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Electrochemical detection of extracellular hydrogen peroxide in *Arabidopsis thaliana*: a real-time marker of oxidative stress

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ABSTRACT

An electrochemical approach to directly measure the dynamic process of H_2O_2 release from cultures of *Arabidopsis thaliana* cells is reported. This approach is based on H_2O_2 oxidation on a Pt electrode in conjunction with continuous measurement of sample pH. For $[H_2O_2]$ <1 mM, calibration plots were linear and the amperometric response of the electrode was maximum at pH 6. At higher concentrations ($[H_2O_2]$ >1 mM), the amperometric response can be described by Michaelian-type kinetics and a mathematical expression relating current intensity and pH was obtained to quantitatively determine H_2O_2 concentration. At pH 5.5, the detection limit of the sensor was 3.1 μM (S/N = 3), with a response sensitivity of 0.16 $AM^{-1} cm^{-2}$ and reproducibility was within 6.1% in the range $1–5 \times 10^{-3}$ M (*n* = 5). Cell suspensions under normal physiological conditions had a pH between 5.5–5.7 and H_2O_2 concentrations in the range 7.0–20.5 μM (*n* = 5). The addition of exogenous H_2O_2, as well as other potential stress stimuli, was made to the cells and the change in H_2O_2 concentration was monitored. This real-time quantitative H_2O_2 analysis is a potential marker for the evaluation of oxidative stress in plant cell cultures.

Key-words: amperometric sensor; plant cells; Pt electrode.

INTRODUCTION

In plants, even under optimal conditions, many metabolic processes, including chloroplastic, mitochondrial and plasma membrane-linked electron transport systems, produce reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl free radical ($HO^{\bullet}$). Among these, H_2O_2 is the most commonly studied in cellular environments due to its stability and penetrability through cellular membranes (Wu *et al*. 2011a). It is an important, naturally occurring ROS that has been gaining recognition in biological sciences as a subcellular signalling molecule (Rhee 2006).

Intra- and intercellular levels of H_2O_2 increase during environmental stresses such as intense light, temperature, desiccation, salt, heavy metal contamination, air pollutants, herbicides, etc. Its accumulation can lead to oxidative stress, characterized by disruption of cellular homeostasis, which triggers cell death. However, H_2O_2 not only leads to cellular damage, but it has also been shown to induce a defensive response during both biotic and abiotic stresses at moderately elevated levels (Dat *et al*. 2000). Thus, a fine-tuned balance between H_2O_2 production and its removal is crucial for plant cell survival. Major H_2O_2-scavenging enzymes of plants include ascorbate peroxidase, catalase, glutathione peroxidase and peroxiredoxin, that together with antioxidant compounds, provide cells with highly efficient machinery for detoxifying H_2O_2 (Mittler *et al*. 2004). In addition, this detoxification is also carried out by the glutathione-ascorbate (GHS-ASC) cycle, which is a complex network of spontaneous, photochemical and enzymatic reactions (Noctor & Foyer 1998; Valero *et al*. 2009). Bearing in mind the great importance of H_2O_2 in living organisms, accurate and sensitive analytical methods to quantify this metabolite are required. Traditionally, H_2O_2 has been detected using several analytical techniques, such as titrimetry (Hurdis & Romeyn 1954), spectrophotometry (Gay, Collins & Gebicki 1999) and chemiluminiscence (Hanaoka, Lin & Yamada 2001). However, these approaches are based on indirect methods that have been shown to be time consuming, difficult to automate and highly prone to interferences. Furthermore, dyes are also widely used to determine H_2O_2 (Rhee *et al*. 2010) although there are some issues and concerns around their use. Veljovic-Jovanonic, Noctor & Foyer (2002) and Queval *et al*. (2008) have very clearly discussed some of the difficulties in accurately measuring H_2O_2 in leaf extracts.

Recently, electrochemical methods have become the techniques of choice because of their low cost, excellent analytical characteristics, simple operation and speed of use (Guascito *et al*. 2008). Among these methods, amperometric techniques have increasingly become more attractive in biological applications because they possess excellent temporal resolution, allowing information about the production and consumption of H_2O_2 in real time (Amatore *et al*. 2008). Most amperometric techniques rely on enzyme-based biosensors that usually depend on the enzymatic reduction of H_2O_2 using peroxidases (Wu *et al*. 2011b) or haemoglobin (Wei *et al*. 2011). One drawback of these biological sensors is a lack of stability originating mainly from the H_2O_2-induced suicide

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inactivation of the protein (González-Sánchez *et al*. 2011a,b) and the special requirements of temperature, concentration and pH. To solve these problems, considerable attention has been paid to developing non-enzymatic electrodes. Many metals such as Pt (Zhang & Wilson 1993), Ag (Flätgen *et al*. 1999) and Pd (Cao *et al*. 2008) have been employed as catalysts for H_2O_2 reactions. Recently, the enhanced electrochemical reduction of H_2O_2 at Ag electrodes modified with surfactants and salt has been reported as a very promising tool in the development of new non-enzymatic sensors (González-Macia *et al*. 2011).

A sensor – particularly one that is developed for the purpose of long-term *in vivo* measurements – requires that the sensor material be robust. Pt is certainly one of the best choices because of its physical stability, inertness and the excellent reproducibility of its redox potentials (Zhang & Wilson 1993). For all these advantages, the Pt electrode was selected here to directly quantify the extracellular H_2O_2 in plant cell suspensions. Xu *et al*. (2010) reported an electrochemical method using poly-*o*-phenylenediamine and a Pt microparticle-modified Pt electrode as a sensor for the *in vivo* detection of oxidative burst induced by Cd^{2+} stress in oilseed rape. However, a detailed pH-dependent quantitative analysis was not performed.

Therefore, our main goal was to use a simple, rapid, direct and sensitive electrochemical method based on a Pt electrode for the quantitative determination of H_2O_2 in plant cell suspensions using *Arabidopsis thaliana* as a model system. This main objective was addressed as follows: (1) to measure the concentration of extracellular H_2O_2 in *A. thaliana* cell suspensions under normal physiological conditions; (2) to evaluate the response of cells against exogenously added H_2O_2 and (3) to quantify the endogenous H_2O_2 produced while cells are subjected to different stress conditions. This evaluation of H_2O_2 content in the plant extracellular media can be an important oxidative burst marker in plant cell cultures.

MATERIALS AND METHODS

Chemical and buffers

Hydrogen peroxide, Murashige and Skoog Basal Medium, sucrose, 1-naphthaleneacetic acid, kinetin (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), ethylendiamino-tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), Triton X-100, protease inhibitor cocktail (for plant cells and tissue extracts, DMSO solution), albumin from bovine serum, Bradford reagent, catalase from bovine liver (2150 units per mg protein), horseradish peroxidase (167 units per mg protein), superoxide dismutase (3780 units per mg protein) from bovine erythrocytes, polygalacturonic acid, 3-amino-1,2,4-triazole and sulphates (aluminium, copper, ferrous, cadmium, zinc) were obtained from Sigma Aldrich (UK). A commercial insecticide containing thiacloprid as active component (0.15 g L^{-1}) and a fertilizer (composition: 10.6% N, 4.4% total P_2O_5, 1.9% P, 1.7% K_2O, 1.4% K) were purchased locally.

A. thaliana cell cultures

Cell suspension cultures of *A. thaliana* were maintained as previously described (Clark *et al*. 2000). Cells were subcultured by transferring 5 mL of a 10-day-old culture into 100 mL of fresh AT3 medium (Murashige and Skoog medium supplemented with 3% (w/v) sucrose, 0.5 mg L^{-1} 1-naphthaleneacetic acid and 0.05 mg L^{-1} kinetin, pH 5.5). Cell cultures were incubated at 20 °C on a rotary shaker under 16/8 h light/dark regime. For the experiments, 7-day-old *A. thaliana* cell suspensions were used. The cell density was determined microscopically using a counting chamber.

To determine total protein content in cell suspensions, cultures were frozen in liquid nitrogen, then ground in a mortar and pestle in extraction buffer. The extraction buffer consisted of 20 mM HEPES (pH 7.7), 1 mM EDTA, 0.4% (w/v) SDS, 0.2% Triton X-100 and a complete protease inhibitor cocktail (Sigma Aldrich). The homogenate was centrifuged at 12 000*g* for 20 min, the supernatant removed and frozen in liquid nitrogen. All the samples were stored at −80 °C and protein concentrations were determined using the Bradford assay (Bradford 1976).

Electrochemical apparatus

Electrochemical measurements were carried out in aerobic conditions using an Autolab potentiostat Model PGSTAT302 N (Eco Chemie b.V., Utrecht, the Netherlands). The system was run by PC via the General Purpose Electrochemical System software for Windows, version NOVA (Eco Chemie b.V.). All electrochemical measurements were performed in a three-electrode system with a Pt electrode as working electrode, an Ag/AgCl (3 M KCl) electrode as reference and a Pt mesh as counter electrode. The pH was controlled with a Hanna Instruments HI 9321 Microprocessor pH-meter (Hanna Instruments Ltd, Bedforshire, UK).

Electrochemical measurements

The Pt working electrode was polished with 0.3 and 0.05 μm alumina slurry paste and any residual abrasive particles were removed by subsequent sonication in 70% (v/v) ethanol and water. The electroactive area of the working electrode used here was 0.0163 cm^2, obtained from Randles-Sevcik relationship. It was calculated by cyclic voltammetry using 2.25 mM ferricyanide in 0.1 M KCl at different scan rates.

For electrochemical measurements of *A. thaliana*, 10 mL of cell suspension culture without any treatment were placed in the electrochemical cell. The cell suspension culture was stirred slowly and continuously. Electrochemical detection of H_2O_2 was performed by chronoamperometry, recording the current under a constant potential of +0.6 V versus Ag/AgCl. The continuous monitoring of pH was performed in all the measurements.

Calibration of the H_2O_2 sensor

Calibration plots were made by successive additions of small volumes of the standard aqueous H_2O_2 solutions (stock

solutions prepared daily) to stirred AT3 medium at the indicated concentrations, recording the current under a constant potential of +0.6 V. The usage of AT3 medium has been made to minimize possible interferences which its components could exert on the current signal. The pH was adjusted using NaOH or HCl. Continuous monitoring of pH during calibration was performed and no appreciable variations were observed after the H_2O_2 additions.

RESULTS AND DISCUSSION

H_2O_2 oxidation at the Pt electrode

It is well known that Pt is able to catalyse the electrochemical oxidation of H_2O_2, which is a two-electron irreversible process involving both kinetic and mass transfer components (Zhang & Wilson 1993). The Pt electrocatalytic characteristics for the oxidation of H_2O_2 in AT3 medium were evaluated at pH 5.5. Figure 1 shows the cyclic voltammograms obtained when the Pt electrode was introduced into AT3, both in the absence and presence of H_2O_2. It can be seen that the AT3 medium did not exhibit the presence of any interfering redox process (curve *b*). The addition of H_2O_2 (curves *c* and *d*) gave rise to an anodic response on the bare Pt electrode at potentials greater than +0.4 V, which resulted from the oxidation of H_2O_2 versus Ag/AgCl in AT3 as indicated in Eqn 1:

$$H_2O_2 \xrightarrow{Pt} O_2 + 2H^+ + 2e^- \qquad (1)$$

In addition, a cathodic peak between 0 and 0.2 V was also observed in curves *b*, *c* and *d*. This peak is associated with the reduction of O_2 on the Pt electrode, as it was absent in cyclic voltammograms performed under anoxic conditions (curve *a*). This peak increased with H_2O_2 concentration (curves *c* and *d*) as a consequence of the spontaneous partial decomposition of H_2O_2.

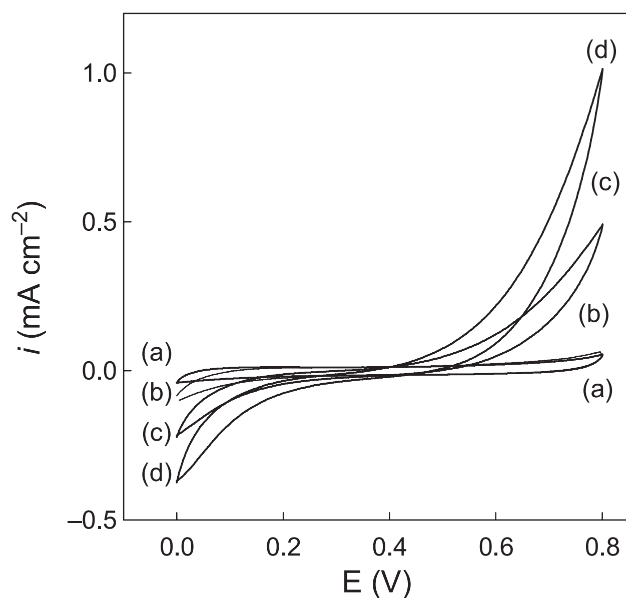

Figure 1. Cyclic voltammograms of the bare Pt electrode at pH 5.5: (a) AT3 deaerated in argon for 10 min; (b) AT3; (c) AT3 containing 2 mM H_2O_2 and (d) AT3 containing 5 mM H_2O_2.

Calibration of the Pt sensor at different pH values

The Pt electrode was applied to the measurement of extracellular H_2O_2 concentrations in *A. thaliana* cell culture suspensions. In cells, unlike the symplast, which is relatively well buffered, homeostasis in the apoplast and extracellular medium is easily perturbed by stimuli or stresses (Pignocchi & Foyer 2003). Extracellular alkalinization has been reported as one of the earliest responses against oxidative burst (Bolwell *et al.* 2002), as a result of the chemical processes on the outer surface of the plasmalemma (Pignocchi & Foyer 2003). Therefore, a possible pH change in the extracellular medium in cell suspensions has to be expected. To avoid this, pH change ionophores and strong buffers could be used. However, these substantially inhibit the oxidative burst (Bolwell *et al.* 2002), modifying the amount of H_2O_2 released by cells. To prevent this effect, measurements in cell suspensions should be made without any pH adjustment. As protons are involved in the H_2O_2 oxidation reaction by the Pt electrode (Eqn 1), there is a strong dependence of both potential and current on the pH of the solution (Zhang & Wilson 1993). Therefore, calibration plots at different pH values should be performed.

Previous studies have showed that when H_2O_2 concentration is low (<1 mM), the reaction indicated in Eqn 1 is the predominant process at the electrode surface (Zhang & Wilson 1993). The situation changes as the H_2O_2 concentration increases (>1 mM), since the decomposition of H_2O_2 is significant and the surface adsorption of either H_2O_2 or O_2 leads to saturation. Our results (*vide infra*) showed a current response with different behaviour depending on the H_2O_2 concentration range studied. Therefore, different calibration plots were constructed for low and high H_2O_2 concentrations at different pH values to allow the quantitative analysis of H_2O_2.

Calibration of the Pt sensor at low H_2O_2 concentrations (<1 mM)

The application of the Pt sensor in AT3 medium for the direct detection of H_2O_2 at low concentrations was assessed. The H_2O_2 concentration-dependent current at different pHs (in the range of 5 to 7) for $[H_2O_2]$ <1 mM is shown in Fig. 2a. The relationship between H_2O_2 concentration and current showed a linear dependence at any pH, but with different response sensitivity. Sensitivity values could be calculated from the slope of the linear plots at the different pH values tested (R^2 = 0.9976 to 0.9996). The data obtained can be seen in Fig. 2b. The maximum sensitivity was obtained at pH 6.0. These results allow the calculation of H_2O_2 concentration at low levels at a known pH, directly from amperometric data by interpolating into the response profile from Fig. 2b. For

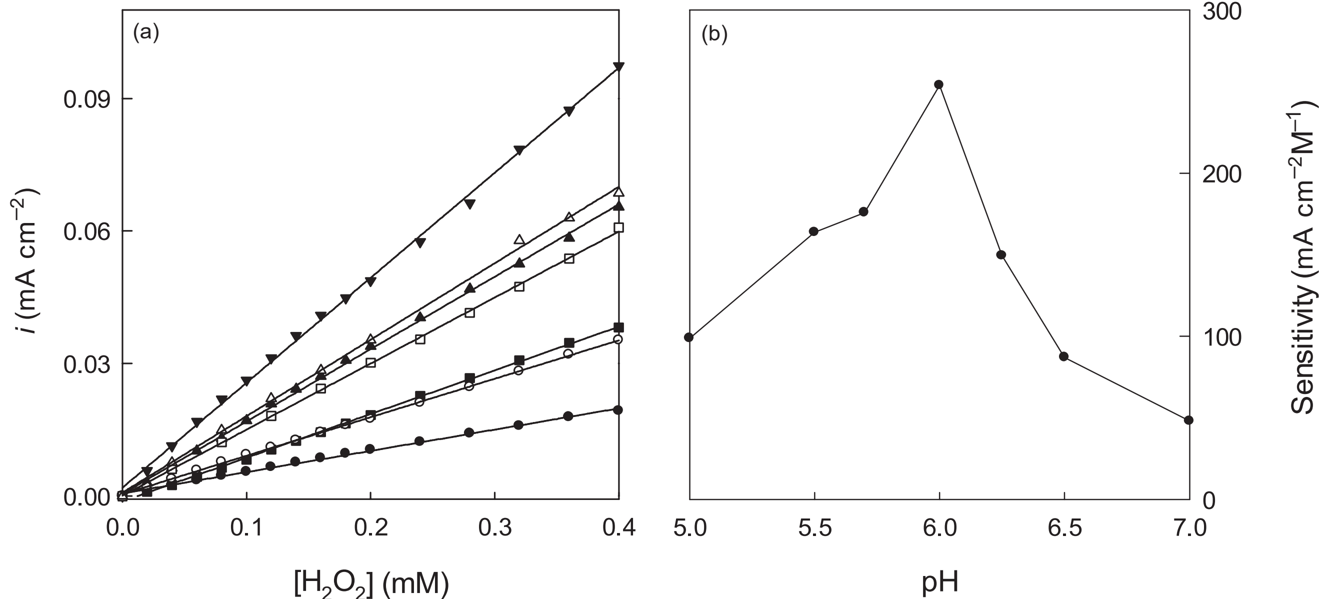

Figure 2. (a) Amperometric response of the Pt electrode in stirred AT3 at low H_2O_2 concentrations (+0.6 V versus Ag/AgCl) at different pH values: 5.00 (■), 5.50 (▲), 5.75 (△), 6.00 (▼), 6.25 (□), 6.50 (○) and 7.00 (●). (b) Dependence of sensitivity to pH.

example, at pH 5.5, the detection limit was 3.1 μM (S/N = 3) and the sensitivity was 0.16 AM^{-1} cm^{-2}.

Calibration of the Pt sensor at high H_2O_2 concentrations (>1 mM)

The H_2O_2 concentration-dependent current at pH from 5 to 7 was also studied in AT3 medium for $[H_2O_2]$ >1 mM (Fig. 3a). As can be seen, in the range of concentrations here studied, the amperometric response increased as the pH was increased, which is in agreement with the results obtained by other authors (Zhang & Wilson 1993). When comparing these data with calibration plots obtained at lower H_2O_2 concentrations, it can be seen that current response shows a different relationship to pH, since at low concentrations, sensitivity peaks at approximately pH 6, whereas at high concentrations, sensitivity increased across the range of pH from 5 to 7. This fact may be due to the different kinetic mechanism of H_2O_2 oxidation at the bare Pt electrode depending on the H_2O_2 concentration range under study. Reproducibility obtained was within 6.1% in the range $1–5 \times 10^{-3}$ M (pH = 5.5, $n = 5$).

In addition, a deviation from linearity was observed at $[H_2O_2]$ >1 mM at all pH values studied (Fig. 3a). Hall, Khudaish & Hart (1998) reported that the current response due to H_2O_2 oxidation is under mixed kinetic and diffusion control and that the mechanism for the oxidation of H_2O_2 can be explained in terms of Michaelian-type kinetics. Therefore, data from Fig. 3a were fitted to the hyperbolic-type equation (Eqn 2):

$$i = \frac{a[H_2O_2]}{b + [H_2O_2]} \quad (2)$$

and very good regression coefficients were thus obtained ($R^2 = 0.9894$ to 0.9931). To obtain an expression relating the current signal (i), H_2O_2 concentration and pH, the coefficients a and b were plotted as a function of pH (Fig. 3b). These data were fitted by non-linear regression analysis to different equations and the best statistical results were obtained for a second-order polynomial and a uni-exponential decay equation, respectively, as follows:

$$a = a_0 + a_1\text{pH} + a_2\text{pH}^2 \quad (3)$$

$$b = b_0 + b_1 e^{-b_2\text{pH}} \quad (4)$$

where a_0, a_1, a_2, b_0, b_1 and b_2 are the parameters obtained from the statistical analysis (Table 1), obtaining R^2 values of 0.9848 and 0.9930 for Eqns 3 and 4, respectively.

Inserting Eqns 3 and 4 into Eqn 2 and solving for $[H_2O_2]$, the mathematical expression in Eqn 5 was obtained, which allows the quantification of H_2O_2 concentrations once the current (i) and the extracellular pH are known:

$$[H_2O_2] = \frac{(b_0 + b_1 \exp(-b_2\text{pH}))i}{-i + a_0 + a_1\text{pH} + a_2\text{pH}^2} \quad (5)$$

The simultaneous measurement of pH and i is possible with a normal potentiostat since most of them offer the possibility to include the integration of a pH electrode.

The selectivity and interference characteristics

The apoplast matrix of cells contains many enzymatic and non-enzymatic components, including solutes, proteins and

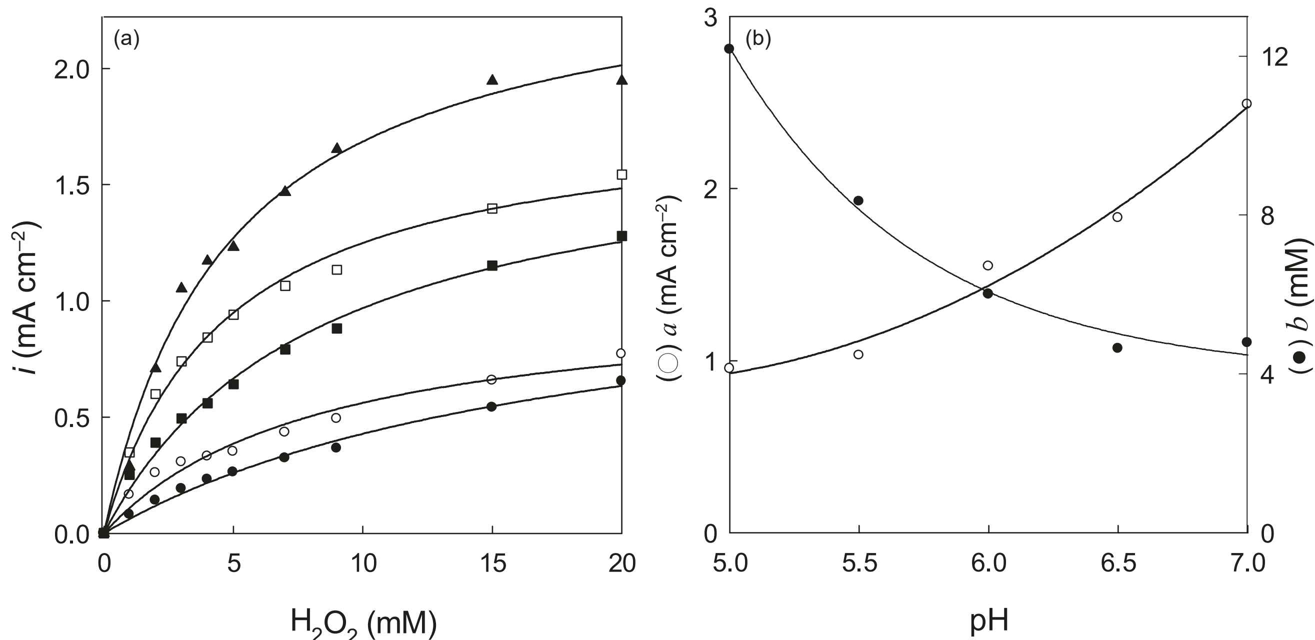

Figure 3. (a) Amperometric response of the Pt sensor in stirred AT3 at H_2O_2 >1 mM at different pH values: 5.0 (●), 5.5 (○), 6.0 (■), 6.5 (□) and 7.0 (▲) (+0.6 V versus Ag/AgCl). The points correspond to experimental data while the lines correspond to data obtained by non-linear regression analysis to Eqn 2. (b) Dependence of parameters *a* (○) and *b* (●) from Eqn 2 upon pH. The points correspond to parameters *a* and *b* obtained from Eqn 2 while the lines correspond to data obtained by non-linear regression analysis to Eqns 3 and 4, respectively.

cell wall constituents (Pignocchi & Foyer 2003). In order to better test the applicability of this electrochemical sensor, interferences from some substances that could usually be found in the apoplast and secreted into the culture medium were examined by amperometry. These were glucose (100 μM), urea (100 μM), mannitol (100 μM), citric acid (100 μM), dehydroascorbic acid (100 μM), quercetin (20 μM), catalase (25 μg mL^{-1}), peroxidase (25 μg mL^{-1}) and superoxide dismutase (10 μg mL^{-1}). Only dehydroascorbic acid produced a mild increase of *i*, which compared to the signal obtained for 100 μM H_2O_2 could be considered negligible (3.7%, data not shown). It is important to point out that the present method has been developed for measuring H_2O_2 in extracellular media, without disruption of cell walls, so less interferences might be expected when using viable cell cultures.

To determine if the current signal obtained was only due to the presence of H_2O_2 in the media, catalase was added to the cell cultures (Fig. 4). Plant suspension cultures treated with this enzyme (curve *b*) showed a current signal similar than that obtained for cell-free AT3 medium (curve *a*). Curve *c* shows the current response of unstressed cells. These results suggest that any effect due to other chemical species in the extracellular medium was negligible and that H_2O_2 was the major species being detected. Thus, a quantitative determination of H_2O_2 could be performed.

Table 1. Values of the parameters from Eqns 3 and 4

Parameter	Value obtained
a_0	6.31 mA cm^{-2}
a_1	–2.39 mA cm^{-2}
a_2	26.40 mA cm^{-2}
b_0	3.93 mM
b_1	7.35 mM
b_2	1.36

H_2O_2 concentration in unstressed cells

The present method is able to measure a steady-state H_2O_2 concentration, being the overall result of the production and removal of this compound by different cell pathways. Thus, this electrode is useful for measuring real-time steady-state levels, which is very important. A continuous and quantitative measurement of H_2O_2 concentration in *A. thaliana* cell cultures (7-day-old, with a cell density of c. 3.4×10^6 cells per mL) has been carried out here using calibration data previously determined for low H_2O_2 concentrations (Fig. 2). Firstly, the Pt sensor was used to evaluate the amount of extracellular H_2O_2 produced by plant cells without any stress stimuli. The results showed that all cellular suspensions under normal physiological conditions had pH in the range 5.5–5.7 and H_2O_2 concentrations in the range of 7.0–20.5 μM ($n = 5$).

Addition of exogenous H_2O_2 to plant cells

It is known that plants are able to tolerate much higher intracellular H_2O_2 concentration than other organisms

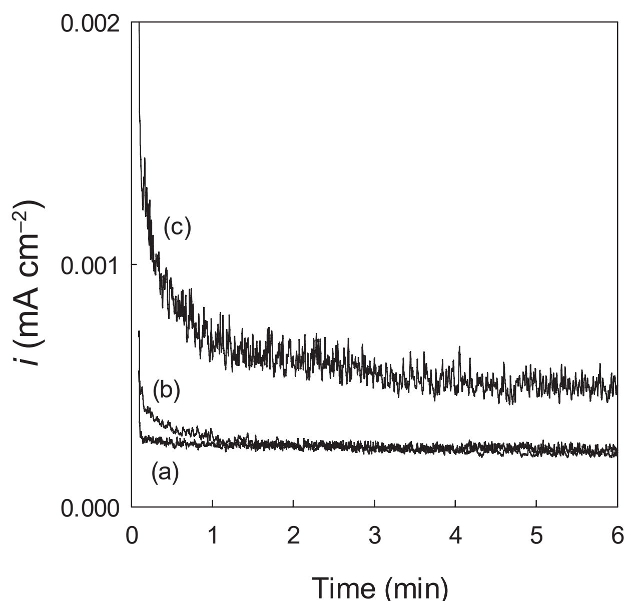

Figure 4. Effect of catalase on the current response of *Arabidopsis thaliana* cell cultures. Amperometric signal of: (a) cell-free AT3 medium, (b) *Arabidopsis thaliana* cell cultures 15 min after the incubation with 0.5 mg mL^{-1} catalase and (c) *Arabidopsis thaliana* cell cultures without any treatment.

(Queval *et al*. 2008). The scavenging of extracellular H_2O_2 is largely controlled by diffusion (Bienert, Schjoerring & Jahn 2006). In addition, enzymes of the apoplast such as peroxidases and catalases protect the plasmalemma from oxidative damage by H_2O_2, decreasing its concentration. Plant cells have a remarkable ability to metabolize H_2O_2, and they can completely consume H_2O_2 concentrations as high as 10 mM in less than 10 min (Levine *et al*. 1994). However, it has also been reported that a 60 min exposure to $[H_2O_2] \geq 5$ mM is sufficient to initiate an irreversible commitment to cell death in *A. thaliana* suspension cultures (Desikan *et al*. 1998).

A. thaliana cell suspensions were used to study the real-time behaviour of cells after H_2O_2 addition. To directly study the consumption of H_2O_2 by the cells, 2 mM H_2O_2 was added to the *A. thaliana* cellular suspensions and current (at +0.6 V versus Ag/AgCl) was monitored. Figure 5a shows the current response and the H_2O_2 concentration variation calculated using Eqn 5, following the addition of H_2O_2. The pH of the medium became progressively more alkaline (data not shown) in agreement with that previously reported (Bolwell *et al*. 2002). The initial bolus of H_2O_2 added was consumed in 7.7–9.8 min ($n = 3$). This consumption of H_2O_2 was corroborated by cyclic voltammetric analysis of the plant cells before and after H_2O_2 addition (inset of Fig. 5a, solid and dashed line). As can be seen (inset of Fig 5a, dotted line), cells subjected to boiling showed no H_2O_2 consumption owing to enzyme denaturation.

Moreover, in cells that were previously incubated with catalase (0.5 mg mL^{-1}, incubation time = 15 min) the same

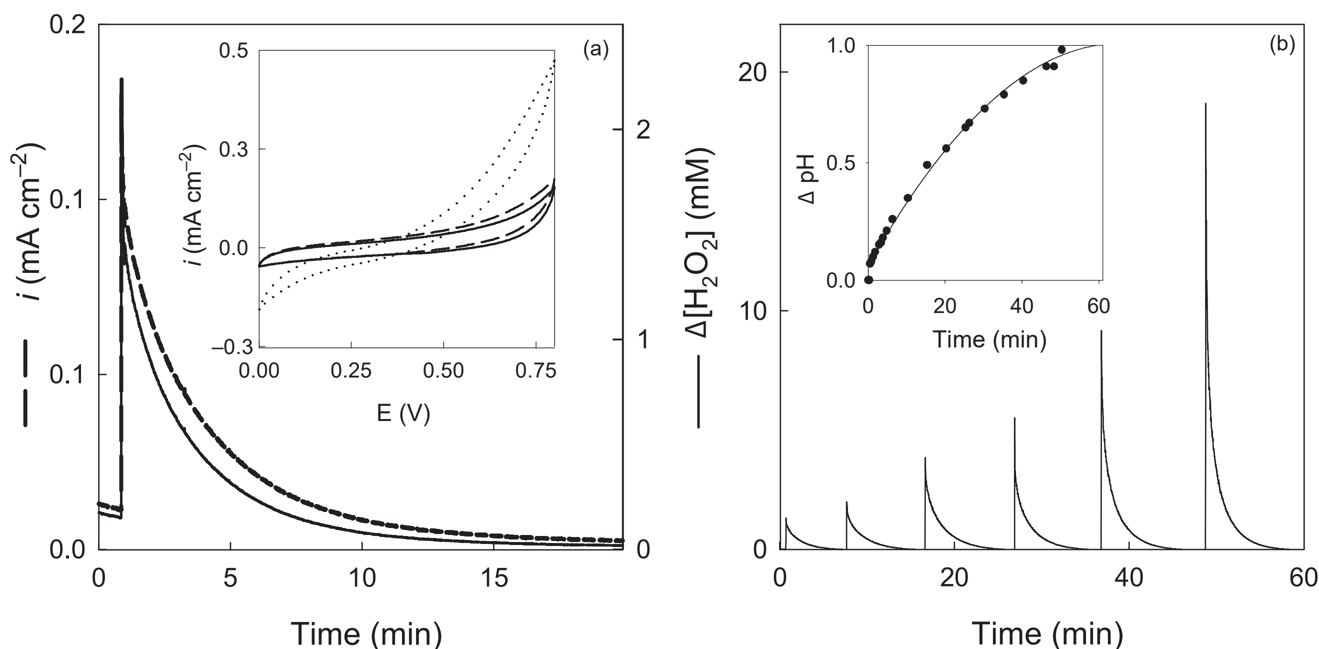

Figure 5. (a) Current intensity (dashed line) and $[H_2O_2]$ variation calculated using Eqn 5 (solid line) upon addition of 2 mM H_2O_2 to *Arabidopsis thaliana* cells. Current intensity was measured using a potential of +0.6V versus Ag/AgCl by amperometry. $[H_2O_2]$ was calculated according to Eqn 5. Inset: Cyclic voltammograms of cells without H_2O_2 exogenous addition (solid line), cells 20 min after the addition of 2 mM H_2O_2 (dashed line) and boiled cells 20 min after the addition of 2 mM H_2O_2 (dotted line). (b) H_2O_2 concentration calculated using Eqn 5 upon successive additions of H_2O_2: 1, 2, 5, 10, 15 and 20 mM (from left to right). Inset: pH variation of the extracellular medium during H_2O_2 additions.

quantity of H_2O_2 (2 mM) was depleted in less than 3 min, whereas in the presence of the catalase inhibitor 3-amino-1,2,4-triazole (2 mM, incubation time = 12 h, dark conditions), H_2O_2 consumption took almost 3 h (data not shown). The pH increased in all cases.

In order to extend the study of cell behaviour with respect to H_2O_2 consumption, the ability of cell cultures to eliminate H_2O_2 upon successive additions was also evaluated (Fig. 5b). As can be seen, H_2O_2 depletion in cells was very rapid due to the significant ability of the antioxidant defence system of plant cellular metabolism to respond to elevated H_2O_2 levels. Even in the case of the addition of 20 mM H_2O_2, this was eliminated by the cells in less than 10 min. The use of the bare Pt electrode allowed the effective monitoring of the H_2O_2 elimination by the cells in real time, without any sample treatment. The inset of Fig. 5b shows the increase of the extracellular pH during successive H_2O_2 additions.

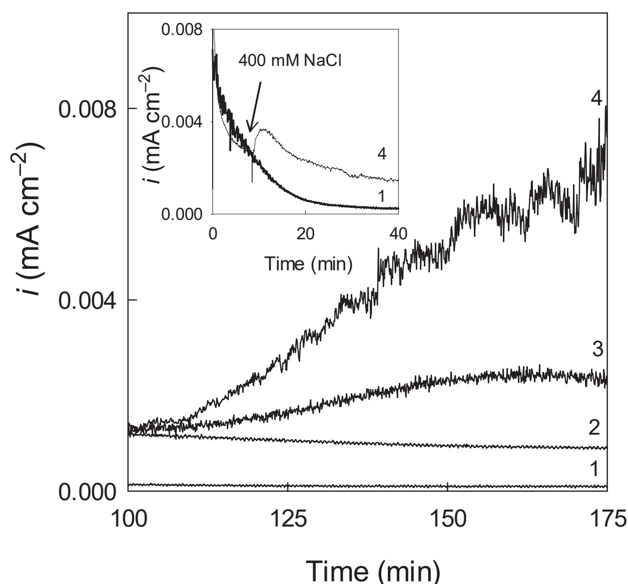

Figure 6. Effect of NaCl treatment on the current response in *Arabidopsis thaliana* after adding 0, 100 mM and 400 mM NaCl for curves 2, 3 and 4, respectively. 400 mM NaCl was added to cell-free AT3 medium as a control, to assess the effect of the addition of NaCl on the electrochemical signal (curve 1). Inset: Addition of 400 mM NaCl to AT3 medium (1) and cells (4) at short times.

Production of endogenous H_2O_2

Hydrogen peroxide has also been shown to be an intercellular signal mediating systemic acquired resistance (Alvarez *et al*. 1998) and systemic acquired acclimation (Karpinski *et al*. 1999). Extracellular levels of H_2O_2 can increase during environmental stresses such as drought, excessive solar radiation, high temperature and in response to other exogenous factors (Miller *et al*. 2010). These factors include different environmental pollutants such as heavy metals, pesticides and salts, which are found in agricultural, urban and industrial wastes. A quantification of extracellular H_2O_2 production by cells during an oxidative burst could be very important. The performance of the sensor for the measurement of steady-state H_2O_2 produced by cells under different possible abiotic stresses was evaluated. For that purpose, several known chemical stimuli were assessed.

Salt stress

It is well known that NADPH oxidase homologues are activated under salt stress (Foreman *et al*. 2003; Ma *et al*. 2011) increasing the concentration of $O_2^{\bullet-}$, whose spontaneous dismutation produces accumulation of H_2O_2. Both $O_2^{\bullet-}$ and excessive H_2O_2 production can damage cell macromolecules including lipids, proteins and nucleic acids (Halliwell & Gutteridge 1986). The effect of salinity on the amount of H_2O_2 generated from *A. thaliana* cells can also be evaluated using the present electroanalytical method. Changes in the oxidative status were monitored by amperometry in real time. Figure 6 shows the results obtained when cells were exposed to 100 mM and 400 mM NaCl (curves 3 and 4, respectively). In the absence of exogenous NaCl, H_2O_2 levels remained relatively constant (curve 2). However, an increase of the current response was observed at about 2 h after the addition of the salt (curves 3 and 4). pH values were found to be 6.25 and 6.50 for cells exposed to 100 mM and 400 mM NaCl, respectively, in contrast to pH 5.5 in unstressed cells. The increases in H_2O_2 concentration (calculated using sensitivity data from Fig. 2b) generated by the cells were 19.7 and 41.43 μM, respectively. The inset in Fig. 6 shows that the addition of NaCl also produced a small, but rapid increase in current intensity immediately upon addition of NaCl (curve 4), which was not observed in cell-free AT3 medium (curve 1), probably due to the immediate production of H_2O_2 by the cells after salt addition, which was subsequently reduced by their antioxidant defence system.

Addition of metals

The contamination of water, soil and sediments with toxic metals has been, and will continue to be a major environmental problem that needs to be dealt with. Exposure of plants to metals can result in oxidative stress as indicated by lipid peroxidation, H_2O_2 accumulation and an oxidative burst (Keunen *et al*. 2011). Based on their chemical and physical properties, different molecular mechanisms of heavy metal toxicity have been proposed (Schützendübel & Polle 2001; Keunen *et al*. 2011).

The amperometric measurement of H_2O_2 was also used here to evaluate the dependence of H_2O_2 release from *Arabidopsis thaliana* cells upon addition of some toxic metals. The results obtained are summarized in Table 2. The addition of different metals to AT3 medium in the absence of the cells did not produce any effect on the signal current (data not shown).

Data obtained showed that Fe^{2+} and Cu^{2+} initially (4.5 h) resulted in a decrease of H_2O_2 concentration, but in the case of Fe^{2+}-exposed cells, rising after 24 h. Fe^{2+} and Cu^{2+} have been shown to be significant in terms of oxidative stress as they undergo a Fenton reaction in the presence of H_2O_2.

Chemical compound[a]	4.5 h after addition pH	4.5 h after addition $\Delta[H_2O_2]$ (μM)	24 h after addition pH	24 h after addition $\Delta[H_2O_2]$ (μM)	Total protein ratio (%)[b]
$CuSO_4$	5.91	−8.45	5.97	−10.69	88
$FeSO_4$	6.43	−6.90	6.30	+10.03	74
$ZnSO_4$	5.45	+5.46	5.33	−0.41	100
$Al(SO_4)_3$	5.04	+25.23	6.23	+18.75	94
$CdSO_4$	5.63	+12.02	5.49	+24.47	94
3-amino-1,2,4-triazole	5.53	+3.81	5.33	−3.03	82
Polygalacturonic acid	5.62	+6.78	5.45	+6.48	97
Insecticide	5.71	−10.69	5.40	−9.58	100
Fertilizer	6.15	−3.74	5.45	+4.70	101

[a]Final concentration of all the active compounds in extracellular medium was 1 mM sulphates and 3-amino-1,2,4-triazole, 3 mg L^{-1} thiacloprid (insecticide) and polygalacturonic acid, and 0.21% N, 0.038% P, 0.0285% K (fertilizer). Control cells (without any addition) had approximately pH 5.5 and $[H_2O_2] = 15$ μM. H_2O_2 concentration variations, $\Delta[H_2O_2]$, were calculated as the difference between $[H_2O_2]$ after the addition of each component and $[H_2O_2]$ in control cells.
[b]Total protein ratio was calculated as the ratio between the total protein content after metal exposure and the total protein content in control cells.

Table 2. H_2O_2 concentrations in the medium of *Arabidopsis thaliana* cell suspensions after the addition of metals, an insecticide and a fertilizer

Toxicity and cellular injury via this mechanism is well documented (Halliwell & Gutteridge 1986; Winterbourn 1995). In this reaction, both hydroxyl radicals and higher oxidation states of metals are formed, which are capable of oxidizing a wide range of substrates and causing biological damage. Biological damage was evident as the colour of the cell suspensions changed from green to yellow-brown (data not shown). In addition, the pH became alkaline in both cases.

Al^{3+}, Zn^{2+} and Cd^{2+} all gave rise to an increase in H_2O_2 production by the cells after 4.5 h. In suspensions containing Zn^{2+}, H_2O_2 gradually recovered to a new balance after 24 h, which was attributed to the effect of ROS-scavenging defence system. This did not occur in the case of Al^{+3} and Cd^{2+} probably because the toxicity of these metals is higher and cellular homeostasis could not recuperate. Both of them are considered to be non-essential metals and generate toxic responses in organisms even at low-exposure concentrations (Keunen *et al*. 2011). In plants, it was suggested that H_2O_2 accumulation after Cd^{2+} exposure is produced because of depletion of cellular glutathione and inhibition of antioxidative enzymes (Schützendübel & Polle 2001). Furthermore, recent studies have suggested that Al^{3+} induces programmed cell death in plants by means of ROS generation and a decrease of mitochondrial transmembrane potential caspase-3-like activation (Li & Xing 2011).

In addition, a decrease in *A. thaliana* cell growth was observed when cells were incubated with Fe^{2+}, Cu^{2+}, Cd^{2+} and also Al^{3+}, as evidenced by the protein levels before and after exposure (Table 2, last column). Furthermore, after 24 h, an increase of pH concomitant with the addition of Fe^{2+}, Cu^{2+} and Al^{3+} was produced, whereas the addition of Zn^{2+} produced a decrease in pH.

Addition of other chemical stimuli

Plant cell cultures were treated with other chemical stimuli including polygalacturonic acid, 3-amino-1,2,4-triazole, a commercial insecticide and a fertilizer. The H_2O_2 concentration produced by cells was also evaluated in each case. The addition of these compounds to cell-free AT3 medium did not produce any effect on the current signal (data not shown).

The presence of polygalacturonic acid produced an increase in H_2O_2 concentration and a mild increase of pH in the first 4.5 h (Table 2). A small decrease in cellular growth was also observed. In plants, polygalacturonic acids are major components of cell wall polysaccharides (pectins), which can be released into cells as a consequence of, e.g. pathogen colonization, giving rise to H_2O_2 and superoxide production (Legendre *et al*. 1993).

The presence of 3-amino-1,2,4-triazole led also to an increase of H_2O_2 concentration in the first 4.5 h, which decreased during the next hours (Table 2). This is in agreement with data reported by May & Leaver (1993), who showed a fourfold increase in the level of reduced glutathione in *A. thaliana* cells in the presence of 3-amino-1,2,4-triazole, to combat the oxidative burst. However, cellular damage was also detected by us since the protein content decreased by about 18% with respect to control cells.

The addition of an insecticide containing thiacloprid as an active component decreased H_2O_2 production by cells (Table 2). This class of insecticides has been reported to enhance plant vigour and stress tolerance in some cases, independently of their insecticidal function (Ford *et al*. 2011).

The addition of a common fertilizer also caused a decrease in H_2O_2 concentration in the first hours, but this had increased 24 h after the addition. With respect to pH changes, an increase of pH was observed in both cases after the first 4.5 h, which recovered 24 h after the addition.

CONCLUDING REMARKS

A bare Pt electrode was successfully applied to study the variation of H_2O_2 concentration in *A. thaliana* extracellular

medium. Calibration plots at low H_2O_2 concentrations were linear with a maximum sensitivity at pH 6.0, whereas at higher H_2O_2 levels, the amperometric response was described by a hyperbolic equation and increased with pH. Cell suspensions under normal physiological conditions had a pH between 5.5–5.7 and H_2O_2 concentrations in the range 7.0–20.5 μM ($n = 5$). To demonstrate how this methodology might be used the effects of the addition of H_2O_2, salt, toxic metals and other chemical treatments on *A. thaliana* cell cultures were evaluated, showing that changes in extracellular H_2O_2 production by these cells could be determined.

The protocol described here is a simple and reliable quantitative approach that has the potential to be used for real-time course analysis of H_2O_2 concentrations in plant cell suspensions. Pt electrochemical detection not only allows the real-time detection of H_2O_2 in biological systems, but also can help to study new insights about the role of H_2O_2 in plant physiological processes. This approach could be a first step in the development of future electrodes for *in vivo* monitoring of H_2O_2 in intact plants, especially in apoplastic fluids, and even in animals due to the existence of parallels between them. Furthermore, the method could also be extendable to micro-organisms and isolated organelles.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Evaluation of H_2O_2 content in extracellular media can be an important oxidative burst marker in plant cell cultures. This paper shows an electrochemical method based on a Pt-electrode which can be used for real-time course analysis of H_2O_2 concentrations in plant cell suspensions. The protocol has been satisfactory applied in *Arabidopsis thaliana* cell suspensions and could be a first step in the development of future electrodes for *in vivo* monitoring of H_2O_2.