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A comparison of colorimetric assays detecting hydrogen peroxide in leaf extracts

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Ifjúság u. 6, H-7624 Pécs Hungary**Abstract**

Hydrogen peroxide (H_2O_2) is a central molecule in plant stress responses as potential oxidizing agent or signal molecule, depending on its localization and cellular concentrations. The present work compared three colorimetric assays assessing H_2O_2 concentrations in terms of sensitivity, linearity with increasing H_2O_2 concentrations, solvent interactions and recovery of exogenous H_2O_2 from leaf extracts. The tested methods were based on the oxidation of one of the following chromophores: potassium iodide (KI), 3,3'-diaminobenzidine (DAB) or xylenol orange (XO). Various solvents were chosen for extraction to represent two different approaches of sampling: extraction into water or water-based buffer providing material for an array of bioassays; and extraction into solutions containing trichloroacetic acid (TCA) or ethanol in order to minimize interactions arising from leaf biochemistry. The most employable technique was the xylenol orange assay in organic solvents, although it is advisable to interpret results against a series of spiked samples rather than a calibration line acquired in solvent only.

Introduction

Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS) generated in a variety of cellular compartments including chloroplasts, mitochondria, peroxisomes and cell walls [1]. H_2O_2 concentrations in leaves vary strongly between and within species and are also affected by age and physiological status [2]. H_2O_2 sources include enzymes, such as superoxide dismutases, nicotinamide adenine dinucleotide phosphate oxidases and cell wall peroxidases, as well as non-enzymatic processes, for example the spontaneous conversion of superoxide radicals to H_2O_2 or glycolate oxidation in peroxisomes [3]. Among ROS, H_2O_2 is the least reactive and has the longest lifetime, approximately 1 ms in soybean (*Glycine max* L.) cell suspension cultures [1] or in HeLa cell cultures [4]. H_2O_2 is assumed to diffuse some μm from its production site [5] and even cross membranes via aquaporins [6]. Because of these characteristics, H_2O_2 is considered as a signaling molecule in growth and development [7], cell cycle [8], flowering [9], senescence [10] and in stress responses [11]. On the other hand, travelling H_2O_2 may spread oxidative damage as the source of highly reactive hydroxyl radicals via Fenton chemistry [12] or direct photo-cleavage by UV-B [13]. Consequently, assessing H_2O_2 concentrations in leaves is of special interest as indicator of physiological status and stress responses.

Various methods are available to measure the concentration of H_2O_2 in plant samples, for example fluorescence [14], chemiluminescence [15], electrochemical [16] or colorimetric [2] assays. Although results may be affected by metabolites other than

H₂O₂, and appear to depend on the choice of method [17, 18], photometric assays based on absorption changes of chromophores upon oxidation by H₂O₂ are still the most widely used techniques due to their simplicity, low cost and rapidity. Probably the most popular assays are the ones using KI [19], DAB [20] or XO [21].

We compared these methods in three steps, in order to examine: (i) the absorption characteristics of the oxidized chromophores, (ii) the concentration range of H₂O₂ detection, (iii) whether leaf metabolites other than H₂O₂ interfered with the assay. For the last step, leaf extracts were spiked with exogenous H₂O₂ which was attempted to recover using the assays. All measurements were repeated using four different solvents: distilled water, potassium-phosphate buffer, trichloroacetic acid (TCA) or ethanol. Solvents were chosen to represent two different approaches in sampling. One is to preserve biological activities and prepare extracts using a pH neutral water-based buffer [22] or distilled water [23]. The advantage of these methods is that extracts are also suitable for determinations of various enzyme activities or pigment concentrations. The disadvantage is that biological activities maintained in such extracts may modify metabolite profiles during extraction. For example, peroxidases may decrease H₂O₂ concentrations. Such interference can be prevented by inactivating biological functions during extraction, for example by the use of TCA [24] or alcohol : water solutions that are frequently applied to study phenolic compounds [25]. This second approach, however, may hinder assays relying on using exogenous enzymes to catalyze chromophore oxidation by H₂O₂.

Materials and methods

H₂O₂ determination

1 M KI, 3.5 mM DAB and 125 μM XO solutions were prepared as described [19-21]. For absorption characterization and H₂O₂ concentration range determination tests, a series of 1-100 nM H₂O₂ solutions were dissolved in 100 μL total volume of distilled water, 100 mM K-phosphate buffer (pH 7.0), 70% (v/v) ethanol or 6% (v/v) TCA and 1 mL of KI, DAB or XO solution were added. For leaf compound interference test, 100 μL of leaf extract (see below) was added to 1 mL of chromophore solution. The DAB assay was performed with the use of 0.4 U horseradish peroxidase (HRP).

Plant material and leaf extract preparation

Our choice of model plant, *Nicotiana benthamiana* is widely applied in plant virology [26] where responses to infection reportedly include increased cellular H₂O₂ concentrations [27]. Plants were grown in growth chambers (Sanyo MLR-352H-PE, Panasonic Healthcare Co., Ltd., Oizumi, Japan) under 90 μmol m⁻² s⁻¹ PAR (16h/8h light/dark, 25°C/20°C, 70% humidity). Freshly harvested leaves from 4 weeks old plants were homogenized in ice cold solvent (one of the above) using pestle and mortar. Extracts were centrifuged (15,000 × g, 10 min, 4°C) (Heraeus Fresco 17 Centrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA) and supernatants were used immediately.

Results and discussion

Absorption characteristics

Chromophore oxidation by H_2O_2 should be accompanied by a characteristic absorption change. Leaf samples contain a plethora of metabolites absorbing UV and/or visible light and concentrations of these may vary in response to the same environmental conditions which are expected to modify H_2O_2 levels. A well-defined peak in the absorption spectrum of the oxidized chromophore is necessary to separate H_2O_2 induced changes from background absorption from other metabolites. The product of the KI and H_2O_2 reaction (triiodide, I_3^-) has maximum absorption at 352 nm, although the assay is usually evaluated using 390 nm data [19]. This peak was detected in pure water, in the water based phosphate buffer and in ethanol but not in TCA solution. Consequently, the KI assay in TCA was not included in further analyses (Table 1). The absorption maximum of oxidized DAB was detected at 465 nm in water, in K-phosphate buffer and in ethanol; and at 455 nm in acidic TCA solution (Table 1). The chemically unknown colored product of the reaction between XO and H_2O_2 had a maximum absorption at 560 nm in all tested solvents (Table 1). This absorption seems ideal for assessing H_2O_2 in chlorophyll containing samples which have low absorption in this spectral region themselves. On the other hand, near UV and blue absorptions of oxidized KI and DAB, respectively, are to be measured over the background of pigments, such as chlorophyll or flavonoids.

Detectable H_2O_2 concentration range

Increase in the chromophore's absorption is required to be linear with increasing H_2O_2 concentrations for a valid quantification. Ranges of H_2O_2 concentrations fulfilling this requirement are listed in the second row of Table 1. When applicable, the assays were sensitive to H_2O_2 concentrations as low as 1 nM. The DAB-based assay had the widest calibration range (up to 80 nM, Table 1) but was not applicable in TCA solution due to the non-linearity of calibration (Supplementary Information). In the ethanol : water solution, DAB was less responsive to low H_2O_2 concentrations. The same solution, however, expanded the calibration range of the KI-based assay, as compared to that in water or buffer (Table 1). The relatively narrow 1-10 nM linear response range of the XO-based assay (slightly wider than the 1-8 nM recommended by the manufacturer [21]) calls for caution when samples with high H_2O_2 concentrations are evaluated.

Interference with leaf compounds

Metabolites present in leaf extracts should not interfere with the rate of chromophore oxidation by H_2O_2 , i.e. should not modify the linearity of the above calibrations. This requirement was only examined in solvents in which calibration was possible (row-(ii) in Table 1). Leaf samples were spiked with various amounts of H_2O_2 during preparation and measuring the efficiency of detecting this exogenous H_2O_2 . Spiking was only possible in TCA and ethanol solutions. All three methods failed to respond to exogenous H_2O_2 in the presence of leaf extracts in water or buffer. This may be explained by the consumption of added H_2O_2 by leaf antioxidants. Neither the KI- nor the DAB-based assay responded to

spiking in the ethanol solution, (Electronic Supplementary Information, Fig. S4). However, when applying the XO assay in organic solvents, spiked samples showed linear increase in 560 nm absorption with increasing exogenous H_2O_2 in both TCA and ethanol solutions (Fig. 1).

Correction of the XO assay

The two fitting lines characterizing the connections between added and recovered H_2O_2 concentrations in TCA and ethanol solutions were different from each other and also from the ideal line corresponding to equality (dashed line in Fig. 1.). Both had non-zero intercepts and their slopes were either higher (TCA) or lower (ethanol) than that of the equality line. The positive intercepts correspond to the H_2O_2 contained in the leaf extract that was relatively low because the model plants were not exposed to stress. However, the XO method evaluated leaf H_2O_2 contents differently, as 1.0 or 2.8 nM (per 100 mg fresh weight), when measured in TCA or in ethanol, respectively. This discrepancy may be explained by assuming that extracting into different solvents resulted in samples containing different amounts of phenolic compounds and ascorbate that may react with H_2O_2 during sample preparation and thus affect detection [17].

As endogenous H_2O_2 adds to exogenous concentrations, ideal recovery of spiking concentrations is expected to shift fitting lines upwards but keeping a slope equal or close to 1. This was not the case in our experiment. Detected H_2O_2 concentrations were all higher than spiking concentrations but slopes were 1.24 and 0.84 when the XO assay was applied in 6% TCA or in 70% ethanol, respectively (Fig. 1). In addition to the applied solvent, the extent of over- or underestimating depended on H_2O_2 concentrations. These results indicate that it is not advisable to rely on a single spiking concentration as reference. Statistical analysis of the two assay conditions as comparison of methods confirmed significant differences (Electronic Supplementary Information, Fig. S5 and Table S1).

Conclusions

Our results show that among the tested conditions extraction into and assaying in TCA or ethanol with XO as chromophore proved the most suitable methods to detect H_2O_2 in plant derived samples. However, it is recommended to calculate cellular concentration relying on a series of H_2O_2 spiked samples rather than a calibration line acquired in the corresponding solvent only.

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Table 1.
Comparison of H₂O₂ detecting colorimetric assays

	KI				DAB				XO			
	water	buffer	EtOH	TCA	water	buffer	EtOH	TCA	water	buffer	EtOH	TCA
(i) Maximum absorption (nm)	352	352	352	n.a.	465	465	465	455	560	560	560	560
(ii) Linearity (nM H ₂ O ₂)	1-20	1-20	1-60	n.a.	1-80	1-80	10-80	n.a.	1-10	n.a.	1-10	1-10
(iii) H ₂ O ₂ recovery (nM H ₂ O ₂)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1-20	1-10

Buffer, 100 mM potassium-phosphate pH 7.0; DAB, 3.5 mM 3,3'-diaminobenzidine; EtOH, 70% (v/v) ethanol; KI, 1 M potassium-iodide; TCA, 6% (v/v) trichloroacetic acid; water; XO, 125 μM xylenol orange; n.a., not applicable. Raw data are in Figs. S1-S3.

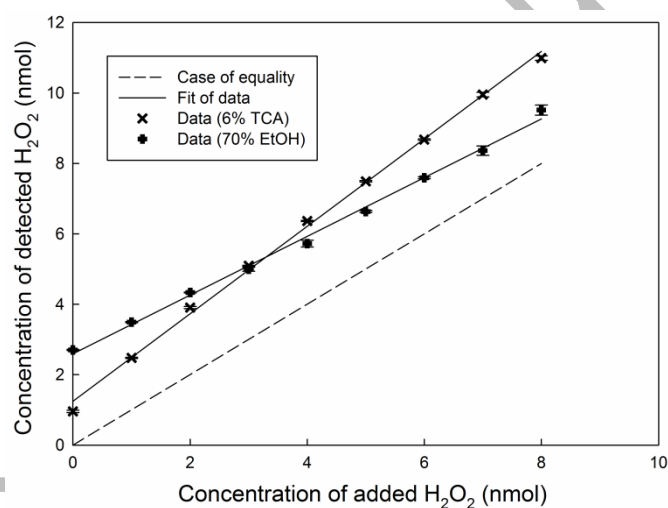


Figure 1.

Detection of exogenous hydrogen peroxide with xylenol orange in 6% (v/v) trichloroacetic acid or 70% (v/v) ethanol based leaf extracts. Data represent the mean (marked with x and + symbols, respectively) and standard deviation of three technical repeats. The dashed line shows the ideal case when the concentration of H₂O₂ detected by the assay would be equal to the concentration added. The solid line corresponds to a linear fit of data characterized by R²=0.998 and R²=0.996 coefficients, respectively.

Electronic supplementary information (ESI) available. Figs S1-S5, Table S1.
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