Calcium Localization in Lettuce Leaves with and without Tipburn: Comparison of Controlled-environment and Field-grown Plants

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Abstract. An electron microprobe was used to determine tissue concentrations of Ca across 20-mm-long leaves of 'Green Lakes' crisphead lettuce (*Lactuca sativa* L.) with and without tipburn injury. Concentrations within the fifth and 14th leaves, counted from the cotyledons, from plants grown under controlled-environment conditions were compared to concentrations within similar leaves obtained from plants grown under field conditions. Only the 14th leaf from plants grown under controlled-environment conditions developed tipburn. Injured areas on these leaves had Ca concentrations as low as 0.2 to 0.3 mg·g⁻¹ dry weight. Uninjured areas of tipburned leaves contained from 0.4 to 0.5 mg·g⁻¹ dry weight. Concentrations across the uninjured 14th leaf from field-grown plants averaged 1.0 mg·g⁻¹ dry weight. Amounts across the uninjured fifth leaves from both environments averaged 1.6 mg·g⁻¹ dry weight. In contrast, Mg concentrations were higher in injured leaves than in uninjured leaves and thus were negatively correlated with Ca concentrations. Magnesium concentrations averaged 4.7 mg·g⁻¹ dry weight in injured leaves compared with 3.4 mg·g⁻¹ dry weight in uninjured leaves from both environments. Magnesium concentrations were uniform across the leaf. Potassium concentrations were highest at the leaf apex and decreased toward the base and also decreased from the midrib to the margin. Potassium averaged 51 mg·g⁻¹ dry weight in injured and uninjured leaves from both environments. No significant differences in K concentration were present between injured and uninjured leaves. This study documented that deficient concentrations of Ca were present in areas of leaf tissue developing tipburn symptoms and that concentrations were significantly higher in similar areas of other leaves that had no symptoms. This study also documented that Ca concentrations were significantly lower in enclosed leaves that exhibited tipburn symptoms than in exposed leaves that did not exhibit tipburn. Also, the amounts of Ca in plants that developed tipburn in controlled environments were lower than in plants of the same cultivar that did not develop tipburn in field plantings. The reduced levels of Ca in plants grown in controlled environments were associated with faster development rates compared with field-grown plants.

Tipburn is a Ca-related disorder of lettuce that involves a collapse and necrosis of the apex and margins of actively growing leaves (Collier and Tibbitts, 1982; Termohlen et al., 1966). The disorder is believed to be due to insufficient Ca transport to enlarging areas of susceptible leaves, rather than insufficient Ca uptake by the roots (Bangerth, 1979). Calcium moves mainly by transpirational mass flow in the xylem (Bell and Biddulph, 1963; Clarkson, 1984). Leaves of lettuce plants that are wholly or partly enclosed as a result of heading are particularly susceptible to the disorder (Collier and Tibbitts, 1982). These leaves do not freely transpire and contain abnormally low levels of Ca (Collier and Huntington, 1983).

Tipburn is most severe in greenhouses and growth rooms, where the injury occurs early in plant development when leaves become enclosed during the beginning stages of head initiation (Collier and Tibbitts, 1982). We have seen tipburn develop on the seventh to ninth true leaves, counted from the cotyledons, in controlled environments as early as 23 days after seeding. When plants are grown under field conditions, tipburn does not occur on leaves this early in plant development. Rather, tipburn

is usually initiated after the head is well formed and close to market maturity (Ryder, 1979).

The reasons for this difference in incidence between field and controlled environments is not completely understood, but it is believed to be related to plant growth rate (Collier and Huntington, 1983; Cox et al., 1976). Tipburn development is associated with environmental conditions that encourage rapid dry-matter accumulation and thus have a high daily requirement for Ca in expanding leaf tissue (Collier and Tibbitts, 1982), conditions commonly present in controlled environments. However, differences in other factors, such as soil temperature, air movement, and vapor pressure deficits, that influence the uptake and transport of Ca to susceptible tissues may occur between these two environments (Bangerth, 1979; Collier and Tibbitts, 1982).

Although Ca deficiency is understood to be the causal factor in injury development, precise determination of Ca concentrations in the injured tissue has not been undertaken. Many workers harvested leaves after injury symptoms were clearly visible, several days or weeks following actual physiological injury, a period during which increased rates of Ca accumulation probably occurred. In addition, samples included uninjured tissue and veins, such as the midrib, which contain moderate amounts of Ca (Collier and Huntington, 1983; Collier and Wurr, 1981). Concentrations of Ca associated with tipburn reported in these studies ranged from 2 to 10 mg·g⁻¹ dry weight (Ashkar and Ries, 1971; Kruger, 1966; Thibodeau and Minotti, 1969; Yanagi et al., 1983). These amounts are significantly higher than the minimum functional requirement of 1 to 2 mg Ca/g dry weight suggested by Loneragan and Snowball (1969). More

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careful studies, involving analysis of entire leaves at the time of injury development, found levels of Ca associated with tipburn ranging from 0.5 to 1.7 mg·g⁻¹ dry weight (Barta and Tibbitts, 1986; Collier and Huntington, 1983; Collier and Tibbitts, 1984). These levels, however, are significantly higher than those found in fruit and storage organs subject to Ca-related injuries (Al-Ani, 1978; Cerda et al., 1979; Chiu and Bould, 1976; Collier et al., 1978; Fallahi et al., 1988).

To stay above the limit of instrument sensitivity when tissue concentrations were low, measurements using traditional plant analysis techniques have required a significant quantity of tissue, which prevented earlier researchers from analyzing discrete, localized areas of leaves expressing symptoms.

Here we report tissue Ca concentrations in selected areas across leaves using wavelength dispersive electron microprobe X-ray analysis, a powerful technique permitting analysis of low Ca concentrations in small areas of tissue (Barta and Tibbitts, 1991; Zeyen, 1982). Concentrations were determined in leaves at a stage of enlargement when tipburn injury is first evident. Concentrations of Ca were compared to concentrations of Mg and K, two minerals known to influence Ca uptake by plants (Kirkby, 1979; Sonneveld and Mook, 1983). Concentrations were determined in leaves from plants grown under controlled-environment conditions and compared to concentrations determined in leaves at a similar stage of development obtained from plants grown under field conditions.

Materials and Methods

Plant culture and leaf harvests

Controlled environment-grown plants. 'Green Lake', a crisphead lettuce, was grown in a walk-in growth chamber (Sherer-Gillett, model CEL 512-37). Six seeds were sown in each 1-liter white plastic pot that contained 50 peat : 50 vermiculite (v/v) growing mix. The pots were watered four times daily to excess with a modified half-strength Hoagland's nutrient solution (Hammer et al., 1978) at a pH of 6.4 containing (mmol·liter⁻¹) 3.0 K, 1.0 Mg, 2.5 Ca, 0.5 H₂PO₄, 7.5 NO₃, 1.0 SO₄, 0.5 Na, 0.5 Cl and (μmol·liter⁻¹) 41.2 Fe, 23.1 B, 4.6 Mn, 0.38 Zn, 0.16 Cu, and 0.05 Mo. Radiation was provided by cool-white fluorescent lamps at a 16 h/8 h (light/dark) photoperiod and a photosynthetic photon flux of 350 ± 35 μmol·m⁻²·s⁻¹. The air was at 20 ± 1°C and 65% ± 5% relative humidity.

After 12 days, the plants were thinned to two per pot, based on the size of the first true leaf. The plants were thinned to one per pot between 14 and 20 days after seeding, by harvest of one of the two plants, when the fifth true leaf, counted from the cotyledons, was 5, 10, 20, or 40 mm long. Twelve plants were selected and harvested at each growth stage. The remaining plants were thinned to one per pot. During enlargement, the fifth leaf became free from enclosure of surrounding leaves and did not develop tipburn. Remaining plants in each pot were harvested between 24 and 31 days after seeding, when the 14th leaf was 5, 10, or 20 mm long. Again, 12 plants were selected and harvested at each growth stage, and the remaining plants were discarded. During enlargement, the 14th leaf was completely enclosed by surrounding leaves and developed tipburn. At each harvest, plants were cut at the cotyledonary node and shoot fresh weight was determined. Only leaves 20 mm long were excised and prepared for electron microprobe X-ray analysis. Two of the fifth leaves and two 14th leaves were used.

Field-grown plants. Field-grown plants of 'Green Lake' let-

tuce were obtained directly from commercial plantings on a muck soil near Friendship, Adams County, Wis., during June and July 1984. Separate plants were harvested when the fifth leaf was 5, 10, 20, and 40 mm long and when the 14th leaf was 5, 10, and 20 mm long, which occurred between 18 and 22, and 28 and 32 days from seeding, respectively. Leaf enclosure patterns under field conditions were similar to those under controlled-environment conditions; however, all leaves were free of tipburn. For harvests when the fifth and 14th leaves were 5 and 40 mm long, five plants were taken at each harvest. When the leaves were 10 and 20 mm long, 10 plants were taken. Plants were harvested by their removal from the soil, with a major portion of the tap roots still intact. Plant samples were placed into an insulated container over crushed ice until they were processed at a laboratory, several hours later. Fresh weights were determined for all plants harvested by cutting at the cotyledonary node. As with leaf harvests from controlled-environment-grown plants, only the 20-mm-long fifth and 14th leaves were excised and prepared for electron microprobe X-ray analysis.

Sample preparation

Freeze-drying. Immediately after excision, leaves were cut into strips 5 mm wide, quick-frozen in liquid nitrogen, and stored at -80°C before drying. Samples were dried under vacuum at -20°C and stored in a desiccator until mounting.

Mounting. Pieces of each leaf sample were attached to 25.4-mm-diameter aluminum mounts using double-stick tape. Samples were positioned so that the abaxial surface would be oriented upward and perpendicular to the electron beam. An edge of each sample was secured to the mount by quick-drying epoxy resin. Samples were coated with evaporated carbon to enhance electron conduction, which prevented charging of the sample.

Microprobe analysis

Instrument conditions. An electron microprobe equipped with wavelength dispersive spectrometers for X-ray separation (Applied Research Laboratories, Sunnyland, Calif., model SEM-Q) was used for analysis of Ca, Mg, and K in the plant tissue. The K_α X-ray lines, mineral standards, and spectrometer crystals used for the various elements are shown in Table 1. A stationary electron beam was used with a diameter of 50 μm. The beam energy was 15 kV and the sample current, measured on wollastonite, was 10 nA. Under these instrument conditions, the lowest detectable concentration of Ca was 0.2 mg·g⁻¹ dry weight (Barta and Tibbitts, 1991).

The large beam area and relatively low beam energy and current were selected to minimize specimen damage during beam exposure. In addition, with a large beam area, several cells were irradiated and analyzed at one time, so that each analysis represents an average concentration of many cells. The actual depth of beam penetration was measured to be between 50 and 55 μm (Barta and Tibbitts, 1991), so that only ≈50% of the leaf profile was analyzed. However, because the concentrations of Ca are relatively uniform in the interveinal tissues of these young leaves, such analyses reflect concentrations throughout the entire leaf cross section (Barta and Tibbitts, 1991).

The K_α X-ray lines and background X rays of each element were counted for a total of 50 sec and quantified to concentration (Barta and Tibbitts, 1991). The determined elemental concentrations in samples were corrected with gelatin standards measured under the same beam conditions to correct for matrix

Table 1. K_{α} X-ray lines, mineral standards, and spectrometer crystals used for electron microprobe X-ray analysis of Ca, Mg, and K.

Element	X-ray line		Standard	Spectrometer crystal
	Voltage (kV)	Wavelength (pm)		
Ca	3.690	335.8	Wollastonite	Lithium fluoride
Mg	1.253	989.0	Magnesium oxide	Thallium acid phthalate
K	3.312	374.1	Microcline feldspar	Pentaerythritol

Table 2. Shoot fresh weight and tipburn incidence of controlled-environment and field-grown 'Green Lake' lettuce at seven stages of plant development based on the length of the fifth and 14th leaves.

Controlled environment				Field		
Leaf length (mm)	Shoot fresh wt (g) ^z	Age (days)	Plants with tipburn (%)	Shoot fresh wt (g) ^z	Age (days)	Plants with tipburn (%)
<i>Leaf 5</i>						
5	0.86 ± 0.07	14	0	0.65 ± 0.09	18	0
10	1.48 ± 0.09	16	0	0.94 ± 0.16	20	0
20	2.62 ± 0.23	16	0	1.67 ± 0.23	20	0
40	4.69 ± 0.25	20	0	3.17 ± 0.22	22	0
<i>Leaf 14</i>						
5	21.15 ± 2.52	25	100	18.10 ± 1.76	28	0
10	32.97 ± 3.84	27	100	32.73 ± 4.21	31	0
20	56.43 ± 6.60	30	100	51.33 ± 7.38	32	0

^z ± SD.

differences between the biological tissue and mineral standards (Barta and Tibbitts, 1991).

Measurements of leaf tissue. Patterns of Ca, Mg, and K were determined across each pair of replicate leaves. Because the patterns of injury on the leaf lamina were similar on each side of the midrib and gradients in mineral concentration were not expected to markedly differ, only one side of each leaf was analyzed. Analyses were performed along four transects. Each extended from the midrib to the margin, roughly perpendicular to the secondary veins. The first measurement transect extended along the upper margin of a leaf. The remaining three were parallel to the first and spaced evenly across the rest of the leaf. Along each transect three to five locations were analyzed for the three elements. At each location, an average concentration was calculated from analysis of two points within 500 μ m of each other. Only areas of interveinal tissue were analyzed. The analyses are plotted on idealized drawings. The results from the two replicate leaves analyzed for each environment and leaf type were combined and are reported as average concentrations.

Results

Tipburn development

Tipburn developed on all plants grown under controlled-environment conditions but was absent in all field-grown plants harvested during the study (Table 2). Tipburn injury appeared as complete collapse and necrosis of the upper half of affected leaves, including both the lamina and midrib. Tipburn was usually evident first on the eighth leaf to develop from the cotyledons, and was found on all successive leaves.

Growth

Plants developed more rapidly under controlled-environment than field conditions (Fig. 1); the fifth and 14th leaves reached

Fig. 1. Shoot growth of 'Green Lake' lettuce grown under controlled-environment and field conditions. Each data point is the average of 12 plants $\pm$ SD.

a length of 20 mm when the former plants were 2 days younger (Table 2). At equivalent lengths of the fifth leaf, shoot fresh weight was higher under controlled-environment than field conditions. However, later in development, at equivalent lengths of the 14th leaf, differences in shoot fresh weight were not significant. The slope of the growth curves in Fig. 1 indicates that growth rate during the exponential phase was similar for controlled-environment and field plants.

Leaf mineral concentrations

The 14th leaf of plants grown in the controlled environment was severely injured by tipburn and had lower concentrations of Ca than the 14th leaf from field-grown plants or the fifth leaf from either controlled-environment or field-grown plants (Fig. 2). Calcium concentrations ranging from 0.2 to 0.4 $\text{mg}\cdot\text{g}^{-1}$ dry weight were present in injured areas. Calcium concentrations in the uninjured lower portion of the leaves ranged from 0.4 to 0.5 $\text{mg}\cdot\text{g}^{-1}$ dry weight. Under field conditions, the 14th leaf did not develop tipburn and contained concentrations of Ca ranging from 0.6 to 1.6 $\text{mg}\cdot\text{g}^{-1}$ dry weight across the lamina, averaging 1.1 $\text{mg}\cdot\text{g}^{-1}$ dry weight.

Leaf 5, a leaf without tipburn, had Ca concentrations ranging from 1.1 to 3.1 $\text{mg}\cdot\text{g}^{-1}$ dry weight, with an average of 1.6 $\text{mg}\cdot\text{g}^{-1}$ dry weight (Fig. 2). This average is three to eight times higher than concentrations found in tipburned leaves. Levels of Ca were generally lowest toward the base and increased toward the upper margin, with the highest levels near the leaf apex. Calcium concentrations and distribution patterns were similar for both growing environments.

Magnesium concentrations were higher in tipburned leaves than in all uninjured leaves (Fig. 3). Concentrations in tipburned leaves ranged from 2.8 to 6.3 mg Mg/g dry weight, with an

Fig. 2. Concentrations of Ca across 20-mm-long 'Green Lake' lettuce leaves expressed as mg Ca/g dry weight. Analysis of leaves 5 and 14, counted from the cotyledons, from plants grown in two environments. Concentrations are averaged for two separate leaves. Shaded area indicates region with tipburn.

Fig. 3. Concentrations of Mg across 20-mm-long 'Green Lake' lettuce leaves expressed as mg Mg/g dry weight. Analysis of leaves 5 and 14, counted from the cotyledons, from plants grown in two environments. Concentrations are averaged for two separate leaves. Shaded area indicates region with tipburn injury.

average of 4.7 mg·g⁻¹ dry weight. Uninjured leaves had Mg concentrations ranging from 2.7 to 5.1 mg·g⁻¹ dry weight, and averaged 3.4 mg·g⁻¹ dry weight. Magnesium was relatively uniform across the uninjured leaves from both growing environments, except for accumulations near the apex of some leaves.

The distribution of K was similar in all leaves regardless of growing environment, leaf number, or presence of tipburn (Fig. 4). Potassium concentrations ranged from 30 to 74 mg·g⁻¹ dry weight with an average of 51 mg·g⁻¹ dry weight. Levels of K were generally highest at the leaf apex and decreased along two axes, from the apex to the base and from the midrib to the margin.

Discussion

Extremely low tissue concentrations of Ca (0.2 to 0.4 mg·g⁻¹ dry weight) were associated with areas expressing tipburn injury symptoms. Concentrations ≤0.4 mg Ca/g dry weight in interveinal areas appear to be critical for injury development. These data support the generally accepted hypothesis that lettuce tipburn results from a localized deficiency of Ca.

The levels of Ca found in tipburn areas of leaves are about

one-tenth the levels reported by Roorda van Eysinga and Smilde (1981) for Ca-deficient tissues of lettuce. The small sample sizes needed for the microprobe allowed analysis of only the injured tissue, unlike determinations of earlier workers whose analyses were affected by the inclusion of uninjured tissue or veins containing higher amounts of Ca. For example, whole inner leaves of mature heads of lettuce having a high incidence of tipburn have been reported to contain from 3.4 to 5.2 mg Ca/g dry weight (Misaghi and Grogan, 1978; Thibodeau and Minotti, 1969; Yangi et al., 1983). Subsamples of the leaf lamina, which removed the influence of midrib but still included some uninjured tissue and minor veins, were reported to contain from 1.1 to 1.7 mg Ca/g dry weight (Collier and Huntington, 1983; Collier and Wurr, 1981), still significantly higher than results obtained in this study.

One factor that affected the results of earlier studies was the time period between the development of symptoms and the harvest for mineral determination. For accurate analyses, leaves must be sampled at the time of injury initiation, when Ca concentrations within the tissue would be at their lowest. Otherwise, there is a chance of additional accumulation of Ca within the tissue, particularly if the leaf becomes exposed and tran-

Fig.4. Concentrations of K across 20-mm-long 'Green Lake' lettuce leaves expressed as mg K/g dry weight. Analysis of leaves 5 and 14, counted from the cotyledons, from plants grown in two environments. Concentrations are averaged for two separate leaves. Shaded area indicates region with tipburn injury.

spires. In this study, analyses were performed on leaves just as tipburn injury was initiated. Initial injury was seen as presence of water soaking, veinal browning, or 0.5-mm spots of latex release at the apex or upper marginal areas of the developing leaves. The leaves were only 20 mm long and were enclosed by surrounding leaves.

The critical concentration of 0.4 mg Ca/g dry weight for tipburn development in lettuce reported in this study is similar to the threshold for injury reported for many Ca-related disorders of fruits, including blossom-end rot of tomato (Cerde et al., 1979), bitter pit of apple (Fallahi et al., 1988), cork spot of pear (Al-Ani, 1978), and internal rust spot of potato (Collier et al., 1978). These plant parts contain a much lower percentage of vascular tissue than leaves. Thus, many nonvascular tissues, the ground tissue of fruits and tubers, and the interveinal tissue of leaves have similar minimum functional requirements for Ca.

Some reports have suggested that tipburn development is a manifestation of a localized Ca deficiency resulting from chelation of Ca by organic acids and other metabolites, lowering the soluble Ca fraction within the leaf (Misaghi and Grogan, 1978; Thibodeau and Minotti, 1969). However, the extremely low concentrations of total Ca detected with electron microprobe

X-ray analyses in this study suggest that total Ca levels are sufficiently low to cause injury.

The higher levels of Ca found in the fifth than in the 14th leaf undoubtedly reflect accumulation resulting from transpirational mass flow of Ca. The fifth leaf, unlike the 14th leaf, was not enclosed by surrounding leaves during early enlargement and was free to transpire. In controlled environments, leaf enclosure has been shown to reduce Ca accumulation sufficiently in developing leaves to induce tipburn (Barta and Tibbitts, 1986).

The higher levels of Ca in the 14th leaves of field-grown plants than in controlled environment-grown plants are consistent with the absence of injury on these leaves, and may be related to development rate. Plants grown under field conditions developed more slowly than plants grown under controlled-environment conditions. The occurrence of tipburn has been correlated with rapid growth rates (Collier and Huntington, 1983; Cox et al., 1976). Leaves of field-grown plants may have been free from injury because leaf enlargement and demand for Ca did not exceed the quantity of Ca that was being taken up by the roots and provided to the leaf tissues. However, many other factors, such as soil temperatures, air movement, vapor pressure deficits, and nutrient levels could have caused the differences in Ca accumulation and tipburn development observed here (Collier and Tibbitts, 1982).

The higher levels of Mg associated with tipburned tissue found in this study have been observed in other Ca-related disorders, including blossom-end rot of tomato (Murray et al., 1972) and bitter-pit of apple (Hopfinger and Poovaiah, 1979). Although the levels of K showed no correlation to tipburn injury, the relatively high tissue concentrations of K along with elevated levels of Mg may have enhanced injury development by straining cell membranes already weakened by Ca stress (Bangerth, 1979; Simon, 1978).

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