Free Radical Research, February 2008; 42(2): 105–113

informa
healthcare

Long-term ozone exposure of potato: Free radical content and leaf injury analysed by Q-band ESR spectroscopy and image analysis

AMPARO ASENSI-FABADO[1], JOSÉ L. CARRASCO-RODRÍGUEZ[1], CARLOS J. GÓMEZ-GARCÍA[2], & SECUNDINO DEL VALLE-TASCÓN[1]

[1]*Departamento de Biología Vegetal, Universidad de Valencia, C/ Doctor Moliner 50, 46100 Burjassot, Valencia, Spain, and* [2]*Instituto de Ciencia Molecular, Departamento de Química Inorgánica, Universidad de Valencia, Pol. La Coma s/n, 46980 Paterna, Valencia, Spain*

Accepted by Dr E. Niki

(Received 23 July 2007; in revised form 22 October 2007)

Abstract

This paper presents Q-band electron spin resonance (ESR) studies on free radicals (FR) generated in potato leaves exposed to different O_3 levels in open-top chambers (OTC), together with a quantitative study of the relationship between FR signal intensity and area of potato leaf damage. The advantages of Q-band when compared to X-band ESR spectroscopy are analysed, the main advantage being an absence of overlapping between Mn(II) and FR signals, allowing a quantitative study of FR signal intensity. This study also reports on a graphical method developed to quantitatively measure the damaged area on leaves caused by ozone exposure. Results indicate a direct relationship between FR signal intensity (measured as area under the signal) and percentage of O_3 damage and clearly demonstrate a close relationship between visible ozone-induced symptoms and permanent FR concentration in potato leaves.

Keywords: *Q-band ESR spectroscopy, potato leaf injury, permanent free radical, open-top chambers, ozone*

Abbreviations: *auc, area under the curve of the ESR signal; ESR, electron spin resonance; FR, free radical(s); I, nuclear spin; id, intensity of damage; R-squared, determination coefficient; RMSE, root mean squared error; OTC, open-top chambers; UTC, universal time coordinated.*

Introduction

Ozone (O_3) is regarded as one of the most damaging air pollutants affecting crops [1]. Responses of vegetation to tropospheric O_3 include: foliar injury, inhibition of photosynthesis, reduction of productivity, decreased growth, changes in crop quality, increased sensitivity to ambient stress, shift in the partition of carbon and increased respiration [2–5].

Ozone enters the leaf through stomata. The O_3 concentration in the intracellular space is close to zero [6], indicating that O_3 is absorbed and rapidly decomposed. Once in the substomatic cavity, O_3 reacts with water [7], with the plasmalemma or the cell wall [8] to form reactive oxygen species (ROS). Highly ROS disrupt membranes integrity, pigments structure and oxidize proteins [9], since they are well known as toxic metabolic products [10]. ROS trigger programmed cell death (apoptosis) [10–12], trigger gene expression of enzymes involved in the synthesis of detoxifying substances [13,14] and act as a signal to initiate or coordinate other processes such as ethylene production, which induces senescence [10,11].

Correspondence: Amparo Asensi-Fabado, Departamento de Biología Vegetal, Universidad de Valencia. C/ Doctor Moliner 50, 46100 Burjassot, Valencia, Spain. Tel: 34 963543786. Fax: 34 963544372. Email: m.amparo.asensi@uv.es

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760701834537

Several research groups have used ESR spectroscopy to study paramagnetic compounds formed during O_3 exposure. The first direct evidence for the formation of FR in plants exposed to O_3 has been reported by Mehlhorn et al. [8]. These authors observed a FR after treating bean plants with the spin-trapping N-t-butyl-α-phenylnitrone and exposing them to O_3; however, the nature of the trapped radical was not identified. During the exposure to O_3 (240 $\mu g/m^3$) in air flowing through the spectrometer cavity [15], the appearance of a signal with the characteristics of superoxide radical has been demonstrated. In freeze-dried wheat leaves a stable FR has been observed [16]. Massive FR generation accompanied the freeze-drying process [17] and thus the stable FR [16] probably corresponded to the freeze-drying leaf treatment. A single-peak FR signal and a large increase of the Fe(III) signal at $g = 4.3$ was observed [18] in wheat leaves fumigated with high O_3 concentrations (150–400 ppb, for 8 h a day, over a period of 3 days). Fumigation with different O_3 concentrations and leaf treatments suggested the possibility that research groups were observing distinct FR.

The aim of this study was to use direct ESR spectroscopy to study paramagnetic compounds formed during long-term (chronic) exposure to O_3 in open-top chambers. The objectives of this study were to (i) compare the extent of ozone-induced paramagnetic compounds formation in OTC, (ii) evaluate the effect of O_3 doses and (iii) determine the relationship, if any, between the presence of paramagnetic signals and the ozone exposure. We tested the hypothesis of different ESR signal intensity for different ozone doses.

Materials and methods

Experimental site

The experiment was conducted at the Centro de Capacitación Agraria of the Generalitat Valenciana, located at Carcaixent. The site is a rural area 40 km south of the city of Valencia in eastern Spain (39°7′N, 0°27′W), 20 m above sea level.

Plant material

Potato tubers (*Solanum tuberosum* L. var. Agria) were sown manually in 20 L-pots (one tuber per pot) in standard potting soil mix (Terraplant 1, BASF). The crop was cultivated from 3 February until 19 May 2005.

Plants developed their whole cycle in OTC under three different ozone concentrations: charcoal-filtered air (CF), in which ~80% ambient ozone is removed, ambient air passing a particle filter only (NF) and NF air plus O_3 (NF^+), 5 h a day from Monday to Friday (08:30–13:30 hours UTC). The NF^+ treatments comprised ambient air plus 80, 100, 120, 100 and 80 ppb of ozone at 8:30–9:30, 9:30–10:30, 10:30–11:30, 11:30–12:30 and 12:30–13:30, respectively.

Samples were harvested when the crop had almost completed its cycle, on 26 April 2005. The third leaflet from the fourth fully expanded leaf in a random shoot of each plant was collected and immediately frozen in liquid nitrogen. The samples were stored at -80°C until measurement.

The open-top chambers facility

The OTC were 2.4 m high (including a 0.4 m frustum) with a diameter of 3 m, each chamber made of three sections of transparent plastic in aluminium frames. A frustum was placed at the top of the chambers forming a 40° inward angle to minimize the influx of ambient air. The chambers were ventilated by electric fans at a rate of approximately four chamber volumes of air per minute. Ozone was produced by an electric discharge generator (GOAC mod S-3003, SIR, Valencia, Spain).

Ozone, sulphur dioxide and nitrogen oxides were monitored continuously throughout the experiments. The air sampling point was located at the centre of each chamber at a height of 1 m. Ozone was determined by UV absorbance (Dasibi mod. 1008 RS), SO_2 by fluorescence (Dasibi mod. 4181) and nitrogen oxides by chemiluminescence (Dasibi mod. 2108).

Sample preparation for ESR spectroscopy

Samples were kept in liquid nitrogen prior to ESR measurement. Two methods were used to select the leaf material. Initially, frozen small leaf pieces of 12–16 mg weight for X-band and 2–7 mg for Q-band measurements were randomly selected (major leaf veins were discarded) and transferred to quartz tubes. In the second method, defrosted leaves were used to select samples for Q-band spectroscopy measurements following a gradation in ozone-damaged leaf surface proportion. Each leaf sample was photographed and immediately stored in liquid nitrogen until ESR measurements were done. During sample selection, samples were kept at room temperature for ~1 min.

ESR spectroscopy

ESR measurements were performed with a Bruker ELEXSYS E-500 computer-controlled spectrometer operating at either X or Q-band frequencies (9.4 or 34 GHz, respectively).

Preliminary measurements were made at different temperatures in the range 10–250 K to find the optimum signal, for both X and Q-bands. The temperature that yielded the best signal quality in

X-band was 10 K, whereas 100 K was found to be the optimum for Q-band measurements.

Spectra were recorded in 1024 points using a modulation frequency of 100 kHz, microwave attenuations of 10–25 dB (giving microwave powers of 19.3–0.6 mW) and modulation amplitudes of 0.2–0.8 mT (1 mT = 10 G). Measurements were made over the range 238–438 mT at X-band or 1160–1260 mT at Q-band. The parameters were selected after determination of the saturation characteristics of the ESR signals to obtain maximum signal intensity without distortion or saturation. Spectra were acquired as the first derivative of the microwave absorption.

The intensities of the Mn signals were measured as the area under the curve (auc) by calculating the sum of the trapezoids formed by the data points and were normalized for sample weight. Free radical signal intensities were measured as the auc and were normalized for sample weight and manganese signal intensity. g-values were determined from the equation of resonance $h\nu = g\beta H$, where h is Planck's constant (6.626×10^{-34} Js), ν is the frequency of the electromagnetic radiation, β is the Bohr magneton (9.274×10^{-24} J/T), H is the resonant magnetic field and g is the gyromagnetic constant that characterizes the resonance point position of the chemical species being investigated. Calculated standard deviations were found to vary between 0.04–0.07% of the mean g-values.

Image analysis

Both intact attached leaves and leaf ESR samples were photographed for visible ozone-injury assessments by image analysis. Intact attached whole leaves were photographed with a digital camera (Olympus Camedia C-5050 Zoom) on 26 April 2005. Leaf ESR samples were photographed with a stereomicroscope attached digital camera (Olympus DP 10) prior to ESR measurements. Digital images were analysed to estimate the percentage of leaf surface visibly injured by ozone. The identification of visual ozone symptoms was based on the examples given by Donnelly et al. [19] and on the experience gained in many ozone fumigations with OTC. The injured areas were selected with the Magic Wand tool in Adobe Photoshop v7.0. The selected areas were then filled with white colour using the Edit options. A selection of the total sample area was also performed following the same procedure. The images were then retrieved with Matlab v6.5 (Image Analysis toolbox). The white foreground pixels in both images (injured and total area) were separated and differentiated from the background using the threshold command. The area occupied by white pixels in both images was measured as number of pixels. Finally, the percentage of visible injury in the sample was calculated as 100 × (injured area/whole sample area).

Statistics

Analysis of variance (ANOVA) followed by post-hoc least significant difference test to check the effects of ozone treatment on Mn and FR signal intensities was performed with SPSS v12.0. Differences were considered significant at $p \leq 0.05$. Polynomial regression was calculated with Matlab v6.5 (Curve fitting toolbox).

Results

Air quality and ozone exposure

The ambient air ozone concentrations were characterized by a fairly high background concentration with the exception of 2 days at the beginning and the end of this experiment period. In the ambient air chamber, the average concentrations (24-h mean and mean daily maximum) were, respectively, 23.8 and 37.76 ppb. Daily, 24-h average of ozone in the three OTC treatments during the experimental period is shown in Figure 1.

Differences in ozone exposures between the treatments were readily apparent as expressed in the AOT40 (accumulated exposure over a threshold over 40 ppb) values. The AOT40 exposure index for ozone within filtered air, ambient air and ambient air enriched with ozone was 33, 4186 and 16 488 ppb.h, respectively.

The concentration of sulphur dioxide was very low and close to the detection limit of the analytic equipment. The levels of nitrogen oxides (NO and NO_2) were also monitored. Their 24-h average mean during the growing season never exceeded 5 ppb for any of the two gasses, levels too low to have a

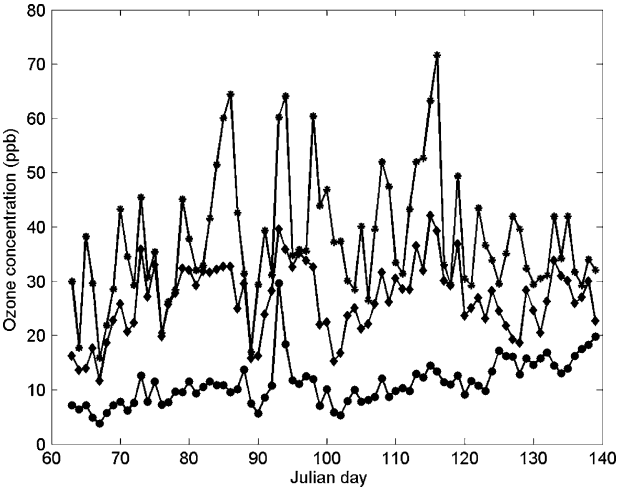

Figure 1. 24-h mean values for ozone exposures within charcoal-filtered (circles, bottom), non-filtered (diamonds, middle) and non-filtered plus extra ozone (asterisks, top) OTC at Carcaixent (Spain) for the period 3 February to 19 May 2005.

substantial effect on O_3 response in these experiments [20].

Severity of ozone injury was assessed as the percentage of damaged leaf area in leaves with foliar O_3 symptoms. Clear injury symptoms caused by ozone exposure were observed in all three OTC. The degree of O_3 injury varied among OTC. Maximum injury was observed in NF^+ chambers. Inside this chamber type, 33.9% injury was observed, whereas 0.7 and 6.5% injuries were observed in CF and NF, respectively.

X-band and Q-band: A comparison

ESR studies of plant leaves were carried out at X and Q-bands. At the beginning of this work, measurements were taken using X-band over the range 238–438 mT. A six-line hyperfine signal was found (Figure 2), resulting from the interaction between the unpaired electrons with the nuclear magnetic moment of the ^{55}Mn nucleus (nuclear spin, $I = 5/2$, natural abundance = 100%) involved in the unpaired electron orbital. The signal had a $g = 2.00119 \pm 0.00089$. This assignment is in agreement with other authors [8,15,18,21–23]. Another signal, corresponding to mononuclear Fe(III) complexes, which has $g = 4.27$ [18], is sometimes found in plant ESR spectra, but this was not present in our samples (data not shown). However, a broad feature underlying the Mn(II) spectra, which some authors have attributed to Fe(III) in storage proteins such as ferritin [18], could be observed in the X-band spectra. The fourth hyperfine component of the signal appeared to be higher than the others, suggesting an overlapping with other signal(s). Because of this strong overlapping it is not possible at present to derive accurate parameters for this extra signal(s). To compare the performance of Q-band, similar studies were carried out with O_3-treated potato leaves.

As shown in Figure 3, the six-line signals were much better resolved at Q-band and therefore signal parameters could be measured accurately: hyperfine splitting of 9.5–9.7 mT, peak-to-peak linewidth of 1.0–1.7 mT and $g = 2.0064 \pm 0.0014$. These parameter values confirm that this signal can be attributed to Mn(II). Moreover, a new signal appeared between the third and fourth hyperfine components, with $g = 2.0105 \pm 0.0014$ and half-height linewidth of 0.7–0.8 mT. This signal can be attributed to one or several FR. It is worth noting that the signal overlapping found at X-band was not observed in Q-band spectroscopy.

The Mn(II) signal intensity was computed for each OTC treatment. The ANOVA test indicated a test statistics (F_s) of 0.809 with two degrees of freedom and a p-value of 0.461: the F_s not being significant. Thus, the sextet signal auc was not affected by the OTC treatment. Since the Mn signal intensity was constant, FR auc was normalized for the Mn(II) signal.

Visible injury and ESR signals

The working hypothesis of this research was initially centred on the supposition that potatoes growing under high ozone concentrations present more intense ozone-induced paramagnetic signals than plants exposed to low concentrations of this pollutant. In Figure 3 the chamber effect on FR signal is presented. The visual inspection of this signal and the ANOVA analysis ($F_s = 0.317$ and $p = 0.735$, two degrees of freedom) demonstrated that FR were not related to the O_3 level in the OTC and thus the working hypothesis should be reformulated. However, when we measured ozone-injured leaf pieces,

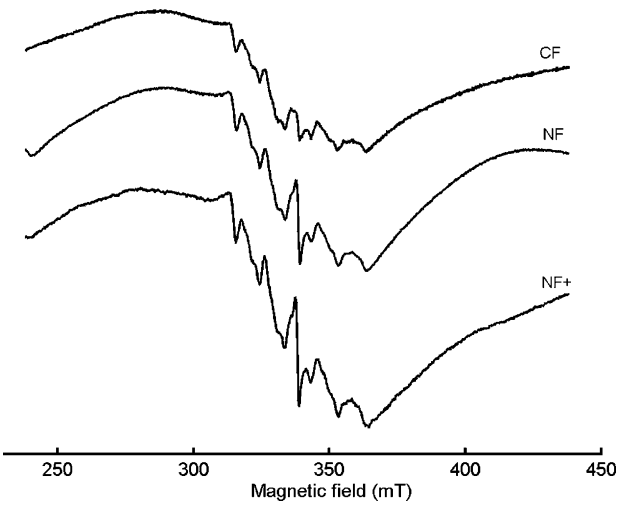

Figure 2. X-band ESR spectra taken at 10 K from potato plants grown within OTC. CF, charcoal-filtered air; NF, non-filtered air; NF+, non-filtered air plus extra ozone.

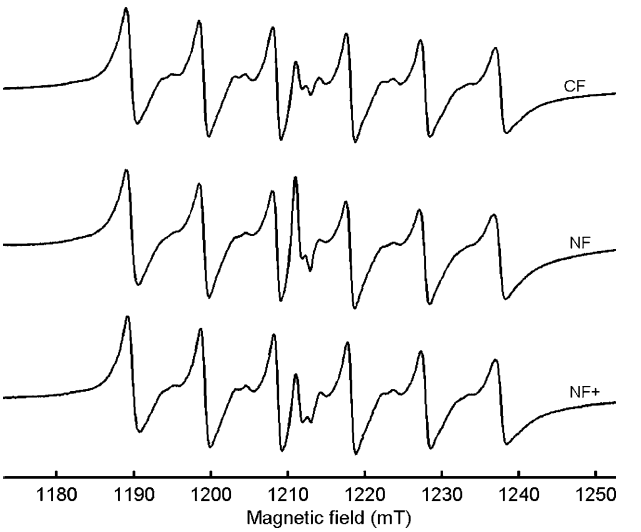

Figure 3. Q-band ESR spectra taken at 100 K from potato plants grown within OTC. OTC treatments as in Figure 2.

their FR signals were higher than those yielded by green pieces from the same leaves (data not shown). This observation suggested that FR signal could be related to visible injury caused by ozone. In fact, although the X-band data in Figure 2 show the expected order in the intensity of the FR signal ($NF^+ > NF > CF$), this is not the case in the Q-band data in Figure 3. A possible reason for this discrepancy is that the leaf pieces used for the X-band spectra are more representative of the whole leaf since they are much bigger than the samples used in the Q-band spectra.

To contrast the new hypothesis we needed to use a different method for sample selection. Before employing this method, we studied signal stability at room temperature. Following immediate ESR measurement, two samples were kept in the quartz tubes at room temperature and measured again after 24 h. Although both Mn(II) and FR signal intensities decreased over time, they could still be detected after 24 h at room temperature, the mean intensity decrease being 21.9% for Mn(II) signal and 41.2% for FR signal (Figure 4). These data revealed that FR and Mn signals were relatively persistent.

Visible injury percentage of every leaf sample was accurately determined by the developed image analysis method. Figure 5 shows, as an example, a piece of potato leaf and the selected damaged areas used for image analysis calculations.

Variations in the free radical signal are presented in Figure 6. The free radical heights showed a progressive increase with the proportion of damaged area. It is worth noting that the free radical signal was not associated with the O_3 concentrations inside the OTC and so it would be interesting to plot the free radical auc against the percentage of O_3 damage.

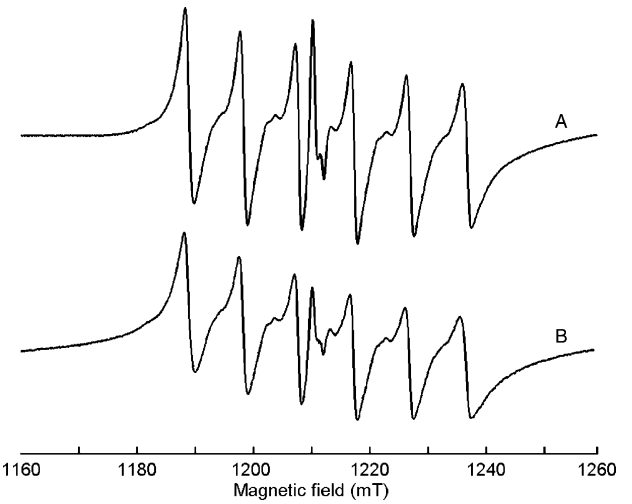

Figure 4. Variation of the Q-band ESR signal after keeping a non-filtered leaf sample at room temperature for 24 h. (A) Sample measured immediately after preparation, (B) same sample measured after 24 h at room temperature.

Figure 7 shows the percentage of visual symptoms caused by ozone plotted against the area under the FR signal: this plot shows the presence of a persistent weak FR signal when the visible injury is below 25% together with an exponential increase of FR intensity as the visual symptoms increase above this threshold of ca. 25%. This relationship suggests that FR are mainly formed when symptoms exceed a certain limit, that can be considered as the self-protection threshold of leaves. Thus, in the present case, leaf areas exhibiting more than ca. 25% symptoms are not able to remove FR and therefore their FR concentration increases in a quite abrupt way.

Discussion

In this study we attempted to investigate the usefulness of Q-band ESR spectroscopy, in combination with the quantitative estimation of the leaf area affected by visible symptoms, to measure paramagnetic species formed during long-term (chronic) exposure to O_3 in potato plants. The initial working hypothesis that the paramagnetic species formation and concentration would be related to ozone exposure was based on the response of antioxidant enzymes (e.g. guaiacol peroxidase, glutathione peroxidase and ascorbate peroxidase) and antioxidant compounds (e.g. ascorbic acid and glutathione) to O_3 exposure. Both enzymatic and non-enzymatic systems are affected by O_3 exposure [10,11,24]. Our initial measurements using the X-band ESR spectroscopy indicated important limitations and uncertainties. In order to overcome these problems a microwave frequency of 34 GHz (Q-band) was used.

Open-top chambers have been extensively used as a standard tool to study O_3 effects on crops. The most evident effect of O_3 on plants is visible foliar damage. The currently used exposure index for O_3 in Europe is the AOT40. Critical levels of air pollutants correspond to the cumulative exposure resulting in significant negative effects on crops. The AOT40 (in ppb.h) for the experimental period was 4186 ppb.h in NF chambers. This AOT40 index was higher than 3000 ppb.h, the critical value proposed by the United Nations Economic Commission for Europe (UNECE) for crops [25]. The results presented here clearly indicate the influence of O_3 on the development of foliar injury on potato leaves.

As the ambient O_3 concentrations were particularly high, charcoal-filtering treatment still resulted in adult leaves receiving a sufficient dose to induce foliar symptoms. However, the extent of injury was much more reduced in comparison to non-filtered (ambient) and fumigated chambers. The highest percentage of ozone injury, measured as injured leaf surface, was observed in plants grown in NF^+ chambers.

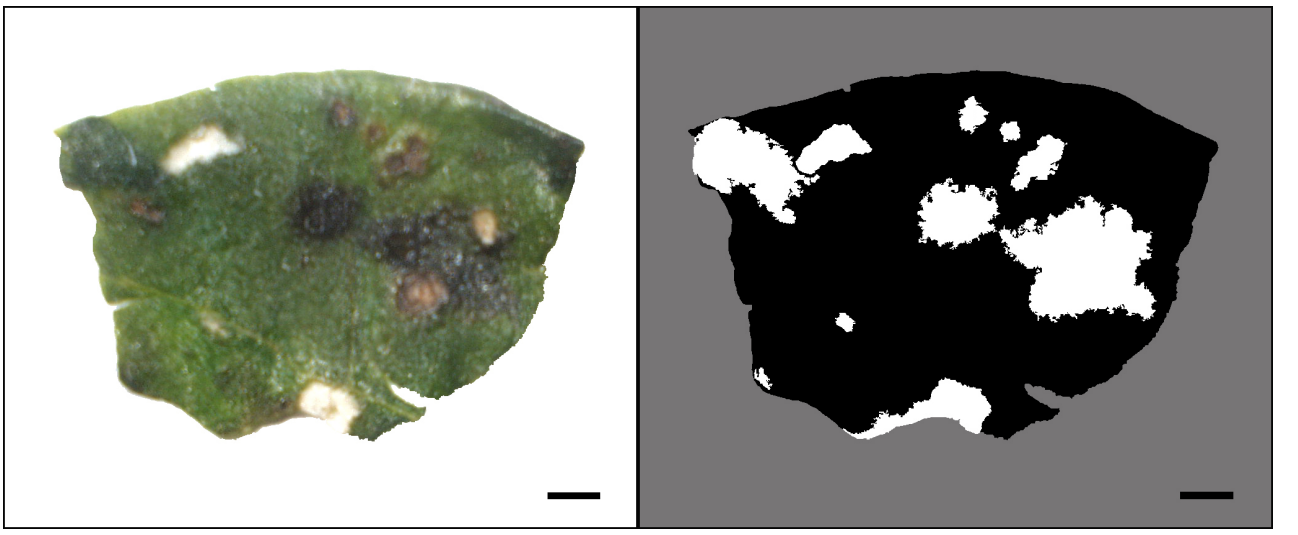

Figure 5. Left: photograph of a non-filtered potato leaf piece with ozone-induced lesions. Right: image of the same leaf piece with the lesions filled with white colour. Bars = 1 mm. According to the analysis, the injured area of this leaf piece comprised 21.1% of the total area.

It has been suggested [26] that O_3 differs from other atmospheric pollutants in the fact that it can injure sensitive species at concentrations just above the maximum natural concentrations (~ 4 ppb). The observed 24-h mean in CF chambers was 7.53 ppb; the exhibition of foliar damage in these chambers indicated that potato plants are sensitive to O_3 injury. Given the current knowledge about the physiological and morphological responses of plants exposed to O_3, there is a general consensus that this exposure can induce a variety of responses in sensitive species. Ozone injury presents a wide range of visible symptoms and their physiological meaning is not well known. The leaf injury severity induced by O_3 is determined by concentration, exposure duration, genetic and many environmental variables. Visible

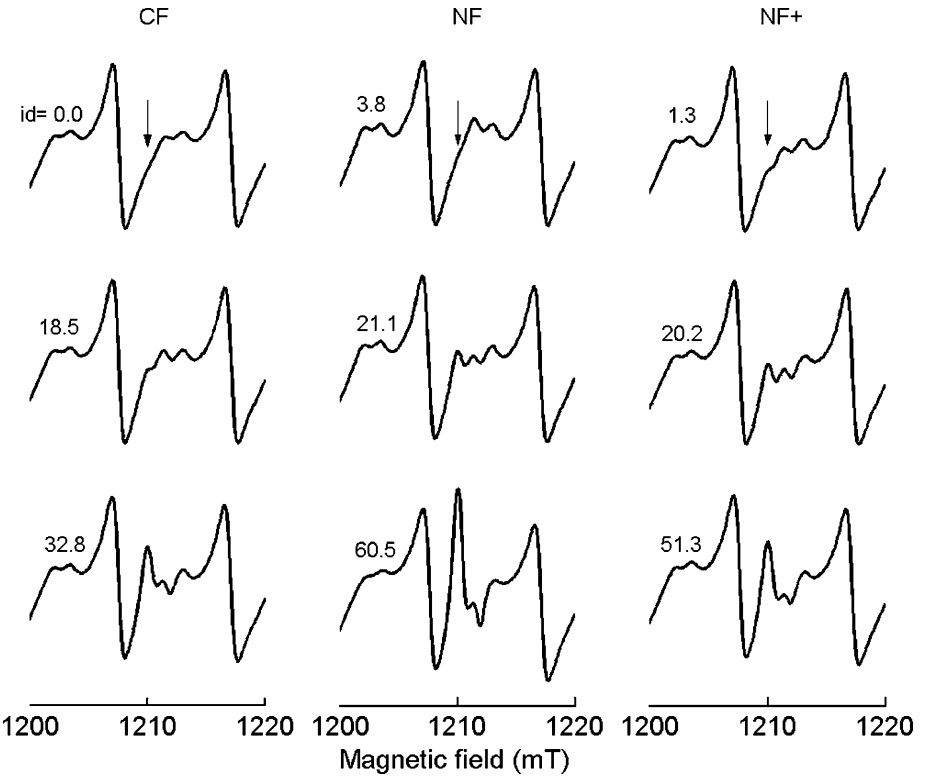

Figure 6. ESR signals from potato leaf pieces grown within OTC (CF, left column; NF, central column; NF+, right column; treatment abbreviations as in Figure 2) exhibiting different percentage of visible injury. The spectra have been shortened to the third and fourth hyperfine components of the Mn(II) signal. The arrows indicate the location of the FR signal (g = 2.011) within each column. id: intensity of damage (% visible injury measured by image analysis).

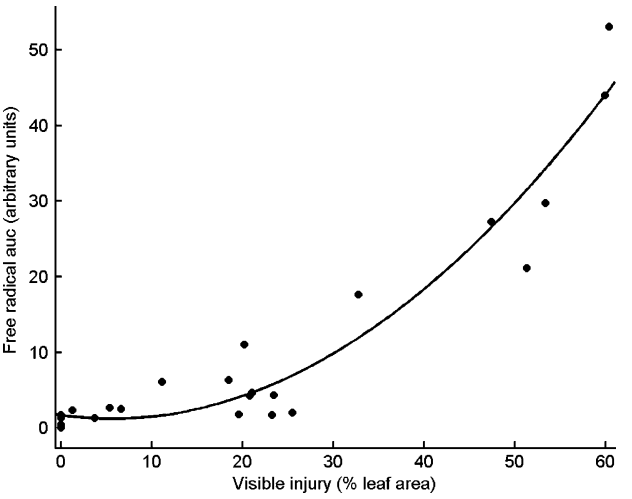

Figure 7. Scatter plot and quadratic regression of the free radical signal area under the curve (g = 2.011) as a function of the percentage of ozone-induced visible symptoms. The robust quadratic regression was accepted as the best fit because the minimum RMSE (2.45) and maximum adjusted *R*-squared (0.97) were observed ($p < 0.0001$).

foliar symptoms consist of the collapse of the palisade mesophyll cells [27] as a result of ROS accumulation and the synthesis of detoxifying compounds.

All ESR studies on plant leaves have been carried out at X-band spectroscopy. For numerous reasons (overlapping of spectral components, noise and resolution) researchers tend to use higher frequencies. Q-band spectroscopy offers higher sensitivity and increased g-factor resolution; thus smaller samples can be measured when compared to X-band. Therefore, Q-band spectroscopy, with a higher magnetic field strength, could yield more information on plant leaves. In order to obtain more information, potato leaves were measured using Q-band at 100 K. The obtained Q-band spectra showed better resolution and lower noise.

Sample size is one advantage of Q-band spectroscopy. Leaf sample surface was 20–50 mm^2 and fresh weight was 2–7 mg. This sample size is relatively small when compared to antioxidant enzyme activity measurements ($\sim$ 80–120 mg fresh weight).

In order to measure FR in leaf pieces with different percentages of visible symptoms, a specific method was developed. It was assumed that ESR signal intensity did not decrease significantly during the short time taken for sample selection and photography, based on the FR stability measurements mentioned above. It is important to note that obtaining samples with a high percentage of visible injury from CF leaves was a difficult task. Moreover, we were able to select few samples with a low degree of ozone injury from NF^+ leaves. This is a consequence of the fact that damaged areas in intact leaves increased with ozone concentration in the OTC.

The Mn(II) paramagnetic signal is typical of plant tissues [28]. Manganese is an essential element for plants and a cofactor for arginases, phosphotransferases, superoxide dismutases and the water splitting complex in photosynthesis [29]. The Mn(II) ESR signal intensity is strong in conifer needles and has been used as a bioindicator of O_3 pollution [23]. The change in the manganese signal intensity has been observed in studies with O_3 exposure, this signal decrease being associated with the PS II disintegration [24,30]. The results of our study, consisting in no change in the Mn(II) signal intensity, are in agreement with the results from wheat plants exposed to air with 80 nmol/mol added O_3 [16]. However, significant decreases in the Mn(II) ESR signal have been reported in perennial rye fumigated with 120 nmol/mol O_3 for 4 weeks [15] and in pine needles grown in areas with high O_3 concentration [30]. The results presented in this paper reporting that the Mn(II) signal was statistically independent of visual symptoms and O_3 exposure has been used to normalize the FR signals. Data not presented demonstrate that the g = 4.27 ESR signal was not observed in potato spectra. These results are in agreement with a very weak signal reported in wheat plants exposed to ambient air supplemented with 80 and 120 mmol/mol O_3 [16].

The results presented here demonstrate a single-peak radical signal with a $g = 2.0105 \pm 0.0014$ and ca. 0.75 mT linewidth. Considerably higher linewidths have been reported for X-band spectroscopy resulting from O_3 treatments [15,16]. The values for g-factor reported in the literature vary over the range 2.001–2.003, these g-values being clearly lower than our g-factor. Owing to the differences in g-factor, linewidths and O_3 treatments (chronic or acute), it is possible that different types of free radicals are formed as a result of O_3 damage. Unfortunately, it is not possible to unambiguously determine the exact nature of the FR generating the observed ESR signal. In fact, previous studies on ozone and other stress processes (senescence and desiccation) have assigned the observed signals to different types of quinone and semiquinone radicals [31], phenoxy and radical centres trapped in macromolecular structures such as proteins [18]. The assignment of a quinone-derived FR has been previously discussed [32].

A number of different assessment methods are available to measure visual symptoms. Currently available techniques for performing foliar injury measurements rely on visual estimation. Visual assessment usually uses scanning systems (e.g. Horsfall and Barrat [33] rating systems). Visual injury assessment is based on a single characteristic or a number of different characteristics that may be combined into a single index of injury [34]. These approaches are limited by observer subjectivity and bias and by intra- and inter-observer variations. Another disadvantage is statistical analysis, since assessments are often made using ordinal scales. The major advantage of

imaging consists of the instantaneous visualization of the heterogeneity in the visual symptoms of the leaf sample. Our method successfully determines the lesion area of the leaf image to evaluate the severity of ozone exposure on potato. The method developed required a stereomicroscope-attached digital camera, a personal computer and two computer programs (Adobe and Matlab) for image analysis and allowed a quantitative assessment. In future research on foliar injury development, our procedure could be used to undertake repeated assessments and also to estimate visual symptoms caused by other air pollutants.

To gauge the importance of visual symptoms for the occurrence of FR signal intensity the experimental data were fitted by the polynomial regression. The statistical parameters of such fitting calculation (adjusted *R*-squared and RMSE) evidenced a good agreement of experimental and calculated data. There was a statistically significant relationship between FR intensity and visual symptoms. To the best of the authors' knowledge, no studies have been reported concerning this relationship.

In conclusion, our results suggest an experimental approach for studying the deleterious effects of O_3 and other air pollutants on crop plants. At a magnetic field of 1.25 T (when compared to 0.36 T at X-band), the Mn(II) sextet was much better resolved and the single-line signal did not overlap with the hyperfine components of the previous signal, thereby minimizing noise, sample size, lower resolution and other limitations of X-band ESR spectroscopy. Q-band is thus much more appropriate for studying plant tissues than X-band. The ESR spectra of potato leaves demonstrated a persistent radical induced by O_3 exposure. The present study reports on the relationship between FR auc and the percentage of foliar visible injury. Q-band ESR spectroscopy represents a valuable tool for detecting ozone-induced paramagnetic species in crop plants.

Acknowledgements

This work was supported by a grant (Project Bio 2005-09252-C02-02) from the Ministerio de Educación y Ciencia, Spain. AAF thanks the Generalitat Valenciana for a predoctoral fellowship. The authors thank Dr Samia Benmansour for her valuable help with the ESR measurements, and Dr Carrasco-Sorlí and Dr Ferrando (University of Valencia) for their helpful comments. We also thank Mr Duncan Gates for improving the English style of the manuscript.

References

[1] Thomson AM. The oxidising capacity of the Earth's atmosphere: probable past and future changes. Science 1992;256:1157–1165.

[2] Runeckles VC, Chevone BI. Crop responses to ozone. In: AL Lefohn, editor. Surface level ozone exposures and their effects in vegetation. Chelsea: Lewis; 1992. p 189–270.

[3] Heath RL, Taylor GE. Physiological processes and plant responses to ozone exposure. In: H Sandermann, AR Wellburn, RL Heath, editors. Forest decline and ozone: A comparison of controlled chamber and field experiments. Berlin: Springer; 1997. p 317–368.

[4] del Valle-Tascon S, Carrasco-Rodriguez JL. Impact of ozone on crops. In: Dris R, Jain SM, editors. Production practices and quality assessment of food crops. Vol 1. Dordrecht: Kluwer; 2004. p 189–208.

[5] Fiscus EL, Fitzgerald LB, Burkey KO. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant Cell Environ 2005;28:997–1011.

[6] Laisk A, Kull O, Moldau H. Ozone concentration in leaf intracellular air spaces is close to zero. Plant Physiol 1989;90:1163–1167.

[7] Grimes HD, Perkin KK, Boss WH. Ozone degrades into hydroxyl radical under physiological conditions. Plant Physiol 1953;72:1016–1020.

[8] Mehlhorn H, Tabner BJ, Wellburn AR. Electron spin resonance evidence for the formation of free radicals in plants exposed to ozone. Physiol Plant 1990;79:377–383.

[9] Mehlhorn H, Seufert G, Smitdt A, Kunert KJ. Effects of SO_2 and O_3 on production of antioxidants in conifers. Plant Physiol 1986;82:363–383.

[10] Schraudner M, Langebartels C, Sandermann H. Changes in the biochemical status of plant cells induced by the environmental pollutant ozone. Physiol Plant 1997;100:274–280.

[11] Pell EJ, Schlagnhaufer CD, Arteca RN. Ozone-induced oxidative stress: mechanisms of action and reaction. Physiol Plant 1997;100:264–273.

[12] Wohlgemuth H, Mittlstrass K, Kschieschan S, Bender J, Weigel HS, Oermeyer W, Kangsjärvi J, Sandermann H, Langebartels C. Action of an oxidative burst as a general feature of sensitive plants exposed to air pollutant ozone. Plant Cell Environ 2002;25:717–726.

[13] Alsher RG, Donauhe JL, Cramer CL. Reactive oxygen species and oxidants: relationships for green cells. Physiol Plant 1997;100:233–244.

[14] Kolb TE, Fredericksen TS, Steiner KC, Skelly JM. Issues in scaling tree size and age responses to ozone: a review. Environ Pollut 1997;98:195–208.

[15] Runeckles VC, Vaartnou M. EPR evidence for superoxide anion formation in leaves during exposure to low levels of ozone. Plant Cell Environ 1997;20:306–314.

[16] Reichenauer TG, Goodman BA. Stable free radicals in ozone-damaged wheat leaves. Free Radic Res 2001;35:93–101.

[17] Pirker KF, Goodman BA, Pascual EC, Kiefer S, Soja G, Reichenauer TG. Free radicals in fruits of three strawberry cultivars exposed to drought stress in the field. Plant Physiol Biochem 2002;40:709–717.

[18] Goodman BA, Reichenauer TG. Formation of paramagnetic products in leaves of wheat (*Triticum aestivum*) as a result of ozone-induced stress. J Sci Food Agri 2003;83:1248–1255.

[19] Donnelly A, Craigon J, Black CR, Colls JJ, Landon G. Does elevated CO_2 ameliorate the impact of O_3 on chlorophyll content and photosynthesis in potato (*Solanum tuberosum*)? Physiol Plant 2001;111:501–511.

[20] Darrall NM. The effect of air pollutants on physiological processes in plants. Plant Cell Environ 1989;12:1–30.

[21] Stegmann HB, Schuler P, Ruff HJ. Investigation of damage in forest by EPR spectroscopy *in vivo*. Photochem Photobiol 1989;50:209–211.

[22] Reichenauer TG, Bolhar-Nordenkampf HR, Erhilich U, Soja G, Postl WF, Halbwachs F. The influence of ambient and elevated ozone concentrations on photosynthesis in *Populus nigra*. Plant Cell Environ 1997;20:1061–1069.

[23] Alberti A, Macciantelli D, Predieri S, Rossi F. Monitoring the physiological activity of plants by means of EPR spectroscopy. Mn(II) signals in *Pinus nigra* Arnold. Trees 2000;14:312–315.
[24] Iqbal M, Abdin MZ, Mahmooduzzafar, Yunus M, Agrawal M. Resistance mechanisms in plants against air pollution. In: Yunus M, Iqbal M, editors. Plant response to air pollution. Chichester: John Wiley & Sons; 1997. p 195–240.
[25] Fuhrer J, Skärby L, Ashmore MR. Critical levels for ozone: effects on vegetation in Europe. Environ Pollut 1997;97:91–106.
[26] Boone R, Westwood R. A forest health study of bur oak (*Quercus macrocarpa* Michx.) and trembling aspen (*Populus tremuloides* Michx.) stands near the Manitoba Hydro Selkirk generating station, Selkirk, Manitoba. Available online at: http://www.gov.mb.ca/conservation/envapprovals/publs/selkgenstn/index.html, accessed July 2003.
[27] Guderian R, Tingey DT, Rabe R. Effects of photochemical oxidants on plants. In: Guderian R, editor. Air pollution by photochemical oxidants. Formation, transport, control and effects on plants. Ecological Studies. Vol 52. Berlin: Springer-Verlag; 1985. p 127–333.
[28] Hepburn HA, Goodman BA, McPhail DB, Matthews S, Powell AA. An evaluation of EPR measurements of the organic free radical content of individual seeds in the non-destructive testing of seed viability. J Exp Bot 1986;37:1675–1684.
[29] Marshner H. Mineral nutrition of higher plants. London: Academic Press; 1986.
[30] Valavanidis A, Zymi M, Stathopoulou D, Georgiou P, Ganotidis M. Monitoring of ozone pollution and the physiological activity of *Pinus halepensis* (Mill.) by electron paramagnetic resonance and other parameters. Trees 2004;18:630–638.
[31] Atherton NM, Hendry GAF, Möbius K, Rohrer M, Törring JT. A free radical ubiquitously associated with senescence in plants: evidence for a quinone. Free Radic Res Comms 1993;19:297–301.
[32] Goodman BA. Do EPR spectra show the presence of a unique and ubiquitous quinone-derived free radical that is associated with senescence in plants? Free Radic Res 1995;23:187–190.
[33] Horsfall JG, Barratt RW. An improved gradient system for measuring plant diseases. Phytopathology 1945;35:655.
[34] Muir PB, McCune B. Index construction for foliar symptoms of air pollution injury. Plant Dis 1987;71:558–565.