ABSTRACT

Plant breeders have recently focused on increasing the sugar content of grasses as a means to improve their nutritional value. The objective of this study was to compare the chemical composition of four annual ryegrass varieties (Lolium multiflorum Lam.): two intermediate tetraploids [L. multiflorum var. italicum, Bandito2, (conventional) and Aberewe, (high sugar)] and two short cycle diploids [L. multiflorum var. westerwoldicum, Lonestar, (conventional) and Enhancer, (high sugar)] grown in greenhouses. Seeds were planted into plastic pots (16 pots per variety) and clipped three times at six-week intervals. Material was weighed, flash frozen, lyophilized and ground (1 mm). Chemical analyses and digestibility at 24 and 48 h were assessed. In vitro DM (IVDMD), OM (IVOMD) and NDF (IVNDFD) disappearance as well as in vitro true DM disappearance (IVTD) were calculated. Results were compared by preplanned orthogonal contrasts as follows: C1, intermediate tetraploids vs annual diploids, C2, conventional vs high sugar varieties. Intermediate tetraploid varieties had lower DM content, lower OM content, lower NDF and hemicellulose content. They also tended to have higher CP content, but no differences were observed in WSC content or WSC:CP. Conventional and high sugar varieties did not differ except for DM content. Intermediate tetraploid had higher in vitro DM and OM disappearance at 24 and 48 h, and higher in vitro true DM disappearance and NDF disappearance at 24 h. Conventional varieties had higher digestibility at 24 h but not at 48 h. No differences in WSC were detected between intermediate tetraploids and annual diploids, or between conventional and high sugar varieties. Differences in forage quality were more important between intermediate tetraploids and annual diploids, but no differences were found between conventional and high sugar varieties. High temperatures at the greenhouse may not have allowed high sugar varieties to accumulate increased levels of WSC.

Keywords: Ryegrass, water soluble carbohydrates, high sugar forages, nutritional quality.

RESUMEN

En años recientes, la selección genética se ha abocado a aumentar el contenido de azúcares de los forrajes como una forma mejorar su valor nutricional. El objetivo de este estudio fue comparar la composición química de cuatro variedades de ryegrass anual (Lolium multiflorum Lam.): dos variedades tetraploides de ciclo in-
The objective of this study was to analyze the digestibility of Lolium multiflorum (
L. multiflorum var. Italicum, Bandito2, (convencional) and Abereve, (alto azúcar)) and
dos variedades de ciclo corto [L. multiflorum var. westerwoldicum, Lonestar, (convencional) y Enhancer, (alto azúcar)], las cuales se
cultivaron en invernáculos. La siembra se hizo en macetas plásticas (16 macetas por variedad y se hicieron
tres cosechas (5 cm de altura) con intervalos de 6 semanas entre cosechas. El material cortado fue pesado
en fresco, congelado en nitrógeno líquido, liofilizado y molído a 1 mm. Se realizaron análisis químicos y las di-
gestibilidades a las 24 y 48 horas. Se calcularon la degradación in vitro de la materia seca, materia orgánica y FDN así como la digestibilidad in vitro real (in vitro true digestibility). Los resultados se compararon por medio
de los siguientes contrastes ortogonales: C1, tetraploides intermedios vs. diploides anuales, C2, variedades
convencionales vs. variedades alto azúcar. Las variedades tetraploides de ciclo intermedio mostraron menor
contenido de materia seca, materia orgánica, FDN y hemicelulosa. Además tendieron a tener mayores conte-
vidos de proteína bruta, sin mostrar diferencias en contenido de hidratos de carbono soluble o en la relación
hidratos de carbono soluble: proteína bruta. Las variedades convencionales y alto azúcar no mostraron diferen-
cias entre ellas, excepto en contenido de materia seca. Con respecto a la digestibilidad, las variedades tetra-
plóides de ciclo intermedio mostraron mayor degradación in vitro de materia seca y materia orgánica a las 24 y 48 h, y mayores valores de digestibilidad in vitro real para la materia seca y la materia orgánica a las 24 horas.
Las variedades convencionales mostraron mayor degradabilidad y digestibilidad in vitro real a las 24 h, pero
no a las 48 h. No se encontraron diferencias para contenido de hidratos de carbono solubles entre tetraploides
intermedios y diploides anuales, o entre variedades convencionales y alto azúcar. Las diferencias en calidad
más importantes se encontraron entre tetraploides intermedios y diploides anuales, pero no entre variedades
convencionales y alto azúcar. Las altas temperaturas en el invernáculo podrían haber impedido la expresión del
potencial de acumulación de hidratos de carbono solubles por parte de las variedades alto azúcar.

**Palabras clave:** Ryegrass, hidratos de carbono soluble, forrajes alto azúcar, calidad nutricional.

**INTRODUCTION**

Annual ryegrass (*Lolium multiflorum* Lam.) is an annual cool season grass cultivated throughout all temperate zo-
nes around the world (Jung et al., 1996; Wilkins and Hum-
prey, 2003). Due to its high digestibility, it is used in cattle
with high nutrient requirements. However, grasses nutrient
balance is not always adequate. Low water soluble carbo-
hydrate (WSC) content or low WSC to crude protein (CP)
ratios (WSC:CP) leads to nutrient imbalance which impairs
the ability of ruminal microorganisms for synthesizing micro-
bial protein (Nocek and Russell, 1988; Kingston-Smith and Theodorou, 2000). Therefore, an improved nutrient balance
in grasses (i.e., a higher WSC:CP ratio) may lead to higher
nitrogen use efficiency by the host animal.

Plant breeders have lately developed high WSC varie-
ties, known as “high sugar grasses” (Smith et al., 2007).
Tetraploid and diploid varieties which express higher con-
centration of fructans in leaves may offer productive advan-
tages for producers. Tetraploids cultivars are associated with higher levels of WSC and higher cell content to cell
wall ratio (Hageman et al., 1993). Miller et al. (2001) repor-
ted milk yield improvement without affecting solid composi-
tion in cows grazing high sugar ryegrass. They also repor-
ted lower amounts of urinary nitrogen excretion. Moorby et
al. (2006) found higher dry matter (DM) intake, higher DM
digestibility, improved microbial protein synthesis and a hig-
her protein yield in dairy cows fed high sugar ryegrass. Lee
et al. (2001) evaluated the performance of suckling lambs
stocked on a high sugar *Lolium perenne* sward and found
increased liveweight gain and higher carrying capacity.

To our knowledge, most of the published research was
carried out evaluating perennial ryegrass. Scientific infor-
mation is scarce for high sugar annual ryegrass varieties,
and the ability of this species to accumulate WSC has only
been tested by a smaller number of researchers (Hopkins
et al., 2002). The objective of this study was to analyze
chemical constituents that affect nutritive value and in vi-
tro digestibility of four ryegrass varieties two intermediate
cycle tetraploids (*L. multiflorum* var. *italicum*, Bandito2,
(conventional) and Abereve, (high sugar)) and two short
cycle diploids (*L. multiflorum* var. *westerwoldicum*, Lonestar,
(conventional) and Enhancer, (high sugar)) grown in greenhouse conditions. Our hypothesis was that interme-
diate tetraploids and high sugar varieties would have hig-
her WSC content, lower cell wall concentration and higher
in vitro digestibility.

**MATERIALS AND METHODS**

The experiment was conducted at a 15 m x 13 m gre-

ehouse at Clemson University, Clemson, South Carolina,
USA. Seeds of annual ryegrass (*Lolium multiflorum* Lam.)
were planted at 0.5 cm depth into plastic pots (3.84 L) con-
taining potting soil. Four annual ryegrass varieties were
evaluated: two intermediate tetraploids [Bandito2, (conven-
tional) and Abereve, (high sugar)] and two annual diploids
[Lonestar, (conventional) and Enhancer, (high sugar)]. All
the varieties were provided by Sucraseed (Oregon, USA).
Sixteen pots per variety were planted. Pots were watered
to saturation and, after germination, plants were watered
daily with tap water and fertilized weekly with 20-10-20 (N-P-K) nutrient solution (Scotts Sierra Horticultural Products Company, Ohio, USA). No artificial light was used. The greenhouse was equipped with an airflow distribution system. Temperature varied from a minimum of 18°C during the night to a maximum of 29°C during the day and relative humidity was maintained at 70%.

Plants were harvested at six-week intervals by clipping at 5 cm height. The intention was to emulate a rotational grazing situation in which resting time was fixed. A total of three cuttings were harvested. All harvests started at 2:30 PM on days with full sunlight, to ensure a higher accumulation of WSC (Mayland et al., 2005). Material was weighed, placed into cloth bags, and flash frozen in liquid nitrogen. Plant material was stored at -20°C until freeze dried (Labconco bulk tray dryer, USA) and ground through a Wiley mill (1 mm), except for an aliquot which was used to estimate DM by drying in the oven at 102°C until constant weight. Ground material was then pooled by variety and sampling. Plant tissue analyses included organic matter (OM) content by placing on muffle furnace (600°C, 6 h), NDF and ADF content which were assessed in the ANKOM fiber analyzer according to Van Soest et al. (1991), acid detergent lignin (ADL) by immersing samples into 72% H$_2$SO$_4$ (Van Soest et al., 1991), water soluble carbohydrate content (WSC) was assessed by colorimetric phenol-sulfuric acid assay according to Dubois et al. (1956), and CP concentration by combustion method on a Leco FB528 analyzer (Leco Corporation, Minessota, USA; AOAC, 1990). Hemicellulose was estimated as the difference between NDF and ADF, and cellulose as the difference between ADF and ADL.

For the estimation of the in vitro DM (IVDMD), OM (IVOMD) and NDF (IVNDFD) disappearances, dry and ground forages (0.50±0.01 g) were weighed into acetone pre-rinsed incubation bags (F57 bags, Ankom, New York, USA) in duplicate for each variety and sampling. Then they were incubated in a Daisy® in vitro incubator (Ankom, New York, USA). Rumen fluid was collected from a cannulated Holstein dairy cow in mid lactation fed a diet comprised of 34% corn silage, 6% grass hay and 60% corn. Liquid and fistfuls of fibrous material were collected from the rumen, kept in pre-warmed thermic bottles and taken to the lab, where it was blended in a in a preheated blender while purged with CO$_2$. Four hundred ml of the filtered rumen fluid was poured into an incubation jar that contained 1600 ml of buffer (KH$_2$PO$_4$, 8.3 g/l, MgSO$_4$*7H$_2$O, 0.41 g/l, NaCl, 0.41 g/l, CaCl$_2$*2H$_2$O, 0.08g/l, urea 0.41 g/l, Na$_2$CO$_3$ 2.5 g/l and Na$_2$S*9H$_2$O, 0.16 g/l) while purging with CO$_2$. In vitro true digestibility (IVTD) was obtained by calculating NDF content in the residue post incubation (Goering and Van Soest, 1970).

**Statistical Analyses.** Chemical composition variables were analyzed by Proc Glimmix of SAS (SAS Institute, Cary, NC) in a model that included variety as fixed factor and cutting date as a random factor. Two pre-planned orthogonal contrasts were used for comparisons: C1, to compare intermediate tetraploids (Bandito2 and Abereve)}

<table>
<thead>
<tr>
<th>Intermediate Tetraploid</th>
<th>Annual diploid</th>
<th>Contrasts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yield (g DM/ pot)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bandito2 (C)</td>
<td>9.57</td>
<td>Abereve (HS)</td>
</tr>
<tr>
<td>Lonestar (C)</td>
<td>10.61</td>
<td>Enhancer (HS)</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>1.252</td>
<td></td>
</tr>
<tr>
<td><strong>C1</strong></td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td><strong>Composition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM content (g kg$^{-1}$ wet)</td>
<td>14.69</td>
<td>16.62</td>
</tr>
<tr>
<td>OM content</td>
<td>90.21</td>
<td>90.31</td>
</tr>
<tr>
<td>NDF</td>
<td>45.33</td>
<td>44.66</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>16.98</td>
<td>17.01</td>
</tr>
<tr>
<td>ADF</td>
<td>28.35</td>
<td>27.65</td>
</tr>
<tr>
<td>Cellulose</td>
<td>26.19</td>
<td>25.37</td>
</tr>
<tr>
<td>ADL</td>
<td>2.16</td>
<td>2.28</td>
</tr>
<tr>
<td>Crude protein</td>
<td>10.24</td>
<td>10.81</td>
</tr>
<tr>
<td>WSC</td>
<td>12.98</td>
<td>15.63</td>
</tr>
<tr>
<td>WSC:CP</td>
<td>1.37</td>
<td>1.63</td>
</tr>
</tbody>
</table>

**Table 1.** Dry matter yield and chemical composition of ryegrass varieties (Bandito2, Abereve, Lonestar and Enhancer) grown in greenhouse conditions

*Presented as g kg$^{-1}$ DM unless stated otherwise.
C: conventional, HS: high sugar.
vs. and annual diploids (Lonestar and Enhancer); C2, to compare conventional (Lonestar and Bandito2) vs high sugar (Enhancer and Aberve). Differences between means with P < 0.05 were considered statistically different, while differences with P < 0.10 were considered as tendencies.

**RESULTS AND DISCUSSION**

The DM content of the intermediate tetraploid varieties was lower than annual diploid varieties (table 1). These differences, obtained under identical environmental conditions and at the same growing intervals, would indicate genetic differences. Several authors have reported that tetraploid grasses have lower DM content (Van Wijk, 1988; Baert, 1994; Wims et al., 2012). Additionally, maturation is faster in annual varieties, reaching a higher DM content due to a more advanced phenological stage. However, we choose fixed-interval cuts to emulate most rotational grazing systems. Intermediate varieties are crosses of annual x perennial varieties; therefore, they show intermediate characteristics (Hannaway et al., 1999). The two high sugar varieties (Aberve and Enhancer) tended (P = 0.06, table 1) to have higher DM content than conventional varieties (Bandito2 and Lonestar). Higher DM contents in high sugar ryegrass varieties have been reported by several authors (Miller et al., 2001, Moorby et al., 2006, Cosgrove et al., 2007). Dry matter content could improve animal performance, through an increase in voluntary intake (John and Ulyatt, 1987).

With respect to the cell wall components analysis, intermediate tetraploid tended to have lower NDF content (P = 0.07), with the hemicellulose fraction being significantly lower (table 1). Since no differences were observed in ADF content, the cellulose fraction resulted higher in the intermediate tetraploid. No differences were found in ADL (table 1). The contrast between high sugar varieties and conventional varieties did not differ. Lower NDF (Wims et al., 2012) and lower hemicellulose content (Morrison, 1980) in tetraploid varieties have been previously reported. The duplication of chromosome number in tetraploid varieties is associated with increased cell size and higher cell content to cell wall ratio, which have a dilution effect on NDF concentration (Hageman et al., 1993). Fiber concentration and dry matter digestibility are usually correlated (Wilkins and Humphreys, 2003). Fiber concentration, due to its filling effect, is important determining forage intake and animal performance (Wilkinson et al., 1982).

Crude protein content tended to be higher in the intermediate tetraploid varieties (P = 0.09, table 1). This agrees with the reports of Cosgrove et al. (2009) and Wims et al. (2012) who reported that tetraploid perennial ryegrass varieties at vegetative stage had higher CP content than diploids. No differences in WSC were found between intermediate tetraploids and annual diploids (table 1). Water soluble carbohydrates and CP are the main components of cell content (Wilkins and Humphreys, 2003). As previously mentioned, tetraploid grasses have higher cell content. This is in turn associated with higher WSC and CP content, as well as proteins and lipids, and improvements in forage digestibility (Hageman et al., 1993; Nair, 2004).

In our experiment, temperature varied between 18°C and 29°C, which might have impaired the expression of the high sugar trait, explaining the lack of differences.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Intermediate Tetraploid</th>
<th>Annual diploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEM</td>
<td>C1</td>
<td>C2</td>
</tr>
<tr>
<td><strong>IVDM disappearance</strong></td>
<td>73.12</td>
<td>70.75</td>
</tr>
<tr>
<td><strong>IVOM disappearance</strong></td>
<td>81.08</td>
<td>77.93</td>
</tr>
<tr>
<td><strong>DM IVTD</strong></td>
<td>81.58</td>
<td>77.40</td>
</tr>
<tr>
<td><strong>IVNDF disappearance</strong></td>
<td>59.55</td>
<td>49.67</td>
</tr>
<tr>
<td><strong>IVDM disappearance</strong></td>
<td>83.30</td>
<td>82.43</td>
</tr>
<tr>
<td><strong>IVOM disappearance</strong></td>
<td>92.37</td>
<td>91.38</td>
</tr>
<tr>
<td><strong>DM IVTD</strong></td>
<td>88.65</td>
<td>87.48</td>
</tr>
<tr>
<td><strong>IVNDF disappearance</strong></td>
<td>75.02</td>
<td>72.13</td>
</tr>
</tbody>
</table>

Table 2. *In vitro* dry matter and organic matter disappearance, *in vitro* dry matter true digestibility and *in vitro* NDF disappearance at 24 and 48 hours of incubation of ryegrass varieties (Bandito2, Aberve, Lonestar and Enhancer) grown in greenhouse conditions.

Presented as g kg⁻¹ DM.

C: conventional, HS: high sugar.

IVDM: *in vitro* dry matter disappearance after incubation in Daisy⁴; IVOM: *in vitro* organic matter disappearance after incubation in Daisy⁴; DM IVTD: *dry matter in vitro* true digestibility. IVNDF: *in vitro* neutral detergent fiber disappearance after incubation in Daisy⁴. SEM: standard error mean. C1: orthogonal contrast intermediate tetraploid varieties vs annual diploid; C2: orthogonal contrast high sugar varieties (Aberve and Enhancer) vs conventional (Bandito2 and Lonestar). Table built based on experimental data.
Research has shown that the expression of the high sugar trait is affected by environmental conditions. Parsons et al. (2004) found that high sugar trait expression needed low night temperatures, which would reduce the ratio of dark respiration to photosynthesis in plant tissues, allowing the accumulation of sugars. Cosgrove et al. (2007) reported slight differences (2 to 4 g/kg DM, depending on the year) between high sugar grasses (diploid and tetraploids) and conventional varieties in spring, but no significant differences when the same varieties were compared in fall. Conversely, working at field paddocks in New Zealand, Lazzarini et al. (2010) found no differences between varieties in spring and slight differences (1.5 g/100 g DM) in fall. Rasmussen et al. (2014) detected effects of growth temperature not only on the ability of varieties to concentrate WSC, but also on the expression of specific fructosyltransferases, which showed a reduced expression at high temperatures. The above mentioned results show that genotype × environment interaction exists in the expression of high sugar trait, which does not express equally in every environmental situation (Halling et al., 2004; Edwards et al., 2007; Rasmussen et al., 2014).

With respect to in vitro disappearance and digestibility data (table 2), intermediate tetraploid varieties tended to have higher IVDMD both at 24 and 48 h of incubation (P = 0.10 and P = 0.08, respectively) and significantly higher IVOMD at both incubation times (P < 0.05). At 24 h, DM IVT and IVNDF disappearance were also higher in intermediate tetraploids, but there were no differences at 48 h of incubation (table 2). These results agree with those obtained by Skaland and Volden (1973) in Norway, Wims et al (2012) in Ireland, and Balochi and López (2009) in Chile, who reported that tetraploid varieties had higher digestibility.

Conventional varieties had higher IVDM disappearance, IVOM disappearance, DM IVTD and IVNDF disappearance at 24 h of incubation than high sugar varieties. These differences disappeared for all variables at 48 h of incubation (table 2). With no differences in composition between conventional and high sugar varieties, results may be explained by a faster digestible fiber fraction in the conventional varieties, especially Bandito2.

Rye grass is the most digestible of all the grass species (Morrison, 1980; Frame, 1991). We reported average IVDMD values of 71.02 g/100 g DM and 82.15 g/100 g DM at 24 and 48 h of incubation, which are close to the values reported by Hopkins et al. (2002). Acid detergent lignin values were very low (2.27 g/100 g DM, on average), which helps to explain the high digestibility (Jung and Allen, 1995; Moore and Jung, 2001).

CONCLUSIONS

Both in terms of chemical compositions and in vitro disappearance and digestibility, intermediate tetraploids showed high nutritive quality. Either no differences or minor significant differences were found when comparing conventional to high sugar varieties. No variety effect was detected in WSC content, possibly due to temperatures higher than optimal. Breeding strategies for high WSC varieties should include the selection of genotypes with the ability to concentrate WSC in a wide range of environments, including warmer temperatures.

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