DISSERTATION

PHENOTYPIC VARIABILITY, COLD HARDINESS AND FLOWERING INDUCTION OF SALTGRASS [*Distichlis spicata* (L.) Greene] CLONES

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY HRVOJE HARRY RUKAVINA ENTITLED, "PHENOTYPIC VARIABILITY, COLD HARDINESS AND FLOWERING INDUCTION OF SALTGRASS [*Distichlis spicata* (L.) Greene] CLONES" BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PHENOTYPIC VARIABILITY, COLD HARDINESS AND FLOWERING INDUCTION OF SALTGRASS [*Distichlis spicata* (L.) Greene] CLONES

With increased population growth and periodic droughts in the semiarid U.S. west, there is interest in developing alternative turfgrass species that are water efficient and tolerate poor quality water. Colorado State University is currently evaluating saltgrass for its potential use as turf. Development of a new turfgrass cultivar requires an understanding of environmental factors that influence traits important for turf quality. Furthermore, cultivar development for northern climates and transition zones necessitates an understanding of cold hardiness. Finally, to enable hybridization throughout the year and make rapid progress in a breeding program, it is important to develop techniques and understand environmental stimuli that induce flowering in saltgrass. This study was initiated to:

1) characterize variation in morphological traits and time of leaf browning in fall among saltgrass clones relative to geographic and climatic variables at the location of clones' origin;

2) examine the relative freezing tolerance of saltgrass clones as related to climatic zone of origin as well as relationship between freezing tolerance and time of leaf browning in fall and

3) examine the influence of sampling time from the field, as well as burning and nitrogen fertilization on flowering induction of saltgrass clones from different environments.

In the first experiment, traits of growth (morphology) and time of leaf browning in fall were measured on 53 saltgrass clones from 42 locations established at one location in Fort Collins, CO. Principal component analysis on the traits of plant morphology extracted the first principal component (PC-1) that explained 78% of variability and was used as the estimate of growth. Principal component analysis was followed by multiple regression of PC-1 and time of leaf browning in fall on the environmental variables at locations of clones' origin. Variation in saltgrass growth (morphology) was related to the seasonal climatic variables of summer drying and fall cooling that explained 50% of variability of morphological traits. Variation in time of leaf browning in fall was related to longitude and minimum winter temperature which together explained 60% of total variability of this trait.

In the second experiment, rhizomes were sampled during 2004 and 2005 mid-winters from 27 saltgrass clones from three cold hardiness zones established in Fort Collins, CO and then subjected to a freezing test. Saltgrass freezing tolerance was highly influenced by the climatic zone of clone origin in both years of the experiment. Clones with greater freezing tolerance turned brown earlier in fall in both seasons. This study indicated that saltgrass clones from northern (cooler) climates had greater freezing tolerance than clones from southern (warmer) areas.

In the first year of the third experiment, three clones (A1540 from Colorado, 1490 from South Dakota and C1660 from Nevada) were sampled from the field twice

(in August and November). In the second year, two additional clones from the Colorado Front Range (A1180 and A1610) and an additional sampling time (January) were included. In the first year, nitrogen fertilization increased number of spikes in saltgrass. Compared with August sampling, sampling in November increased the number of spikes, and had a greater effect on clone A1540 than on clone 1490. The burning treatment increased number of spikes only in plants sampled from the field in August. In the second year, nitrogen fertilization increased the number of spikes to a greater extant when nitrogen was applied with burning than without the burning treatment. In comparison with August sampling, sampling in November increased number of spikes in all clones with the greatest effect in clone A1540. Sampling in January additionally increased number of spikes in clones 1490 and C1610 without a significant effect on number of spikes in clone A1540. Burning treatment had its greatest effect on number of spikes in plants sampled in August, as compared with November and January sampling.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Literature Review:

1.1 Saltgrass Characteristics

Saltgrass [*Distichlis spicata* (L.) Greene] is a perennial grass, native to North America (Uchytil 1990). Recent literature usually makes a distinction between marsh saltgrass *Distichlis spicata* and inland saltgrass *Distichlis spicata* var. *stricta* (Reid, 2001). Marsh saltgrass occurs along the Atlantic (from Nova Scotia to Florida) and Gulf coasts (from Florida to Texas) (Seliskar and Gallagher, 2000; Reid 2001). Inland saltgrass is distributed across the broad range of the western United States and Canada, from Saskatchewan south to California, Texas and Mexico, to its eastern borders of distribution in Nebraska, Kansas and the Dakotas (Uchytil, 1990; Reid, 2001). Marsh saltgrass is found in salt marshes on both United States coasts. The inland variety is common in salty and alkaline soils throughout the western and central parts of the country (Eppley et al., 1998). Saltgrass is a halophytic plant (Gallagher and Seliskar, 1993) that tolerates a very broad range of soil salinity. It inhabits many plant communities but it becomes the dominant vegetation only on salty, moist, fine textured and alkaline soils, with a pH range between 7.5 and 8.5 (Uchytil, 1990).

Marcum et al. (2005) found differences in salinity tolerance among saltgrass ecotypes. Active salt glands (Alshammary et al., 2004) indicate an important mechanism for salinity tolerance. These glands exude salt on the leaf surface and enable plants to maintain adequate osmotic potential. Saltgrass tolerates extended drought periods and has excellent heat tolerance as well (Kopec and Marcum, 2001). Saltgrass is also tolerant to selenium. It can accumulate selenium under saline

conditions and may help reduce soil contamination of soluble selenium (Banuelos et al., 2005). Saltgrass is an important species for stabilization of marsh sites disturbed by storms and for revegetation of contaminated sites (Seliskar and Gallagher, 2000; Uchytil, 1990).

Saltgrass is a useful salt-tolerant crop when farmland becomes salinized (Gallagher and Seliskar, 1993). It provides valuable late-season forage in arid areas (Reid, 2001). Gallagher and Seliskar (1993) tested a large number of saltgrass plants from 14 populations and selected the salt tolerant forage variety 'Seabrook'. This variety is used as pasture, hay or silage. They also developed a tissue culture technique to produce somaclonal variants of saltgrass that have potential for greater forage value (Gallagher and Seliskar, 1993). Bustan et al. (2005) tested many saltgrass ecotypes from several locations in the U.S. which resulted in the selections of six salt tolerant accessions with superior forage quality.

Saltgrass is a dioecious species with possible differences between male and female individuals in growth and survival on saline sites (Gallagher and Seliskar, 1993). Saltgrass sex is genetically determined, and sexes are spatially segregated in natural populations (Eppley et al., 1998) in a way that female individuals are commonly located at much lower elevations than male individuals (Eppley, 2001). Spatial segregation of sexes reduces mating success of both males and females due to the restricted pollen dispersal (Eppley, 2005). Female plants have a much stronger competitive effect than males. Sexual dimorphism in competitive effects varies with changes in environmental conditions and occurs only in more nutrient rich soil where the majority of saltgrass individuals are females (Eppley, 2006).

Saltgrass is a wind-pollinated species with seed dispersion by water. Saltgrass is generally a poor seed producer and propagation mainly occurs asexually by rhizomes (Eppley, 2005). Aggressive spreading by sharp and robust rhizomes makes saltgrass an important colonizer of disturbed marshes (Seliskar and Gallagher, 2000), and a pioneer species in areas that are less favorable for plant growth (Uchytil, 1990; Reid, 2001). Saltgrass is a warm-season grass that turns brown in the winter and greens up in the spring about one month later than cool-season grasses in the same area (Uchytil, 1990; Kopec and Marcum, 2001).

1.2 Turfgrass Potential for Saltgrass

The population growth in the semiarid western United States has increased non-potable water use on golf courses. As the population continues growing, there is interest in the development of unused grasses as turfgrasses that can tolerate reduced irrigation and poor (high saline) water quality (Hughes et al., 2002). Colorado State University and the University of Arizona are in the process of evaluating the potential of saltgrass ecotypes for their use as turf on golf courses and in home lawns. About 10% of saltgrass ecotypes display desirable turf-type growth habit (Kopec and Marcum, 2001) and show turf like appearance with good color, high sod density and short internodes. Some of those ecotypes fill gaps quickly and have good potential for sod production (Kopec and Marcum, 2001). Clones that tolerate low mowing height and respond positively to nitrogen fertilization have also been identified (Koski and Qian, 2005). Saltgrass also appears to tolerate traffic and compaction better than other warm-season grasses (Kopec and Marcum, 2001). Saltgrass does appear to have some

genetic limitation for seed production but Hughes et al. (2002) have identified clones with good seed production.

Breeding of a new turfgrass species requires the evaluation of many traits as well as a clear understanding of environmental factors influencing those traits. Characterization of traits in relation to environmental factors should give a breeder an opportunity to identify particular environments that may be the best for the specific traits of interest. Furthermore, development of saltgrass varieties for the high plains of North America necessitates an understanding of cold hardiness in this warm season species. Finally, in order to make rapid progress in breeding it is important to develop techniques that can be used to induce flowering in the greenhouse and facilitate winter crossings.

1.3 Ecotype Variation Associated with Adaptive Traits

Research into the natural variation of ecologically important or adaptive traits within a plant species is important in understanding the role of natural selection and species' adaptation to their environments, gene flow and subsequent genetic drift that affects distribution of species (Jonas and Geber, 1999). Furthermore, association between plant morphology and environmental factors can be used to explore the morphological variation important for efficient selection in breeding programs (Ram et al., 2004) as well as for use in understanding the molecular basis of quantitative traits through QTL studies (St Clair et al., 2005). Adaptive traits are those that enable plants to adapt to their native environment, such as plant morphology, physiology and environmental stress tolerance (Johnson et al., 2004). The most common way of

examining natural variation within plant species is by growing ecotypes from different geographic regions in a common environment ('common garden'). The use of GIS (Geographical Information System) and climatic models such as PRISM (Parameter-Elevation Regression on Independent Slope Model) enable precise estimation of geographic and climatic factors from locations of an ecotypes' origin. If variation in measured traits among those individuals is related to geographic or climatic factors, traits may have responded to natural selection pressure and may have adaptive importance (Johnson et al., 2004). Such variation is commonly termed ecotypic variation. Variation that occurs gradually across the environmental gradients is termed clinal variation.

A typical statistical analysis used in research that includes a relationship between a set of multiple traits and geographic/climatic variables is principal component analysis on a data set of traits which is followed by multiple regressions of selected principal component scores on geographic and climatic factors (Erickson et al., 2004). St Clair et al. (2005) applied CCA (Canonical Correlation Analysis) approaches in a natural variation study of Douglas fir. CCA, which is actually an extended multiple regression, finds pairs of linear combinations between two sets of variables (canonical variables); measured traits and geographic/climatic variables. Linear combination (correlation) between canonical variables is maximized while other combinations are not correlated with those previously determined.

Intraspecific variation of ecologically important traits relative to geographic and climatic variables has been studied in many plant species. In many of those studies, gradual variation of traits across the environment indicated their adaptive

importance. For example, in a study with Douglas fir [*Pseudotsuga menziesii* (Mirbel) Franco], St Clair et al. (2005) found a strong relationship between bud phenology (bud set, emergence and growth), elevation and winter temperatures. They concluded that winter temperatures and frost dates were the most important factors for Douglas fir adaptation in Western Oregon and Washington. A study with Ponderosa pine (*Pinus ponderosa* L.) plants from four geographic regions (Keller et al., 2004) indicated an association between climatic factors and the temperature coefficient of respiration as well as a positive correlation of temperature coefficients of respiration with elevation and latitude. This study demonstrated that photosynthesis and photorespiration were adapted to the region of plant origin. In a study with the endemic California plant, *Clarkia unguiculata* Lindl., Jonas and Geber (1999) determined that the time required for seed germination and first flower formation varies gradually across the longitudes of plants' origin. Clampan et al. (1998) found that populations of Norway spruce (*Picea abies* L.) differ in their requirements for far-red light needed for photoperiodic control of bud set across the latitudes of origin. Only northern populations of Norway spruce required far-red light for bud set. In a similar study with Scots pine (*Pinus sylvestris* L.), Hurme et al. (1997) found a correlation of bud set timing and frost hardiness development with latitude. In this study, northern populations of Scots pine set buds and developed frost hardiness earlier than southern populations. In a study with sea buckthorn (*Hippophae rhamnoides* L.), Yao end Tigersted (1995) also reported strong clinal variation of populations across the latitudes. Plants gradually showed earlier dormancy, shorter

growth period and reduced plant height as well as greater cold hardiness with higher latitude.

1.4 Cold Hardiness and Freezing

Freezing is the most common environmental stress imposed on warm-season grasses in Colorado (Koski and Qian, 2005). Generally, the freezing temperature is one of the major abiotic factors that reduces yield in crops and limits geographical distribution of both wild and cultivated plant species (Pearce, 2001). Therefore, understanding the causes of freezing injury and the plant mechanism of freezing tolerance is very important.

Plant species from tropical and subtropical regions do not have the ability to survive freezing temperatures. These plants are typically injured by low non-freezing temperatures. This chilling injury is primarily the consequence of decreased fluidity of cell membrane and inactivation of membrane-bound ion pumps (Beck et al., 2004). On the other hand, plants from temperate regions have evolved mechanisms for cold hardiness and increased freezing tolerance upon exposure to a period of low but usually non-freezing temperatures and short day-lengths. This process is known as cold acclimation or cold hardening. It is initiated in nature by decreasing temperature and day length in the fall or early winter, and, depending on plant species, it commonly takes a few days to several weeks (Xin and Browse, 2000). In a study with Scots pine (*Pinus sylvestris* L.), Beck et al. (2004) found both environmental signals of short day and low temperature independently effective for inducing plant freezing

tolerance. Scots pine achieved the greatest freezing tolerance upon exposure to a short period of mild frost.

Freezing temperature typically injures plants by causing cell and tissue dehydration when water between cells freezes (Beck et al., 2004). Freezing temperatures cause ice formation in the space between cells because the fluid between cells has lower solute concentration and therefore a higher freezing point than the cytoplasm (Thomashow, 1999). Common locations of extracellular ice formation are leaves, cortex of woody shoots, bud parts and the subtending stem (Pearce, 2001). Water molecules can form ice either spontaneously (homogenous nucleation) or can be catalyzed by another substance, like INA (Ice Nucleation Active) bacteria (heterogeneous nucleation). Homogenous nucleation usually occurs at temperatures far below 0° C, but heterogeneous nucleation can occur just below 0° C and it is very common in moist climates (Pearce, 2001). Because ice has a lower chemical potential than liquid water, extracellular ice formation causes a sudden drop in water potential outside the cell. Consequently, water from the cytoplasm moves to the intracellular spaces. This severe cellular dehydration can damage cells in many ways, but it primarily damages the cell membrane system (Thomashow, 1999; Xin and Browse, 2000). The main cause of this membrane damage is a structural change in membrane lipids, from a bilayer to a non-bilayer structure (Pearce, 2001).

Therefore, a major factor in freezing tolerance is protection of cell membranes against dehydration associated with freezing (Pearce, 2001). This membrane stabilization includes several mechanisms such as changes in cell membrane lipid composition, the accumulation of soluble sugars, amino acids and hydrophilic

proteins from group LEA (Late Embryogenesis Abundant) in the surrounding cytosol, (Thomashow, 1999; Xin and Browse, 2000; Pierce, 2001).

Alternations in cell membrane lipids' composition have been observed in many plant species after exposure to cold non-freezing temperatures (Pearce, 2001). During cold acclimation the proportion of unsaturated fatty acids and membrane phospholipids increase. An increase in phospholipid content occurs during the early stages of cold acclimation as a result of an increased proportion of two major unsaturated phospholipid groups in the plasma membrane (Uemura et al., 2006; Wang et al., 2006). Lipid composition of cell membranes primarily affects their functional stability. An increased content of phospholipids and unsaturated fatty acids increases membrane hydration (fluidity) and decreases the propensity for freeze-induced lesions in the cell membrane (Uemura et al., 2006).

Soluble sugars accumulate during cold acclimation and the time of their accumulation is correlated with development of freezing tolerance in many plant species (Xin and Browse, 2000; Pearce et al., 2001). Xin and Browse (2000) described a mutational approach which provided the genetic evidence for the role of sugars in freezing tolerance. A mutant of *Arabidopsis thaliana* L. (or shortly *Arabidopsis) sfr4* had impaired ability to cold acclimate and did not accumulate sugar upon exposure to low temperature. Gene *sfr4* is one of the five *SFR* (sensitivity to freezing) genes that have a significant role in cold acclimation in *Arabidopsis* (Thomashow, 1999). In a similar experiment Xin and Browse (2000) used a constitutively freezing-tolerant *Arabidopsis* mutant *esk1* that accumulated sugars at warm temperatures. Constitutively freezing-tolerance mutants of *Arabidopsis* have

the gene *eskimo1* (*esk1*) which has a major role in freezing tolerance. These mutants have better freezing tolerance than wild-type plants without cold acclimation (Thomashow, 1999). Apparently, these soluble sugars function as cryoprotectants for critical enzymes and protect cell membranes by promoting their stability as molecules as well as by binding water as osmolytes to prevent excessive dehydration of essential tissue components (Xin and Browse, 2000). However, there is evidence that soluble sugars alone are not enough for plant freezing tolerance. The mutants of *Arabidopsis sfr2* and *sfr5* were sensitive to freezing but they normally accumulated sucrose during the cold acclimation (Thomashow, 1999). Genetically transformed tobacco with the invertase gene had increased sugar level without an increase in freezing tolerance (Xin and Browse, 2000).

Other solutes, including betaines and proline also accumulate in cytosol during cold acclimation (Pearce, 2001). Glycinebetaine can contribute to recovery of cells injured by freezing (Uemura et al., 2006). Xin and Browse (2000) used genetic transformation to examine the role of betaines in freezing tolerance. *Arabidopsis* naturally does not accumulate betaines. The transferred gene for choline oxidase from bacteria enabled betaine accumulation. Genetically transformed *Arabidopsis* with the gene for choline oxidase had significantly better freezing tolerance. However, betaines alone probably do not promote freezing tolerance since many freezing and chilling sensitive plants accumulate betaines (Xin and Browse, 2000).

Proline is an effective cryoprotectant *in vitro*. The *Arabidopsis* mutant *esk1* had 30-fold higher proline content without cold acclimation than wild-type plants. It is assumed that the significant increase in proline contributed to the freezing tolerance

in this mutant (Thomashow, 1999; Xin and Browse, 2000). Freezing stress can also result in the production of Reactive Oxygen Species or ROS. Oxygen free radicals (e.g. superoxide) cause oxidation and injury of cell membranes and organelles (Alscher et al., 1991). Proline is included in scavenging Reactive Oxygen Species and therefore cell and membrane protection (Uemura et al., 2006).

In addition, plant cells, upon exposure to cold temperatures produce dehydrin group proteins (Pearce, 2001). These proteins accumulate during seed desiccation which has resulted in their commonly accepted name of Late Embryogenesis Abundant or LEA proteins. LEA proteins have unusual hydration properties; they are extremely hydrophilic and remain soluble upon boiling. They are generally composed of very few amino acids or repeated amino acid sequences. Their role in the cell is still unknown but it is suggested that LEA proteins can bind water and protect cells from dehydration associated with freezing (Thomashow, 1999).

1.5 Flowering Characteristics

Many plants are adapted to flower at particular times of year to ensure optimal pollination and seed maturation. In these plants, the timing of flowering is primarily controlled by a variety of interrelated environmental signals that reflect the changing seasons (Levy and Dean, 1998; Simpson et al., 1999). Many plants respond to changes in daylength and extended periods of cold temperature, since they are both predictable and reliable indicators of seasonal progression. For example, *Arabidopsis thaliana* L. flowering is accelerated as the daylength increases, an environmental condition that signals the onset of spring and summer in the higher latitudes. This

process is known as a photoperiod response. *Arabidopsis* flowering is also accelerated following an extensive period of cold treatment, an environmental condition that signals the passage of winter and the onset of spring. This process is known as vernalization (Simpson et al., 1999).

The other factors that can influence flowering induction include light quality (spectral composition), light quantity (photon flux density), and water and nutrient availability. On the other hand, some plant species are less sensitive to environmental variables and appear to flower in response to internal cues such as plant size or number of vegetative nodes (Levy and Dean 1998).

Flowering can also be influenced by stresses such as nutrient deficiency, drought, and overcrowding. This response enables the plant to produce seeds, which are much more likely to survive the stress than is the plant itself. For example, flowering in *Arabidopsis* is stress responsive; light quality changes that accompany shading by near neighbors (enrichment in the far-red wavelengths) will promote flowering (Simpson et al., 1999). Finally, all these interrelated environmental and developmental signals control the production of a floral stimulus, which moves from the leaves and/or roots to the shoot apex. The induction of flowering leads to the specification of flowers at the shoot apex. This later phase can be persistent, even if conditions that induce flowering cease.

Temperature is very important in controlling seasonal responses in plants. One of the most important temperature effects is the initiation of flowering in response to extended exposures to low temperatures. During this vernalization response, flowering of plant species is repressed until they have been exposed to an extended

period of low temperature similar to those experienced in winter. The repression of flowering occurs even if the plant is growing under conditions (e.g. photoperiod) that would otherwise promote flowering. A study with *Arabidopsis* (Samach and Coupland, 2000) demonstrated that exposure to long days promotes early flowering in many varieties. However, those varieties that required low temperature treatments did not flower early even in long day conditions unless they had been previously exposed to winter conditions. Vernalization is generally most effective when plants are exposed to low temperatures for prolonged periods lasting several weeks. For example, exposure of *Arabidopsis* seedlings to 4°C for 6 weeks resulted in a maximal response, while treatment for 2 weeks gave a reduced effect (Samach and Coupland, 2000). This response is considered a form of biological timer that ensures that flowering is repressed until the end of the winter months. Plants, however, differ concerning susceptibility to this treatment. Many naturally occurring *Arabidopsis* will flower very late if they are not exposed to the vernalization treatment. In nature, these varieties do not flower until they have been exposed to winter conditions (Mouradov et al., 2002). On the other hand, many summer annual weeds germinate and flower in the same summer without requirements for vernalization. Winter cereals, like wheat (*Triticum aestivum* L.) and rye (*Secale cerale* L.), possess a facultative (or quantitative) vernalization requirement for flowering, which means that plants subjected to cold temperatures when young will flower earlier than non-vernalized plants, but non-verbalized plants will often eventually flower without exposure to low temperatures (Robertson et al., 1996).

The vernalization response is important in fitting the plant lifecycle to the environment in which it is grown, so it can make the best use of the seasonal opportunities for growth and avoid adverse climatic factors (Mahfoozi et al., 2001). The vernalization requirement is critical to temperate plants because it prevents transition to the reproductive phase in regions with cold winters. Once the vernalization requirement is met and the vegetative phase ends, cool season plants gradually lose their ability to tolerate below-freezing temperatures even when they are maintained at temperatures in the optimum range for low temperature acclimation. Based on this observation, and a previous study (Fowler et al., 1996) it is likely that any factor that influences the length of vegetative growth stage affects the expression of low-temperature tolerance genes in cool season plants exposed to acclimating temperatures. Consequently, timing of the transition from the vegetative to reproductive phase is of fundamental interest in both terms of flowering time and regulation of low-temperature gene expression.

Besides temperature, daylength is one of the most important environmental variables that influence the flowering of plants. The photoperiodic induction of flowering was discovered almost 90 years ago by Julien Tornois in hops (*Humulus lupulus* L.) (*cit.* Hempel et al., 2000). Shortly afterwards, Gaernar and Allard (*cit.* Samach and Coupland, 2000) proposed that the role of daylength is a general phenomenon that controls the initiation of flowering in many plant species. They showed that some plants would flower only in daily cycles in which the light period was longer than a particular threshold length, referred to as a critical daylength. Other plants, however, would flower only if daylength was shorter than a critical daylength.

In these plants, named long-day and short-day species, respectively, the duration of the critical daylength varies among species and also among varieties of the same species adapted to different latitudes. Varieties of the same species grown at different latitudes flower in response to different photoperiod lengths; for example, varieties of cocklebur (*Xanthium strumarium* L.) found in Florida flower when the daylength reaches 14 hours, but further north in Michigan they require 16-hour long days (Moore, 1995).

Different responses to day length require a mechanism for measuring time, and the biological clock that regulates daily rhythms in behavior also acts as the timer in the measurement of daylength. Shortly after the original description of photoperiod more than 60 years ago, Bunning (Samach and Coupland, 2000) proposed the circadian clock, a mechanism by which plants measure the duration of a photoperiod as a prerequisite for the photoperiodic control of flowering time. Features of the circadian rhythms are that the duration of one cycle is approximately 24 hours, that they are synchronized with the day/night cycle by environmental changes in light/dark, and that the rhythm persist when organisms are shifted from light/dark cycles to continuous conditions of light/dark. Parts of the circadian clock and their functions have been extensively studied in *Arabidopsis* (Dunlap, 1999; Kay and Millar, 1995; Samach and Coupland, 2000). These studies considered the circadian clock in three parts: a central oscillator that creates the 24-hour periodicity, input pathways to the oscillator that synchronies the oscillation to the day/night cycle, and outputs from the oscillator that present overt rhythms in gene expression and behavior.

Furthermore, a number of experiments (reviewed by Hempel et al., 2000) have indicated that the production of the photoperiod-induced floral stimulus occurs in the leaves of a wide variety of flowering plants. During the induction of flowering a floral stimulus moves from the leaves to the shoot apex. However, a study with *Arabidopsis* (Hempel et al., 2000) did not confirm whether its further floral specification is generally controlled by the leaves or by the shoot meristem. In other words, stimulus reaching the shoot apex might act on the apical meristem, or it might act directly on developing primordia. If the determination of flowering is controlled by the apical meristem, this implies that the meristem responds directly to the floral stimulus. If the leaves control the commitment to flowering, it is possible that the floral stimulus acts directly on developing primordia and/or on the shoot meristem. On the other hand, a leaf removal experiment confirmed that floral specification in Impatiens is controlled by the leaves.

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Chapter 2: Phenotypic Variation of Saltgrass Clones Relative to Geographic and Climatic Factors

2.1 Abstract

Breeding of a new turfgrass species requires an evaluation of numerous traits as well as an understanding of environmental factors influencing those traits. This study was initiated to characterize variation in saltgrass growth (morphology) and time of leaf browning in fall relative to environmental (climatic and geographic) factors at the source location (geographic location of clones' origin). Growth traits and time of leaf browning in fall were measured on 53 saltgrass clones from 42 locations established at one location (common garden) in Fort Collins, CO. Principal component analysis on the morphological traits extracted the first principal component (PC-1) that explained 78% of the variability. PC-1 and time of leaf browning in fall were related by multiple regressions to climatic and geographic factors at the source locations. Regression analysis of PC-1 indicated that variation in growth traits was related to seasonal climatic variables of summer drying and fall cooling that explained approximately 50% of variability in morphological traits in a two-variable regression model. Variation in time of leaf browning in fall was related to longitude and minimum winter temperature. These two variables explained about 60% of the total variability in time of leaf browning in fall. Information obtained in this study may facilitate selection in saltgrass breeding program by helping breeders identify the best environments for specific traits of interest.

2.2 Introduction

Increased population growth and periods of drought in the semiarid U.S. West have increased demands for potable water. Consequently, many golf courses in dry western regions rely heavily on irrigation with non-potable water (Kopec and Marcum, 2001; Hughes et al., 2002). As population growth continues in the west, there is interest in developing alternative turfgrass species that are water efficient and tolerate poor quality water. Colorado State University is currently evaluating saltgrass [*Distichlis spicata* L. (Greene)] and other native U.S. grasses for their potential use as turf.

Like many other native grass species, saltgrass has niche adaptation to particular environments. It is well adapted to grow in very harsh soil conditions and tolerates highly alkaline soils, extended drought, high salt concentration and certain metals like selenium (Uchytil, 1990; Eppley et al., 1998; Kopec and Marcum, 2001) which enables it to become a dominant plant species in environments with adverse conditions.

Correlations between variation in traits and environmental factors of plants' origins suggest that natural selection has led to adaptive variation to particular environments. Environmental factors are commonly measured directly as climatic or indirectly as geographic variables (St Clair et al., 2005). It is generally thought that adaptation to local environments has generated variability within all turf and other grass species (Casler and Duncan, 2003). Humphreys and Eagles (1988) found a strong positive correlation between perennial ryegrass (*Lolium perenne* L.) freezing

tolerance and mean temperature of the coldest month at the ecotype origin. Genome size of tall fescue (*Festuca arundinacea* L.) was strongly related to the latitude of clone origin (Ceccarelli et al., 1992) which suggests that structural changes in DNA have enabled tall fescue adaptation to different geographical locations. The decline in the presence of orchardgrass (*Dactylis glomerata* L.) in Japan has been related to the mean summer temperature in southern parts of Japan as well as the lowest temperature before snowfall in northeast parts of the country (Sugiyama, 2003). Erickson et al. (2005) reported that longitude of ecotype origin was the most important variable that explained morphological and physiological differences among three geographically distinct groups of the native self-pollinated grass, blue wildrye (*Elymus glaucus* Buckley). Ram et al. (2004) determined that large phenotypic variation occurred among 37 saltgrass ecotypes, but were not able to relate morphological traits to locations of clones' origin. In that study, the relationship between geographic and genetic distance of saltgrass ecotypes was very weak, indicating that genetic interaction existed between geographically distant saltgrass ecotypes.

Efficient selection in the saltgrass breeding program necessitates characterization of germplasm in relation to environmental (climatic and geographic) factors. Determination of environmental factors that are most strongly related to variation in a specific trait can be useful in a breeding program to identify environments that may be the best for traits for particular releases. This study was initiated to characterize variation in growth (morphological traits) and time of leaf

browning in fall among 53 saltgrass clones from 42 locations relative to geographic and climatic variables at location of clones' origin.

2.3 Materials and Methods

A total of 53 clones from 42 different sites (locations) were chosen from a larger collection of saltgrass germplasm. Clones were selected with the intention of covering a broad range of environmental (climatic and geographic) conditions over which saltgrass is distributed in the central and western United States (Table 2.1). Clones were established via rhizomes plugs in the summer of 2003 at the Horticultural Research Center at Colorado State University in Fort Collins, CO. Each clone had two 5 by 5 m plots in a randomized complete block design. The soil was a clay loam. No fertilizers were applied and the field was left unmowed. Irrigation was applied weekly during the summer by using a linear move irrigation sprinkler system.

Latitude, longitude and elevation were obtained for each location of the clones' origin (http//www.topozone.com). The climatic conditions for each location were obtained from GIS (Geographic Information System) layers generated by the PRISM (Parameter Elevation Regressions on Independent Slopes Model) statistical geographical model, which estimates mean monthly and annual temperature and precipitation, mean minimum and maximum monthly temperature, and the average dates of the last and the first frost for 4 x 4 km grid cells. All traits except time of leaf browning in fall were measured in late August/early September of 2004. Clone traits measured were morphological (growth) characteristics, leaf color, chlorophyll content and time of leaf browning in the fall. Traits of morphology or growth included leaf length, leaf width, internode length, canopy height and the angle that leaves projected

with the stem. Earliness in leaf browning in the fall was measured in the last two weeks of October. This trait correlated with greater cold hardiness of the turfgrass clones as noted in Rukavina et al. (2006). Evaluations of time of leaf browning in fall were performed on a scale from 1 (brown) to 9 (green), as previously described (Rukavina et al., 2006). Following preliminary data analysis, some of the traits were removed from the data because they were not correlated with climatic/geographic variables or showed non-significant location effect. Upon removal, four variables were retained for further analysis (Table 2.2).

All statistical analyses were performed with SAS software (SAS Institute, 1999). Analysis of variance (PROC MIXED) was performed to determine the effect of location of clones' origin on measured traits. Locations of clones' origin and clones nested within locations were treated as random factors.

Principal component analysis (PROC PRINCOMP) was performed on the data set of three morphological (growth) variables (leaf length, canopy height and internode length). The first principal component explained most of the variation in traits of growth and was used as the estimate of "growth" in further analysis. Relationships between measured traits and geographic and climatic factors at locations of clones' origin were determined using correlation and regression. Simple and multiple regression models were constructed by regressing growth (PC-1) and average rating of time of leaf browning in fall on geographic and climatic factors. Geographic factors included latitude, longitude and elevation as well as the interaction term between latitude and longitude. Estimated climatic variables included monthly and annual precipitation and temperature (mean, minimum and maximum)

as well as several seasonal rate variables identified in the regression analyses; fall cooling (September maximum temperature – October maximum) and summer drying (June mean precipitation – August mean precipitation). Model building was performed by using the R^2 selection method of the PROC REG procedure, which identify the models with the largest R^2 for the specific number of variables considered.

Table 2.1 Latitudes, longitudes and elevations of sampling sites (locations) of saltgrass clones used in the experiment.

Clone	Location	Latitude (° N)	Longitude (° W)	Elevation (ft)
1220	Ruby Valley, NV	40.360	115.447	6100
1250	Carlin, NV	40.714	116.103	5000
1260	Ruby Lake, NV	40.183	115.471	5962
1280	Deeth, NV	41.066	115.274	5340
1330	Wendover, UT	40.737	114.037	4430
1420	Evanston, WY	41.268	110.963	6700
1440	Green River, WY	41.529	109.466	6250
1460	Sturgis, SD	44.410	103.509	3450
1490	Chamberlain, SD	43.811	99.330	1465
1720	Ansley, NE	41.288	99.381	2400
1840	Lusk, WY	42.763	104.452	5100
C1660	Humboldt Sink, NV	39.973	118.606	3850
C1100	Pueblo, CO	38.254	104.609	4600
A1210	Denver, CO	39.739	104.984	5300
A1350	Denver, CO	39.739	104.984	5300
A1370	Denver, CO	39.739	104.984	5300
A1390	Denver, CO	39.739	104.984	5300
A1410	Denver, CO	39.739	104.984	5300
A1500	Aurora, CO	39.729	104.831	5400
A1530	Aurora, CO	39.729	104.831	5400
A1650	Aurora, CO	39.729	104.831	5400
A1970	Longmont, CO	40.167	105.101	4950
A11070	Longmont, CO	40.167	105.101	4950
A11230	Wellington, CO	40.704	105.008	5200
A11260	Timnath, CO	40.529	104.985	4850
A11370	Timnath, CO	40.529	104.985	4850
130610	Cheyenne, WY	41.140	104.820	4925
130630	Guernsey, WY	42.270	104.741	4440
130760	Scotts Bluff, NE	41.838	103.697	4100
130880	Chadron, NE	42.829	102.999	3360
130920	Hay Springs, NE	42.684	102.689	3830
130960	Gordon, NE	42.805	102.203	3550
131010	Cottonwood Lake, NE	42.914	101.674	3240
131020	Winner, SD	43.377	99.859	600
131130	O'Neill, NE	42.458	98.647	2000
131140	Grand Island, NE	40.925	98.342	1870
131350	Paxton, NE	41.124	101.356	3050
131470	McCook, NE	40.202	100.625	2550
131930	Lamar, CO	38.087	102.620	3610
131980	Las Vegas, NM	35.594	105.223	6880
132090	Albuquerque, NM	35.084	106.651	5050
132180	Garden City, KS	37.972	100.872	2840
132220	Atwood, KS	39.807	101.042	2950
132350	Grand Junction, CO	39.064	108.550	4600
132410	Grand Junction, CO	39.064	108.550	4600
132480	Twin Falls, ID	42.563	114.460	3720
132500	Letha, ID	43.894	116.647	2285
132510	Alkali Lake, OR	42.977	120.027	4200
132570	Lakeview, OR	42.189	120.345	5050
132630	Alturas, CA	41.487	120.541	4370
132650	Burns, OR	43.586	119.053	4150
12320	Fresno, CA	36.748	119.771	300
12560	Fresno, CA	36.748	119.771	300

Table 2.2 Description of saltgrass clones' traits that were included in statistical analyses.

Trait	Abbreviation	Description (unit)
Canopy height	Conhgt	Measured from the ground level to the top of the tallest leaf (cm)
Internodes length	Interlgt	Average value of the three lowest internodes length on three steam (mm)
Leaf length	Lflgtavg	Average value of the three lowest leaves length (measured from the stem to the top of the leaf) on the stem (cm)
Time of leaf browning in fall	None	Leaf color visually evaluated on a scale from 1 (brown) to 9 (green)

2.4 Results and Discussion

Results from analysis of variance indicated that canopy height, internode length and leaf length were significantly influenced by location of clone's origin, while time of leaf browning in fall had borderline significance. Leaf width showed non-significant location effect and was removed from further analysis (Table 2.3).

Table 2.3 The effect of the location of clones' origin on the traits that were measured in the experiment.

Trait	F Statistic	P value
Canopy height	3.71	0.012
Internodes length	6.2	0.001
Leaf length	3.16	0.022
Time of leaf browning in fall	2.45	0.055
Leaf width	1.23	0.38

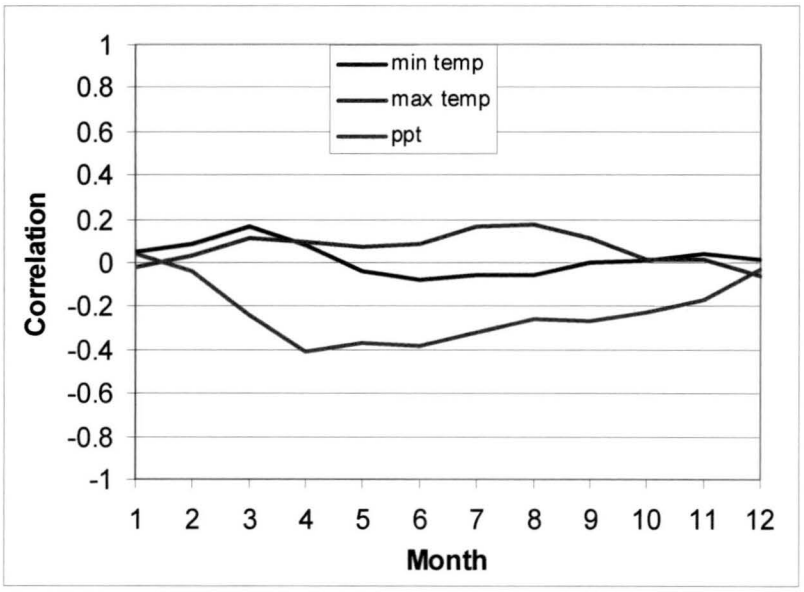
The first principal component from the principal component analysis explained 78% of the variability in the three morphological variables and had an eigenvalue of 2.35. The remaining two principal components had eigenvalues less than one and explained only a small percent of the total variability. Based on these results, only PC-1 was retained for further analyses and represented growth in these clones.

The initial correlation analysis between growth (PC-1) and the climatic and geographic factors only found significant negative correlations of growth with spring and early summer precipitation (Fig. 2.1A). Increased growth that was associated with reduced spring and summer precipitation can be explained by water use efficiency. The plants from arid areas used water more efficiently than the plants from humid climates. In a common garden, water is equally available to all plants, so the plants from drier locations grew more with the water applied than those from wetter

locations. Growth was moderately negative correlated with summer drying ($r = 0.38$, $p = 0.014$) and moderately positive correlated with fall cooling ($r = 0.37$, $p = 0.015$).

Time of leaf browning in fall had a strong positive correlation with longitude ($r = 0.65$, $p<0.0001$) and a moderate negative correlation with latitude ($r = -0.335$, $p = 0.0303$). Winter minimum temperatures were positively correlated with browning as well ($r >0.7$, $p<0.0001$). Longitude and minimum winter temperature were also strongly associated ($r = 0.59$, $p<0.0001$). Time of leaf browning in fall had strong negative correlations with spring and summer precipitation, but correlations were positive with winter precipitation (Fig. 2.1B). This indicated that greater precipitation in spring and summer reduced average browning grade (increased cold hardiness), but greater precipitation in the winter increased browning grade (reduced cold hardiness). Presumably, increased winter moisture is association with active growth during the winter, thereby reducing potential cold hardiness (increased browning grade).

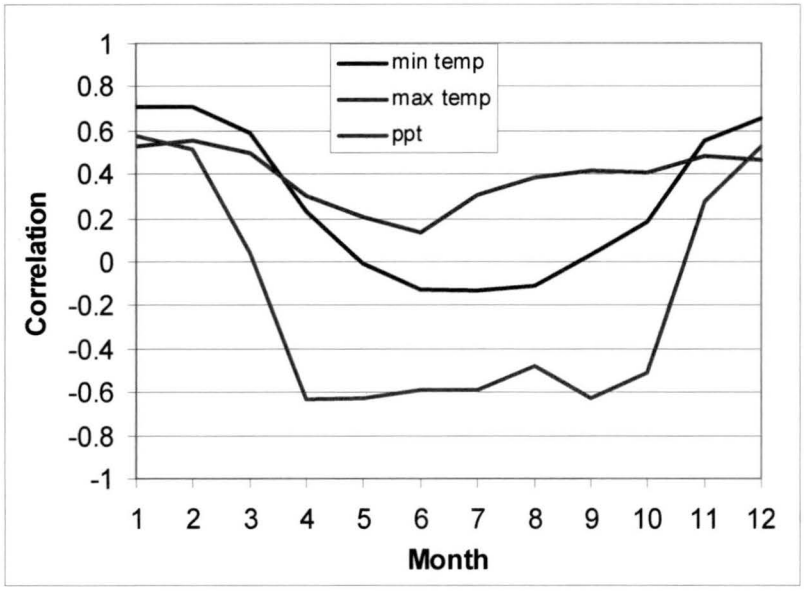
Figure 2.1A Correlations between monthly climatic data* and traits of saltgrass growth.



Levels of statistical significance are r = 0.304 for a p value of 0.05, and r = 0.393 for a p value of 0.01.

* Key to monthly climatic data: min temp = minimum temperature, max temp = maximum temperature, ppt = precipitation.

Figure 2.1B Correlations between monthly climatic data* and time of saltgrass leaf browning in fall.



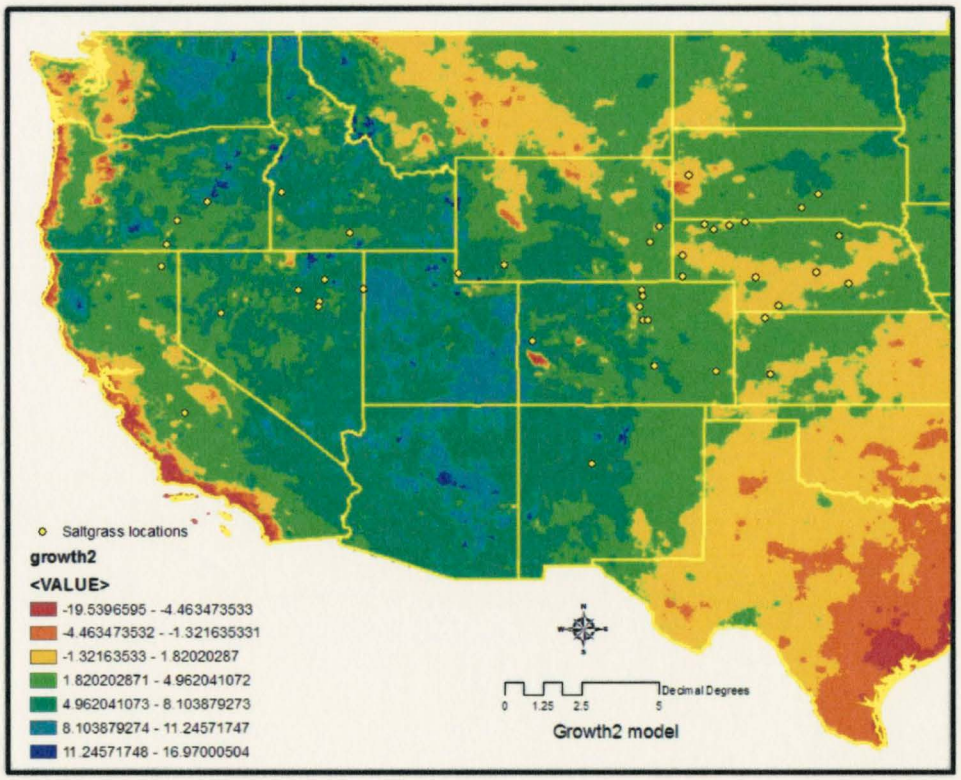
Levels of statistical significance are r = 0.304 for a p value of 0.05, and r = 0.393 for a p value of 0.01.
* Key to monthly climatic data: min temp = minimum temperature, max temp = maximum temperature, ppt = precipitation.

Regression analysis of saltgrass growth indicated that climatic factors contributed to the variability in morphological traits much more than geographic factors. Initially, one of the best four-variable regression models included September and October maximum temperatures and June and August precipitation. However, the sign of the coefficients varied for each of the precipitation and temperature variables. This suggested that rate of change was important for summer precipitation and fall temperatures. As a result, the two variables, summer drying and fall cooling were constructed. Of simple regression models, climatic variables, such as summer drying and precipitation during the spring and summer months, explained a similar amount of variability, between 14-20%. Two-variable regression models suggested that most of the variation in morphological traits was associated with summer drying and fall

cooling (R^2=0.495). Because both summer drying and fall cooling were significantly correlated with PC-1, it seems likely that they are important factors associated with variation in saltgrass morphology. The regression model described above is evident in the map that summarizes variation in saltgrass growth (Fig. 2.2). Growth pattern seems almost a quadratic with low values along the coast and in the mid-west and with high values in the Rockies.

Regression analysis of time of leaf browning in fall indicated that variation in that trait was a function of both geographic and climatic factors. Longitude was the most important geographic factor that explained 42% of variability. Of climatic factors, winter, as well as January and February minimum temperatures explained a similar degree of variability (between 49-51%) in simple models. Of the two-variable models, a number of two-variable combinations explained 60% of the variation. However, based on variability explained in simple regression models, it can be assumed that time of leaf browning in fall is strongly related to the winter months' minimum temperatures and longitude, but precipitation can explain some of the residual variation. The regression model in which time of leaf browning in fall is a function of longitude and February minimum temperature can be seen on the map (Fig. 2.3). As expected, as source locations move from west warmer sites along the coast to east cooler sites in the interior of the country, values for time of leaf browning decrease, and therefore potential cold hardiness increases.

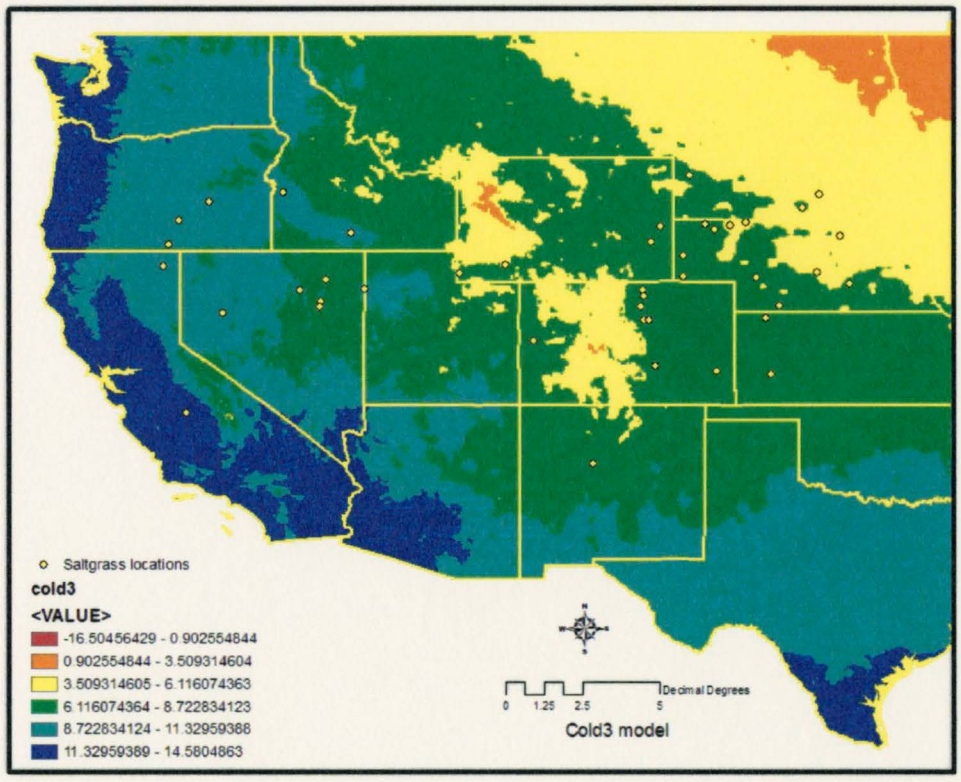
Figure 2.2 Geographical variation observed in saltgrass growth. Map presents multiple regression model where growth is a function of summer drying and fall cooling. Color grouping are in the units of a standard deviation of the 42 original points of saltgrass clones' origin.



Seasonal climatic parameters of summer drying and fall cooling are likely the most important factors for saltgrass growth adaptation in the central and western United States. Decreased precipitation during the summer has likely resulted in natural selection for decreased growth during summer water deficiency. This was presumably an adaptation to summer drought. On the other hand, decreased temperatures in fall (or fall cooling) seem to have resulted in natural selection for increased growth as soon as conditions for growth became favorable. This is probably because lower temperatures in the fall reduce the water limitation. This type of association between growth variation and climatic factors is similar to that found in studies with other plant species. Biondi and Fessenden (1999) studied variation in Lodgepole pine (*Pinus contorta* Dougl.) growth characteristics relative to climatic factors in California. They found a high negative correlation between July temperature and growth of Lodgepole pine. The increased temperature in July caused greater water stress which in turn resulted in decreased growth. In a similar study with Douglas fir [*Pseudotsuga menziesii* (Mirbel) Franco] in western Oregon and Washington, St Clair et al. (2005) found that seedling sources with earlier bud burst produced increased growth before water limitation, and generally came from areas with higher summer temperature and lower summer precipitation. Furthermore, some morphological features, like smaller leaves in grasses that reduce transpiration, have a significant role in adaptation to an arid environment (Erickson et al., 2004).

Geographic variation in cold hardiness is generally considered as an adaptive response to environmental factors, such as temperature, photoperiod and moisture (Balduman et al., 1999). Many plant species have shown latitudinal variation in cold

Figure 2.3 Geographical variation observed in saltgrass time of leaf browning in fall. Map presents multiple regression model where time of leaf browning is a function of longitude and February minimum temperature. Color grouping are in the units of a standard deviation of the 42 original points of saltgrass clones' origin.



This is the first study that provides information about geographic and climatic factors that contribute to the variation in growth (morphology) and time of leaf browning in fall (indication of cold hardiness) among saltgrass clones. The two analyzed variables in this study, growth (PC-1) and time of leaf browning in fall showed different patterns of variation relative to geographic and climatic factors. While morphological traits showed variation associated with fall cooling and summer drying, variation in time of leaf browning in fall was strongly associated with longitude and minimum winter temperatures.

hardiness (Yao et al., 1995; Hurme et al., 1997; Finne et al., 2000; Hannerz and Westin, 2000) or in phenological traits related to cold hardiness such as growth cessation and dormancy (Ingvarsson et al., 2005). In our study, the north-south interval of tested clones might not be broad enough to show strong latitudinal variation in time of leaf browning in fall. Time of leaf browning in fall showed east – west variation which was associated with minimum temperatures during the winter months and rainfall pattern which are highly correlated with longitude. Cold hardiness was greater in clones from eastern locations which is likely due to the differences in winter temperatures and rainfall patterns between east and west sites. The climate on western sites is milder than that on eastern sites and more of the rainfall happens in the winter months. Our results indicate that lower temperatures, particularly temperatures during the winter months, have resulted in selection of greater cold hardiness (lower browning grade) in saltgrass clones from eastern sites. A lower browning grade, associated with greater spring and summer precipitation, occurs in clones from eastern locations (those locations generally associated with more precipitation in spring and summer). Our results correspond to a similar study with Scots pine (*Pinus sylvestris* L.) cold hardiness (Anderson and Fedorkov, 2004). They found a strong association between Scots pine cold hardiness and longitude and interpreted longitudinal effects as due to the differences in temperatures between east and west. Cooler temperatures on eastern sites, especially in October (which is the most important for gaining winter hardiness) enabled faster cold acclimation and greater cold hardiness. In a study with Douglas fir, Balduman et al. (1999) found greater east-west variation in cold hardiness as compared to north-south variation. In

that study, cold hardiness increased with lower winter temperatures. On the other hand, freezing damage in plants in fall increased with lower elevation and higher temperature in January.

In summary, seasonal climatic variables were better than geographic variables in explaining variation in growth of saltgrass ecotypes. Decreased growth may be an adaptation to drought during the summer months in the central and western United States. In the case of decreased growth, it seems likely that natural selection has resulted in morphological traits that relate to drought avoidance. On the other hand, selection for increased growth during fall cooling and therefore a period of lower drought have also resulted in variation among saltgrass clones. Time of leaf browning in fall had a discernable pattern on the landscape. Longitude of clone origin is likely associated with different temperature regimes during the winter, which have resulted in natural selection for cold hardiness in saltgrass clones. Overall, our results indicate variation in saltgrass growth (morphology) and time of leaf browning in fall that is related to the environmental factors of source locations. A saltgrass breeding program should therefore maintain geographical structure with regards to those traits analyzed in this study. Traits of saltgrass growth (morphology) and time of leaf browning in fall showed a relationship with environmental factors at source location, indicating that these traits may be of adaptive importance.

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Chapter 3: Freezing Tolerance of 27 Saltgrass Ecotypes from Three Cold Hardiness Zones

3.1 Abstract

Freezing is the major abiotic stress that limits geographical distribution of warm season turfgrasses. Prior studies have indicated variation in freezing tolerance in saltgrass clones. Therefore, this study examined freezing tolerance of 27 saltgrass clones as related to collection sites in three zones of cold hardiness. Furthermore, these clones were evaluated for time of leaf browning in the fall with the intent to determine if there was a correlation between this trait and freezing tolerance. Rhizomes were sampled during 2004 and 2005 midwinters from clones established in Fort Collins, Colorado, and then subjected to a freezing test in a programmable freezer. Saltgrass freezing tolerance was highly influenced by the climatic zone of clone origin in both years of the experiment. Clones with greater freezing tolerance turned brown earlier in fall in both seasons. Ranking of zones for the average LT_{50} (lethal temperature at which 50% of rhizomes died) was: zone 4, most northern, (-17.2 °C) < zone 5 (-14.4 °C) < zone 6, most southern, (-11.1 °C) in 2004 and zone 4 (-18.3 °C) < zone 5 (-15.7 °C) < zone 6 (-13.1 °C) in 2005. Clones from northern areas tolerated lower freezing temperatures overall. This likely indicates that freezing tolerance is inherited. Large intraspecific variation in freezing tolerance may be effectively used in developing cold hardy cultivars.

3.2 Introduction

Saltgrass [*Distichlis spicata* (L.) Greene] is a North American native that has only recently been used as a forage crop but only now is being looked at for potential for turf adaptation (Kopec and Marcum, 2001). It is a warm season species with excellent drought and salinity tolerance and commonly appears in salty and alkaline soils throughout the central and western United States (Eppley et al., 1998). With continued population growth and periodic drought in the semiarid western United States there is an increased public interest in using turf species that can tolerate reduced irrigation and saline soil.

Freezing is the major environmental stress that limits geographical distribution of warm season turf grass species. Variation in freezing tolerance still exists in most grasses and can be effectively used in breeding (Larsen, 1994). A previous freezing study with six saltgrass clones (Shahba et al., 2003a) indicated variation in freezing tolerance and suggested that freezing tolerance could be associated with origin of clone. Differences in freezing tolerance among cultivars and ecotypes have been found in other warm-season turf species such as seashore paspalum (*Paspalum vaginatum* Swartz) [Cardona et al., 1997; Cyril et al., 2002], zoysiagrass (*Zoysia* spp.) [Dunn et al., 1999] buffalograss [*Buchloe dactyloides* (Nutt.) Engelm.] [Qian et al., 2001], and bermudagrass (*Cynodon* spp.) [Anderson et al., 2003]. On the other hand, Busey (2003) observed variation in freezing tolerance among St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] cultivars at only two out of 24 experimental counties in Florida. In a freezing experiment with buffalograss performed in Colorado, cultivars with better freezing tolerance turned

brown earlier in fall, whereas those less tolerant remained green until mid November (Qian et al., 2001).

In order to develop new cultivars for specific geographic locations, it is important to study freezing tolerance in this species. Thus, this two-year experiment was initiated to examine relative freezing tolerance of 27 saltgrass ecotypes collected in three U.S. zones of cold hardiness but grown in one location and to determine if there is an association between leaf color retention in fall and freezing tolerance.

3.3 Materials and Methods

A total of 27 saltgrass clones collected at different locations in three zones of cold hardiness (USDA zones 4, 5, and 6) were used in this experiment (Table 3.1). Clones were established in small (5 by 5 m) plots on a clay loam with an initial N content of 23 mg kg^{-1} at the Colorado State University Horticultural Research Center, Fort Collins, CO in July 1998. Each clone had two plots in a randomized complete block design. No fertilizers were applied and the field was mowed once a week at the height of 6 cm. Irrigation was applied once a year in July by flooding the field with approximately 12 cm of water. Clones used in this experiment were dug up by a shovel from the semi-frozen soil from mid January to late February in 2004 and 2005 (Fig. 3.1A and B). At that time saltgrass clones were fully cold acclimated (Shahba et al., 2003a). The procedure was repeated three successive times in each experimental year. The equivalent of two one-gallon pots was dug for each clone. After removing soil and inert matter, rhizomes of each clone were washed in cold water and divided into eight groups. Each group of rhizomes was wrapped in moist tissue paper that was then enclosed in aluminum foil. The preparation of samples for freezing was

performed in a cold environment. Following preparation, samples were subjected to freezing treatment in a thermo-controlled freezer (Tenny Jr. Programmable Freezer, Tenny Inc., South Brunswick, NJ). Samples were initially left at -2 C for 16 hours to nucleate ice and then linearly cooled at 2°C/h. Samples were thawed when temperatures reached - 2, -12, -14, -16, -18, -20, -22 and –24 °C. One group of rhizomes of each clone was removed from the freezer when a specific temperature was reached and held at ~ 5°C overnight. Following thawing, 10 individual rhizomes (~ 2 cm long, with 1-2 nodes) of each target temperature were planted in a row of styrofoam cells filled with commercial potting media. Styrofoam trays were then placed under a mist system in the greenhouse. The average temperature in the greenhouse during the experiment was approximately 21°C, with a 14-hour light period. Mist occurred at 10-minute intervals for 12 hours and was turned off during the night. Clones' survival was evaluated by observing shoot re-growth during a 2 month period.

The experiment was analyzed as a completely randomized design with three replicates. The logistic regression procedure (proc logistic) of SAS (SAS Institute, 1996) in which the ratio of survived and total number (10) of rhizomes was the function of temperature was used to predict LT_{50} (lethal temperature resulting in 50% mortality) for each treatment combination. LT_{50} (calculated as -intercept/slope) was then used as a response variable in a nested mixed model ANOVA (proc mixed) in SAS. Cold hardiness zones of clones origin and clones nested within zones were treated as fixed factors, while repetitions and interaction between repetitions and zones were treated as random factors in both years. Differences between least squares

means were used to compare the levels of factors of interest. To test the effect of year on freezing tolerance, data from both years were merged and two new fixed effects, year and interaction between year and zone were included in the analysis.

Leaf color of clones was visually evaluated in the fall by the senior author using a scale from 1 (brown) to 9 (green), as previously described (Qian et al., 2001). Color evaluation was performed in October and November in both experimental years. The relationship between time of leaf browning in fall and saltgrass freezing tolerance was determined by using regression in SAS.

Figure 3.1A The minimum and maximum air temperatures in Fort Collins, CO from January 16 to February 15 in 2004. Crosses indicate sampling dates.

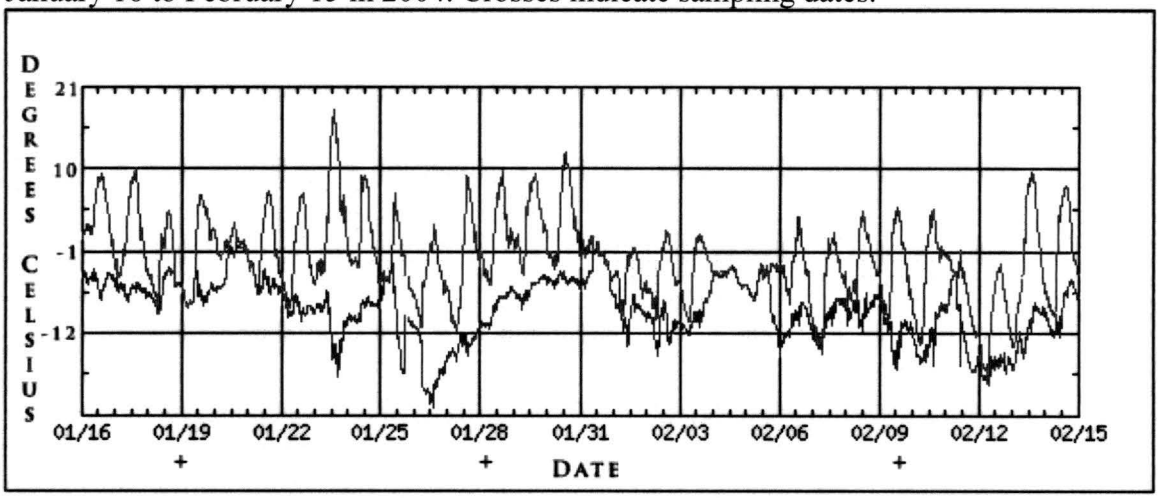


Figure 3.1B The minimum and maximum air temperatures in Fort Collins, CO from January 15 to February 15 in 2005. Crosses indicate sampling dates.

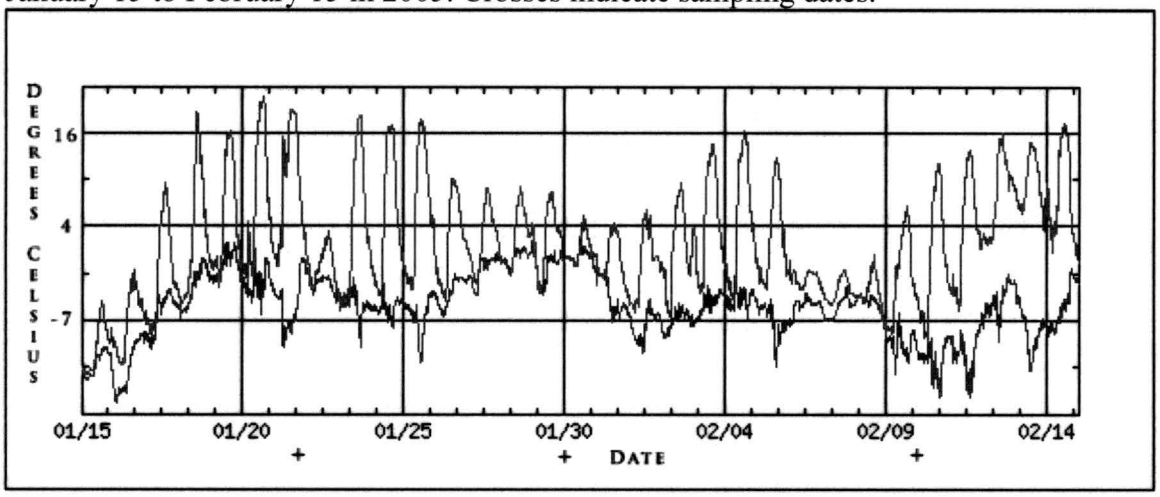


Table 3.1 Saltgrass clones used in the experiment according to their origin and cold hardiness zone.

Clone	Cold-hardiness zone	Place of origin
1500	4	Chamberlain, SD
1650	4	Belle Forche, SD
1720	4	Ansley, NE
1840	4	Lusk, WY
1870	4	Lusk, WY
A1210	5	Denver, CO
A1240	5	Denver, CO
A1290	5	Denver, CO
A1350	5	Denver, CO
A1370	5	Denver, CO
A1390	5	Denver, CO
A1410	5	Denver, CO
A1490	5	Aurora, CO
A1500	5	Aurora, CO
A1530	5	Aurora, CO
A1650	5	Aurora, CO
A1860	5	Longmont, CO
A1970	5	Longmont, CO
A11070	5	Longmont, CO
A11230	5	Wellington, CO
A11260	5	Timnath, CO
A11370	5	Timnath, CO
A11380	5	Timnath, CO
C1120	6	Delta, CO
C1560	6	Aberdeen, ID
C1660	6	Humboldt Sink, NV
C1920	6	De Beque, CO

3.4 Results and Discussion

3.4.1 First year of the experiment (fall 2003 and midwinter 2004)

Cold hardiness zones of clones origin and clones significantly influenced freezing tolerance of saltgrass ($p = 0.0002$ and $p < 0.0001$, respectively), which indicates that variability in freezing tolerance that exist among saltgrass clones could be associated with clones' origin and was likely inherited adaptation.

The average freezing tolerance of saltgrass clones (LT_{50} mean values) was significantly different among all three cold hardiness zones of clones' origin. Clones from zone 4 tolerated lower freezing temperatures (LT_{50} –17.2 °C) as compared to clones from zone 5 (LT_{50} = -14.4 °C). Clones from zone 5 exhibited significantly lower LT_{50} values than clones from zone 6 (LT_{50} = -11.1 °C).

LT_{50} values of saltgrass clones from zone 4 ranged from -17.8 °C (clone 1720) to –17.1 °C (clone 1870) with no significant differences among them. A broader range in freezing tolerance was observed for zone 5, with clones' LT_{50} values ranging from -17.8 °C (clone A1290) to -11.9 °C (clone A11370). Clones A1290, A1530, A11070 and A1500 had better freezing tolerance than the other eight clones from zone 5, with the exception of no significant difference in freezing tolerance between clones A1500 and A11380. Clones from zone 6 exhibited the least freezing tolerance. These clones LT_{50} values ranged from -12.6 °C (clone C1120) to -9.5 °C (clone C1920). Freezing tolerance of clone C1120 was better than that of clones C1660 and C1920. There was no difference in freezing tolerance between clones C1120 and C1560.

A significant positive relationship between LT_{50} and fall color retention was

determined in October and November of 2003 (Fig. 3.2A and B). Those saltgrass clones with lower LT_{50} turned brown earlier (lower color grade scale).

Figure 3.2A Linear regression plot of relationship between LT_{50} (^{o} C) and leaf color retention for 27 saltgrass clones in October 2003. Leaf color was visually evaluated on a 1 to 9 scale where 1 = brown and 9 = green leaf.

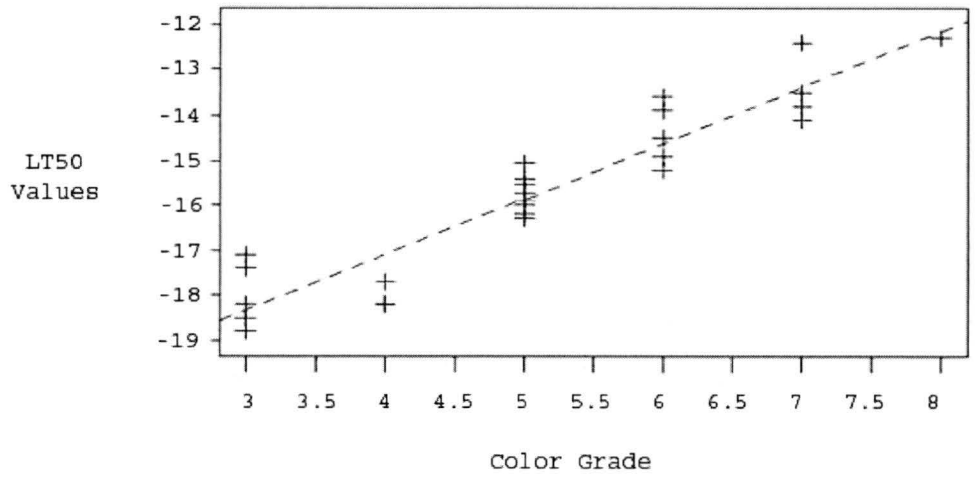
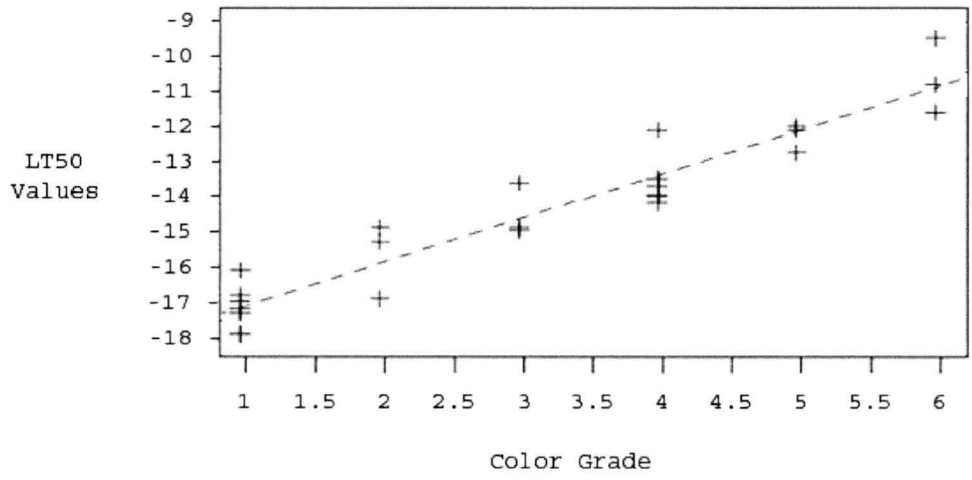


Figure 3.2B Linear regression plot of relationship between LT50 (o C) and leaf color retention for 27 saltgrass clones in November 2003. Leaf color was visually evaluated on a 1 to 9 scale where 1 = brown and 9 = green leaf.



3.4.2 Second year of the experiment (fall 2004 and midwinter 2005)

Saltgrass' freezing tolerance was significantly influenced by climatic zone of clones' origin ($p < 0.0001$) and clones ($p < 0.0001$). These results indicate within species variation in freezing tolerance that is associated with origin of clone.

The average LT_{50} of saltgrass clones were significantly different among zones. These values were slightly lower than those in the first experimental year. Clones from zone 4 had an average LT_{50} of -18.3°C, which was lower than that of clones from zone 5 (-15.75°C). Likewise, LT_{50} values of zone 5 clones were lower than those of clones from zone 6 (-13.1°C).

Similarly to the first year of the experiment, clones from zone 4 did not significantly differ in freezing tolerance. Their LT_{50} values ranged from -18.8°C (clone 1500) to -17.8°C (clone 1650). Clones from zone 5 again had a broader range

of freezing tolerance, with LT_{50} values from -18.6°C (clone A1500) to -13.6°C (clone A11260). Clones A1500 and A1290 showed the greatest freezing tolerance in this year, with no significant difference between clones A1290 an A11070. LT_{50} values of clones from zone 6 ranged from -14.1°C (clone C1560) to -12.3°C (clone C1660). Clones C1560 and C1120 had better freezing tolerance than clones C1920 and C1660 from this zone. Leaf fall color retention and LT_{50} values for the 27 clones were again significantly related in October and November observation (Fig. 3.3A and B). Those clones with lower LT_{50} values turned brown earlier in the fall.

Figure 3.3A Linear regression plot of relationship between LT_{50} (° C) and leaf color retention for 27 saltgrass clones in October 2004. Leaf color was visually evaluated on a 1 to 9 scale where 1 = brown and 9 = green leaf.

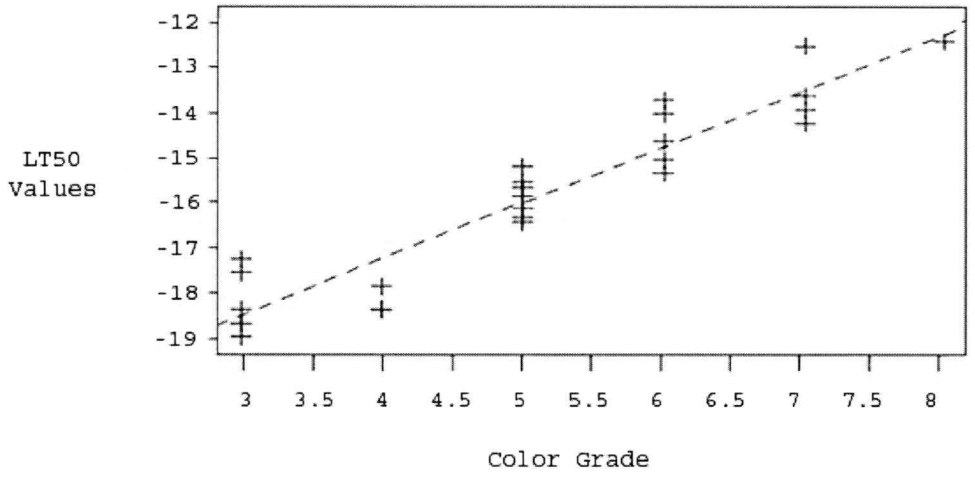
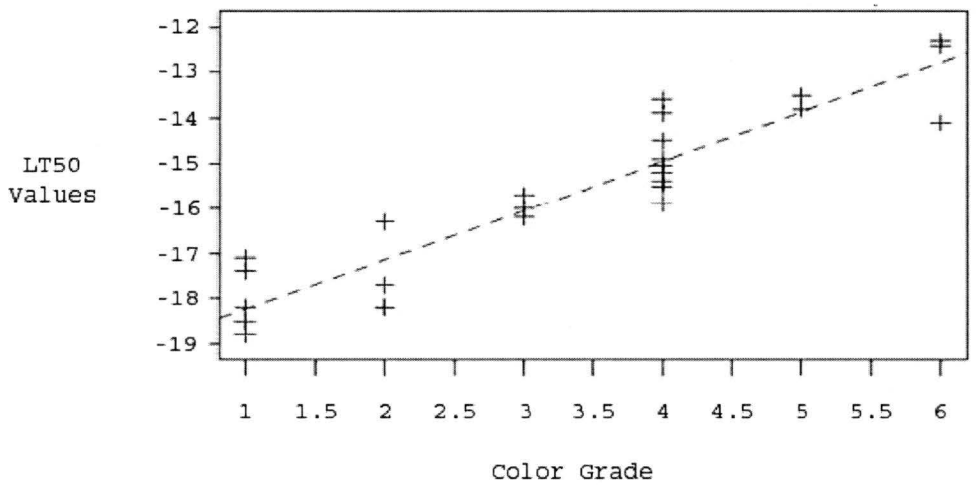


Figure 3.3B Linear regression plot of relationship between LT50 (o C) and leaf color retention for 27 saltgrass clones in November 2004. Leaf color was visually evaluated on a 1 to 9 scale where 1 = brown and 9 = green leaf.



Freezing tolerance was significantly influenced by the experimental year ($p < 0.0001$). The clone ranking for freezing tolerance was the same in both years, but average LT_{50} values of all three cold hardiness zones were lower in the second year (Table 3.2). Overall, all clones showed lower LT_{50} values in the second year. The difference in freezing tolerance between the two experimental years may be related to climatic conditions. Lower temperatures during cold acclimation period (second half of October and first half of November) in the second experimental year allowed saltgrass to achieve greater freezing tolerance. Warm temperatures in October and November in the first experimental year likely reduced the rate of saltgrass cold acclimation and subsequent freezing tolerance. Differences in saltgrass freezing tolerance between two experimental years were also found by Shahba et al. (2003a).

They explained differences in freezing tolerance as due to the differences in freezing temperatures in two years. Permanently frozen soil during the winter in the first year of research by Shahba et al. (2003a) enabled saltgrass to maintain greater freezing tolerance. Saltgrass freezing tolerance was less in the second year due to the cycles of soil freezing and thawing.

Table 3.2 Mean values for LT50 of cold hardiness zones in the both years of the experiment.

Zone	LT50 values (° C) in 2004	LT50 values (° C) in 2005
4	-17.2	-18.3
5	-14.4	-15.7
6	-11.1	-13.1

Significant differences in average LT_{50} values were found among all three climatic zones of clones' origin in both experimental years. Clones that originated in northern (cooler) areas had greater freezing tolerance than clones from southern (warmer) climates. This supports the suggestion from a previous study (Shahba et al., 2003a) that saltgrass' freezing tolerance is associated with clones' origin. The rank of LT_{50} values observed in this study is comparable to that reported by Shahba et al., (2003a). Clone A1290 was one of the most cold hardy clones while clone C1660 was the least cold hardy in both experiments.

Differential response in saltgrass clones' cold tolerance may be related to differences in soluble carbohydrate content in rhizomes. Studies with buffalograss (Ball et al., 2002) and saltgrass (Shahba et al., 2003b) have shown that cultivars and clones with greater freezing tolerance have higher soluble carbohydrate concentration in stolons and rhizomes. Saltgrass freezing tolerance may also be related to unsaturated fatty acids accumulation in rhizomes cell membrane (Cyril et al., 2002), cold regulated protein (COR) synthesis in rhizomes and crowns (Gatschet et al., 1996) and deep rhizome penetration in the soil (Shahba et al., 2003a). These examples may be the basis for freezing (cold) tolerance in saltgrass but were not evaluated in this study.

All clones from cold hardiness zone 4 had similar freezing tolerance. Their adaptation to cooler climates likely enables them to cold acclimate faster and to a higher degree. In general, zone 4 clones became dormant (turned brown) earlier than clones from zone 5 and 6. This is similar to a study with buffalograss cultivars (Qian et al., 2001). All clones with reduced freezing tolerance retained greater leaf color

retention in the fall. In climatic zone 5, considerable variation in freezing tolerance was found among clones in both years of the experiment. These accessions were from the Colorado Front Range, and were primarily collected along the I-25 interstate between Aurora (a South Denver suburb) and Wellington (north of Fort Collins). Clones A1290, A1500, A1530 and A11070 had the greatest freezing tolerance while clones A1490, A11260 and A11370 exhibited the least freezing tolerance in both seasons. Natural variation among clones in this zone appears to be higher than in the other two zones. Some of these clones may actually be native to other (warmer) areas, but accidentally brought to this zone along the highway. Proximity of urban areas that raises environmental temperature might enable survival of these clones as well. However, a broader range of freezing tolerance in zone 5 than in zone 4 and 6 should be taken with caution. There were approximately four times as many clones sampled from zone 5 than from other zones. Clone numbers in zone 4 and 6 might be too small to represent the actual range of freezing tolerance in these zones. Finally, all 4 clones in climatic zone 6 exhibited the lowest freezing tolerance in both years. These clones appear to be adapted to warmer climates (Nevada and Southern Colorado) and exhibit greater winterkill in more northern areas.

In summary, freezing tolerance is the main environmental factor that limits use of warm season turfgrass species in northern climates and in the transition zone. Our results indicate significant variability in freezing tolerance among saltgrass clones that may be used in the development of cold-hardy cultivars. Time of leaf browning in fall may serve as a selection criterion in breeding for cold hardiness. Freezing tolerance is an environmental adaptation inherited by saltgrass clones

associated with specific location. This study indicated that clones from northern (cooler) areas have greater freezing tolerance than those from southern (warmer) climates.

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Chapter 4: Sampling Time, Nitrogen Fertilization and Burning Affect Flowering in Saltgrass Clones

4.1 Abstract

A breeding program for turf-type saltgrass is currently a focus at Colorado State University. Hybridization among accessions in the greenhouse during the winter would facilitate this breeding program. Therefore, the influence of sampling time from the field, nitrogen fertilization and burning on numbers of spikes (flowering) of saltgrass accessions from different environments were evaluated over 2 years. In the first year, 3 clones (A1540, 1490 and C1660 from Colorado, South Dakota and Nevada respectively) were sampled from the field at 2 times (August and November). Clone C1660 did not respond to the flowering induction treatments. For clones A1540 and 1490 nitrogen fertilization increased number of spikes (flowering) by approximately 30%. Sampling in November increased flowering in A1540 and 1490 clones as compared to August sampling, but with a greater effect in clone A1540. Burning increased flowering only in plants sampled from the field in August. In the second year, two additional clones from the Colorado Front Range (A1180 and A1610), and an additional sampling time in January were included. Clones C1660 and A1180 did not respond to flowering induction treatments. Nitrogen fertilization increased the number of spikes to a greater extent when nitrogen was applied in combination with burning treatment as compared to no nitrogen or nitrogen without burning. In comparison with August sampling, November sampling increased number of spikes in all clones with the greatest effect in clone A1540. Compared to sampling in November, sampling in January further increased the number of spikes in clones 1490 and C1610 but with no significant effect on the number of spikes in clone

A1540. The burning treatment had a greater effect on the number of spikes in plants sampled in August as compared to those sampled in November and January.

4.2 Introduction

Many plants, including turfgrass species from temperate regions, are adapted to flower at particular times of year to ensure optimal pollination and seed maturation. These plants respond to changes in daylength and extended periods of cold temperature, since they are both predictable and reliable indicators of seasonal progression (Simpson et al., 1999). Previous studies of the vernalization (cold treatment) and photoperiod requirements of flowering in some turfgrass species have shown a diversity of genotype and clone responses in Kentucky bluegrass (*Poa pratensis* L.) [Carlson et al., 1995; Johnson and White, 1997 a and b], alpine and arctic bluegrass (*Poa alpina* L. and *Poa alpigena* L. respectively), [Heide, 1989; Pahl and Darroch, 1997] as well as red fescue (*Festuca rubra* L.) [Murray et al., 1973]. Nutrients, particularly nitrogen availability, also influence flowering. Nitrogen application positively influences carbohydrates accumulation in crown and leaf tissue of turfgrass species (Pettit and Fegan, 1974). Carbohydrates are important compounds for the induction of flowering in grasses and other plant species (Perilleux and Bernier, 1997). Nitrogen fertilization increased tiller number and subsequent flowering in Kentucky bluegrass and meadow brome grass (Thompson and Clarke, 1993; Loeppky and Coulman, 2001). Fire is another important factor that influences flowering in many plant species (Bowen and Pate, 2004). Flowering following fire (as well as other stress such as drought and overcrowding) enables the plant to produce seeds which are much more likely to survive the stress than is the plant itself (Levy

and Dean, 1998). The burning of Kentucky bluegrass residue produced higher seed yield than mechanical removal and residue-retaining treatments (Lamb and Murray, 1999; Johnson et al., 2003). Bowen and Pate (2004) reported significantly greater growth and flowering in *Stirlingia latifolia* R.Br. following fire as compared to cutting and removal of plant tissue.

Saltgrass (*Distichlis spicata* [L.] Greene) is a warm season grass with excellent drought, heat and salinity tolerance (Kopec and Marcum, 2001). It is native to North America and it commonly grows in saline and alkaline soils across the western United States (Eppley et al., 1998). Because of rapid population growth in the semi-arid U.S. west there is public interest in using new turfgrass species that are water efficient and tolerant of low quality water (Kopec and Marcum, 2001; Hughes et al., 2002). Evaluation of saltgrass accessions for potential turf use is an ongoing program at Colorado State University. The breeding program is focused on development of seeded turf-type saltgrass cultivars. However, hybridization of selected clones is limited because of the short flowering period of many of the clones that commonly occurs in early June in Fort Collins. Development of a flowering induction procedure would enable hybridization in the greenhouse and thus would accelerate cultivar development. Therefore, this study was initiated to evaluate the influence of sampling time from the field (natural cold treatment), nitrogen fertilization and burning treatment on flowering induction of saltgrass clones.

4.3 Materials and Methods

Five saltgrass clones were used in this experiment (Table 4.1). Clones were established in 5 by 5 m field plots at the Colorado State University Horticultural Research Center, Fort Collins, CO in July 1998. Field soil was a clay loam with an initial N content of 23 mg kg^{-1}. Clones were replicated in the field twice in a randomized complete block design. Fertilizers were not applied and the field was mowed once a week at the height of 6 cm. Irrigation was applied once a year in late July by flooding the field with approximately 12 cm of water. Clones A1540, 1490 and C1660 were used in both experimental years. These three clones represented three different cold hardiness zones. Clones A1180 and A1610, from the Colorado Front Range were added in the second year. All clones had similar tiller density, good flower density and good seed production in the field during the first two years of clones' establishment when the field was left unmowed. 7.6-liter (two-gallon) plugs of each clone were sampled (dug up) from the field by a shovel twice (mid August and mid November) in the first year (2003) and three times (mid August, mid November and mid January) in the second year (2004/2005) of the experiment. Following field sampling, plants were established in 7.6-liter (two-gallon) volume pots filled with field soil (clay loam) and maintained in the Colorado State University greenhouse. The average greenhouse temperature during the experiment was approximately 21 $^{\circ}$ C with daylengths extended to 15 hours using 430 watt high-pressure sodium lamps. After one week in the greenhouse, pots were assigned nitrogen fertilization and burning treatments. Plants that received nitrogen fertilization were fertilized with granular 34-0-0 ammonium nitrate (NH_4NO_3). The

equivalent of 200 kg of N ha^{-1} was applied. This was 0.5 g of granular fertilizer dissolved in water and applied to pots (65 cm^2 area per pot). The tops of those plants that received the burning treatment were burned to soil level with a hand held propane burner so that all above ground tissue was consumed by the fire.

The experimental design was a factorial randomized complete block with three blocks (repetitions). Final spike number (generally reflected in increased flowering of tillers) of each clone was determined for each treatment combination after approximately 7 weeks from treatment application. Mixed model analysis of variance (proc mixed) in SAS (SAS Institute, 1999) was used to determine which factors, and which interactions among factors influenced number of spikes (flowering) in saltgrass. Clones, sampling time, nitrogen fertilization and burning treatment were treated as fixed factors, and blocks were treated as a random factor. To ensure linearity and homogeneity of variance, the dependent variable (number of spikes) was transformed by using the natural logarithm scale with an added constant of two.

Table 4.1 Saltgrass clones used in the experiment according to their origin and cold hardiness zone.

Clone	Place of origin	Cold hardiness zone
A1540	Aurora, CO	5
1490	Chamberlain, SD	4
C1660	Humboldt Sink, NV	6
A1610	Aurora, CO	5
A1180	Denver, CO	5

4.4 Results and Discussion

4.4.1 First year of the experiment

Spikes appeared approximately five weeks after application of treatments. Since clone C1660 from Nevada did not flower with any treatment used, it was not included in the statistical analysis. Highly significant effects on spike number were observed with clones, sampling time, nitrogen fertilization and burning treatment while significant interactions occurred between clone and sampling time, clone and burning treatment and sampling time and burning treatment (Table 4.2).

Table 4.2 The effects of clone, sampling time, nitrogen fertilization, burning and their interactions on spike number in saltgrass in the first experimental year.

Source of variability	F Statistic	P value
Clone	112.22	<0.0001
Sampling time	147.33	<0.0001
Clone*Sampling time	25.79	<0.0001
Nitrogen fertilization	29.5	<0.0001
Clone*Nitrogen	1.53	0.2253
Sampling time *Nitrogen	1.37	0.2507
Clone*Sampling time*Nitrogen	1.9	0.1780
Burning	8.99	0.0054
Clone*Burning	20.83	<0.0001
Sampling time*Burning	9.27	0.0048
Clone*Sampling time*Burning	1.57	0.2201
Nitrogen*Burning	0.32	0.5760
Clone*Nitrogen*Burning	0.57	0.4564
Sampling time*Nitrogen*Burning	0.92	0.3440
Clone*Sampling time*Nitrogen*Burning	0.46	0.5023

The level of significance was 0.05. Factors and interactions with a p value smaller than 0.05 were considered significant.

Analysis of significant factors and interactions showed that clones that received nitrogen fertilization were more likely to produce greater number of spikes than those without fertilization (Table 4.3). An increased number of spikes in both

clones A1540 and 1490 occurred in November as compared to August. However, this increase was greater in clone A1540 than in clone 1490. Similarly, the effect of burning on number of spikes was greater in clone 1540 than in clone 1490. Burning treatment significantly increased number of spikes (flowering) only in plants sampled from the field in August. Burning had no significant effect on number of spikes in those plants sampled in November (Table 4.3).

Table 4.3. Differences between selected least square means of nitrogen fertilization and interactions between clone and burning and sampling time and burning in the first experimental year.

Effect	Least square means compared (number of spikes)		Difference (number of spikes)	t Statistic	P value
N Fertilization	N applied (16)	N not applied (8)	8	5.43	<0.0001
Clone*Sampling time	A1540 November (63)	A 1540 August (8)	55	12.17	<0.0001
Clone*Sampling time	1490 November (9)	1490 August (4)	5	4.99	<0.0001
Clone*Burning	A1540 Burning applied (34)	A1540 Burning not applied (13)	21	5.35	<0.0001
Clone*Burning	1490 Burning applied (7)	1490 Burning not applied (5)	2	1.11	0.2767
Clone*Burning	A1540 Burning applied (34)	1490 Burning applied (7)	27	10.72	<0.0001
Sampling time*Burning	August Burning applied (8)	August Burning not applied (4)	4	4.27	0.0002
Sampling time*Burning	November Burning applied (25)	November Burning not applied (24)	1	0.03	0.9737

The level of significance was 0.05. Differences with a p value smaller than 0.05 were considered significant. Values in parentheses present the least square means that were back-transformed following transformation in logarithm scale.

4.4.2 Second year of the experiment

To test if longer exposure to field temperatures stimulated an increase in spike number (induction of flowering) in clone 1490 and if clones originating from the Colorado Front Range differed in flowering induction requirements, an additional sampling time from the field (January) and two additional clones (A1180 and A1610) from the Colorado Front Range were included in the experiment. The latter test was performed because most of the saltgrass clones used in the breeding program are from the Colorado Front Range. However, neither clone C1660 (as noted in the previous year) nor A1180 responded to the flowering induction treatments used and therefore were not included in the statistical analysis.

Spikes appeared approximately five weeks after assigned treatments. Spike number was significantly influenced by clone, sampling time, burning treatment and nitrogen fertilization (Table 4. 4). This was similar to that observed in the previous year. The interactions between clone and sampling time, burning and sampling time and nitrogen fertilization and burning were highly significant. The three-way interaction among clone, sampling time and burning was small but still significant (Table 4.4).

Nitrogen fertilization increased the number of spikes in all clones but was greatest when nitrogen was applied in combination with the burning (Table 4.5). Saltgrass clones responded differently to sampling time (natural cold treatment). The November sampling time significantly increased number of spikes (flowering) as compared to sampling in August in all clones with the greatest increase in clone

A1540. The January sampling time further significantly increase number of spikes in clones 1490 and A1610 above that of those sampled in November. Sampling in January had no significant effect on number of spikes in clone A1540 as compared to sampling in November. Burning treatment increased the number of spikes in all three sampling times and clones but it had the greatest effect on plants sampled in August and in clone A1540 (Table 4.5).

Table 4.4 The effects of clone, sampling time, nitrogen fertilization, burning and their interactions on spike number in saltgrass in the second experimental year.

Source of variability	F Statistic	P value
Clone	714.6	<0.0001
Sampling time	672.68	<0.0001
Clone*Sampling time	33.43	<0.0001
Nitrogen fertilization	104.34	<0.0001
Clone*Nitrogen	0.69	0.5035
Sampling time*Nitrogen	1.29	0.2085
Clone*Sampling time*Nitrogen	2.32	0.0653
Burning	227.48	<0.0001
Clone*Burning	0.05	0.9544
Sampling time*Burning	8.85	0.0004
Clone*Sampling time*Burning	2.84	0.0305
Nitrogen*Burning	27.19	<0.0001
Clone*Nitrogen*Burning	1.01	0.3703
Sampling time*Nitrogen*Burning	0.82	0.4458
Clone*Sampling time*Nitrogen*Burning	2.15	0.0833

The level of significance was 0.05. Factors and interactions with a p value smaller than 0.05 were considered significant.

Table 4.5 Differences between selected least square means of interactions between fertilization and burning, clone and sampling time and sampling time and burning in the second experimental year.

Effect	Least square means compared (number of spikes)		Difference (number of spikes)	t Statistic	P value
N Fertilization*Burning	N applied with burning (18)	N applied without burning (12)	6	6.98	<0.0001
Clone*Sampling time	A1540 November (54)	A 1540 August (9)	45	23.27	<0.0001
Clone*Sampling time	A1540 January (64)	A1540 November (54)	10	2.34	0.0221
Clone*Sampling time	1490 November (8)	1490 August (4)	4	8.23	<0.0001
Clone*Sampling time	1490 January (16)	1490 November (8)	8	7.82	<0.0001
Clone*Sampling time	A1610 November (7)	A1610 August (3)	4	11.10	<0.0001
Clone*Sampling time	A1610 January (15)	A1610 November (7)	8	9.36	<0.0001
Sampling time*Burning	August Burning applied (7)	August Burning not applied (3)	4	12.13	<0.0001
Sampling time*Burning	November Burning applied (18)	November Burning not applied (12)	6	6.74	<0.0001
Sampling time*Burning	January Burning applied (30)	January Burning not applied (20)	10	7.26	<0.0001

The level of significance was 0.05. Differences with a p value smaller than 0.05 were considered significant. Values in parentheses present the least square means that were back-transformed following transformation in logarithm scale.

Overall, there was variation in flowering in the saltgrass clones. Clone C1660 collected from Nevada flowered continuously during the summer in the field in both seasons of the experiment but either did not flower in the greenhouse with any of the treatments or responded with only a very few spikes. Its genetic adaptation to warmer climates is likely related to flowering induction by long daylengths during the late spring and early summer without exposure to cold temperatures. This is similar to that of annual bluegrass (Johnson and White, 1997 a and b). In those experiments, long-day photoperiods induced flowering in some annual bluegrass genotypes that were not sensitive to imposed vernalization treatments.

Flowering of clones A1540 and 1490 in both experimental years was likely stimulated by exposure to low temperatures and/or short day conditions. However, in the first experimental year clone A1540 developed a greater number of spikes than clone 1490. This suggested that different clones may have different environmental requirements to maximize spike number. Indeed, exposure to low temperatures and shorter days in the second experimental season significantly increased the number of spikes in clone 1490 with little increase in clone A1540. This suggests that clone 1490 requires longer exposure to low temperatures in order to maximize the number of spikes. On the other hand, a shorter exposure to low temperature/short day conditions can maximize spike number in clone A1540. Differences between these two clones are likely attributed to their origin. Clone 1490 is from Chamberlain, South Dakota and clone A1540 from the Colorado Front Range (Denver area), a climate with milder winters and a longer growing season. This finding is consistent with results observed in Kentucky bluegrass (Carlson et al., 1995) and alpine

bluegrass (Pahl and Darroch, 1997). In both of these studies, genotypes from warmer climates required a shorter time of low temperatures/short daylengths than genotypes from northern areas in order to maximize flowering.

Clones A1540 and A1610 from the Colorado Front Range differed in their environmental conditions for maximum flowering. While clone A1540 achieved maximum flowering in late November, clone A1610 required an additional period of cold temperatures/short days for maximum flowering. The Colorado Front Range is composed of many microenvironments. These heterogeneous microclimates would likely lead to environmental adaptation of important traits (e.g. requirements for flowering) causing within population variability as similarly observed in alpine bluegrass (Pahl and Darroch 1997). Clone A1180 from the Colorado Front Range did not flower with the flowering induction treatments used. Hughes et al. (2002) reported that some male and female saltgrass clones from the same geographical region flower abundantly while many others do not. This likely reflects the diversity of genetic background relative to flowering.

Our results indicate that nitrogen fertilization is an important factor that stimulates flowering in saltgrass. In this study, nitrogen stimulated an increase in spike number in saltgrass, regardless of the timing of collection from the field or origin of clone. Nitrogen fertilization has been reported to increase tiller density, flowering and seed yield in many grasses (Loeppky and Coulman, 2001). Our findings correspond to results by Lamb and Murray, (1999) who reported that field burning in Kentucky bluegrass management maintains optimal tiller density for seed yield and increases efficiency of fertilizer.

Saltgrass responds to fire by rapidly regenerating new shoots from rhizomes (Uhitil, 1990). The effect of fire (burning treatment) on spike number in saltgrass clones seems to be associated with the season of burning application. Burning treatment had a greater effect on flowering (spike number) in saltgrass clones sampled from the field in August as compared to the burning effect on plants sampled in November and January. Presumably, the factors associated with season of burn, such as climatic conditions experienced after the fire, influence shoot recovery and subsequent flowering. Our findings are similar to flowering induction study in grasstree *Xanthorrhoea preissii* (Lamont et al., 2000). In that study, a summer burn had significantly greater effect on flowering as compared to winter burn. Similarly, summer burn stimulated flowering in *Stirlingia latifolia* to a greater extant than winter burn (Bowen and Pate, 2004).

In summary, our results indicate that environmental adaptation associated with origin of clone is a major factor influencing flowering and spike number in saltgrass. Length of exposure to low temperature was likely the most important factor influencing differences in spike number among saltgrass clones from different geographic regions. Nitrogen fertilization increases number of spikes in saltgrass regardless of clone origin or sampling time from the field. The effect of fire on number of spikes varies with application time. The knowledge of flowering requirements of particular saltgrass clones is important in plant breeding because it may enable breeders to induce flowering of clones and perform crosses during the entire year.

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