Weather and Plant Age Affect the Levels of Steroidal Saponin and *Pithomyces chartarum* Spores in *Brachiaria* Grass

Flavia G. Lima[1*], Mitsue Haraguchi[2], James A. Pfister[3], Victor Y. Guimaraes[1], Diogo D. F. Andrade[1], Carolina S. Ribeiro[1], Gustavo L. Costa[1], Ana Luiza L. Araujo[2], and Maria Clorinda S. Fioravanti[1]

[1]Federal University of Goias, Goiania, GO, Brazil
[2]Biological Institute, Sao Paulo, SP, Brazil
[3]USDA-ARS, Poisonous Plant Research Laboratory, Logan, UT, USA

*Corresponding author: Flavia G. Lima, flaviamedvet@yahoo.com.br

Abstract

Brachiaria species are cultivated worldwide in tropical and subtropical climates as the main forage source for ruminants. Numerous tropical and warm-season grasses cause hepatogenous photosensitization, among them several species of *Brachiaria.* Steroidal saponins present in these plants may be responsible for liver damage. However, sporidesmin and other hepatotoxic mycotoxins are likely to produce synergistic effects, which could explain the sporadic incidence of poisoning. The objective of this study was to evaluate the relation between steroidal saponin (measured by HPLC/ELSD), *Pithomyces chartarum* spores present in plots of *Brachiaria*, and the possible influence of meteorological factors on saponin and spores levels. The saponin detected in *B. brizantha* and *B. decumbens* was protodioscin. The saponin concentration was higher in immature plants during early growth; further, *B. decumbens* had a higher amount of saponin than found in *B. brizantha*. The level of saponin concentration was moderately influenced by weather variables; the two main variables were the maximum sunshine duration and total cumulative precipitation. *P. chartarum* spore count was higher in older plants and did not differ between *B. brizantha* and *B. decumbens*. Spore counting was influenced by maximum precipitation and average evaporation (Class A pan).

Keywords: protodioscin, sporidesmin, tropical grasses

Introduction

Brachiaria species are cultivated worldwide in tropical and subtropical climates due to their high productivity, and these grasses are critical for meat and milk production in many countries as the main forage source for ruminants (Miles et al. 1996).

The extraordinary reliance on *Brachiaria* in Brazil, as in other tropical countries, is justified by the advantages that this grass has over other species. *Brachiaria* spp. provide excellent yields of green matter in soils with low and medium fertility. They are drought tolerant, resistant to pests, and are well adapted to acidic and poor soils. These species are not only valuable plants for erosion control as a ground cover, but they also tolerate heavy grazing. Moreover, they have the ability to adapt to different environments; they are aggressive and compete with the native vegetation. Further, the seeds have resistivity which allows these plants to establish in, and dominate, native vegetation (Dias-Filho 2000, Argel et al. 2007, Hare et al. 2009).

Pithomyces chartarum (Berk & Curt) M. B. Ellis is a broad-based saprophytic fungus found in tropical, subtropical, and temperate regions. It is mostly found in dead plant material in native pastures, primarily those that are cultivated. Numerous conidia (spores) are produced under

favorable environmental conditions and contain the hepatotoxic sporidesmin, which can cause hepatogenous photosensitization. Experimental reproduction of the fungus indicates that most of the samples (isolates) from Australia and New Zealand produce sporidesmin (Di Menna et al. 2009).

Saponins are substances found in at least 400 plant species, many of which are used as food or supplements in ruminant nutrition. Saponins have beneficial effects as growth promoters, but they sometimes are toxic to ruminants. Saponins are derivatives of secondary plant metabolism, related to the plant defense system, and are mainly found in tissues that are most vulnerable to fungal or bacterial attack or insect predation (Wina et al. 2005). *Brachiaria* species and other plants (*Panicum* spp., *Tribulus terrestris*, and *Narthecium ossifragum*) contain steroidal saponins, which are associated with deposition of crystalloid material in the biliary system, cholangitis, and hepatogenous photosensitization (Driemeier et al. 2002, Brum et al. 2007, Fagliari et al. 2007, Uhlig et al. 2007, Botha and Penrithb 2008, Badiei et al. 2009, Lee et al. 2009, Assumaidaee et al. 2010, Saturnino et al. 2010).

The objective of this study was to evaluate the relation between steroidal saponins and *Pithomyces chartarum* spores present in plots of *Brachiaria* and the possible influence of meteorological aspects of the environment on concentration of saponins and spore counts.

Materials and Methods

Twenty-four plots measuring 3x4 m were planted, 12 with *B. decumbens* cv. Basilisk and the other 12 with *B. brizantha* cv. Marandu, at the Federal University of Goiás, Goiânia, Brazil (16°36'11,35"S 49°16'06,50"W; elevation 770 m). Each plot had 14 lines of plants; the outside lines were not harvested, and two inside lines were harvested at each sample period.

Nitrogen fertilization was applied after an initial soil analysis. Nitrogen was applied evenly to all plots at one of three levels (0, 50, 100 kg/hectare), 30 days after planting.

Plant development was followed up for 12 months. The plants were sampled on days 60, 120, 180, 240, 300, and 360 after planting to determine spore concentration and saponin concentration during this period. Plants were clipped near ground level (5cm), and the green material was collected to determine the quantity of spores and saponin concentration. Although the total plant was collected, the leaves and stems were subsequently separated and the analysis was only done on leaf material.

Spore Counting

The morphologic identification of the fungal spores from *Pithomyces chartarum* was based on Dingley´s (1962) description. The technique used for spore counting was adapted from Di Menna and Bailey (1973). Leaves were cut into 2 cm pieces. Sixty grams of fresh grass were mixed by hand in 600 mL of water. After 1 minute of agitation, spores were counted using a microscope counting chamber (hemocytometer). The quantities of spores counted in a 2mm³ are multiplied by 5,000 which provides the quantity of spores in 1g of forage.

Extraction and Isolation of Saponin

The leaves of *B. decumbens* and *B. brizantha* were dried at room temperature and ground in the Wiley mill (3 mm). The dried material was stored in airtight bags and analyzed about 6 months after each sampling.

Extraction of saponin

Dried powdered leaf samples were processed in duplicate. Each sample (1 g) was extracted three times in 50% acetonitrile and water (v/v) using volumes of 8 mL, 5 mL, and 3 mL under ultrasonic agitation for 30 min, 30 min, and 20 min, respectively. Next, the extracts were transferred to a test tube and centrifuged for 30 min at 5,000 rpm at 13ºC. The extracted solution was completed to 10 mL volume for analysis using HPLC/ELSD (high-performance liquid chromatography/evaporative light scattering).

HPLC/ELSD

Saponin was quantified using a modification of the technique of Ganzera et al. (2001). The analysis was done with a Shimadzu HPLC, model LC-10AD coupled to the detector. The evaporative light-scattering detector from Shimadzu model ELSD-LTs was used for quantitative analysis of protodioscin. The column used was a Shim-Pack CLC-ODS (4.6 x150 mm, 5.0 µm) using a gradient system with acetonitrile (B)/water (A), starting at 20% B for 5 min, 35% B for 12.5 min, and 20% B for 5 min; flow 1mL/min. The injection volume was 10 µL using a 10 µL loop.

Determination of Meteorological Parameters

Meteorological dates refer to the daily averages of each period (interval of 60 days). Data were

acquired in Goiânia, Goiás, Brazil, from December 2007 to December 2008 and provided by the meteorological station of the Federal University of Goiás, about 1 km from the plots.

Twenty-five variables were analyzed: the mean, maximum, and minimum of sunshine duration (Campbell-Stokes sunshine recorder); solar radiation; relative humidity; maximum temperature; minimum temperature; evaporation (class A pan); evaporation (Piche evaporimeter); and precipitation (mean, maximum, cumulative/period, and total cumulative). The equipment and measurements used adhered to the international standard (WMO 2008).

Statistical Analysis

Data for spores were tested for normality and were not normally distributed. The evaluation of the spores and saponin concentration between groups were assessed using the non-parametric Mann-Whitney U test with a significance level of 5% (Sampaio 2007). For the analysis within groups at each level of nitrogen, the Kruskal-Wallis test was used. The correlation between spore counts, saponin concentration, and meteorological parameters was examined using Pearson's correlation and multiple regression.

Correlation analysis and multiple regression procedures with influence diagnostics (e.g. residuals, examining outliers, and leverage) were used in SAS (2007) to determine their relationships. After this initial analysis, a stepwise multiple regression was used in an exploratory analysis to screen independent variables, followed by a hierarchical multiple regression with two variables introduced in a specific order for the final regression equations. Multi-co-linearity is loosely defined as a regression model in which two or more predictor variables are highly correlated; this greatly inflates the R^2 value. The Variance Inflation Factor (VIF) is a common method for detecting multi-co-linearity. VIF (the reciprocal of the tolerance) was examined for each model, and models with VIF greater than 4 were discarded (Belsley et al. 1980). A VIF of this magnitude indicates that the variance of the estimated coefficient is more than 4 times larger than it would be if the predictors were not correlated. The condition index was also used as another indicator of co-linearity; a condition index greater than 30 suggests that some variables are highly correlated, and the model should be refit (Yu 2000). The variables were discarded with a condition index greater than 15. Additionally, co-linearity was diagnosed by recognizing variables that had large proportions of variance (0.50 or more) that corresponded to large condition indices.

The best models were defined as those that had a high R^2, were significant at $P < 0.05$, and had a relatively small value of Mallow's *Cp* statistic, where *Cp* was approximately equal to the number of terms in the model. *Cp* tends to find the best subset of the model that includes only the important predictors of the dependent variable. The data were not compared for R^2 between periods and species because the variances of some of the independent variables were not equal.

Because the purpose of this study was to determine if there was a relationship between the weather and concentrations of saponin and the spore counts, there was no cross validation attempted in this study to forecast levels of saponin and spore count in other populations of plants.

Results

The saponin detected in *B. brizantha* and *B. decumbens* was protodioscin. The level of nitrogen fertilization did not influence ($P > 0.10$) the amount of spores or saponin concentration. For this reason the results were grouped by species of *Brachiaria*, independent of the level of fertilization (table 1 and figure 1).

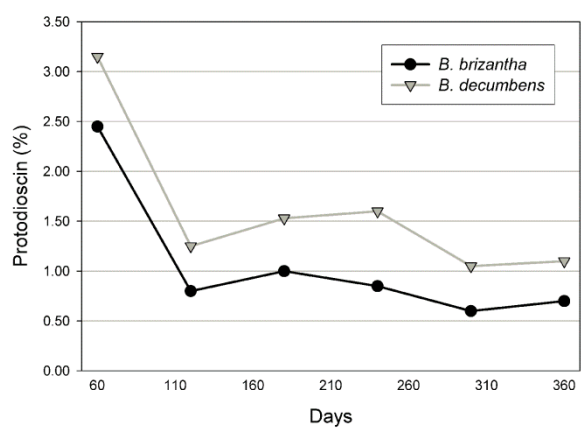

Figure 1. Average saponin concentration (% of dry weight) in *Brachiaria brizantha* and *Brachiaria decumbens*.

The level of saponin detected in *B. decumbens* was higher ($P < 0.05$) than in *B. brizantha* at all time periods. Both of the *Brachiaria* species had the highest average concentration of saponin during the first sampling period at 60 days ($P < 0.05$), then decreased around 70 percent at 120 days and the lowest level was observed at 300 days.

Table1. Saponin average concentration (%) and spore counts (*P. chartarum*) in *Brachiaria brizantha* (BB) *and Brachiaria decumbens* (BD)[1], shown by day of harvest after planting (day 0)

Day/Species	Saponin		Spores	
	BB	BD	BB	BD
60 days	2.39±0.30[a]	3.15±0.52*[a]	3,750±3,750[a]	3,333±3,888[a]
120 days	0.81±0.20[bc]	1.26±0.37*[b]	1,250±1,875[a]	417±763[a]
180 days	0.99±0.48[ab]	1.56±0.50*[b]	8,750±7,083[abc]	7,083±4,236[ab]
240 days	0.83±0.21[bc]	1.65±0.28*[ab]	5,833±3,750[ab]	2,917±2,916[a]
300 days	0.61±0.09[c]	1.06±0.35*[b]	39,167±19,166[c]	36,250±26,458[b]
360 days	0.70±0.21[bc]	1.11±0.30*[b]	24,167±13,472[bc]	34,583±26,111[b]

[1] BB = *B. brizantha*; BD = *B. decumbens*. Plants were sampled at the intervals shown by clipping leaves to near ground level.

[2] Analysis was done using the Kruskal-Wallis test. Asterisks in the same row indicate that the results between plant species differ at $P < 0.05$ *Brachiaria* (BB and BD). Different letters in the same column indicate that the results differ over time ($P < 0.05$) within each species of *Brachiaria*.

The amount of spores ranged from zero to 145,000 spores/g of forage. The spore count in older plants (300 and 360 days) was higher ($P < 0.05$) than in young plants (60 and 120 days) in both species. Several plots of both species had no spores at various time periods, with the exception of day 300, when all the plots had some spores (figure 2).

There was no correlation between the amount of saponin and spores over the periods analyzed ($R^2 = -0.26$). Weather variable means are presented in table 2 to show seasonal variation. The correlations between the main meteorological parameters and the amount of saponin and spores are shown in table 3.

Although the maximum sunshine duration and maximum precipitation were not highly correlated (table 2) with saponin concentration or spore count, these parameters were entered into the multiple regression models and both, with other variables, explained a substantial amount of the variation. Maximum hours of sunshine duration and total cumulative precipitation were related to saponin concentration (R^2=0.55; Cp=3.0) by:

$$y = -0.14 + 0.34\ (\textit{Sunshine Duration Max}) - 0.002\ (\textit{Precipitation Total Cumulative})$$

Maximum precipitation and mean evaporation (Class A pan) were the best 2-variable model (R^2=0.40; Cp=0.32) explaining a substantial portion of the variation associated with spore counts:

$$y = -70923 + 126.8\ (\textit{Precipitation Max}) + 14812\ (\textit{Evaporation Class A Pan Mean})$$

Discussion

For saponin analysis, we used high-performance liquid chromatography. Due to the polar nature of this compound, HPLC is usually chosen for quantitative analysis. The ELSD detector reduces the interference of impurities and the gradient elution of the mobile phase improves the accuracy for saponin determination (Yang et al. 2003).

In the present study, saponin concentrations decreased in both BB and BD plants as they matured. The results are similar to those of Castro et al. (2011). They studied the levels of protodioscin in different *Brachiaria* spp. at various stages of maturity, and they concluded that young plants in the growth phase have a higher amount of saponin than older plants. Castro et al. (2011) reported that protodioscin concentrations of 0.3% to 2.56% were sufficient to induce hepatogenous photosensitization when fed to sheep.

Additionally, *B. decumbens* had higher levels of saponin than *B. brizantha*, which was also reported by Lee et al. (2011). The steroidal saponin protodioscin identified in this study has been described in many plants: *Dioscoera gracillima*, *Melilotus tauricus*, *Dioscoera colletti*, *Asparagus officinalis* L., *Smilax china* L., *Costus speciosus* (Ahmad and Basha 2006), *Panicum virgatum* L. (Lee et al. 2009), and especially in *Brachiaria* spp. (Riet-Correa et al. 2011).

Ferreira et al. (2011) studied the amount of protodioscin in *B. brizantha* as influenced by various levels of maturity, and examined the relationship between saponin, sunshine duration, temperature, and relative humidity. They found that young leaves

Table 2. Weather variables (average per 60-day interval) measured at the Federal University of Goiás, Goiânia, Brazil, near the *Brachiaria* spp. plots. Days correspond to periodic harvesting of *Brachiaria* spp. forage after planting from cultivated plots

Variables	Days	60	120	180	240	300	360
	Months	Dec-Feb	Feb-Apr	Apr-Jun	Jun-Aug	Aug-Oct	Oct-Dec
	Seasons	Summer	Autumn	Autumn	Winter	Winter/ Spring	Spring
Sunshine Duration (hours/Day)	Mean	4.3	5.3	7.9	8.9	8.1	5.6
	Max	9.9	9.7	9.8	9.8	10.2	10.7
	Min	0.0	0.0	0.0	6.2	0.0	0.0
Solar Radiation (MJ/m²)	Mean	17.2	16.8	15.8	17.6	22.5	17.9
	Max	28.9	22.6	20.2	21.2	29.1	29.8
	Min	1.3	8.8	4.1	12.2	5.8	1.6
Relative Humidity (%)	Mean	85.2	85.3	72.0	63.1	57.9	69.5
	Max	95.0	95.0	91.0	73.0	93.0	84.0
	Min	75.0	76.0	54.0	54.0	41.0	53.0
Precipitation (mm)	Mean	9.1	8.9	3.3	0.0	1.6	5.4
	Max	42.0	56.4	100.7	0.0	36.4	80.8
	Min	0.0	0.0	0.0	0.0	0.0	0.0
	Cumulative/ Period	437	527	196	0	97	324
	Total Cumulative	437	963	1159	1159	1256	1580
Max Temperature (°C)	Mean	29.6	30.1	29.7	30.4	32.9	31.6
	Max	33.4	34.2	32.4	34.6	37.2	37.8
	Min	23.8	23.6	20.7	23.2	22.2	26.2
Min Temperature (°C)	Mean	19.1	19.1	14.3	10.2	14.5	19.1
	Max	20.8	20.4	20.3	14.8	21.8	21.8
	Min	17.4	17.0	9.2	5.8	7.8	14.8
Evaporation – Class A pan (mm)	Mean	4.8	4.3	4.5	5.0	7.0	6.1
	Max	9.6	9.8	9.7	8.9	10.6	10.0
	Min	0.7	0.5	0.2	0.9	2.1	2.1
Evaporation – Piche evaporimeter (mm)	Mean	2.7	2.4	1.7	3.2	3.9	2.8
	Max	4.7	6.2	4.2	9.3	18.5	8.0
	Min	0.8	0.1	0.0	1.0	0.1	0.2

Table 3. Pearson correlation between weather variables, saponin concentration, and spore counts for *Brachiaria* forage harvested every 60 days after planting at the Federal University of Goiás, Goiânia, Brazil

	Saponin		Spores	
Variable	r	P	r	P
Sunshine Duration Mean	-0.42	<0.0001	0.10	0.2377
Sunshine Duration Max	0.12	0.1410	0.22	0.0097
Relative Humidity Mean	0.37	<0.0001	-0.43	<0.0001
Relative Humidity Min	0.50	<0.0001	-0.41	<0.0001
Precipitation Max	0.06	0.4502	0.08	0.3288
Precipitation Total Cumulative	-0.56	<0.0001	0.40	<0.0001
Max Temperature Mean	-0.51	<0.0001	0.55	<0.0001
Max Temperature Max	-0.24	0.004	0.49	<0.0001
Evaporation (Class A pan) Mean	-0.37	<0.0001	0.50	<0.0001
Evaporation (Class A pan) Max	0.06	0.4544	0.28	0.0008
Evaporation (Class A pan) Min	-0.38	<0.0001	0.54	<0.0001
Evaporation (Piche) Mean	-0.23	0.0047	0.34	<0.0001
Evaporation (Piche) Max	-0.34	<0.0001	0.49	<0.0001
Evaporation (Piche) Min	0.44	<0.0001	-0.25	0.0025

had higher levels of protodioscin ($P < 0.01$) than did mature and old leaves (3.61±1.12%, 1.94±0.97%, and 1.01±0.79%, respectively); saponin variation in young leaves was related ($r = 0.9$; $P < 0.05$) to sunshine duration and in mature leaves with Meteorological data have suggested that higher levels of saponins in *Brachiaria* spp. are found during periods with greater rainfall (Barbosa et al. 2006, Moreira et al. 2009). In the present study, the duration of sunshine and maximum ambient temperature showed a negative correlation ($r = -0.42$ and $r = -0.51$, respectively) with saponin and a positive correlation with minimum relative humidity mean ($r = 0.50$). There was a negative correlation between saponin concentration and total cumulative precipitation ($r = -0.56$). The harvest at 60 days with high precipitation (437 mm) had the highest percentage of saponin. However, the same result was not repeated at the harvest of 120 days (527mm) and 360 days (324mm) when precipitation was also high. These results suggest that age of the plant is the major influence on saponin levels and not precipitation. These results also suggest that saponin concentrations may be a complex interaction involving several factors, including plant age, geographic location, and cultivation conditions (Yang et al. 2003).

Contrary to our results, Brum et al. (2009) evaluated saponins by a quantitative method (thin layer chromatography and spectrophotometric analysis) and found larger quantities of protodioscin in mature *Brachiaria* during seed shatter. The range of saponin concentration in *B. brizantha* was 0.53% to 2.09%, greater than that found in *B. decumbens* at 0.8% to 1.9%. Outbreaks of hepatogenous photosensitization were evaluated in Pará State, Brazil, where Albernaz et al. (2010) and Silveira et al. (2009) found toxic levels of protodioscin for sheep and goats grazing on *B. brizantha* in an advanced state of maturity during drought period. Saturnino et al. (2010) did not measure the levels of saponin but found that mature *B. decumbens* during seed shatter poisoned sheep.

The toxic dose of the saponin protodioscin from *Brachiaria* spp. for ruminants has not been clearly established (Brum et al. 2009). Outbreaks of hepatogenous photosensitization were detected in sheep that were fed *Brachiaria* containing 0.3% to 2.56% protodioscin (Castro et al. 2011), 0.88% protodioscin (Albernaz et al. 2010), 2.36% protodioscin (Brum et al. 2007), and for goats 1.54% protodioscin (Silveira et al. 2009). However, cattle fed with *Brachiaria* containing 1.09% (Moreira et al. 2009) and 1.63% (Brum et al. 2007) protodioscin did not develop clinical signs of disease.

The ideal conditions for *Pithomyces chartarum* growth have been described as pastures with old leaves and mulch, relative humidity 60%, and ambient temperature from 18 to 27°C. However, the production of sporidesmin is optimal when relative humidity remains at 100 percent and temperature remains around 20 to 25°C. These conditions occur when the rainy season is followed by a dry, hot period, which is ideal for pasture growth (Brook 1964, Bars et al. 1990, Alvariza 1993, Russomanno et al. 2003, Di Menna et al. 2009). This study shows that the periods of grass growth of 300 and 360 days had ideal conditions for fungal growth with plenty of old leaves and decaying material, high temperatures, precipitation, and evaporation. During these periods, the amount of spores was significantly higher ($P < 0.05$) compared with earlier periods with young plants (60 and 120 days).

Moreira et al. (2009) evaluated the spore counts in *Brachiaria* spp. in Goiás State, Brazil, where cattle showed no clinical signs of the disease and observed that in the dry season, counts ranged from 5,000 to 40,000 spores/g of pasture, and during the rainy season the score was higher, up to 50,000 spores/g of pasture. However, pastures throughout these experiments attained a maximum height of 6 cm and also did not have old leaves, conditions that did not favor the growth of the fungus.

Clinical and sub-clinical lesions were observed in cattle grazing on *Brachiaria* spp. containing 0 to 15,000 spores/g of pasture (Fioravanti 1999). The absence of spores or low spore counts do not exclude the fungus as the etiologic agent of sporidesmin toxicosis because there is a lag in the time between when spores appear and another lag from the time of ingestion of sporidesmin and appearance of lesions (Smith 2000, Di Menna et al. 2009).

The sporidesmin detected in plant material corresponded to the number of spores found in the sample in data from Oceania. However, this relationship is not absolute and discrepancies have been reported (Collin et al. 1995, Di Menna et al. 2009).

The fungus *P. chartarum* normally produces sporidesmin but some strains do not produce sporidesmin. Most researchers agree that the isolates from New Zealand and Australia produce more sporidesmin than those 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 sporidesmin (86% of 391 samples), as did those from Australia (67% of 207) and Uruguay (28% of 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 strains from South Africa and the Americas (Beever and Parkes 1993).

Until now the small number of isolates studied from Brazil is not sufficient to conclude that the strains of Brazil do not produce sporidesmin. Future studies should be developed with Brazilian samples to determine conclusively if the type and amount of fungal growth produces sporidesmin. The interaction of saponin type and concentration and toxicity in ruminants also requires further investigation.

Conclusions

The saponin found in *B. brizantha* and *B. decumbens* in Goiás, Brazil, was protodioscin. The saponin concentration was higher in immature plants during early growth; further, *B. decumbens* had a higher concentration of saponin than *B. brizantha*. The most important weather variables that influenced the saponin concentration were maximum duration of sunshine and total cumulative precipitation.

The *P. chartarum* spore count was higher in older plants and did not differ between *B. brizantha* and *B. decumbens*. Spore counting was influenced primarily by maximum precipitation and average evaporation (Class A pan).

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