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### Apoplastic redox metabolism: Synergistic phenolic oxidation and a novel oxidative burst

C. Jacyn Baker

USDA, [jacyn.baker@ars.usda.gov](mailto:jacyn.baker@ars.usda.gov)

Daniel P. Roberts

USDA, [dan.roberts@ars.usda.gov](mailto:dan.roberts@ars.usda.gov)

Norton M. Mock

USDA

Bruce D. Whitaker

USDA

Kenneth L. Deahl

USDA

*See next page for additional authors*

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**Authors**

C. Jacyn Baker, Daniel P. Roberts, Norton M. Mock, Bruce D. Whitaker, Kenneth L. Deahl, and Andrey A. Aver'yanov

## Apoplastic redox metabolism: Synergistic phenolic oxidation and a novel oxidative burst<sup>☆</sup>

C. Jacyn Baker<sup>a,\*</sup>, Daniel P. Roberts<sup>b</sup>, Norton M. Mock<sup>a</sup>, Bruce D. Whitaker<sup>c</sup>,  
Kenneth L. Deahl<sup>d</sup>, Andrey A. Aver'yanov<sup>e</sup>

<sup>a</sup>Molecular Plant Pathology Laboratory, Bldg. 004 Rm. 119, USDA, Beltsville, MA 20705, USA

<sup>b</sup>Sustainable Agricultural Systems Laboratory, USDA, Beltsville, MA 20705, USA

<sup>c</sup>Produce Quality and Safety Laboratory, USDA, Beltsville, MA 20705, USA

<sup>d</sup>Vegetable Laboratory, USDA, Beltsville, MA 20705, USA

<sup>e</sup>Research Institute of Phytopathology, B. Vyazemy, Moscow Region 143050, Russia

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### Abstract

The plant apoplast is an important mediator of communication between the cell cytoplasm and its surroundings. Plant cell suspensions offer a convenient model system to gain insight into apoplastic physiology. Here, we describe a novel phenomenon that took place when two naturally occurring phenolics were added together to either soybean or tobacco cell suspensions. Acetosyringone (AS) and/or hydroxyacetophenone (HAP), phenolics found in the extracellular/apoplast of tobacco cells, were added to soybean or tobacco cell suspensions undergoing an oxidative burst. Individually, AS appeared to be utilized as a typical peroxidase substrate to scavenge hydrogen peroxide, while HAP was utilized at a much lower rate. However, when added together the rate of utilization of both phenolics increased and surprisingly resulted in the production of hydrogen peroxide. We have further characterized this novel phenomenon in suspension cells. This study demonstrates that certain phenolics in plants can cause co-oxidation which, as in animals, could alter the structure and bioactivity of surrounding phenolics.

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**Keywords:** Co-oxidation; Phenolics; *Glycine max*; *Nicotiana tabacum*; *Pseudomonas syringae*; Oxidative burst; Hydrogen peroxide; Apoplast; Redox metabolism; Suspension cells

### 1. Introduction

The apoplast matrix that surrounds the plant cell is a complex and dynamic micro-environment. When outside stresses, such as environmental changes or pathogen attack occur, the micro-environment of the apoplast is likely to be the first line of defense. The cross-linked cell wall polysaccharides provide a backbone to the matrix within

which various proteins, enzymes, metabolites, and inorganic ions are associated. Localized responses to bacterial or fungal attacks often result in structural alterations, such as lignification and papillae formation, involving multiple matrix components including callose, phenolics, and hydroxyproline-rich proteins [1,2], or physiological alterations of the apoplast environment, such as increased pH or production of reactive oxygen species.

Due to the complexity and inaccessibility of the apoplast, examining its rapidly changing stress-related chemistry in vivo remains a challenge. However, analysis of the extracellular/apoplast chemistry of cell suspensions, which mimic stress-related symptoms observed in planta, can provide insight. In our studies of extracellular phenolics during plant/bacterial interactions in suspension cells, we found that qualitative and quantitative changes in

**Abbreviations:** AS, acetosyringone; HAP, 4-hydroxyacetophenone; ROS, reactive oxygen species; HKbac, heat-killed bacteria

<sup>☆</sup>Mention of a trade name, proprietary product, or vendor does not constitute a guarantee of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other vendors that may also be suitable.

\*Corresponding author. Tel.: +1 301 504 5617; fax: +1 301 504 5449.

E-mail address: [bakerc@ba.ars.usda.gov](mailto:bakerc@ba.ars.usda.gov) (C. Jacyn Baker).

phenolic composition were dependent on several factors with the predominant factor being the type of bacteria present [3,4]. When suspension cells were treated with a pathogen that caused an incompatible response and oxidative burst, the concentration of the extracellular phenolics decreased or disappeared as a result of oxidation. We also found that an extracellular phenolic produced by tobacco cells, AS, had a bioactive effect that accelerated the plant/bacterial interaction when added exogenously [5].

In this report, we demonstrate a novel phenomenon that brought new insight to our understanding of apoplastic redox metabolism. In an attempt to protect apoplastic phenolics in soybean suspensions from oxidation prior to their analysis, we added two commercially available plant phenolics, AS and HAP, to serve as antioxidants. Surprisingly, we discovered that the relatively high concentrations of these exogenous phenolics were (1) rapidly oxidized and (2) resulted in a large oxidative burst. We demonstrate that certain phenolics trigger a unique chemistry in the extracellular/apoplast environment of suspension cells that results in a prooxidant environment rather than an antioxidant environment as expected. In addition, we demonstrate that phenolic co-oxidation can occur in the extracellular/apoplast environment of suspension cells. Phenolic co-oxidation is currently an area of intense study and concern in the pharmaceutical industry [6,7] where the co-administration of certain phenolic drugs can cause modification of their structure and bioactivity.

The same principle of phenolic co-oxidation in animals could occur in the plant apoplast, where the introduction of certain phenolics, whether from the cell cytoplasm or invading micro-organisms, could lead to structural and bioactive modification of pre-existing apoplast phenolics. Because this could be insightful for understanding the complexity of apoplastic redox metabolism, we decided to investigate and characterize this phenomenon in suspension cells. The chemical mechanisms involved are also currently being investigated.

## 2. Materials and methods

### 2.1. Chemicals

Horseradish peroxidase (P-8250), soybean peroxidase (P-1432), AS (3,5-dimethoxy-4-HAP, D134406), 4-HAP (278564), and all suspension culture ingredients were purchased from Sigma-Aldrich Chemicals Inc., St. Louis, MO, USA. The peroxidases were purchased and measured in units as described by Sigma; one unit (U) will oxidize 1  $\mu$ mole of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)  $\text{min}^{-1}$ .

### 2.2. Plant material

Soybean (*Glycine max* L. Merr. cv Harosoy) suspension cells were originally isolated from hypocotyl callus and maintained in Gamborg's B-5 medium (Gibco, Grand

Island, NY, USA) augmented with 1  $\text{mg L}^{-1}$  2,4-D, pH 5.0. Suspension cultures of tobacco (*Nicotiana tabacum* L. cultivar Hicks) were derived from pith and maintained on MS media, supplemented with 0.2  $\text{mg L}^{-1}$   $\text{KH}_2\text{PO}_4$ , 0.2  $\text{mg L}^{-1}$  2,4-D and 0.1  $\text{mg L}^{-1}$  kinetin, pH 5.8. Soybean and tobacco cultures were routinely transferred, 10 into 80 mL of fresh media in 250 mL flasks, every 4 days and incubated on a rotary shaker at 150 rpm and 27 °C in the dark.

Suspension cells were washed and suspended in assay buffer, containing 0.5 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{K}_2\text{SO}_4$ , 175 mM mannitol and 0.5 mM MES, pH 6. Then 25 mL of the cell suspensions, 0.05  $\text{g mL}^{-1}$ , were dispensed into 50 mL beakers on a rotary water bath shaker set at 27 °C and 180 rpm to keep cells suspended. Stock solutions, 20 mM, of AS and HAP, were made in assay buffer and were added directly to the cell suspensions using less than 125  $\mu\text{L}$  per beaker to produce final phenolic concentrations ranging up to 100  $\mu\text{M}$ . All experiments were preformed at least twice with two or more replicates per treatment.

### 2.3. Heat-killed bacteria (HKBac) preparation

Cultures of *Pseudomonas syringae* pv. *syringae* 61, isolate WT, were grown for 20 h in Kings B broth, centrifuged, washed, and suspended in deionized water. Based on optical density, the concentration of the suspension was adjusted with water so that addition of about 200  $\mu\text{L}$  of the bacterial suspension to plant cell suspensions resulted in the desired final concentration of about  $10^8$  cfu  $\text{mL}^{-1}$ . The bacterial suspensions were then autoclaved for 15 min and frozen until used.

### 2.4. HPLC-UV quantification of phenolics

One-milliliter samples of cell suspensions were filtered through Miracloth and centrifuged at 12,000g for 5 min prior to HPLC analysis. AS and HAP were separated and quantified using a Onyx monolith C18 analytical column, 100  $\times$  4.6 mm i.d., (Phenomenex, Torrence, CA, USA) with a Waters (Milford, MA) quaternary pump, autosampler, photodiode array detector, and Empower data acquisition on a Dell Pentium 4 computer. Aliquots, 150  $\mu\text{L}$ , of samples were acidified with phosphoric acid (0.1%) and placed in the autosampler using a 10  $\mu\text{L}$  injection volume. An isocratic mobile phase of 30% methanol in 0.01% aqueous phosphoric acid, 2  $\text{mL min}^{-1}$ , separated the phenolics within 4 min. Quantification using peak height was preformed using the UVmax wavelength for each peak, AS, 300 nm, HAP, 276 nm, and calibration with standards.

### 2.5. FOX2 (ferrous oxidation in xylenol orange) assay for hydrogen peroxide

In this spectrophotometric method, ferrous ions are oxidized by hydrogen peroxide to ferric ions, which bind

with xylenol orange to give a colored complex with increased absorbance at 560 nm [8]. The advantage of this technique is that it does not rely on peroxidase which is affected by the exogenous phenolics added during this study. The FOX2 reagent contains 125  $\mu\text{M}$  xylenol orange, 250  $\mu\text{M}$  ammonium ferrous sulfate, 4 mM butylated hydroxytoluene in 90% methanol containing 25 mM sulfuric acid. Using a 96-well plate, 270  $\mu\text{L}$  of FOX2 reagent was added to 30  $\mu\text{L}$  samples that had been filtered through Miracloth to remove cells. After 30 min incubation, the plates were read at 560 nm using a Molecular Devices Versamax micro-plate reader (Sunnyvale, CA, USA). The absorbance change was compared to hydrogen peroxide standards.

### 3. Results

#### 3.1. Metabolism of exogenous AS and HAP by suspension cells

AS and HAP, 100  $\mu\text{M}$ , were added to soybean suspension cells either separately or in combination (Fig. 1). The concentrations of each of the phenolics were monitored periodically by HPLC-UV. The concentration of  $\text{H}_2\text{O}_2$  in the supernatant was measured using a peroxidase-independent spectrophotometric assay (FOX2). Untreated soybean cells, 0.05  $\text{g mL}^{-1}$ , produced a small initial burst of  $\text{H}_2\text{O}_2$ , nearly 20  $\mu\text{M}$ , due to the physical transfer of cells to assay buffer (Fig. 1A). This initial accumulation of  $\text{H}_2\text{O}_2$  was not observed in soybean cells treated with exogenous AS, 100  $\mu\text{M}$ , which is consistent with phenolics serving as peroxidase substrates during the scavenging of  $\text{H}_2\text{O}_2$ . This is supported by HPLC-UV analysis (Fig. 1B), which indicated that the extracellular AS concentration in these suspensions decreased about 30  $\mu\text{M}$  during the first 2 h of the monitoring period. Soybean cells treated with 100  $\mu\text{M}$  HAP produced a burst of hydrogen peroxide similar to untreated cells, suggesting that under these conditions HAP was not as good a substrate for apoplastic peroxidases to efficiently scavenge  $\text{H}_2\text{O}_2$  (Fig. 1A). The concentration of HAP in these suspensions decreased about 7  $\mu\text{M}$  over this monitoring period (Fig. 1B).

Surprisingly, when soybean suspensions were treated with both phenolics, 100  $\mu\text{M}$  each, a greater production of  $\text{H}_2\text{O}_2$  occurred reaching concentrations of 80–90  $\mu\text{M}$  (Fig. 1A). In addition, the loss of both phenolics was greater than when these compounds were added individually, depleting nearly all of the AS within 3 h, and reducing the HAP to 50  $\mu\text{M}$ . Despite the continued presence of  $\text{H}_2\text{O}_2$ , the loss in HAP appeared to nearly stop after 3 h when the AS was nearly depleted.

#### 3.2. Metabolism of AS and HAP by peroxidases

To determine whether extracellular peroxidases may be involved in this phenomenon, we carried out similar experiments in vitro with similar activities of horseradish

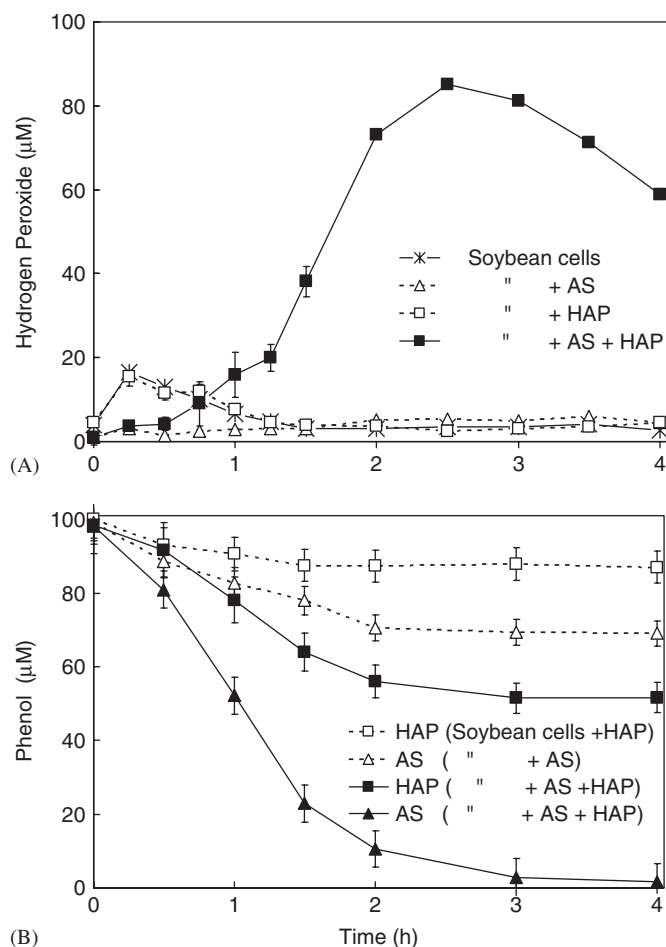


Fig. 1. Monitoring the extracellular  $\text{H}_2\text{O}_2$  levels and phenolic concentrations of suspensions of soybean cells treated with acetosyringone (AS) and hydroxyacetophenone (HAP). The suspensions contained cells, 0.5  $\text{g mL}^{-1}$  and either 100  $\mu\text{M}$  AS or HAP, or both AS and HAP. Samples were periodically removed. (A) Hydrogen peroxide was measured spectrophotometrically @ 560 nm using the FOX2 assay which involved oxidation of xylenol orange. (B) The concentration of the exogenously added phenolics, AS and HAP, was quantified using HPLC-UV. See Section 2 for details.

peroxidase and soybean peroxidase in 25 mL of assay buffer. The phenolics, 50  $\mu\text{M}$ , were added separately or in combination with exogenous  $\text{H}_2\text{O}_2$ , 100  $\mu\text{M}$ . The findings were similar for both, but only the horseradish peroxidase results are shown (Fig. 2). Using peroxidase activities similar to the soybean suspension cells, AS alone was a much better substrate than HAP alone, based on the utilization of  $\text{H}_2\text{O}_2$  (Fig. 2A) and phenolic degradation (Fig. 2B); AS was reduced nearly 70% over the 3 h monitoring period while HAP was reduced less than 5%.

As with the soybean suspension cells, the in vitro degradation of both phenolics was increased when they were present together; more than 90% of the AS was degraded and about 50% of the HAP (Fig. 2B). The utilization of  $\text{H}_2\text{O}_2$  appeared to be about 35  $\mu\text{M}$  (Fig. 2A), about half the total phenolic degradation of AS plus HAP, 70–75  $\mu\text{M}$ . Interestingly, in reactions with HAP alone, the

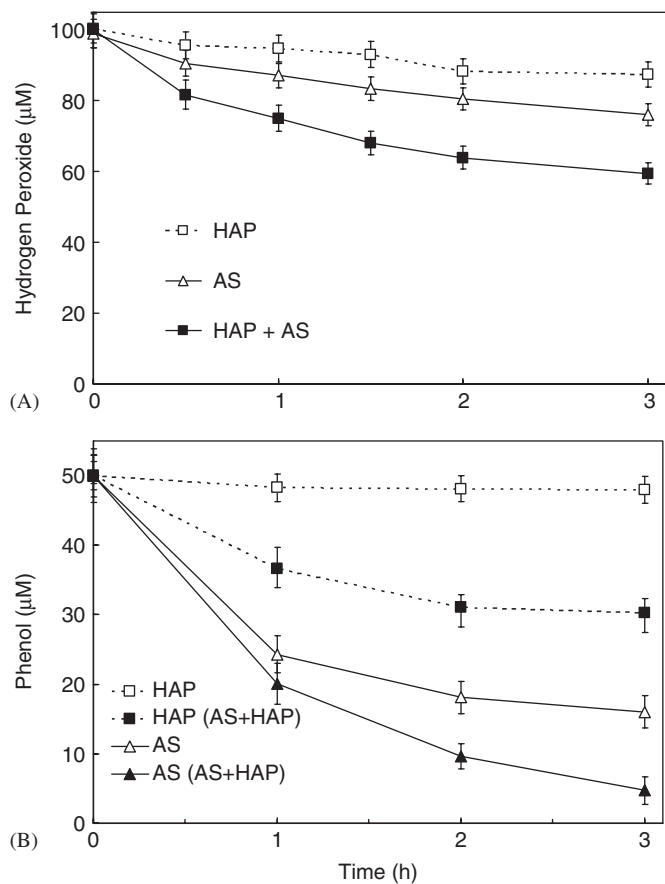


Fig. 2. Monitoring the reaction of AS and/or HAP with horseradish peroxidase and  $\text{H}_2\text{O}_2$ . The reaction mixtures contained  $0.036 \text{ U mL}^{-1}$  horseradish peroxidase in assay buffer, pH 6,  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$ , and  $50 \mu\text{M}$  of either AS or HAP, or  $50 \mu\text{M}$  each AS and HAP. (A) Hydrogen peroxide was measured spectrophotometrically using the FOX2 assay which involved oxidation of xlenol orange. (B) The concentration of the phenolics, AS and HAP, was measured by HPLC-UV. See Section 2 for details.

$\text{H}_2\text{O}_2$  decreased nearly  $10 \mu\text{M}$  (Fig. 2A) while the HAP concentration decreased less than  $3 \mu\text{M}$ .

### 3.3. Need for an initial oxidative burst

The need for an oxidative burst to initiate this reaction was apparent when using tobacco suspension cells. Unlike soybean cells, our tobacco cell suspensions do not produce an oxidative burst upon preparation and transfer to assay buffer. Addition of AS and HAP to tobacco cells did not elicit  $\text{H}_2\text{O}_2$  production or stimulate phenolic degradation (Fig. 3). Previous studies [9] had demonstrated that addition of HKBac to tobacco suspension cells would produce a brief burst of  $\text{H}_2\text{O}_2$ , about  $50 \mu\text{M}$  with these suspensions (Fig. 3A). The same general pattern of  $\text{H}_2\text{O}_2$  production and phenolic degradation that was seen in soybean treatments occurred with the HKBac treated tobacco cells (HKbac-tobacco). HKbac-tobacco suspensions treated with  $100 \mu\text{M}$  HAP responded similarly to

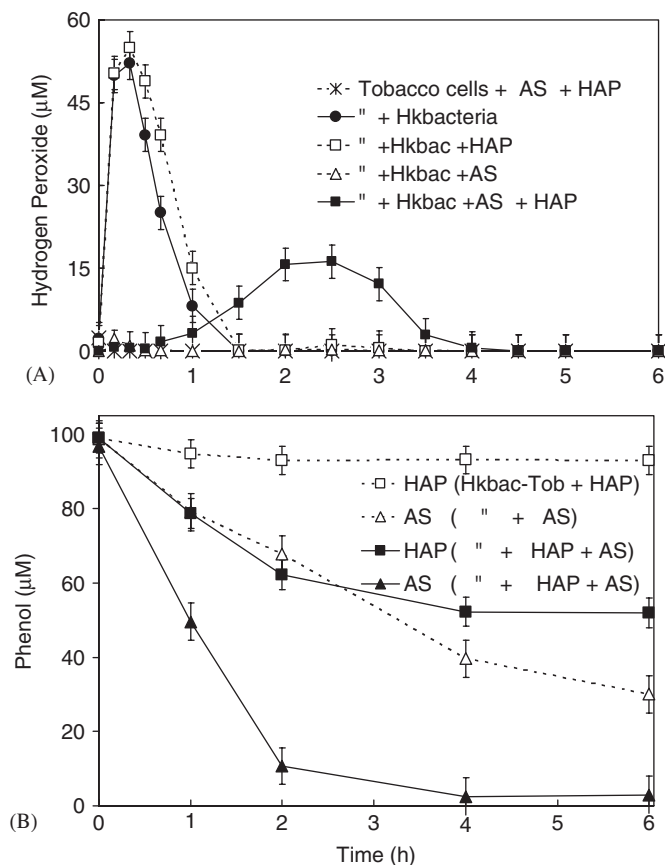


Fig. 3. Monitoring the extracellular  $\text{H}_2\text{O}_2$  levels and phenolic concentrations of suspensions of tobacco cells incubated with AS and HAP after treatment with heat-killed bacteria (HKBac). The tobacco cell suspensions,  $0.5 \text{ g mL}^{-1}$ , were treated with HKBac, equivalent to  $10^{-8}$  cfu, and either  $100 \mu\text{M}$  AS or HAP, or both AS and HAP. Samples were periodically removed. (A) Hydrogen peroxide was measured spectrophotometrically using the FOX2 assay which involved oxidation of xlenol orange. (B) The concentration of the exogenously added phenolics, AS and HAP, was quantified using HPLC-UV. See Section 2 for details.

untreated tobacco cells, producing an initial burst of  $\text{H}_2\text{O}_2$  (Fig. 3A). HPLC-UV analysis indicated that less than  $10 \mu\text{M}$  HAP was degraded over the 6 h period (Fig. 3B), indicating, as in soybean, that HAP was not an ideal substrate for apoplastic peroxidases to scavenge the  $\text{H}_2\text{O}_2$ . HKbac-tobacco suspensions treated with  $100 \mu\text{M}$  AS did not accumulate  $\text{H}_2\text{O}_2$  during the 6 h period (Fig. 3A). Nearly  $40 \mu\text{M}$  AS was metabolized during the 6 h period (Fig. 3B), suggesting that AS can be utilized in tobacco, as well as soybean, to scavenge  $\text{H}_2\text{O}_2$ .

As with soybean cells, when HKbac-tobacco cells were treated with both AS and HAP,  $100 \mu\text{M}$  each,  $\text{H}_2\text{O}_2$  accumulated reaching  $15 \mu\text{M}$  (Fig. 3A), which was less than in soybean (Fig. 1A). The phenolic degradation response of the HKbac-tobacco was similar in magnitude to that of similarly treated soybean cells (Fig. 1B), depleting nearly all of the  $100 \mu\text{M}$  AS within 4 h, and reducing the HAP by  $50 \mu\text{M}$  (Fig. 3B).



### 3.4. Effect of varying the phenolic concentration

To gain insight into the relationship between the two phenolics and  $H_2O_2$  production, soybean cells were treated with varying concentrations of each phenolic. When soybean cells were treated with a constant amount of HAP, 100  $\mu$ M, plus varying amounts of AS, 0–100  $\mu$ M, the amount of  $H_2O_2$  detected increased with the amount of AS present in the initial treatment (Fig. 4A). The initial rate of  $H_2O_2$  accumulation appeared to be the same for the different concentrations of AS. The major effect of the AS concentration was the duration of the  $H_2O_2$  accumulation and therefore the magnitude of the  $H_2O_2$  accumulation (Fig. 4A). The accumulation of  $H_2O_2$  ceased at about the same time the AS concentration was nearly depleted (Fig. 4C), and the HAP degradation decreased (Fig. 4B). For example, the  $H_2O_2$  accumulation in cells treated with HAP plus 50  $\mu$ M AS stopped around 2 h (Fig. 4A), concurrent with the depletion of AS (Fig. 4C) reduced degradation of HAP (Fig. 4B).

When soybean cells were treated with a constant amount of AS, 100  $\mu$ M, plus varying amounts of HAP, 0–100  $\mu$ M, the amount of  $H_2O_2$  detected increased with the HAP concentration in the initial treatment (Fig. 4D). The starting time of  $H_2O_2$  accumulation was similar for the different concentrations of HAP; however, the rate of accumulation increased with HAP concentration. The accumulation of  $H_2O_2$  appeared to cease when AS was nearly depleted (Fig. 4F) and the HAP degradation decreased (Fig. 4E). For example, in treatments with AS plus 100  $\mu$ M HAP, the  $H_2O_2$  accumulation stopped around 3 h (Fig. 4D), concurrent with the depletion of AS (Fig. 4F) reduced degradation of HAP (Fig. 4E).

## 4. Discussion

This study demonstrated two interesting phenomena related to apoplastic metabolism, (1) the synergistic co-oxidation of certain apoplastic phenolics and (2) the subsequent production of ROS in the extracellular environment of suspension cells. When equal amounts of AS and HAP were added to soybean suspension cells undergoing an oxidative burst, and thus producing  $H_2O_2$ , the rate and amount of degradation of each phenolic compound was increased (Figs. 1 and 4). When the same phenolics were added to tobacco suspension cells, a similar synergistic co-oxidation accompanied by the production of ROS occurred, but only after the cells were treated with HKBac to elicit an initial oxidative burst (Fig. 3). The synergistic co-oxidation of AS and HAP could be duplicated *in vitro*, with both horseradish peroxidase (Fig. 2) and soybean peroxidase (data not shown); however, the production of ROS could not be demonstrated. The ROS production by cells treated with these phenolic compounds coincided with a comparable increase in oxygen consumption by the suspension mixture (data not shown).

The mechanism involved in this phenolic co-oxidation would appear to involve peroxidase activity based on the similarity of the synergistic phenolic metabolism that occurred with horseradish peroxidase (Fig. 2B) and the suspension cells (Figs. 1B and 3B). Unlike most biological redox reactions, which require specific cofactors, peroxidases have a wide range of substrates from which they can abstract electrons. The catalytic cycle of peroxidase is shown in Fig. 5a–c. The native enzyme is oxidized by  $H_2O_2$  losing two electrons to form Compound-I. Compound I takes an electron from a reducing phenol, producing a phenolic radical and forming the Compound-II. Compound-II repeats the later step forming another phenolic radical reverting back to the native enzyme. These phenolic radicals can have many fates including disproportionation, dimerization, polymerization, or oxidation of other substrates including other phenolics (co-oxidation) or oxygen (Fig. 5).

In order to aid chemical analysis of the exogenous phenolics added to the cell suspensions, relatively high concentrations, 50–100  $\mu$ M, were used compared to the endogenous concentrations, 2–6  $\mu$ M, detected in previous studies with tobacco cell suspensions [5]. As discussed in the previous study, it seems feasible that the concentration of the phenolics in the cell wall matrix of suspension cells would be much greater than that detected after dilution into the total suspension fluid. In suspensions, the cell volume is about 5% of the total volume and only a fraction of this cell volume is composed of the cell wall matrix. Therefore, although the total amount of exogenous phenolics metabolized by these cells was probably much greater than they would normally encounter, the concentration of the phenolics seems biologically feasible.

Based on the work described in this study as well as other studies of co-oxidation [6,7,10,11], it would appear that AS would be the preferred substrate to react with peroxidase (Fig. 2). The AS radicals are fairly stable [12] and react with either  $AS^*$ ,  $O_2$ , or HAP if present (Fig. 5d–f). The presence of HAP would increase its oxidation by AS, as well as reduce disproportionation of AS (Fig. 5d), which might explain the observed apparent increase in oxidation rate of both phenolics (Figs. 1B, 2B). Consistent with this: (1) the rate of HAP metabolism decreased once the AS was depleted in suspension cell treatments containing both phenolics (Figs. 1–4); (2) the initial rate of HAP metabolism was proportional to the AS concentration (Fig. 4B).

The mechanism of  $H_2O_2$  production observed in this study is not clear. It could occur via the reaction of phenolic radicals with oxygen, producing superoxide and subsequently  $H_2O_2$  (Fig. 5f, h); however, it is likely that other circumstances are involved since this was only observed in suspension cells. It seems likely that the HAP radical was the major component involved in ROS production, since in the absence of HAP, AS was metabolized but did not produce  $H_2O_2$ . Also the initial rate of  $H_2O_2$  production appears dependent on the HAP

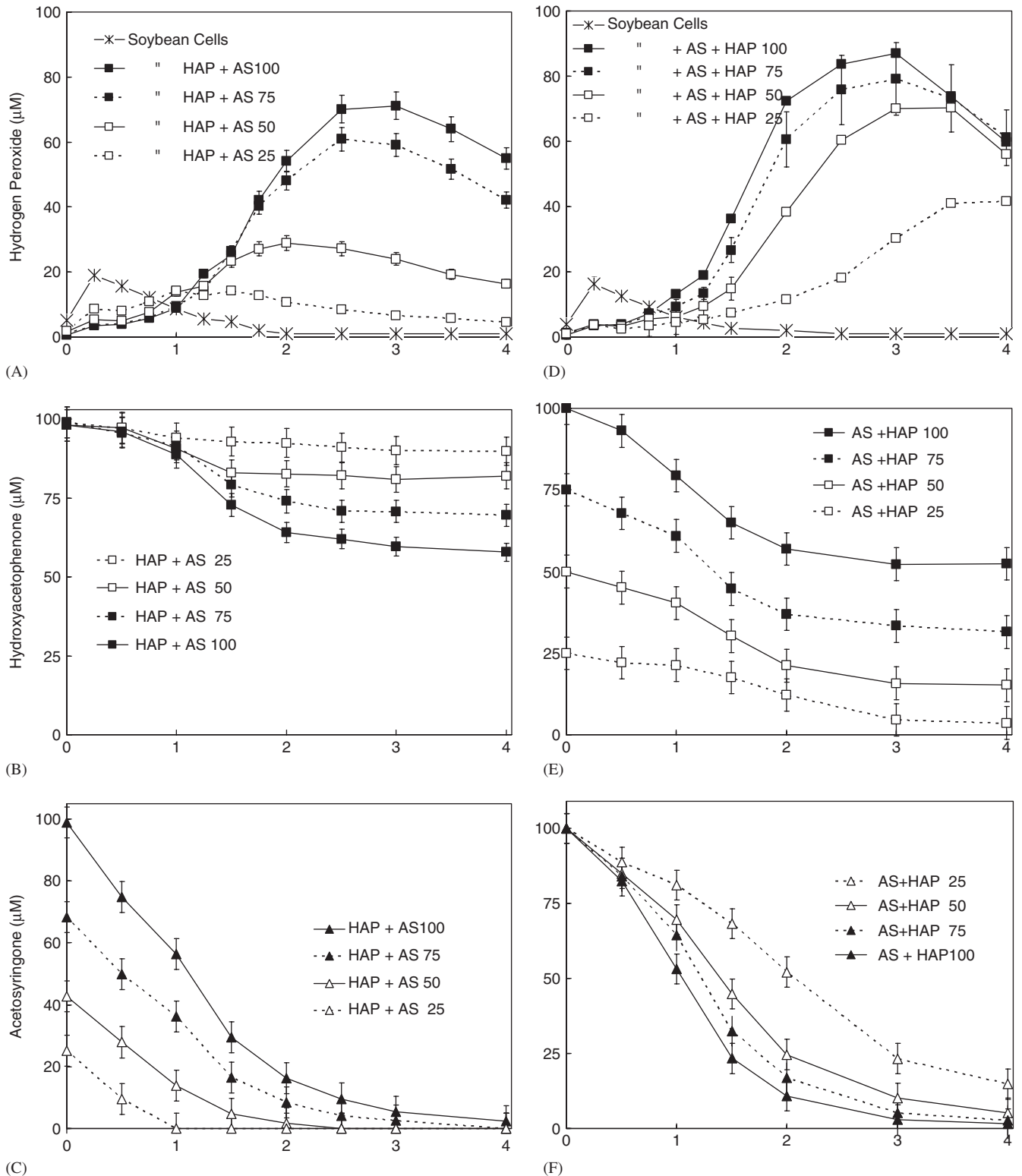


Fig. 4. Effect of varying the concentration of either AS or HAP in soybean cell suspensions on H<sub>2</sub>O<sub>2</sub> accumulation and phenolic degradation. (A–C) Soybean cell suspensions, 0.5 g mL<sup>-1</sup>, in assay buffer were treated with exogenous HAP, 100 μM, plus varying concentrations of AS ranging from 0 to 100 μM as indicated. (D–F) Soybean cell suspensions were treated with exogenous AS, 100 μM, plus varying concentrations of HAP ranging from 0 to 100 μM as indicated. Hydrogen peroxide concentrations in the supernatant were determined with the FOX2 xylenol orange assay (A, D). The concentrations of HAP (B, E) and AS (C, F) were determined by HPLC-UV. See Section 2 for details.



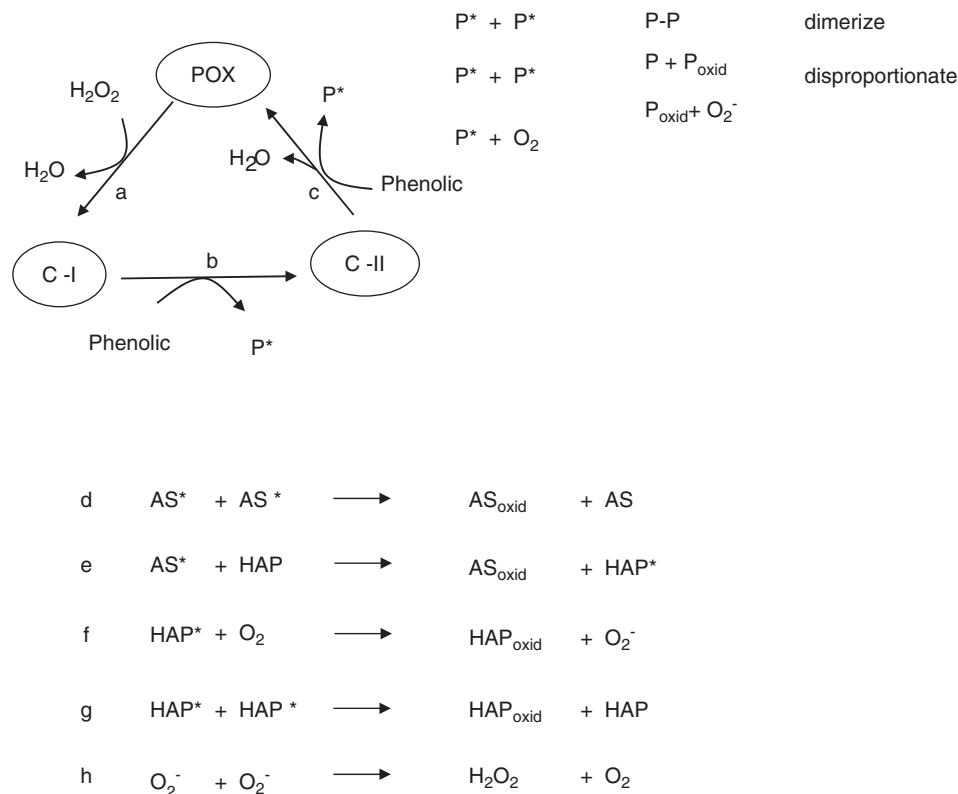


Fig. 5. Traditional peroxidase-related redox mechanisms. (a) The native peroxidase enzyme loses 2 electrons to  $H_2O_2$  forming compound-I (C-I). (b) C-I can gain an electron from a phenolic substrate producing compound-II (C-II) and a phenolic radical ( $P^*$ ). (c) C-II will repeat the later process reverting back to its native state.  $P^*$  may dimerize, disproportionate, or donate an electron to  $O_2$  forming superoxide ( $O_2^-$ ). (d) AS radical ( $AS^*$ ) may disproportionate, or (e) oxidize HAP forming a radical ( $HAP^*$ ). (f)  $HAP^*$  may combine with  $O_2$  forming superoxide, or (g) disproportionate. (h)  $O_2^-$  may form  $H_2O_2$  via superoxide dismutase or disproportionation.

concentration (Fig. 4D). Unfortunately, the accumulation of  $H_2O_2$  cannot be explained by the stoichiometry of these reactions. One cycle of the peroxidase pathway would consume one molecule of  $H_2O_2$  and under the best of conditions produce one molecule of  $H_2O_2$ , since two phenolic radicals would produce two superoxide radicals and subsequently one  $H_2O_2$  (Fig. 5). Additional factors must be involved in the apoplastic  $H_2O_2$  production. Other sources of apoplastic ROS production have been nicely summarized by Bolwell and Wojtaszek [13], however, their connection to the current phenomenon is not clear.

The phenomenon of co-oxidation of phenolics has been an area of intense investigation in the pharmaceutical field where two or more phenolic drugs are prescribed and has revealed effects that could just as likely occur in plants [6,11]. Peroxidases and other redox-active proteins in animals have been shown to metabolize a number of phenolic compounds *in vivo*, leading to modifications of their bioactivity, which could be either beneficial or detrimental [10,14–17]. For example, rifampicin, a broad-spectrum antibiotic used in tuberculosis therapy, inhibits bacterial RNA polymerase, but also has immunosuppressive properties in humans that are attributed to its oxidation product, rifampicin quinone [10]. The quinone results from the oxidation of rifampicin by ROS at the

infection or inflammation site. This oxidative reaction can be increased substantially by co-oxidation with paracetamol, a phenolic painkiller often taken by patients. There is concern that the paracetamol radically reduce the rifampicin concentration to ineffective levels. Another example of phenolic co-oxidation affecting bioactivity involves the interaction of acetaminophen, a common painkiller, and anthracycline drugs used in anticancer therapy [16]. The acetaminophen is a good substrate for endogenous peroxidase when oxidative conditions occur in stressed tissue. The phenolic radical produced will oxidize and degrade the anthracycline drug which normally would not be affected. Because of this finding, the practice of using these drugs together to treat cancer is being reexamined.

We have previously demonstrated the bioactive potential of AS, an apoplastic phenol found in tobacco suspension cells, in plant bacterial interactions [5]. When added exogenously to tobacco or potato cell suspensions treated with bacterial pathogens, AS caused physiological symptoms to occur earlier. In light of the current study, the possibility that the AS might have had a co-oxidative effect on other apoplastic phenolics in these suspension cells thus altering their bioactivity must be considered. Several studies have noted that addition of various phenolic compounds, such as salicylic acid, aromatic monoamines,

and phenylethylamine, have all been found to induce responses similar to pathogens induced responses [18–21]. Although the ROS production is often the focus of these investigations, the co-oxidative effects of these chemicals on the apoplastic phenolics is often overlooked.

Because the apoplast is one of the first arenas in which the molecular interaction between the plant and pathogen occurs, it is essential to understand the molecular processes that take place. It is a complex environment and due to its separation from the cytoplasm, can undergo rapid transitions and conditions that would not be tolerated in the cytoplasm. Because of the complexity of this environment, it has been very difficult to examine the exact nature of many of the events that occur. The cell suspension system described herein allows minimally invasive sampling and analysis of the extracellular/apoplast fluid providing some insight into the apoplastic chemistry.

### Acknowledgment

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