CHAPTER I

REVIEW OF LITERATURE

Introduction

Common and hybrid bermudagrass (*Cynodon dactylon* L. Pers. and *C. dactylon* x *C. transvaalensis* Burtt Davy, respectively) are frequently used in the transition zone, the upper southern region of the United States, where they can provide an excellent surface for golf course fairways and athletic fields. They have high recuperative ability, high density, and excellent drought resistance. These species, however, have a great tendency to be injured or killed by winter temperatures in the transition zone.

Turfgrass managers in the transition zone worry about winter survivability of warm-season grasses yearly (McCarty, 2001). Even in moderate winters, a brief acute freeze can result in a large loss of bermudagrass. In cold winters, exposed bermudagrass is subject to desiccation and death. An example of this occurred during the winter of 1996-97, where 40% of all the bermudagrass in the state of Kentucky was lost (Powell, 1997). This research may assist these managers with options in better managing bermudagrass for improved winter survival.

Winter survival of bermudagrass can be adversely affected by many factors, including direct low-temperature exposure. Bermudagrasses in general are known to be sensitive to cold temperatures. Temperatures as moderate as -5 ^{o}C have been shown to cause death to some cultivars, and cultivars clearly

differ in their sensitivity to lower temperatures. For many years breeders have been selecting for improved turfgrass quality characteristics along with cold tolerance. However, there are very few bermudagrass cultivars that are truly adapted to the climate of the transition zone, and even these cultivars may suffer winter injury as often as one out of five years. Because bermudagrass is found throughout the world, great genetic diversity exists. Bermudagrass discovered growing naturally in cooler climates has been crossed with other strains with excellent turf quality to develop turf-type cold tolerant bermudagrasses.

A process that is thought to be important in the winter survival of bermudagrass is acclimation, or hardening. Levitt (1980) defines acclimation as an adaptation to an environmental stress. Many plants increase their tolerance to cold stress and freezing temperatures during normal exposure to lowered temperatures and/or shorter day lengths during the fall. An acclimation period leading up to chilling and/or freezing temperatures has been shown in bermudagrass to be essential as physiological processes are altered during this time that allow warm-season species to survive stressful conditions.

Fall applications of nutrients such as N, Fe, and possibly anti-senescence hormones may be important for maintaining high turfgrass quality into the fall. As temperatures decline and daylengths shorten, bermudagrass leaves begin to senesce. Applications of N or Fe can increase the period of time in which the grass stays green, as could increased plant levels of cytokinins by interfering with ethylene synthesis. An increased growing season would facilitate extra use for the turf and increase the aesthetic appearance of the area. However, there is

some debate as to whether or not applications of N are affecting the cold acclimation process. Although it may increase turfgrass quality prior to dormancy, it may also negatively affect the cold hardiness of bermudagrass by delaying senescence or reducing stored carbohydrate levels required for survival through the winter.

Some plant growth regulators (PGRs) have been shown to indirectly or directly affect the cold hardiness and/or lipid saturation levels of some plants. Ethephon has been shown to increase cold hardiness in fruit trees, and seaweed extracts (natural products containing cytokinins) have been shown to increase the unsaturation of lipids in Kentucky bluegrass (*Poa pratensis* L.). The effects of these two PGRs could be opposing. Ethephon (which causes evolution of ethylene) may induce senescence and promote earlier dormancy as ethylene is known to cause chlorophyll degradation. This may reduce the amount of sensitive tissue available for damage. Seaweed extract (SWE) may reduce senescence by reducing lipid peroxidation, stabilizing chloroplasts, and upregulating chloroplast DNA and photosynthetic enzymes. These two PGRs therefore present possible chemical approaches to improving cold hardiness of bermudagrass.

Previous research in bermudagrass and other species has shown a direct correlation between increased unsaturation of lipids and cold acclimation. Lipid saturation will decrease as plants go through a hardening period. Increased lipid unsaturation appears to be imperative for membrane and cell function at lower temperatures. Similarly, the osmoregulant proline has been shown in some

species to increase during cold acclimation, decrease during de-acclimation, and increase more in more cold hardy plants. Osmoregulants are important as they act to stabilize membranes and reduce cell dehydration, which can result in cell death. Because winter survival can be problematic in cooler climates such as Virginia's, research aimed at increasing lipid unsaturation as well as osmoregulant levels in bermudagrass plants during acclimation and dormancy may be important for increasing cold hardiness.

Bermudagrass and Winterkill

Winterkill is defined as the death of turfgrass during the winter or early spring. A rapid drop in air temperature to -5 ^{o}C or a less rapid drop to below -12 ^{o}C will often cause injury to warm-season turfgrasses (McCarty, 2001). Turf losses from winterkill results in the need to reestablish, increased pressure from weeds, increased soil erosion, reduced usage, lowered aesthetic value, and lost revenue (Dipaola and Beard, 1992). In the early spring, as warm-season grasses are breaking dormancy, they are especially susceptible to winterkill. This is thought to be because the non-structural carbohydrates (NSC) that help the turfgrass survive the winter are being converted to soluble sugars that promote bud break and the production of new leaves and roots (McCarty, 2001). As warmer spring weather triggers de-acclimation and new growth, stored carbohydrates that were used throughout the winter as fuel for respiration and survival are needed for production of new photosynthesizing tissue to replenish the NSC reserve. This de-acclimated plant with new growth and reduced NSC is

preparing for summer conditions and is unable to withstand a sudden cold snap.

In the turfgrass literature, “winterkill” is used as a collective term for a number of processes that cause lethality in turfgrass plants (McCarty, 2001). The first of these processes is crown hydration. Crown hydration can cause winterkill when turfgrass crowns are in moist areas following a thaw and absorb high levels of water. At the next freezing event, ice crystals are more prone to form intra- or intercellularly and cause cell rupture.

A second winterkill process is desiccation. This cause of winterkill is compounded by high winds and low humidity. As the soil dries and the plant continuesto transpire, desiccation can result. Traffic is a problem during frost events or on frozen or slush-covered turfgrass. The mechanical injury produced by traffic ruptures cells, and frozen crowns are more easily damaged.

Another factor that can lead to turfgrass winterkill is disease. Diseases can reduce plant moisture levels, disrupt cell wall components, or alter normal plant products such as hormones that can change temperature tolerance levels. These weaker plants are more prone to winterkill.

A final cause of bermudagrass winterkill is direct low temperature exposure. Direct low temperature exposure is a measure of the cold hardiness of a plant. A rapid drop in temperature to below -5 ^{o}C can be fatal for bermudagrass plants by ice formation (leading to cell rupture or desiccation) or loss of membrane fluidity and function.

Cold Hardiness Mechanisms

Cold hardiness is typically minimal in plants of recent tropical or subtropical origin. Even for species adapted to temperate zones, freezing injury can occur at temperatures near freezing if the plants do not go through an acclimation period. Some common effects of cold injury include reduced photosynthesis, carbohydrate translocation, respiration, enzyme activity, and protein synthesis. These effects are all likely due to or confounded by membrane dysfunction (Samala et al., 1998; Taiz and Zeiger, 1998).

Membrane Lipid Unsaturation

Plant membranes must be kept in a fluid state to maintain function. When membranes become less fluid or gel-like, their protein components become impaired or non-functional (Samala et al., 1998; Taiz and Zeiger, 1998). Plants maintain membrane fluidity at lower temperatures by increasing the unsaturation of the lipids in the phospholipid bilayer. Unsaturation maintains membrane fluidity as the double bonds in the fatty acids lower the freezing point of the lipid bilayer. Therefore as unsaturation increases, the membrane's freezing or "gel point" decreases.

In cold-sensitive plants, there appears to be a membrane transition from a fluid to a gel state when the temperature reaches that which is critical for chilling injury (Hale and Orcutt, 1987). Membranes in a gel state have inhibited H^+-ATPase activity, transport, energy transduction, and metabolism. Differences in

cold hardiness may be due to differences in membrane fluidity/functionality. In cold-resistant plants, lipid desaturase activity increases during acclimation and results in greater unsaturation (Taiz and Zeiger, 1998).

Saturated fatty acids are said to be saturated as they have the maximum amount of H atoms bonded to them and consequently do not contain any double bonds (McCarty et al., 2003). Unsaturated fatty acids, on the other hand, are not saturated with H atoms, therefore they are able to contain one or more double bonds (usually in the cis configuration) (Stryer, 1995). This cis double bond is important, as it affects molecular structure. Each location in the nonpolar fatty acid "tail" that contains a double bond results in a bend in the hydrocarbon tail; and, due to freedom of rotation around each bond in the tail, there exists the possibility of many conformations (Matthews and van Holde, 1990). These bends produced from unsaturation allow the space around fatty acid side chains to increase, causing the hydrocarbon tails to be less aligned. Attractive forces (hydrogen bonding, et al.) between tails decreases with increasing distance, lowering fatty acid melting points and allowing membranes to remain in a fluid state (McCarty et al., 2003).

Cyril et al. (2001) examined changes in fatty acid composition during cold acclimation in bermudagrass. They discovered that four fatty acids make up over 95% of the fatty acid content in bermudagrass. These consist of two saturated types, palmitic [$CH_3(CH_2)_{14}COOH$] and stearic acid [$CH_3(CH_2)_{16}COOH$], and two unsaturated types, linoleic [$CH_3(CH_2)_4(CH{=}CHCH_2)(CH{=}CH(CH_2)_7COOH$] and linolenic acid [$CH_3(CH_2)_4(CH{=}CHCH_2)(CH{=}CHCH_2)(CH{=}CH(CH_2)_4COOH$]. Cyril

et al. (2001) reported few changes in palmitic and stearic acid; but linoleic acid levels decreased during acclimation, while linolenic acid levels increased. This increase occurred to a greater extent in a cold-tolerant cultivar than in a cold-sensitive cultivar.

Samala et al. (1998) looked at changes in fatty acid composition of 'Midiron' and 'U3' bermudagrasses during cold acclimation. They found in the cold-tolerant cultivar Midiron, a fourfold increase in the unsaturated:saturated fatty acid ratio over the cold-sensitive cultivar U3. They suggested that desaturases are extremely important in controlling membrane fluidity and assisting cold tolerant bermudagrass cultivars in avoiding low temperature damage. In a similar study with the C_4 grass seashore paspalum (*Paspalum vaginatum* Sw.), researchers found that a cold-tolerant cultivar had a significant increase in linolenic acid during acclimation over a cold sensitive cultivar (Cyril et al., 2002). The authors reported that differences in cold tolerance were partly explained by differences in levels of linolenic acid. Based on studies such as these, it seems rational to try to find ways to increase linolenic acid levels during cold acclimation to reduce the threat of bermudagrass winter injury.

Osmoregulants and Plant Cold Hardiness

During cold stress, plants accumulate sucrose and other simple sugars as well as proline and glycine betaine, which have been reported to help stabilize membranes and act as osmolytes that maintain water balances within the cell (Nilsen and Orcutt, 1996; Holmstrom et al., 2000). Proline may have many

functions in stress tolerance, including osmotic adjustment, protein and membrane stabilization, gene induction, reactive oxygen species scavenger, N and C source, and a reduction equivalent source during stress recovery (Rudolph et al., 1986; Delauney and Verma, 1993; Saradhi et al., 1995; Hare and Cress, 1997; Iyer and Caplan, 1998; Brugiere et al., 1999). Proline levels have been shown to increase during cold acclimation, decrease during de-acclimation, and increase to a greater extent in cold-hardy species (Levitt, 1980). As extracellular ice crystals form, water moves from inside the cell to enlarge these extracellular ice crystals. By increasing the solute concentration in the cell, cell osmotic potential could be decreased (made more negative), making water movement out of the cell less likely. This reduction would prevent the enlargement of ice crystals and maintain cell hydration (Rossi, 1997).

Researchers in Australia looked at changes in amino acid levels in wheat (*Triticum vulgare* L.) during cold stress. They found an increase in cellular proline levels, while levels of the proline precursor glutamic acid decreased (Naidu et al., 1991). Koster and Lynch (1992) measured intracellular osmotic potential of rye (*Secale cereale* L.) during cold acclimation and found that the osmotic potential nearly doubled. Glycine betaine and soluble sugars increased dramatically early in the acclimation period, while proline content remained unchanged during the first 3 wk and then increased from 27 to 580 μg g^{-1} over the next 3 wk. A study measuring seasonal changes in proline concentration in halophytic plants showed that highest levels of proline were measured during the coldest part of the year, although not in every species (Murakeozy et al., 2003).

Exogenous applications of proline have also shown to be beneficial to cold tolerance. Leddet and Schaeverbeke (1975), applied proline to Jerusalem artichoke (*Helianthus tuberosus* L.) and found that the applications increased tolerance to freezing temperatures. The artichokes had a lethal temperature of -3 ^{o}C in the controls but -5 to -7 ^{o}C in proline-treated plants.

Accumulations of proline in the cytoplasm are only beneficial when membranes remain intact. In a study investigating cold injury in maize (*Zea mays* L.), researchers found that cells accumulated high amounts of proline (Chen and Li, 2002). They also reported that abscisic acid treatments reduced lipid peroxidation during chilling allowing the cells to retain the accumulated proline.

Although the benefits of proline accumulations are well documented in many species, very little work has been performed on bermudagrass. Proline quantification in bermudagrass could be another method of characterizing cold acclimation potential among the different cultivars.

Carbohydrate Influence on Cold Hardiness

The role of NSC in winter survival is unclear. It is generally known that NSC are energy sources for maintaining metabolic activity throughout the winter and for regrowth in the spring (Anderson et al., 1997). However, there is evidence that NSC may also have cold protection properties. Davis and Gilbert (1970) reported a strong association between carbohydrate reserves and

freezing stress tolerance. But, as Beard and Rieke (1966) state, the role of NSC in the regulation of frost injury remains inconclusive.

Carbohydrates are required for metabolic maintenance in bermudagrass as well as for survival during dormancy. Carbohydrates produced through photosynthesis are used by the plant for energy as well as growth (Turgeon, 1996). These photosynthetically-derived carbohydrates are required for the assimilation of other compounds, such as polysaccharides, cellulose, lignins, and proteins. Some of the carbohydrates are translocated to storage organs for future use. This formation of NSC is more likely to occur when carbohydrates accumulate at a rate that exceeds that used for growth. The periods of greatest NSC accumulation occur when there is high light intensity and minimal shoot growth as well as during the period preceeding winter dormancy (Beard, 1973).

Acclimation to Winter Environments

The optimum temperature range for many warm-season grasses is 27 to 35 ^{o}C (Beard, 1973). Temperatures outside this range may cause stress. Rogers et al. (1975) found an increase in zoysiagrass (*Zoysia japonica* Steud.) cold hardiness during the fall. They reported a reduction in the killing temperature from –5.6 ^{o}C in September to –11.1 ^{o}C in January. Similar results have been observed (Davis and Gilbert, 1970; Dunn and Nelson, 1974) in bermudagrass.

Acclimation primarily occurs because of cooler fall temperatures and shorter photoperiods. However, there is some debate as to what is happening during acclimation. Junttila (1980) and Chen and li (1980) found that in *Salix,*

Betula, and *Solanum* a cold period was required, but acclimation occurred regardless of photoperiod. White and Schmidt (1989) described the period preceding winter dormancy in C_4 grasses as a time when the plant loses its green pigment, alters physiological functions, decreases photosynthetic activity, and transports stored carbohydrates from leaves to stems.

Rogers et al. (1977) looked at cold hardening of bermudagrass and zoysiagrass (*Z. japonica*). They found that zoysiagrass, which is usually thought to be more cold hardy than bermudagrass, had higher photosynthetic rates than bermudagrass in late fall. Although both species had only 10 to 12% green tissue, the zoysiagrass was four to eight times more photosynthetically active. The zoysiagrass also had higher amylolytic enzymatic activity, which is usually found in more cold tolerant plants. The authors suggested that, because zoysiagrass had a higher carbon dioxide exchange rate (CER) than bermudagrass at lower temperatures, it would have an advantage, as most photosynthate would be conserved during a period of little or no growth. But zoysiagrass, with its cold tolerant advantage over bermudagrass, can still be severely damaged by even moderate temperatures if it does not go through a cold acclimation period (Rogers et al., 1975). This cold acclimation is essential in the survival of C_4 grasses over winter.

Dunn and Nelson (1974) looked at changes that took place in bermudagrass plants during cold hardening and also throughout the winter. They found that the 50% lethal temperature for rhizomes and stolons decreased as the fall progressed and then began to increase again in late winter. They also

found that NSC levels had the same trend, as they increased in early and late fall and then decreased during the winter. The authors speculated that the decrease in NSC levels was probably due to low-level respiration, which continues to occur during the winter when frost-killed leaves do not produce new photosynthate. Similar results were found with zoysiagrass (Rogers et al., 1975), where NSC increased during fall and early winter and then decreased in late winter. However, in zoysiagrass the maximum tolerance to cold temperatures was in late winter.

Hardening occurs in species other than grasses, of course. Jung and Smith (1961) found that, with alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.), cold tolerance increased during the fall, maintained this level until late February, and then declined through the spring. There may be differences among C_4 species in the time when the maximum level of hardiness occurs, but researchers agree that a cold acclimation period is essential for survival.

Bermudagrass and Cold Tolerance

Emmons (2000) describes the transition zone as an area that is not ideal for cool or warm-season grasses, as winters can be too cold for C_4 grasses and summers too hot for C_3 grasses. As stated earlier, warm-season grasses are best adapted for growth between 27 and 32 ^{o}C. The optimum soil temperature for bermudagrass root, rhizome, and stolon growth is 24 ^{o}C (Duble, 1989). When temperatures drop below these optima, bermudagrass plants do not operate as

efficiently; and, with temperatures below 12 ^{o}C, plants experience rapid reductions in growth rate and development (Buchanan et al., 2000; Hale and Orcutt, 1987). Beard (1973) explains that bermudagrass discolors with cool fall temperatures, and may remain in a state of dormancy throughout the winter. He describes winter dormancy as a termination of organ growth and leaf death that is brought on by extended periods of low temperature. Warm-season grasses stay in this dormancy state until soil temperatures climb above 10 ^{o}C the following spring.

Until recently, bermudagrass has been vegetatively propagated due to the fact that seeded cultivars established slowly and lacked the aesthetic qualities and cold tolerance of vegetative cultivars. Through breeding efforts, however, seeded cultivars are becoming much more desirable. The popularity and use of seeded bermudagrasses continues to rise as is evident in the most recent National Turfgrass Evaluation Program (NTEP) report, where there are nearly 30 seeded entries and fewer than 15 vegetatively propagated cultivars (Morris, 2002).

Although many of the improved vegetative cultivars are superior to seeded cultivars in texture, color, and overall quality, they are not completely adapted to the environment of the transition zone. In areas farther south, these improved vegetative bermudagrasses are the grasses of choice. In the mountain region of Virginia, however, ratings from the NTEP show that many of these newer seeded cultivars are very late greening up in the spring, and survival over the winter can be less than 50% (Morris, 2002). Vegetative cultivars such as ‘Vamont’ and

‘Midiron’ have superior cold tolerance compared to many other cultivars; but their leaf texture, color, spring transition, wear recovery, and overall quality are inferior to many new bermudagrasses propagated from seed.

Bermudagrass cultivars vary greatly in response to freezing temperatures. The coldest reported killing point for any bermudagrass cultivar was -17 ^{o}C for the cultivar ‘Brookings’ (Ibitayo and Butler, 1981). However, this temperature is unusually cold for bermudagrass, and the normal survival range is from -4 to -11 ^{o}C (Fry, 1990). ‘Midiron’ consistently ranks among the best in terms of freeze tolerance, surviving temperatures as low as -11 ^{o}C (Anderson et al., 1988). ‘Tifway’ has been shown to survive temperatures as low as -7.0 to -7.9 ^{o}C (Shashikumar and Nus, 1993, Anderson et al., 2003). A winter survival test in Mississippi showed that ‘Princess’ had a significantly poorer survival rate than Tifway (Philley et al., 1999). This report is consistent with some recent research out of Oklahoma State University, where they found ‘Princess’ to have a killing temperature of -6.9 ^{o}C (Anderson et al., 2003). This same study showed that Riviera had moderate cold hardiness, surviving down to -8.3 ^{o}C. However, even the most cold-tolerant bermudagrasses may be lost in especially cold winters (Schaffer, 1994).

Common bermudagrass (*C. dactylon* L. Pers. var. *dactylon*) is a highly variable and genetically diverse species with differences occurring in color, texture, density, vigor, and environmental adaptation (Turgeon, 1996; Taliaferro, 2000) It is most commonly a tetraploid plant (2n=4x=36), but some cultivars are diploid (2n=2x=18) (Taliaferro, 2000). African bermudagrass (*C. transvaalensis*

Burtt Davy) (2n=2x=18) is fine-textured and yellowish-green (Taliaferro, 2000). It has small, often red, stolons and produces a high amount of mostly sterile seedheads (Duble, 1989). Midiron, a vegetatively propagated cultivar released by the Kansas Agricultural Experiment Station in the 1960s, has a high degree of cold tolerance. Midiron, an interspecific hybrid of *C. dactylon* and *C. transvaalensis*, has medium texture and is in general use on golf courses and athletic fields where winter injury is a concern (McCarty, 2001). Tifway, another interspecific (*C. dactylon* x *C. transvaalensis*) hybrid and also vegetatively propagated, was developed by Glenn Burton and was released by the University of Georgia in 1960 (Beard, 2002). Princess, an intraspecific (*C. dactylon* x *C. dactylon*) hybrid, was developed by Arden Baltensperger of Seeds West Inc. and is propagated by seed (Rodgers, 2003; C. Rodgers, 2003, personal communication). Riviera (*C. dactylon*), developed by Charles Taliaferro at Oklahoma State University, is a cold-tolerant selection. It is a synthetic cultivar and is the result of a cross among three cold-hardy *C. dactylon* clones (Rodgers, 2003).

As early as the 1960s, breeders were developing cold-tolerant bermudagrasses (Taliaferro, 2000). Since then, research has shown that, during the winter, many biochemical changes occur in cold-tolerant cultivars that do not occur in cold-sensitive cultivars. During the cold acclimation process, biochemical changes occurring in cold-tolerant plants include changes in carbohydrate content and type, changes in proteins and synthesis of new

proteins, levels of cytoplasmic solutes, and changes in membranes including their degree of lipid saturation (Levitt, 1980; Pollock et al., 1993; Stryer, 1995).

Although many methods of determining cold tolerance of warm-season grasses have been employed, Cyril et al. (2002) state that accumulations of linolenic acid partly explain differences in cold-tolerance between cultivars. Research in South Carolina has shown that cold-tolerant bermudagrasses accumulate unsaturated fatty acids to a greater degree than cold-sensitive cultivars (Samala et al., 1998). They showed that Midiron had nearly four times as much of an unsaturated:saturated ratio then U3. In a similar study it was shown that the cold-tolerant cultivar 'Quickstand' had a greater increase in lipid unsaturation than the cold sensitive cultivar 'Arizona Common' during cold acclimation (Cyril et al., 2001).

Many reports in the literature document differences in cold tolerance of bermudagrass cultivars and offer biochemical explanations for the differences. However, although it is accepted that proline concentrations increase in the cytoplasm during cold stress, few reports show differences in proline concentration between cultivars of warm-season species. Munshaw et al. (in press) studied the bermudagrass cultivar 'Princess' and found that freezing tolerance increased with increasing proline concentration. However, as was mentioned, little is known about the effects of cold acclimation on proline concentrations between different bermudagrass cultivars.

Although seeded and vegetative varieties both have strengths and weaknesses, more research is needed to provide turfgrass managers with new

methods of ensuring winter survival of bermudagrass in Virginia and throughout the transition zone.

Seaweed Extract and Cold Hardiness

Natural products such as seaweed (*Ascophyllum nodosum* Jol.) extract contain high levels of cytokinins and auxins as well as moderate levels of other hormones (Mooney and Van Staden, 1986; Crouch et al., 1992). Verkleij (1992) states that the efficacy of these products appears to be due mainly to cytokinins, but may also be due to trace nutrients found in the products. A recent study by Zhang and Ervin (in press) also indicate that beneficial effects of seaweed extract (SWE) applications such as increased levels of antioxidants and increased photochemical efficiency during drought may be due to increased endogenous levels of cytokinins.

Cytokinins promote cell division, shoot growth, and stomatal opening (Buchanan et al., 2000). They also can act to inhibit senescence in leaves by counteracting the effects of ethylene or abscisic acid (Arteca, 1996; Buchanan et al., 2000). Mok (1994) summarizes the role of cytokinins in inhibiting or preventing senescence. He states that cytokinins stimulate the production of chloroplast DNA, photosynthetic enzymes, grana formation, and chloroplast replication. Mok goes on to say that cytokinins prevent the increase of proteolytic enzymes that are known to increase during senescence. Cytokinins may also maintain membrane integrity by reducing lipase and lipoxygenase activity, processes involved in membrane breakdown.

Goatley and Schmidt (1990) reported anti-senescence responses in excised Kentucky bluegrass (*Poa pratensis* L.) leaves after treatment with the synthetic cytokinin benzyladenine. However, White and Schmidt (1990) found that treating bermudagrass with benzyladenine did not consistently affect fall color or quality and did not influence carbohydrate levels. Similarly, Nakamae and Nakamura (1982) did not see an increase in leaf chlorophyll content of *Zoysia matrella* during autumn after applications of 6-benzyladenine.

Although results vary, SWE applications have been shown to increase leaf chorophyll content and growth (Featonby-Smith and van Staden, 1984; Beckett et al., 1994). Research at Virginia Tech has shown the benefits of SWE applications prior to drought episodes (Zhang and Schmidt, 1999; 2000; Zhang and Ervin, in press), but the possible effect of seaweed extract on cold tolerance of turfgrass is not well researched. Schmidt and Chalmers (1993) found that fall applications of SWE fortified with humic acid and thiamine on bermudagrass had no effect on fall color retention. They did find, however, that these fall SWE applications generally improved post-dormancy regrowth.

Yan et al. (1997) studied the effect of SWE on drought-stressed perennial ryegrass (*Lolium perenne* L.) plants and found an increase in lipid unsaturation in the treated plants over the control. They also found an increase in the double bond index (percentage of each fatty acid multiplied by the number of double bonds in the fatty acid) in the SWE treated plants.

Because SWE has been shown to increase lipid unsaturation, an increase in cold hardiness may occur. Also, because cytokinins are known to cause a

delay in senescence, there may be a longer photosynthetic period enabling plants to increase levels of carbohydrates and osmoregulants that could increase the chance of survival over the winter.

Late-season Nutrition and Cold Hardiness

Nitrogen Effects

Nitrogen inputs are essential to proper plant function, as they are required for amino acid, protein, and nucleic acid synthesis as well as being a major component of the chlorophyll molecule and cytokinins (Buchanan et al., 2000). Turfgrasses that are deficient in N will often be chlorotic due to a reduction in chlorophyll production (McCarty, 2001).

The N requirement for turfgrasses is much higher than for other macronutrients (Turgeon, 1996). Depending on length of growing season and use of the turf area, bermudagrass N fertilization can range from 390 to 1172 kg ha^{-1} $year^{-1}$ (McCarty, 2001). A commonly recommended N fertility rate on high quality bermudagrass is 49 to 73 kg ha^{-1} $month^{-1}$ during the growing season (Duble, 1989; Beard, 2002).

However, conflicting reports on proper application timings appear in the literature. Studies in the past have focused on the effect of N on winter survival of bermudagrass. Traditionally, late summer/early fall fertilization of warm-season grasses has not been recommended, as it has been suspected that N may make turfgrasses more susceptible to freezing temperatures and winterkill (Schmidt and Blaser, 1969; Beard, 1973). Interestingly, Beard (1973) makes

these claims to encompass all turf species with very little information as to the effects of N on warm-season species.

More recent research suggests that earlier reports of negative effects of late-season N applications on bermudagrass cold tolerance may not be completely accurate. Goatley et al. (1994) found that late-season N improved fall and spring color and had little effect on NSC levels. Schmidt and Chalmers (1993) reported that the positive effects of late-season N applications (better color, longer color retention) occurred without any negative effects on post dormancy recovery in the spring.

Researchers in Texas looked at the effect of late-fall N fertilization on cold hardiness of 'Tifgreen' bermudagrass (Reeves et al., 1970). They found that plots receiving N later in the fall remained green longer and were quicker to green up in the spring. They also found that N had no effect on survival of bermudagrass artificially frozen in a growth chamber. A study in Mississippi showed that most N sources applied during late-season increased color, while no N source affected NSC (Goatley et al., 1998). The study showed that NH_4NO_3 provided a much greater response than all slow release sources tested. White and Schmidt (1990) also found that bermudagrass plots receiving high amounts of N through September had longer color retention and higher turf quality than plots receiving lower N amounts. They also found that N levels did not affect spring recovery or stolon NSC levels. Richardson (2001) found that late season N fertility had no influence on seeded bermudagrass winter survival. Richardson (2002) also found late-season N applications on Tifway bermudagrass increased

fall color, enhanced spring greenup, and showed no difference from control rhizomes in artificial freeze tests. In a study looking at differences in photosynthesis during cold hardening of zoysiagrass and bermudagrass, Rogers et al. (1977) reported that zoysiagrass remained greener longer in the season than bermudagrass. The authors state that this higher photosynthetic rate in zoysiagrass would be beneficial as carbohydrate could be conserved while little shoot growth was occurring.

Gilbert and Davis (1971) looked at fertility ratios and found that bermudagrass plants that received only N were the least resistant to cold temperatures. With moderate soil fertility (K at 8 me 100 g^{-1}), they found that when N and K levels were approximately equal and 4 to 5 times higher than P, the greatest amount of post-freeze regrowth occurred.

Petit and Fagan (1974) looked at the influence of N on carbohydrates of buffalograss (*Buchloë dactyloides* Nutt.), another warm-season grass. They found that stolons contained the highest levels of carbohydrate reserves when compared to other plant parts. They also found, however, that these carbohydrate reserves decreased with increased N rates. Similar studies evaluating the effect of N on cool-season grasses have been conducted. The response to N is most likely caused by higher shoot growth as well as other demands on photosynthetic energy required in the reduction and assimilation of N (Hull, 1992).

Because there are many varying results as to the effect of fall fertilization it is important to test N to determine the effect on new bermudagrass cultivars. If N

does not negatively affect cold hardiness of bermudagrass as some reports suggest, it may be a desirable practice in the transition zone for prolonged green color.

Late-season N applications have been shown in numerous studies to increase fall color of bermudagrass without adversely affecting NSC levels or freezing tolerance. However, there have not been any studies examining the effects of N on physiological processes such as lipid unsaturation and proline concentration. Examining the effects of N further will give us a greater understanding as to whether early reports of negative N effects can be substantiated.

Potassium Effects

A proper ratio of N:P:K during the fall on bermudagrass seem to be important for cold hardiness. Several studies have looked at the effect of fertilization on bermudagrass carbohydrate levels and winter survival. Miller and Dickens (1996) applied four rates of KCl or K_2SO_4 to bermudagrass during the growing season. They found that with low (36 kg ha^{-1}) or very low (15 kg ha^{-1}) soil K levels applications of high K rates 390 kg ha^{-1} $month^{-1}$) were not beneficial to bermudagrass as they either had no effect on root and rhizome NSC levels or they decreased NSC levels. Trenholm et al. (1998) conducted a similar study looking at the influence of N and K on bermudagrass growth and NSC. They found that N and K reduced NSC levels in two cultivars of bermudagrass. Similar research was performed in Mississippi evaluating the response to late-season

applications of N and K. Potassium did not influence turfgrass color or affect NSC levels at any rate applied with initial soil K level being 168 kg ha^{-1}. Nitrogen, however, improved both fall and spring color, but high rates of N moderately decreased NSC levels (Goatley et al., 1994). Studies with alfalfa (*Medicago sativa* L.) seedlings, however, have shown that carbohydrate concentrations increased with increasing amount of K applied (Matches et al., 1962; Reid et al., 1965).

Iron Effects

Iron is a component of chlorophyll-protein complexes in the chloroplast (Taiz and Zeiger, 1998). Iron is also necessary for the synthesis of heme and cytochrome as well as ferredoxin used in cellular respiration (McCarty, 2001). Iron is the micronutrient that is most often deficient on fairways and putting greens (Christians, 1998; Beard, 2002). Iron deficiency appears initially as interveinal chlorosis in young leaves and eventually spreads to older leaves during extended deficiencies (Beard, 1973; Carrow et al., 2001).

A number of soil conditions can induce Fe deficiency in turfgrass leaves, including an alkaline pH as well as cold and wet soils (Turner and Hummel, 1992). Low soil temperatures cause a reduction in activity of soil microbes often resulting in a reduction in Fe uptake (Duble, 1989). Grasses acquire Fe from the soil by excreting low molecular mass compounds called phytosiderophores. These compounds have a high affinity for Fe^{3+} and solubilize these ions and make them available for uptake (Buchanan, 2000). Schmidt (2003) explains that

phytosiderophores may not be released from roots when bermudagrass is exposed to cool fall temperatures. Because of the reduced acquisition of these compounds in the fall, foliar Fe applications can markedly increase and prolong turfgrass quality and color.

Tests have shown that Fe applications in conjunction with moderate (24 kg ha^{-1} $month^{-1}$) summer N applications can improve the performance of bermudagrass during the fall and improve recovery in the spring (White and Schmidt, 1990). White and Schmidt (1989) also report that Fe maintained the aesthetic quality of cold-sensitive and cold-tolerant bermudagrass cultivars after a chilling period and assisted in CER recovery.

Iron has been shown to assist bermudagrass in color and photosynthesis recovery after a chilling period, but the effect of Fe on the freezing tolerance of bermudagrass cultivars is unknown. It is also apparent from previous literature that Fe does not negatively influence bermudagrass NSC levels. However, as there is no literature examining the influence of Fe on lipid unsaturation and proline concentration, further research is warranted in this area as well.

Ethylene (Ethephon) and its Relation to Cold Hardiness

Yang and Pratt (1978) state that any stress may lead to an increase in ethylene production. Several studies have shown that chilling temperatures cause an increase in 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity, which causes an increase in ACC, the precursor to ethylene (Wang, 1989). High levels of ethylene are known to induce leaf senescence. Taiz and

Zeiger (1998) report that elevated levels of ethylene result in chlorophyll degradation and color fading. They also suggest that senescence is controlled by the relative amounts of ethylene and cytokinins in the plant. Ethylene is also known to increase membrane permeability, perhaps by decreasing the phospholipid content (Lyons and Pratt, 1964; Harber and Fuchigami, 1989).

Studies using the PGR ethephon (2-chloroethylphosphonic acid) show that it is effective in inducing ethylene-related responses such as fruit ripening and abscission of fruits and flowers (Taiz and Zeiger, 1998). Ethephon works by releasing ethylene inside the tissue (Diesburg, 1999). Although there are conflicting reports, some data indicate that exogenous ethylene applications (including ethephon) prior to chilling temperatures increases cold hardiness and substantially reduce development of chilling injury (Wang, 1989; Harber and Fuchigami, 1989). Yu et al. (2001) reported that ethephon-treated winter rye had increased levels of antifreeze activity and concentration of apoplastic protein. The authors concluded that ethylene regulates the antifreeze activity in response to cold temperatures.

Ethylene is an important, perhaps ubiquitous, hormone in terms of plant stress and its production increases with lowered temperatures. Mazur (1988) found that the use of ethephon (Proxy®, Bayer Environmental Sciences) as a transition agent on overseeded bermudagrass did not decrease turf quality from the control, whereas other plant growth regulators did reduce quality. However, Diesburg (2000) reported that multiple ethephon applications can result in reductions in turfgrass quality. Coleman and Estabrook (1992) applied growth

regulators to fruit trees at low rates during acclimation. They found that freezing stress resistance was increased, possibly due to alterations in membrane composition, a reduction in growth during acclimation, and an increase in energy accumulation.

The need for research of ethephon on bermudagrass is great due to the following reasons: i) no data exist for the use of ethephon as an agent for increasing bermudagrass cold hardiness; ii) ethephon may enhance membrane fluidity and affect energy accumulation in other species; iii) ethephon may induce senescence and early dormancy; iv) early dormancy may allow bermudagrass plants to acclimate earlier and avoid chilling and/or freezing temperatures; and v) previous research has not shown reductions in turf quality.

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CHAPTER II

INFLUENCE OF LATE-SEASON IRON, NITROGEN, AND SEAWEED EXTRACT ON FALL COLOR RETENTION AND COLD TOLERANCE OF FOUR BERMUDAGRASS CULTIVARS

INTRODUCTION

Late-season nutrient applications have been previously shown to boost fall color, but there has been some concern that prolonging fall growth may adversely affect bermudagrass survival throughout the winter. Physiological parameters such as non-structural carbohydrates (NSC) seem to be unaffected by fall applications of fertilizer, and an added benefit of earlier spring greenup may occur. However, few physiological parameters have been measured as to the effect that late-season nutrient applications might have on them. This study was designed to examine the effects of fall N and Fe applications on saturation levels of fatty acids as well as the cytoplasmic solute proline at different periods throughout the fall, winter, and spring. An understanding of these factors will help to better define the effects that late-season nutrient applications have on physiological processes occurring during cool weather in the fall.

Seaweed extract (SWE) applications have been shown in a number of turfgrass species to assist the plant during periods of stress. The effect of SWE may be mediated by increasing endogenous levels of cytokinins. It is known that increased plant cytokinin levels reduce senescence. Thus, it may be hypothesized that, if SWE does increase endogenous cytokinin levels, senescence may be reduced and fall color

retention may be prolonged. If SWE is a factor in color retention, it is important to test physiological factors such as lipid unsaturation and proline concentration of treated bermudagrass to examine the effect that SWE has on these factors. The following null hypotheses were tested:

1. Seaweed extract applications will not affect lipid saturation or proline concentrations, and will not have an effect on bermudagrass cold hardiness nor delay senescence and allow the grass to remain green longer into the fall.
2. Fall N and Fe applications will not affect lipid saturation or proline concentrations and will not have an effect on cold hardiness.

If N, Fe, and SWE can extend the bermudagrass growing season without negatively effecting physiological factors known to be important in winter survival, then turfgrass managers across the transition zone can use this information as another tool for prolonging fall color without having to resort to overseeding of cool-season species.

Innate differences in cold tolerance have been shown to exist among bermudagrass cultivars. To examine physiological differences between cultivars during fall, winter, and summer, the following null hypotheses were assumed:

1. Midiron, Tifway, Princess, and Riviera bermudagrass will not become less susceptible to direct low temperature kill following a period of acclimation.
3. The degree of lipid unsaturation in Midiron, Tifway, Princess, and Riviera bermudagrass membranes will not increase during cold acclimation.
4. Proline concentration will not increase during cold acclimation.
5. The bermudagrass cultivars Midiron, Tifway, Princess, and Riviera will not

differ in cold-hardiness.

6. A correlation between cold hardiness and the degree of lipid unsaturation will not be apparent in Midiron, Tifway, Princess, and Riviera bermudagrass cultivars varying in cold hardiness.
7. A correlation between cold hardiness and proline concentration will not be evident in Midiron, Tifway, Princess, and Riviera bermudagrass cultivars varying in cold hardiness.

MATERIALS AND METHODS

Plant Material, Establishment, and Experimental Design

A field study was conducted at the Virginia Tech Turfgrass Research Center in Blacksburg, VA. Plots were established on a Groseclose silt loam (fine, kaolinitic, mesic typic Hapludult) with a pH of 6.8 and a K level of 59 mg kg^{-1}. Plots were 3.1 x 9.1 m and established 20 June 2001 using four bermudagrass cultivars: 'Tifway', 'Midiron', 'Princess', and 'Riviera' (Table 2.1). Princess seed was supplied by Dr. Charles Rodgers (Seeds West Inc., Maricopa, AZ), and Riviera seed was supplied by Dr. Charles Taliaferro (Oklahoma State University, Stillwater, OK). Midiron and Tifway sprigs were supplied by the Virginia Tech Turfgrass Research Center. Seeding rates were 48.8 kg pure live seed (PLS) ha^{-1}, and sprigging rates were 1800 bu ha^{-1}.

Seeded plots were planted under a Remay cover to discourage runoff and to enhance germination and plant development. Plots were mowed three times per week

Table 2.1. Bermudagrass cultivar sensitivity to cold temperatures (Beard et al., 1980; Morris, 2002).

Hardiness	Seeded Cultivars	Vegetative Cultivars
Cold Tolerant	Riviera	Midiron
Cold Sensitive	Princess	Tifway

with a reel mower set at 1.91 cm. Nitrogen was applied in the form of NH_4NO_3 (34-0-0) once monthly at a rate of 48.8 kg N ha^{-1} beginning at establishment and ending 15 August. A complete fertilizer (10-10-10) was applied the following spring (25 May) at a rate of 48.8 kg N ha^{-1}, and monthly N applications began again on 15 June 2002. Irrigation was supplied as needed.

After bermudagrass establishment, three chemical treatments were applied during the period leading to bermudagrass senescence. A non-treated control was also included within each bermudagrass cultivar.

Fall Chemical Treatments

Fall chemical treatments began 15 August in 2001 and 2002 and continued on a 3-wk schedule until apparent dormancy (80 to 100% canopy browning). Final treatment dates were applied 17 October 2001 and 31 October 2002. Seaweed extract was applied at a rate of 0.54 kg ha^{-1} (Zhang et al., 2002); N was applied at a rate of 48.8 kg N ha^{-1} as NH_4NO_3 (White and Schmidt, 1990); Fe was applied at a rate of 1 kg Fe ha^{-1} as $FeSO_4$ (Schmidt and Chalmers, 1993).

The experimental design was a randomized complete block with four replications. The study was conducted during the 2001-02 growing season and repeated during the

2002-03 growing season. Treatments were arranged in a split-split plot design. In cases where sampling times were considered important treatment effects (i.e., fall versus winter or early bermudagrass greenup versus late greenup), sampling times were considered main plots. Where measurements were taken only once or sampling times were not considered treatments effects, bermudagrass cultivars served as main plots. Late-season chemical treatments consisted of N, Fe, SWE, and none and were subplots. In some cases, controlled freezing of bermudagrass samples was conducted to elicit measurable responses. Various freezing temperature regimes were considered subordinate to sampling dates but superior to cultivars in the split-split plot design. The following discussion will explain the techniques used to collect and statistically analyze experimental data.

Fall Quality and Spring Greenup

Visual turf quality ratings were taken (using a 1 to 9 scale, where 1 = brown dormant or dead turf, and 9 = lush green turf) monthly during fall treatments. Spring greenup was visually estimated as a percent of green ground cover.

Data were analyzed using PROC GLM of the SAS statistics package (SAS, 1989), and data were tested for homogeneity of variance by plotting residuals before statistical analysis. To comply with the assumptions of analysis of variance, visual ratings were transformed by the arcsine of the square root (Ott and Longnecker, 2001). However, actual means are presented textually and in tables. Year was considered a random variable and main effects and interactions were tested using the mean square associated with the random variable (McIntosh, 1983). Rating times were considered

main plots, cultivars were subplots, and chemical treatments were subsubplots. Where year effects were significant, data were separated by year; otherwise, data were pooled. Appropriate main effects and interactions were separated using Fishers Protected LSD test at P=0.05.

Controlled Freezing

Freeze chamber analyses were performed on putatively acclimating (fall) and acclimated (winter) 2001-02 and 2002-03. Additional analysis was conducted on samples collected in summer 2003. During November of both years due to the freezing process being time consuming and possible physiological differences occurring in samples between the first and last tests of each sampling period. Samples were analyzed from all plots in winter each year and summer 2003. A 10.2-cm (diameter) cup cutter sample was removed from each plot, cleaned of soil by washing, and divided into four equal subsamples. One of the subsamples was placed in a refrigerator and held at 4 ^{o}C to act as a "control". The other three subsamples were placed in a freeze chamber that ramped from 8 to 1 ^{o}C overnight. The temperature then ramped to -2.8 ^{o}C over 2 hr, stayed at this temperature for 0.5 hr, and a sub sample was removed. This process continued with ramping to -5.0 and then -7.2 ^{o}C over the next 5 hr. After removal, sub samples were held over night at 4 ^{o}C and then placed in a sand medium in the glasshouse at 22 ± 2 ^{o}C on a mist bench. Regrowth was visually estimated as the percent of the sample exhibiting regrowth or appearing green approximately 4 wk after freezing (Schmidt and Chalmers, 1993).

Since only control plots were assessed in November each year and summer samples were only collected in 2003, the data set was not balanced and a combined analysis was not possible. Thus, sampling time by year combinations were considered a single factor having five levels (i.e., Nov. 01, Jan. 02, Nov. 02, Feb. 03, July 03). This single factor was considered the main plot in a split-split-split plot analysis. Subplots were freezing temperature regimes, subsub plots were bermudagrass cultivars, and subsubsub plots were chemical treatments. Main plot sample dates were 2 November 2001, 10 January 2002, 21 November 2002, 24 February 2003, and 20 July 2003. Sub plot temperatures were 4, -2.8, -5.0, and -7.2 ^{o}C. Subsub plot cultivars were Midiron, Riviera, Tifway, and Princess. Subsubsub plot chemical treatments were N, Fe, SWE, and a "zero control". Data were analyzed using PROC GLM of the SAS statistics package (SAS, 1989), and data were tested for homogeneity of variance by plotting residuals before statistical analysis. To comply with the assumptions of analysis of variance, visual ratings were transformed by the arcsine of the square root (Ott and Longnecker, 2001). However, actual means are presented textually and in tables. Appropriate means were separated using Fishers Protected LSD at P=0.05.

Total Lipid Extraction and Fatty Acid Quantification

Tissue samples were removed from the field during acclimation (November), when acclimated (January-February), and when non-acclimated (July) and stored at -80 ^{o}C until analyzed. For each analysis, 1 g of stolon tissue was ground with a mortar and pestle in liquid N_2. This ground tissue was then transferred to a centrifuge tube for total lipid extraction using 3 ml of a buffer containing chloroform:methanol:water (1:2:0.8).

After soaking for 1 hr at room temperature, 1 ml 1% NaCl and 3 ml chloroform were added and centrifugation occurred at 1200 G for 10 min. The lower chloroform layer containing the lipids was transferred to a test tube, and the chloroform addition, centrifugation, and chloroform layer transfer were repeated two more times (Cyril et al., 2001).

In a method described by Goyal (2000) and modified by Shang (2003, personal communication), the chloroform layer was then evaporated under a stream of N_2. After evaporation, 5 ml of 2% NaOH in 90% methanol were added, and tubes were placed in a water bath at 75 to 80 ^{o}C for 30 min. Tubes had an air-cool reflux (small funnels placed in tubes) during this process to facilitate hydrolysis. At the end of the 30 min, the mouths of the tubes were left open to allow for evaporation of the methanol under a fume hood. Next, 2 ml of distilled/deionized (DD) H_2O were added to facilitate dissolution, and the contents were transferred to a 10-ml screw-cap tube. The residue was washed twice more with 2 ml DD H_2O, and contents transferred to a new tube. To this new tube was added 300 µl of 6 M H_2SO_4 to precipitate sodium salts out of the fatty acids. The acid form of the fatty acids was recovered by adding 1 ml hexane and centrifuging at 150 G for 5 min. After centrifugation, the hexane layer containing the free fatty acids was transferred to a new 10-ml screw-cap tube and the process was repeated two more times. After the final centrifugation, the hexane volume was reduced to 100 µl under a gentle stream of N_2 gas, and 100 µl α-bromoacetephenone (10 mg ml^{-1} acetone) and 100 µl triethylamine (TEA) (10 mg ml^{-1} acetone) were added and caps were tightly fastened. Tubes were placed into a water bath at 100 ^{o}C for 15 min. Free fatty acids react with α-bromoacetophenone in the presence of TEA and produce a

derivative that is UV sensitive and can be quantified with a UV detector after HPLC separation. Tubes were then allowed to cool, and 140 µl acetic acid (2 mg ml^{-1} acetone) were added and the tubes were placed back in the 100 ^{o}C water bath for 5 min. After cooling a second time, the content was dried under a stream of N_2 to inactivate the remaining reagents. The residue was dissolved with 500 µl acetonitrile, and the solution was filtered with a 0.2 µm membrane before injection into HPLC.

HPLC Procedures

Chromatographic analyses were performed on an Agilent (Palo Alto, CA) 1100 series HPLC system with a photodiode array detector. An Ultrasphere-C8 (250 × 4.6 mm, 5 µm) analytical column with a C-8 guard column (7.5 × 4.6 mm) was used for chromatographic separation. The mobile phase was 90% acetonitrile in water. Samples were eluted at a gradient rate: from 1 ml min^{-1} up to 2 ml min^{-1} within the first 2 min, at a isocratic elution of 2 ml min^{-1} for 10 min, and then down to 1 ml min^{-1} within the next 8 min. Total elution time per sample was 20 min. The injection volume was 20 µl. The fatty-acid derivatives were quantified at a wavelength of 214 nm. The retention times (minutes) at the described conditions were 4.6, 5.8, 7.3 and 10.8 for linolenic, linoleic, palmitic and stearic acids, respectively. The limit of identification for the above procedure is 0.07 to 0.6 µmole per injection using three times the standard deviation for one-half of the lowest standard.

Fatty acid standards were purchased from Sigma Chemical Co. (St. Louis, MO). External standards prepared from the commercial standards were used for calibration.

Two reference samples, which were pre-weighed from a composite tissue sample, were included in each analysis set.

Data derived from samples collected in July 2003 could not be included in the combined analysis. Therefore, two separate analysis were performed. The first was a combined analysis with year as the random variable. Sampling times (fall and winter) were considered main plots, bermudagrass cultivars were considered subplots, and chemical treatments were considered subsub plots. The second analysis only included the year 2002-03 and was conducted to compare data collected from summer sampling to that of fall and winter. The analysis is identical except "summer" was included as an additional sampling time and the random variable "year" was excluded. Data were analyzed using PROC GLM of the SAS statistics package (SAS, 1989), and data were tested for homogeneity of variance by plotting residuals before statistical analysis. No transformations were required since fatty acid data appeared homogeneous (Ott and Longnecker, 2001). Appropriate means were separated using Fishers Protected LSD at P=0.05.

Proline Determination

Additional stolon samples were removed at the same time as the controlled freezing tests (fall, winter, and summer), placed in liquid N_2 to halt respiration and then placed in a -80^{o}C freezer for later analysis. Stolons were ground with a mortar and pestle in liquid N_2 and approximately 0.20 g stolon material was homogenized in 10 ml of 3% sulfosalicylic acid, and the homogenate was filtered through Whatman # 2 filter paper. Two ml acid ninhidrin and 2 ml glacial acetic acid were added to 2 ml of the

filtrate and incubated at 100°C for 1 hr. The reaction was terminated by placing test tubes in an ice bath. The mixture was extracted with 4 ml toluene and vortexed for 15 to 20 sec. The chromophore containing toluene was warmed to room temperature and absorbance read at 520 nm using toluene as the blank. Proline concentration was determined from a standard curve (Syvertsen and Smith, 1983).

Data were analyzed using the same methods employed for fatty acid data with the exception that proline data were log transformed to achieve uniform variance (Ott and Longnecker, 2001). Although log transformed for analysis, actual means are presented textually and in tables for clarity.

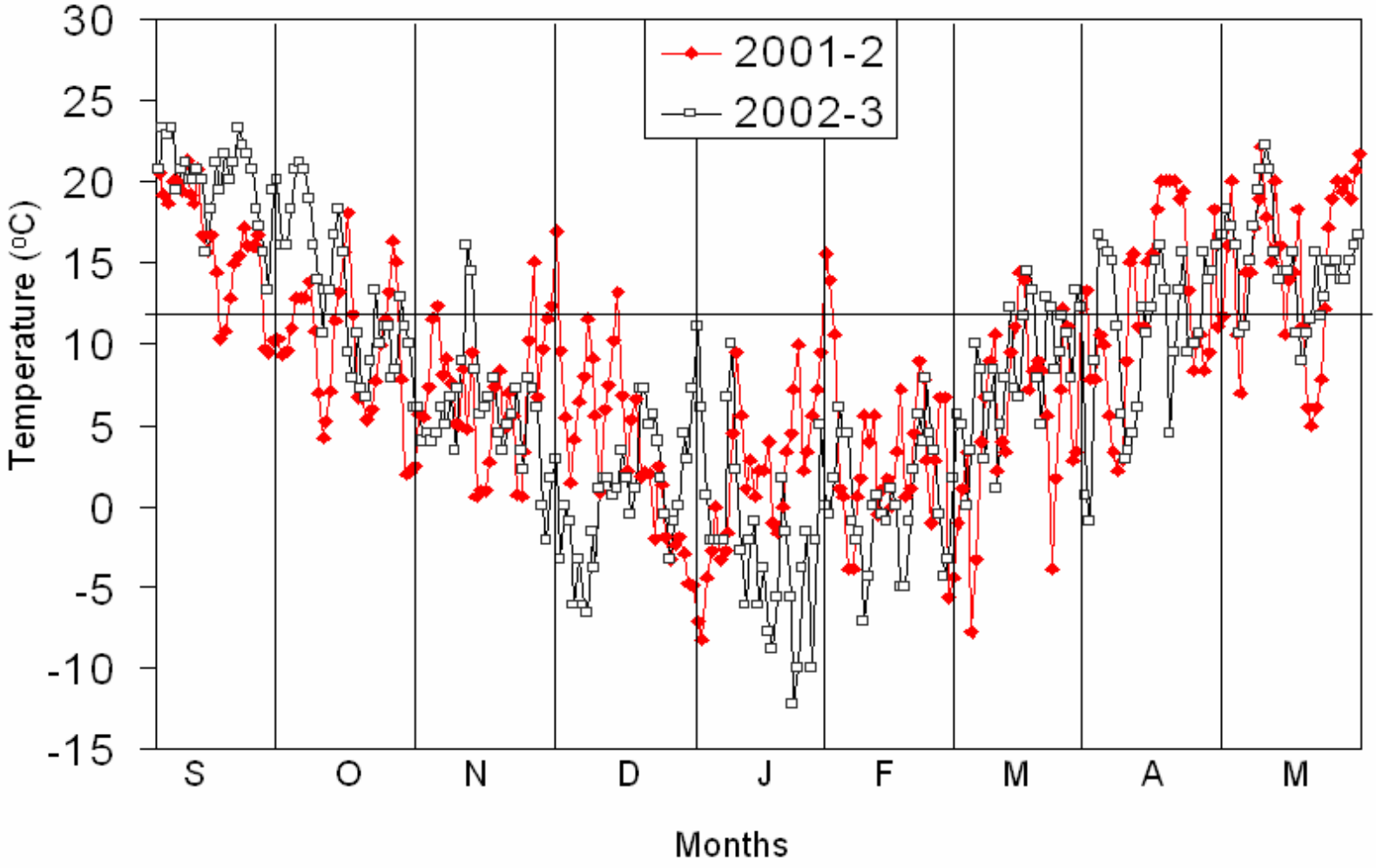
RESULTS AND DISCUSSION

Data presented in this chapter describe the main effects of fall chemical treatment and of cultivar or the interaction of the two. Mean daily temperature data for fall, winter, and spring of each year are shown in Fig. 2.1. In general, air temperatures remained warmer during the fall and early winter in 2001 than in 2002.

Turfgrass Quality

Visual ratings commenced shortly after the initial fall treatment application. Visual quality data for 2001 and 2002 were examined, and the subsequent ANOVA table showed significant main effects as well as interactions (Table 2.2).

Figure 2.1. Daily mean temperature data (September – May 2001-2003). Line at 12 °C indicates bermudagrass growth cessation point.



Year x Observation Date Interaction on Turfgrass Quality

The interaction of year x observation date showed that, in 2001, quality ratings gradually decreased as the fall progressed (Table 2.3). In 2002, August and September

Table 2.2. Analysis of variance for chemical treatment and cultivar effects on bermudagrass visual quality (2001 & 2002).

Source	df	*F* value
Year	1	0.8
Error	6	
Date	3	5998.9**
Year x date	3	1040.2**
Error	18	
Cv	3	256.9**
Date x cv	9	88.8**
Year x cv	3	75.1**
Year x date x cv	9	13.1**
Error	72	
Trt	3	26.4**
Date x trt	9	2.7**
Cv x trt	9	1.4
Date x cv x trt	27	1.5
Year x trt	3	0.4
Year x date x trt	9	1.3
Year x cv x trt	9	3.0**
Year x date x cv x trt	27	1.3
Error	288	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Table 2.3. Year x observation date interaction for visual quality across cultivars and chemical treatments (2001 & 2002).

	----------Observation Date----------				
Year	Aug	Sept	Oct	Nov	LSD[1]
	Visual Quality (1 to 9)				
2001	7.7	7.1	5.8	4.9	0.3
2002	7.8	7.8	7.1	2.9	0.4
LSD[2]	ns	0.2	0.2	0.2	--

[1]LSD = Comparison of observation date within year.
[2]LSD = Comparison of year within observation date.

were not different, but quality ratings decreased through the remainder of the observation period. Quality in August of both years was not different. In September and October, however, quality remained higher in 2002. The November rating showed that quality was higher in 2001. Weather data in both years showed that the fall of 2001 was cooler than 2002 (Figure 2.1). September 2001 had a monthly mean temperature of 15.5 ^{o}C compared with 19.3 ^{o}C in September 2002. October 2001 had a monthly mean temperature that was 3.2 ^{o}C cooler than October 2002. November 2001, however, was 1.6 ^{o}C warmer than November 2002. Hale and Orcutt (1987) and Buchanan et al. (2001) suggest that when temperatures drop below 12 ^{o}C, C_4 plants experience rapid reductions in growth rate and development. The mean October temperature in 2001 was below 12 ^{o}C, which may explain quality differences between years. All cultivars in 2001 were most likely beginning to yellow earlier in the season than in 2002. The rapid drop in visual quality seen in November 2002, but not 2001, may have been due to air temperatures declining more quickly in 2002 than in 2001. This rapid drop in air temperature over October likely allowed senescence to occur quickly as October temperatures were conducive to bermudagrass growth.

Cultivar Quality Over Time

Although the interaction of year x cultivar x fall chemical treatment was also significant, cultivar x observation date and chemical treatment x observation date are presented to highlight differences over time (Tables 2.4 & 2.5). Cultivar differences were noticeable in August, with Riviera having better quality than all other cultivars (Table 2.5). Riviera normally has medium texture and density, and exhibits a darker green color than Midiron. Midiron had similar quality to Tifway, which had better quality

than Princess. Princess, although normally having superior density and texture to the other cultivars tested, was slower in establishing than the other cultivars resulting in poorer density. In September and October there were no differences between Midiron, Riviera, and Tifway, but all were better than Princess. By November, however, Princess had better quality than all other cultivars. Quality for all cultivars decreased over the fall, but to varying degrees (Table 2.4). Midiron, Riviera, and Tifway all had quality reductions of over 50% between August and November, whereas Princess, which had lower visual quality at the outset, showed only a 36% reduction over the same period.

Visual turfgrass quality ratings during the fall of both years showed significant differences between cultivars much before the obvious yellowing effects of senescence began to take place. Generally, Riviera, Midiron, and Tifway had better quality than Princess until the November observation date. Because Princess was slower to establish than other cultivars, density was poor and contributed to decreased quality throughout the majority of the fall. However, Princess had better color than the other cultivars in November. The better color observed in November overrode the issue of

Table 2.4. Breakout of observation date x cultivar interaction for visual quality across all chemical treatments (2001 & 2002).

	----------Observation Date----------				
Cultivar	Aug.	Sept.	Oct.	Nov.	LSD[1]
	Visual Quality (1 to 9)				
Midiron	8.0	7.8	6.6	3.8	0.2
Riviera	8.4	7.7	6.6	3.8	0.2
Tifway	8.1	7.7	6.6	3.8	0.2
Princess	6.6	6.5	6.1	4.2	0.2
LSD[2]	0.3	0.2	0.1	0.2	--

[1]LSD = Comparison of dates within cultivars.
[2]LSD = Comparison of cultivars within dates.

density. These findings are in agreement to a statement by Beard (1973) who generalized that cold-tolerant bermudagrass cultivars discolor earlier in the fall than cold-susceptible cultivars. The response observed here may be due to differences in rate of establishment in 2001 and the fact that Princess plots were heavily damaged by winterkill in 2001-02 and were still recovering in fall 2002. Seeded cultivars produce smaller stolons during the establishment year and lack rhizome production (Hensler et al., 1999; Munshaw et al., 2001). As a result, the first winter after seeding can be extremely stressful on bermudagrass and can sometimes result in the loss of turf, regardless of planting date.

Chemical Treatment Differences Over Time

In both years of the study, quality was not different across cultivars prior to the first application in August of the fall chemical treatments (Table 2.5). In September, N resulted in greater color, which corresponded to a slight increase in quality over the control. In October, all chemical treatments improved turfgrass quality compared to the control, but only N improved turfgrass quality in November. As would be expected, overall quality for all treatments decreased as the fall progressed (Table 2.5). All treatments had the same quality in August, but SWE and control plots had the greatest reductions in quality by the end of the observation period in November.

The ability to prolong fall color of bermudagrass with fall chemical treatments is important in non-overseeded bermudagrass, as the longer growing season may also increase turf functionality. Although the differences between N and the control were not large in November, low-budget athletic fields may benefit from this increased color by allowing activities to continue on somewhat greener grass. As has been reported

previously (Reeves et al., 1970; White and Schmidt, 1990; Schmidt and Chalmers, 1993; Goatley et al., 1994; Richardson, 2001; Richardson, 2002), late-season N applications improved quality and prolonged green color.

Table 2.5. Breakout of observation date x fall chemical treatment interaction for visual quality across all cultivars (2001 & 2002).

Chemical	Observation Date				
Treatment	Aug.	Sept.	Oct.	Nov.	LSD[1]
	Visual Quality (1 to 9)				
N	7.8	7.6	6.7	4.1	0.2
SWE	7.8	7.4	6.4	3.8	0.2
Fe	7.8	7.4	6.4	3.9	0.2
C	7.8	7.4	6.3	3.8	0.2
LSD[2]	ns	0.2	0.1	0.2	--

[1]LSD = Comparison of dates within chemical treatments.
[2]LSD = Comparison of chemical treatments within dates.

Iron did not significantly improve fall color during three of the four evaluation times. White and Schmidt (1990) did not observe any differences with the application of Fe until air temperatures reached freezing. Schmidt and Chalmers (1993) found a 20% improvement in October color due to Fe, but only when no N was applied in September. Rodgers (2003) suggests that the new seeded bermudagrasses use Fe more efficiently than older seeded cultivars. Although no older seeded cultivars were examined in this study, the interaction between cultivar and chemical treatment was not observed. The effect of Fe was minimal on all cultivars. A higher rate of $FeSO_4$ or a chelated form of Fe may have resulted in a greater response through the fall.

Seaweed extract applications did not have any effect on bermudagrass color retention, as quality ratings were very similar to the control in both years of the study. This finding is in agreement with White and Schmidt (1990), who found that synthetic

cytokinin applications did not have a consistent effect on turf quality or color. Seaweed extracts also contain cytokinins and the response on bermudagrass appears to be very similar with the two products.

Controlled Freezing

Cultivar Freeze Tolerance Across Seasons

The ANOVA showed a significant sampling date x temperature x cultivar interaction (Table 2.6). As temperature decreased from 4 to -7.2 ^{o}C, bermudagrass survival generally decreased (Table 2.7). Further, at all winter sampling dates, Midiron typically had the highest amount of survival/regrowth, especially following exposure to the colder temperatures. Riviera normally had the next highest amount of survival, followed by Tifway and finally Princess. These results are in agreement with previous work where Midiron was described as cold-tolerant. Midiron is followed by Riviera with moderate cold tolerance, Tifway somewhat cold susceptible, and Princess having poor cold tolerance (Shashikumar and Nus, 1993; Anderson et al., 2003).

Samples collected after fall 2001 and held at 4 ^{o}C had varying levels of survival, likely due to air temperatures in the field rather than the moderate refrigerator temperature. The first killing frost in 2001 occurred on 8 October and there were many subsequent days with temperatures dropping below 0 ^{o}C. However, with unseasonably warm temperatures occurring in November of 2001 (22.7 ^{o}C on the 2 November sampling date), a high amount of green color remained for all cultivars. Acclimation may have begun in October 2001, but with warmer temperatures in late October and early November, the acclimation process may have slowed. Although samples had

been subjected to some freezing temperatures in the month of October, these temperature events may only have been enough to partially harden field stolons and allow adequate survival at the moderately cold freeze-chamber temperatures of 4 and -2.8 ^{o}C. This is shown by high amounts of survival at both temperatures. If the samples

Table 2.6. Analysis of variance for post-freeze regrowth.

Source	df	*F* value
Rep	3	6.2**
Date	4	72.6**
Date x rep	12	3.2 **
Temp	3	1068.2**
Date x temp	12	74.5**
Error	45	
Cv	3	177.3**
Date x cv	12	25.8**
Temp x cv	9	14.8**
Date x temp x cv	36	2.7**
Error	180	
Trt	3	1.1
Date x trt	6	1.2
Temp x trt	9	0.7
Cv x trt	9	2.2*
Date x cv x trt	18	4.0**
Temp x cv x trt	27	0.4
Date x temp x trt	18	0.7
Date x temp x cv x trt	54	0.7
Error	432	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

were not completely hardened by the November 2001 sampling date, the colder freeze-chamber temperatures of -5.0 and -7.2 ^{o}C should have had a much greater effect on survival than the warmer temperatures. This was in fact what was found after freezing to -5.0 and -7.2 ^{o}C.

In 2002, the first killing frost did not occur until 2 November, and temperatures generally remained cool until the fall sampling date (Figure 2.1). This 3-wk period of temperatures dropping below freezing may have been enough to harden samples to a level not attained in 2001. Further, the summer of 2002 was very dry. If samples were

Table 2.7. Breakout of the sampling date x temperature x cultivar interaction for post-freeze regrowth of bermudagrass stolons across all chemical treatments.

Sampling		----------°C----------				
Date	Cultivar	4	-2.8	-5	-7.2	LSD[1]
		Regrowth (%)				
Fall 01	Midiron	100.0	60.0	29.0	3.8	19.4
	Riviera	100.0	43.8	1.3	0.0	23.4
	Tifway	100.0	33.8	1.8	0.0	17.4
	Princess	100.0	17.5	0.0	0.0	10.6
	LSD[2]	ns	34.1	16.2	3.8	--
Winter 02	Midiron	73.1	59.4	54.4	41.6	10.3
	Riviera	80.3	60.9	28.6	8.1	17.8
	Tifway	54.7	32.2	10.1	0.3	31.9
	Princess	36.3	25.6	1.6	0.0	10.7
	LSD[2]	41.8	35.2	20.8	14.6	--
Fall 02	Midiron	62.5	47.5	28.8	22.0	16.0
	Riviera	85.0	72.5	32.5	1.3	21.4
	Tifway	77.5	57.5	20.0	0.0	18.0
	Princess	72.5	65.0	14.0	0.0	19.3
	LSD[2]	18.5	ns	16.0	13.0	--
Winter 03	Midiron	74.1	68.1	40.6	21.3	15.4
	Riviera	79.4	55.0	27.8	4.2	17.5
	Tifway	55.6	19.7	5.4	0.0	21.4
	Princess	13.1	12.8	0.9	0.0	9.4
	LSD[2]	24.8	24.1	14.1	13.7	--
Summer 03	Midiron	72.2	0.0	0.0	0.0	19.7
	Riviera	97.5	0.0	0.0	0.0	2.3
	Tifway	96.9	0.0	0.0	0.0	2.5
	Princess	75.0	0.0	0.0	0.0	13.9
	LSD[2]	23.1	ns	ns	ns	--

[1]LSD = Comparison of cultivars within a temperature and sampling date.
[2]LSD =.Comparison of temperatures within a cultivar and sampling date.

Table 2.8. LSD comparisons for cultivars and temperatures across all dates in Table 2.7.

	°C			
Cultivar	4	-2.8	-5	-7.2
Midiron	24.5	13.3	37.0	19.5
Riviera	8.2	26.8	19.3	7.1
Tifway	36.6	30.6	14.2	0.4
Princess	16.6	23.0	13.5	0

exposed to drought stress for significant periods throughout 2002, bermudagrass stolons may have not been as fit entering the fall. This factor may further explain differences in survival between the two seasons at 4 and -2.8 °C (Table 2.7). The amount of survival after freezing to -5.0 and -7.2 °C in fall 2002 was much higher than in fall 2001. This indicates that the combination of the 3-wk period in early November and a gradual decline in temperatures throughout the fall were sufficient for acclimation. Gatschet et al. (1994) found that lethal temperatures were lowered 5 °C by acclimating bermudagrass for 4 wk at 8/2 °C day/night temperatures. The 3-wk period in November had a mean temperature of 6.7 °C, with a high of 20 and many lows below 0 °C.

Survival at lower temperatures was generally greater in winter than in fall of each year (Table 2.7). Clearly, acclimation was still occurring after the fall sampling dates. Anderson and Taliaferro (1995) subjected bermudagrass to freezing temperatures at monthly intervals throughout the fall and winter and found survival was higher in mid-winter than in November. There were generally no differences in survival between cultivars in winter 2002 and 2003 (Table 2.7 & 2.8). Table 2.8 shows the comparisons of cultivar and temperature across sampling dates. Air temperatures leading up to the winter 2002 sampling date had reached as low as -14.4 °C, which may have caused regrowth after 4 °C to be less than 100%. Temperatures leading up to the winter 2003

sampling date had reached as low as -16.9 ^{o}C, but due to the insulating effects of snow cover, soil temperatures were actually warmer in 2003 than in 2002, resulting in similar amounts of regrowth for both years.

The summer 2003 freezing episode revealed that chilling temperatures (4 ^{o}C) had differing effects on survival of unacclimated samples with Riviera having more regrowth, than Midiron (Table 2.7). Although seeded bermudagrasses are normally slow to establish, subsequent years after sowing show much greater stolon and rhizome development. This increased density in the summer may have allowed Riviera to become more efficient photosynthetically and to allow the storage of more NSC. Hensler et al. (1998) suggest and Munshaw et al. (2001) report that increased stolon diameter results in higher NSC levels. Although stolon diameters and NSC were not measured in the current study, they may play an important role in freezing survival. None of the cultivars survived even the most moderate of freezing temperatures. Anderson et al. (1988) found that freeze tests conducted on bermudagrass cultivars in June resulted in much less survival than during the winter.

Cultivar and Chemical Treatment Over Time

Sampling date, cultivar, and fall chemical treatment interacted on post-freeze regrowth (Table 2.9). Table 2.10 is shown to highlight differences in cultivars and chemical treatments across dates. As explained above, fall treatment effects were not tested in November of either year. In general, there was little effect of chemical treatment on bermudagrass survival across all temperatures and sampling dates, and no chemical treatment reduced survival relative to the control. Turfgrass managers

growing bermudagrass typically discontinue N use in late summer with the belief that late-season applied N will increase succulence and winter injury. Controlled freezing tests on cold-acclimated bermudagrass samples in this study indicated that, across all temperatures, N treatment did not affect regrowth of any cultivar. Richardson (2002)

Table 2.9. Breakout of sampling date x cultivar x fall chemical treatment interaction for post-freeze regrowth of bermudagrass stolons across all temperatures.

Sampling		Treatment				
Date	Cultivar	N	SWE	Fe	C	LSD[1]
		Regrowth (%)				
Nov/01	Midiron	--	--	--	48.2	--
	Riviera	--	--	--	36.3	--
	Tifway	--	--	--	33.9	--
	Princess	--	--	--	29.4	--
	LSD[2]	--	--	--	10.1	--
Jan/02	Midiron	61.6	69.4	38.1	59.4	30.9
	Riviera	53.0	35.0	46.9	43.1	ns
	Tifway	22.5	26.8	27.5	20.5	6.4
	Princess	17.5	18.8	13.1	14.1	ns
	LSD[2]	40.9	20.8	33.8	22.1	--
Nov/02	Midiron	--	--	--	40.2	--
	Riviera	--	--	--	47.8	--
	Tifway	--	--	--	38.8	--
	Princess	--	--	--	37.9	--
	LSD[2]	--	--	--	ns	--
Feb/03	Midiron	57.8	40.3	55.3	50.6	14.3
	Riviera	37.2	41.9	48.8	38.5	11.0
	Tifway	18.1	26.6	11.8	24.1	13.2
	Princess	5.3	7.8	8.1	5.6	ns
	LSD[2]	16.0	13.2	23.9	15.8	--
July/03	Midiron	17.5	18.4	17.5	18.8	ns
	Riviera	25.0	25.0	24.4	23.1	ns
	Tifway	25.0	25.0	23.8	23.1	ns
	Princess	17.5	20.0	18.1	19.4	ns
	LSD[2]	4.4	ns	ns	ns	--

[1]LSD = Comparison of chemical treatment within cultivar and sampling date.
[2]LSD = Comparison of cultivar within chemical treatment and sampling date.

froze late-season N-treated bermudagrass rhizomes to similar temperatures and did not find any negative effects on regrowth after freezing. Schmidt and Chalmers (1993) evaluated regrowth after controlled freezing and did not find any negative effects of late-season N applications. When equal amounts of N and K were applied, Gilbert and Davis (1971) did not find any negative affect on post-freeze regrowth. Although positive effects of late-season K fertilization in bermudagrass are inconsistent (Goatley et al., 1994: Miller and Dickens, 1996) it appears that as long as K is not limiting, it does not

Table 2.10. LSD comparisons for cultivars and chemical treatments across all dates in Table 2.9.

	Treatment			
Cultivar	N	SWE	Fe	C
Midiron	43.0	13.3	37.0	13.6
Riviera	21.7	11.1	16.4	10.6
Tifway	22.2	21.1	28.4	18.1
Princess	11.3	13.0	17.9	12.8

play a significant role in the process of bermudagrass cold hardiness (Richardson, 2002).

In this study, Fe-treated bermudagrass exhibited no differences in terms of post-freeze regrowth from the control, with the exception of Tifway in winter 2002 and Riviera in winter 2003 (α=0.10) (Table 2.9). Schmidt and Chalmers (1993) reported a 50 to 100% increase in regrowth with Fe treatment relative to the control. Although rates of Fe treatment were the same in both studies, differing Fe sources (Fe DTPA) were used. This may play a role in bermudagrass cold tolerance. Iron DTPA is a chelated form of Fe that has a longer greening effect than other sources (McCarty, 2001). Grasses

acquire Fe from the soil by excreting low molecular mass compounds called phytosiderophores. These compounds have a high affinity for Fe^{3+} and solubilize these ions and make them available for uptake (Buchanan, 2000). Schmidt (2003) explains that phytosiderophores may not be released from roots when bermudagrass is exposed to cool fall temperatures. Because of the reduced release of phytosiderophores in cool weather, non-chelated forms of Fe are being oxidized and are unavailable for plant uptake. This may explain differences in Fe response between the present study and previous studies.

Schmidt and Chalmers (1993) observed improved bermudagrass post-freeze regrowth folloowing applications of SWE fortified with humic acid and thiamine. In the present study, SWE alone did not influence post-freeze regrowth (with the exception of Tifway in winter 2002 [α=0.10]), indicating that the positive results seen by Schmidt and Chalmers may have been due to humic acid, thiamine, or interactions of the three products.

Fatty Acid Analysis

The absolute amounts of key fatty acids in total polar lipids (mainly phospholipids) for each cultivar are shown in Table 2.11. With the exception of fall 2001, individual fatty acid levels were generally fairly constant. Palmitic and stearic acids have been previously reported (Samala et al., 1998; Cyril et al., 2002) to remain somewhat constant during cold acclimation treatments. In the current study, it was found that there were variations in these fatty acids during different times of the year. However, trends were not consistent and did not hold for all cultivars.

It has also been reported (Samala et al., 1998) that linoleic acid levels decrease and linolenic acid levels increase in bermudagrass during cold acclimation. In the current study, only one sampling date occurred during the "acclimation" period each fall and thus differences over time during hardening in these two fatty acids cannot be corroborated. However, there did not appear to be reductions in linoleic acid levels at other times of the year when linolenic acid levels were high, which may indicate that

Table 2.11. Summary of the absolute amounts of the four major fatty acids in total polar lipids of untreated (control) Midiron, Riviera, Tifway, and Princess at all sampling dates.

		Fatty Acid (μg g^{-1})[z]			
Cultivar	Sampling Date	Linolenic	Linoleic	Palmitic	Stearic
Midiron	Fall 2001	115.2 a	204.0 a	329.4 a	47.7 a
	Winter 2002	60.6 ab	135.0 ab	198.4 b	40.5 a
	Fall 2002	39.4 b	85.5 b	124.9 b	19.8 a
	Winter 2003	83.5 ab	149.7 ab	169.8 b	25.0 a
	Summer 2003	63.3 ab	123.7 ab	116.7 b	27.3 a
Rivera	Fall 2001	109.3 a	222.1 a	309.5 a	53.2 a
	Winter 2001	33.0 b	96.7 b	126.5 bc	32.4 b
	Fall 2002	35.4 b	82.5 b	172.1 b	19.8 bc
	Winter 2003	30.5 b	68.8 b	85.0 c	12.6 c
	Summer 2003	34.5 b	84.2 b	107.5 bc	13.0 c
Tifway	Fall 2001	44.6 a	113.1 a	160.8 a	39.4 a
	Winter 2001	25.2 a	100.9 a	140.3 a	36.1 ab
	Fall 2002	24.4 a	107.8 a	245.6 a	25.0 abc
	Winter 2003	40.6 a	107.9 a	131.1 a	17.3 c
	Summer 20	31.7 a	95.9 a	175.7 a	22.7 cb
Princess	Fall 2001	86.4 a	186.6 a	299.9 a	51.6 a
	Winter 2002	34.4 b	64.0 b	104.3 d	21.6 b
	Fall 2002	27.0 b	87.3 b	187.4 bc	26.6 b
	Winter 2003	42.4 b	102.1 b	224.6 b	23.7 b
	Summer 2003	37.5 b	79.9 b	141.2 cd	19.5 b

[z]Values followed by the same letter within the same column and cultivar are not significantly different ($P<0.05$).

triunsaturated fatty acids may not occur at the expense of diunsaturated fatty acids. However, as linolenic acid has been shown in the literature to be very important in cold hardiness, fatty acid levels will be presented in terms of the percent linolenic acid to all fatty acids measured, or the fraction of linolenic acid to total polar lipids.

The ANOVA table is shown in Table 2.12. In the current study, N and Fe were not different from the control even though they increased visual quality. This suggests that these treatments did not have an effect on bermudagrass cold tolerance via a lipid-mediated mechanism.

Table 2.12. Analysis of variance table for fraction of linolenic acid to total polar lipids.

Source	df	*F* value
Year	1	10.8**
Error	6	
Sampling date	1	3.8
Year x sampling date	1	65.5**
Error	6	
Cv	3	22.9**
Year x cv	3	0.5
Year x sampling date x cv	6	3.4**
Error	36	
Trt	3	0.2
Sampling date x trt	3	0.3
Cv x trt	9	1.4
Sampling date x cv x trt	9	2.0*
Year x trt	3	0.9
Year x cv x trt	9	0.9
Year x sampling date x trt	3	1.3
Year x sampling date x cv x trt	9	1.6
Error	144	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Cultivar Differences Over Dates

There was a year x sampling date x cultivar interaction for percentage of linolenic acid to total polar lipids (Table 2.13). Cultivar differences in percentage of linolenic acid in total polar lipids showed that cold-tolerant cultivars generally had higher levels than cold-sensitive cultivars. Although linolenic acid level differences were not always significant, Midiron consistently had the highest levels, followed by Riviera, Princess, and Tifway. Previous studies have shown that linolenic acid levels increase during cold acclimation and increase to a greater degree in cold-tolerant than cold-sensitive cultivars (Samala et al., 1998; Cyril et al., 2001; Cyril et al., 2002). Linolenic acid levels were high in fall 2001, but dropped in winter 2002. Although it was expected that samples would have as high or higher levels of linolenic acid during this time, air

Table 2.13. Breakout of year x sampling date x cultivar interaction for percent linolenic acid across chemical treatments.

		-------Sampling Date------		
Year	Cultivar	Fall	Winter	LSD[1]
		Linolenic Acid (Percent of Total)		
2001-2	Midiron	17.4	12.8	0.8
	Riviera	14.0†	12.2	2.4
	Tifway	12.8†	10.6	1.8
	Princess	15.4†	13.4	7.2
	LSD[2]	1.6	2.6	--
2002-3	Midiron	13.6	18.7†	4.6
	Riviera	9.9	14.2	1.3
	Tifway	7.0	12.1	3.5
	Princess	9.3	12.2	3.6
	LSD[2]	2.7	2.5	--

[1]LSD = Comparison of sampling date within a cultivar and year.
[2]LSD = Comparison of cultivars within a date and year.
†Values followed by a dagger are significantly higher within cultivar and between years ($P<0.05$).

temperature changes in January may at least partially explain this difference. Air temperatures leading up to the sampling date were cool, averaging around 0 to 5 ^{o}C. Several days before sampling, however, the air temperature increased to 10 to 15 ^{o}C and may have had a short-term effect on the degree of lipid saturation. Further, Beard (1973) explains that grasses experience their highest level of hardiness in early winter and may experience a dehardening period in mid- to late-winter. Another possible explanation for high linolenic acid levels in fall 2001 is that samples had much better color than in fall 2002 (Table 2.3). Although quality was not rated in the winter months, plots of all cultivars were completely brown. A greater amount of color would equate to greater amounts of chlorophyll in bermudagrass stolons. Salisbury and Ross (1992) explain that chloroplast pigments encompass 50% of the thylakoid membrane and the fatty acid portion of this membrane has high levels of both linolenic and linoleic acid. Thus, greater amounts of stolon chloroplasts in November 2001 could have resulted in higher linolenic acid levels.

High levels of linolenic acid did not necessarily result in greater amounts of survival following controlled freezing. Although it was found that cold-tolerant cultivars had higher levels of linolenic acid than cold-sensitive cultivars, differences within cultivars and between dates could not be correlated with survival. Riviera, Tifway, and Princess all had higher levels of linolenic acid in fall 2001 than fall 2002, however, there were no differences in survival (across all temperatures) between these dates (Table 2.9 & 2.13). In winter 2003, a significant regrowth x percent linolenic acid correlation ($r=0.28^{**}$) existed at 4 ^{o}C. Percent linolenic acid ($r=0.39^{**}$) correlation at -2.8 ^{o}C was significant. Percent linolenic acid correlation at -5.0 ^{o}C ($r=0.37^{**}$) and -7.2 ^{o}C ($r=0.20^{*}$)

were also significant. But, as can be seen, lipid unsaturation only explains 20 to 37% of regrowth—obviously, many other factors are involved in bermudagrass cold hardiness.

Cultivar and Dates in Season 2

Samples collected in summer were compared during the second season of the study. Variance was uniform for linolenic acid analysis and the ANOVA table is shown in Table 2.14. The main effects of date and cultivar were significant and are presented together in Table 2.15. Linolenic acid levels were higher in winter 2003 than in fall 2002. However, post-freeze survival was generally not different in winter and fall (Table 2.9). Bermudagrass samples removed from the field in winter would naturally be more acclimatized than samples removed in fall and thus should have higher levels of survival. Temperature data shows that a low temperature of -3.5 ^{o}C occurred prior to sampling in fall, while samples removed in winter had been subjected to nearly -17 ^{o}C.

Table 2.14. Analysis of variance table for fraction of linolenic acid to total polar lipids during the second year of the study (2002-03).

Source	df	*F* value
Rep	3	0.2
Sampling season	2	30.2**
Sampling season x rep	6	0.7
Cv	3	22.5**
Sampling season x cv	6	0.6
Error	27	
Trt	3	1.2
Sampling season x trt	6	0.5
Cv x trt	9	1.5
Sampling season x cv x trt	18	0.6
Error	108	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Even with winter samples being more acclimated and having greater amounts of linolenic acid than fall samples, -17 ^{o}C is very cold for bermudagrass and likely reduced survival levels and offset some of the benefits of full acclimation.

Table 2.15. Sampling date and cultivar main effects for percentage of linolenic acid to total polar lipids during the second year of the study (2002-03).

	Sampling date			
Cultivar	Fall	Winter	Summer	LSD[1]
	Linolenic Acid (Percent of Total)			
Midiron	13.6	18.7	18.1	3.4
Riviera	9.9	14.2	15.7	2.8
Tifway	7.0	12.1	11.7	5.0
Princess	9.3	12.2	14.8	3.9
LSD[2]	2.7	2.5	4.6	--

[1]LSD = Comparison of cultivar within date.
[2]LSD = Comparison of date within cultivar.

The high linolenic acid levels in summer were unexpected. As previous work in bermudagrass has shown an increase in unsaturation during the fall cold acclimation period, pre-acclimation levels were expected to be low. However, as air temperatures and light intensity during the summer were conducive to high bermudagrass growth, chloroplast pigments were most likely at higher levels than in other times of the growing season. As was explained above, thylakoid membranes contain high levels of linolenic and linoleic acids (Salisbury and Ross 1992). Although chloroplasts were not examined per se in the present study, they would have contributed significantly to sampled tissues mainly in the summer sampling period. The presence of the unsaturated fatty acids in the thylakoid membrane causes a higher level of fluidity (Salisbury and Ross, 1992).

Proline Concentration

Analysis of variance for proline concentration is shown in Table 2.16. All data were log-transformed before analysis of variance. Year, date, cultivar, and chemical treatment interacted. The complexity of tabulating a four-way interaction is such that the data were divided into two tables. Table 2.17 shows the three-way interaction of sampling date x cultivar x chemical treatment within the first year of the study. Table 2.18 again shows the three-way interaction of sampling date x cultivar x chemical treatment but for the second year. Differences between years will be presented textually.

Table 2.16. Analysis of variance for proline concentration.

Source	df	*F* value
Year	1	15.7**
Error	6	
Month	1	44.6*
Year x sampling date	1	85.5**
Error	6	
Cv	3	48.0**
Sampling date x cv	3	2.6
Year x sampling date x cv	6	4.8**
Error	36	
Trt	3	4.4**
Sampling date x trt	3	4.3**
Cv x trt	9	3.5**
Sampling date x cv x trt	9	1.7
Year x trt	3	11.6**
Year x sampling date x trt	3	2.4
Year x cv x trt	9	1.6
Year x sampling date x cv x trt	9	5.3**
Error	144	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Table 2.17. Breakout of sampling date x chemical treatment x cultivar interaction for proline concentration in the first year of the study (2001-2002).

Sampling Date	Cultivar	Treatment N	Treatment SWE	Treatment Fe	Treatment C	LSD[1]
		Proline Concentration (µg g^{-1})				
Fall	Midiron	168.4	1438.8†	1224.1	693.3	747.9
	Riviera	456.9†	342.5	220.4	550.5	ns
	Tifway	565.1†	934.0†	413.6	219.4	472.7
	Princess	243.0	541.5†	298.4	183.8	162.1
	LSD[2]	180.4	458.5	527.1	ns	--
Winter	Midiron	416.5	402.2	1521.0	167.9	794.8
	Riviera	103.7	323.4	54.3	43.2	258.6
	Tifway	47.9	49.0	360.5	36.3	178.8
	Princess	82.2	85.6	116.1	19.3	84.8
	LSD[2]	227.6	290.1	761.8	143.0	--

[1]LSD = Comparison of cultivar within chemical treatment and sampling date.
[2]LSD = Comparison of chemical treatment within cultivar and sampling date.
†Values followed by a dagger are significantly higher within cultivars and between dates ($P<0.05$).

Table 2.18. Breakout of sampling date x chemical treatment x cultivar interaction for proline concentration in the second year of the study (2002-2003).

Sampling Date	Cultivar	Treatment N	Treatment SWE	Treatment Fe	Treatment C	LSD[1]
		Proline Concentration (µg g^{-1})				
Fall	Midiron	591.7	624.9	524.8	623.3	ns
	Riviera	286.0	381.2†	411.8	444.4	ns
	Tifway	141.3	160.5	146.4	222.3	ns
	Princess	146.4	76.1	140.8	73.4	57.2
	LSD[2]	255.7	353.7	234.8	296.9	--
Winter	Midiron	325.2	504.6	878.0	1204.2	486.8
	Riviera	1303.7†	139.8	404.3	652.5	731.8
	Tifway	451.8	122.5	340.9	214.3	208.3
	Princess	155.1	364.8	175.0	136.6	ns
	LSD[2]	669.8	325.7	475.5	499.8	--

[1]LSD = Comparison of cultivar within chemical treatment and sampling date.
[2]LSD = Comparison of chemical treatment within cultivar and sampling date.
†Values followed by a dagger are significantly higher within cultivars and between dates ($P<0.05$).

Cultivar and Chemical Treatment Differences Over Time

Generally, proline concentration varied with cultivar (Table 2.17 & 2.18). Midiron normally had the highest levels, followed by Riviera, Tifway, and Princess. This finding is significant as proline concentration among cultivars follows the same trend as was shown in post-freeze regrowth (Table 2.9). Most of the previous literature has looked at how late-season fertility affects bermudagrass carbohydrate concentrations and has drawn conclusions on cold tolerance based on these data. There are, however, many likely physiological differences in cold-tolerant and cold-sensitive bermudagrasses including lipid unsaturation (Samala et al., 1998) and cellular proline concentration (Munshaw et al., in press). Proline studies indicate that cold-tolerant cultivars had higher levels than cold-sensitive cultivars. As was shown in maize, cellular proline concentrations increase during cold acclimation (Chen and Li, 2002). As Rossi (1997) points out, an increase in cellular proline concentrations can have a large impact on osmotic adjustment during freezing events, decreasing the possibility of cytoplasm dehydration, extracellular ice formation, and cell rupture.

Increases in proline content should allow cells to remain intact during cold stress and continue functioning. During mid-winter controlled freeze tests on bermudagrass samples, a high concentration of cellular proline was correlated with amount of regrowth after freezing. This result agrees with Munshaw et al. (in press), who showed that more regrowth was evident after freezing when high cellular proline levels were present.

Samples tested in fall 2002 showed a significant proline x regrowth correlation ($r=0.46^{**}$) at -7.2 ^{o}C. In winter 2003, proline x regrowth ($r=0.29^{**}$) at -2.8 ^{o}C was

significant as well as at -5.0 ^{o}C (r=0.36**). There was also a significant proline x regrowth (r=0.38**) correlation at -7.2 ^{o}C.

In the present study, proline concentration was not consistently or significantly affected by Fe, SWE, or N (Tables 2.17, 2.18, & 2.20). There was, however, a large difference in terms of proline concentration between samples that were assumed to be non-acclimated (fall) and acclimated (winter). Munshaw et al. (in press) found that increasing proline concentrations in bermudagrass due to moderate salt applications was correlated with increased regrowth after freezing. Because no previous research has examined the effect of N, SWE, and Fe treatments on proline levels in bermudagrass, correlations must be drawn to the studies that have found very similar results showing no effects of these treatments on carbohydrate concentrations (White and Schmidt, 1990; Goatley et al., 1994; Richardson, 2002). Although fall quality may be affected by these chemical treatments, they appear to have no influence on physiological parameters measured in this study, as well as others.

Cultivar and Chemical Treatment Differences in Year 2

Proline concentrations were assessed in the summer during the second season of the study. Date, cultivar, and fall chemical treatment interacted (Table 2.19). As was mentioned above, cold-tolerant cultivars generally had higher proline levels during the fall and winter (Table 2.20). Midiron again consistently had the highest proline levels, followed by Riviera, Tifway, and Princess.

Table 2.19. Analysis of variance table for proline concentration during the second year of the study (2002/3).

Source	df	*F* value
Rep	3	2.2
Sampling date	2	89.4**
Error	6	
Cv	3	24.9**
Sampling date x cv	6	9.4**
Error	27	
Trt	3	1.0
Sampling date x trt	6	2.3
Cv x trt	9	1.6
Sampling date x cv x trt	18	2.9**
Error	108	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Table 2.20. Sampling date x cultivar x chemical treatment interaction for proline concentration during the second year of the study.

Sampling		----------Treatment----------				
Date	Cultivar	N	SWE	Fe	C	LSD[1]
		Proline Concentration (μg g^{-1})				
Fall	Midiron	591.7	624.9	524.8	623.3	ns
	Riviera	286.0	381.2	411.8	444.4	ns
	Tifway	141.3	160.5	146.4	222.3	ns
	Princess	146.4	76.1	140.8	73.4	57.2
	LSD[2]	255.7	353.7	234.8	296.9	--
Winter	Midiron	325.2	504.6	878.0	1204.2	486.8
	Riviera	1303.7	139.8	404.3	652.5	731.8
	Tifway	451.8	122.5	340.9	214.3	208.3
	Princess	155.1	364.8	175.0	136.6	ns
	LSD[2]	669.8	325.7	475.5	499.8	--
Summer	Midiron	105.0	66.0	82.2	91.2	ns
	Riviera	67.0	72.3	62.5	73.9	ns
	Tifway	96.5	71.3	97.8	97.8	ns
	Princess	71.6	132.1	92.5	89.9	57.1
	LSD[2]	ns	58.7	ns	ns	--

[1]LSD = Comparison of chemical treatment within cultivar.
[2]LSD = Comparison of cultivar within chemical treatment.

Chemical treatment effects were again insubstantial during the second year of the study. The interesting result during the second season was that proline concentrations were much reduced in summer for all cultivar and chemical treatment combinations over samples taken in fall or winter. Post-freeze survival in summer showed similar trends to proline concentrations, in that survival was also much reduced during the summer (Table 2.7). It appears that high levels of lipid unsaturation are required during all times of the year to allow cellular membranes to remain fluid. However, because high levels of lipid unsaturation alone do not fully explain differences in cold-tolerance, other factors such as proline concentration, NSC level, and perhaps many other physiological processes must act in concert to achieve cold-tolerance. This is shown in samples collected in July that had poor freeze tolerance (Table 2.7), high lipid unsaturation (Table 2.15), and low proline concentration (Table 2.20).

Spring Greenup

Percent greenup was rated on three dates in 2002 and 2003. Analysis of variance for visually estimated greenup data is shown in Table 2.21. Data were transformed to the arcsine of the square root before analysis of variance.

Cultivar Differences Over Time

The breakout of year x date x cultivar showed the expected result of increased greenup as the spring progressed (Table 2.22). Midiron and Riviera had greater amounts of greenup early in the spring and across all observation dates than Tifway and Princess. In 2002, Riviera and Midiron were 20 to 25% greener than Tifway and 55

Table 2.21. Analysis of variance for spring greenup.

Source	df	*F* value
Year	1	5414.6**
Error	6	
Observation date	3	3599.2**
Year x observation date	2	2.4
Error	15	
Cv	3	3655.3**
Observation date x cv	9	139.0**
Year x cv	3	46.5**
Year x observation date x cv	6	45.5**
Error	63	
Trt	3	3.1*
Observation date x trt	9	0.6
Cv x trt	9	2.7**
Observation date x cv x trt	27	1.2
Year x trt	3	3.1*
Year x observation date x trt	6	0.6
Year x cv x trt	9	0.3
Year x observation date x cv x trt	18	0.5
Error	252	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Table 2.22. Breakout of year x date x cultivar interaction for spring greenup.

		----------Observation Date----------			
Year	Cultivar	28 April	15 May	1 June	LSD[1]
		Greenup (%)			
2002	Midiron	65.3†	100.0†	100.0†	3.6
	Riviera	72.5†	100.0†	100.0†	5.7
	Tifway	45.0†	74.1†	95.6†	10.1
	Princess	9.9†	24.8†	56.3†	13.7
	LSD[2]	10.7	13.2	13.2	--
2003	Midiron	41.6	89.4	95.9	8.4
	Riviera	58.1	87.0	95.0	9.2
	Tifway	9.4	49.6	63.1	8.9
	Princess	0.2	12.5	14.8	7.4
	LSD[2]	12.4	10.6	14.6	--

[1]LSD = Comparison of observation date within cultivar within year.
[2]LSD = Comparison of cultivar within observation date within year.
†Values followed by a dagger are significantly higher within cultivars and between years ($P<0.05$).

to 75% greener than Princess on 28 April and 15 May (Table 2.22). On 1 June, Riviera, Midiron, and Tifway all had higher percentage greenup than Princess.

In 2003, Riviera had statistically more percentage greenup at the first observation date (28 April) than all other cultivars (Table 2.22), and Midiron had more than Tifway and Princess. Midiron and Riviera had higher amounts of greenup on 15 May and 1 June than Tifway and Princess, and Tifway had more greenup than Princess.

Differences across years showed that all cultivars had higher amounts of greenup at all observation dates in 2002 than in 2003. Daily means in the winter of 2003 were 5 ^{o}C cooler on average than in 2002 (Fig. 2.1). Temperatures in general, were cooler during the winter of 2003 and were likely a factor in greenup differences between years. The previous statement can be made with some confidence due to the fact that the cold-sensitive cultivars were affected by the colder temperatures to a greater degree than the cold-tolerant cultivars. Midiron showed a reduction of 36% in April 2003, while Riviera was only reduced 20%. Tifway (somewhat cold-sensitive) showed a 79% reduction in greenup in 2003, and Princess (cold-sensitive) was reduced 98%.

In both years of the study, the cold-tolerant cultivars Midiron and Riviera rejuvinated earlier and more quickly than the cold-sensitive cultivars Tifway and Princess. Clearly, a large percentage of Princess was lost to winterkill in both years of the study. Munshaw and Ervin (2003) reported that, in the transition zone, Riviera had the best spring greenup of all cultivars tested in the 1997 NTEP trials. The NTEP trial also showed that Tifway had significantly worse greenup than Riviera, and Princess was

Table 2.23. Breakout of year x chemical treatment interaction for spring greenup across all cultivars.

	Treatment				
Year	N	SWE	Fe	C	LSD[1]
	Greenup (%)				
2002	68.6	70.8	70.5	71.2	2.4
2003	39.2	38.6	41.3	39.0	1.5
LSD[2]	ns	ns	ns	ns	--

[1]LSD = Comparison of chemical treatment within year.
[2]LSD = Comparison of year within chemical treatment.

slower to greenup than Tifway (Morris, 2002). These results are identical to the findings in the current study as Midiron and Riviera had better greenup than Tifway and Princess.

Chemical Treatment Effects Over Seasons

There was a significant year x chemical treatment interaction (Table 2.23). However, although statistically there were differences, in practical terms these differences are not biologically significant. In 2002, N treatments resulted in slower/less greenup than control plots. In 2003, Fe-treated plots produced more greenup than all other treatments. Although there was a nearly 30% difference due to N treatment, no statistical differences were noted in greenup due to chemical treatment between years. This difference for N is likely due to large cultivar differences between years. In spring 2002, a positive response of the treatments was not evident during greenup. Goatley et al. (1994) did not see an effect on spring greenup with late-season applied Fe, even with rates in excess of 4 kg ha^{-1}. White and Schmidt (1990), however, saw enhanced recovery after dormancy with fall-applied Fe at 1.2 kg ha^{-1}. Richardson (2002) as well as Schmidt and Chalmers (1993) found that fall N applications promoted early spring greenup. In the current study, survival after controlled freezing was not affected by N

treatment during either winter (Table 2.9). The reduction in greenup of N treated cultivars in 2002 may have been an anomaly and was a very slight difference. As this negative effect was not noticed in 2003, supplementary research would need to be conducted examining the effect of N on spring greenup.

Chemical treatment did not affect greenup of Midiron, Riviera, and Tifway (Table 2.24). Princess control plots had greater amounts of greenup than N treated plots. These data suggest that greenup, in general, is also genetically controlled. Cultivars that are able to prepare for winter better than others by increasing lipid unsaturation and/or proline concentration, are the cultivars that begin greenup earlier in the spring and are less affected by winterkill.

Table 2.24. Breakout of cultivar x chemical treatment interaction for spring greenup across all observation dates and both years.

	Treatment				
Cultivar	N	SWE	Fe	C	LSD[1]
	Greenup (%)				
Midiron	72.3	71.5	73.4	71.6	3.0
Riviera	72.9	73.9	74.4	73.2	1.7
Tifway	48.3	48.7	48.2	47.5	1.9
Princess	13.9	15.5	19.3	19.1	3.6
LSD[2]	4.8	4.6	4.7	4.5	--

[1]LSD = Comparison of chemical treatment within cultivar.
[2]LSD = Comparison of cultivar within chemical treatment.

Summary

In general, cultivars decreased in quality as the fall progressed. Riviera, Tifway, and Midiron had similar quality through October, and all were better than Princess. Princess, however, retained its color better than other cultivars in November. Survival

after freezing showed that Midiron was superior than all other cultivars. This was not surprising as Midiron has been shown in the literature to be very cold hardy. Midiron also consistently had greater amounts of linolenic acid and proline than the other cultivars. These high levels, and Midiron's ability to survive controlled freezing equated to earlier and faster greenup than Tifway and Princess. Riviera was second to Midiron in post-freeze survival, which was supported by high levels of proline during the winter. Riviera also had very good greenup. Tifway followed Riviera in terms of regrowth after freezing, proline concentration, and greenup. Princess generally had lower values for all of these parameters.

Proline concentration appears to be associated very closely with cold hardiness. Higher proline levels consistently resulted in the greatest amount of survival after freezing and the ability to green up early and rapidly. Summer proline levels were also very low. As freezing survival was very low during the summer, it appears that high proline levels are very important in the survival of bermudagrass over the winter.

Lipid unsaturation did not appear to be as linked to freeze survival or greenup as proline. Although linolenic acid levels were high for Midiron, results were highly variable for the other cultivars, and were not closely associated with survival or greenup. Previous literature has suggested that lipid unsaturation is higher in cold-tolerant cultivars. Results generated in this study do not support nor dispel previous findings, with the exception that the thylakoid membrane contains high levels of linolenic acid. Findings in the summer showed very high linolenic acid levels, presumably due to the high amount of chlorophyll present in the plants.

Fall chemical treatments had little effect on bermudagrass cold-tolerance. Nitrogen applications marginally increased color over the control during the fall. This was expected as N is an important component of the chlorophyll molecule. Nitrogen did not affect freeze survival, proline concentrations, or lipid unsaturation. The effect on spring greenup was slightly negative in 2002 and non-existent in 2003. These results indicate that prolonged fall color has little, if any, effect on bermudagrass cold-tolerance. Seaweed extract and Fe also had little effect on all parameters measured. Although it was believed that SWE may reduce the senescence process during the fall and prolong green color, this result was not observed. Iron applications have been suggested to prolong green color during the fall as it too is a component of the chlorophyll molecule. Few differences were noted due to Fe treatment over control plots with the exception of increased greenup in 2003.

CONCLUSIONS

Late-season N applications can have a beneficial effect on bermudagrass color in the fall without negatively affecting cold tolerance. This study confirms this fact and even reverses conventional wisdom that has been published in textbooks (Beard, 1973; Dubel, 1989). Controlled freezing tests showed little effect of N on survival after freezing. Proline concentration and fatty acid unsaturation levels were also unaffected by late-season N applications, again indicating that N fertilization may not cause weaker plants that are more prone to winter injury. No beneficial effect of greenup was noticed with N applications.

The objective to increase the growing season with SWE was not achieved. Seaweed extract applications did not show any differences from control plots in terms of turfgrass quality late in the growing season. There were also no consistent effects on post-freeze regrowth, proline concentration, or lipid unsaturation. Seaweed extract also did not show any effect on spring greenup.

Iron treatments were able to increase turfgrass quality over the control but only late in the growing season. This is a beneficial response, as a prolonged green period was the objective. Late-season Fe treatments had no consistent effect on post-freeze regrowth, proline concentration, or lipid unsaturation. There was, however, a beneficial response of Fe on spring greenup in 2003.

Bermudagrass cultivars vary tremendously in terms of quality, recuperability, and cold tolerance. Although previous research has shown differences between these cultivars in terms of cold tolerance, no physiological explanations have been offered. These results show that cultivars that are known to be cold tolerant produce higher

levels of linolenic acid and proline during fall and winter months. Results from 2002-2003 showed correlations between levels of linolenic acid and proline and regrowth after freezing. This suggests the importance of enhanced levels of these compounds during the winter. However, based on the chemical treatments examined in this study, it appears that proline and lipid unsaturation are genetically controlled and are generally not affected by N, Fe, or SWE. Cold-tolerant cultivars also showed much faster and earlier greenup than cold sensitive cultivars in the spring. It seems logical to conclude that cultivars less affected by stress during fall and winter break dormancy in much better shape physiologically than cold sensitive cultivars.

Recommendations for turfgrass managers in the transition zone based on data generated in this study are the use of cold-tolerant cultivars such as Midiron or Riviera that exhibit good quality during the summer and fall. The use of these cultivars is also important to maximize the likelihood of winter survival. However, because Rivera is propagated by seed, it represents a step forward in bermudagrass utilization. Seeded cultivars are much easier to establish than vegetative cultivars as live plant material is not necessary. Renovation after a particularly cold winter can also be performed with greater ease and less time requirement than a vegetative cultivar. Also, judicial N applications throughout the entire growing season along with maintaining sufficient soil K levels can prolong the growing season. Lengthening the period of greenness through N applications will increase turf use and potential revenue for golf courses and athletic fields. Iron applications may also prolong fall color and increase spring greenup.

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CHAPTER III

EFFECTS OF LATE-SEASON ETHEPHON APPLICATIONS ON BERMUDAGRASS SENESCENCE AND COLD TOLERANCE

INTRODUCTION

Ethylene levels increase in plants in response to stress. Ethylene causes, among other things, a reduction in chlorophyll content and leads to leaf senescence. The PGR ethephon releases ethylene once inside the plant. It is used in turfgrass maintenance programs, mainly on C_3 species, for clipping suppression without visual quality loss. Ethephon treatments on turfgrasses have been shown to have negative effects on turfgrass quality if applied too frequently. Studies on fruit trees have shown that applying ethephon increases cold hardiness and may increase lipid unsaturation. Because no research has looked at the effects of late-season ethephon on bermudagrass cold-hardiness, the following hypotheses were tested:

1. Ethephon applications will cause early leaf senescence.
2. Ethephon applications will increase the degree of lipid unsaturation and/or proline concentration, thereby improving bermudagrass cold hardiness.

Applications of ethephon during the fall may increase bermudagrass cold-tolerance with little cost to turfgrass managers and minimal effect on the environment. Ideally, ethephon will work in conjunction with new bermudagrass cultivars to reduce the threat of winterkill.

MATERIALS AND METHODS

Plant Material, Establishment, and Experimental Design

A field study was conducted at the Virginia Tech Turfgrass Research Center in Blacksburg, VA. Plots were established on a Groseclose silt loam (fine, kaolinitic, mesic typic Hapludult) with a pH of 6.8 and a K level of 59 mg kg^{-1}. Plots were 3.1 x 9.1 m and established 20 June 2001 using four bermudagrass cultivars: 'Tifway', 'Midiron', 'Princess', and 'Riviera'. Princess seed was supplied by Dr. Charles Rodgers (Seeds West Inc., Maricopa, AZ), and Riviera seed was supplied by Dr. Charles Taliaferro (Oklahoma State University, Stillwater, OK). Midiron and Tifway sprigs were supplied by the Virginia Tech Turfgrass Research Center. Seeding rates were 48.8 kg pure live seed (PLS) ha^{-1}, and sprigging rates were 1800 bu ha^{-1}.

Seeded plots were planted under a Remay cover to discourage runoff and to enhance germination and plant development. Plots were mowed three times per week with a reel mower set at 1.91 cm. Nitrogen was applied in the form of NH_4NO_3 (34-0-0) once monthly at a rate of 48.8 kg N ha^{-1} beginning at establishment and ending 15 August. A complete fertilizer (10-10-10) was applied the following spring (25 May) at a rate of 48.8 kg N ha^{-1}, and monthly N applications began again on 15 June 2002. Irrigation was supplied as needed.

After bermudagrass establishment, ethephon treatment was applied during the fall. Three chemical treatments were applied during the period leading to bermudagrass senescence. A non-treated control was also included within each bermudagrass cultivar.

Fall Chemical Treatments

Fall chemical treatments began 15 August in 2001 and 2002 and continued on a 3-wk schedule until apparent dormancy (80 to 100% canopy browning). Final treatment dates were applied 17 October 2001 and 31 October 2002. Seaweed extract was applied at a rate of 0.54 kg ha^{-1} (Zhang et al., 2002); N was applied at a rate of 48.8 kg N ha^{-1} as NH_4NO_3 (White and Schmidt, 1990); Fe was applied at a rate of 1 kg Fe ha^{-1} as $FeSO_4$ (Schmidt and Chalmers, 1993). Ethephon was applied at the recommended label rate of 16 l ha^{-1}.

The experimental design was a randomized complete block with four replications. The study was conducted during the 2001-02 growing season and repeated during the 2002-03 growing season. Ethephon treatments were arranged in a split-split plot design. Chemical treatments were arranged in a split-split-split plot design. In cases where sampling times were considered important treatment effects (i.e., fall versus winter or early bermudagrass greenup versus late greenup), sampling times were considered main plots. Where measurements were taken only once or sampling times were not considered treatments effects, bermudagrass cultivars served as main plots. Ethephon treatments (+/-) were subplots. Late-season chemical treatments consisted of N, Fe, SWE, and none and were subsubplots. In some cases, controlled freezing of bermudagrass samples was conducted to elicit measurable responses. Various freezing temperature regimes were considered subordinate to sampling dates but superior to cultivars in the split-split plot design. The following discussion will explain the techniques used to collect and statistically analyze experimental data.

Fall Quality and Spring Greenup

Visual turf quality ratings were taken (using a 1 to 9 scale, where 1 = brown dormant or dead turf, and 9 = lush green turf) monthly during fall treatments. Spring greenup was visually estimated as a percent of green ground cover.

Data were analyzed using PROC GLM of the SAS statistics package (SAS, 1989), and data were tested for homogeneity of variance by plotting residuals before statistical analysis. To comply with the assumptions of analysis of variance, visual ratings were transformed by the arcsine of the square root (Ott and Longnecker, 2001). However, actual means are presented textually and in tables. Year was considered a random variable and main effects and interactions were tested using the mean square associated with the random variable (McIntosh, 1983). Rating times were considered main plots, cultivars were subplots, ethephon treatments were subsubplots, and chemical treatments were subsubsubplots. Where year effects were significant, data were separated by year; otherwise, data were pooled. Appropriate main effects and interactions were separated using Fishers Protected LSD test at P=0.05.

Controlled Freezing

Freeze chamber analyses were performed on putatively acclimating (fall) and acclimated (winter) bermudagrass samples in 2001-02 and 2002-03. Additional analysis was conducted on samples collected in summer 2003. During November of both years due to the freezing process being time consuming and possible physiological differences occurring in samples between the first and last tests of each sampling

period, only “control” plots were sampled. Samples were analyzed from all plots in winter each year and summer 2003. A 10.2-cm (diameter) cup cutter sample was removed from each plot, cleaned of soil by washing, and divided into four equal subsamples. One of the subsamples was placed in a refrigerator and held at 4 ^{o}C to act as a “control”. The other three subsamples were placed in a freeze chamber that ramped from 8 to 1 ^{o}C overnight. The temperature then ramped to -2.8 ^{o}C over 2 hr, stayed at this temperature for 0.5 hr, and a sub sample was removed. This process continued with ramping to -5.0 and then -7.2 ^{o}C over the next 5 hr and remaining at these temperatures for 0.5 hr before sample removal. After removal, sub samples were held over night at 4 ^{o}C and then placed in a sand medium in the glasshouse at 22 ± 2 ^{o}C on a mist bench. Regrowth was visually estimated as the percent of the sample exhibiting regrowth or appearing green approximately 4 wk after freezing (Schmidt and Chalmers, 1993).

Since only control plots were assessed in November each year and summer samples were only collected in 2003, the data set was not balanced and a combined analysis was not possible. Thus, sampling time by year combinations were considered a single factor having five levels (i.e., Nov. 01, Jan. 02, Nov. 02, Feb. 03, July 03). This single factor was considered the main plot in a split-split-split plot analysis. Subplots were freezing temperature regimes, subsub plots were bermudagrass cultivars, subsubsub plots were ethephon treatments, and subsubsubsub plots were chemical treatments. Main plot sample dates were 2 November 2001, 10 January 2002, 21 November 2002, 24 February 2003, and 20 July 2003. Sub plot temperatures were 4, -2.8, -5.0, and -7.2 ^{o}C. Subsub plot cultivars were Midiron, Riviera, Tifway, and

Princess. Subsubsub plot ethephon treatments were ethephon or no ethephon. Subsubsubsub plot chemical treatments were N, Fe, SWE, and a "zero control". Data were analyzed using PROC GLM of the SAS statistics package (SAS, 1989), and data were tested for homogeneity of variance by plotting residuals before statistical analysis. To comply with the assumptions of analysis of variance, visual ratings were transformed by the arcsine of the square root (Ott and Longnecker, 2001). However, actual means are presented textually and in tables. Appropriate means were separated using Fishers Protected LSD at P=0.05.

Total Lipid Extraction and Fatty Acid Quantification

Tissue samples were removed from the field during acclimation (November), when acclimated (January-February), and when non-acclimated (July) and stored at -80 ^{o}C until analyzed. For each analysis, 1 g of stolon tissue was ground with a mortar and pestle in liquid N_2. This ground tissue was then transferred to a centrifuge tube for total lipid extraction using 3 ml of a buffer containing chloroform:methanol:water (1:2:0.8). After soaking for 1 hr at room temperature, 1 ml 1% NaCl and 3 ml chloroform were added and centrifugation occurred at 1200 G for 10 min. The lower chloroform layer containing the lipids was transferred to a test tube, and the chloroform addition, centrifugation, and chloroform layer transfer were repeated two more times (Cyril et al., 2001).

In a method described by Goyal (2000) and modified by Shang (2003, personal communication), the chloroform layer was then evaporated under a stream of N_2. After evaporation, 5 ml of 2% NaOH in 90% methanol were added, and tubes were placed in

a water bath at 75 to 80 ^{o}C for 30 min. Tubes had an air-cool reflux (small funnels placed in tubes) during this process to facilitate hydrolysis. At the end of the 30 min, the mouths of the tubes were left open to allow for evaporation of the methanol under a fume hood. Next, 2 ml of distilled/deionized (DD) H_2O were added to facilitate dissolution, and the contents were transferred to a 10-ml screw-cap tube. The residue was washed twice more with 2 ml DD H_2O, and contents transferred to a new tube. To this new tube was added 300 µl of 6 M H_2SO_4 to precipitate sodium salts out of the fatty acids. The acid form of the fatty acids was recovered by adding 1 ml hexane and centrifuging at 150 G for 5 min. After centrifugation, the hexane layer containing the free fatty acids was transferred to a new 10-ml screw-cap tube and the process was repeated two more times. After the final centrifugation, the hexane volume was reduced to 100 µl under a gentle stream of N_2 gas, and 100 µl α-bromoacetephenone (10 mg ml^{-1} acetone) and 100 µl triethylamine (TEA) (10 mg ml^{-1} acetone) were added and caps were tightly fastened. Tubes were placed into a water bath at 100 ^{o}C for 15 min. Free fatty acids react with α-bromoacetophenone in the presence of TEA and produce a derivative that is UV sensitive and can be quantified with a UV detector after HPLC separation. Tubes were then allowed to cool, and 140 µl acetic acid (2 mg ml^{-1} acetone) were added and the tubes were placed back in the 100 ^{o}C water bath for 5 min. After cooling a second time, the content was dried under a stream of N_2 to inactivate the remaining reagents. The residue was dissolved with 500 µl acetonitrile, and the solution was filtered with a 0.2 µm membrane before injection into HPLC.

HPLC Procedures

Chromatographic analyses were performed on an Agilent (Palo Alto, CA) 1100 series HPLC system with a photodiode array detector. An Ultrasphere-C8 (250 × 4.6 mm, 5 µm) analytical column with a C-8 guard column (7.5 × 4.6 mm) was used for chromatographic separation. The mobile phase was 90% acetonitrile in water. Samples were eluted at a gradient rate: from 1 ml min^{-1} up to 2 ml min^{-1} within the first 2 min, at a isocratic elution of 2 ml min^{-1} for 10 min, and then down to 1 ml min^{-1} within the next 8 min. Total elution time per sample was 20 min. The injection volume was 20 µl. The fatty-acid derivatives were quantified at a wavelength of 214 nm. The retention times (minutes) at the described conditions were 4.6, 5.8, 7.3 and 10.8 for linolenic, linoleic, palmitic and stearic acids, respectively. The limit of identification for the above procedure is 0.07 to 0.6 µmole per injection using three times the standard deviation for one-half of the lowest standard.

Fatty acid standards were purchased from Sigma Chemical Co. (St. Louis, MO). External standards prepared from the commercial standards were used for calibration. Two reference samples, which were pre-weighed from a composite tissue sample, were included in each analysis set.

Data derived from samples collected in July 2003 could not be included in the combined analysis. Therefore, two separate analysis were performed. The first was a combined analysis with year as the random variable. Sampling times (fall and winter) were considered main plots, bermudagrass cultivars were considered subplots, , ethephon treatments were subsub plots, and chemical treatments were considered subsubsub plots. The second analysis only included the year 2002-03 and was

conducted to compare data collected from summer sampling to that of fall and winter. The analysis is identical except “summer” was included as an additional sampling time and the random variable “year” was excluded. Data were analyzed using PROC GLM of the SAS statistics package (SAS, 1989), and data were tested for homogeneity of variance by plotting residuals before statistical analysis. No transformations were required since fatty acid data appeared homogeneous (Ott and Longnecker, 2001). Appropriate means were separated using Fishers Protected LSD at P=0.05.

Proline Determination

Additional stolon samples were removed at the same time as the controlled freezing tests (fall, winter, and summer), placed in liquid N_2 to halt respiration and then placed in a -80^{o}C freezer for later analysis. Stolons were ground with a mortar and pestle in liquid N_2 and approximately 0.20 g stolon material was homogenized in 10 ml of 3% sulfosalicylic acid, and the homogenate was filtered through Whatman # 2 filter paper. Two ml acid ninhidrin and 2 ml glacial acetic acid were added to 2 ml of the filtrate and incubated at 100^{o}C for 1 hr. The reaction was terminated by placing test tubes in an ice bath. The mixture was extracted with 4 ml toluene and vortexed for 15 to 20 sec. The chromophore containing toluene was warmed to room temperature and absorbance read at 520 nm using toluene as the blank. Proline concentration was determined from a standard curve (Syvertsen and Smith, 1983).

Data were analyzed using the same methods employed for fatty acid data with the exception that proline data were log transformed to achieve uniform variance (Ott

and Longnecker, 2001). Although log transformed for analysis, actual means are presented textually and in tables for clarity.

RESULTS AND DISCUSSION

Turfgrass Quality

Visual ratings of ethephon effects began shortly after the first application in each year of the study. There was a year x date x cultivar x ethephon interaction (Table 3.1). In general, ethephon treatments caused a reduction in visual quality in all cultivars on most observation dates (Table 3.2). Princess seemed to be most negatively affected by ethephon treatments, as visual quality reductions occurred at nearly all observation dates in both years of the study. Riviera also had large quality reductions, but to a greater extent in 2001.

In 2001, cultivars treated with ethephon showed similar trends throughout the observation period (Table 3.2). Generally, Midiron, Riviera, and Tifway all had better quality than Princess at every observation date. In control plots Midiron, Riviera, and Tifway had better quality than Princess at nearly every date. In 2002, trends were similar to those in 2001, except Princess treated with ethephon began the fall with much lower quality than the previous year. However, Princess without ethephon maintained better quality than all other cultivars in November 2002.

Ethephon applications reduced turfgrass quality in both years of the study. This result was expected as increases in endogenous ethylene levels cause leaves of many species to senesce. Diesburg (2000) reported that sequential ethephon applications on Kentucky bluegrass (*Poa pratensis* L.) may reduce turfgrass quality. However, Ervin and Ok (2001) reported no reductions in quality on ‘Meyer’ zoysiagrass (*Zoysia japonica* Steud.) during the growing season.

Table 3.1. Analysis of variance for chemical treatment, ethephon treatment, and cultivar effects on bermudagrass visual quality (2001 & 2002).

Source	df	*F* value
Year	1	25.9**
Error	6	
Date	3	12650.5**
Year x date	3	2405.1**
Error	18	
Cv	3	1412.6**
Date x cv	9	281.8**
Year x cv	3	160.7**
Year x date x cv	9	69.6**
Error	72	
Eth	1	1113.3**
Year x eth	1	14.8**
Date x eth	3	23.4**
Year x date x eth	3	37.8**
Cv x eth	3	229.0**
Year x cv x eth	9	44.9**
Date x cv x eth	9	18.8**
Year x date x cv x eth	9	30.9**
Error	96	
Trt	3	39.7**
Date x trt	9	4.7**
Cv x trt	9	2.0*
Date x cv x trt	27	1.5
Year x trt	3	3.8*
Year x date x trt	9	2.3*
Year x cv x trt	9	4.8**
Year x date x cv x trt	27	2.0**
Eth x trt	3	1.4
Year x eth x trt	3	1.7
Date x eth x trt	9	0.3
Cv x eth x trt	9	0.6
Year x date x eth x trt	9	0.4
Year x cv x eth x trt	9	0.3
Date x cv x eth x trt	27	1.3
Year x date x cv x eth x trt	27	0.5
Error	576	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Table 3.2. Breakout of year x observation date x cultivar x ethephon treatment interaction for visual quality across all chemical treatments (2001 and 2002).

Year	Date	Cultivar	Ethephon +	Ethephon -	LSD[1]
			Visual Quality (1 to 9)		
2001	Aug	Midiron	8.1	8.3†	ns
		Riviera	8.1	8.3	0.2
		Tifway	8.0	7.9	0.1
		Princess	6.0†	6.5	ns
		LSD[2]	0.4	0.4	--
	Sept	Midiron	6.9	7.6	0.2
		Riviera	6.4	7.4	0.3
		Tifway	6.9	7.3	0.3
		Princess	4.0	6.0	0.2
		LSD	0.2	0.3	--
	Oct	Midiron	5.8	6.1	0.3
		Riviera	5.2	6.1	0.2
		Tifway	6.0	6.0	ns
		Princess	4.0	5.0	0.4
		LSD	0.2	0.2	--
	Nov	Midiron	4.9†	4.8†	ns
		Riviera	4.0†	5.1†	0.2
		Tifway	5.0†	5.0†	ns
		Princess	3.5†	4.8†	0.3
		LSD	0.2	0.3	--
2002	Aug	Midiron	8.0	7.8	0.2
		Riviera	8.5†	8.5†	ns
		Tifway	8.3	8.3†	ns
		Princess	4.5†	6.8†	0.2
		LSD	0.3	0.3	--
	Sept	Midiron	7.8†	8.1†	0.3
		Riviera	8.0†	8.0†	ns
		Tifway	8.1†	8.1†	ns
		Princess	5.8†	7.1†	0.3
		LSD	0.3	0.2	--
	Oct	Midiron	6.0†	7.0†	ns
		Riviera	7.1†	7.1†	ns
		Tifway	7.2†	7.2†	ns
		Princess	6.9†	7.2†	ns
		LSD	0.3	0.1	--
	Nov	Midiron	2.0	2.8	0.2
		Riviera	2.4	2.6	0.2
		Tifway	2.1	2.7	0.3
		Princess	2.7	3.6	0.3
		LSD	0.3	0.3	--

[1]LSD= Comparison of ethephon treatments within cultivar, date, and year.
[2]LSD= Comparison of cultivars within ethephon treatments
†Values followed by a dagger are significantly higher within date, cultivar, and ethephon treatment and between years ($P<0.05$).

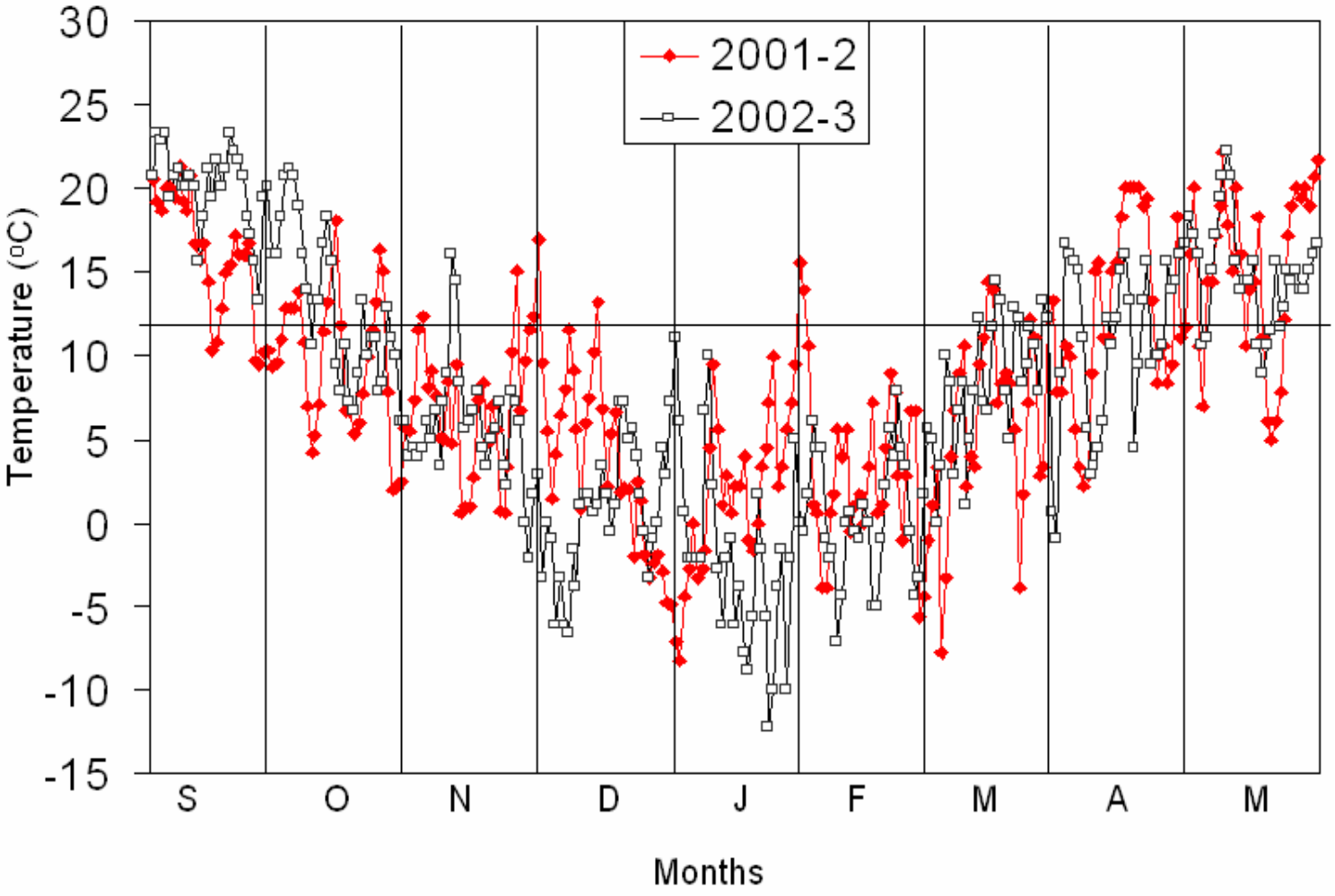
Table 3.3. LSD comparison for cultivars and ethephon treatments over all dates in Table 3.2.

Year	Cultivar	Ethephon +	Ethephon -
2001	Midiron	0.3	0.3
	Riviera	0.2	0.3
	Tifway	0.1	0.2
	Princess	0.4	0.4
2002	Midiron	0.2	0.2
	Riviera	0.3	0.3
	Tifway	0.2	0.3
	Princess	0.4	0.3

Princess was severely damaged during the winter of 2001-2002, causing its quality ratings early in fall 2002 to be much lower than other cultivars. The other seeded cultivar, Riviera, also had greater quality reductions than Midiron and Tifway during the first fall of the study. Richardson et al. (2003) explained that one of the reasons seeded bermudagrasses are susceptible to winter injury in their establishment year is because they are not fully developed, having smaller stolons and lacking rhizomes. If the seeded cultivars were not fully developed during the first year of the study, it is possible that a senescence-promoting PGR could have a greater effect. Richardson et al. (2003) reported that herbicides applied to seedling bermudagrass resulted in injury not observed on mature turf.

In general, early-fall quality ratings were higher in 2002 than in 2001 (Table 3.2). By November, however, all cultivars, with or without ethephon, had higher quality in 2001. Quality ratings during the first 3 months of 2002 were most likely higher than the previous year due to differences in temperatures (Fig. 3.1). Fall 2002 was generally much warmer than the previous fall. Thus, with temperatures remaining above the bermudagrass chilling stress level (12 ^{o}C), yellowing would not occur as rapidly.

Figure 3.1. Daily mean temperature data (September – May 2001-2003). Line at 12 °C indicates bermudagrass growth cessation point.



Air temperatures also explain the late-fall reduction in quality in 2002. Temperatures at the beginning of November 2002 declined quickly and the first killing frost occurred on 2 November. Three weeks of low temperatures prior to the Fall 2002 ratings were likely enough to reduce quality through hastened yellowing.

Controlled Freezing

Ethephon Treatment and Survival

There was a date x temperature x cultivar x ethephon interaction (Table 3.4); so data are presented to show those relationships (Table 3.5). At the two winter sampling dates, Midiron had the greatest amount of survival after freezing, followed by Riviera, Tifway, and Princess.

Generally, differences between ethephon treatments showed that the effect of ethephon on post-freeze regrowth was not significant, with a single exception. In winter 2003, Princess plots treated with ethephon had less post-freeze survival than plots not treated with ethephon. Otherwise, the effect of ethephon on post-freeze regrowth was not significant. These results do not confirm previous reports that ethephon applications prior to chilling temperatures increase cold hardiness and substantially reduce development of chilling injury (Wang, 1989; Harber and Fuchigami, 1989). Although ethephon applications promoted early senescence during the fall as seen in reduced visual quality, this did not appear to affect bermudagrass cold hardiness.

Table 3.4. Analysis of variance for post-freeze regrowth (2001, 2002, & 2003).

Source	df	*F* value
Rep	3	16.6**
Date	4	149.0**
Date x rep	12	5.4 **
Temp	3	1991.2**
Date x temp	12	149.3**
Error	45	
Cv	3	422.2**
Date x cv	12	61.4**
Temp x cv	9	31.1**
Date x temp x cv	36	5.1**
Error	180	
Eth	1	6.7**
Date x eth	4	2.8*
Temp x eth	3	2.1
Date x temp x eth	12	0.3
Cv x eth	3	3.7*
Date x cv x eth	12	2.1*
Temp x cv x eth	9	0.9
Date x temp x cv x eth	36	0.8
Error	240	
Trt	3	0.3
Date x trt	6	0.5
Temp x trt	9	0.5
Cv x trt	9	1.9
Date x cv x trt	18	3.4**
Temp x cv x trt	27	0.6
Date x temp x trt	18	0.7
Date x temp x cv x trt	54	0.8
Eth x trt	3	2.8*
Date x eth x trt	6	3.7**
Temp x eth x trt	9	0.4
Cv x eth x trt	9	2.8**
Date x temp x eth x trt	18	0.6
Date x cv x eth x trt	18	2.5**
Temp x cv x eth x trt	27	0.8
Date x temp x cv x eth x trt	54	1.0
Error		

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Table 3.5. Breakout of sampling date x cultivar x ethephon interaction for post-freeze regrowth across all temperatures.

Sampling Date	Cultivar	Ethephon +	Ethephon -	LSD[1]
		Regrowth (%)		
Fall 01	Midiron	46.7	48.2	ns
	Riviera	39.7	36.3	ns
	Tifway	32.3	33.9	ns
	Princess	31.3	29.4	ns
	LSD[2]	ns	ns	--
Winter 02	Midiron	63.8	40.2	ns
	Riviera	43.8	47.8	ns
	Tifway	23.9	38.8	ns
	Princess	5.4	37.9	ns
	LSD[2]	9.5	ns	--
Fall 02	Midiron	40.6	40.2	ns
	Riviera	55.0	47.8	ns
	Tifway	40.6	38.8	ns
	Princess	37.9	37.9	ns
	LSD[2]	ns	ns	--
Winter 03	Midiron	45.6	51.0	ns
	Riviera	38.0	41.6	ns
	Tifway	16.1	20.2	ns
	Princess	3.1	6.7	3.5
	LSD[2]	8.9	9.2	--
Summer 03	Midiron	18.0	18.0	ns
	Riviera	23.1	24.4	ns
	Tifway	23.9	24.2	ns
	Princess	18.8	18.8	ns
	LSD[2]	ns	ns	--

[1]LSD= Comparison of ethephon treatment within cultivars and sampling dates.
[2]LSD= Comparison of cultivars within ethephon treatment and sampling dates.

Fatty Acid Analysis

The ANOVA table is shown in Table 3.6. Ethephon treatment did not effect linolenic acid percentages and there were no ethephon interactions with other main

effects. Table 3.7 shows ethephon treatments and the lack of a response to the PGR exhibited by all cultivars. The ANOVA table for the second year of the study shows that

Table 3.6. Analysis of variance for ratio of linolenic acid to total polar lipids in fall and winter (2001-2003).

Source	df	*F* value
Year	1	39.6**
Error	6	
Date	1	6.9**
Year x date	1	167.7**
Error	6	
Cv	3	41.2**
Date x cv	3	2.2
Year x date x cv	6	5.3**
Error	36	
Eth	1	1.8
Date x eth	1	0.1
Cv x eth	3	0.7
Date x cv x eth	3	0.9
Year x eth	1	1.7
Year x date x eth	1	0.5
Year x cv x eth	3	0.3
Year x date x cv x eth	3	1.7
Error	48	
Trt	3	0.3
Date x trt	3	0.7
Eth x trt	3	0.5
Cv x trt	9	0.7
Date x eth x trt	3	0.1
Date x cv x trt	9	1.1
Eth x cv x trt	9	1.3
Date x eth x cv x trt	9	0.8
Year x trt	3	0.8
Year x date x trt	3	0.7
Year x cv x trt	9	1.4
Year x eth x trt	3	2.1
Year x date x cv x trt	9	1.6
Year x date x eth x trt	3	0.7
Year x cv x eth x trt	9	0.5
Year x date x cv x eth x trt	9	1.0
Error	288	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

there were no effects of ethephon treatment on percent linolenic acid (Table 3.8).

These findings that percent linolenic acid was not affected by ethephon treatment are in contrast of those of Harber and Fuchigami (1989) and Lyons and Pratt (1964), who found that increased endogenous ethylene levels resulted in an increase in membrane permeability supposedly associated with greater levels of fatty acid unsaturation.

Table 3.7. Breakout of cultivar x ethephon interaction for percent linolenic acid across fall chemical treatments.

Cultivar	Ethephon +	Ethephon -	LSD[1]
	Linolenic Acid (Percent of Total)		
Midiron	15.3	15.6	ns
Riviera	13.0	12.6	ns
Tifway	11.5	10.6	ns
Princess	13.1	12.6	ns
LSD[2]	1.1	1.1	--

[1]LSD= Comparison of ethephon treatment within cultivars.
[2]LSD= Comparison of cultivars within ethephon treatment.

Based on data generated in this study, it appears that ethephon applications do not increase lipid unsaturation or freeze tolerance. Although the specific role of ethylene in cold hardiness is not entirely clear, Kacperska (1993) hypothesizes that the supposed effects of ethylene on membrane permeability and osmotic potential allow for maintenance of lower water potentials, providing the protoplast with higher supercooling ability.

Proline Concentration

Analysis of variance for proline concentration is shown in Table 3.9. There were

significant interactions evident in the ANOVA table, however, they were likely caused by main effects of year, date, and cultivar, rather than a combination of factors.

Table 3.8. Analysis of variance for ratio of linolenic acid to total polar lipids during the second year of the study.

Source	df	*F* value
Rep	3	0.8
Date	2	66.8**
Error	6	
Cv	3	35.2**
Date x cv	6	2.1
Error	27	
Eth	1	0.0
Date x eth	2	0.1
Cv x eth	3	2.6
Date x cv x eth	6	1.7
Error	36	
Trt	3	0.9
Date x trt	6	0.3
Cv x trt	9	1.3
Eth x trt	3	0.8
Date x cv x trt	18	0.6
Date x eth x trt	6	1.1
Cv x eth x trt	9	0.9
Date x cv x eth x trt	18	0.7
Error	216	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Ethephon Treatment and Proline Concentration

Ethephon had no effect on proline concentration (Table 3.10). In winter 2002, Midiron treated with ethephon showed reduced proline concentrations over plots not treated with ethephon. As this was the only occurrence of a ethephon effect, it was likely due to a procedure error.

Cultivars treated with ethephon revealed no discernable trends during the fall and winter 2001-2003 (Table 3.10). When averaged over all chemical treatments, all

cultivars had higher proline levels in fall 2001 than winter 2002. Proline concentrations were similar for all cultivars during the winter of 2002-2003. The reasons for such

Table 3.9. Analysis of variance for proline concentration during fall and winter (2001-2003).

Source	df	*F* value
Year	1	60.9**
Error	6	
Date	1	136.8**
Year x date	1	208.4**
Error	6	
Cv	3	69.4**
Date x cv	3	0.8
Year x date x cv	6	4.8**
Error	36	
Eth	1	4.4*
Date x eth	1	3.4
Cv x eth	3	6.8**
Date x cv x eth	3	10.4**
Year x eth	1	3.8
Year x date x eth	1	0.6
Year x cv x eth	3	3.0*
Year x date x cv x eth	3	8.1**
Error	48	
Trt	3	7.3**
Date x trt	3	2.8*
Cv x trt	9	7.6**
Eth x trt	3	3.1*
Date x cv x trt	9	2.8**
Date x eth x trt	3	2.6
Cv x eth x trt	9	1.4
Date x cv x eth x trt	9	5.0**
Year x trt	3	6.9**
Year x date x trt	3	0.6
Year x cv x trt	9	1.3
Year x eth x trt	3	10.8**
Year x date x cv x trt	9	3.9**
Year x date x eth x trt	3	3.3*
Year x cv x eth x trt	9	1.5
Year x date x cv x eth x trt	9	4.5**
Error	288	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Table 3.10. Breakout of year x sampling date x cultivar x ethephon interaction for proline concentration. Data are averaged over fall chemical treatments.

Year	Sampling Date	Cultivar	Ethephon +	Ethephon -	LSD[1]
			Proline Concentration (μg g^{-1})		
2001-2	Fall	Midiron	904.3	881.1	ns
		Riviera	458.6	392.6	ns
		Tifway	460.3	533.0	ns
		Princess	270.0	316.7	ns
		LSD[2]	277.0	296.1	--
	Winter	Midiron	59.6	626.9	374.6
		Riviera	188.9	131.1	ns
		Tifway	102.5	123.4	ns
		Princess	55.9	75.8	ns
		LSD[2]	82.4	268.3	--
2002-3	Fall	Midiron	583.3	591.2	ns
		Riviera	372.4	380.8	ns
		Tifway	167.8	167.6	ns
		Princess	171.1	109.2	ns
		LSD[2]	126.3	118.1	--
	Winter	Midiron	617.6	728.0	ns
		Riviera	383.3	625.1	ns
		Tifway	230.3	282.4	ns
		Princess	221.3	207.9	ns
		LSD[2]	231.4	292.1	--

[1]LSD= Comparison of ethephon treatment within cultivars.
[2]LSD= Comparison of cultivars within ethephon treatment.

variation in proline concentration between dates are unclear. As proline is known to increase during cold acclimation (Levitt, 1980), cooler fall temperatures in 2001 may have caused the acclimation process to begin at an earlier time. Since there were no differences between ethephon treatments, the increased senescence caused by ethephon during the fall did not seem to interfere with proline increases during acclimation. It is interesting to note that low proline levels in ethephon treated Midiron plots in winter 2002 did not result in less survival after controlled freezing. Results such as this suggest that proline may not be a factor in cold-tolerance. However, there are

likely many biochemical parameters changing during cold acclimation that are required for survival during the winter.

Chemical and Ethephon Treatments on Proline Concentration

Chemical treatments in conjunction with ethephon treatments had little effect on proline concentration (Table 3.11). With the exception of SWE and Fe in winter 2002, there were no differences between ethephon treatments. In winter 2002, both SWE and Fe, when treated with ethephon, had lower proline levels than plots not treated with ethephon. The likelihood of a negative interaction between these chemical treatments and ethephon is slight as this only occurred at one sampling date.

In this study, proline concentration was not consistently or significantly affected by Fe, SWE, or N when receiving ethephon treatments (Table 3.11). However, winter proline levels were generally higher than fall levels. Munshaw et al. (in press) found that increasing proline concentrations in bermudagrass through moderate salt applications was correlated with increased regrowth after freezing. Because no previous research has examined the effect of N, SWE, and Fe treatments on proline levels in bermudagrass, correlations must be drawn to the studies that have found very similar results showing no effects of these treatments on carbohydrate concentrations (White and Schmidt, 1990; Goatley et al., 1994; Richardson, 2002).

Ethephon treatments showed that there were few differences in proline concentration for cultivar and chemical treatment combinations (data not shown). This result is in agreement with Gorin et al. (1989) who found no effect of ethylene treatment on proline concentration in cut roses (*Rosa hybrida* L.). Ozturk and Demir (2003) applied ethephon to spinach (*Spinacia oleracea* L.) and also found no effect on proline

Table 3.11. Breakout of year x sampling date x chemical treatment x ethephon interaction for proline concentration.

Year	Sampling Date	Chemical Treatment	Ethephon +	Ethephon -	LSD[1]
2001-2	Fall	N	665.3	358.4	ns
		SWE	562.3	814.2	ns
		Fe	566.7	539.1	ns
		C	298.9	411.8	ns
		LSD[2]	311.4	310.8	--
	Winter	N	181.0	162.6	ns
		SWE	73.0	215.1	127.0
		Fe	59.4	513.0	397.6
		C	93.6	66.6	ns
		LSD[2]	84.5	291.2	--
2002-3	Fall	N	321.9	291.3	ns
		SWE	360.5	310.7	ns
		Fe	355.6	305.9	ns
		C	256.5	340.8	ns
		LSD[2]	ns	ns	--
	Winter	N	323.5	559.0	ns
		SWE	353.7	282.9	ns
		Fe	495.6	449.6	ns
		C	279.7	551.9	ns
		LSD[2]	ns	ns	--

[1]LSD = Comparison of ethephon treatment within chemical treatment, year, and sampling date.
[2]LSD = Comparison of chemical treatment within ethephon treatment, year, and sampling date.

concentration. As was previously mentioned, ethephon treatment had no discernable effect on bulk tissue proline. There were instances where combinations of ethephon and chemical treatment resulted in higher proline levels, but these instances were infrequent and highly variable. Likewise, plots not treated with ethephon had higher amounts of proline at various sampling dates, but were inconsistent and did not hold for all cultivars.

Ethephon Treatment and Proline Concentration in Season 2

The analysis of variance is shown in Table 3.12. Although date x cultivar x

Table 3.12. Analysis of variance for proline concentration during the second year of the study.

Source	df	*F* value
Rep	3	3.3*
Date	2	92.8**
Error	6	
Cv	3	47.5**
Date x cv	6	14.2**
Error	27	
Eth	1	0.4
Date x eth	2	1.8
Cv x eth	3	3.2*
Date x cv x eth	6	1.5
Error	36	
Trt	3	1.1
Date x trt	6	1.5
Cv x trt	9	5.5**
Eth x trt	3	3.9**
Date x cv x trt	18	5.4**
Date x eth x trt	6	2.0
Cv x eth x trt	9	3.1**
Date x cv x eth x trt	18	1.8*
Error	216	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

ethephon treatment did not interact, data are presented to show the effects over time of ethephon on cultivars (Table 3.13). The sampling date x cultivar x ethephon treatment interaction indicated that there were differences between cultivars during the winter months on plots not treated with ethephon. Midiron had the highest levels, followed by Riviera, Tifway, and Princess. As was discussed previously, ethephon treatment was again not a factor in proline concentration for any cultivar at all dates sampled. The July sampling date showed no differences between cultivars, but proline

Table 3.13. Breakout of sampling date x cultivar x ethephon interaction for proline concentration during the second year of the study. Data are averaged across fall chemical treatments.

Sampling Date	Cultivar	Ethephon +	Ethephon -	LSD[1]
		Proline Concentration (μg g^{-1})		
Fall	Midiron	583.3	591.2	ns
	Riviera	372.4	380.8	ns
	Tifway	167.8	167.6	ns
	Princess	171.1	109.2	ns
	LSD[2]	126.3	118.1	--
Winter	Midiron	617.6	728.0	ns
	Riviera	383.3	625.1	ns
	Tifway	230.3	282.4	ns
	Princess	221.3	207.9	ns
	LSD[2]	231.4	292.1	--
Summer	Midiron	79.2	86.1	ns
	Riviera	71.9	69.0	ns
	Tifway	92.0	90.9	ns
	Princess	125.0	96.5	ns
	LSD[2]	28.5	ns	--

[1]LSD= Comparison of ethephon treatment within cultivars.
[2]LSD= Comparison of cultivars within ethephon treatment.

levels were greatly reduced over winter months. Proline levels appear to be higher in cold-tolerant cultivars and during winter months. This suggests that proline may play a significant role in bermudagrass cold hardiness.

Spring Greenup

The ANOVA (Table 3.14) shows there was a year x date x cultivar x ethephon treatment interaction. In April 2002, ethephon treatment did not change the rate of Midiron greenup (Table 3.15). However, Riviera, Tifway, and Princess treated with ethephon were less green than plots not treated with ethephon. In May 2002, ethephon treatment reduced greenup of all cultivars. In June 2002, Midiron and Riviera were not

Table 3.14. Analysis of variance for spring greenup.

Source	df	*F* value
Year	1	7516.0**
Error	6	
Date	3	7008.5**
Year x date	2	74.2**
Error	15	
Cv	3	9402.1**
Date x cv	9	574.2**
Year x cv	3	142.0**
Year x date x cv	6	69.6**
Error	63	
Eth	1	645.3**
Date x eth	3	32.1**
Cv x eth	3	54.8**
Date x cv x eth	9	24.7**
Year x eth	1	208.4**
Year x date x eth	2	7.3**
Year x cv x eth	3	32.2**
Year x date x cv x eth	6	21.4**
Error	84	
Trt	3	10.1**
Date x trt	9	1.6
Cv x trt	9	4.7**
Eth x trt	3	0.3
Date x cv x trt	27	1.8**
Date x eth x trt	9	0.2
Date x cv x eth x trt	36	0.5
Year x trt	3	5.7**
Year x date x trt	6	1.1
Year x cv x trt	9	0.5
Year x eth x trt	3	0.5
Year x date x cv x trt	18	1.3
Year x date x eth x trt	6	0.7
Year x cv x eth x trt	9	01.3
Year x date x eth x trt	6	0.7
Year x cv x eth x trt	9	0.4
Year x date x cv x eth x trt	18	0.4
Error	504	

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

Table 3.15. Year x date x cultivar x ethephon interaction on spring greenup across all chemical treatments.

Year	Date	Cultivar	Ethephon +	Ethephon -	LSD[1]
			Greenup (%)		
2002	28 April	Midiron	62.2†	65.3†	ns
		Riviera	57.5	72.5†	13.8
		Tifway	26.3†	45.0†	2.4
		Princess	2.5†	9.9†	1.6
		LSD[2]	9.8	10.7	--
	15 May	Midiron	91.9†	100.0†	1.8
		Riviera	90.9†	100.0†	0.7
		Tifway	47.2†	74.1†	2.3
		Princess	3.8	24.8†	3.1
		LSD[2]	20.0	13.2	--
	1 June	Midiron	100.0†	100.0†	ns
		Riviera	100.0†	100.0†	ns
		Tifway	85.3†	95.6†	3.1
		Princess	18.1†	56.3†	7.5
		LSD[2]	17.7	13.2	--
2003	28 April	Midiron	36.6	41.6	4.4
		Riviera	57.8	58.1	ns
		Tifway	3.9	9.4	1.7
		Princess	0.0	0.2	0.1
		LSD[2]	9.2	12.4	--
	15 May	Midiron	87.6	89.4	1.8
		Riviera	86.4	87.0	ns
		Tifway	43.4	49.6	2.8
		Princess	5.9	12.5	4.4
		LSD[2]	5.7	10.6	--
	1 June	Midiron	92.8	95.9	1.1
		Riviera	93.1	95.0	ns
		Tifway	53.8	63.1	3.6
		Princess	7.7	14.8	5.6
		LSD[2]	10.3	14.6	--

[1]LSD= Comparison of ethephon treatment within cultivar, date, and year.
[2]LSD= Comparison of cultivar within ethephon treatment, date, and year.
†Values followed by a dagger are significantly higher within ethephon treatment, cultivar, and date and between years ($P<0.05$).

Table 3.16. LSD comparison for cultivars and ethephon treatments over all dates in Table 3.15.

Year	Cultivar	Ethephon +	Ethephon -
2002	Midiron	10.4	3.6
	Riviera	8.1	5.7
	Tifway	16.3	10.1
	Princess	9.0	13.7
2003	Midiron	8.8	8.4
	Riviera	6.9	9.2
	Tifway	6.3	8.9
	Princess	4.5	7.4

affected by ethephon treatment, while Tifway and Princess both had lower amounts of greenup when treated with ethephon. In April, May, and June 2003, Midiron, Tifway, and Princess had less percentage greenup when treated with ethephon. In contrast, Riviera greenup was not effected by ethephon treatment.

Regardless of ethephon treatment, cultivars showed the same general trends of greenup in all months of both years (Tables 3.15 & 3.16). Midiron and Riviera rejuvenated quicker than Tifway. Tifway had more percentage greenup than Princess. Differences between dates generally show what would be expected (Tables 3.15 & 3.16). All cultivars, regardless of ethephon treatment, had more greenup in June, followed by May and April in both years of the study. Differences across years showed that all dates in 2002 had higher levels of greenup, regardless of ethephon treatment (Table 3.16). In 2003, daily mean temperatures were 5 ^{o}C colder than in 2002 (Fig. 3.1). In general, temperatures were cooler during the winter of 2003, which likely was a factor in greenup differences between years. As cold-sensitive cultivars had delayed

greenup much more than cold-tolerant cultivars, colder winter temperatures likely caused this injury. This delayed greenup was likely a result of winterkill.

Spring greenup was also negatively affected by ethephon applications in all cultivars but to a greater degree in the cold-susceptible cultivars Princess and Tifway. The effect on Princess was so severe that a large majority of the plots did not recover until late in the summer. Chalmers and Schmidt (1979) found that bermudagrass rhizomes had lowered freeze tolerance, with increased dormancy duration. Although proline was not measured during deacclimation, it may be possible that stored carbohydrates were diminishing and cellular proline levels were changing with long dormancy periods, reducing post-dormancy survival. Because the cold acclimation period in the fall was altered by ethephon applications, survival of the bermudagrasses may have been affected. Rogers et al. (1975) reported that zoysiagrass that does not properly acclimate before winter can be severely damaged by even moderate temperatures. Results of the current study suggest that tampering with the natural acclimation process (by inducing fall yellowing) may result in decreased freeze tolerance and delayed greenup in the spring.

Summary

Ethephon increased senescence in all cultivars and visual quality ratings during the fall were substantially reduced. Senescence induction in this study was thought of as a possible method to assist in the acclimation process prior to cold weather. However, ethephon treatments showed little affect on bermudagrass cold hardiness.

Color loss during the fall reduces turfgrass functionality and results in the need for overseeding with a C_3 species.

Post-freeze regrowth was generally unaffected by ethephon application. This result was not surprising as lipid unsaturation and proline concentration were also unaffected by ethephon. Previous studies have shown that fall chemical treatments may enhance fall and spring color without affecting bermudagrass cold hardiness. In this study, ethephon reduced fall and spring color and resulted in an increased amount of winterkill for at least one of the cultivars measured. This result indicates that physiological parameters other than proline concentration and lipid unsaturation that are important in bermudagrass cold hardiness may have been negatively affected by fall ethephon treatments.

CONCLUSIONS

The hypothesis that ethephon applications will reduce the length of the growing season was accepted. Ethephon applications caused early senescence during both years of the study. However, the effect of decreasing the length of the growing season to promote cold tolerance was unsuccessful.

Ethephon applications reduced turfgrass quality during the fall. Although bermudagrass naturally senesces and becomes dormant in mid to late fall in Virginia, keeping high quality turf until this point is very important in non-overseeded turf. Since golfers and athletes expect a green playing surface for optimum performance, shortening the growing season with ethephon applications reduces turf functionality.

No effect of ethephon treatment was noted on post-freeze regrowth in the glasshouse. Ethephon treated bermudagrass cultivars were just as likely to survive freezing temperatures as non-treated samples. The lack of a significant effect on regrowth after freezing suggests that inducing senescence early does not benefit cold tolerance in bermudagrass.

The effect of ethephon treatment on spring greenup was also negative. Plots not receiving ethephon exhibited earlier dormancy break and reached 100% green cover significantly earlier than the ethephon treated plots. There was also a negative interaction with the cold-sensitive cultivar Princess. The effect of ethephon on Princess resulted in significant turf loss and plots did not recover fully until the following fall. This loss of bermudagrass was unacceptable and would result in extensive renovation.

Ethephon treatments had little effect on proline concentration in the cultivars tested. As proline concentration has been linked to cold tolerance, practices to increase

proline levels rather than holding them constant would be wise. This effect was confounded by the negative affects of reduced fall quality and delayed spring greenup.

Finally, the effect of ethephon on bermudagrass membrane lipid unsaturation was not significant. Lipid unsaturation has been shown to be important for bermudagrass winter survival in cooler climates. Although ethylene has been shown to increase membrane permeability in other species, it was not effective in this study. To assist bermudagrass in survival over the winter, practices to increase lipid unsaturation, rather than holding them constant, need to be discovered.

Clearly the application of ethephon on bermudagrass with the intention of increasing cold tolerance is a poor management practice. Although there are uses for ethephon in other fine turf situations, the use on bermudagrass during the fall should not be recommended as a best management practice for increasing winter survival.

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