

# Membrane Polar Lipid Changes in Zoysiagrass Rhizomes and Their Potential Role in Freezing Tolerance

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**ABSTRACT.** Cell membranes play an integral role in freezing tolerance. The objectives of this study were to quantify polar lipids in cold-tolerant ‘Meyer’ zoysiagrass (*Zoysia japonica*) and cold-sensitive ‘Cavalier’ zoysiagrass (*Zoysia matrella*) and to evaluate their potential role in freezing tolerance. Grasses were acclimated outside and sampled once monthly between October and January to determine freezing tolerance and lipid composition in rhizomes. Lowest LT<sub>50</sub>s (temperature resulting in 50% survival) were observed in November for ‘Cavalier’ (–8.5 and –9.6 °C in 2005 and 2006, respectively) and December for ‘Meyer’ (–16.2 and –15.4 °C in 2005 and 2006, respectively). The most abundant lipids in zoysiagrass rhizomes were monogalactosyl diacylglycerol, digalactosyl diacylglycerol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid, which comprised 90% of the polar lipids. Differences in lipid contents and double bond indices (DBI) were detected between ‘Meyer’ and ‘Cavalier’ during cold acclimation, but there were no consistent relationships between lipid classes or DBI and freezing tolerance in zoysiagrass.

Zoysiagrass (*Zoysia* spp.) is a common turfgrass used for golf course fairways and tees in southern and transition zone climates because of its good heat and drought tolerance and low requirement for maintenance (Fry et al., 2008). ‘Meyer’ has been the predominant commercial cultivar used in the transition zone since its release in 1952 because of its relatively fine leaf texture compared with common *Z. japonica*, and its excellent freezing tolerance (Grau, 1952; Grau and Radko, 1951). Several high-quality cultivars, including ‘Cavalier’ (Engelke et al., 2002a), ‘Diamond’ (*Z. matrella*) (Engelke et al., 2002b), and ‘Palisades’ (*Z. japonica*) (Engelke et al., 2002c), have been developed and released by researchers at Texas A&M University; however, poor freezing tolerance has limited their use in the transition zone (Morris, 1996; Patton and Reicher, 2007).

Physiological contributors to freezing tolerance in *Zoysia* spp. have been evaluated, but the results are inconclusive. For example, Rogers et al. (1975) reported accumulation of total nonstructural carbohydrate and starch during cold acclimation in ‘Meyer’ in a field study. In contrast, Patton et al. (2007a) reported decreased starch concentrations in zoysiagrass acclimated under controlled conditions. Fuller et al. (1999) concluded that starch and sucrose were not reliable indicators of zoysiagrass cold tolerance, whereas Patton et al. (2007a) observed a negative correlation between starch content and freezing tolerance. Recently, research showed that proline, proteins, and abscisic acid (ABA) are associated with *Zoysia* freezing tolerance (Patton et al., 2007a, 2007b; Zhang et al.,

2009). More information is needed regarding physiological contributors to freezing tolerance in *Zoysia* spp. to assist in selection and development of high-quality, cold-tolerant cultivars.

Membranes are the primary sites of freezing injury in plants (Steponkus and Wiest, 1978). During freeze-induced dehydration, plasma membranes and chloroplast membranes may form a hexagonal II phase (H<sub>II</sub>), which compromises membrane integrity and cell function (Cullis and De Kruijff, 1979; Uemura and Steponkus, 1997, 1999). Thus, during cold acclimation, changes in lipid composition take place to preserve the membrane bilayer structure, fluidity, and function (Alberdi and Corcuera, 1991). Research has shown that polar lipids change when plants are exposed to stress such as drought, oxidative assaults, or extreme temperatures (Chen et al., 2006; Li et al., 2004; Selstam and Öouist, 1990; Styer et al., 1996; Toivonen et al., 1992; Wang, 2005). Higher concentrations of polar lipids were observed in woody evergreen leaves (*Nothofagus dombeyi*) from plants grown at high altitudes (LT<sub>50</sub> = –10.2 °C) compared with those grown at lower altitudes (LT<sub>50</sub> = –5.0 °C) (Alberdi et al., 1990). Furthermore, dominant lipids in *N. dombeyi*, digalactosyl diacylglycerol (DGDG), monogalactosyl diacylglycerol (MGDG), and phosphatidylglycerol (PG), increased 200% to 400% during cold acclimation. Welts et al. (2002) observed higher phosphatidylcholine (PC) and lower phosphatidic acid (PA) contents in *Arabidopsis thaliana* with enhanced freezing tolerance after cold acclimation at 4 °C for 3 d or exposure to –8 °C. In other studies, no relationship has been reported between membrane changes and freezing tolerance (Senser and Beck, 1984; Uemura and Steponkus, 1994).

Fatty acid unsaturation levels are associated with cold hardiness in plants. Unsaturated fatty acid content was higher

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in bermudagrass (*Cynodon* spp.) and seashore paspalum (*Paspalum vaginatum*) cultivars that were more cold tolerant (Cyril et al., 2001, 2002; Samala et al., 1998). Changes in membrane composition and their association with freezing tolerance in turfgrass, and specifically zoysiagrass, have not been measured. Our objective was to compare differences in rhizome polar lipid classes in less hardy *Z. matrella* 'Cavalier' and more hardy *Z. japonica* 'Meyer', and to determine their potential role in freezing tolerance.

## Materials and Methods

**FREEZING TOLERANCE.** Detailed procedures on 'Meyer' and 'Cavalier' zoysiagrass propagation and maintenance to determine freezing tolerance were published by Zhang et al. (2009). Briefly, 96 containers of 'Meyer' and 'Cavalier' were propagated in cone-containers (8 cm diameter  $\times$  20 cm deep) in a root zone mix and were kept in a greenhouse with average 30/20 °C day and night temperature for 4 months before they were transferred outside to a sand-filled tank on 15 Sept. 2005 and 17 Aug. 2006 at the Rocky Ford Turfgrass Research Center, Manhattan, KS. During that period, grasses were mowed once weekly at 2 cm, watered every 2 d, and fertilized with soluble fertilizer at 10 kg·ha<sup>-1</sup> N once every other week. Soil-encapsulated thermocouple (SET) sensors were assembled following the method of Ham and Senock (1992) to monitor soil surface temperature across the tank. Sensors were placed in contact with two randomly selected crowns near the center and at the perimeter of the tank, and were connected to a data logger (CR-10; Campbell Scientific, Logan, UT). The sensors were used to record daily soil temperature at 1-h intervals and data were downloaded from the data logger once monthly during the study period. The data logger malfunctioned in early autumn each year, and soil surface temperature was reported only from 29 Oct. to 11 Jan. for both study periods. Differences in soil surface temperature between the center and perimeter of the tank were <2 °C over this time period; an average from both sensors is reported (Figs. 1 and 2). Air temperature was also recorded hourly using a weather station located 3 m north of the sand-filled tank from 2 Sept. to 11 Jan. in 2005–06 and 2006–07 (Figs. 1 and 2).

Containers were arranged in the sand-filled tank as a completely randomized design and were sampled once monthly from October to January in 2005 and 2006 to determine freezing tolerance following the procedure of Anderson et al. (1993). Four replicates were randomly sampled and run through the freezing chamber. Replicates one and two were sampled from the field and were subjected to freezing on the dates which follow, and replicates three and four were removed from the field and subjected to freezing 2 d later. Sampling dates were 4 Oct., 5 Nov., 2 Dec., and 4 Jan. in 2005–06; and 2 Oct., 7 Nov., 1 Dec., and 9 Jan. in 2006–07. Differences in average air temperature between the two sampling times (i.e., replicates 1 and 2 sampled 2 d ahead of replicates 3 and 4) were 9.5 and 10.7 °C in October, 5.8 and 7.7 °C in November, 3.2 and 1.2 °C in December, and 5.1 and 6.7 °C in January in 2005 and 2006, respectively (Figs. 1 and 2). Differences in average soil temperature between the two sampling times in 2005 and 2006 were 4.6 and 8.0 °C in November, 2.2 and 0.8 °C in December, and 5.0 and 3.4 °C in January, respectively.

At the sampling of each replicate, one container of each cultivar served as a control and was not exposed to freezing.

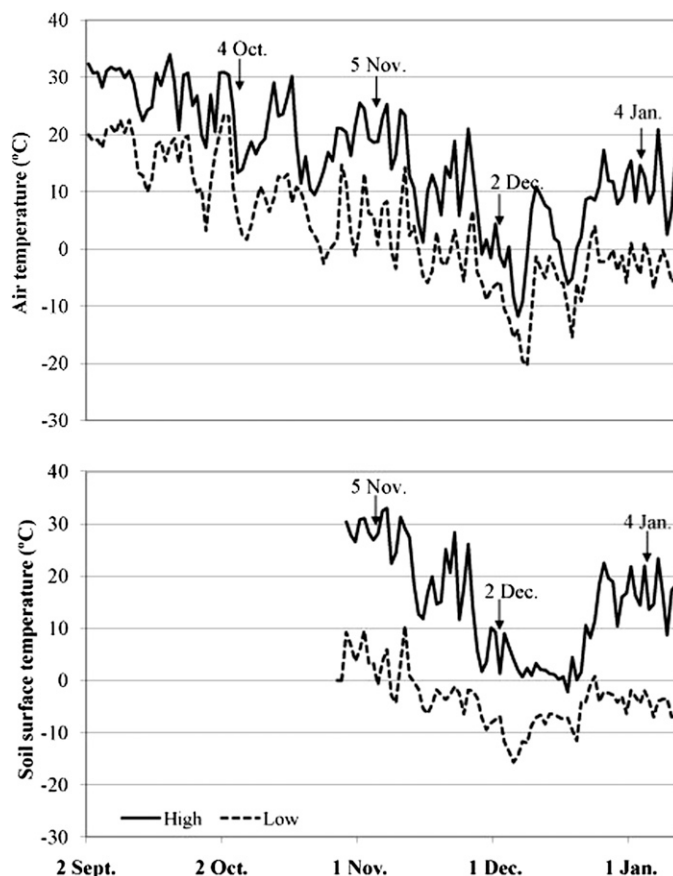


Fig. 1. Air (2 Sept. 2005–11 Jan. 2006) and soil surface temperatures (29 Oct. 2005–11 Jan. 2006). Arrows indicate the dates on which zoysiagrass was sampled for freezing tolerance and lipid analysis. Soil surface temperatures were missing from 2 Sept. to 28 Oct. 2005 due to data logger malfunction.

The other four containers of each cultivar were placed in a freezing chamber (Tenney Benchmaster; Tenney Engineering, Union, NJ). Two SET sensors were placed in two random selected containers at 2 cm below the soil surface to monitor the temperature during the process. Containers holding 'Cavalier' were removed once the soil temperature reached –3, –6, –9, and –12 °C, while those with 'Meyer' were removed at –5, –9, –13, and –17 °C, as previous research showed that 'Meyer' is more cold tolerant than 'Cavalier' (Dunn et al., 1999; Patton and Reicher, 2007). After a 6-week recovery period, a nonlinear regression of freezing temperature versus percentage of green tissue in a container was generated with Excel (Excel 2003; Microsoft, Seattle, WA) from which the LT<sub>50</sub> was calculated.

**LIPID ANALYSIS.** Rhizomes were selected for lipid profiling as their survival is critical during exposure to freezing temperatures (Rogers et al., 1975). Four containers (replicates) of each cultivar were randomly sampled once monthly to profile lipids in rhizomes; each replicate was sampled at the same time as the unfrozen controls in the freezing tolerance evaluation. The root zone mix was washed from rhizomes with water that had been cooled at 4 °C overnight, and roots and leaves were removed from rhizomes. Rhizomes were then immersed in liquid N<sub>2</sub> immediately and stored in a –80 °C freezer until extraction. Lipids were extracted from rhizomes following the method described by Welti et al. (2002) with minor modifications, and a polar lipid profile was generated with electrospray ionization

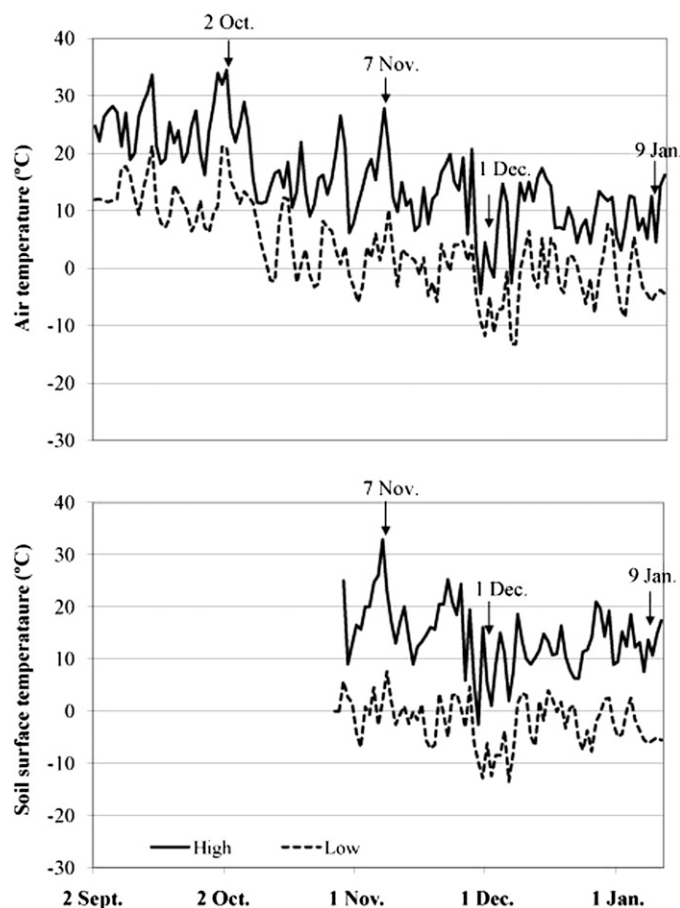


Fig. 2. Air (2 Sept. 2006–11 Jan. 2007) and soil surface temperatures (29 Oct. 2006–11 Jan. 2007). Arrows indicate the dates on which zoysiagrass was sampled for freezing tolerance and lipid analysis. Soil surface temperatures were missing from 2 Sept. to 28 Oct. 2006 due to data logger malfunction.

tandem mass spectrometry (ESI-ES/MS) at the Kansas Lipidomics Research Center, Manhattan, KS. Briefly, the frozen rhizomes were heated for 15 min at 75 °C in a preheated test tube with 3 mL of isopropanol with 0.01% butylated hydroxytoluene. Tissue was then ground in a mortar with 1.5 mL of chloroform and was transferred back to the tube followed by adding 0.6 mL of distilled water. The tube was shaken for 1 h before the lipid layer (upper layer) was transferred to a new tube. Tissue was re-extracted five times with 4 mL of chloroform/methanol (2:1) with 0.01% butylated hydroxytoluene with 30 min agitation each time. All the extracts were combined and washed with 1 mL of 1 M KCl and 2 mL of distilled H<sub>2</sub>O after the final extract. The remaining tissue was dried (105 °C overnight) and weighed. The final extract was dissolved in 1 mL of chloroform after evaporated under nitrogen. The final extract was divided into two aliquots, one for phospholipid analysis and the other for galactolipid analysis.

Phospholipid and galactolipid standards were purchased from Avanti Polar Lipids (Alabaster, AL) and Matreya (State College, PA), respectively, except di24:1-PE and di24:1-PG, which were prepared by transphosphatidylation of di24:1-PC. The samples were combined with standards and were then injected with an auto-sampler (LC Mini PAL; CTC Analytics, Zwingen, Switzerland) to a “triple” quadrupole tandem mass spectrometer (Micromass Ultima; Micromass, Manchester,

UK) equipped for electrospray ionization. Temperatures of the source and the desolvation were 100 and 250 °C, respectively. Argon ( $1.7 \text{ e}^{-3}$ ) was the collision gas, and the voltage for the electrospray capillary and the cone energy were  $\pm 2.8 \text{ kV}$  and 40 V, respectively. The lipids were quantified by correction curves generated by the internal standards.

**DOUBLE BOND INDICES.** Lipid species within a head group (class) were separated by the number of carbon atoms and double bonds. Double bond indices were calculated individually for each class having unique functions. The double bond indices (DBI) of each lipid class was calculated from a modified equation as  $\text{DBI} = \text{sum of } [(\text{each lipid species in a class} \times N) / \text{the class}]$ , where N is the number of double bonds of the species (Cyril et al., 2002).

**DATA ANALYSIS.** Homogeneity of variance was tested using the PROC GLM procedure with the HOVTEST option (SAS, 2004), and showed no significant differences ( $P \leq 0.05$ ) between the two sampling times (i.e., replications 1 and 2 sampled 2 d ahead of replications 3 and 4) in LT<sub>50</sub>, lipid content, or DBI (data not shown). Furthermore, PROC ANOVA (SAS, 2004) showed no effect of sampling time on recovery from exposure to freezing and lipid extract. Therefore, data were analyzed with PROC GLM as four replicates, and means were separated by Fisher’s protected least significant difference (LSD) at  $P \leq 0.05$  when appropriate. PROC CORR (SAS, 2004) was used to evaluate the relationships between polar lipids and LT<sub>50</sub>s in rhizomes. There were interactions between year and freezing tolerance and lipid content; therefore, data were presented in each year separately.

## Results

**FREEZING TOLERANCE.** Detailed presentation of acclimation and freezing tolerance in ‘Meyer’ and ‘Cavalier’ zoysiagrass was published by Zhang et al. (2009). Briefly, ‘Meyer’ had lower LT<sub>50</sub>s than ‘Cavalier’ on all sampling dates (Fig. 3). ‘Cavalier’ reached its maximum freezing tolerance (lowest LT<sub>50</sub>s) in November, while the lowest LT<sub>50</sub> in ‘Meyer’ was observed in December. No LT<sub>50</sub>s were calculated in ‘Cavalier’ in Dec. 2005 and in ‘Meyer’ in Jan. 2006 due to the severe freezing injury (Fig. 3). Higher freezing injury in both cultivars in 2005 than in 2006 was likely due to colder air and soil temperatures in Dec. 2005 compared with Dec. 2006 (Figs. 1 and 2).

**POLAR LIPIDS IN ZOYSIA RHIZOMES.** Lipid profiles were analyzed on the sampling dates when LT<sub>50</sub>s were available in both cultivars. Eleven lipid groups were identified in zoysiagrass rhizomes: DGDG, MGDG, PC, phosphatidylethanolamine (PE), PA, PG, phosphatidylinositol (PI), phosphatidylserine (PS), and three Lyso groups (Lyso-PG, -PC, and -PE). The most abundant lipid groups in zoysiagrass rhizomes were DGDG, MGDG, PC, PE, and PA, which comprised 90% of the polar lipids, and the Lyso-groups were found in trace amounts (Tables 1 and 2).

No differences were observed in DGDG and MGDG between ‘Meyer’ and ‘Cavalier’, except 5 Nov. 2005, where the DGDG and MGDG contents were 143% and 194% higher in ‘Meyer’ than in ‘Cavalier’, respectively (Tables 1 and 2). However, the DGDG to MGDG ratio was not significantly different between the cultivars on the same sampling date (Table 1). On 5 Nov. 2005 and 1 Dec. 2006, ‘Cavalier’ had less unsaturated fatty acids in the galactolipids, DGDG and MGDG, than ‘Meyer’ (Tables 3 and 4).



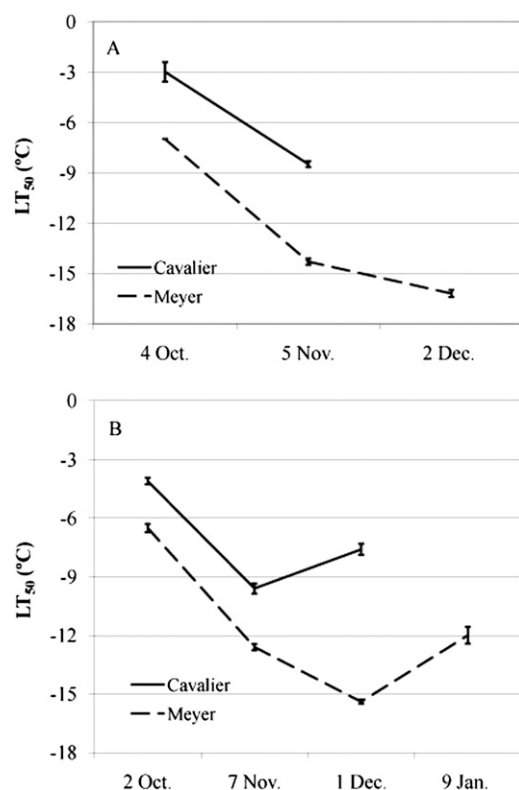


Fig. 3. Freezing tolerance [temperature resulting in 50% survival (LT<sub>50</sub>)] of 'Cavalier' and 'Meyer' zoysiagrass in 2005 (A) and 2006 (B) (Zhang et al., 2009). Freezing tolerance was not available for 'Cavalier' on 2 Dec. 2005 and 9 Jan. 2007 due to severe freezing injury.

Although 'Cavalier' had lower PC and PE contents than 'Meyer' on 5 Nov., 2005, PC and PE were counted for 31% and 19% of total polar lipids in 'Cavalier', respectively, 5% and 3% higher than those in 'Meyer' (Table 1). Phosphatidic acid content in 'Meyer' was 2.95 times as high as that in 'Cavalier' on 5 Nov. 2005, but counted for similar proportion of total polar

lipids (Table 1). The PC to (PE + PA) ratio was higher in 'Cavalier' than in 'Meyer' on the same sampling date (Table 1). 'Cavalier' also had lower PG, PI, and PS levels on 5 Nov. 2005. Reversed relationships were observed on 2 Oct. and 7 Nov. 2006 that 'Meyer' had lower PC, PA, PI and PS levels than 'Cavalier' (Table 2). Higher PA contents in 'Cavalier' (32% of total polar lipids) resulted in lower PC to (PE + PA) ratio than 'Meyer' on 2 Oct. 2006 (Table 2). In contrast, the PC to (PE + PA) ratio was not significantly different between 'Cavalier' and 'Meyer' on 7 Nov. due to higher levels of PC and PA in 'Cavalier' (Table 2). Differences in DBI in phospholipids was observed occasionally between the two cultivars, however, the results were not consistent (Tables 3 and 4). The Lyso-groups were detected in zoysiagrass rhizomes, but the levels were low (<1%). No differences were observed in 'Cavalier' and 'Meyer' in the Lyso-groups, except 5 Nov. 2005 and 2 Oct. 2006 (Tables 1 and 2).

**CORRELATIONS BETWEEN POLAR-LIPIDS AND FREEZING TOLERANCE.** An association between changes in lipid composition, fatty acid unsaturation, and LT<sub>50</sub>s was observed occasionally. For example, LT<sub>50</sub>s were associated with PC ( $r = -0.76$ ,  $P < 0.001$ ), PE ( $r = -0.76$ ;  $P < 0.001$ ), and PA ( $r = -0.84$ ,  $P < 0.001$ ) in 2005. However, in 2006, the correlation exhibited the opposite trend compared with 2005: PC ( $r = 0.20$ ,  $P = 0.40$ ), PE ( $r = 0.17$ ,  $P = 0.47$ ), and PA ( $r = 0.65$ ,  $P = 0.002$ ). No consistent trends were found in the relationships between freezing tolerance and variations in polar lipids and DBI in this study.

## Discussion

The galactolipids, DGDG and MGDG, comprised 20% to 40% of the polar lipids in zoysiagrass rhizomes. Galactolipids are unique to chloroplast membranes; therefore, detection of DGDG and MGDG in zoysiagrass rhizomes indicates that the rhizomes contained plastids or chloroplasts. It is common for stolons to contain chloroplasts, and in zoysiagrass, stolons often penetrate the soil surface, in effect becoming rhizomes, which likely explains the presence of these lipids in rhizome samples. Higher DGDG levels were observed in frost-hardened than

Table 1. Polar lipid content [mean  $\pm$  SE (nmol-mg<sup>-1</sup>)] in 'Cavalier' and 'Meyer' zoysiagrass rhizomes on 4 Oct. and 5 Nov. 2005.

Lipid class <sup>z</sup>	4 Oct.		5 Nov.	
	Cavalier	Meyer	Cavalier	Meyer
DGDG	0.40 $\pm$ 0.05 a <sup>y</sup>	0.40 $\pm$ 0.07 a	0.71 $\pm$ 0.11 b	1.73 $\pm$ 0.28 a
MGDG	0.26 $\pm$ 0.03 a	0.22 $\pm$ 0.03 a	0.36 $\pm$ 0.06 b	1.06 $\pm$ 0.22 a
PC	0.61 $\pm$ 0.11 a	0.51 $\pm$ 0.04 a	0.97 $\pm$ 0.18 b	1.65 $\pm$ 0.11 a
PE	0.43 $\pm$ 0.08 a	0.39 $\pm$ 0.03 a	0.61 $\pm$ 0.11 b	1.05 $\pm$ 0.05 a
PA	0.06 $\pm$ 0.02 a	0.04 $\pm$ 0.01 a	0.22 $\pm$ 0.03 b	0.65 $\pm$ 0.06 a
PG	0.07 $\pm$ 0.03 a	0.07 $\pm$ 0.01 a	0.03 $\pm$ 0.01 b	0.08 $\pm$ 0.00 a
PI	0.04 $\pm$ 0.00 a	0.03 $\pm$ 0.00 a	0.17 $\pm$ 0.03 b	0.37 $\pm$ 0.02 a
PS	0.01 $\pm$ 0.00 a	0.01 $\pm$ 0.00 a	0.05 $\pm$ 0.01 b	0.09 $\pm$ 0.01 a
Lyso-PG	0.00 $\pm$ 0.00 a	0.00 $\pm$ 0.00 a	0.00 $\pm$ 0.00 a	0.00 $\pm$ 0.00 a
Lyso-PC	0.01 $\pm$ 0.00 a	0.02 $\pm$ 0.00 a	0.01 $\pm$ 0.00 a	0.03 $\pm$ 0.00 a
Lyso-PE	0.01 $\pm$ 0.00 a	0.01 $\pm$ 0.00 a	0.01 $\pm$ 0.00 b	0.02 $\pm$ 0.00 a
DGDG/MGDG	1.52 a	1.74 a	1.96 a	1.68 a
PC/(PE + PA)	1.24 a	1.17 a	1.15 a	0.94 b

<sup>z</sup>DGDG = digalactosyl diacylglycerol, MGDG = monogalactosyl diacylglycerol, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PA = phosphatidic acid, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine.

<sup>y</sup>Mean  $\pm$  SE followed by the same letter in a lipid class on a sampling date are not significantly different at  $P \leq 0.05$  level. No lipid analysis was conducted on 2 Dec. 2005 and 4 Jan. 2006 due to severe freezing injury in either of the cultivars.

Table 2. Polar lipid content [mean  $\pm$  SE (nmol-mg<sup>-1</sup>)] in 'Cavalier' and 'Meyer' zoysiagrass from 2 Oct. to 1 Dec. 2006.

Lipid class <sup>z</sup>	2 Oct.		7 Nov.		1 Dec.	
	Cavalier	Meyer	Cavalier	Meyer	Cavalier	Meyer
DGDG	1.32 $\pm$ 0.03 a <sup>y</sup>	1.25 $\pm$ 0.09 a	0.93 $\pm$ 0.03 a	0.86 $\pm$ 0.07 a	0.53 $\pm$ 0.05 a	0.69 $\pm$ 0.02 a
MGDG	0.57 $\pm$ 0.04 a	0.48 $\pm$ 0.04 a	0.39 $\pm$ 0.03 a	0.30 $\pm$ 0.02 a	0.22 $\pm$ 0.04 a	0.31 $\pm$ 0.02 a
PC	1.26 $\pm$ 0.22 a	1.16 $\pm$ 0.09 a	1.24 $\pm$ 0.12 a	0.79 $\pm$ 0.05 b	0.86 $\pm$ 0.12 a	1.06 $\pm$ 0.16 a
PE	1.15 $\pm$ 0.13 a	0.91 $\pm$ 0.08 a	0.96 $\pm$ 0.06 a	0.77 $\pm$ 0.07 a	0.72 $\pm$ 0.09 a	0.93 $\pm$ 0.09 a
PA	2.38 $\pm$ 0.19 a	0.82 $\pm$ 0.10 b	0.74 $\pm$ 0.05 a	0.39 $\pm$ 0.06 b	0.69 $\pm$ 0.07 a	0.77 $\pm$ 0.05 a
PG	0.17 $\pm$ 0.04 a	0.10 $\pm$ 0.02 a	0.05 $\pm$ 0.01 a	0.04 $\pm$ 0.01 a	0.02 $\pm$ 0.00 b	0.04 $\pm$ 0.01 a
PI	0.39 $\pm$ 0.02 a	0.27 $\pm$ 0.03 b	0.29 $\pm$ 0.03 a	0.19 $\pm$ 0.01 b	0.18 $\pm$ 0.02 a	0.21 $\pm$ 0.02 a
PS	0.12 $\pm$ 0.01 a	0.09 $\pm$ 0.01 b	0.08 $\pm$ 0.02 a	0.06 $\pm$ 0.00 a	0.03 $\pm$ 0.01 a	0.02 $\pm$ 0.01 a
Lyso-PG	0.00 $\pm$ 0.00 a	0.00 $\pm$ 0.00 a	0.00 $\pm$ 0.00 a	0.00 $\pm$ 0.00 a	0.00 $\pm$ 0.00 a	0.00 $\pm$ 0.00 a
Lyso-PC	0.06 $\pm$ 0.00 a	0.02 $\pm$ 0.00 b	0.02 $\pm$ 0.01 a	0.02 $\pm$ 0.00 a	0.02 $\pm$ 0.00 a	0.01 $\pm$ 0.00 a
Lyso-PE	0.03 $\pm$ 0.00 a	0.02 $\pm$ 0.00 b	0.02 $\pm$ 0.00 a	0.01 $\pm$ 0.00 a	0.01 $\pm$ 0.00 a	0.01 $\pm$ 0.00 a
DGDG/MGDG	2.36 a	2.66 a	2.44 a	2.86 a	2.48 a	2.22 a
PC/(PE + PA)	0.35 b	0.67 a	0.73 a	0.69 a	0.61 a	0.62 a

<sup>z</sup>DGDG = digalactosyl diacylglycerol, MGDG = monogalactosyl diacylglycerol, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PA = phosphatidic acid = PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine.

<sup>y</sup>Mean  $\pm$  SE followed by the same letter in a lipid group on a sampling date are not significantly different at  $P \leq 0.05$ . No lipid analysis was conducted on 9 Jan. 2007 due to severe freezing injury in 'Cavalier'.

nonhardened pine seedlings (*Pinus silvestris*) (Selstam and Öouist, 1990). Few differences in galactolipids contents were observed between 'Cavalier' and 'Meyer' in our study, suggesting that DGDG and MGDG are merely active during cold acclimation.

Phosphatidylcholine is known to stabilize the membrane bilayer, whereas, PE and PA have been associated with membrane injury resulting from the H<sub>II</sub> phase (Cullis and De Kruijff, 1979; Verleij et al., 1982; Welti et al., 2002). The PC content in *A. thaliana* leaves was highest when freezing tolerance was best (Welti et al., 2002). In our study, the PC content was 70% higher in 'Meyer' than in 'Cavalier' on 5 Nov. 2005 (Table 1). However, higher PE and PA contents were also detected in 'Meyer' on the same sampling date (Table 1), resulting in a lower PC to (PE + PA) ratio in 'Meyer'. A higher

PA content was reported in a wild-type *A. thaliana* with poorer freezing tolerance compared with a freezing-tolerant mutant (Welti et al., 2002). No consistent results were observed between cold hardiness and PA level in this study. For example, 'Meyer' had lower LT<sub>50s</sub> but higher PA contents than 'Cavalier' on 5 Nov. 2005 (Fig. 3, Table 1). In contrast, the PA levels were 190% and 90% higher in 'Cavalier' than in 'Meyer' on 2 Oct. and 7 Nov. 2006, respectively, with lower LT<sub>50s</sub> observed in 'Meyer' on these sampling dates (Fig. 3, Table 2). Variations were found in other lipid groups between the cultivars, yet the functionality of PG, PI, PS, and Lyso groups under stresses is not clear.

Freezing tolerance in grasses is associated with unsaturated fatty acids (Samala et al., 1998; Cyril et al., 2001, 2002). Cyril et al. (2002) observed reduced levels of the di-unsaturated fatty

Table 3. Double bond indices (DBI) in polar lipid groups in 'Cavalier' and 'Meyer' zoysiagrass rhizomes on 4 Oct. and 5 Nov. 2005.

Lipid class <sup>z</sup>	4 Oct.		5 Nov.	
	Cavalier	Meyer	Cavalier	Meyer
DGDG	3.34 a <sup>y</sup>	3.35 a	3.18 b	3.73 a
MGDG	4.70 a	4.61 a	4.56 b	4.96 a
PC	2.83 a	2.92 a	2.83 b	2.95 a
PE	2.77 a	2.75 a	2.95 b	3.07 a
PA	3.52 a	3.11 a	2.83 a	3.06 a
PG	1.20 b	1.57 a	2.28 a	1.95 a
PI	1.71 a	1.82 a	2.18 b	2.23 a
PS	2.11 a	2.17 a	2.14 b	2.23 a
Lyso-PG	0.54 a	0.50 a	0.60 a	0.74 a
Lyso-PC	1.18 a	1.36 a	1.39 a	1.47 a
Lyso-PE	1.09 b	1.22 a	1.27 a	1.37 a

<sup>z</sup>DGDG = digalactosyl diacylglycerol, MGDG = monogalactosyl diacylglycerol, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PA = phosphatidic acid = PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine.

<sup>y</sup>Means followed by the same letter in a lipid group on a sampling date are not significantly different at  $P \leq 0.05$ . No DBI analysis was conducted on 2 Dec. 2005 and 4 Jan. 2006 due to severe freezing injury in either of the cultivars.

Table 4. Double bond indices (DBI) in polar lipid groups in 'Cavalier' and 'Meyer' zoysiagrass rhizomes from 2 Oct. to 1 Dec. 2006.

Lipid class <sup>z</sup>	2 Oct.		7 Nov.		1 Dec.	
	Cavalier	Meyer	Cavalier	Meyer	Cavalier	Meyer
DGDG	3.32 a <sup>y</sup>	3.19 a	2.96 a	3.03 a	2.96 b	3.03 a
MGDG	4.87 a	4.50 a	4.29 a	4.55 a	4.27 b	4.79 a
PC	2.63 a	2.67 a	2.73 b	2.87 a	2.73 a	2.85 a
PE	2.95 a	2.95 a	2.96 a	2.93 a	2.96 a	2.99 a
PA	2.75 a	2.83 a	2.82 a	2.98 a	2.81 a	3.01 a
PG	1.88 a	1.66 b	2.11 a	1.87 a	2.11 a	1.87 b
PI	2.18 a	2.10 a	2.22 a	2.17 a	2.32 a	2.34 a
PS	2.24 a	2.16 a	2.11 a	2.06 a	2.23 a	2.29 a
Lyso-PG	0.66 a	0.29 a	0.26 a	0.50 a	0.67 a	0.00 a
Lyso-PC	1.49 a	1.36 a	1.46 a	1.44 a	1.33 a	1.48 a
Lyso-PE	1.47 a	1.31 a	1.33 a	1.37 a	1.31 a	1.36 a

<sup>z</sup>DGDG = digalactosyl diacylglycerol, MGDG = monogalactosyl diacylglycerol, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PA = phosphatidic acid, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine.

<sup>y</sup>Means followed by the same letter in a lipid group on a sampling date are not significantly different at  $P \leq 0.05$ . No DBI analysis was conducted on 9 Jan. 2007 due to severe freezing injury in 'Cavalier'.

acid, linoleic acid (C18:2), in seashore paspalum with expansion in triunsaturated fatty acid (C18:3). Similar results were reported in bermudagrass that cold-tolerant cultivars had a more rapid and greater increase of C18:3 compared with sensitive ones during cold acclimation (Samala et al., 1998; Cyril et al., 2001). As polar lipids vary in their structural roles in membrane phase transition, it is necessary to study fatty acid saturation within each lipid class. Uemura and Steponkus (1994) observed an increase in total PE content and diunsaturated fatty acids within the PE class in spring oat (*Avena sativa* cv. Ogle) and winter rye (*Secale cereale* cv. Puma) during cold acclimation. The researchers suggested that the large increase in di-unsaturated fatty acids within the PE class overcame its tendency to form nonbilayer structure and helped to stabilize the membrane during freezing. Similarly, Welte et al. (2002) observed an increase in polyunsaturated acyl species, such as 36:6 – PE (di 18:3 PE), after cold acclimation and freezing exposure. In our study, DBI in the galactolipids were higher in ‘Meyer’ than in ‘Cavalier’ on 5 Nov. 2005 and 1 Dec. 2006 (Tables 3 and 4). ‘Meyer’ had higher DBI in phospholipids in 2005, except PA and Lyso-PG and -PC (Table 3). Limited variations in DBI were observed between the two cultivars in 2006 (Table 4). Yet there was no consistent association between LT<sub>50S</sub> and DBI.

In addition to the structural roles in membrane stability, membrane lipids are suggested to be physiological regulators during freezing (Wang et al., 2006a). Phospholipase D, the most abundant lipolytic enzyme in plants, hydrolyzes phospholipids to PA and free-head groups (Welte et al., 2002). During freezing, PA regulates freezing tolerance by inducing reactive oxygen species production, ABA responses, mitogen-activated protein kinases activities, and gene expression (Wang, 2004; Wang et al., 2006b). Zhang et al. (2009) reported that ABA is negatively correlated with freezing tolerance in zoysiagrass. Reduced carbohydrates, proline, and low molecular-weight dehydrins, which can protect membrane function via osmolyte adjustment, are associated with zoysiagrass cold tolerance (Patton et al., 2007a, 2007b). Relationships between polar lipids and other physiological factors under freezing conditions need to be studied.

To summarize, 11 major polar lipid groups were identified in ‘Meyer’ and ‘Cavalier’ zoysiagrass rhizomes. More changes were observed in phospholipids over sampling dates than in galactolipids (DGDG and MGDG) in both cultivars, indicating that phospholipids are more active during cold acclimation. Lipid composition levels and DBI in these cultivars were variable during the 2-year study period and had no consistent association with freezing tolerance. It is clear that we do not fully understand the roles of polar lipids in the response of zoysiagrass species to freezing stress.

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