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High Root-zone Temperatures Influence RuBisCO Activity and Pigment Accumulation in Leaves of 'Rotundifolia' Holly

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Abstract. **Plants of 'Rotundifolia' holly *(Ilex crenata* Thunb.) were grown for 3 weeks with root zones at 30,34,38, or 42C for 6 hours daily to evaluate the effects of supraoptimal root-zone temperatures on various photosynthetic processes. After 3 weeks, photosynthesis of plants grown with root zones at 38 or 42C was below that of plants grown at 30 or 34C. Chlorophyll and carotenoid levels decreased while leaf soluble protein levels increased as root-zone temperature increased. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity per unit protein and per unit chlorophyll responded quadratically, while RuBisCO activity per unit fresh weight increased linearly in response to increasing root-zone temperature. Results of this study suggest that 'Rotundifolia' holly was capable of altering metabolism or redistributing available assimilates to maintain CO_2 assimilation rates in response to increasing root-zone temperatures.**

Supraoptimal root-zone temperatures limit the growth of container-grown plants in the southeastern United States (Fretz, 1971; Ingram et al., 1988; Keever and Cobb, 1984). In Florida, media temperatures as high as 58C in container-grown plants have been recorded (Martin and Ingram, 1988). The critical lethal temperature (T_c) for root of 'Rotundifolia' holly was predicted to be 48 ± 1.5C for a 30-min exposure as determined by electrolyte leakage (Ruter and Ingram, 1991). In a M-liter container under various irrigation regimes, the mean number of degree-hours exceeding 40C was in excess of 3 $h \cdot day^{-1}$ (Martin and Ingram, 1991). The T_c for roots in two species of holly decreased linearly as exposure time increased exponentially (Ingram, 1986). The predicted T_c for a 3-h exposure of 'Helleri' holly *(Ilex crenata* Thunb. 'Helleri') was 45.5 ± 0.5C. Therefore, repeated exposure of holly roots to high root-zone temperatures resulted in root damage that decreased the growth of container-grown holly (Ingram et al., 1988; Ruter and Ingram, 1990).

Little is known about the response of photosynthetic mechanisms to supraoptimal root-zone temperatures. Decreases in photosynthetic rates of woody plants in response to supraoptimal root-zone temperatures have been reported (Gur et al., 1972; Johnson and Ingram, 1984). Foster (1986) reported a nonstomatal decrease in shoot carbon exchange rate when plants of 'Rotundifolia' holly were grown for 1 week with root zones at 36 or 40C rather than 28 or 32C. Greater recovery of carbon exchange rates than of stomatal conductance in 'East Palatka' holly *(Ilex ×attenuata* Ashe) after a 12-week exposure of the root zone to 42C indicated that regulation of photosynthesis may be nonstomatal (Martin et al., 1989).

Loss of photosynthetic activity at high shoot and leaf temperatures has been related to decreased RuBisCO activity (Al-Khatib and Paulsen, 1984; Badger et al., 1982; Monson et al., 1982). Hurewitz and Janes (1987) showed that the RuBisCO activation state was decreased by increasing root-zone temperatures and changes in root sink strength for photoassimilates. Supraoptimal root-zone temperatures influence root carbohydrate content (Foster, 1986; Ingram et al., 1986a), photoassimilate partitioning and carbon loss (Ruter and Ingram, 1990), and root respiratory characteristics (Ruter and Ingram, 1990, 1991).

Loss of chlorophyll and protein in shoots occurred in response to high root-zone temperatures (Kuroyanagi and Paulsen, 1988). Ingram et al. (1986b) found that total protein synthesis decreased and/or protein degradation increased as treatment temperatures for roots of small anise tree *(Illicium parviflorum* L.) increased above 35C. Further research is required to document the effects of supraoptimal root-zone temperatures on physiological responses, particularly nonstomatal limitations related to photosynthesis. Therefore, the objectives of this research were to determine the effects of root-zone temperature on net CO_2 assimilation, RuBisCO activity, and chlorophyll and carotenoid pigment concentrations.

Materials and Methods

Plant material and growth conditions. Rooted, terminal stem cuttings of 'Rotundifolia' holly were potted in 1200-ml clear plastic bags (six drainage holes per bag) using 1000 cm^3 of Metro-Mix 300 (W.R. Grace and Co., Cambridge, Mass) as a growth medium. Plants were fertilized twice weekly with 300 mg N/liter of a soluble 20N–8.8P–16.6K fertilizer (Peters 20-20-20, W.R. Grace and Co.). Plants were grown in a glasshouse (daily air maximum, 34 ± 4C) under natural daylength conditions (Mar.–June 1989) for a minimum of 12 weeks before being transferred to a high-light growth room 3 weeks before the initiation of experiments.

Plants were grown in a 3.0 × 3.6-m walk-in growth room with irradiance supplied by 12, 1000-W phosphor-coated, metal-arc HID lamps (GTE Sylvania Corp., Manchester N.H.). Photosynthetic photon flux (PPF) was 850 ± 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at canopy height measured with a quantum radiometer (LI-COR, Lincoln, Neb.). The photoperiod was 13 h daily (0800 to 2100 HR), with the dark period interrupted for 3 h (0100 to 0400 HR)

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with incandescent light. Air temperature and relative humidity (RH) were maintained at 28 ± 1C and 40% ± 4% during the light period and 21 ± 1C and 90% ± 4% during the dark period. Plants were fertilized twice weekly with 300 mg N/liter of a soluble 20N–8.8P–16.6K fertilizer (Peters 20-20-20) and watered as needed.

Root zones were maintained at 30, 34, 38, and 42C ± 1C for 6 h daily for 21 days for all experiments by use of an electronically controlled root-heating system (Ingram et al., 1990). Plants were arranged using a randomized complete block design. All data were analyzed as general linear models unless otherwise stated.

Extraction and assay of RuBisCO activity. The extraction and assay of HCO_3^-/Mg^{+2}-activated (total) RuBisCO activity (carboxylase activity of the enzyme) from leaf samples was determined using methods from Lorimer et al. (1976). Five replicate plants at each root-zone temperature were used. The most recent fully expanded leaves were collected ≈ 2 h into the light period and frozen at – 80C. Samples (1 g fresh weight) were ground and homogenized in 5 ml of 4C extraction buffer consisting of 100 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1.5% (w/v) PVP-40, and 10 mM D-isoascorbic acid. Samples were centrifuged at 9500× *g* for 6 min at 4C. Assays were performed at 25C using sample vials that contained 400 µl assay medium and control vials that contained 425 µl. No D-Ribulose1,5-bisphosphate (RuBP) was added to control assays. All vials were capped with rubber septa and sealed with masking tape. The assay medium contained 50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM D-isoascorbic acid, and 5.0 mM DTT. Syringes were used to add reactants to each vial. Once 20 µl of 0.5 M NaH^{14}-labeled CO_3 (2 µCi; 1 Ci = 37 GBq) was added to each vial, 50 µl of crude leaf enzyme extract was added to all vials and samples were pre-incubated for 5 min. Assays were initiated by the addition of 25 µl of 5.0 mM RuBP for 1 min and terminated by the addition of 50 µl of 6 N HCl. Controls were terminated after pre-incubation because they lacked RuBP. Samples were uncapped and allowed to dry in a fume hood for 48 h. The incorporation of acid-stable ^{14}C was measured by liquid scintillation spectrometry after the addition of 400 µl distilled H_2O and 4 ml scintillation fluid (ACS-II, Amersham Corp., Arlington Heights, 111.) to each vial. Total soluble protein was determined from extract supernatants according to the method of Bradford (1976).

Measurement of CO_2 assimilation rate. Photosynthesis CO_2 assimilation rates were measured in the laboratory with a portable photosynthesis system (LI-6200, LI-COR). Due to the small leaf size of 'Rotundifolia' holly, canopy gas exchange measurements were made using a custom-designed 7. l-liter plexiglass chamber, which, when mounted to the LI-COR sensing unit, allowed for enclosure of the entire shoot canopy while the root system remained at treatment temperature. The plexiglass chamber was sealed around the stem with foam gaskets and modelling clay. Air was circulated using a LI-COR chamber fan (#6000-16). A high-pressure sodium vapor lamp (Lumalux 400W, GTE Sylvania) was used to provide a PPF of 1100 ± 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at canopy height. Air temperature within the chamber was maintained at 29 ± 1C during measurements by placing a plexiglass water bath between the lamp and the shoot chamber and by providing external air flow using a 1100-V fan. Leaf temperature measurements were made by placing a thermocouple on the abaxial surface of an exposed leaf located about one-third the distance from the top of the plant canopy. RH was maintained at 59% ± 1% for all measurements. After gas exchange measurements were completed, leaf area was determined from a prediction equation using leaf fresh weight [R^2 = *0.99*, leaf area = 5.20 (leaf fresh weight) + 39.37]. Carbon dioxide assimilation rates (CO_2 at 400 ± 5 µl CO_2/liter) were the mean of three measurements per plant. Four replicate plants with uniform foliage canopies at each root-zone temperatures were used (n = 16).

Pigment analysis. Total chlorophyll, chlorophyll a and b, and total carotenoids were determined according to the methods of Bruinsma (1963). Disks from most recently, fully expanded leaves of eight replicate plants at each root-zone temperature were extracted in 80% (v/v) aqueous acetone for 24 h in darkness at 4C before analysis. Analysis of covariance was used for pigment data, using pretreatment measurements as the covariate to account for pretreatment differences.

Results and Discussion

Root-zone temperature significantly influenced CO_2 assimilation rates. Root zones at 30 or 34C induced higher assimilation rates (16.1 ± 0.6 and 17.0 ± 0.9) than those at 38 or 42C (14.7 ± 0.6 and 14.8 ± 0.6 µmol CO_2/sec per m^2, respectively). Leaf area had not been influenced by root-zone temperature after 3 weeks, although there was a trend for decreased leaf area as root-zone temperature increased [62.2 ± 2.1 cm^2 (30C) to 57.5 ± 2.8 cm^2 (42C)].

Soluble protein levels in the leaves increased with increasing root-zone temperature (Table 1). RuBisCO activity per unit protein responded quadratically to increasing root-zone temperature. The highest RuBisCO activity per unit protein occurred at 34C, which corresponded to the highest CO_2 assimilation rate.

RuBisCO activity increased linearly with increasing root-zone temperatures when expressed on a per fresh-weight basis (Table 1). It is possible that the RuBisCO activation state decreased with increasing root-zone temperature. Since RuBisCO comprises ≈ 20% of the soluble protein in leaves (Sharkey, 1985), the increase in soluble protein with increased root-zone temperature may actually have been an increase in RuBisCO protein content. If the RuBisCO activation state were decreased, then an increased RuBisCO protein content would be required to maintain CO_2 assimilation.

Increased root-zone temperatures were shown to affect the RuBisCO activation state in response to altered root sink strength (Hurewitz and Janes, 1987). Thus, the partitioning of photoassimilates between shoots and roots are often influenced by root-zone temperature (Cooper, 1973; Johnson and Ingram, 1984). Supraoptimal root-zone temperatures were found to influence photosynthate partitioning in 'Rotundifolia' holly (Ruter and Ingram, 1990). The N content of pittosporum *[Pittosporum tobira* (Thunb.) Ait.] leaves increased after exposure of their roots to 40C compared to 27 and 32C (Johnson and Ingram, 1984). Since RuBisCO is a major N sink in leaves (Sharkey, 1985), it is possible that 'Rotundifolia' holly was able to reallocate N to the formation of RuBisCO to maintain RuBisCO activity for photosynthesis.

RuBisCO activity, expressed on a per unit chlorophyll basis, responded quadratically and negatively to increased root-zone temperature (Table 1), although the change in RuBisCO activity may have been due to decreased chlorophyll levels (Fig. 1). In tomato *(Lycopersicon lycopersicum* L.), differences in carboxylation efficiency were correlated with chlorophyll content (Augustine et al., 1976). Differences in CO_2 assimilation per unit chlorophyll were not significant (data not shown). Total chlorophyll levels decreased linearly *(R^2 = 0.93)* with increased

Table 1. Effect of increasing root-zone growth temperature on RuBisCO activity in leaves of 'Rotundifolia' holly after 21 days of treatment at 30, 34, 38, or 42C. Values presented are the means (±1 SE) of five replicate plants.

Root-zone temp (°C)	Soluble protein (mg·g^{-1} fresh wt)	RuBisCO activity (μmol CO_2/h per mg protein)	RuBisCO activity (μmol CO_2/h per g fresh wt)	RuBisCO activity (μmol CO_2/h per μg chlorophyll)
30	13.6 ± 1.1	0.572 ± 0.14	8.0 ± 1.3	0.095 ± 0.05
34	13.0 ± 1.1	0.801 ± 0.19	9.9 ± 0.6	0.220 ± 0.11
38	16.5 ± 1.1	0.636 ± 0.14	10.3 ± 1.5	0.141 ± 0.03
42	18.6 ± 1.1	0.580 ± 0.08	10.2 ± 0.7	0.175 ± 0.08
Significance[z]	L*	L^{NS}	L**	L^{NS}
	Q^{NS}	Q*	Q^{NS}	Q*
$P > F$	0.005	0.03	0.03	0.0001
R^2	0.61	0.50	0.50	0.82

[z]L = linear, Q = quadratic.
NS,*,**Nonsignificant or significant at P = 0.05 or 0.01, respectively.

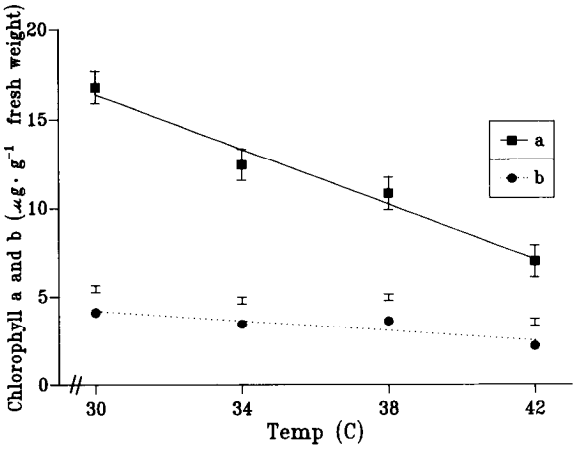

Fig. 1. Chlorophyll a and b concentrations (micrograms per gram of fresh weight) for leaves of 'Rotundifolia' holly after 21 days at root-zone temperatures of 30, 34, 38, and 42C. Each value is the adjusted mean ± 1 SE from analysis of covariance using pretreatment values as the covariate. The relationship between chlorophyll a and root-zone temperature was described as [$R2$ = 0.97, Chl a = 39.64 – 0.774 (T)]. The relationship between chlorophyll b and temperature was described as [R^2 = 0.78, Chl b = 8.360 – 0.139 (T)].

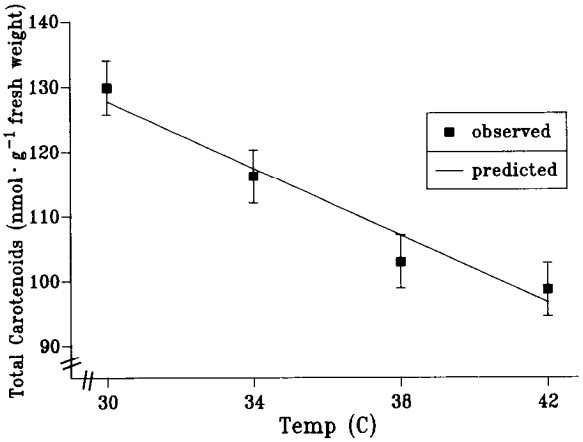

Fig. 2. Total carotenoid concentrations (nanomoles per gram of fresh weight) from leaves of 'Rotundifolia' holly after 21 days at root-zone temperatures of 30, 34, 38, and 42C. Each value is the adjusted mean ± 1 SE from analysis of covariance using pretreatment values as the covariate. The relationship between total carotenoids and temperature was described as [R^2 = 0.94, total carotenoids = 205.37 – 2.58 (T)].

root-zone temperature. Chlorophyll a and b concentrations decreased linearly as root-zone temperature increased. The loss of chlorophyll a was more sensitive to increased root-zone temperature than chlorophyll b (Fig. 1). As a result, the chlorophyll a : b ratios at 38 and 42C (2.91 and 2.97 ± 0.42, respectively) were significantly different from plants grown at 30C (4.33 ± 0.41).

Kuroyanagi and Paulsen (1988) demonstrated that high root-zone temperatures (35C) in wheat *(Triticum* L. spp.) increased the activities of protease and RNase enzymes and increased the loss of chlorophyll, protein, and RNA from shoots. Chlorophyll a and b are important light-harvesting pigments that form various protein–pigment complexes (Lawlor, 1987). Total carotenoids decreased linearly with increased root-zone temperature (Fig. 2). Carotenoids are accessory light-harvesting pigments that are important in photosynthetic energy regulation and dissipation (Lawlor, 1987). Decreased carotenoid levels can lead to photoinhibitory damage and destruction of leaf tissue under high-light conditions. Root-zone temperature had no effect on chlorophyll a fluorescence characteristics (Ruter, 1989), indicating that photosynthetic electron transport systems were not damaged. Since chlorophyll and carotenoid pigments are important light-capturing molecules in photosynthetic processes, differences in CO_2 assimilation may have been due to decreased interception of quanta caused by decreased pigment levels.

In conclusion, various components of photosynthesis responded differently to increasing root-zone temperature. Regulation of photosynthesis in response to environmental stresses is quite complex. High root-zone temperatures have been shown to affect photoassimilate partitioning (Ruter and Ingram, 1990), respiration (Foster, 1986; Ruter and Ingram, 1991), N nutrition (Harrison, 1989), and overall growth of 'Rotundifolia' holly.

Previous results with split-root plants of 'Rotundifolia' holly under different experimental conditions (lower CO_2 and PPF levels than we used) indicated no difference in CO_2 assimilation after 3 weeks of root-zone temperature treatments (Ruter and Ingram, 1990). The general response of CO_2 assimilation to increasing root-zone temperatures up to 42C for 21 days in this study is in agreement with the results of Foster (1986), who found decreased CO_2 assimilation after 7 days at high root-zone temperatures. In our study, chlorophyll and carotenoid concentrations decreased while foliar soluble protein levels increased with increasing root-zone temperatures. Therefore, 'Rotundifolia' holly apparently was capable of altering metabolism or redistributing available assimilates in response to root-zone tem-

perature, which is in agreement with the results of Ruter and Ingram (1990). Recovery of CO_2 assimilation rate in 'East Palatka' holly after exposure to 42C for 12 weeks supported the hypothesis that holly may partially acclimate to supraoptimal root-zone temperatures (Martin et al., 1989). A root-zone temperature of 38C was the upper threshold for several growth and physiological responses in a previous study (Ruter and Ingram, 1990) with 'Rotundifolia' holly. In this study, the highest CO_2 assimilation rate occurred at 34C, while several nonstomatal physiological responses were influenced by increased root-zone temperatures after 3 weeks of treatment. Further research regarding RuBisCO activation state and total RuBisCO concentrations would be necessary to describe the differences in RuBisCO activity that we found.

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