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RESEARCH PAPER

Hydrogen peroxide concentrations in leaves under natural conditions

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Abstract

While H_2O_2 has been implicated in numerous plant environmental responses, normal levels and variabilities are poorly established, and estimates of leaf tissue concentrations span more than three orders of magnitude, even in a single species under similar conditions. Here, leaf tissue H_2O_2 contents under natural conditions are reported after determining (i) that H_2O_2 in extracts was stable with time, (ii) that H_2O_2 added to the extract was recovered quantitatively, and (iii) that the H_2O_2 calibration curve was unaffected (or quantifiably affected) by the extract. The broad applicability of the protocol and variability in leaf concentrations were demonstrated using tissue collected from several habitats in association with three, more extensive, experiments. The first involved nychthemeral studies of the mangrove, *Rhizophora mangle* L. Lowest H_2O_2 levels occurred in the early morning and near sunset, with higher levels both at midday and at night. Second, using five temperate species in Spring, concentrations were compared on a warm, sunny day and a cool, cloudy day. Higher concentrations were found on the warm day for *Aesculus glabra* Willd., *Glechoma hederacea* L., *Plantago major* L., and *Viola soraria* Willd., while there were no differences in *Quercus macrocarpa* Michx. Finally, the effects of elevated CO_2 and ozone were examined in soybean, *Glycine max* L. Pioneer 93B15 under Free Air gas Concentration Enrichment (FACE) conditions. Both supplements led to elevated H_2O_2. Overall, mean leaf, midday, and mid-summer H_2O_2 concentrations ranged from 0.67 μmol $(gFW)^{-1}$ in mangrove to 3.6 μmol $(gFW)^{-1}$ in *A. glabra* Willd. Greatest within-species differences were only 2.5-fold in any of the studies.

Key words: Bur oak, FACE studies, hydroperoxide, Ohio buckeye, oxidative metabolism, red mangrove, soybean, violet.

Introduction

It is now broadly accepted that H_2O_2 is involved in a number of signalling cascades in plants (Neill *et al.*, 2002), including response to pathogen elicitors (Wojtaszek, 1997; Orozco-Cárdenas *et al.*, 2001; Kachroo *et al.*, 2003), extracellular oligogalacturonides (Spiro *et al.*, 1998; Bellincampi *et al.*, 2000), stomatal responses (Chen and Gallie, 2004), systemic acquired resistance (Chen *et al.*, 1993), and programmed cell death (Levine *et al.*, 1994). Understanding responses and mechanisms is complicated, however, in part by the rapid turnover of H_2O_2, and by uncertainties concerning what tissue levels should be considered 'normal'. On the one hand, it is reasonable to think that tissue concentrations should be low: exogenously applied concentrations of 6–8 mM were sufficient to induce the hypersensitive response and cell death in soybean suspension cultures after 2–4 h (Levine *et al.*, 1994), and similar concentrations have other adverse metabolic effects, including inhibition of Fe-SOD (90% at 10 mM) (Bhattacharya *et al.*, 2004), and Rubisco (Badger *et al.*, 1980). On the other hand, plants, like other organisms, have a remarkable ability to metabolize H_2O_2, and cells treated with concentrations as high as 10 mM can completely metabolize the compound in less than 10 min (Levine *et al.*, 1994).

Consistent with the hypothesis that tissue levels should be low, Chen *et al.* (1993) reported a concentration of 0.15 μmol $(gFW)^{-1}$ in tobacco leaves, rising to 0.25 μmol $(gFW)^{-1}$ 24 h after infiltration with 3-aminotriazole or salicylic acid. Similar control values have been reported in tomato leaves, rising to 0.2 μmol $(gFW)^{-1}$ following inoculation with *Botrytis cinerea* (Patykowski and Urbanek, 2003), while in pear fruit tissue, concentrations rose from

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approximately 0.35 to 0.8 μmol $(gFW)^{-1}$ during ripening or when endogenous catalase was inhibited by KCN (Brennan and Frenkel, 1977). H_2O_2 in leaf tissue also responds to other environmental stimuli. For example, in the mangrove, *Bruguiera parviflora* under greenhouse hydroponic conditions, leaf tissue concentrations were reported to increase from 0.067 to 0.089 μmol $(gFW)^{-1}$ following salinization (Parida and Das, 2005). Diurnal fluctuations and accumulations in response to atmospheric ozone have also been demonstrated in greenhouse studies in which conditions were purposefully kept as benign as possible by using charcoal filtered air, large soil volumes, and winter-time growth (Chen and Gallie, 2004, 2005). In those studies, H_2O_2 contents fluctuated diurnally between about 0.03 and 0.06 μmol $(gFW)^{-1}$, and ozone induced H_2O_2 accumulation in guard cell chloroplasts. Interestingly, the highest tissue H_2O_2 levels (10-fold higher than the controls) were found 24 h after the end of an acute ozone exposure.

In contrast to these studies, others have reported much higher H_2O_2 concentrations in leaf tissue. Chaparzadeh *et al*. (2004), for example, reported about 130 μmol H_2O_2 $(gDW)^{-1}$, or 6 μmol $(gFW)^{-1}$, in marigold (*Calendula officinalis*) under growth chamber conditions, rising 26% at high (100 mM) salinity. The relationship between irradiance, temperature and H_2O_2 concentrations in growth-chamber grown *Phragmites australis* leaves showed that brief heat stress (38 °C for 1 h) increased concentrations slightly, while both non-stressed and stressed leaf levels increased with irradiance between 500 and 1500 μmol $m^{-2}s^{-1}$. Overall, tissue concentrations ranged from 5 to 15 μmol $(gFW)^{-1}$ in wild-type plants (Velikova and Loreto, 2005). Water stress and mutationally-restricted proline accumulation capacity in soybeans under otherwise benign growth chamber conditions increased leaf tissue contents from about 1.2 to 4.2 μmol $(gFW)^{-1}$, with recovery upon re-watering, except when proline accumulation was restricted. In that case, there was a secondary increase in H_2O_2 to *c*. 4.2 μmol $(gFW)^{-1}$ associated with the appearance of tissue damage (Kocsy *et al.*, 2005). At the upper extreme, He *et al*. (2005), using Kentucky bluegrass (*Poa pratensis*) of turf origin but after 2 weeks to 2 months under growth chamber conditions, reported leaf tissue concentrations of 1% by dry weight, which, based on the data in their report, is approximately 60 μmol H_2O_2 $(gFW)^{-1}$ (nearly 100 mM on a tissue water basis).

It seems highly likely that some of the variability in reported concentrations, spanning more than three orders of magnitude, reflects methodology and experimental uncertainties rather than biological variation. In maize, for example, concentrations ranging from 20 μmol $(gFW)^{-1}$ under complete nutrient conditions to 75 μmol $(gFW)^{-1}$ under N-deficient conditions, were reported for plants under solution culture in a greenhouse (Tewari *et al.*, 2004), while another maize growth chamber study reported concentrations ranging only between 0.1 and 0.3 μmol $(gFW)^{-1}$ (Kim *et al.*, 2003).

Recently, Veljovic-Jovanovic *et al.* (2002) suggested that reports of tissue H_2O_2 concentrations were, in general, erroneously high due to interferences by ascorbate and phenolics with the peroxide assay itself. They showed, for example, that in the presence of ascorbate, the MTBH-DMAB (3-methyl-2-benzothiazoline hydrazone, 3-(dimethylamino) benzoic acid) assay was significantly less sensitive to H_2O_2 and that ascorbate introduced a significant zero-offset. Interference with the ferricyanide-dependent luminol method by ascorbate or other components of *Arabidopsis* leaf extracts was also demonstrated. Correcting for this by removing the ascorbate enzymatically before the assay, they reported tissue concentrations of 0.74 to 1.2 nmol cm^{-2}, or 0.04–0.12 μmol $(gFW)^{-1}$ in wild-type and catalase-deficient barley under growth chamber conditions.

Nevertheless, it is still not clear that this value is, as a generalization, the 'correct', or even a 'better' estimate of tissue concentrations. For example, despite their suggestion that concentrations should be restricted to 0.1 μmol $(gFW)^{-1}$ for compatibility with cellular biochemistry, one of the authors subsequently reported 6–10-fold higher leaf concentrations in free-growing gingko and birch (Kukavica and Veljovic-Jovanovic, 2004).

The present study began as part of a broader study on the responses of the mangrove, *Rhizophora mangle*, to stresses imposed by its environment and, in particular, of its antioxidant defences (Cheeseman *et al.*, 1997; Cheeseman and Lovelock, 2004; Kandil *et al.*, 2004; Pearse *et al.*, 2005). Preliminary studies aimed at quantifying leaf H_2O_2 levels identified potentially serious interferences due to high concentrations of phenolics, ascorbate and flavologlycans ('slime') and a substantial capacity for tissues and extracts to generate and consume H_2O_2 or other soluble hydroperoxides. [Note: Although all reports of water-soluble hydroperoxides in plants refer to them as H_2O_2, the analytical techniques themselves, with the exception enzymatic methods, are not specific. However, as there are no reports quantifying or detailing aqueous, non-H_2O_2 peroxides, that convention has been accepted here.]

In this paper, therefore, two objectives will be addressed. First, factors inherent in extraction and quantification which affect the reliability of the measured values are examined, and second, a range of leaf tissue H_2O_2 concentrations is established under natural conditions which can serve as a baseline for 'normal' levels. The influence of some environmental and experimental parameters on those estimates will be considered briefly.

Materials and methods

Plant material

Leaf tissue from the mangrove, *Rhizophora mangle* L. was collected in June, 2005 at the Smithsonian research site at Twin Cays, Belize

(Cheeseman and Lovelock, 2004). Soybean (*Glycine max* L. Pioneer 93B15) leaves were collected from the SoyFACE field site at the University of Illinois (Morgan *et al.*, 2004) during July and August, 2005, from plants exposed to ambient CO_2 and O_3, elevated CO_2 or elevated O_3 using Free-Air gas Concentration Enrichment (FACE) technology. Both fumigation treatments were to levels expected for the year 2050, i.e. to 550 $\mu l\ l^{-1}$ ambient CO_2 (Prentice, 2001), and 1.25-times current ambient O_3 (Prather and Ehhalt, 2001). Ozone levels in the rings were dynamically adjusted based on continuous monitoring of ambient levels in control rings. Leaves of five additional native and introduced species were collected locally on two days in the Spring and in July, 2005. These were Ohio buckeye (*Aesculus glabra* Willd.), an understorey tree whose leaf development occurs before that of other species, with leaf senescence beginning by mid-July to early August (Augspurger *et al.*, 2005); *Quercus macrocarpa* Michx. (bur oak), a canopy dominant tree in old-growth forests, characterized by continuous leaf production throughout the growing season; *Plantago major* L., an introduced perennial 'weed' with a competitive growth strategy (sensu Grime, 1977) growing in driveway gravel; *Glechoma hederacea* L. (Creeping Charley), a stoloniferous and highly competitive introduced 'weed' growing in a partly shaded lawn; and *Viola soraria* Willd., a native, perennial violet growing in shade.

Analytical procedure

Tissue was harvested by cutting leaf discs from attached leaves using cork borers, transferring it to HistoPrep Omniset tissue cassettes (Fisher Scientific, Pittsburgh, PA, USA), and submerging it in liquid nitrogen (LN2) until analysis; storage at higher temperatures (−80 °C or –20 °C) resulted in loss of as much as 60% of the H_2O_2 within 7 d. The time between cutting and freezing was minimized (less than 60 s). For analysis, samples were removed from the LN2, quickly weighed without thawing, then ground under LN2 with a prechilled mortar and pestle. For studies under anoxic conditions, while there was still LN2 in the mortar, the sample was poured into a plastic centrifuge tube containing extraction medium which had been degassed by boiling under vacuum. To prevent the introduction of air during the extraction process, before all the LN2 had evaporated, the tube was capped, the cap having a small vent hole and a hole for the insertion of an N_2 bubbling tube, and the remainder of the LN2 was vented through these holes. The N_2 line was then inserted and the headspace vented for an additional 30 s before bubbling started. Solution samples for analysis were removed from the tube with a Hamilton syringe, thus precluding the introduction of air during sampling, and rapidly mixed with the assay medium. If anoxia was not the primary consideration, the frozen, ground material was transferred to the centrifuge tube, and the mortar was rinsed with the extraction medium. In all cases, 3–5 cm^2 leaf area were analysed; tissue weight to solution volume ratios were no greater than 1:50 (for mangroves) or 1:200 (for other species), reflecting differences in specific leaf area.

H_2O_2 determination

A modified ferrous ammonium sulphate/xylenol orange (FOX) method was used to determine H_2O_2 contents of the extracts (Gay *et al.*, 1999; Wei *et al.*, 2002). This assay is the basis for at least four major commercially available H_2O_2 determination kits, due largely to its sensitivity, stability, and adaptability to high-throughput techniques. It should be noted, however, that the FOX method is not strictly specific for H_2O_2, but will detect other water-soluble peroxides with a slightly lower sensitivity (Jiang *et al.*, 1990; Wolff, 1999), and with 90% methanol as an extraction medium and in the assay, also provides a sensitive assay of lipid peroxides (Jiang *et al.*, 1991). The literature on soluble peroxides in plants other than H_2O_2 is extremely limited, however, and the significance of possible interference can not be estimated at this time. Therefore, in this paper, the custom generally adopted for both plant and animal studies (Halliwell and Gutteridge, 1999) is accepted, and total water-soluble hydroperoxides are reported as H_2O_2.

The assay mixture (after addition of the sample) contained 250 μM ferrous ammonium sulphate, 100 μM sorbitol, and 100 μM xylenol orange in 25 mM H_2SO_4. Following control studies on the influence of various solvents for a related study, the assay was modified to include 1% ethanol (EtOH), and designated eFOX. This addition enhanced sensitivity of the assay by about 50%. This method was chosen for a number of reasons: first, it uses a spectrophotometer rather than more expensive, complicated and less portable luminometers or fluorimeters; second, the complete UV and visible spectrum could be monitored continuously with an inexpensive diode array instrument (Ocean Optics S2000, Ocean Optics Inc., Dunedin FL). This allowed continuous monitoring of the peak location, potential interference due to the extreme opacity of the eFOX medium in the 450–500 nm range, and characteristics of the UV portion of the spectrum associated with phenolics from the tissue (peak absorbance at 325 nm). With this instrumentation, the greatest sensitivity and stability of the assay was achieved by measuring the difference in absorbance between 550 and 800 nm at least 15 min after mixing the test solutions with the eFOX reagents; the colour was stable for at least 1 h.

Standards were prepared by dilution of reagent grade, 30% H_2O_2 (Fisher Scientific, Pittsburgh PA). The concentration of H_2O_2 in the reagent was calibrated using absorbance at 240 nm and an extinction coefficient of 43.6 $M^{-1}\ cm^{-1}$.

Results

There are several problems associated with the determination of H_2O_2 from plant tissues, and both the reliability of the measurements and their credibility depend on their solutions. The starting point for this study was the extraction of mangrove leaves (*Rhizophora mangle*) in conjunction with more extensive studies on the environmental physiology of these trees. Mangroves are high in polyphenolics which interfere with at least some H_2O_2 assays (Veljovic-Jovanovic *et al.*, 2002). As strong absorbers in the UV regions used for excitation in fluorescence assays, their potential interference probably extends well beyond the methods considered thus far. *R. mangle* leaves also contain high concentrations of flavologlycans ('slime') which precipitate with titanium in the Jana/Choudhuri assay (Jana and Choudhuri, 1981). Moreover, studies of the Australian relative, *R. stylosa*, showed that they contain on the order of 8–10 mM ascorbate (Cheeseman *et al.*, 1997). Therefore, the analysis of mangrove leaves was expected to present a significant challenge, and solution to the problems with this species would inspire confidence in using a similar approach for other species.

Extraction and stabilization of H_2O_2

Problems associated with extraction of H_2O_2 include the capture of H_2O_2 without releasing other compounds (e.g. phenolics) to the extent that they interfere with the assay, and prevention of either H_2O_2 generation or degradation.

Because H_2O_2 diffuses readily, the approach adopted was to crush the leaves in liquid N_2 (LN2) to a coarse powder, increasing solution access to the cells and reducing diffusion distances, but minimizing release of slime and polyphenolics. The sensitivity of the eFOX assay was such that a very low tissue-to-volume ratio was possible, also reducing the concentrations of interferents.

Distilled water was chosen as the simplest starting medium. Figure 1a shows the apparent concentration of H_2O_2 in mangrove leaf distilled water extracts, mixed during extraction by bubbling with either air or N_2 gas. In both cases, the apparent concentration in the medium was reasonably steady at less than 5 μM, representing <0.5 μmol H_2O_2 $(gFW)^{-1}$ in the tissue. That this stability was not indicative of the actual tissue concentrations was indicated by recovery of a 'spike' of added H_2O_2. The peak concentration 1 min after the spike addition was 80% of the expected, but this was followed by a rapid consumption of the added H_2O_2. Similar results were obtained when leaves were extracted with K-phosphate (KPi, pH 7.0) or Na-acetate (NaOAc, pH 5.2) buffers, and with soybeans and the five perennial, non-cultivated species (data not shown). This is particularly important to note in light of the use of simple KPi buffers for extraction in other studies (Lin and Kao, 2000). The addition of the catalase inhibitor, aminotriazole, to the extraction medium did not change the pattern.

The possibility that, along with the H_2O_2, this protocol extracted enzymatic or non-enzymatic components for consumption or production of H_2O_2 was considered further through use of 5 mM KCN as an inhibitor of catalase, peroxidase and CuZn-superoxide dismutase. As shown in Fig. 1b, in aerated distilled water, this led to a marked increase in the apparent H_2O_2 concentration with time. In this figure, the values are converted to apparent tissue H_2O_2 concentrations (assuming that it represented only release from the tissue) to emphasize the extent to which H_2O_2 accumulated; the concentration in the medium reached 350 μM. Addition of H_2O_2 at 30 min produced the expected step increase, but without changing the slope. When NaOH was substituted for KCN to produce a similar pH (>9.5), the pattern was similar to that shown in Fig. 1a. When N_2 bubbling and KCN treatment were combined, however, both production and consumption of H_2O_2 were inhibited; a stable concentration was often, but not always, achieved within 10 min of grinding, the step change upon spiking was as expected, and a stable state continued thereafter (data not shown).

While these results indicated that metabolism of H_2O_2 was a significant but solvable problem, this solution depended on maintenance of a very low level of O_2 in the medium. Reproducibility was, therefore, problematic and additional studies were performed to identify a more useful solution. The contrasting results obtained with NaOH and KCN at the same pH implied that the result in Fig. 1b was, indeed, related to cyanide itself, perhaps acting as a free radical, leading to the oxidation of H_2O (Halliwell and Gutteridge, 1999). Thus, the KCN effect was expected to be pH dependent, and the behaviour of aerated extracts with KCN was further examined in buffered solutions ranging

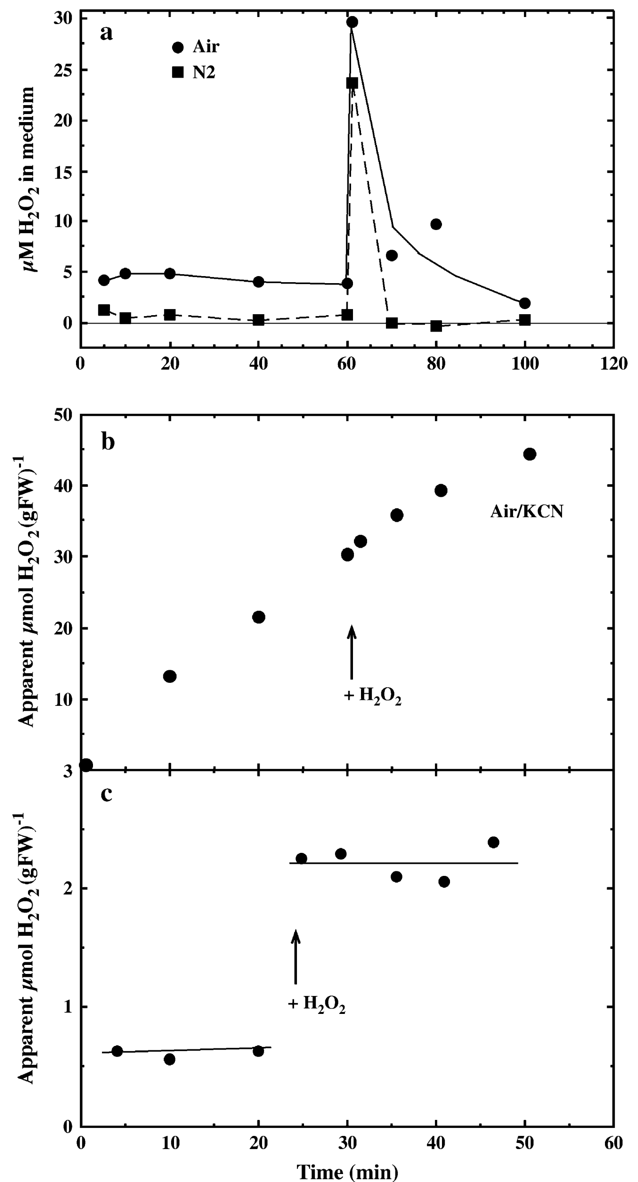

Fig. 1. Effects of extraction conditions on apparent H_2O_2 contents of mangrove (*R. mangle*) leaves. Leaves were harvested from greenhouse-grown plants. (a) Leaves were ground in LN2 and extracted with distilled water bubbled either with air or N_2. H_2O_2 sufficient to raise the concentration in the medium by 30 μM was added at 60 min. (b) Extraction medium was distilled water with 5 mM KCN, bubbled with air. The spike at 30 min was sufficient to raise the concentration in the medium by 30 μM. Results are plotted as a 'apparent' tissue levels to emphasize the magnitude of the increase over time. Absolute concentration of H_2O_2 in the medium reached >350 μM. (c) Extraction medium was 0.1 M K-phosphate (pH 6.4) +5 mM KCN. The spike was sufficient to raise the concentration in the medium by 30 μM. Recovery of the spike was 96% and there was no statistically significant slope either before or after the spike (<0.1% min^{-1}).

from pH 5.2 to >8. The rate of H_2O_2 production was greatest in unbuffered medium and at pH 8 and above. Production decreased with pH to 6.4, and at lower pH there was a net consumption of H_2O_2, possibly reflecting loss of inhibitory activity as HCN volatilized, or auto-oxidation of other compounds in the extracts. A balance of inhibitory activity and stability was achieved at pH 6.4 with 5 mM KCN (Fig. 1c), and under these conditions, anoxia was not required. Therefore, this method was adopted for general use.

Although a complete analysis of all other grinding media was beyond the scope of this study, two alternatives were considered. First, 5% TCA was tested as an extraction medium with mangrove and violet leaf tissue. In both cases, loss of H_2O_2 still occurred, at rates as high as 2.2% min^{-1}; this is less than the loss shown in Fig. 1a, but high enough to make the assay inconveniently time-dependent. Second, 25 mM HCl at the tissue-to-volume ratio used with the other buffers had no effect on the calibration curve, but failed to stabilize H_2O_2: there was a 2.5–4% min^{-1} decline of the apparent tissue concentration between 10 and 20 min after grinding, and similar instability after H_2O_2 spiking. When used at the very high tissue-to-volume ratio used in other studies (Chen and Gallie, 2004, 2005), HCl extracts browned quickly, H_2O_2 was at the lower limit of detection and unstable, and an H_2O_2 spike was consumed within 2 min. The objective here is not to claim that results with these protocols are inaccurate, but to note that they did not work with the species and under the conditions used in this study. Clearly, regardless of the extraction protocol, its suitability must be verified for each condition and species.

H_2O_2 quantification

The second problem to be resolved was associated with the H_2O_2 measurement itself. Based on the report of Veljovic-Jovanovic *et al*. (2002), the concern was that phenolics or other leaf constituents could reduce the sensitivity of the assay, introduce a significant zero-offset to the calibration curve, or both. They showed, for example, that ascorbate, at 4 or 40 μM in the assay medium, changed both the slope and the intercept of the MTBH-DMAB calibration curves and also significantly interfered with the ferricyanide–luminol assay. In the present case, preliminary studies indicated that the eFOX assay was less susceptible to that particular interference: 100 μM ascorbate had no effect on the slope or intercept of the calibration curve when added by itself.

Nevertheless, the problem could not be dismissed on that basis alone, and a more direct demonstration of reliability in the presence of plant material was required. The simplest first step was to use the addition of a 'spike' of a known amount of H_2O_2 to the tissue extract (Fig. 1; Veljovic-Jovanovic *et al.*, 2002) to give an indication of a change in slope of the calibration curve following addition of plant material. Alternately, a more complete internal calibration curve can be used, adding standards to replicate samples when they are mixed with the eFOX reagent. This gives a more reliable estimate of the slope of the H_2O_2 response in the presence of extract for comparison with the standard curve itself. The extrapolation of this line to the intercept provides an estimate the amount of H_2O_2 in the sample, and statistical methods of regression can assign an error estimate to that value. This method does, however, require that the tissue extract itself not introduce a zero-offset in the calibration curve.

Figure 2 shows the results of an experiment to consider this problem directly using soybean leaf samples. The four lines in the main figure represent the external standard curve and internal curves generated with three dilutions of the leaf extract. The insert shows the absorbance of the 'unspiked' sample at each dilution as a function of the dilution factor. If interfering compounds were present in the sample that could offset the intercept, the relationship in the insert would be curvilinear rather than linear. In the present case, the tissue H_2O_2 concentration calculated from the slopes of the internal standard curves, the *y*-axis intercepts and appropriate dilution factors was within 10% of the value calculated from the external standards, and there was no evidence of a zero-offset. Similar results were obtained with the other species, including mangroves at both the tissue-to-volume ratio routinely used and at six times that concentration. This species promised the greatest challenge, both because of its high phenolic contents and because the weight of the standard leaf discs was three to four times that of the other species. Although the tissue

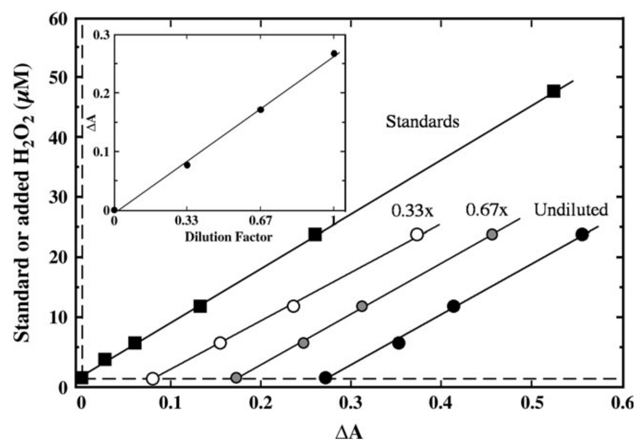

Fig. 2. Standard curves for H_2O_2 concentration as a function of ΔA ($=A_{550}$–A_{800}). This presentation is the inverse of usual practice, reflecting the fact that in actual experiments, absorbance is used to deduce concentration. 'Standards' is the external standard curve, generated in the absence of plant extract. Undiluted, ×0.67 and ×0.33 dilutions of a single extract of an 'elevated ozone' soybean leaf were used in the generation of internal standard curves. Inset: the effect of sample dilution on ΔA in the absence of added internal standard. Interference with the assay by plant-derived compounds should have been manifested by non-linearity of this relationship.

H_2O_2 contents were different from those in soybean leaves (see below), the results were qualitatively the same as those shown in Fig. 2. These results gave confidence in the method which obviated the need to analyse large numbers of internal standards and dilutions for each sample, allowing greater throughput capacity for field studies.

H_2O_2 contents of leaves under natural conditions

Table 1 summarizes the results of tissue analyses for H_2O_2 content using leaves harvested at midday during mid-summer. Mangrove (*R. mangle*) data are the combined values for fringe and dwarf habitats, and sun and shade leaves. These are also the habitats in which photosynthesis, polyphenolic contents, and peroxidase metabolism have been previously studied (Cheeseman and Lovelock, 2004; Kandil *et al*., 2004; Pearse *et al*., 2005). As previously reported for phenolic peroxidases, there were no habitat-related differences in leaf H_2O_2.

Differences between species and their levels of statistical significance depended on the basis of expression of the data. On a fresh weight basis, the mangrove had substantially lower concentrations than the other species, again reflecting the fact that mangrove leaves are much thicker than the others. Differences in specific leaf area were smaller between the temperate species, but still influenced the interpretation. Only soybean was significantly different from other species on the leaf area basis, and, interestingly, its H_2O_2 concentration was highest on both bases. For the remainder of this report, because H_2O_2 is in aqueous solution in plants, the data will be reported on a fresh weight basis. Thus, *R. mangle* and the two 'weeds' appear to have the greatest ability to control their oxidant status, while the understorey tree, *A. glabra*, and soybeans have the least.

For the purposes of this report, three additional illustrations of leaf tissue concentrations serve as examples of questions and results significant to the consideration of plants under field conditions. They also have implications for the extrapolation of laboratory-based models of the role of H_2O_2 in metabolism to plants under field conditions. These are: time-of-day effects (illustrated with mangroves), weather and seasonally related effects (illustrated using the five non-cultivated temperate species), and the effects of elevated CO_2 or O_3 (illustrated using soybean).

Table 1. *Comparison of leaf tissue H_2O_2 contents in the mangrove,* R. mangle*, soybean, and five species of free-growing plants in mid-summer*

Results are reported on both a fresh weight and leaf area basis, and are means ±sem. For mangroves, *n*=39; for all other species *n* ⩾5. Within each column, different letters indicated statistical differences at *P* <0.025.

Species	μmol H_2O_2 $(gFW)^{-1}$	μmol H_2O_2 cm^{-2}
Rhizophora mangle	0.67±0.03 a	0.035±0.001 a
Glycine max	3.47±0.18 b	0.057±0.004 b
Aesculus glabra	3.63±0.13 b	0.044±0.006 a
Glechoma hederacea	1.14±0.14 c	0.022±0.003 a
Plantago major	1.46±0.14 c	0.034±0.003 a
Quercus macrocarpa	2.37±0.30 d	0.044±0.006 a
Viola soraria	2.49±0.13 d	0.034±0.002 a

Figure 3 shows the variation in mangrove leaf H_2O_2 concentration throughout a nychthemeral cycle. As might be expected based on the fact that midday temperatures and light intensities are highest, and in the mangrove habitat, associated with stomatal closure and cessation of net photosynthesis, the concentrations at noon and mid-afternoon were higher than those in early morning or near sunset (08.00 h and 18.00 h). These results are consistent with the pattern reported for tobacco under benign conditions (Chen and Gallie, 2004), and *Phragmites* exposed to various light and temperature conditions (Velikova and Loreto, 2005) although the high-to-low differences in the mangroves were much smaller than those in tobacco. The mean concentrations in mangroves were 10-fold higher than those in the tobacco study, but lower, by approximately the same factor, than those reported for *Phragmites*. Neither the tobacco nor the *Phragmites* study reported levels other than during the light period, but in the mangrove, the high midday concentrations were identical to those well after sunset and before sunrise, indicating that H_2O_2 was not being produced only, or primarily, in chloroplast reactions.

Weather or seasonally-related effects are illustrated in Fig. 4 for the five free-growing, non-cultivated species. Data are shown for two dates in the spring. The first, 23 April 2005, was cool (5 °C) and overcast, while 3 May 2005 was seasonably warm (19 °C) and sunny. Differences between the two dates were significant for *Aesculus, Glechoma*, and *Plantago*, and marginally insignificant for *Viola* (*P* =0.06). Only the oak, *Quercus macrocarpa*,

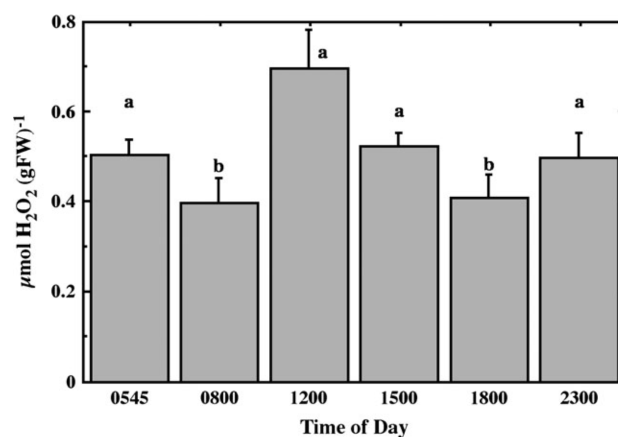

Fig. 3. The concentration of H_2O_2 in leaves of the mangrove, *R. mangle*, sampled in the field at Twin Cays, Belize, June, 2005. Means and sem are shown (*n*=5) and expressed on the basis of leaf fresh weight. The pattern and statistical significance were similar when data were expressed per unit leaf area. Letters above bars indicate statistical differences at *P* <0.05.

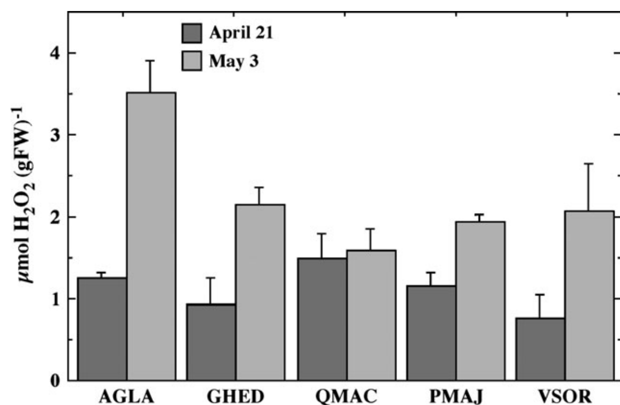

Fig. 4. Comparison of tissue H_2O_2 contents of five species of free-growing plants in Spring. Data are means ±sem for *n*=6. Data are expressed on a fresh weight basis. Abbreviations: AGLA, *Aesculus glabra*; GHED, *Glechoma hederacea*; QMAC, *Quercus macrocarpa*; PMAJ, *Plantago major*; VSOR, *Viola soraria*. 21 April was a cool (5 °C) and overcast day following several unseasonably warm days. 3 May was sunny and seasonably warm (19 °C) following a series of cool days. Note: AGLA and VSOR were growing in shade and GHED was partially shaded.

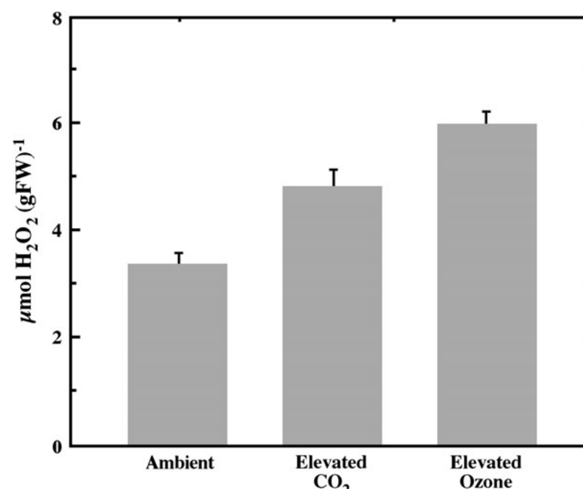

Fig. 5. Leaf tissue H_2O_2 concentrations in soybean (*Glycine max*) under ambient conditions and in FACE rings with elevated CO_2 and ozone. Data are expressed on a fresh weight basis. All means were statistically different at P <0.05. The elevated CO_2 concentration was 550 μl l^{-1}. Elevated ozone levels were to 125% of ambient (expected levels for 2050). See Morgan *et al*. (2002) for a description of the SoyFACE project and treatments.

showed no change. Interestingly, both the *Aesculus* and violet tissue levels increased to the equivalent of their mid-summer levels on the warmer day, while *Glechoma* showed it greatest concentration in the warm, Spring samples (Fig. 4; Table 1). The greatest range of concentrations represented in these analyses was in the violets (2.5-fold) while the smallest was in *Plantago* (1.5-fold). It should also be noted that two of the species, *Aesculus* and *Viola* were growing in the shade and *Glechoma* was partly shaded. Thus, these results are probably more indicative of temperature than light effects, but in any case, further study is warranted.

Finally, Fig. 5 shows the leaf tissue concentrations in mid-summer soybean between 11.00 h and 13.00 h on a sunny day. Samples were collected from FACE rings under ambient conditions and rings enriched with CO_2 and ozone. At this point, the plants were in an early (R_2) reproductive stage (Fehr *et al.*, 1971), at which sensitivity of growth, photosynthesis and future yield to ozone and other stresses is greatest (Morgan *et al.*, 2003). A similar pattern with similar statistical results was seen when data were expressed on a leaf area basis. With respect to the ambient samples, levels in elevated CO_2 and ozone treatments had 42% and 79% higher H_2O_2 contents, respectively. These results contrast with those presented by Chen and Gallie (2005) where 5-fold or greater changes were associated with ozone fumigation. In the present study, however, season-long, naturally-occurring ambient ozone levels were substantial (*c*. 50 nl l^{-1}) and fumigation was only to 125% of ambient (Morgan *et al.*, 2004). While it might be expected that treatment with ozone would increase tissue H_2O_2 levels, it is not immediately clear why elevated CO_2 (from 375 to 550 μl l^{-1}) would have a similar effect, and more detailed studies are in progress. Interestingly, in later stages of pod fill (R_5, R_6), treatment differences disappeared, and mean leaf H_2O_2 levels were approximately the same as those shown for the elevated CO_2 treatment in Fig. 5 (4.52±0.18, *n*=30, and 4.03±0.15, *n*=32 for R_5 and R_6, respectively).

Discussion

H_2O_2 has been proposed as a candidate biomarker for biochemical stress in humans (Halliwell *et al.*, 2000), and with the increased interest in oxidative stress (including that caused by tropospheric ozone) and antioxidative metabolism in plants, a similar need is recognized by plant researchers. At the same time, models based on studies under controlled conditions involving H_2O_2 as a signalling molecule have also become increasingly well supported (see Introduction). In both cases, to move studies from the laboratory to the field, it is clearly important to have accurate baseline knowledge of 'normal' levels in free-growing plants. Only then can the tissue levels of H_2O_2 needed for, or compatible with, these two functions be properly considered.

The suitability of H_2O_2 as an indicator for stress hinges on at least two conditions about which data are still being accumulated. First, it is important to examine the correlation between the size and dynamics of H_2O_2 pool sizes and other measures of stress using field studies which also take into account plant phenology, complex and correlated environmental conditions (such as light and temperature), and normal variations which might be related to the time

of day and night. Studies such as those reported in the second part of this paper address this question. Second, because the turnover of H_2O_2 can be rapid and its metabolism is particularly active in chloroplasts, peroxisomes, and mitochondria, the localization of the H_2O_2 reported by the assays needs to be established. A recent model of antioxidant metabolism in chloroplasts, for example, suggest that concentrations in that organelle should be less than 1 μM (Polle, 2001), although it should be noted that the model did not allow H_2O_2 to diffuse from the compartment (Takahama, 1989) or for serious imbalance between, for example, SOD and APx activities (Cheeseman *et al.*, 1997). On the other hand, the involvement of H_2O_2 in stomatal signalling and accumulation within chloroplasts has recently been supported strongly by direct visualization using fluorescent indicators (Chen and Gallie, 2004), albeit in a way which can not be directly quantified. Generally, however, based on histological studies conducted both in the field (Oksanen *et al.*, 2003) and in controlled conditions (Ranieri *et al.*, 2001), it seems most probable that primary H_2O_2 accumulation is apoplastic, increasing on the cytosolic side of the cell wall, or of the plasma membrane, only under conditions associated with visible damage. A similar conclusion can be drawn from studies examining the localization of H_2O_2 following wounding (Orozco-Cárdenas *et al.*, 2001).

The localization of major H_2O_2 pools also impacts models which assign a signalling role to this molecule. The results of this study would not appear to be compatible with signalling based on dramatic increases in overall leaf contents. Rather, they suggest that it must depend on highly specific, localized activities, such as those which have been well-established for calcium signalling. In that case, understanding of the dynamics of production and consumption, and small fluxes occurring against a background of large, dynamic pools, will need to be incorporated into models.

In this paper, a modified FOX assay has been used for the quantification of soluble hydroperoxides extracted from leaf tissue. In keeping with common practice, the resulting values have been referred to as H_2O_2, although as noted earlier, the assay does not distinguish between this and other hydroperoxides. Nevertheless, as other hydroperoxides are much less soluble than H_2O_2, as the protocol as employed here should not extract lipid peroxides due to lack of organic solvents or detergents, as no data are available in the literature on concentrations of other hydroperoxides in plant tissues, and as all studies of H_2O_2 as a signalling molecule, or a component of pathological responses (e.g. the oxidative burst) suffer, technically, from the same uncertainty, accepting the current convention seems reasonable.

In principle, there are several methods for the quantification of H_2O_2 that should work equally well, and a number of these, in addition to FOX, are available as commercial kits. In practice, however, this aspect of the analysis is considerably less problematic than the extraction and stabilization of the target itself, and its separation from possible interferences or inhibitors of the assay protocol. In my experience, these include precipitation of non-targets in the titanium assay, including flavologlycans (slime) in mangroves, carryover of phenolics with quench fluorescence or change in the sensitivity of the fluorescent reaction (Veljovic-Jovanovic *et al.*, 2002), or inhibitors and interfering compounds affecting enzymatic assays. Indeed, the protocol used to stabilize H_2O_2 in this study would be expected to inhibit the peroxidase used as the basis for enzymatic assays.

This paper contributes to the problem of H_2O_2 (hydroperoxide) quantification in two ways. First, it provides a formalized method to distinguish between important biological variability in H_2O_2 concentrations and variability due to complications with the analytical methods, such as can be inferred from the Introduction to this paper. While there has been one recent analysis of one potential interference in the determination of H_2O_2 using two analytical methods (Veljovic-Jovanovic *et al.*, 2002), the suitability of extraction protocols, of assay techniques, and even of calibration curves for the analysis are, in general, incompletely established. In this study, it has been shown that continued metabolism of H_2O_2 during analysis is potentially as important as other types of interferences, and several control studies have been illustrated to establish confidence that the problem has been eliminated.

With the method established, tissue H_2O_2 levels were analysed for seven plant species, the second contribution of this paper being to establish, with confidence, ranges and variabilities for free-growing plants which can serve as baselines for continued studies, particularly those directed toward the use of H_2O_2 as a marker for oxidative status. Overall, the concentrations in the leaves, on a fresh weight basis, ranged over about one order of magnitude, from 0.6 to 6 μmol $(gFW)^{-1}$. This range would be somewhat smaller if concentrations were expressed on a tissue water basis since the mangroves have a low water content (*c.* 60%). These values were higher than many of those reported for plants grown in controlled environments with low stress potential, and similar to those for free-growing birch/gingko during leaf senescence (Kukavica and Veljovic-Jovanovic, 2004). Differences in leaf tissue H_2O_2 concentration within any one species, whether measured as a function of time of day, season, or environmental exposure were generally less than 2-fold, with the extreme being only 2.5-fold. If this were to increase during direct or indirect stress (e.g. involving the combination of ozone and drought at a sensitive developmental state), excursions from the mean should easily be assessed.

It is concluded, therefore, that it is reasonable to explore the use of H_2O_2 as a biomarker for biochemical stress further. The results also indicated that the species adapted to growth in the most extreme conditions, i.e. the mangrove and the perennial, hard-to-eradicate weeds, also had the

lowest steady-state tissue concentrations. Whether this is due to low rates of H_2O_2 production or high capacity for its removal, this suggests production/consumption capacity as a second potential biomarker. In both cases, these are the subjects for ongoing investigations.

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