CHAPTER FIVE- ROLE OF PROTEINS IN COLD ACCLIMATION AND FREEZE TOLERANCE OF ZOYSIAGRASS CULTIVARS

Abstract

Genetic variation in winter injury exists among zoysiagrass species [*Zoysia japonica* Steud. and *Z. matrella* var. *matrella* (L.) Merr.] and cultivars, but the physiological basis for these differences is not understood. Our objective was to determine the relationships between protein accumulation, polypeptide composition, and freeze tolerance of zoysiagrass. Thirteen cultivars of zoysiagrass with contrasting cold hardiness were identified. Cold-acclimation was induced with four weeks of 8/2˚C day/night cycles and a 10-h photoperiod of 300 μmol m^{-2} s^{-1}. Rhizomes and stolons of zoysiagrass were harvested from non-acclimated and cold-acclimated plants and used for protein analysis. Protein composition was analyzed using SDS-PAGE and immunoblotting with a anti-dehydrin polyclonal antibody. Buffer-soluble protein concentrations were higher among cold-acclimated (7.3 mg g^{-1} dry wt.) than non-acclimated (5.1 mg g^{-1} dry wt.) plants. Though buffer-soluble protein concentrations were similar between *Z. japonica* and *Z. matrella*, concentrations varied among cultivars and ranged from 6.3 to 9.0 mg g^{-1} dry wt. in cold-acclimated plants. SDS-PAGE analysis indicated that cultivars had similar polypeptide composition irrespective of cold acclimation. Immunoblotting indicated that dehydrin polypeptides (23 and 25 kDa) increased during to cold acclimation. Abundance of the 23 kDa dehydrin polypeptide was positively associated (r^2 = 0.41) with genetic variation in freezing tolerance. Our results suggest that dehydrins are associated with zoysiagrass cold-acclimation, but that only the 23 kDa dehydrin plays a role in improving freeze tolerance.

Winter injury varies widely among zoysiagrass [*Zoysia japonica* Steud. and *Z. matrella* var. *matrella* (L.) Merr.] cultivars (Patton and Reicher, 2006). The physiological basis for these differences has only partially been explored (Rogers et al., 1975, 1976, 1977; Akiyama et al., 1994; Fuller et al., 1999; Zhang et al., 2006). Physiological changes that occur during cold acclimation in plants include increased concentrations of sugars, desaturated lipids, organic acids, proline and soluble proteins (Sakai and Yoshida, 1968; Hughes and Dunn, 1990; Harwood et al., 1994). In this paper we will explore changes in soluble proteins before and after cold acclimation among zoysiagrass cultivars.

Polyacrylamide gel electrophoresis has been used in the past to characterize soluble protein changes in warm-season turfgrasses during cold acclimation (Davis and Gilbert, 1970; Palmertree et al., 1973; Gatschet et al., 1994). Soluble protein from cold acclimated centipedegrass [*Eremochloa ophiuroides* (Munro) Hack.] stolons and crowns increased in autumn compared to non-acclimated plants (Palmertree et al., 1973). Certain proteins in rhizomes of hybrid bermudagrass (*Cynodon dactylon* × C. *transvaalensis*) increased during autumn, while others decreased (Davis and Gilbert, 1970; Gatschet et al., 1994). Gatschet et al. (1994) found low molecular weight proteins (14 to 37 kDa) synthesized during cold acclimation were correlated with freeze tolerance in hybrid bermudagrass.

Extracellular voids form in ‘Meyer’ zoysiagrass rhizomes during freezing (Warmund et al., 1998), which causes dehydrative stress on cells (Steponkus, 1980). Certain proteins commonly accumulate during dehydrative stress (drought, low temperature, salinity or seed maturation) of which, dehydrins (late embryogenesis abundant [LEA] D-11 family) are the most common (Close, 1996). Dehydrins are hydrophilic, thermostable, and characterized by a 15 amino acid consensus sequence rich in lysine near the carboxy terminus with repeats occurring within the protein (Close et al., 1993; Close, 1996, 1997). Dehydrins appear to exist in all photosynthetic organisms (Close, 1997) including monocots such as the cereals barley (*Hordeum*

vulgare L.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.) (Close et al., 1993). Dehydrins are also present in the turfgrasses orchardgrass (*Dactylis glomerata* L.) (Volaire, 2002) and tall fescue (*Festuca arundinacea* Schreb.) (Jiang and Huang, 2002) when drought stressed.

Dehydrin accumulation is correlated with freeze tolerance in barley (Zhu et al., 2000), blueberry (*Vaccinius corymbosum* Linn.) (Panta et al., 2001), citrus (*Poncirus trifoliate* L. and *Citrus grandis* L.) (Cai et al., 1995), peach (*Prunus persica* [L.] Batsch) (Artlip et al., 1997), red-osier dogwood (*Cornus sericea* L.) (Karlson et al., 2004), *Rhododendron* spp. (Marian et al., 2003), and wheat (Danyluk et al., 1994). Additionally, overexpressing dehydrin genes in *Arabadopsis* resulted in non-acclimated plants containing similar or higher dehydrin levels than cold-acclimated wild-type plants and with greater freeze tolerance when compared to control plants (Puhakainen et al., 2004). These findings provided evidence that dehydrins contribute to freezing tolerance (Puhakainen et al., 2004) by stabilizing membranes (Close, 1996; Puhakainen et al., 2004), cryoprotection (Wisniewski et al., 1999), antifreeze activity (Wisniewski et al., 1999), or by scavenging free radicals (Hara et al., 2003).

Although the role of dehydrin during freezing stress has been investigated in many plant species, there is no information identifying and understanding its role in cold acclimation and freeze tolerance of turfgrasses. Additional understanding of the role of proteins in freeze tolerance of zoysiagrass will help identify physiological traits that could be exploited by breeders to increase cold hardiness of warm-season turfgrasses. The objective was to determine the relationships between protein accumulation, polypeptide composition, and freeze tolerance of zoysiagrass.

Materials and Methods

Freeze Tolerance

Detailed procedures to determine the freeze tolerance of zoysiagrass cultivars were described previously (Patton, 2006). Briefly, a cold stress simulation chamber was constructed by modifying a 0.55 m^3 chest freezer similar to the method of Beard et al. (1980). A programmable controller (Watlow 981, Watlow Electric Manufacturing Co., St. Louis, MO) and a type T Teflon tipped thermocouple (ThermoWorks, Alpine, UT) connected to the controller were used to control the temperature inside the chamber based upon soil temperatures.

Zoysiagrass plants were established in cone-tainers filled with potting-mix using a phytomere containing root, crown, and shoot material. At least one zoysiagrass cultivar characterized with low, moderate, or high winter injury were selected from each group (*Z. japonica* cultivars established vegetatively, *Z. japonica* cultivars established from seed, and *Z. matrella* cultivars established vegetatively) of commonly used zoysiagrass cultivars based on results from field trials (Patton and Reicher, 2006) (Table 5.1). After 10 wks of establishment in the greenhouse (25 ±5ºC), plants were acclimated for 4 wks using a controlled-environment chamber set at 8/2˚C day/night cycles and a 10-h photoperiod of 300 μmol m^{-2} s^{-1} photosynthetically active radiation (Anderson et al., 1993). Plants were then placed in a cold stress simulator programmed to cool 1˚C h^{-1} after 15 hours at -3˚C. Target temperatures (1˚C intervals, -6˚C to -15˚C) covered a range from complete survival to complete mortality and three cone-tainers were removed at each test temperature for each cultivar. Plants were evaluated for regrowth after freezing and the temperature resulting in no regrowth from 50% of the plants (LT_{50}) was determined by nonlinear regression (Table 5.2). This experiment was conducted six times.

Tissue Preparation

Rhizomes and stolons of zoysiagrass were harvested both before acclimation (non-acclimated) and after cold acclimation from additional sets of plants not used for freeze tolerance testing. Rhizomes and stolons were combined for protein analysis since they have comparable anatomy, freeze tolerance and solute levels in autumn and winter (Rogers et al., 1975, 1976). Rhizomes and stolons were frozen in liquid nitrogen after washing off soil and removing leaves, roots, and stems. Samples were then stored at -80ºC overnight and lyophilized (Lyph Lock, Labconco, Kansas City, MO) the following day. Lyophilized tissues were ground in liquid nitrogen using a mortar and pestle and stored at -80ºC until protein analysis.

Protein Analysis

Methods used to extract and quantify proteins are based on a modification from Li et al. (1996) and carried out at 4ºC or on ice unless stated otherwise. Buffer-soluble proteins were extracted from ~50 mg of lyophilized, ground tissue with 1 mL of 0.1 *M* sodium phosphate buffer (pH=6.8) containing 1 m*M* phenylmethyl sulfonyl fluoride (PMSF) and 10 m*M* 2-mercaptoethanol in 1.5 mL microfuge tubes. Tubes were vortexed 15 s then set in ice a series of four times, centrifuged at 16,000 × *g* for 10 min at 4ºC, and three 25 µL aliquots of supernate removed for protein quantification. The remaining supernate was retained for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Buffer-soluble protein was estimated using the Bradford procedure (Bradford, 1976). Buffer-soluble protein concentrations were determined using bovine serum albumin as a standard and absorbance was read at 595 nm in a spectrophotometer (Stasar II, Gilford Instrument Laboratories, Inc., Oberlin, OH).

Buffer-soluble protein concentrations varied from 0.15 to 0.61 µg/µL in the supernate, so proteins were concentrated with TCA prior to gel electrophoresis (Peterson, 1983). After TCA

precipitation the pellet was washed three times with 1 mL cold (-20ºC) acetone. The acetone was evaporated off and 70 µL of 2× SDS buffer (0.0625 M Tris/HCl pH 6.8, 20% glycerol (v/v), 0.004% bromphenol blue (v/v), 1.28 M 2-mercaptoethanol, and 0.08 M SDS) was added to resuspend the pellet for SDS-PAGE analysis (Laemmli, 1970). Lanes were loaded with 25 µg protein, and proteins were separated in 1.5-mm-thick gels containing 12% (w/v) acrylamide each. Coomassie Brilliant Blue R-250 was used to stain proteins overnight (Merril, 1990). After scanning images of Coomassie gels, gels were rinsed in 50% methanol (v/v) and silver-stained with a modified Wray et al. (1981) method. The SDS-PAGE analysis was conducted for three separate harvests.

Immunoblotting

Methods used for immunoblotting are based on a modification from Cunningham and Volenec (1996). 'White Icicle' radish (*Raphanus sativus* L.) was used as a positive dehydrin control (Close et al., 1993). Proteins from SDS-PAGE gels (15% w/v) were transferred overnight to a nitrocellulose membrane (Protran BA83; Schleicher & Schuell, Inc., Keene) using 60 V, 12 W, and 200 mA (Towbin et al., 1979). Membranes were blocked with tris-buffer saline plus polyethylene glycol sorbitan monolaurate (Tween 20) (TBST) for 30 min. Membranes were incubated with a 1:250 or 1:333 dilution of rabbit anti-dehydrin polyclonal antibody (PLA-100; Stressgen Bioreagents Corp., Victoria, BC Canada) in TBST for 1.5 h. After primary antibody incubation, membranes were washed in TBST for 30 min and then immersed in TBST containing the secondary goat anti-rabbit IgG antibody (dilution 1:3000) conjugated to alkaline phosphate (170-6518; Bio-Rad Laboratories, Hercules, CA) for 1.5 h. Secondary antibodies were detected using 5-bromo-4-chromo-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate solution (Blake et al., 1984). Samples were kept at room temperature throughout all electrophoresis and immunoblotting procedures. Optical density of bands were determined using

analysis software (Quantity One; Bio-Rad Laboratories, Hercules, CA). Immunoblotting was conducted for three separate harvests.

Results and Discussion

Mean buffer-soluble protein concentrations were 5.1 mg g^{-1} dry wt. for non-acclimated (NA) zoysiagrass plants and 7.3 mg g^{-1} dry wt. among cold-acclimated (CA) plants with concentrations ranging from 3.5 to 7.6 mg g^{-1} dry wt. in NA plants and 6.3 to 9.0 mg g^{-1} dry wt. in CA plants (Fig. 5.1). An increase in zoysiagrass buffer-soluble protein concentration during acclimation was expected since proteins are synthesized during cold-acclimation in hybrid bermudagrass (Davis and Gilbert, 1970), centipedegrass (Palmertree et al., 1973) and other crops (Sakai and Yoshida, 1968). Buffer-soluble protein concentrations across acclimation status were similar among *Z. japonica* (6.9 mg g^{-1} dry wt.) and *Z. matrella* (6.2 mg g^{-1} dry wt.) cultivars. Among all the cultivars, 'Zenith', 'J-36', and 'Companion' had the highest concentration of buffer-soluble proteins in both NA and CA plant tissues, while 'Palisades', 'DALZ 0101' and 'DALZ 0102' had the lowest concentration. Zenith, J-36 and Companion all have low winter injury (Patton and Reicher, 2006) and are the only cultivars in this study commercially available by seed. However, LT_{50} was not correlated ($r = 0.35$, $p = 0.25$) with buffer-soluble protein in CA tissues despite the fact that soluble protein content is known to parallel freezing tolerance across seasons (Levitt, 1980).

Since there was no relationship between buffer-soluble protein concentration and zoysiagrass LT_{50}, we explored protein composition and its relationship with freezing tolerance using SDS-PAGE and immunoblotting. The SDS-PAGE analysis revealed several prominent polypeptides using both Coomassie blue (Fig. 5.2A) and silver-staining techniques (Fig. 5.2B). However, levels of only three polypeptides changed in response to cold-acclimation. The amounts of 41 and 75 kDa polypeptides decreased during cold acclimation (Fig. 5.2B), while the amount

of an 18 and 21 kDa polypeptides increased during cold acclimation and were more prominent in silver-stained gels (Fig. 5.2B). Additionally, the amount of a 23 kDa polypeptide increased during cold acclimation and was more prominent in Coomassie gels (Fig 5.2A). The 23 kDa polypeptide was readily visible in 'Companion', J-36' and 'Zenith' all of which have low winter injury (Patton and Reicher, 2006). The relatively few changes in polypeptide composition in zoysiagrass during cold acclimation were unexpected since several changes in polypeptide composition were observed in hybrid bermudagrass or centipedegrass in response to cold acclimation (Davis and Gilbert, 1970; Palmtree et al., 1973; Gatschet et al., 1994).

The anti-dehydrin antibody based on the lysine-rich, 15 amino acid consensus sequence EFFGIMDKIKEKLPG of dehydrins detected six polypeptides (12, 14, 16, 18, 23 and 25 kDa) in CA zoysiagrass, suggesting that these are dehydrins or dehydrin-like polypeptides (Fig. 3). Immunoblotting indicated these dehydrin-like polypeptides were prevalent in CA plants, but were often lacking in NA plants (Figs. 5.2C, 5.3). Although dehydrin-like polypeptides were generally lacking in NA plants, Zorro, Companion and J-36 contained low concentrations in NA rhizomes and stolons (Fig. 5.2C). Two dehydrin-like polypeptides (23 and 25 kDa) were most prominent in CA plants (Fig. 5.3) and thus are displayed for all cultivars across treatments (Fig. 5.2C).

All five *Zoysia matrella* and some *Z. japonica* ('Victoria', DALZ0102, 'El Toro', Palisades) cultivars contain high levels of a 25 kDa dehydrin-like polypeptide, while other *Z. japonica* (Companion, J-36, Zenith, 'Meyer') do not. The difference in abundance of the 25 kDa dehydrin-like polypeptide may be due to cultivars classified as *Z. japonica* that are actually hybrids of *Z. japonica* × *Z. matrella*. Engelke and Anderson (2003) proposed that many commercially available *Z. japonica* cultivars are not a single species as classified by their morphological characteristics, but instead are interspecific hybrids. This is supported by the work of Anderson (2000) who found probable interspecific hybrids based on morphological and restriction fragment length polymorphism fingerprint characterization and by work of Yaneshita

et al. (1997) who found interspecific hybridization of *Zoysia* spp. among natural populations. Cultivars such as Victoria, El Toro and Palisades are likely crosses of *Z. japonica* with *Z. matrella* (Anderson, 2000; Engelke et al., 2002; Gibeault, 2003), but are classified as *Z. japonica* because of their predominant morphological characteristics. Therefore, the genetic makeup of Victoria, DALZ0102, El Toro and Palisades may help explain why they contain high levels of the 25 kDa dehydrin-like polypeptide, while other *Z. japonica* cultivars do not. The 25 kDa dehydrin-like polypeptide is apparently specific to *Z. matrella* or hybrids of *Z. japonica* × *Z. matrella*. This is similar to findings among the genus *Cornus*, *Vaccinium* and *Rhododendron* where dehydrin molecular masses varied depending upon species (Karlson et al., 2004; Panta et al., 2001; Marian et al., 2003).

Immunoblots indicated that the 23 kDa dehydrin-like polypeptide was present in low concentrations in some cultivars (Cavalier, Diamond, Royal, and Victoria) and in larger concentrations in other cultivars (Companion, J-36, Meyer, Zenith, and Zorro) (Figs. 5.2C, 5.3). The 23 kDa dehydrin-like polypeptide concentration mirrored the 23 kDa polypeptide visible in some cultivars when Coomassie blue stain (Fig. 5.2A). Higher optical density values in immunoblots for the 23 kDa dehydrin-like polypeptide were associated ($r^2 = 0.41$, $p = 0.018$) with lower LT_{50} values (Fig. 5.4), whereas optical density values for the 25 kDa dehydrin-like polypeptide were not correlated ($r = 0.47$, $p = 0.11$) with LT_{50} values (Fig. 5.5). These results indicate that the 23 kDa dehydrin-like polypeptide plays a role in improving freezing tolerance, whereas the 25 kDa dehydrin-like polypeptide does not.

Our results suggest that dehydrin-like polypeptides are associated with zoysiagrass cold-acclimation, and that the 23 kDa dehydrin-like polypeptide plays a role in improving freezing tolerance. This is similar to findings in *Arabadopsis*, barley, blueberry, citrus, peach, red-osier dogwood, *Rhododendron* spp. and wheat (Danyluk et al., 1994; Cai et al., 1995; Artlip et al., 1997; Zhu et al., 2000; Panta et al., 2001; Karlson et al., 2004; Marian et al., 2003; Puhakainen et

al., 2004). The contribution of dehydrins to freezing tolerance is achieved by stabilizing membranes (Close, 1996; Puhakainen et al., 2004), cryoprotection (Wisniewski et al., 1999), antifreeze activity (Wisniewski et al., 1999) and by scavenging free radicals (Hara et al., 2003). Additionally, dehydrins are known to form amphipathic α-helices (Ismail et al., 1999) which give them the ability to minimize protein denaturation (Artlip et al., 1997) and preserve enzymatic activity (Rinne et al., 1999).

Jessup et al. (2006) recently identified quantitative trait loci in *Z. matrella* for salinity tolerance. Future marker-assisted breeding could allow for improvement of zoysiagrass tolerance to abiotic stress. As suggested by Houde et al. (1992), dehydrins could be a useful tool for breeders to select freeze-tolerant phenotypes. The 23-kDa dehydrin-like polypeptide could be a potential genetic marker for cold hardiness in zoysiagrass. Additionally, identification and overexpression of transcription activators (C-repeat/dehydration-responsive element binding factor, *CBF1*) or dehydrin genes in zoysiagrass could also be used to enhance freezing tolerance as has been done with *Arabidopsis* (Jaglo-Ottosen et al., 1998; Puhakainen et al., 2004).

Cold-regulated proteins, carbohydrates, lipids, and proline are known to play important roles in the cold-hardiness of hybrid bermudagrass (Dunn and Nelson, 1974; de los Reyes, 2001; Munshaw et al., 2006), but the mechanisms involved in the cold hardiness of zoysiagrass are not as well understood. Zhang et al. (2006) recently discovered that lipid changes in zoysiagrass membranes were correlated with freeze tolerance. This research shows that a 23 kDa dehydrin-like polypeptide improves freezing tolerance of zoysiagrass. We are currently investigating the relationship of carbohydrate and proline levels with genetic variation in freeze tolerance of zoysiagrass. Additional research is needed to determine how management practices such as mowing and fertilization impact zoysiagrass freeze tolerance, and whether the 23 kDa dehydrin-like polypeptide could be used as a diagnostic indicator of the impact of cultural practices on freeze tolerance.

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Table 5.1. Zoysiagrass cultivar and experimental names, species and source of plant material used to test the relationship between freeze tolerance and proteins.

Cultivar	Experimental Name	Species	Source
Cavalier*†	DALZ8507	*Zoysia matrella* (L.) Merr.	M.C. Engelke, Texas A&M Univ.
Companion*	ZMB-2	*Zoysia japonica* Steud.	Seed Research of Oregon, Inc.
DALZ0101	DALZ 0101	*Zoysia matrella* (L.) Merr.	Turfgrass America
DALZ0102	DALZ 0102	*Zoysia japonica* Steud.	Turfgrass America
Diamond*	DALZ 8502	*Zoysia matrella* (L.) Merr.	M.C. Engelke, Texas A&M Univ.
El Toro*	UCR#1	*Zoysia japonica* Steud.	Seedland, Inc.
J-36	J-36	*Zoysia japonica* Steud.	Jacklin Seed Division J.R. Simplot
Meyer*	Z-52	*Zoysia japonica* Steud.	Natl. Turfgrass Evaluation Program
Palisades*	DALZ 8514	*Zoysia japonica* Steud.	M.C. Engelke, Texas A&M Univ.
Royal*	DALZ 9006	*Zoysia matrella* (L.) Merr.	M.C. Engelke, Texas A&M Univ.
Victoria*	Z88-14	*Zoysia japonica* Steud.	West Coast Turf
Zenith*	ZNW-1	*Zoysia japonica* Steud.	Patten Seed Co.
Zorro*	DALZ 9601	*Zoysia matrella* (L.) Merr.	Natl. Turfgrass Evaluation Program

† Cultivar names followed by an asterick (*) are commercially available in 2006.

Table 5.2. Zoysiagrass freeze tolerance (LT_{50}, temperature resulting in no regrowth from 50% of the plants) as determined by nonlinear regression. Plants were grown in a greenhouse for at least 10 wks, cold acclimated in a growth chamber at 8/2˚C day/night cycles and a 10-h photoperiod of 300 µmol m^{-2} s^{-1} for 4 wks and then placed in a cold stress simulator and subjected to temperatures ranging from -6 to -15 ºC. (from Patton, 2006).

Cultivar	Species	LT_{50}†
		-----ºC-----
Diamond	*Z. matrella*	-8.4 ± 0.3‡ a§
Royal	*Z. matrella*	-8.5 ± 0.3 a
Zorro	*Z. matrella*	-9.0 ± 0.4 ab
Victoria	*Z. japonica*	-9.3 ± 0.5 abc
DALZ0101	*Z. matrella*	-10.0 ± 0.3 bcd
DALZ0102	*Z. japonica*	-10.2 ± 0.5 cd
Cavalier	*Z. matrella*	-10.3 ± 0.4 cd
Companion	*Z. japonica*	-10.8 ± 0.7 de
J36	*Z. japonica*	-10.8 ± 0.8 de
El Toro	*Z. japonica*	-10.8 ± 0.7 de
Palisades	*Z. japonica*	-11.0 ± 0.5 de
Zenith	*Z. japonica*	-11.5 ± 0.5 e
Meyer	*Z. japonica*	-11.5 ± 0.8 e

† Freeze tolerance (LT_{50}) was calculated with the following equation: survival = $a + (b-a)/(1 + e^{c(Tm-T)})$, where a is the base line of survival, b is the maximum survival, c is a function of the slope of the line at the inflection point, Tm is the temperature at the inflection point which is also the LT_{50}, and T is the treatment temperature.

‡ Means of six values ± standard error.

§ Within columns, means followed by the same letter are not significantly different according to Fisher's protected LSD (α= 0.05).

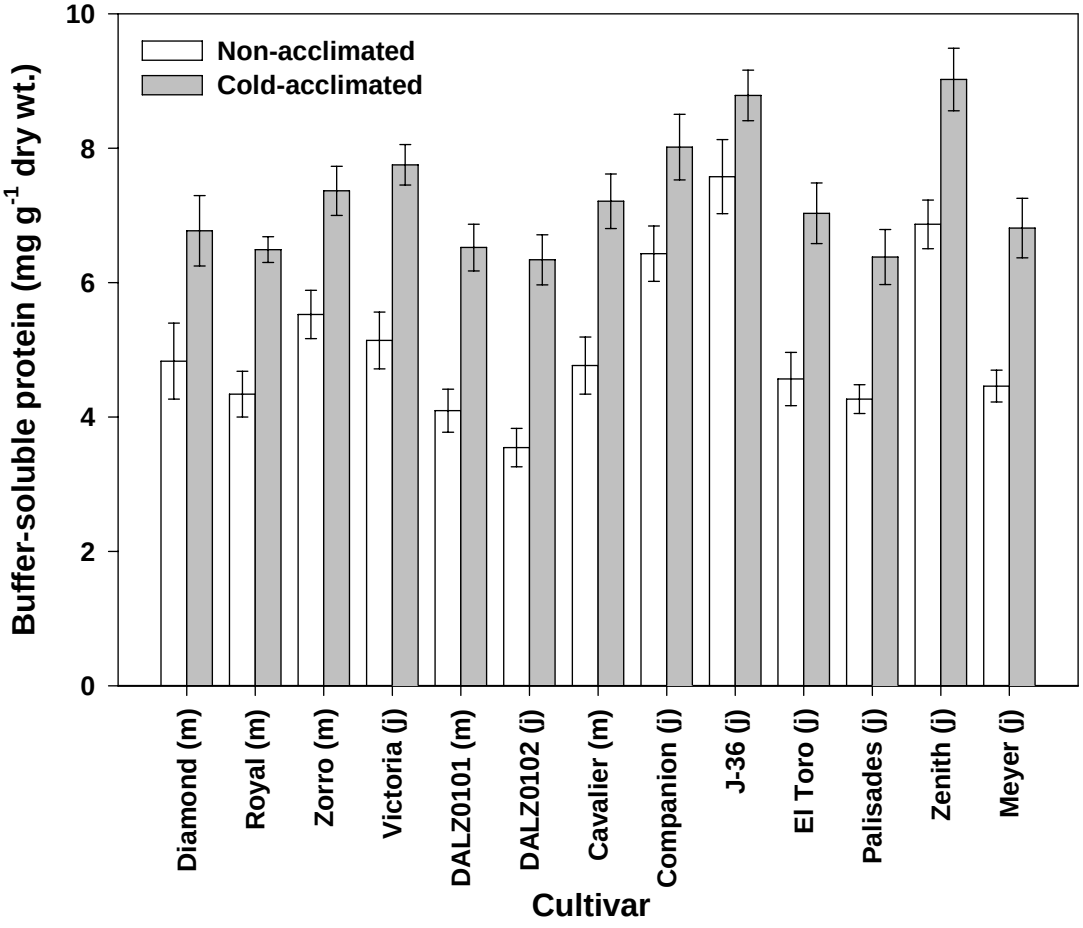

Figure 5.1. Buffer-soluble protein concentrations as influenced by cold acclimation and zoysiagrass cultivar. Error bars represent one standard error of the mean (n=6). Species is indicated by (m) or (j) for *Z. matrella* or *Z. japonica*, respectively.

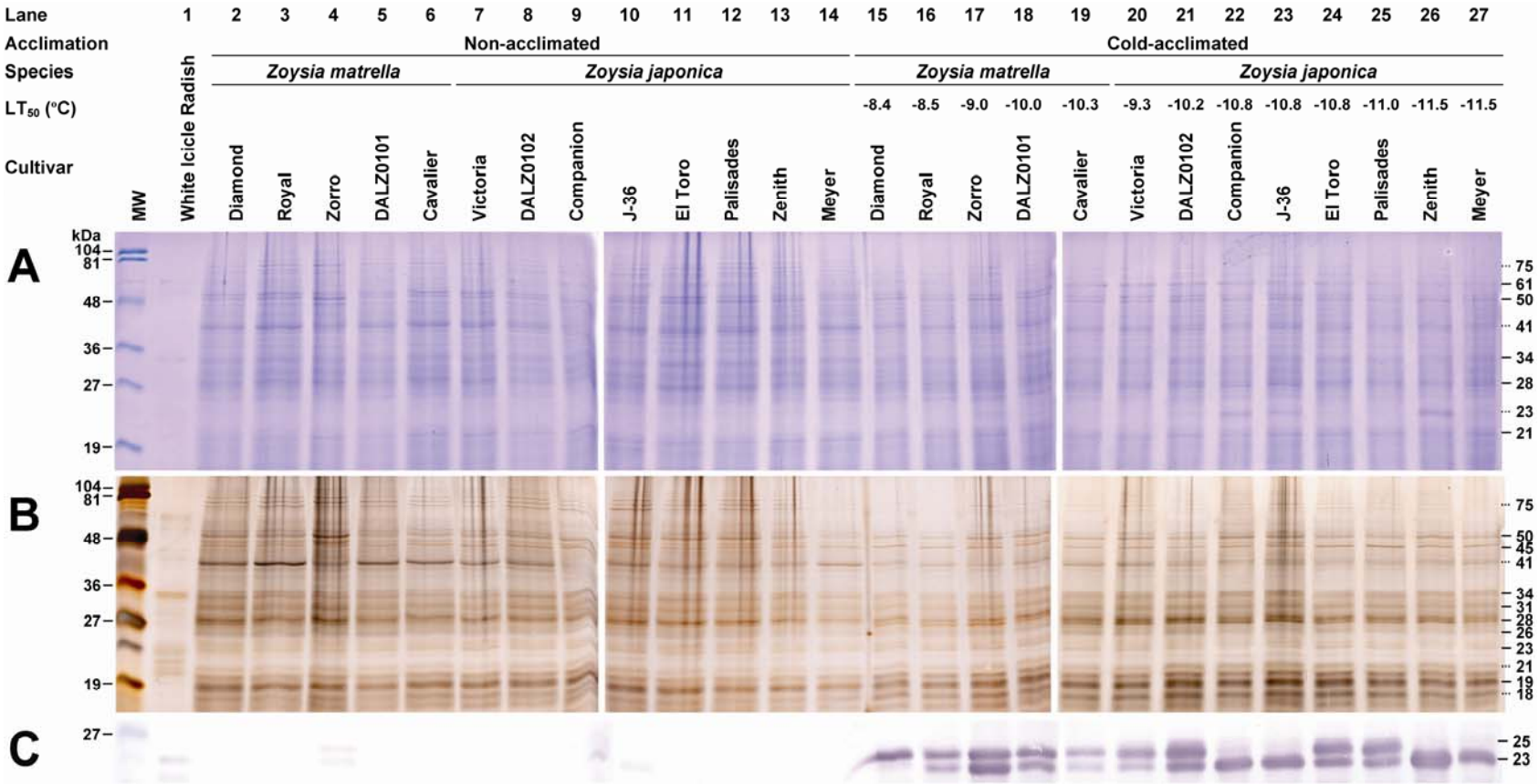

Figure 5.2. SDS-PAGE profiles and immunoblots of buffer-soluble protein from zoysiagrass cultivars with varying freeze tolerances. Gels show protein profiles from non-acclimated (Lanes 2-14) and cold acclimated (Lanes 15-27) zoysiagrass plants. Gels were loaded with equal amounts of protein (25 µg) per lane. Gels were first stained with Coomassie brilliant blue R-250 (A) and then silver-stained (B). Solid lines indicate predominant proteins present in non-acclimated and cold-acclimated plants and dashed lines indicated protein changes in response to cold acclimation with their predicted molecular masses (A and B; far right). Immunoblots show 23 and 25 kDa dehydrin-like polypeptides probed with a 1:250 dilution of rabbit anti-dehydrin polyclonal primary antibody (C). 'White icicle' radish (*Raphanus sativus*) seed was used as a positive control with 3 µg of protein loaded in Lane 1. Solid lines indicate dehydrin-like polypeptides and their predicted molecular masses (C; far right). Molecular weight (MW) markers represent proteins sized 104, 81, 48, 36, 27, and 19 kDa (A, B, and C; far left).

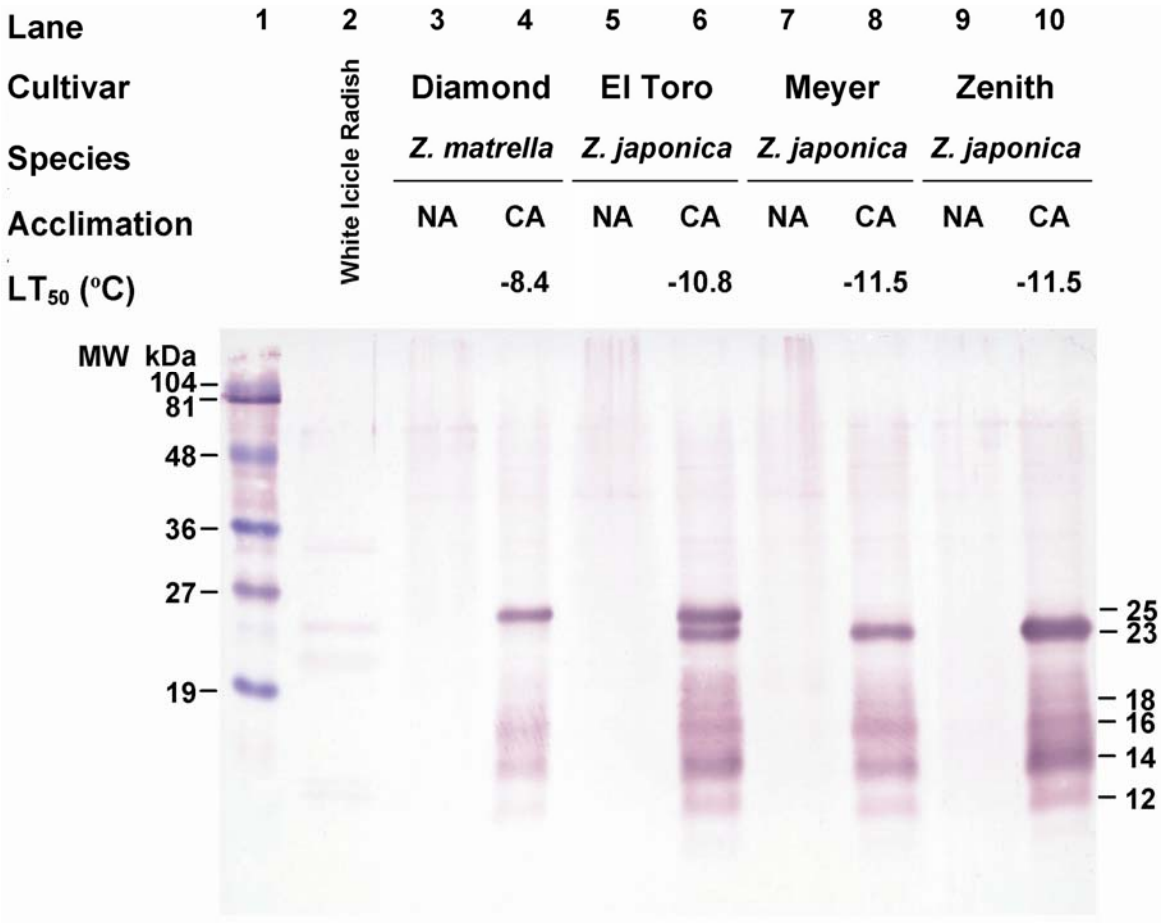

Figure 5.3. Immunoblots of buffer-soluble protein from zoysiagrass cultivars with varying freeze tolerances. Immunoblots show dehydrin-like polypeptides probed with a 1:333 dilution of anti-dehydrin polyclonal primary antibody for non-acclimated (NA)(Lanes 3, 5, 7, and 9) and cold acclimated (CA)(Lanes 4, 6, 8, and 10) zoysiagrass plants. Equal amounts of protein (25 µg) were loaded into each lane. Radish (*Raphanus sativus*) seed was used as a positive control with 10 µg of protein loaded in Lane 2. Dehydrin-like polypeptides and their appropriate size are indicated (far right). Molecular weight (MW) markers represent proteins sized 104, 81, 48, 36, 27, and 19 kDa (Lane 1).

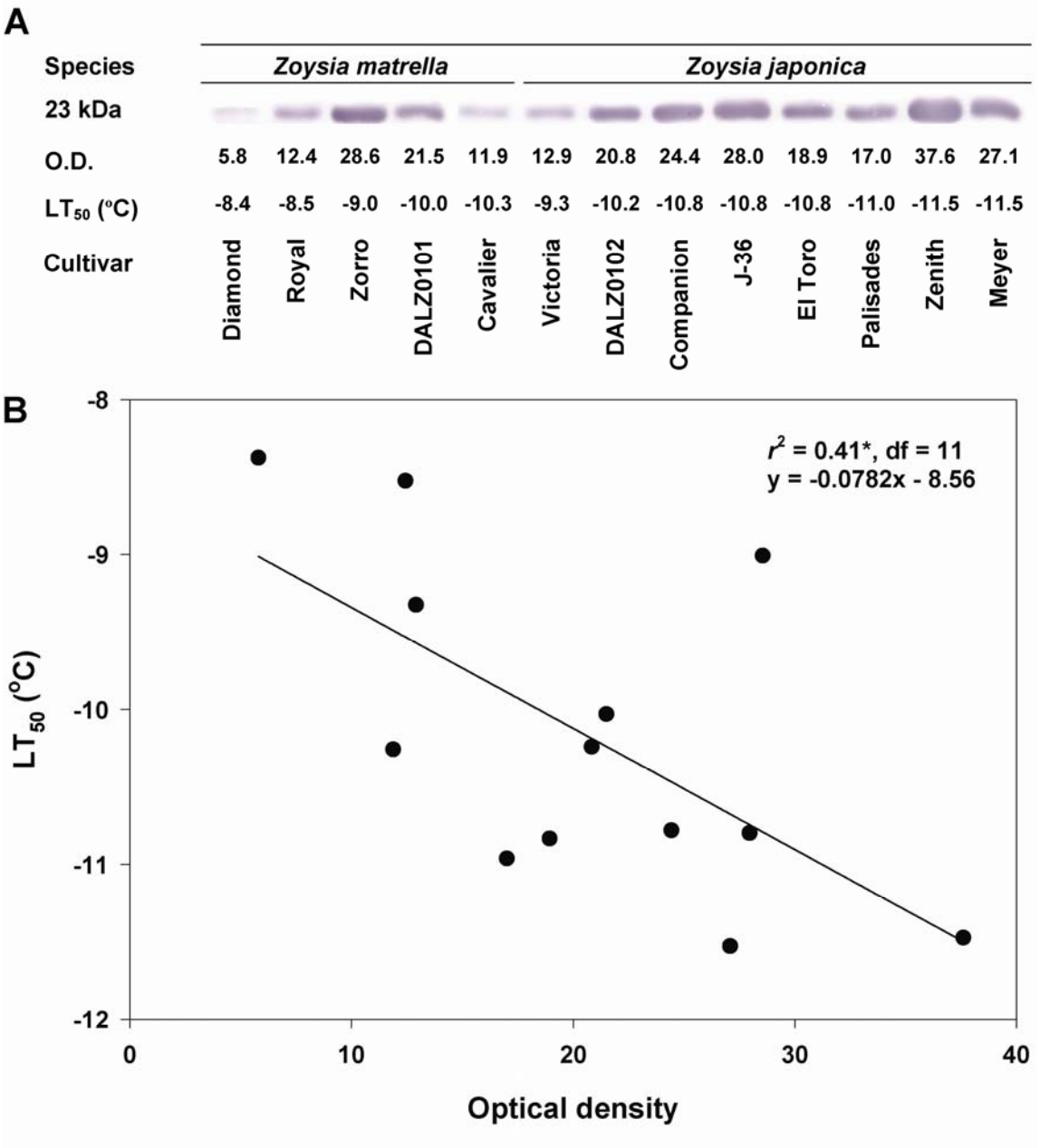

Figure 5.4. Immunoblot of a 23 kDa dehydrin-like polypeptide from zoysiagrass cultivars with varying freeze tolerances (A). Immunoblot shows a 23 kDa dehydrin probed with a 1:250 dilution of anti-dehydrin polyclonal primary antibody for cold acclimated plants. Equal amounts of protein (25 µg) were loaded in each lane. There was a relationship ($r^2 = 0.41$, $p = 0.018$) between freeze tolerance (LT_{50}) and optical density (O.D.) of the 23 kDa dehydrin-like polypeptide band (B).

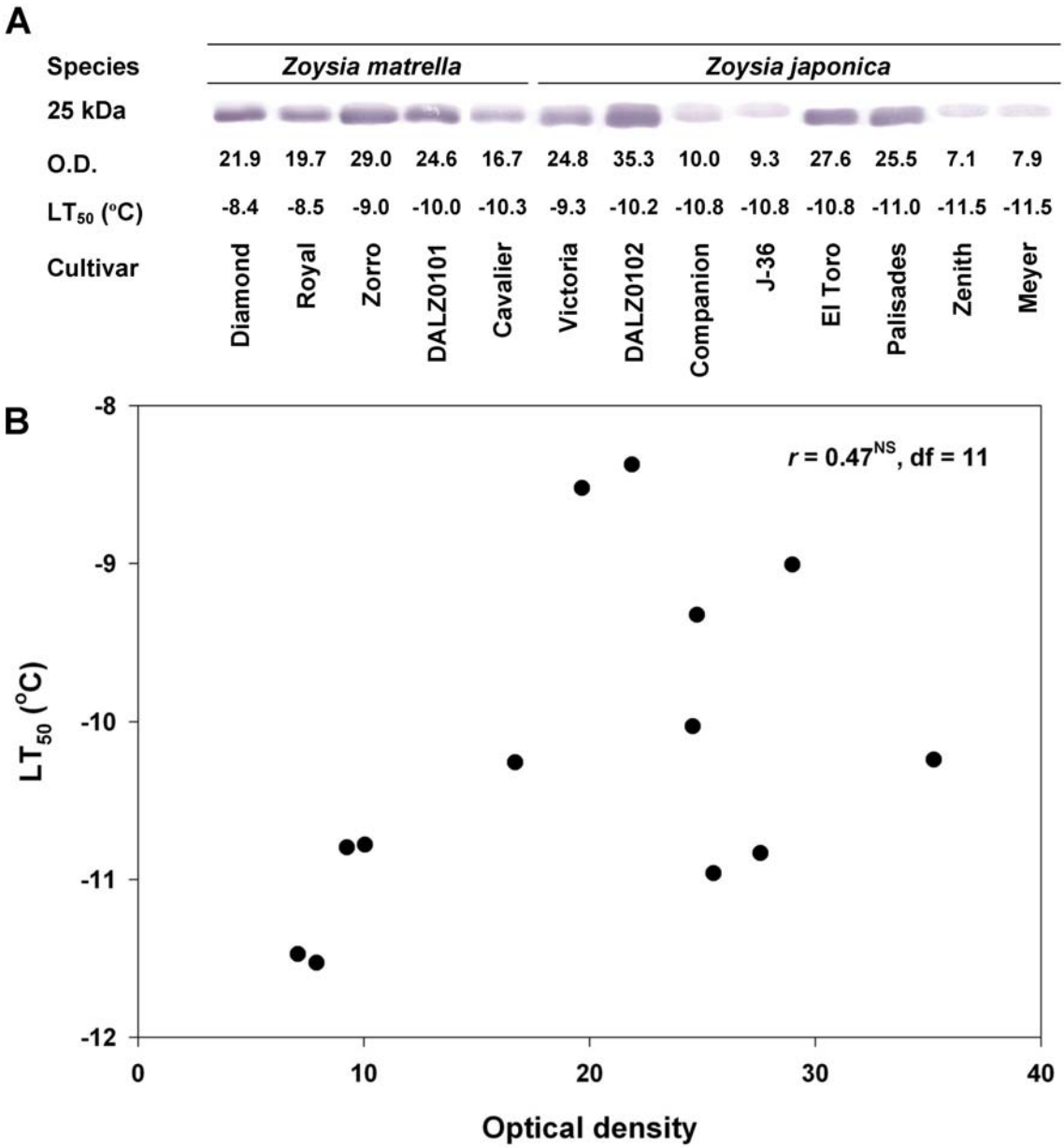

Figure 5.5. Immunoblot of a 25 kDa dehydrin-like polypeptide from zoysiagrass cultivars with varying freeze tolerances (A). Immunoblot shows a 25 kDa dehydrin probed with a 1:250 dilution of anti-dehydrin polyclonal primary antibody for cold acclimated plants. Equal amounts of protein (25 µg) were loaded in each lane. There was no correlation ($r = 0.47$, $p = 0.11$) between freeze tolerance (LT_{50}) and optical density (O.D.) of the 25 kDa dehydrin band (B).