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Characterization of leaf boron injury in salt-stressed *Eucalyptus* by image analysis

J.A. Poss[1,*], S.R. Grattan[2], C.M. Grieve[1] and M.C. Shannon[1]
[1]*US Salinity Laboratory, 450 W. Big Springs Road, Riverside, CA 92507, USA and* [2]*University of California, Department of Land, Air, and Water Resources, Davis, CA 95616, USA*

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Abstract

Symptoms of boron toxicity (i.e., necrosis of leaf tips and margins) have been observed on eucalyptus trees in the San Joaquin Valley of California where the trees are being tested for their effectiveness at reducing the volume of agricultural drainage effluents. In a controlled, outdoor sand-tank study, *Eucalyptus camaldulensis* Dehn., Clone 4544 trees were grown and irrigated with combinations of salinity and B to determine their influence on tree growth and water use. Irrigation water quality treatments were prepared to simulate the Na-sulfate salinity, high B nature of these drainage effluents. Electrical conductivities (EC_{iw}) of the waters ranged from 2 to 28 dS m^{-1} and B concentrations ranging from 1 to 30 mg L^{-1}. As an integral component of this study , we developed a method to quantify and correlate foliar damage with leaf B concentrations. By scanning both injured and uninjured leaves into computer files and processing with image analysis, we were able to simultaneously correlate salinity stress with its overall effect on leaf area as well as to quantify the relative fraction of leaf area affected by specific-ion (i.e., B) injury. Leaf area was unaffected by B stress but was reduced by salinity only in the younger leaves. Boron injury was correlated with increasing irrigation water B only in older leaves. The relative injured area (RIA) of the older leaves was related to the B concentrations of leaves from trees grown at various salinities . A regression equation was developed from injury data obtained from trees grown under boron and salinity stress for 223 days (r^2=0.90). From this relationship, we were able to estimate leaf boron concentrations from injury symptoms in leaves selected at random from main trunk branches of trees grown for 333 days under the same stress conditions. The results suggest that this method may have potential as an effective tool for monitoring the response to toxic levels of boron in eucalyptus, once B toxicity has been established by analytical means. The RIA appears to be mitigated by increased salinity of the irrigation water and is consistent with the general reduction in leaf B by salinity. The interactive effects of boron and salinity on foliar injury depends on the physiological age of the leaf.

Introduction

Unlike specific toxicity, unique foliar symptoms of plants under mild to moderate salinity stress are subtle (Bernstein and Hayward, 1958) or absent, but are primarily manifested as reductions in leaf size (Maas and Nieman, 1978). Diagnosis of specific-ion toxicity via leaf injury symptoms is often descriptive or referenced to published photographs (Winsor, 1987). Photographs by Ashworth et al. (1985) documented that pistachio displayed no foliar injury when B leaf concentrations were 220–235 ppm, but exhibited marginal necrosis when leaf B reached 1000 ppm. Handreck (1990) noted that B-tolerant ornamentals could withstand up to 8.3 mg L^{-1} of soil-extractable B before foliar toxicity symptoms appeared. Photographs of boron toxicity symptoms in eucalyptus leaves caused by different soil application rates of boron fertilizers show marginal reddening and necrosis (Dell, 1996). A visual injury rating scale has been described by Vike and Håbjørg (1995) to relate leaf in-

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[*] FAX No.: 190 934 24963. E-mail: jposs@ussl.ars.usda.gov

jury to foliar fluoride concentrations of several native Norwegian trees. Before analyzing leaves for fluoride, leaf samples were rated for injury on a visual scale of 0 (discoloration) to 9 (100% leaf necrosis).

Reductions in boron accumulation in the presence of high sulfate-salinity have been reported in the stems of several rootstocks of *Prunus* (El Motaium et al., 1994). Trees treated with 1 m*M* B and Na-sulfate salinity at 12 dSm^{-1} reduced the B accumulation in stem tissue by as much as 80%, compared to the non-saline control. Leaf B toxicity symptoms were absent as were significant effects of salinity on leaf B concentrations despite leaf B concentration increases of up to 150% with increasing B in the growth medium from 0.025 m*M* to 1 m*M*. Unlike eucalyptus (Dell, 1996), the absence of leaf injury symptoms in *Prunus* species subjected to high concentrations of B is well documented (Brown and Shelp, 1997).

The reuse of agricultural drainage waters to irrigate eucalyptus, a moderately salt-tolerant species (Marcar and Crawford, 1996), has been proposed as an environmentally-sound method for reducing drainage volumes thereby reducing the area needed for environmentally-sensitive evaporation ponds (San Joaquin Valley Drainage Report, 1990). Eucalyptus groves have been established in various locations of California's San Joaquin valley to test this proposition (Cervinka, 1994). A method to assess salinity and boron stress effects on tree productivity could potentially alert managers that some mitigation may be required to save the forest from lethal concentrations. Perhaps by capitalizing on the time-integrated nature of plant response to salinity stress (Bernstein and Pearson, 1954) and by taking advantage of low boron-mobility in many plant species (Brown and Shelp, 1997), provided good quality water is available for strategic use, concentrations can be attenuated to improve the performance and sustainability of the system.

The effectiveness of eucalyptus trees in concentrating salinity and reducing saline drainage water volumes is dependent on tree survivability, growth rate, and the maintenance of relatively high rates of evapotranspiration. Boron and salinity are two primary stresses present in such systems that can potentially reduce transpiration because of reduced plant growth due to leaf necrosis or reductions in total leaf area. Evaluation of the relative injured area of older leaves of trees grown under salinity and boron stress may be a simple and timely criterion for monitoring tree response.

In this study, we used leaf-image analysis to detect reductions in leaf area due to salinity stress, to quantify the fraction of leaf area showing injury symptoms and to estimate boron concentrations in leaves of eucalyptus given the fraction of leaf area injured. The ability to diagnose differences in ion toxicity or salinity effects on leaf area in tree species at the onset of symptoms may allow the grower enough latitude to initiate remedial action, by irrigating with good quality water temporarily, before tree survival is jeopardized. This method is not a substitute for tissue analysis where B concentrations resulting in leaf injury symptoms are unacceptable. This method does, however, allow one to empirically establish incipient injury leaf-B concentrations that would result in significant injury.

Materials and methods

An experiment was conducted at the US Salinity Laboratory in Riverside, CA where *Eucalyptus camaldulensis* Dehn., Clone 4544 trees were grown under different salinity and boron concentrations in the irrigation water. Two trees were planted 15 June 1995 into each of 23 sand tanks (1.5 m×3 m×2 m deep) filled with sand having an average bulk density of 1.4 mg m^{-3} and a volumetric water content at saturation of 0.34 m^3 m^{-3}. Plants were irrigated once daily with a nutrient solution containing (in mol m^{-3}) 2.5 Ca^{2+}, 1.25 Mg^{2+}, 15 Na^+, 3.2 K^+, 6.9 SO_4^{2-}, 7.0 Cl^-, 5.0 NO_3^-, 0.2 $H_2PO_4^-$, 0.050 Fe as sodium ferric diethylenetriamine pentaacetate (NaFeDTPA), 0.023 H_3BO_3, 0.005 $MnSO_4$, 0.0004 $ZnSO_4$, 0.0002 $CuSO_4$, and 0.0001 H_2MoO_4 made up with city of Riverside municipal water. This base nutrient solution served as the control treatment. The 23 treatments were chosen from 36 possible combinations of six irrigation waters salinities (electrical conductivity (EC_{iw}) = 2, 6, 10, 15, 22, 28 dSm^{-1}) and six boron concentrations (1, 4, 8, 15, 25, and 30 mg L^{-1}). The two-way factorial experimental design was partially replicated with five treatments replicated twice. The irrigation waters were predominately sulfate salts with B added as H_3BO_3 (Grattan et al., 1996). Treatments were imposed beginning on 21 Sep 1995. The pH was uncontrolled but was essentially constant ($\sim$ 7.5) due to chemical equilibrium. Treatment irrigation waters were pumped from 4000 L reservoirs into the sand tanks and returned by gravity through a subsurface drainage system. Water lost to evapotranspiration was replenished daily. Irrigations were applied daily to develop a uniform profile where salt and boron concentrations did not vary with depth. Irrigation volumes were sufficient to maintain a negligible difference in salinity and boron concentration between the irrigation water and the drainage water.

Daily irrigations were sufficiently frequent to avoid water stress. Under peak evapotranspiration (ET), we estimated that less than 15% of the total available water was used daily. A weather station was located on site and collected meteorological data during the course of the study. Average day and night temperatures (± standard error) for the first harvest were: 19.9 (0.1), 13.4 (0.1) C, respectively. For the second harvest, average day and night temperatures were higher, i.e. 23.3 (0.1), 15.5 (0.1) C , respectively. Very little difference in average daily high and low relative humidity was observed between harvests. Average high and low relative humidity was 70% and 50%, respectively. Rainfall was captured into the reservoirs and had a negligible effect on solution concentrations.

On 1 May 1996, one tree in each sand tank was harvested. Several leaves from the lowest fourth and highest fourth of the branches of each tree canopy, relative to its own height, were sampled at the proximal or basal (leaves on branches nearest trunk) and distal or apical (leaves on branches furthest from trunk) locations to examine injury on leaves of differing age. In order of increasing age the sample locations were: high distal $<$ high proximal $<=$ low distal $<$ low proximal.

Once sampled, the leaves were placed on a Hewlett Packard ScanJet II flatbed scanner utilizing Deskscan® software[1], operating on a PC with Windows 3.11 to obtain a scanned image file. The brightness and contrast settings for the black and white scanned image at 300 dots per inch were 115 and 129, respectively. The grid size chosen was 501 pixels wide by 501 pixels high. Calibration grids of 25, 50 and 100 cm^2 area were also scanned with the same settings. The scanned images were saved in a TIFF (8-bit) format. These settings were considered optimum for our conditions but offer considerable flexibility. It should be noted that image acquisition procedures could vary from these outlined and still maintain comparative features.

The image file was then imported into Global Lab Image® version 2.20 image software for analysis. The calibration tool feature was used on the calibration grid image files to calibrate the actual area measured. After calibration the particle tool was used to define the region of interest by defining a particle with minimum and maximum threshold values from a 256 grey scale that correlated with the injury symptoms observable from a particular leaf. Threshold values varied slightly (± 10 grey scale) from image to image to match particle definition with observable injury. The area of the defined particle or injured area was then determined with the area feature within the particle tool. During this process the leaf midrib and petiole were also within the optical thresholds for injury so their area had to be subtracted from the injured area sum. A second area measurement of the total leaf area was taken with threshold values that defined the total leaf as an area particle. A test of total leaf area obtained with a Licor® leaf area meter and those obtained with the image software technique indicated an absolute area difference of less than 5% between the two methods. The Relative Injured Area (RIA) was then calculated as injured area/total leaf area for each leaf image individually. Each leaf image sample was a composite of two, three or four individual leaves and was limited by the scanner area to leaf area ratio. After calibration, sample processing time including the scanning of leaves, total and injured area image processing and subtraction of leaf midrib and petiole areas, is less than five minutes per sample.

Boron concentrations of the same leaf samples were obtained by dry ashing 500 mg ground leaf tissue at 500 C for 4 hours. After cooling, 2.5 mL of 7% nitric acid was added to dissolve the ash. The solution volume was brought to 25 mL, filtered, and analyzed for boron by inductively coupled plasma optical emission spectrometry (ICPOES).

On 11 Nov 1996, the second tree was harvested from the sand tanks. After all the leaves were removed from the tree and placed in bags for fresh weight determination, leaves were removed that exhibited varying levels of foliar injury from some of the treatments without any prior knowledge as to the location of the damaged leaves. Leaves were then processed with the above image analysis procedures and the concentration of boron was estimated from the empirical relationship developed from leaves from the first harvest. Leaves from the second harvest tree were analyzed for boron and compared with the predicted concentration of boron derived from the relationship with RIA and salinity levels as inputs for the model.

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[1] Use of a company or product name is for the convenience of the reader and does not imply endorsement of the product by the USDA to the exclusion of others that may also be suitable.

Table 1. Leaf B concentration of healthy, necrotic, and total eucalyptus leaf tissue sampled from trees in the San Joaquin Valley of California

Location in San Joaquin Valley of California	B concentration, mg kg^{-1}		
	healthy leaf tissue	necrotic leaf tissue	Total leaf
Kern Canal/Lost Hills	302±123[1]	1348±203	930±282
Kern Co. Site #2	333±72	1190±35	823±176
Kern Co. site #3	335±100	1733±899	1033±828

[1] Values are mean±standard error of leaves composited from three different trees at each site April 24, 1995.

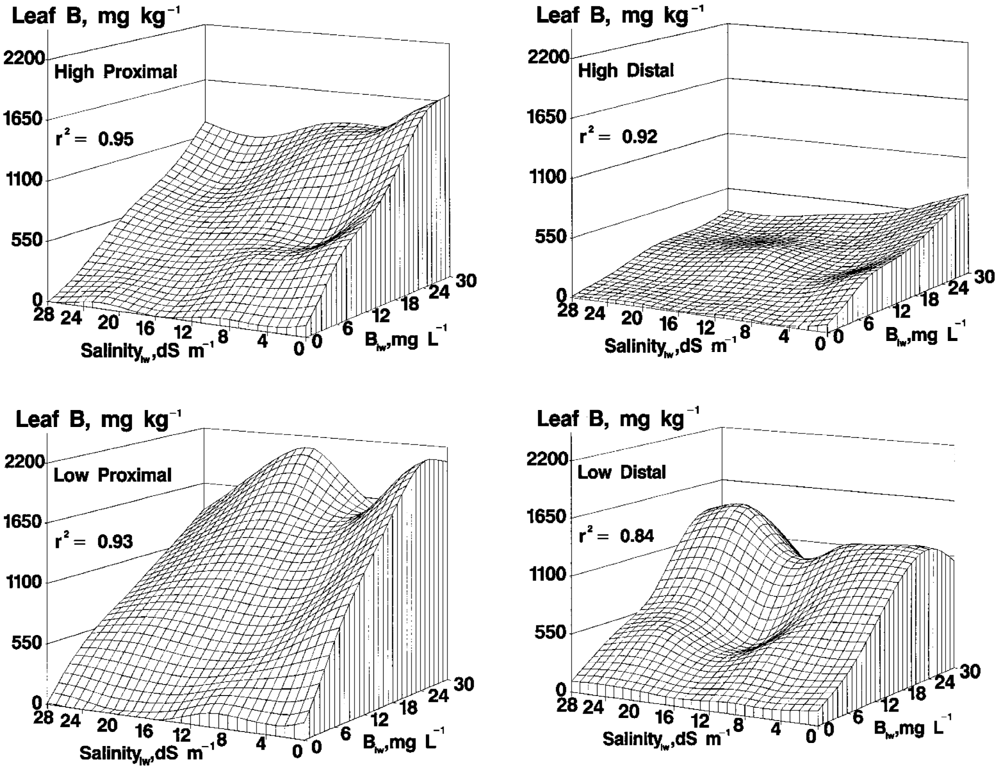

Figure 1. Leaf B concentration of eucalyptus leaves sampled at four canopy locations as a function of irrigation water boron concentrations and salinities.

Results

Influence of Na-sulfate salinity on leaf B concentrations

Leaf B concentration was not only dependent upon the B concentration in the irrigation water, but was also dependent upon position of the leaf in the tree canopy (Figure 1). Depending on leaf location or leaf age, salinity can also influence B concentrations. The lowest B concentrations were measured in the high distal leaves and increased with leaf age. Leaf samples taken from positions between low proximal and low distal in November 1995 indicated that B concentrations were influenced by salinity, whereas B concentrations for those sampled for image analysis in May 1996 were influenced by salinity only in the high proximal leaves. This relationship also provides the measured minimum leaf B concentration related to incipient injury. In our study injury became evident when leaf B concentration exceeded 717±170 mg kg^{-1} dry weight, as determined by fitting the noninjured area as a function of leaf B concentration with a piecewise-linear model

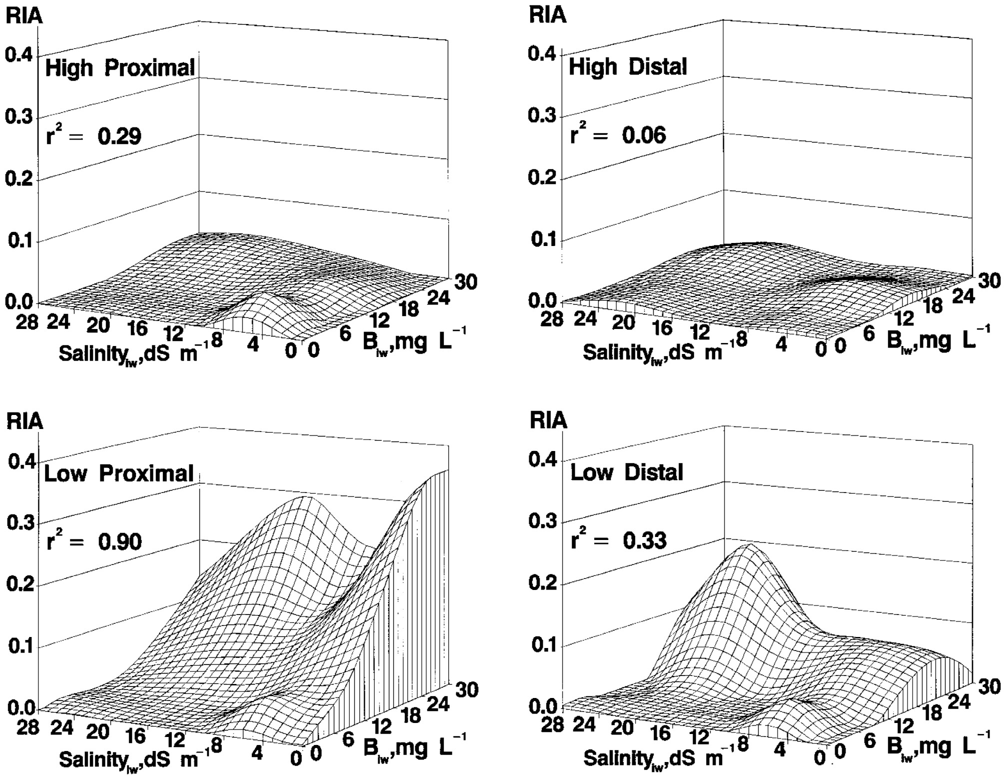

Figure 2. Relative injured leaf area (RIA) of eucalyptus leaves sampled at four canopy locations as a function of irrigation water boron concentrations and salinities.

(van Genuchten and Hoffman, 1984). Concentrations of healthy and necrotic eucalyptus leaf tissue isolated from injured leaf samples in some San Joaquin Valley agroforestry systems were also sampled from trees irrigated with a Na-sulfate waters. At each sampling site, the necrotic tissue and total leaf B concentrations were beyond the fitted threshold (Table 1), indicating the image analysis technique can be related to a field situation.

Relating leaf injury to leaf boron concentrations

In the first harvest, leaf injury was most pronounced on those trees irrigated with the highest levels of boron in the irrigation water and was particularly noticeable on the proximal leaves of lower branches (Figure 2). The percentage leaf surface affected by injury was very low in the low salinity-low boron treatments, except for the 6 dSm^{-1}, 4 mg B L^{-1} treatment that had some (~5%) injury in all but the youngest leaves. The greatest injury (32%) occurred in the oldest tissue in the 2 dSm^{-1} and 25 mg B L^{-1} irrigation solution treatment (Figure 3). Our data also indicate that eucalyptus leaves can tolerate a higher internal leaf B concentration when trees are grown in high Na-sulfate salinity as compared to low salinity (Figure 4). For example, 10% injury is associated with a leaf B concentration of 1100 mg kg^{-1} at low salinity while at high salinity, leaves must attain a concentration of approximately 1300 mg kg^{-1} to produce the same level of injury. Therefore, trees appeared to be less susceptible to B injury in the presence of salinity and more sensitive in the absence of salinity.

Quadratic surface regressions (RSREG procedure, SAS®, 1985) of RIA indicated no significant relationship to salinity or boron with younger branches at either the proximal or distal positions . The relationship was significant for the proximal and distal leaves on the lower or older branches with an $r^2 = 0.90$ for the low proximal leaves (Figure 2). This analysis indicated that visual injury symptoms of only the oldest tissue (low proximal leaves) can be related to the boron and salinity treatments.

To determine a minimally parameterized model, the C_p statistic proposed by Mallows (1964) in the STEPWISE procedure was implemented with the

Figure 3. Leaf boron injury in eucalyptus. From top to bottom: Control (2dS m^{-1}; 1 mg B L^{-1}), 10 dS m^{-1}; 25 mg B L^{-1}, and 2 dSm; 25 mg B L^{-1}.

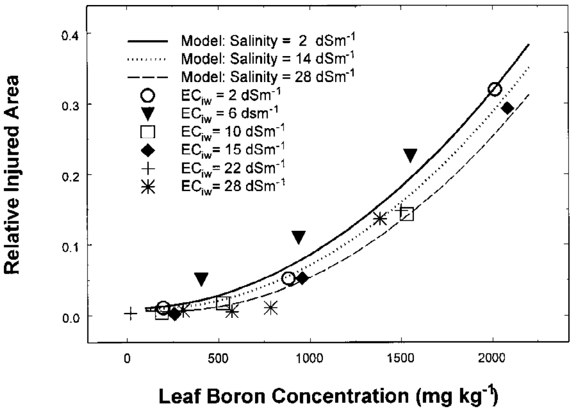

Figure 4. Relative injured leaf area (RIA) as a function of leaf B concentration and predicted relationship from Equation 1.

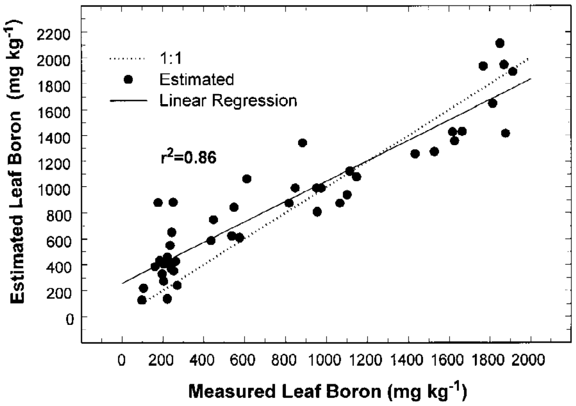

Figure 5. Estimated leaf boron from relative leaf injury symptoms.

maximum r^2 option (SAS®, 1985). The quadratic form was accepted (r^2 = 0.88) with two parameters since increasing to 3, 4, or 5 parameters improved the r^2 to 0.90 at best. Figure 4 shows the final fitted empirical relationship for low, medium and high salinities based on data from the first tree harvest:

$$\mathrm{RIA} = 7.82 \times 10^{-8} * \mathrm{boron}^2 - 1.28 \times 10^{-6} * \mathrm{boron} * \mathrm{salt} + 9.9 \times 10^{-3} \qquad (1)$$

By measuring the relative injured area of leaves grown at a measured irrigation water salinity, boron concentration estimates of those leaves can be generated from Equation 1. This relationship (Equation 1) was then applied to leaves sampled from the second tree harvested. The observed analytical B concentrations were compared to B concentrations estimated from Equation 1. We randomly selected leaves with a wide range of injury from very slightly injured (<0.01 RIA) to severely injured (> 0.3 RIA) from several of the salinity-boron treatment combinations. We could then estimate the leaf B concentration using the relationship above based on the RIA. The estimates generated for leaf B from image analysis, across a range of injury levels, were generally proportional to the measured B in the leaves with significant overestimation of leaf B when damage was low (Figure 5).

Relating yield to leaf injury and leaf boron concentrations

Both leaf B and RIA are correlated with irrigation water boron and salinity for leaves sampled at the low proximal canopy position. Tree fresh weight is also highly correlated with salinity in irrigation water and either leaf B or RIA (Figure 6) for leaves sampled

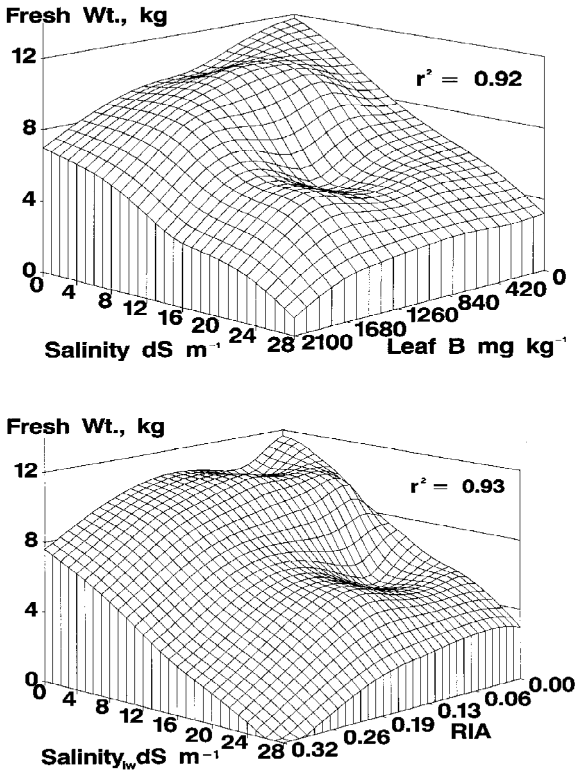

Figure 6. Fresh weight of eucalyptus as a function of irrigation water salinity and either low proximal leaf B concentrations or RIA of low proximal leaves.

from the same canopy position. The number of samples (composed of two to four leaves) that would be required for a 10% difference in population and sample means to be significant at $P = 0.05$ was calculated to be 22 samples based on the coefficient of variation (Lamb, 1976) for the surface regression model. For a 20% difference the sample size could be reduced to six. Based on the relationship between tree fresh weight, irrigation water salinity, and RIA, one would expect a yield reduction of about 30% from control conditions if an RIA of 0.3 were measured when soil water salinity was < 4 dS m^{-1} .

Relating leaf area to salinity

Average leaf areas of younger leaves sampled from trees from the first harvest were significantly reduced by increasing salinity, whereas the individual leaf areas of older leaves were unaffected by salinity treatment. Increased concentrations of B in the irrigation water had no effect on leaf area, therefore leaf areas were averaged across boron levels. Leaf area was proportional to leaf age at all locations with the overall means averaged over salinity and B treatments of 24.2, 29.6, 30.75, and 40.2 cm^2 for youngest to oldest leaves, respectively. Correlation of leaf area as a function of salinity for the younger branches across B treatments was higher for the youngest leaves than the next youngest leaves (Figure 7).

Discussion

The overestimation of boron in eucalyptus leaves showing a low percentage of injury and underestimation of boron concentrations at high injury may be due to variations in age of the leaves sampled from the second harvest. If only the oldest proximal leaves were sampled, then the relationship may have improved. Nevertheless, the relationship explains 86% of the variation regardless of the age and position of the leaves, therefore the estimate is reasonable based solely on empirical RIA-B concentration data obtained from older leaves. Older leaves in the eucalyptus canopy provide the earliest visual indication of boron toxicity in the tissue.

The mitigation of B-leaf injury in older leaves and B concentrations in the youngest leaves in salt-stressed eucalyptus is consistent with the reported salinity-induced reduction in B accumulation in *Prunus* stems (El Motaium et al., 1994) where sulfate-salinity systems were used to evaluate the response of these rootstocks to B. Similarly, Yadav et al. (1989) observed that B concentrations of chickpea were reduced by 28% as salinity was increased from 1.4 to 8.5 dSm^{-1} when salinizing salts were 80% chloride and 20% sulfate. Reductions in plant B concentrations with increasing salinity appears to be independent of the predominant anionic species in solution.

These data also indicate the immobility of B in eucalyptus. The percentage of injured leaf area and related concentrations of B in younger tissue were lower in comparison to older leaves indicating immobility (Brown and Shelp, 1997) of B. An immobilization of B in older tissues of tomato has also been reported (Oertli, 1993) when B stress was removed. This observation is consistent with the proposed mitigative strategy regarding B toxicity in *Eucalyptus*: if it is possible to supply trees with low B irrigation water after detecting significant toxicity via image analysis, then sustained toxicity due to remobilization from older tissues would not be expected to interfere with remedial action for future growth.

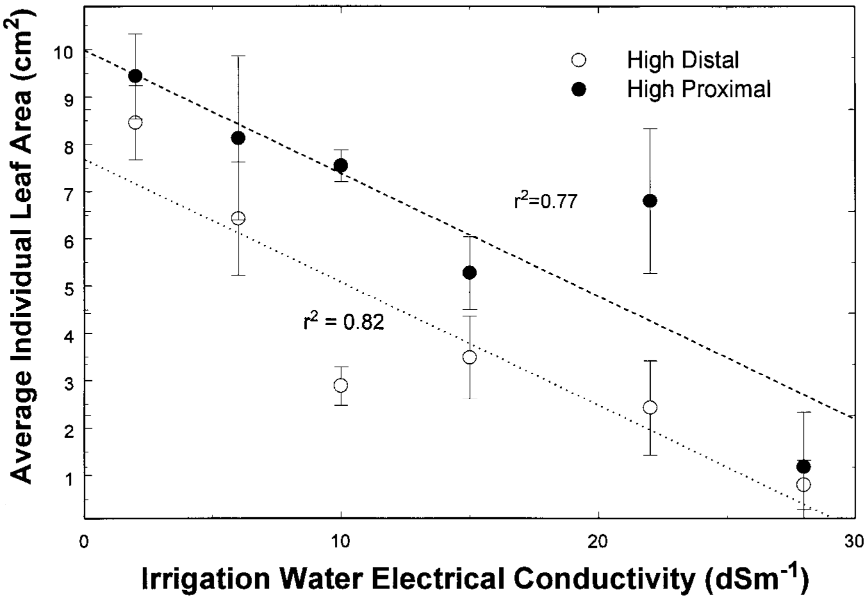

Figure 7. Leaf area of younger leaves of eucalyptus as a funtion of irrigation water salinity. Error bars represent standard error of means averaged across boron treatments.

This method appears to be useful for quantifying the extent of damage due to boron toxicity and may increase the accuracy of characterizing injury symptoms beyond that obtained with qualitative visual rating scales. Computer imaging techniques coupled with tissue analysis for calibration, can lead to conclusions similar to those from tissue ion analysis in the toxic concentration range. The incipient B injury can thus be quantified. This technique may also be useful for quantifying leaf injury due to other abiotic stresses as well that caused by biotic stresses. Leaf area can also be obtained simultaneously which will provide further insight regarding tree health. For example, the lack of correlation between salinity and leaf area in the oldest leaves in our study suggest that these leaves were mature before salinity treatments were imposed. Growth rates based on changes in leaf area may also be possible with this method. Although image analysis is primarily a research tool that should be coupled with other physiological measurements, similar image processing efforts could prove useful in the development and implementation of empirically based management criteria. Tissue analysis cannot be obtained without the help of an independent lab and requires specialized laboratory equipment and use of toxic chemicals. Leaf image analysis coupled with limited tissue analysis for calibration under specific conditions may minimize dependence on tissue analysis although an initial investment in developing the correlation is necessary. Additionally, further improvements in this method would be expected with the incorporation of color scanners and color image analysis software.

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