An Overview of Hydrogen Peroxide Production and Cellular Determination in Plants

Follow this and additional works at: https://digitalcommons.aaru.edu.jo/hujr_a
Part of the Life Sciences Commons

Recommended Citation
Available at: https://digitalcommons.aaru.edu.jo/hujr_a/vol3/iss2/7

This Article is brought to you for free and open access by Arab Journals Platform. It has been accepted for inclusion in Hebron University Research Journal-A (Natural Sciences) - مجله جامعة الخليل للبحوث- أ (العلوم الطبيعيه) - by an authorized editor. The journal is hosted on Digital Commons, an Elsevier platform. For more information, please contact rakan@aaru.edu.jo, marah@aaru.edu.jo, dr_ahmad@aaru.edu.jo.
An Overview of Hydrogen Peroxide Production and Cellular Determination in Plants

*Fawzi A. Razem

Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

Abstract:

Recent advances in biochemical and genetic studies point to the steady-state production of hydrogen peroxide (H$_2$O$_2$) in plant tissues. H$_2$O$_2$ was traditionally viewed as a harmful by-product of reactive oxygen species (ROS) produced during plant metabolism. This view has, however, been changed by mounting evidence for its involvement in biotic and abiotic stress and its role in signal transduction in plants. During stress, such as following pathogen infection and wounding, plants adjust their cellular metabolism and the redox (reduction/oxidation) status of the cell resulting in increased intercellular levels of H$_2$O$_2$. Due to H$_2$O$_2$ toxicity, plants use antioxidants to keep its production under tight control and an abrupt increase in its levels results in a serious imbalance between H$_2$O$_2$ and antioxidant levels that eventually leads to cell death. Increased interest in determining the redox status of stressed plants has led to the development of several methods for H$_2$O$_2$ detection. Levels of H$_2$O$_2$ measured from a variety of plant species show a wide range (e.g., nM to mM) of H$_2$O$_2$ concentrations, a clear indication of technical difficulties in quantifying H$_2$O$_2$ levels. In this brief review, several of the methods used to detect H$_2$O$_2$ levels are discussed, particularly those that

Key words: H$_2$O$_2$ content, H$_2$O$_2$ detection, peroxidase-mediated assays, tissue printing.

*Corresponding author: razemf@cc.umanitoba.ca
are used for quantitative estimations of $H_2O_2$ based on the peroxidase-mediated assays. Because of various problems associated with $H_2O_2$ detection in plants, it is recommended that several methods be used to verify $H_2O_2$ contents before any interpretation of data is made.

**Abbreviations:** DAB, 3, 3'-diaminobenzidine; DAF, 2,7-diaminofluorene; DCP, 2, 4- dichlorophenol; DMAB, 3- dimethylaminobenzoic acid; PXD, peroxidase; $H_2O_2$, hydrogen peroxide; $O_2^*$-, superoxide; ROS, reactive oxygen species; SOD, superoxide dismutase. TMB, 3, 5, 3’5’-tetramethylbenzidine

**Production of ROS in biological systems**

Environmental interactions subject plants to enormous challenges through biotic and abiotic insults. Herbivory and mechanical injuries are examples of the severe stresses a plant may experience in their native environment (Gatehouse 2002). Such insults can cause wounding that may disrupt cellular structure and provide entry to pathogens. Plants have developed an array of defense mechanisms to cope with adverse conditions, one of which is the production of reactive oxygen species (ROS). For example, following pathogen infection and wounding, a significant amount of ROS is produced (i.e., oxidative burst), a process that is likely involved in the direct elimination of pathogens and in the cross-linking of cell wall components. This oxidative burst provides an early line of defense for injured plants (Bolwell 1996; Razem and Bernards 2002).

Reactive oxygen species are free radicals that possess an unpaired electron, which originates from the single electron reduction of molecular oxygen during aerobic metabolism (Vranová et al. 2002). Superoxide ($O_2^*$-) and $H_2O_2$ are the most studied ROS because of their importance in plant development and stress-induced defenses. Plants usually produce ROS in small quantities during normal metabolic activities such as respiration and photosynthesis by reactions that involve electron transport in chloroplasts, mitochondria, and peroxisomes (del Rio et al. 1992; Mehdy 1994; Møller 2001). The production of ROS increased significantly under stress conditions such as pathogen infection, wounding, hormone application (e.g., abscisic acid), high osmotic stress, low water potential, and contamination with heavy metals (Van Breusegem et al. 2001; Razem and Bernards 2003; Razem et al. 2004; Razem and Hill 2007). Several enzymatic systems have been shown to generate $H_2O_2$ during normal plant growth and development and under biotic and abiotic stresses. These ROS-generating systems include peroxidases and NADPH-dependent oxidases (Cross
and Jones 1991; Sutherland 1991; Lamb and Dixon 1997; Wojtaszek 1997; Berne- 
nards et al. 2004).

Cellular contents of $\textit{H}_2\textit{O}_2$

Production of ROS during normal growth and development is at a con-
stant rate at 240 $\mu$M S$^{-1}$ O$_2^*$- with a steady-state level of approxi-
mately 0.5 $\mu$M $\textit{H}_2\textit{O}_2$ (e.g., in chloroplasts) (Polle 2001). The steady-state level is under tight control and higher concentrations may be an indicator of greater inter-
cellular stress (Mittler 2002). Following various types of biotic and abiotic stresses, rates of ROS production increase dramatically, reaching up to 720 $\mu$M S$^{-1}$ O$_2^*$- and steady-state levels of 5-15 $\mu$M $\textit{H}_2\textit{O}_2$ (Polle 2001). Different biotic and abiotic stresses result in increases of the in planta steady-state contents of $\textit{H}_2\textit{O}_2$ that vary by several orders of magnitude (e.g., between nM and mM $\textit{H}_2\textit{O}_2$ concentrations) (Table 1). Although there is a general consent that $\textit{H}_2\textit{O}_2$ levels can rise significantly following certain stresses, it has been always difficult to obtain accurate measurements for this increase due to technical difficulties in quantifying $\textit{H}_2\textit{O}_2$ contents (Neill et al. 2002). These difficulties are apparent from the wide range of $\textit{H}_2\textit{O}_2$ levels observed, even within the same plant species (Table 1). Furthermore, these differences usually lead to misinterpretation of the redox status of stressed tissues. For example, treatment of maize leaves with the plant hormone abscisic acid (ABA) increased $\textit{H}_2\textit{O}_2$ levels to approximately 2.8 $\mu$mol g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


increased $\textit{H}_2\textit{O}_2$ levels to approximately 2.8 $\mu$mol g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$


g$^{-1}$ FW (Jiang and Zhang 2001), whereas up to 310 $\mu$mol $\textit{H}_2\textit{O}_2$.
Assays are usually taken at pH values that are optimal for the peroxidase (usually between pH 4.5 and pH 7.0). The absorbance is compared against a calibration curve prepared using known concentrations of H\textsubscript{2}O\textsubscript{2} or against the extinction coefficient of the chromogen. Commonly used spectrophotometrically peroxidase-based assays are summarized in Table 2. The advantages of these assays are in their abilities to provide quantitative values for H\textsubscript{2}O\textsubscript{2} contents in tissue extracts, in addition to being simple and fast. Other peroxidase-independent spectrophotometrically based assays used to determine H\textsubscript{2}O\textsubscript{2} concentrations in plant extracts use titanium (IV) (Patterson et al. 1984) and xylenol orange (Jiang et al. 1990). The titanium assay follows the formation of a titanium-H\textsubscript{2}O\textsubscript{2} complex spectrophotometrically at 410 nm. The xylenol assay is based on the H\textsubscript{2}O\textsubscript{2}-mediated oxidation of Fe\textsuperscript{2+} to Fe\textsuperscript{3+} (catalyzed by sorbitol) under acidic conditions (Jiang et al. 1990). The resultant Fe\textsuperscript{3+} reacts with the dye xylenol orange and the colour formation is followed at 560 nm. The later method is sensitive to lower concentrations (e.g., < 0.1 \mu M H\textsubscript{2}O\textsubscript{2}).

Spectrophotometric assays require that cell-free plant extracts are prepared, therefore the measurement of H\textsubscript{2}O\textsubscript{2} is taken in vitro. Although there are some problems accompanying such measurements (see section below), these assays are considered quantitative and are reliable if carried out under conditions that minimize the metabolism of H\textsubscript{2}O\textsubscript{2} before measurements.

Cytochemical staining of H\textsubscript{2}O\textsubscript{2} Methods which involve the cytochemical staining of intact tissues (e.g., thin sections) are more reliable in determining the location and the existence of H\textsubscript{2}O\textsubscript{2} producing systems. Although arbitrary units can be given, cytochemical staining methods are not usually considered quantitative. The most commonly used is the starch/KI staining method described by Olson and Varner (1993). This method utilizes potato starch (4% w/v) and KI (0.1 M) applied to thin sections of freshly cut plant tissues. In the presence of H\textsubscript{2}O\textsubscript{2}, iodide is oxidized to iodine before it is complexed by starch to form a blue-purple colour. Though this reaction is independent of peroxidase, it is not suitable for spectrophotometric assays because of the viscosity of starch solution.

Another cytochemical staining method that is commonly used for cytochemical localization of H\textsubscript{2}O\textsubscript{2} is cerium chloride technique (Thomas and Trelease 1981). The formation of cerium perhydroxide from exogenous cerium chloride and endogenous H\textsubscript{2}O\textsubscript{2} can be viewed as dark deposits under electron microscopy (Bestwick et al. 1997; Córdoba-Pedregosa et al. 2003).

Cytochemical staining of H\textsubscript{2}O\textsubscript{2} can also be achieved by utilizing peroxidase-mediated reactions with suitable reductants such as 3,5,3’5’-tetramethylbenzidine-hydrochloric acid (TMB)-HCl or 3,3’-diaminobenzidine (DAB)-HCl. Tissue sections are incubated in a solution containing one of these reagents and the change in colour at the site of H\textsubscript{2}O\textsubscript{2} production can be viewed, e.g., TMB (Ros Barcelo’ 1998; Bernard and Razem 2001; Morohashi 2002).
and DAB (Thordal-Christensen et al. 1997). Diaminobenzidine polymerizes instantly into a coloured precipitate when comes into contact with \( \text{H}_2\text{O}_2 \) in the presence of peroxidase (Thordal-Christensen et al., 1997). It should be noted that the amount of coloured polymer appearing at the sites of \( \text{H}_2\text{O}_2 \) production is indicative of the content of \( \text{H}_2\text{O}_2 \) accumulated over the duration of staining. If the ability of cells to produce \( \text{H}_2\text{O}_2 \) is sought, then removal of pre-existing concentrations of \( \text{H}_2\text{O}_2 \) in tissues by incubation with 1 mM ascorbic acid for 1 min before incubation in the staining solution is necessary. Staining with TMB appears to be more sensitive for lower concentrations of \( \text{H}_2\text{O}_2 \) than DAB.

Chemiluminescence and fluorescence detection of \( \text{H}_2\text{O}_2 \)

The production of \( \text{H}_2\text{O}_2 \) can be determined by using appropriate chemiluminescence reagents, such as luminol (5-amino-2,3-dihydro-phthalazine-1,4-dione) and the resultant emission of light following the oxidation of luminol by \( \text{H}_2\text{O}_2 \) measured with a scintillation counter (Warm and Laties 1981; Jones and Scowen 1987). Although the mechanism of this reaction is not completely understood, it is believed that the light emission is a result of the excitation of 3-aminophthalate generated by \( \text{H}_2\text{O}_2 \)-mediated luminol oxidation in a reaction catalyzed by haem-containing compounds, such as ferricyanide. The emission response is pH dependent (pH 9.5) and gives linear line with increasing concentrations of \( \text{H}_2\text{O}_2 \) (Warm and Laties 1981).

Detection of \( \text{H}_2\text{O}_2 \) can also be achieved by using suitable fluorescent reagents in reactions catalyzed by peroxidases. For example, the oxidation of dichlorofluorescein (DCF) by \( \text{H}_2\text{O}_2 \) is catalyzed by peroxidases and yields highly fluorescent DCF (Cathcart et al., 1983) that can be monitored by laser scanning confocal microscopy (Zhang et al. 2001). Also, spectrofluorimetric determination of \( \text{H}_2\text{O}_2 \) can be achieved by following the oxidative quenching of the fluorescent peroxidase substrate pyranine (8-hydroxypropene-1,3,6-trisulfonic acid trisodium salt; excitation at 405 nm, emission at 512 nm) (Chandra and Low 1995).

Tissue printing of \( \text{H}_2\text{O}_2 \)

Tissue printing is the ability to visualize \( \text{H}_2\text{O}_2 \) produced by thin plant sections on a substrate membrane, commonly nitrocellulose, using appropriate reagents (Varner and Ye 1994; Spruce et al. 1987; Cassab et al. 1988; Schopfer 1994). This implies that the generated \( \text{H}_2\text{O}_2 \) is transferred to a receptive membrane when the cut surfaces of plant sections are pressed against such a membrane (Varner and Ye 1994). Although visualization of the \( \text{H}_2\text{O}_2 \) may utilize virtually any of the stain reagents discussed above, it is preferable to choose one with a low-coloured background, such as starch/KI. Receptive membranes can be prepared by impregnating a nitrocellulose film with a mixture of KI and soluble starch solution before being air-dried. When \( \text{H}_2\text{O}_2 \) is transferred from the cut surface of the plant tissue to the dried membrane, iodide is oxidized to iodine before it is then complexed
by starch to form a colour that can be visualized on the membrane surface. This method, however, is insensitive to low concentrations of H$_2$O$_2$ (Schopfer 1994). Tissue printing can also be achieved by using suitable peroxidase substrates, such as 0-phenylenediamine (Cassab et al. 1988), guaiacol and DAB (Spruce et al. 1987).

Common technical difficulties of H$_2$O$_2$ measurements

Problems that arise during measurement of H$_2$O$_2$ levels in plant tissues are mainly due to reactivity and rapid metabolism of H$_2$O$_2$. For example, in soybean cells 10 mM of exogenously applied H$_2$O$_2$ was disproportionated in approximately 5 min (Levine et al. 1994). Furthermore, the content of endogenous H$_2$O$_2$ declined to more than 40% after 1 h of extraction and 90% following one-day storage (Razem, unpublished data). The endogenous scavenging of H$_2$O$_2$ in experimental assays causes serious problems that affect accuracy and reproducibility of the experiments and can be summarized as follows:

Extraction problems.

Plant tissues contain enzymes and other soluble antioxidants that may remain in cell-free extracts following homogenization. For example, contamination with endogenous catalase can decompose H$_2$O$_2$ and accelerate its disproportionation. Plant tissues also contain various compounds, such as phenolics, that may react with H$_2$O$_2$ and therefore make it difficult to obtain accurate measurements following homogenization. The production of phenolics and other aromatic substrates varies among plant species, causing variation in H$_2$O$_2$ assays. Accuracy becomes even more problematic as plants contain peroxidases with a wide range of substrate specificity, resulting in increased competition for H$_2$O$_2$ in those assays dependant upon peroxidase activity. For example, wounded potato tubers synthesize larger amounts of phenolics (Razem and Bernards 2002; 2003) compared to wounded alfalfa leaves, (unpublished data). Desalting the homogenate, for example by using appropriate size exclusion column chromatography, can remove most of the low molecular weight compounds but it also removes H$_2$O$_2$ from the extract thus preventing its utilization. Unfortunately, it is extremely difficult to prevent the dissipation of H$_2$O$_2$ during extraction, although a proper handling of tissue extract would minimize H$_2$O$_2$ metabolism. Problems associated with extraction would likely cause some of the inconsistencies in the measurements of H$_2$O$_2$ levels as shown in Table 1. To minimize H$_2$O$_2$ metabolism, it is critically important to carry all extraction steps at low temperature (e.g., 4°C) and to carry out the assays immediately following extraction. Extraction at low temperature would minimize the activity of catalase and the disproportionation of H$_2$O$_2$. It is also helpful to adjust the pH of the extraction buffer between pH 7.3 - 8.0, as most peroxidases have acidic pH optima between pH 4.5-7.0. It is not recommended, however, to in-
introduce heme inhibitors (e.g., KCN and NaN₃) to the homogenization buffer as these compounds may interfere with the exogenously applied peroxidase required in some of the assays.

Incubation time.
Choosing appropriate assay for H₂O₂ measurement that is reproducible and stable over incubation time is of great importance. The length of incubation time prior to the start of the reaction is an important factor that should be taken into account when peroxidase-mediated H₂O₂ assays are used. Peroxidase catalyzes a fast reaction and the oxidized products continue to form and cross-react with other compounds in the assay mixture, with resultant changes in the absorption spectra. This instability can alter measurements and therefore it is necessary to check stability against a known concentration of H₂O₂ over different time intervals and set the reaction time identical to the reaction used for calibration. Furthermore, peroxidase and substrate concentrations as well as pH should be optimized so they do not become limiting factors. Optimizing the reaction conditions in terms of pH, reductant concentration, and incubation time is therefore crucial for getting reliable quantitative measurements of H₂O₂ in plant extracts.

Sensitivity to low concentration of H₂O₂
Most of the available peroxidase-based assays are not sensitive to low concentrations of H₂O₂ (i.e., < 1 μM H₂O₂). While TMB is relatively more sensitive to low concentrations (1-100 μM H₂O₂), other assays, such as guaiacol and 0-dianisidine cannot usually be used to detect concentrations below 5 μM H₂O₂ (Table 2). The peroxidase-independent assays, particularly xylenol orange and titanium are more sensitive and are able to detect H₂O₂ to nmolar (>100 nM H₂O₂) concentrations (Table 2). It is therefore recommended to verify the sensitivity of the methods used before commencing the experiment.

Concluding remarks
Determination of H₂O₂ contents in plant tissues is of increasing interest as H₂O₂ concentration is normally used as an indicator for intercellular redox status and stress (Figure 1). Most of the methods that have been developed so far to obtain quantitative values for H₂O₂ levels in plant tissues are dependent on peroxidase-mediated oxidation of various reductants. Due to the great interest in this field, however, it is likely that new methods that are more reliable in determining H₂O₂ contents will be developed. Because of the relative instability of H₂O₂ in aqueous solutions and its fast disproportionation, collection of quantitative data should be taken with more precautions. To avoid errors and irreproducible or highly variable results in such data, it is important that all assays must be optimized and verified with known concentrations of H₂O₂. This includes optimization of incubation time and extraction conditions. It is also recommended that two or more different assays should be used to obtain
more consistent and reliable values.

References


15. Guan, L.M., Zhao, J., Scandalios, J.G., (2000). Cis-elements and trans-factors that regulate expression of the maize CaT1 antioxidant gene in response to ABA and osmotic stress: H₂O₂ is the
An Overview of Hydrogen Peroxide Production and Cellular Determin

Hydrogen peroxide and lignification, Plant J. 4, 887-892.
oxidase in microbodies (glyoxysomes and peroxisomes) of higher plant tissues with the CeCl₃ technique, Protoplasma 108, 39-53.


### Table 1: Estimated physiological and stress-induced contents of H$_2$O$_2$ in plant tissues.

Methods used for H$_2$O$_2$ estimation and type of stress or elicitor are given for comparison.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Normal</th>
<th>Induced</th>
<th>Stress/elicitor</th>
<th>Methods</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis</td>
<td></td>
<td></td>
<td></td>
<td>DMAB</td>
<td>O’Kane et al. 1996</td>
</tr>
<tr>
<td>callus*</td>
<td>0.0012</td>
<td>0.0016</td>
<td>Chilling</td>
<td>Fluorescence</td>
<td>Meinhard et al. 2002</td>
</tr>
<tr>
<td>leaves</td>
<td>0.30</td>
<td>ND</td>
<td>--</td>
<td>Fluorescence</td>
<td>Meinhard et al. 2002</td>
</tr>
<tr>
<td>seedlings</td>
<td>0.07</td>
<td>ND</td>
<td>--</td>
<td>Fluorescence</td>
<td>Meinhard et al. 2002</td>
</tr>
<tr>
<td>Barely leaves</td>
<td>0.032</td>
<td>0.033</td>
<td>Boron toxicity</td>
<td>0-dianisidine</td>
<td>Karabal et al. 2003</td>
</tr>
<tr>
<td>roots</td>
<td>0.078</td>
<td>0.055</td>
<td>Boron toxicity</td>
<td>0-dianisidine</td>
<td>Karabal et al. 2003</td>
</tr>
<tr>
<td>Bean leaves</td>
<td>0.370</td>
<td>ND</td>
<td>--</td>
<td>Titanium</td>
<td>Patterson et al. 1984</td>
</tr>
<tr>
<td>Cotton fibers*</td>
<td>300</td>
<td>5000</td>
<td>Differentiation</td>
<td>Titanium</td>
<td>Potikha et al. 1999</td>
</tr>
<tr>
<td>Cucumber seedlings</td>
<td>1.0</td>
<td>7.5</td>
<td>Ergosterol</td>
<td>Luminol</td>
<td>Kauss and Jeblick 1996</td>
</tr>
<tr>
<td>leaves</td>
<td>0.092</td>
<td>ND</td>
<td>--</td>
<td>Titanium</td>
<td>Patterson et al. 1984</td>
</tr>
<tr>
<td>Maize leaves</td>
<td>1.80</td>
<td>2.80</td>
<td>ABA</td>
<td>Titanium</td>
<td>Jiang and Zhang 2001</td>
</tr>
<tr>
<td>cultured cells</td>
<td>310.0</td>
<td>490.0</td>
<td>ABA</td>
<td>Luminol</td>
<td>Guan et al. 2000</td>
</tr>
<tr>
<td>Rice leaves</td>
<td>0.180</td>
<td>0.375</td>
<td>NaCl</td>
<td>DMAB</td>
<td>Uchida et al. 2002</td>
</tr>
<tr>
<td>roots*</td>
<td>8.50</td>
<td>17.00</td>
<td>ABA</td>
<td>Titanium</td>
<td>Lin and Kao 2001</td>
</tr>
<tr>
<td>Spinach leaves</td>
<td>0.536</td>
<td>ND</td>
<td>--</td>
<td>Titanium</td>
<td>Patterson et al. 1984</td>
</tr>
<tr>
<td>Sunflower leaves</td>
<td>0.160</td>
<td>0.210</td>
<td>Fe deficiency</td>
<td>Titanium</td>
<td>Ranieri et al. 2003</td>
</tr>
<tr>
<td>seeds*</td>
<td>0.85</td>
<td>0.50</td>
<td>Artificial drying</td>
<td>DMAB</td>
<td>Bailly et al. 2004</td>
</tr>
<tr>
<td>Tobacco leaves</td>
<td>9.1</td>
<td>42.1</td>
<td>Cryptogen</td>
<td>Luminol</td>
<td>Rustérucci et al. 1996</td>
</tr>
<tr>
<td>leaves</td>
<td>9.1</td>
<td>36.1</td>
<td>Capsicein</td>
<td>Luminol</td>
<td>Rustérucci et al. 1996</td>
</tr>
<tr>
<td>Wheat leaves*</td>
<td>0.045</td>
<td>0.135</td>
<td>Drought</td>
<td>Titanium</td>
<td>Lin and Wang 2002</td>
</tr>
</tbody>
</table>

DMAB, 3-dimethylaminobenzoic acid

* Dry weight was used
Table 2: Common quantitative assays used for $\text{H}_2\text{O}_2$ determination in plant extracts using chromogenic substrates. The optimal pH, peak absorbance, and concentration range are given. Minimum level of detection is based on experiments using a known concentration of $\text{H}_2\text{O}_2$.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Annm</th>
<th>pH</th>
<th>Sensitivity</th>
<th>PxD-dependence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAF</td>
<td>600</td>
<td>6.0</td>
<td>ND</td>
<td>+</td>
<td>Criquet et al. 2001</td>
</tr>
<tr>
<td>DCP</td>
<td>510</td>
<td>6.5</td>
<td>ND</td>
<td>+</td>
<td>Ishida et al. 1987</td>
</tr>
<tr>
<td>DMAB</td>
<td>590</td>
<td>6.5</td>
<td>ND</td>
<td>+</td>
<td>O’Kane et al. 1996</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>450</td>
<td>7.0</td>
<td>$&gt;5 \mu\text{M H}_2\text{O}_2$</td>
<td>+</td>
<td>Geetha and Shetty 2002</td>
</tr>
<tr>
<td>O-dianisidine</td>
<td>436</td>
<td>7.0</td>
<td>$&gt;5 \mu\text{M H}_2\text{O}_2$</td>
<td>+</td>
<td>Karabal et al. 2003</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>430</td>
<td>6.0</td>
<td>$&gt;5 \mu\text{M H}_2\text{O}_2$</td>
<td>+</td>
<td>Criquet et al. 2001</td>
</tr>
<tr>
<td>Titanium</td>
<td>410</td>
<td>8.0</td>
<td>$&gt;0.1 \mu\text{M H}_2\text{O}_2$</td>
<td>-</td>
<td>Patterson et al. 1984</td>
</tr>
<tr>
<td>TMB</td>
<td>660</td>
<td>5.5</td>
<td>$&gt;1 \mu\text{M H}_2\text{O}_2$</td>
<td>+</td>
<td>Ros Barcelo’ 1998; Razem 2003</td>
</tr>
<tr>
<td>Xylenol Orange</td>
<td>560</td>
<td>5.5</td>
<td>$&gt;0.1 \mu\text{M H}_2\text{O}_2$</td>
<td>-</td>
<td>Jiang et al. 1990</td>
</tr>
</tbody>
</table>

Abbreviations: DAF, 2,7-diminothiazoline; DCP, 2,4-dichlorophenol; DMAB, 3-dimethylaminobenzoic acid; ND, not determined; PxD, peroxidase; TMB, 3,5,3’5’-tetramethylbenzidine.

Figure 1. Schematic representation of $\text{H}_2\text{O}_2$ production in plant tissues. During stress, $\text{H}_2\text{O}_2$ levels rise significantly and if the levels are not controlled by antioxidants, the cellular $\text{H}_2\text{O}_2$ will become toxic and eventually lead to programmed-cell death (PCD). CAT: catalase; APX: peroxidase; GSR: glutathione-s-reductase; ROS: reactive oxygen species.