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Seasonal patterns of leaf H_2O_2 content: reflections of leaf phenology, or environmental stress?

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Abstract. H_2O_2 is an ubiquitous compound involved in signalling, metabolic control, stress responses and development. The compatibility of leaf tissue levels with these functions has, however, often been questioned. The objective here is to document H_2O_2 levels and variability under natural conditions, and their underlying causes. Using the FOX method, bulk H_2O_2 concentrations were analysed in leaf samples from 18 species of herbs and trees throughout the 2006 growing season. Sampling addressing targeted predictions was emphasised in 2007 and 2008. H_2O_2 levels varied 100-fold through the year, with a main peak in spring. Two hypotheses were examined: (H1) that H_2O_2 reflects seasonally variable responses to environmental stresses, and (H2) that it reflects metabolism associated with leaf development. Based on poor or inappropriate correlations between H_2O_2 and indicators of light, temperature or drought stress, support for H1 was minimal. H2 was supported both by seasonal patterns and by targeted analyses of concentration changes throughout leaf development. This study concludes that bulk tissue H_2O_2 concentrations are poor indicators of stress, and are generally too high to reflect either signalling or metabolic control networks. Instead, the linkage of H_2O_2 and leaf phenology appears to reflect the roles of H_2O_2 in cell expansion, lignification and wall cross-linking.

Additional keywords: hydrogen peroxide, leaf expansion, leaf toughness, metabolic signalling, oak, oxidative stress, soya bean, soybean, temperate forest, understorey.

Introduction

The analysis and quantification of H_2O_2 in plant tissues has been a recurring but problematic research topic in recent years (Veljovic-Jovanovic *et al*. 2002; Cheeseman 2006, 2007; Queval *et al*. 2008). On the one hand, it has become well established that H_2O_2 can act as a potent signalling molecule, and the bulk of recent attention has been focussed on this. In general, this function is best accomplished when the background level of the signal is low and stimuli increase it quickly and dramatically (Veljovic-Jovanovic *et al*. 2002). On the other hand, the widely recognised conundrum is that bulk leaf tissue levels often appear to exceed 'low' by even two orders of magnitude. This problem, of course, would go away if it could be shown that the techniques for H_2O_2 determination were faulty. However, based both on analysis of several control experiments (Cheeseman 2006; Queval *et al*. 2008), and on careful comparisons of different techniques (Queval *et al*. 2008), and even on comparisons of field grown plants with the laboratory *Arabidopsis* model (Veljovic-Jovanovic *et al*. 2002; Kukavica and Veljovic-Jovanovic 2004), this objection appears, as a generality, to be unsustainable. Queval *et al*. (2008) also carefully considered ways to reconcile the need for H_2O_2 homeostasis, the enhanced potential production of H_2O_2 under stress conditions, and demands for an efficient signalling system. They concluded that if bulk leaf levels are as high as some of the literature reports indicate, then they 'will be of limited value as indicators of oxidative stress or signalling'.

In a previous study (Cheeseman 2006), the concentrations of H_2O_2 in field grown leaves of five temperate zone plants were reported, along with those for soybeans grown under SoyFACE conditions (ambient, with elevated CO_2 or with elevated ozone), and the mangrove, *Rhizophora mangle* L., also collected in the field. For the different species under ambient conditions, mean leaf levels ranged from 0.67 to 3.63 μmol H_2O_2 $(g\,FW)^{-1}$, or 22 to 57 nmol cm^{-2}. Based on comparisons between 2 days, one being warm and sunny and the other cool and cloudy, the results suggested a correlation with environmental conditions. Moreover, during early reproductive stages in soybean (but not in later ones), leaf concentrations were significantly higher both under elevated CO_2 and ozone than at ambient conditions.

In this paper, the study of H_2O_2 in plant leaves under natural (non-laboratory) conditions is extended. First, the range of natural variabilities is reconsidered by examining season long patterns of leaf H_2O_2 in a broad range of species differing in life history strategies and phenologies. Included among the 18 species surveyed are nine spring ephemerals and understorey perennial herbs whose most active season is the spring; these can experience profound environmental variability in the few months which encompasses their entire aboveground presence for the year.

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The dataset from this survey, supplemented by harvests targeting specific questions, is then used to address two hypotheses and their associated predictions. Because a substantial fraction of the recent literature on oxidative metabolism has concentrated on stress (light, temperature and drought), on the adverse effects of oxidants, and on the importance of maintaining H_2O_2 homeostasis, the first hypothesis is that high or elevated H_2O_2 levels report oxidative stress occurring directly or indirectly as the result of environmental stress. To challenge this, I examine three predictions: first, because a major potential source of oxidant stress is the reduction of oxygen in the photosynthetic electron transport chain, leaf H_2O_2 concentrations will be positively correlated with irradiance at the time of sampling; second, samples taken at temperature extremes and during periods of high temperature variation will show elevated concentrations; and finally, H_2O_2 will increase with time since last rainfall, and the increase will be greater at higher temperatures.

Alternately, because H_2O_2 has specific roles in development, e.g. lignification of xylem and sclerenchyma, and cross-linking of cell walls, the second hypothesis is that H_2O_2 reflects developmental processes dependent on oxidant availability. In this case, the associated prediction is that leaf H_2O_2 concentrations will be elevated during periods of leaf expansion or maturation, and possibly again during senescence, and reduced during periods of low growth activity (e.g. mid-summer quiescence in single-flush trees and understorey perennials).

Materials and methods

Plant material

Eighteen species were selected for this study, including trees, spring ephemeral herbs, spring/summer active herbs, biennials, and herbs actively growing throughout the year. The species, abbreviations used to designate them, and their phenological patterns are shown in Table 1.

Harvesting sites and dates

Plant material was harvested from two sites, Brownfield Woods (a university-owned forest tract ~8 km north-east of the University of Illinois), and a private residential garden ~1 km east of the university. For full list of species and acronyms used see Table 1. ASAC, AGLA, APET, ATRI, ACAN, ASIM, CVIR, HAPP, HVIR, PAME and PPEL were sampled at Brownfield Woods. MVIR, PRUG, QMAC, SCAN, TREC and VSOR were sampled at the residential garden. GHED was sampled at both sites. The harvests covered the full growing seasons in 2006 and 2007, and bur oak (QMAC) studies were continued in the summer of 2008. Also in 2008, in order to access a large sampling pool at known and comparable leaf developmental states, an experiment

Table 1. Species represented in the study

Sample dates are Julian dates. The long-term average for canopy closure is JD 124 (May 4, 10%) and JD 142 (May 22, 90%). Opening starts on JD 289 (October 16) and reaches 90% on JD 317 (November 13). First frost was JD 286 (October 13); first hard freeze was JD 305 (November 1) (C. Augspurger, pers. comm.). Standard errors for LMA were <0.0001 except for ASIM (0.0004) and QMC (0.0003). Sample numbers for the last two columns were identical

Species	Acronym	Common name	Phenology	First sample (2006)	Last sample (2006)	LMA ($g\,DW\,cm^{-2}$) (n)	g DW/g FW
Acer saccharum Marsh.	ASAC	Sugar maple	Tree[A]	108	305[B]	0.0026 (47)	0.165 ± 0.005
Aesculus glabra Willd.	AGLA	Ohio buckeye	Tree[A]	94	213[B]	0.0026 (45)	0.264 ± 0.007
Alliaria petiolata (M. Bieb.) Cavara & Grande	APET	Garlic mustard	Biennial	48	328	0.0023 (54)	0.191 ± 0.006
Arisaema triphyllum (L.) Schott	ATRI	Jack in the pulpit	Spring/summer	108	204[B]	0.0032 (48)	0.384 ± 0.010
Asarum canadense L.	ACAN	Wild ginger	Spring/autumn	108	328[B]	0.0018 (9)	0.293 ± 0.012
Asimina triloba (L.) Dunal	ASIM	Paw Paw	Tree	274[C]	305[B]	0.0014 (30)	0.112 ± 0.003
Claytonia virginica L.	CVIR	Spring beauty	Spring	48	123[B]	0.0029 (26)	0.095 ± 0.008
Glechoma hederacea L.	GHED	Creeping Charley	Spring/autumn	22	328	0.0026 (91)	0.189 ± 0.005
Hydrophyllum appendiculatum Michx.	HAPP	Hairy waterleaf	Biennial[D]	20	328	0.0023 (85)	0.161 ± 0.005
Hydrophyllum virginianum L.	HVIR	Virginia waterleaf	Perennial[E]	69	328	0.0021 (37)	0.198 ± 0.014
Mertensia virginica (L.) Pers. Ex Link	MVIR	Virginia bluebell	Spring ephemeral	90	131[B]	0.0020 (20)	0.144 ± 0.006
Phytolacca americana L.	PAME	Pokeweed	Annual	213[C]	291[B]	0.0016 (45)	0.144 ± 0.006
Plantago rugelii Decne[F]	PRUG	Common plantain	Spring/autumn	92	305[B]	0.0032 (44)	0.195 ± 0.005
Podophyllum peltatum L.	PPEL	Mayapple	Spring/summer	90	244[B]	0.0023 (47)	0.178 ± 0.007
Quercus macrocarpa Michx.	QMAC	Burr oak	Tree[G]	131	305[B]	0.0065 (36)	0.472 ± 0.013
Sanguinaria canadensis L.	SCAN	Bloodroot	Spring ephemeral	92	193[B]	0.0026 (32)	0.190 ± 0.004
Trillium recurvatum Beck	TREC	Purple trillium	Spring ephemeral	90	157[B]	0.0027 (19)	0.166 ± 0.006
Viola sororia L.	VSOR	Violet	Spring/autumn	69	305[B]	0.0028 (66)	0.216 ± 0.005

[A]All leaves appear in a single flush in the spring.
[B]Species was senescing at the time of the last sample.
[C]Sampling not started at beginning of season in 2006.
[D]No leaves for up to 6 weeks in mid-summer.
[E]Up to three leaf cohorts per year.
[F]In the previous study (Cheeseman 2006), PRUG was incorrectly identified as *Plantago major*. The species are distinguished by seeds (*P. rugelii* are smaller) and colouration of leaf bases (*P. rugelii* are red).
[G]Leaves are produced throughout the growing season.

using soybean was conducted under ambient environmental conditions at the SoyFACE experimental site at the University of Illinois (Morgan *et al*. 2004).

For each wild species, sampling began when the plants were first visible above the litter layer or when the first leaves were large enough to sample, and continued through senescence; dates of first and last sampling in 2006 are given in Table 1 (all sampling dates are reported as Julian dates, or JD). In 2007, sampling was, in general, directed at testing specific predictions regarding the patterns observed in 2006. In all cases, samples were collected between 1000 and 1400 hours local standard time. For the final, 2008 experiment on H_2O_2 in soybean first and third trifoliate leaves during expansion, control (ambient environment) leaves were harvested from the time leaves were ~1 cm long until at least two harvests indicated, by leaf size, that full expansion had been achieved.

It should be noted that the two spring sampling periods were different, both climatologically and phenologically. The winter of 2006 was very mild; sampling began in mid-February and continued without interruption through mid-November. In 2007, February sampling was precluded by snow cover, March was warmer than average, and April was marked by an historically unusual week of hard freezes (–4°C) (see Gu *et al*. 2008; Augspurger 2009). Consequently, seasonal, all-species H_2O_2 patterns observed in the 2 years were not comparable.

Concomitant with the sampling, irradiance (PPFD) at the sampled leaf was determined using a quantum sensor (Apogee QMSW-SS, Logan, UT, USA). Other weather data – air temperature, daily maximal, minimal and average temperatures, maximal temperature for the previous day, rainfall in the last 24 h and last 5 days, and days since 2 mm of rainfall – were recorded based on data from a network-accessible 'personal weather station' ~4 km from the sampling sites. This station operated using a Vantage Pro2 Plus (Davis Instruments, Hayward, CA, USA). Given the lack of topography of central Illinois and the homogeneity of surrounding agricultural activities, with the occasional exception of rainfall totals, these data were considered acceptable for basic correlative analyses.

Harvesting and extraction

Each sample consisted of three leaf disks taken with a #1 cork borer (0.46 cm^2 total area, ~20 mg FW). Unless dictated differently by the needs of a specific test of predictions, at each harvest, three samples of each species were collected from different individuals. In the case of clonal species, individuals were selected that were sufficiently distant from each other to assure that they were in different clones. When possible, samples were taken to minimise inclusion of major veins. This was not, however, possible in very young leaves of most species, or in the narrow, lanceolate leaves of CVIR. A corollary to this is that in most cases, it was not possible to repeatedly sample the same individual.

The disks were immediately submerged in 1 mL of acetone acidified with 25 mM H_2SO_4 and frozen in liquid nitrogen. Samples remained frozen over liquid nitrogen until analysis. The acetone quickly penetrated the small disks, effectively dehydrating the tissue and inactivating enzymes which offered potential interference with the assay. It also prevented oxidation of phenolic compounds, as indicated by lack of browning of any samples. Preliminary trials, however, showed that H_2O_2 was stabilised only when the acetone was acidified. H_2SO_4 was chosen for this purpose because it is the solvent in the FOX assay. Tests in which the acidified acetone was spiked with H_2O_2 before tissue sampling confirmed that this prevented changes in H_2O_2 contents, even when thawed samples were allowed to sit at room temperature for 30 to 60 min before mixing with FOX reagents. There are two technical points of importance here. First, the vials used for this were made of polypropylene. Although this is considered stable in acetone, vials filled more than 24 h before sample collection or kept for prolonged periods at room temperature, when mixed with the FOX medium, immediately developed a cloudiness which interfered with the assay, possibly due to solubilisation of plasticizers (D. Seigler, pers. comm.). Second, preliminary experiments showed that H_2O_2 levels were not stable if samples were stored at –80°C or above. Therefore, all vials were filled immediately before sampling forays and all tubes remained at liquid nitrogen temperatures until analysis.

H_2O_2 determination

For analysis, samples were thawed and allowed to stand at room temperature for 45 min with occasional mixing by inversion. Thereafter, 25 μL of extract was mixed with 500 μL of FOX1 medium (Wolff 1994), and incubated for an additional 45 min. The FOX medium was not deoxygenated (as by Queval *et al*. 2008) because O_2 is a reactant in the xylenol orange reduction (Wolff 1994). As the incubation time was longer than needed for full colour development, it allowed analysis of a large number of samples at once without significant development-time differences. H_2O_2 was determined based on the difference in absorption at 550 and 850 nm using a standard curve, and an Ocean Optics S2000 diode array spectrometer (Ocean Optics Inc., Dunedin, FL, USA). H_2O_2 standards covering the range of 0–100 μM were mixed with FOX1 at the same 25 μL to 500 μL ratio as the samples. Full calibration curves were run each day and for each 50 samples. The low tissue to acetone ratio, the 20× dilution of samples during analysis, and use of two wavelengths minimised interference due to chlorophyll or other pigments (Cheeseman 2006). The spectrometer also continuously displays the spectrum of the sample (250–850 nm), allowing a visual check for consistency of the samples.

The stability of H_2O_2 in the extraction medium and during storage was verified by comparing standards and samples spiked with standards, all being handled identically to normal samples. The slopes of these calibration curves were identical, as well as identical to the normal calibration curve (see fig. 2 in Cheeseman 2006). When standards were added to leaf samples, the resulting offset in absorbance (due to H_2O_2 in the leaf material) was consistent for each standard.

Statistical analyses

Statistical analyses and data summaries were performed using JMP 7 (SAS Institute, Cary, NC, USA). Leaf areas were determined for GHED, QMAC and GMAX using scanned images and NIH ImageJ software (http://rsb.info.nih.gov/ij/). Measured areas regressed against a critical leaf dimension (width in GHED,

length in QMAC and GMAX) were used to estimate areas in leaf growth studies.

Results

Seasonal patterns

In a previous study, leaf H_2O_2 contents were reported for two species of temperate trees and three herbs, comparing results on just 2 days, a warm, sunny day in May and a cool, cloudy day 2 weeks earlier. In four of the five cases, the contents were higher on the warm, sunny day. To consider the generality of this result and the causes for these differences, I extended the study, throughout the 2006 growing season, to survey more species having a broad range of phenological patterns.

Table 1 shows the species used, their phenologies, and the periods over which they were sampled. In this paper, all H_2O_2 contents will be reported on a leaf area basis, i.e. the one on which the samples were actually collected. To allow approximate conversion to concentration on a fresh or dry leaf mass basis, Table 1 also includes leaf mass per unit area (LMA) and the dry mass to fresh mass ratios based on parallel sampling over the course of the growing season.

Figure 1 shows the seasonal pattern, including all species, for leaf H_2O_2 in 2006; because of differences in phenology, not all species were represented at all times of year (see Table 1). The pattern was similar in 2007 but the initial peak was less pronounced, probably reflecting the very different weather patterns in the 2 years (see 'Materials and methods').

Within the pattern in Fig. 1 are embedded several other characteristic patterns (see Fig. 2). Figure 2*a* shows the H_2O_2 concentration pattern for spring ephemerals. Spring beauty (CVIR) appeared (sufficiently to sample) by the end of January, and leaf H_2O_2 levels were initially very low. A similar pattern was found in over-wintering biennials (Fig. 2*d*). Virginia bluebells (MVIR) and *Trillium* (TREC) appeared at the end of March and leaves developed very rapidly (within a week in the case of *Trillium*). All three species had fully senesced by the end of May (JD 150). From March through to May, there was a monotonic decline in H_2O_2 concentration with time. At the time of the last sampling, tissue concentrations remained above 20 nmol H_2O_2 cm^{-2}. This may reflect the speed of senescence; plants essentially senesced and disappeared from one weekly sampling time to the next.

Figure 2*b*, *c* shows two alternate patterns of seasonal progress through the spring. Ohio buckeye (AGLA), sugar maple (ASAC) and garlic mustard (APET) (Fig. 2*b*) showed initially high concentrations which changed little between mid-April and early June after which they abruptly decreasing to a level which remained until senescence. Mayapple (PPEL), bloodroot (SCAN) and violets (VSOR), in contrast, showed a linear decline with time (Fig. 2*c*).

Finally, Fig. 2*d* shows the year-long patterns for the biennial *Hydrophyllum* (HAPP, hairy waterleaf), two perennial, weedy herbs (creeping Charlie, GHED, and blackseed plantain, PRUG) and bur oak (QMAC). Both late winter and mid-summer concentrations were very low, while highest levels were measured in spring. A secondary peak in late summer appeared to correspond with increased day-to-day temperature variability, increased late summer rain, or a period of renewed leaf initiation (in the case of the oak, this followed pruning).

Specific factor considerations

In order to further explore these seasonal patterns, I considered two alternative hypotheses: H1 – that H_2O_2 concentrations reflect

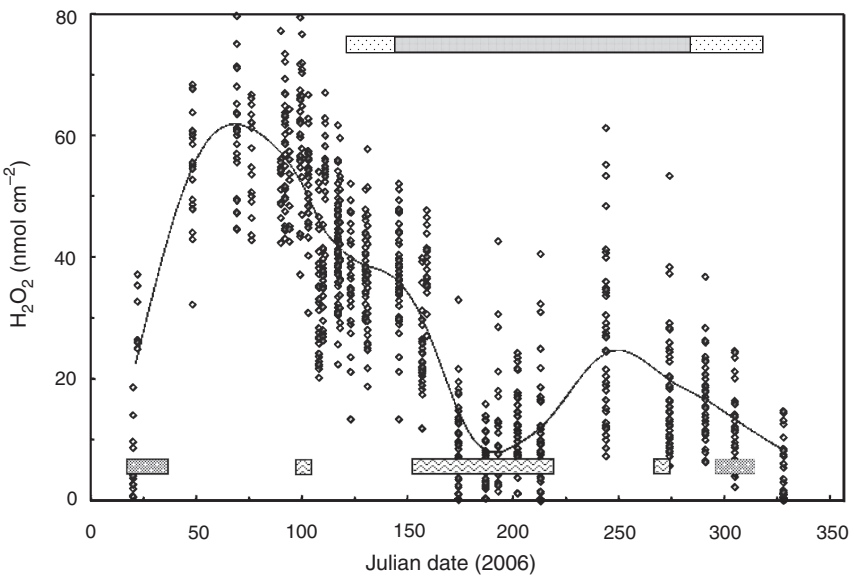

Fig. 1. Leaf H_2O_2 contents (nmol cm^{-2}) for 18 species over the course of the growing season, 2006. A smoothing spline curve with $\lambda = 45\,000$ was inserted to serve as an eye guide. Each point is a single analysis. Individual species are not distinguished in this plot because symbols would not be visible. Along top, lightly shaded areas are periods of canopy closing and opening (10–90%); heavily shaded area designates fully closed canopy. Along bottom, hatched bars are periods below 5°C; wavy bars show periods >25°C.

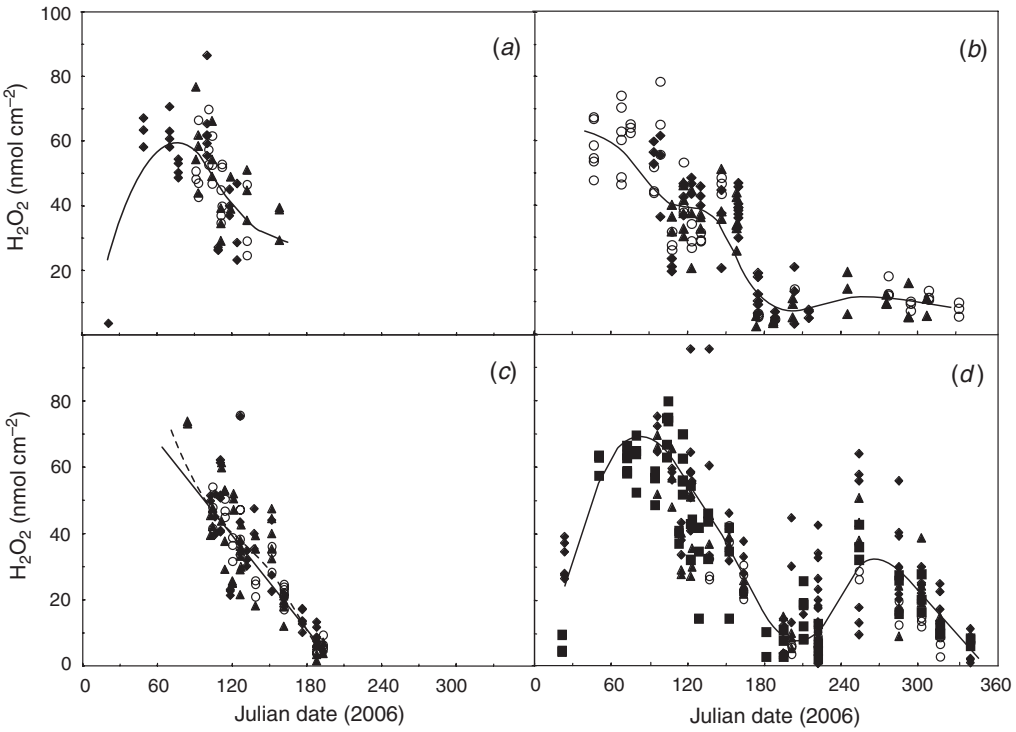

Fig. 2. Variations on the pattern of leaf H_2O_2 contents (nmol cm^{-2}) over the course of the growing season, 2006. In each section, a smoothing spline curve with $\lambda = 45\,000$ was inserted to serve as an eye guide; in (*c*), a linear fit (solid line) is also included. Each point is a single analysis. See Table 1 for explanations of abbreviations. (*a*) Spring ephemerals CVIR (♦), MVIR (○), TREC (△); (*b*) AGLA (♦), APET (○), ASAC (△); (*c*) PPEL (♦), SCAN (○), VSOR (△); (*d*) GHED (♦), QMAC (○), PRUG (△), HAPP (■).

environmental stresses which indirectly led to oxidative stress, or, H2 – that they correspond to periods of high metabolic activity, especially relating to leaf growth. I approached these through a combination of data mining and sampling directed towards specific predictions.

The first hypothesis implies that throughout a growing season, leaf H_2O_2 concentrations will be positively correlated with water stress (e.g. days since last rainfall), and exposure to high light. In the case of temperature, the correlation may be parabolic, i.e. elevated at both high and low temperatures (although none of the target species would be classed as 'chilling sensitive' and T_{max} seldom exceeded 30°C). Concentrations might also be greater when diurnal variations were larger. Three specific predictions were tested.

Prediction – as a major recognised potential source of oxidant stress is reduction of O_2 by the photosynthetic electron transport chain, leaf H_2O_2 concentrations will be positively correlated with irradiance at the time of sampling (cf. Noctor *et al*. 2002).

For nine of the species (ACAN, AGLA, ASAC, CVIR, GHED, HVIR, PRUG, QMAC and VSOR), although a wide range of irradiances was represented in the dataset, there were no significant correlations, or even suggestive trends, between leaf H_2O_2 and irradiance. This was true even after removal of the linear effects of sampling dates by multiple regression, and even if the dataset was restricted to the period before canopy closure in order to reduce over-representation of low irradiance samples for understorey plants.

In contrast, for APET, HAPP and PPEL, in spring of both years, there was a significant positive correlation between H_2O_2 and irradiance, but only up to ~200 μmol photons m^{-2} s^{-1} (Fig. 3). Higher irradiance, which would be expected to exacerbate any photosynthetically associated H_2O_2 production, did not. Moreover, although both APET and HAPP resumed growth in late summer, there were no similar correlations then (PPEL was no longer present, see Table 1).

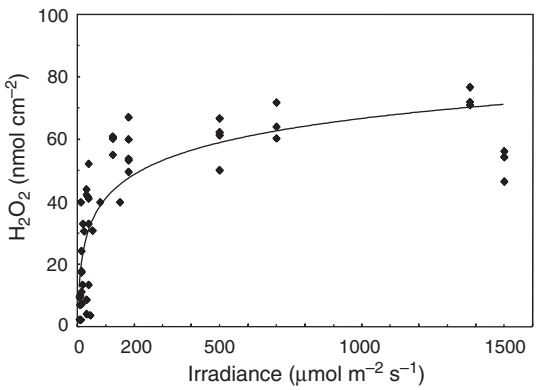

Fig. 3. The relationship between leaf H_2O_2 content and irradiance in HAPP. Line fits the relationship $y = -10.7 + 11.2\log_e x$, $r^2 = 0.69$. Data cover the period between January 20 (JD 020) and July 21 (JD 202), 2006. Qualitatively similar relationships were found for APET and PPEL. Irradiances above 50 μmol m^{-2} s^{-1} were associated with light flecks.

Finally, SCAN (in 'partial shade') showed a positive, linear irradiance response, i.e. without apparent saturation, over the full range of irradiances sampled. In this species, H_2O_2 was also strongly correlated with temperature. Figure 4 shows the linear relationships between H_2O_2 and irradiance and T_{min} (the minimum temperature for the preceding 24 h), combining data from both years. Note, however, that the relationship to T_{min} was negative throughout the entire temperature range, i.e. well beyond the range which might be associated with chilling stress. Similar, but less significant trends were found for other temperature measures (all being highly correlated), and there was no reversal, i.e. no increase in H_2O_2 with temperature, even when the harvest time temperature was >30°C.

An alternative approach to this prediction was taken with PAME (pokeweed) which was located only in or on the edges of light gaps. Leaves were taken at each sampling from plants in the centers of gaps, with sunny day irradiances above 1500 µmol m^{-2} s^{-1}, and from plants on gap edges (<50 µmol m^{-2} s^{-1}). Leaves in the gaps were often wilted, and although it could not be directly measured, leaf temperatures were most likely higher than those of the shaded leaves. These additional factors should have exacerbated any differences associated with light alone. The mean H_2O_2 levels for both shade and gap leaves were, however, identical [17 ± 1.4 (s.e., $n = 99$) nmol cm^{-2}]. Adding samples from overcast days (intermediate irradiances) verified the lack of any relationship with irradiance. Similar results were obtained for fully expanded GHED, PRUG and VSOR during mid summer, comparing data from fully exposed and deeply shaded areas of the residential garden.

Prediction – samples taken at temperature extremes or during periods of high temperature variation (e.g. warm days after very cold periods) will show elevated H_2O_2 concentrations.

To test this, the dataset was restricted to samples for which T_{min} was less than 6°C, and ΔT (the difference between the temperature at the time of sampling and T_{min}) was greater than 8°C. These restricted subsets showed significant simple correlations between ΔT and H_2O_2 for only three species: AGLA, CVIR and QMAC. For AGLA and CVIR, the correlations were, however, negative and all qualifying points were in early spring. In stepwise regressions, when Julian date and T_{min} were included, the partial correlation coefficient for ΔT was no longer significant. For QMAC after mid-September, the correlation was positive. Nevertheless, stepwise regression yielded the same result as for the spring species, i.e. showing no support for the importance of temperature difference *per se*.

Prediction – H_2O_2 levels will increase with time since last rainfall and the increase will be greater at higher temperatures.

The underlying model for this prediction is that, together, these conditions would be associated with reduced soil moisture, more rapid water loss and greater water stress. Increased H_2O_2 in leaves would then reflect associated oxidative stress (Cheeseman 2007).

To test this prediction, 'high temperature' was defined operationally as >25°C at the time of sampling. Restricting the 2-year dataset to high temperatures and to samples taken 5–15 days after the last 2 mm rainfall (the available range at high temperature) effectively removed spring data, and thus, the negative correlation to JD. In only two species, however, was the correlation between rainfall and H_2O_2 significant (r^2 ~0.35) and consistent with the prediction, ACAN and ASAC. However, temperature variables showed no exacerbating effect in multiple regressions. Indeed, in ACAN, while inclusion of temperature at the time of harvest increased r^2 significantly (to 0.58), the slope of the temperature effect was negative.

To extend the prediction to spring ephemerals, a similar analysis was performed at temperatures less than 25°C. As a result, the negative relationship between H_2O_2 and JD was also a factor. In this case, there were significant correlations for ACAN, AGLA, APET, CVIR, HAPP, PPEL, PRUG and VSOR. The correlations between H_2O_2 and days since 2 mm were, however, negative for all species, at least partly reflecting a significant negative correlation between precipitation and JD. In all cases, H_2O_2 was also positively correlated with total rainfall in the last 5 days. For these species, therefore, the correlation analyses actually contradict the prediction.

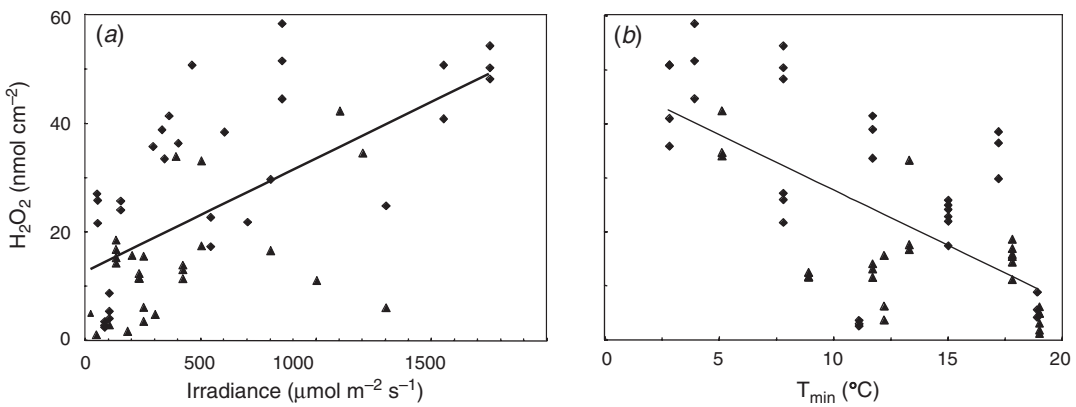

Fig. 4. The linear relationships between H_2O_2 and irradiance ($r^2 = 0.3947$) and T_{min} ($r^2 = –0.4417$), in SCAN, including data from both years. Combining the two parameters in multiple linear regression, $r^2 = 0.5948$. Although, as shown in Fig. 2*c*, H_2O_2 declined linearly with Julian date, including it in MLR increased r^2 by only 0.02, reflecting a high correlation between JD and T_{min} (♦), 2006; (▲), 2007.

The relationship between H_2O_2 contents and leaf growth rate

H2, an alternative to the environmental response hypothesis is that, because H_2O_2 has specific roles in development, H_2O_2 concentrations will reflect developmental processes dependent on oxidant availability. An associated prediction is that leaf H_2O_2 concentrations will be elevated during periods of leaf expansion (e.g. spring or late summer), and reduced during periods of low growth activity (e.g. mid-summer quiescence in understorey perennials). During senescence, if H_2O_2 plays a role in programmed cell death, the concentration might again rise.

I approached this hypothesis, first, using five species which produced leaves in late summer, and in 2008, using cultivated soybeans during early growth. Summer growth was chosen, rather than spring, because overall temperature and light uniformity were greater. Weekly sampling of all leaves on a plant or stem was accompanied by measurement of leaf dimensions for leaf area estimation. For the two species growing in the forest (APET and HAPP), and one in the sun (PAME), no significant patterns or suggestive trends could be discerned.

Results consistent with the prediction were, however, obtained in three cases where more accurate assessment of leaf growth was possible. Figure 5 shows the results for GHED at a lawn/garden interface with a 'partial shade' exposure. GHED is a clonal weed, spreading rapidly via above ground stolons with long internodes. As noted above, there were no differences in leaf H_2O_2 contents in GHED associated with light exposure at the time of harvesting. For the purpose of this analysis, sampling was done during a period in which rapid leaf initiation and growth were clearly happening, although determination of actual expansion rates and repeated sampling of the leaves was precluded by their small size. There was, nevertheless, a clear negative correlation between leaf area and H_2O_2. The degree of scatter was greatest in the smaller, presumably fastest growing, leaves. The overall r^2 (0.49) could be improved to 0.57 in MLR by including the number of days since 2 mm of rain had fallen. This correlation was negative, i.e. the longer since rain, the lower the H_2O_2.

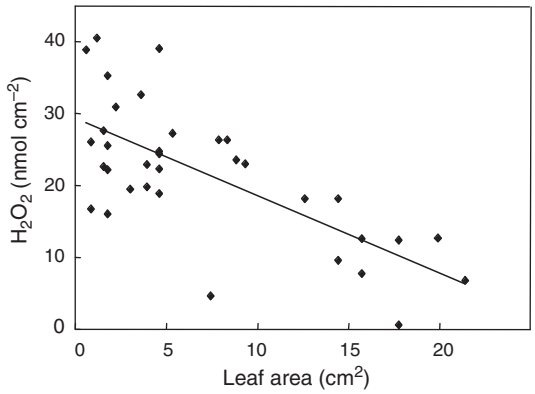

Fig. 5. The relationship between leaf H_2O_2 and leaf area in GHED during a period of rapid stolon extension, leaf initiation and leaf expansion ($r^2 = 0.49$). The plants were growing at a lawn/garden interface with 'partial shade'. Note: the small size of young leaves required that three leaves be pooled for each analysis (diameter <1 cm). With areas <5 cm^2, major veins could not be avoided.

Bur oak (QMAC), because of its large leaves and because leaf production was stimulated by mid-summer pruning, allowed an alternative approach of this prediction. Figure 6 shows the results of a study in which 30 leaves were repeatedly measured. Each was also sampled up to 12 times throughout leaf expansion and for 2 weeks thereafter. The reference date (i.e. day 0) was defined as the date on which each leaf was 95% fully expanded, based

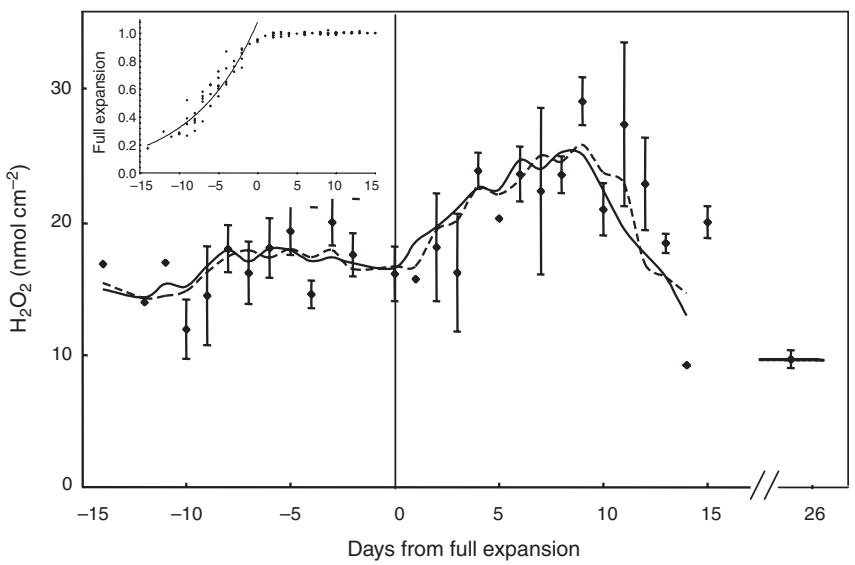

Fig. 6. The relationship between leaf H_2O_2 and leaf growth in bur oak (QMAC). Thirty leaves were repeatedly sampled throughout expansion and maturation during mid-summer, 2008. Day 0 was defined as the date at which each leaf was 95% fully expanded based on a logarithmic plot of its growth. Inset: leaf growth as a function of time for the combined dataset; the fit line is exponential with a slope of 0.13 day^{-1}.

on individual leaf analyses. Until then (inset, Fig. 6), the mean leaf relative growth rates were surprisingly uniform at $0.13\ day^{-1}$ for all leaves. During this expansion period, leaf H_2O_2 levels averaged $16.5 \pm 0.6\ nmol\ cm^{-2}$. Immediately after full expansion, H_2O_2 levels increased by 45% to a mean of $23.9 \pm 1.0\ nmol\ cm^{-2}$, dropping subsequently to $9.7 \pm 0.7\ nmol\ cm^{-2}$ in a final sampling 26 days after full expansion. This experiment suggests it is not growth rate *per se* which is associated with H_2O_2 production, but maturation of leaf tissues following expansion.

Finally, this prediction was addressed using soybean (*Glycine max* (L.) Merr.) under field conditions early in the 2008 growing season. Although repeated leaf sampling during expansion was not possible because of their size, a large cohort of uniform-aged leaves under homogeneous soil and environmental conditions was available due to the cropping situation. The first and third trifoliates were used, after which variability between plants increased, reducing the synchrony of developmental state. Figure 7 shows the daily progression of leaf expansion and leaf H_2O_2 for both trifoliates. In contrast to oak, soybean leaf H_2O_2 increased markedly just before full expansion in both trifoliates. Like the oak, however, it declined thereafter.

Discussion

Since the 1980s, molecular genetics approaches in plant physiology have allowed increasingly complex relationships in biochemistry and gene expression to be examined. It is because of this that attention to H_2O_2 has increased. Unfortunately, the quantification of H_2O_2 contents has proved problematic; established, reliable analytical techniques for H_2O_2 based on chemiluminscence, fluorescence, titanium complex formation or absorbance all tend to respond in unexpected manners when challenged with plant extracts (e.g. Queval *et al*. 2008). Tissue-to-extraction buffer ratios, buffer contents and pH, and post-extraction treatments have all been found to change the apparent tissue concentrations; and although several potential interfering compounds, e.g. phenolics and ascorbate, have been identified, only ascorbate has been examined – even then, the extent to which it is actually a problem remains unknown (Cheeseman 2006; Queval *et al*. 2008).

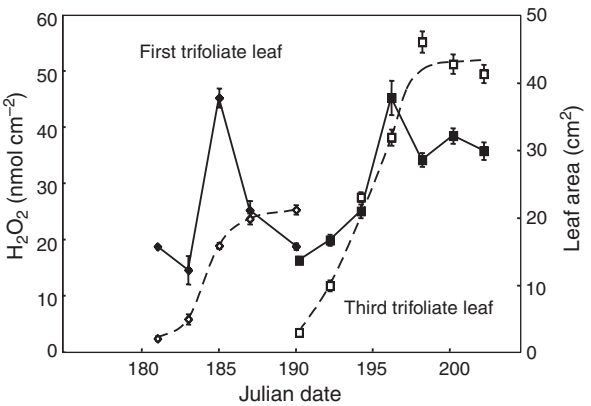

Fig. 7. The relationship between leaf H_2O_2 and leaf growth in soybean (GMAX) based on sampling of first and third trifoliate leaf cohorts under homogeneous soil and environmental conditions. At each date, 15 leaves were sampled, leaf areas were determined, and leaf H_2O_2 was assayed (left hand axis and solid lines). Data points are means ± s.e. Dashed lines show progress of leaf area development (right hand axis).

As studies of H_2O_2 as a signalling molecule, a moderator of metabolic activity, and a player in the overall metabolism of reactive oxygen have expanded, the levels at which H_2O_2 actually occurs in 'normal' plants under natural conditions has remained largely unexplored. In an earlier paper (Cheeseman 2006), leaf H_2O_2 concentrations were compared on 2 days in spring. The results suggested a correlation between H_2O_2 and environmental conditions. A mid-summer analysis of soybean suggested that H_2O_2 was also affected by both ozone and CO_2, although those effects were not measurable later in development.

In this paper, I have extended that study to cover full season patterns and a variety of species with markedly different phenological characteristics. I have used the FOX technique, modifying the harvesting and extraction protocols to increase their stability and repeatability. Despite controls which indicated the reliability of the actual measurements, however, it cannot be unequivocally discounted that uncharacterised interfering compounds were present that varied with species and season. Similarly, it cannot be discounted that interfering compounds in sun and shade exposed leaves, for example, varied dependent upon their growing in gap centers (with high light) or shaded at the edges. Given both the length and breadth of the survey, however, as well as the consistency of the results shown in Figs 5–7 for the H_2O_2 patterns during leaf expansion, and the absence of comparable field surveys, it seems reasonable to accept the dataset and examine its implications.

The results of the present study indicate that the patterns reported earlier were embedded in a broader seasonal progression. Here, more than 2500 samples from 18 species sampled over a period of 2 years were analysed, encompassing wide local environmental variability on and between sampling days. H_2O_2 levels ranged over about two orders of magnitude with the highest levels in the spring, and with a second peak in species showing late summer or autumn leaf initiation. This survey was coupled with two hypotheses designed to examine the phenomena underlying or modulating these patterns. The first hypothesis was approached by mining the dataset of the seasonal surveys; the second was approached more directly, by correlating H_2O_2 with leaf expansion.

H1 – The stress hypothesis

Like other ROS, variation in H_2O_2 contents is associated with metabolic imbalance engendered by environmental stress, particularly involving the photosynthetic and mitochondrial electron transport chains (Møller 2001; Noctor *et al*. 2002; Møller *et al*. 2007; Bettini *et al*. 2008). As Queval *et al*. (2008) noted, 'It is commonly considered that numerous stress conditions promote enhanced production of H_2O_2, and that this leads to increases in its concentration.' It has, however, been challenging to establish this correlation in practice. For example, Chen and Gallie (2004, 2005) reported elevated H_2O_2 in *Arabidopsis* following acute ozone exposure, but only with a 24-h delay. Moreover, chloroplasts, mitochondria, the cytosol, and other organelles, are generally well endowed with antioxidant enzymes which occur in several forms and under the control of

multiple genes. Only when one is inactivated or eliminated genetically, do oxidative stress and damage result. However, even then, complex interactions between defence and gene expression may lead to counterintuitive responses, including stress amelioration (e.g. Rizhsky *et al*. 2002).

Given the expectation that temperature, water and light stress are more likely in mid-summer than spring, Figs 1, 2 appeared incompatible with a purely stress-based model and hypothesis H1, and the three associated predictions were designed to address stress as a modulator of seasonal trends. The scope of this study precluded instrumentation to monitor individual plants and local soil conditions, or direct experimental manipulation of environmental conditions. Testing was, therefore, accomplished *a posteriori*, i.e. by mining the dataset (cf. Cheeseman *et al*. 1991, 1997). One consequence of this approach is that 'treatment' variables are continuous rather than discrete. Apparent responses to one variable (e.g. light), might, therefore, depend on, or be overridden by responses to a second (e.g. temperature). In some cases, multiple regression indicated the significance of both, but in others, alternate, highly correlated parameters could substitute (e.g. other temperature measures for T_{min} in Fig. 4). r^2 could also frequently be greatly improved by inclusion of several parameters (e.g. T, T_{min}, $T_{max\text{-}yesterday}$, and rainfall measures). I have not presented analyses involving more than two independent variables because the regression coefficients for correlated measures were usually in opposite directions (indicating mere tweaking of the model), or because the resultant combinations were physiologically uninterpretable with current understanding.

Support for any of the predictions was more limited than expected based on the previous study (Cheeseman 2006). Correlations between irradiance and H_2O_2, for example, were found in only four species (Figs 3, 4), and in three, the association was limited to low light. Even in gaps and at edges where light stress was exacerbated by temperature and wilting, irradiance had no effect. Temperature effects (second prediction) were supported only for SCAN (Fig. 4). Broad daily temperature swings (ΔT) were a potential factor only for AGLA and CVIR, but for both, the correlations were negative and insignificant when sampling date effects were included. Combining high temperature and conditions favouring drought (prediction three), only ACAN and ASAC could be described as 'suggesting' support. Expanding the temperature range to include spring active species never experiencing 25°C, combined temperature and 'drought' analyses specifically contradicted the prediction.

Thus, even if it were tenable in a restricted or marginal way for some species in some season, the first hypothesis was poorly supported overall by this survey based approach.

H2 – The developmental hypothesis

That H_2O_2 is produced by a plant to serve metabolic purposes and that its concentration reflects metabolic needs and activity, has been a focus for a different research community, in general, than the one addressing stress, signalling and antioxidants (Cheeseman 2007; Queval *et al*. 2008). The alternative hypothesis, therefore, was that H_2O_2 reflects developmental processes dependent on oxidant availability. The prediction was that concentrations would be elevated during leaf expansion, low following leaf maturation, and possibly, elevated again during senescence. The last of these never occurred.

This prediction reflects the fact that in normal development, leaves transition from increasing in size to increasing in 'toughness'. Toughness reflects the lignification of xylem vessels, fibres and sclerenchyma, and cross linking of cell walls which lend rigidity and mechanical protection to the blade. Toughness is, in most species, negatively correlated with folivore success (Marquis *et al*. 2001; Kursar and Coley 2003). Kursar and Coley (2003) noted that although there is considerable interspecific variation in leaf expansion rates, leaf toughening occurs over a short period around full expansion. Marquis *et al*. (2001) noted that both the time to reach full expansion and final leaf toughness increased from herbs to shrubs to trees.

The involvement of H_2O_2 in wall maturation and lignification, wound healing and pathogen-induced wall cross-linking is well established (Livingstone *et al*. 2005; Cona *et al*. 2006). It is produced by polyamine oxidases (PAO) and serves as a substrate in peroxidase-mediated wall stiffening (Angelini *et al*. 1996). In *Arabidopsis*, atao1, which encodes a Cu-containing amine oxidase, is expressed contemporaneously with lignification of tracheary elements, suggesting it as a marker for vascular development (Moller and McPherson 1998). Wall-bound peroxidase (POX) activity associated with cross linking and toughness is also influenced by biotic and abiotic environmental factors, including herbivore feeding and jasmonic acid (Moore *et al*. 2003*a*, 2003*b*). In grasses, drought increases POX activity while reducing both the size of the elongation zone and rates of elongation (Bacon *et al*. 1997).

Although these studies suggest that H_2O_2 might be low in leaves during rapid expansion, cell wall peroxidases, like other peroxidases, are versatile and incompletely understood enzymes (Passardi *et al*. 2005). H_2O_2 production within the elongation zone in maize, for example, has also been implicated in mediating wall loosening during extension growth (e.g. Fry *et al*. 2002; Rodriguez *et al*. 2002; Schopfer and Liszkay 2006). Thus, over the course of leaf expansion, H_2O_2 might, at one time, promote extension, and later, contribute to maturation. Only once full expansion and maturation were complete might the overall tissue concentration decrease significantly.

The results of the present study are largely consistent with this model. Over the course of the full season, H_2O_2 was highest during periods associated with rapid leaf expansion, i.e. the spring for all species, and summer and autumn for those which resumed leaf initiation then (Figs 1, 2). Three species for which good growth data were accessible provided additional support. With GHED, leaf H_2O_2 decreased linearly with expansion, and the relationship was sufficient to explain 50% of the variance in H_2O_2 over a two month period of rapid, summer and autumn stolon elongation and leaf initiation (Fig. 5). Nevertheless, the asynchronous leaf initiation patterns, and leaf sizes which precluded repeated sampling or sampling only between major veins in young leaves, constrained interpretation of the results.

With bur oak (QMAC) and soybean, leaf sizes were less restricting and greater resolution of growth rates was possible.

In oak, this was by repeated sampling of individual leaves, and in soybean by sampling from a large and uniform population of rapidly growing individuals. Again, neither of these was without its complications. With the oak, for example, repeated sampling entailed repeated damage to individual leaves, potentially affecting H_2O_2 levels at subsequent harvests. However, the overall relative growth rates were the same in all leaves (Fig. 6), regardless of the eventual leaf size or position on a branch. This suggests that sampling did not significantly alter expansion *per se*. Peak H_2O_2 concentrations occurred during the period in which leaf toughness was palpably increasing.

By comparison, in soybean, the age uniformity of the leaf population was the critical factor, and beyond the third trifoliate, establishing this limited the analysis. Nevertheless, both trifoliates 1 and 3 showed a sharp H_2O_2 peak just before, rather than just after, full expansion (Fig. 7). Based on the discussion above of toughening, this difference from oak may reflect the fact that soybeans are herbs whose leaves never develop the toughness that oaks do.

Conclusions

Queval *et al*. (2008), in the title of their paper, asked, 'Why are literature data for H_2O_2 contents so variable?' Their answer focussed heavily on analytical technique. Although technique is undoubtedly one of the causes and might explain much of the variations they summarised between studies (see also Cheeseman 2007), the present study, using a single, consistent, analytical method, still shows high variability. Moreover, that variability has a clear seasonal and developmental pattern.

Thus, a reasonable, alternative answer is – the data appear variable because H_2O_2 levels really are variable. Unfortunately, as Queval *et al*. (2008) anticipated, the results also imply that it is unlikely that measured bulk tissue H_2O_2 concentrations can ever reliably be used to indicate stress, signalling, or involvement in metabolic control of such processes as stomatal closing: the tissue levels were substantially higher than would be reasonably expected for intercellular concentrations based both on the sensitivity of intercellular processes to H_2O_2 and the ubiquitous and effective intercellular metabolic systems for detoxifying all ROS.

Instead, this study points to an important role of leaf phenology in determining H_2O_2 levels, with leaf concentrations reflecting developmental state and the role of the compound as a metabolite. As the relationships between physiology and phenology have received relatively little attention, the biochemical and genetic bases underlying the patterns and their control remain to be explained fully, but must certainly include cell wall-based enzymes, including polyamine oxidases and cell wall peroxidases.

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