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# Involvement of putative chemical wound signals in the induction of phenolic metabolism in wounded lettuce

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**Cutting leaves of Romaine lettuce (*Lactuca sativa* L. cv. Longifolia) produces a wound signal that induces the synthesis of phenylalanine ammonia lyase (PAL, EC 4.3.1.5) and the accumulation of phenolic compounds in cells up to 2 cm from the site of injury, and tissue browning near the site of injury. The response of leaves within a head of Romaine lettuce to putative chemical wound signals [abscisic acid (ABA), jasmonate (JA) and methyl jasmonate (MeJA)] differed significantly with leaf age. Exposure of harvested heads of lettuce to ABA, JA, MeJA, or salicylic acid (SA) did not induce changes in PAL activity, the concentration of phenolic compounds or browning in mature leaf tissue that was similar to the level induced by wounding. Methyl jasmonate applied as vapour (10, 100 or 1000 $\mu l\,kg^{-1}$ FW), or as an aqueous spray or dip (0.01–100 $\mu M$) at 5 or 10°C did not produce an effect on PAL activity or browning that differed significantly from the untreated controls. In contrast, JA, MeJA and SA did induce elevated levels of PAL activity in younger leaves. However, the levels induced were far lower than those induced by wounding. Wound induced phenolic metabolism in mature leaves appears to be induced by different signals than those functioning in young leaves.**

## Introduction

There are two classes of putative wound signals in plants: (1) chemical compounds transmitted in the phloem or xylem (Peña-Cortes et al. 1995), and (2) hydraulic (Malone and Alarcon 1995), and bio-electrical waves (Wildon et al. 1992, Peña-Cortes et al. 1995). Jasmonates [methyl jasmonate (MeJA) and jasmonic acid (JA)], abscisic acid (ABA), ethylene, and salicylic acid (SA) are considered to be some of the primary chemical wound signals (León et al. 2001). MeJA, which is a natural constituent of the fragrance spectrum of plants [i.e. *Jasminum grandiflorum* (reviewed by Reinbothe et al. 1994)], appears to be part of the wound signal complex (Creelman et al. 1992, Farmer and Ryan 1990, Peña-Cortes and Willmitzer 1995), as well as having different physiological functions in plants (reviewed by Creelman and Mullet 1997). Exogenous application of MeJA induces an increase in the mRNA of wounded-induced genes (Creelman et al. 1992), such as phenylalanine ammonia lyase (PAL, McConn et al. 1997, Taguchi et al. 1998), that is similar to that of wounding. Conversely, MeJA has also been reported to decrease the browning of cut celery and green bell peppers (Buta and Moline 1998). The involvement of ABA is implicated in some wound responses, but its function as a primary signal has recently been questioned (Birkenmeier and Ryan 1998). JA and SA are both important components of the signal pathway and can interact to promote or inhibit wound responses (Preston et al. 1999, Shenk et al. 2000). However, most of the research on these compounds has been done with cell cultures or young plants. Mature differentiated tissues and organs often have a different physiological response to stress than immature and undifferentiated tissues and organs.

Mechanical wounding (i.e. abrading, crushing, cutting) induces an increase in the production of wound repair and defense compounds (Saltveit 1997). For example, wounding induces an increase in the synthesis and activity of PAL and other enzymes involved in phenylpropanoid metabolism such as polyphenol oxidases

*Abbreviations* – 1-MCP, 1-methylcyclopropene; ABA, abscisic acid; MeJA, methyl jasmonate; PAL, phenylalanine ammonia lyase; POD, peroxidase; PPO, polyphenol oxidase; SA, salicylic acid.

(PPO) and peroxidases (POD) (Ke and Saltveit 1989a). The response of wounded plant tissue is analogous to the physiological changes induced in minimally processed fruits and vegetables (Brecht 1995, Saltveit 1997). Increased levels of phenolic compounds are synthesized and accumulated in lettuce tissue following wounding and exposure to hormonal levels of ethylene (Tomás-Barberán et al. 1997).

The undesirable development of tissue browning follows the accumulation of the newly synthesized phenolic compounds (Couture et al. 1993, Lopez-Galvez et al. 1996) and causes a significant loss of quality in minimally processed lettuce (Lopez-Galvez et al. 1996). The rise in PAL activity precedes the accumulation of phenolic compounds and browning in wounded tissue (Peiser et al. 1998). Depending on the temperature, the accumulation of phenolic compounds and browning lags behind the rise in PAL activity by 4–24 h (Ritenour et al. 1995).

Experiments reported in this paper were done to study the effect of exposure to jasmonates and other putative wound signals on the activity of phenolic metabolism in harvested lettuce and to compare the physiological effects of exogenous applications of such compounds to that of wounding. We show that phenylpropanoid metabolism is enhanced in young leaves, but not in mature leaves by some of these wound signals, but that the level of induction is far less than that induced by mechanical wounding.

## Materials and methods

### Plant material

Heads of Romaine lettuce (*Lactuca sativa* L. cv. Longifolia) were obtained from commercial sources, transported to the University of California, Davis, USA, and held at 0.5°C until used. Whole heads, uninjured leaves, or excised tissues were used in the experiments. In some experiments leaves were segregated into size classes of 7 $\pm 2$, 14 $\pm 2$, and 21 $\pm 2$ cm in length. Wounding was applied by cutting the achlorophyllous (i.e. white) midrib tissue into $10 \times 10$-mm pieces with a stainless steel razor blade. Tissue was immediately assayed for PAL activity or held in a dark or light, humid atmosphere at 5, 10 or 25°C until analysed.

### Application of treatments

Tissue for analysis was excised 4 cm from the base of leaves from whole heads or from previously excised leaves to prevent wounding from the initial excision from affecting the results. In preliminary experiments we found that the wound signal did not stimulate phenylpropanoid metabolism beyond 3 cm from the cut surface. Unless indicated, all chemicals were purchased from Sigma, St Louis, MO, USA.

The kinetics of wound-induced changes in PAL activity were studied by periodically assaying wounded midrib (10 x 10 mm) tissue for PAL after holding at 5, 10 and 25°C for up to 6 days.

Exogenous application of methyl jasmonate (MeJA, Aldrich, Milwaukee, WI, USA) was by solution and vapour. Freshly cut midrib tissue from mature leaves were dipped in aqueous 0.01, 1.0 or 100 $\mu M$ solutions of MeJA containing 0.05% Tween 20 for 4 min at 18°C, and then held at 5°C in a flow of humidified, ethylene-free air for up to 72 h. Pieces of midrib dipped in water plus surfactant without MeJA were the control. Whole heads of lettuce (approximately 0.5 kg) were exposed to vapours from 10 to 1000 µl MeJA $kg^{-1}$ FW in 17-l glass containers at 10°C for 24 h. Controls were either non-injured heads or leaves, or wounded lettuce leaf tissue held in air at 10°C. After exposure, all tissue was held in humidified, ethylene-free air at 10°C.

Whole mature leaves were sprayed to run-off with an aqueous 100 $\mu M$ solution of MeJA containing 0.05% Tween 20. Controls consisted of uninjured leaves sprayed with water plus surfactant. POD, PPO and PAL activity were assayed. In a parallel experiment, enough 1-methylcyclopropene (1-MCP, Biotechnologies for Horticulture, SC, USA) was added to the containers before sealing to give a physiological effective concentration of 0.5 µl $l^{-1}$ in 17-l glass containers. After 6 h of 1-MCP treatment, the leaves were cut in 1 cm pieces and stored at 10°C for 24 h and assayed for PAL, POD and PPO. Pieces of wounded lettuce without 1-MCP exposure were the control. Another group of whole non-wounded leaves were preincubated for 6 h with 1 µl $l^{-1}$ of 1-MCP and then sprayed with a 50-$\mu M$ aqueous solution of MeJA. The absorbance of methanolic extracts from the non-wounded control and the sprayed leaves were compared after 8 h at 25°C.

Whole mature leaves were sprayed to run-off with an aqueous solution of abscisic, salicylic and jasmonic acid and methyl jasmonate at concentrations of 1, 10, 100 or 1000 $\mu M$. The leaves were stored at 10°C for 2 days after treatment and analysed for changes in the amount of phenolic compounds.

Treatments designed to inhibit the synthesis of jasmonates were performed as follows: mature lettuce leaf bases were stood for 8 h in light conditions, in 200 ml of a 10-m$M$ phosphate buffer (pH 7) containing 50 $\mu M$ *n*-propyl gallate at 25°C in a 40-l plastic container. PAL activity was measured in tissue excised from the leaf bases after 8 h.

Accumulation of chlorogenic acid was studied by spraying whole mature leaves to run-off with aqueous solutions of 10 $\mu M$ ABA or SA, or 10, 100 or 1000 $\mu M$ MeJA. The leaves were stored at 10°C for 2 days prior to extraction and quantification of chlorogenic acid by HPLC (see below).

Leaves of different maturities were excised and sorted into three size classes of 21 $\pm 2$ cm (large, mature, fully expanded), 14 $\pm 2$ cm (medium, expanding), 7 $\pm 2$ cm (small, rapidly expanding) in length. They were sprayed to run-off with aqueous solutions of 1.0 or 0.1 m$M$

MeJA, JA, SA or ABA containing 0.05% Tween 20. Leaves were stored at 10°C for 24 h before PAL activity was assayed. In other experiments, small leaves (7 ± 2 cm in length) attached to the stem were sprayed to run-off with the same solutions mentioned above. PAL activity was analysed after 24 h at 10°C.

### Enzyme assay

Midrib tissue was used for enzyme assays. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity was measured as previously described by Ke and Saltveit (1986), with some modifications. Briefly, 4 g of tissue was homogenized with an Ultra-Turrax (Takmar, Cincinnati, OH, USA) (high speed for 5 min) with 16 ml of 50 m*M* borate buffer (pH 8.5) containing 5 m*M* 2-mercaptoethanol and 0.4 g polyvinylpolypyrrolidone. The homogenate was filtrated through 4 layers of cheesecloth and centrifuged at 20 000 *g* at 4°C for 20 min. The supernatant was assayed for PAL activity after the addition of 0.55 ml of 50 m*M* L-phenylalanine and incubated at 40°C for 30 min. The absorbance was measured at 290 nm before and after incubation. One unit of PAL activity equals the amount of PAL that produced 1 μmol of cinnamic acid in 1 h, and is expressed as $\mu mol\,g^{-1}\,FW\,h^{-1}$.

Polyphenol oxidase (PPO, EC 1.10.3.2) was extracted as described by Sirinphanic and Kader (1985), with modifications performed by Loiza-Velarde et al. (1997). Briefly, 4 g of tissue was ground as described above, except that 12 ml of 50 m*M* phosphate buffer (pH 6.5) was used instead of citrate phosphate buffer. The homogenates were filtered and centrifuged as previously described. PPO activity was assayed as reported by Couture et al. (1993), 0.5 ml of enzyme extract was incubated with 0.2 ml of 0.1 *M* caffeic acid ethanolic solution and the absorbance was recorded at 480 nm over a period of 5 min. The reaction rate was calculated using the linear portion of the curve. The enzymatic activity was expressed as a percentage of the control (Loiza-Velarde et al. 1997).

Peroxidase (POD, EC 1.11.1.7) activity was assayed as described by Loiza-Velarde et al. (1997), 4 g of lettuce tissue was ground in 12 ml 50 m*M* phosphate buffer (pH 6.2), filtered and centrifuged, similarly to PPO quantification. The reaction mixture was 2.55 ml 50 m*M* phosphate buffer, 0.1 ml enzyme extract, 0.25 ml 0.1 *M* guaiacol and 0.25 ml of 0.25% $H_2O_2$. The quantification of POD activity was done as for PPO, but the absorbance was read at 420 nm.

### Chlorogenic acid assay

The chlorogenic acid analyses were performed based on Tomás-Barberán et al. (2001, in press) with modifications. A description of the method is as follows. Frozen material (5 g) was ground using the Ultra Turrax homogenizer (high speed for 5 min) with 10 ml of water-methanol (2:8) solution containing 2 m*M* NaF. The homogenates were centrifuged at 20 000 *g* at 5°C for 20 min. The supernatant was filtered (0.45 μm, Osmonics/MSI Cameo Nylon Filters, Fisher, CA, USA) before analysis. The samples were directly injected into a Hewlett Packard HPLC system with photodiode array detector (DAD model 1040M, Series II). The separation was conducted with a Nucleosil C-18 reverse phase column (150 × 4.6 mm; 5 μm particle size) (MetaChem Technologies, Inc. Torrance, CA, USA). The mobile phase was 5% methanol in water with 5% formic acid (solvent A), water and methanol (88:12) and 5% formic acid (solvent B) and water and methanol (20:80) and 5% formic acid (solvent C) and methanol (solvent D). The flow rate of the mobile phase was $ml/min^{-1}$. A composite gradient system was used: 0–5 min 100% A; 5–10 min a gradient to reach 100% B; 10–13 min isocratic with 100% B; 13–35 min a linear gradient was used to reach 75% B and 25% C, then 35–50 min to obtain 50% B and 50% C; and 50–52 min to get 100% C; 52–57 min was maintained isocratic with 100% C; the next 57–60 min was isocratic with 100% D for washing the column and then 60–65 min 100% A (equilibration). The chromatograms were recorded at 340 nm. This wavelength was selected after running a spectrum analysis of an authentic standard of chlorogenic acid. All the analyses were performed in triplicate.

### Phenolic compounds analysis

The concentration of phenolic compounds was measured as described by Ke and Saltveit (1988). Briefly, 10 g of tissue were ground in 20 ml of HPLC grade methanol with the Ultra-Turrax tissue homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15000 *g* for 20 min. As described by Loiza-Velarde et al. (1997), the absorbance of an aliquot of the supernatant was read at 320 nm using an UV-VIS spectrophotometer (Shimadzu UV-160 A, Scientific Instruments, Columbia, MD, USA).

### Ethylene and respiration analysis

Lettuce heads that had been treated with different concentrations (10, 100, or 1000 $\mu l\,kg^{-1}$ FW) of MeJA vapour at 10°C, were enclosed in 17-l glass containers. Head space gas samples (1 ml) were taken after 1 h for carbon dioxide and ethylene analyses. An infrared analyser (PIR 2000, Horiba) was used for respiration measurements (Saltveit and Strike 1989), while a gas chromatograph (Model 8000, Carle Instruments, HACH/CARLE, Loveland, CO, USA) equipped with alumina column and a flame ionization detector was used for measuring ethylene (Saltveit and Yang 1987).

### Statistics

Each experiment was repeated at least twice with similar results. All treatments were replicated at least twice within each experiment. Means and standard errors were calculated from pooled data. When present in the figure,

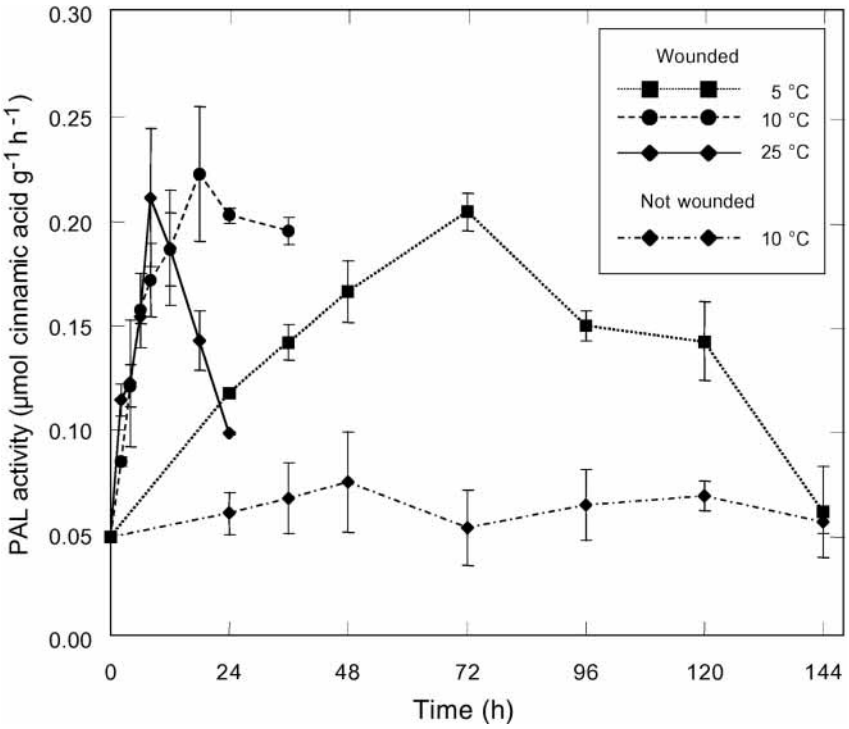


Fig. 1. Phenylalanine ammonia-lyase (PAL) activity in excised 10 × 10 mm midrib pieces of Romaine lettuce. The tissue was immediately assayed for PAL or held at 5, 10 or 25°C in air until analysed. Values are means ± SE (n = 3).

the vertical line associated with each point or on the top of each bar represents the standard error.

## Results and discussion

### PAL activity and browning

Wounding midrib tissue of harvested Romaine lettuce induced a rapid increase in PAL activity (Fig. 1). This increase was most evident at 25°C, where the maximum was reach 8 h after wounding. The maximum was reached later at lower temperatures, e.g. after 18–24 h at 10°C, and 72 h at 5°C. The reduction in maximal PAL activity with increasing temperature that was reported for wounded lettuce leaves (Lopez-Galvez et al. 1996) or for lettuce leaves exposed to ethylene (Hyodo et al. 1978, Ritenour et al. 1995) was not observed in these experiments. These differences may have been due to variety dependant characteristics; we used Romaine lettuce while iceberg (i.e. crisphead) lettuce was used in the other studies. However, we did notice that there was a rapid decline from maximum PAL activity with increasing temperature as previously reported (Lopez-Galvez et al. 1996). The decrease in PAL activity may have resulted from reduced PAL synthesis, or from the increased activity of a PAL inactivating factor at the higher temperatures (Tan 1980, Ritenour and Saltveit 1996).

PAL is the first committed step in phenylpropanoid metabolism and it catalyses the deamination of L-phenylalanine to produce trans-cinnamic acid and ammonia (Koukol and Conn 1961). An increase in PAL activity is the key to production of many defense compounds (Dixon and Paiva 1995). There is a consistent increase in the concentration of phenolic compounds following the wound-induced increase in PAL activity (Ke and Saltveit 1989b). The major phenolic compounds produced by wounded and stressed lettuce were characterized by Tomás-Barberán et al. (1997). They include 5-caffeoylquinic (chlorogenic acid), 3,5-dicaffeoylquinic (isochlorogenic), caffeoyltartaric and dicaffeoyltartaric acid. Phenolic compounds are involved in lignin synthesis and the strengthening of cell walls (Ke and Saltveit 1989a), and the production of phytoalexins (Dixon and Paiva 1995). However, another fate for these phenolics is their oxidation and production of slightly coloured compounds (i.e. quinones) by the action of PPO and POD (Ke and Saltveit 1989a). These quinones can polymerize to produce coloured compounds typically seen in browning processes (Amiot et al. 1997).

The rise in PAL activity from 8 to 24 h after wounding at 10°C preceded the accumulation of phenolic compounds (absorbance of a methanolic extract at 320 nm) measured 12 h later (Fig. 2). Within these time intervals, PAL activity is given by the linear equation: PAL activity = [0.606 × absorbance at 320 nm (measured 12 h later)] − 0.497, with an $R^2$ of 0.98 (Fig. 3). The relationship between PAL activity and the absorbance of a methanol extract taken from tissue 12 h later was periodically confirmed in subsequent experiments. The major phenolic compound accumulated in wounded iceberg lettuce is chlorogenic acid (Tomás-Barberán et al. 1997) and it increased over 14-fold (0.58–8.21 $\mu g\,g^{-1}$ FW) 48 h after wounding (Fig. 4).

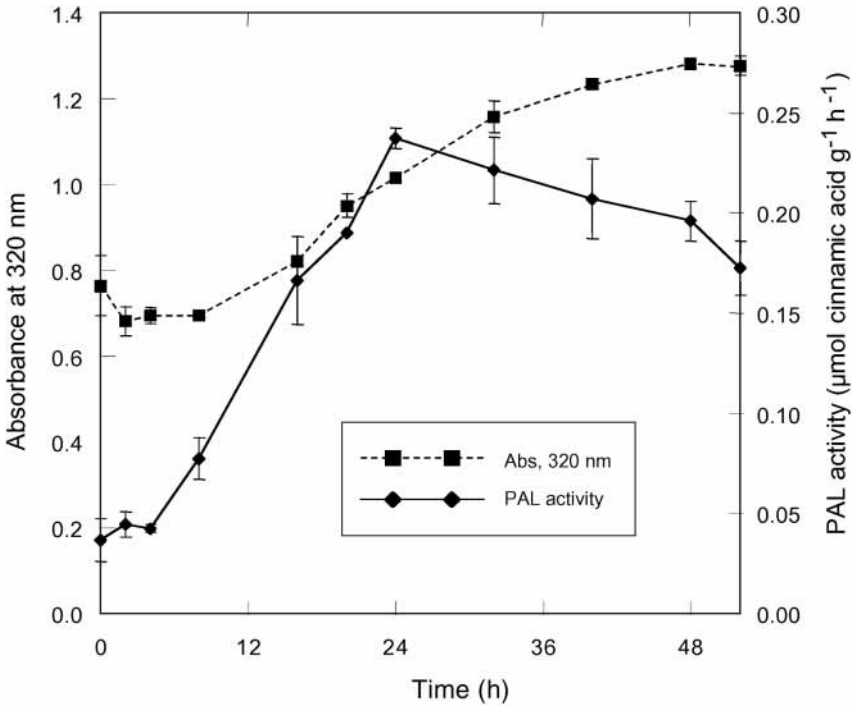


Fig. 2. Phenylalanine ammonia-lyase (PAL) activity and the absorbance of a methanol extract (abs 320 nm) taken from 10 × 10 mm midrib pieces of Romaine lettuce held at 10°C for varying lengths of time. Values are means ± SE (n = 3).

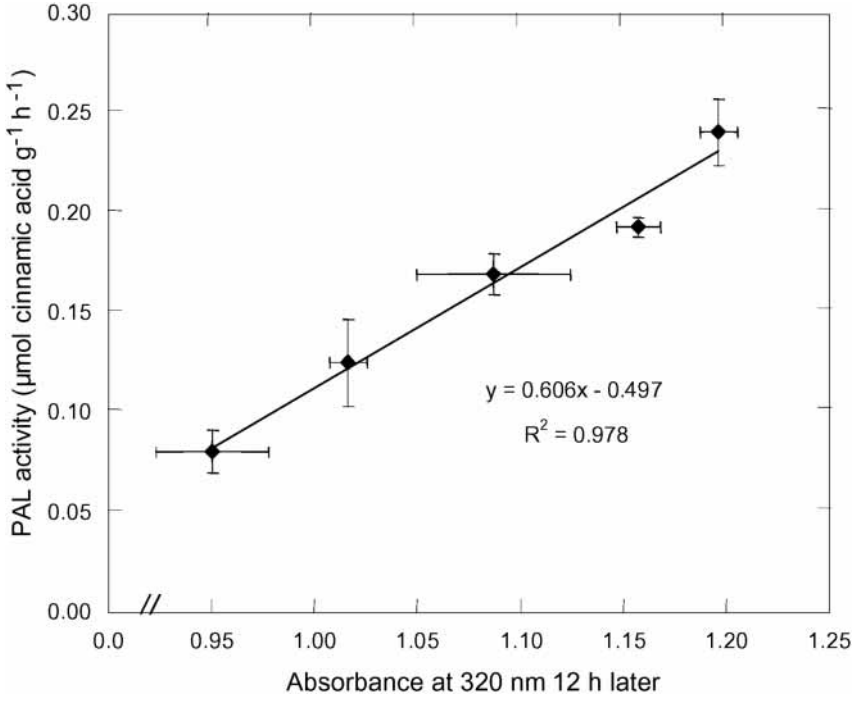


Fig. 3. Correlation between wound-induced phenylalanine ammonia-lyase (PAL) activity and the absorbance of a methanol extract (abs 320 nm) taken 12 h later from 10 × 10 mm midrib pieces of Romaine lettuce held at 10°C for varying lengths of time. Values are means ± SE (n = 3).

### Effect of methyl jasmonate and ethylene on phenolic metabolism

Spraying whole, non-wounded mature lettuce leaves with 100 µM MeJA did not induce significant changes in PAL activity or other enzymes involved in phenolic oxidation (e.g. POD and PPO) compared with the controls (Fig. 5). These results are different from the findings of Ellard-Ivey and Douglas (1996) with parsley cell cultures, or of Gundlach et al. (1992) with soybean cell cultures. In both of their reports, treatment with MeJA induced PAL transcription, and in the case of soybean, PAL activity was correlated with the increase of PAL mRNA. There could be differences among species ac-

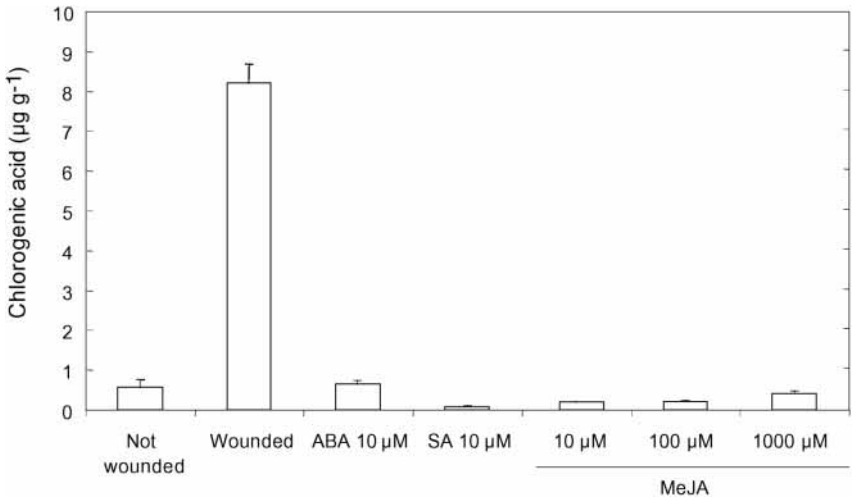


Fig. 4. Concentration of chlorogenic acid extracted from 10 × 10 mm midrib pieces of Romaine lettuce held at 10°C in air for 48 h. Whole mature leaves were treated with 10 µ*M* ABA and SA, or 10, 100 or 1000 µ*M* MeJA. Values are means ± SE (n = 3).

counting for this variability. Walker-Simmons and Ryan (1977) showed that proteinase inhibitor activity did not increase in lettuce tissue after application of a 'wound hormone' (i.e. a proteinase inhibitor-inducing factor) as it did in plants from many of the other families assayed. However, another explanation is that there are differences in response with developmental changes (e.g. between mature, fully expanded leaves and immature, rapidly expanding leaves or suspension cultures of undifferentiated cells). Thipyapong and Steffens (1997) reported MeJA and JA induced PPO/F promoter in young tomato leaves, but not in older ones.

There are reports that describe an interaction or synergy between MeJA and ethylene in the induction of specific events in plants (Emery and Reid 1996, Penninckx et al. 1998). In order to examine the possible interaction between MeJA and ethylene, whole, non-wounded lettuce leaves were exposed to 0.0 or 0.5 µl$l^{-1}$ of 1-MCP (an inhibitor of the ethylene receptor, Sisler et al. 1996) 6 h before wounding (Fig. 5). There was no statistical difference in PAL, POD, or PPO activity among the treatments. The lack of a response may have been because there was insufficient 1-MCP to totally block the ethylene receptor, but that is unlikely since treatment of carnation flowers with 0.5% of the highest concentration we used (i.e. 2.5 n$l^{-1}$ of 1-MCP for 6 h) was effective in protecting the flowers from the effects of ethylene for 4 days (Sisler et al. 1996).

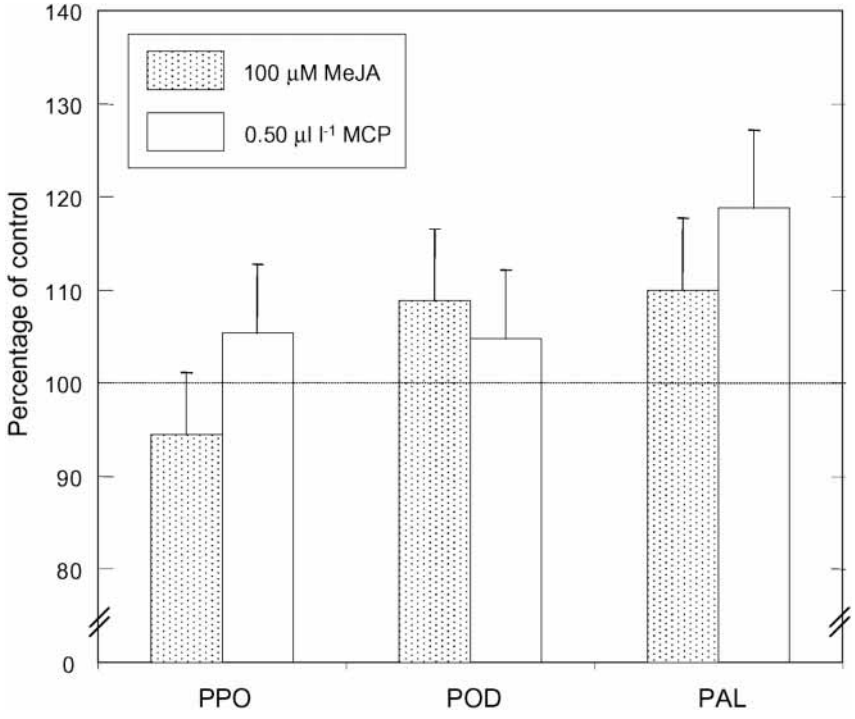


Fig. 5. Comparison of phenylalanine ammonia lyase (PAL), peroxidase (POD), and polyphenol oxidase (PPO) activities in extracts from midrib tissue taken from non-wounded lettuce leaves treated with 100 µ*M* MeJA or from wounded leaves treated with 0.50 µl$l^{-1}$ of 1-MCP. The non-wounded leaves were sprayed with 100 µ*M* MeJA 24 h before being sampled. non-wounded leaves were exposed to 1-MCP for 6 h before the midrib tissue was excised and held for 24 h. Tissue in each treatment was stored at 10°C for 24 h. The results are expressed in percentage of the control. Values are means ± SE (n = 3).

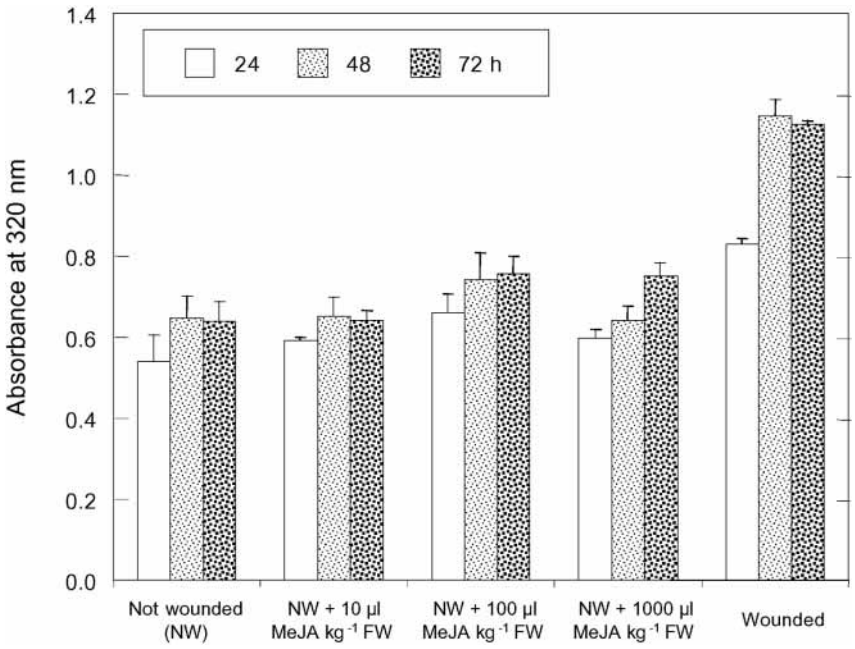


Fig. 6. Absorbance of a methanol extract (abs 320 nm) taken from 10 × 10 mm midrib pieces of Romaine lettuce. Whole heads of lettuce (approximately 0.5 kg) were exposed to 10–1000 µl methyl-jasmonate (MeJA) $kg^{-1}$ FW as vapour in a 17-l glass container at 10°C for 24 h. Controls consisted on uninjured heads or wounded lettuce tissue held in air at 10°C. After exposure, heads were subsequently held in air at 10°C. Values are means ± SE (n = 3).

Our second approach was to investigate the combined effect of MeJA and ethylene on wound-induced phenolic metabolism. Whole non-wounded leaves were preincubated for 6 h with 1 µl $l^{-1}$ of 1-MCP and then sprayed with a 50-µ*M* aqueous solution of MeJA. The absorbance of methanolic extracts from non-wounded control tissue, and tissue exposed to 1-MCP or MeJA was similar for all three treatments (i.e. 0.114 ± 0.005 OD 320 nm). The combination of 1-MCP and MeJA increased the accumulation of phenolic compounds by 17% compared with the controls (0.145 ± 0.006 vs 0.120 ± 0.005), but the increases were much smaller than the doubling seen in wounded tissue (e.g. 0.23 ± 0.01). The lack of an effect by blocking ethylene receptors are in accordance with results obtained by Ke and Saltveit (1989b) who used a kinetic analysis to show that ethylene is not part of the wound signal that stimulates PAL in lettuce. They showed that while continuous exposure to hormonal levels of ethylene induces elevated PAL activity, wound-induced ethylene synthesis is too low and transitory to increase PAL activity in lettuce. In addition, Tomás-Barberán et al. (1997) showed that ethylene exposure did not mimic the effect of wounding on either the amount of phenolics produced or on the kinetics of their synthesis in comparison to wounded tissue. Differences in tissue response to wounding and to ethylene is also shown by the observation that both ethylene and wounding stimulate phenylpropanoid metabolism and the accumulation of phenolic compounds in lettuce, but only ethylene also induces a physiological disorder called russet spotting. In this disorder the accumulated phenolic compounds give rise to oval brown lesions on the leaves (Hyodo et al. 1978, Ke and Saltveit 1989a).

One important concern with the exogenous application of compounds is whether the treatment increased endogenous concentrations over a specific threshold to trigger a response. We used MeJA vapour to ensure uniform distribution and penetration of the leaf tissue. Exposure of whole, non-wounded heads of lettuce to 10, 100, or 1000 µl MeJA $kg^{-1}$ FW vapour for 24 h did not induce any significant difference in phenolic compounds between the treated and control tissue when assayed after 24, 48 or 72 h (Fig. 6). During that period, the phenolic concentration in wounded tissue increased about 75%, while it only increased a non-significant 17% in lettuce treated with 100 or 1000 µl MeJA $kg^{-1}$ FW. Since chlorogenic acid showed the highest accumulation after wounding in Romaine, Butter leaf and iceberg lettuce cultivars (Tomás-Barberán et al. 1997), we focused our analyses on that compound.

Wounding induced a 14-fold increase in the concentration of chlorogenic acid, while there was no significant change induced by exposure of non-wounded leaves to 10, 100 or 1000 µ*M* MeJA as previously described (Fig. 4). These results are similar to those derived from measurement of the absorbance of methanol extracts at 320 nm (Fig. 6), and show that the accumulation of simple phenolic compounds is a significant part of the processes triggered by wounding (Dixon and Paiva 1995). These results again show the minimal effect of MeJA on phenylpropanoid metabolism in mature Romaine lettuce leaves.

The rate of respiration and ethylene synthesis were measured after exposing whole heads of lettuce to MeJA vapour (data not shown). Since MeJA induces the synthesis of the proteinase inhibitor (Pin) in species of the Solanaceae and Fabaceae (Farmer and Ryan 1990, Farmer et al. 1992), it may also produce other changes in metabolism that would be reflected in a rise in respiration. However, MeJA vapour did not induce an in-

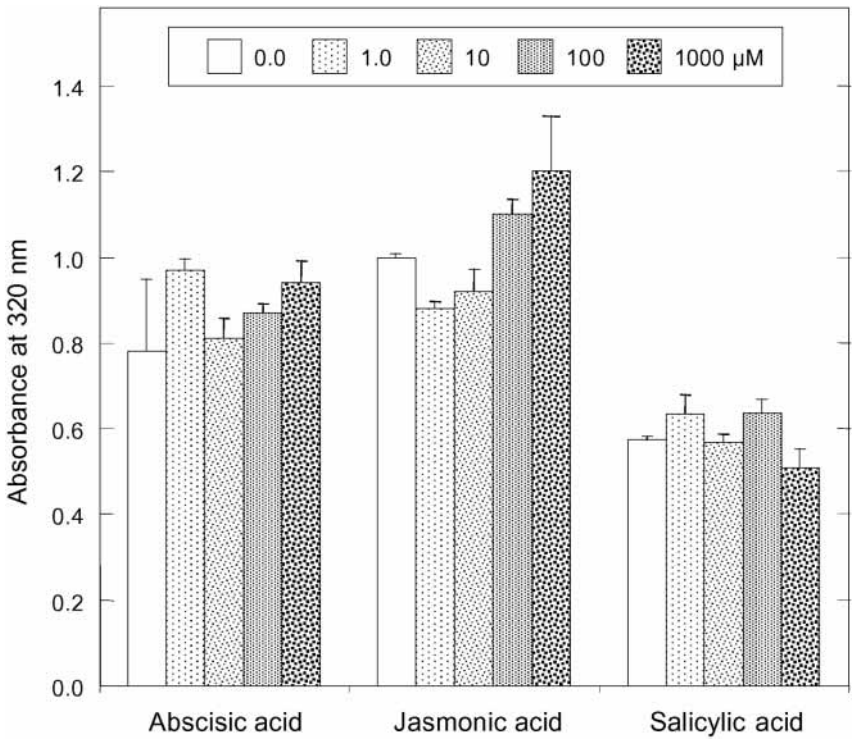


Fig. 7. Absorbance of a methanol extract (abs 320 nm) taken from unwounded Romaine lettuce leaves. Whole, excised mature leaves were sprayed to run-off with an aqueous 1–1000 µ*M* solution of abscisic, jasmonic or salicylic acid. Controls consisted of leaves sprayed with water. Leaves were held in air at 10°C for 48 h. Tissue for analysis was excised 4 cm from the base of the leaf. Values are means ± SE (n = 3).

crease in either carbon dioxide or ethylene production over the control (data not shown). Saniewski and Czapski (1985) reported that MeJA application increased ethylene production by tomato fruit. However, Penninckx et al. (1998) did not observe a significant increase in ethylene production after MeJA application to *Arabidopsis* plants. Perhaps the responsiveness of Asteraceae species is different from Solanaceae species.

### Effect of abscisic, salicylic and jasmonic acid on phenolic metabolism

It is thought that stress signals work in a coordinate way – some complementary and others antagonistic – to generate a highly organized plant defense system (Schenk et al. 2000). ABA is known to be a necessary element in the wound signal complex (Peña-Cortes et al. 1989, Hildmann et al. 1992). Harvested commodities, although kept in a high relative humidity environment, can undergo water stress that could trigger a cascade of signals involving ABA. Exogenous application of ABA induces increased PAL activity and phenylpropanoid metabolism in grape and strawberry fruit (Revilla and Gonzalez-San Jose 1997, Kondo et al. 1998). However, ABA can also act as a suppresser of phenylpropanoid metabolism in soybean (Graham and Graham 1996). SA is a signal molecule associated with the systemic acquired response (SAR) in plants (Gaffney et al. 1993). One of the first changes associated with SAR is an increase in PAL activity (Smith-Becker et al. 1998). SA also acts as an antagonist (Seo et al. 1997) and an inhibitor of JA biosynthesis (Peña-Cortes et al. 1993). However, Schenk et al. (2000) recently reported that SA and MeJA work in a coordinated, rather than an antagonistic manner.

Because of the interrelation among these putative stress signals, we felt it was important to study the effect of ABA and SA together with jasmonates. Whole mature non-wounded lettuce leaves were sprayed with aqueous solutions of 10 µ*M* of ABA or SA, then they were assayed by chlorogenic acid concentration after 48 h (Fig. 4). In addition, leaves treated with 1.0–1000 µ*M* of ABA, JA or SA at 10°C and the concentration of phenolic compounds were analysed after the same period (Fig. 7). These signalling molecules did not cause an increase in the chlorogenic acid accumulation or extractable phenolics compounds when they were compared with the control. As mentioned before, wounding greatly stimulated the accumulation of chlorogenic acid that was not mimicked by exogenous applications of ABA, JA or SA.

The lack of differences between the non-wounded controls and the ABA or SA treatments were confirmed by the phenolic data (Fig. 7). The optical density of the methanolic extract from lettuce treated with 0–1000 µ*M* ABA was 0.87 ± 0.06, while it was 0.58 ± 0.02 for lettuce treated with the same range of salicylic acid concentrations. In contrast to these fairly stable levels that occurred across the wide range of concentrations of ABA and salicylic acid, the concentration of extractable phenolic compounds increased as the concentration of JA increased above 10 µ*M*. The optical density was 0.93 ± 0.06 for tissue treated with 0.0–10 µ*M* JA and then it increased to 1.1 ± 0.04 and 1.2 ± 0.13 as the concentration of JA in-

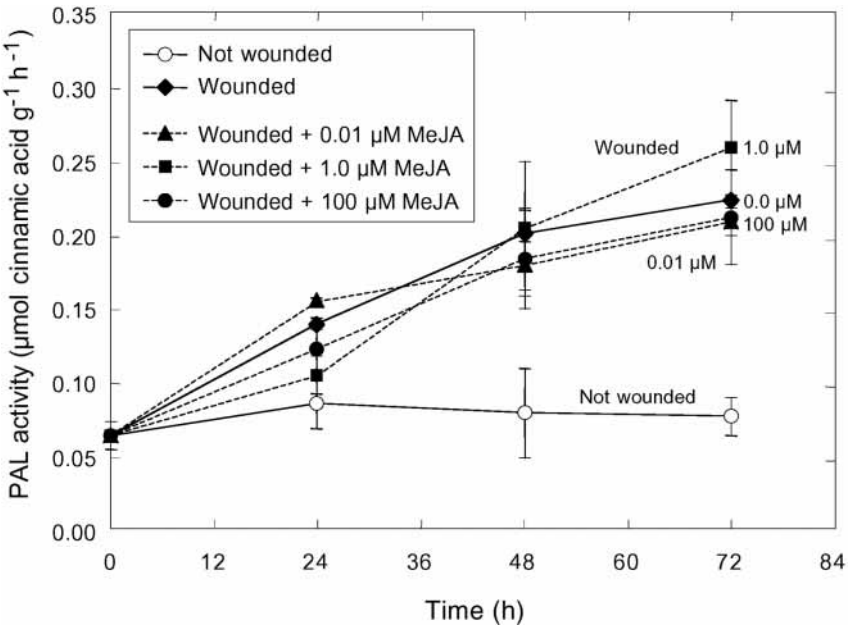


Fig. 8. Effect of methyl-jasmonate (MeJA) on wound-induced phenylalanine ammonia-lyase (PAL) activity in excised 10 × 10 mm midrib pieces of Romaine lettuce held at 5°C in air. Freshly excised midrib tissue was dipped in aqueous solutions of 0.01, 1.0 and 100 µ*M* MeJA for 4 min at 18°C. Values are means ± SE (n = 3).

creased to 100 and 1000 µ*M*. However, the biologically active concentration of JA in plant tissues is much lower (Creelman and Mullet 1995, McConn et al. 1997).

Plant growth regulators can enhance the effect of a specific stress. For example, PAL activity in wounded iceberg lettuce can be further increased by exposure to high concentrations of ethylene (Lopez-Galvez et al. 1996). If MeJA is part of the wound signal in cut lettuce tissue, then applying it in combination with wounding may increase PAL activity over that of wounding alone. However, dipping wounded midrib tissue in aqueous solutions of 0.01, 1.0 or 100 µ*M* MeJA for 4 min at 18°C did not significantly affect the activity of PAL induced by wounding (Fig. 8). These results are consistent with those reported earlier.

Interfering with the synthesis of JA did not alter the wound response of mature lettuce leaves. The synthesis of MeJA is inhibited by *n*-propyl gallate, an inhibitor of lipoxygenase activity (Ellard-Ivey and Douglas 1996). Application of 50 µ*M* *n*-propyl gallate did not alter the pattern or level of wound-induced PAL activity (data not shown).

### Effect of tissue age

Several papers that have reported a participation of jasmonates in the wound signal complex have used young plant material (cotyledons, seedlings) or cell cultures. We explored the possibility that the age of the tissue is an important factor in its responsiveness to exogenous applications of plant growth regulators. Basal levels of PAL activity are correlated with the age of the tissue. The youngest leaves (7 ± 2 cm long) had over twice the basal level of PAL activity than the other two stages tested (Fig. 9). High PAL activity should be associated with young tissue since developing leaves require compounds derived from primary and secondary metabolism, and PAL is a key regulatory enzyme in phenylpropanoid metabolism.

The high level of PAL activity in young leaves was further increased by the application of 1.0 m*M* MeJA or SA (Fig. 9). Since young leaves were more responsive than older leaves, and excising the leaves could have modified their responsiveness, a new series of experiments were designed to test the effects of ABA, JA, MeJA and SA on nonexcised young leaves. Whole heads were trimmed of older leaves, treated as before, and the 7 ± 2 cm long leaves were analysed for PAL activity after 24 h at 10°C. In addition, a wounding treatment was included to compare with the effect of the applied chemicals.

Wounding caused a 4.3-fold increase in PAL activity (Fig. 10). Application of 1.0 m*M* JA, MeJA or SA increased PAL activity by an average of approximately 50% (from 0.18 to 0.25, 0.28, and 0.26 for JA, MeJA and SA, respectively). The young tissues used in these experiments were more responsive than older tissue (Fig. 9), but even in this responsive tissue high concentrations of applied chemicals were unable to duplicate the inductive effect of wounding. We could speculate that mature tissue may have a higher capacity than younger leaves to inactivate, conjugate or compartmentalize the applied plant growth regulators, or that there is a different threshold to trigger a response in these tissues; but more research is needed to select among these possibilities.

However, there is already information that the responsiveness to certain treatments is developmentally regulated. Ke and Saltveit (1989a) reported that ethylene-induced PAL activity in iceberg lettuce, and development of the postharvest disorder russet spotting were under

developmental control. Emery and Reid (1996) working with MeJA and ethylene in sunflower (another species of Asteraceae), showed that leaves were less responsive than cotyledons or hypocotyls to applied MeJA. They concluded that a developmental control exists over the response to MeJA. Thipyapong and Steffens (1997) working with PPO in tomato plants postulated that the developmental stage is a critical factor in the capacity to respond to MeJA applications. Cipollini and Redman (1999) mentioned that differences in tomato plant response to JA-induced activity of PPO and POD was due to plant age. Constabel et al. (2000) reported a differential responsiveness in leaves of hybrid poplar, where PPO mRNA was induced more strongly in young leaves than in old ones by MeJA spray treatments.

The data presented in this paper allowed us to conclude that in harvested Romaine lettuce leaf tissue, MeJA does not appear to play a significant role of the wound signal that induces PAL activity, the accumulation of phenolic compounds and tissue browning. Our use of commercially mature lettuce may have precluded its response to concentrations of MeJA that have induced significant changes in phenylpropanoid metabolism in other species. We did observe that the puta-

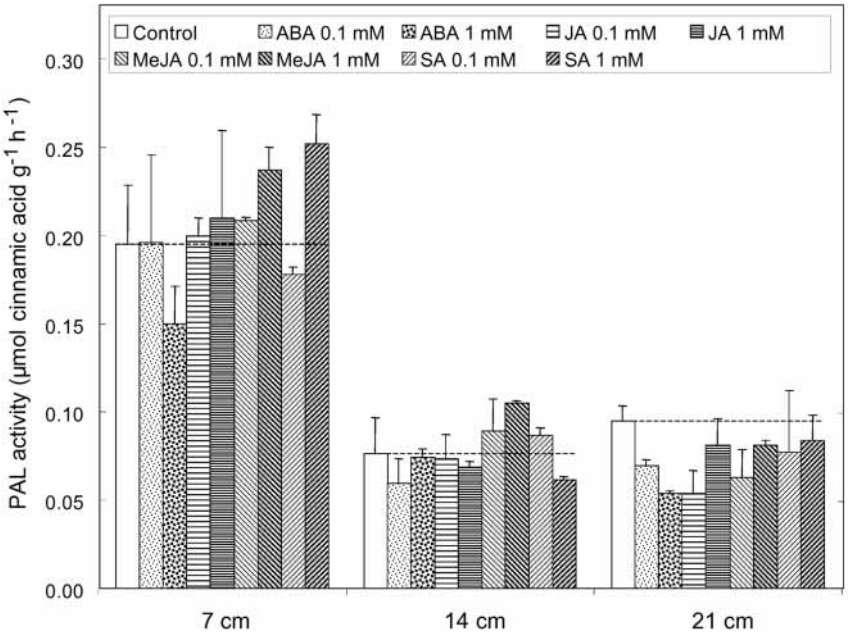


Fig. 9. Effect of tissue age on induction of phenylalanine ammonia-lyase (PAL) activity. Whole, excised leaves were segregated into groups that were 7 ± 2, 14 ± 2, or 21 ± 2 cm in length. Each leaf was sprayed to run-off with an aqueous 0.1 or 1.0 m*M* solution of abscisic acid (ABA), jasmonic acid (JA), methyl jasmonate (MeJA), or salicylic acid (SA). Controls consisted of leaves sprayed with water. Leaves were held at 10°C for 24 h. Tissue for PAL analysis was excised 4 cm from the base of the leaf. Values are means ± SE (n = 3).

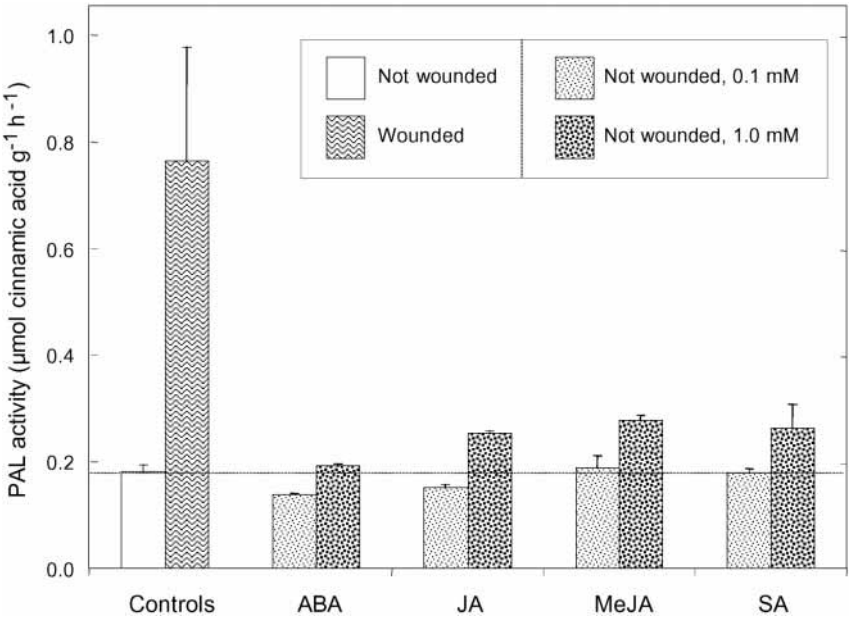


Fig. 10. Activity of phenylalanine ammonia-lyase (PAL) in young leaves. Whole heads were trimmed of older leaves and sprayed to run-off with an aqueous 0.1 or 1.0 m*M* solution of abscisic acid (ABA), jasmonic acid (JA), methyl jasmonate (MeJA), or salicylic acid (SA). Controls consisted of heads sprayed with water or wounded 7 ± 2 cm long leaves. Heads and wounded leaves were held at 10°C for 24 h. Tissue for PAL analysis was excised 4 cm from the base of the non-injured leaves. Values are means ± SE (n = 3).

tive role of jasmonates in stimulating the defense system by increasing phenylpropanoid metabolism could be detected in young leaves. However, young leaves are a small percentage of the whole lettuce head used in commercial operations. We cannot exclude the possibility that jasmonates could be enhancing the action of another unknown signal molecule in mature leaves. However, in the light of our results, jasmonates do not appear to be involved in the increase in phenylpropanoid metabolism associated with the level of wounding ordinarily produced during the commercial preparation of fresh-cut lettuce. The opportunity to explore a novel mechanism of wound signalling may be possible with mature lettuce leaves.

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