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Antioxidative responses in roots and shoots of creeping bentgrass under high temperature: Effects of nitrogen and cytokinin

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ABSTRACT

It has been previously reported that either nitrogen (N) or cytokinin (CK) applications can alleviate heat stress injury on creeping bentgrass, with some studies reporting enhanced antioxidant metabolism being related to stress protection. The objective of this research was to investigate the simultaneous effects of CK and N on the antioxidant enzyme activity and isoforms of heat stressed creeping bentgrass. 'L-93' creeping bentgrass treated with three rates of CK (trans-zeatin riboside, *t*ZR, 0, 10 and 100 μM, designated by CK0, 10, and 100) and two nitrogen rates (2.5 and 7.5 kg N ha^{-1} biweekly, low and high N) in a complete factorial arrangement was maintained in a 38/28 °C (day/night) growth chamber for 28 d and then harvested. Grass grown at high N (averaged across CK rates) had higher O_2^- production, H_2O_2 concentration, and malondialdehyde content in roots. The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and guaiacol peroxidase (POD) in roots were enhanced 19%, 22%, and 24%, respectively, by high N relative to low N. Twenty-eight days of heat stress resulted in either the development of new isoforms or enhanced isoform intensities of SOD, APX, and POD in roots compared to plant responses prior to heat stress. However, no apparent differences were observed across treatments. Both SOD and POD showed different isoform patterns between roots and shoots, suggesting the function of these isoforms could be tissue specific. Interestingly, no CK effects on these antioxidant parameters were found in this experiment. These results demonstrate the impacts of N on antioxidant metabolism of creeping bentgrass under heat stress with some differences between roots and shoots, but no simultaneous impacts of CK and N.

Introduction

Heat stress is usually defined as a period when temperatures are high enough for sufficient time to cause injury to plant function or development. Heat stress inhibition of photosynthesis in chloroplasts results in an imbalance of the electron-transfer chain and promotes production of reactive oxygen species, including singlet oxygen ($^1O_2$), superoxide radical (O_2^-), and hydrogen peroxide (H_2O_2) (Smirnoff, 1993). Although ROS can function as signal molecules for plant growth and development, excess ROS are detrimental and can cause the autocatalytic peroxidation of membrane lipids and pigments, leading to loss of membrane semi-permeability and modification of function (Senthil-Kumar et al., 2007; Wahid et al., 2007). Due to this biological paradox, levels of ROS are generally well regulated by their rate of generation and degradation as influenced by the scavenging capacity of two systems, the enzymatic system (antioxidant enzymes) and non-enzymatic system (small molecular antioxidants) (Asada, 1999; Blokhina et al., 2003). Superoxide dismutase (EC 1.15.1.1) constitutes the first line of defense against ROS by dismutating the superoxide anion to H_2O_2. Next, H_2O_2 is finely regulated by catalase (EC 1.11.1.6) and an array of peroxidases localized in almost all compartments of the plant cell, such as ascorbate peroxidase (EC 1.11.1.11) and guaiacol peroxidase (EC 1.11.1.7) (Blokhina et al., 2003). There is mounting evidence suggesting that tolerance of adverse environments is correlated with an increased capacity to scavenge or detoxify ROS, and protection against oxidative stress is thought to be an important component in determining the survival of a plant under heat stress (Smirnoff, 1993; Maestri et al., 2002).

As an important plant hormone, cytokinin has been targeted in many plant species to improve their tolerance to different environmental stresses (Barna et al., 1996; Huynh et al., 2005; Havlova et al., 2008; Zhang and Ervin, 2008). Exogenous application of CK has been shown to have potential in alleviating heat injury in various higher plants (Skogqvist, 1974; Liu and Huang, 2002; Schrader, 2005). For example, retarded leaf senescence and reduced cell membrane lipid peroxidation in creeping bentgrass was observed via exogenous zeatin riboside application, and enhanced

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; DAT, day after initial treatment application; POD, guaiacol peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TQ, turf quality; *t*ZR, trans-zeatin riboside.

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antioxidant response was suggested as a possible mechanism for the observed reductions in heat injury (Liu and Huang, 2002). Zhang and Ervin (2008) also indicated that CK is thought to protect plants under stress via its antioxidant properties.

Nitrogen is the mineral that most often limits plant growth because relatively large quantities of N are required for incorporation into numerous organic compounds that are crucial for plant growth and development, such as proteins, nucleic acids, and some plant hormones (Pessarakli, 2002). A proper level of nitrogen nutrition is essential to maintain adequate plant growth to withstand heat stress. Tawfik et al. (1996) suggested that plants receiving N fertilization during heat stress benefited from greater rhizospheric N levels compared to plants receiving N fertilization before heat stress as indicated by greater fresh and dry weights, and significantly higher membrane thermostabilities. A more recent study reported that higher N helped to maintain higher photosynthetic N-use efficiency and photosynthesis in maize (*Zea mays* L.) under heat stress (Wang et al., 2008). In heat stressed cool-season turfgrasses, additional foliar N supply was found to be beneficial (Fu and Huang, 2003; Zhao et al., 2008). Maintenance of the scavenging ability of antioxidants and inhibition of lipid peroxidation by foliar application of NH_4NO_3 is thought to be related to improved heat tolerance (Fu and Huang, 2003).

Creeping bentgrass is a widely used cool-season turfgrass on golf greens and fairways in temperate and sub-tropical climate zones, where heat stress continues to be the primary factor limiting its summer performance (Zhang and Ervin, 2008). Some studies have reported that high temperature stress affected antioxidant responses in creeping bentgrass and other cool-season turfgrass species (Liu and Huang, 2000; Jiang and Huang, 2001; He et al., 2005; Xu et al., 2006). For instance, a recent study on heat-acclimated versus non-acclimated cool season turfgrass species indicated that the ability to protect against oxidative stress is an important component in determining the heat stress tolerance of a plant (Xu et al., 2006). In addition, several studies reported either the effects of CK or N on creeping bentgrass, with antioxidant response as one of the mechanisms accounting for less damage (Liu and Huang, 2002; Fu and Huang, 2003). However, there have been no studies regarding how CK and N simultaneously affect antioxidant metabolism in roots and shoots of creeping bentgrass under heat stress. In addition, changes in the amount of a particular isoform of an antioxidant enzyme can be more important than alterations in the total activity (Mullineaux and Creissen, 1997). Pinhero et al. (1997) have suggested that synthesis of new antioxidant enzyme isoforms could be more beneficial for antioxidant metabolism than mere enhancement of the activity. Thus, the analysis of isoform changes could provide additional information about antioxidant responses in creeping bentgrass. However, studies on the isoform patterns of antioxidant enzymes in cool-season turfgrasses under heat stress are very limited.

The objective of this study was to investigate the effects of N and CK on the antioxidant response of heat stressed bentgrass, including any changes of antioxidant enzyme isoforms. Knowledge of antioxidant metabolism would provide valuable information for understanding the mechanisms underlying creeping bentgrass responses to heat stress, perhaps leading to enhanced summer management programs.

Materials and methods

Plant materials and growing conditions

'L-93' creeping bentgrass (*Agrostis stolonifera* L.) was planted in late July 2007, at 49 kg PLS (pure live seed) ha^{-1} in plastic pots (14-cm diameter, 14.5-cm depth) filled with calcined clay (heat-treated montmorillonite clay mineral, 0.015% N, Profile LLC, Buffalo Grove, IL). The grass was fertilized with Bulldog fertilizer (28-8-18, 1% ammoniac N, 4.8% nitrate N, and 22.2% urea N; SQM North America, Atlanta, GA) at 5 kg N ha^{-1} every week over the first two months, then reduced to 2.5 kg N ha^{-1} biweekly. Three months after growing on a greenhouse bench with a mist system ($25 \pm 3/15 \pm 2\,^{\circ}C$, day/night), the grass was transferred into a growth chamber set at: 38/28 °C (day/night), relative humidity 70/85% (day/night), photosynthetically active radiation (PAR) at 450 μmol s^{-1} m^{-2} and a 14-h photoperiod. Grass was cut to 12 mm three times a week with an electric clipper during the project, except the last week in order to collect enough tissue samples for further analysis.

Treatments

Sub-irrigation (a 18-cm pan was placed under each pot in which water or nutrient solution was applied) to supply nitrate treatments (60 mL Hoagland's solution per pot) (Epstein and Bloom, 2005) and foliar spray treatments of CK (*trans*-zeatin riboside) in 0.05% Tween 20 solution (500 μL per pot) was applied biweekly (0 d and 14 d). Both KNO_3 and $Ca(NO_3)_2$ were used as the nitrate sources in the Hoagland's solution. KCl and $CaCl_2$ were added into low N treatment solutions to equalize the potassium and calcium levels across N rates. Thus, all levels of other nutrients were the same, except higher Cl^- concentration in the low N treatment solution relative to the high N treatment solution. Potted grasses were sub-irrigated daily to prevent drought stress.

The treatments included:

1) Nitrate N at 2.5 (low N) kg N ha^{-1}, no CK (CK0).
2) Nitrate N at 2.5 (low N) kg N ha^{-1}, CK at 10 μM (CK10).
3) Nitrate N at 2.5 (low N) kg N ha^{-1}, CK at 100 μM (CK100).
4) Nitrate N at 7.5 (high N) kg N ha^{-1}, no CK (CK0).
5) Nitrate N at 7.5 (high N) kg N ha^{-1}, CK at 10 μM (CK10).
6) Nitrate N at 7.5 (high N) kg N ha^{-1}, CK at 100 μM (CK100).

Sampling and measurements

Turf quality (TQ) was rated weekly based on a visual scale of 1–9, with 1 indicating yellow, dead leaves, 9 the best possible quality, and 6 minimum acceptable quality according to NTEP (National Turfgrass Evaluation Program). Canopy photochemical efficiency of photosystem II (PSII) ($Fv/Fm = (Fm_{690} - F0_{690})/Fm_{690}$) were recorded after each TQ reading by using a dual wavelength chlorophyll fluorometer (OS-50II, Opti-sciences, Tynsboro, MA). Shoots were harvested at d 28 (28 days after initial treatment application plus heat stress). Roots were washed free of soil at the same time. All samples were immediately frozen with liquid nitrogen and stored at −80 °C until analysis.

To extract the soluble protein, a frozen sample (0.5 g) from the entire root or shoot tissue was ground into fine powder using liquid nitrogen and 4-mL of extraction buffer (50 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% polyvinylpyrrolidone (PVP), 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl (PMSF), pH 7.8) was added. Samples were then centrifuged at $15{,}000 \times g$ for 15 min at 4 °C, and supernatant was collected for enzyme assay. The protein content was determined using the Bradford method (Bradford, 1976). All enzyme assays were performed the same day as the Bradford assay using a Spectronic Genesys 10 Bio Spectrophotometer (Thermo Electron Corporation, Waltham, MA).

Total SOD activity was measured according to the method of Giannopolitis and Ries (1977). The assay medium contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM p-nitro blue tetrazolium chloride (NBT), 2 μM riboflavin, 0.1 mM EDTA, and 30–40 μL of enzyme extract. A reaction mixture was illuminated under 80–90 μmol m^{-2} s^{-1} for 10 min. The reaction mixture