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Postharvest Biology and Technology 37 (2005) 47–55
Postharvest Biology and Technology
www.elsevier.com/locate/postharvbio

# Wound-induced phenolic accumulation and browning in lettuce (*Lactuca sativa* L.) leaf tissue is reduced by exposure to *n*-alcohols

Young-Jun Choi [a], Francisco A. Tomás-Barberán [b], Mikal E. Saltveit [a,*]

[a] *Mann Laboratory, Department of Plant Sciences, University of California, One Shields Avenue, Davis, CA 95616-8631, USA*
[b] *Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS-CSIC, P.O. Box 164, Murcia 30100, Spain*

Received 20 November 2004; accepted 5 March 2005

**Abstract**

A wound signal originates at the site of injury in lettuce (*Lactuca sativa* L.) leaf tissue and propagates into adjacent tissue where it induces a number of physiological responses which include increased phenolic metabolism with the de novo synthesis of phenylalanine ammonia lyase (PAL, EC 4.3.1.5), the synthesis and accumulation of soluble phenolic compounds (e.g., chlorogenic acid), and subsequent tissue browning. Exposing excised mid-rib leaf tissue to vapors (20 μmol/(g FW)) or aqueous solutions (100 mM) of *n*-alcohols inhibited this wound-induced tissue browning by 40 and 60%, respectively. Effectiveness of the alcohol increased linearly from ethanol to the 7-carbon heptanol, and then was lost for the longer *n*-alcohols 1-octanol and 1-nonanol. The 2- and 3-isomers of the effective alcohols did not significantly reduce wound-induced phenolic accumulation at optimal 1-alcohol concentrations, but significant reductions did occur at much higher concentrations (100 μmol/(g FW)) of the 2-, and 3-isomers. The active *n*-alcohols were maximally effective when applied during the first 2 h after excision, and were ineffective if applied 6 h after excision. Phospholipase D (PLD) and its products linolenic acid (LA) and phosphatidic acid (PA) are thought to initiate the oxylipin pathway that culminates in the production of jasmonic acid, and PLD is specifically inhibited by 1-butanol, but not by 2-, or 3-butanol. These results suggest that PLD, LA, PA, and the oxylipin pathway may be involved in producing the wound signal responsible for increased wound-induced PAL activity, phenolic accumulation and browning in fresh-cut lettuce leaf tissue.


*Keywords:* Abiotic stress; Fresh-cut; Jasmonic acid; Octadecanoid pathway; Oxylipin pathway; Phospholipases D; Wounding

## 1. Introduction

Wounding (e.g., cutting) increases phenolic metabolism in lettuce leaf tissue with the production and accumulation of soluble phenolic compounds (mainly hydroxycinnamic acid conjugates such as chlorogenic acid) that subsequently react to produce wound-induced tissue browning (Tomás-Barberán et al., 1997). An initial response to wounding in lettuce (and many other biotic and abiotic stresses) is the de novo synthesis and increased activity of phenylalanine ammonia lyase (PAL, EC 4.3.1.5) (Campos-Vargas

* Corresponding author. Tel.: +1 530 7521815; fax: +1 530 7524554.
*E-mail address:* mesaltveit@ucdavis.edu (M.E. Saltveit).

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doi:10.1016/j.postharvbio.2005.03.002

et al., 2004), the first committed enzyme in the phenylpropanoid pathway (Dixon and Paiva, 1995). The soluble phenolic compounds produced by this enhanced PAL activity are sequestered in the vacuole and only participate in browning reactions when the disruption of membranes (e.g., depolarization following wounding or during senescence) allows the substrates and enzymes (e.g., polyphenol oxidase, peroxidase) to mix. Various compounds (e.g., antioxidants, calcium) and treatments (e.g., low oxygen, heat-shock) reduce wound-induced tissue browning by interfering with either the synthesis or oxidation of precursor phenolic compounds (Brecht, 1995; Saltveit, 1997).

The wound signal that transduces a physical wound into a physiological response (e.g., the de novo synthesis of PAL and accumulation of phenolics) is unknown in lettuce. Research has characterized the signal, but not identified its components. It appears that a signal is produced at the site of injury in lettuce leaves and migrates or propagates into adjacent, non-injured tissue where it induces increased phenolic production (Ke and Saltveit, 1989). Both wounding and ethylene increase PAL activity and tissue browning (Tomás-Barberán et al., 1997), and ethylene is thought to be involved in the wound signal complex in some plants (Abeles et al., 1992). However, a kinetic analysis of wound induction of ethylene and of PAL in lettuce indicated that the increase in wound-induced PAL activity did not proceed through the induced synthesis and action of ethylene (Ke and Saltveit, 1989). Evaluation of a number of putative wound signal chemicals (i.e., abscisic acid, jasmonic acid, methyl jasmonate, salicylic acid) failed to identify one of them as the wound signal in lettuce (Campos-Vargas and Saltveit, 2002).

Products of the oxylipin pathway [e.g., jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA)] are part of the wound signal complex in a number of plants (Peña-Cortés and Willmitzer, 1995; Creelman and Mullet, 1997; Turner et al., 2002). Mechanical wounding lead to increased levels of JA in soybean (Creelman et al., 1992), tomato and potato plants (Peña-Cortés et al., 1993, 1995), and *Arabidopsis* (Wang et al., 2000). Critical reactions in this pathway include the production of phosphatidic acid (PA) and release of free linolenic acid from phospholipids in membranes as a direct or indirect consequence of phospholipase D (PLD) activation (Creelman and Mullet, 1997; Ryu and Wang, 1996, 1998; Wang et al., 2000; Leon et al., 2001). Subsequent steps in the synthesis of JA produce a myriad of phytoactive compounds that participate in tissue responses to biotic and abiotic stresses (Farmer and Ryan, 1992; Farmer et al., 1998; Munnik, 2001; Schaller, 2001).

The activity of PLD is selectively inhibited by *n*-butanol (1-butanol) with the formation of phosphatidyl butanol instead of phosphatidic acid, while 2-, and 3-butanol have no such inhibitory effect (Lee et al., 2001; Gardiner et al., 2003). The effectiveness of PLD inhibition by *n*-alcohols increased with length of the molecule from ethanol to 1-heptanol, whereas 1-octanol and longer *n*-alcohols were ineffective (Ella et al., 1997).

Research reported in this paper was undertaken to further characterize the wound signal in lettuce that leads to wound-induced increases in PAL activity, phenolic accumulation and tissue browning, and to investigate the influence of exogenously applied alcohols on the tissue's response to wounding.

## 2. Materials and methods

### 2.1. Plant material

Romaine lettuce (*Lactuca sativa* L. cv. Longifolia) was purchased from local commercial vendors. Outer leaves were discarded, and undamaged leaves were carefully detached from the stem. The leaf blade was removed and segments of the mid-rib tissue from 2.5 to 20 mm in length were excised with a stainless steel razor blade starting 20 mm from the base and extending 80 mm up the mid-rib. The freshly excised segments were randomly distributed among treatments, and 10 g of segments were placed in each pre-weighed 20 mm × 100 mm diameter plastic Petri dish. Each dish constituted a replicate.

### 2.2. Alcohol treatments

Freshly excised mid-rib segments were either immersed in shaken aqueous solutions of the various alcohols, or exposed to vapors of the pure compound. After immersion with gentle shaking for 2 h in 20 or 100 mL of the appropriate solution, the segments were

drained, blotted with paper tissues to remove excess moisture and placed in pre-weighed 20 mm × 100 mm diameter Petri dishes. The dishes were placed in 20 cm × 15 cm × 10 cm plastic tubs lined with wet paper towels, the top of each tub loosely covered with aluminum foil, and the tubs placed in a 10 °C incubator. The low solubility of the higher molecular weight alcohols limited the concentrations that could be applied as aqueous solutions. Alcohols were alternatively applied as vapors to overcome this problem.

Tissue was exposed to alcoholic vapor by placing the bottom portion of a 7 mm × 20 mm diameter plastic Petri dish in the center of the 20 mm × 100 mm diameter Petri dish and applying the appropriate amount of alcohol (μmol/(g FW)) to a filter paper disk inserted in the smaller dish. The top of the larger dish was put in place and the dish enclosed in a zip-lock plastic bag, which was sealed shut. The assembled dish and bag were put one layer deep on a shallow tray and the tray placed in a 10 °C walk-in cold-room. The dish was removed from the plastic bag after an appropriate length of time, the smaller dish with filter paper removed, and the dish replaced on the tray, which was then covered with wet paper towels and returned to the 10 °C incubator. Air movement past the exposed dishes facilitated diffusion of the previously applied alcohols away from the treated tissue.

### 2.3. Measurement of phenolic content

Tissue from each Petri dish (3 or 4 g) was put into a 50 mL plastic centrifuge tube along with 20 mL of methanol. The tissue was ground and the absorbance at 320 nm of a clarified aliquot was measured (Ke and Saltveit, 1989; Loaiza-Velarde et al., 1997; Campos-Vargas and Saltveit, 2002) and expressed as $A_{320}$ per gram fresh weight ($A_{320}$/(g FW)). This measure is highly correlated with the chlorogenic acid content of wounded lettuce leaf tissue (Tomás-Barberán et al., 1997).

### 2.4. Measurement of PAL activity

Phenylalanine ammonia-lyase activity was measured as previously described by Ke and Saltveit (1989), with slight modifications (Campos-Vargas and Saltveit, 2002).

### 2.5. Statistical analysis

Each experiment had at least three replicates of each treatment and all experiments were run at least twice. Measurements from all the replicates were combined and treatment effects subjected to an ANOVA, and 5% LSD values calculated when significant treatment differences were detected.

## 3. Results

### 3.1. Wound-induction of PAL activity and phenolic accumulation

Excision (i.e., wounding) of de-bladed mid-rib tissue segments induced a rise in PAL activity within 4 or 8 h for the 5- or 20-mm thick segments, respectively (Fig. 1). PAL activity peaked with a seven-fold increase at 18 or 36 h for the 5- or 20-mm thick segments, respectively, and then declined to near initial

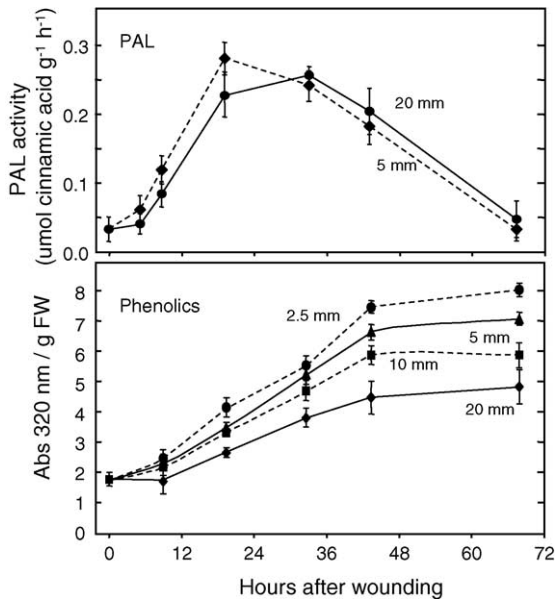


Fig. 1. Wound-induced changes in PAL activity and phenolic content over time for Romaine lettuce mid-rib segments of various thicknesses. Data for PAL activity are not shown for the 2.5 and 10 mm segments in the top panel to improve clarity of presentation because of significant overlap with presented data. The vertical line associated with each point represents the standard deviation about that mean.

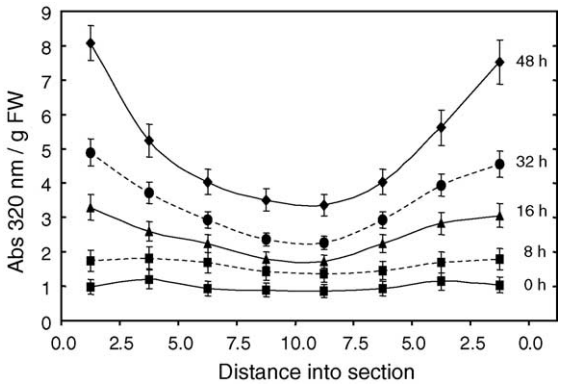


Fig. 2. Changes in phenolic content within an excised Romaine mid-rib segment. Excised 20 mm mid-rib segments were sectioned into 2.5 mm at 0, 8, 16, 32, and 48 h after wounding. The vertical line associated with each point represents the standard deviation about that mean.

levels at 70 h. Phenolic concentration ($A_{320}$/(g FW)) increased in concert with the rise in PAL activity, and then leveled off as PAL activity declined. The rise was more rapid in 2.5-mm thick sections than in 20-mm thick sections, but all thicknesses reached fairly stable levels by 70 h, albeit at different concentrations (Fig. 1).

Thicker sections had lower phenolic concentrations because less of the tissue was induced to accumulate high levels of phenolic compounds (Fig. 2). Wounding induced an increase in phenolic content in all the tissue in a 20-mm thick segment, but the greatest induction was in the 2.5 mm adjacent to the cut surface. Subsequent experiments used 5-mm thick segments because of the more rapid induction of PAL activity, the greater accumulation of phenolic compounds, and the better penetration of applied chemicals into these tissue segments.

Accumulation of wound-induced phenolic compounds was reduced in excised mid-rib tissue by either immersion in aqueous solutions of 1-butanol for 2 h or exposure to 1-butanol vapors for 12 h (Fig. 3). The level of inhibition was maximized at 40% for exposure to 20 μmol/(g FW) vapors for 12 h, and at 60% for immersion in 100 mM 1-butanol for 2 h.

### 3.2. Timing of alcohol application

A 2 h exposure to 1-butanol vapors (20 μmol/(g FW)) produced the greatest inhibition of wound-induced phenolic compounds when applied during the

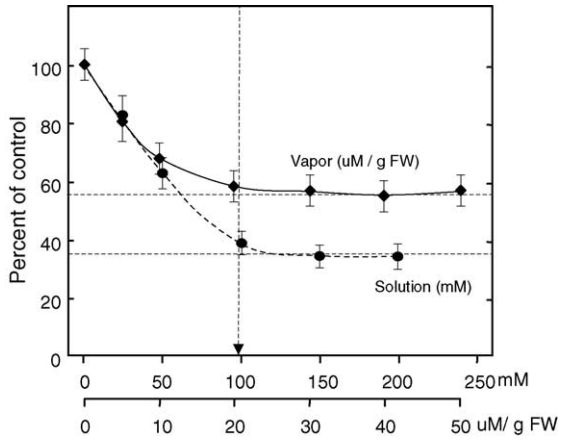


Fig. 3. Phenolic content of excised Romaine mid-rib segments exposed to 1-butanol as vapor, or aqueous solution. Segments were exposed to vapors for 12 h at 10 °C, or to solutions for 2 h at 20 °C. Phenolic content was determined by measuring the absorbance of a clarified methanol extract at 320 nm after holding the tissue for 48 h at 10 °C after excision. Vertical lines associated with each point represent the standard deviation about that mean.

first 2 h after excision (Fig. 4). Delaying exposure rapidly decreased the effectiveness of the treatment from 55 to 18% and 13% when the 2 h exposure started 2 and 4 h after excision, respectively. The treatment became ineffective when applied 6 h after excision. Similar results were obtained with tissue exposed to the same concentration of 1-hexanol (data not shown).

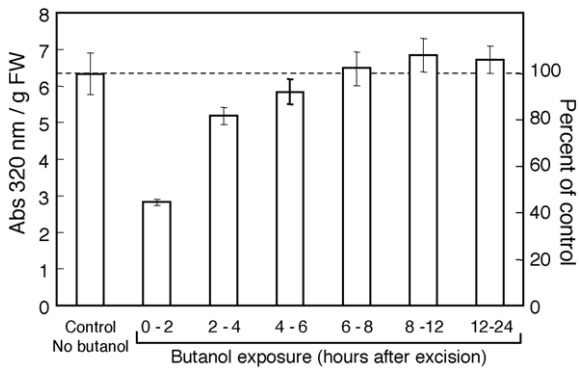


Fig. 4. Phenolic content of excised 5-mm Romaine mid-rib segments exposed to 20 μmol 1-butanol per gram FW at various times after excision. Phenolic content was determined by measuring the absorbance of a clarified methanol extract at 320 nm after holding the tissue for 48 h at 10 °C. Vertical lines associated with each bar represent the standard deviation about that mean.

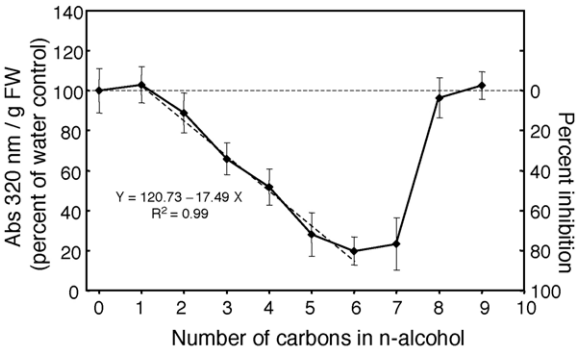


Fig. 5. Phenolic content of excised 5-mm Romaine mid-rib segments exposed to vapors from *n*-alcohols (from 1-carbon methanol to 9-carbon nonanol). Tissue was exposed to 20 μmol of the alcohol per gram FW for 12 h at 10 °C. The regression line and linear equation for the normal (1-alcohol) alcohols from 1 to 6 carbons is shown. The vertical line associated with each point represents the standard deviation about that mean.

### 3.3. Reduction of wound-induced phenolic accumulation by alcohols

Wound-induced phenolic accumulation was not affected by methanol, but was progressively reduced by exposure to 20 μmol/(g FW) vapor of the *n*-alcohols from ethanol to 1-hexanol (Fig. 5). The linearity of the inhibition had an $R^2$ of 0.99 when phenolic content was compared to the number of carbons in the alcohol molecule, or to the molecular weight of the alcohol (data not shown). About 80% inhibition was produced by application of 20 μmol/(g FW) of the 5-, 6-, and 7-carbon *n*-alcohols. Increasing the length of the alcohol by one more carbon to the 8-carbon 1-octanol, completely eliminated the inhibitory property of the *n*-alcohols. The 9-carbon 1-nonanol was also ineffective. Increasing the concentration of the ineffective 1-octanol up to 100 μmol/(g FW) had no significant inhibitory effect on phenolic accumulation, which remained constant at $6.5 \pm 0.8$ throughout the range of applied alcohol concentrations. Similar increases in applied 1-nonanol concentrations had no effect on wound-induced phenolic accumulation (data not shown).

### 3.4. Differential effect of alcohol isomers

Straight chain (normal) butanol, pentanol and hexanol (Fig. 6) are effective inhibitors of wound-induced

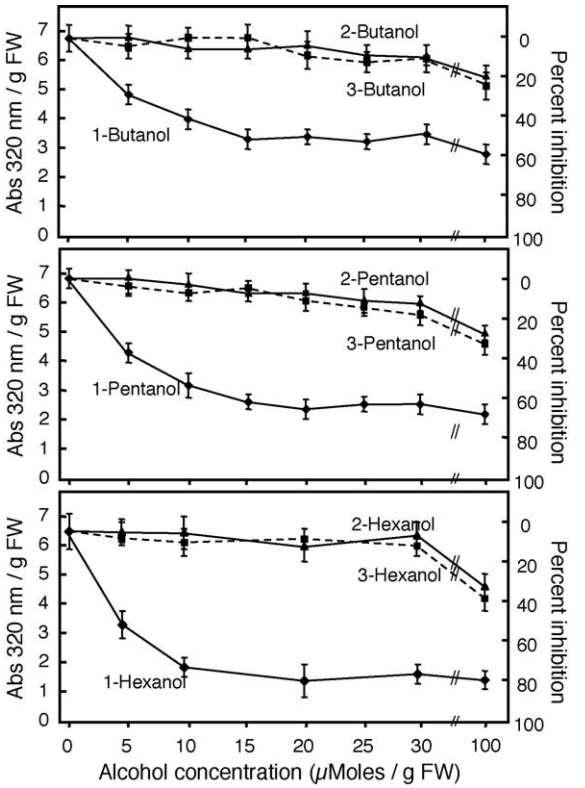


Fig. 6. Phenolic content of excised 5-mm Romaine mid-rib segments exposed to various concentrations of butanol, pentanol, and hexanol isomers for 12 h at 10 °C. Phenolic content was determined by measuring the absorbance of a clarified methanol extract at 320 nm after holding the tissue for 48 h at 10 °C. The vertical line associated with each point represents the standard deviation about that mean.

synthesis and accumulation of phenolic compounds. Exposure to 5 μmol/(g FW) of these 1-alcohols for 12 h inhibited phenolic accumulation by 30, 40, and 50% for 1-butanol, 1-pentanol, and 1-hexanol, respectively. Increasing the concentrations to 10 μmol/(g FW) increased the inhibition to 45, 55, and 75% for the respective 1-alcohols. The level of inhibition was saturated by a concentration of 20 μmol/(g FW) at which level the inhibition reached 55, 65 and 80% for the respective 1-alcohols.

These same concentrations of the 2-, and 3-alcohols had no significant inhibitory effect on wound-induced phenolic accumulation (Fig. 6). Increasing the concentrations of the 2- and 3-alcohols produced linear declines ($R^2 > 0.98$) in phenolic accumulation, which suggest a chemical effect, rather than the quadratic

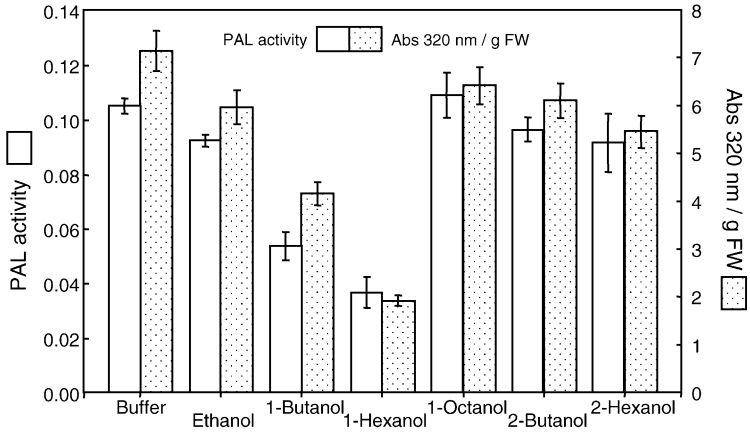


Fig. 7. Phenolic content and PAL activity of excised Romaine mid-rib segments exposed to alcohol vapors. Excised 5-mm thick segments were exposed to 20 μmol of various alcohols for 12 h at 10 °C. PAL activity was assayed 24 h after excision, while phenolic content was determined 48 h after excision. Vertical lines associated with each bar represent the standard deviation about that mean.

decline produced by the physiological effect of the *n*-alcohols. Even 100 μmol/(g FW) produced only a 23, 30, and 36% level of inhibition, for the respective 2-, or 3-alcohols of butanol, pentanol and hexanol. This level of inhibition was far less than the 60–80% inhibition produced by the same concentration of the 1-alcohols.

### *3.5. Effect of butanol on PAL activity in vitro*

A PAL enzyme extract was prepared from 5-mm mid-rib tissue segments held for 24 h at 10 °C after excision. PAL assays were run with sufficient 1- or 2-butanol added to the reaction mixture to mimic a tissue concentration of 0, 10, or 20 mM butanol in the assayed tissue. In a representative experiment, PAL enzyme activity averaged $0.197 \pm 0.006$ μmol $g^{-1}$ $h^{-1}$ over all three concentrations of both isomers. The tissue had no alcoholic odor when the Petri dishes were opened just prior to preparation for analysis. If all the 1-butanol had been retained in 10 g of tissue treated with 20 μmol/(g FW), the alcohol concentration in the PAL assay mixture would have been 5 mM.

### *3.6. Relationship between PAL activity and phenolic accumulation*

There was a strong correlation ($R^2 = 0.94$) between wound-induced PAL activity and phenolic accumulation measured 48 h after excision in tissue exposed to 20 μmol/(g FW) of various alcohols for 6 h (Fig. 7).

## 4. Discussion

Wounding increased PAL activity and the accumulation of phenolic compounds in 5-mm thick segments of de-bladed Romaine mid-rib leaf tissue. The rate of PAL induction and the level of phenolic accumulation increased as the thickness of the mid-rib tissue segments decreased from 20 to 2.5 mm (Fig. 1). Excision promotes phenolic accumulation in all the tissue in a 20-mm long mid-rib segment, but the level of induction and greatest accumulation occur within the first 2.5-mm of the cut (Fig. 2). The progressive accumulation of phenolic compounds over time and the reduced accumulation in tissue distant from the excision cut suggest that an inductive signal originates at the site of excision and decreases in strength as it propagates into the non-wounded tissue.

The origin of a wound signal at the site of excision is further supported by the great effectiveness of 1-heptanol (C7), an alcohol with very low water solubility (0.015 mol $L^{-1}$) and high vapor pressures (0.22 mm Hg at 25 °C) (Schwarzenbch et al., 1993). Its low water solubility would greatly limit is ability to dissolve and diffuse into cells distant from the exposed site of excision, while its high vapor pressure would prevent high concentration from being transferred from the moistened filter paper disc to the excised tissue during the usual 12 h exposure at 10 °C.

Experiments were done using 5-mm segments because penetration of the applied alcohols would be

faster than into thicker segments, and because wound-induced increase in PAL activity and phenolic accumulation would be larger and faster. Alcohols were applied as vapors instead of aqueous solutions to overcome limitation of water solubility with the higher molecular weight alcohols. Treatment with either aqueous solutions (100 mM) or vapor (20 μmol/(g FW)) inhibited wound-induced phenolic accumulation (Fig. 3).

The use of phenolic content to approximate induced PAL activity was confirmed by analyzing tissue at different times after excision (Fig. 1) and tissue treated with isomers of the different alcohols. PAL activity measured 0–24 h after excision was highly correlated with phenolic content ($A_{320}$/(g FW)) measured 24 h later (Fig. 7). This confirms similar findings (Loaiza-Velarde et al., 1997; Campos-Vargas and Saltveit, 2002).

The alcohols themselves did not have a significant effect on PAL activity, so the ability of the 1-alcohols from the 2-carbon ethanol to the 7-carbon heptanol to reduce PAL activity and the accumulation of phenolic compounds probably resulted from an inhibition of the induction and/or synthesis of PAL, not from inactivation of the PAL enzyme synthesized. It should be remembered that most of the wound-induced increase in PAL activity occurred after the effective 2 h exposure to the *n*-alcohols (Fig. 4), and that there was little PAL activity in the tissue at the time of exposure to the alcohols (Fig. 1). The treatments could not, therefore, have directly reduced the activity of PAL enzyme in the tissue at the time of treatment, but had to reduce the capacity of the tissue to increase PAL activity, either through decreased synthesis or increased destruction, in response to wounding.

The oxylipin pathway is involved in wound responses in a number of plants. Octadecanoid-derived compounds formed in this pathway (i.e., JA and MeJA, and their biosynthetic C18 precursors) are linolenic acid-derived oxylipins and represent plant compounds that function as regulators in various plant physiological processes such as mechano-transduction, growth inhibition (Dathe et al., 1981) and senescence promotion (Ueda and Kato, 1980). While treatment of whole non-injured lettuce leaves with JA did induce slight increases in PAL activity in mid-rib tissue, the increase was small in comparison to that induced by wounding (Campos-Vargas and Saltveit, 2002). This does not eliminate the oxylipin pathway as the source of the wound signal, since other phytoactive compounds are also produced during the synthesis of JA. Mechanical wounding of potato plants that had been transformed to have elevated levels of JA lead to the additional accumulation of JA and wound gene expression, but elevated endogenous levels of JA alone did not induce constitutive expression of wound-response genes (Harms et al., 1995).

The first committed step in the oxylipin pathway is the release of linolenic acid from cellular membranes as a consequence of the action of PLD and the production of phosphatidic acid (Ryu and Wang, 1998; Wang et al., 2000). The production of phosphatidic acid by PLD is selectively inhibited by 1-butanol, but not by 2-, or 3-butanol (Munnik et al., 1995; Gardiner et al., 2003). PLD-dependent signaling in plants has been studied using 1-butanol. Abscisic acid and gibberellic acid signaling in barley aleurone (Ritchie and Gilroy, 1998) and ABA-induced closure of stomatal guard cells (Jacob et al., 1999) were inhibited by 1-butanol blockage of PA production. PLD-mediated pollen germination and tube growth in tobacco was stopped by 1-butanol, whereas 2-, and 3-butanol were ineffective (Potocky et al., 2003). We show similar specificity with the inhibition of wound-induced phenolic accumulation being inhibited by 1-butanol, 1-pentanol, and 1-hexanol, whereas the 2-, and 3-isomers of these alcohols were far less effective (Fig. 6). However, the effective isomers may be affecting other pathways besides those currently identified.

The ability of the *n*-alcohols to inhibit PLD activity increased with increasing chain length up to 1-octanol, while secondary and tertiary alcohols were ineffective (Ella et al., 1997). A similar pattern was seen with the ability of *n*-alcohols from propanol to heptanol to inhibit wound-induced phenolic accumulation (Fig. 5). Ripening of tomato fruit pericarp discs was likewise inhibited by alcohols, and the level of inhibition increased with increasing chain length of the *n*-alcohols (Saltveit, 1989). While ripening inhibition was ascribed to the alcohols effect on ethylene synthesis and action, these alcohol treatments may also have affected lipid signaling pathways that affect fruit ripening as well as responses to biotic and abiotic stresses (Munnik, 2001; Turner et al., 2002).

The wound signal, which propagates from the site of injury into adjacent tissue where it induces the de novo synthesis of PAL, increased PAL activity and accu-

mulation of phenolic compounds, remains unknown. However, results presented in this paper suggest that PLD and the oxylipin pathway may be involved in producing the wound signal responsible for browning in fresh-cut lettuce.

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