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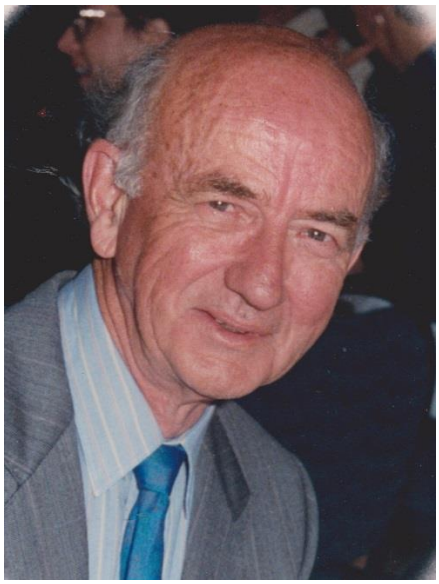
This issue is dedicated to the memory of **Ronald (Ron) J. Williams (1930-2017)**, Australian plant geographer and pasture scientist, a pioneer in tropical forage plant collection, introduction and evaluation. His friends and colleagues throughout the tropical world will not forget him for his remarkable personality and intimate knowledge, vision and enthusiasm for tropical forage plant genetic resources.



Editors

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Principal Contacts

Rainer Schultze-Kraft
Centro Internacional de Agricultura Tropical (CIAT)
Colombia
Phone: +57 2 4450100 Ext. 3036
Email: r.schultzekraft@cgiar.org

Technical Support
José Luis Urrea Benítez
Centro Internacional de Agricultura Tropical (CIAT)
Colombia
Phone: +57 2 4450100 Ext. 3354
Email: CIAT-TGFT-Journal@cgiar.org

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Research Papers

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Research Paper

Temporal differences in plant growth and root exudation of two *Brachiaria* grasses in response to low phosphorus supply

Diferencias en el crecimiento y exudaciones radiculares de dos especies de Brachiaria *en respuesta a baja disponibilidad de fósforo*

ANNA E. LOUW-GAUME[1], NOEL SCHWEIZER[1], IDUPULAPATI M. RAO[2], ALAIN J. GAUME[3] AND EMMANUEL FROSSARD[1]

[1]*Group of Plant Nutrition, Institute of Agricultural Sciences, ETH Zurich, Lindau, Switzerland.* www.plantnutrition.ethz.ch
[2]*International Center for Tropical Agriculture (CIAT), Cali, Colombia.* www.ciat.cgiar.org
Presently: Plant Polymer Research Unit, National Center for Agricultural Utilization Research, ARS, USDA, Peoria, IL, USA. www.ars.usda.gov
[3]*Syngenta Crop Protection, Münchwilen AG, Stein, Switzerland.* www.syngenta.com

Abstract

Exploiting the natural variability of *Brachiaria* forage germplasm to identify forage grasses adapted to infertile acid soils that contain very low available phosphorus (P) is an important research objective for improving livestock production in the tropics. The objective of this study was to determine the differences in the release of root biochemical markers, i.e. carboxylates and acid phosphatases (APases), during the development of P deficiency in signalgrass and ruzigrass. We used the hydroxyapatite pouch system in hydroponics to simulate conditions of low P supply in acid soils to test the response of well-adapted signalgrass (*Brachiaria decumbens* cv. Basilisk, CIAT 606) and less-adapted ruzigrass (*B. ruziziensis* cv. Kennedy, CIAT 654). We monitored shoot and root growth and other physiological and biochemical components that are important for root functionality at weekly intervals for 3 weeks. We found that monocarboxylate exudation was not associated with the plant's physiological P status, while exudation of oxalate and secreted-APases increased with declining plant P concentrations in both grasses. Ruzigrass showed higher exudation rates and grew faster than signalgrass, but could not maintain its initial fast growth rate when P concentrations in plant tissue declined to 1.0 mg P/g dry matter. Oxalate was the dominant exuded carboxylate for signalgrass after 21 days of growth and this response might confer some eco-physiological advantages in signalgrass when grown in low-P acid soils.

Keywords*:* Acid phosphatases, leaf expansion, oxalate, phosphate uptake and use, root elongation.

Resumen

El aprovechamiento de la variabilidad natural en germoplasma del género *Brachiaria* para identificar variedades forrajeras adaptadas a suelos ácidos de baja fertilidad y bajo contenido de fósforo (P) disponible, es un objetivo de investigación importante con el fin de mejorar la producción ganadera en áreas tropicales. En el estudio se evaluaron las diferencias en la exudación de carboxilatos y fosfatasas ácidas como marcadores bioquímicos radiculares durante el desarrollo de deficiencia de P en 2 especies de *Brachiaria.* Para el efecto, se utilizó el sistema hidropónico de bolsas con

Correspondence: Idupulapati M. Rao, Plant Polymer Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA.
Email: i.rao@cgiar.org; rao.idupulapati@ars.usda.gov

hidroxiapatita para simular condiciones de baja disponibilidad de P en suelos ácidos, con el fin de identificar diferencias entre una gramínea adaptada (*Brachiaria decumbens* cv. Basilisk, CIAT 606) y otra menos adaptada (*B. ruziziensis* cv. Kennedy, CIAT 654). Monitoreamos el crecimiento de las partes aéreas y las raíces de las plantas, así como algunos componentes fisiológicos y bioquímicos importantes para la funcionalidad de las raíces, cada semana en un período de 3 semanas. Los resultados mostraron que la exudación de monocarboxilatos no estaba asociada con el estado fisiológico de P de la planta, mientras que la exudación de oxalato y fosfatasas ácidas aumentó con la disminución de las concentraciones de P en ambas gramíneas. *Brachiaria ruziziensis* mostró tasas de exudación más altas y creció más rápido que *B. decumbens*; no obstante su tasa de crecimiento rápido inicial se redujo cuando las concentraciones de P en el tejido vegetal disminuyeron a 1.0 mg/g de materia seca. El oxalato fue el carboxilato exudado prevalente para *B. decumbens* después de 21 días de crecimiento, una respuesta que aparentemente confiere algunas ventajas ecofisiológicas a esta gramínea cuando se cultiva en suelos ácidos de bajo contenido de P disponible.

Palabras clave*:* Absorción y uso de fósforo, elongación radicular, expansión foliar, fosfatasas ácidas, oxalato.

Introduction

Adoption of *Brachiaria* forage grasses over the past 4 decades had a revolutionary impact on livestock productivity in the tropics (White et al. 2013). Both signalgrass [*Brachiaria* (now: *Urochloa*) *decumbens* cv. Basilisk, CIAT 606] and ruzigrass [*Brachiaria* (now: *Urochloa*) *ruziziensis* cv. Kennedy, CIAT 654] are grown on infertile acid soils that contain very low available phosphorus (P) levels, and are used for livestock production in the tropics (Miles et al. 2004).

A comparative study by Louw-Gaume et al. (2010a; 2010b), using signalgrass and ruzigrass, analyzed the role of morphological and physiological responses of roots, as plant mechanistic components to enhance P acquisition and P recycling within the plant. More specifically, plants of both grasses grown under low-P conditions had higher root biomass fractions and higher root tissue levels of acid phosphatases (APases) and phytases than plants grown under high-P conditions. Interestingly, root morphological traits of signalgrass were not responsive to variation in P supply, while lateral root growth in ruzigrass was significantly increased in plants grown at low P supply in hydroponic growth conditions.

Veneklaas et al. (2003) suggested that the key factor in plant-soil interactions might be rhizosphere chemistry, rather than root morphology. Mechanisms to increase inorganic P (Pi) availability in the rhizosphere include carboxylate exudation and APase secretion by plant roots (Gaume et al. 2001; Lambers et al. 2006; Neumann and Römheld 2012). The induction of APases is a general response of plants to Pi starvation and correlations between the intracellular and/or extracellular APase activity and cellular Pi status have been found (Vance et al. 2003; Nanamori et al. 2004).

Carboxylates enhance Pi release through the dissolution of calcium (Ca), iron (Fe) or aluminum (Al) phosphates. However, little in vivo evidence for P mobilization by carboxylates exists, except that most P-deficient plants release higher amounts than P-sufficient plants (Ström et al. 2002). The organic acid anions most effective at mobilizing P in soils are, in descending order, tricarboxylate citrate and the dicarboxylates, oxalate and malate (Neumann and Römheld 2000; 2012). Carboxylate release also requires the counter release of a cation to maintain charge balance. In the case of P deficiency, the rhizosphere pH has been shown to decline concurrently with carboxylate release, suggesting a balancing role for proton (H^+) efflux via H^+-ATPases (Hinsinger et al. 2003; Neumann and Römheld 2012). Two other likely candidates in terms of counter ions are potassium (K) and magnesium (Mg). Increased K^+ concentrations in root exudates suggest that carboxylate- and K^+-effluxes are coupled (Ryan et al. 2001), while Zhu et al. (2005) reported the involvement of Mg^{2+} in P-limiting carboxylate release in white lupin. Benefits for P uptake resulting from the coupling of carboxylate release to K^+-efflux have been shown by Palomo et al. (2006) as rhizosphere alkalinization by K-citrate-enhanced P mobilization in a high P-fixing acid soil.

The objective of this study was to determine the differences in the release of root biochemical markers, i.e. carboxylates and APases, during the development of P deficiency in signalgrass and ruzigrass. Our hypothesis was that exudation rates of both biochemical markers of P deficiency will be augmented in P-deficient plants of both grasses, but the 2 grasses could differ qualitatively and quantitatively in their response, when grown for a short period of 21 days at low P supply. We used the hydroxyapatite pouch system in hydroponics (Sas et al.

2001) to simulate low P supply conditions of infertile tropical soils (Louw-Gaume et al. 2010a) and to investigate whether the release of carboxylates and APases from roots is part of a temporally coordinated and targeted response to P limitation in signalgrass and ruzigrass. In addition to these physiological responses and associated differences in plasticity, related mechanistic components such as exuded counter-ions for charge balance and tissue levels of carboxylates were investigated at weekly intervals for 3 weeks. Finally, as responses in leaf and root growth of *Brachiaria* grasses might differ at low P supply (Rao et al. 1996; Louw-Gaume et al. 2010a, 2010b), we also examined these morphological responses in order to obtain a whole-plant perspective that might contribute to understanding diversity in plant attributes for tolerance to low-P acid soils that exists in *Brachiaria* germplasm (Rao et al. 1998; Miles et al. 2004; Rao 2014).

Materials and Methods

Plant growth and harvests

The experimental protocol for the germination of seeds and growth of a tetraploid, apomictic signalgrass [*Brachiaria* (now: *Urochloa*) *decumbens* cv. Basilisk, CIAT 606] and a diploid sexual ruzigrass [*Brachiaria* (now: *Urochloa*) *ruziziensis* cv. Kennedy, CIAT 654] in nutrient solution at pH 5.5, using the hydroxyapatite (HAP)/dialysis pouch system, was reported by Louw-Gaume et al. (2010a). Seeds were surface-sterilized and germinated in the dark (25 °C) for 3 to 4 days on filter paper saturated with deionized water. Seedlings were grown for one week in sand culture (with nutrient supply in mg/kg of sand: 2.6 P, 2.5 N, 3.1 K, 1.0 Ca, 0.38 Mg, 0.38 S, 0.02 Zn, 0.03 Cu, 0.001 B, 0.001 Mo) in growth chambers with a day/night cycle of 12 h at 25 °C and 12 h at 18 °C, 60% relative humidity and a photon flux density of 250 µmol/m^2/sec. These conditions for early seedling growth were used since *Brachiaria* grasses do not display rapid early seedling growth level due to their small seed size. Selected seedlings of each grass with similar development were further grown in aerated nutrient solution (in mM: 0.25 NH_4NO_3, 0.53 KNO_3, 0.75 $Ca(NO_3)_2$, 0.33 $CaCl_2$, 0.42 $MgSO_4$, 0.17 NaCl, 0.01 FeNaEDTA; in µM: 30 H_3BO_3, 5 $ZnSO_4$, 0.2 $CuSO_4$, 10 $MnCl_2$, 0.1 Na_2MoO_4) under the same controlled conditions. The hydroxyapatite/dialysis pouch system in hydroponics was used to induce high-P (5 g of hydroxyapatite) and low-P (1 g of hydroxyapatite) conditions (Louw-Gaume et al. 2010a). The current study included only the low-P treatment to further characterize physiological and biochemical responses of both grasses to low P supply. Plant responses were monitored at 3 time intervals, i.e. day 7 (D7), day 14 (D14) and day 21 (D21) after inducing low P supply to one-week-old seedlings transferred to nutrient solution. This growth period was selected to focus on root-level mechanisms for P uptake during vegetative growth.

Phosphate release in control containers (n = 6) without plants was monitored and measured as 0.33 ± 0.02 µM Pi/d. The mean concentration of Pi on day 0 (day before introducing seedlings) was 1.00 ± 0.11 µM (n = 16), a Pi level that is in agreement with the value of 1 µM Pi used by Wenzl et al. (2003) to simulate Pi level in soil solutions of highly weathered acid soils. On a daily basis, the pH and Pi concentration of hydroponic solutions were measured, while the HAP-containing pouch was checked daily for potential leakage and visible evidence of bacterial growth. The complete nutrient solution, including the pouches, was renewed on days 8 and 15 (D8 and D15). As described before (Louw-Gaume et al. 2010a), each hydroponic tank contained 2 replicates of each grass and each replicate consisted of 3 plants. The number of replicates was 10 for each grass (that is, 30 plants in total). The experiment was repeated and the data from the second experiment are reported, since this experiment included all measurements. Similar results were observed in both experiments on biomass production, carboxylate composition and exudation rates for both grasses.

Three destructive harvests were performed following the collection of root exudates at D7, D14 and D21, starting at the same time of day for each harvest, as it has been reported that rhizosphere processes for P mobilization exhibit a temporal variability (Neumann and Römheld 2012). The dry matter (DM) per young seedling (n = 10) before inducing low-P treatment was slightly higher for ruzigrass than for signalgrass (37 vs. 32 mg DM). The shoot mass density of signalgrass seedlings was higher than for ruzigrass (0.16 vs. 0.13 g DM/g fresh biomass). The nutrient concentrations (% of dry weight) of the seedlings before low-P treatment were: 0.16 P, 0.19 S, 1.14 N and 44.5 C for the shoot tissue of signalgrass; and 0.23 P, 0.31 S, 4.29 N and 42.0 C for the shoot tissue of ruzigrass. For root tissue of the seedlings, the concentrations were: 0.07 P, 0.12 S, 1.14 N and 44.5 C in signalgrass; and 0.08 P, 0.13 S, 1.36 N and 39.3 C in ruzigrass. Plant material was dried for 4 d at 45 °C before DM determination. Leaf area was recorded with a leaf area meter (Li-COR Model 3100, Lincoln, USA). The complete root system was scanned and root length was analyzed using WinRHIZO V3.09b root imaging software (Regent Inc., Quebec, Canada). The relative

growth rate was calculated for each harvest interval, according to the method of Hoffmann and Poorter (2002). Rates of leaf expansion and root elongation between harvests were calculated as the change in leaf area (in cm^2) or root length (in m) per day for the three 7-day growth periods. For the determination of the plant P, K and Mg concentrations, dried and milled plant material was incinerated at 550 °C, followed by solubilization in 65% HNO_3 and analysis with ICP-emission spectroscopy (Louw-Gaume et al. 2010b).

Collection of root exudates and pH measurement in $CaCl_2$ traps

At each sampling plants were removed from hydroponic containers and root systems were washed twice in 0.1 mM $CaCl_2$ solution (pH 5.5, adjusted with HCl) to eliminate possible interference from remaining nutrients close to root systems during the exudation steps. Great care was taken when handling root systems to avoid tissue damage. The first exudation step was performed for 6 h in aerated 0.1 mM $CaCl_2$ (pH 5.5) solution containing 0.01% (v/v) protease inhibitor cocktail (Sigma, P2714) under the same growth chamber conditions as for plant growth. The second step was carried out for 1 h at 4 ºC in 0.1 mM NaCl (pH 5.5, adjusted with HCl) with the same inhibitor cocktail. Exudation volumes were adjusted at each harvest time to compensate for different plant sizes. For example, at D7, the exudation volume was 30 ml per bunch of 3 plants for both grasses and 80 ml and 110 ml for signalgrass and ruzigrass, respectively, at D21. The pH was measured at the end of the 6-h period in the $CaCl_2$ solutions. Exudates were centrifuged at low speed (4 ºC) for 3 min, filtered through 0.2-µm syringe filters and stored at -80 ºC until assayed. These steps were in line with recommendations by Gaume et al. (2001) and Neumann and Römheld (2000).

Carboxylate extraction and determination

The roots were washed with de-ionized water and blotted dry with paper towels, frozen in liquid nitrogen and stored at -80 ºC until extraction. The method of Zindler-Frank et al. (2001) was slightly modified and soluble oxalate was extracted by grinding frozen leaf and root material in warm (50 ºC) deionized water, followed by heating at 80 ºC for 30 min, bench-top centrifugation, filtration through 0.2-µm syringe filters and acidification with HCl to pH 3–4. These extracts were also used for the determination of glycolate.

The carboxylates in vacuum-concentrated $CaCl_2$ solutions were analyzed by ion chromatography (Dionex DX 500 System, Dionex Corporation, USA). An Ion Pac AS10 column, in combination with suppressed conductivity, was used and the eluent was 50 mM NaOH with a flow rate of 1 ml/min. The exudate samples were dried and the pellets re-suspended in nanopure water prior to injection. The identification of carboxylates was confirmed by spiking with standards and carboxylate release rates were expressed per unit of root length, i.e. nmol/m/h.

Effluxes of H^+, K^+, Mg^{2+} and NO_3^-

At each harvest time, the pH of $CaCl_2$-exudate solutions increased from 5.5 to values above 6 for both grasses over the 6-h exudation period. These increases were converted into proton equivalents and expressed as the relative change in protons. Efflux rates of K^+ and Mg^{2+} were determined by analyzing the K and Mg concentrations in the $CaCl_2$ solutions with ICP-emission spectroscopy. The $CaCl_2$ solutions were also analyzed for the presence of nitrate (NO_3^-) using a flow injection analyzer (SKALAR San++ System, Netherlands). The relative change in protons and efflux rates of K^+, Mg^{2+} and NO_3^- were expressed per unit of root length, i.e. µmol protons, K^+, Mg^{2+}/m/h and nmol NO_3^-/m/h.

Acid phosphatase and phytase activity

Acid phosphatase activities detected in the $CaCl_2$ and NaCl solutions were grouped as secreted APases (sAPases) and cell-wall-associated APases (cwAPases), respectively. Root exudate solutions were concentrated with centrifugal filters (Amicon Ultra-15, Millipore, USA) for the detection of phytase activity. The activities of acid phosphomonoesterases and phytases were determined as described by Louw-Gaume et al. (2010b). Enzyme activities were expressed as enzyme units (U) per unit root length, where 1 U releases 1 µmol Pi/min.

Statistical analysis

The Welch two sample t-test was used to determine differences between species and between harvest intervals (R Core Team 2014).

Results

Biomass production and plant P concentrations

The total biomass production increased between sequential harvests for both grasses, but ruzigrass produced more biomass at each harvest time (Figure 1A).

Figure 1B shows that ruzigrass could not maintain its relative rate of biomass production after D14 (14 days after inducing low-P treatment) and the relative growth rate declined by 30%, to a level similar to that maintained by signalgrass throughout. Rate of leaf expansion increased strongly between D7 and D14, with a smaller increase in rate from D14 to D21, while rate of root elongation for ruzigrass was much greater between D14 and D21 than for the other periods (Figures 1C and 1D, respectively). Root diameter was not affected by decreasing plant-P concentrations, although signalgrass had thinner roots at each harvest time (results not shown).

Plant-P concentrations in both grasses declined with age with a greater reduction for ruzigrass than for signalgrass (Figure 2). P concentration in ruzigrass at D7 was much greater than for signalgrass (3.8 vs. 2.1 mg/g DM) but levels were similar for both grasses at subsequent harvests, reaching about 1.0 mg P/g DM at D21.

Carboxylates in root exudates and in tissues

Figure 3A shows exudation rates and composition of organic acid anions, i.e. acetate, glycolate, formate, lactate (monocarboxylates) and oxalate (dicarboxylate). Citrate (tricarboxylate) and malate (dicarboxylate) could not be detected in the root exudates of either grass. The combined exudation rates of all carboxylates for ruzigrass were 115% greater than those for signalgrass at D7, 240% greater at D14 and only 55% greater at D21. The temporal patterns of monocarboxylate exudation did not differ between grasses, with rates decreasing after D7 (Figure 3B), but then increasing slightly between D14 and D21. In contrast,

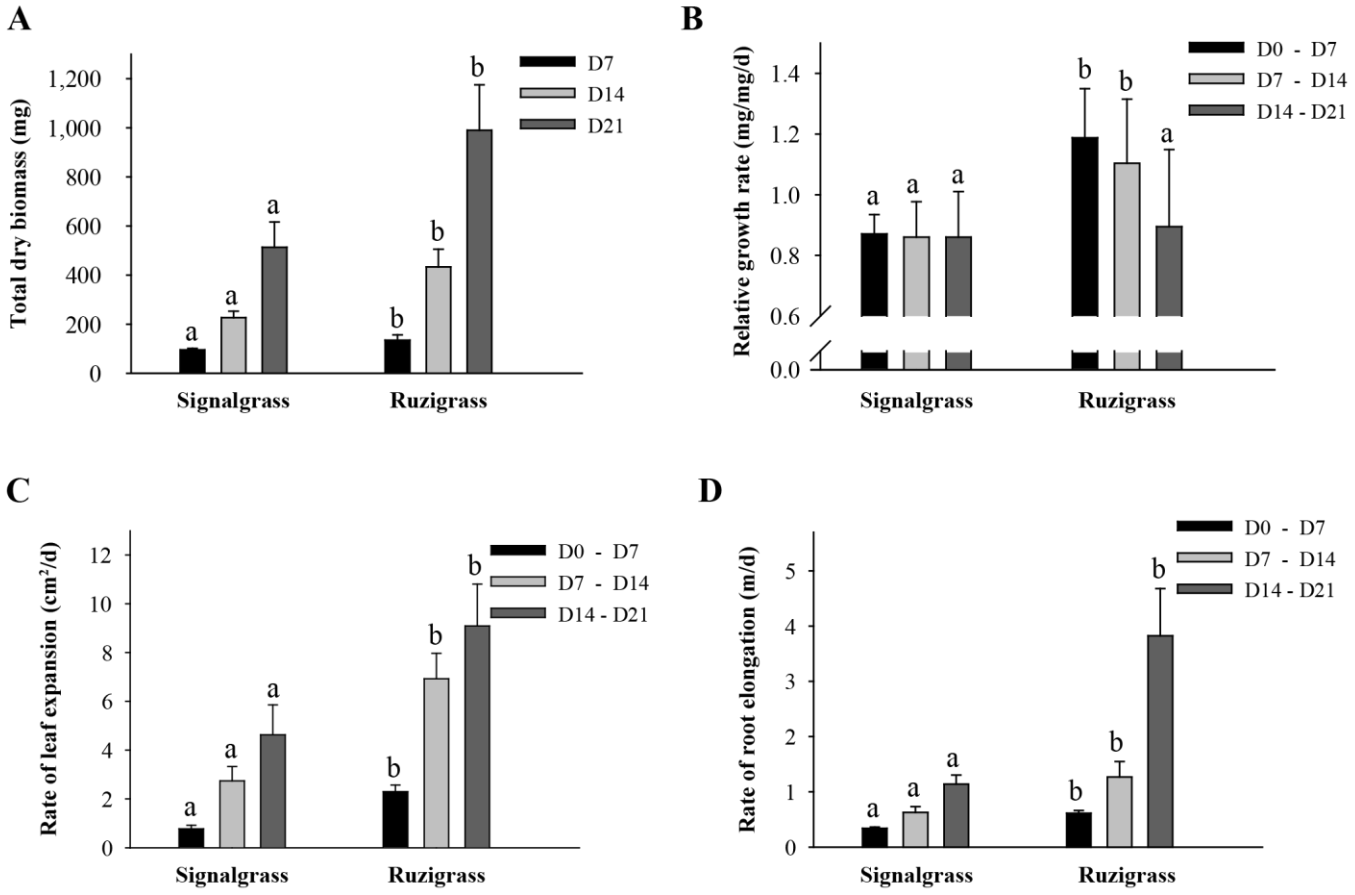

Figure 1. Morphological attributes of signalgrass and ruzigrass at day 7 (D7), day 14 (D14) and day 21 (D21) after inducing low-P treatment under hydroponic conditions. (**A**) Total dry biomass (DM). (**B**) Relative growth rate (mg/mg/d) for each of the 3 harvest intervals, i.e. D0-D7 (first harvest interval), D7-D14 (second harvest interval) and D14-D21 (third harvest interval), where D0 refers to the start of the experiment and the day on which young seedlings were prepared for experimental use. (**C**) Rate of leaf expansion (cm^2/d). (**D**) Rate of root elongation (m/d). Means for a specific harvest time or harvest interval with different letters indicate significant differences between grasses ($P<0.05$).

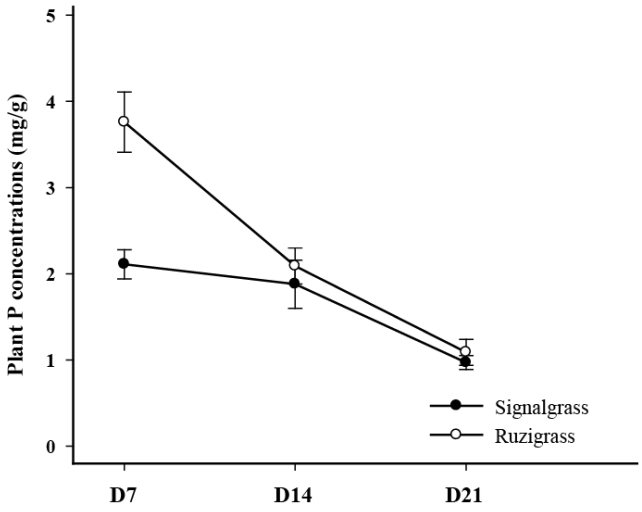

Figure 2. Plant-P concentrations of signalgrass and ruzigrass at day 7 (D7), day 14 (D14) and day 21 (D21), expressed as mg P/g DM, after inducing low-P treatment under hydroponic conditions. Vertical bars represent ± s.e. (n = 30).

patterns of oxalate exudation differed between grasses (Figure 3C) with rate increasing throughout for signalgrass but peaking at D14 for ruzigrass. Final levels at D21 were similar for both grasses.

The carboxylate composition of root exudates changed over time for both grasses (Figure 3A). The oxalate fraction at D7 and D14 was greater for ruzigrass than for signalgrass (5 and 31% vs. 1 and 17%, respectively). By D21 the level in signal grass had increased to 45%, while the level in ruzigrass remained at 31%. Patterns of lactate exudation were similar in both grasses, being high at both D7 and D21 with very low levels at D14. At D21 signalgrass had a higher oxalate:lactate ratio than ruzigrass (1.7 vs. 0.5). For both grasses, the temporal patterns for glycolate and formate fractions were similar; absolute levels of exudation did not vary over time as much as levels of acetate, oxalate and lactate but the percentages of total exudation fluctuated because of changes in the other components.

Tissue concentrations of soluble oxalate and glycolate are shown in Table 1. For each grass, leaf and root oxalate concentrations did not change between D7 and D14. At these harvest times, signalgrass had greater leaf:root oxalate ratios than ruzigrass due to oxalate concentrations in signalgrass being higher in leaves and lower in roots than those of signalgrass. Leaf oxalate concentrations in signalgrass decreased after D14, while for ruzigrass, both leaf and root oxalate concentrations declined. Glycolate concentrations were up to 30 times those of oxalate in both grasses. In addition, leaf glycolate concentrations showed moderate temporal variation, with lowest values at D14, while root glycolate levels changed in a similar way to oxalate levels in each grass.

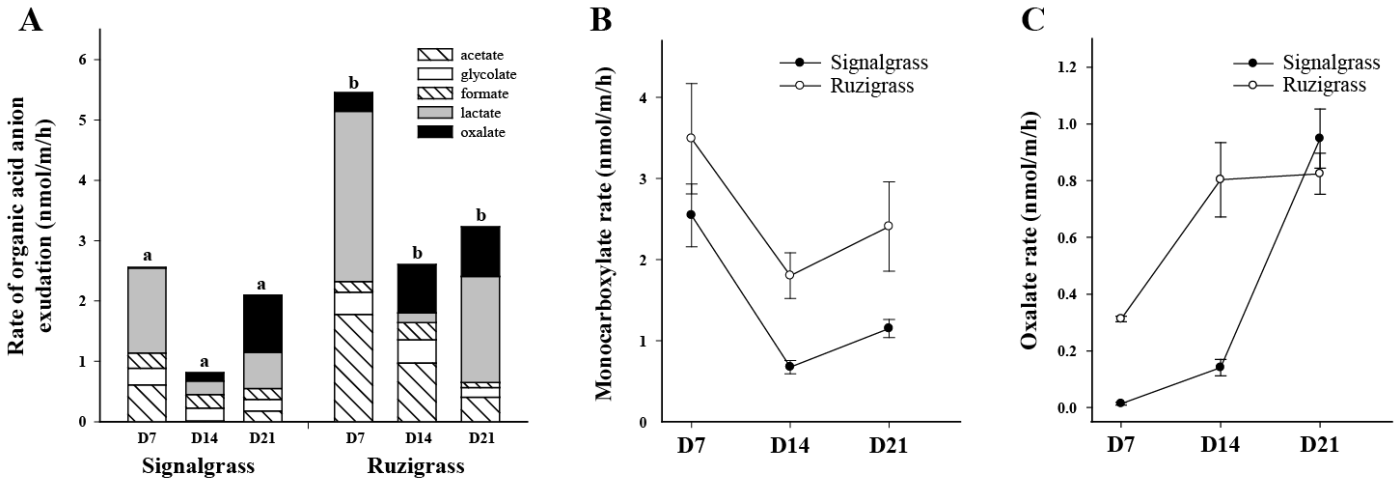

Figure 3. Root exudation rates (nmol/m/h) of carboxylates by signalgrass and ruzigrass at day 7 (D7), day 14 (D14) and day 21 (D21) after inducing low-P treatment under hydroponic conditions. (**A**) Total root exudation rates and carboxylate composition. Different letters indicate significant differences between grasses for total root exudation rate at a specific harvest. (**B**) Rates of monocarboxylate root exudation, including acetate, glycolate, formate and lactate. (**C**) Rates of oxalate root exudation.

Table 1. Tissue concentrations (nmol/g fresh mass) and leaf:root ratios of oxalate and glycolate for signalgrass and ruzigrass at day 7 (D7), day 14 (D14) and day 21 (D21) after inducing low-P treatment under hydroponic conditions.

Carboxylate	Harvest	Signalgrass			Ruzigrass		
		Leaf (L)	Root (R)	L:R ratio	Leaf (L)	Root (R)	L:R ratio
Oxalate	D7	53a[1]	17a	3.1	44b	22b	2.0
	D14	61a	16a	3.8	45b	27b	1.7
	D21	39a	18a	2.2	33a	13b	2.5
Glycolate	D7	1,379a	415a	3.3	1,354a	646b	2.1
	D14	960a	471a	2.0	1,215b	814b	1.5
	D21	1,364a	448a	3.0	1,601a	329b	4.9

[1]Within rows and plant parts, values followed by different letters are different ($P<0.05$).

Changes in proton equivalents and efflux rates of K^+, Mg^{2+} and NO_3^- and root concentrations of K and Mg

The relative change in proton equivalents (Figure 4A) was greater for signalgrass than for ruzigrass at D7, but grasses did not differ at D14 and D21. For both grasses the lowest values were recorded at D21 (i.e. pH increased to a lesser extent from the value of 5.5 after D14). Rates of efflux of NO_3^- (Figure 4B), K^+ (Figure 4C) and Mg^{2+} (Figure 4D) generally increased with time, while root concentrations of K and Mg declined over time (Figures 4E and 4F).

Temporal patterns of APase secretion and phytase proportion

Rates of secretion of sAPases were similar in both grasses at D7 and D21, but ruzigrass had a much higher secretion rate at D14 than signalgrass (Figure 5A). For both grasses, cwAPase release rates increased only after D14, by 2-fold in signalgrass and 6-fold in ruzigrass (Figure 5B). Compared with sAPases at D21, rates of cwAPases were 3-fold higher in signalgrass and 10-fold higher in ruzigrass. Extracellular phytases could be detected only at D21 in both the $CaCl_2$- and NaCl-collections. Phytase proportions (as a percentage of the total APase pool) were low for both grasses, but were slightly higher in the cwAPase pool than in the sAPase pool.

Discussion

Low P supply reduced biomass production and leaf expansion in ruzigrass

In agreement with earlier findings (Louw-Gaume et al. 2010a), ruzigrass was a faster-growing grass and produced more biomass than signalgrass at low P supply during this short experimental period of 21 days that focused on plant mechanisms and associated plasticity for P uptake during early vegetative growth. However, the growth of ruzigrass was compromised during the development of P deficiency; while ruzigrass grew very fast initially, it could not maintain its relative growth rate and strong leaf expansion after D14. In addition, plant-P concentrations declined after D14 to below 2.0 mg P/g DM, indicating that ruzigrass started to economize on Pi. Veneklaas et al. (2012) suggested that the reduction in growth is not a direct consequence of low shoot-P status, but of signaling events that can be genetically controlled. Lambers et al. (2008) also reported that roots sense that nutrients such as N and P are limiting well before leaves experience deficiency symptoms, indicating that shoot growth is regulated in a feed-forward manner.

Critical shoot-P concentrations in *Brachiaria* grasses are around 1.0 mg P/g DM (Rao 2001). For ruzigrass, the key factor responsible for high P uptake and high initial P concentrations might be a faster growth rate as suggested by Lambers and Poorter (2004). In contrast, signalgrass had lower P uptake and P concentration in tissue initially, resulting in lower growth rates. This balanced growth rate may ensure that nutrient demand does not exceed its supply.

Release of oxalate and APase are linked to decreasing plant-P concentrations

Our results suggest that oxalate and APases are involved in the P-nutrition of both *Brachiaria* grasses as temporal associations between decreases in plant-P concentrations and increases in the exudation of these biochemical attributes were evident. The release of both components might also form part of a coordinated adaptive strategy and functional synergy between oxalate and APases, which could improve acquisition of P in low-P acid soils

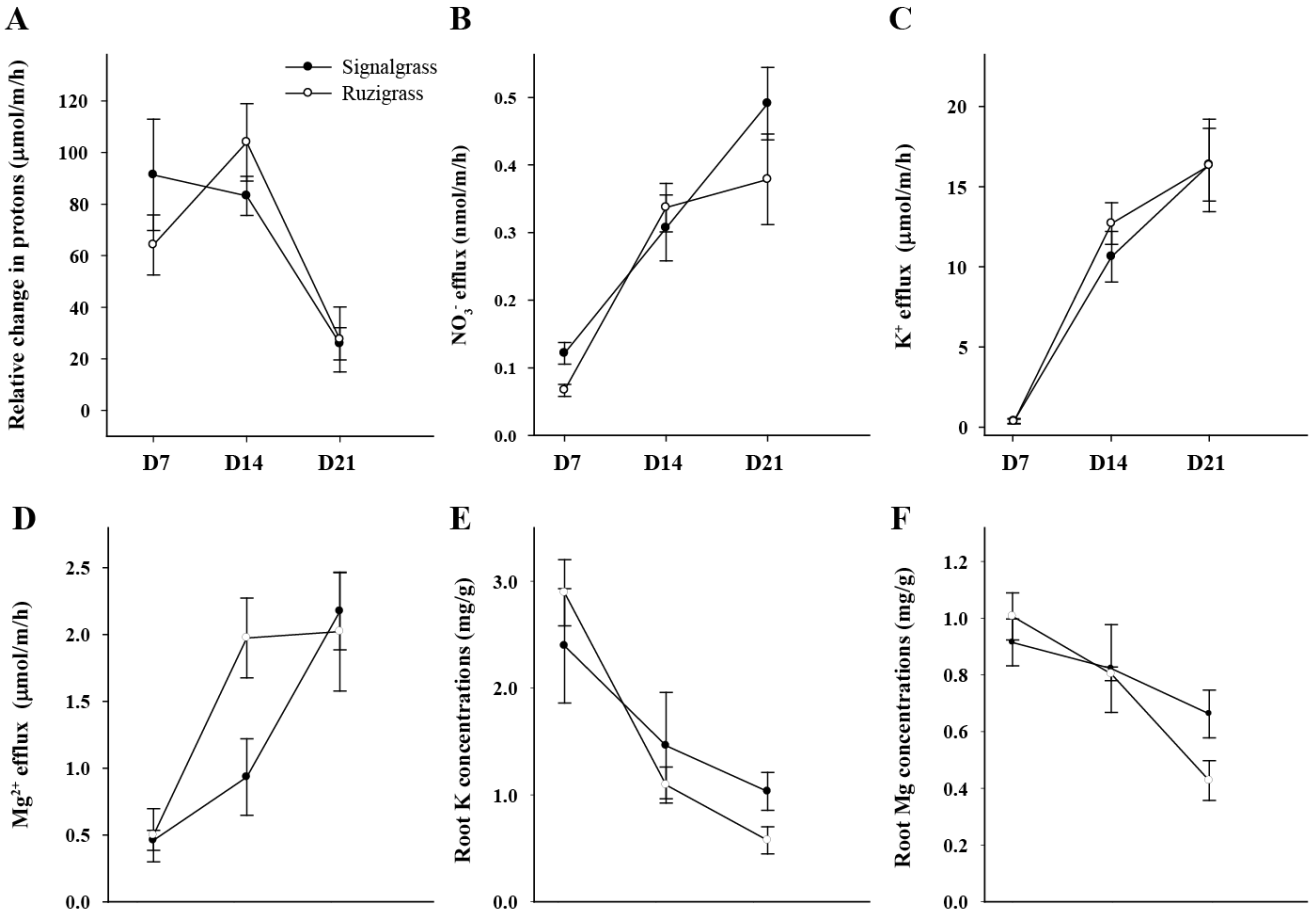

Figure 4. Relative change in proton equivalents (µmol/m/h) and efflux rates of NO_3^- (nmol/m/h), K^+ (µmol/m/h) and Mg^{2+} (µmol/m/h) and K and Mg concentrations (mg/g) in roots of signalgrass and ruzigrass at day 7 (D7), day 14 (D14) and day 21 (D21) after inducing low-P treatment under hydroponic conditions. (**A**) Change in pH (from pH 5.5) expressed as relative change in proton equivalents. (**B**) Rate of NO_3^--efflux. (**C**) Rate of K^+-efflux. (**D**) Rate of Mg^{2+}-efflux. (**E**) Root K concentrations. (**F**) Root Mg concentrations.

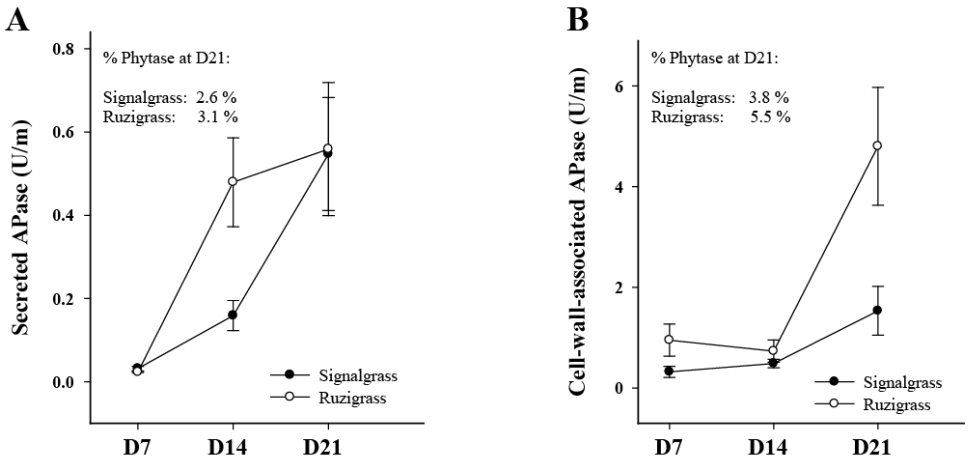

Figure 5. Root exudation of APases (U/m) by signalgrass and ruzigrass at day 7 (D7), day 14 (D14) and day 21 (D21) after inducing low-P treatment under hydroponic conditions. (**A**) Rate of secreted APases collected in $CaCl_2$ solution. (**B**) Rate of cell-wall-associated APases collected in NaCl solution. Activity of extracellular phytase was detected only at D21 and its percentage of the total pool of APases is indicated in the left upper corner of each graph.

as carboxylates can enhance the solubility of not only inorganic P, but also organic soil-P forms, which are subsequently hydrolyzed by phosphatases (Vance et al. 2003; Jones et al. 2004; Playsted et al. 2006).

The increase in exudation of oxalate and sAPases by roots could be associated with decreases in P concentrations in each grass. These traits increased during early growth, together with the first decline in plant-P concentrations in ruzigrass, supporting as well a higher growth demand for P in this grass. While rate of exudation of carboxylate and sAPase by ruzigrass peaked at D14, secretion of cwAPase increased sharply during the next 7 days, when plant-P concentrations declined further and biomass production would have been compromised. It is important to emphasize that, although root exudation responses of ruzigrass after D14 appeared to level off, root elongation increased strongly after D14. Thus, if root growth was stimulated while exudation rates were maintained during P-limited growth, the key factor to consider was total below-ground output of carboxylates, which might be higher for ruzigrass. Louw-Gaume et al. (2010a) also showed that lateral root growth was stimulated in ruzigrass only when grown at low P supply. Interestingly, Hütsch et al. (2002) reported that cultivar differences in total amounts of root-released C could be attributed to root length.

Furthermore, it appears that root morphological plasticity in ruzigrass is associated with a high level of root physiological plasticity as evident from the strong induction of cwAPases by plant P concentrations below 2 mg P/g DM. This finding also suggests a dependence on a critical threshold of Pi depletion as a signal for enzymatic cwAPase induction (Jain et al. 2007). In white lupin, secretory APases were produced not only by tap root epidermal cells, but also in the cell walls and intercellular spaces of lateral roots. Such apoplastic phosphatases are protected from inactivation by various soil processes, but effectiveness depends on the presence of soluble organophosphates in soil solution (Neumann and Römheld 2007; 2012). Although the enzymatic hydrolysis of root-secretory phosphatase is limited by the low solubility of organic P forms in soils (Neumann and Römheld 2012), higher phosphatase activities in the rhizosphere have been reported to contribute to the depletion of organic P from Oxisols containing very low available P (George et al. 2006). In signalgrass, exudation responses of all 3 biochemical markers for P limitation, i.e. oxalate and both groups of APases, were temporally coordinated and increased only after D14.

Acquisition of P from phytate by phytases could potentially provide plants with an alternative organic P source (Richardson et al. 2005). Louw-Gaume et al. (2010b) reported higher root tissue levels of APases and phytases for plants grown under low-P conditions with phytase proportions representing less than 1% of the total APase pool in root tissue, while the present study found higher phytase proportions in both the sAPase and cwAPase pools (2 and 5%, respectively). Interestingly, the grasses we studied did not differ with regard to phytase fractions in either study. Our results are at variance with the findings by Li et al. (1997), who reported high levels of phytase secretion in P-deficient *B. decumbens* plants. The experimental system used in simulating low-P supply conditions in the hydroponics growth medium might explain these differences. Our results support the observations of Hayes et al. (1999), who reported that phytase activity constituted only a small component (less than 5%) of the total APase activity in various plants.

Oxalate exudation may enhance P acquisition in acid tropical soils

Oxalate exudation in response to P deficiency has been reported in sugarbeet (Gerke et al. 2000), soybean (Dong et al. 2004), rice (Hoffland et al. 2006) and *Banksia* species (Denton et al. 2007) and our results for both *Brachiaria* grasses are consistent with these observations. Pentanedioic acid and oxalic acid were also dominant exuded organic acids in P-deficient elephantgrass (*Pennisetum purpureum*), another tropical forage grass (Shen et al. 2001). Dong et al. (2004) also noted that exudation of oxalate rather than other carboxylates may present higher physiological efficiency, as less C and energy are consumed during exudation.

Hydroponic experiments provide only indirect evidence and the functional significance of carboxylate exudation in a real soil environment remains unknown (Jones et al. 2004; Neumann and Römheld 2012). Observed exudation rates cannot be compared with those of leguminous plants as their carboxylate effluxes are 10 to 50 times higher than for graminaceous species (Gerke et al. 2000). In soils with low P-availability, competition by carboxylates for P-sorption sites might be of greater significance than P-desorption mechanisms, which require high concentrations of carboxylates such as citrate and oxalate. Huguenin-Elie et al. (2003), using a modeling approach, showed that low release rates of

citrate could account for 90% of the P uptake of rice grown under aerobic conditions. Furthermore, average values integrated over the whole root system can be misleading and may result in erroneous conclusions about nutrient relationships in the rhizosphere, due to spatial variability of exudation along the root axis (Neumann and Römheld 2000; 2012).

Fox and Comerford (1992) suggested that the cumulative oxalate loading rate contributes to the solubilization of large amounts of P on an annual basis and this might be relevant for the survival of the grasses used in our study. Furthermore, the effectiveness of oxalate was shown in both calcareous and acid soils treated with monocalcium phosphate and phosphate rock (Fox and Cromerfold 1992; Ström et al. 2002), suggesting that oxalate exudation by signalgrass and ruzigrass might have significance for enhanced P-acquisition in acid soils. Application of rock phosphates to acid soils has been suggested (Fardeau and Zapata 2002) and their suitability as P fertilizer for signalgrass has been demonstrated (Lopes et al. 1991). Araújo et al. (2003) also reported greater importance for the acid-soluble P fraction than for the NaOH-extractable fraction in a pot experiment using *B. decumbens*. As signalgrass is better adapted to and more persistent on infertile acid soils that contain very low available P than ruzigrass (Miles et al. 2004; Rao 2014), signalgrass might have a selective ecophysiological advantage over the long term due to the dominance of oxalate plus its slower and more balanced growth rate and associated implications for higher plant carbon (C) use efficiency (Louw-Gaume et al. 2010b). Leaf oxalate concentrations were also higher for signalgrass, consistent with higher oxalate levels reported for slower-growing plants (Libert and Franceschi 1987).

Although lactate appears to be commonly exuded by plant species that are adapted to acid soils (Tyler and Ström 1995), the finding that lactate was the dominant exuded carboxylate in ruzigrass at D21 was unexpected, as the presence of lactate has also been linked to detoxification that could be associated with cytoplasmic acidosis (Neumann and Römheld 2000). It is possible that, despite its high biomass production, high P uptake and high exudation rates of biochemical traits important for P-mobilization in acid soils, ruzigrass might start to experience metabolic complications in maintaining cellular Pi homeostasis over a longer growth period (Veneklaas et al. 2012). In addition, C-costs related to exudation (Dilkes et al. 2004) might have been substantial in ruzigrass, as faster-growing grasses deposit more C than species adapted to infertile soils (Warembourg et al. 2003).

The higher rate of formate exudation in signalgrass was also interesting, as Tanaka et al. (1995) suggested that formate could solubilize Fe-P forms due to its strong reducing capacity, based on observations of increased formate secretion in P-deficient *Arachis hypogaea*. Dinkelaker et al. (1995) also proposed that increased reductive capacity in roots may be another P-adaptive response.

Oxalate exudation might be an important strategy for Al resistance, as Al-toxicity and P-deficiency co-exist in acid soils and both are major constraints for productivity of *Brachiaria* pastures (Miles et al. 2004). Carboxylate exudation could not be linked to external Al detoxification in either grass (Wenzl et al. 2001), but a low-P background might have obscured responses (Liao et al. 2006). Interestingly, phytosiderophore-mediated iron release from goethite is also enhanced by oxalate (Marschner et al. 2011) and thus, oxalate might also be important for Fe uptake from iron oxides in both *Brachiaria* grasses.

Are Mg^{2+} ions involved in charge balance during oxalate exudation?

Our observations reiterate that interpretation of pH changes in the rhizosphere should be considered with caution (Hinsinger et al. 2003; Neumann and Römheld 2012). The greater pH of $CaCl_2$-containing root exudates may be attributed to lower Ca^{2+} uptake (versus Cl^-) (Hinsinger et al. 2003). In *B. dictyoneura* Hylander and Ae (1999) also reported an increase in the rhizosphere pH due to higher amounts of basic cations and proton neutralization. However, the pH of nutrient solutions with growing plants declined over time and ruzigrass showed greater capacity to lower the pH (observed in pre-experiments), so we adopted the practice of growing both grasses in the same hydroponic container to eliminate interferences from the addition of KOH that was used for pH control. Proton release has also been linked to differential cation/anion uptake (Hinsinger et al. 2003), consistent with the report by Logan et al. (2000), who found that plant-induced acidity by *B. humidicola* and *B. brizantha* was not due to low P-availability, but to adequate supply of nutrients for growth.

Our study focused on the most likely counter-cation candidates to accompany carboxylate efflux (Ryan et al. 2001; Zhu et al. 2005). Interest in K^+-efflux and root-K levels also stems from the finding that the K or sodium salt of oxalate is predominantly found in grasses (Jones and Ford 1971). The two grasses did not differ in the

pattern of K^+-efflux, which increased strongly after D7. Marschner et al. (1997) reported that K^+ functions in charge balance, especially in NO_3^--fed plants, participating as well in translocation of carboxylates and soluble sugars. Interestingly, an increase in NO_3^--efflux after D14 was observed only in signalgrass, supporting higher NO_3^--efflux rates as reported in slow-growing plants (Nagel and Lambers 2002). Root-K concentrations decreased for both grasses as reported during P deficiency in white lupin (Sas et al. 2002) and the *Brachiaria* hybrid cv. Mulato (Watanabe et al. 2006).

Despite these uncertainties for H^+ and K^+, Mg^{2+} appears to be a counter-ion for oxalate efflux as its efflux pattern corresponded well with the release curves of oxalate in both species. Zhu et al. (2005) reported that Mg^{2+} was involved in carboxylate release of white lupin during P deficiency. Increases in Mg^{2+}-efflux by roots also corresponded in a timely manner with decreases in root concentrations of Mg in each grass.

Another consideration is that cation-efflux rates were higher than those of carboxylates. Deficiency of P enhances membrane leakiness (Neumann and Römheld 2007), suggesting that the likelihood of higher non-specific efflux during P limitation cannot be excluded.

Glycolate might be an oxalate precursor

As expected, monocarboxylate exudation could not be related to the plant-P status in the current study, but our results on monocarboxylate composition and exudation patterns could have significance for C utilization. Oxalate can be formed from photorespiratory glyoxylate via glycolate, catalyzed by glycolate oxidase (Franceschi and Nakata 2005). In both grasses we used glycolate might be an oxalate precursor, as leaf glycolate levels were significantly higher than those of oxalate and, in addition, leaf oxalate levels decreased after D14, while leaf glycolate levels increased. Interestingly, Ueno et al. (2005) studied 28 C4 grasses (ruzigrass not included) and found activity of glycolate oxidase was greatest in *B. brizantha* and *B. decumbens.*

While oxalate exudation increased after D14 in signalgrass and also ruzigrass (when increases in root elongation are considered), leaf oxalate concentrations decreased for both grasses, suggesting that leaves might be the site of oxalate biosynthesis, as reported by Ji and Peng (2005). Root levels of oxalate and glycolate did not change over time in signalgrass, but both decreased strongly in P-deficient ruzigrass plants. Carboxylate efflux has been correlated with intracellular root concentrations, but Ryan et al. (2001) pointed out that membrane processes appear to be the key step for efflux.

Our results support the notion that increased carboxylate biosynthesis in plants is a physiological alteration associated with the preferential root exudation of carboxylates with highest efficiency in P mobilization under conditions of P limitation (Neumann and Römheld 2007).

Conclusions

The experimental approach used in this study highlights the importance of adopting an eco-physiological perspective to understand developmental, physiological and biochemical aspects of adaptation to low-P stress in *Brachiaria* grasses. Furthermore, a comparison of species differences in adaptation to limiting P supply became feasible by studying, simultaneously, temporal responses of: (i) whole-plant growth together with variations in both root and leaf attributes; and (ii) root-induced changes in the rhizosphere that determine nutrient availability and influence plant growth. Results from this study indicate that growth may be faster for ruzigrass than for signalgrass during early establishment in low-P soils but ruzigrass may demand higher P supply to sustain its higher growth rate. Further research is needed on soil-grown plants of both grasses to characterize changes in rhizosphere induced by exudation of organic acids and phosphatases from roots.

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Research Paper

Effect of pollination mode on progeny of *Panicum coloratum* var. *makarikariense*: Implications for conservation and breeding

Efecto del modo de polinización sobre la progenie de Panicum coloratum *var.* makarikariense*: Implicaciones para conservación y fitomejoramiento*

LORENA V. ARMANDO[1,2], MARÍA A. TOMÁS[1], ANTONIO F. GARAYALDE[2,3] AND ALICIA D. CARRERA[4]

[1]*Instituto Nacional de Tecnología Agropecuaria (INTA), EEA Rafaela, Santa Fe, Argentina*. www.inta.gob.ar
[2]*Centro de Recursos Naturales Renovables de la Zona Semiarida, CERZOS-CCT-CONICET, Bahía Blanca, Buenos Aires, Argentina*. www.cerzos-conicet.gob.ar
[3]*Departamento de Matemática, Universidad Nacional del Sur, Bahía Blanca, Buenos Aires, Argentina*. www.matematica.uns.edu.ar
[4]*Departamento de Agronomía, Universidad Nacional del Sur, Bahía Blanca, Buenos Aires, Argentina*. www.uns.edu.ar/deptos/agronomia

Abstract

Panicum coloratum var. *makarikariense*, a perennial grass native to Africa, is adapted to a wide range of soil and climatic conditions with potential to be used as forage in tropical and semi-arid regions around the world. Our objective was to understand how the pollination mode affects viable seed production and further survival of the progeny. We evaluated self- and open-pollinated progenies from different accessions by measuring the seed production of the parents and their germination performance, germination rate and seedling survival. Parents and progeny were also fingerprinted with Simple Sequence Repeats (SSR). Progeny produced through open-pollination resulted in significantly more filled seeds and superior seedling survival than self-pollination. These results indicate that accessions studied here rely heavily on cross-pollination, whereas the contribution of self-pollinated offspring to the population is likely to be low. SSR profiles showed that, on average, 85% of the progeny (arising from cross-pollination) possessed paternal specific markers and 100% of them were genetically different from the maternal genotype. All plants examined had 4x = 36 chromosomes. Overall, our findings indicate that var. *makarikariense* is able to generate highly polymorphic progeny through segregation and recombination. This study provides reference information for the formulation of appropriate strategies for pasture germplasm management, conservation and development of breeding programs.

Keywords*:* Breeding systems, pollination, genetic variation, germination, polyploidy, seed production.

Resumen

Panicum coloratum var. *makarikariense* es una gramínea perenne nativa de África. Se adapta a un amplio rango de ambientes y posee uso potencial como forraje en distintas regiones tropicales y semiáridas del mundo. El estudio tuvo como objetivo evaluar el efecto del modo de polinización sobre la producción de semilla viable y la supervivencia de la progenie. Se evaluaron progenies de autopolinización y de polinización cruzada en diferentes accesiones midiendo la producción de semillas, germinación, tasa de germinación y supervivencia de plántulas, y se obtuvieron perfiles moleculares con Secuencias Simples Repetidas (SSR). La progenie obtenida mediante polinización cruzada mostró

Correspondence: L. Armando, Instituto Nacional de Tecnología Agropecuaria, INTA-EEA, 2300 Rafaela, Santa Fe, Argentina.
E-mail: larmando@criba.edu.ar.

significativamente mayor producción de semillas llenas y supervivencia de plántulas que la de autopolinización. Esto indica que las accesiones evaluadas dependen en gran medida de la alogamia y que la contribución de la descendencia por autofertilización a la población sería escasa. Los perfiles moleculares SSR mostraron que, en promedio, 85% de la progenie (obtenida a partir de polinización cruzada) presentó marcadores específicos paternos y 100% de ella difirió del genotipo materno. Todas las plantas examinadas presentaron 4x = 36 cromosomas. En conjunto, los resultados indican que la var. *makarikariense* puede generar progenie altamente polimórfica a través de la segregación y recombinación. Este estudio provee información útil para el diseño de estrategias de conservación, manejo del germoplasma y programas de mejoramiento.

Palabras clave*:* Germinación, polinización, poliploidía, producción de semilla, sistema de reproducción, variación genética.

Introduction

The amount of genetic variability within a species and, therefore, adaptability of their progeny to the environment, are mostly determined by the breeding system. Autogamous and asexual species produce populations with little evolutionary flexibility and high local specialization (Stebbins 1950), whereas outcrossing species produce more genetically diverse and ecologically variable offspring. Grasses display an extraordinary diversity of breeding systems including outcrossing, selfing or mixed-breeding, and a mixture of asexual and sexual reproduction (Quinn 1998). Many plant species have developed different ecological, morphological and physiological mechanisms that reduce the degree of self-fertilization to promote cross-pollination (Eckert 1994), most likely motivated by the increase in individual and average population fitness caused by heterosis.

The frequency of outcrossing is an important determinant of population genetic structure, affecting both genetic diversity within populations and genetic differentiation among them (Barrett and Harder 1996). Methods commonly employed for assessing the mode of reproduction in forage grasses include cytological and embryological analyses of the mother plant and screening for morphologically aberrant progeny. Molecular marker analysis, in particular, Simple Sequence Repeat (SSR) or microsatellite, is a tool now widely used in a variety of fundamental and applied fields of biology, including the identification of selfed, outcrossed or apomictic progeny in several grass species (Chistiakov et al. 2006; Liu and Wu 2012). SSRs are loci ubiquitously distributed within genomes that show a high level of polymorphism, environmental independence and rapid detection protocols. The reproductive system and the ploidy level of a species determine the transmission of genes across generations, the pattern of inheritance and gene flow, and influence the genetic structure of plant populations and their evolutionary potential. This information is critical when planning and developing conservation and breeding programs.

Panicum coloratum L., a perennial grass native to Africa, is adapted to a wide range of soil and climatic conditions, and has been used as forage in Australia, Japan, USA, Mexico and South America (Cook et al. 2005). This species has been classified into mainly 2 botanical varieties, var. *makarikariense* Gooss. and var. *coloratum*, distinguished by morphological traits and environmental preferences (Bogdan 1977; Armando et al. 2013). The var. *makarikariense* is particularly well adapted to heavy clay soils that fluctuate between drought and waterlogged conditions, whereas var. *coloratum* develops well in sandy soils, is tolerant of salinity and performs well at higher latitudes or elevations, as it thrives under low temperatures, withstanding some frost (Tischler and Ocumpaugh 2004). In Argentina, a breeding program and research activities involving var. *makarikariense* were initiated by the National Institute of Agricultural Technology (INTA) in 2006, with the purpose of developing new pasture cultivars adapted to marginal (drought, waterlogging, salinity or thermal stress) and less productive environments where livestock production has been displaced, with expansion of cropping into the most productive paddocks and planting of soybeans.

Panicum coloratum botanical varieties have been described as mainly allogamous (Brown and Emery 1958; Hutchison and Bashaw 1964), although the degree of self-fertilization has not been quantified and apomictic mechanisms have been suggested (Hutchison and Bashaw 1964). Unlike previous reports, which focused on the female parts of flowers and embryo sac development, our main interest is the analysis of the particular effects of different pollination systems on viable seed production and the survival of subsequent progeny. In addition, cytogenetic studies in var. *makarikariense* showed variable numbers of chromosomes: 2n = 18, 36, 45, 49

and 63 (Hutchison and Bashaw 1964; Pritchard and De Lacy 1974).

In the present work, progeny of *P. coloratum* var. *makarikariense* derived from self- and open-pollinated panicles were studied through the stages of seed production, germination and progeny survival. Additional data were obtained from SSR marker analysis, and chromosome number was also determined. This study attempted to provide information regarding the reproductive behavior of *P. coloratum* var. *makarikariense*, with utility for conservation and breeding.

Materials and Methods

Plant samples

Panicum coloratum was introduced into Argentina in the 1990s but has not been used widely as forage, although it has been conserved at various locations as collections or in small paddocks. Details of introductions are often limited, with many coming from different parts of the world. A collection of *P. coloratum* var. *makarikariense* (Table 1) was established in a common garden at the INTA Rafaela Experiment Station (31°11'41" S, 61°29'55" W) in Argentina in 2006, as a breeding population. Pre-breeding studies demonstrated a high level of variability in morphological and molecular markers, both among and within accessions, which justified the initiation of a breeding program (Armando et al. 2013). In fact, a cultivar from the program was released recently: Kapivera INTA (Giordano et al. 2013). The collection comprised 6 accessions of 32 plants each and 15 clonally propagated genotypes (IFF) obtained by selection on agronomic characteristics. The 32 plants of each accession were placed at 0.6 m intervals in an 8 × 4 matrix plot, with plots 15 m apart, while the 15 IFF genotypes were clonally propagated 8 times and arranged linearly in an 8 × 15 matrix plot at a distance of 0.6 m.

Seed production

Seed production of 3 plants (only 2 plants for accession DF), selected at random from each of the UCB, MR, BR, ER and CM accessions and 1 clone of each of the 15 IFF genotypes (Table 1) (a total of 32 plants) of *P. coloratum* var. *makarikariense*, was measured in the field from March to May 2009. Unfortunately, 1 plant of the DF accession was damaged and data were unavailable. For each plant, 2 panicles were selected at random: 1 for self-pollination and 1 for open-pollination. Only a single panicle was enclosed in each seed trap, the ones for self-pollination before anthesis and the ones for outcrossing when 2/3 of the panicle was in anthesis. Seed traps were used in order to facilitate seed collection and to prevent losses by seed shattering. Traps were therefore put in place at different stages of development for self- and open-pollinated treatments, but within the same treatment attempts were made to select panicles at the same stage of development. In self-pollinated treatments seed traps were covered with a white cotton bag to prevent pollen arrival from other sources without precluding light interception and photosynthesis of glumes (Figure 1). Self- and open-pollinated seeds were collected simultaneously once a week and manually separated from the glumes and other residuals. Eventually, the total number of seeds per inflorescence was counted, i.e. dark brown seeds (comprising lemma and palea containing a caryopsis). Small light-weight whitish seeds (hereafter referred to as "empty seeds") were also produced and

Table 1. Accessions of *P. coloratum* var. *makarikariense* and their collection site description.

Accession code	Description	Site of preservation	Coordinates	Province
DF	Twelve-year-old pasture Under heavy cattle grazing	Dean Funes (150 km Northwest from Córdoba city)	30°26' S, 64°21' W	Córdoba
UCB	Ungrazed pasture	Catholic University of Córdoba; collected in South Africa	31°25' S, 64°11' W	Córdoba
MR	Ungrazed pasture	Catholic University of Córdoba; collected in South Africa	31°25' S, 64°11' W	Córdoba
BR	Ten-year-old pasture under cattle grazing	Mercedes Experiment Station (INTA); introduced from Brazil	29°11′ S, 58°02′ W	Corrientes
ER	Five-year-old pasture under cattle grazing	Private farm near Mercedes	29°03' S, 57°49' W	Corrientes
IFF 1–15	Clonal materials	CIAP-INTA Institute of Physiology and Plant Genetic Resources	31°24' S, 61°11' W	Córdoba
CM	Seeds commercially distributed by a private company	cv. 'Bambatsi'; imported from Australia		-

counted as immature florets and/or spikelets with premature shattering from the inflorescences. Empty seeds show poor germination capacity, while dark brown seeds show a high germination percentage (Maina et al. 2017). The final numbers of seeds produced under self- and open-pollinated conditions were compared.

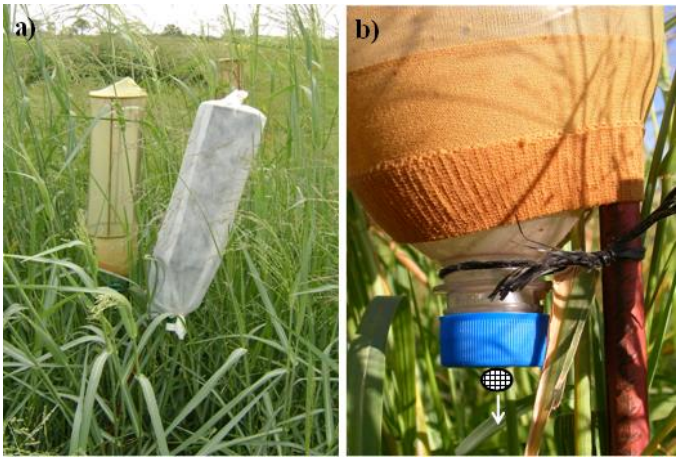

Figure 1. Seed traps enclosing inflorescences consisting of an iron cylindrical structure covered by a nylon stocking (modified from Young 1986). **a)** Open-pollination trap (left) and self-pollination trap with a white cloth bag (right). **b)** Detail of the lower part of the trap (water drainage). Seeds (= mature florets) were trapped and funneled into a cap as they shattered from the panicle.

Seed germination and seedling survival

Harvested seeds were naturally air-dried and stored at room temperature in paper bags for 1 year before testing for seed germination to ensure dormancy was already overcome (Tischler and Young 1987). Of the 32 plants evaluated, only 11 produced filled seeds under self-pollination. In each accession, only plants producing a good quantity of filled seeds (UCB_3, MR_1, BR_1, ER_1, CM_2, IFF_{10}; see Figure 2) were used to evaluate germination and seedling survival (n = 6). Thirty filled seeds per panicle from the same plants in both self- and open-pollinated treatments were placed in 10-cm diameter Petri dishes separately with filter paper at the bottom moistened with distilled water, and incubated in a programmed germination chamber at 42% humidity and 27 °C (Tomás et al. 2015) at a 16-hour photoperiod (light photon flux density: 48 mmol/s/m^2). Dishes from different pollination treatments and different plants were randomly arranged in the chamber. The number of germinated seeds per dish was counted daily and seed germination percentage (% G) was recorded on day 7 after the initiation of the germination trial. Studies by Tomás et al. (2015) showed that maximum germination has been reached by day 7. A seed was considered germinated when the radicle emerged through the seed coat. Eight-day-old seedlings were individually transplanted into 0.5 L plastic containers filled with a soil-sand-perlite mix (1:1:1 v/v), placed in a greenhouse at 28 °C and watered as needed, usually every 2 to 3 days. Seedling survival percentage (% Ss) was recorded when seedlings were 15 and 40 days old.

Progeny test

In order to analyze genetic composition of the offspring, a random sample of 12–15 seedling descendants from 3 female parents, UCB_3, ER_1 and IFF_{10}, was genetically characterized. These plants were selected to represent the observed range in the number of seeds produced within var. *makarikariense* (see Figure 2). Progeny test was performed only on seeds produced via open pollination as only a limited number of progeny were obtained from selfing. In addition, progeny obtained from open-pollinated traps resembled more natural pollination conditions.

DNA extraction was carried out using a modified SDS method (Edwards et al. 1991). Approximately 150 mg of leaf tissue (from plants >1 year old) was homogenized in liquid nitrogen. A 700 µL volume of extraction buffer containing: 50 mM Tris pH 8, 10 mM EDTA pH 8, 100 mM NaCl, 10 mM β-mercaptoethanol and 10% SDS, was added and incubated at 65 °C for 20 min. After adding 200 µL of 5 M potassium acetate pH 4.8, the sample was incubated on ice for at least 20 min and then centrifuged at 13,000 rpm for 20 min. This was followed by precipitation with 700 µL of iso-propanol incubated at -20 °C for 10 min, and centrifugation at 13,000 rpm for 4 min. The resulting pellet was washed with ethanol 70% and dissolved in 100 µL of 1 x TE buffer. DNA quality was evaluated in agarose gel and the quantity was determined by spectrophotometry.

In previous work, out of 40 heterologous SSR loci evaluated in *P. coloratum* var. *makarikariense*, 10 primer pairs were successfully amplified showing polymorphic and clear banding patterns (Armando et al. 2015). From these, the 5 most variable ones were chosen for analysis both of mother plants and offspring (Table 2). Amplification reactions were performed in 20 µL final volume containing: 30 ng of DNA template, 2.5 mM $MgCl_2$, 0.125 mM of each dNTPs, 10 pmol of each primer and 1 U of Taq DNA polymerase in 1.6x buffer. Negative

Table 2. Simple sequence repeat (SSR) loci used for progeny analysis and polymerase chain reactions (PCR) conditions.

Repeat motif	Source	Sequence (5´ → 3´)	TD/Tm
1- $(AG)_8T(AG)_7$	EST- *Panicum maximum*	F: TGTATGAGCTGAGTCGC	63–53/58
		R: TGGTAATCTAGTTGATATTC	
2- $(AG)_8$	EST- *Panicum maximum*	F: CCCGAGGCGATCCGATTCGTT	63–53/58
		R: TACGCCGACGACGAGGACGA	
3- $(AT)_{13}$	EST- *Panicum virgatum*	F: TCCAGATGACTCCCAGGAAC	50–40/45
		R: TCATCACTCGATTCCTCAAGC	
4- $(GT)_{38}$	Genomic- *Panicum virgatum*	F: GCAACCATGACAAGAAGCAT	63–53/58
		R: ATACAAACCGGGGTGCTAAG	
5- $(CGT)_n$	EST- *Eragrostis curvula*	F: TCTCCAACACGCCACGAC	63–53/58
		R: CAATCCACTACAAGAAACCAC	

SSR **1** and **2** (Ebina et al. 2007); **3** (Tobias et al. 2006); **4** (Wang et al. 2011); **5** (Cervigni et al. 2008).
TD/Tm: Touchdown/Annealing temperature; F: forward primer; R: reverse primer.

controls (no DNA template) were also included. Polymerase chain reactions (PCR) were carried out in an MJ Research Thermal Cycler. The optimum annealing temperature (Tm) was determined for each locus (Table 2). By touchdown PCR (TD), the annealing temperature was decreased to 1 °C starting with 5 °C over the set annealing temperature. Initial denaturation step of 95 °C for 3 min was followed by 10 touchdown cycles of 94 °C for 30 sec, touchdown annealing temperature for 30 sec and 72 °C for 45 sec. PCR products were subsequently amplified for 34 cycles at 94 °C for 30 sec, annealing temperature for 30 sec, and 72 °C for 45 sec with a final extension at 72 °C for 20 min. Amplifications were initially checked on 1% agarose gels. PCR products were analyzed on 6% denaturing polyacrylamide gel, with a TBE 1x electrophoresis buffer at 50W for 1 h and 45 min to 2 h. Bands were visualized by silver staining and scanned.

Chromosome number

To confirm the chromosome number of *P. coloratum* var. *makarikariense*, 8 different plants were evaluated. One plant from each accession was chosen at random and considered representative of each accession (DF, UCB, MR, BR, ER and CM), while 2 plants were selected from IFF.

Root tips of 3-month-old plants were pretreated with 8-hydroxyquinoline (0.002 ml/g) for 5 h. The roots were fixed in a freshly prepared mixture of ethanol:glacial acetic acid (3:1 v/v) for 48 h at room temperature and then placed in 70% ethanol at 4 °C for several weeks. Treated roots were put in 95, 70 and 40% ethanol and distilled water for 15 min each, hydrolyzed in 1 N HC1 at 60 °C for 8 min, transferred to distilled water for 2 min and stained in leuco-basic fuchsin for 1 h at room temperature in the dark. The 3–4 mm deeply stained root tips were removed from the roots, placed in a drop of 2% hematoxylin with 2% ferric citrate used as mordant, and squashed (modified from Núñez 1968). Cells with fully contracted and well spread metaphase chromosomes were photographed using a digital camera.

Data analyses

Average number of filled and empty seeds, germination percentages at day 7, time at which 50% of seeds had germinated (T50) and survival of 15- and 40-day-old seedlings were recorded for self- and open-pollinated conditions. The mean values obtained for the 2 forms of pollination were compared through one-tailed Student's t-test for paired samples, since a higher number of filled seeds was expected in cross-pollination. Analysis of variance (ANOVA) for the mean number of seeds was performed and accessions were compared by Fisher's LSD tests. Statistical analyses were performed using Infostat software (Di Rienzo et al. 2008).

Based on the number of seeds, germination and survival data per panicle, probability of seed production, and probability of seedling survival for self- or cross-pollinated conditions were estimated by using the conditional probability and Bayes Theorem (Quinn and Keough 2002). The probability of seedling survival (SS) was calculated as: $P(SS) = P(SS/SPs)*P(SPs) + P(SS/SPo)*P(SPo)$, where $P(SS/SP_S)$ and $P(SS/SP_O)$ are the conditional probabilities of seedling survival from seeds produced under self- and open-pollinated conditions, respectively; P(SPs) and P(SPo) are the probabilities of seed production under self- and open-pollination; and P(SS) is the probability of seedling survival. The conditional probability of finding a plant given that it was produced under self- or open-pollination was calculated

as: P(SPs/SS) = P(SS/SPs)*P(SPs)/P(SS) and P(SPo/SS) = P(SS/SPo)*P(SPo)/P(SS), respectively.

In the progeny test, offspring's DNA fingerprints were individually compared with the maternal pattern. The occurrence of a sexual reproduction event was defined as when progeny DNA fingerprints revealed a deviation from the maternal profile. Each band was considered as an independent locus, and polymorphic bands were scored visually as either absent (0) or present (1) for each of the 45 plants. Only those bands consistently scored were considered for analysis. By combining the markers, unique profiles were obtained for each individual. Genetic diversity in the progeny was estimated using the total number of alleles, number of alleles shared with female parents (maternal alleles) or deriving from male parents (paternal alleles), and percentage of polymorphic loci (%P).

The genetic dissimilarity between the maternal parents and their progeny was analyzed using a genetic binary distance (GD) according to Huff et al. (1993). An UPGMA cluster was obtained from GD matrix. Analyses of molecular data were performed using GenAlEx 6, Genetic Analysis in Excel (Peakall and Smouse 2012) and Infostat programs (Di Rienzo et al. 2008).

Results

Effect of pollination method on progeny number and seedling survival

The mean number of filled seeds per panicle under open-pollination was considerably higher than those produced under self-pollination (P<0.001, Table 3). In contrast, the mean number of empty seeds (empty perfect florets and/or caryopses unable to germinate) did not differ significantly between the 2 forms of pollination (P = 0.1518, Table 3). In general, the number of filled seeds per panicle was highly variable among plants, registering values from 0 to 72 (CV = 149%) for self-pollinated panicles and from 14 to 795 (CV = 61%) for open-pollinated panicles (Figure 2). Mean number of filled seeds differed significantly among accessions for both self- and open-pollination methods (Figure 3). According to Fisher´s LSD test, the commercial variety (cv. Bambatsi) had the highest mean number of filled seeds under open-pollination, while the lowest number was from accession BR (Figure 3).

Regarding progeny performance, the seed germination percentages were above 80% and together with T50 no significant differences were observed in filled seeds obtained from both self- and open-pollinated conditions (Table 3). In all the plants evaluated T50 values showed that at least half of the seeds (range 50–97%) had germinated by the 3rd day after the trial started, except for one plant where T50 extended to the 5th day (data not shown). The survival of both self- and open-pollinated seedlings decreased over time, but the survival of seedlings derived from open-pollination was significantly higher (P<0.001) than those obtained from selfing at both 15 and 40 days of age. In particular, survival of 40-day-old seedlings from self-pollinated panicles was much lower than that from out-crossing (4.8 vs. 35.7%) (Table 3).

Estimates of total seed production and seedling survival of plants from accessions of var. *makarikariense* are shown in Table 4, differentiating those obtained from the different methods of pollination. Of the total number of filled seeds produced per panicle, the probability of them being produced by open-pollination was greater (92%) than by self-pollination (8%). The estimated survival at 40 days of a seedling from a seed obtained by self-pollination was lower (6%) than the survival of a seedling coming from a seed obtained by open-pollination (32%). In addition, the estimated probability of plant survival for this period of time, independently of whether it came from self- or open-

Table 3. Comparison of the average number of filled (n° Fs) and empty (n° Es) seeds per panicle, seed germination percentage (% G), time needed for seeds to reach 50% germination (T50) and seedling survival percentage (% SS) of 15- and 40-day-old seedlings, between self-pollinated and open-pollinated panicles, using the student t-test in *P. coloratum* var. *makarikariense.*

	n° Fs	n° Es	% G	T50	% SS 15	% SS 40
n	32	32	6	6	6	6
Mean Self	10.9	176.1	88.5	80.1	38.1	4.78
Mean Open	279.2	207.6	81.7	85.6	77.6	35.73
Mean difference	-268.3	-31.5	6.83	-5.48	-39. 5	-31.0
T	-9.13	-1.05	0.91	-1.94	-10.15	-5.80
P	<0.0001	0.1518	0.7980	0.0551	0.0001	0.0011

n: number of genotypes evaluated.

T: Student t-values. P: p value of student t-test.

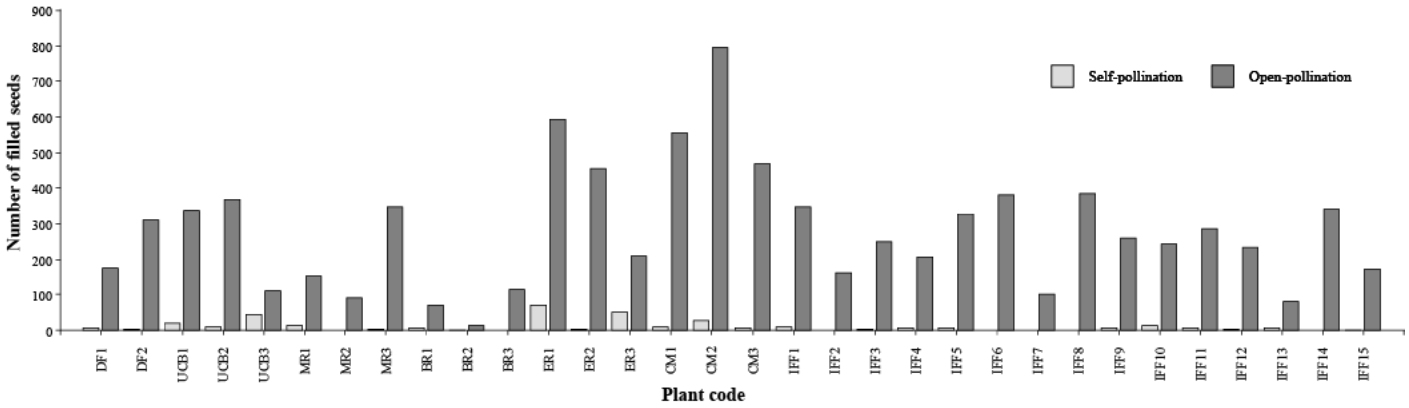

Figure 2. Number of filled seeds produced per panicle under self-pollination and open-pollination in 32 individuals of *P. coloratum* var. *makarikariense.*

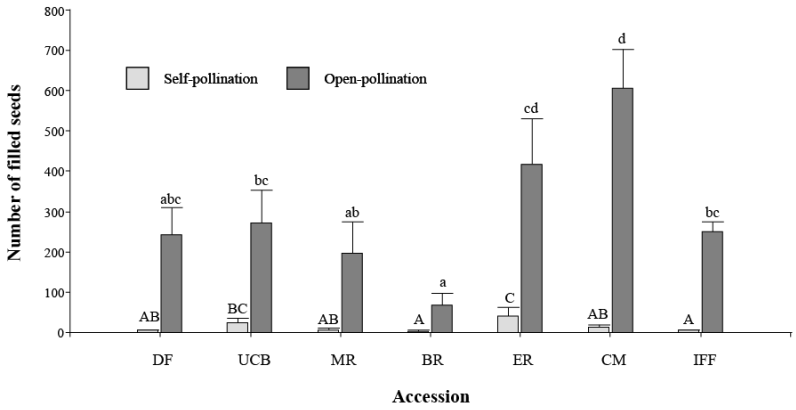

Figure 3. Mean number of filled seeds produced per panicle under self-pollination and open-pollination in accessions of *P. coloratum* var. *makarikariense*. In each type of pollination, different letters indicate significant differences between accessions ($P<0.001$) using Fisher's LSD tests and bars represent standard errors of means (lower case letters refer to open-pollinated and upper case to self-pollinated).

Table 4. Probabilities of seed production (SP) under self- ($_S$) or open-pollination ($_O$) and seedling survival (SS) in *P. coloratum* var. *makarikariense*.

Pollination	Probabilities	UCB	MR	BR	ER	CM	IFF	Mean
Self	$p(SP_S)$	0.29	0.09	0.09	0.11	0.03	0.05	0.08
	$p(SS/SP_S)$	0.13	0.00	0.00	0.03	0.00	0.10	0.06
	$p(SP_S/SS)$	0.15	0.00	0.00	0.01	0.00	0.02	0.03
Open	$p(SP_O)$	0.71	0.91	0.91	0.89	0.97	0.95	0.92
	$p(SS/SP_O)$	0.30	0.23	0.36	0.53	0.17	0.37	0.32
	$p(SP_O/SS)$	0.85	1.00	1.00	0.99	1.00	0.98	0.97
-	p(SS)	0.25	0.21	0.32	0.48	0.16	0.35	0.30

$p(SS/SP_S)$ and $p(SS/SP_O)$: conditional probability of seedling survival given that seed production was under self- or open-pollination, respectively.

p(SPs/SS) and $p(SP_O/SS)$: conditional probability of finding a plant given that it was produced under self- or open-pollination, respectively.

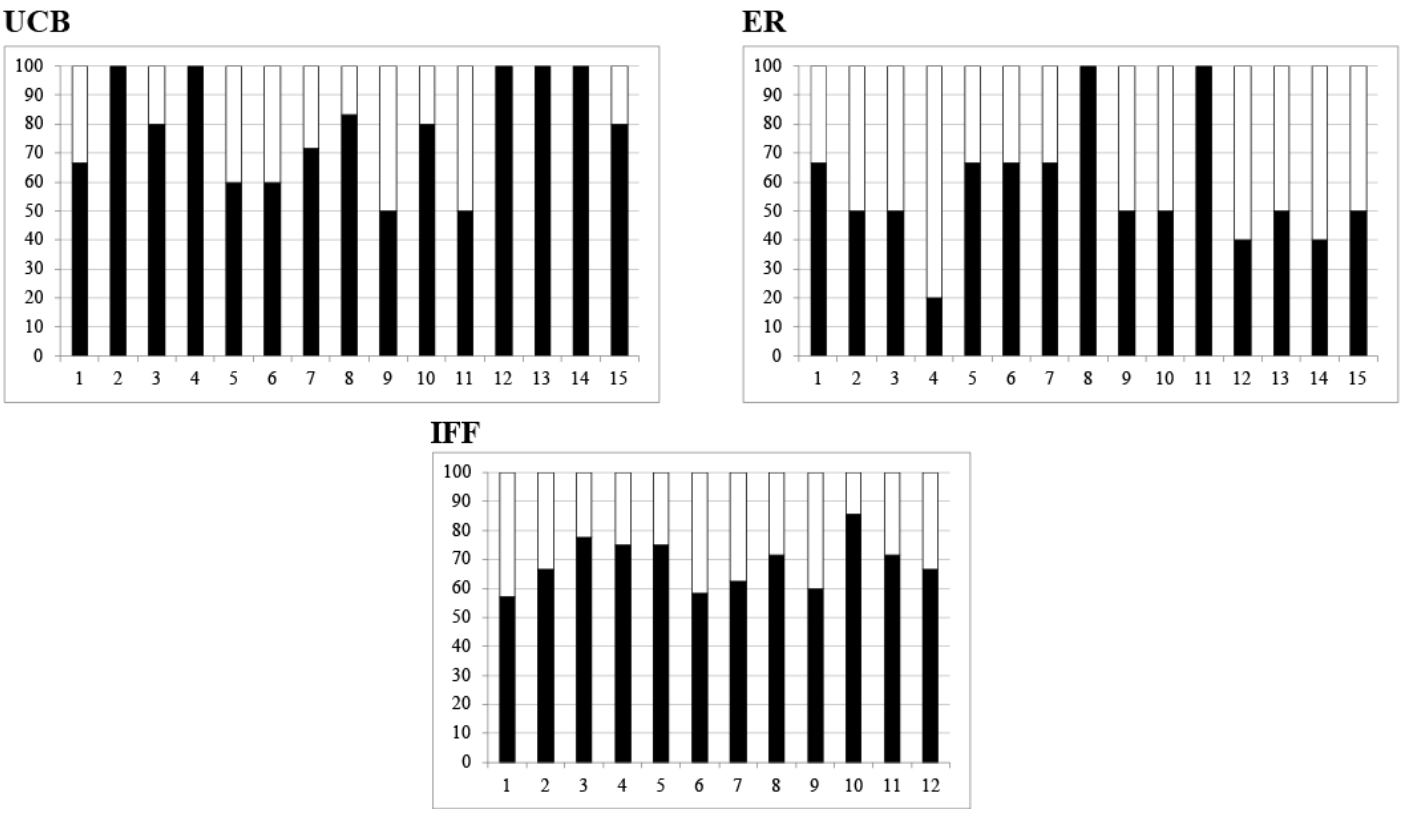

Figure 4. Percentage of SSR alleles attributed to female (■) and male (□) parents in progenies of *P. coloratum* var. *makarikariense* from 3 accessions (names are detailed in Table 1).

pollination, was 30%. Combining data, the estimated probability of finding a plant obtained by open-pollination was significantly higher (97%) than those produced by self-pollination (3%).

Effects of pollination method on genetic variability and contrast with female parents

Progeny from 3 female parents were analyzed with 5 SSR markers. Offspring plants were classified as identical when patterns were the same as the female parents at all the evaluated SSR loci or distinct when any band differences between female parent and progeny were observed. The SSR analysis showed values of percent polymorphic loci (%P) over 50% in progenies, and maternal and paternal alleles could be recognized in individual progeny (Table 5). On average, 85% (range 67–100%) of individual progeny presented alleles not found in the female parent (Figure 4). Additionally, offspring were not identical with their maternal patterns but they were closely grouped in the dendrogram according to families (Figure 5). Moreover, the 3 female plants were genetically distinct.

Chromosome numbers in the P. coloratum *var.* makarikariense *collection*

In all mitotic cells observed, 36 chromosomes were counted at metaphase. This number remained stable for all 8 *P. coloratum* var. *makarikariense* plants evaluated.

Table 5. Genetic diversity in 3 progeny of *P. coloratum* var. *makarikariense* assessed by 5 simple sequence repeat markers (SSR).

Female parent	N° progeny	Total alleles	Maternal alleles	Paternal alleles	%P
UCB_3	15	22	14	8	50.0
ER_1	15	23	12	11	56.3
IF_{10}	12	25	16	9	62.5

%P: percentage of polymorphic SSR loci in progeny.

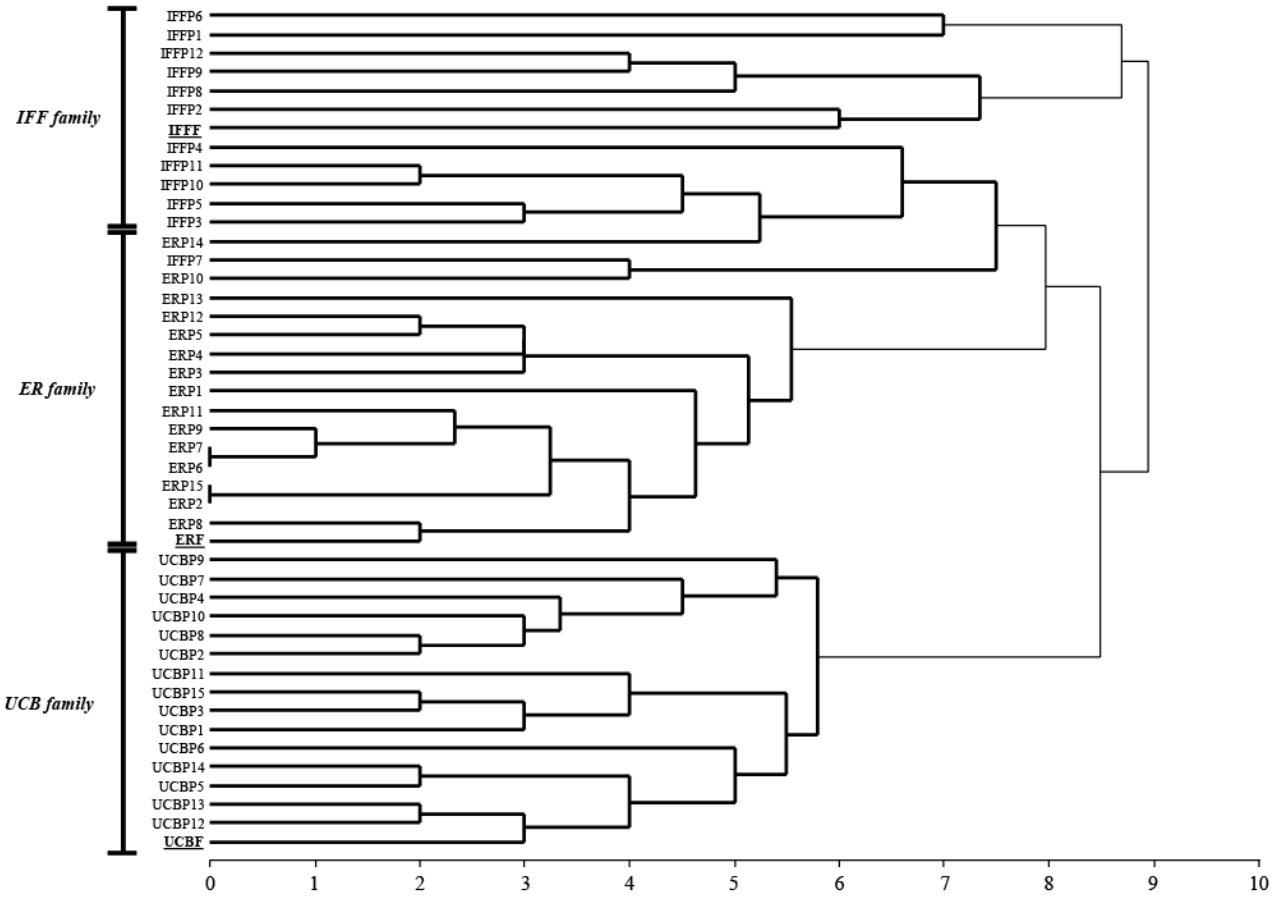

Figure 5. Unweighted pair-group method with arithmetical average (UPGMA) dendrogram based on the GD SSR matrix of 3 female parents (F) and their progeny (P) in families of *P. coloratum* var. *makarikariense*. The female plants are underlined.

Discussion

There is an increasing need for a better understanding of the reproductive biology in *P. coloratum*, given that this is an extremely important prerequisite to develop breeding strategies and also for germplasm management and conservation purposes. In this study, we sought to determine the preponderant mode of reproduction of one collection of the var. *makarikariense* used for breeding purposes both by evaluating seed production in open and forced-to-inbreed panicles and by a progeny test using SSR markers. The evaluated plants in this study were selected to cover the variability present in the germplasm collection at INTA EEA Rafaela, which comprises accessions collected within semi-arid temperate mesic and subtropical zones, both grazed and non-grazed areas, and a commercial variety. Although we analyzed only 3 panicles per accession, our results corroborate previously reported studies pointing out extensive variability in seed production per panicle among genotypes from different accessions in the collection (Barrios et al. 2010). These accessions are also distinctive in other morphological characters, both vegetative and reproductive (Giordano et al. 2013; Armando et al. 2013; 2015). In addition, seed yield is a complex character and additional variability arises as seed set is the culmination of a series of processes at canopy level including radiation interception, biomass production and partitioning. Therefore, variability among individuals may be the result of both genetic variation and the integration of these different genetic backgrounds with the multiple environmental influences (Boelt and Studer 2010). The considerable differences in seed production we observed among plants and accessions suggest a promising scenario for selection to increase seed production by means of augmenting the number of seeds per panicle.

The results from seed production, germination and seedling survival assessments in var. *makarikariense*

indicated that open-pollination is by far the most frequent form of pollination in the reproductive biology of this variety. A similar behavior is suggested for var. *coloratum* based on a small sample of 3 plants (data not shown). Additionally, as only a few panicles produced good quality seeds by self-pollination, a limited number of plants could be analyzed. However, the results from the combined probabilities clearly showed that progeny from selfing make low to no contribution to the population over time since a mean of only 6% of the plants obtained from self-pollinated seeds survived. Although seeds obtained under self-pollination were not weighed, they were visibly much smaller than and had a different color (white vs. brown) from those produced by open-pollination, which could explain the lower vigor of the seedlings (Tomás et al. 2007). The fact that we did obtain seeds from selfing demonstrates the existence of some degree of self-compatibility. Burson and Young (1983), using fluorescence microscopy, demonstrated that, when var. *coloratum* was self-pollinated, 90% of the pollen germinated within minutes after the pollen grain came in contact with the stigma, but only 2% of the pollen tubes actually grew into the ovary and entered the micropyle within 1 hour after pollination, suggesting active self-incompatibility mechanisms. A gametophytic S-Z incompatibility system has been found in related species (Martinez-Reyna and Vogel 2002) and is common for most of Poaceae (Baumann et al. 2000). In the majority of the grasses this system is not absolute, and some seeds may be obtained from self-fertilization. Further, selfed progeny of highly heterozygous genotypes could result in fitness reduction due to inbreeding (Eckert 1994), a point that we cannot prove with only one generation of selfing.

Brown and Emery (1958) observed typical sexual 8-nucleate embryo sacs in 82 ovules of var. *makarikariense* and var. *coloratum* plants indicating both varieties reproduced sexually. Hutchison and Bashaw (1964) also observed 8-nucleate embryo sacs in both varieties but about 2% of the older ovules had large vacuolated cells, which appeared similar to multiple embryo sacs. They considered this as possible evidence of apospory, but developed embryos were not observed in these cells, which dispelled the possibility of apomixis in this species. The high amount of variation expressed in open-pollinated *makarikariense* progeny rules out the possibility of apomictic reproduction in the species. In our study, molecular progeny tests revealed that an average of 85% of offspring possessed paternal alleles; this is indicative of cross-pollination and 100% of them were genetically distinguishable from the maternal genotype. Progeny without clear paternal contribution were probably derived from self-pollination or open-pollination involving parents with the same molecular pattern, although this value may be reduced with an increased number of markers. In addition, the level of genomic DNA polymorphism of the analyzed progeny suggests sexuality and genetic recombination, and provides additional evidence that *P. coloratum* is mainly an allogamous species.

All our analyses accumulated evidence pointing to *P. coloratum* var. *makarikariense* as a species with mainly sexual reproduction that depends primarily on open-pollination to obtain viable offspring. All var. *makarikariense* plants evaluated in this study had 36 chromosomes, and the same number was observed in 3 plants of var. *coloratum* (data not shown). Considering a basic number of x = 9 (Hamoud et al. 1994), these plants are tetraploids. This ploidy level is one of the most frequently reported for *P. coloratum* (Hutchison and Bashaw 1964; Pritchard and De Lacy 1974). Apomixis occurs throughout the plant kingdom and is always associated with polyploidy as was reported in *Panicum maximum* and *Paspalum notatum* (Warmke 1954; Quarin et al. 2001). However, sexuality also occurs at the 4x and 6x ploidy levels as in *Panicum virgatum* and *Brachiaria humidicola* (Barnett and Carver 1967; Pagliarini et al. 2012). The natural distribution of diploid and tetraploid levels in *P. coloratum* at its center of origin appears to occur throughout central and South Africa, while the hexaploid level was confined to East Africa (Pritchard and De Lacy 1974).

Knowledge of the chromosome number and ploidy level of the germplasm is necessary for developing an efficient strategy of preservation of this promising forage species and is crucial for use in the *P. coloratum* breeding program. The ploidy level of this species may partially explain the high level of genetic variability in the species and the highly polymorphic progeny. New genetic combinations seem to be obtained relatively easily through segregation and recombination. This fact may represent an interesting means to increase germplasm variability and may be an important factor to consider when releasing new materials resulting from selection and breeding. In addition, the mode of reproduction in a given species must be clear to the plant breeder to accomplish crop improvement. Knowledge about how a plant reproduces naturally helps the breeder to predict the behavior under field conditions and to establish the most appropriate selection method, which markedly differs between self- and cross-pollinated crops. Knowledge of

breeding systems is also of benefit for germplasm banks. Based on our results, the number of individuals destined for seed increase should be large enough to include the original variability in genetic diversity. Moreover, isolation distances need to be taken into account in order to prevent gene flow among accessions and thus preserve the genetic identity of each accession.

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Research Paper

Screening of salt-tolerance potential of some native forage grasses from the eastern part of Terai-Duar grasslands in India

Evaluación de la tolerancia a la sal de algunas gramíneas forrajeras nativas de la parte oriental de los Terai-Duar Grasslands en la India

SWARNENDU ROY[1,2] AND USHA CHAKRABORTY[1]

[1]*Plant Biochemistry Laboratory, Department of Botany, University of North Bengal, Siliguri, Darjeeling, West Bengal, India.* www.nbu.ac.in
[2]*Department of Botany, Kurseong College, Dow Hill Road, Kurseong, Darjeeling, West Bengal, India*. www.kurseongcollege.net
Presently: Molecular & Analytical Biochemistry Laboratory, Department of Botany, University of Gour Banga, Malda, West Bengal, India. www.ugb.ac.in

Abstract

The salt tolerance of 12 native forage grasses from the eastern part of Terai-Duar grasslands was assessed using a rapid method of leaf disc senescence bioassay. Samples of these grasses were grown in untreated water as well as 100 and 200 mM NaCl solutions for periods of 3, 6 and 9 days. Discs of fresh leaf were then placed in untreated water as well as in 100 and 200 mM NaCl solutions for 96 hours. Quantitative effects were measured as the effects on chlorophyll concentration in leaves in response to exposure to the varying solutions. From these results, the salt sensitivity index (SSI) of the individual grasses was determined. The SSI values indicated that *Imperata cylindrica*, *Digitaria ciliaris* and *Cynodon dactylon* were most salt-tolerant of all grasses tested. Further characterization of the grasses was done by observing the changes in 6 biomarkers for salinity tolerance: relative water content, total sugar concentration, proline concentration, electrolyte leakage, membrane lipid peroxidation and H_2O_2 concentration following exposure to 100 and 200 mM NaCl concentrations for 3, 6 and 9 days. Finally, hierarchical cluster analysis using the software CLUSTER 3.0 was used to represent the inter-relations among the physiological parameters and to group the grasses on the basis of their salinity tolerance. The overall results indicated that *Imperata cylindrica*, *Eragrostis amabilis*, *Cynodon dactylon* and *Digitaria ciliaris* were potentially salt-tolerant grasses and should be planted on saline areas to verify our results. On the other hand, *Axonopus compressus*, *Chrysopogon aciculatus*, *Oplismenus burmanni* and *Thysanolaena latifolia* were found to be highly salt-sensitive and would be unsuitable for use in saline areas.

Keywords*:* Biomarkers, hierarchical cluster analysis, leaf disc senescence bioassay, salinity tolerance.

Resumen

En la University of North Bengal, Siliguri, India, utilizando a nivel de laboratorio un método rápido de bioensayo de senescencia de discos foliares, fue evaluada la tolerancia a salinidad de 12 gramíneas forrajeras nativas de la parte oriental de los Terai-Duar Grasslands en la India nororiental. Las gramíneas fueron cultivadas tanto en agua no tratada como en soluciones de 100 y 200 mM NaCl durante 3, 6 y 9 días. Después se colocaron discos de hoja fresca tanto en agua no tratada como en soluciones de 100 y 200 mM NaCl durante 96 horas. Los efectos cuantitativos se midieron como la

Correspondence: Usha Chakraborty, Plant Biochemistry Laboratory, Department of Botany, University of North Bengal, Siliguri - 734013, Darjeeling, West Bengal, India.
Email: ucnbu2012@gmail.com

concentración de clorofila en las hojas en respuesta a la exposición a las diversas soluciones. Los resultados, con base en un índice de sensibilidad a la sal, mostraron que *Imperata cylindrica*, *Digitaria ciliaris* y *Cynodon dactylon* fueron las gramíneas más tolerantes a la salinidad. Además se realizó una caracterización de las gramíneas mediante la determinación de los cambios en 6 biomarcadores para la tolerancia a la salinidad: contenido relativo de agua; concentración de azúcar total; concentración de prolina; pérdida de electrolitos; peroxidación lipídica de membrana; y concentración de H_2O_2 después de la exposición a concentraciones de 100 y 200 mM NaCl durante 3, 6 y 9 días. El análisis de conglomerados jerárquicos utilizando el software CLUSTER 3.0 para representar las interrelaciones entre los parámetros fisiológicos y agrupar las gramíneas sobre la base de su tolerancia a la salinidad mostró que, en general, que *Imperata cylindrica*, *Eragrostis amabilis*, *Cynodon dactylon* y *Digitaria ciliaris* fueron gramíneas potencialmente tolerantes a la sal que deberían ser cultivadas en suelos salinos para verificar nuestros resultados. Por otra parte, *Axonopus compressus*, *Chrysopogon aciculatus*, *Oplismenus burmanni* y *Thysanolaena latifolia* resultaron ser altamente sensibles a la sal y no son especies apropiadas para uso en áreas salinas.

Palabras clave*:* Análisis de conglomerados jerárquicos, bioensayo de senescencia de discos foliares, biomarcadores, tolerancia a la salinidad.

Introduction

In India, available fodder for stock is estimated to be 40–50% below requirements, and this scenario is gradually worsening due to the concomitant decrease in grass coverage and increase in livestock population (Indian Council of Agricultural Research 2009). Global climate change in the last decade has been correlated with changes in the productivity of forage grasses and is likely to have a detrimental effect on the overall grass coverage in the long term (Abberton et al. 2008). A huge proportion of land in the country is classified as wasteland due to the problems of soil salinity, alkalinity and waterlogging. The selection of grass germplasm for salinity tolerance is critical for more efficient utilization of these degraded lands by establishing stress-tolerant grasses in non-arable marginal areas (Ashraf 2006). Species that are relatively salt-tolerant show greater endurance and adaptability among the native species (Squires 2015). Therefore there is an urgent need to: identify salt-tolerant traits in wild forage grasses; evaluate their potential for enhancing the productivity of grasslands in their native habitats; and utilize them for the rejuvenation of grasslands and croplands with reduced or lost productivity.

Abiotic stresses, in particular water and salinity stress, play a major role in disrupting the growth and development of grasses including cereals (Tester and Bacic 2005). Salinity limits plant growth and productivity through the toxic effects of Na^+ and Cl^- ions, which leads to ionic imbalances, osmotic and oxidative stress (Munns and Tester 2008). Native grasses, however, show variable degrees of NaCl tolerance, especially those belonging to the subfamilies Panicoideae and Chloridoideae (Bromham and Bennett 2014; Roy and Chakraborty 2014). Salinity tolerance is a complex trait, governed by several physiological and biochemical parameters and these parameters greatly influence the normal growth and development of plants (Zhu 2000). Salt tolerance of any individual species is demonstrated as the ability to maintain an optimal physiological and biochemical equilibrium under NaCl treatment (Sairam and Tyagi 2004). Ashraf and Harris (2004) suggested different biomarkers as indicators of salinity tolerance, including soluble sugars, proteins, amino acids, ammonium compounds, polyamines, polyols, antioxidants and ATPases.

In the present study however, 6 biochemical markers, viz. relative water content (RWC), proline and soluble sugar concentrations, membrane lipid peroxidation (malondialdehyde, MDA), electrolyte leakage (EL) and H_2O_2 concentration were selected for use in screening for salinity tolerance of the selected grasses. Increase in leaf RWC in the halophyte *Atriplex nummularia* with increasing salinity indicated an efficient mechanism to adjust cell cytosol osmotically (Araújo et al. 2006). Accumulation of osmolytes like proline, soluble sugars and glycine betaine and elevated levels of antioxidative enzymes play a vital role in conferring salt tolerance in grasses (Roy and Chakraborty 2014). Accumulation of glycine betaine in *Cynodon* and *Spartina*, proline in *Paspalum* and myo-inositol in *Porteresia* has been found to confer salinity tolerance (Wyn Jones and Storey 1981; Marcum and Murdoch 1994; Sengupta et al. 2008). Accumulation of proline, fructans and soluble carbohydrates was also correlated with salinity tolerance in salt-tolerant cultivars of wheat (Kafi et al. 2003). MDA concentration has been proposed as an indicator of oxidative damage and a lesser accumulation of the same in root tissues was employed for screening the salt-

tolerant genotypes of *Cenchrus ciliaris* (Castelli et al. 2009). Electrolyte leakage as an indicator of cell membrane stability of durum wheat cultivars under osmotic stress was demonstrated, with level of electrolyte leakage being inversely related to degree of salt tolerance of cultivars (Bajji et al. 2002).

In addition to the characterization of 12 forage grasses that are widely grazed by and fed to livestock in the eastern parts of the Terai-Duar grasslands by observing the changes in 6 biomarkers for salinity tolerance, the objective of our study was to evaluate the salt-tolerance potential of those grasses by using a rapid screening technique where the inherent tolerance of saline conditions was assessed as a precursor to selective propagation in varied environmentally challenged wastelands.

Materials and Methods

Study area and plant materials

Twelve native grasses were collected from the different regions of the eastern part of the Terai-Duar grasslands (88.22–89.66° E, 26.45–26.86° N; Figure 1). These grasses are widely grazed by livestock and harvested by local people for feeding to domestic animals, viz. *Arundo donax* L. of the subfamily Arundinoideae; *Axonopus compressus* (Sw.) P. Beauv., *Capillipedium assimile* (Steud.) A. Camus, *Chrysopogon aciculatus* (Retz.) Trin., *Digitaria ciliaris* (Retz.) Koeler, *Arundinella bengalensis* (Spreng.) Druce, *Imperata cylindrica* (L.) Raeusch., *Oplismenus burmanni* (Retz.) P. Beauv., *Setaria pumila* (Poir.) Roem. & Schult. and *Thysanolaena latifolia* (Roxb. ex Hornem.) Honda of the subfamily Panicoideae; and *Cynodon dactylon* (L.) Pers. and *Eragrostis amabilis* (L.) Wight & Arn. of the subfamily Chloridoideae. In the subsequent text only the generic names are used.

Experimental design and NaCl treatment

A rapid screening protocol was implemented for the differentiation of salt-tolerance potential of the forage grasses. The grasses were collected from their natural habitats and placed in small flasks containing 0.1X Hoagland solution with their roots intact, before being transferred to the plant growth chamber in the laboratory of the Department of Botany, University of North Bengal, Siliguri. Before NaCl treatment, the roots were gently washed with sterile dH_2O to remove any mud and then again transferred to conical flasks containing 0.1X Hoagland solution. The plants were then allowed to acclimatize for 48 hours in the growth chamber, with a standard temperature of 20–25 °C, RH 65–70% and 16 h photoperiod. Following acclimatization, 2 groups of plants were grown in NaCl treatments of 100 and 200 mM for 9 days, while the third group remained as control and the effects of NaCl on the plants in terms of several biomarkers after 3, 6 and 9 days of treatment were analyzed.

Three individual samplings from 3 different locations (Figure 1) were completed for each grass and the results were expressed as mean ± SD for all parameters analyzed. For grasses with broad leaves like *Thysanolaena* and *Arundo*, 3 plants were taken per sampling site, whereas for grasses with small narrow leaves, 5–6 plants were taken per sampling site.

Salt sensitivity index (SSI)

The youngest healthy fully expanded leaves from the plants were briefly washed in deionized water and 1 cm diameter leaf discs were finely cut and floated in a 5 ml solution of NaCl (100 and 200 mM) for 96 hours. Leaf discs floated in sterile dH_2O served as the experimental control for the bioassay (Fan et al. 1997). The effects of salt treatment on leaf discs were assessed by observing the phenotypic changes and the extent of NaCl effect in terms of SSI, which was quantified by estimating the chlorophyll concentration in NaCl-treated and control sets. Briefly, the leaf discs were crushed in 80% acetone and the absorbance was recorded in a UV-VIS spectrophotometer at 645 and 663 nm and the chlorophyll concentration was calculated using Arnon's formulae (Arnon 1949). SSI values were then calculated at 100 and 200 mM NaCl as the percent decrease in chlorophyll concentration of the NaCl treatment in comparison with the untreated leaf discs using the following formula:

$$\text{SSI} = \frac{\text{Chlorophyll conc. of NaCl-treated leaf discs}}{\text{Chlorophyll conc. of untreated leaf discs}} \text{ x } 100$$

Biochemical markers for assessment of NaCl tolerance

For an alternative screening of grasses for their salt-tolerant attributes, 6 different biochemical parameters were chosen, viz. relative water content (RWC), proline and soluble sugar concentrations, membrane lipid peroxidation (malondialdehyde, MDA), electrolyte leakage (EL) and H_2O_2 concentration. For these experiments, the first 3 fully expanded leaves from the top of each grass subjected to the various growth solutions were collected.

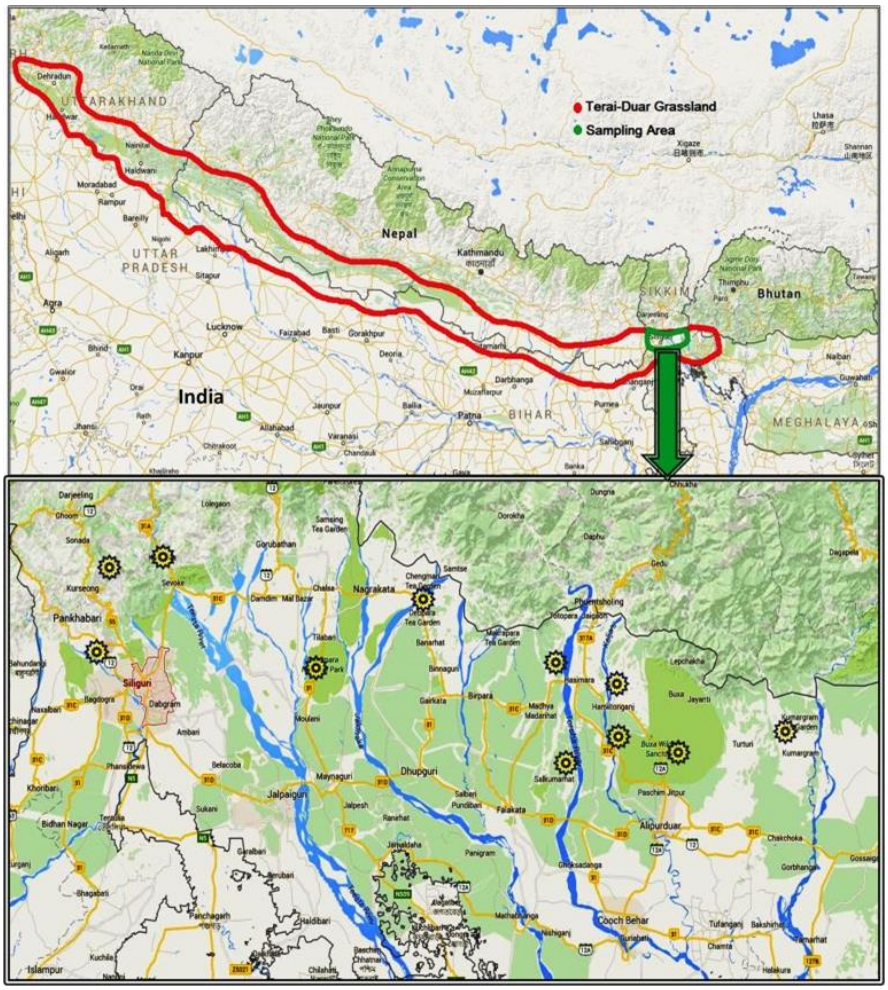

Figure 1. Geographical location of the Terai-Duar grasslands and the sampling area. Sampling area (enlarged view) with major locations from which the forage grasses were collected.

Relative water content. RWC was measured following the protocol of Barr and Weatherley (1962). Briefly, fresh leaf samples from control and different treatment sets were weighed to obtain fresh weight (FW). The samples were then immediately hydrated to full turgidity for 4 h, dried of surface moisture and weighed to obtain fully turgid weight (TW). Samples were then oven-dried at 80 °C for 24 h and weighed to determine dry weight (DW). RWC was calculated by the following equation:

$$RWC\ (\%) = [(FW - DW) / (TW - DW)] \times 100$$

Proline. Extraction and estimation of proline were done by the method of Bates et al. (1973). Leaf tissue was homogenized in 3% sulfosalicylic acid. Ninhydrin reagent was used for the estimation of proline in the extract, which was separated in a separating funnel using toluene, prior to recording the absorbance at 520 nm.

Total sugar. Soluble sugar in leaves was extracted in 95% ethanol following the method of Harborne (1973). Anthrone reagent was used to estimate total sugar following the method of Plummer (1978). Briefly, 4 ml of anthrone reagent was added to 1 ml test solution and kept over boiling water bath for 10 min, after which the absorbance was taken at 620 nm. Total sugar was finally calculated using a standard curve of D-glucose.

Membrane lipid peroxidation. Membrane lipid peroxidation was measured in terms of concentration of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction, following the method of Heath and Packer (1968). Leaves were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and estimation was done with 0.5% (w/v) TBA in 20% TCA. The absorbance of the reaction mixture was determined at 532 and 600 nm and the MDA content was calculated using an extinction coefficient of 155 mM/cm.

Electrolyte leakage. Electrolyte leakage (EL) was measured as described by Lutts et al. (1996). Leaves were washed thoroughly with deionized water and placed in culture tubes containing 10 ml of deionised water on a rotary shaker for 24 h. Subsequently, the electrical conductivity of the solution (L_t) was determined and the samples were then autoclaved at 120 °C for 20 min and cooled to room temperature before determining the final electrical conductivity (L_0). EL was calculated as follows:

$$\text{Electrolyte leakage (\%)} = (L_t / L_0) \times 100$$

H_2O_2 concentration. The extraction and estimation of H_2O_2 were done by the method given by Jana and Choudhuri (1981) with slight modification. Leaf tissue was homogenized in 50 mM phosphate buffer (pH 6.5) and mixed with 0.1% titanium sulphate in 20% (v/v) H_2SO_4 and centrifuged at 6,000 rpm for 15 min. Absorbance was measured at 410 nm and H_2O_2 concentration was measured using the extinction coefficient of 0.28 µmol/cm.

Hierarchical cluster analysis

For cluster analysis of the grasses for their NaCl tolerance, the data for fold change values of RWC, proline, soluble sugar, MDA, EL and H_2O_2 after NaCl treatments for 3, 6 and 9 days with respect to the control sets were taken. Hierarchical cluster analysis was performed using the CLUSTER 3.0 program by the uncentered matrix and complete linkage method following the protocol of de Hoon et al. (2004). The resulting tree figure was displayed using the software package, Java Treeview, as described by Chan et al. (2012).

Statistical analysis

All experiments were repeated with sampling from 3 different locations (n = 3) for each species. Species and treatment means were statistically analyzed using Least Significant Difference ($P \leq 0.05$) for a completely randomized design.

Results

Salt sensitivity index (SSI) of grasses

Chlorophyll concentration in fresh untreated leaves varied from 0.72 mg/g (*Capillipedium*) to 1.45 mg/g (*Oplismenus*). SSIs of grasses determined by leaf disc assay and represented in terms of % decrease in chlorophyll concentration in the leaf discs floated in 100 mM and 200 mM NaCl solutions relative to the control sets, i.e. leaf discs kept in sterile dH_2O, are shown in Table 1. At 100 mM NaCl, the senescence assay indicated that *Setaria, Thysanolaena, Imperata* and *Cynodon* were least affected with SSI values of 0.45–7.36. At the same time, *Capillipedium, Axonopus* and *Arundinella* were much more sensitive (SSI values of 24.20–18.37). However, at 200 mM NaCl, *Imperata, Digitaria* and *Cynodon* were least affected by salt concentration (SSI values of 6.59–15.00). Interestingly, *Thysanolaena* and *Setaria* were more affected by 200 mM NaCl, showing marked increases in SSI values (23.38 and 57.98, respectively). *Capillipedium* showed the highest sensitivity to both 100 and 200 mM NaCl with SSI values of 24.20 and 61.93, respectively. This result was also reciprocated by the phenotypical changes in the leaf discs floated in NaCl solutions, which can be clearly observed in Figure 2.

Effect of NaCl on biochemical markers for analysis of salinity tolerance

Relative water content. Leaf RWC values were found to decrease in all grasses with both increase in NaCl concentration and duration of treatment (Table 2). The fold change values of RWC in plants subjected to 100 and 200 mM NaCl in comparison with the control sets revealed the smallest changes in *Cynodon* and *Imperata* and the largest changes in *Chrysopogon* and *Digitaria* (Figure 3a).

Proline concentration. Proline concentration in fresh untreated leaves varied from 11.6 µg/g (*Chrysopogon*) and 12.4 µg/g (*Setaria*) to 63.1 µg/g (*Imperata*) and 64.5 µg/g (*Digitaria*). During the first 3 days of NaCl treatment (100 and 200 mM), proline concentration in fresh tissue increased with increase in NaCl concentration in all grasses except *Axonopus*, where levels of proline declined (Table 3; Figure 3b). The largest increases (on a percentage basis) were recorded in *Cynodon, Arundinella* and *Imperata*. Similarly after 6 and 9 days of treatment, proline concentrations increased as NaCl concentration increased in all grasses except *Axonopus*, *Chrysopogon*, *Thysanolaena* and *Oplismenus*, where concentrations declined with increasing NaCl concentration. The largest percentage increases in proline concentration were observed in *Cynodon* and *Arundinella* (1.8–3-fold increase).

Table 1. Chlorophyll concentration in detached leaf discs of grasses dipped in 0, 100 and 200 mM NaCl solutions and salt sensitivity index expressed as relative % decrease of chlorophyll concentration of detached leaves at 100 and 200 mM NaCl.

Grass	Chlorophyll concentration (mg/g fresh weight of tissue, fwt)			Salt sensitivity index (% decrease in chlorophyll conc.)	
	Concentration of NaCl (mM/L)			Concentration of NaCl (mM/L)	
	0	100	200	100	200
Arundo	1.22 ± 0.21	1.00 ± 0.07	0.75 ± 0.05	18.37	38.11
Axonopus	1.00 ± 0.12	0.78 ± 0.04	0.60 ± 0.01	21.72	39.94
Capillipedium	0.72 ± 0.09	0.55 ± 0.02	0.27 ± 0.01	24.20	61.93
Chrysopogon	0.78 ± 0.11	0.70 ± 0.04	0.53 ± 0.02	10.29	32.49
Cynodon	1.17 ± 0.22	1.09 ± 0.08	1.00 ± 0.03	7.36	15.00
Digitaria	0.91 ± 0.08	0.8 ± 0.04	0.78 ± 0.03	11.86	14.11
Arundinella	1.29 ± 0.08	1.02 ± 0.08	0.71 ± 0.05	21.33	44.58
Eragrostis	0.94 ± 0.07	0.82 ± 0.07	0.65 ± 0.01	12.61	30.33
Imperata	1.35 ± 0.14	1.28 ± 0.11	1.26 ± 0.08	5.67	6.59
Oplismenus	1.45 ± 0.17	1.22 ± 0.15	1.12 ± 0.12	15.82	22.35
Setaria	0.80 ± 0.11	0.80 ± 0.04	0.33 ± 0.01	0.45	57.98
Thysanolaena	0.81 ± 0.08	0.80 ± 0.02	0.62 ± 0.02	1.52	23.38

Values for chlorophyll concentration are mean ± SD (n = 3). Greater values of salt sensitivity index denote greater sensitivity or susceptibility to NaCl, whereas lower values denote lesser sensitivity.

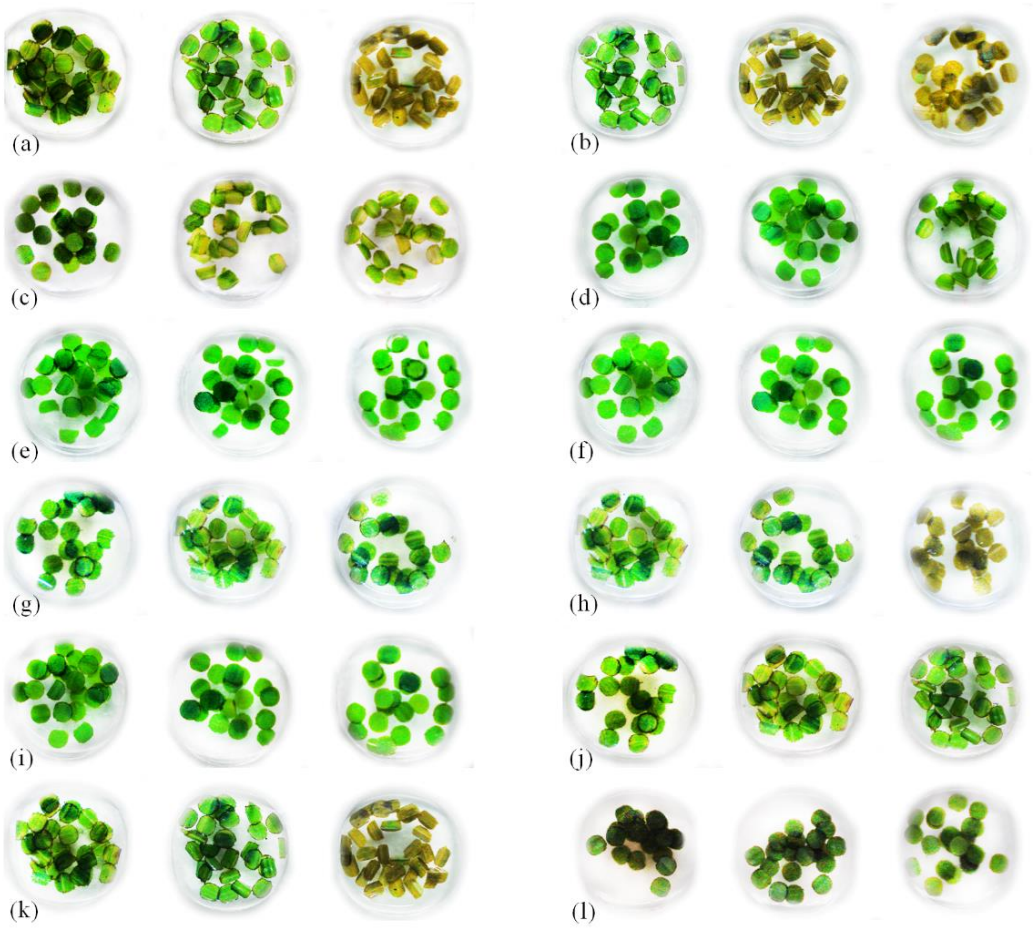

Figure 2. Leaf disc senescence bioassay: Phenotypic changes observed as chlorophyll bleaching occurs in response to 0, 100 and 200 mM NaCl treatment (left to right) after 96 h. (a) *Arundo*; (b) *Axonopus*; (c) *Capillipedium*; (d) *Chrysopogon*; (e) *Cynodon;* (f) *Digitaria*; (g) *Arundinella*; (h) *Eragrostis*; (i) *Imperata*; (j) *Oplismenus*; (k) *Setaria*; and (l) *Thysanolaena*.

Table 2. Relative water content (%) of grasses under treatment of 0, 100 and 200 mM NaCl solutions for 3, 6 and 9 days.

Grass	Concentration of NaCl (mM/L) and duration of treatment								
	3 days[1]			6 days[2]			9 days[3]		
	0	100	200	0	100	200	0	100	200
Arundo	85.2 ±1.1	80.6 ±2.1	78.5 ±1.1	84.1 ±0.9	77.5 ±0.6	71.2 ±1.4	84.6 ±1.2	70.2 ±0.8	66.5 ±2.2
Axonopus	84.5 ±1.2	78.6 ±2.3	74.1 ±1.7	83.2 ±1.5	76.5 ±0.7	74.2 ±0.9	83.2 ±1.3	73.1 ±1.1	68.6 ±0.4
Capillipedium	85.2 ±1.4	77.3 ±1.8	76.5 ±1.3	86.6 ±1.2	75.4 ±1.2	72.3 ±0.8	85.5 ±2.1	75.5 ±1.1	70.1 ±0.9
Chrysopogon	82.1 ±0.9	74.3 ±1.2	72.1 ±2.3	81.8 ±0.8	73.2 ±1.5	69.4 ±0.6	82.6 ±2.2	66.5 ±1.5	60.7 ±1.1
Cynodon	91.5 ±0.8	89.6 ±1.5	87.2 ±2.5	90.2 ±1.3	87.2 ±1.1	82.9 ±1.8	90.7 ±1.2	84.2 ±1.7	81.5 ±0.8
Digitaria	84.1 ±1.2	78.6 ±2.4	74.5 ±1.2	83.9 ±2.1	77.2 ±0.8	68.9 ±0.9	85.8 ±1.5	74.3 ±1.3	61.2 ±0.6
Arundinella	80.1 ±2.1	75.5 ±1.2	72.5 ±2.5	81.5 ±2.3	73.2 ±1.2	70.8 ±0.7	80.6 ±1.5	70.4 ±2.1	65.4 ±1.4
Eragrostis	85.1 ±1.9	81.2 ±1.1	79.6 ±2.6	83.2 ±1.8	76.7 ±1.6	72.1 ±1.2	84.1 ±1.8	71.2 ±2.4	63.1 ±0.7
Imperata	82.5 ±1.4	80.2 ±0.9	78.2 ±1.6	81.9 ±0.9	79.2 ±1.8	77.6 ±1.8	80.5 ±0.9	76.1 ±1.5	75.9 ±0.9
Oplismenus	87.3 ±0.8	80.5 ±0.9	77.6 ±1.4	86.5 ±1.4	78.2 ±0.8	74.6 ±1.9	85.9 ±1.1	76.1 ±0.8	72.3 ±1.1
Setaria	82.4 ±0.6	77.5 ±1.2	74.1 ±0.7	80.5 ±1.2	74.1 ±1.1	70.6 ±0.3	80.5 ±2.1	71.1 ±0.6	62.3 ±1.3
Thysanolaena	86.5 ±1.1	80.5 ±1.7	76.2 ±1.2	87.1 ±2.2	74.5 ±0.7	70.2 ±1.6	85.2 ±1.5	70.7 ±1.3	64.2 ±1.8

[1]LSD (P≤0.05) Species = 2.23; Treatment = 1.12. [2]LSD (P≤0.05) Species = 3.41; Treatment = 1.7. [3]LSD (P≤0.05) Species = 5.19; Treatment = 2.59. Values represent mean ± SD, where n = 3.

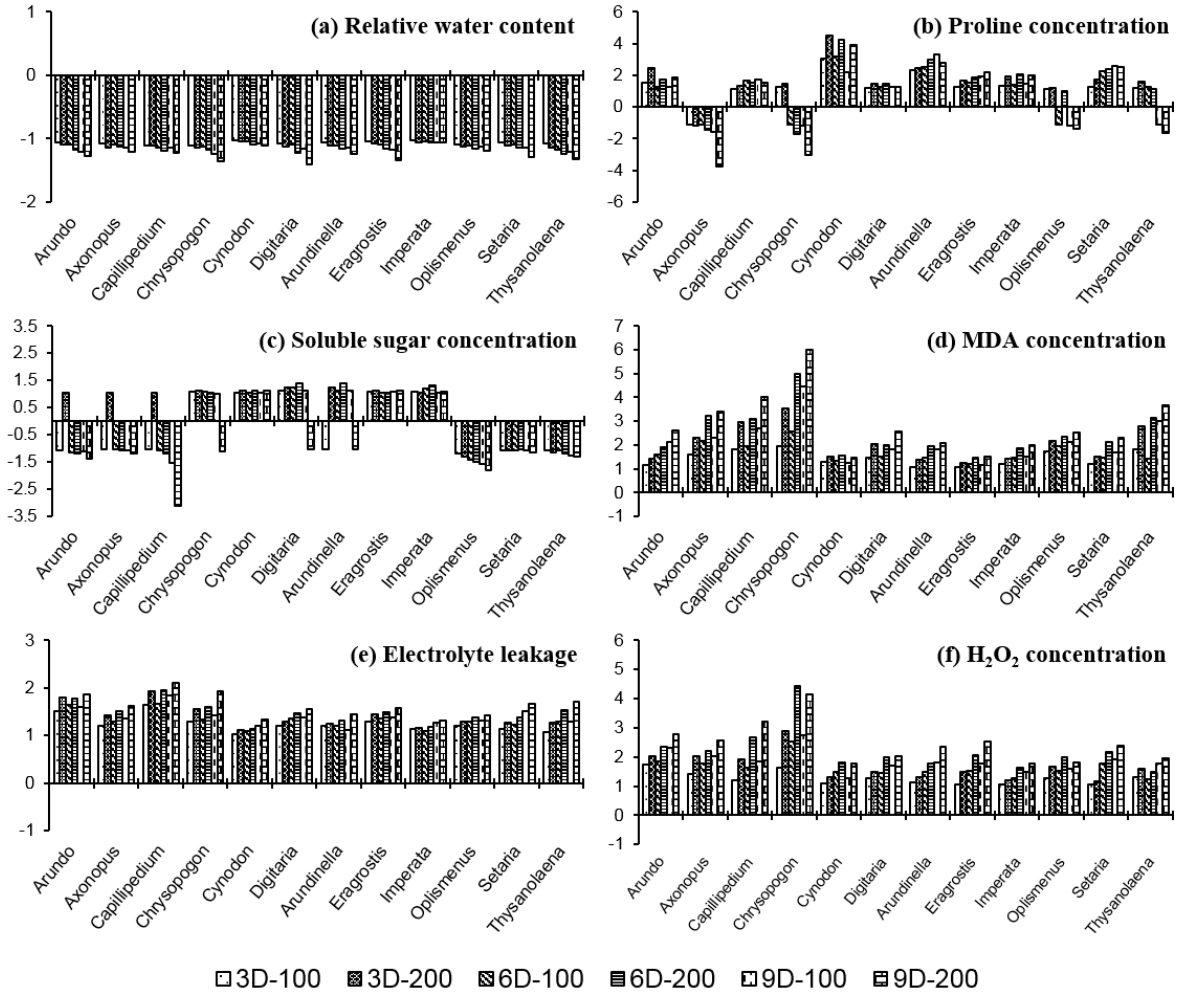

Figure 3. Fold change values of the biochemical markers in grasses subjected to NaCl stress. (a) Relative water content; (b) Proline concentration; (c) Soluble sugar concentration; (d) MDA concentration; (e) Electrolyte leakage; and (f) H_2O_2 concentration. 3D, 6D and 9D represent the duration of exposure to NaCl solutions (days) and 100 and 200 represent the concentrations of NaCl (mM/L).

Table 3. Proline concentration (µg/g fwt) in grasses under treatments of 0, 100 and 200 mM NaCl solutions for 3, 6 and 9 days.

Grass	Concentration of NaCl (mM/L) and duration of treatment								
	3 days[1]			6 days[2]			9 days[3]		
	0	100	200	0	100	200	0	100	200
Arundo	40.5 ±0.8	60.8 ±0.3	98.3 ±0.1	45.2 ±0.4	57.9 ±0.5	78.2 ±0.9	42.5 ±0.2	55.2 ±0.4	78.4 ±1.6
Axonopus	32.3 ±0.7	29.8 ±0.1	27.4 ±0.7	30.2 ±0.3	26.5 ±0.2	20.7 ±0.1	30.5 ±0.3	19.7 ±0.1	8.2 ±0.1
Capillipedium	39.3 ±0.7	45.2 ±0.2	51.3 ±0.7	35.9 ±0.4	60.4 ±0.6	55.2 ±0.8	36.6 ±0.1	64.2 ±0.2	56.1 ±1.1
Chrysopogon	12.2 ±0.2	15.3 ±0.2	17.8 ±0.1	11.9 ±0.1	10.8 ±0.1	7.1 ±0.5	10.6 ±0.7	8.8 ±0.4	3.5 ±0.2
Cynodon	48.1 ±1.2	145.3 ±2.1	215.1 ±2.5	50.1 ±0.7	160.2 ±1.5	210.8 ±2.3	45.8 ±0.2	100.3 ±1.3	178.2 ±1.4
Digitaria	65.3 ±1.1	78.9 ±1.7	95.6 ±1.5	63.2 ±1.1	80.6 ±1.1	90.7 ±1.5	66.1 ±0.2	82.6 ±1.4	85.1 ±0.9
Arundinella	30.5 ±0.8	70.1 ±1.1	75.2 ±1.5	34.2 ±0.6	86.1 ±0.9	102.5 ±1.6	32.1 ±0.1	107.1 ±1.5	90.2 ±1.3
Eragrostis	40.5 ±0.7	51.2 ±0.9	68.7 ±1.1	42.5 ±0.6	65.4 ±0.8	79.8 ±0.9	44.4 ±0.2	86.5 ±1.5	97.3 ±1.5
Imperata	63.3 ±0.9	83.5 ±1.1	120.2 ±0.9	60.7 ±0.1	85.2 ±0.9	125.3 ±1.3	65.4 ±0.4	96.9 ±1.6	132.1 ±1.1
Oplismenus	23.1 ±0.5	25.6 ±0.7	27.1 ±0.5	22.7 ±0.3	20.1 ±0.5	23.5 ±0.3	20.9 ±0.1	17.6 ±0.4	15.2 ±0.5
Setaria	12.2 ±0.1	15.5 ±0.3	20.8 ±0.4	14.3 ±0.3	32.1 ±0.2	34.5 ±0.1	10.6 ±0.7	27.6 ±0.4	26.7 ±0.1
Thysanolaena	25.6 ±0.3	30.8 ±0.4	41.1 ±0.8	23.2 ±0.2	28.7 ±0.3	26.2 ±0.5	20.2 ±0.6	17.8 ±0.7	12.5 ±0.1

[1]LSD (P≤0.05) Species = 40.82; Treatment = 20.41. [2]LSD (P≤0.05) Species = 41.82; Treatment = 20.91. [3]LSD (P≤0.05) Species = 40.15; Treatment = 20.07. Values represent Mean ± SD, where n = 3.

Total sugar concentration. Concentration of sugars in untreated fresh leaves varied from 16.1 mg/g (*Capillipedium*) to 56.9 mg/g (*Eragrostis*). Changes in concentration followed no consistent pattern across the various grasses subjected to NaCl treatments (Table 4; Figure 3c), with some showing decreases while a few showed increases. Those showing greatest decreases were *Capillipedium* (69% decrease) and *Oplismenus* (45% decrease), with most of the grass species showing little change in sugar concentration over the 9 days, even at 200 mM NaCl.

Membrane lipid peroxidation. MDA concentration in untreated fresh leaves varied from 2.2 mM/g (*Chrysopogon*) to 11.9 mM/g (*Arundo*). Concentrations showed a consistent pattern, increasing across all concentrations and durations of NaCl treatment in all grasses with greater responses to increasing concentration than to increasing duration of exposure (Table 5; Figure 3d). After 9 days, greatest increases in MDA concentration occurred in *Chrysopogon* (5-fold), *Capillipedium* (3-fold) and *Axonopus* (2.4-fold).

Table 4. Soluble sugar concentration (mg/g fwt) in grasses under treatments of 0, 100 and 200 mM NaCl solutions for 3, 6 and 9 days.

Grass	Concentration of NaCl (mM/L) and duration of treatment								
	3 days[1]			6 days[2]			9 days[3]		
	0	100	200	0	100	200	0	100	200
Arundo	35.2 ±0.7	33.1 ±0.3	36.7 ±0.1	34.1 ±0.2	30.2 ±0.1	28.9 ±0.1	33.9 ±0.1	31.1 ±0.1	24.6 ±0.1
Axonopus	50.1 ±1.5	48.9 ±1.5	52.1 ±0.6	47.8 ±1.4	46.8 ±0.8	45.1 ±1.2	47.5 ±0.9	44.3 ±1.2	40.1 ±1.1
Capillipedium	15.6 ±0.2	14.9 ±0.1	16.5 ±0.2	16.1 ±0.1	15.1 ±0.1	13.4 ±0.3	16.7 ±0.1	10.9 ±0.1	5.4 ±0.1
Chrysopogon	32.1 ±0.1	34.4 ±0.4	36.7 ±0.3	30.5 ±0.2	33.1 ±0.2	31.6 ±0.2	30.9 ±0.2	31.5 ±0.2	27.8 ±0.1
Cynodon	40.1 ±0.1	42.1 ±1.4	45.3 ±0.2	41.8 ±0.5	43.2 ±0.8	46.3 ±1.4	40.5 ±0.9	42.6 ±1.1	44.9 ±1.2
Digitaria	35.4 ±0.2	40.1 ±0.6	44.3 ±1.4	34.6 ±0.3	43.2 ±1.3	47.6 ±1.6	36.1 ±0.2	40.5 ±1.3	35.5 ±0.2
Arundinella	29.8 ±0.1	28.6 ±0.1	36.5 ±0.5	27.6 ±0.1	31.5 ±0.2	38.7 ±0.2	30.5 ±0.2	34.2 ±0.3	29.9 ±0.1
Eragrostis	56.1 ±1.1	60.3 ±0.7	62.3 ±0.7	57.8 ±1.3	60.5 ±1.5	61.4 ±0.2	56.8 ±0.6	61.3 ±0.5	63.3 ±0.3
Imperata	33.2 ±0.9	36.1 ±0.5	35.3 ±0.2	30.8 ±0.2	36.6 ±0.4	40.9 ±1.5	33.3 ±0.3	34.5 ±0.6	35.7 ±0.7
Oplismenus	40.5 ±0.2	34.5 ±0.3	31.2 ±0.3	43.2 ±0.5	30.6 ±0.2	28.7 ±0.2	41.9 ±1.4	26.7 ±0.3	23.2 ±0.2
Setaria	49.2 ±1.1	46.5 ±1.2	45.5 ±1.5	47.8 ±1.2	44.4 ±1.1	46.5 ±0.3	47.7 ±0.5	44.3 ±0.3	41.1 ±1.2
Thysanolaena	36.5 ±0.5	34.2 ±0.3	31.3 ±0.1	35.5 ±0.2	33.3 ±0.2	29.8 ±0.3	37.7 ±0.2	30.1 ±0.2	28.9 ±0.3

[1]LSD (P≤0.05) Species = 4.96; Treatment = 2.48. [2]LSD (P≤0.05) Species = 7.24; Treatment = 3.62. [3]LSD (P≤0.05) Species = 6.92; Treatment = 3.46. Values represent Mean ± SD, where n = 3.

Table 5. MDA concentration (mM MDA/g fwt) of grasses under treatments of 0, 100 and 200 mM NaCl solutions for 3, 6 and 9 days.

Grass	Concentration of NaCl (mM/L) and duration of treatment								
	3 days[1]			6 days[2]			9 days[3]		
	0	100	200	0	100	200	0	100	200
Arundo	12.1 ±0.1	14.2 ±0.8	17.3 ±0.4	11.3 ±0.2	18.2 ±0.8	21.6 ±0.3	12.3 ±0.1	26.1 ±0.6	32.3 ±0.2
Axonopus	10.1 ±0.1	16.2 ±0.2	23.1 ±0.2	10.6 ±0.4	23.1 ±0.4	34.2 ±0.3	11.1 ±0.3	25.6 ±0.4	37.6 ±0.4
Capillipedium	5.6 ±0.2	10.1 ±0.3	16.7 ±0.1	5.7 ±0.1	11.1 ±0.1	17.6 ±0.2	4.9 ±0.3	13.2 ±0.6	19.8 ±0.6
Chrysopogon	2.2 ±0.7	4.3 ±0.1	7.8 ±0.1	2.1 ±0.5	5.4 ±0.2	10.5 ±0.8	2.2 ±0.1	9.8 ±0.1	13.2 ±0.2
Cynodon	10.2 ±0.6	13.2 ±0.2	15.6 ±0.2	10.5 ±0.1	14.1 ±0.1	16.4 ±0.1	11.2 ±0.1	13.9 ±0.2	16.5 ±0.4
Digitaria	3.5 ±0.4	5.1 ±0.1	7.2 ±0.7	4.1 ±0.9	6.2 ±0.7	8.1 ±0.6	3.7 ±0.9	6.7 ±0.3	9.5 ±0.8
Arundinella	4.8 ±0.8	5.1 ±0.1	6.7 ±0.1	4.5 ±0.1	6.7 ±0.1	8.8 ±0.2	4.1 ±0.8	7.5 ±0.7	8.5 ±0.9
Eragrostis	8.6 ±0.6	9.1 ±0.1	10.7 ±0.6	8.1 ±0.4	9.7 ±0.3	11.8 ±0.4	8.8 ±0.5	10.1 ±0.1	13.4 ±0.6
Imperata	5.4 ±0.3	6.5 ±0.1	7.8 ±0.5	4.8 ±0.8	7.1 ±0.2	8.9 ±0.3	5.1 ±0.2	7.7 ±0.3	10.1 ±0.2
Oplismenus	9.8 ±0.5	17.1 ±0.2	21.3 ±0.7	9.5 ±0.1	18.6 ±0.1	22.5 ±0.1	10.1 ±0.2	21.3 ±0.3	25.4 ±0.4
Setaria	10.1 ±0.3	12.1 ±0.3	15.4 ±0.3	9.7 ±0.2	14.3 ±0.2	20.5 ±0.1	10.2 ±0.2	17.3 ±0.7	23.7 ±0.4
Thysanolaena	3.1 ±0.6	5.6 ±0.2	8.7 ±0.4	3.4 ±0.3	4.9 ±0.6	10.7 ±0.3	3.6 ±0.5	10.8 ±0.6	13.2 ±0.1

[1]LSD (P≤0.05) Species = 3.34; Treatment = 1.67. [2]LSD (P≤0.05) Species = 5.07; Treatment = 2.53. [3]LSD (P≤0.05) Species = 6.25; Treatment = 3.12. Values represent Mean ± SD, where n = 3.

Electrolyte leakage. Electrolyte leakage levels in untreated fresh leaves varied from 5.1% (*Arundinella*) to 15.5% (*Setaria*) and increased across all concentrations and durations of NaCl treatment in all grasses (Table 6; Figure 3e). *Arundo* and *Capillipedium* showed the greatest increases in electrolyte leakage with exposure to NaCl treatment with a much greater response to increasing concentration (80–90%) than to duration of exposure (10–24%). The lowest responses occurred with *Cynodon* and *Imperata*.

H_2O_2 concentration. Concentrations of H_2O_2 in untreated fresh leaves ranged from 2.4 µmol/g (*Chrysopogon*) to 11.8 µmol/g (*Digitaria* and *Thysanolaena*) and increased across all concentrations of and durations of exposure to NaCl solutions for all grasses (Table 7; Figure 3f). The most responsive grasses were *Chrysopogon*, *Capillipedium* and *Arundo*, while the least responsive were *Cynodon* and *Imperata*.

Hierarchical cluster analysis for the evaluation of NaCl tolerance

Based on the variable effects of NaCl treatment on biochemical parameters, the grasses were grouped according to their NaCl tolerance through hierarchical cluster analysis, where the fold change values of all parameters were taken into consideration (Figures 3a–3f).

Table 6. Electrolyte leakage (%) of grasses under treatments of 0, 100 and 200 mM NaCl solutions for 3, 6 and 9 days.

Grass	Concentration of NaCl (mM/L) and duration of treatment								
	3 days[1]			6 days[2]			9 days[3]		
	0	100	200	0	100	200	0	100	200
Arundo	14.1 ±0.5	21.2 ±0.1	25.4 ±0.5	14.1 ±0.6	23.1 ±0.3	24.9 ±0.2	14.3 ±0.2	22.9 ±0.4	26.7 ±0.3
Axonopus	10.1 ±0.7	12.2 ±0.3	14.3 ±0.3	10.3 ±0.3	13.4 ±0.2	15.6 ±0.3	10.6 ±0.3	14.3 ±0.6	17.2 ±0.3
Capillipedium	8.7 ±0.1	14.3 ±0.5	16.7 ±0.6	9.1 ±0.3	15.1 ±0.3	17.8 ±0.3	8.8 ±0.3	16.2 ±0.6	18.6 ±0.3
Chrysopogon	5.2 ±0.5	6.7 ±0.3	8.1 ±0.3	6.1 ±0.5	8.2 ±0.4	9.7 ±0.3	5.5 ±0.3	7.8 ±0.3	10.6 ±0.2
Cynodon	11.9 ±0.9	12.1 ±0.4	13.2 ±0.5	12.2 ±0.4	13.2 ±0.5	13.9 ±0.3	10.8 ±0.4	12.9 ±0.4	14.3 ±0.4
Digitaria	11.2 ±0.8	13.4 ±0.3	14.5 ±0.3	10.7 ±0.7	14.5 ±0.5	15.6 ±0.3	11.1 ±0.4	15.2 ±0.2	17.2 ±0.3
Arundinella	5.2 ±0.6	6.2 ±0.2	6.5 ±0.2	5.1 ±0.2	6.1 ±0.3	6.7 ±0.1	4.9 ±0.3	5.5 ±0.3	7.1 ±0.3
Eragrostis	10.1 ±0.9	13.1 ±0.2	14.5 ±0.3	10.4 ±0.3	14.2 ±0.4	15.4 ±0.2	10.6 ±0.3	14.5 ±0.2	16.7 ±0.4
Imperata	14.3 ±1.1	16.1 ±0.3	16.5 ±0.3	14.5 ±0.4	15.8 ±0.3	17.2 ±0.3	14.9 ±0.4	18.8 ±0.3	19.7 ±0.3
Oplismenus	13.4 ±0.7	16.1 ±0.4	17.2 ±0.4	13.1 ±0.3	16.8 ±0.2	18.1 ±0.4	13.4 ±0.2	17.5 ±0.2	19.2 ±0.6
Setaria	15.1 ±0.8	17.2 ±0.2	19.3 ±0.4	15.4 ±0.2	18.9 ±0.4	21.3 ±0.4	16.1 ±0.3	24.3 ±0.3	26.7 ±0.5
Thysanolaena	7.6 ±0.6	8.1 ±0.1	9.7 ±0.5	7.3 ±0.3	9.5 ±0.3	11.2 ±0.3	7.8 ±0.4	10.1 ±0.4	13.4 ±0.4

[1]LSD (P≤0.05) Species = 2.6; Treatment = 1.3. [2]LSD (P≤0.05) Species = 2.53; Treatment = 1.27. [3]LSD (P≤0.05) Species = 2.82; Treatment = 1.41. Values represent Mean ± SD, where n = 3.

Table 7. H_2O_2 concentration (µmol/g fwt) in grasses under treatment with 0, 100 and 200 mM NaCl solutions for 3, 6 and 9 days.

Grass	Concentration of NaCl (mM/L) and duration of treatment								
	3 days[1]			6 days[2]			9 days[3]		
	0	100	200	0	100	200	0	100	200
Arundo	6.5 ±0.1	11.2 ±0.2	13.1 ±0.3	6.6 ±0.4	12.3 ±0.2	15.6 ±0.1	6.7 ±0.3	15.4 ±0.3	18.7 ±0.5
Axonopus	7.2 ±0.3	10.2 ±0.3	14.5 ±0.2	8.1 ±0.1	14.3 ±0.2	17.8 ±0.2	8.3 ±0.1	16.7 ±0.1	21.3 ±0.3
Capillipedium	4.5 ±0.2	5.4 ±0.4	8.7 ±0.2	4.1 ±0.2	6.7 ±0.1	10.9 ±0.3	4.8 ±0.3	8.8 ±0.1	15.4 ±0.5
Chrysopogon	2.5 ±0.8	4.1 ±0.2	7.2 ±0.4	2.1 ±0.3	5.3 ±0.2	9.3 ±0.4	2.7 ±0.1	7.4 ±0.5	11.2 ±0.2
Cynodon	10.1 ±0.7	11.2 ±0.2	13.2 ±0.5	9.7 ±0.4	14.5 ±0.3	17.6 ±0.3	10.3 ±0.4	13.2 ±0.5	18.1 ±0.3
Digitaria	12.1 ±0.8	15.4 ±0.3	17.8 ±0.3	11.7 ±0.5	17.1 ±0.4	23.1 ±0.5	11.9 ±0.1	20.1 ±0.4	24.3 ±0.2
Arundinella	6.8 ±0.5	7.6 ±0.5	8.9 ±0.2	6.6 ±0.6	9.9 ±0.4	11.7 ±0.4	6.5 ±0.4	11.7 ±0.4	15.3 ±0.5
Eragrostis	4.5 ±0.4	4.7 ±0.2	6.7 ±0.2	4.1 ±0.3	6.2 ±0.5	8.4 ±0.1	4.3 ±0.2	7.6 ±0.3	10.9 ±0.2
Imperata	8.7 ±0.3	9.1 ±0.7	10.3 ±0.3	8.2 ±0.3	10.3 ±0.3	13.4 ±0.3	8.6 ±0.4	12.9 ±0.5	15.2 ±0.3
Oplismenus	11.3 ±0.4	14.3 ±0.2	18.7 ±0.3	10.9 ±0.2	16.5 ±0.2	21.8 ±0.1	11.1 ±0.1	17.6 ±0.3	20.1 ±0.5
Setaria	8.5 ±0.5	9.1 ±0.3	9.8 ±0.2	8.1 ±0.3	14.3 ±0.2	17.6 ±0.2	7.9 ±0.8	15.1 ±0.2	18.9 ±0.3
Thysanolaena	11.1 ±0.9	14.5 ±0.2	17.6 ±0.1	12.2 ±0.6	15.2 ±0.3	18.1 ±0.3	12.1 ±0.7	21.5 ±0.3	23.8 ±0.1

[1]LSD (P≤0.05) Species = 2.1; Treatment = 1.05. [2]LSD (P≤0.05) Species = 2.19; Treatment = 1.09. [3]LSD (P≤0.05) Species = 2.42; Treatment = 1.21. Values represent Mean ± SD, where n = 3.

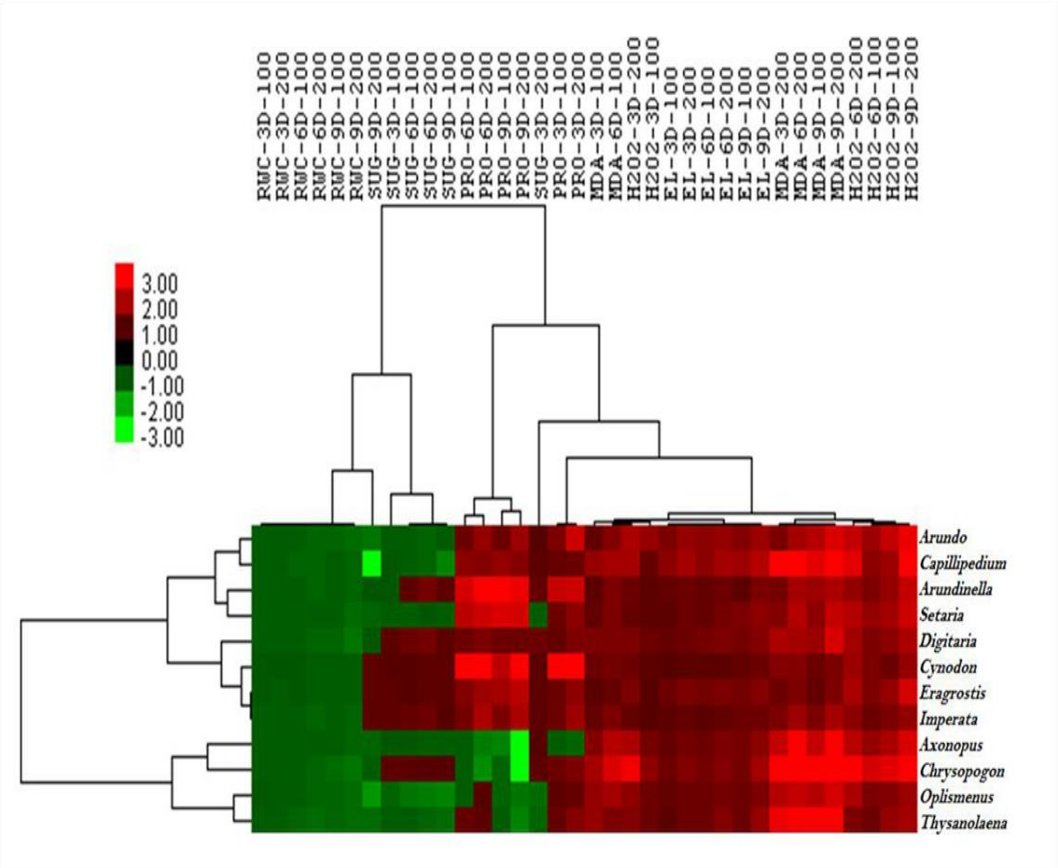

Figure 4. Hierarchical cluster analysis of the grasses using the fold change values of relative water content (RWC); proline concentration (PRO); soluble sugar concentration (SUG); membrane lipid peroxidation (malondialdehyde, MDA); electrolyte leakage (EL); and H_2O_2 concentration after NaCl treatments (100 mM and 200 mM) for 3, 6 and 9 days. Resulting tree figure was displayed using Java Treeview after hierarchical cluster analysis through CLUSTER 3.0. The color grids in the cluster analysis represent the relative fold change values (-3 to +3 shown by different colors) of the specific biochemical markers for each of the individual grasses. For the analysis of salt tolerance, the greenness of the grids for biomarkers like MDA, EL and H_2O_2 and redness for RWC, PRO and SUG was considered; which means a species for which the grids are more reddish for RWC, PRO and SUG and less greenish for MDA, EL and H_2O_2 could be considered the most tolerant of all. However, this was easily recognized in the cluster analysis due to grouping of the studied species on the basis of their responses to biochemical markers.

The ranges of fold change values in the clusters are represented by the colored bars. Results suggested the probable interrelations among biochemical parameters subjected to NaCl stress and variable salt tolerance between all grass genera.

Based on their salt sensitivities, the grasses formed 2 distinct groups (Figure 4). One group was comprised of *Axonopus*, *Chrysopogon*, *Oplismenus* and *Thysanolaena*. The remaining grasses with varying response patterns to NaCl solutions formed the second group and were classified into 3 subgroups: *Arundo* and *Capillipedium; Arundinella* and *Setaria*; and *Digitaria*, *Cynodon*, *Eragrostis* and *Imperata*.

Discussion

This rapid screening for salinity tolerance in the forage grasses has been attempted as a simple method of identifying the most salt-tolerant grasses for introduction into areas with increasing soil salinity and decreasing productivity. Previously, Zulkaliph et al. (2013) in their studies with turfgrasses ranked the different species of grasses for salinity tolerance on the basis of shoot and root growth, leaf firing, i.e. yellowing of leaves resulting from cell death due to osmotic imbalances, turf color and turf quality. We estimated salinity tolerance of the grasses primarily by a salt sensitivity index (SSI), determined by evaluating the effects of NaCl solutions on leaf discs over 96 hours. This type of bioassay has been used previously in several transgenesis experiments to evaluate the tolerances of transgenic plants relative to the wild type plants from which they were bioengineered (Bhaskaran and Savithramma 2011; Yadav et al. 2012).

The amount of chlorophyll leached out from the leaf discs into the NaCl solution was used as an indicator of the effect of NaCl on leaf tissues. The decrease in chlorophyll concentration in plants subjected to NaCl treatment has been inversely correlated with salinity tolerance. For instance, the decrease in Chlorophyll a: Chlorophyll b ratio in salt-tolerant *Najas graminea* was lower than in *Hydrilla verticillata* and *Najas indica* (Rout et al. 1997). In the present study, we quantified the amount of chlorophyll in the leaf discs in both control and treatment sets and the values were used to reciprocate the sensitivity of grasses towards NaCl treatment. Greater salt sentivity index values denoted greater susceptibility of the grasses towards NaCl. Overall, the results of the bioassay indicated that among the grasses tested, *Imperata*, *Cynodon* and *Digitaria* could be considered as less sensitive or resistant on the basis of SSI values at 100 and 200 mM NaCl. SSI therefore presents an easy and rapid technique to screen out the potential salt-tolerant forage grasses.

The 6 biomarkers we selected to analyze the salt-tolerance potential of the forage grasses, namely relative water content (RWC), proline and soluble sugar concentrations, membrane lipid peroxidation, electrolyte leakage and H_2O_2 concentration, proved useful in indicating differences between species in ability to tolerate saline conditions both simply and rapidly.

While RWC of any plant always decreases with the increase in NaCl concentration, a lower decrease in RWC is a valuable marker in the selection of salt-tolerant species (Ziaf et al. 2009). In our study, lowest decreases in RWC were observed in *Cynodon*, *Eragrostis* and *Imperata* across all concentrations and durations of NaCl treatments, identifying them as salt-tolerant species. In contrast, accumulation of proline and soluble sugars is considered to be positively correlated with salinity tolerance (Karsensky and Jonak 2012; Hayat et al. 2012). Accumulation of higher levels of proline has been reported in the halophytes, *Mesembryanthemum crystallinum* and *Sporobolus virginicus* when compared with the glycophytes carrot and rice (Thomas et al. 1992; Tada et al. 2014). In the present study, apart from *Axonopus*, *Chrysopogon* and *Oplismenus*, proline accumulation increased in all grasses subjected to NaCl treatment. We also observed that soluble sugar accumulation decreased in *Arundo*, *Axonopus*, *Capillipedium*, *Oplismenus*, *Setaria* and *Thysanolaena* across all concentrations of NaCl and durations of exposure. In contrast, accumulation of soluble sugars increased in *Digitaria*, *Imperata* and *Arundinella* subjected to NaCl treatments for 3, 6 and 9 days. Nedjimi (2011) also correlated the accumulation of greater amounts of soluble sugars in the forage grass *Lygeum spartum* with osmotic adjustment and protection of membrane stability that conferred salinity tolerance.

Increase in malondialdehyde (MDA) concentration, an indication of lipid peroxidation, is considered unfavorable for plant health, and plants, which show little increase in MDA concentration when exposed to NaCl, are considered to be salt-tolerant (Miller et al. 2010). Marked increases in MDA concentration were observed in *Axonopus*, *Capillipedium*, *Chrysopogon* and *Thysanolaena*, following exposure to salt. However, minimal increase was observed in *Cynodon* and *Eragrostis* across all concentrations and durations of treatment.

Similarly, low electrolyte leakage (EL) and limited increase in H_2O_2 concentration in response to NaCl treatment are also considered as markers of the salt

tolerance of plants (Mostafa and Tammam 2012). Accumulation of H_2O_2 in plants interferes with the normal biochemical processes inside plants. In the present study, EL in all grasses increased with the increase in NaCl concentration and duration of treatment. Least EL was observed in *Cynodon*, *Imperata* and *Arundinella*, which could be considered salt-tolerant species in comparison with the other grasses. The high increases in H_2O_2 concentration observed in *Arundo*, *Axonopus*, *Capillipedium* and *Chrysopogon* indicate that these species can be considered susceptible to salination on the basis of this trait. Comparatively, low increases in H_2O_2 concentration observed in *Imperata*, *Setaria* and *Cynodon* indicate that they can be considered salt-tolerant.

Finally, hierarchical cluster analysis using the software CLUSTER 3.0 was used to represent the inter-relations among the physiological parameters and to align the grasses on the basis of their salinity tolerance as a similar type of hierarchical cluster analysis has been performed to evaluate the natural variation in drought tolerance in bermuda grass (Shi et al. 2012) and the variation in salt tolerance in rice cultivars (Chunthaburee et al. 2016). In the present study we utilized the relative fold change values of all the parameters in forming clusters. Based on the variations of the physiological parameters, all grasses were grouped according to their NaCl tolerance that could be interpreted with the aid of the fold change values denoted by colored bars. The relationships between the physiological parameters themselves was also illustrated in the cluster analysis. The grasses were clearly divided into 2 groups - a susceptible group (*Axonopus*, *Chrysopogon*, *Oplismenus* and *Thysanolaena*) and a relatively salt-tolerant group containing the remaining grasses. Critical analysis of the second group revealed 3 subgroups of less tolerant (*Arundo* and *Capillipedium*), moderately tolerant (*Arundinella* and *Setaria*) and tolerant grasses (*Digitaria*, *Cynodon*, *Eragrostis* and *Imperata*). These results are in accordance with the findings of other workers who reported the use of some of these and other related, tolerant grasses for the reclamation and utilization of saline soils and increased forage production (Kaffka 2001; Weber and Hanks 2006).

Based on the results of hierarchical clustering, we conclude that *Imperata cylindrica*, *Eragrostis amabilis*, *Cynodon dactylon* and *Digitaria ciliaris* were relatively salt-tolerant. SSI values individually pointed towards the superior salt-tolerance of *Imperata*, *Digitaria* and *Cynodon*, whereas proline concentration indicated marked tolerance in *Cynodon*, *Arundinella*, *Imperata*, *Eragrostis* and *Setaria*. If we consider the MDA concentrations, *Cynodon*, *Arundinella*, *Imperata* and *Eragrostis* could be considered salt-tolerant. Thus, while individual biochemical markers provide good indications of the degree of salt tolerance of a species, cluster analysis, which incorporates the results with several biomarkers, provides a much more reliable indication. However, SSI values can provide an easy and rapid tool for the screening of salt tolerance. Based on our screening results, we consider that the selective propagation of the most salt-tolerant species could be utilized for the rejuvenation of native grasslands and also for the reclamation of salinity infested wastelands.

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Research Paper

Reduction of sward height in the fall and winter as a strategy to improve the structure of marandu palisadegrass (*Urochloa brizantha* syn. *Brachiaria brizantha* cv. Marandu)

Reducción de la altura del pasto en otoño e invierno como estrategia para mejorar la estructura de una pastura de Urochloa *(sin.* Brachiaria*)* brizantha *cv. Marandu*

MANOEL E.R. SANTOS[1], MIRIÃ G. SIMPLÍCIO[1], GUILHERME P. SILVA[2], HERON A. DE OLIVEIRA[1], LUDIÊMILEM K.P. DA COSTA[1] AND DIOGO O.C. DE SOUSA[1]

[1]*Faculty of Veterinary Medicine, Federal University of Uberlândia, Uberlândia, MG, Brazil.* www.ufu.br
[2]*Animal Science Department, ESALQ, University of São Paulo, Piracicaba, SP, Brazil.* www.esalq.usp.br

Abstract

The objective of this study was to identify defoliation strategies that might improve the structure of *Urochloa brizantha* (syn. *Brachiaria brizantha*) cv. Marandu (marandu palisadegrass). The following 3 defoliation strategies were compared in a plot study: sward kept at 15 cm in fall and winter (W) and 30 cm in spring (Sp) and summer (Su) (15W-30Sp-30Su); sward kept at 30 cm during the entire experimental period (30W-30Sp-30Su); and sward kept at 45 cm in fall and winter and 30 cm in spring and summer (45W-30Sp-30Su). The experimental design was completely randomized, with 4 replicates. Plots were cut with shears to the appropriate height weekly in winter and twice weekly in spring, summer and fall. Tiller density, mean tiller weight, leaf area index, forage mass, percentage of live leaf blades and percentage of stems were measured every 28 days. Forage mass in winter was directly related to pasture height ($P<0.05$) but differences had disappeared by summer ($P>0.05$). Mean tiller density was independent of cutting height but was higher in spring and summer than in winter ($P<0.05$). Mean tiller weight in winter was directly related to cutting height ($P<0.05$) but differences had disappeared by summer. The percentage of live leaf blades in the swards was affected by season with spring>summer>winter and by cutting height in fall/winter with leaf percentage inversely related to cutting height. Stem percentage in the swards in winter was directly related to cutting height. Grazing studies seem warranted to determine if these plot results are reflected under grazing conditions and what the impacts are on animal performance.

Keywords: Herbage mass, leaf area index, morphological composition, tillering.

Resumen

El objetivo del estudio, conducido en Uberlândia, Minas Gerais, Brasil, fue identificar estrategias de defoliación con el fin de mejorar la estructura de una pastura de *Urochloa brizantha* (sin. *Brachiaria brizantha*) cv. Marandu. Se compararon 3 estrategias: (1) mantener el pasto a una altura de 15 cm en otoño e invierno (W) y de 30 cm en primavera (Sp) y verano (Su) (15W-30Sp-30Su); (2) mantener el pasto a una altura de 30 cm durante todo el período experimental (30W-30Sp-30Su); y (3) mantener el pasto a una altura de 45 cm en otoño e invierno y de 30 cm en primavera y verano (45W-30Sp-30Su). El diseño experimental fue completamente al azar, con 4 repeticiones. Las parcelas se cortaron con tijeras a la altura respectiva semanalmente en invierno y 2 veces por semana en primavera, verano y otoño. Cada 28 días

Correspondence: D.O.C. de Sousa, Faculty of Veterinary Medicine, Federal University of Uberlândia, Campus Umuarama, Av. Pará 1720, Uberlândia CEP 38400-902, MG, Brazil.
Email: diogoolimpio@hotmail.com

se midieron la densidad de brotes, el peso medio de los brotes, el índice de área foliar, la masa de forraje, el porcentaje de hojas vivas y el porcentaje de tallos. La masa forrajera en invierno se relacionó directamente con la altura del pasto (P<0.05), pero las diferencias desaparecieron en verano (P>0.05). La densidad media de los brotes fue independiente de la altura de corte, pero fue mayor en primavera y verano que en invierno (P<0.05). El peso medio de los brotes en invierno estuvo directamente relacionado con la altura de corte (P <0.05), pero las diferencias desaparecieron en verano. El porcentaje de hojas vivas en la pastura se vio afectado por la estación del año, con primavera>verano>invierno y por la altura de corte en otoño/invierno cuando el porcentaje de hojas estuvo inversamente relacionado con la altura de corte. El porcentaje de tallos en invierno estuvo directamente relacionado con la altura de corte. Estudios de pastoreo parecen justificados para determinar si estos resultados, obtenidos a nivel de parcela de corte, se reflejan bajo condiciones de pastoreo, y cuáles son los impactos en la producción animal.

Palabras clave: Composición morfológica, índice de área foliar, masa forrajera, rebrotes.

Introduction

Pasture structure is a function of how the organs of the aerial parts of forage plants are distributed in the pasture, both vertically (Zanini et al. 2012) and horizontally (Barthram et al. 2005). Some parameters used to describe pasture structure are: sward height, forage mass, volume and density (Carvalho et al. 2009).

Pasture height is highly correlated with forage mass and morphological composition (Paula et al. 2012; Nantes et al. 2013), in addition to being a cheap, easy and quick measurement. For this reason, average pasture height has been recommended as a management criterion for when to commence and cease grazing (Silva and Nascimento Júnior 2007). Studies on grazing management strategies, based on pasture height, enable the understanding of variations in pasture structure, as well as the responses of animals and plants to these variations (Trindade et al. 2007; Fonseca et al. 2012, 2013).

Sbrissia et al. (2010) suggested that the optimal height range for management of marandu palisadegrass (*Urochloa brizantha* syn. *Brachiaria brizantha* cv. Marandu) under continuous grazing during the rainy season was 20–40 cm. However, Santos et al. (2013) suggested that pasture height should be adjusted according to the season of the year to optimize the productivity of the pasture. Other studies, e.g. Sbrissia and Silva (2008) and Giacomini et al. (2009), indicated that plant development is often affected by interactions between defoliation management strategies and season of the year, which suggests that the success of a particular management strategy might differ between seasons. On the basis of these findings, we conclude that grazing management strategies should be flexible over the year and vary with seasonal conditions.

Maintaining the sward shorter during winter, the season with adverse climate and in which the plant has the lowest rate of photosynthesis (Lara and Pedreira 2011a), could result in lower maintenance respiration by the plants, which would provide greater energy and carbon balance in the sward (Taiz and Zeiger 2012). In contrast, keeping pasture tall in winter would increase the energy needs for survival of individual plants, precisely when photosynthesis is at its lowest point.

Moreover, Santana et al. (2014) suggested that the greater shading at the plant base, inherent in taller pastures, would lead to greater leaf senescence at the lower canopy stratum, which might inhibit tillering in early spring. On the other hand, pasture grazed short in winter would permit greater incidence of light at the base of the sward in spring, which should stimulate the appearance of young tillers (Paiva et al. 2012) with better structural traits (Barbosa et al. 2012).

We therefore hypothesize that, by varying sward height during fall and winter, it may be possible to modify physiological processes such as photosynthesis and respiration as well as plant development, e.g. tillering and leaf senescence. All these processes, in turn, may change sward structure not only in fall and winter, the seasons in which plant height is changed, but also in subsequent ones.

This study was conducted to characterize the structural changes of a marandu palisadegrass sward maintained at various sward heights in fall and winter, and kept at a constant height in spring and summer. This knowledge should prove beneficial in formulating recommendations regarding defoliation strategies for this forage plant throughout the year.

Materials and Methods

The experiment was conducted from March 2013 to March 2014, on the Capim Branco farm, belonging to the Faculty of Veterinary Medicine of the Federal University of Uberlândia, in Uberlândia, MG, Brazil (18º53’19” S, 48°20’57” W; 776 masl). The climate in the region of

Uberlândia, according to the Köppen (1948) classification, is a Cwa altitude tropical type, with mild and dry winters and well defined dry and rainy seasons. The average annual temperature is 22.3 ºC, with mean maximum and minimum values of 23.9 and 19.3 ºC, respectively. Average annual precipitation is 1,584 mm.

The experiment was developed on a pasture of *Urochloa brizantha* syn. *Brachiaria brizantha* cv. Marandu (palisadegrass), established in the year 2000, and well managed with cattle. Twelve plots (experimental units) with an area of 12 m^2 each were used. A border area of 0.25 m wide was discarded leaving a usable area of 8.75 m^2 on each plot for data collection.

Climatic conditions during the experimental period were monitored at the meteorological station, located approximately 200 m from the experimental area (Figures 1 and 2).

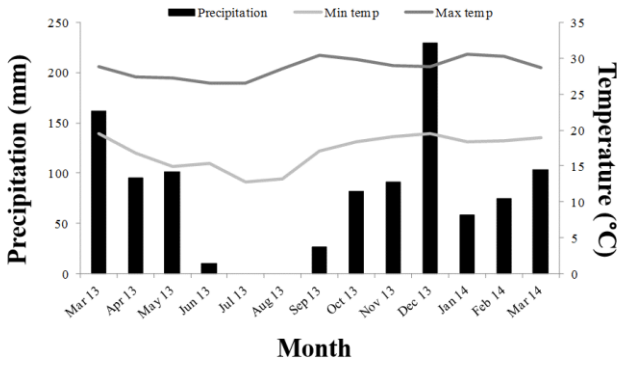

Figure 1. Monthly mean minimum and maximum temperatures and precipitation from March 2013 to March 2014. The seasons are: winter, July–September 2013; spring, October–December 2013; and summer, January–March 2014.

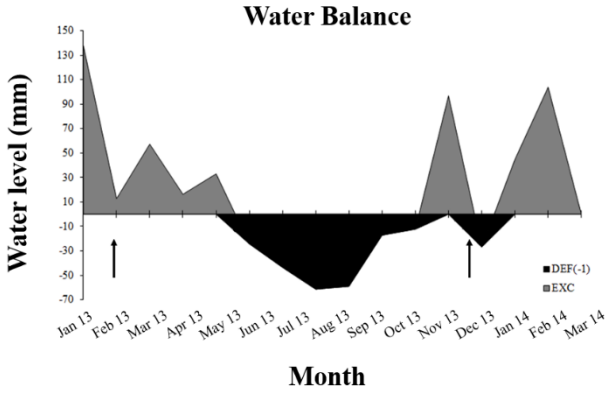

Figure 2. Summary of the water balance in the soil from January 2013 to April 2014. Arrows indicate the time when fertilizer was applied. The seasons are: winter, July–September 2013; spring, October–December 2013; and summer, January–March 2014. DEF (-1) = Deficit; EXC = Excess.

Before the experiment commenced, soil samples from the 0–10 cm layer were collected and analyzed, revealing the following chemical properties: pH in H_2O - 6.1; P - 9.4 mg/dm^3 (Mehlich-1); K^+ - 156 mg/dm^3; Ca^{2+} - 5.5 $cmol_c/dm^3$; Mg^{2+} - 1.7 $cmol_c/dm^3$; Al^{3+} - 0.0 $cmol_c/dm^3$ (KCl 1 mol/L); effective CEC - 7.6; CEC at pH 7.0 - 10.3; and base saturation - 74%. Based on these results, 35.5 kg P/ha as single superphosphate, 50 kg N/ha as urea and 41.5 kg K/ha as KCl were broadcast on the plots in February 2013. These same amounts were applied again in January 2014.

Three defoliation strategies were evaluated, characterized by the heights at which the marandu palisadegrass sward was maintained during fall and winter (15, 30 and 45 cm), with a standard height of 30 cm during spring and summer. To maintain the grass at these heights, the swards were cut with pruning shears once a week in winter and twice a week during spring, summer and fall. This approach aimed to ensure that the actual heights of the canopies remained within 100–110% of the desired values. The first strategy, with marandu palisadegrass maintained at 15 cm in fall and winter and 30 cm in spring and summer, equated with heavy defoliation during winter and moderate defoliation subsequently. For the second strategy the pasture was maintained at 30 cm during the entire experimental period, according to the recommendations of Sbrissia and Silva (2008), i.e. moderate defoliation throughout. The third strategy consisted of maintaining the grass at 45 cm in fall and winter, i.e. only light defoliation, and at 30 cm in spring and summer.

The experimental period during which pasture measurements occurred was divided into winter (July–September 2013), spring (October–December 2013) and summer (January–March 2014). The experimental design was completely randomized, with 4 replicates.

The fall (March–June 2013) was considered the period of acclimation of the plants to the particular sward heights. From June 2013, at 28-day intervals, tiller density was evaluated by counting the live tillers within two 50 × 25 cm metal frames randomly located in each experimental unit. The data were grouped according to season.

Monthly, in each season of the year and on each plot, a sample of 50 tillers with average length similar to the sward height was chosen. These tillers were harvested at ground level and divided into live leaf blade, dead leaf blade and live stem (stem + leaf sheath). Parts of the leaf blade that did not show signs of senescence (green organ) were incorporated into the live leaf blade fraction. Any part of the leaf blade with a yellowish tone and or necrosis was considered dead leaf blade. Each sub-sample (live leaf blade, dead leaf blade and live

stem) from the 50 tillers was collected in a single paper bag, dried in an oven at 65 ºC for 72 h and then weighed together, in order to obtain the masses of the morphological components, and the mean weight of tillers was calculated. The masses of the sward morphological components were obtained by the following formula: FM = NT × TM, in which FM is the forage mass or the mass of the plant morphological component (kg DM/ha); NT is the number of tillers/10,000 m^2; and TM is the mass of the morphological component of the tiller (kg DM/tiller). The masses of the plant morphological components were expressed as percentages of the total forage mass.

After harvesting the tillers in each plot, 50 live leaf blades were also collected at random and placed in plastic bags. A small portion of the extremities of the leaf blades (apex and base) was cut and discarded, so as to generate an approximately rectangular leaf blade segment. The width and length of each segment were measured, and the leaf area of the leaf blade segments was calculated as the product of these dimensions. These segments were placed in a forced-ventilation oven at 65 ºC for 72 h and then weighed. With these data, the specific leaf area (cm^2 leaf blade/g dry leaf blade) was calculated. The leaf area index of each tiller was calculated as the product of the specific leaf area and the live leaf blade mass of the tiller. The pasture leaf area index, however, was obtained by multiplying the leaf area of the tiller by the number of tillers per ha.

For the data analysis, the results were grouped according to the season of the year (winter, spring and summer). Initially, the dataset was analyzed to check if it met the assumptions of the analysis of variance (normality and homogeneity). The data were then analyzed using the MIXED procedure (mixed models) of the SAS® (Statistical Analysis System) statistical package, version 9.2. The variance and covariance matrix was chosen using Akaike's Information Criterion (Wolfinger 1993). The treatment means were estimated using the "LSMEANS" option, and compared with each other by Student's t test at 5% probability.

Results

Tiller density in the palisadegrass was influenced only by season of the year (P = 0.035), with fewer tillers in winter than in spring and summer (Figure 3).

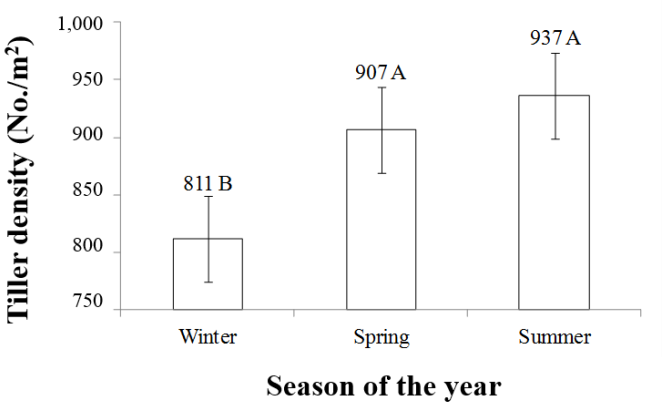

Figure 3. Effects of time of year on mean tiller density in palisadegrass swards.
Means followed by the same letter do not differ (P>0.05).

Mean tiller weight was influenced by defoliation strategy (P = 0.016) and by the interaction between this factor and season of the year (P = 0.024). In winter, tiller weight was greater in the sward maintained at 45 cm in fall/winter than in that at 15 cm, while in spring, the sward kept at 45 cm in fall/winter produced heavier tillers than that at 30 cm in fall/winter. However, by summer, mean tiller weight was similar for all defoliation strategies in fall/winter (Figure 4). The sward maintained at 45 cm in fall/winter produced similar sized tillers throughout (P>0.05), while the 30 cm sward in winter produced its smallest tillers in spring (P<0.05) and the 15 cm sward in winter produced progressively bigger tillers from winter to summer (P<0.05).

Forage mass in the marandu palisadegrass was influenced by season of the year (P = 0.013) and by the interaction between this factor and defoliation strategy (P = 0.009). In winter, forage mass was greatest in the sward maintained at 45 cm, intermediate in the sward maintained at 30 cm, and lowest in the sward maintained at 15 cm in fall/winter. In spring, forage mass in the sward maintained at 45 cm in fall/winter was greater than in that kept at 30 cm in fall/winter. However, forage mass in summer was independent of defoliation strategy in fall/winter (Figure 5).

The percentage of live leaf blades (PLLB) in the forage mass was influenced by both season of the year (P<0.0001) and defoliation strategy (P = 0.010). Overall PLLB followed the order: spring>summer>winter (Figure 6A), and was inversely related to height in winter (Figure 6B).

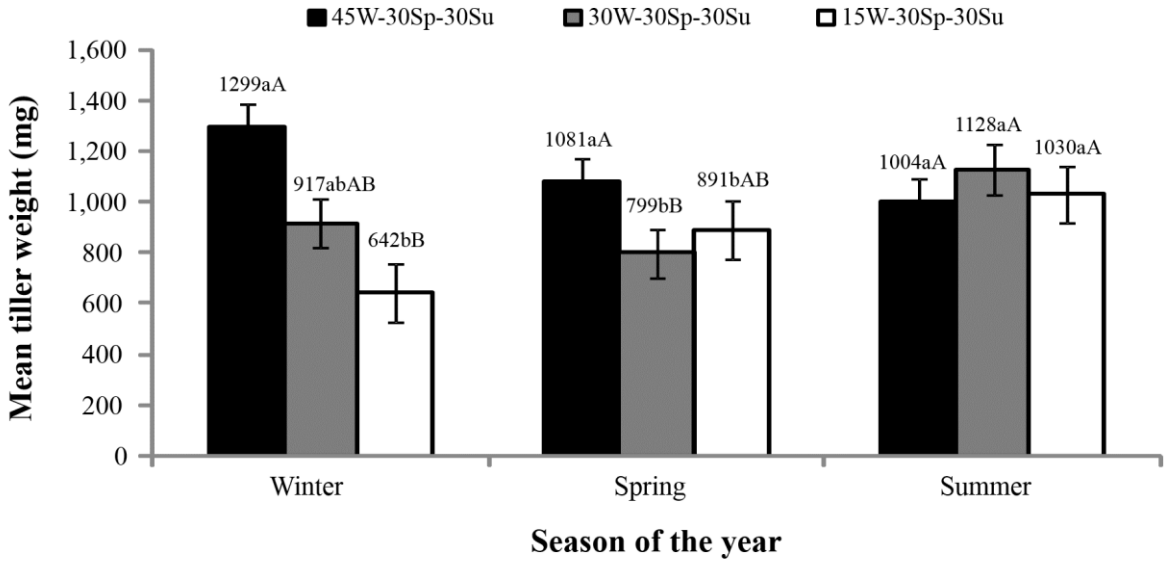

Figure 4. Effects of time of year and defoliation management on mean tiller weight in palisadegrass swards.
45W-30Sp-30Su: sward kept at 45 cm in winter and 30 cm in spring and summer; 30W-30Sp-30Su: sward kept at 30 cm in winter, spring and summer; and 15W-30Sp-30Su: sward kept at 15 cm in winter and 30 cm in spring and summer. Lowercase letters compare defoliation strategies within seasons of the year, and uppercase letters compare seasons of the year within each defoliation strategy. Means followed by the same letter do not differ (P>0.05).

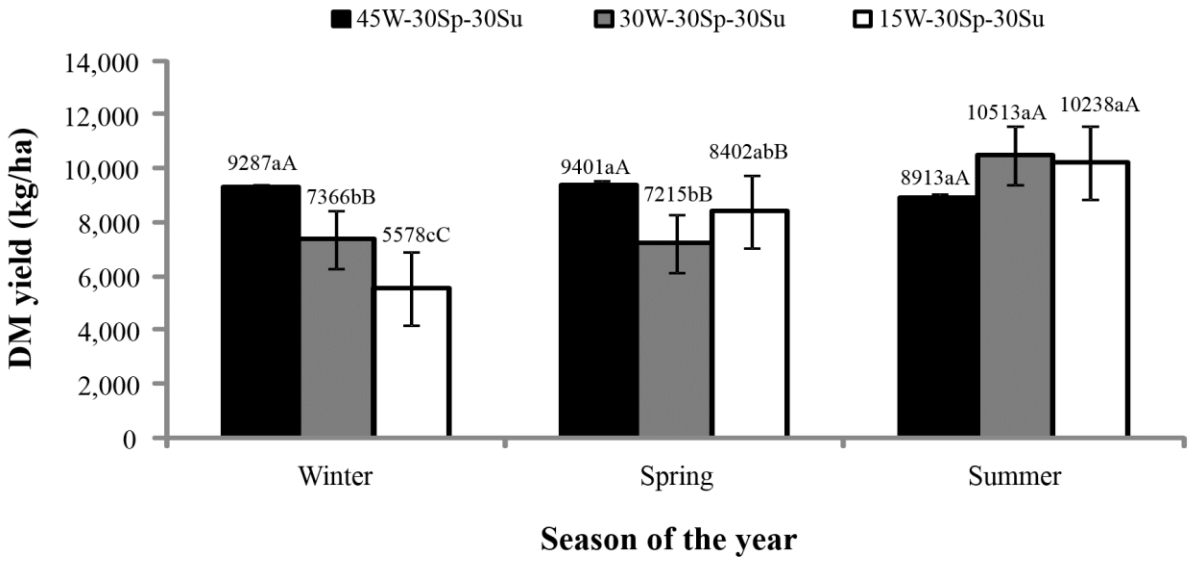

Figure 5. Effects of time of year and defoliation strategy on forage mass in palisadegrass swards.
45W-30Sp-30Su: sward kept at 45 cm in winter and 30 cm in spring and summer; 30W-30Sp-30Su: sward kept at 30 cm in winter, spring and summer; 15W-30Sp-30Su: sward kept at 15 cm in winter and 30 cm in spring and summer. Lowercase letters compare defoliation strategies within each season of the year, and uppercase letters compare seasons of the year within each defoliation strategy. Means followed by the same letter do not differ (P>0.05).

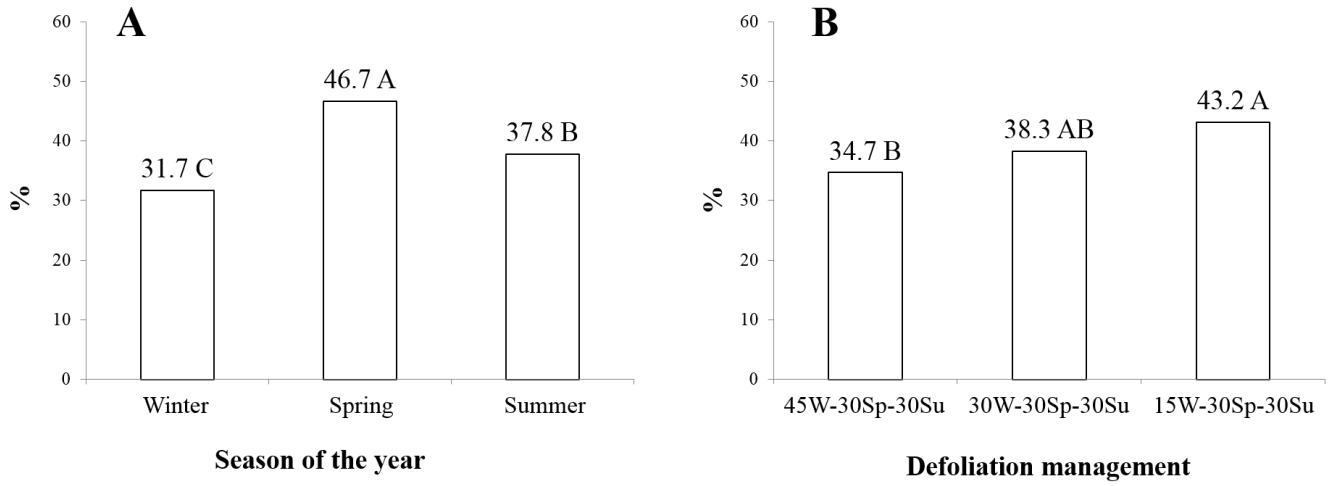

Figure 6. Percentage of live leaf blades in the forage mass of palisadegrass according to season of the year (A) and defoliation management strategy (B).
45W-30Sp-30Su: sward kept at 45 cm in winter and 30 cm in spring and summer; 30W-30Sp-30Su: sward kept at 30 cm in winter, spring and summer; and 15W-30Sp-30Su: sward kept at 15 cm in winter and 30 cm in spring and summer. In each graph, means followed by the same letter do not differ (P>0.05).

The percentage of stems (PS) was influenced by season of the year (P<0.0001), defoliation strategy (P = 0.0002) and the interaction of these factors (P = 0.007). In winter, the sward kept at 15 cm in fall and winter displayed a lower PS than those kept at 45 and 30 cm. During spring and summer, PS was independent of the sward height during the fall/winter period (Figure 7).

The percentage of dead material was not influenced by season of the year (P = 0.191), defoliation strategy (P = 0.575) or by the interaction of these factors (P = 0.305), averaging 23%.

Season of the year affected leaf area index (LAI) (P<0.0001), with a lower value in winter than in spring and summer (Figure 8).

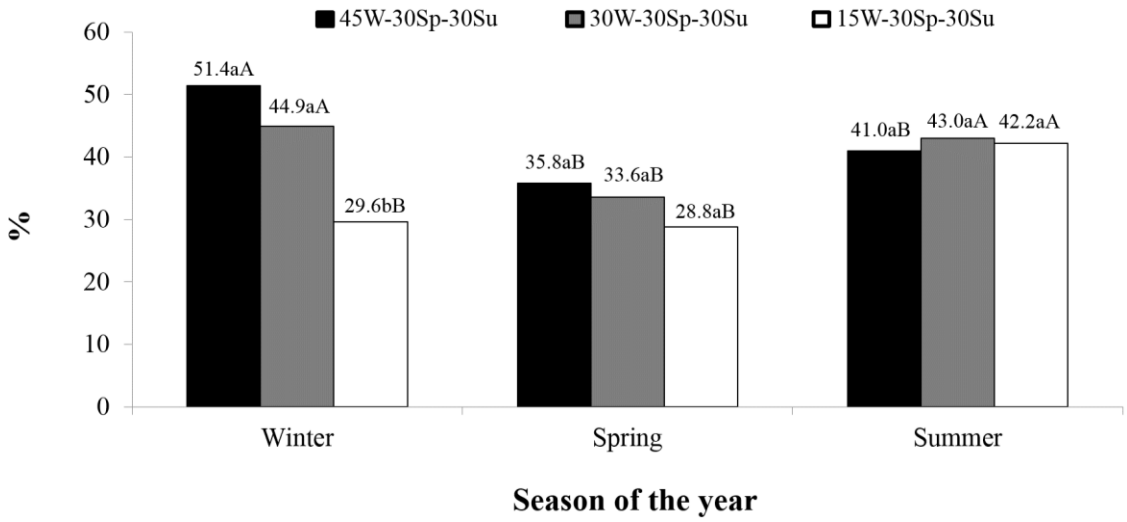

Figure 7. Percentage of live stems in the forage mass of palisadegrass according to time of year and defoliation strategy.
45W-30Sp-30Su: sward kept at 45 cm in winter and 30 cm in spring and summer; 30W-30Sp-30Su: sward kept at 30 cm in winter, spring and summer; 15W-30Sp-30Su: sward kept at 15 cm in winter and 30 cm in spring and summer. Lowercase letters compare defoliation strategies within each season of the year, and uppercase letters compare seasons of the year within each defoliation strategy. Means followed by the same letter do not differ (P>0.05).

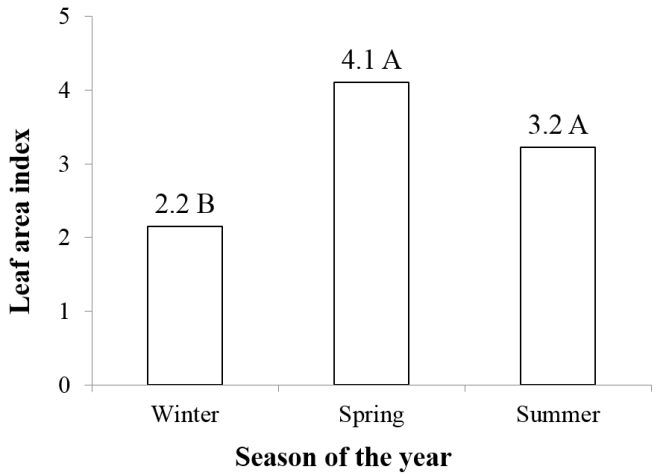

Figure 8. Leaf area index of palisadegrass according to season of the year.
Means followed by the same letter do not differ (P>0.05).

Discussion

This study has provided further valuable information on how the height, at which a marandu palisadegrass pasture is maintained in winter, spring and summer, affects the structure and composition of the pasture. This will be of use in explaining why pastures behave differently and have different levels of production under differing grazing strategies, especially in winter.

We hypothesized that keeping pasture short in winter would allow greater light penetration to the base of the sward, which might stimulate greater tiller development in spring as reported by Matthew et al. (2000) and Sbrissia et al. (2010). However, the defoliation strategy in fall/winter did not influence the number of tillers in the sward in spring and summer, which demonstrates the flexibility of marandu palisadegrass to variations in height in the fall and winter. During fall/winter tiller density was similar on all pastures regardless of sward height and increased following the onset of better conditions for growth in spring. Climatic conditions seemed to be the overriding factor. There was very little precipitation in June and no rain in July and August, with mean minimum temperature below 15 ºC (Figure 1). When the temperature is below 15 ºC, the lower threshold temperature for marandu palisadegrass (Mendonça and Rassini 2006), the rate of photosynthesis is impaired, which compromises tillering in the pasture. Sbrissia and Silva (2008), in a study with marandu palisadegrass under continuous stocking, also observed lower tiller density in winter than in spring and summer.

The adverse climatic conditions for plant growth in winter (Figure 1) might also have resulted in a lower percentage of live leaf in the forage mass in this season as compared with spring and summer (Figure 6A). Low temperatures and water deficit, typical of winter conditions, decrease leaf appearance and elongation rates (Lara and Pedreira 2011b), which would reduce the percentage of live leaves in the forage mass. A similar lower percentage of live leaves during winter was observed by Paula et al. (2012) in palisadegrass pastures continuously grazed at 15, 30 and 45 cm throughout the year.

The low tiller density in winter (Figure 3) was partially responsible for the low forage mass in swards maintained at 15 and 30 cm in fall/winter (Figure 5), as well as for the lower leaf area index (LAI) in all swards (Figure 8) in winter. Three structural traits could potentially change the sward LAI: tiller density, number of leaves per tiller and leaf blade size. Of these, tiller density has the greatest potential to change the LAI (Matthew et al. 2000). According to Fagundes et al. (2005), the low LAI of the pastures in winter would be a result of the lower number of live leaves per tiller and the shorter final length of the leaves at that time.

On the other hand, in spring and summer, the increase in temperature and occurrence of rainfall (Figure 1) provided favorable conditions for tillering, resulting in increased numbers of tillers (Figure 3), a typical response pattern observed in other research studies with forage grasses of the genus *Brachiaria* (Sbrissia and Silva 2008; Calvano et al. 2011). Lara and Pedreira (2011b) recorded twice as many tillers in summer as in winter in cvv. Marandu, Xaraés, Arapoty and Capiporã of *Urochloa brizantha* (syn. *Brachiaria brizantha*) and cv. Basilisk of *U. decumbens* (syn. *B. decumbens*).

The greater number of tillers in spring and summer (Figure 3) resulted in a higher LAI of the swards in these seasons (Figure 8). Since increased LAI increases interception of light by the sward (Pedreira et al. 2007), which is a premise for the occurrence of photosynthesis (Taiz and Zeiger 2012), this results in increased growth rate of the pasture.

As a consequence of the accumulated effects of rainfall, temperature and solar radiation as the seasons progressed, a larger number of tillers was expected in summer than in spring. This response pattern did not occur, possibly due to the lower than normal rainfall experienced in January and February 2014 (Figure 2). Additionally, the similar LAI in spring and summer (Figure 8) might also have contributed to tiller density remaining stable in these seasons (Figure 3). The LAI controls, in part, the amount of solar radiation that reaches the soil surface, such that a larger LAI is associated with

higher light interception by the sward (Giacomini et al. 2009) and in fact, with lower penetration of light to the soil. Since the amount of light received at the base of plants has a significant influence on degree of tillering (Martuscello et al. 2009), the constancy of LAI in spring and summer might have provided similar levels of luminosity close to the soil surface, resulting in similar numbers of basal buds developing into new tillers. The maintenance of marandu palisadegrass at a constant height in spring and summer also resulted in similar tiller weight in these seasons to the swards managed at 15 and 45 cm in fall/winter (Figure 4).

On swards maintained at 15 and 30 cm in fall/winter, the greater forage mass in summer than in the other seasons of the year (Figure 5) might have been a consequence of the onset of flowering of the palisadegrass in this season (Calvano et al. 2011). With flowering, the leaf:stem ratio in the plant is reduced (Santos et al. 2009), which explains the lower percentage of live leaves in the forage mass in summer as compared with spring (Figure 6A). Since stem is a denser organ than leaf (Pereira et al. 2010), its greater proportion in the sward should result in a larger forage mass. Furthermore, with flowering, compounds from root reserves are translocated to the aerial parts of the forage plant (Silva et al. 2015), which also contributes to increasing the sward forage mass.

It should be noted that we might have overestimated the forage mass values (Figure 5) in this study. To obtain this response variable, we multiplied average tiller weight by the number of tillers. It is possible that some young tillers, shorter than the average sward height, were counted along with the taller ones. However, to determine mean tiller weight, we harvested only those with height similar to the sward height, so the average tiller weight would have been overestimated, with an equal effect on forage mass.

Considering that the tiller is the basic growth unit of forage grasses (Hodgson 1990), the stability of tiller density in the swards subjected to variable defoliation regimes in fall and winter indicates that their perenniality was not compromised and that the growth potential of the pasture was probably not impaired.

In winter, variations in mean weight of tillers (Figure 4) and forage mass (Figure 5) were a consequence of the modification of the sward height in this season. When the sward heights were similar (30 cm) in all swards, differences in tiller weight and forage mass between the swards declined and had disappeared by summer (Figure 4). Moreover, in the sward kept at 45 cm in fall and winter, there might have been more competition for light among the tillers (Sbrissia et al. 2010), which can lead to greater stem elongation and consequently a greater tiller weight (Figure 4), as well as a higher percentage of live stems in the forage mass (Figure 7). This high relative contribution of live stem in winter resulted in a reduction in the percentage of live leaves during the entire experimental period in the sward kept at 45 cm in fall/winter as compared with that kept at 15 cm (Figure 6B). Nevertheless, in spring, when all swards were kept at the same height (30 cm), the highest one (45 cm) in fall and winter continued to present a greater tiller weight. Thus, a residual effect of the management employed in fall and winter was detected in the subsequent season. Contrastingly, maintaining the sward lower (15 cm) in fall and winter resulted in lower tiller weight in winter (Figure 4), as well as a lower percentage of live stems in the forage mass during winter (Figure 7). These results allow us to infer that the structure of the marandu palisadegrass kept shorter in winter would be more favorable for forage intake by grazing animals.

The effect of a particular defoliation strategy in a particular season of the year on tiller growth in the following season is partially due to the phenotypic plasticity of the forage plant, i.e. to the change in the morphogenetic and structural traits of the plant in response to environmental variations, including the defoliation environment (Silva and Nascimento Júnior 2007). This is a gradual process, and, therefore, does not occur in the short term; when the defoliation management in a sward is changed, there is a carry-over effect and effects of the previous management are displayed in the subsequent periods.

Conclusions

This study has shown that: 1) *Urochloa brizantha* (syn. *Brachiaria brizantha*) cv. Marandu (marandu palisadegrass) shows limiting structural traits in winter as compared with spring and summer; 2) both pasture height and season affect pasture structure of Marandu; and 3) managing Marandu at 15 cm in fall and winter and 30 cm in spring and summer will result in a leafier pasture with lower percentage stems than keeping it at 30 or 45 cm in winter.

Grazing studies seem warranted to determine whether the effects demonstrated in this experiment hold under grazing and how varying pasture height in different seasons compares with maintaining a fixed grazing height. Furthermore, how the sward height variation affects pasture yield and quality and translates into animal performance should be monitored before recommendations should be made.

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Research Paper

Evaluation and strategies of tolerance to water stress in *Paspalum* germplasm

Evaluación y estrategias de tolerancia a estrés hídrico en germoplasma de Paspalum

CRISTIANA DE G. PEZZOPANE[1], ARTHUR G. LIMA[2], PEDRO G. DA CRUZ[3], TATIANE BELONI[4], ALESSANDRA P. FÁVERO[5] AND PATRÍCIA M. SANTOS[5]

[1]*Centro Universitário Central Paulista - UNICEP, São Carlos, SP, Brazil* www.unicep.edu.br
[2]*Universidade Estadual Paulista (UNESP), Rio Claro, SP, Brazil* www.unesp.br
[3]*Embrapa Rondônia, Porto Velho, RO, Brazil* www.embrapa.br/rondonia
[4]*Universidade Federal de São Carlos, Araras, SP, Brazil* www.ufscar.br
[5]*Embrapa Pecuária Sudeste, São Carlos, SP, Brazil* www.embrapa.br/pecuaria-sudeste

Abstract

The evaluation of genetic resources in germplasm banks of *Paspalum* can contribute to their use in breeding programs and for advanced research in biotechnology. This study evaluated the tolerance of 11 *Paspalum* accessions to abiotic stress caused by soil water deficit in a greenhouse experiment at Embrapa Pecuária Sudeste, São Carlos, state of São Paulo, Brazil. The variables analyzed were: dry biomass of green matter, dead matter and roots; leaf area; leaf water potential; number of days to lose leaf turgor (wilting); soil moisture at wilting; and number of tillers per pot. The results showed high genetic variability for all traits, not only among species but also within species, and also reflected the existence of different strategies of response and potential adaptation to water deficit events. For breeding programs, when the aim is to produce materials better adapted to the occurrence of prolonged drought, 5 accessions from this group seem to have good potential: *P. malacophyllum* BGP 289, *P. quarinii* BGP 229, *P. regnellii* BGP 112, *P. conspersum* BGP 402 and *P. urvillei* x *P. dilatatum* BGP 238. Conversely, when the goal is to select materials for short-term water stress conditions, 6 accessions stand out: *P. atratum* BGP 308, *P. regnellii* BGP 215, 248 and 397, *P. dilatatum* BGP 234 and *P. malacophyllum* BGP 293.

Keywords*:* Abiotic stress, genotypes, germplasm bank, water deficit.

Resumen

La evaluación de recursos genéticos en bancos de germoplasma de *Paspalum* constituye una gran ayuda en programas de mejoramiento genético y de investigación avanzada en biotecnología. En un experimento en macetas en Embrapa Pecuária Sudeste, São Carlos, estado de São Paulo, Brasil, se evaluó la tolerancia de 11 accesiones de varias especies de *Paspalum* al estrés abiótico causado por el déficit hídrico en el suelo. Las variables analizadas fueron: biomasa seca de la materia verde, materia muerta y raíces; área foliar; potencial hídrico foliar; número de días hasta la pérdida de la turgencia foliar (marchitamiento); humedad del suelo al momento del marchitamiento de las plantas; y número de brotes por planta. Los resultados mostraron tanto una alta variabilidad genética para todos los parámetros, no solo entre las especies, sino también dentro de las especies, como la existencia de diferentes estrategias de respuesta y potencial adaptación a eventos de déficit hídrico. Para los programas de fitomejoramiento, cuando el objetivo es producir materiales mejor adaptados a la sequía

Correspondence: P.M. Santos, Embrapa Pecuária Sudeste, Rodovia Washington Luiz, km 234 s/nº, Fazenda Canchim, São Carlos CEP 13560-970, SP, Brazil.
Email: patricia.santos@embrapa.br

prolongada, las accesiones con mayor potencial fueron: *P. malacophyllum* BGP 289, *P. quarinii* BGP 229, *P. regnellii* BGP 112, *P. conspersum BGP 402* y *P. urvillei x P. dilatatum BGP 238.* Por el contrario, cuando el objetivo es seleccionar materiales para condiciones de estrés hídrico de corta duración, se destacan las accesiones: *P. atratum* BGP 308, *P. regnellii* BGP 215, 248 y 397, *P. dilatatum* BGP 234 y *P. malacophyllum* BGP 293.

Palabras clave*:* Banco de germoplasma, déficit hídrico, estrés abiótico, genotipos.

Introduction

According to predictions from the fifth assessment report (AR5) of the Intergovernmental Panel on Climate Change (IPCC 2013) global temperature may increase by up to 4.8 °C by 2100, with increased variability and occurrence of extreme events. In Brazil, a regionalized projection suggests trends for increasing maximum and minimum extremes of temperature and high spatial variability for precipitation when analyzing different emission scenarios (Marengo et al. 2009). The challenges presented by the effects of climate change scenarios on agriculture are adaptation of production systems and mitigation of greenhouse gas emissions.

Plant breeding programs are designed to incorporate relevant traits, such as high dry matter yield, high nutritional value and increased resistance to or tolerance of biotic and abiotic factors into elite genetic resources, with the aim of releasing cultivars better suited to the conditions of use. Knowledge of these characteristics in genotypes conserved in gene banks provides fundamental information that allows the selection of appropriate accessions for use both in breeding programs and in biotechnology research.

The characterization of accessions conserved in germplasm banks is essential to ensure their efficient use for different purposes. For example, the study of responses of forage plants to stress caused by water deficit is of utmost importance, since moisture restriction can greatly reduce forage production and persistence of pasture (Guenni et al. 2002; Melo et al. 2003; Araújo et al. 2012; Volaire et al. 2014).

The genus *Paspalum* belongs to the family Poaceae and includes several grasses with forage potential. More than 330 species have been identified (Zuloaga and Morrone 2003), occurring widely in South America (Quarín et al. 1997), including the Pampas, where the grass is grazed by cattle, in particular. Nevertheless, its use in cultivated pastures is still low in Brazil, while in other countries, like the USA, many species of *Paspalum* that occur in Brazil, such as *Paspalum notatum*, *P. dilatatum*, *P. plicatulum* and *P. guenoarum*, are used successfully as forages (Acuña et al. 2011). The number of accessions and species conserved has been growing in recent years, and the Germplasm Bank (GB) of Embrapa Pecuária Sudeste contains more than 340 accessions of 49 different species of *Paspalum*, most belonging to the informal group Plicatula.

Batista and Godoy (2000) evaluated the dry matter (DM) production of 217 accessions of *Paspalum* from the *Paspalum* GB of Embrapa Pecuária Sudeste, using *B. decumbens* and *Andropogon gayanus* cv. Baetí as controls. While 58 accessions (27%) showed DM production equal to or higher than the cultivars used as controls, the selection and development of new cultivars should also take into account the plasticity in the response of the genotype to specific conditions.

Some species of *Paspalum*, such as *P. vaginatum* (Shahba et al. 2014) and *P. notatum* (Acuña et al. 2010), have characteristics of interest in relation to drought tolerance. While some species have high forage value, no controlled experiments checking the tolerance to water stress of species like *P. atratum*, *P. conspersum*, *P. dilatatum*, *P. malacophyllum*, *P. quarinii* and *P. regnellii* have been conducted. According to Zuloaga and Morrone (2003), *P. malacophyllum* is found from Mexico to northern Argentina, Paraguay, Brazil and Bolivia, at elevations from sea level to 3,000 m. It is found in agricultural fields, roadsides and woodlands. In turn, *P. regnellii* is distributed from the center to the south of Brazil, northeastern Argentina and eastern Paraguay. Both *P. conspersum* and *P. regnellii* are recorded in forest edges or disturbed sites, in heavy clay soils which are subject to waterlogging. Accession BGP 238 used in this study is a natural hybrid derived from a cross between *P. urvillei* and *P. dilatatum*. Since *P. urvillei* has sexual reproductive behavior, it is common to observe hybrids in populations where these species coexist.

This study evaluated the tolerance to soil moisture stress in some germplasm accessions of *Paspalum*, aiming at identifying genes for drought-tolerance and transferring them to other plant families in future breeding programs.

Materials and Methods

The experiment was conducted in a greenhouse, at Embrapa Pecuária Sudeste, in São Carlos, state of São Paulo (21°57' S, 47°50' W; 860 m asl). We evaluated 11 accessions of 7 distinct species of *Paspalum* belonging to 5 different informal groups. Seeds were obtained from the germplasm bank of Embrapa Pecuária Sudeste (Table 1).

The accessions were chosen after a previous study identified genotypes more suitable for forage production. Among these genotypes, 2 belonged to the informal botanical group Dilatata (BGP 234, *Paspalum dilatatum* Poir. biotype Uruguaiana and BGP 238, a natural hybrid between *P. urvillei* Steud. and *P. dilatatum*), 2 to the group Malacophylla (BGP 289 and BGP 293, *Paspalum malacophyllum* Trin.), 1 to the group Plicatula (BGP 308, *Paspalum atratum* Swallen), 1 to the group Quadrifaria (BGP 229, *Paspalum quarinii* Mez) and 5 to the group Virgata (BGP 402, *Paspalum conspersum* Schrader and BGP 112, 215, 248 and 397, *Paspalum regnellii* Mez).

Seedlings were grown on trays filled with organic substrate Plantmax® and transplanted to pots at the 3-leaf stage with 2 plants per pot. Pots with capacity of 8.5 L were filled with 7 kg sieved soil, with the following chemical and physical characteristics: pH_{CaCl2} 5.4, OM 25 g/dm^3, P_{resin} 6 mg/dm^3, SO_4-S 21 mg/dm^3, K 1.3 mmolc/dm^3, Ca 26 mmolc/dm^3, Mg 14 mmolc/dm^3, H+Al 24 mmolc/dm^3, Al 0 mmolc/dm^3, CEC 66 mmolc/dm^3, base saturation 63%, sand 417 g/kg, silt 253 g/kg and clay 330 g/kg.

Each pot was fertilized with 1.07 g N as urea, 1.4 g P as simple superphosphate, 0.53 g K as potassium chloride, following the recommendations of Malavolta (1980) for experiments in pots.

The experimental layout was an 11 (accessions) x 2 (water conditions) x 3 (replications) factorial in a complete randomized block design. The 2 watering treatments were unwatered and irrigated regularly. When the plants had at least 3 tillers, irrigation of pots in the treatment with water stress was suspended, while irrigation of pots in the control treatment continued with a daily amount of water equivalent to the air evaporative demand as measured by several Piche evaporimeters located at random in the greenhouse.

Plants of particular accessions in the unwatered treatment were harvested when the first leaf blade displayed wilting in the predawn period, so different accessions were collected on different days. Concomitantly, in the same block, we collected a pot with 2 plants of the same accession from the control treatment. Therefore, 2 pots were collected on each occasion for each accession, 1 from the stressed treatment showing symptoms of wilting and another with well-watered plants from the control.

Table 1. Identification codes (BGP and collection), species names, collection sites and informal botanical groups of *Paspalum* accessions evaluated in this study.

Site code (BGP)	Collection code	Species	Collection site	Botanical group
112	VDBdSv 10073	*P. regnellii* Mez	Praia Grande - Santa Catarina - Brazil	Virgata
215	Lr 2	*P. regnellii* Mez	Itirapina - São Paulo - Brazil	Virgata
229	VTsDp 14220	*P. quarinii* Morrone & Zuloaga	São Miguel das Missões - Rio Grande do Sul - Brazil	Quadrifaria
234	VTsDp 14251	*P. dilatatum* Poir. biotipo Uruguaiana	Uruguaiana - Rio Grande do Sul - Brazil	Dilatata
238	VTsZi 14285	*P. urvillei* x *P. dilatatum*	Xangri-lá - Rio Grande do Sul - Brazil	Dilatata
248	VTsRcRm 14424	*P. regnellii* Mez	Capão Alto - Santa Catarina - Brazil	Virgata
289	VRcMmSv 14582	*P. malacophyllum* Trin.	Aral Moreira - Mato Grosso do Sul - Brazil	Malacophylla
293	VRcMmSv 14606	*P. malacophyllum* Trin.	Japorã - Mato Grosso do Sul - Brazil	Malacophylla
308	VRcMmSv 14525	*P. atratum* Swallen	Terenos - Mato Grosso do Sul - Brazil	Plicatula
397	-	*P. regnellii* Mez	unknown origin	Virgata
402	-	*P. conspersum* Schrader	unknown origin	Virgata

Collectors: Bd = I.I. Boldrini; D = M. Dall´Agnol; Dp = Dario Palmieri; Lr = L.A.R. Batista; Mm = M.D. Moraes; Rc = Regina Célia de Oliveira; Rm = R. Miz; Sv = Glocimar P. da Silva; Ts = T. Souza-Chies; V = José Francisco M. Valls; Zi = F. Zilio.

When the plants were harvested, the following parameters were measured: leaf water potential (MPa), determined in the last expanded leaf, in the pre-morning period, with the aid of a psychrometer (Wescor micro-meter Psypro model and sample chamber model C52), where a microvoltmeter is connected to chambers where, after being calibrated with NaCl standard solution, 25 mm diameter leaf discs are placed to be measured; green biomass; dead biomass; and root biomass determined after each of the parts was packed in paper bags and dried in a circulation oven at 65 °C until reaching constant weight; total leaf area, measured using the LI-COR leaf area integrator, model LI-3100; days to turgor loss (wilting); soil moisture at wilting determined by weighing wet soil and then drying to constant oven weight at 105 °C; and number of tillers per pot.

At the completion of the harvests, data were analyzed using the PAST software (Hammer et al. 2001), using principal component analysis. This analysis is based on grouping assessments to determine the genetic differences (Cruz 2006).

Results

There were no significant interactions among genotypes and watering treatments for any of the variables. There was an increase ($P<0.0001$) in dry biomass of dead material of shoots (Figure 1A) in all studied accessions under water restriction, especially for *P. regnellii* BGP 215, which showed 62% more dead material than the control. There was no significant difference among genotypes ($P = 0.09$).

Despite the lack of significant differences in green biomass between accessions ($P = 0.066$), there was wide variation among accessions in response to drying ($P<0.0001$). Dry biomass of green matter of accessions *P. malacophyllum* BGP 293 and *P. regnellii* BGP 248 was reduced by only 7 and 8%, respectively, as a result of moisture stress, while accessions *P. regnellii* BGP 215 and BGP 112 showed decreases of 36 and 40% (Figure 1B).

Root biomass varied among accessions ($P = 0.0004$) as did responses to drying (Figure 1C). Under irrigated conditions, accessions *P. urvillei* x *P. dilatatum* BGP 238 and *P. conspersum* BGP 402 produced the highest root yields, while *P. malacophyllum* BGP 289 and BGP 283 produced the lowest. Drying out under moisture stress produced quite variable responses in root biomass, with a range from an increase of 34% in root biomass for *P. regnellii* BGP 215 to a decrease of 42% for accession *P. conspersum* BGP 402. For this variable, there was no significant difference among treatments ($P = 0.3099$).

Moisture stress caused a reduction ($P<0.0001$) in leaf area (Figure 1D) in all accessions, reaching 85% in *P. regnellii* BGP 215, with no significant differences among genotypes ($P = 0.43$).

Drying caused significant differences ($P<0.0001$) in leaf water potential values in all accessions (Figure 1E), with no significant differences among accessions ($P = 0.33$). In contrast, the number of tillers per pot (Figure 1F) was affected differently by drying for different genotypes ($P<0.0001$). Responses ranged from an increase in the number of tillers under water stress conditions of 19% for *P. quarinii* BGP 229 to a decrease of 34% in tiller numbers for *P. malacophyllum* BGP 293. There was considerable variation among accessions in time to wilting following the cessation of watering, with a range from 9 days for *P. regnellii* BGP 215 to 22 days for *P. malacophyllum* BGP 289 (Figure 1G) ($P<0.0001$). However, most accessions wilted between 17 and 22 days after watering ceased. At the point of wilting for all accessions, soil moisture levels were about 12% (Figure 1H).

Principal Component Analysis (PCA) was performed to group accessions according to the variables that had most influence on their responses. Figure 2A illustrates the PCA comparing the accessions under both drought and well-watered conditions, and considering all the variables recorded in this study. The cumulative variance of the first 2 components was 73.9%. The x-axis was characterized by leaf area and the y-axis by the number of tillers. Two distinct groups were formed, one consisting of accessions under water restriction (to the left) and the other composed of non-stressed accessions (to the right), indicating differences between the groups; water restriction was critical in changing the main characteristics of plants.

The principal component analysis run only with accessions under water stress, indicated that the variables that explained best the distribution of genotypes were soil moisture on the x-axis, and dry biomass of roots on the y-axis (Figure 2B). The cumulative variance for the 2 axes was 68.4%. In Figure 2B, accessions were grouped according to certain characteristics in main number of tillers, wilting days, leaf area, water potential and soil moisture. Accession *P. regnellii* BGP 215 stood out among other accessions by the higher dry biomass of roots, *P. malacophyllum* BGP 293 by the larger leaf area, *P. regnellii* BGP 248 by the higher dry biomass of green matter and soil moisture and accession *P. urvillei* x *P. dilatatum* BGP 238 by dry biomass of dead matter and roots. The variable water potential grouped the accessions *P. dilatatum* BGP 234, *P. regnellii* BGP 397 and *P. atratum* BGP 308, while number of tillers and days to wilting determined the group formed by *P. malacophyllum* BGP 289, *P. quarinii* BGP 229, *P. conspersum* BGP 402 and *P. regnellii* BGP 112.

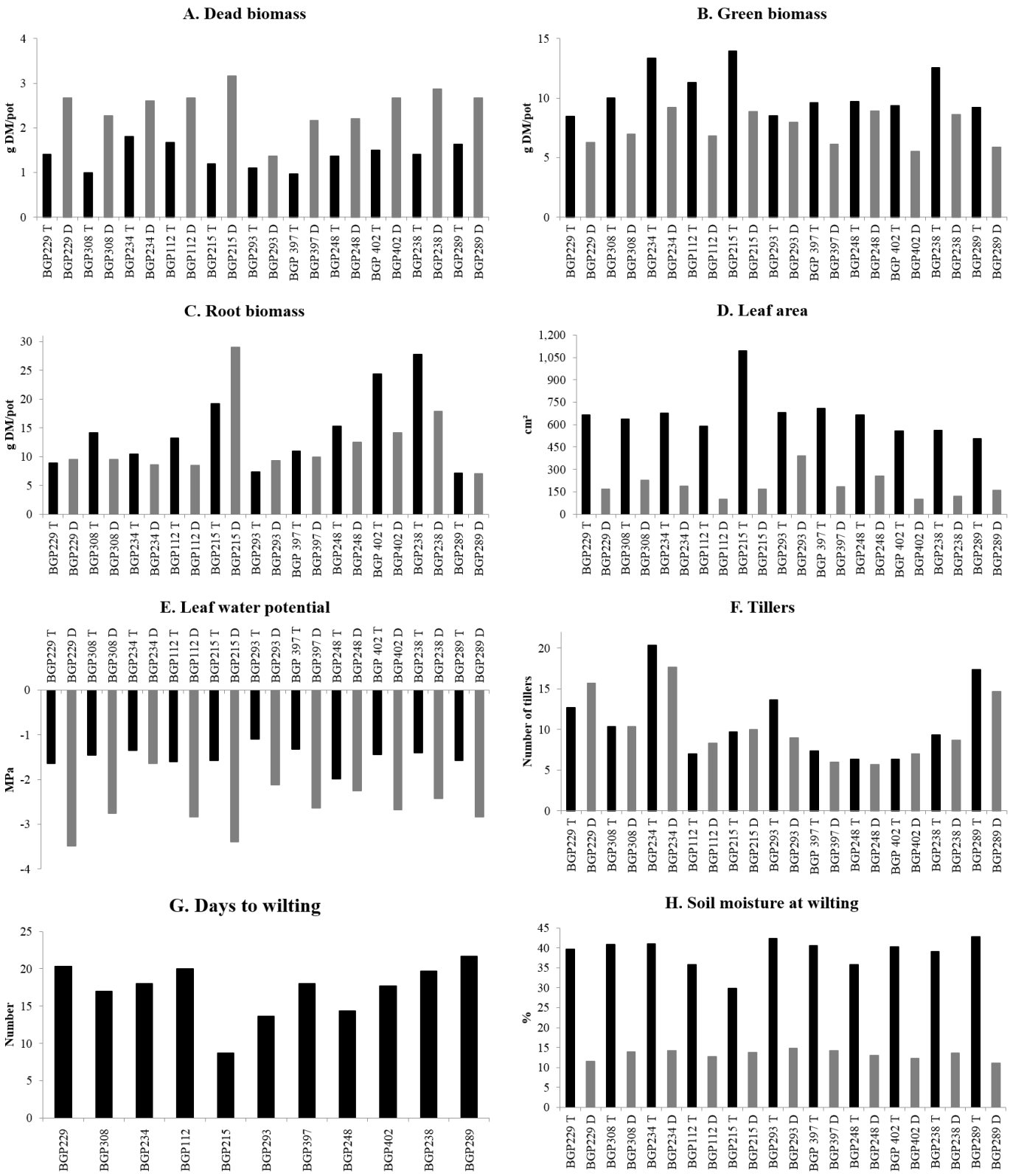

Figure 1. Mean values of the measured variables for the *Paspalum* accessions used in this study. Black bars indicate non-stressed plants and grey bars indicate plants under water stress. **A.** Dead biomass (g DM/pot); **B.** Green biomass (g DM/pot); **C.** Root biomass (g DM/pot); **D.** Leaf area (cm^2); **E.** Leaf water potential (MPa); **F.** Number of tillers; **G.** Days to wilting; **H.** Soil moisture at wilting (%).

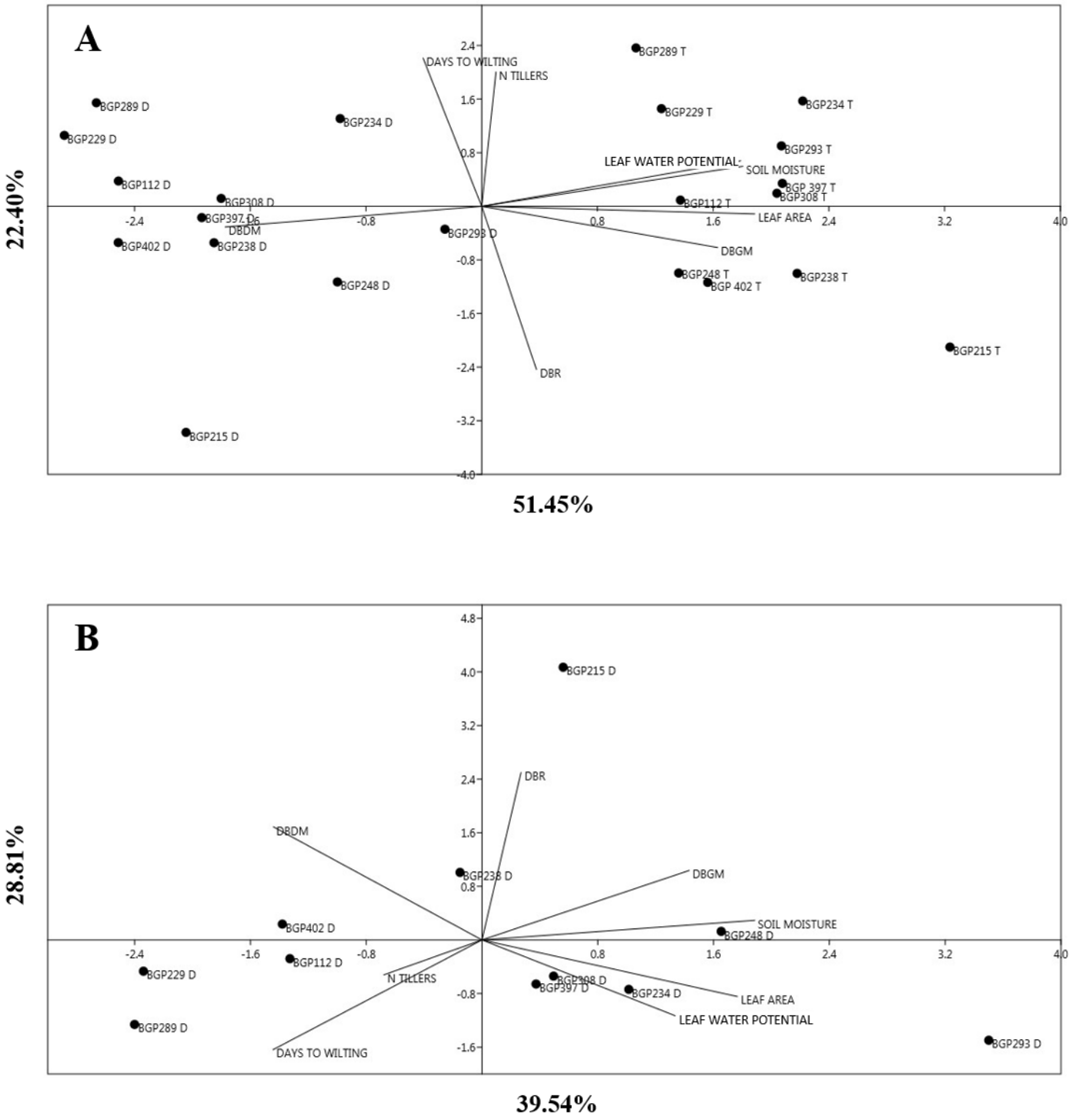

Figure 2. **A** – Principal component analysis of accessions of *Paspalum* subjected (represented by letter D) or not (represented by letter T) to water stress. **B** – Principal component analysis with only accessions of *Paspalum* subjected to water stress. Analyses were made considering all variables evaluated (dry biomass of: dead matter - DBDM, green matter - DBGM and roots - DBR; leaf area; leaf water potential; number of tillers; soil moisture at wilting; and days to wilting).

Discussion

This study has provided interesting data on the comparative tolerances of a range of *Paspalum* accessions to low soil moisture situations. As such it provides indications of which accessions might be appropriate for inclusion in breeding programs with specific aims. However, more real differences between accessions might exist than appear from our results. The number of significant differences obtained between accessions may have been limited by the low numbers of plants examined for each treatment combination as large differences in treatment means in some cases proved to be non-significant (P>0.05). If larger numbers of plants had been included per treatment, more differences might have been recorded as significant.

Mechanisms of tolerance to stress by water deficit in Paspalum

Physiological responses of plants to drought conditions are considered primary characteristics because they are rapidly triggered in the presence of stress (Sherrard et al. 2009). According to Garcez Neto and Gobbi (2013), all effects caused by water stress lead to production loss and possible adjustments should be achieved for ecological sustainability and productivity of forage grasses grown in environments with eventual or permanent water restrictions.

The increase in dry biomass of dead matter in unwatered treatments was not surprising as death of plant parts as a result of moisture stress is well recognized (Figure 1A, 1B and 1D). Mattos et al. (2005) studied 4 species of *Urochloa* subjected to low water availability and observed a decrease in leaf elongation rate and increased senescence of leaf blades for all species.

Some species lose leaves as drought is intensified, which is known as an avoidance mechanism. This strategy allows water savings, because the smaller leaf area reduces transpiration of water by the plant, favoring the maintenance of turgor, plus some photosynthetic activity and carbon gain for a longer time period (Givnish 1987; Lamont et al. 2002; Escudero et al. 2008). Reduction in water loss and protection of meristems can also ensure regrowth and survival of plants when drought conditions occur, and thus represent a strategy that some plants use to tolerate drought (Volaire and Lelièvre 2001; Munne-Bosch and Alegre 2004; Volaire et al. 2014).

Besides decreasing the production of plant biomass, drought can change the photoassimilate partitioning in plants. Studies with *Urochloa* and *Paspalum* subjected to water deficit demonstrated a greater allocation of photoassimilates to the root system enabling the exploitation of a larger volume of soil for water absorption, maintaining hydration levels in the tissue for longer (Baruch 1994; Casola et al. 1998). The results of biomass partitioning showed that some accessions invested in root biomass to a greater extent than others.

The decrease in leaf water potential with decreasing soil moisture (Figure 1E) was observed previously by Mattos et al. (2005) in *Urochloa* species, where the leaf water potential reduced by a factor of 8 in *U. mutica* and by a factor of 4 in the other species studied, *U. humidicola*, *U. decumbens* and *U. brizantha*. The reduction in leaf water potential is the consequence of losing water from stomata, which is not compensated for by water extraction from the soil. Osmotic adjustment is considered as a physiological mechanism to maintain turgor at low leaf water potentials. The decrease in the osmotic potential, due to the accumulation of sugars, organic acids and ions in the cytosol, allows the plant to continue to absorb and translocate water to the shoot under conditions of lower water availability (Bray 1997).

In this experiment, the effect of water restriction on number of tillers varied according to genotype (Figure 1F). This result suggests a variation among *Paspalum* genotypes in relation to the capacity to protect meristematic tissues from dehydration during periods of water restriction. The reduction in the number of tillers is related to lower activity of cell division in the meristematic zone, responsible for leaf initiation (Skinner and Nelson 1995), which also influences the activation of axillary buds in the formation of new tillers, prioritizing existing tillers (Garcez Neto and Gobbi 2013).

The ability of accessions *P. quarinii* BGP 229, *P. regnellii* BGP 112, *P. urvillei* x *P. dilatatum* BGP 238 and *P. malacophyllum* BGP 289 to delay dehydration longer than others would have been partially due to the reduction in leaf area and water potential, which would have led to energy savings.

Grouping and classification of Paspalum *genotypes according to tolerance to drought*

Among the accessions there is genetic diversity, as seen in the PCA. However, there was no grouping per species or per botanical group, which reflects the high genetic variability that may be present not only among species but also within each species of this genus. Our results suggest that these *Paspalum* accessions can be grouped according to response strategies to stress caused by water restriction. The first group comprised of *P. regnellii* BGP 215, 248

and 397, *P. malacophyllum* BGP 293, *P. dilatatum* BGP 234 and *P. atratum* BGP 308 showed the best values in variables of development; the second group made up of accessions *P. malacophyllum* BGP 289, *P. quarinii* BGP 229, *P. regnellii* BGP 112 and *P. conspersum* BGP 402 were characterized by the greatest number of days to lose turgor in the predawn period and genotype *P. urvillei* x *P. dilatatum* BGP 238 was not grouped with the others, forming a specific group. Apparently, there was no correlation between collecting site and strategy used by plants to overcome water deficit.

Accessions that stood out in terms of development variables can be further divided into 3 subgroups: *P. regnellii* BGP 215 (group 1); *P. malacophyllum* BGP 293 (group 2); *P. atratum* BGP 308, *P. regnellii* BGP 397, *P. dilatatum* BGP 234 and *P. regnellii* BGP 248 (group 3).

According to PCA and the mean values of variables represented in it, accession *P. regnellii* BGP 215 was the first to wilt, despite increased root system biomass and reductions in leaf area. This result suggests that this accession is able to maintain productivity under mild water stress by expanding the root system and exploration of a greater volume of soil, but is not tolerant of severe drought. Pérez-Ramos et al. (2013) found that accessions with a more aggressive survival strategy based on increased acquisition of resources, when in deep soils, reduce the rate of dehydration of the meristem by deepening the root system and increasing the absorption of water.

Santos et al. (2013) studied forage plants of the genus *Urochlo*a under water stress and also observed different behavior among the cultivars, which presented different strategies of survival. *Urochloa brizantha* cv. Piatã decreased vegetative development, consequently reducing production, indicating a conservative strategy, lowering metabolism for its survival; *U. brizantha* cv. Marandu presented a more aggressive strategy, which did not reduce productive development, but maintained high productivity, which, according to the authors, promoted advantages under mild stress, but under conditions of severe stress, survival may be compromised because there was no reduction of metabolism.

Accession *P. malacophyllum* BGP 293 presented a distinct response (Figure 1); even though wilted at 14 days, it maintained a relatively high leaf area and little biomass of dead matter at the time of harvest (Figures 1A and 1D). The high leaf water potential (Figure 1E) indicates that osmotic adjustment is not among the main mechanisms of tolerance to water stress of this genotype, because early stomatal closure helps control water loss. On the other hand, little change in root biomass (Figure 1C), along with the other observed results, suggests that it may use stomatal control mechanisms to reduce water loss and delay tissue dehydration.

Accession *P. urvillei* x *P. dilatatum* BGP 238 behaved similarly to accession *P. regnellii* BGP 215 because it also has high values of biomass of dead matter and roots but, unlike BGP 215, the moisture stress had a negative effect on root biomass, with 35% reduction compared with the control (Figure 1C). Time to wilting of BGP 238 was relatively long, being similar to that of genotypes that were grouped by this characteristic (*P. regnellii* BGP 289, *P. quarinii* BGP 229, *P. regnellii* BGP 112 and *P. conspersum* BGP 402; Figures 2 and 1G), but the biomass of green matter was higher, suggesting that this accession has good potential for use under conditions where there is risk of severe drought (Figures 2 and 1B).

Accessions *P. malacophyllum* BGP 289, *P. quarinii* BGP 229, *P. regnellii* BGP 112 and *P. conspersum* BGP 402, which were characterized by the greatest number of days to wilting (Figures 2 and 1G), presented a more conservative strategy of use of natural resources, which provided high tolerance to conditions of severe water stress. More conservative genotypes in the use of resources have smaller leaf area, maintain turgor and activate osmoregulation mechanisms at the leaf blade level during moderate drought, and under reduced water availability, they prioritize meristems and tips of the roots, ensuring the recovery of plants after the elimination of stress (Volaire and Lelièvre 2001; Volaire et al. 2014). This is because meristems exhibit higher osmotic adjustment than other tissues during drought (Munns et al. 1979; Matsuda and Riazi 1981; West et al. 1990) and therefore have potential for regeneration when the aerial part of the plant is dead (Van Peer et al. 2004).

The *Paspalum* accessions evaluated in this study can be categorized according to their strategies in response to abiotic stress due to imposed water restriction. Knowledge of these survival strategies, which may focus on reduced development or maintenance of productivity, will contribute to the creation and selection of genotypes for use in the *Paspalum* breeding program. This assumes greater importance as more severe global climate change scenarios are forecast.

Under the conditions of this experiment, where the evaluation assessment was interrupted when the genotype's shoots wilted in the predawn period and no recovery period was allowed, it is suggested that accessions be separated into 2 groups so that they can be used in breeding programs aimed at tolerance to drought.

For environments subjected to the occurrence of prolonged droughts, the most promising candidates appear to be: *P. malacophyllum* BGP 289, *P. quarinii* BGP 229, *P. regnellii* BGP 112, *P. conspersum* BGP 402 and *P. urvillei* x *P. dilatatum* BGP 238, as they adopt strategies in which survival under adverse conditions is prioritized. On the other hand, for cases of mild-moderate water stress, priority should be given to accessions *P. atratum* BGP 308, *P. regnellii* BGP 215, BGP 248 and BGP 397, *P. dilatatum* BGP 234 and *P. malacophyllum* BGP 293, where productivity losses during water restriction are lower.

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Research Paper

Screening of common tropical grass and legume forages in Ethiopia for their nutrient composition and methane production profile in vitro

Composición nutricional y producción de metano in vitro de algunas gramíneas y leguminosas forrajeras comunes en Etiopía

ABERRA MELESSE[1,2], HERBERT STEINGASS[2], MARGIT SCHOLLENBERGER[2] AND MARKUS RODEHUTSCORD[2]

[1]*School of Animal and Range Sciences, Hawassa University, Hawassa, Ethiopia.* www.hu.edu.et
[2]*Institute of Animal Science, University of Hohenheim, Stuttgart, Germany*. www.uni-hohenheim.de

Abstract

A study was conducted to assess the nutrient composition, in vitro gas production (GP) characteristics and methane (CH_4) production potential of some common Ethiopian grass and legume forages. Crude protein (CP) concentration was lower in grasses than in legumes, while the reverse was observed for neutral detergent fiber (aNDFom) and acid detergent fiber (ADFom) concentrations. Within the 9 grasses tested, *Cynodon dactylon* had the highest CP concentration (187 g/kg DM), while *Panicum coloratum* and *Cenchrus ciliaris* had the lowest (70 and 82 g/kg DM, respectively) values. *Chloris gayana* contained the highest aNDFom (651 g/kg DM) concentration, while *Avena sativa* had the lowest (484 g/kg DM). Among the 3 legumes tested, *Vicia sativa* had the highest CP concentration (346 g/kg DM). The aNDFom and ADFom concentrations were highest in *V. sativa* and lowest in *Medicago sativa*. In grasses, *Brachiaria mutica* had the highest calcium, magnesium, iron and manganese concentrations, while in legumes the highest concentrations of phosphorus, potassium and zinc were observed in *V. sativa*. Methane production was generally higher ($P<0.05$) in grasses than in legumes. *Panicum coloratum* produced the lowest ($P<0.05$) CH_4 levels within the grasses followed by *B. mutica,* while *Desmodium intortum* produced the lowest ($P<0.05$) CH_4 levels within the legumes. *Panicum coloratum* and *D. intortum* appear to have potential as suitable forage species for ruminants, resulting in reduced CH_4 emissions. Studies with animals are needed to verify these in vitro findings.

Keywords*:* In vitro gas production, minerals, nutrient profiles, tropical pastures.

Resumen

En el laboratorio de Hawassa University, Etiopía, se realizó un estudio para evaluar la composición nutricional, la producción de gas (PG) in vitro y el potencial de producción de metano (CH_4) de 9 gramíneas y 3 leguminosas forrajeras comunes en Etiopía. Como era de esperar, la concentración de proteína cruda (PC) fue menor en las gramíneas que en las leguminosas, mientras que las concentraciones de fibra detergente neutro (FDN) y fibra detergente ácido (FDA) fueron más altas en las primeras. Entre las gramíneas evaluadas, *Cynodon dactylon* presentó la mayor concentración de PC (187 g/kg de MS), mientras que *Panicum coloratum* y *Cenchrus ciliaris* presentaron los valores más bajos (70 y 82 g/kg de MS, respectivamente). *Chloris gayana* presentó los valores más altos de FDN (651 g/kg de MS) y *Avena sativa* los más bajos (484 g/kg de MS). Entre las leguminosas, *Vicia sativa* presentó las mayores concentraciones de PC (346

Correspondence: Aberra Melesse, School of Animal and Range Sciences, Hawassa University, P.O. Box 05, Hawassa, Ethiopia.
Email: a_melesse@uni-hohenheim.de

g/kg de MS) y de ambas fibras, mientras que las concentraciones más bajas de FDN y FDA se registraron en *Medicago sativa*. Respecto a minerales, *Brachiaria mutica* presentó las mayores concentraciones de calcio, magnesio, hierro y manganeso entre las gramíneas, mientras que en las leguminosas se observaron en *V. sativa* las mayores concentraciones de fósforo, potasio y zinc. La producción de CH_4 fue generalmente mayor ($P<0.05$) en las gramíneas que en las leguminosas. Entre las gramíneas, *P. coloratum* presentó los niveles de CH_4 más bajos ($P<0.05$), seguido por *B. mutica*, mientras que entre las leguminosas *Desmodium intortum* produjo los niveles de CH_4 más bajos ($P<0.05$). Con miras a emisiones reducidas de CH_4, *P. coloratum* y *D. intortum* parecen tener potencial como especies forrajeras amigables con el medio ambiente. Se requieren estudios con animales rumiantes para verificar estos hallazgos obtenidos in vitro.

Palabras clave*:* Minerales, pastos tropicales, perfiles nutricionales, producción de gas in vitro.

Introduction

Developing countries in general and African nations in particular are increasingly becoming victims of climate change as global temperatures rise. The Intergovernmental Panel on Climate Change (IPCC) has attributed the temperature increases to human activities, including releases of the greenhouse gases, carbon dioxide, methane and nitrous oxide into the atmosphere. They have requested nations to quantify the amounts of gases they produce and to develop research to limit further gaseous emissions (Moss et al. 2000).

Ruminants are a major source of methane (CH_4) emissions, and France et al. (1993) estimated that the world's cattle emit about 100 Mt of CH_4 into the atmosphere annually, constituting 12.5–20% of the total global CH_4 emissions. More than half of the global cattle population are located in the tropics (McCrabb and Hunter 1999), a large proportion of which are supported on relatively low-quality, highly fibrous feed resources. This constitutes a significant source of global CH_4 emissions. Moreover, enteric CH_4 emissions in ruminants represent a loss of 2–12% of gross energy of feeds (McCrabb and Hunter 1999). As a result, CH_4 emissions from livestock have become a focus of research activities, especially in countries where agriculture is an important economic sector.

A wide diversity of forage sources are used in feeding livestock in the tropics. Improving the feed resource base by identifying alternative and more nutritious feeds with low CH_4 production would both reduce greenhouse gas emissions and increase the efficiency of energy utilization in forage. There is a lack of data describing and identifying those tropical grass and legume forages with low CH_4 production potential when fed to ruminant animals.

Since in vivo studies of methanogenesis by ruminants are time-consuming and expensive, and require large-scale specialized facilities and resources, there has been growing interest in using in vitro techniques to simulate the in vivo process (Blümmel et al. 2005; Bhatta et al. 2008; Soliva et al. 2008; Melesse et al. 2013). Use of in vitro gas-production techniques allows the screening of significant numbers of species rapidly and at relatively low cost (Soliva et al. 2008; Singh et al. 2012).

We used in vitro techniques to assess: a) the chemical and mineral compositions; and b) ruminal fermentation characteristics and CH_4 emission potentials, of some common Ethiopian green forages (9 grasses and 3 legumes) for their subsequent use in formulating diets for ruminants with lower potential emissions of CH_4.

Materials and Methods

Feed sample collection

Samples of grasses and legumes were collected during the small rainy season (March–May) in 2013. Samples of *Avena sativa* and *Vicia sativa* were collected from the first stage of growth on the forage farms of College of Agriculture, Hawassa University, Hawassa (7°03'43.38" N, 38°28'34.86" E; 1,700 masl). Samples of *Pennisetum purpureum, Chloris gayana, Panicum maximum, Panicum coloratum, Hyparrhenia cymbaria, Desmodium intortum* and *Medicago sativa* were collected at the pre-flowering stage of plants from ILRI's (International Livestock Research Institute) Forage Seed Multiplication Center located at Debre-Zeit (8°45'8.10" N, 38°58'42.46" E; 2,006 masl). Samples of *Brachiaria mutica*, *Cenchrus ciliaris* and *Cynodon dactylon* were collected at the pre-flowering stage of plants from ILRI's Forage Seed Multiplication Center located at Zeway (7°55'59.99" N, 38°43'0.01" E; 1,640 masl). All samples were dried on plastic sheets kept in shade, ground to pass a 1 mm sieve and transported in air-tight plastic containers to the University of Hohenheim, Germany, for analyses.

Chemical analyses

Chemical analyses of proximate nutrients, fiber fractions and minerals were performed as outlined by Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA 2007). The samples were analyzed at the Institute of Animal Science, University of Hohenheim, for dry matter (DM, method 3.1), ash (method 8.1), crude protein (CP, method 4.1.1; N x 6.25), petroleum ether extract (EE, method 5.1.1) and crude fiber (CF, method 6.1.1). Neutral detergent fiber (aNDFom) was assayed on an organic matter basis after amylase treatment (method 6.5.1) and acid detergent fiber on an organic matter basis (ADFom, method 6.5.2). Acid detergent lignin (ADL) was analyzed according to method 6.5.3. Cellulose and hemicellulose were computed as ADFom minus ADL and aNDFom minus ADFom, respectively. Non-fiber carbohydrate (NFC) concentration was calculated as 100 – (aNDFom + CP + crude fat + ash) according to NRC (2001). Nitrogen free extract (NFE) was computed as OM – (CF + EE + CP). Minerals [Ca, P, magnesium (Mg), potassium (K), sodium (Na), iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn)] were determined according to methods 10 and 11 of VDLUFA (2007) using an Inductively Coupled Plasma spectrometer (ICP-OES).

Four species with possible anti-nutritional factors (*Chloris gayana, Desmodium intortum, Medicago sativa* and *Vicia sativa*) were selected from the collection and analyzed for concentrations of total phenols and non-tannin phenols using the Folin-Ciocalteu method [Jayanegara et al. (2011) with modifications as described by Wischer et al. (2013)]. Extractable condensed tannins were analyzed according to Jayanegara et al. (2011). Concentrations of tannin phenols were then calculated as differences between total phenol and non-tannin phenol concentrations. The absorbance of total phenols and non-tannin phenols was recorded at 725 nm using a UV-VIS spectrophotometer (Perkin Elmer Instruments, Norwalk, CT, USA). Condensed tannins were analyzed by the butanol-HCl-iron method according to Jayanegara et al. (2011). The absorbance was read at 550 nm using the same UV-VIS spectrophotometer as for total phenols and non-tannin phenols and was expressed as leucocyanidin equivalents.

All analyses were run in duplicate and were averaged. If deviation between duplicates was above the level specified for each analysis, the analyses were repeated.

In vitro gas production

Gas production (GP) was determined according to the VDLUFA official method (VDLUFA 2007, method 25.1) (Menke and Steingass 1988). About 200 mg of feed sample was weighed and transferred into 100 ml calibrated glass syringes, fitted with white Vaseline-lubricated glass plungers.

A buffer solution was prepared and maintained in a water bath at 39 °C under continuous flushing with CO_2. Rumen fluid was collected before the morning feeding from 2 rumen-cannulated, lactating Jersey cows, fed a total mixed ration consisting (DM basis) of 20% maize silage, 20% grass silage, 20% hay and 40% dairy concentrate. The rumen fluid from both cows was mixed, filtered and added to the buffer solution (1:2 v/v) under constant stirring. Thirty mL of buffered rumen fluid was injected into each syringe, which was then immediately placed into a rotating disc and oven-incubated at constant temperature of 39 °C. Three syringes with only buffered rumen fluid, termed as blanks, plus 3 syringes with hay standard and 3 with concentrate standard with known GP were included in each run. The GP of samples, blanks and standards was recorded at 2, 4, 6, 8, 12, 24, 32, 48, 72 and 96 hours of incubation. The plunger of the syringe was re-set to 30 ml after the 6 and 24 hour readings. For metabolizable energy (ME) estimation, the GP of the feed samples was recalculated as 24 h GP on 200 mg DM using results from the blanks, with the corrections determined by the standards of hay and concentrate, the sample weight and its DM concentration.

The estimations of organic matter digestibility (OMD) and ME were carried out according to Menke et al. (1979) and Menke and Steingass (1988) by using the following equations:

ME (MJ/kg DM) = 1.68 + 0.1418*GP + 0.0073*CP + 0.0217*XL – 0.0028 XA

OMD (%) = 14.88 + 0.889*GP + 0.0448*CP + 0.0651*XA

where: GP, CP, XL and XA are 24 h gas production (ml/200 mg DM), crude protein, crude fat and ash (g/kg DM) of the incubated feed samples, respectively.

The corrected GP recorded between 2 and 96 h of incubation and the kinetics of GP were described by using the exponential equation: $y = b*(1-e^{(-c(t-lag))})$, which assumed one pool of asymptotic GP (b, ml/200 mg DM) with a constant fractional rate of GP (c, per hour) with a lag phase (lag, hours) in the onset of GP; parameter "y" is GP at time "t" (Blümmel et al. 2003; 2005).

Methane production

For CH_4 determination, 6 separate in vitro runs were performed. Based on the previous in vitro GP results for each feed sample, we calculated the quantity of each feed sample to be incubated for 24 h without having to remove the gas produced in the syringes during the incubation

period. After 24 h of incubation, total GP was recorded, and the incubation liquid was carefully decanted, while leaving the gas inside the syringes. The CH_4 content of the total gas in the syringes was then analyzed using an infrared methane analyzer (Pronova Analysentechnik, Berlin, Germany) calibrated with a reference gas (13.0% CH_4 by volume, Westfalen AG, Münster, Germany). Syringes were directly connected to the analyzer and about 20 ml of gas was injected for about 20 seconds until the displayed CH_4 concentration was constant. The CH_4 produced by each sample was corrected by the amount of CH_4 produced by blank syringes (containing only the rumen fluid) and by the factors of reference hay and concentrate feed, which were included in each run.

Statistical analyses

Results of chemical and mineral composition are expressed as means of duplicate analyses of a bulked sample. Model fitting for gas production kinetics and parameter estimation was done according to Blümmel et al. (2003) by using the computer program GraphPad Prism 5.0 (2007) for Windows (GraphPad Software Inc., La Jolla, CA, USA). Data on 24 h gas and methane productions were subjected to the GLM of the Statistical Analysis System (SAS 2010). Analysis of variance was conducted according to the following model: $yij = \mu + Pi + Rj + eij$, where: yij is the independent variable, μ is the overall mean, Pi is the effect of the i^{th} plant material, Rj is the effect of the j^{th} experimental run of the i^{th} plant material and eij is the residual error. All multiple comparisons among means were performed with Duncan's multiple range tests.

Results

Crude nutrients and anti-nutritional factors

Crude nutrient concentrations in the studied grass and legume plants are presented in Table 1. The CP concentrations in grass species ranged from 70 g/kg DM (*Panicum coloratum*) to 220 g/kg DM (*Avena sativa*), while those in legumes ranged from 257 g/kg DM (*Medicago sativa*) to 346 g/kg DM (*Vicia sativa*). For grasses, CF concentrations ranged from 281 g/kg DM (*A. sativa*) to 322 g/kg DM (*Cenchrus ciliaris* and *Chloris gayana*), while values for legumes ranged from 213 g/kg DM (*Desmodium intortum*) to 249 g/kg DM (*M. sativa*). Similarly, grasses contained more aNDFom (484–651 g/kg DM) than legumes (364–404 g/kg DM). Concentrations of ADL in grasses (25.3–41.3 g/kg DM) were lower than those in legumes (48.2–89.8 g/kg DM).

As shown in Table 2, concentrations of total phenols were comparable for *M. sativa*, *V. sativa* and *C. gayana*, while those for *D. intortum* were higher by a factor of 10. No tannin phenols or extractable condensed tannins were detected in either *M. sativa* or *V. sativa*, while *C. gayana* contained very low concentrations of these compounds. Both tannin phenols and extractable condensed tannins were at high concentrations in *D. intortum.*

Table 1. Crude nutrient concentrations (g/kg DM) in some common grass and legume forages grown in Ethiopia.

Forage species	Ash	CP[1]	EE	CF	NFE	aNDFom	ADFom	ADL	Cellulose	Hemi-cellulose	NFC
Grasses											
Avena sativa	133	226	31.2	281	263	484	326	25.3	301	159	126
Brachiaria mutica	167	159	12.2	229	382	504	277	29.8	247	227	158
Cenchrus ciliaris	154	82	14.5	322	380	601	373	26.7	346	228	149
Chloris gayana	131	135	13.3	322	341	651	370	41.1	329	281	70
Cynodon dactylon	125	187	14.7	272	357	609	323	41.3	282	286	64
Hyparrhenia cymbaria	105	156	11.6	299	375	605	333	33.7	299	272	122
Panicum coloratum	102	70	21.0	292	461	633	322	26.3	296	312	174
Panicum maximum	140	140	12.5	284	373	566	344	27.1	317	222	142
Pennisetum purpureum	173	121	10.9	315	325	599	372	33.4	339	227	96
Legumes											
Desmodium intortum	91.7	258	9.3	213	365	396	319	89.8	229	77.2	245
Medicago sativa	151	257	13.3	249	282	364	302	48.2	254	61.7	215
Vicia sativa	147	346	17.2	218	214	404	336	56.6	279	67.9	86

[1]CP = crude protein; EE = crude fat; CF = crude fiber; NFE = nitrogen free extract; aNDFom = neutral detergent fiber on organic matter basis after amylase treatment; ADFom = acid detergent fiber on organic matter basis; ADL = acid detergent lignin; NFC = non-fiber carbohydrates.

Table 2. Concentrations (g/kg DM) of total phenols, tannin phenols and extractable condensed tannins in some selected forage species in Ethiopia.

Species	Type of forage	Total phenols	Tannin phenols	Extractable condensed tannins
Medicago sativa	Legume	6.7	nd[1]	nd
Desmodium intortum	Legume	77.7	57.6	77.6
Vicia sativa	Legume	7.6	nd	nd
Chloris gayana	Grass	6.8	1.3	0.2

[1]nd = not detected.

Minerals

As presented in Table 3, among the grasses *Brachiaria mutica* had the highest concentrations of Ca, Mg, Fe and Mn, while P concentration was highest in *A. sativa*, *Pennisetum purpureum* and *Panicum maximum.* In leguminous forages, *M. sativa* and *V. sativa* had Ca concentrations of about 9 g/kg DM, while *V. sativa* had the highest P concentration (5.6 g/kg DM). Sodium concentrations varied widely in both legumes and grasses, with ranges of 0.06–8.01 g/kg DM for grasses and 0.10–4.26 g/kg DM for legumes.

In vitro gas production profiles and fermentation kinetics

As shown in Table 4, metabolizable energy (ME) concentrations in grasses ranged from 7.4 MJ/kg DM in *Panicum coloratum* to 10.6 MJ/kg DM in *A. sativa* and in legumes from 8.1 MJ/kg DM in *D. intortum* to 10.2 MJ/kg DM in *V. sativa*. Organic matter digestibility in grasses ranged from 56.1% in *P. coloratum* to 79.6% in *A. sativa*, and from 64.6 to 82% in legumes. The highest asymptotic GP (parameter b) values for grasses were observed in *Hyparrhenia. cymbaria* (58.6 ml) and *P. maximum* (59.2 ml) with the lowest in *B. mutica* (49.2 ml). Values for legumes were generally lower with a range of 39.0–45.2 ml. The fractional rates of GP per hour (parameter c) for grasses ranged from 0.0387 (*P. coloratum*) to 0.0667 (*A. sativa*). The range for legumes was 0.0537 (*D. intortum*) to 0.0851 (*V. sativa*). As shown in Table 4, the values for the goodness of fit (R^2) of the exponential model were above 94% for all species.

Patterns of gas production for the grasses are shown in Figure 1 and for legumes in Figure 2.

Table 3. Mineral composition of some common grass and legume forages grown in Ethiopia.

Forage species	Major (g/kg DM)					Trace (mg/kg DM)			
	Ca[1]	P	Mg	K	Na	Fe	Cu	Mn	Zn
Grasses									
Avena sativa	3.69	5.24	2.18	38.0	8.01	104	2.44	46.9	78.4
Brachiaria mutica	8.55	3.49	4.63	26.0	1.83	716	7.02	84.7	29.9
Cenchrus ciliaris	4.30	2.54	2.95	24.3	0.45	430	4.21	31.7	18.6
Chloris gayana	3.88	2.94	1.98	35.6	0.50	210	4.75	68.7	28.9
Cynodon dactylon	5.11	2.07	2.46	24.9	0.15	181	5.09	57.5	35.0
Hyparrhenia cymbaria	4.27	1.40	2.41	20.6	0.06	150	5.87	44.3	28.1
Panicum coloratum	3.20	2.78	3.66	11.0	1.82	191	3.98	21.7	15.2
Panicum maximum	4.14	4.56	4.38	23.9	3.54	420	8.27	40.6	26.3
Pennisetum purpureum	3.09	4.49	3.49	38.9	0.16	267	7.50	27.5	25.5
Legumes									
Desmodium intortum	6.85	2.37	6.25	18.8	0.10	486	7.14	58.7	34.8
Medicago sativa	9.32	3.24	3.02	45.9	4.26	494	2.42	55.2	65.3
Vicia sativa	9.05	5.57	3.12	46.0	2.64	441	3.37	61.3	388

[1]Ca = calcium; P = phosphorus; Mg = magnesium; K = potassium; Na = sodium; Fe = iron; Cu = copper; Mn = manganese; Zn = zinc.

Table 4. In vitro estimates of metabolizable energy (ME), organic matter digestibility (OMD) and kinetics of gas production (ml/200 mg DM) in some common grass and legume forages grown in Ethiopia.

Forage species	ME (MJ/kg DM)	OMD (%)	b[1]	c	Lag time (h)	R^2
Grasses						
Avena sativa	10.6	79.6	54.7	0.0667	1.17	97.3
Brachiaria mutica	8.0	66.7	49.2	0.0448	0.20	97.3
Cenchrus ciliaris	8.5	66.9	57.7	0.0469	0.94	98.2
Cynodon dactylon	9.7	75.2	56.9	0.0537	1.47	97.7
Chloris gayana	9.0	70.3	56.4	0.0511	1.53	97.7
Hyparrhenia cymbaria	10.0	75.6	58.6	0.0613	1.39	97.7
Panicum coloratum	7.4	56.1	55.4	0.0387	0.81	96.2
Panicum maximum	9.7	75.2	59.2	0.0548	0.96	97.8
Pennisetum purpureum	8.4	68.8	54.4	0.0475	1.25	97.4
Legumes						
Desmodium intortum	8.1	64.6	39.0	0.0537	0.37	94.7
Medicago sativa	9.5	77.2	45.2	0.0839	0.94	97.9
Vicia sativa	10.2	82.0	45.0	0.0851	0.89	96.1

[1]b = total asymptotic gas production (ml/200 mg DM); c = the rate at which b is produced per hour with a lag phase in the onset of gas production.

Methane production

Most grass species produced significant amounts of methane during digestion, but *P. coloratum* produced about half that of other species (P<0.05) (Table 5). Total gas production followed a similar pattern with highest values for *H. cymbaria*, *P. maximum*, *A. sativa* and *Cynodon dactylon* and lowest for *P. coloratum* (P<0.05). Methane:total gas ratios (CH_4:GP) ranged from 0.18:1 (*B. mutica*) to 0.11:1 (*P. coloratum*) (P<0.05).

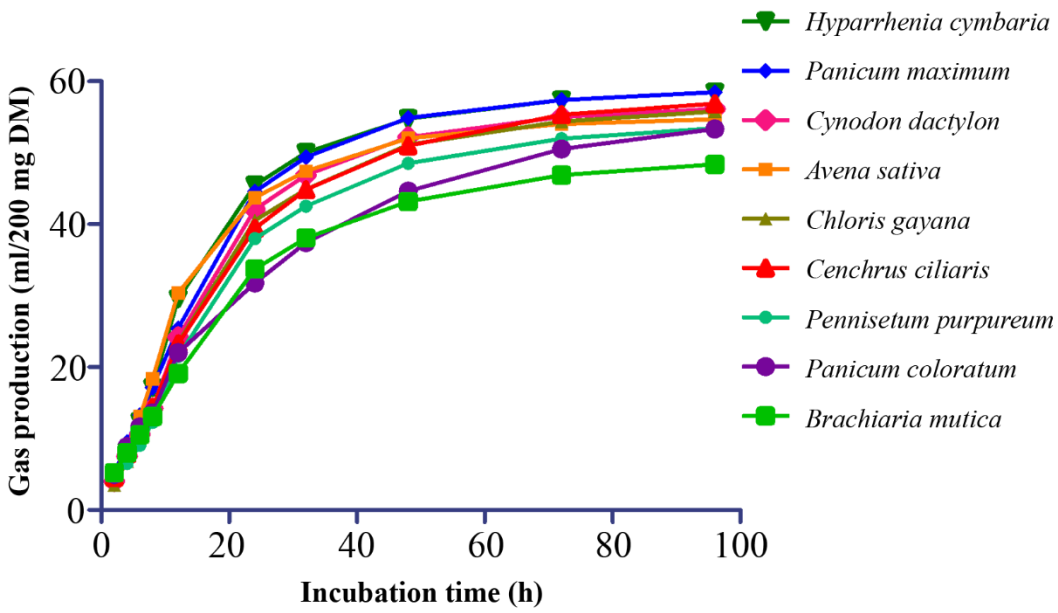

Figure 1. Patterns of gas production of some tropical grass forages during in vitro incubation for 96 h.

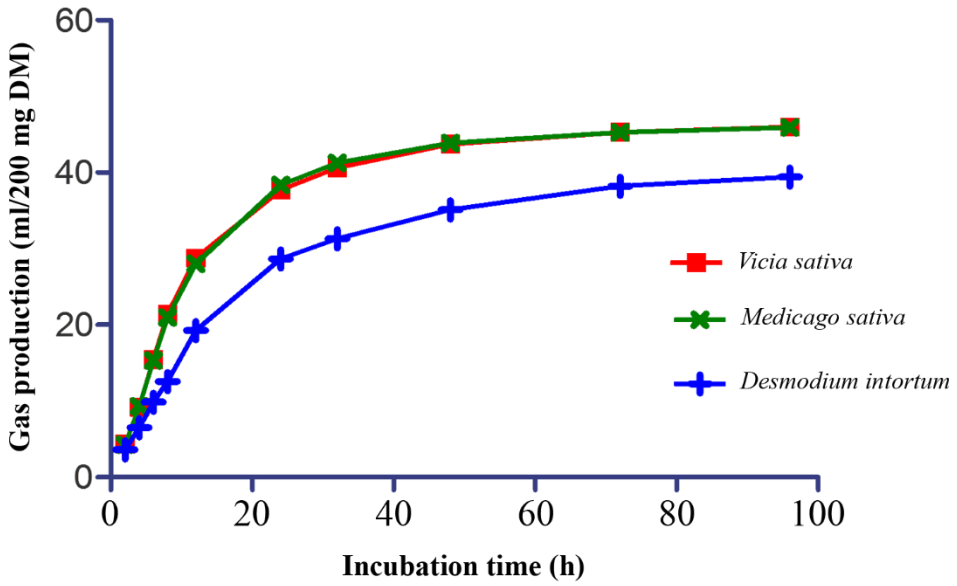

Figure 2. Patterns of gas production of some tropical legume forages during in vitro incubation for 96 h.

Desmodium intortum produced much less CH_4 than *V. sativa* and *M. sativa* (P<0.05) differing significantly from these other legumes (Table 5). While differences between species for total gas production were not so marked, the lowest CH_4:GP ratio was observed in *D. intortum* (0.12:1), which differed significantly from those for the other 2 legumes (0.16:1). In general, grasses produced comparatively higher (P<0.05) GP, CH_4 and CH_4:GP ratios than legume forages.

Table 5. *In vitro* methane and total gas production profiles (±SD) in some common grass and legume forages grown in Ethiopia.

Forage species	CH_4 (ml/200 mg DM)	GP (ml/200 mg DM)	CH_4:GP (v:v)
Grasses			
Avena sativa	6.16±1.00ab[1]	44.0±5.34abc	0.140d
Brachiaria mutica	5.80±0.53b	33.5±1.41e	0.178a
Cenchrus ciliaris	6.92±0.45a	40.9±1.62cd	0.169b
Chloris gayana	6.44±0.68ab	41.8±3.20bcd	0.159bc
Cynodon dactylon	7.01±0.53a	43.6±4.38abc	0.161bc
Hyparrhenia cymbaria	6.78±0.68a	47.0±2.10a	0.145d
Panicum coloratum	3.18±0.47c	31.4±1.02e	0.107e
Panicum maximum	7.01±1.06a	44.9±2.00ab	0.159bc
Pennisetum purpureum	6.94±1.06a	39.7±2.15d	0.165bc
Legumes			
Desmodium intortum	3.67±0.39b	29.9±1.03b	0.123b
Medicago sativa	5.90±0.72a	37.5±2.86a	0.157a
Vicia sativa	5.73±0.51a	36.1±3.25a	0.159a
Grasses vs. legumes			
Grasses	6.23±1.42a	40.3±5.52a	0.156a
Legumes	5.34±1.21b	36.9±6.15b	0.144b

[1]Means within columns and plant types followed by different letters differ significantly (P<0.05).
CH_4 = methane production; GP = total gas production at 24 h incubation of feed samples.

Discussion

Crude nutrient and mineral concentrations

The generally lower CP concentrations in the grasses than in the legumes were consistent with the reports of Singh et al. (2012) for Indian green forages. Consistent with the reports of Tessema and Baars (2006), all forages studied had protein concentrations above 8%, suggested by Van Soest (1982) as the critical level, below which intake may fall due to lack of sufficient nitrogen for effective proliferation of rumen micro-organisms. Higher CP values in leguminous forages than in grasses might be related to the N-fixing abilities of the legumes. Our current findings are in good agreement with those of Tessema and Baars (2006) from Ethiopia, that pure legume stands and grass-legume mixtures produced forage with higher CP and lower fiber concentrations than pure stands of grass. The CP concentrations in *C. gayana* and *P. maximum* in the present study are lower than those reported by Tessema and Baars (2006). However, those authors also reported lower CP for *M. sativa* than found in the present study. These differences in CP concentrations could be explained due to stage of maturity, N profile of the soils where they had been grown and differences in efficiency of protein accumulation during growth. Moreover, differences in nutrient concentrations in the feeds may be due to variations in the stage of growth and plant parts (i.e. twigs, leaves, soft stem) when sampled.

All leguminous forages had higher lignin concentrations than grasses as reported by Singh et al. (2012). This might be explained by the fact that the leguminous forages synthesize lignin for strength and rigidity of plant cell walls. Singh et al. (2012) reported 310 and 58.8 g/kg DM for ADF and ADL concentrations, respectively, in *M. sativa*, which are comparable with the present findings.

Except for *B. mutica*, grass species in the present study had higher aNDFom, ADFom, cellulose and hemicellulose concentrations than legumes, which is in accordance with the findings of Tessema and Baars (2006) and Singh et al. (2012). The threshold level of NDF in tropical grasses, beyond which DM intake of cattle is affected, is suggested to be 600 g/kg DM (Meissner et al. 1991) and all legumes and some grasses (*B. mutica*, *P. maximum*, *P. purpureum* and *C. ciliaris*) had lower NDF values than this critical level. Since animals, when allowed to selectively graze, can select a better quality diet than feed on offer, these issues may not be a major problem under a grazing situation. However, where a cut-and-carry system operates, they become quite relevant. All leguminous forages contained less fiber than grasses, which might be explained in part by lower hemicellulose concentration in the legumes at comparable levels of cellulose (Table 1). Cellulose and hemicellulose in forages represent the main sources of energy to ruminants (Merkel et al. 1999).

The aNDFom, ADFom and ADL concentrations in *V. sativa* were comparable with those reported by Berhane et al. (2006) from the lowlands of northern Ethiopia. While the CP concentration in *P. coloratum* was similar to the observation of the same authors, they reported higher NDF, ADF and ADL values than those we found. Such variations might be induced by the stage of maturity of the forage at harvest as grasses increase stem proportions with age, resulting in higher NDF, ADF and lignin and lower CP values (Mero and Udén 1997; 1998).

Except for *P. coloratum*, lipid concentrations in the forages investigated here were much lower than observations reported by Singh et al. (2012) and Pamo et al. (2007). These variations might be attributed mainly to stage of maturity of the forage at the time of sampling and different environmental conditions. While lipids do not constitute a major source of energy from forages, forages with high lipid concentrations may be a tool to modify milk fatty acid profile towards more long-chain and unsaturated fatty acids (Elgersma 2015).

Phosphorus is one of the most important minerals for many metabolic processes in animals and a deficiency of P in the diet can retard growth and reproductive performance of livestock (Paterson et al. 1996). While *V. sativa* and *A. sativa* were found to be the richest sources of P in our study (>0.5% P), all forages had P concentrations above 0.2%. *Brachiaria mutica*, *V. sativa* and *A. sativa* proved to be the richest sources of Ca, which is closely related to P metabolism in the formation of bones. The calculated average Ca:P ratio for legume forages in the present study was 2.0:1, while for grasses it was 1.7:1, both of which fall within the recommended range for Ca:P ratio in feedstuffs of 1:2 to 2:1 (NRC 2001), indicating that the studied forages are likely to be a well-balanced source of both minerals.

In vitro gas and methane production

The study has shown that methane production from all forages tested was relatively uniform, with the exception of *P. coloratum* and *D. intortum*, which produced much less CH_4 than the remaining species. The observed low in vitro GP pattern in *D. intortum* (Figure 2) might be explained by the presence of high concentrations of total

phenols (77.7 g/kg DM) and condensed tannins (77.6 g/kg DM), which have the ability to complex with protein and are a major cause of the resistance of this legume to bacterial decomposition.

Consistent with the current observations, Mero and Udén (1998) reported in vivo OMD values between 61.5 and 64.8% for *C. ciliaris* hay harvested at 6 weeks of age. They also reported comparable OMD values for *P. coloratum* harvested at 6 weeks of age. Berhane et al. (2006) reported values of 65.5 and 68.3 ml for in vitro GP (parameter b) of fresh-cut *V. sativa* and *P. coloratum*, respectively, which were higher than those observed in the current study. *Panicum coloratum* in the present study had the lowest ME and OMD values. Except for *D. intortum*, legumes produced more gas than grasses within 96 h of incubation, which is consistent with the findings of Singh et al. (2012).

The observed variations in CH_4 production among the investigated forages may be due to variations in their chemical composition. Such variations in in vitro CH_4 production have been observed in straws, forages and food industry byproducts (Santoso and Hariadi 2009; Singh et al. 2012). In the current study, except for *P. coloratum* and *B. mutica*, all investigated grasses had higher CH_4 values than leguminous forages, which is in agreement with the findings of Boadi et al. (2004) and Navarro-Villa et al. (2011). At 12 h fermentation, Widiawati and Thalib (2007) found that in grasses CH_4 production per unit of OM degraded was twice that in legume forages. Moreover, hydrolysis of legumes such as lucerne and red clover generates less CH_4/g DM than hydrolysis of grasses (Ramirez-Restrepo and Barry 2005). The lower CH_4 values in legumes vs. grasses might be attributed to less extensive in vitro rumen fermentation of legumes as suggested by Navarro-Villa et al. (2011). When CH_4 emissions are expressed as a proportion of gross energy intake (Waghorn et al. 2006), values are lower for animals fed forage legumes (Waghorn et al. 2002) than for those receiving a predominantly grass diet. Beauchemin et al. (2008) proposed that the lower CH_4 emissions of legume-fed animals is a result of a combination of factors including the presence of condensed tannins, lower fiber concentration, higher DM intake and an increased passage rate from the rumen. In the current study, no extractable condensed tannins were detected in *M. sativa* and *V. sativa*. Beauchemin et al. (2008) also reported that, although differences in CH_4 emissions reflect compositional differences between grasses and legumes, stage of maturity at the time of harvest can be a confounding factor.

Fermentation of cell wall carbohydrates produces more CH_4 than fermentation of soluble sugars, which produce more CH_4 than fermentation of starch (Johnson et al. 1996) and legume forages are digested more quickly than grasses. This was demonstrated for *M. sativa* and *V. sativa* in the current study, which means that intake and productivity on leguminous pasture can be higher than on grasses. In tannin-containing forages, excess plant proteins that become bound to tannins leave the rumen without being digested. However, some leguminous forages containing tannins, such as *D. intortum*, can release these proteins in the abomasum in response to low pH. This allows the protein to be digested and absorbed in the small intestine (Waghorn et al. 1987), resulting in high productivity in both sheep (Douglas et al. 1995) and cattle (Wen et al. 2002).

Legumes contain higher CP than grasses at the same stage of maturity and protein fermentation in vitro has been shown to be associated with lower CH_4 production than fermentation of carbohydrates (Cone and Van Gelder 1999). In vitro studies conducted by Soliva et al. (2008), Tiemann et al. (2008), Bekele et al. (2009) and Archimède et al. (2011) have shown that a large portion of the variability of CH_4 production in legumes can be associated with the presence of secondary metabolites (condensed tannins, saponins) in some legume species, which can inhibit CH_4 formation (Beauchemin et al. 2007; Jouany and Morgavi 2007). In the present study, *D. intortum* had the highest phenols and extractable condensed tannins, which possibly contributed to the reduction of CH_4 production in this species. In other studies, prolonged feeding of tanniniferous forage legumes showed that animals receiving *D. intortum* had the lowest total worm burdens, the lowest female:male parasite ratios, the lowest numbers of eggs in the uterus of each female worm and the lowest per capita fecundity (Debela et al. 2012). There is high variability among legumes, particularly regarding the presence of secondary metabolites such as tannins, which are more common in tropical legumes (Waghorn 2008).

Tropical legumes show promise as a means of reducing CH_4 production, partly because of their lower fiber concentration and faster rate of passage than grasses, and in some cases, the presence of condensed tannins as observed in *D. intortum* in this study. Various studies have reported that condensed tannins in legume forages are able to suppress ruminal methanogenesis directly through their antimethanogenic activity and indirectly through their antiprotozoal activity (Goel and Makkar 2012). Patra and Saxena (2010), Pellikaan et al. (2011)

and Goel and Makkar (2012) indicated that condensed and hydrolyzable tannins extracted from a diverse array of plant materials reduced CH_4 production in vitro. Similarly, Puchala et al. (2005) demonstrated that the presence of condensed tannins in forages can decrease CH_4 production in vivo. This was confirmed by Animut et al. (2008), who observed decreased CH_4 emissions in sheep fed a ration supplemented with different condensed tannin sources.

The CH_4 values measured at 24 h in vitro for *M. sativa*, *P. purpureum* and *P. maximum* reported by Singh et al. (2012) are generally higher than those obtained from the current study. These variations could be due to quality and maturity stage of the forages, soil type and climate in which forages have been grown.

Enteric CH_4 production could be influenced by the nature of carbohydrates fermented, such as cellulose, hemicelluloses and soluble residues of the diets. In the present study, grasses had higher aNDFom, ADFom, cellulose and hemicellulose concentrations than legumes and produced more CH_4 per unit weight. Similarly, Moss et al. (1994) reported that digestible ADF, cellulose and hemicellulose are important fiber fractions influencing CH_4 production in the rumen.

Many studies (Santoso et al. 2003; Santoso and Hariadi 2009; Singh et al. 2012) have reported correlations between chemical constituents and CH_4 production. In the current study, CH_4 was negatively correlated with non-fiber carbohydrates (NFC) only (data not shown) as fermentation of NFC produces less hydrogen due to relatively higher propionate production. Accordingly, increments in NFC in forages should depress CH_4 production. This has been clearly observed in the current study, in which both *P. coloratum* and *D. intortum* had high NFC values and produced lower CH_4 levels than other forage species. These results are consistent with the observations of Grainger and Beauchemin (2011), who reported that increasing NFC levels in feeds reduces CH_4 production by lowering pH and increasing rate of ruminal passage to favor propionate production, and reduce rumen protozoal populations.

Conclusions

The CP concentrations were lower in the grasses than in the legumes, while the reverse was the case for aNDFom, ADFom and cellulose. Methane production was numerically higher in grasses than legumes. Thus, feeding of grasses in combination with legumes should result in enhanced productivity, while reducing CH_4 emissions by ruminants, especially per unit of product. Despite their lower OMD, it appears that *P. coloratum* and *D. intortum* could be fed alone or in combination to supplement tropical feed resources for practical mitigation of CH_4 emissions from ruminants. We recommend animal-based experiments to validate the actual feeding values of these forages, which showed reduced CH_4 production in vitro, and to assess their production potential.

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