Root Respiratory Characteristics of 'Rotundifolia' Holly under Supraoptimal Temperatures

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Abstract. Respiration of excised *Ilex crenata* (Thunb.) 'Rotundifolia' roots as influenced by root-zone growth temperature and buffer solution temperature was measured in the presence and absence of salicylhydroxamic acid (SHAM) and potassium cyanide (KCN). Respiration rates of roots excised from plants grown for 3 weeks with root-zones at 30, 34, 38, or 42C decreased linearly with increased root-zone growth temperatures when the buffer solution was maintained at 25C. When the buffer solution was the same temperature as the root growth temperature, respiration rates were similar. Respiration in roots from plants grown with the root zone at 30C was maximal with the buffer solution at 34C and decreased to a minimum at 46C. Above 46C, a presumably extra-mitochondrial stimulation of O₂ consumption occurred. The activity of the CN-resistant pathway was fully engaged ($P' = 0.99$) when roots were grown at 30C and buffer solution was at 25C (30-25). CN-resistant pathway activity decreased with 'the buffer solution at 46C.

High root-zone temperatures are known to have profound effects on growth, assimilate partitioning, and respiratory mechanisms of plants (Cooper, 1973). Root growth is often retarded by temperatures in excess of 30C, with cessation of top growth or necrosis occurring at 40C or above (Wong et al., 1971). Decreases in root dry weight (Barr and Pellett, 1972) and root growth (Johnson and Ingram, 1984) have been reported for several woody species exposed to root-zone temperatures above 40C.

Temperatures as high as 45C have been shown to increase respiration rates of actively growing tissues (Janes et al., 1988; Levitt, 1980). Temperature can have significant effects on the maintenance costs of plant cells (Penning de Vries, 1975). Dark and maintenance respiration have a Q_{10} of two or more in a variety of plants as temperature increases (Hunt and Loomis, 1979; McKree, 1974). Respiration rate increases initially in response to supraoptimal root-zone temperatures, but decreases over time due to the progressive degradation of enzymatic processes (Langridge and McWilliam, 1967). Janes et al. (1988) found respiration rates in cultured tomato roots to be highest between 40 and 45C after a 30-min exposure, which suggests that respiratory enzymes are functioning at supraoptimal temperatures.

Temperature differentially affects the electron transport pathways of plant mitochondria (Dizengremel and Chauveau, 1978). Two mitochondrial electron transport terminal oxidases are known, cytochrome c oxidase, which is cyanide-sensitive (CN-sensitive), and the less-understood alternative oxidase, which is CN-resistant. The CN-resistant pathway in plant mitochondria is considered to be an alternative, nonphosphorylating pathway that branches from the respiratory chain at ubiquinone (Henry and Nyns, 1975). Salicylhydroxamic acid (SHAM) is a substituted hydroxamic acid that specifically inhibits the CN-resistant pathway in isolated mitochondria and can be used to assess Un-resistant respiration (Schonbaum et al., 1971). Although concentrations of SHAM up to 25 mM can be used to estimate the

activity of the CN-resistant pathway in plant tissue (Lambers et al., 1983), SHAM has been shown to stimulate O₂ consumption in roots (de Visser and Blaquiére, 1984; Spreen Brouwer et al., 1986). The stimulation of O₂ consumption at low SHAM concentrations (≤ 5 mM) is believed to be caused by a CN-sensitive peroxidase (Spreen Brouwer et al., 1986; van der Plas et al., 1987). The CN-resistant pathway is engaged under a variety of environmental stresses. In leaves of *Fatsia japonica* the CN-resistant respiratory pathway functioned between 6 to 30C, but declined significantly with temperatures up to 40C (Burgos et al., 1987).

Working with *Ilex crenata* 'Rotundifolia', Foster (1986) found root respiration to increase after 7 days with root-zone temperatures up to 40C. Although total CO₂ respired from 'Rotundifolia' holly roots was not influenced by increasing root-zone temperature up to 42C, increases in respiration of current photoassimilates at 38C were detected (Ruter and Ingram, 1990). Increased respiration can affect long-term plant growth and vigor in reaction to supraoptimal root-zone temperatures. Our studies with 'Rotundifolia' holly were conducted to determine: 1) the effect of high temperatures on respiratory rates of roots grown at ambient (25C) or supraoptimal root-zone temperatures, and 2) the effect of high temperature on the function of the CN-resistant and CN-sensitive respiratory pathways.

Materials and Methods

Rooted stem-tip cuttings of *Ilex crenata* 'Rotundifolia' were potted in 1200-ml clear plastic bags using 1000 cm³ of Metro-Mix 300 (W.R. Grace, Cambridge, Mass.). Plants were grown in a glass greenhouse under natural daylength conditions for a minimum of 12 weeks before being transferred to a high-light growth room 3 weeks before the initiation of each experiment.

Experiments were conducted in a 3.0 × 7.6 m walk-in growth room with irradiance supplied by eighteen 1000-W phosphor-coated metal-arc HID lamps (GTE Sylvania, Manchester, N.H.). Photosynthetic photon flux density as measured with a quantum radiometer (LI-COR, Lincoln, Neb.) was 500 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ at canopy height. The photoperiod was 13 hr daily (0700 to 2000 HR) with the dark period being interrupted for 3 hr (2200 to 0100 HR) with incandescent light. The air was maintained at 28 ± 1C during the light period and 21 ± 1C during the dark period. Relative humidity was maintained at ≈40% during the light period and 90% during the dark period. Plants were fertilized twice weekly with solution that contained 300 mg N/liter

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(5.6% nitrate, 4.0% ammonium, 10.4% urea) from soluble 20N-8.8P-16.6K fertilizer (Peters 20-20-20, W.R. Grace).

Two experiments were designed to evaluate the effects of temperature on respiration in roots of 'Rotundifolia' holly. In expt. 1, the plants were grown with the root-zone at 30, 34, 38, or 42C for 6 hr daily (28 ± 1 C base temperature) for 3 weeks before respiration of excised roots was measured in buffer solution corresponding to the growth temperature (30, 34, 38, or 42C) or at 25C. Also, plants were grown with the root-zone at 30C, and respiration measurements were made at buffer solution temperatures of 25 to 70C. For expt. 2, effects of temperature on the CN-resistant respiratory pathway and the CN-sensitive pathway were determined for the same treatments as in expt. 1 and from 25 to 54C using different root tissue samples. For all experiments, root-zone temperature treatments were imposed for 21 days in a completely randomized block design replicated with a minimum of five plants per treatment. Root-zone temperatures were maintained within ± 1 C using an electronically controlled root-heating system described by Foster (1986).

Respiratory measurements were made with a Clark-type oxygen electrode (Hansatech, Kings Lynn, England). The temperature of the reaction vessel was maintained by circulating heated water through the water jacket. The buffer solution consisted of 0.3 M mannitol and 25 mM Tes buffer adjusted to pH 7.1 with KOH plus appropriate concentrations of SHAM (0, 5, 10, and 15 mM) and KCN (0, 0.5, and 1.0 mM) when used. Samples (100 mg) of uniform, fibrous white lateral roots ≈ 1.0 cm long were used. Root samples were pooled from a minimum of three plants per root-zone temperature treatment and were kept moist (maximum period of 10 min) until measurements were made. Rates of O_2 uptake were determined between 8 and 10 min after steady state uptake was achieved and were expressed on the basis of root fresh weight.

V_{res} is that portion of V_T (total respiration) that is insensitive to inhibitors of the CN-sensitive and CN-resistant pathways and is considered to be extra-mitochondrial. The activity of the CN-resistant alternative pathway was determined using the method described by Lambers et al. (1983). Effects of SHAM on the CN-sensitive cytochrome pathway were determined by reverse titration of roots with KCN in the presence and absence of SHAM (de Visser and Blacquiére, 1984; Lambers and Day, 1987; Theologis and Laties, 1978).

Electrolyte leakage procedures for the determination of a critical temperature for 'Rotundifolia' holly roots were performed as described by Ingram and Buchanan (1981). Excised roots (1-g samples) were placed in test tubes and exposed for 30 min to temperatures ranging from 30 to 55C in a thermostatically controlled circulating polyethylene glycol bath. Twenty-five milliliters of deionized water were added to each sample before ice bath incubation for 24 hr. Conductivity of the incubation solution was determined and the samples were killed by autoclaving at 120C for 20 min. Samples were incubated for 24 hr before the final conductivity reading was taken. Electrolyte leakage was expressed as the ratio of incubation solution conductivity after treatment to the conductivity after autoclaving. Data for all experiments were analyzed as a general linear model (SAS Institute, Cary, N.C.), unless otherwise noted.

Results and Discussion

Effects of temperature on respirator rate. When the plants were grown with root zone at 30 to 42C and O_2 consumption was determined at 25C, respiration rate decreased linearly with

increasing root-zone growth temperature [$R^2 = 0.98$, $VT = 0.189 - 0.0033$ (Temp)] (Fig. 1). However, root-zone temperatures did not affect respiration rate when the buffer solution temperature was equal to the growth temperature. The volatility of O_2 decreases with increasing buffer solution temperature and could potentially limit respiration. These results showed that decreased respiration was due to root-zone growth temperature and not to decreased O_2 availability. Solution temperature up to 42C did not adversely affect respiration rates due to decreasing O_2 levels in the buffer solution.

In *Agave deserti*, a succulent desert perennial, root respiration increased several fold from 5 to 40C and decreased 2-fold as temperature increased from 45 to 55C (Palta and Nobel, 1989). Increased respiration rates up to 45C have generally been attributed to increased maintenance respiration and/or changes in enzyme activation energy relative to changes in temperature. Variation in respiration rates at different buffer solution temperatures (Temp/Temp at 38 and 42C vs. Temp/25 at 38 and 42C) in expt. 1 (Fig. 1) may be due to increased maintenance respiration costs when the roots were exposed to higher buffer solution temperatures. Maintenance respiration increases with increasing temperature to produce energy for maintaining cellular structure and ion gradients and for making physiological adaptations necessary to maintain cellular integrity in changing environments.

Enzyme-catalyzed reactions are sensitive to small changes in temperature in the biologically active range. Many enzymes are labile in the range of 40 to 50C. Above an optimum temperature, catalytic effectiveness decreases due to enzyme inactivation or denaturation. Root respiration in 'Rotundifolia' holly increased when plants were exposed to temperatures up to 40C for 1 week (Foster, 1986). The decrease in respiration at 25C when root-zone growth temperatures were maintained for 3 weeks at 30 to 42C possibly may be explained by the loss of catalytic effectiveness of certain respiratory enzymes after repeated, prolonged exposure to elevated temperatures. The elevated respiratory rates during growth at 38 or 42C when the buffer solution

Fig. 1. Influence of root-zone growth temperature and buffer solution temperature combinations on the respiratory rate (V_T) of *Ilex crenata* 'Rotundifolia' roots. Temp/25 (*, $R^2 = 0.98$) indicates plants were grown with their root zone at 30, 34, 38, or 42C and respiration was measured at 25C. Temp/Temp ($\square$, $R^2 = 0.65$) indicates plants were grown and respiration measurements were made at the same temperature. 30/Temp ($\times$) indicates plants were grown with their root-zone at 30C and respiration was measured at and is shown for different temperatures (25 to 60C). Respiration rates from 25 to 46C responded quadratically ($R^2 = 0.94$) while rates from 46 to 60C responded linearly ($R^2 = 0.76$). Each value represents the mean of six independent determinations.

was also 38 or 42C, respectively, could be due to decreased activation energy requirements at the buffer solution temperatures noted when compared to the requirements at 25C.

Two responses were seen as roots grown at 30C were exposed for 10 min to buffer solutions held in the range of 25 to 60C (Fig. 1). A quadratic response described the relationship of respiration to temperature in the range of 25 to 46C [$\text{Pr} > F = 0.01$, $R^2 = 0.94$, $\text{VT} = 0.3559 - 0.0349 (\text{Temp}) + 0.0014 (\text{Temp})^2$]. Maximum O_2 consumption occurred at 34C ($0.121 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \text{FW}$) while respiration decreased by $\approx 50\%$ at 46C in relation to the rate at 25C. Consumption of O_2 increased linearly in the range of 46 to 60C [$\text{Pr} > F = 0.01$, $R^2 = 0.76$, $V_T = -0.2965 + 0.0074 (\text{Temp})$]. Variation in O_2 consumption values $> 54\text{C}$ may have been due to degeneration of root tissue. At 65 and 70C, O_2 consumption (0.057 and $0.032 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \text{FW}$, respectively) decreased below the control value ($0.088 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \text{FW}$).

Consumption of O_2 in the range of 25 to 46C was probably due to mitochondrial respiratory processes, while increased O_2 consumption at temperatures $> 46\text{C}$ may have been due to lipid oxidation caused by peroxidases (Levitt, 1980). Peroxidases are extremely heat-stable enzymes that have been implicated in the SHAM stimulation of O_2 uptake in intact roots of several species (de Visser and Blacquiere, 1984; Spreen Brouwer et al., 1986). Electrolyte leakage procedures have been shown to be an effective method for determining supraoptimal, temperature thermostability of woody plant roots (Ingram and Buchanan, 1981). Lethal temperatures for woody plant roots can be determined from the midpoint of a sigmoidal response curve of electrolyte leakage vs. temperature (Ingram and Buchanan, 1984). The lethal temperature of 'Rotundifolia' holly roots was predicted to be $48 \pm 1.5\text{C}$ for a 30-min exposure as determined by electrolyte leakage (Fig. 2). This lethal temperature corresponds with the temperature initiating an increase in O_2 consumption (46 to 48 C). Baaziz (1989) found that a 20-min exposure to 60C did not completely inactivate all peroxidase isozymes from date palm leaves. In preliminary studies, 'Rotundifolia' holly roots exposed to 44 to 60C for 10 min in buffer solution continued O_2 consumption after the roots were removed. In barley roots, peroxidase was easily washed from the roots but was not active unless exogenous NADH was added

(Bingham and Farrar, 1987). Since an apparent loss of membrane integrity occurs in 'Rotundifolia' holly near 48C, reductants such as NADH necessary for the functioning of a peroxidase may have leaked out into the buffer solution above 46C.

Effect of temperature on the CN-sensitive and CN-resistant pathways. The effect of inhibitor concentrations on root respiration was determined for plants grown with the root-zone at 30C, but measuring respiration in a buffer solution at 25C. Maximum inhibition of respiration (66% decrease) occurred with 1.0 mM KCN and 15.0 mM SHAM. SHAM alone (15.0 mM) decreased respiration (V_T) by $\approx 40\%$ while residual respiration (V_{res}) accounted for 33% of total respiration. The inhibition caused by SHAM indicates the CN-resistant pathway was engaged in vivo in holly roots (Moller et al., 1988). Potassium cyanide, the inhibitor of the CN-sensitive cytochrome pathway, decreased respiration by $\approx 22\%$ at 1.0 mM. The p values (slope of the resulting regression relationship) obtained by reverse filtration for KCN at 0.5 and 1.0 mM were 1.48 and 1.05, respectively. According to de Visser and Blacquiere (1984), a p value > 1 indicates that SHAM inhibits the flow of electrons through the CN-sensitive cytochrome pathway. When $p = 1$, SHAM had no effect on the cytochrome pathway. Accordingly, the concentrations of SHAM used in this study could be used in conjunction with 1.0 mM KCN to inhibit the cyanide-resistant pathway in 'Rotundifolia' holly roots since p at 1.0 mM KCN was not different from 1.

Percent SHAM inhibition ranged from a low of 26.7% for the 30/40C (root-zone growth temperature – buffer solution temperature) treatment to a high of 58.7% for the 30/25C treatment (Table 1). That portion of respiration resistant to KCN (percent CN resistance) ranged from a high of 93.3% (30/46C) to a low of 53.9% (42/42C). Lambers et al. (1983) reported a range of 0% to 44% SHAM inhibition and 32% to 64% CN resistance for intact roots of several agronomic annual species. Residual respiration (V_{res}) accounted for up to 40% of total respiration in crowns of frost-sensitive and frost-tolerant winter wheat cultivars (Rybka, 1989). Lambers and Day (1987) noted that residual respiration rarely exceeds 10% in roots. In our study, residual respiration ranged from a low of 27.9% (34/25C) to a high of 70.4% (30/46 C). This high degree of residual respiration indicates the presence of extra-mitochondrial oxidases that are resistant to both SHAM and KCN or, else, poor penetration of inhibitors into the roots. Large residual respiration values in certain tissues may be caused by poor penetration of inhibitors into certain parts of tissues (Lambers and Day, 1987). Greater suberization in 'Rotundifolia' holly root segments compared to succulent root tips used in other studies could have decreased inhibitor penetration and may have accounted for some of the variation obtained in this study (Table 1). Oxygen consumption did not increase at low concentrations of SHAM for intact holly roots in our study, thus indicating that peroxidases were not stimulated.

The activity of CN-resistant respiration (P'), as calculated from the ratio of SHAM inhibition divided by CN resistance (Lambers et al., 1983), was fully activated ($P' = 0.99$) in plants grown with the root zone at 30C when respiration was measured in buffer solution at 25C. No differences in CN-resistant pathway activity were detected when the temperatures of the root growth zone and of the buffer solution were the same. When the buffer solution was maintained at 25C, the 34 and 38C root-zone treatments exhibited decreased CN-resistant pathway activity compared to the control (30C).

In leaves of *Fatsia japonica*, the capacity of the CN-resistant

Fig. 2. Electrolyte leakage from excised roots of 'Rotundifolia' holly after a 30-min exposure to temperatures in the range of 30 to 55C. The critical temperature ($48.0 \pm 1.5\text{C}$) was determined from a fitted sigmoidal curve as described by Ingram and Buchanan (1984). Each point is the mean of eight replications.

Table 1. Effects of root-zone temperature and increasing buffer solution temperature on the CN-sensitive and CN-resistant respiratory pathways in holly roots. Activity of CN-resistant respiration (P') was calculated as the ratio of (SHAM inhibition)/(CN-resistance). Values presented are the means $\pm$ SE of at least five independent determinations. The concentrations of effectors used were: SHAM (15 mM) and KCN (1.0 mM).

Growth (°C)	Buffer (°C)	S H A M inhibition (%)	CN resistance (%)	P'	v_{res}
30	30	35.7 (10.9)	57.0 (2.4)	0.63 (0.21)	39.8 (2.4)
34	34	34.0 (12.6)	58.7 (11.2)	0.58 (0.22)	35.6 (8.5)
38	38	28.7 (9.8)	60.2 (13.0)	0.48 (0.20)	39.5 (4.5)
42	42	35.5 (6.1)	53.9 (6.1)	0.66 (0.20)	42.9 (4.5)
30	25	58.7 (6.1)	59.0 (8.3)	0.99 (0.19)	34.3 (8.9)
34	25	27.4 (5.2)	41.3 (2.1)	0.67 (0.12)	27.9 (5.6)
38	25	39.4 (9.2)	62.1 (8.1)	0.63 (0.14)	44.0 (8.9)
42	25	38.8 (8.5)	55.1 (8.8)	0.70 (0.32)	33.8 (4.0)
30	25	58.7 (6.1)	59.0 (8.3)	0.99 (0.19)	34.3 (8.9)
30	30	35.7 (10.9)	57.0 (2.4)	0.63 (0.21)	39.8 (2.4)
30	34	50.4 (7.2)	61.4 (8.3)	0.82 (0.22)	41.6 (3.4)
30	38	37.5 (5.2)	59.6 (6.7)	0.63 (0.17)	48.0 (6.5)
30	42	38.5 (11.1)	66.1 (8.3)	0.69 (0.23)	53.2 (5.1)
30	46	26.7 (11.4)	93.3 (6.4)	0.29 (0.12)	70.4 (6.4)
30	50	42.3 (5.0)	63.9 (8.8)	0.66 (0.16)	40.5 (5.8)
30	54	39.0 (5.8)	79.0 (7.7)	0.49 (0.20)	52.3 (7.8)

pathway was high between 6 and 30C (0.81 to 0.98) and declined significantly at 36 and 40C (Burgos et al., 1987). In our study, the activity of the CN-resistant pathway was fully engaged at 25C, ranged from 63% to 82% activation between 30 to 42C and decreased to 29% activation at 46C when roots were grown at 30C and then exposed to the higher buffer solution temperatures. Several authors have proposed that decreased O_2 availability at high temperatures due to decreased O_2 solubility may explain why the alternative pathway accounts for a greater portion of total respiration at low than at higher temperatures (Cook and Cammack, 1985; McCaig and Hill, 1977; Purvis, 1988). Temperature did not have a significant effect on the activity of the CN-resistant pathway at buffer solution temperatures up to 46C, indicating that decreased O_2 solubility with increasing temperature did not limit respiratory functions during this short exposure period. The decrease in CN-resistant pathway activity at 46C corresponds to a decrease in SHAM inhibition and an increase in CN resistance (93.3%). These differences may also be due to thermal effects on the oxidases themselves or temperature-induced alterations in membrane structure. The loss of CN-resistant activity at 46C corresponded to the temperature ($48 \pm 1.5C$) disrupting holly root membranes, thus leading to the disorganization of the electron transport system. We speculate that the "recovery" of activity at 50C was due to an uncharacterized oxidase that appeared to be stimulated by temperatures $> 46C$.

Kiener and Bramlage (1981) have proposed that the CN-resistant pathway serves a protective role against temperature extremes, functioning in reserve in case of failure of the more sensitive CN-sensitive pathway. Since activity did not differ for CN-resistant pathway activity in the range of 25 to 42C, we conclude that engagement of the CN-resistant pathway did not depend on exposure to temperature extremes. Lambers (1982) suggested that the CN-resistant pathway functions as an "energy overflow" that removes excess carbohydrates when substrate levels in the cell are high and the need for carbon skeletons and ATP is low. de Visser et al. (1986) proposed the concept of the

CN-resistant pathway acting as an "overcharge overflow", where under circumstances of increased cellular need for ATP, the CN-resistant pathway would be engaged as an inefficient method for supplementing ATP produced through the cytochrome pathway. Further research would be required before the views of Lambers (1982) or de Visser et al. (1986) could be distinguished relative to the respiratory response of the CN-resistant pathway to increasing root-zone temperature in 'Rotundifolia' holly.

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