University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Publications from USDA-ARS / UNL Faculty | U.S. Department of Agriculture: Agricultural Research Service, Lincoln, Nebraska

2002

Continuous production of extracellular antioxidants in suspension cells attenuates the oxidative burst detected in plant microbe interactions

C. Jacyn Baker
USDA, jacyn.baker@ars.usda.gov

Nichole R. O'Neil
USDA

Kenneth Deahl
USDA

John Lydon
USDA

Follow this and additional works at: https://digitalcommons.unl.edu/usdaarsfacpub

Part of the Agricultural Science Commons

Baker, C. Jacyn; O'Neil, Nichole R.; Deahl, Kenneth; and Lydon, John, "Continuous production of extracellular antioxidants in suspension cells attenuates the oxidative burst detected in plant microbe interactions" (2002). *Publications from USDA-ARS / UNL Faculty*. 339.
https://digitalcommons.unl.edu/usdaarsfacpub/339

This Article is brought to you for free and open access by the U.S. Department of Agriculture: Agricultural Research Service, Lincoln, Nebraska at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Publications from USDA-ARS / UNL Faculty by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

ELSEVIER

Plant Physiol. Biochem. 40 (2002) 641–644

Plant Physiology and Biochemistry

www.elsevier.com/locate/plaphy

Continuous production of extracellular antioxidants in suspension cells attenuates the oxidative burst detected in plant microbe interactions[§]

C. Jacyn Baker [a,*], Nichole R. O'Neill [a], Kenneth Deahl [b], John Lydon [c]

[a] *Molecular Plant Pathology Laboratory, Plant Sciences Institute, USDA, Beltsville, MD 20705, USA*
[b] *Vegetable Laboratory, Plant Sciences Institute, USDA, Beltsville, MD 20705, USA*
[c] *Sustainable Agricultural Systems Laboratory, Plant Sciences Institute, USDA, Beltsville, MD 20705, USA*

Received 7 December 2001; accepted 7 March 2002

Abstract

Suspension cells of *Solanacearum tuberosum* and *Nicotiana tabacum* placed in fresh buffer rapidly produce and maintain significant pools of extracellular antioxidants. The extracellular antioxidant was detected by first adding a known amount of exogenous H_2O_2 to samples and then immediately measuring the remaining H_2O_2. The difference between the amount added and amount remaining was used to determine the antioxidant capacity of the sample. This extracellular antioxidant pool attenuates levels of hydrogen peroxide produced during plant–bacterial interactions. When tobacco cells were inoculated with an isolate *Pseudomonas syringae* pv. *syringae* that causes a hypersensitive response much of the antioxidant capacity had been expended neutralizing the oxidative burst characteristic of such plant–microbe interactions. After a brief delay, the levels of extracellular phenolics increased commensurate to antioxidative capacity in freshly transferred cells within 2–4 h. The strong UV absorbance of these extracellular phenolics within 250 and 350 nm was used to follow oxidation upon reaction with H_2O_2. This extracellular antioxidant pool is an important consideration in cell suspension studies of the plant–microbe oxidative burst. This study demonstrates that the true magnitude and timing of the oxidative burst in cell suspensions is masked by extracellular antioxidants. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Antioxidants; Cell wall; Hydrogen peroxide; Phenolics; Potato; Suspension-cells; Tobacco

1. Introduction

Extracellular phenolics play important functions in plants ranging from cell wall reinforcement to antibiotics and antioxidants. Lignin and polysaccharide bound phenolics cause physical and chemical changes in the plant cell wall that protect it from physical challenges and attack by various pathogens [4,6,7,9,12]. Antibiotic phenols can be both constitutive and induced at the site of infection [3,8,11]. A key cell wall metabolite is ascorbic acid, which in association with other components, is an antioxidant that can protect plants and cells from oxidative damage derived from many sources including pollution, pathogens, photosynthesis and the aerobic environment [5,14,16]. A unique and critical role of the ascorbic acid and other phenolic antioxidants in the apoplast along with peroxidases may help to regulate the redox potential of the apoplast [15,16]. This phenomenon has serious implications regarding the triggering of other mechanisms and how the cell will react to various stimuli.

In this study, we report the antioxidative buffering capacity of this extracellular phenolic pool and its affect on our ability to detect the plant–microbial oxidative burst. It had been noticed in the previous study that suspension cells of tobacco that were treated with non-hypersensitive response (HR)-causing bacteria accumulated an antioxidant capacity that could rapidly scavenge exogenously added hydrogen peroxide. At that time, the source of this activity and its implications were not understood. In the current study, we demonstrate the potential antioxidative buffering

Abbreviations: cfu, colony forming units; HR, hypersensitive response

* Corresponding author.
E-mail address: bakerc@ba.ars.usda.gov (C. Jacyn Baker).

[§] 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.

© 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.
PII: S 0 9 8 1 - 9 4 2 8 (0 2) 0 1 4 1 3 - 4

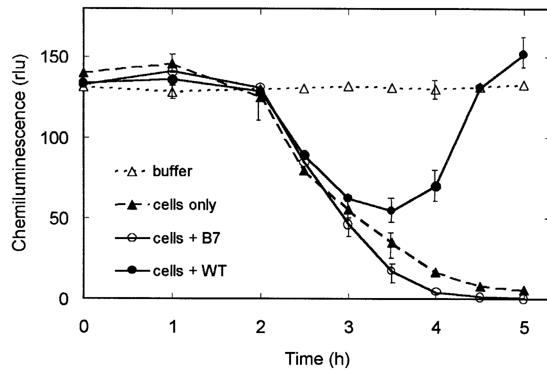

Fig. 1. Tobacco cell suspensions were inoculated with *P. syringae* pv. *syringae*, 2.5×10^7 cfu ml^{-1}, and monitored for antioxidative capacity. Exogenous H_2O_2, 50 µM, was added to samples of treated cells and the remaining H_2O_2 was immediately determined by luminol-dependent-chemiluminescence, as described in the Methods. Treatments included buffer control, cells with no treatment, and cells inoculated with either HR-causing (WT) or non-HR-causing (B7) isolates of *P. syringae* pv. *syringae*. Chemiluminescence is shown in relative light units (rlu).

capacity which can buildup rapidly in cell suspensions and cause an underestimation of the actual oxidative stress.

2. Results

Monitoring antioxidative capacity of cell suspensions could be achieved by the addition of exogenous H_2O_2 followed by an immediate measurement of the remaining H_2O_2 (Fig. 1). The buffer control to which 20 µM H_2O_2 are added remained relatively constant throughout the monitoring period. Cells with no treatment or treated with the non-HR-causing isolate, *P. syringae* pv. *syringae* B7, were able to consume increased amounts of exogenous H_2O_2 as time increased. While the initial consumption of H_2O_2 in treatments with the HR-causing isolate, *P. syringae* pv. *syringae* (WT), was similar to B7, after 3 h the consumption of exogenous H_2O_2 decreased relative to the other treatments. This would be consistent with previous work that demonstrates H_2O_2 is produced during HR reactions [1]. The H_2O_2 produced by these treatments was scavenged by the antioxidants and therefore less exogenous H_2O_2 could be consumed. This experiment indicates a method by which an oxidative burst may be detected even though H_2O_2 does not accumulate.

The antioxidant capacity is defined as the amount of H_2O_2 consumed by a quantity of cells as described in the Methods. The antioxidant capacity of a suspension of tobacco and potato cells increased over a 5 h incubation period (Fig. 2). Phenolics from the same cells were found to increase over the same time period (Fig. 3). The antioxidant capacity as well as the amount of phenolics reached higher levels in tobacco than in potato cell suspensions.

The antioxidant capacity was associated with the extracellular fluid (Fig. 4). The supernatant from suspensions that had been incubated for 5 h had nearly 95% of the activity of

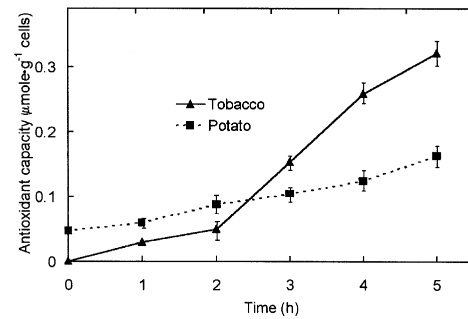

Fig. 2. Cell suspensions of potato and tobacco were monitored for antioxidant capacity over a 5 h period. Details of the procedures are described in the Methods. Exogenous H_2O_2, 20 µM for potato and 50 µM for tobacco, was added to samples of non-treated cells and the remaining H_2O_2 was immediately determined by luminol-dependent-chemiluminescence. The amount consumed per gram cells is defined as the antioxidant capacity.

the cell suspension, while the cell residue retained less than 5% antioxidant capacity. The extracellular antioxidant was heat stable, retaining more than 80% activity after autoclaving for 10 min.

The increase in antioxidant activity in the suspension cells paralleled an increase in absorbance in the 250–350 nm range characteristic of phenolics (Fig. 5A, B). Samples of fresh cell suspensions were scanned hourly in a spectrophotometer. Peaks at 278 and 318 nm were predominant in potato samples and peaks at 288 and 314 nm were predominant in tobacco samples. Incremental addition of

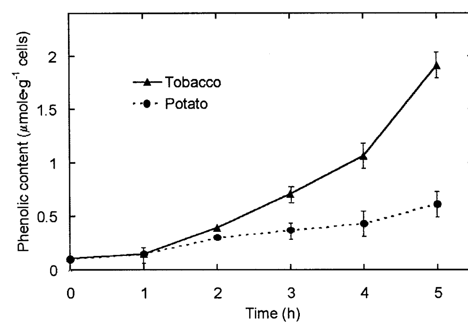

Fig. 3. Cell suspensions of potato and tobacco were monitored for phenolic substances over a 5 h period. Details of the procedure are described in the Methods.

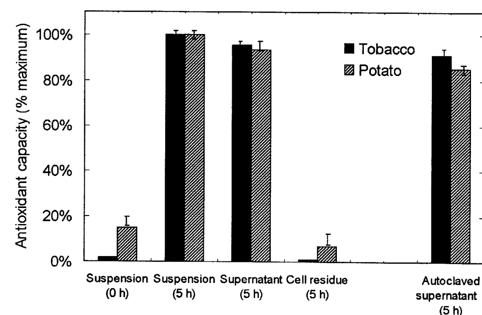

Fig. 4. Antioxidant capacity of various cell suspension treatments were determined using procedures described in the Methods.

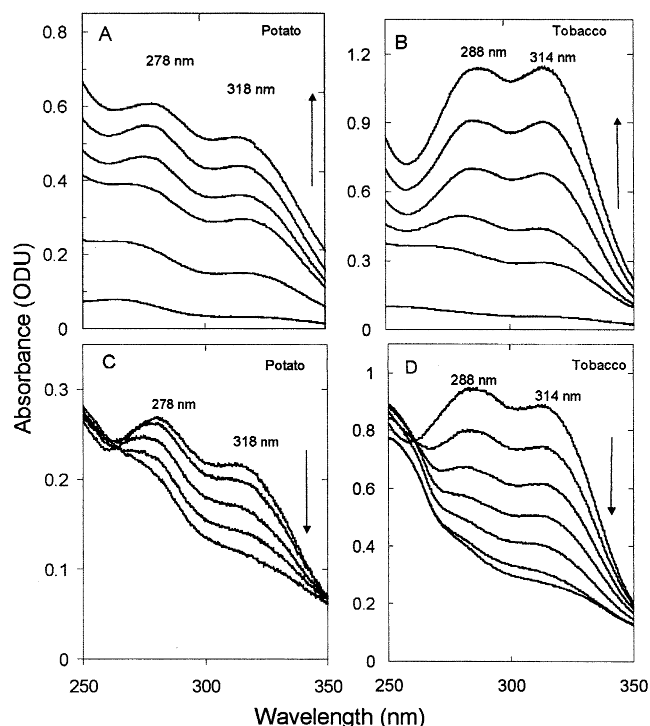

Fig. 5. Absorbance scans of cell suspensions were monitored from 250 to 350 nm. The increase in predominate peaks of potato (**A**) and tobacco (**B**) were observed over a 5 h period. The 5 h supernatant was scanned after being treated with sequential additions of H_2O_2: 5 or 10 μM additions for potato (**C**) or tobacco (**D**) supernatant, respectively.

H_2O_2 to the 5 h supernatant samples of both cells caused an incremental decrease in absorbance of these peaks (Fig. 5C, D).

Peroxidase appears to be required for this antioxidant phenomenon. Addition of H_2O_2, without peroxidase, to the autoclaved 5 h supernatants did not cause a decrease in absorbance. To determine if peroxidase activity increases significantly during this period we followed peroxidase activity using a standard guaiacol assay as described in the Methods. Peroxidase activity remained about 7×10^{-8} and 35×10^{-8} katals g^{-1} cells for potato and tobacco, respectively, over the 5 h period.

3. Discussion

The results suggest that healthy suspension cells of tobacco and potato have significant extracellular pools of phenolic compounds that can attenuate the oxidative burst resulting from stresses such as microbial attack. The extracellular phenolic content of suspension cells of either tobacco or potato increased concurrently with scavenging or antioxidant capacity (Figs. 2, 3). The scavenging activity of the extracellular phenolic extracts could be followed spectrophotometrically by decreased absorbance in the 270–320 nm range after addition of hydrogen peroxide (Fig. 5).

When cells of tobacco were inoculated with a pathogen, *Pseudomonas syringae* pv. *syringae*, it was clear that part of the oxidative burst was buffered by the extracellular phenolics (Fig. 1). Untreated cells or cells treated with the non-HR-causing isolate B7 scavenged more of the exogenously added H_2O_2 compared to the treatment with WT. This we believe was due to the oxidative burst produced in the WT treatment, which consumed or oxidized the extracellular antioxidants, thus reducing the antioxidant capacity of these samples. It appears that free H_2O_2 will not accumulate in the extracellular supernatant of cell suspensions until the antioxidant buffer pool has been consumed.

The antioxidant capacity of the leaf apoplast has been studied extensively especially in regard to ascorbic acid and ozone tolerance [15,17]. Ozone will produce a chronic stress of reactive oxygen species in the apoplast. Ascorbic acid and extracellular phenolics along with peroxidases attenuate the level of reactive oxygen species encountered by the leaf cells. It appears likely that phenolic compounds react directly with peroxidase and H_2O_2 while the ascorbic acid serves to regenerate the oxidized phenolic compound. A similar scenario is likely to be occurring in the suspension cell model. It is apparent that a family of phenolics are being produced and the exact composition varies depending on the cell type and age (unpublished data).

This study demonstrates that the oxidative burst caused by HR-causing bacteria in suspension cells is initiated prior to the detection of free H_2O_2. Under certain conditions, an oxidative burst may not exceed the antioxidative capacity of the cell suspension. It would be undetectable if free reactive oxygen species were used as the measure. We are currently developing a technique to measure the total oxidative burst in cell suspensions. It will need to account for the oxidative component that is reduced by the extracellular antioxidants as well as the free H_2O_2 detected in the supernatant. This information and technique would be applicable to areas in which the oxidative burst is being studied.

4. Methods

4.1. Plant material and bacteria

Suspension cells of tobacco (*Nicotiana tabacum* L. cv. Hicks) and potato (*Solanum tuberosum* cv. Kennebec) were maintained and prepared as previously described [2] except that tobacco callus and suspension cells were maintained on MS media (supplemented with 0.2 mg l^{-1} KH_2PO_4, 0.2 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin). Four-day-old cultures, which were in log phase, were transferred, 8×80 ml of fresh media in 250 ml flasks. Cells from 4-day-old cultures in log phase growth were washed and suspended in assay buffer (175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 0.5 mM MES, pH 6) for a final cell concentration of 0.1 g ml^{-1}. The assay buffer is prepared in bulk and autoclaved to allow storage. It was found that the autoclaved assay buffer should be allowed to equilibrate with air for

1 h prior to use to minimize perturbation of the cells. Cell suspensions, 25 ml, contained in 50 ml beakers were equilibrated on a rotary shaker at 180 rpm and 25 °C for 0.5 h. Bacterial cultures of *P. syringae* pv. *syringae* 61 were maintained and prepared as previously described [1]. The wild-type (WT) isolate caused an HR reaction on tobacco while the Tn5 insertion mutant (B7) did not. The cultures were grown on Kings B agar plates and suspended in deionized water and added to cell suspension for a final concentration of 2.5×10^7 cfu ml^{-1}.

4.2. ROS measurement

The luminol-dependent chemiluminescent assay was used to measure H_2O_2 as an estimate of ROS accumulation [10]. Samples, 0.4 ml, of treated suspension cells were dispensed into tubes, placed into the measuring chamber of an EG&G Berthold Autolumat 953 luminometer (Bad Wildbad, Germany), and automatically mixed with horseradish peroxidase, final concentration 0.72 U ml^{-1}, followed by luminol, final concentration 85 µM, for immediate measurement of luminescence. Standard curves were prepared with dilutions of H_2O_2 in assay buffer (pH6). Antioxidant capacity was calculated by determining the µmol of H_2O_2 consumed per gram of cells. Exogenous H_2O_2, 20 µM for potato cells or 50 µM for tobacco cells, was added to samples by the luminometer, and the remaining H_2O_2 immediately measured. The exogenous H_2O_2 was injected after the peroxidase and prior to the luminol. The difference between the samples and buffer with no cells was considered the amount of H_2O_2 consumed by the antioxidant.

4.3. Phenol assay

The phenolic concentration was determined using the Folin-Ciocalteu method of Singleton and Rossi [13] as described by Booker and Miller [3]. We used 200 µl of sample plus 70 µl of the 2 N Folin-Ciocalteu followed 3 min later with 1 M Na_2CO_3. The samples were read 1 h later at 724 nm using 4-coumaric acid as a standard.

4.4. Spectrophotometric scans

Absorbance scans were carried out on the supernatant of the cell suspension treatments. Samples were filtered through cheesecloth into 1 ml quartz cuvettes. Scans were performed hourly on a Beckman Model DU-650 spectrophotometer between 210 and 400 nm. The 5 h supernatant was scanned after being treated with sequential additions of H_2O_2: 5 or 10 µM additions for potato or tobacco supernatant, respectively.

4.5. Peroxidase assay

Peroxidase activity was assayed using a modified guaiacol assay. Samples of cell suspension treatments were assayed for enzyme activity in a reaction mixture containing 1 mM guaiacol, 50 mM MES buffer (pH6) and 1 mM H_2O_2. The change in absorbance was measured at 475 nm in a spectrophotometer at 27 °C.

References

[1] C.J. Baker, G.L. Harmon, J.A. Glazener, E.W. Orlandi, A noninvasive technique for monitoring peroxidative and H_2O_2—scavenging activities during interactions between bacterial plant pathogens and suspension cells, Plant Physiol. 108 (1995) 353–359.

[2] C.J. Baker, N.M. Mock, K. Deahl, J. Domek, Monitoring the rate of oxygen consumption in plant cell suspensions, Plant Cell, Tissue Organ Cult. 51 (1997) 111–117.

[3] F.L. Booker, J.E. Miller, Phenylpropanoid metabolism and phenolic composition of soybean [*Glycine max* (L.) Merr.] leaves following exposure to ozone, J. Exp. Bot. 49 (1998) 1191–1202.

[4] C.T. Brett, G. Wende, A.C. Smith, K.W. Waldron, Biosynthesis of cell-wall ferulate and diferulates, J. Sci. Food Agric. 79 (1999) 421–424.

[5] F. Castillo, H. Greppin, Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in *Sedum album* L. leaves after ozone exposure, Environ. Exp. Bot. 28 (1988) 231–238.

[6] R. Franke, S.C. Fry, H. Kauss, Low-molecular-weight precursors for defense-related cell wall hydroxycinnamoyl esters in elicited parsley suspension cultures, Plant Cell Rep. 17 (1998) 379–383.

[7] S.C. Fry, Cross-linking of matrix polymers in the growing cell walls of angiosperms, Annu. Rev. Plant Physiol. 37 (1986) 165–186.

[8] H. Kauss, R. Franke, K. Krause, U. Conrath, W. Jebllick, B. Grimmig, U. Matern, Conditioning of Parsley (*Petroselinum crispum* L.) suspension cells increases elicitor-induced incorporation of cell wall phenolics, Plant Physiol. 102 (1993) 459–466.

[9] R.L. Nicholson, R. Hammerschmidt, Phenolic compounds and their role in disease resistance, Annu. Rev. Phytopathol. 30 (1992) 369–389.

[10] E.W. Orlandi, S.W. Hutcheson, C.J. Baker, Early physiological responses associated with race-specific recognition in soybean leaf tissue and cell suspensions treated with *P. syringae* pv *Syringae*, Physiol. Molec. Plant Pathol. 40 (1992) 173–180.

[11] A. Schmidt, D. Scheel, D. Strack, Elicitor-stimulated biosynthesis of hydroxycinnamoyltyramines in cell suspension cultures of *Solanum tuberosum*, Planta 205 (1998) 51–55.

[12] P. Schopfer, Hydrogen peroxide-mediated cell-wall stiffening in vitro in maize coleoptiles, Planta 199 (1996) 43–49.

[13] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, Am. J. Enol. Viticult. 16 (1965) 144–158.

[14] N. Smirnoff, The function and metabolism of ascorbic acid in plants, Ann. Bot. 78 (1996) 661–669.

[15] U. Takahama, Redox state of ascorbic acid in the apoplast of stems of *Kalanchoe daigremontiana*, Physiol. Plant 89 (1993) 791–798.

[16] U. Takahama, Ascorbic acid-dependent regulation of redox levels of chlorogenic acid and its isomers in the apoplast of leaves of *Nicotiana tabacum* L, Plant Cell Physiol. 39 (1998) 681–689.

[17] E. Turcsanyi, T. Lyons, M. Plochl, J. Barnes, Does ascorbate in the mesophyll cell walls form the first line of defence against ozone? Testing the concept using broad bean (*Vicia faba* L.), J. Exp. Bot. 51 (2000) 901–910.