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Mono-carboxylic acids and their salts inhibit wound-induced phenolic accumulation in excised lettuce (*Lactuca sativa*) leaf tissue

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Wounding, as during excision and preparation of lettuce (*Lactuca sativa* L.) leaf tissue for salads, induces the synthesis and accumulation of phenolic compounds that participate in subsequent reactions that cause tissue browning. Exposure of excised 5-mm mid-rib segments of romaine lettuce leaf tissue to vapors of mono-carboxylic acids or aqueous solutions of mono-carboxylic acids or their salts inhibited wound-induced phenolic accumulation (WIPA) and subsequent tissue browning. The decline in phenolic content followed a quadratic curve with increasing concentration, reaching a maximum inhibition after 60 min of 74 ± 8% for 50 m*M* sodium acetate (2 carbons, C2) and 91 ± 4% for 20 m*M* sodium decanoate (capric acid, C10). Respiration (i.e. carbon dioxide production) was unaffected by concentrations of formic, acetic, or propionic acids that reduced wound-induced phenolic content or that increase ion leakage from the tissue into an isotonic mannitol solution. However, WIPA was suppressed up to 70% at concentrations (20 m*M* acetate) that did not increase ion leakage over that of water controls. Various acetate salts (i.e. ammonium, calcium, magnesium, and sodium) all produced the same level of inhibition. The effectiveness of the compounds increased with increasing number of carbons in the molecule from 1 to 10, and was unaffected by whether the carbons were a straight chain or branched or whether the treatment was delayed by up to 6 h. The effectiveness of butyrate (C4) in reducing WIPA (27% reduction at 20 m*M*) was less than that predicted from the response of the two adjacent mono-carboxylates similarly applied: propionate (C3) (62%) and valerate (C5) (73%). It appears that, unlike the n-alcohols, mono-carboxylates are not interfering with the synthesis or propagation of a wound signal but are interfering with subsequent steps in the production and accumulation of wound-induced phenolic compounds.

Introduction

Mechanical injury of plant tissue during growth, harvest, or preparation for consumption induces an increase in phenolic metabolism with the production and accumulation of soluble phenolic compounds (e.g. chlorogenic acid) (Tomás-Barberán et al. 1997b). A wound signal originates at the site of injury and

Abbreviations – Abs, absorbance; F.W., fresh weight; PA, phosphatidic acid; PAL, phenylalanine ammonia lyase; PLD, phospholipase D; POD, peroxidase; PPO, polyphenol oxidase; TCA, tricarboxylic acid cycle; WIPA, wound-induced phenolic accumulation.

propagates into adjacent tissue where it induces the *de novo* synthesis and increased activity of phenylalanine ammonia lyase (PAL, EC 4.3.1.5), the first committed enzyme in the phenyl propanoid pathway (Dixon and Paiva 1995, Hahlbrock and Sheel 1989). In lettuce leaf tissue, this wound signal arises within 30 min of wounding (e.g. excision of mid-rib segments) and propagates into adjacent non-injured tissue at 5 mm h^{-1} (Ke and Saltveit 1989). Enhanced PAL activity results in an increased production and accumulation of soluble phenolic compounds that are sequestered in the vacuole and participate in browning reactions when membranes become disrupted. Various compounds (e.g. antioxidants, calcium) and treatments (e.g. low oxygen, heat-shock) reduce wound-induced tissue browning by either interfering with the synthesis or oxidation of precursor phenolic compounds (Brecht 1995, Saltveit 1997).

A variety of phospholipid-derived molecules have been implicated as signaling molecules, and the oxylipin biosynthetic pathway (i.e. the pathway producing jasmonic acid from phospholipids) has been implicated in the generation and/or propagation of the wound signal in lettuce (Choi et al. 2005). An initial step in the oxylipin pathway is the release of phosphatidic acid from membrane phospholipids by the action of phospholipase D (PLD, EC 3.1.4.4) (Leon et al. 2001, Turner et al. 2002). Subsequent steps in the pathway produce a myriad of phytoactive compounds which participate in tissue responses to biotic and abiotic stresses (Farmer et al. 1998, Schaller 2001). The activity of PLD is selectively inhibited by n-butanol (1-butanol), whereas 2- and 3-butanol have no such inhibitory effect (Lee et al. 2001). The effectiveness of the n-alcohols in reducing wound-induced phenolic accumulation (WIPA) increased with length of the n-alcohol from ethanol (C2) to 1-heptanol (C7) and then became ineffective at 1-octanol (C8) and longer n-alcohols (Choi et al. 2005). Delaying exposure of excised tissue to inhibitory levels of the active n-alcohols for 1 h appears to allow the appearance of a wound signal that induces the accumulation of elevated levels of phenolic compounds in non-wounded adjacent tissue. Delaying the application of a compound or treatment that reduced WIPA could be used to determine whether it was interfering with generation of the wound signal or some subsequent step in enhanced phenolic metabolism and tissue browning.

We previously showed that aqueous solutions of dilute acetic acid (i.e. vinegar) were effective in preventing browning of lettuce tissue (Tomás-Barberán et al. 1997a). Research reported in this paper was undertaken to further characterize the wound signal in lettuce that leads to wound-induced tissue browning and to investigate the influence of exogenously applied mono-carboxylic acids and their salts on the tissue's response to wounding.

Materials and methods

Plant material

Romaine lettuce (*Lactuca sativa* L. Longifolia) was purchased from local commercial vendors. Outer leaves were discarded, and the undamaged leaves were carefully detached from the stem. The leaf blade was removed, and 5-mm segments of the mid-rib tissue were excised starting 10 mm from the base and extending 8 cm up the mid-rib. The freshly excised segments were randomly distributed among treatments and placed in plastic Petri dishes. This randomization was done to minimize the slight difference in wound response along the length of the mid-rib. To further minimize variation within an experiment, each experiment was designed to be done with a single head of lettuce.

Treatments

Freshly excised mid-rib segments were either exposed to vapors of the mono-carboxylic acids or immersed in shaken aqueous solutions of the various acids or their salts. Tissue was exposed to vapor by placing a 7 × 20 mm diameter plastic Petri dish in the center of the larger (20 × 100 mm diameter) dish and applying the appropriate amount of compound to a filter paper disk in the smaller dish. The top of the larger dish was put in place and the dish enclosed in a plastic bag that was sealed shut. The assembled dish and bag were put on a tray one layer deep and the tray placed at 10° C. The dish was removed from the plastic bag after 2 h, 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 10° C. Carbon dioxide concentrations in the bag did not exceed 0.5% during treatment. Tissue concentrations of the compounds applied as vapors were calculated assuming 90% absorption of the compound by the tissue (all the material had evaporated from the filter paper, and it was dry when removed), equal distribution of the compound in the tissue, and a water content equal to the weight of the tissue. For example, concentrations of 0–50 m*M* should have been produced in tissue from exposure to 0–30 µl per 10 g fresh weight (FW) of glacial acetic acid.

Freshly excised tissue was also immersed in gently shaken aqueous solutions of the various chemicals. Solutions were made in 25 m*M* potassium phosphate

buffer (pH 7.0). Higher molecular weight mono-carboxylic acids have limited water solubility, and C10 to C16 compounds were initially made up in methanol as a bridging solvent. At most, 1 ml of this methanol solution was added to 100 ml of the buffer. The resultant concentration of methanol (25 m*M*) did not have a significant effect on WIPA (data not shown; Choi et al. 2005). In some experiments, a 60-min immersion was done immediately after excision or delayed for up to 5 h. In most experiments, the buffered solutions were adjusted to pH 7.0 with HCl or NaOH, but in some, the pH of the solutions was adjusted to pH 3, 5, 7, or 9. The pH of the solutions after treatment was within ± 0.1 units of the pH before treatment.

After immersion, the tissue was drained, blotted with paper tissues to remove excess moisture, and placed in tarred Petri dishes. The dishes were placed in $20 \times 15 \times 10$-cm plastic tubs lined with wet paper towels, the top of the tubs was loosely covered with aluminium foil, and the tubs were placed at 10° C for 48 h.

Measurement of phenolic content

After 48 h at 10° C, tissue from each Petri dish (3 g) was put into a 50-ml plastic centrifuge tube along with 20 ml of methanol. The tissue was ground, and the absorbance of a clarified aliquot was measured at 320 nm (Campos-Vargas and Saltveit 2002, Ke and Saltveit 1989, Loaiza-Velarde et al. 1997) and expressed as absorbance per gram fresh weight (Abs 320 nm g^{-1} FW).

Measurement of ion leakage

The slope of ion leakage was calculated as previously described (Saltveit 2002, 2005), with slight modifications. The conductivity of a 20 ml isotonic bathing solution (0.2 *M* mannitol) containing 2 g of tissue was periodically measured, and a regression line (R^2 of 0.95 or better) was fitted to the data from 30 to 120 min. Total leakage was measured at room temperature after two cycles of freezing and thawing. Leakage is expressed as a slope with values of percent of total conductivity per minute.

Measurement of carbon dioxide production

Carbon dioxide production was calculated from changes in the head space concentration as previously described (Saltveit 2005), with slight modifications. Tissue (2 g) was enclosed in a 50-ml plastic centrifuge tube for 1 h at 20° C. One-milliliter gas samples were withdrawn through a rubber serum stopper inserted in the tube's lid and analyzed using an infrared carbon dioxide analyser as previously described.

Chemicals used

All chemicals used were reagent grade or better and were purchased from Sigma-Aldrich (St. Louis, MO). Additional butyric acid and sodium butyrate were purchased from ACROS Organics (Morris Plains, NJ) and Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

Statistical analysis

Each experiment contained two replicates of each treatment, and all experiments were run at least three times. Means and standard deviations were calculated from all the replicates, and where appropriate, the data were combined and treatment effects subjected to further analysis.

Results

Mono-carboxylic acid vapors reduce wound-induced phenolic accumulation

Exposure of 5-mm segments of mid-rib tissue to vapors of formic or acetic acid produced a quadratic reduction in WIPA (Fig. 1). The reduction in WIPA was accompanied with a rise in the leakage of ions from the treated tissue (i.e. slope). At the lowest applied concentration, however, there was a significant decline in WIPA before there was a significant increase in leakiness.

Reduction in WIPA could be caused by a general disruption of metabolism brought about by increased membrane permeability. Alternatively, the carboxylic acids could serve as a readily available substrate for oxidation in the tricarboxylic acid (TCA) cycle. Either possibility would alter rates of respiration. However, the rate of carbon dioxide production by 5-mm mid-rib segments exposed to levels of formic, acetic, or propionic acid vapors that would have resulted in concentration of 0–20 m*M* in the tissue and that decreased WIPA while increasing leakage (Fig. 1) remained fairly stable across all concentrations (Fig. 2). It appears that these carboxylic acids were not altering some general aspect of metabolism.

Salts of mono-carboxylic acids reduce wound-induced phenolic accumulation

Because the pH of the tissue would have been significantly altered by exposure to vapors of the volatile mono-carboxylic acids and pH changes could obscure possible effects of the chemicals themselves,

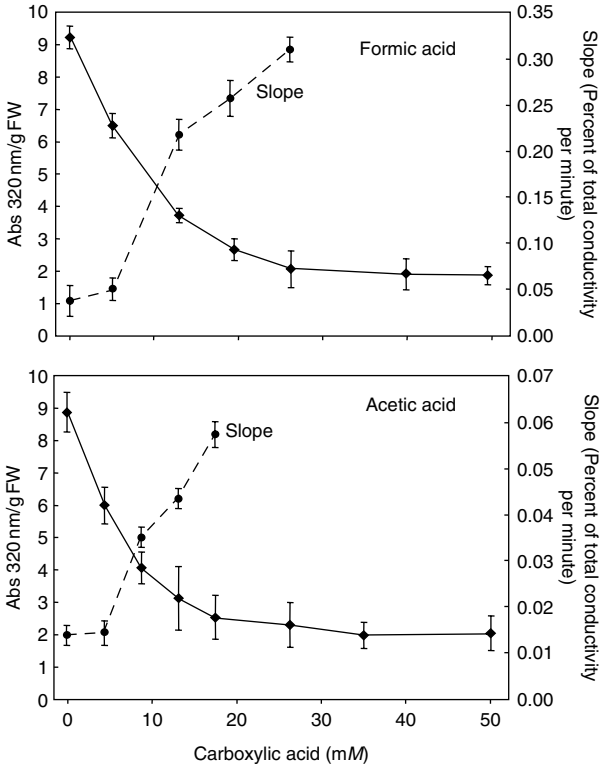

Fig. 1. Phenolic content and slope of ion leakage from excised Romaine lettuce leaf mid-rib segments exposed to vapors of formic or acetic acid. Excised 5-mm mid-rib segments were exposed to vapors from 0 to 30 µl per 10 g fresh weight (FW) of tissue (corresponding to 0–50 m*M*) for 2 h at 10° C. Absorbance of a clarified methanol extract was read at 320 nm after holding the tissue for 48 h at 10° C. Slope of ion leakage was calculated from changes in conductivity in a bathing isotonic 0.2 *M* mannitol solution from 0.5 to 2 h. The vertical line associated with each point represents the standard deviation about that mean.

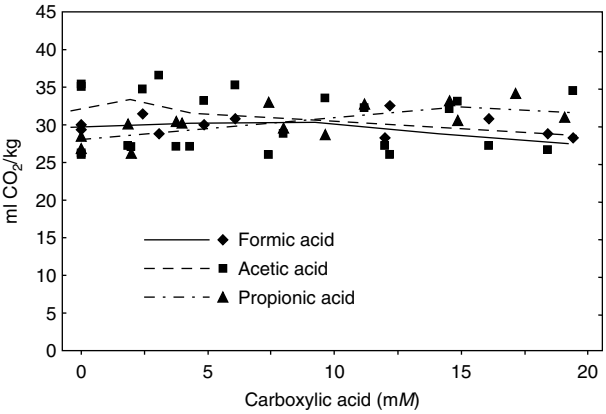

Fig. 2. Carbon dioxide production by excised Romaine lettuce leaf mid-rib segments exposed to vapors of formic, acetic, or propionic acid. Excised 5-mm mid-rib segments were exposed to concentrations of the carboxylic acids as vapors that would produce from 0 to 20 m*M* in the tissue for 2 h at 10° C. Production was calculated from changes in carbon dioxide concentration in the headspace of 50-ml containers in which 2 g of tissue was enclosed for 1 h at 10° C.

experiments were continued with pH 7 buffered solutions of sodium mono-carboxylates. Sodium acetate was effective in reducing WIPA (Fig. 3). The decline in phenolic content (Abs 320 nm g^{-1} FW) followed a quadratic decline for both concentrations (Fig. 3A) and duration of immersion (Fig. 3B). A 60 min soak in 50 m*M* sodium acetate produced a 74 ± 8% decline in WIPA.

The pH of the solution had an affect on WIPA, with the more acidic (pH 3 or 5) solutions producing greater inhibition than a neutral (pH 7) or alkaline (pH 9) solution (Fig. 4). This trend was evident in the 25 m*M* buffer and in the 50 m*M* sodium formate and sodium acetate solutions. The reductions in WIPA caused by the sodium mono-carboxylates became less, as the pH increased from 3 to 9. Both concentrations and salts caused the same 80% inhibition of WIPA at pH 3. At pH 9, however, 50 m*M* sodium formate and 25 m*M* sodium acetate caused 47% reductions, whereas 50 m*M* sodium acetate reduced WIPA by 63%.

Other acetate salts were equally effective at reducing WIPA. Calcium acetate solutions from 10 to 100 m*M* at pH 7 were as effective as sodium acetate in reducing WIPA (Fig. 5). WIPA was reduced by 70 ± 3.4% (from 10.7 ± 0.6 to 2.7 ± 0.6 Abs 320 nm g^{-1} FW) by exposure for 60 min to 50 m*M* solutions of ammonium acetate, calcium acetate, magnesium acetate, or sodium acetate, whereas exposure to the same concentration of their corresponding chloride (e.g. ammonium chloride) did not reduce WIPA (data not shown). In fact, the chlorides increased WIPA by 18% over the water control. All compounds increased the rate of ion leakage over the water control (0.037% of total conductivity per minute), with the chlorides being 43% higher (0.053 ± 0.006) and the acetates being 60% higher (0.087 ± 0.010) than the water control.

Relation between reduced wound-induced phenolic accumulation and ion leakage

Immersion of 5-mm mid-rib segments in low concentrations (2.5–20 m*M*) of sodium acetate for 1 h reduced phenolic content (64%; from 11.3 for water to 4.1 for 20 m*M* sodium acetate) without increasing membrane permeability (0.033 ± 0.008) (Fig. 6). Higher concentrations caused a further decline in WIPA (74% and 83% reductions by 40 and 80 m*M*, respectively), but these reductions were accompanied by 58 and 152% increases in the rate of leakage (i.e. the slope), respectively.

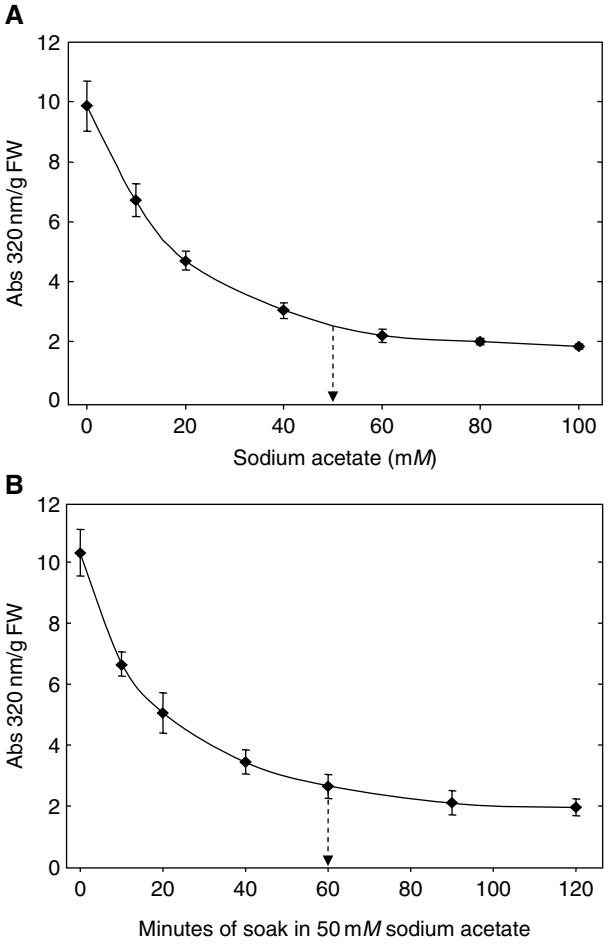

Fig. 3. Phenolic content of excised Romaine mid-rib segments exposed to aqueous solutions of sodium acetate. Excised 5-mm mid-rib segments (10 g) were immersed in 40 ml of buffered (pH 7) (A) 0–100 m*M* sodium acetate for 60 min or (B) 50 m*M* sodium acetate for 0–120 min at 20° C. Absorbance of a clarified methanol extract was read 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.

Effectiveness of mono-carboxylates related to size and isomer

The effectiveness of the C1 to C10 mono-carboxylates in reducing WIPA increased with increasing concentration and number of carbons in the molecule (Fig. 7). The inhibitory effect followed a quadratic decline for all C1–C10 mono-carboxylates tested (except for butyrate); a representative curve for nonanoate (C9) is shown (Fig. 7, insert). There was a strong correlation ($R^2 > 0.95$) between the levels of inhibition for all the mono-carboxylates (except butyrate) and the number of carbons in the molecule. Butyrate inhibits WIPA in a concentration-dependent fashion similar to the other compounds (data not shown), but the level of inhibition produced by a specific concentration

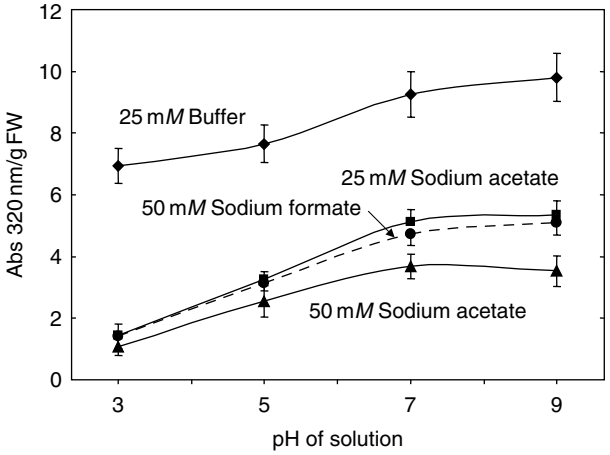

Fig. 4. Phenolic content of excised Romaine mid-rib segments exposed to aqueous solutions of sodium formate or acetate at different pH values. Excised 5-mm mid-rib segments (10 g) were immersed in 40 ml of buffered 50 m*M* sodium formate or 25 or 50 m*M* sodium acetate at pH 3, 5, 7, or 9 for 60 min at 20° C. Absorbance of a clarified methanol extract was read 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.

of butyrate (C4) was less than that produced by the same concentration of the C3 or C5 mono-carboxylate. The difference was most evident at concentrations below 50 m*M*, because the inhibitory effect became saturated at higher concentrations of the compounds tested.

Mono-carboxylates with straight chains longer than nine carbons are relatively insoluble in water, and

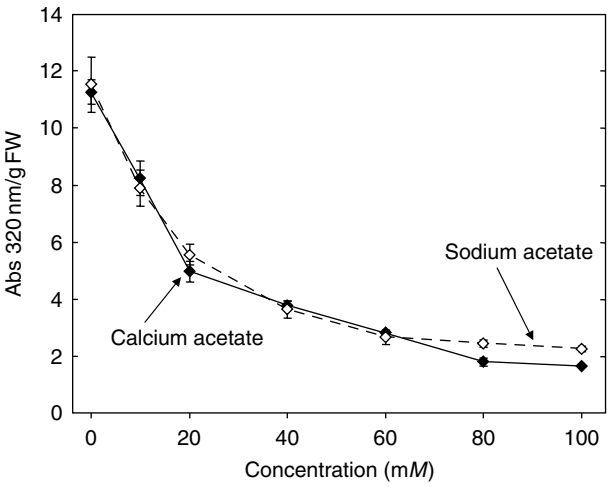

Fig. 5. Phenolic content of excised Romaine mid-rib segments exposed to aqueous solutions of calcium or sodium acetate. Excised 5-mm mid-rib segments (10 g) were immersed in 40 ml of buffered (pH 7) 0–100 m*M* calcium or sodium acetate for 60 min at 20° C. Absorbance of a clarified methanol extract was read 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.

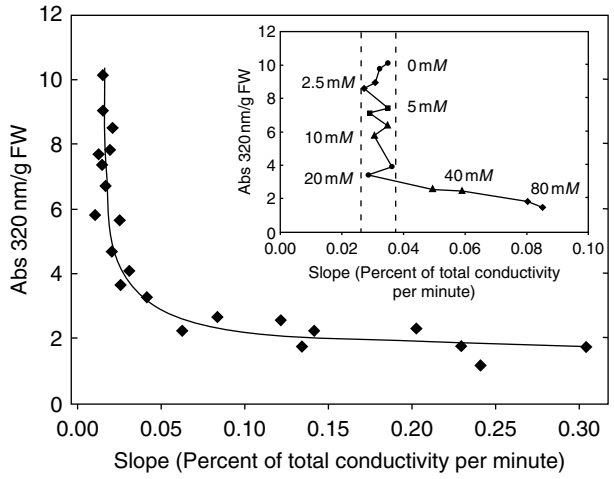

Fig. 6. Phenolic content and rate of ion leakage from excised Romaine mid-rib segments exposed to aqueous solutions of sodium acetate. Excised 5-mm mid-rib segments (10 g) were immersed in 40 ml of 0–80 m*M* sodium acetate at pH 7 for 60 min. Absorbance of a clarified methanol extract was read at 320 nm after holding the tissue for 48 h at 10° C. Slope of ion leakage was calculated from changes in conductivity in a bathing isotonic 0.2 *M* mannitol solution from 0.5 to 2 h.

methanol solutions were made to facilitate preparation of the treatment solutions. The solubility of the C8, C10, and C12 carboxylic acids in 20° C water are 68, 15, and 6 mg, respectively, which equate to concentrations of 4.7, 0.87, and 0.28 m*M*. The sodium salts were far more soluble and using methanol as a bridging solvent allowed preparation of 20 m*M* C10, 1 m*M* C12, 0.6 m*M* C14, and 0.3 m*M* C16. Methanol was used as a bridging solvent, because, at the resultant solution concentration of 25 m*M*, it does not significantly affect WIPA (Choi et al. 2005).

Carboxylates longer than C8 first exhibited a stimulatory and then an inhibitory effect on WIPA. For example, as the concentration of C10 increased from 0 to 1.5 m*M*, there was a complementary quadratic ($R^2 = 0.98$) stimulation in WIPA reaching 30% at 1.5 m*M* (Fig. 8). Increasing C10 concentration further produced a quadratic decline ($R^2 = 0.99$) in WIPA. The stimulatory effect was also seen at low concentrations of C12, C14, and C16 (Fig. 9). The limited solubility of these compounds in the aqueous buffer solution used to apply the treatments prevented the application of higher concentrations that may have followed a similar trend as the shorter, more soluble carboxylates that inhibited WIPA.

Butyrate was less effective at reducing WIPA than would be expected from the effectiveness of the two adjacent compounds. While 20 m*M* butyrate (C4) reduced WIPA by 27%, similar concentrations of propionate (C3) and valerate (C5) reduced WIPA by 62 and 73%, respectively (Fig. 7). This anomaly was confirmed by using butyric acid and sodium butyrate obtained from different sources and at various concentrations and durations of application (data not shown).

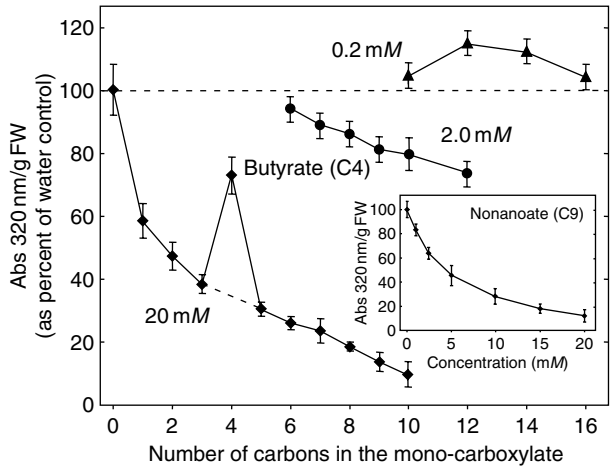

Fig. 7. Phenolic content of excised Romaine mid-rib segments exposed to aqueous solutions of sodium salts of formate (C1) to hexadecanoate (palmitate) (C16). Excised 5-mm mid-rib segments (10 g) were immersed in 100 ml of pH 7 solutions for 60 min. The concentrations were 20 m*M* for C1–C10, 2.0 m*M* for C6–C12, and 0.2 m*M* for C10–C16. Methnaol was used as a bridging solvent for the 2.0 and 0.2 m*M* solutions. Insert for nonanoate (C9) shows the typical form of the curve describing the relation between concentration and Abs 320 nm. Absorbance of a clarified methanol extract was read 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.

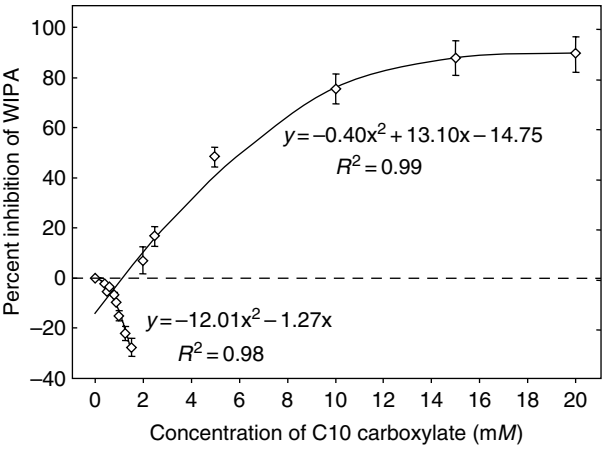

Fig. 8. Phenolic content of excised Romaine mid-rib segments exposed to aqueous solutions of sodium decanoate (C10). Excised 5-mm mid-rib segments (10 g) were immersed in 100 ml of a buffered solution at pH 7 for 60 min. Methanol was used as a bridging solvent. Absorbance of a clarified methanol extract was read 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. WIPA, wound-induced phenolic accumulation.

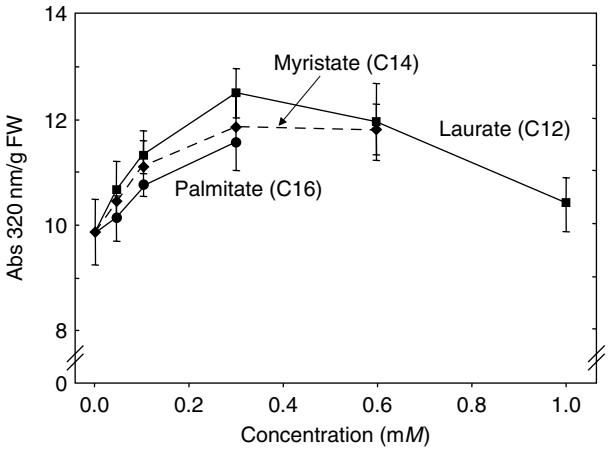

Fig. 9. Phenolic content of excised Romaine mid-rib segments exposed to aqueous solutions of sodium laurate (C12), myristate (C14), or palmitate (C16). Excised 5-mm mid-rib segments (10 g) were immersed in 100 ml of a buffered solution at pH 7 for 60 min. Methanol was used as a bridging solvent. Absorbance of a clarified methanol extract was read 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.

Effectiveness of mono-carboxylates related to isomer

The effectiveness of the C4, C5, and C6 carboxylates was similar for the straight chain molecule and its methyl isomer (Fig. 10). Immersion in 50 m*M* solutions of normal and iso-butyrate reduced WIPA by 63 ± 9%, normal and iso-valerate reduced WIPA by 66 ± 5%, whereas normal and iso-hexanoate reduced WIPA by 74 ± 7%. The slopes (i.e. rates of ion leakage) were similar for water and the butyrates (0.066 ± 0.009% of total conductivity per minute), 26% higher for the valerates (0.083 ± 0.005), and 117% higher for the hexanoates (0.143 ± 0.015). Treatment with 20 m*M* solutions of the three carboxylates also failed to produce significant differences between the isomers, and neither the normal or methyl isomer increased ion leakage (data not shown).

Period of application does not affect WIPA reduction

Immersion in 20 m*M* sodium acetate or hexanoate at pH 7 for 60 min starting immediately after excision (0–1 h) or at 1 h intervals produced the same level of reduction in WIPA whether applied immediately or applied up to 5 h after excision (Fig. 11).

Discussion

Excision (i.e. wounding) of 5-mm segments of romaine mid-rib tissue induced a rise in PAL activity that was detected after 4 h at 10° C, peaked at 18 h, and then declined to near initial levels by 72 h (Choi et al. 2005). After an 8 h delay during which the phenolic content (Abs 320 nm g^{-1} FW) remained relatively constant, it

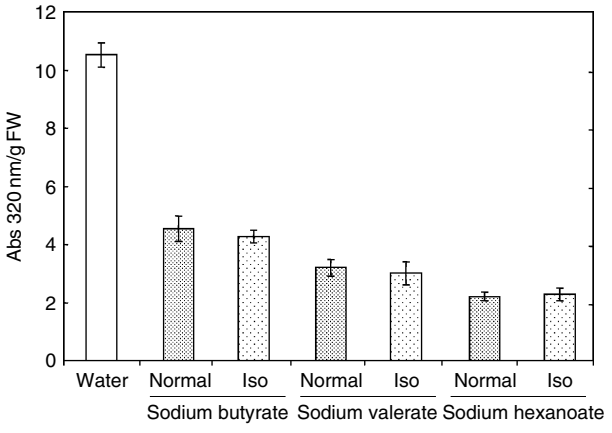

Fig. 10. Phenolic content of excised Romaine mid-rib segments exposed to aqueous solutions of isomers of butyrate, valerate, or hexanoate. Excised 5-mm mid-rib segments (10 g) were immersed in 40 ml of 50 m*M* sodium butyrate (C4), iso-butyrate (2-methylpropionate), valerate (C5), iso-valerate (3-methylbutyrate), hexanoate (C6), and iso-hexanoate (4-methylpentanoate) at pH 7 for 60 min. Absorbance of a clarified methanol extract was read at 320 nm after holding the tissue for 48 h at 10° C. The vertical line atop each bar represents the standard deviation about that mean.

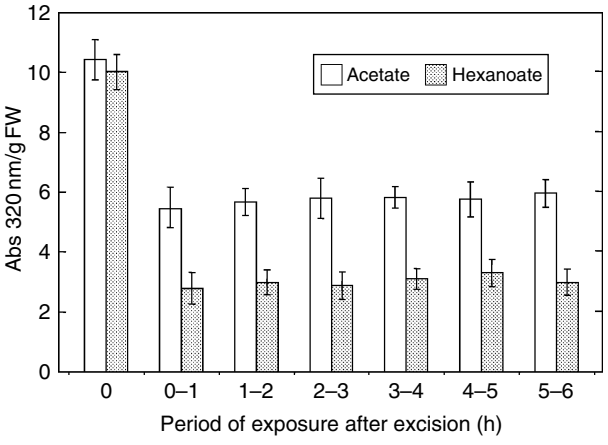

Fig. 11. Phenolic content of excised Romaine mid-rib segments exposed to aqueous solutions of sodium acetate or sodium hexanoate at various times after excision. Excised 5-mm mid-rib segments (10 g) were immersed in 100 ml of buffered 20 m*M* sodium acetate or hexanoate at pH 7 for 60 min starting immediately after excision (0–1 h) and every 60 min thereafter for 5 h (5–6 h). Absorbance of a clarified methanol extract was read at 320 nm after holding the tissue for 48 h at 10° C. The vertical line atop each bar represents the standard deviation about that mean.

increased linearly to 48 h and then remained fairly constant to 72 h after excision. The high correlation ($R^2 = 0.95$) between PAL activity measured after 24 h at 10° C and phenolic content measured after 48 h at 10° C is consistent with previous reports (Campos-Vargas and Saltveit 2002). Absorbance readings (Abs 320 nm) were stable with measurements taken immediately after preparation being highly correlated ($R^2 > 0.99$) with readings of the same samples taken after 24 h at 20° C (data not shown).

Acetic acid was previously shown to be an effective inhibitor of WIPA in lettuce (Tomás-Barberán et al. 1997a). We have expanded that study by showing that C1–C10 mono-carboxylates are effective inhibitors of WIPA (Fig. 7). The mode of application affected the response of the tissue. Exposure of 5-mm mid-rib segments to vapors of formic and acetic acid that would have produced uniform cellular concentrations of 20 m*M* produced significant increases in ion leakage (slope) (Fig. 1), whereas immersion of tissue in aqueous 20 m*M* solutions at Ph 7 did not produce significant increase in ion leakage (Fig. 6). The 20 m*M* concentration in tissue exposed to vapors is a computed average, and compounds applied as vapors would have been at higher concentrations in tissue at the cut surface than in internal tissue. The much higher concentration of formic and acetic acid in cells at the cut surfaces could have produced greater rates of ion leakage than was seen in tissue exposed to similar concentrations as aqueous solutions. For example, the 20 m*M* aqueous solutions would have been at 20 m*M* at the cut surface and lower in cells internal to the cut surface. Also, the vapors were of carboxylic acids, whereas the solutions were pH 7 sodium carboxylates.

The mode of action by which the carboxy group reduces WIPA does not appear to be through an alteration of general metabolism, because the rate of carbon dioxide production was unaffected by compounds and concentrations that significantly suppressed WIPA while increasing ion leakage (Fig. 2). While pH had an effect on the level of WIPA, sodium salts of formate and acetate were effective suppressors at pH values from 3 to 9 (Fig. 4). Calcium salts reduce tissue browning, but there was no added benefit of calcium over sodium acetate in reducing WIPA (Fig. 5). The suppression of WIPA occurred at concentrations of sodium acetate that did not increase ion leakage over that of the water control (Fig. 6).

Except for butyrate, the effectiveness of the mono-carboxylates increased with increasing number of carbons in the molecule (Fig. 7) or molecular weight (data not displayed in that manner). Increasing chain length should have increased lipid solubility and may have facilitated penetration of the compound into the cell. A similar increase in effectiveness of n-alcohols to reduce WIPA with increasing chain length from C2 to C7 has been reported (Choi et al. 2005). They proposed that the n-alcohols were interfering with wound signal production through the jasmonic acid pathway by reacting with PLD to form the inactive phosphatidic alcohol instead of phosphatidic acid (PA). Their line of reasoning was supported by the fact that n-alcohols above C7 were ineffective, that the 2- and 3-isomers of the effective alcohols were ineffective, and that the effectiveness of the alcohols was reduced if their application was delayed for even 1 h after excision. A wound signal is rapidly produced at the site of injury and moves into adjacent tissue at a speed of 5 mm h^{-1} (Ke and Saltveit 1989). A delay in the application of inhibitors of the wound signal would reduce their effectiveness in suppressing the wound response, because a portion of the signal would have been produced and would have induced increased phenolic metabolism in adjacent tissue. Compounds that interfere with other aspects of the synthesis and accumulation of wound-induced phenolic compounds would not be so affected by delays in their application.

Three lines of evidences suggest that the inhibitory effect of mono-carboxylates on WIPA is not acting through the synthesis or propagation of a wound signal generated by the oxylipin biosynthetic pathway. Unlike the n-alcohols, the effectiveness of the molecules containing the carboxyl group increased with increasing number of carbons beyond the eight at which the n-alcohols became ineffective (Fig. 7); the methyl isomers of the effective carboxylates were equally effective at reducing WIPA as were the straight chain molecules (Fig. 10), and their effectiveness in reducing WIPA was not diminished by delaying application for up to 5 h after excision (Fig. 11).

Sequential events in the jasmonate-signaling cascade have been proposed to proceed through calcium activation of protein kinases and cytosolic acidification (Suhita et al. 2004). Undissociated weak acids, such as mono-carboxylic acids, partitions into the lipid phase of the cell and rapidly enter the cell, predominantly as the undissociated acid (Reid et al. 1989). The degree of partitioning is dependent on the length of the lipophilic carbon chain (Wilson et al. 2000). In the cytoplasm, they dissociate to yield high concentrations of the carboxylate anion which causes a rapid reduction in cytoplasmic pH.

The carboxylic acids are weak acids and not highly dissociated in dilute aqueous solutions (a 20 m*M* acetic acid solution has a pH of 3.2, and only 3% is dissociated). As the pH of the aqueous solution of a mono-

carboxylic acid is neutralized by the addition of NaOH, more of the acid becomes dissociated until it is all dissociated when an equal molar amount of NaOH has been added, and the pH is at its pKa value. Apart from formic and acetic acids which have a pKa of 3.75 and 4.76, respectively, the remaining C3–C10 mono-carboxylic acids have an average pKa of 4.86 ± 0.03. Hence, at a pH of 7.0, all the mono-carboxylic acids would be completely dissociated. The reduction in efficacy of formic and acetic acids as the pH of the treatment solution was increased from 3 to 7 (Fig. 5) probably reflects more rapid penetration of the cell by the larger percentage of undissociated forms of these molecules at the more acidic pH.

It is unlikely that acidification alone caused the reduction in WIPA. While we did not measure the cell's internal pH, cellular acidification caused by external carboxylic acid solutions of pH 5 is small (<0.3 pH units) and transient. Changes in pH recovered within an hour for *Sinapis alba* (L) root hairs treated with 10 m*M* acetic acid (Herrmann and Felle 1995) or suspension cultures of tobacco BY-2 cell exposed to 2 m*M* butyric acid (Tena and Renaudin 1998). Induced cellular acidification could not have reduced the activity of wound-induced PAL, because it takes a few hours after excision before wound-induced PAL is synthesized *de novo* and fully 24 h before it reaches maximum activity (Campos-Vargas et al. 2004).

Other enzymes involved in enzymatic browning are present in the tissue before wounding and treatment. However, polyphenol oxidase from lettuce (PPO; EC 1.14.18.1) and cabbage (EC 1.14.18.1; PPO) and cabbage (*Brassica oleracea*) (EC 1.10.3.1) have broad optimal pH ranges (5–8) (Fujita et al. 1995, Heimdal et al. 1994), and cabbage peroxidase (POD; EC 1.11.1.7) had a similar broad pH optimum and was more stable than PPO (Fujita et al. 1995). Peroxidase activity in lettuce also had a broad pH optimum (5–7) when assayed with the most abundant phenolic compounds in lettuce (caffeic and chlorogenic) (Bestwick et al. 1998, Tomás-Barberán et al. 1997b).

Some cellular processes (e.g. stress-induced calcium gradients) depend on the presence of pH gradients. The pH gradient and the underlying calcium gradient can be dissipated by treatment with membrane-permeant weak acids such as propionic acid (Gibbon and Kropf 1994). Changes in cellular pH alone or in conjunction with altered calcium levels modulated the activity of membrane transport proteins (Grabov and Blatt 1997), PAL activity, and the production of secondary metabolites (Crawford et al. 1994, Hagendoorn et al. 1994, He et al. 1998) and MAP kinases (Tena and Renaudin 1998, Morris 2001). However, in these tissues, induced acidification stimulated, rather than diminished PAL activity and the production of secondary metabolites (Crawford et al. 1994, Hagendoorn et al. 1994, He et al. 1998).

The reduced effectiveness of butyrate (C4) compared with either propionate (C3) or valerate (Fig. 7) was unexpected and is difficult to explain. The solubility of butyrate is consistent with its molecular structure. The pKa is almost the same for propionic, butyric, and valeric acids (they are 4.87, 4.82, and 4.82, respectively). Sodium butyrate is completely miscible in water as is sodium propionate and much more soluble than valeric acid (5 g/100 ml water at 20° C), yet its effectiveness is significantly less (27% reduction in WIPA for 20 m*M*) than these two adjacent straight chain mono-carboxylic acids (63% for propionate and 73% for valerate) (Fig. 7). Butyrate has various effects in plants: it stimulated the activity of phosphoenolpyruvate carboxykinase in cucumber plants (Chen et al. 2004), whereas it inhibited stomatal closure caused by ABA or methyl jasmonate in *Arabidopsis* (Suhita et al. 2004).

Treatment of excised tissue with effective concentrations of mono-carboxylate inhibitors of WIPA (e.g. 20 m*M* sodium acetate) were equally effective whether applied immediately after excision or after a delay sufficient to allow synthesis and propagation of the wound signal (Fig. 11). This suggests that the mono-carboxylates are affecting events subsequent to the actual synthesis of the wound signal, possibly the induction of wound-induced PAL activity, membrane permeability, or phenolic synthesis, accumulation, or oxidation. The mode of action by which the mono-carboxylic acids and their salts inhibits WIPA awaits clarification.

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