Plant Hormone Interaction and Phenolic Metabolism in the Regulation of Russet Spotting in Iceberg Lettuce

Dangyang Ke and Mikal E. Saltveit, Jr*
University of California, Department of Vegetable Crops, Mann Laboratory, Davis, California 95616

ABSTRACT

Russet spotting (RS) is a physiological disorder induced in iceberg lettuce (Lactuca sativa L.) by exposure to parts per million levels of ethylene at 5 ± 2°C. Ethylene induced phenylalanine ammonia-lyase and ionically bound peroxidase activities that correlated with development of RS symptoms. The ethylene-treated tissue had significantly higher lignin content than air control tissue with lignification localized in walls of RS-affected cells. Ethylene also caused the accumulation of the flavonoids (+)-catechin and (-)-epicatechin and the chlorogenic acid derivatives 3-caffeoyl-quinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. These soluble phenolic compounds were readily oxidized to brown substances by polyphenol oxidase isolated from RS tissue. Ethylene substantially increased ionically bound indole-3-acetic acid (IAA) oxidase activity, while IAA application greatly reduced ethylene-induced phenylalanine ammonia-lyase, peroxidase, and IAA oxidase activities, soluble phenolic content, and RS development.

MATERIALS AND METHODS

Plant Tissue Preparation. Iceberg lettuce (Lactuca sativa L.) was obtained from a local wholesale market and stored at 0°C. Wrapper and cup leaves were discarded and the next 8 to 12 uninjured leaves were used. Previous studies had shown that basal midrib tissue was most sensitive to RS. Therefore, midrib tissue segments, about 2 cm wide and extending 4 cm up the leaf, were excised starting 1 cm from the base of each leaf. Segments excised from a number of heads were randomly distributed among treatments. Segments were submerged in water or treatment solutions for 10 min, blotted dry with soft paper towels, and one segment was placed in each chamber of four-chambered 15 × 100 mm plastic Petri dishes. Each dish served as one treatment replicate and each treatment had at least two replicates. Each experiment was repeated at least twice with similar results. Dishes were placed in 35-L metal boxes which were held at 5°C and flushed with a 100 L/h flow of humidified air with or without 3 ppm ethylene. The ethylene concentration was periodically monitored by gas chromatography.

Estimation of RS Development. After an appropriate period of time, midrib tissue was visually scored to estimate the extent of RS development. A rating scale of 0 to 9 was used as previously described (7). A score of 0 meant no RS, while a score of 9 indicated severe RS.

Assays of Enzyme Activity. Fresh midrib tissue was used for enzyme extraction and assays. PAL activity was assayed as previously described (7). Soluble and bound POD and IAA oxidase were extracted according to the procedure of Gibson and Liu (3) and Thomas et al. (18). Bound POD and IAA oxidase included ionically bound and covalently bound fractions. Preliminary experiments showed that lettuce tissue had negligible amounts of covalently bound POD activity and no covalently bound IAA oxidase activity (data not shown). Therefore, subsequent measurements of bound POD and IAA oxidase activities were confined to the ionically bound fraction to simplify the experimental procedure. The POD assay used guaiacol as the phenolic substrate, and IAA oxidase activity was assayed according to Sequeira and Mineo (15). PPO activity was assayed according to Siriphanich and Kader (16) with caffeic acid as the phenolic substrate. The browning potential of phenolic compounds was estimated at equivalent substrate concentration (10 μM) with both commercially purified PPO and PPO extract from lettuce tissue. The increase in A480 over time was used as an indication of the browning potential for each phenolic compound.

Determination of Phenolic Compounds. The procedure for the extraction and measurement of total soluble phenolic content from fresh midrib tissue was that of Hyodo et al. (5) with p-coumaric acid as the standard. The lignification staining procedure with phloroglucinol was described by Gahan (2). Very thin sections were cut from fresh midrib tissue with a razor blade and were soaked in water. The sections were degassed by vacuum
infiltration, drained, and immediately soaked in an ethanolic solution of phloroglucinol (10 g/95 mL) for 3 min. The sections were drained, placed on a glass plate, and a few drops of concentrated HCl solution were added. After 1 min, the solution was blotted with a paper tissue and a few drops of glycerol were added. The glass plate was covered with a cover glass and mounted on a light microscope. Lignin stained red with the reagent and the red stained areas showed black in the photocopy. The extraction and spectrophotometric measurement of lignin from fresh midrib tissue were according to Fukuda and Komamine (1). The procedure for measuring flavonoid content from fresh midrib tissue was that of Kramling and Singleton (8).

The identification and quantification of individual phenolics were accomplished by HPLC. Ten g of fresh midrib tissue were homogenized with an Ultra-Turro Tissue Homogenizer in 20 mL of methanol (HPLC grade). The homogenate was filtered through four layers of cheesecloth, centrifuged at 25,000 g for 15 min, and the supernatant decanted and flushed with nitrogen gas until dry. The residue was dissolved in 1 mL of methanol plus 4 mL of 0.1 M ammonium phosphate (HPLC grade) buffer (pH 2.8). The sample was then filtered through a 0.45-μm membrane filter before injection. The HPLC measurements were done with Bio-Rad equipment: an HPLC pump model 1330, a 25 × 0.4 cm reverse phase C-18 column (Bio-Sil ODS-5S), and a UV detector model 1306. A linear gradient program from 80% mobile phase solvent A plus 20% mobile phase solvent B to 20% solvent A plus 80% solvent B was used to separate and purify the individual phenolics. Solvent A was 0.1 M ammonium phosphate buffer (pH 2.8) and solvent B was 80% methanol plus 20% of the phosphate buffer. The solvents were degassed by continuous flushing with helium. Each run took 30 min. About 30 authentic chemicals from various groups of phenolic compounds were used as external and/or internal standards for the identification and quantification of individual phenolics.

Separation of the Brown Substances from RS Tissue by Gel Filtration. Several Sephadex gels (G-10, G-15, G-25, G-50, G-75, G-100, G-150, and G-200) with mol wt exclusion limits from 700 to 30,000 were used to separate the brown substances extracted from RS tissue. The column was 50 × 1 cm and the elution solvent was 0.05 M NaCl.

RESULTS AND DISCUSSION

Changes in PAL activity and total soluble phenolic content during RS development in iceberg lettuce (Lactuca sativa L.) were measured to confirm the observations by Hyodo et al. (5). As expected, ethylene treatment induced RS development and caused an accompanying increase in PAL activity and total soluble phenolic content. For example, on day 0 the RS score, PAL activity, and total soluble phenolic content were 0.0, 0.10 μmol/(h·g FW), and 74 μg/g FW, respectively; whereas after 7 d of ethylene treatment, the values had increased to 6.8, 0.56 μmol/(h·g FW), and 268 μg/g FW, respectively. The increase in PAL activity and total soluble phenolic content suggested that phenolic metabolism may be involved in RS symptom development. However, the product of the PAL reaction can give rise to several classes of compounds, including lignins, flavonoids, and hydroxycinnamic esters. Therefore, it was necessary to identify the pathways related to the appearance of RS symptoms.

Lignification as Related to Cell Wall Thickening in RS Tissue. Cell wall thickening is one of the major anatomical changes associated with RS development (6, 10). Walls of RS-affected cells gave a positive reaction for lignin with the phloroglucinol-HCl reagent (Fig. 1A). This indicated that lignification occurred in RS cell walls, which resulted in cell wall thickening, an RS symptom. Ethylene-treated tissue contained significantly higher levels of lignins than tissue exposed only to air. In a typical experiment, for example, the lignin content in RS tissue and air control tissue was 83 and 46 μg/g dry weight, respectively. This increase in lignin content underestimated the level of induced lignin synthesis in RS-affected cell walls, because air control tissue also contained lignified xylem cells (Fig. 1B) which were not related to RS formation yet contributed to the background readings.

Peroxidase is involved in the oxidation of free phenolic precursors in the lignin pathway (4, 11, 12). Therefore, changes in POD activity were measured during RS development (Fig. 2). On day 0, ionically bound POD activity was very low. Ionically bound POD activity increased more than fivefold after the appearance of RS in ethylene-treated tissue on d 3 and continued to increase as RS symptoms developed. There was no significant change in ionically bound POD activity and no RS was observed in the air control. In ethylene-treated tissue, the increase in ionically bound POD activity was highly correlated with the increase in RS score (the correlation coefficient r = 0.93). This increase in ionically bound POD activity is consistent with the increase in lignin biosynthesis in RS cell walls, since free phenolic precursors must be oxidized by POD to form lignin polymers. Several researchers (3, 17, 18) have suggested that ionically bound POD exists in plant cell walls.
Soluble POD activity was very high in both the air control and ethylene-treated tissues (Fig. 2C). It increased in ethylene-treated tissue after d 5 but had not increased by d 3 when RS was rapidly developing. Therefore, the increase in soluble POD activity can not be an initial factor in RS appearance. The role of soluble POD in RS development is unclear. It seems that soluble POD is not involved in the process of cell wall lignification, since cytochemical studies have shown that soluble POD exists in the inner face of the tonoplast (17, 18), whereas the oxidation of phenolic precursors for lignin formation occurs in the cell wall (4, 11, 12).

**Accumulation and Oxidation of Soluble Phenolic Compounds in Relation to Brown Discoloration in RS Tissue.** Cell discoloration is the most obvious symptom of RS. If phenolic metabolism is related to cell discoloration of RS tissue, then discoloration could result from the accumulation of soluble phenolic compounds that are oxidized to quinones which spontaneously polymerize to form brown polymers.

Since it has been reported that flavonoid compounds have very high browning potentials (8), the change in flavonoid content was measured during RS development (Fig. 3). Ethylene treatment increased flavonoid content threefold during the incubation period. This was accompanied by a parallel increase in RS score. Flavonoid content and RS score were highly correlated ($r = 0.95$). Soluble nonflavonoid phenolic content also increased in ethylene-treated tissue from 50 $\mu$g/g FW on d 0 to 76 $\mu$g/g FW on d 3, and continued to increase to 106 and 157 $\mu$g/g FW on d 5 and d 7, respectively. There was no significant change in the flavonoid content, soluble nonflavonoid phenolic content or RS score in the air control tissue during the 7 d of incubation.

The major flavonoid and nonflavonoid phenolic compounds were identified and quantitated by HPLC (Table I). Ethylene induced the accumulation of two flavonoids: (+)catechin and (-)epicatechin. A small amount of phloridzin was also isolated from ethylene-treated tissue. Chlorogenic acid (3-cafeoylquinic acid) was the most concentrated phenolic compound. Ethylene also induced accumulation of the chlorogenic acid derivatives, 3,5-dicafeoylquinic acid and 4,5-dicafeoylquinic acid. Caffeic acid is the precursor for the synthesis of chlorogenic acid derivatives and its concentration was increased by ethylene treatment.

**Fig. 2.** Changes in peroxidase activity ($\mu$mol/[min g FW]) during russet spot development. A, Changes in RS score; B, changes in ionically bound POD activity; C, changes in soluble POD activity. Vertical bars represent standard deviation. Air (---); 3 ppm C$_2$H$_4$ + air (-----).

**Table 1. Major Phenolic Compounds in Lettuce**

<table>
<thead>
<tr>
<th>Phenolic Compound</th>
<th>Air Control</th>
<th>Ethylene Exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)Catechin</td>
<td>6.4</td>
<td>14.0$^*$</td>
</tr>
<tr>
<td>(-)Epicatechin</td>
<td>0.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Phloridzin</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>70.6</td>
<td>277.8</td>
</tr>
<tr>
<td>3,5-Dicafeoylquinic acid</td>
<td>3.3</td>
<td>70.9</td>
</tr>
<tr>
<td>4,5-Dicafeoylquinic acid</td>
<td>4.9</td>
<td>34.3</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>1.8</td>
<td>10.8</td>
</tr>
</tbody>
</table>

* All data were significant at the 1% level of F value.

The concentrations of the major phenolic compounds in RS tissue were in the $\mu$M range. Hyodo et al. (5) identified the accumulation of chlorogenic acid in ethylene-treated tissue by TLC and paper electrophoresis. However, they were unable to quantify the amount of chlorogenic acid or to identify other phenolic compounds due to the limited sensitivity and resolution capacity of the techniques employed. Neither were they able to identify the connection between the accumulation of chlorogenic acid and the appearance of RS symptoms.

The browning potentials of the phenolic compounds identified in RS tissue were determined to ascertain the connection between the accumulation and oxidation of these compounds and cell discoloration in RS tissue. The activity of PPO was 2.2 and 2.3 $\mu$mol/(min g FW) in air control and ethylene-treated tissues, respectively. Although ethylene treatment did not enhance PPO activity, the activity of this constitutive enzyme was always over 100-fold higher than PAL activity and would be sufficient for the oxidation of the endogenous levels of the phenolic compounds observed. Both commercially purified PPO and PPO extract from lettuce tissue were used to oxidize the phenolic compounds identified in RS tissue. Polyphenol oxidase oxidized (-)epicatechin, (+)catechin, chlorogenic acid, caffeic acid, 3,5-dicafeoylquinic acid, and 4,5-dicafeoylquinic acid to brown substances within minutes. These results indicate that the browning reaction in RS tissue may be largely due to the accumulation and oxidation of two groups of phenolics: flavonoids and chlorogenic acid derivatives. In evaluating the contribution of a phenolic compound to the
browning reaction in RS tissue, both the concentration and the browning potential of the compound should be considered. For example, the concentration of (−)-epicatechin was lower than that of chlorogenic acid, but the browning potential of the former was higher than the latter. Both compounds may contribute significantly to the browning reaction in RS tissue. Since phloridzin occurred in small amounts and had very low browning potential, it may not contribute significantly to the browning reaction in RS tissue. Ethylene also induced the accumulation of several minor phenolic compounds whose identities and browning potentials have not been determined.

Both PPO and POD oxidized phenolic compounds to brown substances. However, the brown products from the PPO reaction were stable for weeks, while the brown products from the POD reaction disappeared after 1 d at room temperature. The stability of the brown color in RS tissue suggests that PPO instead of POD is involved in the discoloration.

The brown substances extracted from RS tissue were separated into a high mol wt fraction (mol wt between 5,000 and 30,000) and several low mol wt fractions (mol wt < 700) by gel filtration. The high mol wt fraction was darker brown in color than the low mol wt fractions. The low mol wt fractions are probably the quinone precursors for the formation of the brown polymers in the high mol wt fraction. Supporting this was the observation that the visible and UV spectra of the low mol wt fractions became similar to that of the high mol wt fraction after several days at room temperature (data not shown).

Regulation of Phenolic Metabolism and RS Development by Plant Hormone Interaction. Ethylene induced phenolic metabolism and RS development, whereas both auxin (IAA) and the auxin-type synthetic growth regulator (2,4-D) inhibited ethylene-induced RS development (7). However, 2,4-D was more effective than IAA in inhibiting RS. It seems that there is an interaction between ethylene and auxin in regulating RS development. Preliminary experiments showed that other plant hormones did not significantly affect RS development (data not shown).

Ethylene increased ionically bound IAA oxidase activity from 0.08 unit on d 0 to 1.21, 2.25, and 3.00 units on d 3, d 5, and d 7, respectively (Fig. 4A). Increased IAA oxidase activity could have enhanced the oxidation of endogenous IAA in lettuce tissue. Ethylene-induced ionically bound IAA oxidase activity and RS score were highly correlated ($r = 0.97$). Since exogenously applied IAA inhibits RS development, probably through increasing endogenous IAA levels, the oxidization of endogenous IAA due to IAA oxidase activity could have reduced endogenous IAA levels thereby increasing RS development. This may also explain why 2,4-D was more effective than IAA in inhibiting RS development, since IAA but not 2,4-D can serve as substrate for IAA oxidase.

There was no significant difference in soluble IAA oxidase activity between ethylene-treated and air control tissues (Fig. 4B). The role of soluble IAA oxidase is unclear. Since both air control tissue and ethylene-treated tissue had the same level of soluble IAA oxidase activity, this enzyme activity is not specific for RS development. Thomas et al. (18) have suggested that soluble IAA oxidase probably exists in the vacuole and may not contribute to IAA oxidation in healthy plant tissue.

The application of 1.0 mm IAA greatly reduced PAL activity, total soluble phenolic content, and flavonoid content (Table II). IAA application also reduced POD activity, especially ionically bound POD activity. This indicated that IAA inhibited ethylene-induced phenolic metabolism and thereby inhibited RS development. IAA application also reduced ethylene-induced ionically bound IAA oxidase activity, although it did not reduce soluble IAA oxidase activity. It is interesting that IAA inhibits the oxidation of itself by reducing ethylene-induced IAA oxidase activity.

The application of IAA did not increase ethylene production, while the application of ACC dramatically enhanced ethylene synthesis. For example, ethylene production rates were 0.33, 0.24, and 5.91 nL/(h·g FW) for air control, 1.0 mm IAA, and 1.0 mm ACC treatments, respectively. This suggested that lettuce tissue had an intrinsically high level of ethylene-forming enzyme activity, while IAA treatment did not induce ACC formation. In the aspect of IAA action on ethylene biosynthesis, lettuce seems to be quite different from many other plant systems where IAA can induce ACC synthase activity and greatly increase ethylene production (19).

The interaction between ethylene and IAA in lettuce tissue may be generalized as follows: ethylene induces IAA oxidation, while IAA inhibits ethylene action but does not stimulate ethylene biosynthesis. RS development may be thought of as a rapid senescence process induced by ethylene but inhibited by IAA. Another example of senescence in lettuce is tissue yellowing, which is a gradual process occurring in mature lettuce tissue during incubation in either air or air plus ethylene. Ethylene promoted while IAA retarded this senescence process. For example, 84% of air control tissues but only 19% of IAA-treated tissues became slightly yellow after 7 d of incubation at 5°C; in the same period of time, 100% of ethylene-treated tissues became

![Figure 4](image)

**Figure 4. Changes in IAA oxidase activity ($A_{450}$ [h·g FW]) in lettuce tissue. A, Changes in ionically bound IAA oxidase activity; B, changes in soluble IAA oxidase activity. Vertical bars represent standard deviation. Air (-----); 3 ppm C3H6 + air (----).**

**Table II. Effects of IAA on Ethylene Action**

All measurements were done on the 7th d of incubation. Units: PAL activity (μmol/[h·g FW]); total soluble phenolic content (μg/g FW); flavonoid content (μg/g FW); POD activity (μmol/[min·g FW]); IAA oxidase activity ($A_{450}$ [h·g FW]).

<table>
<thead>
<tr>
<th>Enzyme Activity or Phenolic Content</th>
<th>Ethylene</th>
<th>Ethylene + 1.0 mm IAA</th>
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<tbody>
<tr>
<td>PAL activity</td>
<td>0.81</td>
<td>0.06*</td>
</tr>
<tr>
<td>Total soluble phenolic content</td>
<td>238</td>
<td>76*</td>
</tr>
<tr>
<td>Flavonoid content</td>
<td>97</td>
<td>35*</td>
</tr>
<tr>
<td>Ionically bound POD activity</td>
<td>0.84</td>
<td>0.32*</td>
</tr>
<tr>
<td>Soluble POD activity</td>
<td>0.71</td>
<td>0.37*</td>
</tr>
<tr>
<td>Ionically bound IAA oxidase activity</td>
<td>3.48</td>
<td>1.36*</td>
</tr>
<tr>
<td>Soluble IAA oxidase activity</td>
<td>0.93</td>
<td>1.02*</td>
</tr>
</tbody>
</table>

*Significant at the 1% level of $F$ value.  
†Not significant at the 1% level of $F$ value.
yellow, while only 28% of the tissues treated with ethylene plus IAA became slightly yellow. The opposite effects of ethylene and IAA on this senescence process were very obvious.

Results from previous research (5–7) and this paper were used to construct a possible metabolic pathway for the regulation of RS development (Fig. 5). Ethylene has a profound effect on this pathway since it induces PAL activity and the production of cinnamic acid and its derivative compounds. Some of these products are directed to the lignin pathway, and the ethylene-induced ionically bound POD activity correlates with lignin formation. Cell wall lignification results in cell wall thickening, which is one of the major anatomical symptoms of RS. Other products are largely directed to the flavonoid and chlorogenic acid pathways. The oxidation of flavonoids and chlorogenic acid derivatives by PPO results in the formation of brown substances which cause cell discoloration; the most obvious RS symptom. Furthermore, the accumulation of toxic quinones may be a contributing factor for cell death. In severe cases, pit-like depres-
sions of RS lesions could result from the death and collapse of RS affected cells.

The regulation of RS development is largely through the actions and interaction of ethylene and IAA. Ethylene not only induces phenolic metabolism and RS development but also induces IAA oxidase activity which causes the oxidation of internal IAA. Reduced endogenous levels of IAA could make the lettuce tissue more sensitive to RS. On the other hand, application of IAA or transport of IAA from other tissues could increase the internal IAA level. This could inhibit ethylene action on phenolic metabolism, resulting in reduced PAL and POD activities, reduced production of phenolic compounds, and reduced RS development. Increased internal IAA level could also reduce ethylene-induced IAA oxidase activity and make the lettuce tissue more resistant to RS.

RS is a typical physiological disorder and is an excellent model system for studying the regulation of phenolic metabolism at both the molecular and cellular levels. RS development is also a model system for studying the modes of action and interaction of ethylene and auxin.

![Fig. 5. The pathways related to the regulation of russet spot development. Symbols: ——, activation ———, inhibition.](image)

**LITERATURE CITED**

12. MADER M, R FUSIL 1982 Role of peroxidase in lignification of tobacco cells. II. Regulation by phenolic compounds. Plant Physiol 70: 1132–1134