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Winters can be severe enough to cause extensive winterkill to bermudagrass, especially in the transition zone. Scientists at Oklahoma State University are using a multi-discipline approach to develop seed- and vegetatively-propagated bermudagrasses with high turf quality and improved freeze tolerance. *(Photo credit: Dr. Michael Richardson)*

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PURPOSE

The purpose of *USGA Turfgrass and Environmental Research Online* is to effectively communicate the results of research projects funded under USGA's Turfgrass and Environmental Research Program to all who can benefit from such knowledge. Since 1983, the USGA has funded more than 225 projects at a cost of $25 million. The private, non-profit research program provides funding opportunities to university faculty interested in working on environmental and turf management problems affecting golf courses. The outstanding playing conditions of today's golf courses are a direct result of ***using science to benefit golf***.

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Freeze Tolerance and Low Temperature-induced Genes in Bermudagrass Plants

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SUMMARY

Bermudagrass is susceptible to freeze damage, especially in the northern boundary of the transition zone between warm- and cool-season grasses. Freeze tolerance is enhanced via traditional breeding approaches such as recurrent selection, and targeted approaches including gene transfer. As part of an integrated approach to improving freeze tolerance in bermudagrass, researchers at Oklahoma State University have refined laboratory-based methods to determine relative freeze tolerance of bermudagrasses to indicate progress in breeding programs and assist turfgrass managers in selecting adapted cultivars. Their research findings also include:

- Considerable variability in freeze tolerance exists within bermudagrasses.
- 'Midlawn', 'Patriot' and 'Quickstand' were among the hardiest vegetatively-propagated cultivars examined.
- 'Riviera' and 'Yukon' were among the seed-propagated cultivars least likely to experience winterkill.
- Putting green bermudagrasses were freeze susceptible, with 'Tifgreen', 'Tifdwarf' and 'TifEagle' being the most hardy
- Longer lasting freezes cause more damage than shorter freezes, even at the same minimum temperature.
- Two genes associated with freeze tolerance in bermudagrass have been identified.

Turfgrass managers spend a considerable amount of time and energy to establish and maintain turfgrasses for various aesthetic, environmental, and recreational purposes. In addition to routine activities like mowing, fertilizing, and irrigating, we periodically deal with biotic stresses that may require applications of pesticides or alterations in management practices.

If these challenges during the growing season are not enough, we then come under the mercy of old man winter. Some years are relatively mild and cause little or no damage, even in the transition zone between warm- and cool-season grasses. Other winters are sufficiently severe to cause extensive winterkill to warm-season grasses such as bermudagrass. The costs, in terms of loss of use and dollars to re-establish following winterkill can be substantial.

Our long-term goal is to develop seed- and vegetatively-propagated bermudagrasses with high turf quality and improved freeze tolerance (12). The breeding efforts at Oklahoma State

Above, Dr. Jeff Anderson inserts a thermocouple into a pot of improved bermudagrass as he prepares it for artificial freeze testing. Such testing is necessary to select for germplasm with improved cold hardiness.

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University have been linked with physiological and molecular genetic studies to reveal changes that take place during acclimation to cold, and to determine the role they play in increased freeze tolerance.

Freeze Tolerance

Bermudagrass germplasm improvement programs require rapid, reproducible means to evaluate freeze tolerance. Freeze tolerance information is also beneficial to turfgrass managers selecting bermudagrasses for the transition zone. The most straight-forward way to compare relative freeze tolerance of a group of cultivars is to establish them in the field, then wait for cold temperatures to sort them out. Unfortunately, there are several limitations to this approach. During any particular winter, low temperatures may not be cold enough to kill any cultivars of interest and no progress would be achieved. If evaluations were conducted at a northern or high elevation location, low temperatures may kill most or all of the bermudagrasses.

Since the prevailing weather conditions before a freeze can vary and may have a profound influence on the acclimation state of the plants, relying on test winters makes it difficult to repeat studies over time and across climatic locations. Another factor that comes into play during natural freezes is the nature of the freeze itself. Whether or not a snow cover is present and the length of time the temperature remains below freezing can have marked influences on plant survival.

Developmental and morphological features can also be factors in winter survival. The presence of rhizomes can contribute to freeze avoidance by being sufficiently deep in the soil profile to avoid temperature extremes. The increased susceptibility of newly seeded bermudagrasses is well known and may involve physiological and/or morphological factors such as stolon density (11).

Mimicking Mother Nature in the Laboratory

To overcome the unpredictable occurrence of test winters and to extend evaluations year-

Figure 1. Refrigerated bath modified for use with potted bermudagrass plants. Plants were placed inside plastic tubes glued to a plywood frame

round, laboratory-based methods to measure freeze tolerance have been developed. Plant material has been acclimated in growth chambers, followed by exposure to a range of temperatures in a freeze chamber (1). The combined approach with grasses acclimating in the field, followed by laboratory-based exposure to sub-freezing temperatures also has been used effectively (9).

Laboratory-based freeze tolerance evaluations generally correspond well with field observations (10), and have provided useful information on relative freeze tolerance of turfgrasses. Therefore, our objective was to quantify freeze tolerance of advanced lines, recently released cultivars, and standard varieties using laboratory-based methods. Standardized, quantitative information on bermudagrass freeze tolerance is vital to scientists to track progress in developing new cultivars. Freeze tolerance data are also beneficial to turfgrass managers selecting turfgrasses for the transition zone.

A number of experiments were conducted to evaluate relative freeze tolerance levels, or to characterize factors contributing to bermudagrass survival at low temperatures. In one series of experiments, freeze tolerance evaluations were divided into three groups based on intended use: vegetatively propagated fairway types, seed-propagated cultivars, and vegetatively-propagated bermudagrasses used for putting greens. A follow-up study examined freeze tolerance of a group of emerging cultivars, both seed- and vegetatively-propagated.

Bermudagrass plants were established and acclimated in growth chambers. After plants had acclimated to fall-like temperatures, they were placed in a freeze chamber with a temperature sensor in each pot. The chamber was programmed to slowly cool the plants, allowing them to be removed over a range of temperatures. Ideally, no damage would occur at the warmest temperatures, and all plants would be killed by exposure to the coldest temperatures. After being removed from the freeze chamber, plants were thawed and returned to the growth chamber to observe regrowth. Evaluating the temperature-survival curve allowed estimation of a Tmid value, similar to the LD_{50} (lethal dose for 50% of the subjects) in a toxicity screen.

A second type of experiment was designed to determine how survival was affected by the length of time plants were held at the minimum temperature. In this study, plants were established and acclimated in growth chambers as described above. However, instead of being placed in a freeze chamber, plants were held at constant, sub-freezing temperatures for various periods of time in plastic tubes in a modified refrigerated bath

Fairway		**Seeded**		**Putting Green**	
Genotype	T_{mid} (EC)	Genotype	T_{mid} (EC)	Genotype	T_{mid} (EC)
GN-1	-5.9 a*	AZ Common	-5.6 a	Champion	-4.8 a
Baby	-6.7 ab	Mirage	-6.1 ab	Floradwarf	-4.9 a
Tifway	-6.7 ab	Jackpot	-6.3 abc	MS-Supreme	-5.2 ab
TifSport	-7.2 bc	Guymon	-7.4 bc	MiniVerde	-5.8 bc
Quickstand	-8.0 cd	Yukon	-7.6 c	TifEagle	-6.0 cd
Midlawn	-8.4 d			Tifdwarf	-6.5 d
				Tifgreen	-6.5 d

* Column means are separated by Duncan's New Multiple Range Test at P = 0.05.

Table 1. Freeze tolerance of fairway, seeded, and putting green bermudagrasses. The T_{mid} values represent the midpoint of the survival-temperature response curve. Adapted from Anderson et al. (2).

(Figure 1).

Genotype	T_{mid} (EC)
Princess	-6.9 a*
Tifway	-7.9 b
TifSport	-7.9 b
Riviera	-8.3 bc
U-3	-8.9 cd
Patriot	-9.7 de
Midlawn	-10.3 e

* Column means are separated by Duncan's New Multiple Range Test at P = 0.05.

Table 2. Freeze tolerance of turf bermudagrasses. T_{mid} values represent the midpoints of survival-temperature response curves. Adapted from Anderson et al. (3).

Bermudagrasses Vary Considerably in Freeze Tolerance

The vegetatively propagated fairway types included 'GN-1', 'Baby', 'Tifway', 'TifSport', 'Quickstand', and 'Midlawn'. 'GN-1' was significantly less hardy than 'TifSport', 'Quickstand', and 'Midlawn' (Table 1). The second set of bermudagrasses comprised the seed-propagated varieties: 'Arizona Common', 'Mirage', 'Jackpot', 'Guymon', and 'Yukon'. 'Arizona Common' was significantly less freeze tolerant than 'Guymon' and 'Yukon'. 'Mirage' and 'Jackpot' were not significantly hardier than 'Arizona Common'.

The third series of plants included vegetatively-propagated bermudagrasses used for putting greens: 'Champion', 'Floradwarf', 'MS-Supreme', 'MiniVerde', 'Tifeagle', 'Tifdwarf', and 'Tifgreen'. 'Tifdwarf' and 'Tifgreen' were significantly hardier than all of the other putting green bermudagrasses tested except 'Tifeagle'. We observed a significant amount of variability in freeze tolerance comparing bermudagrass cultivars within a use category, as well as between categories.

It is important to distinguish between T_{mid} temperatures determined in the laboratory and air temperatures experienced during a natural freeze. In the laboratory, conditions are set up to ensure that plants reach the target temperatures. Critical tissues, such as crowns, of plants in the field will usually be considerably warmer than air temperature due to the thermal buffering capacity of the soil.

In another series of experiments, relative freeze tolerance of seven emerging cultivars was determined. 'Tifway' and 'TifSport' were significantly hardier than 'Princess', but had less freeze tolerance than 'U-3', 'Patriot', and 'Midlawn' (Table 2). 'Riviera' was significantly hardier than 'Princess', but less freeze tolerant than 'Patriot' and 'Midlawn'. 'Midlawn' was significantly hardier than all cultivars except 'Patriot'. Results should be useful in selecting appropriate genotypes for the transition zone of turfgrass adaptation.

In order to mimic freezes of different duration, acclimated plants were held at constant, subfreezing temperatures for various periods of time in plastic tubes in a refrigerated bath. Survival of 'U-3' and 'Riviera' decreased to 25% or less when exposed to -7.0° C for two or five days, compared with 100 and 83% survival, respectively, when plants were removed from the refrigerated bath immediately after reaching at -7.0° C.

'Princess' exhibited 89% survival when plants were removed immediately after equilibrating at -5.4° C, but survival after 2, 24, and 72 hours was 67, 30, and 11%, respectively (Figure 2). Although minimum exposure temperature is a primary determinant of survival, freeze damage to turf bermudagrasses increased as exposure duration increased. Therefore, longer duration freezes are likely to cause more damage than shorter freezes, even if the plants are exposed to the same minimum temperature.

Getting the Genes Out of the Bottle

We have been interested in the changes that take place in bermudagrass plants as they make the transition from active growth in the summer to a dormant, freeze tolerant state during the winter. Earlier studies indicated that changes in gene expression take place, leading to alter-

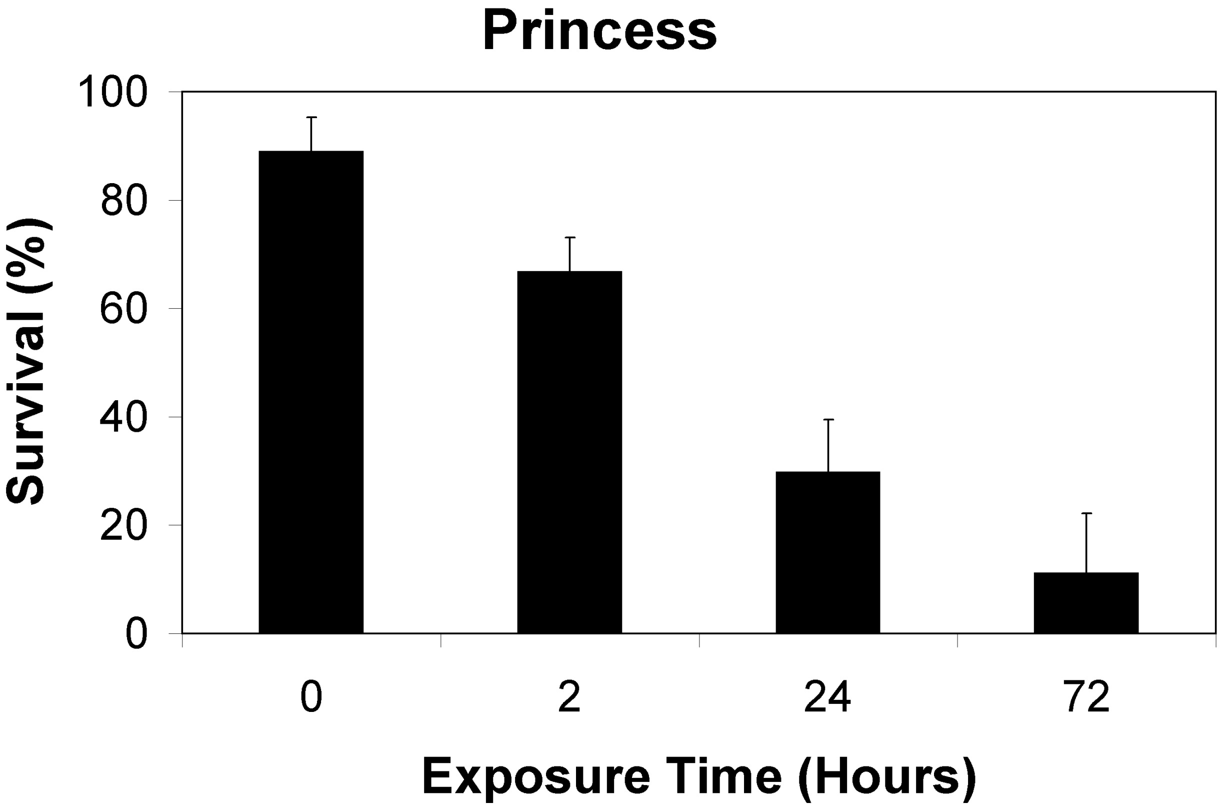

Figure 2. Survival of 'Princess' bermudagrass after being held at -5.4EC for 0, 2, 24, or 72 hours. The 0 hour samples were removed from the bath immediately after reaching the target temperature.

ations in patterns of protein production. Some proteins are produced in relatively greater amounts while others decrease. The challenges lie in determining which changes are important in freeze tolerance, what role they play, and can they be transferred to plants that have excellent turf quality, but poor freeze tolerance?

Early studies showed that bermudagrasses had increased levels of chitinase protein during acclimation (7). Chitin is an extremely abundant polymer, contributing to the hard shells of insects and crustaceans, and to fungal cell walls. While it was not a surprise that chitinase proteins play a role in breaking down fungal cell walls and limiting disease, it was interesting to learn that some chitinases also possessed antifreeze activity (8). Chitinases and other antifreeze proteins may work by binding to small ice crystals, inhibiting growth.

Two chitinase genes were isolated and sequenced from bermudagrass (6), allowing detailed studies of factors affecting how much protein was produced. More chitinase protein was found after low temperature treatments, consistent with a role in freeze tolerance. In addition, application of absisic acid, a plant hormone, increased production. This is significant because scientists have shown abcissic acid can trigger freeze toler-

Figure 3. Floral dip method of transforming *Arabidopsis* plants. A chitinase gene from bermudagrass was transferred to *Arabidopsis* using this procedure.

ance, even in the absence of low temperatures (4).

In order to determine directly whether chitinases are important in increasing bermudagrass freeze tolerance, we need to generate plants that overproduce the gene product. Due to current limitations in transforming bermudagrass, we are using the plant *Arabidopsis* as an intermediate step. It is anticipated that progress made with this model system will be transferred to bermudagrass, since parallel studies are working out details of gene transfer.

The bermudagrass chitinase gene, CynCHT1, was introduced into *Arabidopsis* using a floral dip procedure (Figure 3). Now that we have transgenic *Arabidopsis* plants containing the chitinase gene, we can compare freeze tolerance levels to plants without the chitinase gene. Positive results will pave the way for using this approach to increase bermudagrass freeze tolerance.

An added bonus may be increased resistance to fungal diseases such as spring dead spot. It may not be a coincidence that there is a significant correlation between resistance to spring dead spot and freeze tolerance (5). Chitinase proteins may play a dual role, breaking down the cell walls of fungal pathogens and acting as antifreeze proteins. Increases in bermudagrass freeze tolerance via traditional breeding, and the potential for increased hardiness through selected gene transfer make the prospects of getting through severe winters a bit brighter.

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