Calcium Localization and Tipburn Development in Lettuce Leaves during Early Enlargement

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ADDITIONAL INDEX WORDS. *Lactuca sativa*, physiological disorder, deficiency, magnesium, potassium, electron microprobe, x-ray analysis

ABSTRACT. Tissue concentrations of Ca, Mg, and K were determined across immature leaves of lettuce (*Lactuca sativa* L. 'Buttercrunch') at different stages of enlargement using electron microprobe x-ray analysis. The analysis was with a wavelength dispersive spectrometer to permit detection of low concentrations of Ca. Patterns of mineral accumulation in immature leaves that were exposed were compared to patterns of accumulation in leaves that were enclosed within a developing head. The leaves developing without enclosure were free to transpire and developed normally whereas leaves developing with enclosure were restricted in transpiration and developed an injury that was characteristic of Ca deficiency. In the exposed leaves, Ca concentrations increased from an average of 1.0 to 2.1 mg·g⁻¹ dry weight (DW) as the leaves enlarged from 5 to 30 mm in length. In the enclosed leaves, Ca concentrations decreased from 1.0 to 0.7 mg·g⁻¹ DW as the leaves enlarged from 5 to 30 mm in length. At the tips of these enclosed leaves a larger decrease was found, from 0.9 to 0.3 mg·g⁻¹ DW during enlargement. Necrotic injury first became apparent in this tip area when the concentration was ≈0.4 mg·g⁻¹ DW. Magnesium concentrations across the exposed leaves were similar to concentrations across the enclosed leaves, and did not change with enlargement. Magnesium concentrations averaged 3.5 mg·g⁻¹ DW in both enclosed and exposed leaves during enlargement from 5 to 30 mm. In both exposed and enclosed leaves, K concentrations increased during enlargement from 40 to ≈60 mg·g⁻¹ DW. Potassium concentrations were highest toward the leaf apex and upper margin where injury symptoms occurred, and this may have enhanced injury development. This research documents the critical low levels of Ca (0.2 to 0.4 mg·g⁻¹ DW) that can occur in enclosed leaves of plants and which apparently leads to the marginal apex necrosis of developing leaves seen frequently on lettuce and other crops.

Enclosure of leaves at the growing point of plants leads to reduced Ca concentrations and frequently a tipburn injury of these leaves. (Barta and Tibbitts, 1986; Bradfield and Guttridge, 1979; Palzkill et al., 1980; Tibbitts et al., 1983; Van Berkel, 1988). The enclosure reduces transpiration and thus reduces Ca transport (Collier and Tibbitts, 1982; Marschner, 1995), as most Ca transport in plants occurs mainly in the xylem by mass flow (Clarkson, 1984). This mass flow results from several factors including transpiration, root pressure, and diurnal changes in water stress (Marschner, 1983). Although Ca is generally considered phloem immobile, the phloem does contain low concentrations of Ca (Bangerth, 1979; Clarkson, 1984) and thus provides some Ca to tissues.

Enlarging leaves have a high requirement of Ca for formation and expansion of cell walls (Clarkson, 1984; Demarty et al., 1984; Marschner, 1995). When young leaves become photosynthetically competent, the rate of nitrate reduction is initially high, leading to the formation of organic acids which have a high affinity for Ca (Kirkby and Knight, 1977; Van Egmond and Breteler, 1972). Thus developing tissues require a continuous, adequate supply of Ca during enlargement.

It was the purpose of this study to determine Ca concentrations in areas of developing lettuce leaves during the early stages of leaf enlargement, and compare these to concentrations of K and Mg in these same areas of the leaves. Patterns of nutrient accumula-

tion in exposed leaves that can transpire freely were compared to patterns in enclosed leaves that were restricted in transpiration and subject to Ca deficiency injuries. An analysis procedure was used that permitted an accurate determination of the anticipated low concentrations of Ca in small discrete areas of these leaves.

Materials and Methods

Plants of *Lactuca sativa* 'Buttercrunch', a butterhead lettuce, were grown in a walk-in growth chamber (model CEL 512-37; Sherer-Gillett, Marshall, Mich.). Six seeds were sown in each of 60 1-L white plastic pots, containing a medium of 1 peat : 1 vermiculite (v/v). The pots were watered four times daily to excess with a modified, half-strength Hoagland nutrient solution (Hammer et al., 1978) at a pH of 6.4. Positions of the pots within the growth room were randomly changed several times during the course of the experiment. A 16-h photoperiod was provided by cool-white fluorescent lamps at a photosynthetic photon flux of 350 ± 35 μmol·m⁻²·s⁻¹, averaged across the pots over the length of the experiment. Air temperature and relative humidity averaged 20 ± 1 °C and 65% ± 5%, respectively, during both the light and dark periods.

After 12 d, the pots were thinned to the modal two plants based on the size of the first true leaf. The pots were thinned to one plant per pot by harvests when leaf 5, the outer exposed leaf, reached a length of 5 mm to 30 mm (Fig. 1). Twelve plants were harvested at about daily intervals to obtain plants with leaf 5 at lengths of (average ± range) of 5 ± 0.5, 10 ± 0.5, 20 ± 1.0, and 30 ± 0.5 mm. The remaining plant in each pot was grown for an additional period of ≈5 d and plants harvested again at about daily intervals when leaf 14, an enclosed leaf, reached lengths (average ± range) of 5 ± 1.1, 10 ± 1.0, 20 ± 2.0, and 30 ± 2.5 mm. At each harvest, the individual leaves on each plant were observed for the presence of injury symptoms. Only one leaf from each of two plants from each harvest was selected that had a length closest to the target

Received for publication 26 Apr. 1999. Accepted for publication 31 Jan. 2000. This work was supported by the College of Agricultural and Life Sciences, Univ. of Wisconsin-Madison, the Graduate School, Univ. of Wisconsin-Madison, and NASA Grant NCC2-136. The advice and facilities for analytical procedures, provided by Everett Glover, Dept. of Geology and Geophysics, Univ. of Wisconsin-Madison, is gratefully acknowledged. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.
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Fig. 1. Elongation of exposed and enclosed lettuce leaves of developing lettuce plants. Each symbol represents a leaf from separate plants harvested on the indicated days from seeding.

harvest size. These selected leaves were excised for electron microprobe x-ray analysis.

In previous studies it was found that the 14th leaf, counted from the cotyledons, developed Ca deficiency symptoms with high frequency under these environmental conditions. This leaf enlarged within the enclosure of several surrounding leaves that minimized its potential for transpiring. The 5th leaf was exposed and always devoid of symptoms. This leaf was exposed to light and the ambient chamber atmosphere early in its development and thus could transpire freely.

Immediately after excision, the selected leaves were cut into 5-mm-wide strips, 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. The dried pieces of each selected leaf were attached to 25.4 mm diameter aluminum mounts using double-stick tape. 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.

An electron microprobe (model SEM-Q; Applied Research Laboratories, Inc., Sun Valley, Calif.) equipped with wavelength-dispersive spectrometers for x-ray separation was used for analysis of the plant tissue. Analysis was made for Ca, Mg, and K following procedures detailed in Barta and Tibbitts (1991b).

To establish concentration gradients across the leaves, analyses were performed along four transects on one-half of each leaf. Each transect extended from the midrib to the margin, roughly perpendicular to the secondary veins. The first measurement transect extended along the upper margin. The remaining three were spaced evenly across the rest of the leaf. Along each transect, three to five locations were analyzed for Ca, Mg, and K. At each location an average concentration was calculated from analysis of two points not more than 500 μm apart. Only interveinal locations were selected to avoid confounding effects caused by both incomplete beam penetration on veinal areas and by higher concentrations present in vascular tissue.

The depth of beam penetration was determined to be between 50 and 55 μm and the beam irradiated $\approx 1 \times 10^{-4} \text{ mm}^3$ of tissue. Thus, each analysis involved measurement of the average concentration of element in 10 to 30 cells. The beam did not penetrate

the leaf completely and only 40% to 70% of the leaf cross-section was analyzed. However, initial studies had shown that concentrations within interveinal areas of these immature leaves were similar throughout the leaf cross-section (Barta and Tibbitts, 1991b). These similar concentrations within the leaf were also found in beech (*Fagus sylvatica* L.) leaves with electron microprobe analysis (Thiec et al., 1995). Analyses were performed through the lower epidermis, with the leaf surface parallel to the mount and perpendicular to the electron beam. This orientation was found to be most useful for estimates of the average concentration of Ca, Mg, and K across the leaf profile.

The measured concentrations of each element are presented on illustrations of idealized leaves drawn from leaf clearings showing locations of major veins. The results from the two replicate leaves were combined and are reported as average concentrations at each location across the leaf.

Results

Tipburn injury began to develop in plants at 22 d after seeding. Marginal injury first occurred on each plant on the ninth, tenth or eleventh leaf and continued on all successive leaves that developed. Injury symptoms were first apparent on leaves when they were 20 to 30 mm in length. When the enclosed 14th leaves were 20 mm long, 33% had tipburn, and when 30 mm long, 83% showed injury. Injury was restricted to the leaf apex and distal margin and was characterized by watersoaking, laminal and veinal necrosis, and laticifer rupture. None of the exposed leaves exhibited any injury symptoms.

Calcium concentrations across the sampled exposed and enclosed leaves are presented in Fig. 2. When 5 mm long, Ca concentrations were relatively uniform across both leaves, and averaged $1.0 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$. At 10 mm in length, a slight reduction in Ca concentrations was observed in the distal portion of both leaves, and this decrease was more pronounced in the enclosed leaves. By 20 mm long, Ca concentrations in the exposed leaf had increased dramatically, particularly toward the apex, reversing the earlier pattern of greater accumulation toward the base. Calcium concentrations were highest along the midrib and upper margin. The highest concentration of Ca in the 20 mm leaf, $3.1 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$, was found at the terminus of the midvein, at the leaf apex. At 30 mm in length, a further increase in Ca across the exposed leaf was noted. Calcium was highest at the upper margin and along the midrib, and generally decreased both laterally and basipetally. In contrast, Ca concentrations across the enclosed leaves continued to decrease during enlargement, particularly in the distal marginal areas. At 30 mm in length, Ca concentrations as low as $0.2 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$ were found along the margin associated with areas of injury development as noted by the shading in these figures. Calcium concentrations in basal areas remained relatively unchanged during this development period. Average Ca concentrations for the leaves were 1.0, 0.9, 1.6, and $2.1 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$ for the exposed leaves and 0.9, 0.7, 0.7, and $0.7 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$ across the enclosed leaves, when 5, 10, 20, and 30 mm long, respectively. Thus, exposed leaves exhibited a large increase in Ca over this period of leaf enlargement whereas enclosed leaves exhibited no increase.

Magnesium concentrations across the sampled exposed and enclosed leaves when 5, 10, 20, and 30 mm long are presented in Fig. 3. Magnesium concentrations were relatively uniform across both leaves throughout enlargement, most values were between 2.5 and $4.5 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$. There were no apparent differences in

Fig. 2. Calcium concentrations, $\text{mg}\cdot\text{g}^{-1}$ dry weight, in enclosed and exposed lettuce leaves during enlargement. Concentrations are averages of two replicate leaves. The leaf length is indicated at the bottom of each leaf. Areas showing collapse and tipburn injury are shaded.

concentration between the exposed and enclosed leaves, with the exception that some severely injured areas on the 30-mm-long enclosed leaves had higher Mg concentrations. Average Mg concentrations for the leaves were 3.8, 3.6, 3.2, and 3.5 $\text{mg}\cdot\text{g}^{-1}$ DW for the exposed leaf and 3.4, 3.6, 3.3, and 3.4 $\text{mg}\cdot\text{g}^{-1}$ DW for the enclosed leaf, when 5, 10, 20, and 30 mm long, respectively.

Potassium concentrations across the sampled exposed and enclosed leaves when 5, 10, 20, and 30 mm long are presented in Fig. 4. In contrast to Mg, large gradients in K concentration developed across both leaves during enlargement. Potassium concentrations tended to be greater in an acropetal direction but less laterally from the midrib. The gradients were small when leaves were 5 mm long, and became greater during enlargement. At 30 mm in length, K levels in the apical regions were almost 2-fold higher than in the base. The highest K concentrations were found at the terminus of the midvein at the leaf apex. The gradient of decreasing K from midrib to margin was less consistent in the enclosed leaves than the exposed leaves.

Average K concentrations for the leaves were 46, 50, 56, 64 $\text{mg}\cdot\text{g}^{-1}$ DW for the exposed leaves and 38, 42, 46, and 50 $\text{mg}\cdot\text{g}^{-1}$ DW for the enclosed leaves, when 5, 10, 20, and 30 mm long, respectively.

Discussion

The tissue concentration of Ca found in this study to encourage injury to lettuce was $\leq 0.4 \text{ mg}\cdot\text{g}^{-1}$ DW and is considerably less than the critical levels reported previously for calcium-related injuries in leaf tissues by most researchers (Cresswell, 1991; Huett, 1994; Thibodeau and Minotti, 1969). However these previous analyses

of leaf tissue were confounded by 1) increases in Ca concentrations occurring after initiation of injury and before sampling for analyses, 2) unavailability of procedures to analyze small discrete areas of tissue, and 3) inclusion of veinal tissue which had higher concentrations of Ca (Barta and Tibbitts, 1991a). This Ca concentration of $0.4 \text{ mg}\cdot\text{g}^{-1}$ is considerably below the concentration (1 to 2 $\text{mg}\cdot\text{g}^{-1}$ DW) that Loneragan and Snowball (1969) proposed as a functional minimum for normal growth of dicotyledonous plants. Nonetheless these low concentrations of Ca are similar to the concentrations reported for Ca-related physiological disorders of fruit and certain storage organs (Al-Ani, 1978; Cerda et al., 1979; Fallahi et al., 1988). Fruit and storage tissues, as potatoes (*Solanum tuberosum* L.), provide large amounts of uniform tissue for effective analysis of low concentrations of Ca by atomic absorption spectrometry.

The declining gradient in Ca concentration from base to apex in Ca deficient leaves described in this paper is consistent with observations of patterns of ^{45}Ca -deficient leaves reported by others (Chiu and Bould, 1976; Millikan and Hanger, 1964; Pressman et al., 1993), with Ca accumulation generally confined to vascular tissues in the basal portion of the leaf. Tissues at the apex and distal margin are first to mature, because cell elongation and maturation occur basipetally during leaf ontogeny, once venation has been established (Olson et al., 1969). The supply of Ca is apparently not sufficient to sustain normal development at the apex and distal areas once the leaf initiates rapid elongation and maturation. Injury development also follows this basipetal progression of deficiency.

Reasons for the decrease in Ca concentration in marginal areas

Fig. 3. Magnesium concentrations, $\text{mg}\cdot\text{g}^{-1}$ dry weight, in enclosed and exposed lettuce leaves during enlargement. Concentrations are averages of two replicate leaves. The leaf length is indicated at the bottom of each leaf. Areas showing collapse and tipburn injury are shaded.

Fig. 4. Potassium concentrations, $\text{mg}\cdot\text{g}^{-1}$ dry weight, in enclosed and exposed lettuce leaves during enlargement. Concentrations are averages of two replicate leaves. The leaf length is indicated at the bottom of each leaf. Areas showing collapse and tipburn injury are shaded.

of the enclosed leaves during enlargement are likely associated with restricted transport of Ca into these tissues. These enclosed leaves were encircled by surrounding leaves during the study and thus would not be expected to have any significant transpirational water movement for accumulation of Ca. Alternative modes of Ca transport, as by root pressure during the dark period or by water movement with turgor cycling over successive light and dark periods (Marschner, 1983; Tibbitts and Palzkill, 1979), must not have occurred to any significant degree in the plants of this study.

In the exposed leaves, rapid accumulation of Ca did not occur until the leaf was between 10 and 20 mm in length, even though the leaf was not enclosed and transpiration was not restricted from the beginning of its development. It is likely that the vascular and stomatal tissues necessary to support transpiration did not mature until the leaf reached this size (Olson et al., 1969). The highest Ca levels in the exposed leaves were first observed at the leaf apex, concomitant with the apex to basipetal trend in leaf maturation.

This research measured the total quantity of Ca (soluble + insoluble forms) in lettuce tissues. Thus, the low concentrations of total Ca found contradict evidence by Misaghi and Grogan (1978) and Thibodeau and Minotti (1969) that tipburn results from chelation or binding of Ca by organic acids or other cellular metabolites. Chelation or binding would reduce soluble Ca but not affect the total Ca concentration.

The concentrations of Mg and K found in the leaves are typical for lettuce grown under controlled environment conditions (Berry et al., 1981). The locally high levels of Mg associated with some injured areas in this study also was found in apples [*Malus*

sylvestris (L.) Mill. var. *domestica* (Borkh.) Mansf.] (Hopfinger and Poovaiah, 1979) and potato tissues (Barta, personal observations) exhibiting Ca deficiency injuries. This high Mg concentration may be due to Mg accumulation resulting from cellular dysfunction during injury development. High levels of K also were associated with sites exhibiting Ca deficiency symptoms. The high levels of K in marginal areas may aggravate the Ca-related disorder by causing a higher requirement for Ca to maintain membrane integrity (Bangerth, 1979). However it must be recognized that the injured tissue would also have had some collapse and thus x-ray penetration may have involved a greater proportion of the leaf, causing increases in the measured concentrations.

The relative concentrations of K:Mg:Ca ($\text{K} \gg \text{Mg} > \text{Ca}$), in leaves 5 to 30 mm long, is consistent with concentrations that would be expected when mineral transport occurs through the apoplast and exchange reactions control the concentration balance of these elements (Bell and Biddulph, 1963; Demarty et al., 1984). Exchange sites within the cell walls of the xylem and other tissues are assumed to absorb cations in relation to their position in the Hofmeister and lyotropic series. These exchange sites have the highest affinity for Ca, less for Mg and least for K. Thus Ca, and to a lesser extent Mg, would be removed preferentially from the apoplastic fluid during transport and would be less available for accumulation in developing leaves and other distal organs. However, the rather similar concentrations of Mg in the enclosed and exposed leaves, indicates that other mechanisms must also be controlling the relative proportions of cations found in the lettuce tissues.

The ratio of K:Mg:Ca concentrations found in the 5- to 10-mm-long leaves (44:4:1), is similar to ratios found in phloem sap of several species (45:2:1), but not the apoplastic fluid within the xylem (2:0.2:1) (Hocking, 1980; Jeschke and Pate, 1991; Pate and Hocking, 1978). The similarity in ratios of K:Mg:Ca in the young developing tissue and in the phloem suggests that the phloem may be supplying a significant portion of the Ca early in leaf ontogeny. A leaf is heterotrophic early in its development, depending upon phloem transport for carbon, water, and many essential mineral nutrients for growth. Phloem elements differentiate ahead of the tracheary elements in lettuce (Olson et al., 1969), and are the first functional conducting tissue available to cells in a developing leaf. Because the Ca requirement of the interveinal tissues of the 5 to 10 mm long leaves is very low, $\approx 1 \text{ mg}\cdot\text{g}^{-1}$ DW, the low levels of Ca in the phloem sap could be adequate to sustain early leaf growth before xylem differentiation is completed.

Further research is needed to understand how physiological and developmental changes in young growing leaves affect the magnitude and mode of Ca transport. It is of particular importance to understand how these changes interact under the environmental conditions that encourage Ca-related injuries.

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