Ion amount in *Zoysia matrella* Merr. leaves during the winter season in the saline environments

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Abstract: The purpose of the present study was to evaluate the ion amounts characteristics of green leaves and brown leaves in *Zoysia matrella* (L.) Merr. during the winter season in the saline environments. Plants were treated with four concentrations of NaCl solution (0, 7.5, 15 and 30 g/L NaCl) for 165 days during the winter season. In the 30 g/L NaCl group, all leaves turned brown (leaf mortality ratio was 100%). In the 7.5 and 15 g/L NaCl groups, Na⁺ amounts of brown leaves were 1.4-fold and 2.0-fold higher than in green leaves, respectively. Furthermore, in the 7.5 and 15 g/L NaCl groups, Cl⁻ amounts of brown leaves were 1.4-fold and 2.0-fold higher than in green leaves, respectively. However, K⁺ amount exhibited an inverse relationship, with higher K⁺ amount in green leaves than in brown leaves. In the 0, 7.5, and 15 g/L NaCl groups, K⁺ amounts of green leaves were 2.3-fold, 1.8-fold, and 1.8-fold higher than in brown leaves, respectively. Furthermore, the results of K⁺/Na⁺ ratio in the green leaves showed higher value compared with brown leaves with significant difference in 7.5 g/L (2.4-fold higher) and 15 g/L (3.5-fold higher) NaCl treated groups. From the results of completely different traits in ion amount and K⁺/Na⁺ ratio in green and brown leaves indicated that *Z. matrella* could accumulate harmful NaCl in senescing leaves against salinity stress in the winter season.

Key words: salt tolerant turf grass, green leaves, brown leaves, sodium ion, potassium ion, K⁺/Na⁺ ratio

杉浦総一郎・高橋新平：冬季塩分環境下におけるコウライシバ (*Zoysia matrella* Merr.) 葉身部のイオン濃度

摘要：冬季塩分環境下におけるコウライシバ (*Zoysia matrella* (L.) Merr.) の健全葉および枯死葉のイオン濃度特性を明らかにするため、冬季 165 日間にわたり本種に 4 段階の NaCl 溶液 [0 g/L (Control), 7.5 g/L, 15 g/L, 30 g/L] を施用した。その結果、30 g/L 区では、全ての葉身部の枯死が確認された (枯死率 100%)。葉身部の Na⁺ 濃度は健全葉より枯死葉で高く、7.5 g/L 区で 1.4 倍、15 g/L 区で 2.0 倍高い値であった。Cl⁻ 濃度も同様に枯死葉で高い値を示し、7.5 g/L 区で 1.4 倍、15 g/L 区で 2.0 倍、健全葉より高い値を示した。一方、K⁺ 濃度は健全葉で高く、Control 区で 2.3 倍、7.5 g/L 区で 1.8 倍、15 g/L 区で 1.8 倍、枯死葉より高い値を示し、Na⁺、Cl⁻ 濃度と逆の傾向を示した。さらに、K⁺/Na⁺ 比は、枯死葉より健全葉で高く、7.5 g/L 区、15 g/L 区においてそれぞれ 2.4 倍、3.5 倍高い値を示した。枯死葉と健全葉のイオン濃度と K⁺/Na⁺ 比に明らかな違いが見られたことから、コウライシバは枯死に至る前の老化した葉に多くの Na⁺、Cl⁻ を蓄積させることで、冬季における塩ストレスに対応していると考えられた。

キーワード：耐塩性芝草、健全葉、枯死葉、ナトリウムイオン、カリウムイオン、K⁺/Na⁺ 比

1. Introduction

Zoysia matrella (L.) Merr. is a popular and common turf grass used as a ground cover plant for green spaces in Japan (Asano & Namihisa, 1999; Fukuoka, 2000). This turf grass also has been recognized as salt-tolerant species (Marcum, 2006; Uddin *et al.*, 2009). Regarding salt tolerance of *Z. matrella*, salt excretion from salt gland and leaf surface (Yamamoto *et al.*, 2016; Sugiura, 2020), salt compartment in the vacuole (Chen *et al.*, 2015) and osmotic adjustment in

cells (Marcum & Murdoch, 1994; Akamine *et al.*, 2002) have been reported. For the characteristics of salt tolerance, the species has been noticed as cost efficient turf grass in landscape area with saline water irrigation (Chen *et al.*, 2009), and it was also considered important greening plants for coastal area with storm surge or tsunami damage may occur.

However, the plant leaves die (turn to brown) in the winter season (new leaves develop from the surviving rhizomes in the spring season). Furthermore, the species is called

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warm-season turfgrass, delicate for low temperature stress, the optimum temperature is 25°C–35°C (Sato, 1995). For utilizing the turf grass sustainably in saline environments such as coastal area, the salinity stress tolerance mechanism in winter season of *Z. matrella* was considered important to understand.

It has been known that the senescing leaves have important role in plants physiology in winter season. Generally for ion reuse, deciduous trees undergo ion translocation from the leaves in the winter season prior to the leaves dying and falling. A prior study reported that in *Quercus serrata*, leaves exhibit low ion amount, including nitrogen, phosphate, potassium and magnesium, in the winter season (Hiraizumi *et al.*, 1996). Especially, potassium is an important ion for osmotic control and tolerance of environmental stress for plants (Turgeon, 2007). Moreover, it was referred that turfgrass undergoes leaf amino acid translocation as the process of leaves dying (Turgeon, 2007).

Senescing leaves also have an important role in plant salinity stress tolerance. Previous reports indicated that the salt tolerant species can accumulate NaCl in leaves, which then die and fall, removing harmful salt (Imada *et al.*, 2009; Kanai *et al.*, 2014). It was demonstrated that *Z. matrella* accumulate large amounts of salt in its leaves compare with other parts of the plant (rhizomes and roots) in saline environments (Sugiura *et al.*, 2017).

From the above, we hypothesized that *Z. matrella* senescing leaves have important role for salinity stress tolerance in winter season as accumulating salinity in senescing leaves. However, there is presently no report evaluating ion amounts of senescing leaves in herbaceous plant including *Z. matrella* with detailed quantitative data. Therefore it considered valuable to clarify ion amounts in brown leaves for understand the plants physiology and strategy for salinity stress in low temperature stress.

To clarify ion amount of brown leaves, we treated *Z. matrella* with different concentrations of NaCl solution during the winter season, and measured ion amount (Na^+ , Cl^- and K^+) in green leaves and brown leaves, together with leaf mortality ratio and K^+/Na^+ ratio. The objective of the present study was to evaluate ion amount characteristics in green and brown leaves in a saline environment during the winter season, and to understand the role of senescing leaves for salinity stress.

2. Materials and Methods

2.1 Species and experimental design

Zoysia matrella (L.) Merr. was purchased as sod turf from the Tottori Lawn Product Association (Tottori, Japan). On June 7, 2015, three stolon segments (3 cm/each) were

planted in 200 cm² Wagner pots filled with 1.0 kg gravel as a drainage layer and 4.0 kg river sand as bed soil. Each pot was irrigated with tap water for 133 days until the plants were well-established (observed dense leaves, and leaves height around 15 cm in each pot). During the tap water irrigation, no fertilizer was applied.

Four concentrations of NaCl solution were prepared [0 (as Control), 7.5, 15, and 30 g/L], with 3 repetitions. For the possibility of utilizing the plant in coastal area, the maximum concentration of the NaCl solution (30 g/L) was set to sea water level, as referring previous experiment (Sugiura *et al.*, 2017). The total number of pots prepared were 12 (4 concentrations of NaCl solution $\times$ 3 repetitions). As for avoiding rain effects, each pot containing a plant was placed at random in a greenhouse (non-heated) at the Tokyo University of Agriculture, Tokyo, Japan.

2.2 NaCl treatment

After irrigating the pots with tap water for 133 days to establish the plants, NaCl solution was added. NaCl (99.5%) was mixed with tap water at the above-specified concentrations in plastic tanks. Thereafter, 600 mL NaCl solution was used to treat to each pot from October 19 2015 to April 1 2016 (165 days). NaCl treatment was conducted when the soil surface was dry, including Oct. 19, Nov. 5 and 22, Dec. 9, 2015, Jan. 19, 2016 and Feb. 29, 2016 to yield six treatments. Tap water was not treated during the NaCl solution treatment period. To avoid adding NaCl treatment directly to *Z. matrella* leaves, a funnel was used, and NaCl solution was added directly to the soil. The average temperature and humidity in the non-heated greenhouse during NaCl solution treatment are illustrated in Fig. 1.

Fig. 1 Daily average greenhouse temperature and humidity during the NaCl treatment period

Data were measured from October 19, 2015 to April 1, 2016 (165 days). Greenhouse was not heated during experiment

2.3 Measurement of leaf dry weight and leaf mortality ratio

After 165 days NaCl treatment, turf leaves were trimmed to a height of 2 cm from the soil surface to measure ion amount and dry weight (DW). To quantify dry weight, the leaves were dried in an oven at 90°C for 24 h and divided into green leaves and brown leaves, followed by measurement of biomass. The leaves were not washed with water prior to ion measurement in order to avoid ion outflow, especially in brown leaves (Japan Soil Association Ed., 2001). Therefore in the present study, the data regarding ion amounts include inside and outside ion of the leaves. Leaf mortality ratio was calculated using green leaf DW and brown leaf DW, according to the following formula:

$$\text{Leaf mortality ratio (\%)} = [\text{brown leaf DW} \div (\text{green leaf DW} + \text{brown leaf DW})] \times 100$$

2.4 Leaf ion measurement

Dried leaves were milled using beads in a centrifuge tube with a homogenizer (BMSA 20 TP, Bio Medical Science Co., Ltd., Tokyo, Japan). Measurement of ion amounts in the leaves was conducted as referring previous experiment (Russo & Karmarkar, 1998). Milled dried leaves were transferred to a 2-mL centrifuge tube containing distilled water. The tube was then shaken thoroughly by hand for 30 sec., and the solution was subsequently filtered through a 0.45- μm membrane filter (NY 025045, Membrane Solutions Limited). The quantities of each ion (Na^+ , Cl^- and K^+) were measured using an ion analyzer (IA-300, DKK-TOA Corporation, Tokyo, Japan).

2.5 Statistical analysis

Considering the small sample sizes (three repetitions) in this study, we used the nonparametric methods (Hoskin, 2010); Mann-Whitney U Test to evaluate significant differences between green and brown leaves, Bonferroni's multiple comparison test for comparison of four different NaCl treatments. Statistical analysis were conducted using the add-in analysis software in Microsoft Excel (BellCurve for Excel, Social Survey Research Information Co, Ltd.).

3. Results and Discussion

3.1 Leaf dry weight and mortality ratio

After 165 days NaCl treatment, green leaf dry weight was decreased according to NaCl concentrations treated. At 30 g/L, no green leaves remained (Table 1). However brown leaves dry weight did not differ between NaCl treatment groups. Leaf mortality ratios were 56.2, 76.5, 89.0, 100% in ascending order of NaCl concentration.

The daily temperature in the green house was recorded below 5°C on January 13 (Fig. 1). At the end of the study, the control group, which was not subjected to NaCl stress, ex-

Table 1 Leaf dry weight and mortality ratio

Leaf mortality ratio was calculated using the following formula: Leaf mortality ratio (%) = [brown leaf DW $\div$ (green leaf DW + brown leaf DW)] $\times$ 100. Data were measured after 165 days NaCl treatment. Data are expressed as mean values $\pm$ SD of triplicate analyses. Values sharing the same letter were not significantly different ($p=0.05$), as measured by a Bonferroni's multiple comparison test in comparison of four different NaCl treatments

Treated NaCl solution concentration (g/L)	Leaf dry weight (g/200 cm ²)			Leaf mortality ratio (%)
	Green leaves	Brown leaves	Green leaves and Brown leaves	
Control	1.9 $\pm$ 0.5 a	2.6 $\pm$ 1.3 a	4.5 $\pm$ 1.7 a	56.2 $\pm$ 5.0
7.5	1.2 $\pm$ 0.3 ab	4.4 $\pm$ 1.7 a	5.6 $\pm$ 1.8 a	76.5 $\pm$ 9.6
15	0.4 $\pm$ 0.1 bc	3.2 $\pm$ 1.6 a	3.6 $\pm$ 1.8 a	89.0 $\pm$ 1.5
30	0.0 $\pm$ 0.0 c	4.5 $\pm$ 1.7 a	4.5 $\pm$ 1.7 a	100.0 $\pm$ 0.0

hibited a high mortality ratio of 56.2%. This suggested that low temperature alone decreased green leaf dry weight, which was further exacerbated by salinity stress (Table 1). For the data of green leaves and brown leaves dry weight, there were no significant differences in all four NaCl treated groups (Table 1). Especially as the control (no-NaCl treated group) showed no significant difference, it was indicated that the low temperature halted leaves growing during the experiment in winter season.

3.2 Leaf ion amounts

In NaCl-stressed plants, both green and brown leaves showed large amounts of NaCl. However, in brown leaves, Na^+ and Cl^- amounts were higher than in green leaves [Fig. 2 (a), (b)]. In the 7.5 and 15 g/L NaCl groups, Na^+ amounts in brown leaves were 1.4-fold and 2.0-fold higher significantly than in green leaves, respectively. Similarly, in the 7.5 and 15 g/L NaCl groups, Cl^- amounts in brown leaves were 1.4-fold and 2.0-fold higher than in green leaves, respectively.

These results demonstrated that *Z. matrella* could accumulate large amounts of NaCl in senescing leaves, consistent with previous reports (Imada *et al.*, 2009; Kanai *et al.*, 2014). And it was considered that *Zoysia matrella* can remove NaCl by accumulating those ions in senescing leaves in a saline environment. However, in the salt tolerant plants, it was reported that the old cell develops vacuole to accumulate more salt in it (Takahashi, 1997, Maeda, 2012). Further study such as investigating relationship between leaves age and ion amounts in *Z. matrella* was considered in need.

Leaf K^+ amount exhibited a different trend than that of Na^+ and Cl^- , with higher K^+ amount in green leaves relative to brown leaves (Fig. 2). In the 0 (Control), 7.5, and 15 g/L NaCl groups, green leaves K^+ amounts were 2.3-fold, 1.8-fold, and 1.8-fold higher significantly than K^+ amount of

Fig. 2 Green leaves and brown leaves ion amounts (a) Na⁺, (b) Cl⁻, (c) K⁺. Ion amounts (Na⁺, Cl⁻ and K⁺) were measured after 165 days NaCl treatment. Data are expressed as means. Vertical bars represent SDs of triplicate analyses. Ion amounts include inside and outside ion of leaves. Statistically significant differences between green and brown leaves are denoted as: * $p < 0.05$. No mark denotes lack of statistical significance. Differences were assessed using a Mann-Whitney U Test. Values sharing the same letter were not significantly different ($p = 0.05$), as measured by a Bonferroni's multiple comparison test in comparison of four different NaCl treatments. Small letter: green leaves, large letter: brown leaves. All leaves were observed as brown leaves in the 30 g/L NaCl treated condition, indicated as N/A

brown leaves, respectively. The results of the present study suggest that during the winter season, *Z. matrella* is capable of translocating K⁺ from senescing leaves to other parts of the plant prior to the leaves' dying as previously reported in *Quercus serrata* (Hiraizumi *et al.*, 1996). Moreover, most of potassium remains as ion in the plant cells and not be transformed other substances (Nakamura *et al.*, 1993). Those reports and low K⁺ amount of brown leaves in the present study results [Fig. 2(c)] suggested our consideration. However, for more detail on ion accumulate trait in leaves, additional experiment such as using isotope tracking techniques was considered in need.

3.3 K⁺/Na⁺ ratio of green and brown leaves.

K⁺/Na⁺ ratio (mass value) in both green and brown leaves showed low value accordance with treated NaCl concentration increased. However, the green leaves showed higher value compared with brown leaves with significant difference in 7.5 g/L (2.4-fold higher) and 15 g/L (3.5-fold higher) NaCl solution treated groups (Table 2).

Usually, plants in the salinity stress lose K⁺ with inclusion of Na⁺ in the cell, that phenomenon accelerates salt stress. In salt tolerant plants, it was reported K⁺ retention in the cell in the salinity stress, and the K⁺/Na⁺ ratio is important indicator for evaluating salt tolerance in plant (Schachtman *et al.*, 1989; Takahashi, 1997; Maeda, 2012). For one of the salinity tolerance mechanism in *Zoysia* species is related to Na⁺ compartment in vacuole, and it was functioned by

Table 2 K⁺/Na⁺ ratio (mass value) of green and brown leaves against four different concentration of NaCl solution treatment

Data were measured after 165 days NaCl treatment. Data are expressed as mean values $\pm$ SD of triplicate analyses. Ion amounts include inside and outside ion of leaves. Statistically significant differences between green and brown leaves are denoted as: * $p < 0.05$, ns denotes lack of statistical significance. Differences were assessed using a Mann-Whitney U Test. Values sharing the same letter were not significantly different ($p = 0.05$), as measured by a Bonferroni's multiple comparison test in comparison of four different NaCl treatments. All leaves were observed as brown leaves in the 30 g/L NaCl treated condition, indicated as N/A

Treated NaCl solution concentration (g/L)	K ⁺ /Na ⁺ ratio		Significant differences
	Green leaves	Brown leaves	
Control	1.26 $\pm$ 0.41 a	1.32 $\pm$ 0.22 a	ns
7.5	0.46 $\pm$ 0.10 b	0.19 $\pm$ 0.06 b	*
15	0.28 $\pm$ 0.02 b	0.08 $\pm$ 0.02 b	*
30	N/A	0.06 $\pm$ 0.01 b	N/A

Na^+/H^+ antiporter on bio membrane (Du *et al.*, 2010; Chen *et al.*, 2015).

The results of ion amount and K^+/Na^+ ratio in the present study indicated completely different with green and brown leaves (Fig. 2, Table 2). From the results of the present study and previous reports, for one of the salt tolerance mechanism of *Z. matrella* in winter season, it was considered that the senescing leaves has important role for accumulation of harmful salinity in the senescing leaves against salinity stress.

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