Quantification of hydrogen peroxide in plant tissues using Amplex Red

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Abstract

Reactive oxygen species (ROS) are by-products of photosynthesis and respiration in plant tissues. Abiotic and biotic stressors also induce the production and temporary accumulation of ROS in plants, including hydrogen peroxide (H₂O₂), whereby they can act as secondary messengers/chemical mediators in plant defense signaling and lead to programmed cell death. H₂O₂ acts as a hub for critical information flow in plants. Despite such key roles in fundamental cellular processes, reliable determination of H₂O₂ levels in plant tissues is hard to achieve. We optimized an Amplex Red-based quantitation method for H₂O₂ estimation from plant tissue lysate. The standard limit of detection and quantitation was determined as 6 and 18 picomol respectively. In this study we also quantified constitutive and/or induced levels of H₂O₂ in three model plants, *Pinus nigra* (Austrian pine), *Oryza sativa* (rice), and *Arabidopsis thaliana*. Overall, assay sensitivity was in the nmol g⁻¹ FW range. Commonly used additives for H₂O₂ extraction such as activated charcoal, ammonium sulfate, perchloric acid, polyvinylpolypyrrolidone, and trichloroacetic acid either degraded H₂O₂ directly or interfered with the Amplex Red assay. We also measured stability of Amplex Red working solution over one month of storage at -80 °C and found it to be significantly stable over time. With appropriate modifications, this optimized method should be applicable to any plant tissue.

Keywords: Hydrogen peroxide; Amplex Red; Analytical method; Fluorescence; ROS

List of abbreviations: ROS: Reactive oxygen species, RFU: Relative fluorescence units (Ex/Em: 560 nm/590 nm), AC: Activated charcoal, AR: Amplex Red, PE: Plant extract, PVPP: Polyvinylpolypyrrolidone, TCA: Trichloroacetic acid, NTCA: Neutralized trichloroacetic acid, PB: Phosphate buffer, DMSO: Dimethyl sulfoxide, HRP: Horseradish peroxidase, LoD: Limit of detection, LoQ: Limit of quantitation, CV: Coefficient of variation, % RSD: Percent relative standard deviation (CV), SE = Standard error of the mean

1 Introduction

Reactive oxygen species (ROS) are mainly byproducts of aerobic metabolism such as respiration and photosynthesis in plants [1-3]. ROS production is compartmentalized in chloroplasts, mitochondria, peroxisomes, and in the apoplast [4]. ROS include highly reactive singlet oxygen ($^1\text{O}_2$), superoxide anion radical ($\text{O}_2^{\cdot-}$), peroxy radical ($\text{RO}_2^{\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{HO}^{\cdot}$). All ROS have relatively short half-lives and are capable of oxidizing various biomolecules including, but not limited to, proteins, lipids, and DNA [5-7]. Production and depletion of ROS in cells are tightly regulated by enzymatic and non-enzymatic processes. Among all ROS, H_2O_2 has been widely studied due to its critical importance as a signaling molecule in response to external stimuli [8, 9] and as a key component of growth and developmental processes [10].

Both biotic and abiotic stress elevate ROS levels in plants [4]. Abiotic stress, such as drought or salt stress, induces cell apoptosis by simultaneously manipulating cellular synthesis and detoxification of ROS. Biotic stressors increase intra-cellular ROS, not only by altering the functionality of cell wall or membrane bound receptors for radical scavenging, but also by activating membrane-associated oxidases to synthesize extracellular superoxide, which is rapidly converted to membrane-permeable cytotoxic H_2O_2 [4, 11, 12]. Much information related to the general roles of H_2O_2 in plants is available; however, understanding the exact functional roles of H_2O_2 and other ROS in plant defense necessitates that low levels of ROS, and in particular H_2O_2 , can be effectively quantified *in planta*.

Excellent reviews and commentaries are available on *in vitro* and *in vivo* estimation of H_2O_2 in plants and animals [13-18]. Among the most widely used methods are luminescence-based [19-

22] and spectrophotometric techniques [23, 24]. However, both can be inadequate at detecting specific ROS [22, 25]. Fluorimetric dye-based detection provides a more robust platform [13-15], with fluorescence enhancement-based methods being more sensitive than fluorescence quenching-based methods [26].

Dichlorofluorescein based dyes have been widely used in previous studies for relative and absolute estimation of ROS [27-29] and homovanillic acid has been used for quantitative estimation of H₂O₂ [30, 31]. Recently, boronate-based dyes, such as Peroxy Crimson and Peroxy Green have been adopted to image H₂O₂ in cellular compartments [32-34]. All of the mentioned assays have advantages and disadvantages. For example several caveats and shortcomings of measuring H₂O₂ using dichlorofluorescein have been discussed in previously published commentaries [35, 36], while homovanillic acid excitation and emission spectra (320 – 450 nm) are close to the near visible region and could be influenced by undesirable matrix effects [29, 37].

Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) has been used for qualitative estimation or *in vivo* imaging of ROS distribution in leaf tissues of plants such as tomato, tobacco, and the fern *Pteris vittata* L. [38-41]. Amplex Red undergoes one electron oxidation and yields the fluorescent product resorufin, a reaction with 1:1 (Amplex Red: H₂O₂) stoichiometry, in which concentration of horseradish peroxidase (a critical component that degrades H₂O₂), light, and pH play important roles [42, 43]. At high H₂O₂ concentrations, resorufin is further oxidized to the non-fluorescent product resazurin [26].

The methods used to extract H₂O₂ from plant tissues vary widely [17, 29, 44] and extraction buffers should be carefully considered because of soluble proteins and interfering substances [29]. Additives such as activated charcoal, polyvinylpolypyrrolidone (PVPP), and trichloroacetic

acid (TCA) have been used in the past, either individually or in combination with one another, to remove interfering substances from plant tissues [21, 24]. However, some of these components, such as activated charcoal, degrade H_2O_2 on contact and should not be used at all.

The main aim of this study was to develop and optimize an Amplex Red-based method for the fluorimetric quantification of H_2O_2 in plant tissues, including a model gymnosperm (Austrian pine, *Pinus nigra* Arnold), a model dicot (*Arabidopsis thaliana* (L.) Heynh.), and a model monocot (rice, *Oryza sativa* L.). Specifically, we determined analytical merit (limit of detection, limit of quantitation, and linear dynamic range), investigated the effects of commonly used extraction additives using H_2O_2 standards; devised a systematic fluorimetric approach to quantify H_2O_2 in plant cell lysate; and performed stability tests on Amplex Red working solution.

2 Materials and methods

An Amplex® Red assay kit (Amplex Red, DMSO, Horseradish peroxidase and phosphate buffer (5X)) was obtained from Life Technologies, Invitrogen, Grand Island, NY USA. Hydrogen peroxide, potassium iodate, potassium iodide, ammonium molybdate, ammonium bicarbonate, potassium carbonate and starch were obtained from Sigma-Aldrich (St. Louis, MO, USA). In all experiments, ultrapure (Milli-Q) water was used (Millipore system, Bedford, MA, USA). The H_2O_2 assay was performed using a Victor² Wallac fluorometer plate reader (Perkin Elmer, Waltham, MA, USA), using 560 ± 10 nm excitation and 590 ± 10 nm emission filters (Omega Optical, VT, USA). Multiplexing was achieved using 96-well Immulon flat-bottom black microfluor plates (Thermoscientific, Waltham, MA, USA). H_2O_2 concentration in commercially available stock solution was measured every six months using conventional iodometric titration to ensure reliability [45].

2.1 Preparation of Amplex Red working solution

Amplex red was prepared as per manufacturer's technical note. In short, 154 µg of Amplex Red solid was dissolved in 60 µL DMSO. Out of this DMSO stock, 50 µL was pipetted out and added to 4.85 mL of 1X phosphate buffer (pH 7.4) and 100 µL horseradish peroxidase (HRP, 10 U/mL), for a final volume of 5 mL (Amplex Red cocktail). Final Amplex Red concentration was 24.94×10^{-6} M (~ 25 µM). We imposed pseudo-first order kinetics for the Amplex Red and H₂O₂ reaction in routine analysis (optimized highest H₂O₂ concentration for standard was 5 µM). To minimize the effect of potentially interfering substances in the plant extracts, we restricted our H₂O₂ or plant extract addition volume to 5 µL/well. Individual components were added to each well of the microfluor plate in the following order: 1X phosphate buffer (pH 7.4) (45 µL), H₂O₂ standard or plant sample (5 µL), and Amplex Red reagent (50 µL), for a total of 100 µL per well. Following addition of all components, the plate was covered using aluminum foil, shaken for 1 min at 450 rpm, and incubated at 30 °C for 30 minutes. For each plate, a blank (containing 50 µL phosphate buffer (pH 7.4) and 50 µL Amplex Red reagent) was run as a negative control.

2.2 Plant material

2.2.1 Pine trees

Accumulation of H₂O₂ was induced in Austrian pine by subjecting trees to a drought treatment. Twenty trees were maintained in a polyhouse on the Ohio State University campus (40°00' N, 083°02' W). The four-year-old saplings were grown in pots of 36 cm (top diameter) × 25 cm (height) in a medium containing pine bark and composted manure (Com-Til) (three parts pine bark to one part Com-Til). The trees were removed from the polyhouse at the beginning of June, 2011 to a greenhouse, placed in a completely randomized design and allowed to acclimate at a daytime temperature of 28 °C (15 hrs) and night temperature of 20 °C (9 hrs) for two weeks.

After two weeks of acclimation, a no water treatment was applied to half of the trees while the other half received 400 mL of water daily. Pre-dawn needle water potential was monitored weekly on three randomly picked needles (old growth) per tree, using a PMS pressure bomb to ensure that the trees were becoming water stressed [46]. Three new shoots per tree were collected after five weeks and needles were removed. The shoots were flash frozen and ground into a powder in a mortar using liquid nitrogen. Powdered samples were stored at -80°C until analysis. Approximately 100 ± 2 mg tissue were used for H_2O_2 extraction.

2.2.2 Rice plants

Oryza sativa ssp. *indica*, variety IR64 and lesion mimic mutant *spotted leaf11* (*spl11*) (GR5717: IR64 background) were utilized in our study. The lesion mimic mutant was shown to be a spontaneous cell death phenotype and has high levels of ROS [47, 48]. The IR64 and GR5717:IR64 background were germinated in 1/2 strength MS medium and the seedlings were transplanted into potting mixture 7 days after sowing. Rice plants were grown in a Conviron growth chamber (Winnipeg, Manitoba, Canada) at a daytime temperature of 26°C with light (3-MHAL, 400 watt; 1-HPSodium, 400 watt) for 11 h and a night temperature of 20°C (13 h), at 80% humidity. Four days post transplanting, 40 ± 3 mg of leaf tissue from two biological replicates of each line, was ground in liquid N_2 using Qiagen TissueLyser (Qiagen Corp., Valencia, CA, USA) with steel balls at 20 Hz frequency and subsequently extracted in phosphate buffer. They were then centrifuged and supernatant was collected for H_2O_2 analysis. Samples were stored at -80°C until analysis.

2.2.3 Arabidopsis plants

Arabidopsis thaliana, specifically wild type Col-0 and an *fls2* mutant were utilized in our study. The *fls2* mutant is a T-DNA insertion that does not express the FLS2 pattern recognition receptor

involved in Arabidopsis innate immune responses. FLS2 is a pattern recognition receptor that produces a ROS burst when activated by an epitope within bacterial flagellin or the synthetic flg22 peptide [49-51]. Our study did not include flg22 peptide. Col-0 and *fls2* mutant were grown in Metro Mix 360 (Sun Gro Horticulture). The plants were grown under short day conditions (8 hr light, 16 hrs dark) with a daytime temperature of 24 °C and a nighttime temperature of 16 °C. When the plants were 4-5 weeks old, two leaves were collected from three plants of each line. Two leaf discs were obtained from each leaf using a size 6 cork borer (11 mm) for a total of twelve leaf discs per plant line. The leaf discs were weighed (66.1 ± 3.1 mg and 67.5 ± 1.8 mg for Col-0 and *fls2* respectively), placed into 1.5 ml micro-centrifuge tubes, and macerated in phosphate buffer (pH 7.4) using sterile polypropylene micro-centrifuge tube sample pestles (USA Scientific, Ocala, FL).

2.3 H₂O₂ extraction and quantification

2.3.1 Effect of common additives on standard H₂O₂

Standard H₂O₂ (10 µM) was extracted using a range of commonly used additives, including activated charcoal (AC), saturated ammonium sulfate, 1M perchloric acid, polyvinylpolypyrrolidone (PVPP), and trichloroacetic acid (TCA) to test stability of H₂O₂ in presence of the additives. All additives used, i.e. 12% AC, 5% TCA, 5% PVPP, were prepared in 1X phosphate buffer (pH 7.4). For TCA, 350 µl of 5% TCA was neutralized with 150 µl of 2.1 M ammonium bicarbonate while for perchloric acid, 400 µL 1M perchloric acid was neutralized with 50 µL of 5M K₂CO₃ and 50 µM phosphate buffer. The standards were prepared by adding H₂O₂ to the buffers, shaking at room temperature for 30 minutes followed by a 5 min centrifugation at 12,000 g to remove the AC or PVPP. Three technical replicates per treatment

were analyzed. From the preliminary results, we identified two top candidates (i) NTCA and (ii) 1X phosphate buffer (pH 7.4, from Amplex Red kit) for further testing.

2.3.2 Baseline responses and critical considerations

Baseline responses were measured to understand the reactivity of the buffers, dye, enzyme and H₂O₂. In a series of experiments we incubated Amplex Red dye (i) with or without H₂O₂ and (ii) in presence or absence of HRP. Similar experiments were performed without the Amplex Red dye, but we did not perform a HRP-only experiment with 1 μ M H₂O₂, since it is unlikely that degradation of H₂O₂ would produce any fluorescence at 590 nm in the absence of a reacting dye. All experiments were performed in two different buffer systems (NTCA and phosphate buffer) and with three technical replicates per treatment.

Extraction of plant tissue in phosphate buffer has been discussed widely in the literature for possible interference caused by dissolved peroxidases and small molecules. To investigate this issue, we compared the effects of plant extracts on Amplex Red oxidation in the presence and absence of HRP. The latter condition showed only a negligible increase in fluorescence (0.33-fold) when droughted pine tissue extracts were mixed with Amplex Red, compared to droughted pine tissue extracts and HRP combined (19.9-fold). From these studies, it became apparent that the unaltered phosphate buffer was the best candidate for H₂O₂ extraction (lowest interference).

2.3.3 Extraction of H₂O₂ from plant tissues using buffer

Powdered shoot tissues from pine and leaf tissues from rice and Arabidopsis were used for H₂O₂ extraction. We extracted plant derived H₂O₂ from 100 mg FW plant mass into 500 μ L phosphate buffer. After addition of phosphate buffer to the powdered samples or maceration of Arabidopsis leaves in the buffer, the suspended tissue was vortexed for 10 seconds and shaken

continuously at room temperature for 30 minutes. Samples were centrifuged at room temperature for five minutes at 12,000 g. Supernatant was transferred to new pre-labeled tubes. The collected supernatant was re-centrifuged for an additional 2 min. Supernatant was further transferred to new tubes and stored on ice until the analysis was conducted on that day. A simplified analytical approach schematic is shown in Figure 1.

All standards were run with three technical replicates on each plate. Stock H₂O₂ standards (2, 20, 50, 100 µM) were prepared in 1X phosphate buffer (pH 7.4). Standard calibration curves were prepared using 5 µL of the stock H₂O₂ standards to yield in-plate H₂O₂ concentrations of 0.1, 1.0, 2.5, and 5 µM, and were accepted with an $R^2 \geq 0.99$. Response from negative control was considered as a true signal and was used as the intercept for each unique calibration curve. To remove potential instrument bias, H₂O₂ from a specific plant samples was quantified against the standard curve associated with that specific plate.

Limit of detection (LoD) and limit of quantitation (LoQ) were calculated using the least square method [52]. A total of three independent experiments were conducted using H₂O₂ plate concentrations of 0.05, 0.1, 1.0, 2.5 and 5 µM (3 technical replicates per level). Statistically calculated LoD was 6 pmol while LoQ was 18 pmol.

2.3.4 Response linearity with plant extract

To assess the efficacy of the method, we added variable volumes (1, 2 and 5 µL, respectively) of the pine shoot tissue extracts obtained from three droughted and three watered trees to Amplex Red cocktail. A linear increase in response with respect to volume was expected and observed (Figure 6).

2.4 Stability of Amplex Red working solution at –80 °C

We conducted a stability test for the Amplex Red working solution over a period of one month. We avoided photo-oxidation of Amplex Red working solution in stability studies by wrapping the storage tube in aluminum foil. Three independent experiments were conducted using freshly prepared standard solutions of H₂O₂ against Amplex Red working solution stored at -80 °C. Six different in-plate concentrations of H₂O₂ standards (0.025, 0.050, 0.100, 1.00, 2.50, 5.00µM) were used. After the first test on day 0, the remaining Amplex Red working solution was stored at –80 °C and was freeze-thawed to be used on the third and thirtieth day of the experiment. A pink coloration in Amplex Red working solution was observed beyond thirty days (indicative of auto-oxidation).

2.5 Statistical analyses

All statistical analyses were conducted using R statistical programming [53]. Univariate statistical analyses were employed to model effect of additives on H₂O₂ extraction (F-test, $\alpha = 0.01$), effect of buffer, HRP, Amplex Red and H₂O₂ (F-test, $\alpha = 0.01$). We also applied univariate statistics to model the treatment or genetic effect on total H₂O₂ (independent t-test, $\alpha = 0.01$) concentrations. Required normality testing methods were employed, homogeneity of variance was verified and Q-Q plots were prepared before performing any univariate statistical methodologies where appropriate. Data visualization was prepared using ‘ggplot2’ package [54] and post-hoc analysis was performed using ‘agricolae’ package in R ($\alpha = 0.01$) [55].

3 Results

3.1 Effect of additives on H₂O₂ estimation in the absence of plant extract

The fluorescence response of Amplex Red to 10 μ M H₂O₂ in the presence of various additives is depicted in Figure 2 and the test statistic is represented in Table 1. All additives either directly or indirectly interfered with H₂O₂ standard estimation. As expected, activated charcoal degraded H₂O₂ significantly. Both ammonium sulfate and perchloric acid produced significantly lower responses in presence of H₂O₂ compared to phosphate buffer and NTCA. PVPP on the other hand, increased resorufin production under no-peroxide condition, hence it was excluded. Due to these effects, only neutralized trichloroacetic acid and phosphate buffer (commercially supplied with Amplex Red kit) were considered for further extraction of H₂O₂ from plant material.

3.2 Baseline response

Baseline responses are critically important in assay optimization. We investigated each individual component in our reaction cocktail to understand how they influence the oxidation of Amplex Red. Statistical analysis showed some main effects and interactions to be significant, however, neither main effect of buffer nor the interaction of Amplex Red, buffer, HRP and peroxide was significant (Table 2 and Figure 3). For the most part, the results showed limited or no change in the control experiments (Figure 3).

Similarly, under no HRP conditions, plant tissue samples extracted in phosphate buffer showed higher RFU than NTCA extracted samples, perhaps because of unknown interactions between Amplex Red and dissolved peroxidases (Figure 4), but the change was minimal compared to addition of HRP (0.33 fold vs. 19.9 fold). Since TCA anion induces oxidative stress in mammalian models, it was eventually excluded from our study, due to its unpredictable

influence on the Amplex Red cocktail [56, 57]. Unfortunately, statistical conclusions cannot be drawn from this experiment because of limited number biological replicates and unbalanced dataset.

3.3 Calibration curves and assay optimization

A robust linear best fit line was observed when Amplex Red was oxidized using 0 to 5 μM H_2O_2 ($R^2 \geq 0.99$) (Figure 5). A coefficient of variation (CV or %RSD) for the slopes from three different days were determined as 6.82%. Increasing the concentration of H_2O_2 to 7.5 and 10 μM showed departure from linearity (Figure 5, inset) ($R^2 = 0.98$). It is apparent that high concentrations of peroxide appear to affect the pseudo-first order kinetics of Amplex Red oxidation and hence should be avoided from the calibration curve. In order to understand the dynamic range of the assay, pine extracts were incrementally added to Amplex Red cocktail. A clear linear trend indicated viability of the assay. Within plant variability was minimal, but inter-plant variability was observed with the drought treatment, as would be expected (Figure 6).

3.4 Quantification of H_2O_2 in plant extracts.

With pine, five weeks of no water treatment resulted in a significant, more than 1.5-fold increase in H_2O_2 in the shoots of droughted trees ($35.5 \pm 3.8 \text{ nmol g}^{-1} \text{ FW}$) compared to the well-watered trees ($21.4 \pm 2.6 \text{ nmol g}^{-1} \text{ FW}$) (Figure 7) [$t_{18} = 3.03$, $p = 0.003$]. The *spl11* lesion mimic mutant of rice showed a 6.5-fold higher H_2O_2 content compared to wild type ($28.8 \pm 0.8 \text{ nmol g}^{-1} \text{ FW}$ and $4.5 \pm 1.1 \text{ nmol g}^{-1} \text{ FW}$, respectively) (Figure 7) [$t_{10} = 17.95$, $p = 3.08 \times 10^{-9}$]. Arabidopsis *fls2* mutant had similar constitutive amounts of H_2O_2 compared to Col-0 ($23.8 \pm 2.2 \text{ nmol g}^{-1} \text{ FW}$ and $30.6 \pm 1.4 \text{ nmol g}^{-1} \text{ FW}$, respectively) [$t_4 = 2.63$, $p = 0.029$] (Figure 7), consistent with the absence of the elicitor of the *fls2* pattern recognition receptor.

3.5 Amplex Red stability experiment

There was no apparent difference in fluorescent product formation with H₂O₂ standards at three different time points per level during the course of 30 days (Figure 8). The dataset violated ANOVA assumptions of normality and heteroscedasticity, even after data transformation. We believe statistics is not necessary because the magnitude of changes are clear and obvious, with minimal variation. Therefore, a simple mean $\pm$ SE representation was used in the visualization of the data. A pink discoloration was observed in the working standard solution beyond the 30 day time period.

4 Discussion

Our study describes analytical procedures based on Amplex Red for *ex vivo* quantification of H₂O₂ in plants. While oxidation of Amplex Red could result from the activities of different ROS, H₂O₂ was specifically targeted in this study by using horseradish peroxidase. We show that this method is capable of quantifying available H₂O₂ at levels as low as 18 pmol. Considering the complexity of the matrix, and the typical H₂O₂ concentrations *in planta* (~low μ M), this value is well within the reasonable limit of quantitation. We could clearly differentiate between plants in which H₂O₂ accumulation is induced experimentally (pine) or plants that are known, or are expected, to produce different or similar amounts of H₂O₂ based on genetic mutations (rice and Arabidopsis, respectively). In a series of experiments we showed the repeatability and robustness of the method and finally, we show that there is no need to prepare fresh Amplex Red solutions for repeated assays over a period of at least 30 days if the stock is shielded from light and preserved at -80°C .

Amplex Red has been used extensively in animal models, either to quantify H₂O₂ from tissues or to study distribution of H₂O₂ within tissue compartments. In plants, this probe has previously been used in a few investigations, mostly to study localization of H₂O₂ in leaf tissues using fluorescence imaging [38-40]. However, imaging H₂O₂ *in vivo*, while being quite useful, cannot provide absolute quantitation of this important molecule. This is a critical aspect, as many studies have made it clear that ROS levels within a cell are of seminal importance for how a plant responds to abiotic stress, such as drought, or interacts with microbes [4, 12, 58].

Due to the diversity of soluble compounds in plant matrices, H₂O₂ levels should be determined with caution [17, 29, 44]. Thus, plant samples are often treated with additives to remove interfering substances such as proteins, chlorophyll, and/or phenolic compounds [17, 21, 24]. Results from our experiments show that additives should be used with great caution when Amplex Red assay is optimized for a specific plant. For example, AC degrades or otherwise removes H₂O₂ and should be avoided altogether for H₂O₂ extraction from plant samples (Figure 2), as trace amounts of AC were shown to degrade H₂O₂ in water [59, 60]. In absence of H₂O₂, PVPP alone directly oxidized Amplex Red and increased the background signal substantially and hence should not be used. Ammonium sulfate and perchloric acid showed lower response compared to phosphate buffer upon oxidation perhaps due to in-solution interactions with Amplex Red.

Since resorufin formation from Amplex Red is a pH-dependent reaction, we neutralized TCA and prepared a buffer to maintain a pH of 7.4. During the baseline response experiment, under no peroxide condition, NTCA produced similar response compared to phosphate buffer in the presence of HRP, which compelled us to study both NTCA and phosphate buffer with plant extracts (Figure 3). It must be noted here that phosphate buffer is also somewhat limited in the

fact that oxidized Amplex Red response was obtained even when Amplex Red and plant extracts were mixed without HRP. However, this auto-oxidation was very low and measurable, and the pattern could be explained if plant derived peroxidases were extracted in the phosphate buffer and they bound to Amplex Red. Comparing data presented in Figures 3 and 4 we can conclude that extraction of H₂O₂ in phosphate buffer and TCA will produce similar results. We encourage other researchers to perform similar pilot control experiments to estimate the effect of the plant extract matrices on Amplex Red before adapting it to a large-scale analysis. Based on our experience with the Amplex Red assay and survey of existing literature, we excluded NTCA and proceeded with phosphate buffer for H₂O₂ extraction.

Determination of H₂O₂ from plant extracts is quite challenging because of the complexity of the matrix. Aqueous extracts from different plant matrices may contain antioxidants such as ascorbate and glutathione, which interfere with assays in a concentration-dependent manner [17, 61]. Amplex Red undergoes a one-electron oxidation process involving the enzyme HRP, which may also be affected by the matrix components. Figure 4 indicates that the plant extract's matrix components have a minimal effect on Amplex Red. Additionally, to minimize the effective concentration of any interfering material, including antioxidants, we used only 5 µL plant extract in a 100 µL reaction mixture (20-fold dilution). A strong response linearity was observed when different volumes of pine tree extracts were used for quantitation of H₂O₂.

We determined different levels of H₂O₂ in differentially treated pine trees and genetically modified rice plants. A 1.5-fold difference in pine and a 6.5-fold difference in the rice plants were observed. As expected, constitutive levels of H₂O₂ did not differ significantly between Col-0 (wild type) and *fls2* mutant. Notably, the wild type Col-0 plants produce higher levels of H₂O₂

relative to the *fls2* mutant upon infiltration with flg22 peptide, which was not used in our experiments [62].

It has been reported that photooxidation and trace amounts of resorufin in Amplex Red stock solution can contribute to degradation of the dye in storage [43]. However, our stability tests with seven different calibration standards and three technical replicates showed that Amplex Red working solution does not have to be consumed within a day of preparation. In fact, Amplex Red will be stable for at least a month at pH 7.4 if wrapped in aluminum foil and stored in a –80 °C freezer (Figure 8). Visual inspection did not reveal any pink coloration (which is indicative of photooxidation), even though the solution was freeze-thawed at least twice during one month.

5 Conclusions

In this study we investigated the analytical merit of Amplex Red dye and optimized a quantitative fluorimetric approach to determine actual H₂O₂ concentrations in plant tissues. Considering the complexity of the plant extract matrices, we investigated the effect of each individual component during assay optimization. We investigated the efficacy of commonly used additives for H₂O₂ extraction from plants. All of them, except phosphate buffer, interfered either directly with H₂O₂ extraction or indirectly with the Amplex Red assay. We wish to emphasize that control experiments are critical during adaptation of this method. A no-HRP control experiment should be performed early on to estimate the direct effects of the extracted plant matrix on Amplex Red. Overall, our data show that this method is cost-effective, efficient, rigorous and repeatable when used with some important model plants and can contribute significantly to our understanding of H₂O₂ dynamics in plant tissues.

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List of figure captions

Figure 1. Simplified schematic of the analytical approach used for determination of H₂O₂ from three different plant species using the optimized Amplex Red method.

Figure 2. Amplex Red fluorescence in response to presence/absence of 10 μ M H₂O₂ and various additives (mean $\pm$ SE, n = 3 technical replicates). Different letters represent statistically significant differences (Tukey's HSD test, α = 0.01).

Figure 3. Amplex Red oxidation in the presence/absence of HRP and 1 μ M H₂O₂ (mean $\pm$ SE, n = 3 technical replicates). Two buffers were tested, NTCA and phosphate (from the Amplex Red kit). Different letters indicate significantly different levels of response across the treatment combinations (Tukey HSD test, α = 0.01). N.D = Not determined, we do not expect HRP to produce any meaningful response at 590 nm in presence or absence of H₂O₂.

Figure 4. Effects of droughted Austrian pine shoot tissue extract on Amplex Red oxidation in NTCA and PB, in the presence or absence of HRP. For each NTCA experiment, 1 biological replicate (n = 3 technical replicates) was used, whereas 2 biological replicates (n = 3 technical replicates each) were used for the phosphate buffer measurements. Each bar represents mean $\pm$ SE.

Figure 5. A representative calibration curve for the overall assay. Similar calibration curves were used to determine LoD (6 pmol) and LoQ (18 pmol). Each data point in the curve represents mean $\pm$ SE of 3 independent experiments. The error bars are too small to visualize, however, they are inside the circles. Linear regression was highly significant ($R^2 \geq 0.99$). Inset shows a departure from linearity at higher concentrations of H₂O₂ ($R^2 = 0.98$).

Figure 6. Incremental addition of Austrian pine shoot extracts to Amplex Red cocktail. Three trees from droughted and watered category were used, respectively. Each data point represents mean $\pm$ SE of 3 technical replicates.

Figure 7. H₂O₂ quantitation by Amplex Red in a conifer tree (n = 10 biological replicates/treatment), a monocot (n = 2 biological replicates/treatment and 3 technical replicates per biological replicates/treatment), and a dicot (n = 3 biological replicates/treatment). Mean concentrations were compared within species and different letters indicate significantly different means (Student t-test, α = 0.01). Bars are SE of the mean.

Figure 8. Amplex Red working solution stored at -80°C was stable for 30 days. Fresh H₂O₂ stock and seven standard dilutions were prepared for each of the three time points. Blank represents Amplex Red and HRP only (no peroxide). Each bar represents mean $\pm$ SE of 3 technical replicates.

List of table captions

Table 1. Results from the linear model testing the effects of additives and H₂O₂ on oxidized Amplex Red production.

Table 2. ANOVA table showing the effects of Amplex Red, HRP, Buffers and H₂O₂ on Amplex Red oxidation in the control samples. All factors and interactions were significant ($P < 0.01$).

Pine

Rice

Arabidopsis

Day Day1 Day3 Day30

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Additive	5	3154279001	630855800	7273.119	0
Peroxide	1	9875026253	9875026253	113848.904	0
Additive $\times$ Peroxide	5	2293215183	458643037	5287.683	0
Residuals	24	2081712	86738	NA	NA

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Amplex Red	1	98970640	98970640	5586.66	0.0000
HRP	1	132747300	132747300	7493.27	0.0000
Buffer	1	66482	66482	3.75	0.0629
Peroxide	1	141576200	141576200	7991.65	0.0000
Amplex Red $\times$ HRP	1	40267490	40267490	2273.01	0.0000
Amplex Red $\times$ Buffer	1	20585	20585	1.16	0.2903
HRP $\times$ Buffer	1	1373	1373	0.08	0.7828
Amplex Red $\times$ Peroxide	1	49102400	49102400	2771.72	0.0000
HRP $\times$ Peroxide	1	144319800	144319800	8146.52	0.0000
Buffer $\times$ Peroxide	1	146125	146125	8.25	0.0077
Amplex Red $\times$ HRP $\times$ Buffer	1	40334	40334	2.28	0.1425
Amplex Red $\times$ Buffer $\times$ Peroxide	1	16350	16350	0.92	0.3449
HRP $\times$ Buffer $\times$ Peroxide	1	93625	93625	5.28	0.0292
Residuals	28	496035	17716	NA	NA