Research Note

Modulation of Wound-Induced Hydrogen Peroxide and Its Influence on the Fate of *Escherichia coli* O157:H7 in Cut Lettuce Tissues

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MS 12-208: Received 14 May 2012/Accepted 19 July 2012

ABSTRACT

Wounding of lettuce tissue has been examined previously in regard to browning reactions, and treatments to modulate wounding responses were evaluated for reduction of browning. However, the wounding process also releases oxygen radicals such as hydrogen peroxide. This study focused on the evaluation of two treatments that reduce hydrogen peroxide at cut surfaces (heat treatment and pyruvate addition) and one treatment that enhances its production (infusion with the fungal elicitor harpin). Hydrogen peroxide changes in response to treatment were also associated with resultant survival of *Escherichia coli* O157:H7, which was inoculated onto the lettuce before cutting. Heat-treated lettuce produced significantly less hydrogen peroxide, and microbial analysis showed that *E. coli* O157:H7 survival on packaged, heat-treated lettuce was higher than on non-heat-treated controls. Lettuce was also cut under a solution of sodium pyruvate (a well-known hydrogen peroxide quencher), and *E. coli* O157:H7 survival was found to be enhanced with that treatment. When lettuce was infused with harpin before cutting, hydrogen peroxide production was enhanced, and this was associated with reduced survival of *E. coli* O157:H7. These results collectively support the hypothesis that modulation of wound-generated hydrogen peroxide can have an influence on *E. coli* O157:H7 survival on cut and packaged romaine lettuce.

Wounding-induced quality changes have been extensively studied for fresh-cut produce (9). However, an aspect of the wound response that has been studied only to a limited degree is that of oxygen radical production (12, 17, 18). Plant tissues have been shown to generate oxygen radicals in response to infection by microorganisms, notably fungi (2). While much is known about the infection response and production of oxidative metabolites in relation to plant stress and pathology (3, 11), little work has been published on oxygen radical production in response to cutting of fresh-cut vegetables. Nonetheless, wound-associated oxygen radicals are considered to potentially play an important role in quality decline in fresh-cut fruits and vegetables (17). Oxygen radicals may also have a significant impact on the fate of microbial contaminants on fresh-cut surfaces. Human pathogens including *Escherichia coli* O157:H7 are sensitive to reactive oxygen species such as hydrogen peroxide (10). The reported concentrations of hydrogen peroxide required to inhibit *E. coli* O157:H7 are not consistent, ranging from 0.1 to 0.7 mM (8, 10, 15, 19). A large part of this variation in inhibitory concentration may be related to artifacts leading to high background levels of peroxide in microbiological media and/or scavenging of hydrogen peroxide by oxidatively active constituents in the media (13). It has not yet been established whether endogenous levels produced in response to wounding incurred by cutting of the tissues during processing can affect the fate of colonizing cells, primarily since hydrogen peroxide is difficult to measure in situ. The most successful approach has been to modulate levels with scavengers to evaluate the biological significance of the hydrogen peroxide levels produced in situ (12).

We have found that warm-water heat treatments (47°C for 3 min) inhibit hydrogen peroxide levels produced in response to cutting and that application of harpin, a fungal elicitor of the hypersensitive plant response (6), enhances the intensity of the reaction by a mechanism known as systematically acquired resistance (1, 6). In addition, pyruvate is a known hydrogen peroxide quencher (13) that could be used to detoxify wound-induced hydrogen peroxide if lettuce tissues were submerged in a pyruvate solution during the cutting process. These approaches to reliably modulate hydrogen peroxide levels permitted a direct, in vivo assessment of the significance of hydrogen peroxide concentrations on the fate of *E. coli* O157:H7 in cut lettuce tissues. The goal of this work was to evaluate whether differences in endogenously produced levels of hydrogen peroxide generated at the cut surfaces of lettuce were associated with measurable differences in *E. coli* O157:H7 survival.

MATERIALS AND METHODS

In situ measurement of hydrogen peroxide generated in cut lettuce leaves. In order to capture hydrogen peroxide at the
time of cutting, leaf tissue from romaine lettuce (see below) was excised into disks that could fit in a reaction well. A plastic cutter was used to remove a 10-mm-diameter disk from the lettuce leaf from the midleaf, approximately halfway between the midvein and the exterior edge of the leaf. Disks were washed for 2 min in 50 ml of deionized water on a platform shaker set at 150 rpm and then placed on moistened filter paper in a petri dish for 40 min to allow initial wound-induced oxygen radical production to subside (7) on the outer edge of the disk. Aged leaf disks were then placed into the wells of a ceramic spot plate containing 500 μl of the Bioxtych H₂O₂-560 reagent (OXIS International, Inc., Foster City, CA) and on top of a silicon rubber disk backing. One drop of 0.05% Triton X-100 (Sigma Chemical Co., St. Louis, MO) was added to the sample to break surface tension. The disks were immersed in the reagent, and a new cut was made with a small, 5-mm-diameter plastic cutter. After the cut was complete, the disk was held under the surface of the reagent for 40 min, ensuring all wound-induced hydrogen production had subsided (7). After 40 min, the reacted Bioxtych reagent was removed from the well with a Pasteur pipette, and absorbance was read at 560 nm using a Cary 100-Bio UV-Visible Spectrophotometer (Varian Canada Inc., Mississauga, Ontario, Canada). Data were expressed in micromoles of H₂O₂ per liter.

Microbiological methods. Lettuce leaves were inoculated with a nalidixic acid-resistant nontoxicogenic strain of E. coli O157:H7 (ATCC 700728). Working cultures were maintained at 4°C on tryptic soy agar (TSA) supplemented with 10 μg of nalidixic acid per ml (Sigma). Inocula for experiments were grown in tryptic soy broth containing 6 g of yeast extract per liter (Difco, Detroit, MI) for 20 h under continuous agitation at 37°C. The cells were spun in a centrifuge at 3,000 x g for 10 min, the pellet was resuspended in sterile buffered peptone water, and the suspension was spun again. The final cell suspension was prepared in citric-phosphate buffer adjusted to pH 7.2. The optical density of the inoculum was adjusted spectrophotometrically (at 600 nm) to achieve the desired cell level in the final cell suspension. The latter was prepared by mixing the inoculum with sterile distilled water at room temperature to obtain approximately 10⁵ or 10⁷ CFU of E. coli O157:H7 per ml.

For experiments requiring inoculation, wrapper (outer) leaves were removed from heads of whole romaine lettuce. Several inner leaves were removed from the core and were suspended from clips fastened to a circular frame made of stainless steel. The leaves suspended by the frame were lowered into a glass beaker containing the inoculating solution, in which they remained fully immersed for 2 min. The inoculated lettuce leaf clipped to metal frames was then transferred to an empty beaker for 1 h to permit attachment of the cells. Loose or unattached cells were removed by gentle agitation of the leaves in a beaker containing sterile distilled water, and the frames were held in a biosafety cabinet for 30 min before the start of the experiments. Leaves were then cut into approximately 5-cm² squares with a sterile stainless steel paring knife. The leaves were then scored in 1-cm bands with the paring knife to maximize the level of injury to the cut leaf tissue.

E. coli O157:H7 populations in cut lettuce were measured by placing the cut and scored 5-cm² squares into 50-ml capped plastic tubes in 25 ml of 0.1% (wt/vol) buffered peptone water. The tubes were vortexed, and the resultant wash was used for dilution and spreading onto plates. Dilutions were prepared in 0.1% (wt/vol) peptone, and aliquots (0.1 ml) were spread onto duplicate plates of TSA (Oxoid, Basingstoke, England) supplemented with 25 mg liter⁻¹ nalidixic acid (TSANA) and then incubated for 24 h at 37°C. Population densities were expressed as either CFU per gram or CFU per square centimeter of lettuce leaf tissue.

Effect of heat treatment on wound-induced H₂O₂ production by lettuce leaves and the fate of E. coli O157:H7. Romaine lettuce was grown for 14 weeks and harvested on 12 March from a crop produced in a growth chamber at the Pacific Agri-Food Research Centre. Fully expanded inner leaves were split in half along the midrib. One half of a leaf was held at room temperature, and the other half was immersed in water at 47°C for 3 min as described by Delaquais et al. (4, 5). Hydrogen peroxide production was measured in leaf tissue disks after 2 and 7 days in cold storage at 4°C. In a separate experiment using the same lot of lettuce, leaves were inoculated with E. coli O157:H7 as described above several minutes after heat treatment, and viable cell populations were measured on cut tissues at 20°C at 0, 1, 3, and 20 h after cutting.

Effect of harpin elicitor infusion on wound-induced H₂O₂ production by lettuce leaves and the fate of E. coli O157:H7. The same lettuce as that described above was used in this experiment. Harpin is a protein elicitor of the wound response (6). Leaves were split in half along the midrib. One half was infused for 5 min under 4 in. of vacuum with 90 mg liter⁻¹ harpin (EBC-351, Plant Health Care, Inc., Pittsburgh, PA) dissolved in distilled water at 20°C. A paired half leaf was left untreated. The leaves were then held in loosely closed polyethylene bags for 4 days at 13°C. Hydrogen peroxide production was measured on treated and untreated disks as described earlier. Leaves were inoculated with E. coli O157:H7 as described above, and viable cell populations were measured on the cut leaf tissue after sitting for 1 h at 20°C.

Effect of a hydrogen peroxide-­quenching agent on the fate of E. coli O157:H7 in vitro. The same lettuce as the one described above was used in an experiment to determine the effect of a hydrogen peroxide-quenching agent on E. coli populations in vitro. Leaves of freshly harvested romaine lettuce were inoculated with E. coli O157:H7 as described above. Each leaf was divided along the midrib, and one half was cut while submerged under 0.4 g liter⁻¹ sodium pyruvate in sterile distilled water; sodium pyruvate is an agent used to quench hydrogen peroxide in microbiological media (13). The other half was cut under sterile distilled water. E. coli O157:H7 populations were measured 0, 30, and 60 min after cutting. Prior to selecting a concentration of sodium pyruvate, an experiment was conducted to confirm its scavenging capacity in relation to a hydrogen peroxide concentration measured in cut romaine lettuce. The test solution contained 5 μmol of hydrogen peroxide per liter, and amounts of sodium pyruvate were added such that the final concentration ranged between 0 and 0.5 g/liter. After 1 h, residual hydrogen peroxide was measured using the Bioxytech H₂O₂-560 reagent, following the instructions in the kit for liquid samples.

Statistical analyses. Analysis of variance (ANOVA) was performed using the PROC GLM procedure (SAS Institute Inc., Cary, NC). Data are expressed as means ± standard errors of five replicates, and statistical significance levels between treatments and significance of interactions are presented in the tables.

RESULTS AND DISCUSSION

Hydrogen peroxide accumulation was significantly lower in cut romaine lettuce tissues prepared from heat-treated than from untreated leaves (Table 1). The similarity in yields after 7 days suggested that the reduction in
hydrogen peroxide production in response to heat treatment was maintained in whole leaves stored at low temperatures for several days. In a separate experiment with the same lot of lettuce, populations of E. coli O157:H7 inoculated onto leaves after heat treatment and prior to cutting were lower for heat-treated lettuce (Table 2). However, surviving populations on control leaves were lower than in the heat-treated lettuce if cut 1 h after treatment (Table 2). Therefore, hydrogen peroxide production in response to heat treatment (47°C for 3 min) is delayed by at least 1 h. Induction of changes in metabolic pathways in plants, such as the reduction of hydrogen peroxide production capability, is not immediate and would require a matter of hours to become expressed after the treatment (11). Hence, a burst in hydrogen peroxide production by wounded lettuce tissues appears to release sufficient amounts of hydrogen peroxide to injure or inactivate E. coli O157:H7. Wound-generated hydrogen peroxide is transient in nature, declining within hours after wounding (7, 12), suggesting that there would not be any residual antimicrobial activity after a few hours.

The modulation of hydrogen peroxide production in lettuce tissues by heat led us to seek a means to enhance production and to verify effects on E. coli O157:H7. Production of hydrogen peroxide was significantly enhanced in cut tissues prepared from leaves infused with harpin to elicit the oxidative stress response (6) (Table 3). The increase in hydrogen peroxide production had a consequent effect on populations of E. coli O157:H7, which were significantly reduced (Table 3). Enhancement of wound-generated hydrogen peroxide was associated with reduced E. coli O157:H7 populations in cut romaine lettuce tissues, even though the reduction in populations was less than 1 log. A final experiment confirmed a clear link between the rapid accumulation of hydrogen peroxide immediately after cutting and the fate of E. coli O157:H7. Cell populations did not change when lettuce leaves were submerged in a solution containing pyruvate. In contrast, populations declined by 2 log CFU g⁻¹ on lettuce leaves cut under distilled water (Table 4). Since pyruvate is a well-known quencher of reactive oxygen species such as hydrogen peroxide (13), increased survival can be ascribed to a reduction in the accumulation of these potentially lethal antimicrobial compounds in the tissues. Preliminary studies conducted in our laboratory have demonstrated that the concentration of pyruvate used would almost completely inhibit the growth of E. coli O157:H7 on fresh-cut romaine treated or not with sodium pyruvate.

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**TABLE 1. Concentrations of hydrogen peroxide produced by heat-treated and nontreated romaine lettuce in response to cutting**

<table>
<thead>
<tr>
<th>No. of days after treatment</th>
<th>Control samples</th>
<th>Heat-treated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. of hydrogen peroxide (µmol/liter)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.617 ± 0.116</td>
<td>0.466 ± 0.059</td>
</tr>
<tr>
<td></td>
<td>0.729 ± 0.104</td>
<td>0.479 ± 0.023</td>
</tr>
</tbody>
</table>

Significanceb (probability > F)  
- Treatment: 0.035  
- Day: 0.474  
- Treatment × day: 0.569

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**TABLE 2. Fate of E. coli O157:H7 in romaine lettuce leaves heat treated or not treated before inoculation and cutting**

<table>
<thead>
<tr>
<th>E. coli O157:H7 populations (log CFU/g) in:</th>
<th>Control samples</th>
<th>Heat-treated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after cutting (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.08 ± 0.02</td>
<td>6.77 ± 0.07</td>
</tr>
<tr>
<td>1</td>
<td>6.89 ± 0.04</td>
<td>6.95 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>7.02 ± 0.01</td>
<td>7.08 ± 0.05</td>
</tr>
<tr>
<td>20</td>
<td>7.66 ± 0.04</td>
<td>7.75 ± 0.05</td>
</tr>
</tbody>
</table>

Significanceb (probability > F)  
- Treatment: 0.7132  
- Time: <0.0001  
- Treatment × time: 0.0009

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**TABLE 3. Effects of infusion with harpin elicitor on the production of hydrogen peroxide by romaine lettuce in response to cutting and on the fate of E. coli O157:H7 4 days after the application of harpin**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydrogen peroxide concn (µmol/liter)</th>
<th>E. coli O157:H7 population (log CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.98 ± 0.14</td>
<td>7.12 ± 0.01</td>
</tr>
<tr>
<td>Harpin</td>
<td>1.74 ± 0.20</td>
<td>6.96 ± 0.03</td>
</tr>
</tbody>
</table>

Significanceb (probability > F)  
- Treatment: 0.0353  
- Day: 0.0337

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**TABLE 4. Fate of E. coli O157:H7 on fresh-cut romaine treated or not with sodium pyruvate**

<table>
<thead>
<tr>
<th>E. coli O157:H7 population (log CFU/cm²)</th>
<th>Control samples</th>
<th>Pyruvate-treated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after cutting (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.46 ± 0.0</td>
<td>5.46 ± 0.0</td>
</tr>
<tr>
<td>30</td>
<td>4.01 ± 0.69</td>
<td>5.37 ± 0.04</td>
</tr>
<tr>
<td>60</td>
<td>2.73 ± 0.01</td>
<td>4.85 ± 0.12</td>
</tr>
</tbody>
</table>

Significanceb (probability > F)  
- Treatment: 0.0113  
- Time: 0.0384  
- Treatment × time: 0.1569

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treatment was capable of reducing or increasing hydrogen peroxide levels sufficiently to cause a biologically important change in E. coli O157:H7 survival.

This work does not imply that the response could be relied upon to significantly reduce E. coli O157:H7; however, it may contribute to selective pressures that determine whether it survives the cutting and sanitation process. Awareness of this interaction has immediate implications for the design of laboratory-based investigations of the fate of E. coli O157:H7 during the processing or storage of fresh-cut lettuce or other leafy vegetables. While hydrogen peroxide may be inhibitory to microbial survival, it can also lead to tissue damage and breakdown (softening and browning) if the levels and duration of production are great enough. Wound-associated hydrogen peroxide can also be associated with a quality decline of fresh-cut lettuce as well as bacterial survival on the cut surfaces.

ACKNOWLEDGMENTS

The authors thank Steve Orban for his technical assistance and Katherine East and Marie Malone for their guidance in conducting the experiments. The authors also thank Plant Health Care, Inc., Pittsburgh, PA, for providing a commercial formulation of harpin (EBC-351).

REFERENCES

13. Long, L. H., and B. Halliwell. 2009. Artefacts in cell culture: pyruvate as a scavenger of hydrogen peroxide generated by ascorbate quenching experiment, the hydrogen peroxide at the cut surfaces. Hydrogen peroxide was made 1 h after adding the sodium pyruvate.

The results of these experiments provide evidence that the fate of microbial contaminants such as E. coli O157:H7 is influenced by reactive oxygen species liberated by cut lettuce tissues. Hydrogen peroxide was monitored in this work, but it is possible that other reactive oxygen species generated in response to wounding are also produced, including superoxide anion and hydroxyl radical (19). The analyses herein yielded hydrogen peroxide concentrations in whole-tissue samples calculated on a sample weight basis. Since wound-associated reactions occur primarily at the site of injury, concentrations at the cut edge were potentially much higher. Hence, bacteria such as E. coli O157:H7, which have been shown to attach preferentially to cut lettuce tissues (16), may be exposed to stressful and potentially lethal concentrations on intrinsically derived reactive oxygen species during the initial interaction between the bacterial cell and plant tissue. The present study is the first report that lettuce tissues release antimicrobial factors that can influence the fate of human pathogens during a critical unit operation in fresh-cut processing. The changes in hydrogen peroxide production in experiments relating to heat treatment and harpin infusion were less than onefold, and this difference was associated with <1-log difference in E. coli O157:H7 survival. However, in the pyruvate quenching experiment, the hydrogen peroxide at the cut surface would have been completely scavenged. In the harpin experiment, hydrogen peroxide was reduced by nearly 100% by the added pyruvate, and that reduction was associated with an ~3-log difference in survival between treatment and control by 60 min. This suggests that cut-induced hydrogen peroxide production must be increased or reduced at least onefold in magnitude in order to get a significant difference in E. coli O157:H7 survival. While significant differences were found between treatments and control, neither the warm water nor the harpin infusion

FIGURE 1. Effect of sodium pyruvate concentration on the residual concentration of hydrogen peroxide in a solution initially containing 5 μmol of hydrogen peroxide per liter. Measurement of hydrogen peroxide was made 1 h after adding the sodium pyruvate.


