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Antioxidant Capacity of Lettuce Leaf Tissue Increases after Wounding

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Wounding induced the accumulation of phenolic compounds in Iceberg and Romaine lettuce leaf tissue. Phenolic concentrations were quantified after holding the leaf tissue at 10 °C for 48 h as the absorbance of a methanol extract at 320 nm, and by the Folin−Ciocalteu method. Heat-shock treatments applied by immersing tissue in 45 °C water for 2.5 min before or after wounding reduced the accumulation of phenolic compounds. Compared to the nonwounded, nonheat-shocked controls, these and other wounding and heat-shock treatments produced leaf tissue with a 4-fold range in phenolic content. The antioxidant capacity of the tissue, measured as DPPH (α,α-diphenyl-β-picrylhydrazyl)-radical scavenging activity, or as ferric-reducing antioxidant power (FRAP), increased after wounding. The increase was linearly correlated with the increase in phenolic compounds in Iceberg ($R^2 > 0.97$) and in Romaine ($R^2 > 0.95$) lettuce leaf tissue. Increased consumption of diets rich in phenolic antioxidants may contribute to reducing human diseases. Treatments that reduce the browning of wounded lettuce leaf tissue by preventing the oxidation of the accumulated wound-induced phenolic compounds may produce a healthier fresh-cut product than treatments that prevent the wound-induced synthesis and accumulation of phenolic compounds with antioxidant properties.

KEYWORDS: DPPH; FRAP; fresh-cut; heat-shock; lettuce; phenolic compounds; wounding

INTRODUCTION

Lettuce leaf tissue contains small amounts of phenolic compounds when the plants are grown under nonstressful conditions (*1*). The activity of the phenylpropanoid pathway increases under stressful conditions (e.g., mechanical injury) and phenolic compounds are synthesized and accumulated (*2*). Physical injuries that occur during the preparation of fresh-cut lettuce for packaged salads induce the activity of phenylalanine ammonia lyase (PAL, EC 4.3.1.5), the first and rate-controlling enzyme in the phenylpropanoid pathway. Browning of fresh-cut lettuce is a detrimental indicator of the oxidation of the accumulated phenolic compounds.

A great deal of effort has been expended to prevent browning of fresh-cut fruits and vegetables. One approach is to inhibit the browning reactions of the accumulated phenolic compounds by excluding oxygen, adding antioxidants, or inhibiting the activity of the responsible enzymes. Another approach is to prevent the synthesis and accumulation of the wound-induced phenolic compounds. We have previously shown that a 2.5 min heat-shock at 45 °C diminishes the ability of wounding to induce increased PAL activity, the synthesis and accumulation of phenolic compounds, and subsequent tissue browning (*3*, *4*). However, heat-shock treatments are only effective in plant tissue where the initial levels of phenolic compounds are low and increase as the result of wound-induced increases in enzyme synthesis.

Many phenolic compounds are antioxidants that may contribute to reducing human diseases (e.g., cancer and heart diseases). The beneficial effect of eating a diet rich in fruits and vegetables has been partially attributed to the increased consumption of phenolic compounds with antioxidant properties (*5*, *6*). These compounds reduce the oxidative damage that has been linked to arteriosclerosis (*7*), brain disorders (*8*), and cancer (*9*).

Several recent studies of natural antioxidant phytochemicals in foods have produced conflicting results because general methods were used to evaluate antioxidant activity (*10*). Most natural phytochemicals with antioxidant properties are multifunctional, and a reliable antioxidant protocol should measure more than one relevant property. Two effective methods to measure antioxidant capacity in foods is the DPPH-radical scavenging method and the ferric reducing antioxidant power (FRAP). The DPPH-radical scavenging method was used to compare the antioxidant activity of the major polyphenolic compounds in grape juices and wines (*11*). In subsequent work, they found a high linear correlation ($r = 0.95$) between antioxidant activity (DPPH-assay) and the total polyphenol content (*12*). The health benefit of fruit juices has been ascribed, in part, to phenolic antioxidants. The antioxidant potential of a range of fruit juices was assessed as their ability to reduce

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Fe(III): their FRAP (*13*). The relationship between antioxidant capacity, as determined by the FRAP assay, and total phenols was linear ($r = 0.96$) (*14*). The total antioxidant capacity of tea (as measured with the FRAP assay) also showed a strong linear correlation ($r = 0.96$) with the total phenolic content of tea (*15*).

If processing fruits and vegetables (e.g., lettuce) for fresh-cut increases the antioxidant capacity of the tissue through the induced synthesis and accumulation of phenolic compounds, then techniques that reduce browning by inhibiting their oxidation may produce a more healthful product than those techniques that inhibit the synthesis and accumulation of beneficial phenolic compounds. We have previously shown that wounding increases the synthesis and accumulation of phenolic compounds in lettuce (*1*), and that their synthesis and accumulation can be suppressed by a heat-shock (*4*). Research reported in this paper was done to see if wound-induced increases in phenolic compounds were correlated with an increased antioxidant capacity in cut lettuce leaf tissue, and whether the decreased production of wound-induced phenolic compounds in heat-shocked lettuce was correlated with reduced antioxidant capacity.

MATERIALS AND METHODS

Plant Material. Heads of Iceberg (Salinas type crisphead) and Romaine leaf lettuce were obtained from commercial sources and stored at 0 °C until used. After discarding wrapper leaves, the next six to eight noninjured leaves were carefully removed and 5 × 7 cm midrib segments were excised starting 3 cm from the base of the leaf. Randomized samples of at least three midrib segments were used as replicates in each treatment.

Treatments. Randomly selected groups of three excised midrib segments were rinsed in water at room temperature before treatment. Heat-shock treatments (i.e., immersion in 45 °C water for 2.5 min) were applied to whole leaves 30 min before mid-rib excision, 30 min after excision, or 12 h after excision. A home salad spinner was used to remove excess water. Treated segments were held at 10 °C in a flow of ethylene free, humidified air (~95% RH) sufficient to keep the CO_2 level below 0.15%. After 48 h, replicated samples were removed for analysis.

Determination of Phenolic Compounds. *Absorbance at 320 nm.* The concentration of phenolic compounds was measured as described by Ke and Saltveit (*2*); briefly, 10 g of mid-rib tissue was homogenized in 20 mL of HPLC grade methanol using an Ultra-Turrax tissue homogenizer (Takmar, Cincinnati, OH) at moderate speed (setting of 60) for 30 s. Lettuce tissue is about 98% water, so the extract was about 70% methanol. Extracts were also prepared in the same way using a phosphate buffered saline (PBS) solution (10 mM phosphate buffer pH 8.0 containing 2.7 mM potassium chloride and 137 mM sodium chloride) (*16*). The homogenate was filtered through four layers of cheesecloth and centrifuged at 15000*g* for 15 min at 20 °C. The absorbance of an aliquot of the supernatant was read at 320 nm using a UV−vis spectrophotometer (Shimadzu UV-160A) (*3*).

Folin−Ciocalteu Method. The total phenolic content in the methanol extracts was determined according to the Folin−Ciocalteu procedure (*17*). The Folin−Ciocalteu method is commonly used to measure phenolic content although it is not completely specific for phenolic compounds (i.e., it is effected by other constituents), and not all phenolic compounds exhibit the same level of activity in the assay. However, it does give a good general measure of phenolic content. A 100 μL aliquot of the supernatant was combined with 500 μL of Folin−Ciocalteu's reagent and 400 μL of sodium carbonate (7.5%). The tubes were mixed for 15 s and then allowed to stand for 30 min at 20 °C. Absorption was measured at 765 nm using the UV−vis spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram of tissue.

Antioxidant Measurement. *DPPH (α,α-Diphenyl-β-picrylhydrazyl) Radical Scavenging Activity.* A 0.5 mL aliquot of the methanol extract prepared above was mixed with 0.25 mL of an ethanolic 0.5 mM DPPH solution and 0.5 mL of 100 mM acetate buffer (pH 5.5). The tubes were mixed for 15 s, and after standing for 30 min, the absorbance of the mixture was measured at 517 nm (*18*).

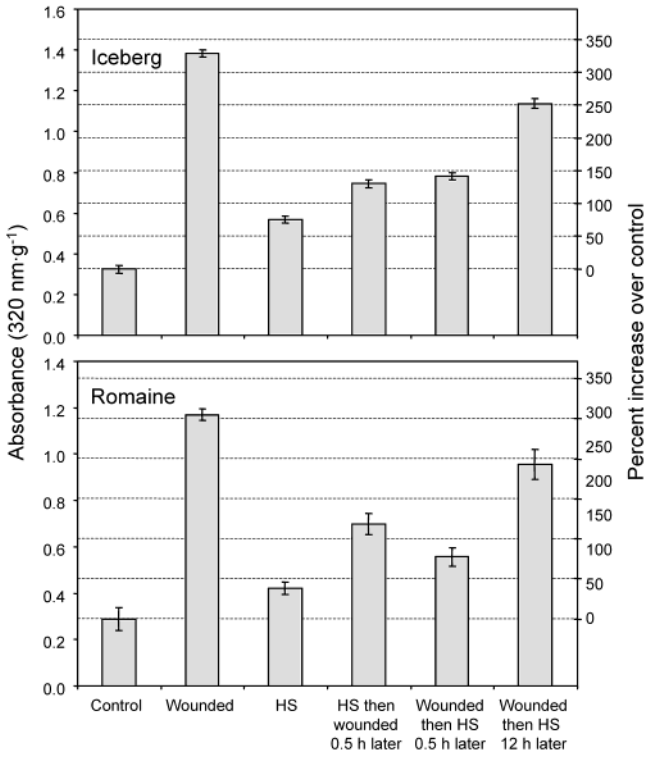

Figure 1. Effect of wounding and/or heat-shock on the phenolic content of Iceberg and Romaine lettuce leaf tissue. Phenolic content was measured as the absorbance at 320 nm of the methanol extract per gram FW. Leaf tissue was held for 48 h at 10 °C after treatment. The 45 °C for 2.5 min heat treatment was applied 30 min before, 30 min after, or 12 h after wounding. Values are expressed as absorbencies at 320 nm g^{-1} and as percent of the nonwounded, nonheat-shocked control. The line on top of the vertical bar represents ± SD ($n = 8$).

Ferric-Reducing Antioxidant Power (FRAP) Assay. The assay was based upon the methodology of Benzie and Strain (*19*). The FRAP reagent was prepared fresh so that it contained 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 2 mM ferric chloride in 0.25 M sodium acetate at pH 3.6. A 100 μL aliquot of the methanol extract prepared above was added to 900 μL of FRAP reagent, and the they were mixed. After the mixture stood at 20 °C for 4 min, the absorbance at 593 nm was determined against a water blank. Calibration was against a standard curve (50−1000 μM ferrous ion) produced by the addition of freshly prepared ammonium ferrous sulfate. FRAP values were calculated as micromolar ferrous ion (ferric reducing power) from three determinations and are presented as percentages of the control. The FRAP assay has been successfully used with 50% ethanol or methanol extracts (*20*, *21*), with extracts in an aqueous buffer (*14*), and with distilled water extracts (*15*). We ran assays with the methanol extract, and with extracts prepared using the phosphate buffered saline (PBS) solution.

EXPERIMENTAL PROCEDURES DESIGN

Each experiment was repeated at least twice with similar results. All treatments were replicated at least four times within each experiment. Means and standard errors were calculated from pooled data.

RESULTS AND DISCUSSION

Phenolic Content. Wounding induced an increase in the phenolic content in both Iceberg and Romaine lettuce (**Figures 1** and **2**). Phenolic content measured as absorbance of the methanol extract at 320 nm increased 330% for Iceberg and 305% for Romaine held at 10 °C for 48 h (**Figure 1**). When

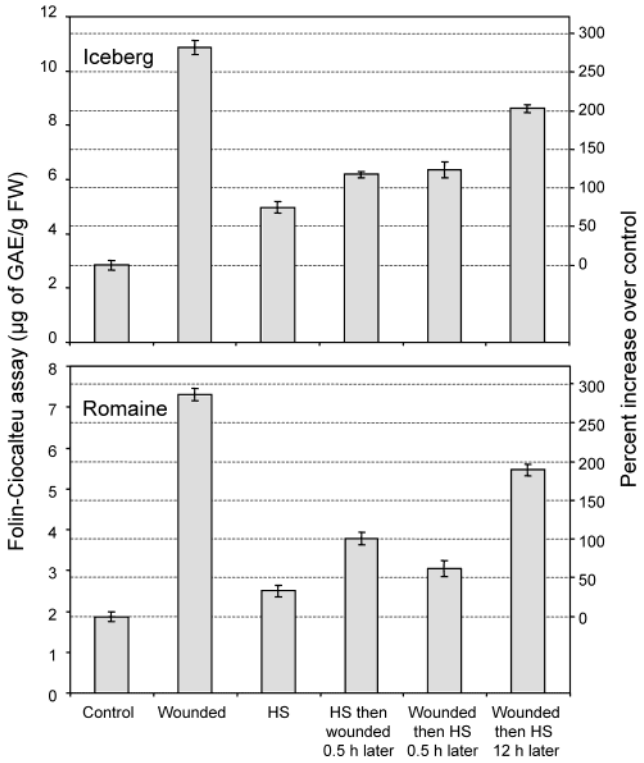

Figure 2. Effect of wounding and/or heat-shock on the phenolic content of Iceberg and Romaine lettuce leaf tissue. Phenolic content was measured using a Folin–Ciocalteu assay of the methanol extract. Leaf tissue was held for 48 h at 10 °C after treatment. The 45 °C for 2.5 min heat treatment was applied 30 min before, 30 min after, or 12 h after wounding. Values are expressed as micrograms of gallic acid quivalents per gram FW and as a percent of the nonwounded, nonheat-shocked control. The line on top of the vertical bar represents ± SD ($n = 8$).

measured by the Folin−Ciocalteu method, wounding increased phenolic content by 280% in Iceberg lettuce and 290% in Romaine lettuce (**Figure 2**).

A 2.5 min heat-shock at 45 °C increased phenolic content in nonwounded Iceberg lettuce by 76% or 74%, and in nonwounded Romaine lettuce by 45% or 34% when measured as absorbance at 320 nm (**Figure 1**), or Folin−Ciocalteu method (**Figure 2**), respectively. These heat-shock induced changes were significant, but small in comparison to the much larger increases induced by wounding.

Applying the heat-shock either 30 min before or 30 min after wounding produced the same level of reduction in wound-induced phenolic synthesis in Iceberg lettuce whether measured as absorbance at 320 nm (a 45% reduction) (**Figure 1**) or by the Folin−Ciocalteu method (a 42% reduction) (**Figure 2**). In contrast, applying the heat-shock 30 min after wounding was significantly more effective in reducing phenolic accumulation in wounded Romaine lettuce by both assays than applying the heat-shock 30 min before wounding whether measured as absorbance at 320 nm (a 52% reduction versus a 40% reduction) (**Figure 1**) or by the Folin−Ciocalteu method (a 58% reduction versus a 48% reduction) (**Figure 2**).

Delaying the heat-shock for 12 h after wounding reduced its effectiveness, but it still produced a significant decrease in phenolic accumulation in both types of lettuce as measured by both assays. In Iceberg lettuce, the wound-induced increase in phenolics as measured by absorbance at 320 nm was reduced 17% (**Figure 1**), and the Folin−Ciocalteu value was reduced 21% (**Figure 2**). In Romaine lettuce the reductions as measured

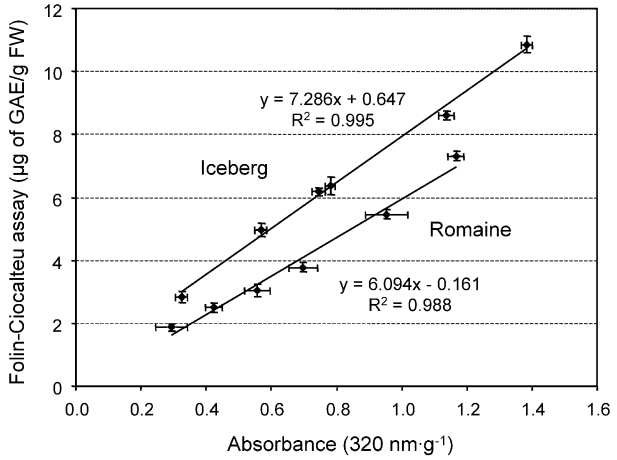

Figure 3. Relationship between phenolic content as measured as absorbance at 320 nm and by the Folin–Ciocalteu assay of the methanol extract of tissue subjected to the treatments described in **Figures 1** and **2** for Iceberg and Romaine lettuce leaf tissue. Values are expressed as absorbencies at 320 nm g^{-1} fresh weight and as micrograms of gallic acid quivalents per gram FW. Data were taken from **Figures 1** and **2**. Vertical and horizontal lines associated with each data point represents the SD ($n = 8$).

as absorbance at 320 nm was 45% (**Figure 1**), and 42% as measured by the Folin−Ciocalteu method (**Figure 2**).

Wounding and the heat-shock treatments produced a 4-fold range of phenolic contents in both types of lettuce. These data were used to evaluate the two methods used to measure phenolic content, and the relation between phenolic content and antioxidant capacity. The phenolic content of the lettuce measured as absorbance of the methanol extract at 320 nm was highly correlated with the Folin−Ciocalteu measurement (**Figure 3**). The Folin−Ciocalteu value is given by the equation [(7.286 × Abs 320) + 0.647], with an R^2 of 0.995 for Iceberg lettuce, and by the equation [(6.094 × Abs 320) − 0.161], with an R^2 of 0.988 for Romaine lettuce. Absorbance at 320 nm was a very reliable measure of total phenolics in both Iceberg ($R^2 = 0.994$) and Romaine ($R^2 = 0.977$) lettuce leaf tissue (data not shown). However, each type of lettuce generated slightly different relationships between the two methods. These differences could be the result of the synthesis and accumulation of different phenolic compounds or the presence of other absorptive compounds (e.g., carotenoids, tocopherols, ascorbic acid, and reduced glutathione) in the lettuce tissue. However, lettuce usually has low levels of these antioxidant compounds, and the major phenolic compounds detected in wounded Iceberg and Romaine lettuce were chlorogenic, isochlorogenic, caffeoltartartic, and dicaffeoltartartic acids (*1*).

Antioxidant Capacity. Wounding increased the antioxidant capacity of both types of lettuce. The DPPH assay indicated that wounding increased the antioxidant capacity of Iceberg and Romaine lettuce by 140% and 255%, respectively (**Figure 4**). The heat-shock alone also increased the antioxidant capacity of Iceberg and Romaine lettuce, but the increase was only 33% and 40%, respectively. Applying a heat-shock either 30 min before or after wounding depressed the increase in wound-induced antioxidant capacity by 35% in Iceberg lettuce. In Romaine lettuce the timing was important with application of the heat-shock 30 min before wounding producing a 37% reduction and a 56% reduction if applied 30 min after wounding. Delaying the heat-shock treatment for 12 h after wounding

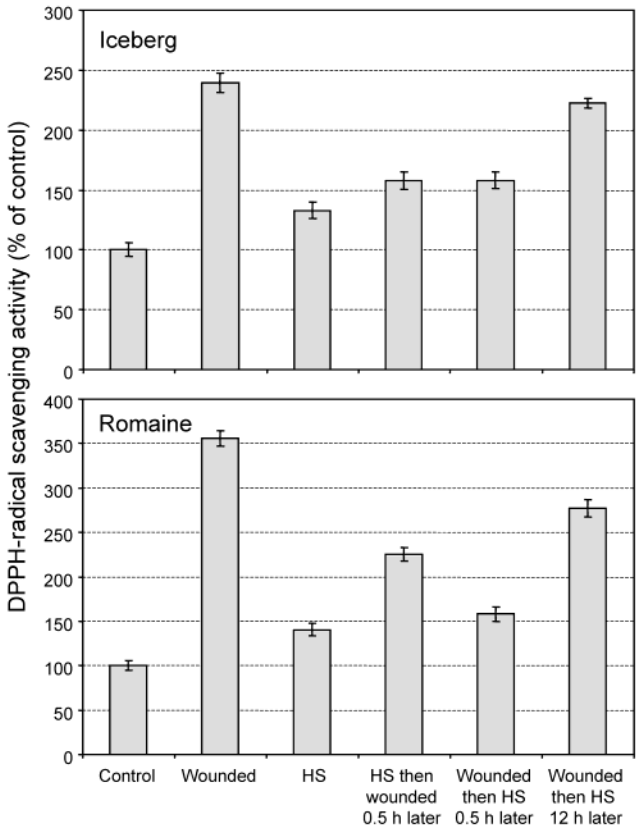

Figure 4. Effect of wounding and/or heat-shock on the antioxidant capacity of Iceberg and Romaine lettuce leaf tissue. Antioxidant capacity was measured as the DPPH-radical scavenging activity of the methanol extract. Leaf tissue was held for 48 h at 10 °C after treatment. The 45 °C for 2.5 min heat treatment was applied 30 min before, 30 min after, or 12 h after wounding. Values are expressed as percent of the nonwounded, nonheat-shocked control. The line on top of the vertical bar represents ± SD ($n = 8$).

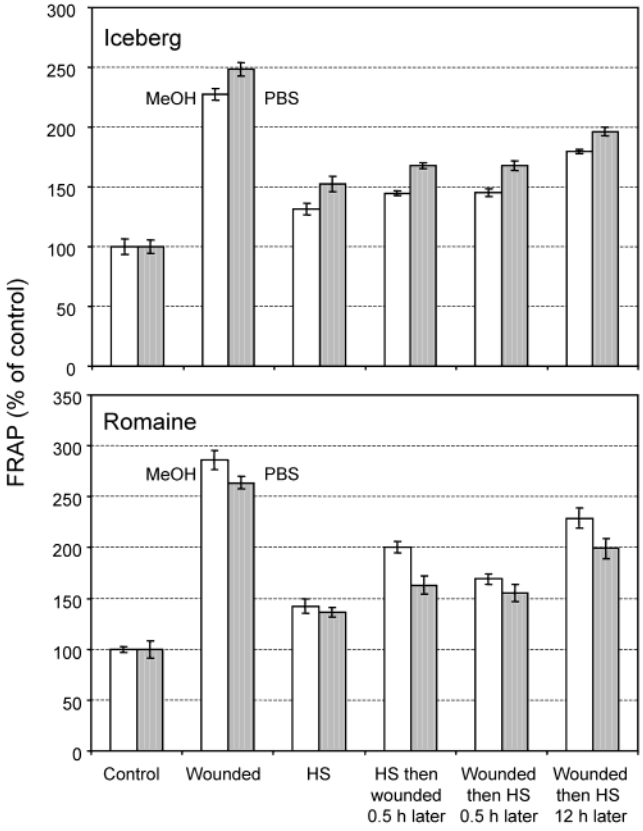

Figure 5. Effect of wounding and/or heat-shock on the antioxidant capacity of Iceberg and Romaine lettuce leaf tissue. Antioxidant capacity was measured using the FRAP assay of the methanol (FRAP MeOH), or phosphate buffered saline (PBS) solution (FRAP PBS) extract. Leaf tissue was held for 48 h at 10 °C after treatment. The 45 °C for 2.5 min heat treatment was applied 30 min before, 30 min after, or 12 h after wounding. Values are expressed as percent of the nonwounded, nonheat-shocked control. The line on top of the vertical bar represents ± SD ($n = 8$).

reduced its effectiveness so that the wound-induced level was only reduced by 7% in Iceberg lettuce and by 22% in Romaine lettuce.

Although different in magnitude, both the FRAP/PBS and FRAP/MeOH assays gave results similar to those obtained with the DPPH assay for the various wound and heat-shock treatments (**Figure 5**). In the following discussion, FRAP/PBS and FRAP/MeOH values are averaged because they were similar for each lettuce type and treatment. Wounding Iceberg and Romaine lettuce leaf tissue produced a 137% and a 175% increase, respectively, in FRAP values compared to the nonwounded control. The heat-shock treatment alone increased the FRAP value by 42% in Iceberg and 39% in Romaine lettuce. Applying the heat-shock to Iceberg lettuce tissue either 30 min before or after wounding produced the same level of reduction in wound-induced increases in the FRAP values. In contrast, applying the heat-shock treatment 30 min after wounding Romaine lettuce leaf tissue was more effective in reducing the wound-induced increase in FRAP than applying it 30 min before wounding. Applying the heat-shock 12 h after wounding still reduced the wound-induced increase in FRAP in both types of lettuce, but the reduction was only about half of that produced when it was applied 30 min after wounding.

Relation between Phenolic Content and Antioxidant Capacity. The increase in phenolic content in the lettuce tissue following the various treatments was linearly correlated with the antioxidant capacity of both types of lettuce (**Figure 6**). As the absorbance at 320 nm increased from 0.32 for control tissue to 1.38 for wounded Iceberg leaf tissue, the DPPH value increased linearly ($R^2 = 0.994$) from 100% to 240% of control for all treatments including the heat-shock treatments. The values for both FRAP/MeOH and FRAP/PBS showed a similar linear increase from 100% of control to 227% ($R^2 = 0.993$) and 248% ($R^2 = 0.976$) of control, respectively, for all treatments including the heat-shock treatments. A similar linear relationship was observed between the absorbance at 320 nm as it increased from 0.29 to 1.17, and the corresponding values for DPPH ($R^2 = 0.988$), FRAM/MeOH ($R^2 = 0.980$), and FRAP/PBS ($R^2 = 0.958$) for Romaine lettuce.

Wounding increased the phenolic content (absorbance at 320 nm) in Iceberg lettuce by 330%, whereas it only increased the antioxidant capacity by 140%. In contrast, wounding increased the phenolic content of Romaine lettuce by 305% and the antioxidant capacity by 255%. It appears that the wound-induced phenolics produced in Romaine lettuce had more antioxidnat capacity than the phenolics induced by wounding in Iceberg lettuce. However, the presence of other compounds with antioxidant capacity in these two types of lettuce could have influenced the antioxidant assay.

Effect of Reducing Tissue Browning on Antioxidant Capacity. Both measures of phenolic content (absorbance at 320 nm and Folin−Ciocalteu) were highly correlated ($R^2 > 0.94$) with both measures of antioxidant capacity (DPPH and FRAP) in leaf tissue from both types of lettuce (Iceberg and

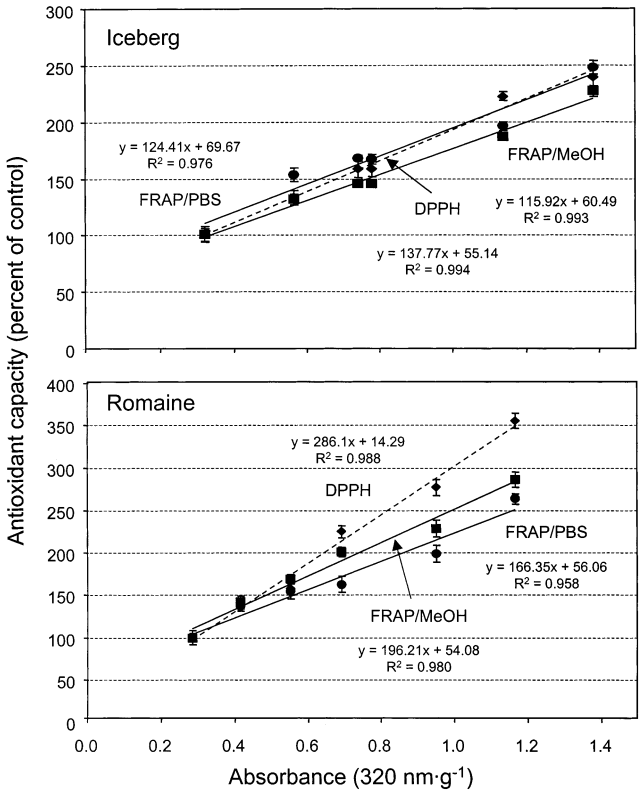

Figure 6. Relationship between phenolic content and antioxidant capacity of the lettuce leaf tissue. Phenolic content was measured as the absorbance at 320 nm of the methanol extract. Antioxidant capacity was measured as the DPPH-radical scavenging activity of the methanol extract, and the FRAP assay of the methanol (FRAP MeOH), or phosphate buffered saline (PBS) solution (FRAP PBS) extract. Values are expressed as percent of the nonwounded, nonheat-shocked control. Data were taken from **Figures 4** and **5**. The line on top of the vertical bar represents ± SD ($n = 8$).

Romaine). Modulating the phenolic content in lettuce leaf tissue by a combination of wounding and heat-shock treatments produced similar changes in the antioxidant capacity of the tissue as in the phenolic content. It appears that accumulation of the phenolic compounds 5-caffeoylquinic (chlorogenic acid), 3,5-dicaffeoylquinic (isochlorogenic), caffeoyltartaric, and dicaffeoyltartaric acid that are induced by wounding Iceberg and Romaine lettuce (*1*) could improve the antioxidant capacity of fresh-cut lettuce. However, the accumulation of these phenolic compounds is associated with subsequent tissue browning, and browning of fresh-cut lettuce reduces its quality and shelf life (*22*−*25*).

A number of treatments have been developed to reduce browning of fresh-cut lettuce. Some, such as treatments with antioxidants and calcium solutions, and exclusion of oxygen, reduce browning by reducing the oxidation of the accumulated wound-induced phenolic compounds (*25*). Others, such as using sharp knifes to minimize wounding, storage at 0 °C, and heat-shocks, reduce browning by reducing the synthesis and accumulation of phenolic compounds. Treatments that prevent the browning of fresh-cut lettuce with elevated levels of phenolic compounds induced by wounding should produce a healthier product than anti-browning treatments that work by reducing the synthesis and accumulation of wound-induced phenolic compounds.

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