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Stable Free Radicals in Ozone-damaged Wheat Leaves

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Chlorophyll fluorescence measurements were performed on attached leaves of wheat plants (*Triticum aestivum* L. cv. Nandu) that were exposed to ambient air and to air supplemented with 80 and 120 nmol mol^{-1} ozone. Decreases in the "current photochemical capacity" were observed that were dependent on both the ozone concentration and duration of exposure. Electron paramagnetic resonance (EPR) spectra on freeze-dried samples from the same batches of plants showed the presence of an unidentified stable free radical, whose spectra had similarities to that of the ubisemiquinone radical. The intensity of this radical signal increased with the duration of ozone exposure in leaves that received an additional 120 nmol mol^{-1} ozone. In contrast, with exposure to air with 80 nmol mol^{-1} added ozone, there was little if any change in free radical signal intensity over the 4 week period of the experiment. The increase in intensity of the EPR signal occurred later than the chlorophyll fluorescence changes, which suggests that it is associated with permanent leaf damage.

Keywords: Ozone, wheat, chlorophyll fluorescence, EPR spectroscopy, free radicals, Mn(II)

INTRODUCTION

Free radicals are regarded as important mediators of the impairment of function of leaf cells by ozone. They have been implicated in early events following ozone exposure,[1] in addition to being associated with cell damage following prolonged exposure.[2] Evidence for the generation of free radicals associated with the phytotoxic effects of ozone comes mostly from indirect measurements of changes in the content of antioxidants and enzymes of the antioxidant scavenging machinery.[2] There have, however, been a few studies where the formation of free radicals has been detected directly in ozone-exposed leaf tissue using EPR spectroscopy.[3–5]

The generation of free radicals prior to the observation of visible injury in leaves of pea and bean plants that had been exposed to air containing

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70 and 300 nl l^{-1} ozone has been demonstrated by Mehlhorn *et al.*[3] These authors observed exposure-dependent EPR signals from plants that had been pre-treated with the spin trap phenyl *t*-butylnitrone (PBN) in specimens that had received as little as 4 h ozone exposure. The nature of these spectra was unusual, however, in that they consisted of single peak signals ($g = 2.0037$–2.0039) and did not have the hyperfine structure that is usually associated with free radical adducts of nitrone spin traps. Pre-treatment of leaves with aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, resulted in a much smaller EPR signal, a result which was interpreted as demonstrating that reaction between ozone and ethylene is the cause of ozone toxicity.

The formation of ozone-induced free radicals has been observed *in vivo* by Runeckles and Vaartnou[4] in leaves of perennial ryegrass. These authors found an increase in the free radical signal, accompanied by a large decrease in the Mn(II) signal, after 14 hours exposure to air containing 1000 µl l^{-1} ozone under white light. The same authors later concluded that this free radical signal corresponded to the superoxide radical anion ($O_2^{-\bullet}$) on the basis of its *g*-value (2.001 high field minimum) and line width (≈1 mT).[5] They also found that the appearance of the EPR signal could be delayed by infiltrating leaves with ascorbic acid (AA) prior to ozone exposure and that the signal disappeared within 16 minutes of the end of ozone exposure.

In the EPR-based studies described above, the ozone-induced free radicals were of a transient nature and were generated using very high ozone levels. In contrast, many biochemical studies have provided evidence for free radical generation following prolonged periods of more modest exposure. In the present work, we have attempted to go some way towards bridging this gap by investigating the effects of moderate ozone levels on the formation of stable free radical species. We have also investigated the relationship between the magnitudes of the EPR signals from these radicals and the "current photochemical capacity" of PSII in leaves of similar ozone exposed plants.

MATERIAL AND METHODS

Plant Material

Wheat caryopses (*Triticum aestivum* L. cv. Nandu) were pre-germinated at room temperature and then vernalised at 1 °C for two months. Seedlings were planted into 12 L-pots in a standard planting substrate (Frux Einheitserde ED 73, Gebr. Patzer KG, Sinntal-Jossa, Germany) and were cultivated in a greenhouse. One day before the start of fumigation, four pots were put into each of six open-top chambers (OTC).

Ozone Treatment

Six OTCs (diameter 1.2 m, height of 1.65 m), that were constructed of acrylic-glass and built in the Austrian Research Center Seibersdorf, were set up in a greenhouse. The base of each OTC contained an ozone inlet and a fan. It was separated from the upper acrylic part by an iron grid, which was the bottom of the chamber where the plant pots were placed. The top of each OTC was covered by a flap consisting of two half circles that could be opened to adjust the air flow through the chamber. Ozone was produced from pure oxygen by an ozone generator (Fischer, Model 502, Meckenheim, Germany) and was supplied daily from 9 a.m. to 5 p.m. It was injected into the base of the OTC, where it was mixed by the fan with air coming from outside. Two OTCs were supplied with ambient air only, two were supplied with ambient air plus 80 nmol mol^{-1} ozone, and two with ambient air plus 120 nmol mol^{-1} ozone.

Chlorophyll Fluorescence

Measurement of chlorophyll fluorescence was performed at 1-week intervals with a non-modulated

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fluorimeter ("Plant Efficiency Analyser", PEA, Hansatech Instruments Ltd., Kings Lynn, England). The tip of the uppermost just-fully-developed leaf was marked with a tape and the same leaf was measured periodically throughout the experiment. Prior to the measurement the adaxial side of the leaf was dark-adapted for 30 minutes using leaf-clips, after which the fast kinetics were measured for 1 second.

F_v/F_m was calculated from the maximum fluorescence (F_m) and the ground fluorescence (F_0) according to the equation $F_v/F_m = [(F_m - F_0)/F_m]$. The dark adapted F_v/F_m was taken as a value for the "current photochemical capacity".[6,7]

Sample Preparation for EPR Spectroscopy

Leaves were harvested after 2, 3 and 4 weeks of ozone fumigation and immediately frozen in liquid nitrogen. They were subsequently freeze-dried at -1°C and 10 kPa (Hetosic CD4, Hetolab Equipment) for three days, homogenised (Mikro-Dismembrator II, Braun, Germany) then stored at -20 °C until use. For the EPR measurements, weighed amounts of the freeze-dried powders were transferred to 4 mm diameter quartz tubes, which were carefully placed in the spectrometer so that the samples were evenly distributed around the centre of the microwave cavity.

EPR Spectroscopy

All EPR measurements were performed at ambient temperature (20 ± 2 °C) with a Bruker ESP300E (Bruker UK Ltd., Coventry) computer-controlled spectrometer operating at X-band frequencies and using an ER4103TM/9202 cylindrical cavity. Microwave generation was by means of a klystron (ER041MR) and the frequency was measured with a built-in frequency counter. All spectra were recorded in 1024 points using 100 kHz modulation frequency. Measurements were made over the range 50–450 mT using 10 mW microwave power and 0.5 mT modulation amplitude, and in the range 345–351 mT using 3 mW microwave power and 0.3 mT modulation amplitude. The parameters for the narrow scan range were selected after determination of the saturation characteristics of the free radical signals in control and ozone-treated specimens. The spectra acquired over the narrow scan range were recorded as both 1st and 2nd derivatives of the microwave absorption, whereas the wide scan spectra were just recorded as 1st derivatives. Signal heights were measured directly using the WIN-EPR software on the spectrometer and then normalised for unit weight of sample. g-Values were determined by using diphenylpicrylhydrazyl (DPPH) ($g = 2.0036$) as an external standard.

RESULTS

In the present experiments, ozone exposure led to symptoms typically associated with ozone damage in wheat leaves; chlorosis, followed by necrotic spots and premature ageing (data not shown).

The "current photochemical capacity" (F_v/F_m) showed a progressive decrease in leaves exposed to air with 120 nmol mol^{-1} added ozone, detectable from the time of the 1st measurement, i.e. 7 days after the start of ozone fumigation (Figure 1A). In leaves exposed to air supplemented with 80 nmol mol^{-1} ozone, a smaller decrease was observed and could only be detected from the 2nd measurement 14 days after the onset of ozone fumigation. The onset of the decrease in F_v/F_m and the observation of symptoms of leaf injury appeared simultaneously.

The ground fluorescence (F_0) was increased significantly in leaves exposed to elevated ozone concentrations from 14 days after onset of fumigation onwards (Figure 1B). In leaves exposed to air supplemented with 80 nmol mol^{-1} ozone the increase was roughly linear with respect to time, but in leaves exposed to air with 120 nmol mol^{-1}

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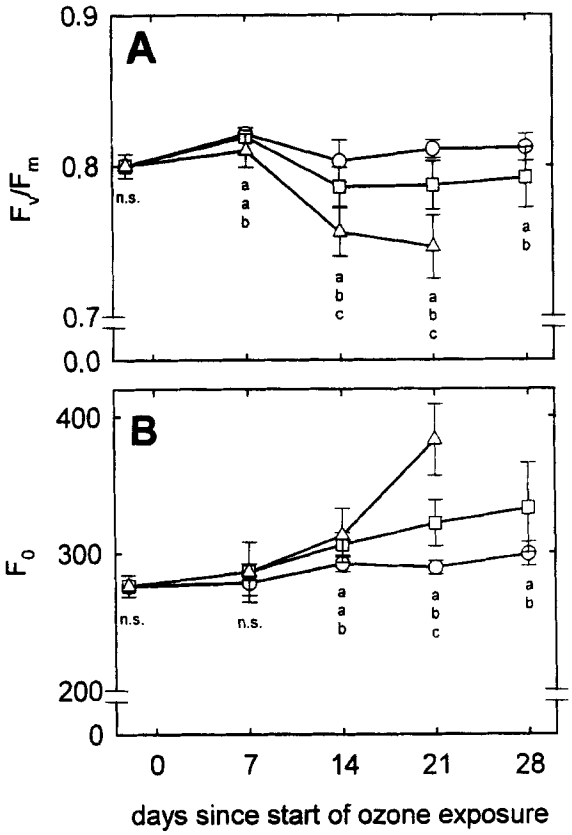

FIGURE 1 Chlorophyll fluorescence parameters of wheat leaves: (A) current photochemical capacity = F_v/F_m, and (B) ground fluorescence = F_0. Symbols: (○) control; (□) 80 nmol mol^{-1} ozone; (△) 120 nmol mol^{-1} ozone. Letters correspond to the symbols in the same vertical line. Significant differences among different treatments are indicated by different letters (Duncan's multiple range test, $p < .05$); n.s.: not significant.

added ozone there was an accelerated increase in F_0.

EPR spectra recorded over the widest scan range showed a number of distinct signals (not shown), typical of those seen in plant tissues;[4,8,9] a very broad resonance with $g = 2.0$ from Fe(III) in magnetically-interacting clusters, probably storage protein, a narrow single peak at 348 mT ($g = 2.003$) from (unidentified) free radical species, a sextet component from Mn(II) with peak separations (hyperfine splitting) of ~9.5 mT and $g \approx 2.00$, and a (near) isotropic feature at ~160 mT ($g = 4.27$) from Fe(III) in an environment with (near) rhombic symmetry. In all samples, the $g = 4.3$ Fe(III) signal was very weak and has not been considered further in this paper; neither has the $g = 2.0$ Fe(III) signal, the characteristics of which are largely determined by magnetic exchange interactions in iron oxide clusters. The Mn(II) signal (measured as the height of its 1st (low field) peak) showed no significant difference between control leaves and leaves exposed to air with 80 nmol mol^{-1} added ozone, but was decreased in leaves exposed to air supplemented with 120 nmol mol^{-1} ozone (Figure 2A). In all samples the Mn(II) signal intensity decreased with increasing leaf age.

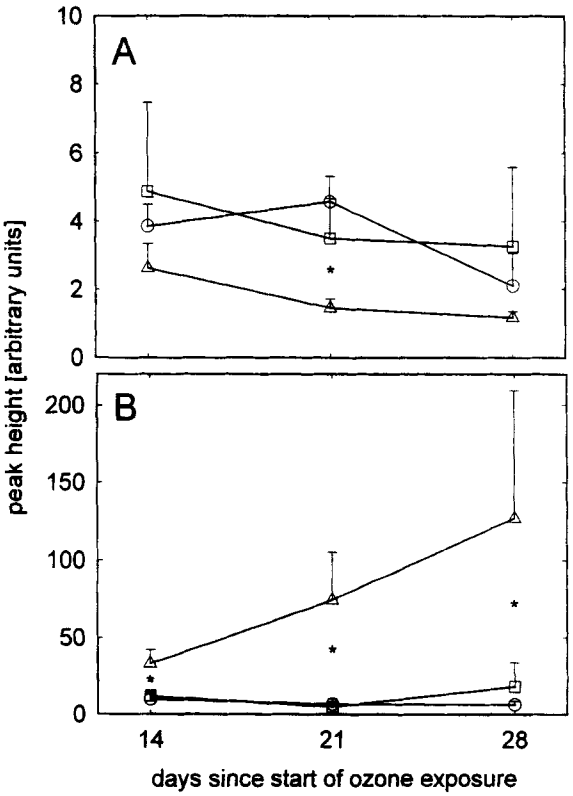

FIGURE 2 Variation with time since the start of ozone exposure of the intensities of (A) the Mn(II) signal, and (B) the overall free radical signal in freeze-dried wheat leaves. Symbols: (○) control; (□) 80 nmol mol^{-1} ozone; (△) 120 nmol mol^{-1} ozone. An asterisks between two symbols indicates significant differences between these symbols (Duncan's multiple range test, $p < .05$).

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The saturation characteristics of the free radical signals, determined from 1st derivative spectra obtained over the narrow scan range, are shown in Figure 3. The curves for the control and ozone-treated samples are very similar and indicate that similar relaxation processes exist in both types of sample. The broader curve for the sample from the ozone-treated leaves (B) is consistent with a wider distribution of saturation behaviour than for the control sample (A). The free radical signals were not symmetrical and 2nd derivative recordings showed the existence of two distinct features with g-values of 2.0053 and 2.0028 (in 36 samples the ranges of measured values were 2.0047–2.0058 and 2.0023–2.0030 for the two peaks). Examples of these spectra are shown in Figure 4 for samples from the three treatment groups 28 days after the start of the ozone exposure. In leaves exposed to air with 120 nmol mol^{-1} added ozone, the overall intensity of the 1st derivative spectra increased progressively with time from a relative height of 33 ± 8 after 2 weeks to 127 ± 71 after 4 weeks (Figure 2B). At all measurement points, the spectral intensity was significantly higher in leaves of plants treated with air containing 120 nmol mol^{-1} added ozone than in either the control plants or those exposed to air supplemented with 80 nmol mol^{-1} ozone. The intensities of the signals from these latter samples were essentially identical until the end of the 4th week of treatment. At that time one of the four samples that had been exposed to air with 80 nmol mol^{-1} added ozone showed a 4-fold increase in signal intensity compared to the other three samples and the controls.

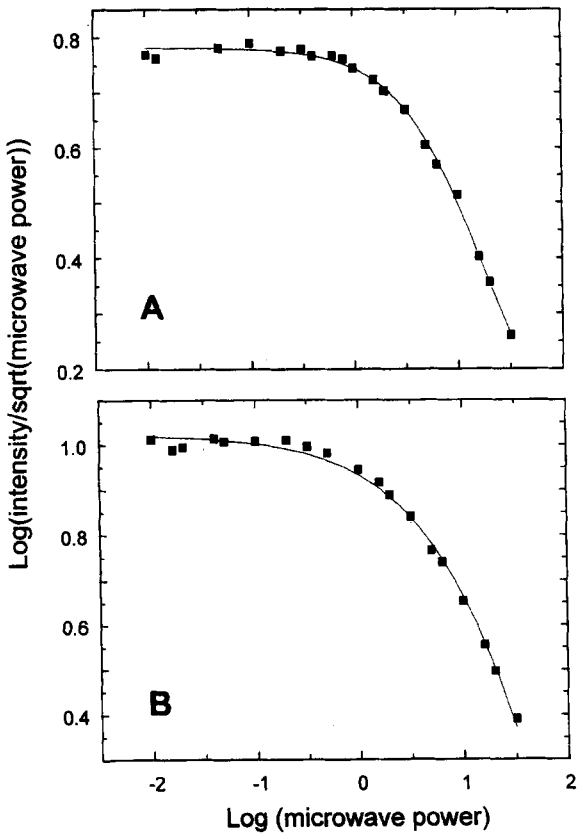

FIGURE 3 Saturation curves for the free radical EPR signals from (A) a control sample, and (B) a sample which had received the 120 nmol mol^{-1} ozone treatment for four weeks prior to harvesting.

The intensity of the high field free radical peak (Peak B) was much smaller than that of peak A in the samples treated with air containing 120 nmol mol^{-1} added ozone and, because of overlap, it was difficult to measure its intensity accurately in these samples. In the other samples its intensities were comparable to those of Peak A. Thus Peak B is much less sensitive to ozone exposure than Peak A and its intensity showed little if any change with time of exposure (data not shown).

The low field free radical signal intensities (peak A) were related in a non-linear manner to the magnitudes of F_v/F_m (Figure 5). On the left side of this curve, which corresponds to control leaves and leaves from plants exposed to air with 80 nmol mol^{-1} added ozone, there was little change in EPR signal intensity with decreasing F_v/F_m. The right side of the curve, which corresponds to leaves exposed to air supplemented with 120 nmol mol^{-1} ozone, shows the opposite trend with large increases in free radical intensity for small additional decreases in F_v/F_m.

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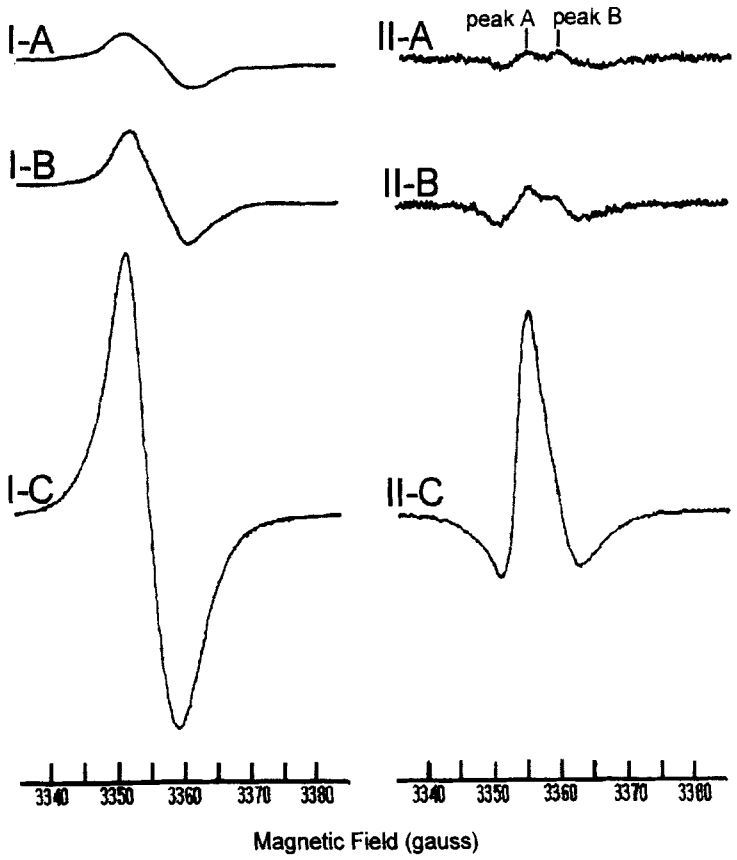

FIGURE 4 First derivative (I) and 2nd derivative (II) recordings of the free radical EPR signal in freeze-dried wheat leaves: (A) control, (B) treated with 80 nmol mol^{-1} ozone, and (C) treated with 120 nmol mol^{-1} ozone for 4 weeks.

DISCUSSION

Wheat plants react to elevated ozone levels by premature ageing of leaves, which in the present experiment was detected by visible injury and a significant decrease in "current photochemical capacity" measured by chlorophyll fluorescence of dark-adapted leaves. Effects related to ozone exposure were also detected in the intensities of the free radical and, to a lesser extent, the Mn(II) signals in the EPR spectra of freeze-dried samples.

One of the free radical components in the EPR spectra was elevated considerably in the samples that had been exposed to air containing

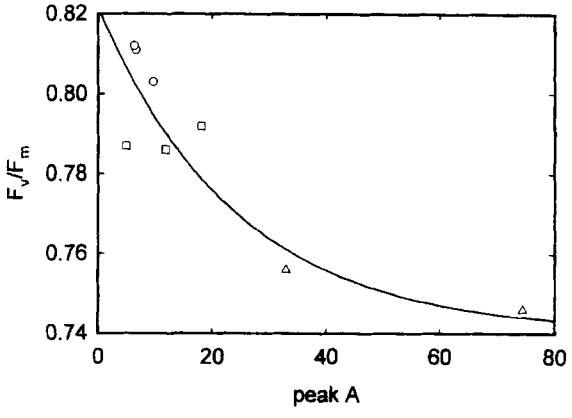

FIGURE 5 Relationship between the overall free radical signal intensity and "current photochemical capacity" for freeze-dried wheat leaves: Symbols as in Figures 1 and 3; $y = 0.740 + 0.081 \exp(-x/24.190)$; $r^2 = .839$.

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120 nmol mol^{-1} added ozone compared to the other samples. Because of the histories of these samples, this signal corresponds to a stable free radical and consequently is a different type of radical from those previously reported to be formed as a consequence of ozone exposure.[3,5] Mehlhorn *et al.*[3] interpreted their EPR results in terms of a radical intermediate formed during lipid peroxidation, but their free radical signal was observed only after reaction with a spin trap. The superoxide radical anion that was reported by Runeckles and Vaartnou,[5] was unstable and disappeared rapidly after the end of ozone exposure. Nevertheless, the *g*-value of 2.0053 for Peak A suggests that it might be an oxygen-centred radical, and its spectral characteristics are similar to that of the radical assigned to ubisemiquinone in a range of tissues from plants subjected to oxidative stress.[12] Leprince *et al.*[13] have suggested that this radical is formed in mitochondria as a consequence of termination of the electron transport chain. Elevated levels of Peak A were only observed in leaves which showed irreversible damage to the electron transport chain in the chloroplasts, as indicated by a progressively decreasing "current photochemical capacity" and increasing F_0. A decrease in F_v/F_m that is paralled by an increase in F_0 indicates an accumulation of non-functional photosystem II ($PSII_{NF}$).[10] The formation of $PSII_{NF}$ is one of the later steps in the sequence of damage of the photosynthetic apparatus by ozone.[11] In leaves exposed to 80 nmol mol^{-1} ozone for 14 days, F_v/F_m was decreased and F_0 increased relative to control leaves (Figure 1), but these changes did not progress further over the following 14 days. This result can be interpreted in terms of the establishment of a steady-state situation in these leaves, where increased impairment of the electron transport chain of the chloroplasts is balanced by an increase in the rate of repair mechanisms. In agreement with this interpretation, these leaves showed no increase in the intensity of Peak A in their EPR spectra.

The high field free radical Peak B was relatively insensitive to ozone treatment. Its *g*-value of 2.0028 is similar to that observed in tissue damaged by a range of biotic and abiotic processes (BAG – unpublished results). This *g*-value is consistent with a C-centred radical, but it is not definitive, and could arise from a completely different type of radical species.

The response of the Mn(II) signal to ozone was less marked than that of the free radical Peak A, and interpretation of these results is complicated by the fact that the signal intensity decreased with leaf age. Nevertheless, the intensity was significantly lower in leaves of plants exposed to air supplemented with 120 nmol mol^{-1} ozone than in the corresponding controls and leaves of plants exposed to air containing 80 nmol mol^{-1} added ozone (Figure 2A). Mn(II) is a component of the O_2-evolving complex that is associated with PSII. Consequently, the decrease in the Mn(II) signal may be associated with the disintegration of the photosynthetic apparatus caused by ageing of the leaf[16] and by ozone exposure,[11] and the differences in the EPR spectra of the leaves exposed to 80 nmol mol^{-1} and 120 nmol mol^{-1} ozone support the above interpretation of the F_v/F_m and F_0 data. A much greater decrease in intensity of the Mn(II) signal in response to ozone exposure was reported by Runeckles and Vaartnou,[4] but the ozone concentration used in that work was much higher than in the present experiment. Although it appears to be associated with the photosynthetic apparatus, the Mn(II) seen in the EPR spectra is not that of the active centre of PSII, which contains a tetranuclear cluster[17,18] and produces multipeak EPR spectra at low temperatures.[19] Its decrease as a result of ageing or high levels of ozone exposure would, however, appear to be the result of oxidative processes, which lead to the generation of EPR-silent species (such as e.g. Mn(III) or a polynuclear species).

The involvement of free radicals with much shorter half-lives in the mediation of ozone injury has already been established, and the

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present work demonstrates that stable free radical species are also generated in plant tissues. On the basis of the present results and publications in the earlier literature, we can speculate about the mechanism of radical formation, which leads to the generation of Peak A. If this corresponds to a semiquinone radical as suggested by Hendry *et al.*[12] the sequence of events leading to its formation might be as follows. Firstly, the photosynthesis dark reaction is impaired by ozone, leading to a decreased demand of "assimilatory power" (ATP and $NADPH + H^+$). This results in an increased back-pressure of electrons in the electron transport chain of the chloroplast (equivalent to the photosynthesis light reaction).[14] The chloroplast can deal with this back-pressure of electrons by energy dissipation and repair of $PSII_{NF}$ to a limited extent[15] (indicated by a reduced F_v/F_m, that is constant over time in leaves exposed to 80 nmol mol^{-1} ozone). If the back-pressure becomes too high (as in older leaves exposed to 120 nmol mol^{-1} ozone), this leads to termination of the electron transport chain. The electrons might then be transferred to O_2, leading to the formation of active oxygen species (AOS). These AOS would then transfer electrons to plastoquinone (which is part of the chloroplast's electron transport chain), which results in the generation of semiquinone radicals. On the basis of these spectroscopic results, however, we cannot exclude the possibility that ubiquinone from mitochondria features in the generation of the EPR signal, or indeed that the observed free radicals are the result of a completely different process.

It is also noteworthy that the ozone-induced free radical survived the freeze-drying treatment. Consequently it might be useful as a marker for the detection of a stress history in processed plant material. Moreover, if similar environmentally-generated free radicals survive processing in other plant tissues, there may be implications for food quality, both from the point of view of the radicals themselves and the antioxidants which inhibit their formation. Such work will be the subject of future investigations.

CONCLUSIONS

Stable free radicals are generated in wheat leaf tissue as a consequence of exposure to elevated ozone levels. These radicals are formed after damage to the photosynthetic apparatus and are different from those generated during the early stages of ozone toxicity that have been reported previously.

Acknowledgments

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