pigment SA between samples (Tables 3, 4) could not be related to changes in the permeability of the plant cell to radioactive precursors resulting from the ozone treatment (Table 2).

In the plants incubated with ^{14}C -ALA, the Chl *a* labelling increased after ozone treatment by 94, 560 and 785% after 24 h of pulse-labelling, and after 10 and 24 h of chase duration, respectively, in *P. halepensis*, and by 336 and 181% after 10 and

24 h chase duration, respectively, in *P. brutia* (Table 3). Carotene labelling decreased significantly, by 81, 82 and 81%, after 24 h of pulse-labelling, and after 10 and 24 h chase duration, respectively, in *P. halepensis* and by 80, 80 and 91% after the same pulse and chase durations, respectively, in *P. brutia* (Table 3).

Seedlings of both species of pine were incubated with ^{14}C -ALA, ^{14}C -Glu and ^{14}C -MVA (Table 4). When the source of the label was ^{14}C -ALA or ^{14}C -Glu, a significant decrease in the SA of carotene appeared in both species after ozone treatment. The labelling decreased by a factor of 6 to 7 in plants incubated with ^{14}C -ALA, and by a factor of 2 to 4 in plants incubated with ^{14}C -Glu (Tab 4). However when the seedlings were incubated with ^{14}C -MVA, no significant difference in labelling was found between the control and ozone treated seedlings (Table 4).

The Pheide and phytol component of Chl *a* were separated by acid hydrolysis (Weinstein and Beal 1983). The difference between the SA of Chl *a* and Pheide represents the SA of the Chl *a* phytol. After incubation of control seedlings with ^{14}C -ALA or with ^{14}C -Glu, only 48 to 49% (total Chl *a* SA - 100%) of the ^{14}C from ^{14}C -ALA and 39 to 46% of that from ^{14}C -Glu was incorporated into Pheide (Table 6),

Table 6. Label incorporation in Pheide after 24 h incubation with ^{14}C -ALA and ^{14}C -Glu as a percentage of total SA of Chl *a*. The purity of Chl *a* was monitored by HPLC. Results are means of three replications $\pm$ SD. LSD between treated and control plants, * $P \leq 0.05$, ** $P \leq 0.005$.

Precursor	Treatment	(Pheid/Chl <i>a</i>) $\times$ 100	
		<i>P. halepensis</i>	<i>P. brutia</i>
^{14}C -ALA	control	47.9 $\pm$ 8.3	49.1 $\pm$ 7.1
	ozone	97.5 $\pm$ 4.3 **	99.6 $\pm$ 11.4 **
^{14}C -Glu	control	45.8 $\pm$ 9.4	39.1 $\pm$ 7.6
	ozone	105.9 $\pm$ 15.3 *	99.4 $\pm$ 6.9 **

indicating that about 50% of the precursors label was incorporated into phytol. Ozone treatment changed the distribution of the ^{14}C between the tetrapyrrole and phytol moieties of Chl *a*. After ozone treatment, ca. 100% of the ^{14}C from ^{14}C -ALA and ^{14}C -Glu, which was incorporated into Chl *a*, was incorporated into Pheide (Table 6).

Discussion and conclusions

Pine seedlings are capable of synthesising Chl and thylakoid membrane proteins in the dark as well as in the light (Bogorad 1950, Shinohara et al 1992, Drumm-Herrel and Mohr 1994, Ou and Adamson 1995). The thylakoids of dark-greening chloroplasts contain components of PS I and PS II, but are unable to evolve oxygen until they are “photoactivated” by light (Kamachi et al 1994). It has been suggested that light is required to complete the formation of the water-oxidising complex of PSII (Raskin and Marder 1997, Tamura et al 1997). In gymnosperms, such as pine and spruce, the chloroplast genome encodes the light-independent protochlorophyllide (Pchl_{id}) reductase (POR), while the nuclear genome encodes light-dependent POR (Wettstein et al. 1995). Cotyledons of conifers contain both the dark (light-independent) and light-dependent pathways for Chl biosynthesis (Selstam and Widell 1986). Forreiter and Apel (1993) showed that in a cell-free extract from 2-week-old pine seedlings, the light-independent reduction of exogenous ¹⁴C-Pchl_{id} reaches similar levels when either dark-grown seedlings or seedlings that had previously been illuminated for 16 h are used as the POR source.

Significant labelling of Chl *a* with ¹⁴C was detected in seedlings that, following 2 weeks of germination and growth in the dark, were incubated for 48 h with ¹⁴C-ALA in the dark (Table 5). The ¹⁴C-labelled precursor was incorporated into Chl *a* during the dark-greening of the seedlings. In cotyledons of *Pinus pinea*, Ou and Adamson (1995) found that onset of rapid light-independent greening occurs between 8 and 12 days after planting. In 5-week-old light-grown seedlings (germination and growth for 2 weeks in the dark followed by 3 weeks in the light), incorporation of ¹⁴C from ¹⁴C-ALA into Chl *a* and carotene was significant only when the incubation took place in the light but not in the dark (Table 5). These results suggest that although dark-grown pine seedlings are capable of synthesising Chl in the dark, the main route for Chl and carotenoid synthesis in light-grown needles is actually light-dependent (Drumm-Herrel and Mohr 1994). von Lintig et al (1997) showed that a significant increase in carotenoid content occurs only under light conditions that induce Chl biosynthesis.

Chl accumulation in cotyledons and primary needles of light-grown pine seedlings has previously been shown to reach a steady state ca. 30 days after planting (Ou and Adamson 1995). Similarly, in our experiment, Chl *a* contents in *P. halepensis* and *P. brutia* cotyledons and needles were 1.7 and 1.5 mg (g fresh weight)⁻¹, respectively,

and no significant increase was observed between two consecutive measurements at 30 and 36 days after planting. We can therefore conclude that most of the ^{14}C -labelling in pigments resulted during pigment turnover and not from their net accumulation (Shlyk 1970, Raskin et al 1995).

Pine seedlings of both species were able to incorporate ^{14}C -ALA into Chl *a* efficiently after 4 days of fumigation with ozone. This suggested that the biosynthetic pathway from ALA to Chl *a* remains largely intact. The observed decline in Chl *a* concentration in the seedlings during the ozone treatment may be attributed mainly to destruction of Chl *a* directly by reactive oxygen intermediates, or indirectly by ozone-induced damage to thylakoid membranes (Sakaki et al 1994). Moreover the possibility of ozone affecting the availability of ALA precursors can also be ruled out, since extensive Chl *a* labelling was observed when pine seedlings were fed with ^{14}C -acetate, following ozone treatment (data not shown).

The two-fold increase in Chl *a* SA during the incubation with ^{14}C -ALA (24 h pulse-labelling) (Tables 3, 4) in the ozone pre-treated seedlings of *P. halepensis* can be attributed to inhibition of the reversible biosynthetic pathway from ALA to Glu. As a result, ALA is channelled towards Chl instead of to the non-tetrapyrrole biosynthetic pathway. In contrast to Chl, ^{14}C -labelling of carotene in the ozone pretreated seedlings was inhibited when ^{14}C -ALA and ^{14}C -Glu were used as the pigment precursors, but not when ^{14}C -MVA was the precursor (Table 4). This difference suggests that ozone adversely affects one of the the enzymes or intermediates in the remobilization of ALA and Glu to general metabolism (non-tetrapyrrole). This could be either by deactivation of one of the involved enzyme or by removal of an essential intermediate of carotene synthesis in a non-mevalonate plastid pathway. A non-mevalonate pathway for the biosynthesis of IPP has recently been identified (Lichtenthaler 1999). The inhibition of carotene labelling with ^{14}C following the ozone treatment was markedly greater when the seedlings were fed ^{14}C -ALA vs ^{14}C -Glu (Table 4).

Dilution of the ^{14}C label during incubation with non-labelled ALA in chase experiments was significantly smaller in the ozone-treated seedlings than in the controls (Table 3). Whereas the Chl *a* SA in control samples from *P. halepensis* decreased ca. fivefold after 10- and 24-h chase durations, in the ozone-treated

samples, only a 1.5-fold dilution was observed after the 10-h chase. The 2.4- and 1.5-fold increases in labeling of Chl *a* in *P. brutia* seedlings during the 10- and 24-h chase durations, respectively, following labelling with ^{14}C -ALA (Table 3), may result from gradual recovery of the plant from the ozone stress.

The conversion of Glu to ALA in the C_5 pathway takes place in the chloroplasts (Beale 1999), and this biosynthesis pathway has been suggested to be reversible (Duggan et al 1982). Troxler and Brown (1975) found differences in the extent to which different algal species utilise ALA via the porphyrin pathway.

In seedlings incubated with ^{14}C -ALA and ^{14}C -Glu, the amount of ^{14}C -labelled Pheide more than doubled relative to the total labelled Chl *a*, following 4 days of exposure to ozone (Table 6). This may indicate specific inhibition of phytol biosynthesis from metabolites of ^{14}C -ALA and ^{14}C -Glu in a non-mevalonate pathway and appearance of other sources of non-labelled phytol. It has been shown that for isolated chloroplasts (containing endogenous chlorophyllides) supplied with exogenous ^{14}C -IPP, the label is channelled towards chlorophyllide esterification at the expense of carotenoid biosynthesis (Dogbo et al 1984).

The absence of inhibition of carotene labelling by ozone pre-treatment when ^{14}C -MVA was used as the label suggests that the plastid isoprenoid biosynthesis pathway was inhibited, but that the cytosolic pathway of IPP biosynthesis from MVA was not. Thus, transport of isoprenoid precursors from the cytosol into the chloroplast compartment cannot be excluded.

In summary, we present evidence of ozone's direct effect on pigment biosynthesis, as well as on the metabolism of C_5 precursors, thus affecting the allocation of resources among various metabolic pathways. Inhibition of Chl *a* phytol labelling by ^{14}C -ALA and ^{14}C -Glu indicates that the biosynthetic pathway leading to phytol synthesis from ALA and Glu was totally blocked by the ozone treatment. The inhibition of carotene biosynthesis via the chloroplast pathway by ozone may reinforce the destruction of Chl by decreasing the protective components of chloroplast membranes. The increase in Chl *a* tetrapyrrol labeling from ^{14}C -ALA following ozone treatment could have appeared as a plant response to Chl decrease by ozone treatment.

The labelling of carotene provides evidence of ALA and Glu conversion to precursors of the plastid isoprenoid pathway. The fate of the administered ALA which we traced in pine seedlings in the present study raises questions concerning the influence of ozone fumigation on the factors that regulate endogenous ALA consumption via the porphyrin pathway. The simultaneous labelling and extraction of Chl and carotene proved a useful tool for investigating the effects of ozone on pigment metabolism.

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