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Chemical composition and *in vitro* digestibility of annual ryegrass varieties grown in greenhouse conditions

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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 cyclethe chemical composition of four annual ryegrass varieties (Lolium multiflorum Lam.): two intermediate 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 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 24h. 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-

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termedio [L. multiflorum var. Italicum, Bandito2, (convencional) y Abereve, (alto azúcar)] y 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 molido a 1 mm. Se realizaron análisis químicos y las digestibilidades 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 contenidos 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 diferencias entre ellas, excepto en contenido de materia seca. Con respecto a la digestibilidad, las variedades tetraploides 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 zones around the world (Jung et al., 1996; Wilkins and Humprey, 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 carbohydrate (WSC) content or low WSC to crude protein (CP) ratios (WSC:CP) leads to nutrient imbalance which impairs the ability of ruminal microorganisms for synthetizing microbial 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 varieties, known as “high sugar grasses” (Smith *et al*., 2007). Tetraploid and diploid varieties which express higher concentration of fructans in leaves may offer productive advantages 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) reported milk yield improvement without affecting solid composition in cows grazing high sugar ryegrass. They also reported 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 higher 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 information 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 vitro* 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 intermediate tetraploids and high sugar varieties would have higher 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 greenhouse 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) containing potting soil. Four annual ryegrass varieties were evaluated: two intermediate tetraploids [Bandito2, (conventional) 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 18ºC during the night to a maximum of 29ºC 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 -20ºC 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 102ºC 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 (600ºC, 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_2SO_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[II] *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_2PO_4, 8.3 g/l, $MgSO_4$*$7H_2O$, 0.41 g/l, NaCl, 0.41 g/l, $CaCl_2$*$2H_2O$, 0.08g/l, urea 0.41 g/l, Na_2CO_3, 2.5 g/l and Na_2S*$9H_2O$, 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)

	Intermediate Tetraploid		**Annual diploid**			**Contrasts**	
	Bandito2 (C)	**Abereve (HS)**	**Lonestar (C)**	**Enhancer (HS)**	**SEM**	***C1***	***C2***
Yield (g DM/ pot)	9.57	8.57	10.61	10.33	1.252	0.04	0.30
Composition*							
DM content ($g.kg^{-1}$ wet)	14.69	16.62	17.98	17.95	2.056	<0.01	0.06
OM content	90.21	90.31	90.82	90.78	0.915	0.02	0.89
NDF	45.33	44.66	46.04	45.90	1.396	0.07	0.39
Hemicellulose	16.98	17.01	18.75	18.23	1.221	<0.01	0.38
ADF	28.35	27.65	27.29	27.67	0.799	0.11	0.57
Cellulose	26.19	25.37	24.93	25.41	0.732	0.03	0.47
ADL	2.16	2.28	2.36	2.26	0.521	0.31	0.95
Crude protein	10.24	10.81	9.93	10.00	2.182	0.09	0.30
WSC	12.98	15.63	15.32	13.97	1.456	0.60	0.33
WSC:CP	1.37	1.63	1.75	1.55	0.388	0.16	0.73

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.
DM: dry matter, OM: organic matter, NDF: neutral detergent fiber, ADF: acid detergent fiber, ADL: acid detergent lignin, WSC: water soluble carbohydrates, WSC:CP: water soluble carbohydrates to crude protein ratio. SEM: standard error mean. C1: orthogonal contrast intermediate tetraploid varieties vs annual diploid. C2: orthogonal contrast high sugar varieties (Abereve and Enhancer) vs conventional (Bandito2 and Lonestar).

vs. and annual diploids (Lonestar and Enhancer); C2, to compare conventional (Lonestar and Bandito2) vs high sugar (Enhancer and Abereve). 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 tetraploids 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 (Abereve 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 and 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 18ºC and 29ºC, which might have impaired the expression of the high sugar trait, explaining the lack of differences.

	Intermediate Tetraploid		Annual diploid			Contrast	
	Bandito2 (C)	Abereve (HS)	Lonestar (C)	Enhancer (HS)	SEM	*C1*	*C2*
24 h incubation							
IVDM disappearance	73.12	70.35	71.32	69.30	2.375	0.10	0.009
IVOM disappearance	81.08	77.93	78.57	76.40	3.128	0.04	0.008
DM IVTD	81.58	77.40	77.83	77.32	2.279	0.02	0.005
IVNDF disappearance	59.55	49.67	52.15	51.02	3.979	0.06	0.002
48 h incubation							
IVDM disappearance	83.30	82.43	81.00	81.87	1.964	0.08	1.00
IVOM disappearance	92.37	91.38	89.27	90.22	2.895	0.03	0.98
DM IVTD	88.65	87.48	86.62	87.17	1.926	0.11	0.67
IVNDF disappearance	75.02	72.13	71.17	72.09	2.071	0.20	0.51

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, Abereve, 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 Daisy[II], IVOM: *in vitro* organic matter disappearance after incubation in Daisy[II], DM IVTD: dry matter *in vitro* true digestibility. IVNDF: *in vitro* neutral detergent fiber disappearance after incubation in Daisy[II]. SEM: standard error mean. C1: orthogonal contrast intermediate tetraploid varieties vs annual diploid; C2: orthogonal contrast high sugar varieties (Abereve 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 x 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.

Ryegrass 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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