# Evaluation of Progress in Annual Ryegrass (*Lolium multiflorum* Lam.) Selected for Increased Winter Productivity

by

Hari Prasad Poudel

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Approved by

Edzard van Santen, Chair, Professor of Crop, Soil and Environmental Sciences
David B. Weaver, Professor of Crop, Soil and Environmental Sciences
David I. Bransby, Professor of Crop, Soil and Environmental Sciences
Karen R. Harris-Shultz, USDA Research Geneticist

## Abstract

Forage availability in the southeastern USA is limited during winter and supplemental feeding increases management costs. Therefore, development of a suitable cultivar with increased winter productivity would be a valuable contribution to animal agriculture. A phenotypic recurrent-selection breeding program was initiated in 2005 to improve the winter productivity of annual ryegrass (*Lolium multiflorum* Lam.). This study was conducted to evaluate the progress from the selection to determine the worth of a newly developed population before releasing it as a cultivar. In a grazing trial, Cycle 2 appears to have higher ADG than Gulf and Marshall during winter months, which may be associated with higher biomass yield during those months. In the correlated study, we found selection for increased winter productivity resulted in more erect plants with early heading date and homogenous ploidy. In the soluble carbohydrates study, the plants outside the greenhouse had higher level of TNC than plants inside the greenhouse but there was no difference in TNC among cultivars within locations. Further grazing evaluations considering early seeding and grazing will be helpful to determine the worth of cycle 2 under grazing during the low forage availability months (December – February).

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## Table of Contents

## List of Tables

## List of Figures

# Literature Review and Introduction

## Species description

*Lolium multiflorum* Lam., also known as annual ryegrass, is an important cool season pasture, forage or turfgrass belonging to the family Poaceae. The origin of Poaceae has been dated back some 70-75 million years (Kellogg, 2001) and members of this family dominate earth's flora, covering approximately 20% of the total land surface (Shantz, 1954). This family consists of about 785 genera with about 10,000 species and directly or indirectly contributes the major portion of the human and domestic animal diet. The plants are herbaceous (Nelson and Moser, 1995) and are easily digestible by ruminants.

As the common name for this species suggests, *L. multiflorum* Lam. completes the cycle from seed to seed within a year; in agronomic situations with periodic complete defoliation it may actually behave like a short-lived perennial (Jung *et al.*, 1996). This species is thought to have originated on the Apennine Peninsula in Italy thus also called Italian ryegrass and then spread to other parts of the world. It is now grown on vast areas in Australia, New Zealand, Mexico, Canada and the United States east of the Mississippi. About 90% area of this grass under cultivation is used as winter pasture in the USA. Annual ryegrass is generally established by over-seeding a warm season perennial grass pasture in autumn to increase forage quality and extend the grazing season (Balasko *et al.*, 1995). Sod seeding of ryegrass into warm season grasses such as bermudagrass (*Cynodon dactylon*

(L.) Pers.) results in efficient utilization of pastureland. Growing annual ryegrass in a mixture with other grasses such as wheat (*Triticum aestivum* L.) and/or rye (*Secale cereale* L.) provides winter grazing for fall-weaned calves in Arkansas that reduces the consumption of hay and feed supplements (Beck *et al.*, 2007). The same study also demonstrated a 17% increase, on average, in forage biomass when annual ryegrass is grown in mixtures with small grains rather than in monoculture.

## Culture and management

Annual ryegrass grows in diverse soil conditions but performs well on fertile and well-drained soils. It is tolerant to a wide range of acidic to alkaline soils but grows best on soil with pH 5.5-7.5 (Hannaway *et al.*, 1999). Germination of annual ryegrass is highest at 5-10°C night temperature and 10-30°C day temperature (Young *et al.*, 1975). This species can be easily established as a pasture grass as it has vigorous growth. The root system is highly branched and deep rooted (Balasko *et al.*, 1995). The seed does not need to be vernalized (Watson and McLean, 1992) but vernalization treatment delays floral initiation.

The seeding rate for annual ryegrass varies from 22 to 39 kg $ha^{-1}$. In Alabama the recommended seeding rate is 22.4 kg $ha^{-1}$ (Glass, 2000). Increasing the seeding rate above this increases early season production but reduces yield after February (Evers *et al.*, 1997). The mean single seed mass for annual ryegrass is 2.6 mg for diploid and 4.8 mg for tetraploid cultivars (Venuto *et al.*, 2002). The seed needs to have good contact with the soil and germinates well when the seed is planted at 0.6 -1.3 cm depth (Balasko *et al.*, 1995).

Annual ryegrass, as a normal practice, is generally sown in autumn. In an experiment to study the seasonal distribution of production in October sown annual ryegrass, it was found that 40% of the production occurred in December to February and the remaining 60% in March to May. It was also found that 30% of the total yield occurred in April alone (Redfearn *et al.*, 2002). As the temperature begins to rise and precipitation increases, annual ryegrass rejuvenates and produces higher biomass. This increased yield of cool season grass in early spring is called spring flush (Belesky and Fedders, 1994).

At any given time ryegrass has three actively-growing leaves per tiller (Davies, 1965) and with the initiation of fourth leaf the oldest leaf senesces (Davies, 1971). Thus, grazing ryegrass pasture younger or older than three leaves per tiller results in lost quality and quantity (Fulkerson and Donaghy, 2001). Maximum pasture yield and utilization is achieved when the pasture is grazed or cut about 5 cm above the soil surface (Parsons and Chapman, 2000). Defoliating *Lolium* spp. below 5 cm removes the available carbohydrate storage which reduces the re-growth and tillering process (Fulkerson and Slack, 1995). Defoliating above 10 cm from the soil surface left enough herbage to cause shading and reduction of incident light interception. This reduces the efficiency of photosynthesis to produce new tillers and new leaves (Hunt and Brougham, 1967). Thus, optimum stubble height should be maintained for efficient utilization and persistence of the forage.

## Photosynthetic mechanisms in forage grasses

Photosynthesis is the mechanism by which green plants convert solar energy into chemical energy and store it in the form of sugar. Different plants store the reserve sugar in different parts such as the seed in cereals, tubers in potato (*Solanum tuberosum* L.), bulb

in onion (*Allium cepa* L.), roots in sugar beet (*Beta vulgaris* L.), etc. In forage, the economically important part is the aboveground biomass and the sugars are used to develop the leaf and stem in the form of digestible cell walls and soluble cell content.

The plants in the family Poaceae have one of two photosynthetic mechanisms, called the $C_3$ and $C_4$ pathways. They are easily distinguished by their leaf anatomy (Waller and Lewis, 1979). The diagnostic feature of $C_4$ plants is the Kranz leaf anatomy, which has vascular bundle surrounded by organelle-rich bundle sheaths, which are further surrounded by undifferentiated palisade mesophyll cells. In $C_3$ plants, the bundle sheath contains relatively few organelles (Dengler and Nelson, 1999) and a greater number of mesophyll cells are differentiated into palisade and spongy cells (Jiang *et al.*, 2011). Tissue of $C_4$ grasses, with denser vascular bundles are slow to break down in the rumen of animals, whereas $C_3$ grass tissue with more mesophyll cells containing thin cell walls are easily digested (Nelson, 1995).

Cool season grasses, such as annual ryegrass, use the $C_3$ carbon pathway in the chloroplast of mesophyll cells in which $CO_2$ is fixed by the enzyme RUBISCO to form the three-carbon intermediate 3-phosphoglycericacid. This undergoes a series of reductions to finally form the six-carbon molecule glucose or its isomer fructose. At higher temperature, photorespiration occurs in the $C_3$ pathway, resulting in a loss of energy. It is estimated that 15-40% of the light energy captured by $C_3$ plants is wasted in photorespiration (Nelson, 1995). Photorespiration occurs on hot, dry days when the plant closes its stomata to prevent excess water loss. If the plant continues to fix carbon dioxide when its stomata are closed, the carbon dioxide level inside the leaf drops and the oxygen level increases. When the

carbon dioxide levels drop to 50 ppm, the enzyme RUBISCO catalyzes the reaction of oxygen with ribulose-bisphophate creating the toxic molecule 2-phosphoglycolate, which the plant must spend energy to convert this compound to a usable form. In $C_4$ plants, phosphoenolpyruvate (PEP) and $CO_2$ are combined by phosphoenol pyruvate carboxylase (PEPc) to form a four carbon intermediate compound oxaloacetate in the mesophyll cells and is transported to bundle sheath where it is decarboxylated to 3 carbon compound. The three carbon acid pyruvate is again transported back to the mesophyll cells for the regeneration of PEP (Sage, 2002). This mechanism reduces the possibility of photorespiration but is energetically expensive.

Accumulation of plant biomass is a function of metabolic processes such as photosynthesis and respiration. The $C_3$ pathway requires 18 molecules of ATP whereas the $C_4$ pathway requires 30 molecule of ATP to synthesize one molecule of glucose. The distribution of $C_3$ plants decreases from north to south in USA. $C_3$ plants are generally dominant at high altitude and high latitude (Sage and Kubien, 2007). Terri and Stowe (1976) predicted 54% of the graminoid species found in Alabama, USA (latitude 30° 13’ N to 35° and longitude 84° 51’ W to 88°28’ W) possess the $C_4$ photosynthesis pathway. $C_3$ species generally can perform their photosynthetic activity without harm between 0 and 30°C. In the case of annual ryegrass grown close to the Gulf coast, the maximum growth is attained at average daily temperature of 18°C and the growth ceases below 6°C (Weihing, 1963). In a study of photosynthesis of forage crops in Japan, Murata and Iyama (1963) found 10 -15°C air temperature optimum for photosynthesis in annual ryegrass.

## Seed production

Unlike other row crops such as corn (*Zea mays* L.), soybean (*Glycine max* L.) or peanut (*Arachis hypogaea* L.) where seed and agronomic production generally take place in the same geographic area, the situation for cool season forage crops is different. The production of high quality seed of annual ryegrass needs mild temperature with a moist winter and spring followed by a dry summer for seed maturation and harvesting (Balasko *et al.*, 1995). Seeds stored under hot and humid summers (southeastern USA) have lower germination percentage and lose their viability after nine months whereas seeds stored in cool dry summers (Pacific Northwest) have higher germination rate and remain viable for 2 years (Evers *et al.*, 1997). The average seed yield of annual ryegrass in the Willamette Valley of Oregon was 2000 kg $ha^{-1}$ (Young and Barker, 1997), whereas it was no more than 1000 kg $ha^{-1}$ in southeastern USA (Weihing and Evatt, 1960). The sultry climate in the southeastern USA during summer creates favorable conditions for pathogens to cause disease in annual ryegrass. Thus, annual ryegrass seed used in the southeastern USA is grown in the Pacific Northwest.

Stratton and Ohm (1989) conducted an experiment to compare the seed yield of orchardgrass (*Dactylis glomerata* L.), which was selected in Indiana, between two locations Indiana and Oregon. They found genetic variation for seed yield components between these two locations. Panicle number and seed yield per panicle showed a positive phenotypic correlation of 0.46-0.77 and 0.17-0.66 with seed yield, respectively. The genotypic and location interactions were significant but genotypic and phenotypic interaction showed very low correlation for seed attributes between two locations. Genetic correlation for seed yield between these locations was nearly zero. Their study indicated

that seed yield of orchardgrass in Oregon cannot be predicted from the seed yield data at Indiana. Therefore, to minimize g × e interaction it is always a good idea to select annual ryegrass for forage breeding purpose in the area of its utilization (southeastern USA) than in the seed producing area (Pacific Northwest).

## Diseases and toxicity

The major diseases affecting productivity and nutritive value of annual ryegrass in the southeastern USA are crown rust and blast. Crown rust is caused by parasitic fungus *Puccinia coronata* and blast is caused by *Pyricularia grisea*. The rust causes loss of leaves and retarded growth. In the crown rust susceptible cv. Lemtal, the rust infection caused reduced dry matter (DM) yield by 23%, 17%, and 13% for the first three harvests respectively (Potter, 1987). The loss of nutritive quality of crown rust infected ryegrass is associated with water soluble carbohydrates (WSC) level. Latch *et al.* (1977) reported decrease of WSC from 15.2% to 11.1% of DM in rust infected Lematal ryegrass.

Annual ryegrass toxicity is a lethal disease of livestock caused by the ingestion of the corynetoxin contaminated annual ryegrass seed heads that have been infected by *Rathayibacter toxicus* (Kowalski *et al.*, 2004). The vector seed gall nematode (*Anguina funesta*) was found to transfer the bacterium from the soil in Australia. These toxins inhibit glycoprotein synthesis and cause damage to the reticulo-endothelelial system (Cheeke, 1995). This problem has been mostly reported in Australia and South Africa. In the USA, annual ryegrass staggers (farm animals that lose their balance) was observed many years ago in Oregon (Galloway, 1961) but it hasn't been observed in recent years.

## Genetics and breeding of annual ryegrass

Annual ryegrass in its natural state is a diploid with 2n = 2x =14. It is self-incompatible and the gametophytic incompatibility is regulated by two multi-allelic unlinked loci S and Z (Fearon *et al.*, 1983). The percentage of self-compatibility is 7.8 in *L. multiflorum* and seed set from interspecific hybridization is 47.5 in *L. perenne* × *L. multiflorum* and 26.7 in reciprocal crosses (Arcioni and Mariotti, 1983). Experiments with hybridizing *Lolium* and *Dactylis* indicated that it is possible to create allo-tetraploid hybrids (Oertel *et al.*, 1996). Artificial tetraploids of *Lolium spp*. have been created through colchicine treatment (Shalygin, 1941). Chromosome doubling provides favorable possibilities of improving forage quality (Wit, 1958). Tetraploid cultivars generally have larger seed size, higher seedling vigor, more cold tolerance and higher concentration of sugar and digestible organic materials than diploid cultivars (Borreani and Tabacco, 1998; Pfahler *et al.*, 1986; Sugiyama, 1998).

There is an increasing demand on the forage to meet the animal nutrient requirements over years. Annual ryegrass forage production can be increased either by a crop management or a plant breeding approach. The former is rather simple and quick. Early or late planting, selecting high yielding disease resistant cultivars, irrigating when required and fertilizing to optimum amounts are some of the ways to increase yield through agronomic means. The latter approach exploits the genetic characteristics for the specific trait. Breeding for improving certain traits in forage is a complex and long process, which will take at least 5 years. For a successful breeding program, the plant breeder should have sufficient knowledge of genetics and reproductive behavior of the species of interest, which provide useful information in determining suitable breeding methods and strategies. As in

the case of annual ryegrass, which is self-incompatible even in presence of perfect flowers, the most common breeding method includes increasing the frequency of a desired allele controlled by additive effects. Recurrent selection, mass selection and hybridization between elite cultivars are some of the traditional plant breeding methods used by ryegrass breeders. Developing single or double hybrids is difficult, however, the option to produce semi hybrids by crossing two populations is feasible in forage crops which exploits the partial heterotic gain (Brummer, 1999).

The progress of plant breeding in forage crops is very slow accounting for 4% genetic gain per decade compared to 13.5% in grain crops (Humphreys, 1997). The success of forage breeding is measured in terms of animal performance and forage quality. In many cases of forage breeding, total annual yield is considered to be of secondary importance to seasonal yield distribution. Under the Irish system of dairy production, breeding for improved winter yield is considered worth up to five times the yield in spring or summer months (McEvoy *et al.*, 2010). Thus, a forage-breeding program should consider several factors such as animal performance, seasonal yield, stress tolerance and persistency in addition to total annual yield.

## Recurrent selection

Recurrent selection is one of the breeding methods used by plant breeders for population improvement and cultivar development. It is used in both cross- and self-pollinated crops but is more common in the former. It is a cyclical improvement technique for increasing the frequency of favorable alleles while maintaining genetic variability for future selections. All recurrent selection procedures are comprised of three phases:

development of selection units, evaluation of selection units in replicated or non-replicated trials, and recombination of superior selected units (Hallauer and Darrah, 1985).

Recurrent selection schemes can be broadly classified into phenotypic and genotypic recurrent selection. The selection of the superior plants in the latter method is based on progeny testing. The progeny development step in genotypic recurrent selection requires at least one additional year or off-season nursery production, which increases the time required for each breeding cycle. There are four types of genotypic recurrent selection each of which is suitable for a different purpose. Recurrent selection for general combining ability (RSGCA) uses wide genetic base cultivars as a tester while recurrent selection for specific combining ability (RSSCA) uses inbred lines or narrow genetic base cultivars as tester. The reciprocal recurrent selection (RRS) exploits both specific and general combining ability. Simple recurrent selection also known as phenotypic recurrent selection does not use any tester and selection is based on the phenotype. It is mostly suitable for traits with high heritability.

Recurrent selection increases the frequency of desirable alleles without reducing genetic variance. This advantage accompanied with simplicity in methodology makes recurrent selection the most popular method of improving crops. Recurrent selection was developed in relation to heterosis breeding. The idea of recurrent selection is to shift the mean of selected trait in the direction of selection. The expected genetic gain (ΔG) of the desired trait can be predicted by a general formula:

$$\Delta G = \frac{C_i V_A}{\gamma \sigma_P}$$

Where, $C_i$ is the selection intensity, $V_A$ is the additive genetic variance, $\gamma$ is the number of years per cycle, and $\sigma_P$ is the phenotypic standard deviation among the units of selection (Acquaah, 2008).

Repeated mass selection, a form of phenotypic recurrent selection, is a traditional and the easiest method of forage improvement. This method involves selection of plants for easily observable characters and is useful for traits with high heritability. Gardner (1961) was able to increase the maize grain yield by 23% in four generations of mass selection indicating its possibility to improve traits with low heritability ($h^2 < 30\%$). Inspired by this result, Burton (1974) used repeated mass selection with some modification to increase the forage yield of Pensacola bahiagrass (*Paspalum notatum* Flüggé var. *saurae* Parodi). He used a grid restriction in which the field is divided into several small sub plots each of which contained 5 × 5 plants. The grid restriction helped to reduce the environmental variation due to soil heterogeneity within a field. He selected 5 plants from each subplot (20% selection intensity) and allowed recombination of the selects. This restriction was beneficial in advancing the progress by double than through mass selection because both the female and male genetic sources were controlled (Burton, 1992). Cycle four of his selection was superior by 16 - 19% to commercial bahiagrass for forage yield. This method is referred to phenotypic restriction recurrent selection (RRPS) and is regarded as a successful method of improving forage yield.

## Forage quality

Characteristics such as high yield, high palatability, and high digestibility makes annual ryegrass a popular cool season forage among livestock farmers. Furthermore, since

the grass is an open pollinated species having a determinate flowering habit, extending the growing season by delaying the flowering date could maintain a higher proportion of leaves to stem for longer periods. This would increases nutritive value, digestibility, and palatability of the forage (McLean and Watson, 1992).

Forage quality is also associated with plant developmental stage. In temperate grasses, the nutritive value and digestibility decreases with the age of stand (Collins and Casler, 1990). The decrease in digestibility is due to an increase in fiber concentration and increased lignification of cell walls (Morrison, 1980). In annual ryegrass, organic matter digestibility decreased from 928 to 576 g $kg^{-1}$ as the forage ages (Valente *et al*., 2000).

The most reliable method of forage quality evaluation is measuring the production output from the animals consuming the forage. Animal performance is a function of voluntary intake and digestibility of a particular forage species (Coleman and Moore, 2003). Voluntary intake is the amount of forage consumed by animals when available *ad libitum* (Marten, 1970). Digestibility is the portion of dry matter in forage that is digested by the animal at certain intake level. However, due to difficulty and variation in measuring intake and digestibility, the prediction of animal performance is often less accurate and less precise. Intake contributes up to 70% to the variability in forage quality (Crampton *et al.*, 1960) and is considered more important than digestibility in determining animal performance (Lippke, 1980). The accurate way to measure forage quality is by measuring average daily gain (ADG) under grazing. In a breeding program many entries need to be screened and animal trials become impractical and/or far too expensive (Casler, 1997). Thus, animal performance is generally predicted based on routine analysis based on

chemical composition, *in vitro* bioassays and near infrared reflectance spectroscopy (NIRS).

The routine chemical analysis of forage includes crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), total non-structural carbohydrates (TNC), ether extract (fats), and ash. ADF is comprised of cellulose, lignin, and fiber bound nitrogen. The NDF fraction includes hemicellulose in addition to ADF. Neutral detergent fiber and ADF are measured as the residue remaining after the forage is digested with either neutral detergent or acid detergent, respectively. TNC measures starch and sugar in forages whereas CP measures non-protein nitrogen (amino acids, urea) and true proteins. Nitrogen content in the forage is measured using acid hydrolysis (Kjeldahl method) and the value multiplied by 6.25 to determine CP content in the forage. The ether extract is the amount of lipids in the forage (Ball *et al.*, 2001).

Proteins and carbohydrates comprise the major portion of the nutrients required for the animal production. CP supplies N for rumen microorganisms and amino acids to the intestine for absorption in ruminant animals. The intake of forage by ruminants is low if the N requirements for microbial activity are not satisfied. Thus, the voluntary intake of forage increases as the CP in forage increases. The ruminants consume an optimum amount of forage if the CP content of the forage is at least 8% of the dry matter (Moore *et al.*, 1999). Dairy cows can consume 1.2% NDF of their body weight (BW) per day and the intake potential can be predicted as: BW × 1.2/fraction of NDF in forage dry matter (Mertens, 1992). This equation represents the intake potential but actual intake may differ depending on animal energy demands and physiological processes (Rohweder *et al.*, 1978).

Based on the routine analysis, Goering and Van Soest (1971) proposed an equation to estimate dry matter digestibility (DMD) based on DM percentage of neutral detergent soluble (NDS) and NDF as $\%DMD = -12.9 + 0.98 * NDS + Dc * \%NDF$, where, -12.9 is a constant indicating loss of DM during digestion, 0.98 is a true digestibility of the NDS, and Dc is the digestibility of the NDF.

The concentration of TNC present in forage is a major source of energy for ruminant animals. Ruminants have higher intake and better nutrient utilization capacity if grazed on forage containing higher TNC levels then on lower TNC levels (Miller *et al.*, 2002; Smit *et al.*, 2006).

## Annual ryegrass and animal grazing

The ultimate aspect of measuring forage quantity and quality is a grazing trial under controlled conditions. It involves the *ceteris paribus* principle, i.e., excluding the effect of all factors except the ones of interest. Grazing trials are difficult to conduct due to the time and cost involved. Different chemical methods to estimate forage quality have been devised. However, variation in forage intake accounts for the inaccuracy to predict animal performance based on lab analysis. So, an animal grazing trial is an indispensable part of evaluating the worth of a new forage cultivar (Fisher *et al.*, 1995) because it involves the interaction among plant composition and growth, the grazing behavior of the animals and management inputs of the producer. The level of animal production is a reflection between the forage quality and quantity consumed by the animals (Briske and Heitschmidt, 1991).

The available forage mass at any time in a pasture is an important factor to consider in grazing experiments. Herbage mass highly correlates with weight gain and overall

livestock production (Guerrero *et al.*, 1984). Forage mass can be determined by either clipping or non-destructive methods; pasture ruler, disk meter, and capacitance meter are the common non-destructive methods. The advantage of using non-destructive methods is the ease of collecting a large number of observations in a short period of time. However, these methods need constant calibration to maintain accuracy and precision of the prediction equations (Gonzalez *et al.*, 1990). The level of calibration error in measuring forage mass using a plate meter was found to be about 10% (Rayburn and Rayburn, 1998). The height measured through non-destructive methods has to be regressed onto forage mass to determine the available forage biomass (Whitney, 1974). Pasture ruler is based on the positive relationship between sward height and forage yield. A disk meter is a more reliable and accurate method than measuring plant height with ruler because it integrates both height as well as density of the forage which is referred to as bulk density (Bransby *et al.*, 1977; Michalk and Herbert, 1977).

Cool season annual grasses in the southeastern USA are usually grown by overseeding a bermudagrass sod. Beck *et al.* (2007) observed significant increase in weight gain $ha^{-1}$ in grazing stockers on annual ryegrass overseeded paddocks. The addition of annual ryegrass over small grain sod pasture increased the dry matter production from 1350 $kg^{-1}$ to 1582 $kg^{-1}$ (17%) in spring. Since annual ryegrass has better growth and development during spring, the sod seeding of annual ryegrass over a small grain pastures can extends the grazing period.

Grazing intensity is one of the most important grazing strategies and is useful in determining the forage mass and herbage allowance on pasture (Burns *et al.*, 2002).

Stocking rate is considered an important treatment variable in animal grazing trials. In general, weight gain per unit area increases up to certain point and then begins to decline with increase in stocking rate. The reverse is the case for gain per animal. Riewe (1961) in a study in Texas, determined that the optimum stocking rate for maximum weight gain was 2.9 animals $ha^{-1}$ with a corresponding live weight gain of approximately 234 kg $ha^{-1}$ during spring.

Annual ryegrasses can produce high quality forage from late fall to spring. Many cultivars are being marketed for commercial cultivation; cvs. Marshall and Gulf being the most preferred by farmers in southeastern USA. Marshall is more cold tolerant than Gulf; thus Gulf is preferred to Marshall in mildly-cold regions (Redfearn *et al.*, 2002). There is a vast difference in weight gain by cattle grazing different annual ryegrass cultivars. In a grazing trial in Alabama, cattle grazing cv. Marshall gained 50% more weight than cattle grazing cv. Gulf (Bransby *et al.*, 1997). But in a similar study in Louisiana, weight gain on cv. Marshall was only 16% more (Wyatt and Granger, 2001) than cv. Gulf. Cultivars Gulf, Jackson, Rio, and Surrey yielded at least 274 kg DM $ha^{-1}$ more in December than cv. Rustmaster. Cultivars Marshall and Jackson are better than Gulf by producing 243 kg DM $ha^{-1}$ more forage in May harvest (Redfearn *et al.*, 2002). This late season additional production is important to make hay or stored forage.

## TNC and annual ryegrass

Forages contain non-structural carbohydrates, structural carbohydrates, proteins, lipid, and minerals. During the process of photosynthesis simple carbohydrates like glucose and fructose are formed and then combined to form complex molecules such as starches

and fructans (Salisbury and Ross, 1992). Non-structural carbohydrates are the photosynthetic products that provide the main energy for the growth and maintenance (Danckwerts and Gordon, 1987). The simple sugars, fructans (stored in the stem), and starch (seed reserve) comprise the total non-structural carbohydrates (TNC) of the temperate ($C_3$) forage (Holt and Hilst, 1969). Temperate grasses predominantly store reserve carbohydrates in the form of fructans, whereas tropical grasses store reserve carbohydrates in the form of starch (Smith, 1973). Storage carbohydrate concentrations show diurnal variation (Holt and Hilst, 1969) as well as seasonal variation (Waite and Boyd, 1953). Warm season forages ($C_4$) usually have lower TNC concentration than cool season forages (Chatterton *et al.*, 1989). The concentration of soluble carbohydrate reserve in ryegrass is the basis for the efficient management of ryegrass pasture. The re-growth of the pasture after defoliation is proportional to the reserve water soluble carbohydrates present in the forage before defoliation (Donaghy and Fulkerson, 1997).

Myer *et al*. (2010) conducted a two-year study to determine the seasonal changes in soluble carbohydrate concentration of annual ryegrass grown in the southeastern USA. Water-soluble carbohydrate (WSC) concentration decreased linearly from 35-33% for January-February harvest to 15-12% for May harvest. There was a small but inconsistent difference of WSC among cvs. Gulf, Marshall, and Jumbo. Cultivar Gulf had the lowest WSC concentration in the first year compared to cultivars Marshall and Jumbo but in the second year cv. Jumbo had the least concentration of WSC.

WSC concentration in annual ryegrass in New Zealand was found to be 47% of DM in stubble, 27% of DM in leaves, and 11% of DM in roots (Vartha and Bailey, 1980).

Thus, it is obvious that the storage part of temperate grass is stubble rather than roots (Fulkerson and Slack, 1994). The analysis of annual ryegrass straw based on the Van Soest analytical scheme contained 29.6% cell soluble matter, 36.8% cellulose, 27.1% hemi-cellulose, 5.4% lignin, and 2% ash with digestibility 64.5%, 45.6%, 42.8%, 0%, and 0% respectively (Han *et al.*, 1975).

There was a significant loss of digestible components in annual ryegrass during senescence. The *in vitro* dry matter digestibility (IVDMD) of the plant decreased by 22% from anthesis to 69 days after anthesis. This decrease in IVDMD was associated with the loss of soluble neutral detergent (NDS) of the third stem inter-node. The digestibility of the NDS blade decreased from 80-95 to 45 % in leaf blade and from 35 to 19 % in stem as annual ryegrass proceeds from anthesis to senescence (Ballard *et al.*, 1990). Thus, as annual ryegrass ages the fiber content increases whereas digestibility and TNC level decreases.

## Ploidy and annual ryegrass

The Federal Seed Act (1995) of the United States has a provision that cultivars for domestic sale should be at least 98 % of the reported ploidy level and accurate determination of ploidy level is required for the certification and release of cultivar. Therefore, it is necessary to determine the ploidy level of any newly developed cultivar before it is released. Ploidy homogeneity is important because natural hybridization between cultivars of different ploidy level may result in genetic instability, poor germination, infertility, and lower seed set (Griffiths *et al.*, 1971).

Traditionally, ploidy level is determined by counting chromosome numbers either in roots or microspore mother cells, a labor-intensive process. Flow cytometry is a newer

and more rapid technique to determine ploidy level in plant species compared to traditional cytological methods (Barker *et al.*, 2001).

Flow cytometry was initially developed for the analysis of mammalian blood cells and has been widely used in clinical diagnosis. Its use in studying higher plants genome was first reported in early 1970's (Heller, 1973) but was not widely used until Galbraith *et al*. (1983) developed a simple chopping technique for the preparation of the intact nuclei suspension in 1983. Flow cytometry works on the principle of scattering or absorbing light when it strikes nuclei in the flowing suspension. The nuclei suspension is stained with a DNA specific fluorochrome. Stained nuclei are allowed to flow within a capillary liquid stream through the focus of intense light. The stained cells scatter the light and emit fluorescence signals, which are quantified and recorded by the flow cytometer. The results are displayed in the form of histogram of relative fluorescence intensity, which represents relative DNA content (Dolozel and Bartos, 2005; Galbraith, 2012).

Tetraploid annual ryegrass cultivars generally have larger seed size, higher seedling vigor, cold tolerance, and higher concentration of sugar and digestible organic materials than diploid cultivars (Borreani and Tabacco, 1998; Pfahler *et al.*, 1986; Sugiyama, 1998). But in a trial comparing forage yield of 2x and 4x cultivars in the southeastern USA, diploid cultivars had the advantage over tetraploids. In trials in the northern parts of Georgia, Alabama, and Mississippi diploid cultivars produced 9% more forage than tetraploids. In the southern trials, tetraploid cultivar yielded 4%, 5%, and 12% more than diploids in Georgia, Louisiana, and Texas, respectively, while in Alabama and Mississippi, diploids had a comparative advantage of 4% and 7 %, respectively (Nelson *et al.*, 2006).

## Statement of problem

In the southeastern USA, forage production during winter is relatively low and beef cattle are fed stored forages and purchased feed to meet their nutritional requirement. This results in increased management costs for animal farmers. Natural forage and pasture grassland are predominantly occupied by warm season grass which are available mostly from April to September and the growth ceases as the temperature declines in autumn (Bartholomew and Williams, 2008). The growing period of cool season forage grasses is from early autumn to late spring with minimal production during early winter. Thus, the two months December and January are known as lean season months in terms of forage availability. The forage of annual ryegrass is found to be of higher quality in winter than in spring (Balasko *et al.*, 1995). So, by improving early growth characteristics, we can make more annual ryegrass forage available during these months.

Feeding animals using stored hay and/or purchased feed is economically expensive compared to grazing on green forage. A study in North Carolina showed different cost involved in various types of forage on dry matter basis. The permanent pasture costs 4.40 cents $kg^{-1}$, summer annuals costs 4.62 cents $kg^{-1}$, winter annuals costs 5.06 cents $kg^{-1}$ and hay costs 10.78 cents $kg^{-1}$ of DM. These figures showed hay is twice as expensive as winter annuals (Benson, 2012). In a comparative study for the production economics of grazing cereal rye - annual ryegrass and perennial tall fescue system, Islam (2011) found the cereal rye - annual ryegrass system to be more economical than the tall fescue system. In that five-year grazing trial, grazing stocker cattle in the cereal rye - annual ryegrass grazing system and tall fescue produced 1.05 and 0.93 kg $d^{-1}$ average daily gain (ADG), respectively. The total grazing days for rye - annual ryegrass was greater by 63 days per

season than tall fescue. The net profit was greater for the cereal rye - annual ryegrass grazing system by \$62 $ha^{-1}$ indicating better economic return from the cereal rye - annual ryegrass system.

## Goal of this study

Development of a suitable cultivar with increased winter productivity would be a valuable contribution to animal agriculture. A breeding program was initiated in 2005 to improve the winter productivity of annual ryegrass. A random-mating base population was subjected to two cycles of phenotypic recurrent selection for increased biomass accumulation during winter. Cycle 2 ($C_2$) produced higher DM yield in winter than $C_0$, $C_1$, Gulf, and Marshall in a two-year, five-location study (Dhaliwal, 2009) but this result was not sufficient to release $C_2$ as a cultivar. Therefore, this current study was conducted to evaluate the progress from the selection and to determine the worth of $C_2$ developed under grazing before releasing it as a cultivar. The specific objectives of my research were: 1) to evaluate the performance of animals under grazing on annual ryegrass selected population $C_2$; 2) to determine the change in correlated traits and ploidy level in annual ryegrass selected populations $C_0$ – $C_7$; and 3) to determine the effect on total non- structural carbohydrates (TNC) content of annual ryegrass populations selected for increased winter productivity under two different environments.

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# Grazing Evaluation of Annual Ryegrass (*Lolium multiflorum* Lam.) Selected for Increased Winter Productivity


## Abstract

Forage availability in the southeastern USA is limited during winter and supplemental feeding increases management costs. Therefore, a phenotypic recurrent selection program was initiated in 2005 to improve the winter productivity of annual ryegrass. In a preliminary study, Cycle 2 produced higher dry matter (DM) yield in winter than check cultivars but the results were not sufficient to release Cycle 2 as a cultivar. Thus, this study was conducted to evaluate the progress from selection and to determine the worth of Cycle 2 under grazing. We conducted a grazing trial in Central Alabama at the Beef Cattle Unit of the E.V. Smith Research Center in Milstead, AL at multiple stocking rates of 3.7, 4.9, and 6.1 steers $ha^{-1}$ for two years. Cycle 2 supported higher average daily gain (ADG) (1.37 kg $d^{-1}$; $P = 0.107$) compared to Gulf (1.26 kg $d^{-1}$) and was consistently better than Gulf and Marshall in ADG at all SR in 2011 but there was no statistically significant difference in ADG among cultivars and cultivars × stocking rate interaction in 2012. The higher ADG on Cycle 2 compared to Gulf was significant ($P \leq 0.085$) in January - March grazing periods in 2011, showing better winter performance. Available forage mass was higher ($P \leq 0.009$) in Cycle 2 than Gulf in February and March. There were no significant differences in nutrient content among cultivars and cultivar × SR interactions except lignin

which was greater in Cycle 2 than Marshall ($P \leq 0.084$). Further grazing evaluations will focus on early seeding and grazing during the low forage availability months (December – February).

## Introduction

Annual ryegrass is a cool season ($C_3$) annual grass widely grown in the southeastern USA. However, the availability of standing forage is limited during winter and cattle are fed stored forage or grains, which increases management cost (Ball *et al.*, 2002). As a general practice in the southeastern USA, annual ryegrass is sown in autumn, which yields maximum growth in spring (Redfearn *et al.*, 2002). Gulf, an early maturing cultivar with crown rust resistance (Weihing, 1963) and Marshall, a late maturing cultivar with cold tolerance (Arnold *et al.*, 1981) are the most commonly used cultivars in the southeastern USA. Several cultivars of annual ryegrass with cold tolerance and early maturity are registered and marketed in southeastern USA. The tetraploid cvs. Nelson and TAMBTO (2n = 4x = 28) released by Texas AgriLife Research have good winter hardiness and productivity in Gulf Coast region of Texas (Nelson *et al.*, 2011; Nelson *et al.*, 2007). Similarly, the diploid cv. TAM 90 was released for winter production and has potentially good yield in Gulf Coast when over seeded onto warm season perennial grass (Nelson *et al.*, 1992). However, there are no annual ryegrass cultivars with increased winter productivity for more northern areas of the southeastern USA.

Shifting the DM yield to the winter months through selection and breeding may alleviate the winter deficiency of standing forage in this region. For that purpose, a phenotypic recurrent selection program was initiated in 2005 at Auburn University.

Crossing six top-performing cultivars from Alabama cultivar performance trials created the base population. The first harvest of the second selection cycle (Cycle 2) resulted in increased DM production by an average (five evaluation locations) of 300 kg $ha^{-1}$, 400 kg, $ha^{-1}$, and 300 kg $ha^{-1}$ than the base population (Cycle 0), Gulf, and Marshall respectively (Dhaliwal *et al.*, 2009).

Although, the breeding program is making progress in terms of DM productivity under mechanical harvesting, it may not necessarily reflect the corresponding increase under grazing. Chemical methods used to estimate nutrient composition from mowed forage may not necessarily predict the forage quality accurately and precisely because of variation in forage intake. It has been demonstrated that the plant response to grazing is species specific (Gao *et al.*, 2008). There are several lines of evidence for compensatory as well as over-compensatory growth of forage due to grazing at moderate intensities (Agrawal, 2000; McNaughton, 1983). The increased growth or fitness was believed due to the presence of thiamine and various growth factors in mammalian saliva. In a recent study by Liu *et al.* (2012), a symbiotic relationship was found to exist between the biomass yield in perennial grasses and animals grazing on it.

Grazing intensity is one of the most important grazing strategies and is useful in determining the forage mass and herbage allowance on pastures (Burns *et al.*, 2002). Thus, stocking rate is considered an important treatment variable in grazing trials. In multiple stocking rate experiments, weight gain per unit area usually increases up to a certain point and then begins to decline with an increase in stocking rate. The reverse is the case for gain per animal. Riewe (1961) in a study on annual ryegrass grazing at Texas obseved that the

optimum stocking rate for maximum weight gain to be 2.9 animals $ha^{-1}$ and a corresponding body weight (BW) gain of approximately 234 kg $ha^{-1}$ during spring. Bransby *et al* (1988), in an experiment on bermudagrass grasses (*Cynodon dactylon* (L) Pers) reported a high linear correlation ($r > 0.9$) among ADG, stocking rate (SR), and available herbage mass. The same study showed the quadratic relationship between SR and gain per unit area.

The available forage mass at any time in a pasture is an important factor to consider in grazing experiments. Herbage mass highly correlates with weight gain and overall livestock production (Guerrero *et al.*, 1984). Forage mass can be determined by either clipping or non-destructive methods. Pasture ruler, disk meter, and capacitance meter are the common non-destructive methods to determine forage mass. The advantage of using non-destructive methods is the ease of collecting a large number of observations in a short period of time. However, these methods need constant calibration to maintain accuracy and precision (Gonzalez *et al.*, 1990). The calibration error in measuring forage mass using a plate meter was found to be about 10% of the mean pasture mass (Rayburn and Rayburn, 1998). The height measured through non-destructive methods has to be regressed onto forage mass to determine the available forage biomass (Whitney, 1974). The pasture ruler method is based on the positive relationship between sward height and forage yield. A disk meter is a more reliable and accurate method than measuring plant height with a pasture ruler because the former integrates both height and density of the forage, which is also referred to as bulk density (Bransby *et al.*, 1977; Michalk and Herbert, 1977).

The nutritive value of the forage determines intake, utilization and performance of ruminants. Protein and carbohydrates comprise the major portion of the nutrients required

for animal productivity. Crude protein (CP) supplies N for rumen microorganisms and amino acids to the intestine for absorption. The intake of forage by ruminants is low if the N requirements for microbial activity are not satisfied. Thus, the voluntary intake of forage increases as CP in forage increases. The ruminants will consume an optimum amount of forage if the CP content of forage is at least 8% of the dry matter (Moore *et al.*, 1999). Soluble carbohydrates present in forage are the major source of energy for ruminant animals. Ruminants have a higher intake and better nutrient utilization capacity if grazed on forage containing higher TNC levels (Miller *et al.*, 2002; Smit *et al.*, 2006).

A grazing trial involves the interaction among plant composition and growth, the grazing behavior of the animals and management inputs of the producer. An animal grazing trial is considered an indispensable part of evaluating a new forage cultivar (Fisher *et al.*, 1995). The reliable way to measure forage quality is by measuring average daily gain and gain per area under grazing. Therefore, the worth of newly developed forage cultivar can be evaluated through animal performance under grazing and it depends upon forage quantity, quality, and grazing behavior of the animals (Moore, 1994). Thus, the objective of this study was to evaluate the performance of Cycle 2 along with cvs. Gulf and Marshall under grazing at multiple stocking rates. Average daily gain, gain $ha^{-1}$, available forage, and nutritive value of population Cycle 2 was assessed and compared to standard cvs. Gulf and Marshall. This research will provide the basis for the release decision of a cultivar selected with increased winter productivity.

## Materials and Methods

This study was conducted according the protocol approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

A 2-yr grazing trial was conducted beginning in January of 2011 (yr 1) and 2012 (yr 2) at the Beef Cattle Unit (BCU), E.V. Smith Research Center, Milstead, AL (32º 45' N lat., 85º 88' W long., 76 m. elev.). Replicated paddocks (0.81 ha) containing cultivars Gulf, Marshall, and SWIPAR Cycle 2 were established during mid October in both years at seeding rate of 22.5 kg $ha^{-1}$. The experiment was set at 3×3 factorial (cultivar × stocking rate) CRD with 3 replicates for 2011 and RBD with three blocks for 2012. Paddocks were stocked with 3, 4 or 5 steers with initial body weight 262 ± 23.9 kg (535 ± 45.9 lbs) in 2011 and 214 ± 21.5 kg (471 ± 47.4 lbs) in 2012 and were continuously grazed for 140 d. All steers had *ad-libitum* access to water and a salt-mineral mix.

Each steer on trial was weighed at the beginning of the trial and every 28 d thereafter for the duration of the grazing season. A rising plate disc meter was used to determine available forage. Forage rising plate meter height was determined at 25 locations within each paddock using a random walk approach. Similarly, forage mass inside a 0.45 m (18 inch) diameter ring was clipped 5 cm above ground surface as the calibration samples from 5 random but representative sites covering the range of sward height observed during the random walk approach. These calibration samples were weighed for green mass determination, oven dried at 60 ºC for 48 hours, and then dry matter mass were recorded. These samples were also used for forage quality evaluation. To determine the TNC content, separate samples from each paddock were clipped from 5 random spots, bulked in a Ziploc

bags, and placed in a -20˚C freezer. These samples were then dried using freeze dryer in preparation for analysis. Samples in the field were taken at 28 days interval during the growing season.

**Analysis of forages for TNC**

Samples were freeze-dried to remove all the moisture and then ground with 1-mm mesh screen Cyclotec™ 1093 sample mill (Foss Analytical, Hoganas, Sweden). Ground samples (0.20-0.25 g) were taken in beaker, mixed with 50 mL of 0.05N $H_2SO_4$, and reflux-boiled in a fiber rack for 15 minutes. Then 5 mL of extraction acid was added and the samples were heated under reflux for 45 additional minutes. Samples were then allowed to cool to room temperature. Afterwards, the pH was adjusted to 4.5 ± 0.1 using different concentrations of acid or base. One mL diluted amyloglucosidase (*Aspergillus niger*, Sigma-Aldrich Inc., St. Louis, MO) was added, thoroughly stirred and incubated for 1 hour at 60°C. The solution was then filtered into 250 mL volumetric flask, 1N NaOH was added, and diluted by $dH_2O$. Ten mL of the solution was taken in a 25×200 mm test tube and 10 mL of Shaffer-Somogyi carbonate reagent (AOAC, 1995) was added. The resulting solution was boiled for 15 minutes, cooled in ice water, mixed with 2 mL KI, 10 mL 1N $H_2SO_4$, and 1 mL starch (1 gm per 100 gm starch powder) solution. Finally the resulting mixture was titrated with 0.02 N sodium thiosulfate until the contents of the tube turned an icy blue in color. Concentration of TNC in samples was calculated as the amount of reducing sugar in the sample, multiplied by product of dilution factor times 100, and divided by sample weight. For every new batch of Shaffer-Somogyi solution, a glucose standard curve was constructed.at varying concentrations. A linear regression model was then fitted to predict the TNC level of each sample based on calculated titer value.

**Forage quality analysis**

Due to the large number of samples (~900), near infrared reflectance spectroscopy (NIRS) was used to determine the forage quality. All oven dried samples were ground with 1-mm mesh screen Thomas Wiley® Laboratory mill (Thomas Scientific, NJ, USA). Ground samples were scanned in a FOSS 5000 NIRS system (FOSS Analytical, Hilleroed, Denmark) with ISIscan™ and WinISI 4 software. The NIRS system 5000 scans the region with wavelength 1100-2500 nm. The spectra were ranked to the *Global Mahalanobis* distance (GH). Thirtyfive spectra with a GH value greater than 3 were removed as outliers. Eighty representative samples from year 2011 were chosen for wet chemistry analysis; 60 samples were used for developing the prediction equation and 20 were used as a validation set. The concentration of ADF, NDF, and ADL for standardization samples were determined according to the procedures of Goering and Van Soest (1970). The N concentration was determined by using Elementar Macro CNS total combustion analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) as described in AOAC, official method of analysis (1995).

**NIR calibration**

For the development of calibration equation, the Standard Normal Variate and Detrend (SNV and Detrend) scatter correction was selected. The math treatment 1,4,4,1 and modified partial least square (modified PLS) regression method was used as a regression method such that the standard error of calibration (SEC) and standard error of cross validation (SECV) were minimum with highest $R^2$ values (Table 2.01). The prediction model developed through this process was tested with the validations set to check the accuracy of the prediction equation. There was no significant difference between

the actual and NIR predicted values, which indicated the acceptable performance of the NIR models.

**Statistical analyses**

All data were analyzed with linear mixed models methodology as implemented in SAS 9.2 (SAS Inst. Inc., Cary, NC) taking into account the repeated measure nature of the data. Cultivar, SR, and period were considered as fixed factors. Since our experiments were set up in different designs (RCBD in 2011 and CRD in 2012) the random factors were different in two years. R-side modeling using the TYPE option of the random statement was used to account for covariance structures and the best covariance structure was selected based on smallest corrected akaike information criterion (AICC) value. I investigated the normality assumption and checked for outliers by inspecting the residual student panel. I did not find any serious outlier except for few observations in forage calibration that were removed before analysis. Least squares means were calculated using the SLICEDIFF option and Dunnett's adjustment was made to compare the means of Cycle 2 with the controls. The LS means were calculated for cultivar, SR, period and their interactions when protected by a F-test significant at $\alpha$=0.10.

Available forage biomass per paddock was estimated using PROC MIXED in SAS 9.2 by regressing calibration samples DM onto pasture height. The estimate for cultivar × SR and height (cultivar × SR) were determined by indicating SOLUTION and NOINT options. The former estimates the intercept and latter the slope to determine the separate regression equation for each cultivar × SR combination, except for the initial pre grazing assessment, where SR did not play a role.

## Results and Discussion

### Average Daily Gain (ADG)

ADG is a prominent indicator to determine the worth of a newly developed forage cultivar. We compared the ADG for test population Cycle 2 with cvs. Gulf and Marshall at multiple stocking rates at periodic interval of 28 days during the grazing season. Cycle 2 (1.37 ± 0.02 kg BW $d^{-1}$) supported higher ADG *(P* = 0.107*)* compared to Gulf (1.26 ± 0.02 kg BW $d^{-1}$) and was consistently better than Gulf and Marshall at all SR in 2011 (Table 2.04). But there was no significant difference among cultivars and cultivar × SR in 2012. The ADG on Cycle 2 compared to Gulf was significantly higher ($P \leq 0.086$) in grazing period 1 (January/February) and period 2 (February/March) in 2011, showing better winter performance. The mean difference of at least 0.16 kg BW $d^{-1}$ ($P > 0.443$) supporting higher ADG for Cycle 2 was observed in period 1 in 2012 (Table 2.05). Cycle 2, which was selected for increased winter productivity and was expected to perform better than other cultivars during December – February. Our results were in accordance with the expectation. Cycle 2 supported greater ADG during grazing period 1 in 2012 and grazing period 1 and 2 in 2011.

Average daily gain for all cultivars in period 1 in 2012 was greater than in 2011. This may be due to compensatory growth associated with lower initial BW in 2012 (262 ± 23.86 kg in 2011 vs. 214 ± 21.53 kg in 2012). The ADG for feedlot finishing cattle is higher for lower initial BW (Zinn *et al.*, 2008). The other probable reason may be because of higher accumulation of growing degree days (GDD) (757 ºC in 2012 vs. 613 ºC in 2011) at the initiation of grazing. The average air temperature in December/ January in 2012 was

9.1 º C, which was 4.1 º C higher than in 2011. The higher GDD contributed more forage accumulation and in turn higher ADG for 2012 during that period.

Consequently, the higher temperature followed by 10 d later commencement of grazing in year 2 resulted in rapid decline in ADG for subsequent periods. Thus, seasonal ADG was lower in 2012 than in 2011. For all cultivars, ADG decreased from period 1 to 2, then increased in period 3 and finally decreased to the lowest on period 4 (final) in both years. The highest gain was observed on period 3 (March/April) for all cultivars in both years expect for Cycle 2 in 2012, which had highest ADG at period 1 (Table 2.05). The lowest gain was observed in period 4 in year 2. The low precipitation in March and April in 2012 may have resulted in less compensatory growth of forage for May grazing (Table 2.02). Like the results reported by Wyatt and Granger (2001) in a grazing experiment in Louisiana, our trial did not find any significant difference in ADG between Gulf and Marshall.

**Gain per unit area**

There was no significant difference in gain per unit area among cultivars on both years. However, Cycle 2 had a comparative benefit to Gulf and Marshall of more than 30 kg BW $ha^{-1}$ in 2011 (Table 2.06). Gain $ha^{-1}$ increased from lowest SR to intermediate SR and then decreased in highest SR in 2011 (Figure 2.01) following the quadratic relationship as noted by Bransby *et al.* (1988). In 2012, gain $ha^{-1}$ increased with increasing SR for all cultivars (Figure 2.02). Cycle 2 had a gain of 758 ± 38 kg BW $ha^{-1}$ which was greater than Gulf by 103 BW $ha^{-1}$ at highest SR in 2011. Similarly, in 2012, Cycle 2 had higher gain than cv. Marshall by 130 kg BW $ha^{-1}$ at highest SR (Table 2.06).

**Available Forage**

The overall average forage availability was higher in Cycle 2 than Gulf and Marshall in 2011. In 2011, there was no significant difference in pre grazing DM yield among cultivars. The available forage during grazing on Cycle 2 was 476, 544, and 393 kg DM $ha^{-1}$ more ($P \leq 0.09$) than Gulf in periods 1, 2, and, 3 respectively (Table 2.08). The available forage was significantly greater for Cycle 2 ($P \leq 0.002$) than Gulf and Marshall by 594 and 731 kg DM $ha^{-1}$ respectively at the intermediate SR (Table 2.07). At the lowest SR, Marshall had more available forage than Cycle 2 by 483 kg DM $ha^{-1}$ ($P = 0.011$) but there was no statistically significant difference among cultivars at the highest SR. These results indicated better performance of Cycle 2 at intermediate SR during the cooler months. However, in 2012 there was no significant difference in available forage among cultivars and cultivar × SR interaction but still the cycle 2 had higher ($P < 0.103$) forage availability during period 2 than cvs. Gulf and Marshall.

The cultivars had the highest available forage mass in March/April for 2011, which is in support of the result by Redfearn *et al.* (2002), who reported 60% of the annual ryegrass production occurred in March – May in Louisiana. They also stated that Gulf was superior to Marshall by 243 kg DM $ha^{-1}$ in May, but our result found no difference in available forage between Gulf and Marshall. The intake by steers depends upon the available forage and it has been reported that intake on wheat pasture was limited at less than 1100 kg $ha^{-1}$ available DM (McCollum *et al.*, 1992). In our study, the available forage was higher at most of the periods in SR 3.7 and 4.9 $ha^{-1}$ but was lower than 1100 kg DM $ha^{-1}$ in paddocks with the highest SR during the final period of the grazing.

**Nutritive value**

We evaluated TNC content only in 2012 due to time and resource constraints. There was no difference in TNC content among cultivars, Cultivar × SR, and Cultivar × SR × period interactions. The TNC decreased linearly from 28.01 in period 0 to 13.79 % of DM in period 4 ($P < 0.001$). At period 2, Cycle 2 had higher TNC than the check cultivar Gulf ($P = 0.019$, Table 2.09). Our results agreed with Myer *et al.* (2010), who found no consistent difference in TNC among the annual ryegrass cultivars.

There was a cultivar effect on % CP but the result was not consistent over the two years of the study. In year 1, Marshall had higher CP than Cycle 2 ($P = 0.039$) by 3.06 % DM. Cycle 2 had higher ADL content than Marshall in both years ($P \leq 0.084$) and higher NDF content than Marshall in 2012 ($P = 0.069$) but these values were not significantly different. The differences were more prominent during later periods corresponding to months April and May of the grazing trial. There was no difference in nutritive values between Cycle 2 and Gulf at any period in any year except at period 3 in 2012, where ADL of Gulf is significantly lower than Cycle 2 ($P = 0.018$, Table 2.10). Cycle 2, which was selected for improved winter productivity had earlier heading date by 8 d than the base population (Dhaliwal *et al.,* 2009) and that may be the reason for the higher ADL concentration.

In temperate forage species fiber concentration increases and CP concentration decreases with maturity (Collins and Casler, 1990). The average CP averaged over all cultivars decreased linearly from January to May (39 to 10 % in 2011 and 27 to 19% in

2012) and the NDF increased from 46 to 73% in 2011 and 55 to 77% in 2012. Similarly, the ADF and ADL concentration linearly increased with maturity.

## Summary

Cycle 2 supported higher ADG in steers during January-March grazing period in 2011. We did not get consistent result in 2012, which may be due to late initiation of grazing. Animal weight gain per unit area in Cycle 2 was higher than the control cultivars at all SR in both years except for lowest SR in 2012. The available forage was significantly greater in Cycle 2 than Gulf and Marshall at intermediate SR. These results indicate better performance of Cycle 2 under grazing at intermediate SR during the cooler months. There was no difference in TNC content among cultivars, Cultivar × SR as well as Cultivar × SR × Period interaction means. There was no difference in nutritive value among cultivars in both years except for ADL, where Cycle 2 had higher ADL concentration than Marshall during the late grazing periods.

Cycle 2 was expected to have higher productivity and performance in winter and our results showed the better performance of Cycle 2 at January/February grazing. The performance was more profound in 2011 than 2012. It may be because of earlier initiation of grazing accompanied with lower average temperature during December/January in 2011. There was a vast elevation of temperature during our experiment period in both years from the 30-year normal. Therefore, more grazing experiments starting in cooler seasons is necessary to justify the worth of Cycle 2 under grazing.

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Table 2.01. Mean, SD, RSQ, SEC, and SECV of analytical values for calibration sets

| Constituents | N | Mean | SD | SEC† | RSQ | SECV‡ | 1-VR§ |
|---|---|---|---|---|---|---|---|
| N | 59 | 3.79 | 1.75 | 0.22 | 0.98 | 0.32 | 0.97 |
| ADF | 57 | 32.44 | 9.60 | 1.14 | 0.99 | 1.38 | 0.98 |
| ADL | 51 | 3.15 | 1.60 | 0.21 | 0.98 | 0.31 | 0.96 |
| NDF | 56 | 57.40 | 11.44 | 3.02 | 0.93 | 3.95 | 0.88 |

† Standard error of calibration

‡ Standard error of cross validation

§ One minus variance ratio

Table 2.02. Monthly average temperature and precipitation during the months of September through May for two grazing seasons and the 30-year Normal in Tallassee, AL

| | Air temp. (°c) | Prec. (mm) | Air temp. (°c) | Prec. (mm) | Air temp. (°c) | Prec. (mm) |
|---|---|---|---|---|---|---|
| Months | 2010/2011† | | 2011/2012† | | 30 yrs Normal ‡ | |
| October | 18.7 | 31 | 16.4 | 25 | 11.6 | 78 |
| November | 13.5 | 52 | 13.3 | 83 | 6.5 | 125 |
| December | 5.1 | 59 | 9.4 | 123 | 2.9 | 113 |
| January | 5.4 | 57 | 9.2 | 111 | 1.5 | 119 |
| February | 10.1 | 100 | 10.9 | 101 | 3.2 | 126 |
| March | 14.9 | 139 | 17.6 | 81 | 6.7 | 154 |
| April | 19.2 | 49 | 18.2 | 24 | 10.1 | 102 |
| May | 21.7 | 56 | 22.5 | 176 | 15.6 | 103 |
| **Average** | 13.6 | 68 | 14.7 | 90 | 7.3 | 115 |
| **Sum** | | 543 | | 724 | | 919 |

† (http://www.awis.com/)

‡ (http://www.noaa.gov/)

Table 2.03. Important grazing dates, calibration periods, and yearly average

| Description | Year 2011 | Year 2012 |
|---|---|---|
| Seed sowing date | Mid October, 2010 | Mid October , 2011 |
| Grazing initiation date | January 20, 2011 | January 30, 2012 |
| Initial weight, kg | 262 ± 23.86 | 214 ± 21.53 |
| Fertilizer/ Irrigation | None | None |
| Grazing/ Calibration periods | | |
| Initial | Sowing-January19 | Sowing-January 29 |
| 1 | January 20-February 17 | January 30-February 29 |
| 2 | Feb 18- March 17 | Feb 29-March 28 |
| 3 | March18-April 14 | March 29-April 24 |
| Final | April 15-May 12 | April 25-May 22 |
| **Yearly average** | | |
| ADG (kg BW $d^{-1}$) | 1.32 | 1.20 |
| Gain $ha^{-1}$ (kg BW) | 717 | 639 |
| Forage availability (kg DM $ha^{-1}$) | 9304 | 8249 |
| TNC % DM | | 17.91 |
| Protein % DM | 20.54 | 22.97 |
| NDF % DM | 60.28 | 65.12 |
| ADF % DM | 35.56 | 38.03 |
| ADL % DM | 3.61 | 3.82 |

Table 2.04. Average Daily Gain (ADG) of the stockers grazing annual ryegrass cultivars at multiple stocking rate (SR) at the Beef Cattle Unit, Milstead, AL for two years.

| Cultivar/ population | 2011 | | | | 2012 | | | |
|---|---|---|---|---|---|---|---|---|
| | SR 3.7 | SR 4.9 | SR 6.1 | Mean | SR 3.7 | SR 4.9 | SR 6.1 | Mean |
| | ---------- kg $d^{-1}$ ---------- | | | | | | | |
| Cycle2 | 1.52 | 1.50 | 1.10 | 1.37 | 1.32 | 1.14 | 1.12 | 1.19 |
| Gulf | 1.38 | 1.47 | 0.95 | 1.26 | 1.42 | 1.10 | 1.10 | 1.20 |
| Marshall | 1.44 | 1.41 | 1.08 | 1.31 | 1.38 | 1.32 | 0.92 | 1.21 |
| SE | 0.072 | 0.066 | 0.075 | 0.04 | 0.117 | 0.116 | 0.112 | 0.05 |
| ***Difference vs. Cycle2*** | | | | | | | | |
| Gulf | 0.145 | 0.035 | 0.145 | 0.11 | -0.098 | 0.041 | 0.028 | -0.01 |
| Marshall | 0.082 | 0.092 | 0.013 | 0.06 | -0.058 | -0.178 | 0.200 | -0.01 |
| ***Dunnett's P vs. Cycle2*** | | | | | | | | |
| Gulf | 0.288 | 0.896 | 0.317 | 0.107 | 0.745 | 0.946 | 0.973 | 0.991 |
| Marshall | 0.639 | 0.540 | 0.985 | 0.411 | 0.898 | 0.408 | 0.312 | 0.986 |

Table2.05. Average Daily Gain (ADG) of the stockers grazing on annual ryegrass cultivars during January-May at the Beef Cattle unit, Milstead, AL for two years

| Cultivar/ population | 2011 | | | | 2012 | | | |
|---|---|---|---|---|---|---|---|---|
| | Period1 | Period2 | Period 3 | Final | Period 1 | Period 2 | Period 3 | Final |
| | ---------- kg $d^{-1}$ ---------- | | | | | | | |
| Cycle 2 | 1.44 | 1.36 | 1.65 | 1.04 | 1.58 | 1.35 | 1.39 | 0.46 |
| Gulf | 1.20 | 1.14 | 1.61 | 1.11 | 1.40 | 1.40 | 1.45 | 0.57 |
| Marshall | 1.32 | 1.27 | 1.46 | 1.20 | 1.42 | 1.30 | 1.50 | 0.61 |
| SE | 0.08 | 0.08 | 0.08 | 0.08 | 0.12 | 0.12 | 0.12 | 0.12 |
| ***Difference vs. Cycle 2*** | | | | | | | | |
| Gulf | 0.25 | 0.22 | 0.04 | -0.07 | 0.18 | -0.05 | -0.05 | -0.11 |
| Marshall | 0.12 | 0.09 | 0.19 | -0.15 | 0.16 | 0.05 | -0.10 | -0.14 |
| ***Dunnett's P vs. Cycle 2*** | | | | | | | | |
| Gulf | 0.046 | 0.086 | 0.913 | 0.762 | 0.443 | 0.936 | 0.919 | 0.708 |
| Marshall | 0.428 | 0.638 | 0.129 | 0.269 | 0.525 | 0.944 | 0.742 | 0.572 |

Table 2.06. Gain ha$^{-1}$ of the stockers grazing on annual ryegrass cultivars at multiple stocking rates (SR) at the Beef Cattle Unit, Milstead, AL for two years.

| Cultivar/ population | 2011 | | | | 2012 | | | |
|---|---|---|---|---|---|---|---|---|
| | SR 3.7 | SR 4.9 | SR 6.1 | Mean | SR 3.7 | SR 4.9 | SR 6.1 | Mean |
| | kg ha$^{-1}$ | | | | | | | |
| Cycle 2 | 631 | 830 | 758 | 740 | 549 | 602 | 786 | 645 |
| Gulf | 571 | 811 | 655 | 679 | 588 | 608 | 769 | 655 |
| Marshall | 597 | 779 | 750 | 708 | 570 | 725 | 655 | 650 |
| SE | 38 | 38 | 38 | 38 | 61 | 61 | 61 | 61 |
| ***Difference vs. Cycle 2*** | | | | | | | | |
| Gulf | 60 | 19 | 103 | 61 | -39 | -6 | 17 | -9 |
| Marshall | 34 | 51 | 9 | 31 | -21 | -123 | 130 | -4 |
| ***Dunnett's P vs. Cycle 2*** | | | | | | | | |
| Gulf | 0.432 | 0.906 | 0.121 | 0.487 | 0.867 | 0.997 | 0.973 | 0.946 |
| Marshall | 0.746 | 0.532 | 0.980 | 0.753 | 0.960 | 0.290 | 0.254 | 0.501 |

Table 2.07. Available forage during paddock grazing on annual ryegrass cultivars at multiple stocking rates (SR) at the Beef Cattle Unit, Milstead, AL for 2011 and 2012

| Cultivars/ Population | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | SR 3.7 | SR 4.9 | SR 6.1 | SR 3.7 | SR 4.9 | SR 6.1 |
| | ----------Kg ha$^{-1}$---------- | | | | | |
| Cycle 2 | 1923 | 1993 | 926 | 2877 | 1626 | 1832 |
| Gulf | 1945 | 1399 | 1044 | 1966 | 2345 | 1672 |
| Marshall | 2406 | 1262 | 1079 | 2537 | 2306 | 1399 |
| SE | 120 | 120 | 120 | 389 | 389 | 389 |
| ***Difference vs. Cycle 2*** | | | | | | |
| Gulf | -21 | 594 | -117 | 912 | -719 | 160 |
| Marshall | -483 | 731 | -153 | 340 | -680 | 432 |
| ***Dunnett P's vs. Cycle 2*** | | | | | | |
| Gulf | 0.988 | 0.002 | 0.712 | 0.172 | 0.310 | 0.934 |
| Marshall | 0.011 | 0.000 | 0.571 | 0.743 | 0.346 | 0.627 |

Table 2.08. Available forage from annual ryegrass cultivars grown in paddocks during January-May at the Beef Cattle Unit, Milstead, AL for 2011 and 2012.

| Cultivar/ Population | 2011 | | | | | 2012 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | Period 1 | Period 2 | Period 3 | Final | Initial | Period 1 | Period 2 | Period 3 | Final |
| | ----------Kg DM $ha^{-1}$---------- | | | | | | | | | |
| Cycle 2 | 1241 | 1578 | 1682 | 2368 | 2321 | 970 | 2303 | 2916 | 2257 | NA |
| Gulf | 1260 | 1102 | 1138 | 1975 | 2682 | 1443 | 1945 | 2166 | 2422 | NA |
| Marshall | 1203 | 1394 | 1431 | 2109 | 2600 | 1821 | 1915 | 2085 | 2502 | NA |
| SE | 57 | 114 | 112 | 138 | 149 | 279 | 279 | 764 | 471 | NA |
| ***Difference vs. Cycle 2*** | | | | | | | | | | |
| Gulf | -19 | 476 | 544 | 393 | -361 | -473 | 358 | 750 | -165 | NA |
| Marshall | 38 | 184 | 251 | 260 | -279 | -851 | 388 | 832 | -245 | NA |
| ***Dunnett P's vs. Cycle 2*** | | | | | | | | | | |
| Gulf | 0.961 | 0.009 | 0.003 | 0.090 | 0.163 | 0.027 | 0.103 | 0.686 | 0.941 | NA |
| Marshall | 0.854 | 0.420 | 0.210 | 0.317 | 0.321 | 0.000 | 0.073 | 0.632 | 0.874 | NA |

Table 2.09. Total non-structural carbohydrates content of annual ryegrass during a January-May grazing period at the Beef Cattle Unit, Milstead, AL for 2012.

| Cultivar/Population | Initial | Period 1 | Period 2 | Period 3 | Final |
|---|---|---|---|---|---|
| | ---------- | ---------- | % DM | ---------- | ---------- |
| Cycle 2 | 26.72 | 19.98 | 13.04 | 16.14 | 14.46 |
| Gulf | 27.61 | 18.13 | 9.92 | 16.16 | 13.10 |
| Marshall | 29.69 | 18.76 | 12.02 | 19.22 | 13.82 |
| Average | 28.01 | 18.96 | 11.66 | 17.18 | 13.79 |
| SE | 1.69 | 2.55 | 1.08 | 1.45 | 0.94 |
| ***Dunnett P's vs Cycle 2*** | | | | | |
| Gulf | 0.890 | 0.818 | 0.019 | 1.000 | 0.237 |
| Marshall | 0.311 | 0.916 | 0.578 | 0.159 | 0.696 |

Table 2.10. Periodic Nutritive value (CP, NDF, ADF, and ADL) of annual ryegrass pastures under grazing at the Beef Cattle Unit, Milstead, AL. for two grazing years. Values are predicted through Near Infrared Reflectance Spectrometry (NIRS).

| Cutivar/ Population | 2011 | | | | 2012 | | | |
|---|---|---|---|---|---|---|---|---|
| | CP | ADF | NDF | ADL | CP | ADF | NDF | ADL |
| | ----------% DM---------- | | | | | | | |
| ***Initial*** | | | | | | | | |
| Cycle 2 | 31.95 | 46.87 | 23.89 | 2.14 | 27.54 | 55.35 | 26.89 | 2.28 |
| Gulf | 32.54 | 48.03 | 24.61 | 2.22 | 25.63 | 56.40 | 29.00 | 2.35 |
| Marshall | 33.48 | 47.64 | 25.52 | 2.11 | 27.25 | 55.41 | 26.77 | 2.28 |
| SE | 0.31 | 1.51 | 0.95 | 0.16 | 0.20 | 1.51 | 1.03 | 0.15 |
| ***Dunnett P's vs Cycle 2*** | | | | | | | | |
| Gulf | 0.920 | 0.699 | 0.770 | 0.902 | 0.459 | 0.815 | 0.235 | 0.930 |
| Marshall | 0.575 | 0.846 | 0.276 | 0.983 | 0.980 | 0.999 | 0.994 | 1.000 |
| ***Period 1*** | | | | | | | | |
| Cycle 2 | | | | | 24.90 | 57.13 | 34.24 | 2.74 |
| Gulf | | | | | 22.78 | 56.79 | 34.90 | 2.68 |
| Marshall | | | | | 22.74 | 55.11 | 35.28 | 2.64 |
| SE | | | | | 0.21 | 1.52 | 1.04 | 0.15 |
| ***Dunnett P's vs Cycle 2*** | | | | | | | | |
| Gulf | | | | | 0.384 | 0.977 | 0.852 | 0.931 |
| Marshall | | | | | 0.382 | 0.492 | 0.684 | 0.836 |

| Cutivar/ Population | 2011 | | | | 2012 | | | |
|---|---|---|---|---|---|---|---|---|
| | CP | ADF | NDF | ADL | CP | ADF | NDF | ADL |
| | ----------% DM---------- | | | | | | | |
| ***Period 2*** | | | | | | | | |
| Cycle 2 | 20.51 | 55.37 | 32.28 | 2.64 | 24.52 | 69.75 | 40.83 | 3.90 |
| Gulf | 22.61 | 55.88 | 33.41 | 2.55 | 25.59 | 67.81 | 40.15 | 3.52 |
| Marshall | 26.51 | 54.11 | 32.34 | 2.42 | 28.29 | 64.81 | 38.35 | 3.34 |
| SE | 0.31 | 1.55 | 0.97 | 0.17 | 0.25 | 1.90 | 1.33 | 0.18 |
| ***Dunnett P's vs Cycle 2*** | | | | | | | | |
| Gulf | 0.390 | 0.936 | 0.544 | 0.862 | 0.798 | 0.561 | 0.865 | 0.160 |
| Marshall | 0.001 | 0.647 | 0.998 | 0.429 | 0.148 | 0.089 | 0.278 | 0.050 |
| ***Period 3*** | | | | | | | | |
| Cycle 2 | 15.62 | 65.92 | 39.34 | 3.85 | 19.23 | 71.53 | 41.89 | 5.01 |
| Gulf | 15.94 | 65.92 | 38.72 | 3.70 | 19.59 | 69.35 | 40.87 | 4.41 |
| Marshall | 17.61 | 62.59 | 36.78 | 3.51 | 20.06 | 65.98 | 38.51 | 4.04 |
| SE | 0.31 | 1.50 | 0.93 | 0.16 | 0.26 | 1.93 | 1.35 | 0.19 |
| ***Dunnett P's vs Cycle 2*** | | | | | | | | |
| Gulf | 0.974 | 1.000 | 0.815 | 0.639 | 0.974 | 0.488 | 0.727 | 0.018 |
| Marshall | 0.404 | 0.077 | 0.057 | 0.138 | 0.898 | 0.054 | 0.112 | 0.001 |

| Cutivar/ Population | 2011 | | | | 2012 | | | |
|---|---|---|---|---|---|---|---|---|
| | CP | ADF | NDF | ADL | CP | ADF | NDF | ADL |
| | ----------% DM---------- | | | | | | | |
| ***Final*** | | | | | | | | |
| Cycle 2 | 9.88 | 74.02 | 47.37 | 6.11 | 17.82 | 77.99 | 48.62 | 6.35 |
| Gulf | 8.39 | 74.96 | 47.99 | 6.38 | 18.56 | 77.80 | 48.36 | 6.22 |
| Marshall | 11.39 | 72.00 | 44.41 | 5.68 | 20.09 | 75.67 | 45.87 | 5.58 |
| SE | 0.33 | 1.68 | 1.09 | 0.19 | 0.25 | 1.87 | 1.31 | 0.18 |
| ***Dunnett P's vs Cycle 2*** | | | | | | | | |
| Gulf | 0.667 | 0.831 | 0.861 | 0.388 | 0.895 | 0.994 | 0.979 | 0.786 |
| Marshall | 0.632 | 0.414 | 0.046 | 0.091 | 0.456 | 0.534 | 0.210 | 0.005 |
| ***Mean*** | | | | | | | | |
| Cycle2 | 23.11 | 57.50 | 32.51 | 3.32 | 22.80 | 66.35 | 38.49 | 4.06 |
| Gulf | 23.38 | 58.47 | 33.24 | 3.35 | 22.43 | 65.63 | 38.65 | 3.84 |
| Marshall | 26.19 | 56.46 | 31.59 | 3.08 | 23.69 | 63.39 | 36.95 | 3.57 |
| SE | 1.67 | 1.20 | 0.64 | 0.11 | 0.87 | 1.04 | 0.67 | 0.10 |
| ***Dunnett P's vs. Cycle 2*** | | | | | | | | |
| Gulf | 0.957 | 0.542 | 0.476 | 0.900 | 0.925 | 0.733 | 0.967 | 0.198 |
| Marshall | 0.039 | 0.480 | 0.306 | 0.085 | 0.690 | 0.070 | 0.188 | 0.012 |

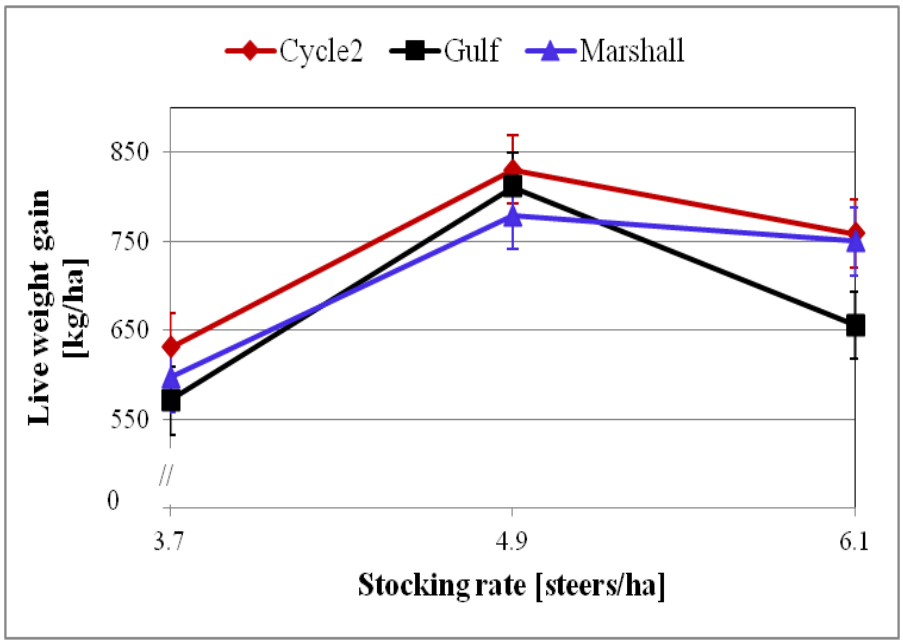


Figure 2.01. Animal gain $ha^{-1}$ on annual ryegrass cultivars under grazing at multiple stocking rate at the E.V. Smith Research Center, Beef Cattle Unit, AL for grazing 2011

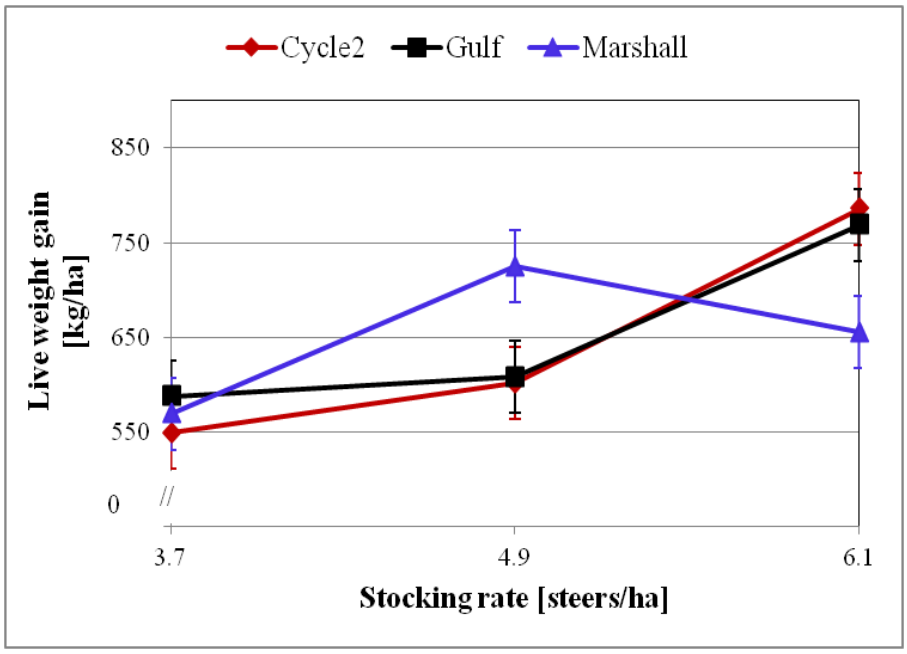


Figure 2.02. Animal gain $ha^{-1}$ on annual ryegrass cultivars under grazing at multiple stocking rate at the E.V. Smith Research Center, Beef Cattle Unit, AL for grazing 2012.

# Correlated Response and Ploidy Level in Annual Ryegrass (*Lolium multiflorum* Lam.) Selected for Improved Winter Productivity.


## Abstract

The availability of forage during winter is limited in the southeastern USA. Thus animals need to be fed with stored feed which increases the management costs. Therefore, a phenotypic recurrent selection breeding approach was used to improve the winter productivity in annual ryegrass which will be of great benefit to cattle producers. So far seven selection cycles have been completed. Selection for a targeted trait may alter other non-target traits that may be genetically correlated. The understanding of change in non-targeted physiological traits as a result of selection pressure provides important information for breeding improved cultivars. Thus, this study was conducted to explore the change in heading date, tiller angle and ploidy level among the populations selected for increased winter productivity. The ploidy level was a matter of concern because the parental cultivars used for recombination consisted of a mixture of diploid and tetraploids. The data for tiller angle and heading date were recorded from a seed increase nursery which contained two replicated blocks of 8 cycles arranged randomly in 8 plots with 210 subsamples per plot. The tiller angle (angle made by tiller with horizontal surface) increased linearly for the first three cycles ($P < 0.0001$) and remained constant afterward. The heading date of the selection cycles followed a linear-plateau-slope model. It decreased linearly for first

selection cycle ($P < 0.0001$) and then remained constant for the next three selection cycles and again decreased linearly for the last three cycles ($P = 0.0116$). The ploidy was tested using BD Accuri flow cytometry for selection cycle 0 and 2. Not a single tetraploid plant was found in Cycle 2 ($C_2$) and only 17 tetraploids were detected in $C_0$. Selection for increased winter productivity resulted in elimination of tetraploids by $C_2$. These results provide important information that the $C_2$ population are homogenous in ploidy (diploid) and are suitable for release as a cultivar. Genetic differentiation of dry matter (DM) yield in response to selection for increased winter productivity could have resulted in more erect plants with early heading date and homogenous ploidy.

## Introduction

Annual ryegrass is a widely cultivated, fast growing cool season forage crop in southeastern USA. However, availability of standing forage is limited during the winter months and cattle are fed stored forage or grain, which increases management costs (Ball *et al.*, 2002). In the southeastern USA, annual ryegrass is generally sown in autumn but attains maximum growth in spring (Redfearn *et al.*, 2002). Shifting the DM yield to winter months through plant breeding may alleviate the winter deficiency of standing forage in this region. For that purpose, a phenotypic recurrent selection program was initiated in 2005 at Auburn University.

Dry matter yield and seed yield are quantitative traits governed by multiple loci that have predominantly additive genetic variability (Moll and Stuber, 1974). Therefore, selection for DM yield may alter other non-selected agronomic traits as well. Such change in a non-selected trait while selecting for the trait of interest is called a correlated response.

A study in annual ryegrass showed a low negative correlation (r = -0.194 to -0.045) between heading date and lodging score at all growth stages and a high negative correlation (r = -0.240) with tiller number (Inoue *et al.*, 2004). Similarly in other studies, positive correlations of r = 0.36 (de Araujo *et al.*, 2002) and r = 0.27 (de Araujo and Coulman, 2004) existed between DM yield and seed yield in meadow brome grass (*Bromus riparius* Rehm) progenies and clones, respectively. The erectness of a population as well as an individual plant is controlled by genetic factors (Warwick and Briggs, 1978). Cultivars with erect leaves have a higher optimum leaf area index (LAI) than those with horizontal leaves. Canopy respiration increases curvilinearly and photosynthesis increases linearly with LAI. Plants with an erect growth habit accumulate more DM yield due to the exposure of optimum leaf area to sunlight. In rice, the erect tillering plants had higher yield than spreading types grown at close spacing (Tanaka *et al.*, 1966).

The first harvest of the second cycle ($C_2$) of selection resulted in increased DM production by an average of 300 kg $ha^{-1}$, increased erectness, and decreased heading date compared to the base population ($C_0$) (Dhaliwal *et al.*, 2009), but the effect on subsequent selection cycle populations is unknown. Thus, the first part of this study was to determine change in tiller angle and heading date on selection cycles $C_0$- $C_7$ selected for higher winter DM production in annual ryegrass.

Annual ryegrass is a self-incompatible and a highly cross-pollinated species (Fearon *et al.*, 1983). It is naturally diploid (2n = 2x = 14), however, tetraploids (2n = 4x = 28) have been developed to improve its forage quality and productivity. Artificial tetraploids in annual ryegrass can be formed by colchicine treatment (Shalygin, 1941). The

induced tetraploids produced less dry matter yield than their diploid counterparts if no selection was imposed. However, they produced equal dry matter to that of mother strains in the third generation of mass selection and were superior by 8% both in green and dry matter yield in the second generation of maternal line selection (Wit, 1958).

Traditionally, ploidy level has been determined by counting the chromosome number in meristematic tissue or microspore mother cells of individual plants. It can also be estimated by observing the pollen size or length of the epidermal guard cells (DeLaat *et al.*, 1987). These processes are labor intensive and less accurate especially when the ploidy difference is small. Flow cytometry is a more rapid and accurate technique to determine ploidy and nuclear DNA content in plant cells (Barker *et al.*, 2001; Galbraith *et al.*, 1983) than pollen size or guard cell length. The use of flow cytometry also provides an opportunity for analyzing plants in groups (bulk analysis), which is highly advantageous to screen large numbers of samples quickly.

The Federal Seed Act of USA (1995) has the provision that a cultivar for domestic sale has to be at least 98% of the reported ploidy level. Therefore, it is necessary to determine the ploidy level of any newly developed cultivar before it is released. Ploidy homogeneity is important because natural hybridization between cultivars of different ploidy level may result in genetic instability, poor germination, infertility and lower seed set (Griffiths *et al.*, 1971).

The dry matter productivity of tetraploid and diploid cultivars is region specific. In a study done by Nelson *et al.* (2006) to compare forage yield of diploid and tetraploid cultivars in southeastern USA they observed mixed results. Tetraploid cultivars were

superior in forage biomass in the southern part and diploids produced more in the northern part of Georgia, Louisiana, and Texas. But in Alabama, diploid cultivars produced higher forage biomass than tetraploids both in northern and southern trials.

We are now in the last stage of the evaluation phase and in the process of releasing $C_2$ as a cultivar. But, the parental cultivars that were initially used contained diploid and tetraploids in a ratio of 2:1. It raised the concern of a possible mixture of diploids and tetraploids in the selection cycles. Therefore, the second part of this study was to determine the ploidy of different selection cycles using flow cytometry and to rogue tetraploids (if any) from $C_2$ and onwards before anthesis to make homogenous diploid populations.

## Materials and Methods

### Establishment of seed increase nursery

In September 2012, $C_0$-$C_7$ populations were seeded in conetainers filled with mixture of peat and sand (1:1 by volume) in the greenhouse at the Plant Science Research Center, Auburn, AL. A single seedling was maintained in each conetainer by thinning 15 days after seeding. In November 2012, 420 seedlings from each selection cycle were transplanted to the seed increase nursery plots at the E.V. Smith Research Center, Plant Breeding Unit, Tallassee, AL. Each population ($C_0$ - $C_7$) with 210 plants as subsamples was randomly assigned to one of eight plots within each of two replicate blocks. Seedlings were planted at 90 cm center spacing. We established a 10 m border surrounding all sides of each plot by sowing cereal rye (*Secale cereale L.* cv. Wren's Abruzzi), which is at least 60 cm taller than annual ryegrass at the time of anthesis. This prevented pollen flow among populations.

## Traits measured

### *Heading date*

Heading date for individual plants was noted by visiting the field every three days during the boot stage (April-May). The heading date for a plant was the day when at least five spikes had fully emerged out of the boot.

### *Tiller angle*

Tiller angle for each plant was measured after anthesis in May with the help of a protractor mounted at the end of a stick. The angle made by outermost tiller with the horizontal ground surface was regarded as the tiller angle of that particular plant.

### *Ploidy*

We used flow cytometry to determine tetraploid plants in our selection cycle. A detailed analysis was conducted for cycles $C_0$ and $C_2$ using plants from the seed increase nursery plots. This work was done in cooperation with USDA-ARS Research Geneticist, Dr. Karen R Harris-Shultz at Crop Genetics and Breeding Research Unit, Tifton, GA.

Samples for ploidy testing were collected from the seed increase nursery plots in March 2013. Approximately 5 cm of leaf blade was clipped from individual plants from both plots of cycle ($C_0$ and $C_2$) and then kept in a polythene bag. The samples for internal standards (Marshall and Tetrastar) were raised inside the greenhouse at the Plant Science Research Center, Auburn, AL. The seeds were sown in polythene pots (15 cm diameter) filled with mixture of peat and sand (1:1 by volume) during the last week of November 2012. A handful of leaves were clipped from Marshall and Tetrastar and were used as

internal standards. All the samples were immediately kept in an ice cooler to prevent loss of moisture during transportation from field to lab.

We followed the modified procedure described by Galbraith *et al.* (2009) for the determination of ploidy in our selection cycles $C_0$ and $C_2$. Twenty to thirty mg of plant samples were cochopped with Marshall or Tetrastar in a 60 mm petri-dish and one ml of freshly prepared Tris-$MgCl_2$ nuclei extraction buffer supplemented with 0.1% w/v Triton (Pfosser *et al.*, 1995) was added to it. The samples were chopped using a double-edged razor blades. The suspension was then filtered through 50 µm Partec CellTrics filters (Partec GmbH, Münster, Germany) to separate debris from the nuclei and were collected in 12 × 75 mm culture tubes. Then 0.5 ml of Rnase/Propidium Iodide solution (BD Biosciences, San Jose, CA, USA) was added to the filtrate and mixed thoroughly. The solution was allowed to stand on ice for 15 min so that the nuclei would be properly stained.

A BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA) was used for ploidy testing. The machine was set at a custom flow rate of 11µL $min^{-1}$. The gating was done on the first run of Marshall co-chopped with Tetrastar, such that it included events that showed a high correlation between FL2-A and FL3-A signals. At least 30,000 events were collected for each run.

Five plants were initially bulked with Marshall per run. Equal amounts of leaf (about 1 cm) from each of the samples and internal standard Marshall (about 1 cm) were chopped together to prepare homogenate such that the ratio of samples to standard was 5:1 by weight. Repeated blind preliminary tests with either six samples of Marshall or five samples of Marshall plus one part Tetrastar indicated that the sample containing the

tetraploid was identified in every case. The mean and CV of FL2-A were recorded for Gap1 (G1) and Gap2 (G2) for each run. Ploidy level was determined by comparing number of counts under each peak and mean FL2-A position of the G1 and G2 peaks.

The presence of tetraploid/s in bulk analysis was suspected if the G2 peak attained a height one fifth or more than that of the G1 peak. The count of nuclei in each peak, which is directly proportional to the height, provided more precise criteria for determining the presence of tetraploidy in a group. For a group of five for which a presence of tetraploid plants was suspected, each individual sample from the bulk was analyzed again on its own. An equal amount of leaves from individual sample and Marshall (1:1) were co-chopped and then prepared to run in the flow cytometer. Diploid samples produced only two peaks (G1, G2) and were detected easily as the G1 peak had a fluorescence signal far larger than $G_2$ peak. The run which produced three peaks of which first two peaks were of nearly same height or second peak higher than first one indicated the sampled plant was a tetraploid. The first peak corresponds to the G1 of diploid, the second peak corresponds to the combined G1 peak of tetraploid and the G2 of the diploid, and the third one corresponds to the G2 peak of the tetraploid. Co-chopping a suspected tetraploid with the internal standard Tetrastar was the confirmatory test. Presence of a single G1 peak confirmed the plant to be a tetraploid.

**Statistical analyses**

The experiment was designed in two replicated blocks of 8 cycles arranged randomly in 8 plots with 210 plants as subsamples per plot. Even though we took observations from each plant individually, the analysis was done on a plot mean basis.

Since the rate of change in response (heading date and tiller angle) was not constant over the cycles, fitting data on simple linear regression model showed lack of fit. Therefore, segmented linear mixed model approach was applied to fit the model. The heading data were fitted in Linear plateau slope (LPS) model and tiller angle data were fitted in linear plateau (LP) model using NLMIXED procedure in SAS 9.2 (SAS Inst. Inc., Cary, NC) as described by Schabenberger and Pierce (2002). This approach gave a better fit than the SLR model as indicated by smaller AICC values.

## Result and Discussion

### Tiller angle

Selection for increased winter productivity in annual ryegrass affected the heading date and tiller angle (angle made by outer tiller with the horizontal surface) in subsequent generations/ cycles. The tiller angle (angle made by tiller with horizontal surface) increased linearly for first three cycles (Figure 3.01, $P < 0.0001$) and remained constant afterward. There was an abrupt increase in tiller angle from 40° in base population to 47° in $C_3$ and remained almost constant from $C_4$ onwards. This pattern indicates that selection for higher winter productivity resulted in more erect plants. A similar pattern was obtained in a study done by Dhaliwal *et al*. (2009) for the first three selection cycle in annual ryegrass.

Since plant DM is associated with tiller angle, our selection protocol which is based on DM yield should have extemporaneously selected for more erect plants. In a study of competitive ability of cowpea genotypes, erect plant type resulted in more biomass yield because of its taller stature and greater height growth rate (Wang *et al.*, 2006a; Wang *et al.*, 2006b). Erect families showed the tendency to flower earlier than prostrate families in *Poa. annua* L. (Warwick and Briggs, 1978). Also selection for early maturity resulted in a

prostrate type plant habit in orchardgrass (*Dactylis glomerata* L.) (Inoue *et al.*, 2004; Short and Carlson, 1989).

**Heading date**

The heading date of the selection cycles followed a linear plateau slope model. It decreased linearly for first selection cycle ($P < 0.0001$), remained constant for next four selection cycles, and then decreased linearly for last three cycles (Figure 3.02, $P = 0.0116$). Heading date (from January first) for the base population was 118 days, for second cycle was 114 days, and for seventh cycle was 110 days (Figure 3.02). Dhaliwal *et al*. (2009) selecting on annual ryegrass found a rapid decrease in heading days from the base population to $C_1$ by 8 days and no significant change in cycle 2 and 3. In our study, the later decrease in heading date may be because of differences in the method of selection and selection intensity. The first five cycles were selected on DM basis with 4% selection intensity whereas the later three cycles were selected on green mass (GM) basis with a 2.8% selection intensity.

Dry matter weight had a high genetic correlation (r = - 0.99) with heading date in annual ryegrass (Fujimoto and Suzuki, 1975a; Fujimoto and Suzaki, 1975b). Our selection was based on higher DM yield for early productivity, which indirectly excluded late maturing plants from being selected for recombination. The non-synchronized flowering behavior of annual ryegrass prevents pollen from late maturing plants to contribute for the seed set. Thus, the selection of DM for early productivity contributed to early-heading populations.

**Ploidy**

Ploidy level of all individual plants in $C_0$ and $C_2$ were tested using a BD Accuri flow cytometer. Out of 420 total plants in $C_0$, 17 plants were tetraploid. Selection for early DM eliminated all tetraploid plants in $C_2$. All of the 420 plants in $C_2$ were purely diploids (Table 3.01).

When the diploid and tetraploid populations of perennial ryegrass were allowed to open pollinate together, no triploids were identified in the $C_2$ progeny. There are no barriers to fertilization between diploid and tetraploid plants but the seed and endosperm development of the resulting triploids has been shown to be poor (Griffiths *et al.*, 1971). This indicates that genetic incompatibility of x and 2x gametes for producing viable zygotes exists (Galbraith *et al.*, 1983). Thus, the triploids were eliminated from the population by natural selection.

The forage yield of diploid cultivars in Alabama was greater than that of tetraploids (Nelson *et al.*, 2006). Consequently, the selection criteria we imposed based on DM yield may have eliminated tetraploids from selection cycles as early as the second generation. Since all the plants in $C_2$ were diploid, plants selected from $C_2$ should have the same ploidy level and further testing on subsequent cycles was not done.

## Summary

The understanding of the indirect changes in traits as a result of selection pressure is essential for managing genetic resources and developing improved cultivars. Phenotypic recurrent selection for increased winter productivity in annual ryegrass resulted in erect plants with earlier heading date. The rapid change in heading date from base population to

$C_1$ could be due to exclusion of the late maturing seeds from $C_0$ to form $C_1$. The later decline in heading date from $C_4$ after gaining constant heading date might be because of change in selection method from DM 4% intensity to GM 2.8% intensity. Erectness in plant habit may be associated with selection for higher yield. Generally, erect plants are photosynthetically more active than prostrate ones. Diploid cultivars perform better in Alabama than tetraploids and selection for higher DM eliminated tetraploids from the population in $C_2$.

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Table 3.01. Number and percentage of diploid and tetraploid plants identified by flow cytometry in selection cycle 0 and cycle 2 selected for improved winter productivity in annual ryegrass.

| Population/ Plot no. | Total N | Diploid | | Tetraploid | | 4x plants identified by Column and Row in each plot |
|---|---|---|---|---|---|---|
| | | N | % | N | % | |
| ***Cycle 0*** | | | | | | |
| 103 | 210 | 196 | 93.3 | 14 | 6.7 | C1R14, C2R4, C2R15, C4R13, C6R6, C6R12, C9R8, C9R10, C9R13, C10R14, C10R15, C13R6, C13R8, C13R13 |
| 202 | 210 | 207 | 98.6 | 3 | 1.4 | C5R12, C10R15, C14R12 |
| Total | 420 | 403 | 96.0 | 17 | 4.0 | |
| ***Cycle 2*** | | | | | | |
| 106 | 210 | 210 | 100 | 0 | 0 | |
| 207 | 210 | 210 | 100 | 0 | 0 | |
| Total | 420 | 420 | 100 | 0 | 0 | |

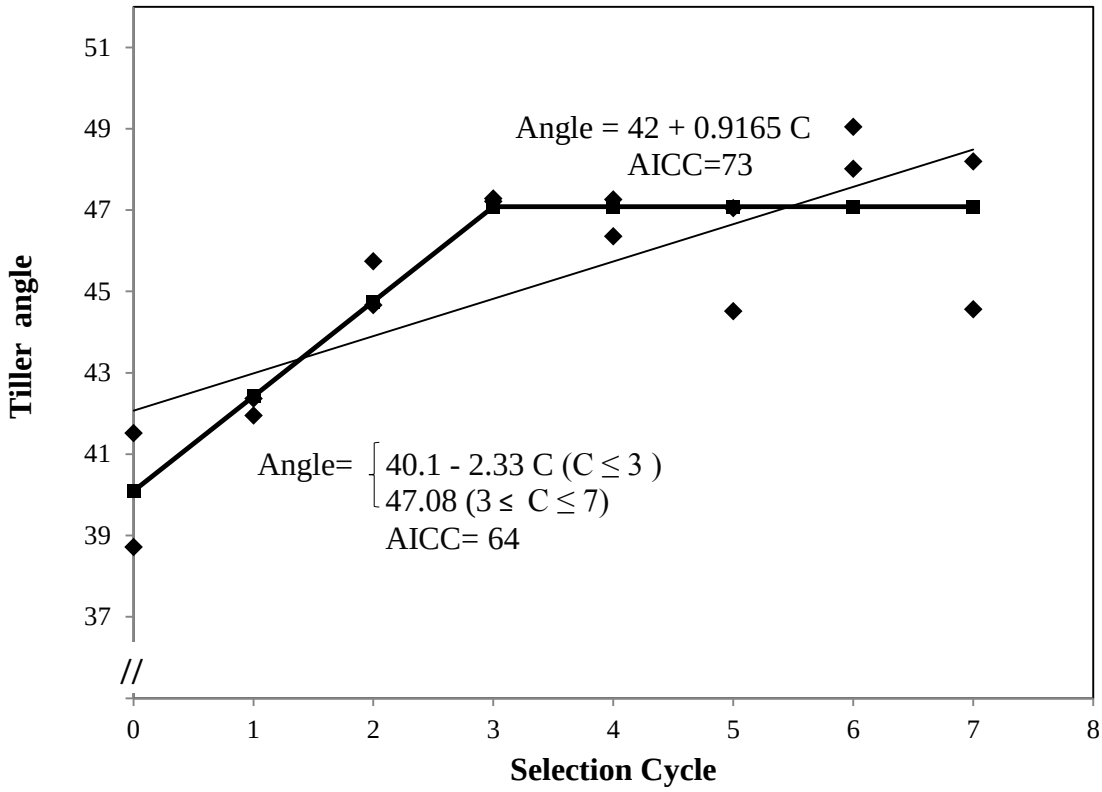


Figure 3.01. Changes in tiller angle of annual ryegrass over seven cycles of phenotypic recurrent selection for winter dry matter or winter green matter yield observed at seed increase nursery in year 2013. The 'C' in the figure indicates selection cycle.

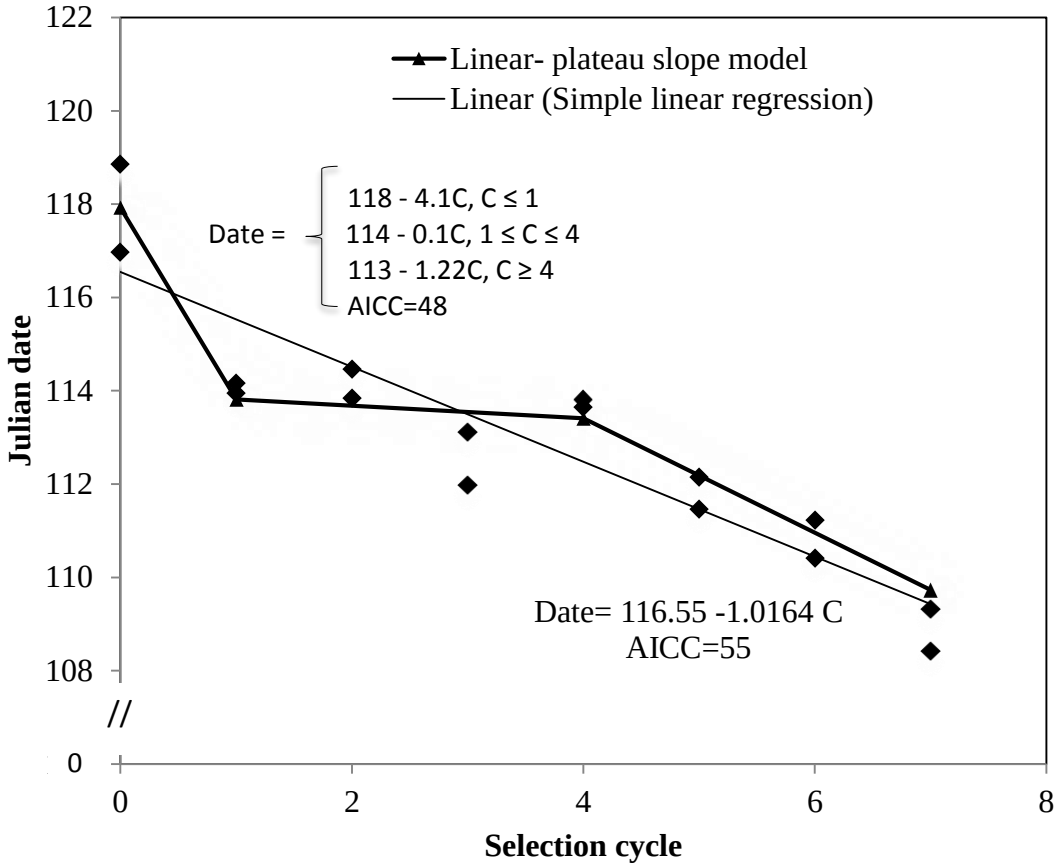


Figure 3.02. Changes in heading date of annual ryegrass selected for increased winter productivity observed at seed increase nursery in year 2013. The 'C' in the figure indicates selection cycle.

# Effect on TNC Content on Annual Ryegrass (*Lolium multiflorum* Lam.) Selected for Increased Winter Productivity


## Abstract

The availability of forage during winter is limited in southeastern USA, which increases the management costs. Therefore, a phenotypic recurrent selection breeding approach was used to improve the winter productivity in annual ryegrass. The grazing trial conducted on Cycle 2 ($C_2$) from this program supported higher average daily gain (ADG) in steers than cvs. Gulf and Marshall which may be due to higher soluble sugar content in $C_2$ populations. Thus, this study was conducted to observe the effect on total non-structural carbohydrate content due to selection for increased winter productivity. The experiment was conducted at the Plant Science Research Center, Auburn, AL in randomized block design at two locations with consideration of repeated measure. Total non-structural carbohydrates (TNC) content of selection cycles $C_0$-$C_6$ populations along with check cvs. Gulf, Marshall, and Shiwasuaoba were determined inside and outside a greenhouse. There was no significant change in TNC and green biomass content due to selection. The TNC and green biomass were highly affected by location with higher TNC in plants grown outside a greenhouse and higher green biomass with plants grown inside greenhouse. There were no differences in seasonal TNC content between $C_2$ and Marshall / Shiwasuaoba at either location. Marshall contained higher TNC than $C_2$ and cv. Gulf on third harvest at both locations. The TNC content of $C_2$ did not differ from the commercial cultivars nor

was there any differences seen on subsequent selection cycles. Thus, the higher ADG on steers grazing on $C_2$ was likely not associated with TNC content.

## Introduction

Total non-structural carbohydrates are the end product of photosynthesis and are found in plants in various forms from simple sugars to complex sugar linkages. Total non-structural carbohydrates are comprised of water soluble carbohydrates (WSC) and water insoluble carbohydrates (Holt and Hilst, 1969). WSC are the photosynthetic products that provide readily available dietary energy for the growth and maintenance of ruminants (Danckwerts and Gordon, 1987). Water insoluble carbohydrates also known as stored carbohydrates (fructans and starch) are used for regrowth by the plant. Temperate grasses predominantly store reserve carbohydrates in the form of fructans (Smith, 1973). They usually contain higher TNC (Chatterton *et al.*, 1989) and consequently result in better nutrient utilization in ruminants than warm season forages (Burns *et al.*, 2005; Lee *et al.*, 2002; Moorby *et al.*, 2006).

The concentration of storage carbohydrates in forages show diurnal variation (Holt and Hilst, 1969) as well as seasonal variation (Waite and Boyd, 1953). The concentration of soluble carbohydrate reserves is the basis for efficient pasture management of perennial ryegrass. The regrowth of the pasture after defoliation is proportional to the reserve carbohydrates present in the forage before defoliation (Donaghy and Fulkerson, 1997).

Harvesting forage at the proper stage is important for efficient forage utilization and re-growth. Ryegrass attains its maximum vegetative dry matter (DM) production and

stores the highest reserve carbohydrates at the three leaves per tiller stage (Davies, 1965). With initiation of the fourth leaf the oldest leaf senesces (Davies, 1971). Thus, grazing ryegrass pasture younger or older than three leaves per tiller results in a loss of pasture quality as well as quantity (Fulkerson and Donaghy, 2001). Similarly, forage quality and quantity depend on defoliating height. Maximum pasture yield and utilization is achieved when the pasture is grazed or cut 5 cm above the soil surface (Parsons and Chapman, 2000). Defoliating *Lolium* spp. below 5 cm removes stored carbohydrate reserves resulting in reduced re-growth and tillering (Fulkerson and Slack, 1995). Consequently, the forage from regrowth after defoliation below 5 cm has a high potassium and nitrate content but low in WSC (Fulkerson and Donaghy, 2001). On the other hand, defoliation above 10 cm from the soil surface reduces the interception of incident light and thus the number of tillers and green leaf decreases and the proportion of dead material increases (Hunt and Brougham, 1967). Temperate grasses store reserve carbohydrate in stubble (Fulkerson and Slack, 1994) and it varies in different plant parts in addition to stubble (Vartha and Bailey, 1980). The WSC content, which is a subset of TNC, was found inconsistent among annual ryegrass cultivars but it decreased consistently as the growing season progressed from December to May (Myer *et al.*, 2010).

The concentration of TNC in forages is not generally considered as a selection criterion in forage breeding programs (Wilkins and Humphreys, 2003) but it may be indirectly affected by selection for other traits. Cycle 2 of a selection program for increased winter productivity in annual ryegrass supported higher ADG in steers. One probable reason for increased ADG may be associated with increased TNC level. Thus, the objective of this study was to determine the effect on TNC content of annual ryegrass populations

selected for increased winter productivity. Progress from selection was evaluated and Cycle 2 was compared to check cvs. Gulf, Marshall, and Shiwasuaoba. Gulf, an early maturing cultivar with crown rust resistance (Weihing, 1963) and Marshall, a late maturing cultivar with cold tolerance (Arnold *et al.*, 1981) are the most commonly cultivated cultivars in the southeastern USA. Shiwasuaoba, an early maturing cultivar was included as a treatment because of its early flowering behavior (Kindiger *et al.*, 2004) and is suitable for early spring productivity.

## Materials and Methods

### Establishment and management of plants

Selection cycles $C_0$ - $C_6$ populations along with check cultivars Gulf, Marshall and Shiwasuaoba were seeded in 8" diameter polythene pots filled with mixture of peat and sand (1:1 by volume) during first week of September in 2011 inside a greenhouse at Plant Science Research Center, Auburn, AL. Seeding rate was 50 seeds per pot and thinning was done 15 days after sowing. Initially, all plants were raised inside a greenhouse in ten replicated blocks. Following the first harvest, five out of ten blocks were transferred to a pad outside the greenhouse. During the early growth stages irrigation was supplied twice a day and then once daily after the plants were fully established. Fertilization was done after each harvest at the rate of 0.9 g/pot (equivalent to 56 kg $ha^{-1}$) with 20:10:20 NPK Scotts peat-lite fertilizer (The Scotts Company, Marysville, Ohio). Thrips (*Heliothrips spp)* were noticed on the plants inside greenhouse during November-December but the damage was not devastating.

**Harvesting procedures**

Harvesting was done at the three leaves per tiller growth stage during the growing season (September - April). The green herbage was harvested manually by clipping at 5 cm above soil surface. Harvesting was conducted at 14.00 h on clear sunny days to minimize diurnal variation in TNC. The green weight was recorded immediately. These samples were frozen immediately and then freeze-dried in preparation for TNC analysis.

**Laboratory procedures**

The samples were freeze dried to remove all the moisture and then ground with 1-mm mesh screen Cyclotec™ 1093 sample mill (Foss Analytical, Hoganas, Sweden). Ground samples (0.2-0.25 g) were taken in a beaker, mixed with 50 mL of 0.05N $H_2SO_4$, and reflux-boiled in a fiber rack for 15 minutes. Then 5 mL of extraction acid was added and the samples were heated under reflux for 45 additional minutes. Samples were then allowed to cool to room temperature. Afterwards, the pH was adjusted to 4.5 ± 0.1 using different concentrations of acid or base. One mL diluted amyloglucosidase (*Aspergillus niger*, Sigma-Aldrich Inc., St. Louis, MO) was added, thoroughly stirred, and then incubated for 1 hour at 60°C. The solution was then filtered into 250 mL volumetric flask, 1N NaOH was added, and diluted by $dH_2O$. Ten mL of the solution was taken in a 25×200 mm test tube and 10 mL of Shaffer-Somogyi carbonate reagent (AOAC, 1995) was added. The resulting solution was boiled for 15 minutes, cooled in ice water, mixed with 2 mL KI, 10 mL 1N $H_2SO_4$, and 1 mL starch (1 g starch in 100 ml $H_20$) solution. Finally, the resulting mixture was titrated with a 0.02 N sodium thiosulfate solution until the contents of the tube turned an icy blue in color. Concentration of TNC in samples was calculated as the amount of reducing sugar in the sample, multiplied by product of dilution factor times 100, and

divided by sample weight. For every new batch of Shaffer-Somogyi solution, a new glucose standard curve was constructed for varying concentrations. The linear regression model was fitted to predict the TNC level of each sample based on the calculated titer value.

**Statistical analyses**

Data was analyzed using a mixed models methodology as implemented in SAS PROC GLIMMIX (version 9.2; SAS Inst. Inc., Cary, NC). Population, harvest, and location (inside and outside greenhouse) were considered as fixed factors. Block(Location), Population × Block(Location), and Harvest × Block(Location) were considered random factors. Normality and outliers were investigated using StudentPanel option of the above mentioned procedure. Because this experiment has a repeated measures nature, R-side modeling was used to account for the covariance structure; the best covariance structure was selected based on smallest corrected akaike information criterion (AICC) value. The least squares means location × harvest were compared using the simulation option to account for multiple comparisons made from the same body of data. Cycle 2 was compared to cvs. Marshall and Shiwasuaoba and cv. Gulf with cv. Marshall. The progress from selection were calculated by regressing selection cycles onto response in SAS PROC GLIMMIX. The estimate for Location ×Harvest and cycle (Location × Harvest) were determined indicated by SOLUTION and NOINT options. The former estimates the intercept and latter the slope to determine separate regression equations for each cycle.

## Results and Discussion

### Biomass yield

All populations had significantly higher ($P \leq 0.01$) green matter yield inside the greenhouse than outside showing the effect of location. The green matter yield inside the greenhouse was higher ($P \leq 0.01$) than outside the greenhouse for all harvests except harvests two and seven. The average seasonal yield inside the greenhouse was 1.23 kg $m^{-2}$ whereas outside the greenhouse was 0.97 kg $m^{-2}$. The green matter yield was highest in February harvest inside the greenhouse whereas it was maximum in March harvest outside the greenhouse (Table 4.02). The greater yield inside the greenhouse during winter was due to higher accumulation of growing degree days (GDD) (Table 4.01).

The seasonal green matter yield for cv. Shiwasuaoba was higher ($P = 0.01$) than Cycle 2 for plants grown inside the greenhouse but the difference was not significant for plants grown outside the greenhouse. The green matter yield between $C_2$ and Marshall, and Gulf and Marshall did not show any significant differences at any locations (Table 4.03). The result is similar to the results by Syfrett (2003) who did not find any difference in dry matter yield between cvs. Gulf and Marshall grown inside a greenhouse and clipped at 2 and 6-week intervals. Green biomass yield among the selection cycles showed a very small but consistent increase with increasing selection cycles at both locations except for harvest 6 inside the greenhouse (Table 4.05).

### Total non-structural carbohydrates

There was environment × harvest interaction and harvest × population interactions for TNC content. The total seasonal TNC for plants grown outside the greenhouse was higher than that grown inside the greenhouse by 7.5 % DM. The maximum TNC was found

in harvest 3 (November) at both locations (Table 4.02). The higher TNC outside the greenhouse may be explained on the basis that plants reserve carbohydrates which act as osmo-protectants under stressful conditions. Because the temperature outside the greenhouse was lower during winter, plants responded by forming non-reducing carbohydrates such as trehalose (Garg *et al.*, 2002). Furthermore, at higher temperature, carbohydrates content was found to be depleted due to increased respiration and growth (Archibald, 1961; Auda *et al.*, 1966). Also the photon flux density outside the greenhouse was higher than inside the greenhouse, which may be another reason for higher TNC content outside the greenhouse.

The seasonal TNC content between $C_2$ and Shiwasuaoba was not different at either location. Marshall had a higher TNC than $C_2$ outside the greenhouse ($P = 0.07$). Marshall contained higher TNC than cv. $C_2$ and Gulf at the third harvest at both locations (Table 4.04). This result was fairly consistent with Syfrett (2003) who found no difference in TNC between Gulf and Marshall grown in a greenhouse. Similarly, Myer *et al.* (2010) in a two year study, reported inconsistent differences in TNC content among annual ryegrass cultivars grown in small plots in Florida. TNC content remained constant across selection cycles (Table 4.06, $P < 0.95$). Harvest 3 (November) had higher TNC content than other harvests at both locations (Table 4.06). The TNC showed bimodal distribution with highest content during November and another peak during January/ February harvest at both locations. Myer *et al.* (2010) observed a linear decrease in WSC content in October seeded annual ryegrass from January to May. TNC is the highly sensitive and variable nutritive component in forage species which is influenced by several seasonal as well diurnal factors such as temperature, day length, solar radiation, rainfall, maturity stage, and clipping height

(Archibald, 1961; Auda *et al.*, 1966). Even though we tried to maintain consistency in sampling, there were practical difficulties on determining harvest date at the 3 leaves per tiller stage because the stage differed among location and cultivar. Most of the time our decision was based on cv. Shiwasuaoba inside the greenhouse, which flowered earlier. In our study, the possible reason for the increase in TNC in February may be associated with lower temperature during December/ January and at low temperature plants responded by storing carbohydrates as osmo-protectants.

## Summary

Although the differences in TNC and green biomass yield among cultivars were not significant, location had a great influence. Since the growing season was from September to May, TNC and yield had variable response over the growing season owing to the change in temperature and season. Both the response showed bimodal distribution as plants aged. Harvest 3 corresponding to November cut had highest TNC level at both locations for all populations. There was no change in TNC across annual ryegrass population selected for increased winter productivity.

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Table 4.01. Harvest date, maximum, average and minimum temperature and GDD inside and outside greenhouse (GH) during the 2011-2012 growing season at the Plant Science Research Center, Auburn AL.

| Harvest | Date | Days between harvests | Max Temp | | Average Temp | | Min Temp | | GDD | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Inside GH | Outside GH | Inside GH | Outside GH | Inside GH | Outside GH | Inside GH | Outside GH |
| Sowing | 8/31/2011 | | | | | | | | | |
| 1 | 9/22/2011 | 22 | 26.1 | 27.7 | 23.3 | 23.0 | 20.5 | 18.3 | 802 | 595 |
| 2 | 10/11/2011 | 19 | 26.7 | 26.4 | 23.0 | 21.2 | 19.3 | 15.9 | 585 | 591 |
| 3 | 11/15/2011 | 35 | 27.0 | 21.9 | 20.6 | 16.5 | 14.3 | 11.2 | 1056 | 867 |
| 4 | 12/19/2011 | 34 | 25.0 | 17.8 | 21.3 | 14.4 | 17.7 | 11.0 | 1068 | 644 |
| 5 | 1/19/2012 | 31 | 25.2 | 15.8 | 22.5 | 13.2 | 19.7 | 10.7 | 1037 | 521 |
| 6 | 2/22/2012 | 34 | 25.3 | 17.5 | 22.6 | 14.0 | 19.9 | 10.5 | 876 | 619 |
| 7 | 3/24/2012 | 31 | 26.5 | 22.8 | 23.3 | 18.2 | 20.0 | 13.5 | 1082 | 796 |

Table 4.02. Least square means for green biomass yield (g $m^{-2}$) and TNC (% DM) content of annual ryegrass for harvests 2-7 inside and outside greenhouse at the Plant Science Research Center, Auburn University, AL.

| Harvest | GM Yield (g $m^{-2}$) | | TNC (% DM) | |
|---|---|---|---|---|
| | Inside GH | Outside GH | Inside GH | Outside GH |
| 2 | 1087a† | 788b | 11.73y | 17.37x |
| 3 | 968a | 987a | 32.50y | 48.73x |
| 4 | 1004a | 746b | 12.72y | 20.42x |
| 5 | 1396a | 836b | 15.02y | 33.21x |
| 6 | 1536a | 954b | 15.60y | 35.69x |
| 7 | 1421a | 1507a | 13.47y | 21.51x |
| Mean | 1235a | 970b | 16.85y | 29.49x |
| SE | 50.03 | 50.03 | 0.85 | 0.85 |

† Within rows and response variable, means followed by same letters are not significantly different ($P \leq 0.05$).

Table 4.03. Comparison of green matter yield (g $m^{-2}$) between selected $C_2$ and commercial cultivars of annual ryegrass for harvests 2-7 inside and outside greenhouse (GH) at the Plant Science Research Center, Auburn University, AL.

| | Harvest | | | | | | |
|---|---|---|---|---|---|---|---|
| Loc/cultivar | 2 | 3 | 4 | 5 | 6 | 7 | Avg. |
| **Inside** | | | | | | | |
| $C_2$ | 955 | 987 | 853 | 1254 | 1510 | 1197 | 1126 |
| Gulf | 1009 | 1014 | 1099 | 1315 | 1705 | 1460 | 1267 |
| Marshall | 1098 | 898 | 969 | 1386 | 1563 | 1420 | 1222 |
| Shiwasuaoba | 1111 | 823 | 1051 | 1632 | 1653 | 1544 | 1302 |
| SE | 115 | 115 | 115 | 115 | 115 | 115 | 57 |
| ***Difference*** | | | | | | | |
| $C_2$ vs Marshall | -143 | 89 | -116 | -133 | -53 | -223 | -96 |
| $C_2$ vs Shiwasuaoba | -156 | 164 | -198 | -378 | -143 | -347 | -176 |
| Gulf Vs Marshall | -89 | 116 | 130 | -72 | 142 | 41 | 45 |
| ***P value*** | | | | | | | |
| $C_2$ vs Marshall | 0.35 | 0.57 | 0.45 | 0.39 | 0.73 | 0.15 | 0.13 |
| $C_2$ vs Shiwasuaoba | 0.31 | 0.29 | 0.20 | 0.01 | 0.35 | 0.02 | 0.01 |
| Gulf vs Marshall | 0.57 | 0.45 | 0.40 | 0.64 | 0.36 | 0.79 | 0.48 |
| **Outside** | | | | | | | |
| $C_2$ | 717 | 884 | 736 | 891 | 904 | 1412 | 924 |
| Gulf | 712 | 898 | 654 | 743 | 876 | 1488 | 895 |
| Marshall | 802 | 897 | 662 | 717 | 811 | 1541 | 905 |
| Shiwasuaoba | 828 | 1016 | 762 | 714 | 929 | 1032 | 880 |
| SE | 115 | 115 | 115 | 115 | 115 | 115 | 57 |
| ***Difference*** | | | | | | | |
| $C_2$ vs Marshall | -86 | -14 | 74 | 174 | 93 | -129 | 19 |
| $C_2$ vs Shiwasuaoba | -112 | -132 | -26 | 177 | -25 | 380 | 44 |
| Gulf vs Marshall | -90 | 0 | -9 | 26 | 65 | -53 | -10 |
| ***P value*** | | | | | | | |
| $C_2$ vs Marshall | 0.58 | 0.93 | 0.63 | 0.26 | 0.55 | 0.40 | 0.77 |
| $C_2$ vs Shiwasuaoba | 0.47 | 0.39 | 0.87 | 0.25 | 0.87 | 0.01 | 0.49 |
| Gulf vs Marshall | 0.56 | 1.00 | 0.96 | 0.87 | 0.67 | 0.73 | 0.87 |

Table 4.04. Comparison of TNC (%DMB) content between selected $C_2$ and commercial annual ryegrass cultivars for harvest 2-7 inside and outside greenhouse (GH) at the Plant Science Research Center in Auburn, AL.

| | Harvest | | | | | | |
|---|---|---|---|---|---|---|---|
| Loc/cultivar | 2 | 3 | 4 | 5 | 6 | 7 | Avg. |
| **Inside** | | | | | | | |
| $C_2$ | 11.60 | 28.29 | 15.26 | 14.55 | 12.73 | 9.08 | 15.25 |
| Gulf | 11.83 | 27.83 | 9.05 | 10.95 | 14.33 | 13.25 | 14.54 |
| Marshall | 11.75 | 38.40 | 13.86 | 14.52 | 14.92 | 10.49 | 17.32 |
| Shiwasuaoba | 11.24 | 27.16 | 12.88 | 16.72 | 15.71 | 17.16 | 16.81 |
| SE | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 1.00 |
| ***Difference*** | | | | | | | |
| $C_2$ vs Marshall | -0.15 | -10.11 | 1.40 | 0.03 | -2.19 | -1.41 | -2.07 |
| $C_2$ vs Shiwasuaoba | 0.36 | 1.13 | 2.38 | -2.17 | -2.98 | -8.08 | -1.56 |
| Gulf Vs Marshall | 0.08 | -10.57 | -4.81 | -3.57 | -0.59 | 2.76 | -2.78 |
| ***P value*** | | | | | | | |
| $C_2$ vs Marshall | 0.96 | < 0.01 | 0.68 | 0.99 | 0.50 | 0.66 | 0.12 |
| $C_2$ vs Shiwasuaoba | 0.91 | 0.73 | 0.46 | 0.50 | 0.36 | 0.01 | 0.24 |
| Gulf vs Marshall | 0.98 | < 0.01 | 0.16 | 0.27 | 0.86 | 0.39 | 0.04 |
| **Outside** | | | | | | | |
| $C_2$ | 18.20 | 44.89 | 20.01 | 33.15 | 32.62 | 19.92 | 28.13 |
| Gulf | 15.54 | 46.63 | 21.48 | 32.24 | 36.46 | 23.01 | 29.23 |
| Marshall | 21.01 | 53.36 | 22.48 | 35.96 | 36.59 | 13.80 | 30.53 |
| Shiwasuaoba | 17.51 | 46.95 | 14.18 | 30.94 | 35.53 | 12.96 | 26.34 |
| SE | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 0.99 |
| ***Difference*** | | | | | | | |
| $C_2$ vs Marshall | -2.81 | -8.46 | -2.47 | -2.81 | -3.97 | 6.12 | -2.40 |
| $C_2$ vs Shiwasuaoba | 0.69 | -2.06 | 5.83 | 2.20 | -2.91 | 6.97 | 1.79 |
| Gulf vs Marshall | -5.47 | -6.72 | -1.00 | -3.72 | -0.14 | 9.20 | -1.31 |
| ***P value*** | | | | | | | |
| $C_2$ vs Marshall | 0.39 | 0.01 | 0.45 | 0.39 | 0.22 | 0.06 | 0.07 |
| $C_2$ vs Shiwasuaoba | 0.83 | 0.53 | 0.07 | 0.50 | 0.37 | 0.03 | 0.18 |
| Gulf vs Marshall | 0.09 | 0.04 | 0.76 | 0.25 | 0.97 | < 0.01 | 0.32 |

Table 4.05. Least square means of TNC (%DMB) content of selected cycles 0-6 for increased winter productivity on annual ryegrass and regression coefficients for harvest 2-7 grown inside and outside greenhouse in Plant Science Research Center, Auburn, AL.

| | Selection Cycles (TNC) | | | | | | | Regression Analysis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Loc/Harvest | 0 | 1 | 2 | 3 | 4 | 5 | 6 | Intercept | Probt | Slope | Probt |
| **Inside** | | | | | | | | | | | |
| 2 | 10.9 | 11.4 | 11.6 | 13.7 | 11.7 | 11.9 | 11.2 | 11.57 | < 0.001 | 0.071 | 0.87 |
| 3 | 32.4 | 33.6 | 28.3 | 37.0 | 32.0 | 34.0 | 34.4 | 31.97 | < 0.001 | 0.373 | 0.40 |
| 4 | 10.1 | 10.6 | 15.3 | 11.7 | 18.9 | 14.9 | 10.0 | 11.78 | < 0.001 | 0.431 | 0.33 |
| 5 | 17.1 | 14.6 | 14.5 | 15.1 | 14.4 | 15.7 | 16.6 | 15.33 | < 0.001 | 0.029 | 0.95 |
| 6 | 17.1 | 15.7 | 12.7 | 17.5 | 15.1 | 16.0 | 17.2 | 15.40 | < 0.001 | 0.151 | 0.74 |
| 7 | 14.6 | 14.9 | 9.1 | 11.6 | 18.8 | 7.3 | 17.6 | 12.98 | < 0.001 | 0.140 | 0.75 |
| SE | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 1.75 | | 1.683 | |
| **Outside** | | | | | | | | | | | |
| 2 | 18.0 | 13.8 | 18.2 | 16.8 | 18.2 | 16.1 | 18.6 | 16.43 | < 0.001 | 0.220 | 0.62 |
| 3 | 52.6 | 45.6 | 44.9 | 49.6 | 50.5 | 50.0 | 47.2 | 48.85 | < 0.001 | -0.075 | 0.867 |
| 4 | 25.4 | 19.8 | 20.0 | 22.1 | 21.2 | 16.6 | 20.9 | 22.86 | < 0.001 | -0.665 | 0.13 |
| 5 | 32.6 | 33.3 | 33.1 | 32.0 | 33.4 | 33.4 | 35.1 | 32.43 | < 0.001 | 0.288 | 0.51 |
| 6 | 35.6 | 35.0 | 32.6 | 38.2 | 36.9 | 34.8 | 35.5 | 35.14 | < 0.001 | 0.128 | 0.78 |
| 7 | 23.9 | 26.6 | 19.9 | 18.4 | 27.6 | 23.1 | 25.9 | 22.89 | < 0.001 | 0.243 | 0.58 |
| SE | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 0.45 | | 0.455 | |

Table 4.06. Least square means of green biomass yield (g $m^{-2}$) of selected cycles 0-6 for increased winter productivity on annual ryegrass and regression coefficients for harvest 2-7 grown inside and outside greenhouse in Plant Science Research Centre, Auburn, AL.

| | Selection cycles (Yield) | | | | | | | Regression Analysis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Loc/Harvest | 0 | 1 | 2 | 3 | 4 | 5 | 6 | Intercept | Probt | Slope | Probt |
| **Inside** | | | | | | | | | | | |
| 2 | 1174 | 940 | 1176 | 1177 | 1068 | 1071 | 1257 | 1025 | < 0.001 | 23 | 0.26 |
| 3 | 865 | 921 | 987 | 1130 | 900 | 1061 | 1082 | 902 | < 0.001 | 30 | 0.13 |
| 4 | 893 | 966 | 853 | 1148 | 885 | 1035 | 1141 | 891 | < 0.001 | 33 | 0.10 |
| 5 | 1499 | 1371 | 1254 | 1296 | 1215 | 1454 | 1549 | 1347 | < 0.001 | 10 | 0.62 |
| 6 | 1672 | 1555 | 1510 | 1546 | 1287 | 1478 | 1396 | 1621 | < 0.001 | -43 | 0.03 |
| 7 | 1424 | 1445 | 1197 | 1419 | 1407 | 1426 | 1473 | 1364 | < 0.001 | 11 | 0.57 |
| SE | 115 | 115 | 115 | 115 | 115 | 115 | 115 | 83 | | 20 | |
| **Outside** | | | | | | | | | | | |
| 2 | 784 | 841 | 717 | 838 | 774 | 818 | 775 | 794 | < 0.001 | -1 | 0.98 |
| 3 | 1061 | 831 | 884 | 1014 | 1066 | 1072 | 1141 | 913 | < 0.001 | 32 | 0.11 |
| 4 | 760 | 704 | 736 | 726 | 787 | 734 | 941 | 699 | < 0.001 | 23 | 0.24 |
| 5 | 792 | 732 | 891 | 809 | 1013 | 993 | 964 | 761 | < 0.001 | 41 | 0.04 |
| 6 | 925 | 863 | 904 | 1014 | 1073 | 1128 | 1027 | 883 | < 0.001 | 36 | 0.07 |
| 7 | 1719 | 1601 | 1412 | 1553 | 1456 | 1606 | 1665 | 1585 | < 0.001 | -4 | 0.85 |
| SE | 115 | 115 | 115 | 115 | 115 | 115 | 115 | 83 | | 20 | |