

Comparative Study on Antioxidant Enzymes and Lipid Peroxidation Related Low Temperature Tolerance in Overwintering Zoysiagrass and Creeping Bentgrass

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월동기간 중 Zoysiagrass와 Creeping Bentgrass의 저온내성에 대한 항산화 효소 및 지질과산화의 비교 연구

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요 약

월동기간 동안 zoysiagrass [*Zoysia matrella* (L.) Merr]와 creeping bentgrass (*Agrostis palustris* Huds)의 뿌리에서 저온 스트레스에 대한 생리학적 반응을 규명하기 위해 지질과산화 정도와 효소적 항산화 반응 및 탄수화물의 변화를 조사하였다. 1월 말 Creeping bentgrass의 뿌리 고사율은 zoysiagrass 보다 약 20% 현저히 높게 나타났다. Creeping bentgrass의 뿌리 성장은 온도변화와 거의 평행하게 변화하였으나 zoysiagrass는 뿌리 성장에 거의 변화가 없이 4월 말까지 유지되었다. Polyssacharide (starch + fructan)의 함량은 두 품종에서 별다른 차이는 발견되지 않았으나 zoysiagrass의 총비구조적 탄수화물이 creeping bentgrass에 비해 약 10% 높게 나타났다. Malondialdehyde (MDA)의 농도는 creeping bentgrass에서 현저히 높게 나타났다. Creeping bentgrass의 peroxidase의 활성은 zoysiagrass에 비해 약 4.2 배 높았으나 superoxide (SOD)와 catalase (CAT)의 활성은 각각 22%와 67% 낮았다. 이상의 결과들은 creeping bentgrass 보다 zoysiagrass가 월동 기간 중 저온 내성 메커니즘을 우선적으로 작동시키며 저온 스트레스에 대해 덜 민감하다는 것을 잘 나타내고 있다.

(Key words : Creeping bentgrass, Zoysiagrass, Overwintering, Lipid peroxidation, Antioxidant enzymes)

I. INTRODUCTION

Zoysiagrasses (*Zoysia japonica*, *Zoysia matrella* and *Zoysia tenuifolia*) and creeping bentgrass (*Agrostis palustris*) are being widely used for golf course green and fairway in Korea. Creeping bentgrass, is known as cool-season turfgrass, for polystand on fairways successfully

provide a playing surface during winter period in Korea. The optimal growth temperature ranged from 15 to 24°C for creeping bentgrass (Beard, 1973) and 27 to 35°C for zoysiagrass (Youngner, 1961). Thus, zoysiagrass suffers from low temperature stress during winter season in Korea when aerial temperatures are often lower than 5°C.

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Many physiological factors could be involved in the cold stress injury to overwintering turfgrasses. In some plant species, cold injury induces oxidative stress, resulting from the production and accumulation of active oxygen species such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl free radical (OH^-), and singlet oxygen (1O_2) (Okuda et al., 1991; Scebba et al., 1998; 1999; Sahoo et al., 2001). The active oxygen species produced during stress may damage many cellular components including lipids, proteins, carbohydrates, and nucleic acids (Monk et al., 1989; Rice-Evans et al., 1991). To scavenge active oxygen species, plants have evolved enzymatic antioxidant systems such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Scandalios, 1993; Scebba et al., 1999). SOD, which dismutates superoxide (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen, is a major scavenger of superoxide. H_2O_2 is eliminated by catalase and peroxidase, which both enzymic and non-enzymic H_2O_2 degradation (Peltzer et al., 2002). CAT removes the bulk of H_2O_2 , whereas ascorbate peroxidase (APOD) can scavenge H_2O_2 that is inaccessible for CAT because of their higher affinity for H_2O_2 and their presence in different subcellular locations. APOD along with CAT and SOD are considered as key enzymes within the antioxidative defense mechanism, which directly determine the cellular concentration of O_2^- and H_2O_2 . However, the function of scavenging enzymes can be interrupted by excessive stress, which can result in increase in lipid peroxidation and consequent membrane damage (Jagtap and Bhargava, 1995; Norman et al., 2001).

Although susceptibility to freezing temperatures has been pointed out as a major factor for winter damage of turfgrasses in golf course, little information exists on physiological response to cold temperature with regard to freezing tolerance and winter adaptation.

In this study, malondialdehyde (MDA) production was measured to evaluate the level of lipid peroxidation, and antioxidant responses were monitored by measuring the activities of enzymatic antioxidants including POD, SOD and CAT for investigating cold tolerance in response to naturally occurring winter freezing stress between zoysiagrass and creeping bentgrass, often used for golf course in Korea.

II. MATERIALS AND METHODS

1. Sampling and collecting site

Sampling was made from green and fairway, established in 1998, of Muan Country Club (35°05'32" N, 126°17'12" E) in Korea. The turfgrasses were maintained by general winter management. Minimum daily temperature in green field ranged from -2.8 to 11.0°C during the experimental period. It reached to 0°C at mid-December, remained at below freezing temperature until end-February. Maximum daily temperature ranged from 5.1 to 20.6°C (Fig. 1).

Creeping bentgrass and zoysiagrass were sampled using a hole cutter (Ø108mm) at five randomly selected sites, from 20 November, 2000 to 23 April, 2001 with about one-month interval. Root samples were obtained to a depth of approximately 20 cm. Roots were severed from crown, packed with ice, and transported to

the laboratory. Roots were washed free of soil under a stream of cold water, immediately frozen in liquid nitrogen, and lyophilized. Freeze-dried samples were finely ground and stored under vacuum for further analysis.

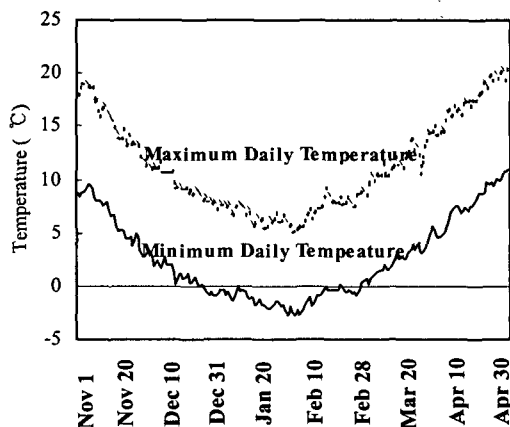


Fig. 1. Daily maximum temperature (---) and minimum temperature (—) during the winter of 2000~2001 at Muan city, Korea.

2. Root growth and root mortality

Dry weight (DW) of lyophilized root sample was weighted to estimate root growth. Root mortality was measured using the modified method of Knievel (1973). The fresh roots (500 mg) were incubated with 10 ml of 0.6% 2,3,5-triphenyltetrazolium chloride in 0.05M phosphate buffer, pH 7.4, for 24h in the dark at 30°C. Roots were then rinsed twice with deionized water. Formazan was extracted twice from the roots with 95% ethanol at 70°C for 4h. Combined extracts was adjusted to a final volume of 50 ml with 95% ethanol. Absorbance was read at 490 nm. A standard curve was made using different proportions of living roots

and killed roots to calculate root mortality. Root mortality was expressed as percentage dead root DW of the total root DW.

3. Lipid peroxidation

The lipid peroxidation levels were determined in term of malondialdehyde (MDA) content. TBA-MDA levels was estimated by correcting for compounds other than MDA which absorbance at 532 nm by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA, as previously described by Hodges et al. (1999). Plant tissue samples were homogenized with 80% (v/v) ethanol, followed by centrifugation at room temperature for 10 min at 3,000 g. A 1 mL aliquot of appropriately diluted sample was added to a test tube with 1mL of either (i) TBA solution comprised of 20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene (ii) +TBA solution containing the above plus 0.65% TBA. Samples were then mixed vigorously, heated at 95°C in a block heater for 25 min, cooled, and centrifuged at room temperature for 10 min at 3,000 g. Absorbances were read at 440 nm, 532 nm, and 600 nm. Malondialdehyde equivalents were calculated in the following manner:

$$1) [(Abs_{532+TBA}) - (Abs_{600+TBA}) - (Abs_{532-TBA} - Abs_{600-TBA})] = A$$

$$2) [(Abs_{440+TBA} - Abs_{600+TBA}) 0.0571] = B$$

$$3) MDA \text{ equivalents (nM mL}^{-1}\text{)} = (A-B/157,000)10^6$$

4. Enzyme extract and POD, SOD, CAT activity assay

For extraction of antioxidant enzymes, 500 mg of fresh roots were homogenized with 1.5 mL of 100 mM phosphate buffer solution (pH 7.0) containing 2 mM of EDTA, 1% polyvinylpyrrolidone (PVP) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 14,000 g for 20 min at 4°C. The supernatant was used for the determination of soluble protein and enzyme activities.

The activity of superoxide dismutase (SOD) was determined by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977) with some modifications (Liu and Huang, 2000). A 3 mL of reaction mixture contained 63 μ M NBT, 1.3 μ M riboflavin, 13 mM methionine, 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), and 20 to 50 μ L enzyme extract. Test tubes containing the reaction mixture were irradiated under a light bank at 78 μ mol photos $s^{-1} m^{-2}$ (15 fluorescent lamps) for 10 min. The absorbance of the irradiated and nonirradiated solution at 560 nm was determined. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT photoreduction at 560 nm.

Activities of POD and CAT were measured using the method of Chance and Maehly (1955). For POD, the oxidation of guaiacol was measured by the increase in absorbance at 470 nm for 1 min. The reaction mixture contained 50 μ L of 20 mM guaiacol, 2.8 mL of 10 mM phosphate buffer (pH 7.0), and 50 μ L enzyme extract. Reaction was initiated by adding 20 μ L of 40 mM H₂O₂. Activity was calculated using

the extinction coefficient (26.6 mM⁻¹ cm⁻¹ at 470 nm) for tetraguaiacol. POD activity was expressed as the concentration of tetraguaiacol produced per min.

For CAT, the decomposition of H₂O₂ was measured by the decline in absorbance at 240 nm for 1 min. The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H₂O₂, and 50 μ L enzyme extract. Activity was calculated using the extinction coefficient (36 M cm⁻¹ at 240 nm) for H₂O₂. CAT activity was expressed as μ M H₂O₂ decomposed per min.

The activity of each enzyme was expressed on a protein basis. Protein concentrations of the crude extract were estimated using protein dye-binding (Bradford, 1976).

III. RESULTS

1. Root mortality and root growth

Root mortality of creeping bentgrass and zoysiagrass were 68% and 48% at January, respectively (Fig. 2A). When temperature increased, root mortality in both species was lowered and not found the difference. Root dry weight of creeping bentgrass was 7 g per hold cutter at January and then increased 2 times at the end of April, in parallel with air temperature (Fig. 2B). However, root dry weight of zoysiagrass was less changed within 5 g per hold cutter. These results indicated that root growth of creeping bentgrass responded much sensitively to low temperature during winter compared to zoysiagrass.

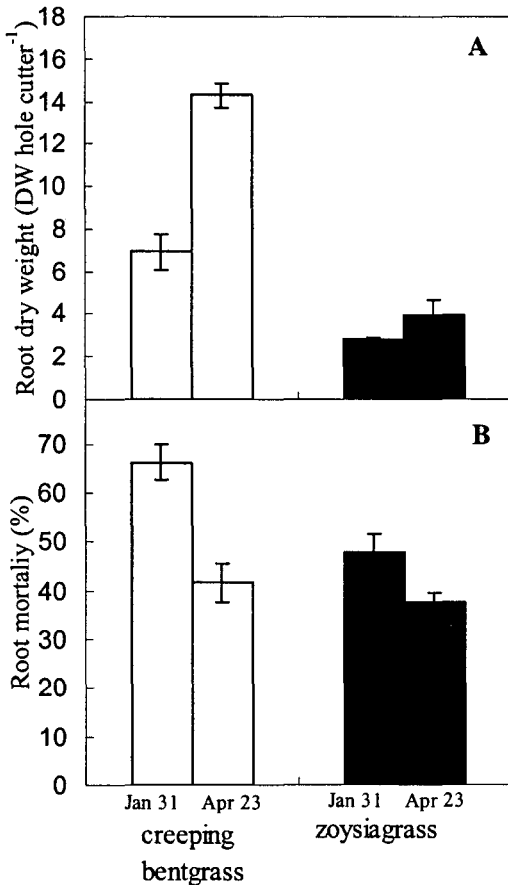


Fig. 2. Root dry weight (A) and root mortality (B) of overwintering creeping bentgrass and zoysiagrass. Each value is the mean \pm s.e. for n=5.

2. Carbohydrates concentration

Changes in carbohydrates in roots of creeping bentgrass and zoysiagrass are shown in Fig. 3. The starch concentration of zoysiagrass was 2.6 times higher than creeping bentgrass (Fig. 3A). In contrast, the fructan concentration was 2.1 times higher in creeping bentgrass than zoysiagrass (Fig. 3B). The concentration of total non-structural carbohydrate (TNC) was slightly higher for zoysiagrass than for creeping bentgrass (Fig. 3C).

3. Lipid peroxidation and antioxidant enzymes

MDA content was measured to assess the extent of lipid peroxidation resulting from oxidative stress caused by low temperature during winter (Fig. 4). The MDA content in creeping bentgrass was 2 times higher than that in zoysiagrass. These results indicated that creeping bentgrass root suffered much severely from oxidative stress compared to zoysiagrass, when the minimum temperature was below 0°C.

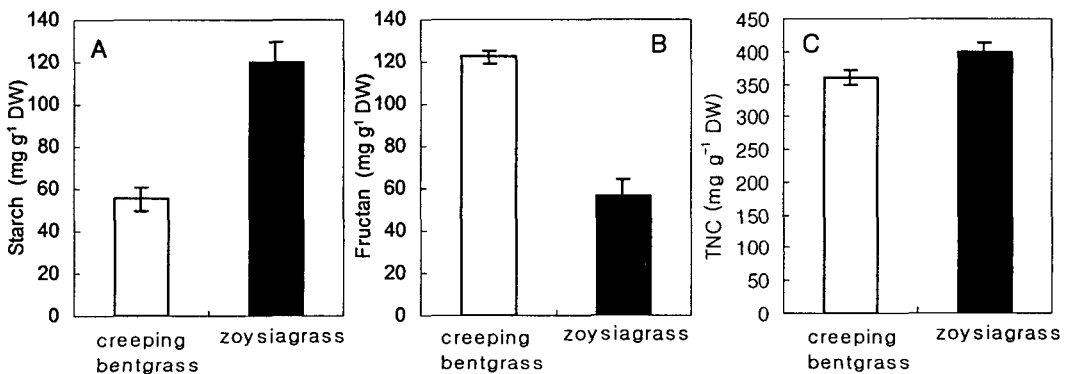


Fig. 3. Changes in carbohydrates concentration of overwintering creeping bentgrass and zoysiagrass roots. (A) starch, (B) fructan and (C) TNC. Each value is the mean \pm s.e. for n=5.

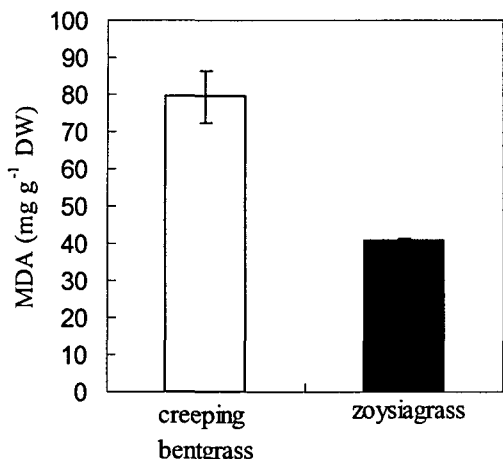


Fig. 4. Changes in malondialdehyde (MDA) contents in roots of overwintering creeping bentgrass and zoysiagrass. Each value is the mean±s.e. for n=5.

The activities of POD, SOD and CAT in roots of creeping bentgrass and zoysiagrass in January were measured to compare the antioxidative activity for freezing tolerance (Fig. 5). The POD activity of creeping bentgrass was 4.2 times higher than that of zoysiagrass (Fig. 5A). In contrast, the activities of SOD and CAT in creeping bentgrass lowered by 22% and 67%,

respectively, compared to zoysiagrass (Fig. 5B and 5C).

IV. DISCUSSION

Low temperature is a major limiting factor in the growth of warm-season plants. As temperature increased after wintering, growth condition in zoysiagrass was little changes but creeping bentgrass increased 2 times at April compared to January (Fig. 2B). In contrast at January, root mortality was higher in creeping bentgrass than zoysiagrass and then it similarly decreased to zoysiagrass at April. This showed that creeping bentgrass might be more susceptible to cold stress.

The main forms of carbohydrate accumulated in reserve tissues were starch for zoysiagrass and fructan for creeping bentgrass, respectively (Kim et al., 2005). In our experiment, the concentrations of starch plus fructan were similar to 176.2 ± 8 mg g⁻¹ DW in zoysiagrass and 177.7 ± 4 mg g⁻¹ DW in creeping bentgrass

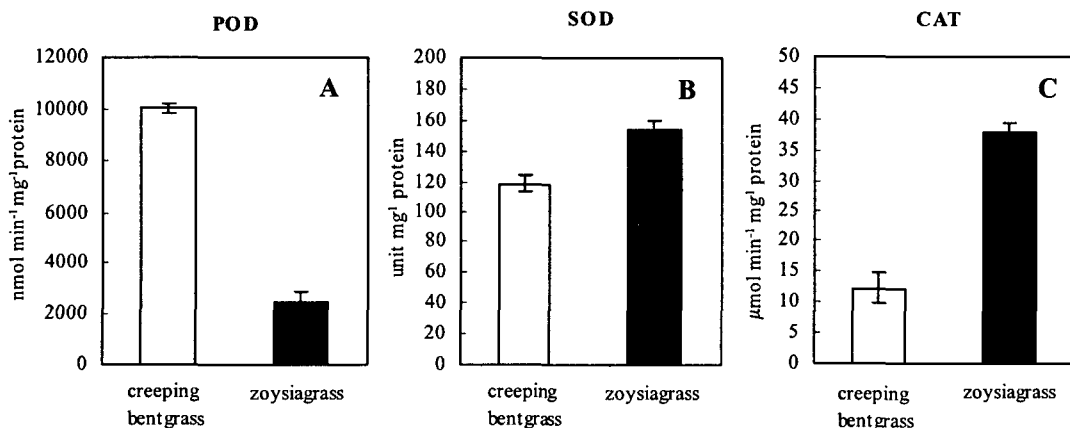


Fig. 5. Activities of antioxidant enzymes in roots of creeping bentgrass and zoysiagrass on 31 January. (A) peroxidase (POD), (B) superoxide dismutase (SOD) and (C) catalase (CAT). Each value is the mean±s.e. for n=5.

(Fig 3A and 3B). However, total non-structure carbohydrate was slightly higher in zoysiagrass than in creeping bentgrass (Fig. 3C). Carbohydrate levels and composition may influence the sensitivity of plant tissues to low temperature (Levitt, 1980). In some gramineae, fructan accumulation is thought to be involved in increased resistance to low temperature (Pontis and Del Cmpillo, 1980). As suggested by Pollock et al. (1988) for other plants (C_3 grasses and cereals), it is likely that the accumulation of fructans and sucrose would enhance survival of *D. antarctica* because they are readily accessible energy reserves during growth periods with negative carbon balance. Bouchart et al. (1998) have also reported that high starch content is prerequisite for winter survival of white clover and a greater starch concentration in stolons confers a better capacity of regrowth through its mobilization in spring. These polysaccharide accumulations in plants may be associated with the acclimation to low temperature. Therefore, zoysiagrass having higher TNC might be have a high cold tolerance than creeping bentgrass.

Low temperature disrupts cellular membranes (Raison and Chapman, 1976) and it causes the break down of the plant cell, and therefore can severely affect cellular function. The level of lipid peroxidation has been used as an indicator of free radical damage to cell membranes under stress conditions. Malondialdehyde (MDA) is a final product of peroxidation of unsaturated fatty acids in phospholipids that is often used as an indicator of the extent of lipid peroxidation resulting from oxidative stress (Smirnov, 1993; Liu and Huang, 2000; Fu and Huang, 2001).

The MDA contents in creeping bentgrass were largely higher than that of zoysiagrass (Fig. 4), indicating that creeping bentgrass was more susceptible to oxidative stress caused by low temperature. Such increases have been found in other species under heat stress (Liu and Huang, 2000; Dionne et al., 2001) and drought stress (Fu and Huang, 2001). Dhindsa (1981) reported that the increase in MDA content indicated membrane lipid peroxidation and it was responsible for cell membrane damage.

Peroxidase activity in January, when the plants were severely exposed to freezing temperature, was also higher in creeping bentgrass (Fig. 5A). High POD activity suggests an accelerated production of active oxygen species in tissues (Okuda et al., 1991), and is likely induced by increased levels of superoxide radicals (Kato and Shimizu, 1985). The higher MDA content and POD activity in creeping bentgrass could be associated with the lower activities in SOD and CAT activities (Fig. 5), in agreement with the results of other studies (Liu and Huang, 2000; Fu and Huang, 2001; Huang et al., 2001). Chilling causes an increase of superoxide (O_2^-) production in chloroplast and mitochondria, especially in chilling sensitive species (Hodgson and Raison, 1991). These lower activities of SOD and CAT induced by freezing temperature in January in creeping bentgrass favor accumulation of O_2^- and H_2O_2 , which can result in lipid peroxidation (Fu and Huang, 2001). Cell membrane stability has been shown to be affected by lipid peroxidation caused by active oxygen species under stress conditions (Dhindsa et al., 1981). The lower activities of SOD and CAT in creeping bent

grass (Fig. 5B and 5C) suggest that the root of creeping bentgrass is less able to curtail lipid peroxidation and is more susceptible to freezing temperature stress compared to zoysiagrass.

In conclusion, the present results indicated that cold tolerance mechanism occurred much actively in roots of zoysiagrass during winter when compared to creeping bentgrass, as manifested by lower intensity of lipid peroxidation, higher activities of antioxidant enzymes.

V. ABSTRACT

To investigate the physiological responses to winter freezing stress naturally occurring, the level of lipid peroxidation and enzymatic antioxidant responses were compared between zoysiagrass and creeping bentgrass during overwintering. Root mortality of creeping bentgrass was significantly higher than zoysiagrass at January. Root growth of creeping bentgrass was nearly parallel with temperature fluctuation, while zoysiagrass showed little changes in root growth until the end of April. Total non-structural carbohydrate of zoysiagrass was 10% higher than creeping bentgrass. Malondialdehyde (MDA) content in creeping bentgrass was 2-fold higher than that of zoysiagrass. The peroxidase (POD) activity of creeping bentgrass in January was 4.2 times higher, while superoxide (SOD) and catalase (CAT) activities lowered 22% and 67%, respectively, compared to zoysiagrass. These results suggest that zoysiagrass roots much properly operate cold tolerance mechanism and are less susceptible to cold stress in comparison to creeping bentgrass.

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