Changes in Freezing Tolerance and its Relationship with the Contents of Carbohydrates and Proline in Overwintering Centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.)

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Abstract: The objective of this study was to clarify the changes in the contents of endogenous carbohydrates and proline in the stolons and leaves of centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.), during the natural cold acclimation (hardening) and de-acclimation (dehardening) in relation to freezing tolerance in the field at the transition zone between temperate and subtropical areas in China. The contents of carbohydrates and proline, and freezing tolerance estimated by LT\(_{50}\), which is the temperature at which 50% of the electrolytes in the organ was measured in the leachate, were determined at 10-day intervals from October 1, 2001 to April 18, 2002. It was indicated that the freezing tolerance of stolons increased (LT\(_{50}\) of stolons decreased) quickly, as temperature dropped before winter, but that of leaves which senesced along with the drop in temperature did not. The freezing tolerance of stolons decreased gradually along with the rise in temperature above 5 °C in spring, when the overwintered plants started to grow. The contents of proline and soluble carbohydrates, including sucrose, fructose and glucose, increased as LT\(_{50}\) decreased when temperature dropped below 5 °C before winter, and decreased as LT\(_{50}\) increased in early spring. Correlation analysis revealed that the freezing tolerance of stolons of centipedegrass significantly and positively correlated with the contents of proline and soluble carbohydrates, and the ratio of the soluble carbohydrates to starch. Thus, the freezing tolerance of stolons, which are critical organs that determine the winter surviving ability, largely depended on the content of soluble carbohydrates and the ratio of soluble carbohydrates to starch in centipedegrass. The possible relationship between freezing tolerance and carbohydrate metabolism was also discussed.

Key words: Carbohydrates, Centipedegrass, Freezing tolerance, LT\(_{50}\), Overwintering, Proline, Stolon.

Centipedegrass, *Eremochloa ophiuroides* (Munro) Hack., a C\(_4\) perennial grass originating in China, is characterized by its dwarf phenotype with high resistance to drought and shade and is extensively planted as a turf grass around the world. However, its sensivenes to low temperature, especially freezing injury, together with poor overwintering ability and short green-span, greatly limited its utilization in northern China (Li and Mao, 2000). Carbohydrates are known to play an important role in the resistance of plants to freezing injury. The accumulation of soluble carbohydrates, such as sucrose, fructose and raffinose, and proline during the cold acclimation, and the relation of the accumulation with the increase in freezing tolerance was examined in detail (Fry et al., 1991 and 1993; Wanner and Junttila, 1999). Recently, some reports revealed that a large difference in freezing tolerance existed among different organs, such as leaves, roots and stems in plants (Samala et al., 1998; Wanner and Junttila, 1999). Tamura and Moriyama (2001) demonstrated that non-structural carbohydrates in the roots rather than those in the tops mainly regulated the wintering ability in several temperate perennial grasses. In our previous study on tall fescue, the relationship between freezing tolerance and the contents of carbohydrates was different in leaves and crowns (Wang et al., 2003). Freezing tolerance is an important factor influencing the overwintering ability of plants, while little is known about environmental and physiological factors affecting the winter survival in centipedegrass (Fry et al., 1993). Cold tolerance and freezing tolerance of plants have usually been estimated by the changes in the LT\(_{50}\) (Fry et al., 1991 and 1993; Griffith and McIntyre, 1993; Rife and Zeinali, 2003). In some studies, LT\(_{50}\) was determined as the subfreezing temperature resulting in 50% mortality in plants or organs, and in some studies as the temperature that caused 50% electrolyte leakage from the plants or organs. Previously, we compared the freezing tolerance determined by the percentage

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Abbreviations: LT\(_{50}\), Lethal temperature of 50%; TSC, Total soluble carbohydrate.
of plant survival and regrowth of organs, with that determined by electrical conductivity of the leachate in tall fescue, and obtained a series of relatively coherent and uniform results (Wang et al., 2002). Thus, it was confirmed that the freezing tolerance could be exactly and conveniently estimated by the temperature at which 50% of the total conductivity in organs was measured in the leachate. It is necessary to evaluate the freezing tolerance of different organs, and also to investigate the role of different components of carbohydrates in freezing tolerance. In this study, therefore, the freezing tolerance and contents of sucrose, fructose, glucose, total soluble carbohydrates and proline were monitored together in leaves and stolons from October 1, 2001 to April 18, 2002. We conducted a series of experiments, (1) to determine the freezing tolerance in different organs; (2) to understand precisely the quantitative changes in total and individual contents of carbohydrates and proline in different organs, and (3) to elucidate the relations of the freezing tolerance with the contents of proline, carbohydrates and their components, and to determine the function of different organ and the individual carbohydrates in protecting the centipedegrass from freezing injury. These studies should provide useful data for the field management and turfgrass breeding for enhancing the freezing tolerance of centipedegrass.

Materials and Methods

1. Plant material and growth conditions

The experiment was undertaken at the experiment station in Wei-Gang campus of Nanjing Agricultural University (118° 28’ E, 32° 02’ N and 1013 mm of average rainfall per year) from 1998 to 2002. A popular centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) cv. Redstem was supplied by Snow Brand Seed Co., Ltd. (Hokkaido, Japan), and sown in May 1998. Watering was done every day until the fourth new leaves expanded. Thereafter, a conventional field management was conducted with mowing once in spring and fertilized twice every year with a compound fertilizer (N: P: K = 1 : 1 : 1) at a density of 6 g N m$^{-2}$. After the lawn was established for 3 years, the leaves and stolons of centipedegrass were randomly sampled every 10 days from October 1, 2001 to April 18, 2002. There were 3 replications for the field block design and 9 samples for each sampling.

2. Determination of freezing tolerance

The freezing tolerance of stolons and leaves was determined by the LT$_{50}$ (subfreezing temperature resulting in 50% mortality), used by Griffith and McIntyre (1993). Nine sections of stolons and leaves at approximately 10-15 mm in length were wrapped in a wet tissue and placed in test tubes in a refrigerated circulating freezing bath (model HC2010B, Experiment Instruments, Sida, Chongqing). Each tube was seeded with an ice chip and equilibrated at $-1^\circ$C for 1 h at the initial freezing and then cooled down at a rate of 2 $^\circ$C h$^{-1}$ from 0 to $-20^\circ$C. Tubes were removed from the freezing bath at 2 $^\circ$C intervals and allowed to thaw at 5 $^\circ$C for 12 hours. Samples were equilibrated in deionized water (20 ml) and the water was evaporated adequately in vacuo. Then the samples were kept at room temperature for 24 h. Electrical conductivity of the leachate was measured with a conductivity meter (model DDS-IIA, Experiment Instruments, Leichi, Shanghai), before and after the stolons and leaves were boiled. The temperature at which 50% of the total conductivity was measured in the leachate was defined as the LT$_{50}$. All samples were measured in duplicate and data presented are average values of at least three independent experiments.

3. Determination of the contents of carbohydrates and proline

The stolons and leaves were sampled randomly in 3 field blocks, washed several times in a deionized water, dried at 105 $^\circ$C for 1 hr and at 80 $^\circ$C for at least 1 day. After grinding and re-drying, they were weighed. Soluble carbohydrates in 50 mg each of
Ground stolon and leaf tissues were extracted using 80% (v/v) ethanol at 80 °C for 40 min. Glucose, fructose and sucrose were quantified using the microtitration method described by Hendrix (1993). The carbohydrate content was determined in triplicate on three separate extractions. Starch was extracted from the ethanol-insoluble residue and quantified as the glucose released following digestion with amyloglucosidase (MacRae, 1971).

Proline content was estimated using the acid-ninhydrin method (Bates et al., 1973). About 0.5 g leaf and stolon samples were severally taken from each block and were ground in an ice bath. Ten ml of sulphasalicylic acid (3%, w/v) was added and the extract was then vacuum filtered through filter paper (No. 2). Then, 2 ml of acid-ninhydrin and acetic acid were added to 2 ml of the filtered extract. The mixture was oven incubated at 100 °C for 1 h and the reaction was finished in an ice bath. The reaction mixture was extracted with 4 ml toluene and absorbance at 517 nm with a spectrophotometer was determined, using toluene as a blank. The proline concentration was calculated using L-proline to make the standard curve.

4. Statistical analysis

The experiments were repeated three times with three replications of each sample for all analyses, LT50 and the contents of carbohydrates and proline. The results were analyzed and compared using one-way analysis of variance.

Results

1. Changes in air temperature and the freezing tolerance in overwintering centipedegrass

Both mean and minimum air temperatures rapidly decreased twice; the first during the first ten days in November and the second, during the third ten days in December 2001 (Fig. 1A). The mean air temperature was below 10 °C from December to next February. The lowest minimum air temperature was −3.6 °C at the beginning of January. Thereafter, the minimum air temperature rose with fluctuation to around 5 °C in February and reached a non-chilling level of 10 °C in March 2002. LT50 of stolons rapidly decreased in late November soon after the first decrease in air temperature in November 2001. Thereafter, centipedegrass acclimated to the cold temperature and exhibited the highest freezing resistance with the LT50 of stolons below −15 °C. The minimum temperature maintained continuously below 5 °C from mid December to mid February, although LT50 undulated along with the fluctuated temperature. The LT50 of stolons reached the lowest level of −23.9 °C in early January 2002 after the second rapid decrease in the minimum air temperature in December 2001 (Fig.1B). In March, the LT50 of stolons increased dramatically accompanied with the increase in air temperature. Note that the freezing tolerance of
stolons of centipedegrass markedly increased during the period of leaf senescing before overwintering, and decreased rapidly during the emergence of new leaves after overwintering along with the increase in air temperature. The freezing tolerance existed of leaves, did not change both before and after wintering. During the wintering periods, the decrease and increase in LT$_{50}$ of stolons were closely associated with the drop and increase in air temperature, respectively (Fig. 1). There was a significantly positive correlation between LT$_{50}$ and mean air temperature ($r=0.842$, $P<0.001$).

2. Changes in the content of soluble carbohydrates in overwintering centipedegrass

The content of soluble carbohydrates in stolons, but not in leaves of centipedegrass, characteristically changed with the progress of growth stages (Fig. 2A). Before leaves senesced in early November 2001, the content of total soluble carbohydrates (TSC) in stolons ranged from 62.47 to 101.36 mg g$^{-1}$ DW, but after the leaf senescence, it increased rapidly as air temperature dropped and reached the highest level of 167.11 mg g$^{-1}$ DW in late November. After the mean air temperature dropped below 10 °C, the TSC contents maintained the high level ranging from 157 to 170 mg g$^{-1}$ DW until early February. When the temperature started to increase after mid February, the TSC content decreased even to the level below the initial 60 mg g$^{-1}$ DW in mid April. The TSC content of leaves slightly increased before they senesced in autumn and slightly decreased in April.

Sucrose and fructose were major components of TSC in stolons of centipedegrass, occupying 90% of the TSC, and the changes in the contents of sucrose and fructose were similar to that of TSC during the overwintering (Figs. 2B and 2C). The content of glucose was very low and ranged from 0.1 to 0.66 mg g$^{-1}$ DW (0.01-0.066%). Thus, glucose could be ignored in the studies on the change in TSC content (Fig. 2D).

LT$_{50}$ of stolons of centipedegrass during the overwintering negatively and closely correlated with the contents of soluble carbohydrates ($r=-0.811$, $P<0.001$). A rapid decrease in LT$_{50}$ occurred soon after the increase in the content of soluble carbohydrates in November (Figs.1B and 2A). On the contrary, the leaves sensitive to freezing were characterized by the poor capacity to accumulate soluble carbohydrates.

3. Changes in starch content in overwintering centipedegrass

Before the full senescence of leaves, starch accumulated in stolons of centipedegrass up to 245.6 mg g$^{-1}$ DW in late October 2001 (Fig. 3A). Thereafter, it was decreased to 172.4 mg g$^{-1}$ DW in late November accompanied by the increase in TSC content, and from late November, there was no notable change in starch content during the period to early March 2002. From early April, the starch content fluctuated corresponding to the start of leaf growth.

Though the change in starch content was not as great as that in soluble carbohydrates, the change in the ratio of TSC to starch in stolons synchronized well with that in TSC contents ($r=-0.985$, $P<0.001$) (Fig. 3B). This ratio increased rapidly to reach almost 1, which means that the content of soluble carbohydrates was the same as starch content in late November. Thereafter, this ratio was maintained around 1 until early February, and tended to decrease in spring.

4. Changes in proline in overwintering centipedegrass

Unlike the smooth change in the contents of carbohydrates, which increased and decreased soon after the drop and rise of air temperature, respectively, the proline content greatly fluctuated during the overwintering period. However, in general,
the content of proline increased and decreased before and after overwintering, respectively (Fig. 4). More than five-fold increase in proline content was observed in stolons from late November to mid December 2001. The increase in proline content of stolons before overwintering may be one of the factors which improved the freezing tolerance of stolons. The proline content in leaves was negligible with no marked change either before or after overwintering.

**Discussion**

Rife and Zeinali (2003) reported that seedlings of oil seed rape could be used to examine the freezing process in the relation to cold tolerance. In our study in centipedegrass, the freezing tolerance of stolons, but not leaves, was enhanced and weakened along with the pre-wintering acclimation and post-wintering de-acclimation to cold, respectively. Such effects could not be found before the stolons had formed in the young centipedegrass (unpublished data). From this point of view, the fully developed stolons are very important for the winter survival and spring regrowth in centipedegrass. The senescence of leaves indicates the start of the overwintering state in centipedegrass. After leaf senescence, the stolons begin to accumulate carbohydrates and undergo complicated transformation between starch and soluble carbohydrates. However, the seedlings of centipedegrass have no ability to overwinter, due to the absence of stolons. Therefore, it is inferred that the most apparent difference in cold tolerance between matured plants and seedlings is whether they have regrowth organs including stolon, crown and rhizome or not.

A series of studies on the relationships between soluble carbohydrates and freezing tolerance in plants has been reported (Koster and Lynch, 1992; Bouchart et al., 1998; Palonen et al., 2000). Shahba et al. (2003) indicated that sucrose was the major sugar in saltgrass, but it hardly showed any seasonal trend and had no correlation with freezing tolerance. On the other hand, fructose, glucose, raffinose and stachyose showed clear seasonal changes and reached the highest concentrations in midwinter in this grass. Higher concentrations of fructose, glucose and raffinose coincided with the lower LT$_{50}$. In contrast, the lowest sugar concentration was related to the susceptibility to low temperatures. They summarized that fructose, glucose, raffinose and stachyose may play important roles in freezing tolerance in saltgrass (Shahba et al., 2003). However, Fry et al. (1993) observed a positive correlation between sucrose level and the number of surviving stolons in centipedegrass. Higher sucrose concentrations in the stolons acclimated to the plants at the low temperature. In general, freezing tolerance includes the resistance to osmotic stress. The accumulation of soluble carbohydrates decreases the freezing point of cytoplasm, and thus protects the protein and bio-membrane from freezing injury, maintains the osmotic homeostasis of cells and promotes the capacity of cell to retain the water molecules. When cells underwent extreme dehydration, the hydroxyl of carbohydrates could partly substitute for the water molecule, and stabilize the protein and bio-membrane in cell (Steponkus, 1984; Guy, 1990; Crown et al., 1993; Hopkins, 1999). In our experiment with centipedegrass in the field, soluble carbohydrates increased dramatically at the onset of natural cold weather, followed by the enhancement of freezing tolerance in stolons. Analysis showed that there was a remarkable correlation between soluble carbohydrates and LT$_{50}$ in stolons. Among carbohydrates, sucrose was the most closely correlated with LT$_{50}$ ($r=-0.822$, $P<0.001$), and the second was TSC ($r=-0.811$, $P<0.001$). Thus, accumulation of soluble carbohydrates was an important factor, for freezing tolerance in centipedegrass, under the assumption that LT$_{50}$ estimated by the electrolyte leakage was the same as that estimated by the lethality of plants under the field conditions as in the case of tall fescue (Wang et al., 2002). Starch has long been noted as the determinant factor for freezing tolerance, and this was confirmed by the negative correlation between LT$_{50}$ and starch content ($r=-0.627$, $P<0.001$) in this study. Thus, the starch stored in stolons, which could be consumed during the overwintering, can improve the overwintering ability of centipedegrass, because starch is transformed to soluble carbohydrates in late autumn in parallel to the increase in osmotic potential resulting in a rapid increase in freezing tolerance (decrease in LT$_{50}$) (Fry et al., 1993; Savitch et al.,
2000). There were two complicated aspects for the relationships between the cold tolerance and proline content. Xin and Browse (1998) reported that in a mutant arabidopsis, eskI, conferred increased cold tolerance with the increase in proline content. The increase in proline content led to the decrease in osmotic potential, which might enhance the cold tolerance. However, Murelli et al. (1995) and Wanner et al. (1999) found no direct relationships between the cold tolerance and proline content, neither in the cold-acclimation experiment nor in a comparative study on barley varieties differing in proline content. In this study, the increase of proline content in stolons corresponded with the decrease in temperature, but it might not be an acclimation response. Even though the increase in proline content of stolons was markedly lower than that in fructose content, proline content responded sensitively to the change in temperature.

In conclusion, our results demonstrated that carbohydrates and its metabolism as well as proline play important roles in freezing tolerance of centipedegrass, and stolon is the critical organ, which would determine the freezing tolerance of centipedegrass under natural conditions.

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