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Whole-plant carbon relations and root respiration associated with root tolerance to high soil temperature for *Agrostis* grasses

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Abstract: Plant tolerance to high soil temperature may be related to the adjustment in carbon production and utilization. The objective of this study was to determine changes in whole-plant carbon balance and root respiration rate in relation to root tolerance to high soil temperature for two *Agrostis* grass species varying in heat tolerance. Plant tolerance to high soil temperature was compared between *Agrostis scabra*, a thermal grass species adapted to chronic high-temperature soils in the geothermal areas in Yellowstone National Park, and creeping bentgrass (*Agrostis stolonifera*), a cultivated grass species adapted to cool climatic regions. Plant roots were exposed to low soil temperature (20°C) or high soil temperature (37°C) for 17 days in water baths placed in a controlled-environment growth chamber. Root biomass and cell membrane stability were determined to evaluate root thermotolerance of both species. Canopy photosynthetic rate (Pn), whole-plant respiration rate, root respiration rate, and total non-structural carbohydrate (TNC) content were measured to assess changes in carbon production and utilization in response to high soil temperature. Root biomass and cell membrane stability declined with increasing soil temperature, but the decline was much less for *A. scabra* than *A. stolonifera*, suggesting that roots of *A. scabra* were more tolerant to heat stress. Canopy Pn decreased and whole-plant respiration rate increased for *A. stolonifera*, but canopy Pn and respiration rate were unchanged for *A. scabra* in response to increasing soil temperature. After 17 days of high soil temperature treatment, *A. stolonifera* exhibited carbon deficit at the whole-plant level, whereas *A. scabra* maintained positive carbon gain. Root respiration of plants previously grown at 20°C increased after a short-term treatment (24 h) at 37°C, but the increase was

significantly lower for *A. scabra* than for *A. stolonifera*. TNC content in roots did not show response to short-term (24 h) changes in temperature and did not exhibit species variations. Leaves of *A. scabra*, however, maintained TNC content under both low and high temperature regimes. Our results suggest that root thermotolerance of cool-season grasses could be related to the maintenance of positive whole-plant carbon balance, and down-regulation of whole-plant and root respiration rates in response to increasing soil temperature.

Keywords: *Agrostis scabra*; *Agrostis stolonifera*; Carbon; Heat stress; High soil temperature

Introduction

High soil temperature is a major environmental factor limiting growth and productivity for cool-season plants (Kuroyanagi and Paulsen, 1988; Ruter and Ingram, 1990, 1992; Udomprasert et al., 1995; Xu and Huang, 2000a,b, 2001). Roots may play critical roles in plant survival in environments with high soil temperature, because of their low optimum temperature range for growth (Nielsen, 1974) and many root functions are sensitive to heat stress (McMichael and Burke, 1999). Shoot responses to high temperature stress have been studied extensively. However, roots have been investigated much less than above-ground parts because soil limits their accessibility, in spite of their importance to whole plant responses to environmental stresses (McMichael and Taylor, 1987). The mechanisms associated with root tolerance to high soil temperature are not well understood.

Among the physiological factors that influence root growth and functions, carbohydrate metabolism of roots, including utilization, is fundamentally important, because roots are completely dependent on carbon assimilates supplied by leaves (Lambers et al., 1999). The disturbance of carbohydrate metabolism in roots has been suggested as a primary factor responsible for growth inhibition and dysfunctions of roots grown at high soil temperature (Du and Tachibana, 1994). In order to survive prolonged high soil temperature, roots must maintain carbon supply from shoots and/or must be efficient in carbon utilization when carbon availability is limited. Studies on creeping bentgrass

(*Agrostis stolonifera*) found that removal of photosynthesizing leaves by mowing grass to a low canopy height (3 mm) reduced root life span, especially during times of high soil temperature (Liu and Huang, 2002), which could be attributable to the reduced carbohydrate availability in roots and carbon supply from shoots (Xu and Huang, 2003). A tissue may die if its maintenance requirements are not met or if respiration is so high that sugar reserves are depleted. Elevated root respiration has been observed at high soil temperature, possibly resulting in carbohydrate depletion and root senescence (Klock et al., 1997; Xu and Huang, 2000b).

The optimum soil temperature for root growth of most C3, cool-season grasses range from 10 to 18°C (Fry and Huang, 2004) and root death was observed when soil temperature reached 23°C in cool-season creeping bentgrass (*A. stolonifera* var. *pulastris*) (Pote et al., in press). However, some C3 grass species are able to survive chronically high soil temperature in geothermally-heated areas of Yellowstone National Park (YNP) (Stout and Al-Niemi, 2002). *Agrostis scabra* (“thermal” rough bentgrass) is one of the predominant grass species in the thermal areas (Tercek et al., 2003). Plants in geothermal areas in YNP are commonly exposed to chronic soil temperatures of up to 50°C, even though air temperature is typically below 25°C (Stout and Al-Niemi, 2002). It is unclear how roots of these thermal grasses can survive or even grow at such high soil temperature, while other cool-season grasses cannot survive at these temperature levels. Specifically, whether changes in whole-plant carbon balance and carbon utilization in roots are associated with heat tolerance of cool-season grass species is not well documented. Therefore, the objective of this study was to examine changes in whole-plant carbon balance and root respiration rate in relation to root tolerance to high soil temperature for two *Agrostis* grass species varying in heat tolerance. We hypothesized that thermal *A. scabra* was better able to adjust whole-plant carbon balance and root carbon utilization through respiration in response to increasing soil temperature, in comparison to a heat-sensitive cool-season turfgrass species, *A. stolonifera*.

Materials and methods

2.1. Plant material and growth conditions

Tillers from *A. stolonifera* (cv. L-93) plants and a thermal *A. scabra* ecotype ('FTAS') collected in Yellowstone National Park, Wyoming were transplanted into clear polyethylene bags (5 cm in diameter and 40 cm in length) filled with sand (0.2–0.5 mm particle size). The plants were first maintained in the greenhouse for approximately 60 days for leaf canopy and root establishment, and then transferred into water baths, which were maintained in a controlled-environment growth chamber. The polyethylene bags (with drainage holes at the bottom) were placed in polyvinylchloride (PVC) tubes with same diameter and length, and then installed inside a water bath (see detailed description below). The growth chamber was set at 20/16°C (day/night), photosynthetic photon flux density of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and photoperiod of 12 h. Grass canopy was maintained at a 2 cm height by cutting weekly and fertilized weekly with 50 mL full-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Plants were irrigated daily before imposing high soil temperature and twice daily during the high temperature treatment to maintain soil moisture at field capacity.

2.2. Treatments

Roots of plants were exposed to 20°C (low temperature control) or 37°C (high soil temperature) in water baths for 17 days, while shoots were exposed to an ambient temperature of 20°C. The high temperature treatment was repeated in four water baths. The design of the soil-temperature controlled water bath was previously described by Xu and Huang (2001). Briefly, the bath was made from PVC sheets and had dimensions of 75 cm (length) $\times$ 72 cm (width) $\times$ 40 cm (height). Inside the box, PVC tubes (5 cm in diameter, 43 cm in length) were installed through holes cut from the bottom PVC sheet to allow for drainage. The box was filled with water and water temperature was regulated by an immersion circulating heater (MR 25-1-X, Process Technology, Mentor, OH) and an aquarium pump for water circulation to maintain uniform temperature of the water bath. Sand-filled polyethylene bags containing plants were then placed inside the PVC tubes installed in the water bath. The design allowed for the water bath to maintain constant, predetermined root temperature, while maintaining canopy temperature close

to the ambient level in the growth chamber. The actual canopy temperature averaged 23°C during the treatment period.

For the determination of root respiration and carbohydrate status in response to short-term changes in root temperature, roots that were previously grown at 20 or 37°C in the water bath were then transferred into nutrient solution for 24 h at 20 or 37°C. The short-term temperature treatments were composed of (1) growth temperature at 20°C and measurement temperature at 20°C (LL); (2) growth temperature at 20°C and measurement temperature at 37°C (LH); (3) growth temperature at 37°C and measurement temperature at 37°C (HH); and (4) growth temperature at 37°C and measurement temperature at 20°C (HL).

2.3. Measurements

Canopy net photosynthetic rate (P_n) and whole-plant respiration rate were measured using an infrared gas-exchange analyzer (LiCor 6400, LiCor Inc., Lincoln, NE) with a custom-designed leaf canopy chamber at 17 days of treatment prior to destructive sampling for other analysis. The leaf canopy chamber was constructed of a clear acrylic tube (8 cm diameter, 10 cm in height) and a PVC joining-collar that fit tightly over the PVC tubes containing plants in the water bath. The top of the canopy chamber was covered with transparent propa-film (Licor Inc., Lincoln, NE) for light penetration during photosynthesis measurement. Canopy P_n was measured at approximately 5 h into the 12 h photo period and whole-plant dark respiration rate was measured at approximately 3 h into the dark period. Canopy P_n , whole-plant respiration rate, and all following measurements were performed on four replicates per treatment for each plant species.

Following the gas exchange measurements, plants were removed from the water bath and roots were washed free of sand for measurement of root respiration. The whole plant was then transferred into 250 mL Erlenmyer flasks, where the root system was submerged in 1/4-strength Hoagland's solution while shoots were exposed to the ambient temperature. The hydroponic solution in each flask was aerated via a circulating pump (Apollo Enterprises Inc., Oxnard, CA, USA) maintaining an open-flow system. Flasks were submerged in a water bath to maintain root temperature at either 20 or 37°C, while shoots were exposed to the ambient air temperature (20°C) of the

growth chamber. After a 24 h period, a closed-flow system was created by connecting the exit air from the Erlenmyer to a circulating pump. Roots were sealed airtight inside the flask using a rubber stopper around the shoot base and vacuum grease in order to measure O₂ depletion rate due to root consumption. A 5 mL aliquot of incubation solution was removed from the flask every 10 min for 60 min and injected into a temperature-controlled oxygen electrode (Kansatech, PPI Systems, Burlington, MA) to determine O₂ consumption rate or root respiration rate. Root respiration rates were calculated from the linear portion of the O₂ depletion graph for each sample.

Cell membrane stability was determined by measuring amino acid leakage from root cells. Fresh root samples weighing 0.1 g were immersed in 10 mL of deionized water and placed in a vacuum at 48 kPa for 1 h. The samples were shaken for 24 h, and a 2 mL sample was then taken from the incubation solution to measure amino acid levels. Following reaction with ninhydrin (Rosen, 1957), the absorbance was read at 570 and 440 nm using a spectrophotometer (Spectronic Genesys Series, Spectronic Instruments Inc., Rochester, NY) to determine the amount of amino acids in the incubation solution leaked from the roots (Larkindale and Huang, 2004). Amino acid leakage is expressed as a percent of the total amino acids in the incubation solution before and after temperature treatments.

Root biomass was determined after roots were dried in an oven at 80°C for 72 h. Dried roots and shoots were ground into fine powder for the analysis of total non-structural carbohydrates (TNC). TNC content was measured for two sub-samples per replication using the method described by Ting (1959) with modifications. Briefly, 40 mg of ground tissues were transferred to glass tubes containing 2.5 mL of 5.0% amylase and incubated at 37°C for 24 h. After 24 h incubation period, 0.5 mL of 0.6 N HCl was added to the solution for an additional 18 h. The solution was then neutralized with 10 N NaOH and diluted to 50 mL with distilled water and filtered. Reducing sugars were measured by taking 1.0 mL of the solution and adding 1.5 mL of alkaline ferricyanide solution. The mixture was heated for 10 min in a 100°C water bath and quickly cooled under running water. The pH of the solution was partially neutralized with 3.0 mL of 2 N H₂SO₄. Finally, 1.2 mL of arsenomolybdate solution was added and the total volume was adjusted to

25 mL with distilled water. Absorbance of the solution was measured at 515 nm using a spectrophotometer (Spectronic Genesys Series, Spectronic Instruments Inc., Rochester, NY) and compared to a standard curve to determine TNC content.

2.4. Statistical analysis

The experiment was a completely randomized block design with four replicates. Analysis of variance was conducted (PROC MIXED SAS 8.02, SAS Institute, Cary, NC, USA) to determine the effects of root temperature on root biomass, cell membrane stability, root respiration rate, TNC content, and canopy gas exchange rates. Treatments were separated using the least significant difference (LSD) test at $P = 0.05$.

3. Results

3.1. Root thermotolerance

Root biomass for both *A. stolonifera* and thermal *A. scabra* declined at the high soil temperature (37°C), compared to the control at 20°C (Fig. 1). However, the reduction in root biomass was lower for thermal *A. scabra* (a 8% reduction) than for *A. stolonifera* (a 15% reduction). In general, thermal *A. scabra* plants had greater root biomass than *A. stolonifera*. Root cell membrane leakage, measured as amino acid leakage, of *A. stolonifera* increased significantly at 37°C, as shown by the increased percent of amino acid leakage (Fig. 2). While increased membrane leakage also occurred in roots of thermal *A. scabra* at the high temperature, this change was not significantly different compared to that of the control plants at 20°C. In addition, the maximum leakage for thermal *A. scabra* was 50%, while the maximum leakage for *A. stolonifera* was 80% when both species were exposed to the high temperature (Fig. 2).

3.2. Whole-plant carbon balance under prolonged high soil temperature

Canopy net photosynthetic rate (P_n) did not change with increasing soil temperature for thermal *A. scabra*, but decreased significantly for *A. stolonifera* at 37°C after 17 days of treatment (Fig. 3A). Canopy dark respiration rate was not affected by increasing temperature for thermal *A. scabra*, but increased significantly for *A. stolonifera* (Fig. 3B). The most profound differences between the two species in terms of changes in canopy photosynthetic and respiration rates with increasing soil temperature can be seen when

total carbon consumption through respiration was subtracted from the total carbon fixation in photosynthesis (Fig. 3C). At 20°C, both species maintained a positive carbon gain; however, at 37°C, there was a negative carbon budget for *A. stolonifera*, as opposed to a positive carbon gain for *A. scabra* (Fig. 3C).

Fig. 1. Root biomass for *A. stolonifera* and *A. scabra* in response to high soil temperature. Different lowercase letters indicate significant differences in root biomass between different temperature treatments ($P \leq 0.05$) within a given species based on a LSD test. Different uppercase letters indicate differences between *A. stolonifera* and *A. scabra* at a given temperature treatment ($P \leq 0.05$) based on a LSD test.

Fig. 2. Root cell membrane stability expressed as percent amino acid leakage for *A. stolonifera* and *A. scabra* in response to high soil temperature. Different lowercase letters indicate significant differences in cell membrane stability between temperature treatments ($P \leq 0.05$) within a given species based on a LSD test. Different uppercase letters indicate differences between *A. stolonifera* and *A. scabra* at a given temperature treatment.

Fig. 3. Canopy photosynthetic rate (A), respiration rate (B), and carbon balance (C) for *A. stolonifera* and *A. scabra* in response to high soil temperature. Different lowercase letters indicate differences in canopy photosynthetic rate, respiration rate, and carbon balance between temperature treatments ($P \leq 0.05$) within a given species based on a LSD test. Different uppercase letters indicate differences between *A. stolonifera* and *A. scabra* at a given temperature treatment ($P \leq 0.05$) based on a LSD test.

Fig. 4. Root respiration expressed as O₂ consumption rate for *A. stolonifera* and *A. scabra* for plants grown at 20 or 37°C and measured at 20 or 37°C. HH—grown and measured at 37°C, HL—grown at 37°C and measured at 20°C, LH—grown at 20°C and measured at 37°C, LL—grown at 20°C and measured at 20°C. Different lowercase letters indicate significant ($P \leq 0.05$) difference between temperature treatments within a given species based on a LSD test. *Differences between *A. stolonifera* and *A. scabra* at a given temperature (LSD test, $P < 0.05$).

3.3. Root respiration rate in response to short-term temperature changes

Short-term temperature responses of root respiration were evaluated by measuring oxygen consumption rate at 20 and 37°C after the plants grown at 20 or 37°C were transferred into nutrient solutions. Short-term temperature changes demonstrated the difference in the capacity of respiratory control between the two species (Fig. 4). Root respiration rates of *A. scabra* grown at 37°C did not change significantly when transferred to either 20 (HL) or 37°C (HH) (Fig. 4). Root respiration rate declined for *A. stolonifera* when grown at 37°C and transferred to 20°C. However, when plants previously grown at 20°C were transferred to high temperature (37°C) (LH), root respiration rate increased dramatically for *A. stolonifera*. Root respiration rate for thermal *A. scabra* was less responsive to short-term temperature changes than that for *A. stolonifera* (Fig. 4). Thermal *A. scabra* maintained lower root respiration rates than *A.*

stolonifera when previously grown at 20°C and transferred to 37°C (LH). Both *A. stolonifera* and thermal *A. scabra* exhibited lower respiration rates when previously grown and maintained at 37°C (HH) than when previously grown at 20°C and transferred to 37°C (LH).

Fig. 5. Total non-structural carbohydrates in leaves (A) and roots (B) for *A. stolonifera* and *A. scabra* for plants grown at 20 or 37°C and measured at 20 or 37°C. HH—grown and measured at 37°C, HL—grown at 37°C and measured at 20°C, LH—grown at 20°C and measured at 37°C, LL—grown at 20°C and measured at 20°C. Different lowercase letters indicate significant difference ($P \leq 0.05$) between temperature treatments within a given species based on a LSD test. *Differences between *A. stolonifera* and *A. scabra* at a given temperature treatment (LSD test, $P \leq 0.05$).

3.4. Carbohydrate content in response to short-term temperature changes

Compared with *A. stolonifera*, *A. scabra* had higher TNC content in leaves under all temperature regimes (Fig. 5A). In addition, *A. stolonifera* showed a significant decrease in leaf TNC content after the short-term shift from 20 to 37°C (LH), whereas leaf TNC

did not change in thermal *A. scabra* with the temperature increase (LH), compared to plants previously grown and maintained at 20°C (LL). TNC content of roots did not vary between the two species at 20 (LL) or 37°C (HH), and both species grown for 17 days at 37°C (HH) had significantly less TNC in roots than those at 20°C (LL) (Fig. 5B). The shortterm change in temperature had no effect on TNC reserves in roots for both species.

4. Discussion

Cell membrane stability is positively related to the ability of cells to tolerate environmental stresses, including heat stress, and is widely used as a stress indicator (Rachmilevitch et al., 2006). The integrity of membranes is essential for many biochemical reactions, including respiration and carbon metabolism. Our data demonstrated that thermal *A. scabra* maintained higher root mass and membrane stability than *A. stolonifera* under high soil temperature, confirming that roots of thermal *A. scabra* were more thermotolerant.

Root thermotolerance of thermal *Agrostis* species could be related to efficient utilization and production of carbon. The importance of adequate carbon for growth and maintenance of roots has been well documented in many plant species, and shortage of assimilates due to high respiratory losses has long been proposed to be a primary factor responsible for root growth inhibition and dysfunction (Youngner and Nudge, 1968). Previous studies have suggested that root death of cool-season grass species at high temperature could be attributed to a net loss of carbon from the plant primarily resulting from excessive consumption of carbon through increased respiration and a reduction in carbon assimilation in photosynthesis (Carrow, 1996; Huang and Gao, 2000; Xu and Huang, 2000b; Liu and Huang, 2001). In a study with three bluegrass species (*Poa pratensis* L., *Poa trivialis* L., *Poa compressa* L.), photosynthetic rate was reduced by 47% and dark respiration was increased by 42% by increasing temperature from 23/15 °C (day/night) to 35/25 °C (Watschke et al., 1972). Increases in dark respiration rates have also been associated with lower biomass production in tall fescue (*Festuca arundinacea* L.), suggesting that excessive dark respiration may adversely affect growth rates (Volenc, 1984). In the present study, it is interesting to find that

thermal *A. scabra* exposed to 37°C for 17 days maintained canopy net photosynthetic rate (Pn) and respiration rate at the same levels as those plants exposed to 20°C. In contrast, Pn decreased and respiration rate increased with increasing soil temperature from 20 to 37°C for *A. stolonifera*. In addition, under high soil temperature conditions, thermal *A. scabra* had a positive carbon gain, whereas *A. stolonifera* had a net loss of carbon, which may have resulted in carbon starvation and death of roots. Our results suggest that the maintenance of high carbon fixation, low carbon consumption rate, and a positive carbon balance could be a critical factor controlling root survival of thermal grass species when exposed to prolonged periods of high soil temperature. However, the underlying factors for high thermal stability of photosynthesis and respiration processes in thermal *A. scabra* are unknown, which deserves further investigation.

Heat tolerant roots may be able to control respiration rate or use efficient respiratory pathways. Increasing temperature from 16 to 20°C caused a 30% increase in root respiration rates in three perennial grasses, *Bellis perennis*, *Dactylis glomerata*, and *Poa annua*, but *B. perennisthat* was acclimated to higher temperature maintained a lower respiration rate than the other species under high temperature conditions (Gunn and Farrar, 1999). In this study, short-term temperature responses of root respiration demonstrated that thermal *A. scabra* was able to down-regulate or control respiration rates at a lower level, which exhibited the same rate of respiration in roots grown and maintained at 37°C as those plants grown at 20°C but transferred to 37°C for 24 h. Furthermore, thermal *A. scabra* grown at 20°C maintained a relatively low respiration rate when transferred to 37°C, compared to *A. stolonifera* that exhibited a more dramatic increase in root respiration rate in response to the short-term temperature elevation. In general, root respiration rate for thermal *A. scabra* was less responsive to short-term temperature changes than that for *A. stolonifera*. Down-regulation of root respiration may contribute to the thermal grass adaptation to elevated temperature.

The ability of a plant to maintain root viability is regulated by both the total amount of carbon assimilated by foliages during photosynthesis and by the amount of carbon allocated to the belowground system (Bloomfield et al., 1996). A reduction in carbohydrate reserves in roots has been correlated with decreased root growth in

several plant species (Aldous and Kaufmann, 1979; Sweeney et al., 2001; Xu and Huang, 2003). In this study, root TNC content was significantly lower in plants grown at 37°C compared to those at 20°C for both species. The reduction in root TNC content corresponded with decreased root biomass at the high soil temperature in both *Agrostis* species. However, root TNC content was not different between the two species at low or high temperature and did not change with the short-term (24 h) temperature change. This suggested that while root TNC levels could be important for root survival of long-term high soil temperature, short-term responses of TNC to changes in temperature could not explain the differences in root thermotolerance between the two species. However, thermal *A. scabra* exhibited significantly greater amount of TNC in leaf tissues than *A. stolonifera*, particularly when grown at low temperature and transferred to high temperature. It should be noted that only roots were exposed to high soil temperature while shoots were exposed to its optimum growth temperature (20°C) in our study. Therefore, more TNC accumulation in leaves that were exposed to the optimal temperature may aid in the whole-plant survival under high soil temperature conditions.

In summary, the ability of thermal *A. scabra* to down-regulate or control root respiration at a relatively lower level under long-term or short-term heat stress, compared to heat-sensitive *A. stolonifera*, may be evidence of an acclimation mechanism allowing it to conserve carbon in response to increasing temperature. This mechanism, coupled with the ability to maintain high canopy photosynthesis and low whole-plant respiration rates, may be essential in the adaptation of cool-season, perennial grasses to prolonged periods of high soil temperature. Such mechanisms could be utilized for selection of improved heat tolerance in heat-sensitive cool-season grass species, such as *A. stolonifera*.

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