SCREENING SWITCHGRASS (*Panicum virgatum* L.)

FOR WATER STRESS TOLERANCE

By

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List of Abbreviations

2, 4-D ....2, 4-Dichlorophenoxyacetic acid
BA...Benzyl adenine
Ci..Intercellular CO_2 concentration
Cond..Stomatal conductance
ETR...Photosynthetic electron transport rate
Fv′/Fm′ ....Efficiency of energy harvesting by oxidized (open)
                                                                    PSII reaction centers in the light
GHG…………………………………..Greenhouse gas emission
LFDRWT ....Leaf dry weight
MPa. ……………………….………… Megapascal.
NDNO..Node number
PEG...Polyethylene glycol
PhiCO2...Quantum yield of CO_2 Fixation
PhiPS2..PS II efficiency
PLHT..Plant height
Pn ....Net photosynthesis rate
qP ....Photochemical quenching
STDRWT ....Stem dry weight
T1 …………………………….…….60% of field capacity
T2……………………….………..20% of field capacity
TLNO...Tiller number
TOTBIO...Total biomass
Tr...Transpiration rate
R.Alamo…………………….….Regenerated Alamo
R.Forestburg………………….…..Regenerated Forestburg
RTDRWT..Root dry weight

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CHAPTER I

INTRODUCTION

GENERAL INTRODUCTION

Energy supply and environmental protection are currently important global issues that need to be addressed for a sustainable future (Berndes, 2002). Efforts are being made in several countries finding alternative sources of liquid fuels to satisfy energy demand while being environmentally friendly. Wind, tidal, solar, and biofuel energy sources are being developed, and they are competing favorably in price and environmental advantages (Ayhan, 2009; Hall and Scrase, 1998). About 80 to 95% of the primary energy consumption in the world is from fossil fuel sources (petroleum products) and 57.7% of that amount is used to satisfy the transport sector demands (Ajanovic and Haas, 2010; Ayhan, 2009; Escobar et al., 2009). However, several studies concluded that fossil fuels are responsible for the emission of a significant amount of pollutants in the atmosphere, including greenhouse gases (GHG) (Ajanovic and Haas, 2010; Escobar et al., 2009).

Studies across the globe have suggested that with current world energy policies and management, the world market of energy consumption is expected to increase by 53 % from 2008 to 2035 (EIA, 2011). At the same time, GHG emissions will increase (EIA, 2011; Ghimire and Craven, 2011). To maintain energy security, reduce environmental footprint, and foreign exchange savings, biofuel can be a possible solution, especially that its market is expected

to grow rapidly over the next decade (Ayhan, 2009). The production of biofuels can be achieved through utilization of agricultural residues and dedicated feedstock as raw materials in a biorefinery (Cherubini and Ulgiati, 2010) or through different crops that are currently used for commercial energy farming such as corn and sugar cane. Other potential energy crops include woody crops and grasses/herbaceous plants (all perennial crops), starch and sugar crops and oilseeds. In general, the characteristics of the ideal energy crop are, high yield, low energy input, low cost, composition with the least contaminants, and low nutrient requirements (Peter, 2002). For liquid biofuels, perennial grasses are a promising source of bioenergy; however, using warm season grasses such as switchgrass (*Panicum virgatum* L.) as a bioenergy crop can have additional environmental benefits including reducing soil erosion and flooding (Epplin, 1996). Perennial grasses grown for bioenergy biomass production can contribute to decreasing CO_2 level by their CO_2 recycling process and efficient C sequestration mechanism. Perennial grasses can outperform other sources of biofuels through low input and low costs of agricultural biomass yield (Lynd et al., 1991; Schmer et al., 2008). Economical, biological, and environmental advantages should be considered when decisions are being made for biofuel crops for long term production and sustainability. Among many candidate crops, switchgrass has received more attention and been well-studied due to its characteristics as a native grass, various ecotypes that are adapted to different environments, and ability to grow in marginal lands with high biomass productivity (David and Ragauskas, 2010; McLaughlin et al., 2006; Parrish and Fike, 2005).

To achieve higher biomass yield while using marginal land with low input, more research should focus on plant traits that can contribute to significant biomass yield. Utilizing the wide genetic diversity of switchgrass due to its adaptation to several eco-regions in the continental US can be useful in switchgrass improvement. If a single abiotic stress is to be identified as the most common environmental factor in limiting the growth of crops worldwide, it is most probably water stress (Araus et al., 2002; Boyer, 1982). Because of the major effect of drought on yield in areas where

switchgrass will be produced, water use efficiency (WUE) and drought tolerance are essential traits that need to be studied and improved (Sade et al., 2011). The National Biofuels Action Plan (NBAP, 2008) identified that feedstocks such as switchgrass should be developed in order to increase water stress tolerance in addition to increase fertilizer and water use efficiencies.

Screening of switchgrass cultivars at multiple levels of water stress can provide physiologists and breeders with beneficial information about these cultivars' behavior under stressful conditions. Simultaneous screening for physiological and morphological traits under water stress conditions and analyzing the results can lead to identification of traits for improved tolerance. Traits at the whole plant level or at the cellular level need to be studied and correlated to switchgrass drought tolerance. Hence, screening methods at both the whole plant level in greenhouse experiments and the cellular level in *in-vitro* experiments were evaluated in the study with the following objectives.

Objectives

The objectives of this study were (1) to evaluate growth and physiological parameters and identify switchgrass traits that can contribute to water stress tolerance and increased water use efficiency, (2) to study the effect of water stress at the cellular level using *in vitro* culture and identify cell lines that can survive water stress and (3) to assess morpho-physiological traits of plants derived from water stress tolerant cell lines**.** The first chapter covers the general introduction, the second chapter presents an overview of the literature, and in the third, fourth and fifth chapters the greenhouse experiment, the *in vitro* culture experiment, and the evaluation of regenerated plants are addressed respectively. The last chapter provides the general discussion and conclusions.

CHAPTER II

REVIEW OF LITERATURE

Alternative fuel sources are necessary due to fossil fuel induced climate change (IPCC, 2007), declining fossil fuel reserves (EIA, 2012) and increasing crude oil price with increased demand. Global warming has received special attention in recent decades that led to more focus on using biomass as an alternative energy source. Biomass currently contributes to 13.4% of world energy and is projected to reach 30% by 2030 (Blanco-Canqui, 2010). An advantage of biomass is its ability to produce energy while only releasing carbon to the atmosphere that has been captured during the growing cycle. In contrast, fossil fuels emit carbon that has been accumulated for millions of years (Blanco-Canqui, 2010; García et al., 2011).

The improvement in maize ethanol and soybean biodiesel market in the USA occurred in response to the decline in crude oil reserves and production rates. Moreover, it can help increase dependency on imported oil by investing in bioethanol production from maize, which also has positive effects of stimulating the agricultural economy and can lead to utilizing more areas of marginal lands. However, low production costs must be achieved for biomass production to compete with low priced fossil fuels. That is key for bioenergy to remain the highest contributor to global renewable energy in the short to medium term with dedicated energy crops set to provide a larger proportion of the biomass feedstock in the coming decades (Sims et al., 2006).

To maintain a sustainable environment, marginal lands should be utilized for energy crops to eliminate additional greenhouse gas emission and prevent food insecurity. Furthermore, improving and utilizing new technologies to improve biomass production per unit area can help satisfy energy demand and maintain sustainability. Switchgrass can be a good candidate for use in unfavorable environmental and soil conditions due to low input requirements and good adaptation to environmental stresses (Blanco-Canqui, 2010; Campbell et al., 2008).

The United States needs a liquid fuel replacement for oil in the future due to limiting resources in the next 40 to 50 years, expected an increases in oil use, and alternative liquid fuels from different sources have been tried for many years (Youngquist and Duncan, 2003; Youngquist, 1997). For ethanol production from switchgrass, average energy input per hectare was determined to be about 3.8 million kcal yr^{-1} with a maximum yield of 10 t ha^{-1} yr^{-1} and an estimated cost of producing a liter of ethanol using switchgrass is 54¢ or 9¢ L^{-1} higher than the 45¢ L^{-1} for corn ethanol production. The two major energy inputs for switchgrass conversion into ethanol were steam and electricity production. However, cost of ethanol from wood is slightly higher than for ethanol produced using switchgrass, 58¢ L^{-1} and 54¢ L^{-1}, respectively (Pimentel and Patzek, 2008).

The leading candidates for biofuel crops are perennial rhizomatous grasses that have environmental tolerance, high growth rates, high biomass yields, grow on poor soils, and have few natural enemies. More needs to be investigated about the physiological ecology of these species (Robertson et al., 2008). Commercial production of these crops may require genetic modification and basic physiological studies for improvement, thus leading to environmental sustainability and better utilization of these potentially important crops.

Switchgrass became an important bioenergy feedstock in the south central US as it is native and can be grown in marginal areas. It is classified ecologically, into upland and lowland

ecotypes (Stroup et al., 2003). Upland ecotypes are octoploids (2n=8x=72) and lowland ecotypes are tetraploids (2n=4x=36) (Brunken and Estes, 1975; Hultquist et al., 1996). Upland cultivars mostly occur in dry regions whereas lowland cultivars occur in wetter regions. Morphologically, upland cultivars have smaller leaves and thinner stems than lowland cultivars. However, lowland cultivars (Alamo and Kanlow) performed better than upland cultivars (Blackwell and Caddo) under drought conditions (Stroup et al., 2003). Lowland cultivars are vigorous and give higher biomass and have lower nutrient requirements, especially nitrogen (Porter, 1966). Lowland plants have a later heading date and are taller with larger and thicker stems.

Some pitfalls, such as environmental impacts can be associated with biofuel production. Thus, environmental pollution costs associated with ethanol production should be carefully considered. These are estimated to be more than 6¢ L^{-1} of ethanol produced (Pimentel, 2003; Pimentel and Patzek, 2005). United States corn production causes more total soil erosion than any other U.S. crop (Pimentel and Patzek, 2008; Pimentel et al., 1995). Corn production also requires more herbicides and insecticides than any other crop produced in the U.S. thereby causing more water pollution than any other crop. Further, corn production uses more nitrogen fertilizer than any other crop produced and therefore is a major contributor to groundwater and river water pollution. All these factors suggest that the environmental system in which U.S. corn is being produced is being rapidly degraded. It has been concluded that the U.S. corn production system is not environmentally sustainable now or in the future, unless major changes are made in the cultivation of this major food/feed crop. Corn is the current raw material for ethanol production, but cannot be considered as a sustainable renewable energy source in the future (Pimentel and Patzek, 2008).

Major air and water pollution problems are also associated with the production of ethanol in the chemical plant. Another pollution problem is the large amount of wastewater that each ethanol plant produces. For each liter of ethanol produced using corn, about 13 L of wastewater

are produced. Ethanol contributes to air pollution problems when burned in automobiles (Youngquist, 1997). In addition, the fossil fuels expended for corn production and later in ethanol plants amount to expenditures of 6,597 kcal of fossil energy per 1,000 L of ethanol produced. The consumption of fossil fuels release large amounts of pollutants to the atmosphere. Furthermore, carbon dioxide emissions released from burning these fossil fuels contribute to global warming and have serious consequences (Pimentel and Patzek, 2008).

Switchgrass as a multipurpose crop species

Switchgrass is a native tallgrass prairie species in non-forested areas in the United States (Hitchcock and Chase, 1971) and over time it became a crop when it was intentionally planted or managed (Anderson, 2000; Coppedge et al., 1998). Indeed, the ecology of switchgrass is inextricably bound with grazing, trampling animals (Eom et al., 2001; Wallace, 1987) and periodic, intense wildfires (Cuomo et al., 1998; Knapp, 1985; Rice and Parenti, 1978). It was a transitional step for switchgrass when it began to be used for nourishing ruminants brought from the Old World. Many of the early scientific reports on switchgrass were botanical descriptions or evaluations of phenotypic variation among accessions (Cornelius et al., 1941; Eberhart and Newell, 1959; Nielsen, 1947). Relatively, little work has been done on switchgrass as a crop species. In general, switchgrass is one of the best biomass species for cellulosic ethanol production because of its positive environmental attributes such as:

- Can be produced for many years once established (McLaughlin and Walsh, 1998).
- High yields of cellulose with low input such as nutrient and pesticide requirements (Powlson et al., 2005).
- Cultivars that are locally adapted and relatively available (Mulkey et al., 2006b).
- Carbon sequestration through its extensive and very deep root system that increases soil organic matter (SOM) contributing to soil conservation (McLaughlin and Kszos, 2005).
- Tolerance of poor soils and wide variations of soil pH (Rinehart, 2006).

- Drought and flood tolerance and efficient water use (depending on the ecotype and variety) (Rinehart, 2006).

- More stable yields during stress years due to much energy stored in the root system (McLaughlin and Walsh, 1998).

- Fields of switchgrass provide excellent habitat for birds and other wildlife (Renz et al., 2009).

- Switchgrass and the combustion of biomass fuel is truly a green energy source (McLaughlin et al., 1999).

All of these advantages make switchgrass one of the most desirable biomass energy crops. It is well adapted and native to North America, easing concerns of invasiveness, and it can produce high yields with minimal inputs. In addition, utilization of marginal land, ease of establishment from seed, an existing seed industry, long productive life, and enhanced environmental quality, all make switchgrass potentially profitable and encourages farmers to grow it.

Production and agronomics of switchgrass as a perennial grass must be well established in the first year (Perrin et al., 2008). However, weed competition during crop establishment should be monitored due to the harmful effect of weeds on switchgrass stand (Schmer et al., 2008b). After switchgrass cultivars are well established, limited herbicides may be required. Nitrogen fertilizer in the first year is not recommended since it can negatively impact the switchgrass crop by enhancing weed growth and also increase establishment cost (Mitchell et al., 2008; Mitchell et al., 2010). Year after year, a late single harvest after frost maximizes switchgrass yields while minimizing inputs by remobilizing the nutrients to the rhizomes (Mitchell et al., 2008). Switchgrass can be harvested and baled using commercially available haying equipment (Larson et al., 2010). Round bales tend to have less storage losses than large square bales when stored outside uncovered, but square bales tend to be easier to handle and load without road width restrictions (Caddel et al., 2010) After harvest, poor switchgrass storage conditions can result in

storage losses of 25% in a single year and can reduce biomass quality. Covered storage is necessary to protect the harvested biomass (Caddel et al., 2010; McLaughlin and Kszos, 2005).

Potential yield and production costs

Switchgrass yield is affected by annual precipitation, soil nutrients, location, and genetics. Generally, upland ecotype is inherently lower yielding than lowland ecotype. Potential yield of Cave-In-Rock, Shawnee, Summer, and Trailblazer decreased when they were grown on marginal lands under natural rain-fed conditions (Mitchell et al., 2008). The F1 hybrids of Kanlow and Summer produced 9.4 tons per acre annually. That was 68% higher than Summer and 50% higher than Shawnee (Schmer et al., 2008a). Nebraska has a potential ethanol yield averaging 372 gallons per acre. This was equal to or higher than that for no-till corn (grain + stover) on a rain-fed site with marginal soils (Varvel et al., 2008). These results were based on switchgrass cultivars developed for grazing. Significantly greater yields are expected by the next generation of biomass-specific cultivars.

Switchgrass has many characteristics that lead to potential adoption including profitability for the producer, ability to fit within existing farming systems, ease of storage and delivery, and availability of extension information for management practices. These characteristics attract many farmers to adopt this crop in their farming programs. Large scale switchgrass farming may raise some concerns of disease and insect pests, but since it is a native component of U. S. grasslands these negative issues should be limited.

Switchgrass is a tall grass that grows from 0.5 to 3.0 m in height, with rooting depths of up to 3 m (Mitchell et al., 1997; Porter, 1966). Switchgrass leaves tend to be erectophile and have stomata on both sides (amphistomic); rhizomes vary in their growth, with consequences for general plant habit (Downing et al., 2011; Parrish and Fike, 2005b). Under greenhouse conditions, switchgrass has good tolerance to water stress conditions. It is reported that in both

lowland and upland ecotypes seed germination, establishment of plants, and flowering can accrue under high soil moisture ≤−0.3 MPa. Lowland types outperformed upland under imposed drought conditions (Barney et al., 2009). However in the same study, both lowland and upland types showed severe reductions (75–80%) in biomass yield, tiller number, and leaf area with water stress at −4 MPa compared to the control plants. It was concluded that, lowland ecotypes have the ability to survive broad soil moisture conditions, are more productive under a wide range of moisture conditions, and may be better candidates for future genetic and agronomic improvement. The same study reported significant reduction in both lowland and upland switchgrass ecotype performance, but they survived and reached flowering stage at soil water potentials below −4 MPa. Both ecotypes produced new tillers and added biomass at soil water potentials below −2 MPa. At the physiological level, a reduction of 50% of the net photosynthetic rate was observed across switchgrass ecotypes at soil water potentials of −1.5 MPa. Upland ecotypes in this study did not maintain higher photosynthetic rates under drought conditions. Photosynthetic water-use-efficiency differed little among soil moisture treatments. Under water stress conditions, switchgrass had lower transpiration rates and stomatal conductance. Switchgrass leaves tend to adjust osmotically to deal with low soil water potentials (Barker et al., 1993; Knapp, 1984). Ecotypic differences were not found for stomatal conductance under stress treatments despite inherent soil moisture preferences. Both ecotypes experienced reduced shoot and root biomass production under drought stress compared to those in control treatments, though drought individuals had higher root-to-shoot ratios. Results also suggest that despite a dramatic decrease in biomass and tiller production, currently available switchgrass cultivars can survive in environments with very low soil moisture availability once established. However, these reductions will likely prevent a sustainable biomass crop in dry areas without additional irrigation or improvement of switchgrass ecotypes.

Adaptation to drought

Water availability for biomass production will be a challenge with increasing energy demand as feedstocks will be grown in marginal and water limited environments with minimum inputs. Genetic diversity helps switchgrass cultivars to adapt to a wide range of soil and diverse climatic conditions however water and nitrogen can have a negative effect on productivity not only on switchgrass cultivars but also in most perennial grasses (Epstein et al., 1996a). Drought as an abiotic stress is a main concern in agriculture and crop production due to its serious consequences on biomass production (Berndes, 2002; Koshi et al., 1982a). Water use efficiency (WUE) can be a good trait contributing to increased biomass production. The WUE varies among cultivars and can help cultivars cope with lack of water during the growing season (Koshi et al., 1982a; Sashidhar et al., 1986a). Switchgrass is considered a dominant species in conservation planting (Koshi et al., 1982b). The plant is an immense biomass producer (27 Mg ha^{-1} year^{-1}) with high cellulosic content (40%) making it better than many alternative crops for ethanol production as well as a combustion fuel source for power production (McLaughlin et al., 1999). Switchgrass has an extensive and deep root system. It uses nitrogen very efficiently, and maintains a beneficial symbiotic relationship with microscopic soil fungi. It is also an excellent plant for use in riparian buffer strips. Its root system prevents soil erosion, by slowing the travel of surface water, decreasing run-off from agricultural fields, and allowing for greater water infiltration (Mulkey et al., 2006a).

To adapt to drought, plants need to combine more than one of these characteristics: reduced leaf area, short growing season, extensive root system, dynamic osmotic adjustment, control stomatal and non-stomatal water loss from leaves to enhance water use efficiency (Sivamani et al., 2000). Due to cultivar genetic diversity, switchgrass can combine heat, cold, and drought tolerance within the species resulting in adequate adaptation for wide ecosystems including arid conditions (Casler et al., 2004; Hitchcock and Chase, 1951). Withholding water

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decreased shoot dry weight and increased root dry weight, resulting in an increased root/shoot (R/S) ratio. High R/S ratio under water stress conditions enables switchgrass to adapt to drought by expanding roots to get more water from the soil as a perennial crop. Whole plant dry biomass of switchgrass also increased after drought which is related to the plants' adaptation-to-drought stress characteristics. Among C_4 grasses, switchgrass has the highest R/S ratio, which would benefit its growth under dry conditions once the seedlings are established (Xu et al., 2006). Switchgrass cultivars differ in water use efficiency (Kiniry et al., 2011; Kiniry et al., 2012) however; using selection for single traits may not result in the desired benefit.

Usually, the first symptoms observed on water stressed plants are inhibition of shoot and root growth, partial or complete stomatal closure resulting in reductions in transpiration and CO_2 uptake for photosynthesis. However, more stress leads to interrupted reproductive development, premature leaf senescence, wilting, desiccation and finally death (Griffiths and Parry, 2002; Schulze, 1986). (Lu and Neumann, 1999) found that moderate water stress reduced cell production and cell expansion in maize, rice, and barley.

Drought in general, is classified to intermittent or terminal (Neumann, 2008). During terminal drought, the soil moisture decreases progressively resulting in premature plant death. Intermittent drought is a function of inadequate irrigation during the growing season and might not affect the plant's life. During mild drought, total water potential can be maintained by osmotic adjustment. Sugars can serve as compatible solutes permitting osmotic adjustment, although many other compounds usually associated with salt stress are also active, such as proline, glycine betaine, and pinitol (Chaves et al., 2003; Chen and Murata, 2002). Sugars may help protect the cells during drought conditions using different mechanisms such as glass formation of solutes crystallizing in the presence of sugars supersaturated liquid to improve mechanical properties of the cell and prevent cellular collapse. Also, one important consequence

of drought and many other stresses is the production of activated oxygen molecules that cause cellular injury (Ingram and Bartels, 1996).

Adaptation to drought is complex. It results from genetic and environmental interactions that have evolved a large number of environmental, anatomical, physiological, biophysical, biochemical and developmental factors. At the cellular level, plants respond to water deficit using mechanisms to perceive and transmit stress signals to cellular activities that lead to adaptive responses (Stroup et al., 2003). During water stress, plant growth is regulated through involvement of long-distance chemical signaling resulting in stomatal closure to maintain shoot water content. In this process, abscisic acid seems to be a major chemical root to shoot stress signal in plants during stress. Abscisic acid can be synthesized in the cytosol of all root cells at low moisture conditions for adjustment during stress and regulating plant development under normal conditions (Christmann et al., 2007; Davies et al., 2005).

A chemical signal produced by the root cells has been proposed that causes stomatal closure under stress conditions. This signal is often associated with increased ABA in xylem sap, prior to a detectable increase in leaf ABA. Similar results can occur by feeding detached leaves with synthetic ABA resulting in stomatal closure (Holbrook et al., 2002).

One of many ways that enable plants to cope with drought is increased water-use efficiency in either rain-fed or irrigated grain crops. A coping mechanism can be achieved by moving more available soil water to the crop while minimizing water loss from the soil surface or drainage, and acquiring more photosynthate in exchange for each unit of water transpired during CO_2 fixation by the crop. Furthermore, partitioning more of the cell growth rate and its inhibition by water deficit is regulated by a complex, multigenic series of metabolic processes that are not simply a function of water availability for turgor maintenance (BarnabÁS et al., 2008; Biamah, 2005; Fischer et al., 2011; Kusvuran, 2012; Michelozzi et al., 2011; Sanchez et al., 2012).

Ongoing research into key mechanisms regulating plant growth should make directed alteration of plant growth responses to drought via genetic manipulations an increasingly realistic goal. However, stress adaptation increases the chances of plants surviving stressful conditions by decreasing or inhibiting growth that will result in decreasing plant size and finally decreasing the yield (Neumann, 2008)

Root and shoot growth may respond differently when exposed to water stress. Leaf and stem growth is rapidly inhibited due to a decrease in cell-wall-yielding properties. However roots can continue growth to reach deeper water as an adaptation to stress (Wu et al., 1996). Other methods of avoidance include closing stomata to minimize water loss, adjusting sink/source allocation by increasing root growth, and decreasing canopy by reducing growth and shedding of older leaves (Rivero et al., 2007)

Condon et al. (2004) listed some objectives for breeders toward increasing water-use efficiency in drought conditions : (1) obtain more of the available water while minimizing (a) evaporation from the soil surface, (b) drainage beyond the root zone, and (c) water left behind in the root zone after harvest; (2) acquire more photosynthate in exchange for each unit of water transpired during CO_2 fixation by the crop; (3) partition more of the acquired photosynthate into harvestable product (Condon et al., 2004; Wullschleger et al., 1996). In a greenhouse experiment results demonstrated a high WUE in wheat grown under stress and control conditions resulting in better yield under stress (Blum et al., 1983). C_4 species appear to have greater WUE by requiring only 50% as much water to produce a gram of dry matter as C_3 species. This appears to be attributed to high photosynthetic capacity of C_4 species due to their high response to environmental changes. In one study, when three C_3 species, wheat *(Triticum aestivum* L.), barley *(Hordeum vulgare* L.), and dandelion *(Taraxacum officinale* L.), and three C_4 species, maize *(Zea mays* L.), green foxtail *(Setaria viridis* L.), and pigweed *(Amaranthus retroflexus* L.), were treated with different CO_2 concentrations and light intensities, the stomata of C_3 species are

less responsive to environmental changes than stomata of C_4 species (Akita and Moss, 1972). In a field study, leaves of C_4 species exposed to high light intensity exhibited a higher rate of photosynthesis than leaves of C_3 species in the same environmental conditions. With similar transpiration rates in both types of the leaves, the C_4 leaves produce more dry matter per gram of water transpired. The C_4 pathway is able to maintain greater photosynthetic, water, and N use efficiencies and greater tolerance to heat, drought, and N stress leading to high biomass production of C_4 species including switchgrass. These characteristics help switchgrass adapt to environments with limited water and nutrient availability.

Some reports indicate that photosynthetic rates in switchgrass become greater with increased ploidy of the cultivar. These differences correspond to greater activity of enzymes such as ribulose-1,5-bisphosphate carboxylase and phosphoenolpyruvate carboxylase (Warner and Edwards, 1993; Warner et al., 1987). Wullschleger et al., 1996 reported greater photosynthetic rates in tetraploid (lowland) cultivars than in octaploid (upland) cultivars early in the growing season, but the trend reversed later in the season. They speculated that, either lowland cultivars cannot maintain photosynthetic rates into the growing season, or upland cultivars are better able to recover from drought stress. (Gunter et al., 1996). Other reports suggested that photosynthetic responses are linked to stress resistance (Parrish and Fike, 2005a; Sanderson and Reed, 2000). Upland switchgrass ecotypes are generally considered more drought tolerant (Nickell, 1973a; Porter, 1966; Stroup et al., 2003) They have higher CO_2 exchange rates under drought stress, greater leaf water potential during stress, and recover faster following drought than lowland types (Nickell, 1973a) As observed previously, switchgrass populations or cultivars differ in their response to soil moisture and water deficit. Sanderson and Reed (2002) reported that photosynthesis and xylem pressure potentials in Alamo are reduced at soil moisture tensions less than -0.045 MPa; however, transpiration efficiency (biomass produced per mass of water transpired) is not affected by drought. Differences among cultivars in water-use efficiency do,

however occur and this may be a useful trait to select for if the region of switchgrass production is to expand (Byrd and May, 2000).

Results of breeding for greater biomass production are contradictory. For example, Das et al. (2003) found a positive correlation between tiller number and yield in lines they were developing in Oklahoma, whereas Smart et al. (2003a) and Smart et al. (2004) working in Nebraska found the relationship to be inverse. It may be significant because Das et al. (2004) were working with inherently coarser-stemmed lowland lines, while (Smart et al., 2004; Smart et al., 2003a; Smart et al., 2003b) worked with upland-derived material. Choice of cultivar for a biofuels planting should be made with good knowledge of the site and the cultivar's cytotype and provenance. The higher, sustained yields ($\geq$20 Mg ha^{-1} year^{-1}) observed in recent years have been in systems managed for maximum sustained biomass (Parrish and Fike, 2005a).

Plant growth begins with meristematic cell division followed by subsequent massive expansion of young cells toward full size and function. Cell expansion is a result of biophysical changes, including a regulated loosening of primary cell walls and subsequent yielding to the hydrostatic (turgor) pressure generated by solute and water uptake into the cells (Cosgrove, 1997; Neumann, 1995). Water stress inhibits cell growth rate through a complex, multigenic series of metabolic processes that are not simply a function of water availability for turgor maintenance (Bassani et al., 2004; Fan and Neumann, 2004). To reach reproductive stage, plant growth might require modifications in growth patterns to cope with terminal drought environments such as a shortened growth cycle that limits vegetative growth, flowering, and seed production. It is undesirable developmental growth style that can give the expected potential yield from agricultural crops. However, a moderate shortening of the vegetative growth period can be associated with acceleration of the onset of flowering that might be helpful to obtain desirable yield from a crop grown without supplemental irrigation in terminal-drought environments.

Abscisic acid (ABA) accumulated as a result of water stress has a clear effect on stomatal closure and is suggested to promote root growth in maize seedlings grown in low water potential (Saab et al., 1990; Sade et al., 2011). Exogenous ABA increased root hydraulic conductivity in soybean (Boru et al., 2003; Glinka, 1980). In terminal drought, leaf senescence can be a beneficial way to transfer nutrients from leaves to growing seeds (Foulkes et al., 2007a; Foulkes et al., 2007b; Zhang et al., 2006) In contrast, senescence of premature leaves will limit photosynthetic capacity by reducing total leaf area resulting in leaf death.

Roots found to be sensitive to water deficits in the rhizosphere induce appropriate adaptive responses by transmitting chemical, hydraulic or electrical signals to the shoot resulting in stomatal closure and/or shoot growth inhibition. (Davies et al., 2002; Kang et al., 1998) Cooperative research between plant genomics, proteomics, transcriptomics, metabolomics, organomics and systems' biology increase understanding and manipulating of plant physiological responses to water deficit which can be utilized in breeding programs (Neumann, 2008).

One study revealed that several molecular compounds, such as inositol, proline, glucose, fructose, and sucrose were accumulated in the leaves of sunflower seedlings when they were grown under drought conditions. Seedlings that showed acclimation to drought stress have alterations in the cellular chemistry as a result of changes in gene expression. Use of PEG 6000 gives the best simulation of drought-stress in *in vitro* culture conditions. Sunflower seedlings grown at -0.6 MPa exhibited strong reduction of hypocotyl length that seems helpful in increasing drought survival. Inhibition of leaf growth maintains some essential solutes from growth requirements to stress-related functions such as osmotic adjustment, to improve cell water and turgor maintenance (Neumann, 2008) In addition to shoot growth, root growth was inhibited under drought stress in *in vitro* culture but to a lesser extent (approximately 50% of control), as reflected by an increased root / shoot ratio. Plants in water stressed conditions tend to have an enlarged root system to increase water uptake capacity. Shoot growth inhibition could also be due

to sugar accumulation (used as osmolytes) possibly contributing to osmotic adjustment under drought stress (Bartels, 2005). Osmotic adjustment through the accumulation of compatible solutes has been considered an important process in plant adaptation to drought, primarily in roots enabling stable water uptake under decreasing soil water availability. Osmotically active compounds synthesized include aminoacids, e.g. proline, methylated quaternary ammonium compounds, e.g. glycine betaine, carbohydrates, e.g. glucose and sucrose, and cyclitols, e.g. inositol (Chaves et al., 2003; Chen and Murata, 2002). Besides osmotic adjustment, Chen and Murata (2002) found that compatible solutes help protect plants against damage by scavenging reactive oxygen species (ROS) and by their chaperone-like activities in maintaining protein structure and function. Soluble sugars are believed to interact with polar head groups and to replace water molecules.

Screening methods for drought tolerance

Plant response to drought stress is considered very complex as it involves different climatic, soil and agronomic factors combined with added variation in timing of occurrence, duration and severity or intensity. Drought has worse consequences under rainfed conditions in marginal lands by irregular and unpredictable rainfall during the growing season, occurring simultaneously with high temperatures and high solar radiation especially in areas with poor soil characteristics (Ceccarell et al., 2007). This complexity has made it difficult to identify specific physiological traits required for improving crop performance under drought to enhance crop drought tolerance and maintain high biomass production. Drought management strategies aim to maximize extraction of available soil moisture and improve the efficiency of its use in crop establishment, growth, and final desired yield (Blum, 2005). From agronomic aspects, the functional definition of drought should consider not only survival of the crop but also yield stability under water deficits and must be researchable and obtainable from wide heritable genetic variation, linked to yield.

Screening of physiological traits corresponding to crop productivity may require full control of specific environmental conditions such as temperature, relative humidity, and soil moisture. Furthermore, using effective instruments such as LI-3050A (Li-Cor, Lincoln, NE) for physiological parameters measurements is very important for collecting good data. Screening procedures should differentiate among genotypes or cultivars the degree of yield reduction under stress conditions and investigate traits that can be related to productivity. Attaining good data requires applying uniform stress at a standardized soil moisture profile, identical pre-stress crop growth, and a consistent water application. These conditions might be available only in growth chambers or greenhouses to mimic natural environmental conditions. This procedure should be repeatable and fully controlled, so stress can be applied at any growth stage and the severity and duration of stress can be easily managed. In the control treatment or well watered plants (WW), soil moisture should be maintained at a level of water that is freely available for plants and both stomatal conductance and water loss are not limited by soil moisture availability. At a given water level, the transpiration rate can be determined by the surrounding environment of the leaves. At moderate stress, the rate of water uptake cannot match the potential transpiration rate. Transpiration rate is limited by declining stomatal conductance and water uptake is equivalent to transpiration rate thereby maintaining water balance. Beyond this stress level of water stress, stomata are not able to match the transpiration rate with the water uptake from the soil. At this point, the plant must develop other mechanisms of drought tolerance to survive (Blum, 2011b).

Physiological parameters such as gas exchange and chlorophyll fluorescence should be determined when evaluating plant drought tolerance due to their important role in the early detection of environmental stress (Guidi et al., 1997). Stomatal conductance regulates the partial pressure of CO_2 inside leaves (Ci) which inturn affects CO_2 assimilation rate. that drives (Sharkey et al., 1982). Stomata control the exchange of gases, in and out of plant leaves, that include transpiration and CO_2 assimilation in the photosynthesis process (Haag-Kerwer et al.,

1999). Leaf level fluorescence parameters such as photochemical quenching (qP) and intrinsic efficiency of PSII (Fv′/Fm′) contribute to PSII photochemical capacity (Oxborough and Baker, 1997). The different soil-water availability levels were better reflected by midday electron transport rate (ETR) than by any other gas-exchange parameters (Medrano et al., 2002). Midday net CO_2 assimilation and ETR clearly reflect the level of water stress applied on plants due to the high correlation between ETR $_{max}$ and pre-dawn water potential (ψ_{PD}) (Flexas et al., 1999). Therefore, photosynthesis capacity can be negatively affected by stomatal closure or by decreasing the capacity of mesophyll cells to CO_2 assimilation as observed by Guidi et al. (1997).

The most important plant characteristics affecting crop yield including leaf photosynthetic rate, leaf expansion and growth are inhibited late in stage I or in stage II of soil drying. The main focus in stage III is survival and developing water conservation mechanisms that affect final yield. Although, much research has been conducted to study drought tolerance in several crops; little progress has been reported in terms of genetic improvement of crop productivity under water deficit environments. Cooperative research between breeders and crop physiologists is needed to test the viability/validity of the trait-based methods for drought tolerance improvement (Richards, 1991; Richards, 1996; Richards, 2004). An appropriate screening trait for drought stress tolerance should meet the following criteria: (i) a strong link with higher or more stable desirable yield in the target stress environment, (ii) a high level of heritability, and (iii) the expression of tolerance must be easily measurable, with adequate replication.

In plant physiology, drought resistance is referred to as the ability to survive or grow in a water-stressed environment, whereas from an agronomic perspective, it is more concerned with crop yields in drought conditions. It should be considered as crop survival ability and production capacity under drought conditions. Drought resistance of crops needs to evolve three important physiological characteristics. First, maintain a high plant water status; second, maintain

physiological functions; third, ability to recover water status and function after drought stress (Blum et al., 1999a; Blum et al., 1999b; Bruce et al., 2002; Luo, 2010).

Drought resistance can be expressed in three aspects (Luo et al., 2001a; Luo et al., 2001b; Zhang et al., 2005): (i) Dehydration avoidance (DA) which indicates the plant's ability to maintain water status by water uptake or reducing water loss at low moisture conditions. Dehydration avoidance can occur by developing a large and deep root system to absorb sufficient water from the soil along with stomatal closure or developing leaf cuticle to reduce transpiration. Screening can be conducted on morphological traits (such as root length, root diameter, and root volume, etc.) and physiological traits (such as stomatal conductance, leaf water potential, leaf relative water content, water loss rate, photosynthetic rate, and canopy temperature, etc). (ii) Dehydration tolerance (DT) is correlated to the capacity of plants to maintain function under low leaf water status. It reflects the ability of plants to maintain osmotic adjustment in plant cells, thus increasing the capacity of osmotic adjustment resulting in maintaining a high turgor. It also increases the capability of plants to remove accumulated harmful substances such as anti-oxidants. The measure of this capacity includes several physiological traits such as osmotic adjustment, ABA content, proline content, soluble sugar content, peroxidase or superoxide dismutase activity, and chlorophyll content, etc. (iii) Drought recovery (DR) refers to the recovery capability of a plant after a period of severe drought which causes the complete cessation of growth, a complete loss of turgor, and leaf desiccation. Though DA, DT, and DR possess various connotations, they are usually involved together in plant function. Dehydration avoidance is the major factor in drought-resistant performance, but drought tolerance (dehydration tolerance) occurs after dehydration avoidance (Blum, 2005b; Luo, 2010).

Yield potential is considered as the maximum yield that can be produced under non-limiting conditions (Blum, 2011a). Environmental stress, especially drought, is considered the most important factor causing yield decrease and plants must tolerate it to maintain yield potential

(Ribaut et al., 1997). Blum (2005) stated that drought resistance in physiological concept is determined by 'dehydration avoidance' and/or 'dehydration tolerance'. Water Use Efficiency is mostly discussed in terms of plant production rather than gas exchange. Levitt (1985) indicated that yield under water-limited conditions can be determined by the genetic factors controlling yield potential, and/or drought resistance, and/or WUE. Plants can resist drought by either dehydration avoidance or by dehydration tolerance. Drought resistance in terms of the physiology involved interacts with the magnitude and timing of the stress. Timing refers to the stage of plant development when stress occurs. For example, drought resistance in seedlings grown in a pot has nothing to do with drought resistance during grain filling in the field (Blum, 2005a). Also, Blum (2005) defined dehydration avoidance as the plant's ability to maintain high plant water status or cellular hydration under drought conditions to maintain plant functions and avoid tissue dehydration. At the crop plant level avoidance of dehydration can be achieved by using effective water absorption, decreasing water loss, and maintaining cellular hydration (Blum, 2005a; Chaves et al., 2004)).

Mitchell et al. (1998) found that Shoot/root dry weight ratio decreases under drought stress conditions and the decrease is corresponded to greater decrease in shoot mass rather than an increase in root mass. They also found that root length and depth also may increase in a drying soil even when total root mass was reduced. In rice, a deep root seems to be associated with a limited number of adventitious roots, resulting from reduced tillering which is an important component of high yield potential. Research indicates that reduced plant size, leaf area, and leaf area index (LAI) are major mechanisms for moderating water use and reducing drought stress injury (Mitchell et al., 1998). Reduced growth duration is associated with reduced leaf number (Blum, 2005b; Blum, 2009).

Sorghum plants tend to selectively kill older leaves under stress while remaining young leaves retain turgor, stomatal conductance, and assimilation (Blum and Arkin, 1984), as a result

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of high osmotic adjustment in the younger leaves. This demonstrates an opportune window for manipulating water use against plant production under stress at the Photosystem II reaction center that reduces photosynthetically active radiation (PAR) absorption and subsequently water use. Such varieties were found adapted to dry and cold conditions (Watanabe et al., 1995). Some reports suggest a correlation between high rate of osmotic adjustment (OA) and sustained yield or biomass under drought conditions in different crop plants (Ali et al., 1999). Increased deep-soil moisture extraction is a major contributor to OA in sorghum (Rontein et al., 2002; Wright et al., 1983). Beyond the effect on cellular hydration, other putative roles of OA have been recently assembled under the vague term of 'osmoprotection'(Rontein et al., 2002). Such a possible role for cell compatible osmolytes in protecting enzymes against heat inactivation was indicated a while ago (Paleg et al., 1981). Associations between OA and cellular membrane stability under drought stress were suggested more recently (Babu et al., 2004; Riga and Vartanian, 1999). Crops and native vegetation that are adapted to water limited conditions in terms of growth and productivity achieve adaptation mainly by dehydration avoidance and escape rather than by desiccation tolerance. At the cellular level, osmotic adjustment is a major cellular drought-responsive trait that contributes to cellular dehydration avoidance and yield under stress (Blum, 2005b).

CHAPTER III

GREENHOUSE EXPERIMENT

Introduction

Water availability for biomass production will be a challenge with increasing needs for human consumption. In addition, biomass feedstocks are to be produced in marginal areas with limited rainfall. Large scale cultivation of bioenergy feedstocks will increase evapotranspiration. Perennial grasses, with relatively low water requirements, are a promising source of bioenergy that do not result in increased CO_2 levels. Using warm season grasses such as switchgrass (*Panicum virgatum* L.) as a bioenergy crop in grass and marginal lands can have additional environmental benefits such as reducing soil erosion and flooding (Epplin, 1996a; Epplin, 1996b). Perennial grasses grown for bioenergy biomass production will not impact increasing atmospheric CO_2 concentration; in contrast it can contribute to decreasing this concentration by increasing photosynthesis rates. Better competition with other energy sources can be reached through high net energy from low input and low costs of agricultural biomass yield (Kiss and Wolf, 2001; Lynd et al., 1991b; Schmer et al., 2008a).

Switchgrass is a tallgrass prairie warm season perennial C_4 grass grown in the central and North American Great Plains for forage and grazing. It has received more attention as a bioenergy crop than any other alternative crop in recent three decades in the U.S. It is classified ecologically, depending on ploidy level and habitat preference, into upland and lowland types (Stroup et al., 2003). Upland types are primarily octoploids (2n=8x=72) and lowland types are

tetraploids (2n=4x=36) (Brunken and Estes, 1975; Hultquist et al., 1996). Upland types are mostly found in dry regions; whereas, lowland types are found in wetter regions. Morphologically, upland cultivars have smaller leaves and stems than lowland cultivars. Due to their environment, upland cultivars are adapted to drought, are less susceptible to water stress, and have higher photosynthetic rates than lowland cultivars (Nickell, 1973b). In contrast lowland cultivars are vigorous and give higher biomass and have lower nutrient requirements especially nitrogen (Porter, 1966). Genetic diversity helps switchgrass cultivars to adapt to a wide range of soils and diverse climates. Water has a significant effect on productivity in most perennial grasses (Epstein et al., 1996b).

Drought as an abiotic stress is a concern in agriculture, and it can have significant effects on biomass production (Berndes, 2002; Koshi et al., 1982b). Water use efficiency (WUE) can be a trait contributing to biomass production. Water use efficiency varies among cultivars and it helps some cultivars to cope with water shortage during the growth season (Koshi et al., 1982b; Sashidhar et al., 1986b). Switchgrass has many advantages such as adaption to different climates and soils, use in conservation planting, and high biomass production with high cellulosic content. This makes it a leading crop among many alternative candidate crops to be used for ethanol production as well as a combustion fuel source for clean energy production (Koshi et al., 1982b; McLaughlin et al., 1999). Switchgrass has an extensive and deep root system that allows it to use water and nutrients very efficiently, and it maintains a beneficial symbiotic relationship with microscopic soil fungi. It is also an excellent plant for use in riparian buffer strips. Its root system prevents soil erosion, slowing the travel of surface water, resulting in decreased run-off from agricultural fields, and allowing for greater water infiltration (Lee and Boe, 2005). Switchgrass cultivars differ in WUE (Hartman, 2011; Kiniry et al., 2011). However, selection for a single trait may not result in the desired benefit. Hence, an experiment was conducted with switchgrass cultivars to identify the biomass and physiological traits that contribute to increased WUE.

Screening of morphological and physiological traits contributing to plant tolerance to water stress can help to identify most correlated and contributing traits to plant tolerance. These could be used as good indicators to choose cultivars with high biomass yield during stressful times or in drought regions. The hypothesis of this experiment was that upland cultivars are more tolerant to water stress than lowland cultivars. Accordingly, the objective of the study was to identify switchgrass traits that are related to drought tolerance and increased WUE through whole plant level screening.

Materials and methods

Plant culture

This experiment was conducted in a greenhouse facility at Oklahoma State University in the summer of 2009. Seeds of 13 switchgrass cultivars (Table 1) including three lowland cultivars, (Carthage,Alamo, and Kanlow) and 10 upland cultivars, (Southlow, Cave-in-Rock, Forestburg, Blackwell, Nebraska 28, Shelter, Shawnee, Dacotah, Sunburst, and WI Ecotype), were obtained from a commercial source (ERNST Meadville, PA). Carthage cultivar was included to lowland according to Lemus et al., 2002 and Alexopoulon et al., 2008 while it was included with upland according to Cortese et al., 2010. Seeds were sown in 12 L pots (314 cm^2), with pure fine masonry sand as the growing medium. Five pots with 20 seed each. Seeds were sown for each cultivar then the number of pants was reduced to four in each pot. An automated drip irrigation system was used to supply Hoagland's nutrient solution (Hoagland and Arnon, 1950). Emergence occurred 7 to 10 days after sowing depending on the variety and WI ecotype emerged 14 days after sowing. Temperature in the greenhouse was maintained at 30± 2 °C and relative humidity at 70 ± 5%. Light was 10% less than outside in the greenhouse.

Treatments

After 80 days of growth with adequate moisture, water stress was induced by decreasing the amount of water supplied to stress treatments to create 60% and 20% of well watered pots. Water needed for 'container' or field capacity was determined by deducting the weight of the pots with dry sand from the weight of the pot after dripping stopped. Stress treatments were then determined by reducing the moisture to 60% and 20% of water according to amount needed for container capacity. Three treatments were included – Well Watered (Control-WW-100%), (T1-60%WW), and (T2-20% WW). The 60% and 20% treatments were imposed by reducing the dripper timing. In the control treatment, each dripper emitted Hoagland's nutrient solution at a rate of 75 mL min^{-1} and the timer was adjusted for 1, 3, and 5 minutes, three times a day (800, 1200, and 1700 h), to generate T1, T2, and control respectively. Delta T devices TH2O portable soil moisture meter (Delta-T Devices Ltd, Cambridge, England) was used to keep track of soil moisture in each pot.

Measurements

Plant measurements included plant height, tiller number, and node number measured weekly when plants reached the fourth leaf stage. After 74 day of applying stress, plants were harvested and plant height, tiller number, and number of leaves on the main tiller were determined. Plants were severed at the soil surface and leaf area was measured using a leaf area meter LI-3050A (Li-Cor, Lincoln, NE). Leaves and stems were separated and dried in a drying oven for three days at 65 °C (150 °F) then weighed. Roots were washed then dried for three days in a drying oven at 65 °C (150 °F) then weighed. Other bulk shoots and roots were dried and then dry weight was determined. Biomass and partitioning traits such as, total biomass, stem dry weight, leaf dry weight, and root dry weight were measured at harvest.

For the photosynthesis measurements, the LI-6400 (Li-Cor, Lincoln, NE) photosynthesis system was set up and calibrated as described by the Li-Cor instructional reference manuals. The instrument was turned on for 20 minutes for warming up and calibration and to allow the system electronics to stabilize. Net photosynthesis and fluorescence parameters were measured weekly between 1000 and 1400 h using LI-6400 portable photosynthesis measurement system when weather conditions were appropriate. Instantaneous WUE (Net photosynthesis/transpiration) was calculated. The uppermost fully expanded leaf, on the main tiller in each pot, was selected for measurements. The LI-6400 system fitted with a 6400-40 leaf chamber flourometer (LCF) that included the LED-based fluorescence illumination source. After the instrument reached stable condition as indicated the CV of the sensors (<1%), chamber environmental conditions were adjusted as following, concentration of CO_2 of reference cell set at 400 µmol mol^{-1}, block temperature at 30°C, constant light intensity of 1500 μ mol photons m^{-2} s^{-1} and flow rate of 500 μ mol s^{-1} was fixed for all net photosynthesis measurement. Relative humidity was maintained at 60 %. The portable LI 6400 infra-red gas analyzer (IRGA) was matched in order to set initial conditions in the chamber. For fluorescence measurements (Msr), the instrument parameters were set at Msr intensity =10, Msr Modulation =0.25 kHz, Msr filter =5 Hz, Gain=10 Gn; To generate the flash for saturating the open reaction centers the settings were Flash type = Single, Flash duration = 0.8 s, Flash intensity = 10, Blue LEDs = no change, Msr modulation = 20 kHz and Msr Filter = 50kHz; For generating the dark pulse the settings were Duration= 6 s, Far-Red intensity= 8, Far-Red Pretime=1 s, Far-red Posttime = 1 s, Msr modulation = 1 kHz and Msr Filter = 0.5 kHz. The ETR was inferred from the actual flux of photons (µmol m^{-2} s^{-1}) driving PS II as in following equation, ETR= $(F_m'-Fs/F_m')$ fI α_{leaf} where f is the fraction of absorbed quanta that is used by PS II and assumed to be 0.4 in switchgrass a C4 plants. The efficiency of energy harvesting by oxidized (open) PSII reaction center in the light (F_v'/F_m'), fraction of absorbed photons that is used for photochemistry for a light adapted leaf (Φ_{PSII}), the photochemical

quenching (qP), and electron transport rate (ETR) were determined by LI-6400 internal algorithms. Three leaves of each cultivar from each treatment were used for measurements.

Experimental design

A Completely Randomized Design (CRD) was applied using thirteen switchgrass cultivars in two water stress treatments in addition to control (WW) with three replicates in each cultivar within each treatment. Two-way ANOVA in SAS was used to analyze main effects of cultivar and treatment and cultivar x treatment interactions. A Principal Component Analysis (PCA) was used on the biomass and physiological traits to compute the contribution of each parameter and to identify cultivars with water stress tolerance and traits that can contribute to enhanced water stress tolerance.

Results and discussion

Water stress decreased plant height in most switchgrass cultivars at T2-20% WW whereas with T1-60%WW few differences occurred likely due to the ability of some switchgrass cultivars to cope with mild stress by enhancing more root and shoot growth. Plant height in Alamo as a lowland cultivar was affected by stress with T2-20% WW compared to upland cultivar Forestburg (Fig. 1)

Stem elongation rate decreased with both T1-60% WW and T2-20% WW compared to control plants (Fig. 2). Treatment interacted with cultivar. Kanlow and Cave-In-Rock were most affected by stress while Forestburg, Blackwell, and WI Ecotype were less affected. At T1-60% WW Shawnee, Alamo, and WI Ecotype were less affected by water deficit than other cultivars. Stem elongation which is a result of cell division and cell enlargement was affected by water stress. Both of these bioprocesses require sufficient water in plant tissue to be accomplished.

Cultivars differed in leaf/node number per plant. Lowland cultivars Alamo and Kanlow had higher plant leaf and node numbers than upland cultivars (Fig. 3). The 60%WW treatment enhanced leaf/node number in Alamo, Kanlow, Cave-In-Rock, and WI Ecotype whereas 20%WW decreased leaf/node number in most cultivars. Tiller number per plant decreased with increased stress in most cultivars except WI ecotype. Cultivars also differed in plant height in response to water stress (Fig. 3). Alamo and Kanlow were the tallest at 140 cm for Alamo and 150 cm for Kanlow whereas Dacotah was the shortest at 70 cm. Plant height for the newly produced tillers decreased withwater deficit especially at T2-20% WW ($p = 0.05$). At WW or normal conditions plant height averaged 103 cm across all cultivars. In lowland cultivars Alamo (139 cm) and Kanlow (147 cm), but it averaged 115 cm in Alamo, and 102 cm in Kanlow at T2-20% WW. Among upland cultivars Dacotah had the lowest average with T2-20% WW (71.3 cm), whereas plant height average was 100.7 cm across plants and treatments. No increase in plant height was observed after one month of applying water stress which indicates the shift in the partitioning strategy that plants performed to survive stress by utilizing all photosynthetically produced sugars in respiration.

Moderate water stress T1-60%WW was tolerated by switchgrass cultivars and positively affected switchgrass cultivars to tolerate water deficit. These results are similar to those of Kiss and Wolf (2001), who found that preconditioning switchgrass plants helps improve photosynthetic capacity.

Water stress decreased leaf, stem, and root dry weight in most cultivars compared to control plants (Fig. 4). The 60%WW enhanced stem, root, and total biomass of Blackwell and Shelter. Total biomass was severely reduced by water stress at T2-20% WW and a significant interaction existed between cultivars and water stress treatments. Forestburg had greater total biomass, leaf, root and stem dry weights with water stress than when well watered. Detailed analysis of partitioning parameters showed similar results (Fig. 5). At T1-60% WW, most

cultivars had increased partitioning. Root dry weight is a good indicator of drought tolerance because switchgrass as a perennial crop tends to extend the root system deeper into the soil to capture moisture. These results are similar to what Xu et al., (2006) found in switchgrass compared with other grasses.

Principal component analysis analyzed most morphological parameters to better understand the contribution of each parameter to drought tolerance (Fig. 6). It is appropriate to calculate the total percentage of variation that indicates a scientific contribution to water stress tolerance. The PC1 and PC2 accounted for 77% of variation among cultivars based on PC scores. Among the biomass and growth traits, total biomass, stem dry weight and root dry weight, contributed the most to treatment and cultivar differences.

The PCA was carried out on a combined control with T2-20% WW data set to determine best parameter contributing in total variation toward water stress tolerance. The PC1 and PC1 accounted for more than 72% of variation among cultivars based on PC scores. Among biomass and growth traits, total biomass, stem dry weight and plant height, contributed to treatment and cultivar differences. The PCA carried out on the differences between control and 20%WW treatments (Fig. 7) indicated that Forestburg, Blackwell, and Sunburst were most tolerant to water stress, whereas Carthage, Cave-in-Rock and Kanlow were most sensitive to water stress. Among the traits studied, PC1 had high loadings for leaf and stem dry weight and stem elongation rate, while PC1 had highest loadings for plant height and root dry weight.

In the PCA of all photosynthesis parameters for all treatments, PC1 accounted for 69.76% of variation and PC2 for 17.10% variation (Fig. 8). The PCA of all photosynthesis parameters with data from all treatments indicated that leaf Pn, ETR, PhiPS2 and PhiCO$_2$ played a major role in separating the genotypes and treatments. Chlorophyll fluorescence is commonly used to assess the photochemical performance of plants under stress conditions. It reflects the

photosynthetic efficiency of the leaves under different environmental conditions. Photosynthesis readings indicated the efficiency of CO_2 assimilation utilizing absorbed photons in tested cultivars while ETR reflected the actual flux of photons driving PSII. Determining PhiPS2, quantum yield of PSII calculated from fluorescence, and PhiCO2, quantum yield calculated form CO_2 assimilation needs calculation of net CO_2 assimilation in the light and assumption of dark respiration and absorbed PAR. Also F_v'/F_m' is a useful parameter to describe energy dissipation in order to estimate efficiency of energy harvesting by oxidized (open) PSII reaction center in the light (Fv´/Fm´). Strong relationships between these photosynthetic parameters associated with photosynthesis yield and be a good indicator for better performance of these factors showed the adaptation to drought in switchgrass cultivars.

The PCA in Figure 9 indicated a difference between control and T2-20%WW for all parameters measured. In this PCA, PC1 accounted for 53.47% of variability, while PC1 accounted for 21.71% variability. Cultivars Carthage, Forestburg, and Blackwell were most tolerant to decreased photosynthesis under water stress.

At the physiological level, photosynthesis was decreased by increased water stress. During the first seven days of water stress no difference occurred between 100% WW and T1-60% as T1-60%WW did not severely affect plant performance. In contrast stress decreased photosynthesis with T2-20% WW compared to the control. After 12 days of stress application, differences between the three treatments existed (Fig. 10). Photosynthesis was severely reduced at T2-20%WW at seven and 12 days of stress and a slight decrease or increase, depending on cultivar was observed under 60%WW conditions. Stomatal conductance was tightly correlated with decreased photosynthesis and impacted net photosynthesis. Stomatal conductance decreased with increased water stress and with time from seven to 12 days resulting in decreased photosynthesis in all treatments. Stomatal control of water losses was a result of stress conditions

to maintain plant moisture, but it affected photosynthesis by limiting CO_2 uptake (Chaves et al., 2002; Tezara et al., 1996).

Electron transport rate decreased linearly ($P<0.0001$) with increase in stress (Fig. 11). At the same time more electrons were needed during stress for CO_2 fixation compared with control conditions (Fig. 11), affecting total carbon needed for growth resulting in total biomass reduction.

Conclusions

Water stress affected switchgrass cultivars morphologically and physiologically by decreasing plant growth and photosynthesis. Genotypic differences existed for the studied traits in response to water stress. However, upland genotypes such as Forestburg and Blackwell exhibited biomass and photosynthetic trait tolerance to water stress more than other tested cultivars. Cultivars Forestburg, Blackwell, and Sunburst were most tolerant to water stress, whereas Carthage, Cave-in-Rock and Kanlow were most sensitive to water stress. Growth traits such as plant height or stem elongation rate can be used for screening switchgrass germplasm. Finally, photosynthetic and fluorescence parameters such as Fv'/Fm' or PhiCO2 can be very helpful for quick screening of traits for tolerance to water stress toward improving these traits.

CHAPTER IV

IN VITRO CULTURE EXPERIMENT

Introduction

Using warm season grasses such as switchgrass as a bioenergy crop on marginal lands requires cultivars with enhanced tolerance to water stress. Close focus on screening switchgrass for water stress tolerance and improving water use efficiency (WUE) could be helpful to reach such goals (Epplin, 1996a). Switchgrass cultivars differ in water stress response as shown in Chapter III and reported from other studies (Blum, 2005a; Koshi et al., 1982a; Sashidhar et al., 1986a; Xu et al., 2010). Whole plant screening is a useful method to improve plant tolerance and to obtain desirable variation by looking for traits that are correlated with high biomass production under stress conditions. Screening at the cellular level might be more effective under specific water stress conditions by providing a system effectively differentiating the cultivars for desired traits (Gopal and Iwama, 2007; Zhang and Donnelly, 1997). It saves cost and effort needed for field based screening programs (Zhang and Donnelly, 1997).

Developing crops better adapted to abiotic stress is important and requires screening of promising cultivars for resistance to this stress (Aazami et al., 2010; Tewary et al., 2000). *In vitro* culture allows a quick screen of cultivars that need to be improved rapidly, and these cultivars exhibit their capacity to tolerate stress in different growth and development stages (Gosal and Bajaj, 1984). Cell lines for salt and drought tolerance have been isolated using *in vitro* culture

techniques from many crop species such as rice (*Oryza sativa* L.), citrus (*Citrus sp*.), carrot (*Daucus carota* ssp.), grape (*Vitis vinifera* L.), mungbean (*Vigna radiate* L.) and alfalfa (*Medicago sativa* L.) (Aazami et al., 2010; Dami and Hughes, 1997a; Gosal and Bajaj, 1984; Handa et al., 1986b; Kishor and Reddy, 1985; Tewary et al., 2000) Both morphological and physiological traits can be used to identify desired cell lines. Morphological traits such as tiller number, plant height, biomass yield and physiological traits such as proline content, photosynthesis, stomatal conductance, ETR, and fluorescence can be good indicators of water stress tolerance (Aazami et al., 2010; Gosal and Bajaj, 1984; Rauf et al., 2007).

Screening of switchgrass cultivars at the cellular level using *in vitro* methods can enhance water stress tolerance. A quick *in vitro* procedure was used in several crops to induce somaclonal variation and tolerant cell lines for water stress were identified (Bairu et al., 2011; Dami and Hughes, 1997b; David and Ragauskas, 2010; Miyao et al., 2012; Shomeili et al., 2011; Snyman et al., 2011). The plantlets developed from the tolerant cell lines demonstrated increased tolerance to water stress as identified by an increase in some traits such as higher epicuticular wax, differences in leaf epidermal cell configuration in grape plants (Dami and Hughes, 1997b) and induced mutants in rice (*Oriza stiva* L.) (Miyao et al., 2012) and potato (*Solanum brevicaule* L.) (Afrasiab and Iqbal, 2012). Therefore, the natural variation that switchgrass cultivars have along with induced variation resulting through tissue culture procedures can be effectively used to obtain switchgrass lines that have enhanced water stress tolerance.

The hypotheses of this experiment were that (1) an optimized growth regulator combination induces callus in all switchgrass cultivars, and (2) *in vitro* water stress treatments produce more drought tolerant cell lines. The objectives of this project were (1) to determine the

best growth regulator combination for callus formation, (2) to investigate the response of switchgrass cultivars to *in vitro* culture, and (3) to determine the effect of water stress caused by PEG in tissue culture media on morphogenesis processes.

Materials and Methods

This experiment was conducted in the Bioenergy Crop Production Laboratory at Oklahoma State University. An *in vitro* culture experiment was carried out to develop an appropriate protocol to induce callus formation and variation in regenerated switchgrass plants with useful physiological changes (Aazami et al., 2010) Genetic variation at the cellular level could be beneficial for improving water stress tolerance. For the first objective, two switchgrass cultivars (Alamo and Forestburg) were used. Alamo was chosen as a high biomass producer representing lowland cultivars and Forestburg was chosen due to water stress tolerance characteristics that it showed in the greenhouse experiment Chapter II). For the second objective, 12 switchgrass cultivars were used; three lowland cultivars (Carthage, Alamo, and Kanlow) and 9 upland cultivars (Southlow, Cave-in-Rock, Forestburg, Blackwell, Nebraska 28, Shelter, Shawnee, Dacotah, and Sunbrust). Seeds were treated with Daconil fungicide (TechPac, LLC. Lexington, KY) 1 mL L^{-1} (Shields et al., 1984) then surface sterilized using 70% ethanol for 1 min followed by 2% sodium hypochlorite and 1% Triton-X for 20 min. Seeds were then rinsed three times for five minutes with double distilled sterile water. Seeds were placed on MS media (Murashige and Skoog, 1962) obtained from Caisson Laboratories Inc. (North Logan, UT, USA) (McLaughlin and Kszos, 2005). The MS media, supplemented with 3% maltose and agar, was added at a rate of 8 g L^{-1} and the media was autoclaved. The media was cooled and separated into six flasks for adding growth regulators. Six combinations of the two growth regulators, 2, 4-dichlorophenoxyacetic acid (2,4-D) – an auxin, and benzyl adenine (BA) – a cytokinin, 11 µM and 15 µM, 11 µM and 45 µM, 28 µM and 15 µM, 28 µM and 45 µM, 45 µM and 15 µM, and 45

µM and 45 µM, respectively, were added to each flask. Each cultivar was tested on all growth regulator combinations for callus formation efficacy.

For the third objective, seeds from Alamo and Forestburg cultivars were sterilized using the protocol described above and then placed on MS media (McLaughlin and Kszos, 2005) supplemented with 3% maltose, and a combination of auxin and cytokinin (45 µM and 45 µM) (2,4-D, BA) that produced best callus development in previous research (Denchev and Conger, 1995). The pH was adjusted to 5.6 with 0.1 N NaOH and the media were solidified with 8 g L^{-1} agar. Twenty five seeds were cultured in each petri dish (100 x 15 mm) with 5 dishes of each growth regulator combination for each cultivar (Conger, 2002; Huang Tao, 2002). Petri dishes were incubated in a dark growth camber at 25˚C for callus development and callus mass was observed weekly.

For identifying water stress tolerant lines, callus was placed on solidified MS media supplemented with PEG. New calli were divided and sub-cultured four times with 30 day duration for each subculture (to get sufficient callus) prior to placing them on MS+PEG media. Two water stress levels (-0.6 MPa and -1.7 MPa) were imposed *in vitro* in addition to the control (Ben-Hayyim, 1987; Bressan et al., 1981; Handa et al., 1986b; Sumaryati et al., 1992). The PEG solution was prepared using PEG 6000 (Sigma-Aldrich Co., St Louis, MO, USA) in desired concentrations along with MS media and autoclaved without agar. This was essential as PEG inhibits the polymerization of agar. To obtain the desired final PEG concentration in the solidified MS media, a MS+PEG solution with twice the osmotic potential   (-1.2 MPa for -0.6 MPa and -3.4 MPa for -1.7 MPa) was used. Osmotic potential of the MS solution after adding PEG was adjusted using a vapor pressure osmometer (Osmometer 5520, Wescor, Inc., UT, USA). In addition, another batch of MS media (with no PEG) was also prepared with agar, autoclaved; 15 mL was poured in to petri plates (100 x 15 mm) and allowed to solidify. A 15 mL of autoclaved solution of MS + PEG solution was poured on top of each plate. The MS+PEG solution was

added to the solidified MS media as described by Dami and Huges (1997a) and var der Weele et al. (2000).The petri plates were left overnight to allow for obtaining an equilibrium state and the osmotic potential of the solution was measured.

The sub-cultured callus was placed on the MS+PEG media for 30 days, then moved to PEG free MS media for another 30 days, and was again allowed to grow on MS+PEG media for 30 days. This resulted in a total of 60 days on MS+PEG media. The 30 days on PEG free MS media was essential to identify and multiply the surviving calli subjected to stress treatments. Callus survival and development were monitored during this period. Survival of callus, mass of callus, and growth were observed. Callus surface area was determined by measuring callus dimensions to compare callus mass formed under control with callus mass formed under stress. After the 60 day stress-treatment period, calli were transferred to growth regulator free MS media and incubated in a growth chamber at 16/8 h light/dark regime at 25°C to induce morphogenesis (Bhaskaran et al., 1985; Handa et al., 1986a). Shoots initiated were then transferred to larger containers with growth regulator free MS media supplemented with 3% maltose for further growth and root initiation. After shoot (3-5 leaves) and root formation, rooted plantlets were transferred to small pots filled with peat moss and moved to the growth chamber for evaluation (Chapter V).

Experimental design

For the first objective, a completely randomized design (CRD) with two cultivars Alamo and Forestburg was used. Six different growth regulator combinations as mentioned in the materials and methods were prepared with MS media and sterilized seeds from both cultivars were distributed on plates containing these combinations. Six plates were used for each growth regulator combination with 20 seeds per plate. For the second objective, the design was completely randomized design (CRD). Seeds from 12 switchgrass cultivars were distributed on

MS media sublimated with 3% maltose and 45 µM 2,4-D and 45 µM BA. Five (5) plates were used for each cultivar and each plate contained 20 seeds. For the third objective, the design was a completely randomized design (CRD) with two water stress treatments (on MS+PEG media) in addition to the control (PEG free MS media). Callus of two cultivars of switchgrass, Alamo and Forestburg, was used. Cultivar for each treatment had 10 plates and each plate contained 4 calli. Two way ANOVA analyses in SAS was used to analyze cultivar, treatment and cultivar x treatment interactions.

Results and Discussion

In vitro culture for callus formation switchgrass cultivars produced clean and uncontaminated culture from all tested switchgrass cultivars. Among the six growth regulators combinations, 45 µM 2,4-D and45 µM BA, respectively produced greatest callus mass when Alamo and Forestburg cultivars were used (Conger, 2003; Denchev and Conger, 1995). Growth regulator combination affected callus development and callus mass in Alamo and Forestburg by enhancing cell division at different rates (Alexandrova et al., 1996; Gupta and Conger, 1998). When seeds of twelve switchgrass cultivars were placed on MS media supplemented with 3% maltose and with the growth regulator combination of 45 µM 2,4-D and 45 µM BA. .callus were successfully produced from all cultivars (Fig. 12). After 14 days of incubation, callus formation was observed from Alamo, Blackwell, and Dacotah in different mass sizes. At day 30, Alamo, Blackwell, and Dacotah produced the largest callus mass. Other cultivars, Kanlow, Forestburg, Cave-In-Rock, and Shawnee took 20 days to initiate callus, whereas Carthage, Southlow, Nebraska, Shelter, and Sunbrust needed 23 days to initiate callus. Differences between cultivars in response to growth regulator combination for callus formation were expected due to differences in hormone requirements.

For the second objective callus surface area was determined by measuring the final length and width after sub-culturing on stress media. Callus surface area was larger on control media than on media with PEG (Fig. 13 and 14). Control treatments in both cultivars differed from T1 and T2. Also, a difference (P <0.05) was observed between Alamo and Forestburg in control plants. Alamo under control conditions produced larger callus than Forestburg. Both cultivars had good callus development on MS media amended with PEG, indicating the ability to survive the stress indicating a greater possibility of somaclonal variation. However, Alamo developed better callus than Forestburg when exposed to T1 stress level while similar callus growth was observed for both cultivars under T2 (Fig. 14). The 30 day growth on the PEG free MS media during the middle of the 60 day stress treatment was essential to test the stability of the callus to survive stress treatments. This step also allowed for producing enough fresh callus for subsequent organogenesis step utilizing the induced variation and for further evaluation of plants (Fig. 15 and 16).

Shoot number and shoot length of the regenerated plantlets were determined before transferring to the growth chamber. Forestburg callus had more shoots per callus than Alamo, but Alamo had longer shoots. Forestburg in the control treatment produced more shoots compared to those in stress treatments, but Alamo had a stable shoot number in T1 and shoot number was reduced in T2 (Fig. 17 and 18). Both cultivars developed root and shoot systems sufficient to obtain individual plantlets to be grown in the growth chamber.

Shoot length was also measured at the time of transferring to pots. Forestburg plantlets were shorter and shoots stuck together whereas Alamo shoots were less numerous and grew separately and taller. Shoot growth was affected by stress due to the need for water for cell division and cell enlargement. Shoot length of Alamo was decreased with water stress. Less decrease was observed in Forestburg between control and stress treatments suggesting less affect

of stress on Forestburg in shoot length. Shoots of Alamo and Forestburg initiated roots using MS free growth regulator media (Fig. 19 and 20).

Conclusions

Alamo and Foretburg produced callus using MS media supplemented with 3% maltose and six growth regulator combinations and best callus mass was obtained using 45 µM 2,4-D and 45 µM BA. All 12 switchgrass cultivars produced callus demonstrating effective culture initiation using MS media supplemented with 3% maltose and combination of growth regulators (45 µM 2,4-D and 45 µM BA). Alamo and Forestburg callus survived a water stress of -1.7 MPa and enhanced variation in the callus cells resulted in Alamo. Regenerated plants can be obtained from switchgrass cultivars using *in vitro* procedures. Successful callus production of these cultivars can lead to more molecular and genetic studies. Furthermore, results help to determine the optimum combination of growth regulators that gives maximum callus yield. Finally, quick production and screening tools such as *in vitro* culture can be utilized to obtain more water stress tolerant plants and maintain somaclonal variant plants through in vitro culture propagation.

CHAPTER V

EVALUATION OF REGENERATED PLANTS

Introduction

In the early history of plant tissue culture used for plant propagation, some variation was observed in plants derived from *in vitro* propagation methods (Cohen, 2011). The variation was introduced as phenotypic variation and defined as somaclonal variation that could be genetic or epigenetic. A review of the literature revealed that plant cell culture itself generates genetic variability (somaclonal variation) when totipotent cells are directly placed on an appropriate culture medium (Larkin and Scowcroft, 1981). Somaclonal variation is also defined as the genetic variation displayed in tissue culture regenerated plants. A single genotype can produce different phenotypes under the same *in vitro* culture conditions that can be utilized for plant improvement (Cohen, 2011; Miguel and Marum, 2011). Epigenetic variation might be considered an adaptation process to unfavorable environmental conditions. This epigenetic mechanism is affected by genetic changes such as DNA methylation, histone modifications, and RNA interference. Generating whole plants from differentiated cells through dedifferentiation and then redifferentiation using tissue culture techniques reflects the plasticity of these cells in responding to specific environmental signals to achieve tolerance by going through differentiation ending with a new developmental pathway. Changes in chromatin and reprogramming of gene expression led by epigenetic regulation

(Cohen, 2011; Miguel and Marum, 2011; Sato et al, 2011). Clear evidence indicates that DNA methyltransferases, histone modification enzyme, and many regulatory proteins play critical roles in plant growth and development (Miguel and Marum, 2011). Many factors enhances mutations in tissue culture such as plant growth regulators, lighting conditions, imbalance of media components, high humidity and transpiration relationship, saline stress, oxidative stress, and nutrient deficiency (Sato et al., 2011).   This variation, along with the corresponding changes observed in tissue culture per se, has been documented through tissue culture reviews (Larkin and Scowcroft, 1981; Sato et al., 2011).

Plant *in vitro* culture is a useful technology to assist plant breeders by decreasing the required time and creating new variation for plant improvement. Somaclonal variation refers to any genetic, cytogenetic, or molecular change produced during tissue culture or plant regeneration and propagation (Karp, 1995; Scandalios, 1992) Changes are affected by the meristem type, dedifferentiated state, and different explant sources that are used for tissue culture establishment. Dedifferentiation is important for tissue and it can result in adapted variation in some desirable traits (Lee and Phillips, 1988a; Scandalios, 1992)

In vitro screening methods require sufficient generated variation of the trait of interest. Variation can be increased with the duration of *in vitro* cultural cycles through various frequent subcultures (Larkin and Scowcroft, 1981; Lee and Phillips, 1988b; Sato et al., 2011). Variation in chromosome number and structure was indicated among plants derived from cultured cells (Lee and Phillips, 1988a). Many treatments can be used to induce somaclonal variation and many procedures can be followed to achieve adequate variation. For example, meristem culture, micropropagation through many subcultures, direct organogenesis from leaf, callus culture, and subsequent plant regeneration, and somatic embryogenesis can be performed. That should be followed by a screening procedure for somaclones in the regenerated plant population (Biswas et al., 2009).

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Proline is one of the most common compatible solutes in plant tissues under water stress (Maggio et al., 2002). It is considered a reliable indicator of water stress in plants in environmental research, and leaf proline content increases in plants subjected to unfavorable environmental conditions and in stressed leaves compared with non-stressed ones (Claussen, 2005). Proline concentration increased faster than any other amino acid in plants under water stress possibly because of its role in osmotic adjustment, and it can be determined in the vegetative parts (Bates et al., 1973; Shah and Dubey, 1997).

The hypothesis of this experiment was that plants regenerated from drought tolerant cell lines perform better than their parental cell lines. The objective was morpho-physiological evaluation of switchgrass plants derived from *in vitro* culture.

Materials and Methods

For this experiment, seeds from Alamo and Forestburg were sown in plastic pots 45 cm in height and 20 cm in diameter. Six pots for each cultivar were placed in a row and a distance of 30 cm was maintained between the rows and placed in a growth chamber (Conviron, PGC20, Pembina, ND, U.S.A). Each pot was filled with gravel at the bottom to maintain proper drainage and the rest of the pot was filled with pure, fine sand. Emergence was observed after 5 days for both cultivars. Plants were irrigated using a drip irrigation system supplying standard Hoagland's nutrient solution. Irrigation was provided three times a day for three minutes at 0800, 1200, and 1700 h and controlled with a timing device. After emergence, each pot was thinned to four plants that were at similar growth stage and physiological age. Regenerated plantlets from *in vitro* treatments, of Alamo (R.Alamo) and Forestburg (R.Forestburg), were grown *in vitro* at the final steps of root initiation that took 10-15 days. The seed and regenerated plants will be referred to as plant types in this chapter. Plants obtained from the *in vitro* procedure were moved to a larger

growth chamber (Fig. 21) to be grown along with plants obtained from seeds. All plant types were grown in the same environmental conditions before applying water stress treatments.

Treatments

After 60 days of growth under adequate soil moisture, water stress was induced by decreasing the amount of water supplied to stress treatments to create T1-60% WW, and T2-20% WW of well watered pots. Water needed for field capacity was determined by deducting the weight of the pots with dry sand from the weight of the pot after dripping stopped. Stress treatments were then determined by reducing the moisture to 60% and 20% of water amount needed for container capacity. Three treatments included – Well Watered (Control-WW-100%), T1-60%WW and T2-20% WW. The 60% and 20% were imposed by manually adding 280 mL, 168 mL, and 56 mL for control, T1-60% WW and T2-20% WW respectively. This amount of Hoagland's nutrient solution (Hoagland and Arnon, 1950) was addedfour times each day, 0800, 1000, 1200, and 1700 h. Temperature was maintained at 30/22 ± 2 ℃ (day/night) and relative humidity was maintained at 70% ± 5 throughout the study.

Measurements

The purpose of this experiment was to compare plants regenerated through *in vitro* culture with plants grown from seed. When plants reached the fourth leaf stage, morpho-physiological properties including plant height, tiller number, and node number were measured weekly. Physiological measurements including photosynthesis, electron transport rate, and stomatal conductance using LI-6400 portable photosynthesis measurement system (Li-Cor, Lincoln, NE) were measured weekly between 1000 and 1400 h as described in Chapter III. The uppermost fully expanded leaves, on the main tiller in each pot, were selected for photosynthetic measurements.

Above ground shoot was severed and leaves of main tillers were separated and their area measured using a leaf area meter (LI-3050A Li-Cor, Lincoln, NE). Each plant shoot was kept separately and dried in a drying oven for three days at 65 °C (150 °F) and then weighed. Roots were washed then dried for three days in a drying oven at 65 °C (150 °F) then weighed. Bulk roots were dried and then dry weight was determined. Biomass and partitioning traits such as, shoot dry weight, and root dry weight were measured at harvest.

Proline determination

Leaf samples (from the still growing fourth leaf from the shoot tip) were collected from plants in each treatment and stored in liquid nitrogen. Proline was extracted following the acidic ninhydrin reagent as described by Bates et al (1973). Samples of 0.5 g leaf FW were ground in a mortar after the addition of a small amount of liquid nitrogen. Acid ninhydrin was prepared by warming 1.25 g ninhydrin in 30 mL glacial acetic acid added to 20 mL 6 M phosphoric acid, with agitation to dissolve, then kept at -4 °C. The 0.5 g plant material was homogenized in 10 mL of 3% aqueous sulfosalicylic acid. The homogenate was filtered through number two Whatman filter paper. Two milliliter of filtrate was added to 2 mL acidic ninhydrin and 2 mL glacial acetic acid in a test tube. After 1 hour at 100 °C, the reaction was terminated in an ice bath. Four milliliters of toluene were added and mixed vigorously using a test tube and a magnetic stirrer for 15-20 sec. The toluene containing the color reagent was aspirated from the aqueous phase and warmed to room temperature and absorbance read at 520 nm on a spectrophotometer (Beckman Coulter Inc., Fullerton, Calif., U.S.A.) using toluene for a blank. The proline concentration was determined from a standard curve and presented on a fresh weight basis (Bates et al., 1973; Claussen, 2005).

Experimental design

A completely randomized design (CRD) was used with four plant types (two seed – Alamo and Forestburg, and two *in vitro* - regenerated Alamo (R.Alamo) and regenerated Forestburg (R.Forestburg) of switchgrass, in two water stress treatments T1-60% WW and T2-20% WW in addition to control (100%WW) with three replicates of each plant type in a pot and 5 pots (replicates) of each plant type within each treatment (3 treatments x 4 cultivars x 3 replicates x 4 plants= 144 pots). Two way ANOVA analyses in SAS was performed to analyze plant type, treatment and plant type x treatment interactions. Principal Component Analysis was carried out to compare photosynthesis and seven additional parameters (Cond, Ci, Fv´, Fm, Fs, Fv´/Fm´, and PhiPS2) with plant types and treatments to identify the most water stress tolerant plant type.

Results and Discussion

Differences between plant types were most evident in tiller number (Fig. 22). Tiller number (p =0.05), was greatest in R.Alamo (27.43 cm), intermediate in Alamo (15.63 cm), and lowest for Forestburg and R.Forestburg (8.18 and 7.4 cm), respectively. Tiller number reflected the total biomass. R.Alamo had greater dry matter compared to plants in other treatments. Water stress decreased tiller number in R.Alamo in both T1-60% WW and T2-20% WW treatments compared with the control. Among the treatments, control plants had the highest tiller number, and Tiller number decreased with increased stress. The effect of treatments on tiller number is presented in Figures 23 and 24. Leaf area was affected by water stress in all plant types and an interaction existed between plant type and stress treatment (Fig. 25). Smaller leaf area is one possible response when plants are affected by water stress. Both R.Alamo and seed Alamo had the same trend of decreasing leaf area when stress was applied and both had the lowest leaf area in T2-20% WW treatment. However R.Forestburg and seed Forestburg acted similarly at the stress levels and the percentage of decreasing leaf area was less than in seed Alamo and R.Alamo.

This finding supports the ability of the upland cultivar to tolerate stress more than the lowland cultivar in terms of morpho-physiological parameters measured in this experiment. However, lowland Alamo and R.Alamo at T1-60% WW and T2-20% WW treatments still had similar or higher leaf area compared to control Forestburg.

Plants of R.Alamo had the highest shoot dry weight with the control treatment, and dry weight decreased with increasing water stress. Plant types behaved differently to water stress. No difference between control Alamo and T1-60% Alamo was observed. Plants of R.Alamo at T1-60% WW had a similar dry weight to Alamo under control treatment. This showed that somaclonal variation induced through *in vitro* stress treatments improved R.Alamo tolerance to water stress. Both Alamo and R.Alamo produced higher shoot dry weight under T1-60% WW and T2-20% WW treatments than R.Forestburg and Forestburg, which supports Alamo switchgrass as a better biomass producer (Stroup et. al., 2003). An interaction between plant types and treatments occurred with respect to superiority of R.Alamo and Alamo in shoot biomass production. A similar trend was observed in root dry weight reflecting the vigorous shoot growth of R.Alamo under control treatment and water stress treatments (Fig. 26). Root dry weight per pot of R.Alamo was the highest among plant types and treatments (Fig. 27).

Height of all switchgrass plant types was affected by water stress as water is essential for cell division and cell expansion. Plant height decreased with increased water stress in all plant types (Fig. 28 and 29). In control treatment Alamo and R.Alamo were taller than Forestburg and R.Forestburg.

Stem elongation, calculated by measuring the plant height twice a week, increased linearly of the stem elongation rate before water stress was applied. The stem elongation rate decreased after imposing both T1-60% WW and T2-20% WW treatments until a stable phase was reached in all treatments (Fig. 30, 31 and 32). Similar to other growth and development

parameters, control R.Alamo plants had the highest vegetative growth compared to T1-60% WW and T2-20% WW plants (Fig. 33).

To better understand the effects of somaclonal variation and water stress on switchgrass plant types, proline concentration was determined using procedures of Bates et al. (1973). Results showed increased proline with increased stress (Fig. 34). Proline concentration was higher in T1-60% WW and T2-20% WW R.Alamo compared to control treatment. Proline concentration in T2-20% WW treatment of R.Alamo was similar to T2-20% WW Forestburg and was double that of Alamo in all treatments indicating greater adaptation to water stress. R.Forestburg had a higher proline concentration with T1-60% WW treatment than T1-60% WW of Forestburg but less in T2-20% WW treatment. Overall R.Alamo responded better to water stress than Alamo and R.Forestburg supporting findings on other growth and developmental traits recorded in this study.

Photosynthetic rate was highest in R.Alamo with a mean of 27 µmol of CO_2 $m^{-2}s^{-1}$, while Forestburg was the lowest in photosynthesis with mean of 16 µmol of CO_2 $m^{-2}s^{-1}$ under T2-20% WW treatment (Fig. 35). Under T1-60% WW all plant types decreased in photosynthesis except for R.Forestburg. Photosynthesis decreased to less than 20 µmol of CO_2 $m^{-2}s^{-1}$ for all plant types with T2-20% WW treatment. R.Alamo had higher photosynthesis (27 µmol of CO_2 $m^{-2}s^{-1}$) than Alamo (23 µmol of CO_2 $m^{-2}s^{-1}$) under control conditions, but both R.Alamo and Alamo decreased in photosynthesis under T1-60% WW and T2-20% WW water stress treatments. Principal Component Analysis carried out to compare photosynthesis and seven additional parameters (Cond, Ci, Fv´, Fm, Fs, Fv´/Fm´, and PhiPS2) separated R.Alamo from Alamo, R.Forestburg, and Forestburg. This result provides evidence that R.Alamo identified through *in vitro* studies had better tolerance to water stress due to its enhanced morphological and physiological traits compared to all other plant types (Fig. 36).

Conclusions

Results demonstrated the effect of water stress on switchgrass plant types. Among plant types, R.Alamo exhibited more biomass and higher photosynthetic rates than Forestburg plant types and seed Alamo. Somaclonal variation in R.Alamo might have contributed to high performance as demonstrated by many morphological and physiological parameters measured. Finally, the study demonstrates that *in vitro* methods can be used to rapidly screen and identify switchgrass plants with improved water stress tolerance.

Table1. Switchgrass cultivars, area of origin, ecotype, and corresponding number works with all figures in this dissertation.

Number	Cultivar	Area of origin	Ecotype
1	Carthage	NC	Lowland
2	Alamo	TX	Lowland
3	Kanlow	OK	Lowland
4	Southlow	NC	Upland
5	Cave-In-Rock	IL	Upland
6	Forestburg	SD	Upland
7	Blackwell	OK	Upland
8	Nebraska 28	NE	Upland
9	Shelter	WV	Upland
10	Shawnee	WV	Upland
11	Dacotah	ND	Upland
12	Sunburst	SD	Upland
13	WI Ecotype	-	-

(Source: Jimmy Carter Plant Materials Center, Americus, GA, 2011)
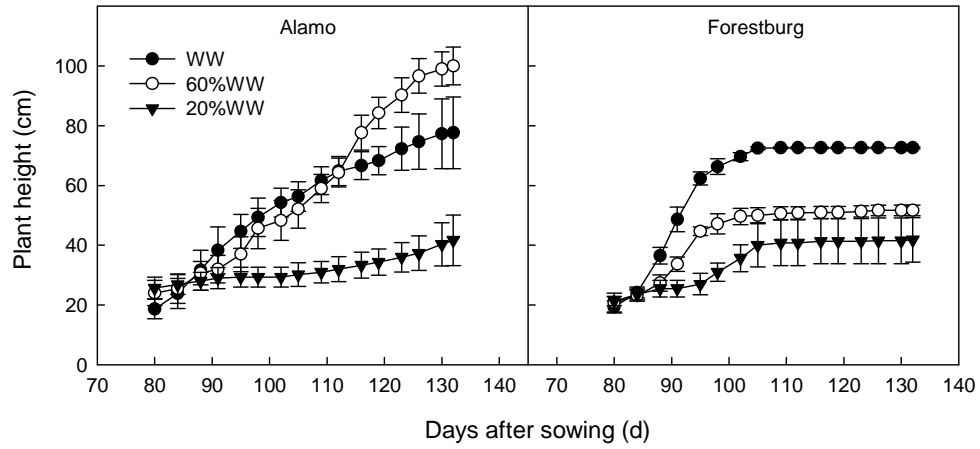
Fig.1. Effect of water stress on plant height (cm) in Alamo and Forestburg cultivars. Vertical bars denote standard errors of mean.
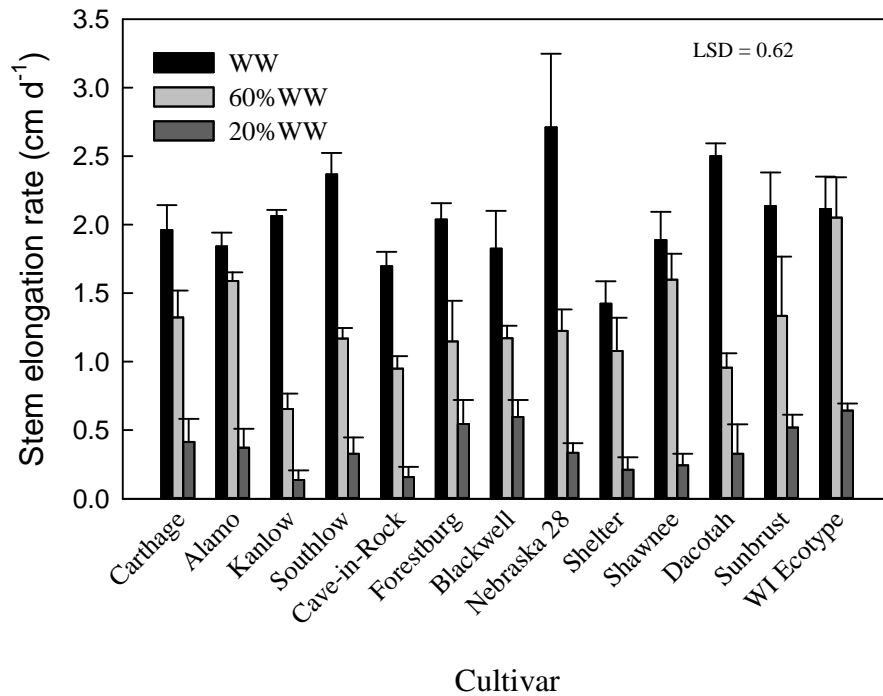
Fig.2. Effect of water stress on stem elongation rate. Vertical bars denote the standard errors of mean.
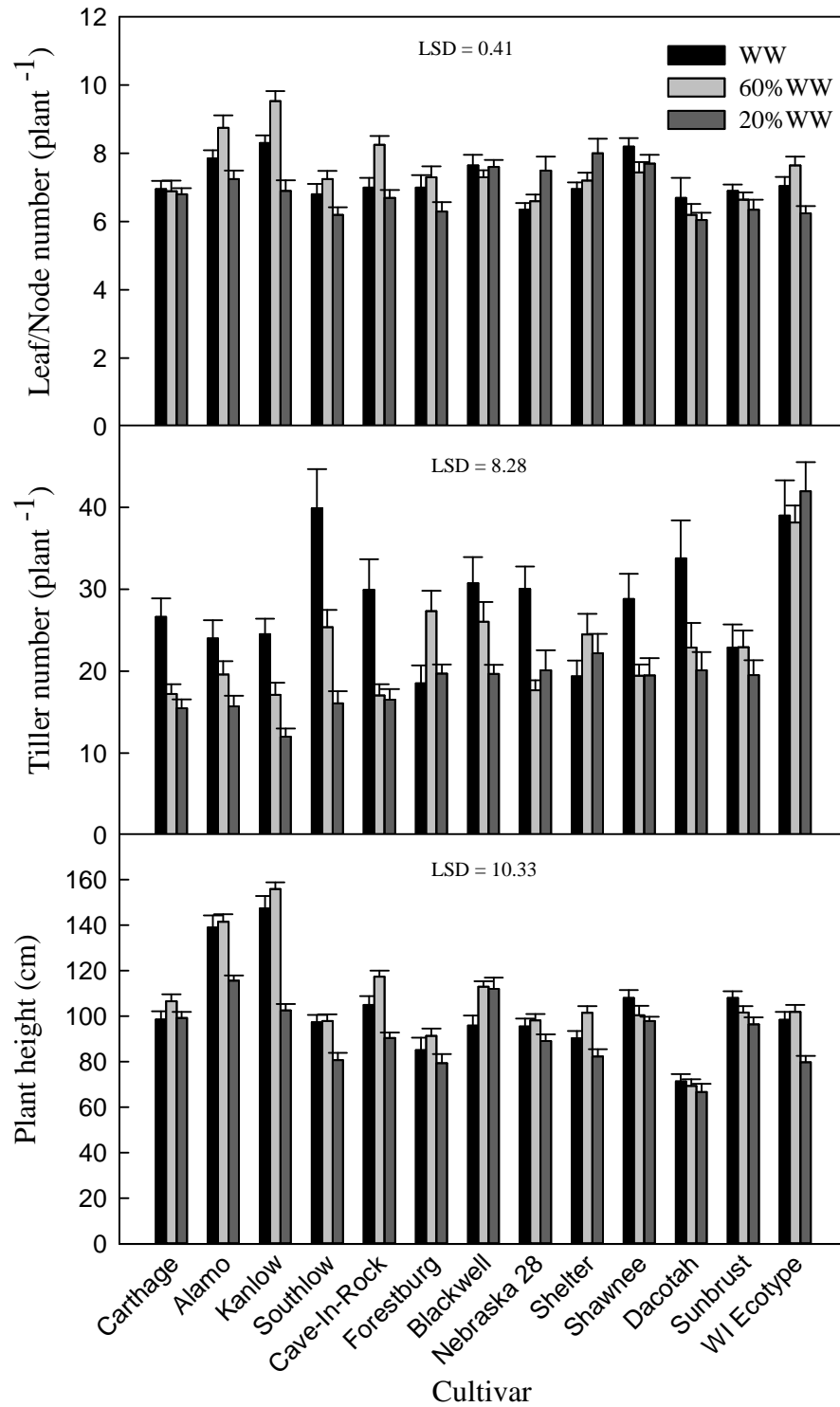
Fig.3. Effect of water stress on leaf/node, tiller number, and plant height in switchgrass cultivars. Vertical bars denote the standard errors of mean.
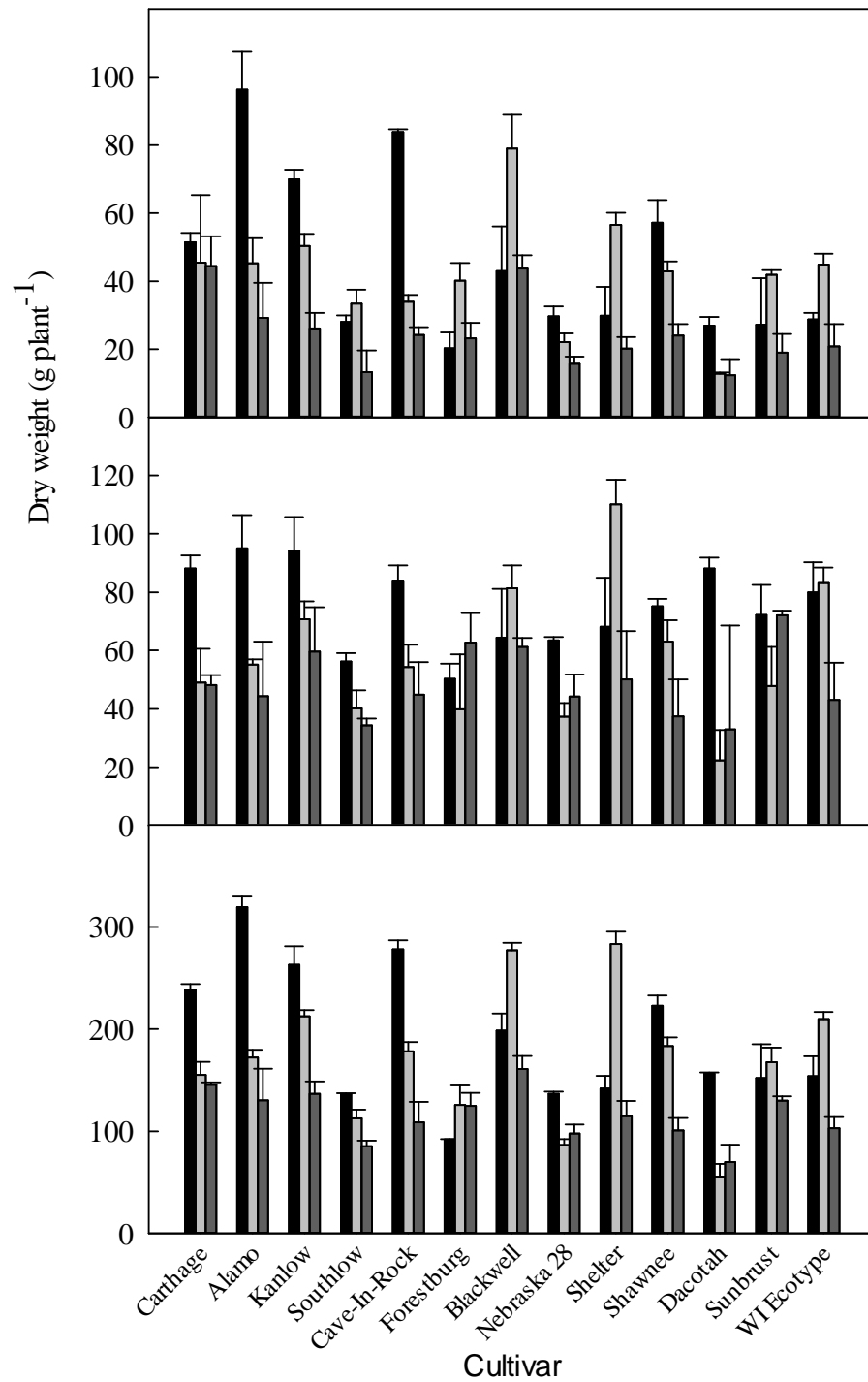
Fig.4. Effect of water stress on leaf, stem, root and total dry weight in switchgrass cultivars. Vertical bars denote the standard errors of mean.
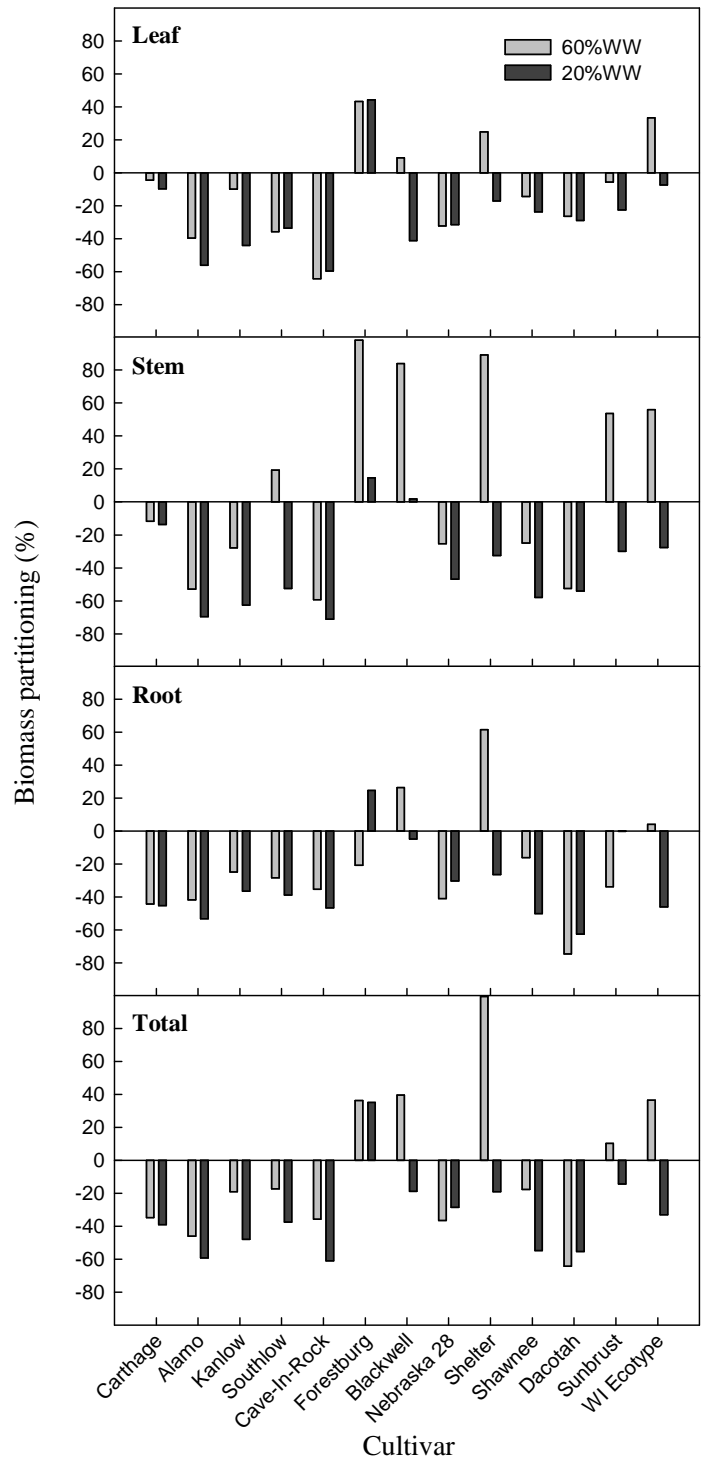
Fig.5. Effect of water stress on biomass partitioning in switchgrass cultivars.
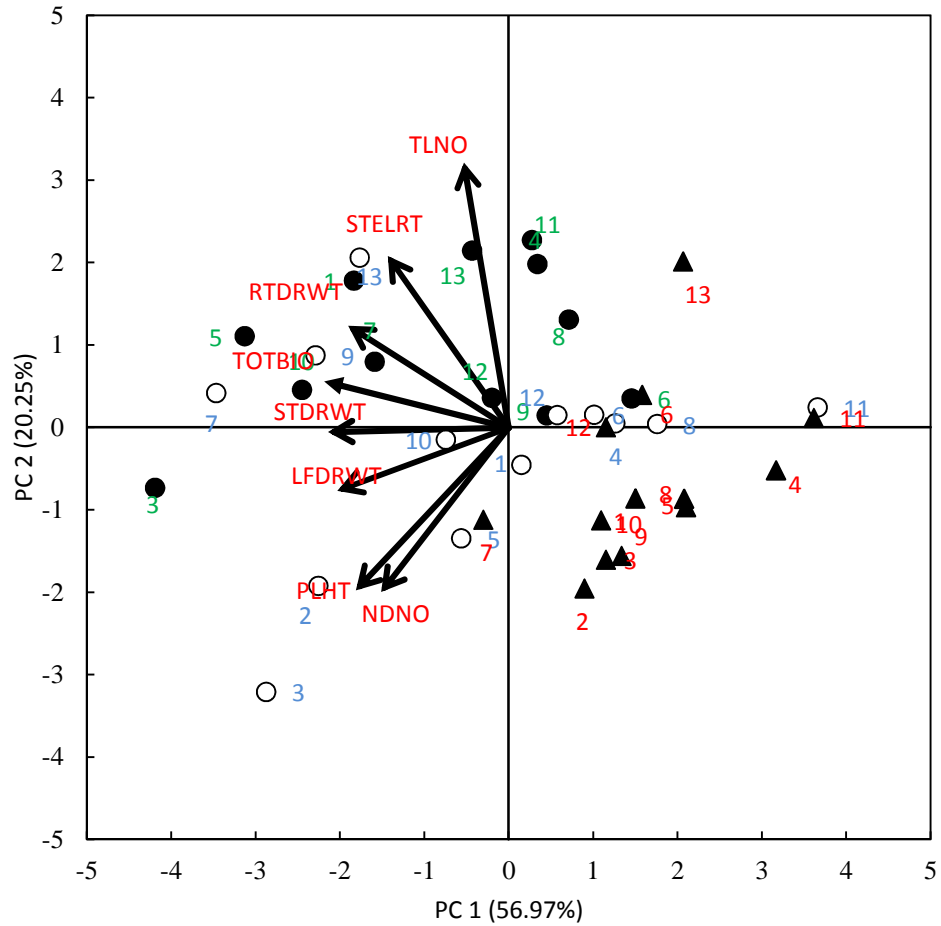
Fig.6. Effect of water stress on eight different morphological traits in switchgrass cultivars using PCA. The parameters presented on per plant basis in the graph are: TLNO – tiller number, STELRT – stem elongation rate, RTDRWT – root dry weight per plant, TOTBIO – total biomass dry weight including root weight, STDRWT – stem dry weight, LFDRWT – leaf dry weight,   PLHT – plant height, NDNO – node number. The numbers in the graph indicate cultivars: 1. Carthage, 2. Alamo, 3 Kanlow, 4. Southlow, 5. Cave-In-Rock, 6. Forestburg, 7.Blackwell, 8.Nebraska 28, 9. Shelter, 10. Shawnee, 11. Dacotah, 12. Sunbrust, 13. WI Ecotype.
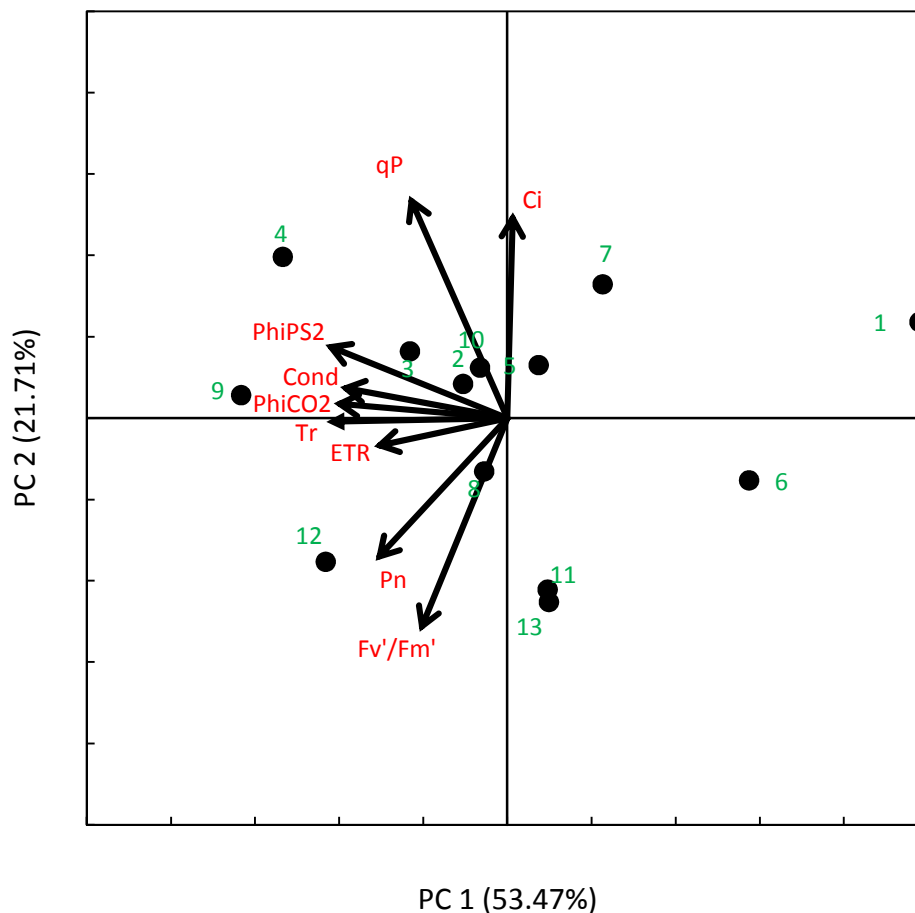
Fig.7. Effect of water stress on eight different morphological traits in switchgrass cultivars using PCA for difference between control (WW) and 20%WW treatments. The parameters presented on per plant basis in the graph are: TLNO – tiller number, STELRT – stem elongation rate, RTDRWT – root dry weight per plant, TOTBIO – total biomass dry weight including root weight, STDRWT – stem dry weight, LFDRWT – leaf dry weight, PLHT – plant height, NDNO – node number. The numbers in the graph indicate the cultivars: 1. Carthage, 2. Alamo, 3 Kanlow, 4. Southlow, 5. Cave-In-Rock, 6. Forestburg, 7.Blackwell, 8.Nebraska 28, 9. Shelter, 10. Shawnee, 11. Dacotah, 12. Sunbrust, 13. WI Ecotype.
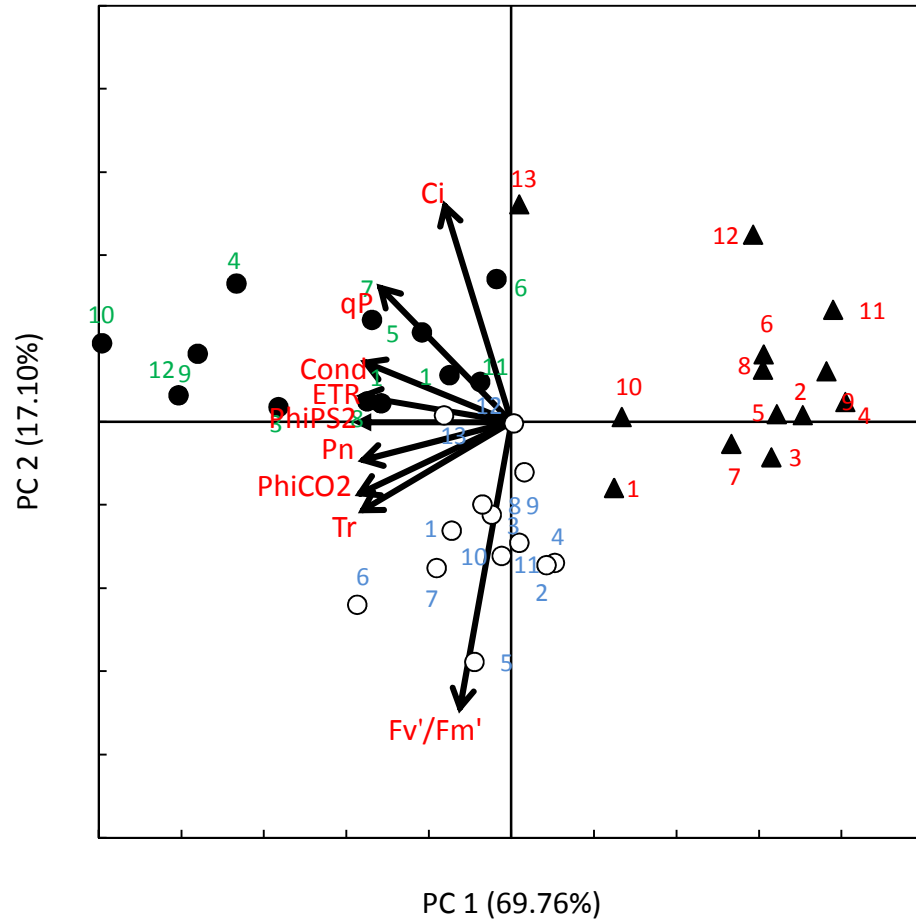
Fig.8. Effect of water stress on nine different photosynthesis traits in switchgrass cultivars using PCA. The parameters presented in the graph are: : Ci – intercellular CO_2 concentration, qP – photochemical quenching, PhiPS2– PS II efficiency, Cond – Stomatal conductance, PhiCO2 – quantum yield of CO_2 Fixation , Tr – transpiration rate, ETR– photosynthetic electron transport rate, Pn – net photosynthesis rate, and - Fv′/Fm′- efficiency of energy harvesting by oxidized (open) PSII reaction centers in the light. The numbers in the graph indicate the cultivars: 1. Carthage, 2. Alamo, 3 Kanlow, 4. Southlow, 5. Cave-In-Rock, 6. Forestburg, 7.Blackwell, 8.Nebraska 28, 9. Shelter, 10. Shawnee, 11. Dacotah, 12. Sunbrust, 13. WI Ecotype.

Fig.9. Effect of water stress on nine different photosynthesis traits in switchgrass cultivars using PCA for difference between control (WW) and 20%WW treatments. The parameters presented in the graph are: Ci – intercellular CO_2 concentration, qP – photochemical quenching, PhiPS2– PS II efficiency, Cond – Stomatal conductance, PhiCO2 – quantum yield of CO_2 Fixation , Tr – transpiration rate, ETR– photosynthetic electron transport rate, Pn – net photosynthesis rate, and and - Fv′/Fm′- efficiency of energy harvesting by oxidized (open) PSII reaction centers in the light. The numbers in the graph indicate the cultivars: 1. Carthage, 2. Alamo, 3 Kanlow, 4. Southlow, 5. Cave-In-Rock, 6. Forestburg, 7.Blackwell, 8.Nebraska 28, 9. Shelter, 10. Shawnee, 11. Dacotah, 12. Sunbrust, 13. WI Ecotype.
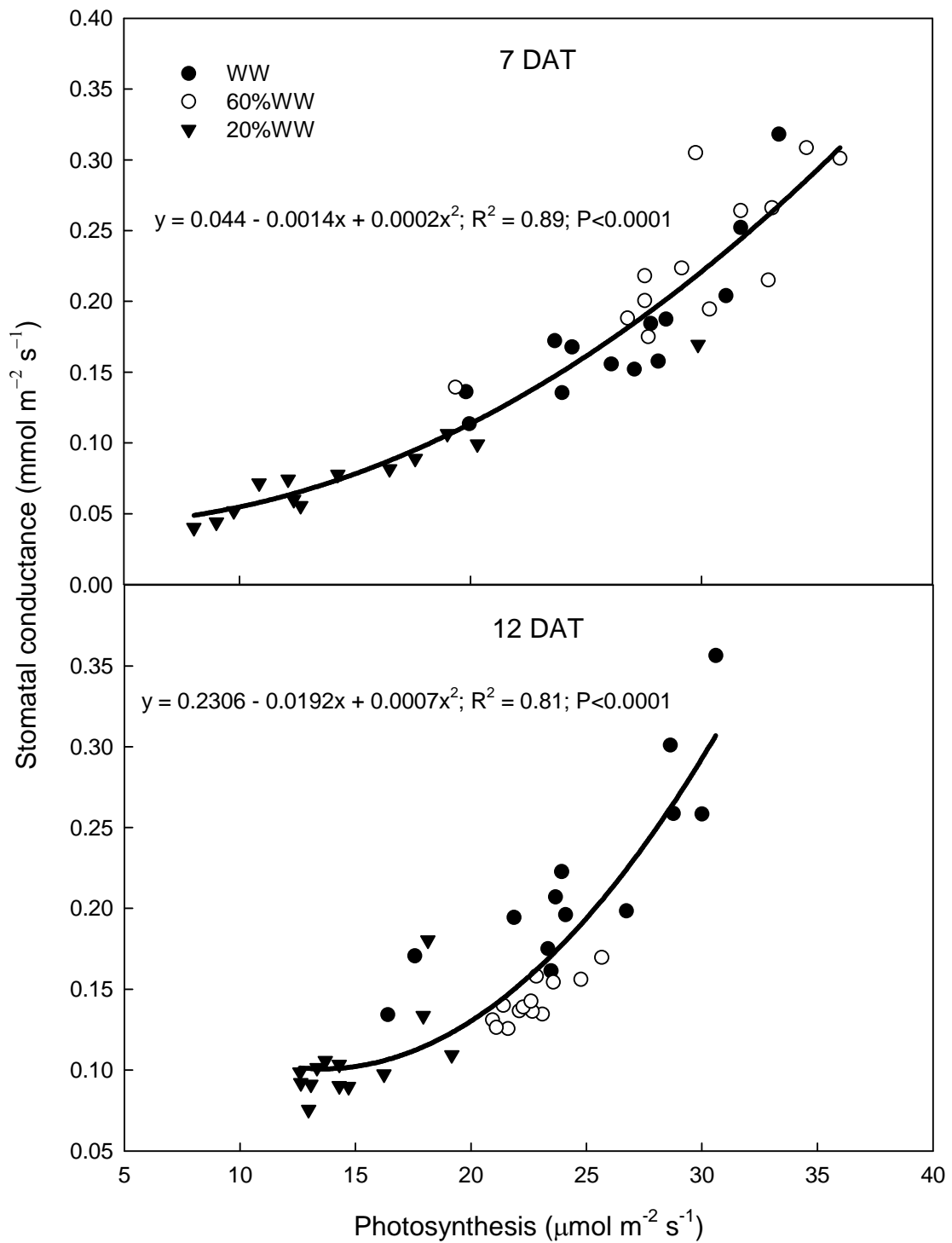
Fig.10. Effect of water stress on stomatal conductance and photosynthesis after 7 day and 12 days of stress.
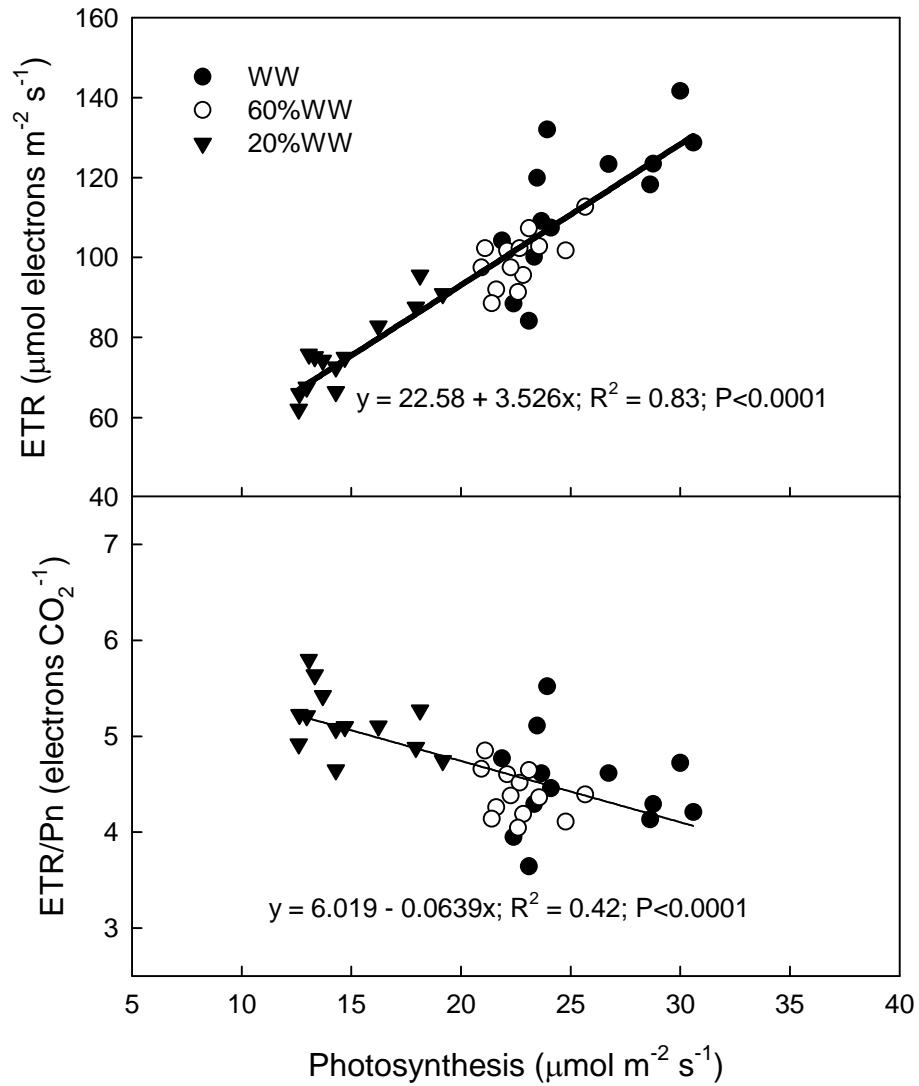
Fig.11. Effect of water stress on electron transport rate (ETR) and electrons per CO_2 fixed.
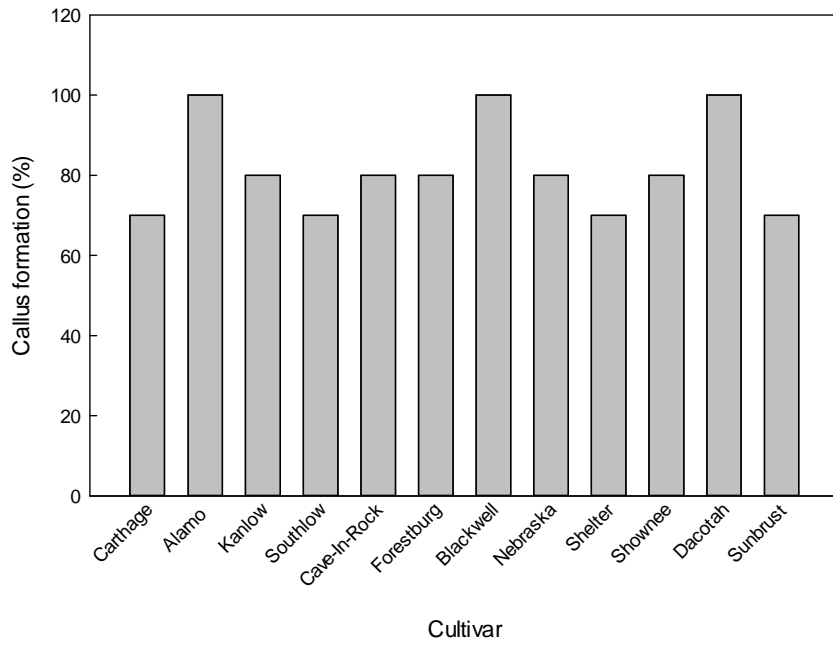
Fig.12. Percentage of callus formation in different switchgrass cultivars based on visual observation and no statistical analysis was applied
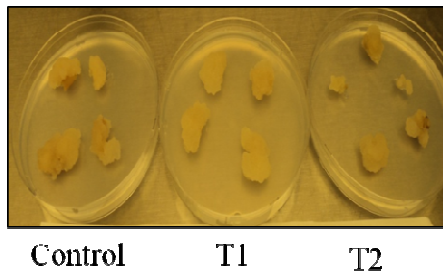

Control   T1   T2

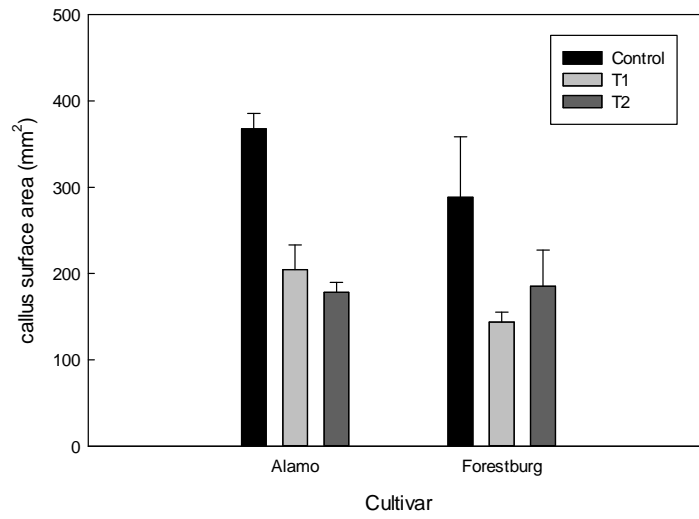Fig.13. Subculture of callus on water stress media imposed using PEG and supplemented by 3%

maltose

Fig.14. Callus formation of two switchgrass cultivars grown on MS media

Fig.15. Morphogenesis of switchgrass callus after 4 subcultures.

Control   T1   T2
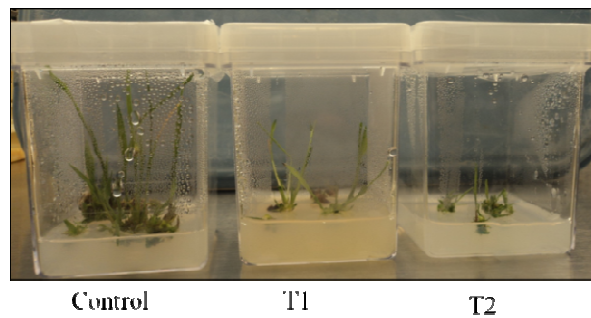
Fig.16. Organogenesis of R.Alamo switchgrass regenerated on water stress treatments
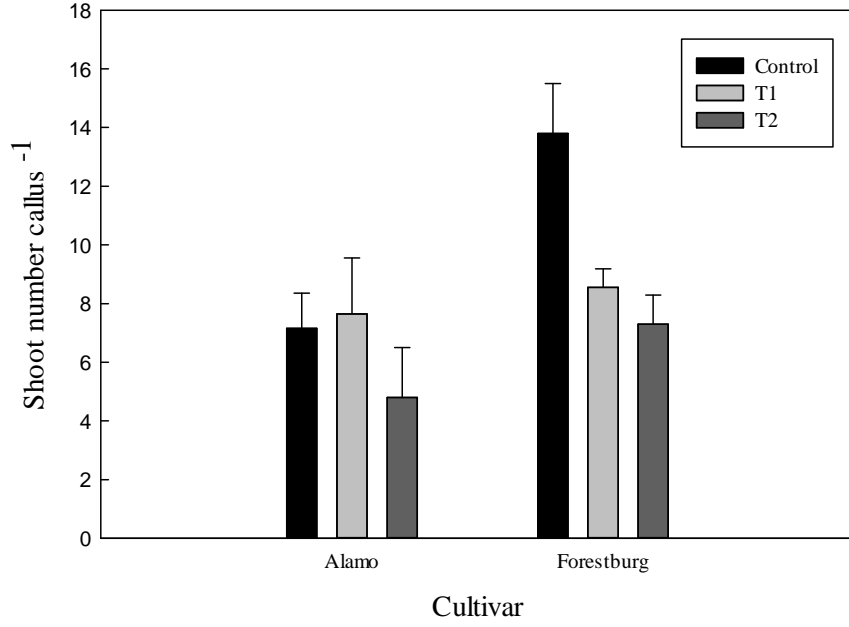
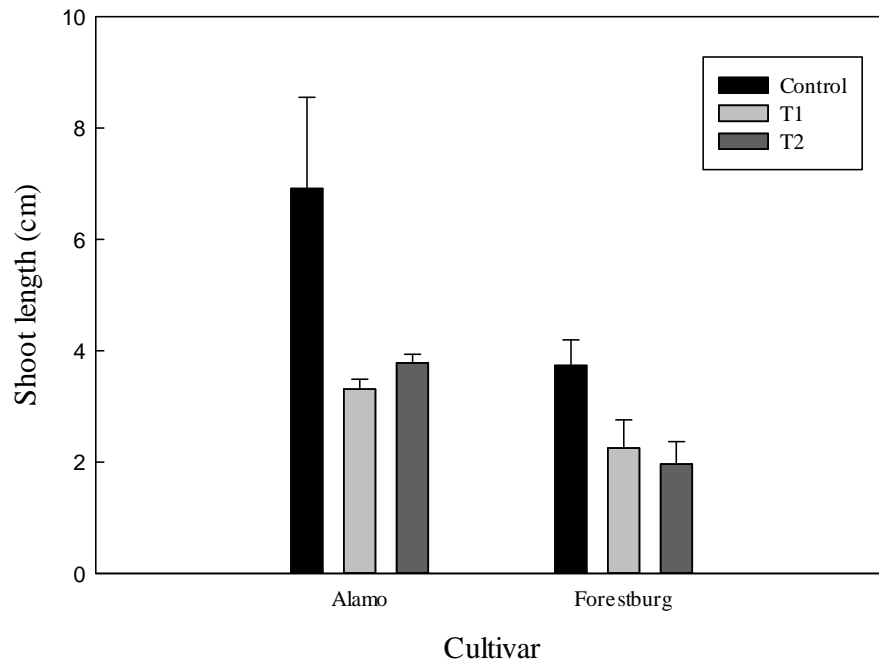Fig.17. Shoot number per callus of two switchgrass cultivars grown on MS media

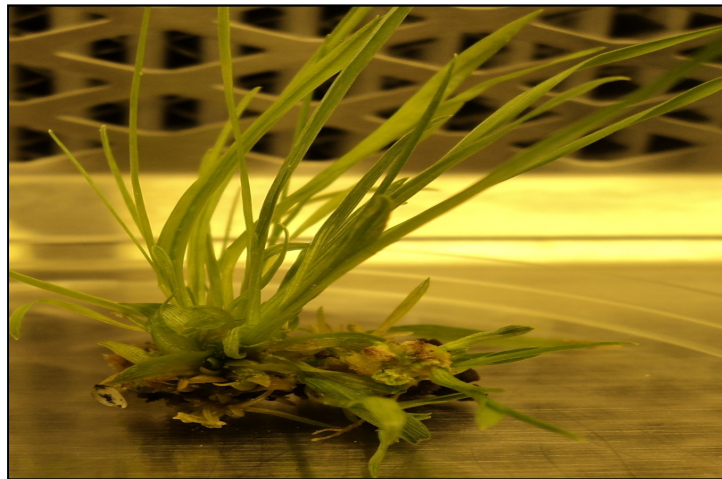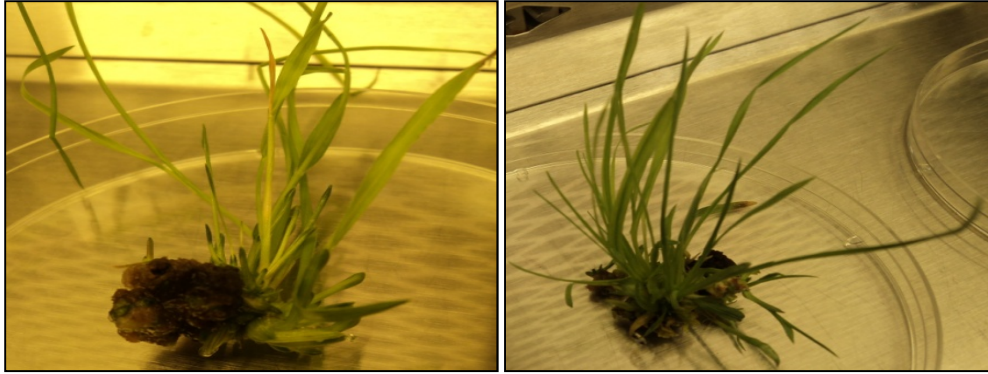Fig.18. Shoot length (cm) of two switchgrass cultivars grown on MS Media

Fig.19. Organogenesis, shoot formation

Fig.20. Organogenesis, Root initiation

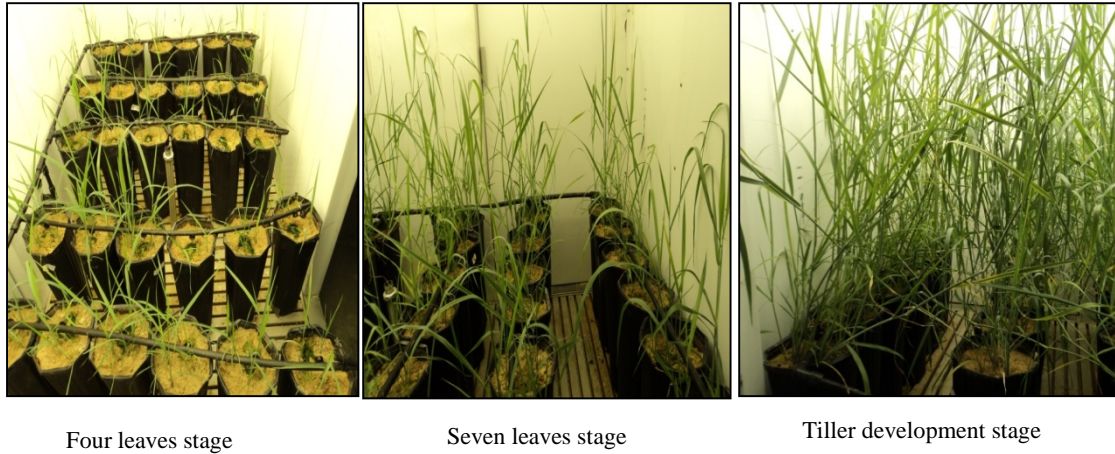| Four leaves stage | Seven leaves stage | Tiller development stage |

Fig.21. Evaluation of regenerated switchgrass cultivars (R.Alamo) and regenerated Forestburg (R.Forestburg) compared to Alamo and Forestburg grown from seed in different growth stages.
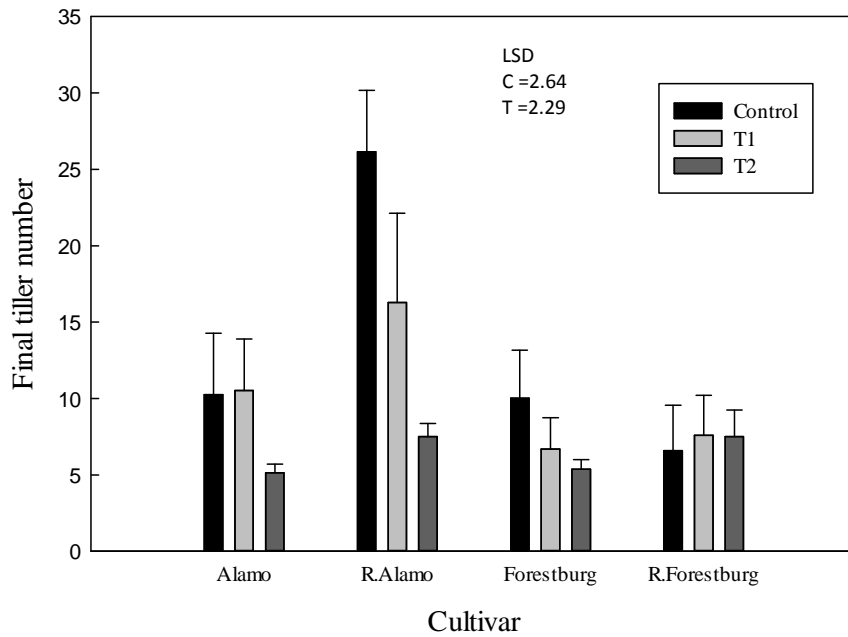

Fig.22. Final tiller number of two regenerated switchgrass cultivars compared to two seed

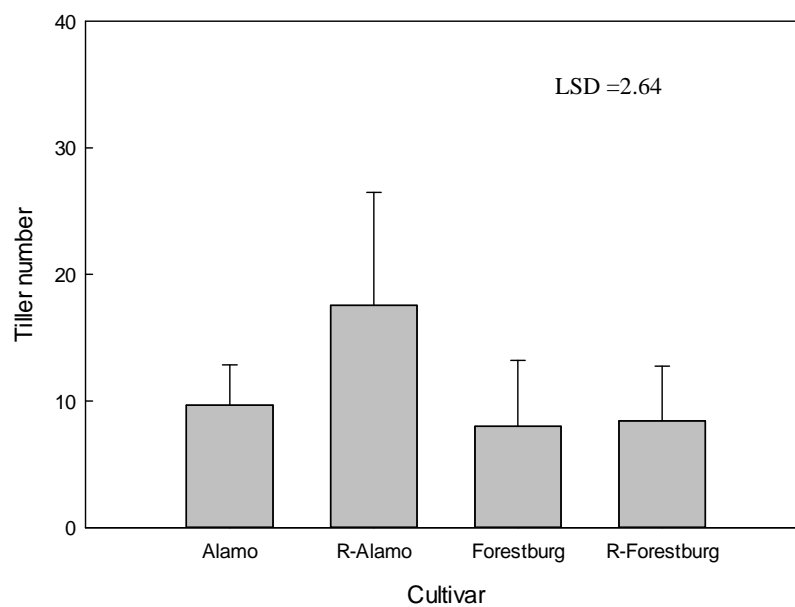cultivars (T1 = T1-60% WWT1-T1-60% WW and T2 = T2-20% WW).

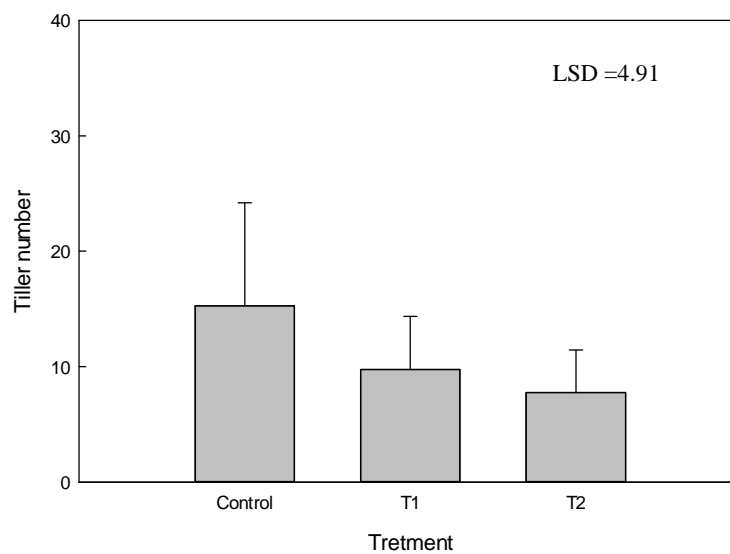Fig.23. Differences between switchgrass cultivars in tiller number

Fig. 24. Effect of water stress treatments on switchgrass cultivars tiller number
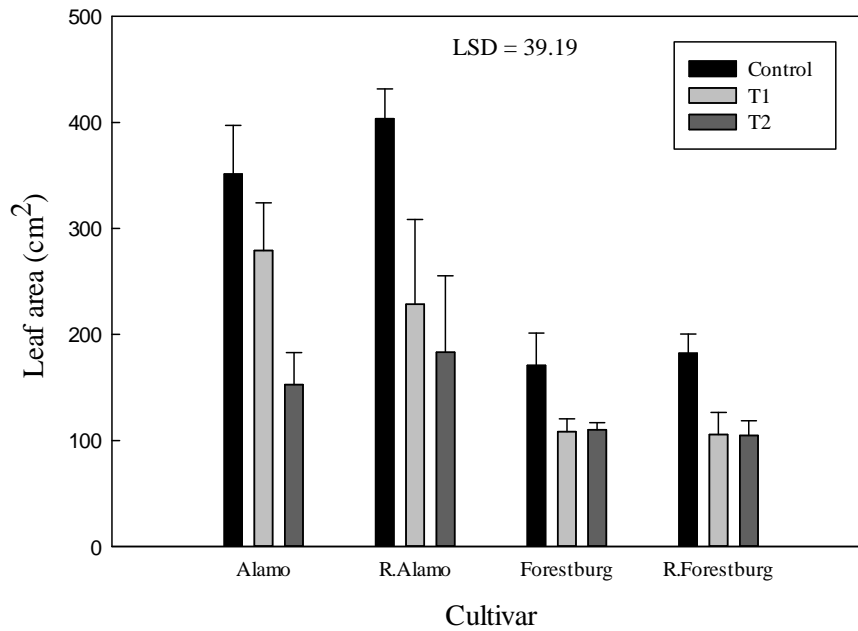
(T1 = 60% WW and T2 = 20% WW).

Fig.25. Leaf area (cm^2) of two regenerated switchgrass cultivars compared to two seed cultivars

(T1 = T1-60% WWT1-T1-60% WW and T2 = T2-20% WW).

Fig.26. Shoot dry weight g plant $^{-1}$ of two regenerated switchgrass cultivars compared to two seed

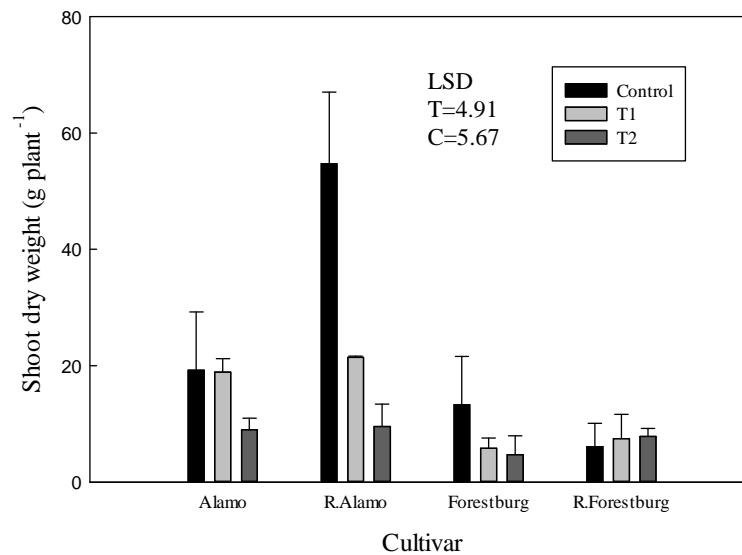cultivars (T1 = T1-60% WWT1-T1-60% WW and T2 = T2-20% WW).
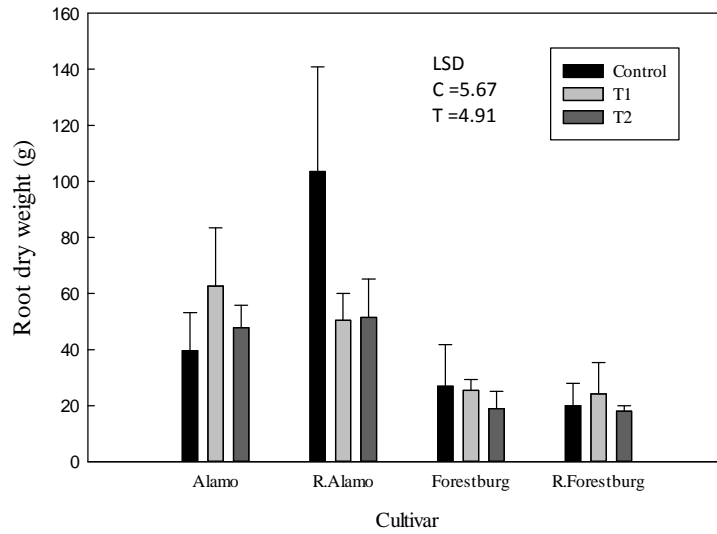
Fig.27. Root dry weight g pot $^{-1}$ of two regenerated switchgrass cultivars compared to two seed

cultivars (T1 = T1-60% WWT1-T1-60% WW and T2 = T2-20% WW).
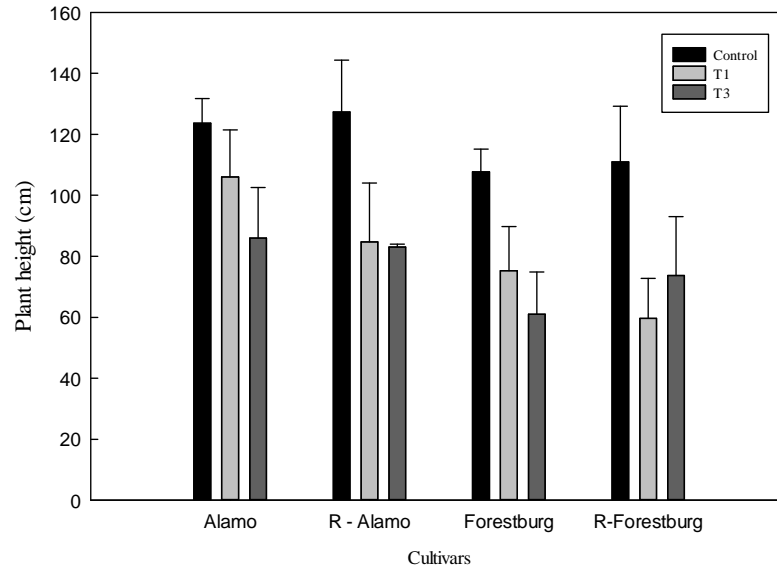
Fig.28. Plant height (cm) of two regenerated switchgrass cultivars compared to two the same

cultivars from seed (T1 = T1-60% WWT1-T1-60% WW and T2 = T2-20% WW).
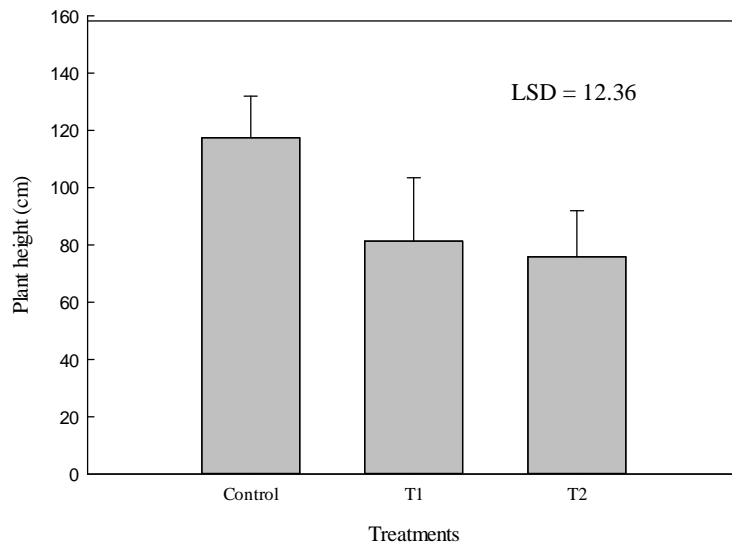
Fig.29. Effect of water stress on plant height of two regenerated switchgrass cultivars compared

to two seed cultivars (T1 = 60% WW and T2 = 20% WW).
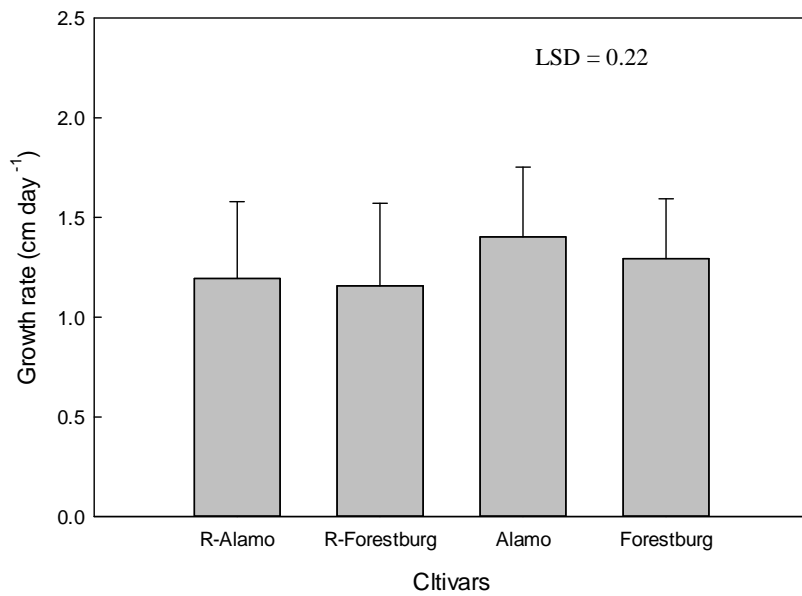
Fig.30. Differences between switchgrass cultivars in growth rate
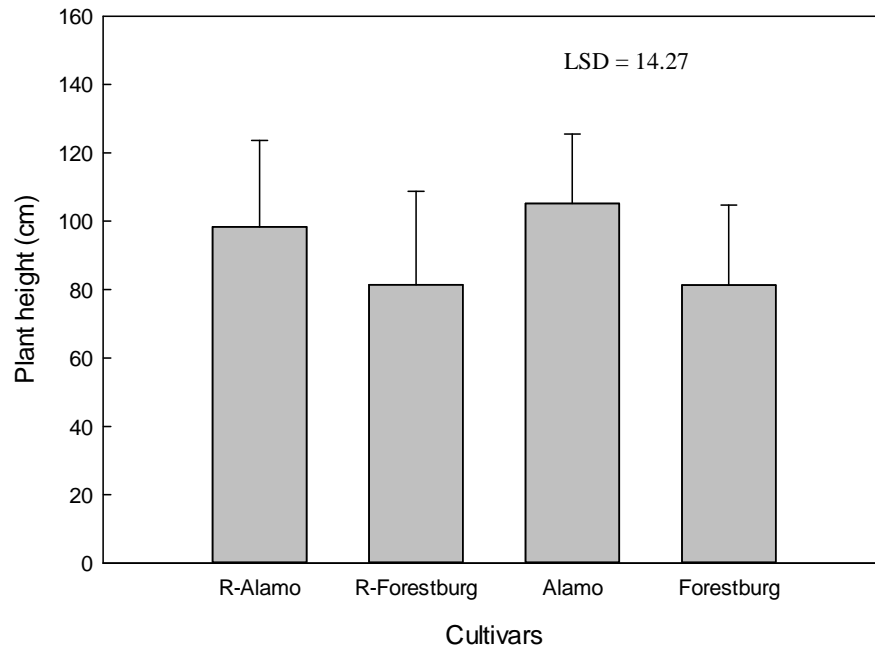
Fig.31. Differences between switchgrass cultivars in plant height.
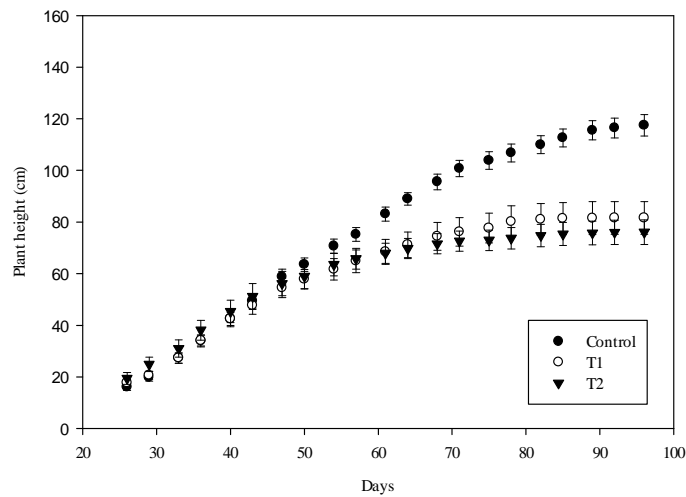
Fig.32. Effect of water stress on plant height on switchgrass cultivars across treatments, stress was applied on day 40 (T1 = T1-60% WWT1-T1-60% WW and T2 = T2-20% WW).

Control T1 T2

Fig.33. R.Alamo cultivar in the control and water stress treatments
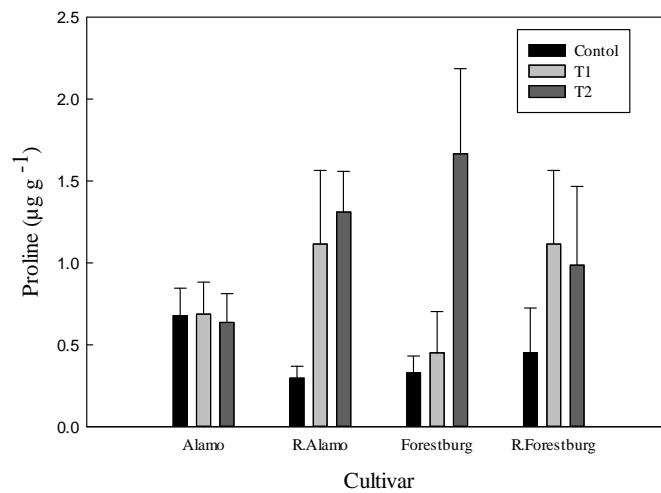
(T1 = -60% WW and T2 = 20% WW).

Fig.34. Effect of water stress on proline concentration of two regenerated switchgrass cultivars

compared to two seed cultivars (T1 = 60% WW and T2 = 20% WW).
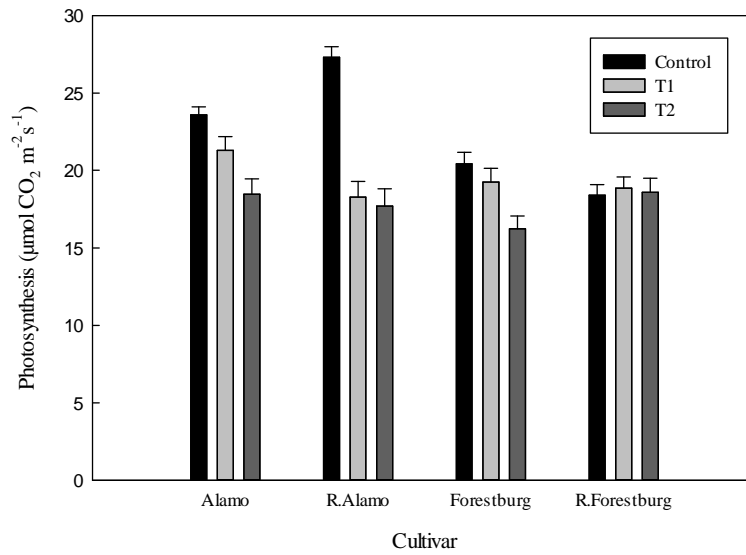
Fig. 35. Affect of water stress on photosynthesis in switchgrass cultivars
(T1 = 60% WW and T2 = 20% WW).
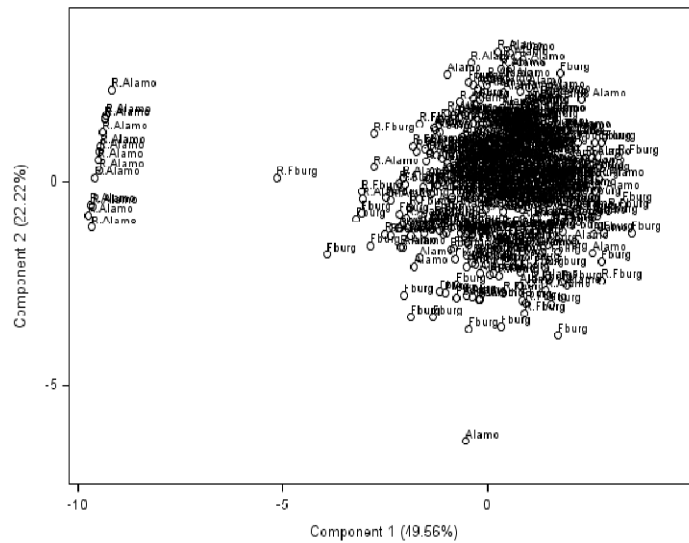
Fig.36. Affect of water stress on eight different photosynthesis traits in switchgrass
cultivars using PCA for control, T1=60% WW, and T2=20% WW treatments.

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Scope and Method of Study: Switchgrass can benefit from enhanced water stress tolerance as it will be grown in marginal areas. Whole plant and *in vitro* culture techniques can help to improve switchgrass biomass production and water stress tolerance. Identifying specific traits in switchgrass that contribute to water stress tolerance can provide useful information to breeders to increase productivity. The objectives of this study are (1) to evaluate growth and physiological parameters and identify switchgrass traits that can contribute to water stress tolerance and increased water use efficiency, (2) to study the effect of water stress at the cellular level using *in vitro* culture and identify cell lines that can survive water stress, and (3) to assess morpho-physiological traits of plants derived from water stress tolerant cell lines. Under Objective 1, 13 genotypes were screened using control (well watered - WW), 60%WW and 20%WW treatments, and growth and physiological traits, total biomass, and biomass components were measured. Under Objective 2, 12 genotypes were evaluated using an *in vitro* culture procedure to investigate callus formation. Two cultivars, Alamo and Forestburg, selected from Objective 1 and from the initial *in vitro* water stress screening were used to induce variation under water stress using MS media + Polyethylene glycol. For Objective 3, regenerated Alamo and Forestburg were compared to seed plants of the same cultivars.

Findings and Conclusions: The tested switchgrass cultivars differed in tolerance to water stress. In the greenhouse study, the effect of water stress on total biomass was genotype dependent. Alamo (lowland) and Forestburg (upland) were identified as most tolerant to water stress. Morphological traits such as stem elongation rate and total dry weight, and physiological traits such as stomatal conductance, transpiration and photosystem efficiency were identified to help rapidly screen switchgrass populations.The *In vitro* study identified 45 μM 2, 4-D + 45 μM BA as the best combination of growth regulators for inducing callus formation in switchgrass. Results from *in vitro* studies revealed that both Alamo and Forestburg callus survived the stress induced and produced callus. Regenerated Alamo had better morpho-physiological traits and proline concentration compared to plants of regenerated Forestburg and seed plants of Alamo and Forestburg. In conclusion, whole plant screening can identify cultivars and traits for increased water stress tolerance and *in vitro* culture can be used to rapidly screen and improve switchgrass for abiotic stress tolerance.

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