Survival and Recovery of 'Meyer' Zoysiagrass Rhizomes after Extracellular Freezing

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ABSTRACT. Rhizomes of 'Meyer' zoysiagrass (*Zoysia japonica* Steud.) were subjected to temperatures below 0 °C and were subsequently placed in a growth chamber with air at 34 °C day/28 °C night to determine the rate of shoot growth from nodes. Rhizomes exposed to subzero temperatures produced shoots steadily up to 16 days after freezing (DAF), but subsequent shoot growth from rhizomes was minimal. At 32 DAF, shoots were present on 68% and 44% of the nodes of unfrozen control (2 °C) rhizomes and those frozen to -7 °C, respectively. In another study, samples were frozen to a sublethal temperature (-7 °C) to examine the distribution of extracellular ice voids near the apical meristems of rhizomes and to characterize tissue recovery. Extracellular voids were present within the leaf tissue and between the leaves in samples prepared for scanning electron microscopy (SEM) immediately after freezing to -7 °C. By 12 DAF, most of the remaining voids were observed in older leaves. Nearly all extracellular voids in the leaves were absent by 20 DAF. However, by 28 DAF, some rhizomes still had small voids between leaves. Although the structure of zoysiagrass rhizomes subjected to -7 °C was temporarily disrupted, tissues recovered from extracellular freezing and new shoot growth was produced following exposure to warm temperatures.

Zoysiagrass is a high-quality turf that is typically used for lawns and golf fairways and tees in warm or transitional climates due to its excellent heat and wear tolerance. During autumn in the northern hemisphere, zoysiagrass discolors in response to temperatures ≤ 12 °C, but it turns green in the spring with warmer temperatures. Although zoysiagrass has good low-temperature tolerance compared to other warm-season grasses, injury can occur after a severe winter, especially in the absence of snow cover (Beard, 1973; Dunn, 1989; Rogers et al., 1977). Generally, low-temperature injury is not detected or assessed until spring when the turf is rated for survival (Bughrara et al., 1998).

Ice formation in turfgrass rhizomes and subsequent recovery and regrowth after exposure to low temperatures has not been studied. Light and scanning electron microscopy of leaves (Pearce, 1988; Pearce and Ashworth, 1992) and crowns (Olien, 1964) of various cereal crops have shown that extracellular tissue voids were formed from the presence of ice during low-temperature exposure. Winter cereal crops survive exposure to freezing temperatures (about -25 °C) by tolerating intercellular ice formation (Burke et al., 1976).

While zoysiagrass and the cereal crops are monocots, the spreading growth habit of rhizomes and meristematic regions of zoysiagrass differ from those of cereal grasses. Thus, the objectives of these studies were to 1) determine the rate of shoot growth from rhizomes of 'Meyer' zoysiagrass after exposure to various temperatures below 0 °C; 2) examine the distribution of extracellular ice in rhizomes exposed to -7 °C; and 3) characterize tissue recovery after exposure to sublethal temperatures.

Materials and Methods

'Meyer' zoysiagrass sod was cut at a 25-mm thickness and removed from the Univ. of Missouri Turfgrass Research Center

(Columbia, Mo.) on 2 Oct. 1995, before a natural autumn freeze occurred in 1995. Sod was placed in 53 × 27-cm flats in the greenhouse at ≈ 22 °C and maintained until experiments were performed. Before each experiment was initiated, a section of sod was removed from the greenhouse, shoots were removed, rhizomes (≈ 2 mm in diameter) were washed free of soil, and five-node sections of this tissue were cut. Samples were then wrapped in a paper towel moistened with distilled water, sealed in a plastic bag, and placed in cold storage at 0 °C for cold acclimation for 1 week.

LOW-TEMPERATURE EXPOSURE OF RHIZOMES. Following storage at 0 °C, eight rhizomes were placed in moist cheesecloth and wrapped in aluminum foil for each of eight test temperatures. A 0.01-mm-diameter (30-gauge) copper-constantan thermocouple was attached to a rhizome to monitor tissue temperature and to record the ice nucleation temperature. Thermocouple output was read on a digital thermometer (Omega Engineering, Stamford, Conn.). Samples were placed in a programmable freezer (Tenney Engineering, Union, N.J.) at -2 °C for 2 h and then cooled at 1 °C/h to temperatures estimated to result in tissue injury. Five, eight-rhizome replicates were removed from the freezer at -4, -5, -6, -7, -8, -9, or -11 °C. These samples were then placed in a refrigerator at 2 °C, and allowed to thaw slowly for 24 h. Unfrozen control samples were held at 2 °C while other samples were exposed to subzero temperatures.

SHOOT GROWTH FROM RHIZOMES. After thawing, each rhizome was planted in a vermiculite-filled flat in a 3.8 × 3.8-cm cell. Flats were then placed in a growth chamber at 85% RH, 34 °C day/28 °C night cycle, and a 14-h photoperiod at a photosynthetic photon flux of $\approx 500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the surface of the vermiculite. The number of visible shoots that grew from rhizomes was recorded at 2-d intervals for 32 d. The percentage of shoots that grew each day from nodes of eight rhizomes per test temperature was calculated, and data were transformed to the arcsine square root for analysis (Snedecor and Cochran, 1989). Shoot growth data were analyzed as a two-way factorial arrangement of treatments in which the linear statistical model contained the main effects of DAF and exposure temperature, and the interaction of these main effects. The general linear model procedure of SAS (SAS Institute, Cary, N.C.) was used for the statistical analysis.

SEM STUDY. Additional plants were sampled and prepared for exposure to low temperatures as described above. These rhizomes

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Table 1. Percentage shoot growth from 'Meyer' zoysiagrass rhizomes following exposure to selected low temperatures.^z

Days after freezing	Exposure temp (°C)							
	Unfrozen control	-4	-5	-6	-7	-8	-9	-11
2	5	5	0	0	0	0	0	0
4	16	14	19	18	13	7	1	1
6	32	35	36	30	22	17	10	3
8	42	41	43	39	25	25	16	6
10	56	49	43	44	38	35	18	6
12	57	54	50	48	40	35	21	7
14	58	56	53	49	42	36	22	7
16	58	58	53	52	42	38	26	7
18	59	58	54	52	42	39	27	7
20	60	59	54	52	42	39	27	7
22	60	59	54	52	42	39	28	7
24	61	59	56	52	43	39	29	7
26	61	59	57	52	43	40	29	9
28	61	59	57	52	44	42	30	9
30	65	59	57	52	44	42	30	9
32	68	59	57	52	44	43	30	9

^zPercentage shoot growth = number of visible shoots ÷ 40 nodes. Values are the means of five 8-rhizome replications. Each sample had five nodes per rhizome. Interaction LSD(0.05) = 4.

were also held at -2°C for 2 h and then cooled at 1°C/h to -7°C . Immediately after low-temperature exposure, 10 rhizomes were cross-sectioned near the distal apical meristem and prepared for SEM (Warmund, 1984). Tissue was fixed in 3% glutaraldehyde and 1% osmium tetroxide (OsO_4) in 0.1 M phosphate buffer (pH 7.0). Tissue was then dehydrated in a graded ethanol series, critical point dried, sputter-coated with gold palladium, and examined on a JEOL JSM-35 microscope (JEOL, Peabody, Mass.) operated at 10 kV. Ten unfrozen controls were also fixed for SEM. Additional rhizomes exposed to -7°C were thawed at 2°C for 24 h, planted in vermiculite-filled flats, and placed in the growth chamber. Ten of these rhizomes were removed from the growth chamber every 4 d and were prepared for SEM over a period of 28 d.

Results

LOW-TEMPERATURE SURVIVAL. Although ice nucleation occurred on the surface of the rhizomes at about -2.8°C , rhizome samples that were exposed to test temperatures less than or equal to -4°C produced shoots (Table 1). By 2 d after freezing (DAF), regrowth had occurred from rhizomes exposed to -4°C and the unfrozen control samples. In contrast, shoot regrowth from rhizomes exposed to -5 to -11°C was delayed until 4 DAF. Regrowth from nodes of rhizomes exposed to most subfreezing temperatures doubled from 4 to 6 DAF. Moreover, shoot regrowth of frozen samples generally increased at each date up to 10 DAF and continued at a slow rate thereafter. After 16 DAF, additional shoot regrowth was minimal. At 32 DAF, shoots were present on 68% of the nodes of unfrozen control (2°C) rhizomes. About half of the nodes of rhizomes cooled to -6°C regrew by 32 DAF. While rhizomes survived exposure to -11°C , shoot regrowth by 32 DAF was minimal (9%) when compared to that at -9°C (30%).

SEM. The diameter of the apical meristem of 'Meyer' rhizomes was $\approx 58\text{ }\mu\text{m}$. New shoots near the apical meristem were visible (Fig 1). There were generally four leaves surrounding the basal portion of the meristem (Fig. 2). Tissues of unfrozen control rhizomes appeared intact without visible injury (Fig. 2A). In contrast, extracellular voids were apparent among thin-walled parenchyma cells in young and old leaf tissue and between leaves

in rhizome samples prepared for SEM immediately after freezing to -7°C (Fig. 2B). Several cells in the outer layers of leaves that were adjacent to voids were compressed (Fig. 3). Most cell walls adjacent to voids that were within leaves appeared intact. By 4 DAF, extracellular voids appeared smaller (Fig. 2C) than those in rhizomes sampled immediately after low-temperature exposure (Fig 2B). Most of the voids within leaves were confined to older ones by 12 DAF (Fig. 2D). Nearly all extracellular voids in leaves

Fig. 1. Scanning electron micrograph of three shoots (s) near the apical meristem (m) of a rhizome of 'Meyer' zoysia. Bar = $10\text{ }\mu\text{m}$.

Fig. 2. Scanning electron micrographs of 'Meyer' zoysia rhizomes that were (A) unfrozen, or (B) cooled to -7°C and immediately fixed in glutaraldehyde and OsO_4 , or subsequently grown in the growth chamber for (C) 4, (D) 12, (E) 20, or (F) 28 d before fixation. Bar = 100 μm . Extracellular voids (arrows) were visible within (B, C, D) and between leaves (E) of rhizomes examined after exposure to -7°C . Voids were absent in most rhizomes examined at 28 d after freezing.

Fig. 3. Rhizomes of 'Meyer' zoysia that were cooled to -7°C and fixed in glutaraldehyde and OsO_4 . Extracellular voids (v) were visible in the leaf tissue. Cellular compression (arrows) was apparent adjacent to ice voids that formed between leaves. Bar = 100 μm .

were absent by 20 DAF (Fig. 2E). However, by 28 DAF, a few rhizomes still had small voids between leaves, but they were no longer apparent in most samples (Fig. 2F).

Discussion

Although the mean ice nucleation temperature of zoysiagrass rhizomes was -2.8°C , this was not the lethal temperature for tissue injury. On some plants, ice nucleation may be caused by ice-nucleation-active (INA) bacteria, such as *Pseudomonas syringae* van Hall, *P. fluorescens* Migula, or *Erwinia herbicola* (Löhnis) Dye (Lindow, 1983). Alternatively, nonbacterial nucleators may incite ice formation on plant surfaces. In some frost-sensitive tissues, such as foliar tissue of corn (*Zea mays* L.) (Lindow et al., 1982b), tomato (*Lycopersicon esculentum* Mill.), eggplant (*Solanum Melongena esculentum* L.), pumpkin (*Cucurbita pepo* L.), cucumber (*Cucumis sativus* L.), marigold (*Tagetes patula* L.) (Lindow et al., 1982a), frost injury was associated with high bacterial densities of INA bacteria. Warmund and English (1998) found that the ice nucleation temperature represents the lethal temperature for strawberry (*Fragaria xananassa* Duch.) flowers at full bloom. Others (Andrews et al., 1986; Gross et al., 1984) reported that frost-sensitive peach [*Prunus persica* (L.) Batsch] and cherry (*P. avium* L.) flowers were injured by ice formation induced by INA bacteria or nucleators of plant origin. It is unknown if rhizomes we studied were colonized with INA bacteria. Nevertheless, zoysiagrass rhizomes survived exposure to the ice nucleation temperature.

'Meyer' zoysiagrass rhizomes recovered relatively quickly from exposure to subzero temperatures. All rhizomes exposed to all test temperatures produced shoot growth by 4 DAF (Table 1). Thereafter, shoot regrowth continued. However, from 16 to 32 DAF, there was $\leq 5\%$ additional shoot regrowth from rhizomes exposed to subfreezing temperatures. Bughara et al. (1998) demonstrated that shoot regrowth from rhizomes measured 6 weeks after low-temperature exposure in a freezing chamber was useful in identifying 'Meyer' zoysiagrass cold tolerance. While only one

zoysiagrass cultivar was used, results from this study indicate that the period of regrowth for assessing the cold tolerance of 'Meyer' zoysiagrass may be reduced considerably to only about 16 DAF.

SEM micrographs from this study confirmed the presence of extracellular voids near the apical meristem of zoysiagrass rhizomes after exposure to a sublethal temperature. Ice was not observed in this study due to the rapid thawing of the rhizomes after their removal from the freezing chamber. However, tissue voids were most likely the result of extracellular ice formation during low-temperature exposure. Extracellular ice masses have been observed in crowns of barley (*Horeum vulgare* L.) (Olien, 1964), strawberry (Warmund et al., 1993) and rye (*Secale cereale* L.) (Pearce, 1988) and wheat (*Triticum aestivum* L.) leaves (Pearce and Ashworth, 1992). Electron microscopy studies of plant tissues confirmed that extracellular voids were the result of ice formation. In these plants, water migrated from inside cells to extracellular ice as the temperature dropped below the melting point of the cell sap. Lethal injury may be caused by dehydration stress, physical impairment of cell membranes, reduced cellular volume below a critical size, or concentration of cell sap, resulting in the denaturation of proteins (Chen et al., 1995; Levitt, 1980).

As the number of days after low-temperature exposure increased, there were fewer extracellular voids observed within the zoysiagrass leaves. In strawberry crowns that were subjected to a sublethal temperature, extracellular voids were smaller after plants were grown in the greenhouse for 8 weeks than those examined immediately after freezing (Warmund, 1993). Cell division and enlargement were also observed near extracellular voids in strawberry crowns at 8 weeks after freezing. In our study, cell division was difficult to evaluate in cross sections of rhizomes. However, the reason that the extracellular tissue voids were absent in young leaves of zoysiagrass rhizomes by 12 DAF is most likely due to cell division and/or enlargement. Older leaves apparently were slower to recover from extracellular ice formation than younger ones. Additionally, an absence of sloughed off leaf tissue in the cells in which rhizomes were planted after freezing to -7°C also indicates tissue recovery.

During exposure to subfreezing temperatures, cellular contraction occurs as cellular water is redistributed to extracellular ice (Johansson and Krull, 1970; Levitt, 1980). Gusta et al. (1982) suggested that, as the critical temperature is reached, the plasma membrane of dehydrated, collapsed cells undergoes a phase transition, resulting in a loss of semipermeability. It is unclear in this study if the compressed cells observed in the outer layers of leaves are a result of loss of cell volume due to dehydration or simply mechanical pressures induced by the formation of an ice mass between the leaves.

In conclusion, rhizomes of 'Meyer' zoysiagrass survived exposure to -11°C . Although extracellular tissue voids were observed near the apical meristem of zoysiagrass rhizomes following exposure to -7°C , tissue structure was disrupted only temporarily and rhizomes were not permanently injured. Within 4 DAF, shoot growth resumed. By 20 DAF most of the extracellular tissue voids within leaves were absent or reduced considerably in size. Thus, 'Meyer' zoysiagrass rhizomes subjected to -4 to -11°C tolerated extracellular freezing and produced new shoot growth following exposure to warm temperatures.

Literature Cited

- Andrews, P.K., E.L. Proebsting, Jr., and D.C. Gross. 1986. Ice nucleation and supercooling in freeze-sensitive peach and sweet cherry tissues. J. Amer. Soc. Hort. Sci. 11:232-236.
- Beard, J.B. 1973. Turfgrass: Science and culture. Prentice-Hall, Inc.,

- Englewood Cliffs, N.J.
- Dunn, J.H., S.S. Bughara, M.R. Warmund, and B.F. Fresenburg. 1998. Identifying winter hardy zoysiagrasses by freezing tests. *HortScience* (in press).
- Burke, M.J., L.V. Gusta, H.A. Quamme, C.J. Weiser, and P.H. Li. 1976. Freezing and injury in plants, p. 507–528. In: W.R. Briggs, P.B. Green, and R.L. Jones (eds.). *Annual review of plant physiology*. Annual Reviews Inc., Palo Alto, Calif.
- Chen, T.H.H., M.J. Burke, and L.V. Gusta. 1995. Freezing tolerance in plants: An overview, p. 115–135. In: R.E. Lee, Jr., G.J. Warren, and L.V. Gusta (eds.). *Biological ice nucleation and its applications*. APS Press, St. Paul, Minn.
- Dunn, J.H. 1989. Observations on 'Meyer' zoysiagrass after 20 years of research. *Golf Course Mgt.* 57(6):64–76.
- Gross, D.C., E.L. Proebsting, Jr., and P.K. Andrews. 1984. The effects of ice-nucleation-active bacteria on temperatures of ice nucleation and freeze injury of *Prunus* flower buds at various stages of development. *J. Amer. Soc. Hort. Sci.* 109:375–380.
- Gusta, L.V., D.B. Fowler, and N.J. Tyler. 1982. Factors influencing hardening and survival in winter wheat, p. 23–40. In: P.H. Li and A. Sakai (eds.). *Plant cold hardiness and freezing stress*. vol 2. Academic Press, New York.
- Johansson, N.O. and E. Krull. 1970. Ice formation, cell contraction and frost killing of wheat plants. *Natl. Swedish Inst. Plant Protect. Contr.* 14:343–362.
- Levitt, J. 1980. Responses of plants to environmental stresses. vol 1. Chilling, freezing, and high temperature stresses. 2nd ed. Academic Press, New York.
- Lindow, S.E. 1983. The role of bacterial ice nucleation in frost injury to plants. *Annu. Rev. Phytopathol.* 21:363–384.
- Lindow, S.E., D.C. Arny, and C.D. Upper. 1982a. Bacterial ice nucleation: A factor in frost injury to plants. *Plant Physiol.* 70:1084–1089.
- Lindow, S.E., S.S. Hirano, W.R. Barchet, D.C. Arny, and C.D. Upper. 1982b. Relationship between ice nucleation frequency of bacteria and frost injury. *Plant Physiol.* 70:1090–1093.
- Olien, C.R. 1964. Freezing process in the crown of 'Hudson' barley, *Hordeum vulgare* (L., emend. Lam.) Hudson. *Crop Sci.* 4:91–95.
- Pearce, R.S. 1988. Extracellular ice and cell shape in frost-stressed cereal leaves: A low temperature scanning-electron-microscopy study. *Planta* 175:313–324.
- Pearce, R.S. and E.N. Ashworth. 1992. Cell shape and localisation of ice in leaves of overwintering wheat during frost stress in the field. *Planta* 188:324–331.
- Rogers, R.A., J.H. Dunn, and C.J. Nelson. 1977. Photosynthesis and cold hardening in zoysia and bermudagrass. *Crop Sci.* p. 727–732.
- Snedecor, G.W. and W.G. Cochran. 1989. *Statistical methods*. 8th ed. Iowa State Univ. Press, Ames.
- Warmund, M.R. 1984. Anatomical and biochemical responses of grain sorghum (*Sorghum bicolor*) seedlings to flurazone and alachlor. PhD diss., Univ. of Missouri, Columbia (Diss. Abstr. 84-06252.)
- Warmund, M.R. 1993. Ice distribution in 'Earliglow' strawberry crowns and tissue recovery following extracellular freezing. *J. Amer. Soc. Hort. Sci.* 118:644–648.
- Warmund, M.R. and J.T. English. 1998. Ice nucleation, freezing injury, and colonization of 'Totem' strawberry flowers with ice-nucleation-active bacteria. *J. Amer. Soc. Hort. Sci.* 123: 234–238.