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Gontijo de Lima, Flavia; Thomas Lee, Stephen; Pfister, James Alan; Sayuri Miyagi, Eliane;
Lage Costa, Gustavo; Dias da Silva, Roberta; Soares Fioravanti, Maria Clorinda

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The effect of ensiling and haymaking on the concentrations of steroidal saponin in two *Brachiaria* grass species

O efeito da ensilagem e fenação sobre as concentrações de saponina esteroide em duas espécies de gramínea *Brachiaria*

Flavia Gontijo de Lima^{I*} Stephen Thomas Lee^{II} James Alan Pfister^{II} Eliane Sayuri Miyagi^I
Gustavo Lage Costa^I Roberta Dias da Silva^I Maria Clorinda Soares Fioravanti^I

ABSTRACT

Brachiaria (signalgrass) is now the most widely used tropical grass genus in Central and South America. However, *Brachiaria* spp. can cause hepatogenous photosensitization in livestock. Steroidal saponins, specifically protodioscin, present in *Brachiaria* spp. may be responsible for liver injury and subsequent photosensitization. The objective of this study was to determine the effect of ensiling *Brachiaria decumbens* and *Brachiaria brizantha* or making hay from *Brachiaria decumbens* on the concentrations of steroidal saponin in these grasses. *Brachiaria* grass had no detectable levels of the saponin protodioscin after 24 days of ensiling. In addition, in *Brachiaria decumbens*, the concentration of the protodioscin decreased 48% over the first three days after haymaking and then remained constant. These results suggest that livestock consuming *Brachiaria* either as silage or hay may have reduced risk of intoxication by protodioscin.

Key words: haymaking, hepatogenous photosensitization, saponin, protodioscin, signalgrass, silage.

RESUMO

Brachiaria atualmente é o gênero de gramínea tropical mais amplamente utilizado na América Central e do Sul. Entretanto, as espécies de *Brachiaria* podem causar fotossensibilização hepatógena em animais de produção. Saponinas esteroides, especificamente protodioscina, presentes em *Brachiaria*, podem ser responsáveis pelo dano hepático e consequentemente fotossensibilização. O objetivo deste estudo foi determinar o efeito da ensilagem de *Brachiaria decumbens* e *Brachiaria brizantha* e fenação de *Brachiaria decumbens* sobre a concentração de saponina esteroide. As amostras de *Brachiaria* ensiladas não tiveram concentrações detectáveis de saponina protodioscina depois de 24 dias de ensilagem. Assim como em *Brachiaria decumbens*, a concentração de protodioscina reduziu 48% nos três primeiros dias após a fenação e posteriormente manteve-se constante. Estes resultados sugerem que animais de

produção, ao consumirem *Brachiaria* tanto como silagem ou feno, podem ter o risco de intoxicação por protodioscina reduzido.

Palavras-chave: fenação, fotossensibilização hepatógena, saponina, protodioscina, braquiária, silagem.

INTRODUCTION

In most parts of the world, forage conservation is a key element for productive and efficient ruminant livestock operations. Forage conservation methods such as ensiling and haymaking allow a more reliable supply of quality feed when forage production is low or dormant (MUCK & SHINNERS, 2001).

In tropical and subtropical countries, techniques for making silages from forages and by-products are continually improving, and it is probable that the importance of silage will increase, particularly in areas where the markets for milk and milk products are well developed (WILKINS, 2005). Silage represents a convenient and economical source of feed for the dairy and the beef industry. Haymaking also offers a number of advantages: harvesting can largely be mechanized; hay stores well when adequately protected; and hay can meet the nutritional requirements of most classes of livestock (BALL et al., 1991).

Brachiaria spp. (signalgrass) is now the most widely used tropical grass genus, especially in

^IUniversidade Federal de Goiás (UFG), 74001-970, Goiânia, GO, Brasil. E-mail: flaviamedvet@yahoo.com.br. *Corresponding author.

^{II}United States Department of Agriculture (USDA), Poisonous Plant Research Laboratory, Logan, Utah 84341, USA.

Central and South America. In Brazil alone, about 40 million hectares of *Brachiaria* pastures have been planted, more than 85% of which consist of *B. decumbens* cv. 'Basilisk' and *B. brizantha* cv. 'Marandu' (MILES et al., 1996). Silage and hay made from *Brachiaria* spp. provide quality feed and adequate nutrition for ruminants (FERNANDES et al., 2001; JAYME et al., 2011).

However, *Brachiaria* spp. can also cause hepatogenous photosensitization in livestock (BRUM et al., 2007). Steroidal saponins, specifically protodioscin, present in these plants may be responsible for liver injury (CASTRO et al., 2011). Saponins are high-molecular-weight glycosides, consisting of a sugar moiety linked to a triterpene or steroid aglycone (HOSTETTMANN & MARTSON, 1995). In addition, sporidesmin and other hepatotoxic mycotoxins may act in synergy with the steroidal saponins. These complicating factors may explain the sporadic incidence of poisonings (TOKARNIA et al., 2002).

There is little information available about the effect of ensiling or haymaking on saponin concentrations in *Brachiaria* spp. Thus, the objective of this study was to determine if ensiling and haymaking would change the levels of saponin in *Brachiaria* grass species.

MATERIALS AND METHODS

Silage making

Signal grasses (*Brachiaria brizantha* and *Brachiaria decumbens*) were cultivated in the municipality of São Francisco de Goiás, in the state of Goiás, west central Brazil (15°55' 51" S, 49°15' 39" W), on a farm of the Federal University of Goiás. The forages were cut for silage 90 days after seeding emergence. Harvested plant material was chopped to 1cm by a mechanical harvester and ensiled in small laboratory silos (i.e., plastic bucket) with a 15-liter capacity. A layer of sand and gravel (4kg) were placed in the bottom of the silo to drain the effluent. A piece of fabric was placed between the sand and the grass. The fresh forage was introduced into the silos in small amounts and compacted by a manual piston. When completely full the silos were immediately sealed hermetically using a rubber stopper with a Bunsen valve in the center and an additional metallic cover, then stored in a cool and well-ventilated area (VENDRAMINI et al., 2010).

Fresh plants were sampled for saponin analysis before ensiling (Day 0), and after ensiling. The silos were opened on days 4, 8, 12, 16, 20 and

24. A complete randomized design was followed, with a factorial distribution of 2x7 (grasses x storage period), with three replicates each.

Haymaking

Plots of *Brachiaria decumbens* were cultivated on the same farm. The forages were cut for haymaking 150 days after seeding emergence. After harvesting, the cut forage was air-dried for three days and then bales (1mx0.4mx0.3m) were made mechanically and stored in a well-ventilated barn.

The bales were sampled for saponin analysis (LEE et al., 2009) before haymaking (fresh plant) and on days 1, 2, 3, 4, 5, 6, 7, 8, 14 and 21 after bales were made. There were five replicates for each day and the samples were collected from multiple bales each period, using a 45cm drill-type probe that collected a core sample that included exterior and interior material.

Plant preparation

The samples of *B. decumbens* and *B. brizantha* silage, and *B. decumbens* hay were dried in a forced-air oven at 65°C for 72 hours, ground to pass a 2-mm screen in a Wiley mill, and stored in airtight bags at room temperature until analyzed. The plant material stored were dried once more at 105°C for 16 hours to the calculation and adjustments to total dry matter.

Extraction

Extraction of plant material for protodioscin analysis (LEE et al., 2009) was accomplished by weighing 100mg of ground plant material into a 13mL screw top test tube equipped with Teflon lined caps (Pierce, Rockford, IL, USA). Methanol (MeOH, 5mL) was added to each test tube and placed in a mechanical shaker for 30min, then centrifuged to separate the plant residue and MeOH extract.

The MeOH extract was transferred to a clean 20mL test tube. The grass residue was extracted two more times with 5mL MeOH for 30min and all MeOH extracts combined for a total of 15mL. A 0.1mL aliquot of the MeOH extract was transferred to a 1mL autosample vial containing 0.9mL 0.1% formic acid:acetonitrile (90:10) and mixed thoroughly prior to HPLC-ESI-MS analysis.

HPLC-ESI-MS

Samples were injected (5µL) onto a Betasil C-8 reversed phase column (100x2.1mm i.d.) (Thermo Electron Corporation, Waltham, MA, USA) protected by a guard column of the same phase. The

total HPLC run time was 3.0min with protodioscin eluting at 1.9min. The saponin was eluted from the column with an isocratic flow (0.500ml min⁻¹) of 72:28 (0.1% formic acid:acetonitrile) mobile phase. Flow from the column was connected directly to a Thermo Finnigan (San Jose, CA USA) LCQ ion trap mass spectrometer via an electrospray ionization (ESI) source. Full scan mass data were collected for a mass range of 300-1300amu. Protodioscin standard (ChromaDex Irvine, CA USA) was prepared in a solution of 0.1% formic acid:acetonitrile (90:10) to give a seven point standard curve over the range of 0.78µg mL⁻¹-50.0µg mL⁻¹. Peak areas of protodioscin were determined from reconstructed ion chromatograms of the MH⁺-H₂O ions ($m/z=031$), according to LEE et al. (2009).

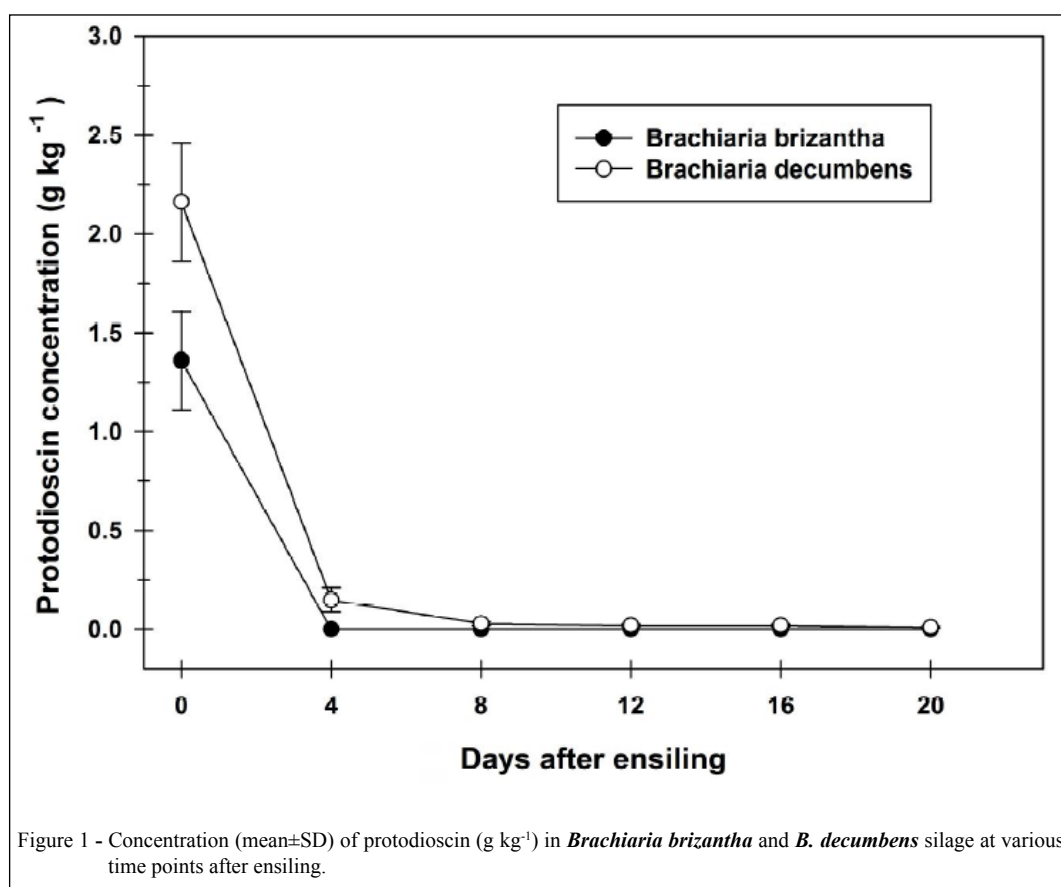
Statistical analysis

Data analyses were performed using SAS/STAT® software (SAS 2011) by ANOVA procedures for a completely random design in a 2x7 factorial treatment arrangement, with the factors grass variety and storage.

RESULTS AND DISCUSSION

Silage was made using both *B. decumbens* and *B. brizantha*, however only *B. decumbens* was used to make hay, because *B. brizantha* hay was previously analyzed by LIMA et al. (2012). The protodioscin results were expressed on dry matter basis. The levels of protodioscin in both *Brachiaria* spp. rapidly decreased after ensiling. The concentration of protodioscin in *B. brizantha* decreased from 1.41±0.92g kg⁻¹ on Day 0 (fresh plant) to 0g kg⁻¹ after 4 days ($P<0.01$). The concentration of protodioscin in *B. decumbens* also decreased by 94% from 2.28±1.04g kg⁻¹ to 0.14±0.23g kg⁻¹ ($P<0.01$) over the first 4 days and then decreased to 0g kg⁻¹ over the next 20 days (Figure 1).

The concentration of protodioscin in fresh *B. decumbens* was 10.6±0.25g kg⁻¹ and this decreased by approximately 48% after 3 days as hay ($P<0.01$). For the next 5 days (Day 3 to 8) the concentration remained relatively constant at approximately 5.5-7.3g kg⁻¹ ($P<0.01$), then had a light increase to



$8.67 \pm 0.64 \text{ g kg}^{-1}$ on Day 14 ($P > 0.01$) and $8.24 \pm 1.31 \text{ g kg}^{-1}$ on Day 21 ($P < 0.01$), in comparison to the fresh plant (Figure 2).

Protodioscin in *Brachiaria brizantha* and *B. decumbens* was degraded under the silage conditions employed in this study. Similar research has been performed with alfalfa silage. And KALAC et al. (1996) measured saponin changes in alfalfa over 7 and 90 days of ensilage, and saponin losses ranged from 38% to 100% depending on the specific saponin. OLIVEIRA et al. (2003) also reported saponin degradation of 55-90% in alfalfa silage over 33 days. In addition, PINOS-RODRIGUEZ et al. (2008; 2009) also found that ensiling *Agave salmiana* led to reductions in saponin levels.

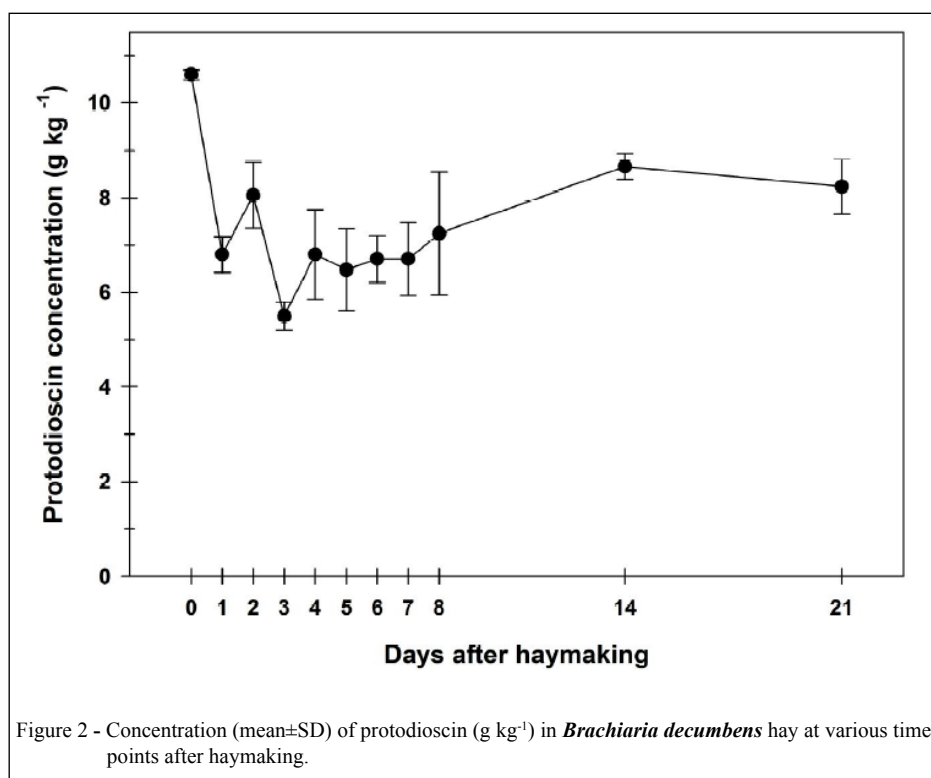
Harvesting forage initiates a complex chain of events that can affect quality and concentrations of secondary compounds. Forage quality declines during the post-harvest phase because plant enzymatic activity and microbial growth continue (MOSER, 1980). Aerobic respiration may result in the loss of some sugars in the first stage of the ensiling process. The second stage involves anaerobic bacterial action and the fermentation reactions of the ensiling process, when carbohydrate fermentation occurs (MOSER, 1980).

Losses of sugars during hay wilting and the first step of ensiling, by plant respiration and microbial

growth could explain the reduction of protodioscin in the present study. The typical steroidal saponin molecule has an aglycone or non-saccharide portion (sapogenin) attached to one or more sugar chains containing glucose, galactose, xylose, arabinose, rhamnose or glucuronic acid (HOSTETTMANN & MARSTON, 1995; WINA et al., 2005). The extensive enzymatic activity could explain the decrease in the level of intact saponins, by the cleavage of the sugar side chains (KALAC et al., 1996).

In a previous study, LIMA et al. (2012) found that the haymaking process and storage reduced the levels of protodioscin in *Brachiaria brizantha* samples by about 46%. The amount of protodioscin in the fresh plant was of 18.7 g kg^{-1} in contrast to 8.6 g kg^{-1} in the hay. Their results in hay are similar to the 48% reduction in protodioscin concentration observed after Day 3 of *Brachiaria decumbens* hay in this study.

The reduction of saponin during wilting in haymaking calls into question the methodology of studies that quantified steroidal saponins in grasses. There is no standardized methodology for drying grass samples before analysis. Several experiments have dried grass samples using various methods: air-drying, drying at room temperature, freeze-drying, or drying in a forced-air oven. Furthermore,



some studies do not report the drying method used (BRUM et al., 2007; CASTRO et al., 2011). For this reason, the amount of steroidal saponins described in the current literature may be underestimated when compared to the fresh plant and with what animals are really being fed. Studies are needed to determine the effect of drying method on quantification of steroidal saponins.

The spores of *Pithomyces chartarum* fungus can contain the hepatotoxic sporidesmin that can cause hepatogenous photosensitization like *Brachiaria* poisoning. *P. chartarum* normally produces sporidesmin, but some strains do not produce sporidesmin. Most researchers agree that the isolated forms of New Zealand and Australia produce more sporidesmin than that from the Americas. LEMOS et al. (1996) examined 30 isolated cases of *P. chartarum* from *B. decumbens* with a history of outbreaks in sheep in Brazil, and only one isolate produced sporidesmin. They concluded that the disease was caused by *B. decumbens* and not by the toxin from the fungus. COLLIN et al. (1998) evaluated 51 isolates of *P. chartarum*, including 9 samples from Brazil, only a single Brazilian sample produced sporidesmin (2%), while isolates from New Zealand produced 86% (391), from Australia 67% (207) and from Uruguay 28% (182). HALDER et al. (1981) analyzed samples from North America, and did not observe the production of sporidesmin. DNA analysis proved that the strains producing sporidesmin from New Zealand are different than those non-producing from South Africa and the Americas (BEEVER and PARKER, 1993). Until now, there is not a strong evidence that strains from Brazil produce sporidesmin and the causes of hepatogenous photosensitization are mainly attributed to *Brachiaria* poisoning.

The periodic incidence of liver toxicity in ruminants grazing on *Brachiaria* spp. pasture is problematic. However, the use of ensiling and haymaking to preserve *Brachiaria* forage may reduce the incidence of toxicity in ruminants fed with these prepared forages compared to ruminants grazing on *Brachiaria* pastures. Animal toxicity studies will be necessary to verify these results for the livestock industry.

CONCLUSION

The process of ensiling and making hay reduced the levels of the steroidal saponin protodioscin in *Brachiaria brizantha* silage and in *Brachiaria decumbens* silage and hay.

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