UNIVERSITY OF SÃO PAULO
FACULTY OF ANIMAL SCIENCES AND FOOD ENGINEERING

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Primary and secondary metabolites production in signal grass around the year under nitrogen fertilizer

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Thesis presented to the faculty of Animal sciences and Food engineering of Uni. of Sao Paulo for obtaining the title of Doctor in science

Specialization: Animal Production and Quality

Supervisor: Dr. Valdo Rodrigues Herling

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SYEDA MARYAM HUSSAIN

سیدہ مریم حسین

DEDICATION

PARENTS

I just owe almost everything to my father [and] it's passionately interesting for me that the things that I learned in a small town, in a very modest home, are just the things that I believe have won the election.

Margaret Thatcher

ماں کے نام

یہ کامیابیاں، عزت، یہ نام تم سے ہے
خدا نے جو بھی دیا ہے مقام، تم سے ہے
تمھارے دم سے ہیں میرے لہو میں کھلتے گلاب
میرے وجود کا سارا نظام، تم سے ہے
کہاں بساطِ جہاں، اور میں کمسن و ناداں
یہ میری جیت کا سب اہتمام، تم سے ہے
جہاں جہاں ہے میری دشمنی، سبب میں ہوں
جہاں جہاں ہے میرا احترام، تم سے ہے

"Indeed, ALLAH Subhanahu Wa Ta'ala will not change the condition of a people until they change what is in themselves."

[Qur'an, 13:11]

ABOUT THE AUTHOR

SYEDA MARYAM HUSSAIN, daughter of SYED SULTAN HUSSAIN and MUKHTIAR BEGUM, born in Mardan, KPK – Pakistan on 30th May 1984.

In 1998, she completed Secondary education in Services Public School, Mardan getting 2nd position in school over all.

In 2000, she finished faculty of science (Pre-Medical) (intermediate) in Govt. girl's degree college Mardan and was the top student of the year for English and Urdu literature.

In January 2001, she gets admission in Animal Husbandry (B.Sc (HONS)) at Agricultural uni. Peshawar and awarded as Silver Medalist in 2005. She continues her (M.Sc. (HONS)) in Animal Nutrition in the same university.

In the following year, she opted for English Literature and Economics from Uni. of Peshawar for a sideline education.

In March 2006, she joined the Livestock and Dairy Development Board (Civil Veterinary Hospital, Abbottabad) as Livestock Production Officer along with continuing her M.Phil.

In 2007, was selected for outstanding yearly achievements by South Asian publications (SAP) and in the same year; she gained Indigenous Scholarship for Ph.D. by Higher Edu. Commission of Pakistan.

Upon completion of the master; she started short term consultancies (Livestock & veterinary, Water and sanitation) for training conductions and manuals preparation for USAID (US), World Vision (CANADA), Church World Services (CWS), Relief international (UK) and Islamic Relief (UK) and Swiss development corporation (SWC) and many national NGO's.

In September 2009; she was selected as female Livestock instructor for AHITI (animal husbandry in-service training institute) –Livestock and Dairy Dev. Board, Govt. of KPK.

Right back she joined again the NGO's sector in 2010 and consultancies on Women and minority rights, Water Sanitation, and Hygiene.

In Dec. 2012; she got the CNPq-twas fellowship for Brazil in the area of Animal Production and Quality at Faculty of Animal Science and Food Engineering, University of São Paulo, conducting research in the area of pastures and Forage Crops. She passed his synopsis board of studies on 6th October 2014.

In, Sept. 2015, she went IUNG, Poland (Deptt. of Biochemistry) for 04 months for abroad research component.

She defended her Ph.D. on May, 31st, 2016.

ABSTRACT

HUSSAIN; Syeda Maryam. Primary and secondary metabolites production in signal grass around the year under nitrogen fertilizer application. Thesis (Ph.D.) 108 p. – Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, 2016.

Plants produce a number of substances and products and primary and secondary metabolites (SM) are amongst them with many benefits but limitation as well. Usually, the fodder are not considered toxic to animals or as a source having higher SM. The *Brachiaria decumbens* has a considerable nutritional value, but it is considered as a toxic grass for causing photosensitization in animals, if the grass is not harvested for more than 30 days or solely. The absence of detailed information in the literature about SM in *Brachiaria*, metabolites production and its chemical profile enable us to focus not only on the nutritive value but to get answers in all aspects and especially on toxicity. The study was conducted in the period of december 2013 to december 2014; in greenhouse FZEA-USP. *B. decumbens* was used with two cutting heights (10 and 20 cm) and nitrogen doses (0, 150, 300 and 450 kg ha^{-1}) in complete randomized block design. The bromatological analysis were carried out on near infrared spectroscopy. Generally, the application of 150 kg ha^{-1} N was sufficient to promote the nutritional value in *B. decumbens* but above it the nitrogen use efficiency decline significantly. The highest dry matter yield (99.97 g/pot) was observed in autumn and the lowest was in winter (30.20 g/pot). While, as per nitrogen dose the average highest dry matter yield was at 150 kg ha^{-1} (79.98 g/pot). The highest crude protein was observed in winter (11.88%) and the lowest in autumn (7.78%). By the cutting heights; the 10 cm proved to have high CP (9.51%). In respect of fibrous contents, the highest acid detergent fiber was noted in summer (36.37%) and lowest in winter (30.88%). While the neutral detergent fiber was being highest in autumn and lowest in spring (79.60%). The highest in vitro dry matter and organic matter digestibilities were noted at 300 kg ha^{-1} N; being 68.06 and 60.57%; respectively; with the lowest observed in without N treatments (62.63% and 57.97), respectively. For determination of the classes, types and concentration of SM in *B. decumbens*, phytochemical tests, thin layer and liquid chromatography-mass spectrometry and nuclear magnetic resonance analysis were carried out. Height, nitrogen and seasons significantly ($P < 0.0001$) affected the secondary metabolic profile. A new protodioscin isomer (protoneodioscin (25S-)) was identified for first time in *B. decumbens* and is supposed to be the probable

toxicity reason. Its structure was verified by 1D and 2D NMR techniques (^{1}H, ^{13}C) and 1D (COSY-45, edited HSQC, HMBC, H2BC, HSQC -TOCSY, NOESY and 1 H, 1 H, J). All factors influence the metabolic profile significantly (P <0.0001). The lowest phenols were at 300 kg ha^{-1} while the lowest flavones were at 0 kg ha^{-1}. Season wise the highest phenols occurred in autumn (19.65 mg/g d.wt.) and highest flavones (28.87 mg/g d.wt.) in spring. Seasons effect the saponin production significantly (P <0.0001) and the results showed significant differences in the protodioscin (17.63±4.3 – 22.57±2.2 mg/g d.wt.) and protoneodioscin (23.3±1.2 – 31.07±2.9 mg/g d.wt.) concentrations. The highest protodioscin isomers concentrations were observed in winter and spring and by N doses the highest were noted in 300 kg ha^{-1}. Simply, all factors significantly played their role in varying concentrations of secondary metabolites.

Keywords: Protoneodioscin, metabolites, photosensitization, nitrogen, season, Basilisk

RESUMO

HUSSAIN; Syeda Maryam. Produção de metabólitos primários e secundários em capim-braquiária em adubação nitrogenado. Tese (Doutorado) 108 f.- Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, Pirassununga, de 2016.

As plantas produzem diversas substâncias e produtos e os metabólitos primários e secundários (MS) estão entre eles, apresentando tanto efeitos benéficos como limitação de utilização. Geralmente, a forrageiras não são consideradas tóxicas aos animais ou como fonte de MS. As espécies de braquiárias são caracterizadas pelo alto valor nutricional, entretando a *Brachiaria decumbens* é a espécie mais tóxica para causar a fotossensibilização. A ausência de informações detalhadas na literatura a respeito de MS, sua produção e perfil químico nos faz focar não somente no aumento da produção de matéria fresca e no valor nutritivo da planta mas, em obter respostas em todos os aspectos e na redução de sua toxicidade. O estudo foi conduzido no período de dezembro 2013 ate dezembro 2014, em casa de vegetação localizada na FZEA-USP. Utilizou-se duas alturas de corte (10 e 20 cm) e quatro doses de nitrogênio (0, 150, 300 e 450 kg·ha^{-1}) em delineamento de blocos casualizados (4x2).

Altura, doses de N e estação afetaram significativamente o perfil de MS. Houve aumento na produção de saponina nas estações da primavera e outono devido ao estresse. A análise bromatológica foi feita por espectroscopia de infravermelho próximo. Geralmente, a aplicação de 150 kg·ha^{-1} de N foi suficiente para promover o valor nutricional na *B. decumbens*, entretanto acima desse valor a eficiência de uso de Nitrogênio decai significativamente. A maior produção de matéria seca (MS) (99,97 g/vaso) foi observada no outono e a menor foi no inverno (30,20 g/vaso). Embora, de acordo com a dose de nitrogênio, o maior rendimento médio de matéria seca foi de 150 kg·ha-1 (79,98 g/vaso). Observou-se que o maior teor de proteína bruta (CP) foi no inverno (11,88%) e o menor foi no outono (7,78%). Pelas alturas de corte, os 10 cm provaram ter alta CP (9,51%). A respeito do conteúdo fibroso, o maior teor de fibra detergente ácida foi observado no verão (36,37%) e o teor mais baixo no inverno (30,88%). Por outro lado, o teor da fibra em detergente neutro foi maior no outono e o menor teor na primavera (79,60%). As maiores digestibilidades in vitro da matéria seca e matéria orgânica foram observadas em 300 kg·ha-1 de N, sendo 68,06% e 60,57% com o menor valor observado em tratamentos sem N (62,63% e 57,97%), respectivamente.

Para determinação das classes, tipos e concentração de MS em *B. decumbens*, foram realizados testes por fitoquímico, cromatografia de camada fina, cromatografia líqu acoplada à espectrometria de massa e ressonância magnética nuclear.

Foi identificado um novo isômero de protodioscina (protoneodioscina (25S-) pra primeria vez na *B. decumbens* que é supostamente a provável razão da toxicidade. Sua estrutura foi verificada pelo 1D e 2D (1H combinação de 1D (1H, 13C) e a técnica 1D RMN (COSY-45, editado HSQC, HMBC, H2BC, HSQC-TOCSY, NOESY e 1H, 1H, J) como O-α-L -rhamnopyranosyl- (1 4) -O-β-D- glucopiranosil- (1 6) -O-β-D-6-O-acetylglucopyranosyl-] (1 2) - p-D-glucopiranosil-28 medicagen.

Todos os fatores influenciaram o perfil metabólico significativamente (P <0,0001). Para flavonas, a menor produção foi observada em outono (19,65 mg/g peso seco (p.s)) e a maior na primavera (28.87 mg/g p.s). As concentrações de saponina foram afetadas significativamente (P<0,0001) pelas estações e os resultados mostraram diferenças na protodioscina (17,63+4,3 - 22,57+2,2 p.s) e protoneodioscina (23,3+1,2 - 31,07+2,9 p.s). Os maiores teores da concentração dos isômeros de protodioscina foram observados no inverno e na primavera e em relação ao N aplicado, o maior teor foi de 300 kg·ha-1. Simplesmente, todos os fatores influenciaram significativamente a variação das concentrações dos metabólitos secundários.

Palavras-chaves: Basilisk, Protoneodioscin, metabólitos secundarios, fotosensibilização, nitrogênio, estação.

TABLES

FIGURES

ABBREVIATIONS

Acetonitrile	CH_3CN
Acid detergent fiber	ADF
Brachiaria decumbens	Bd
Carbon	C
Carbon dioxide	CO_2
Carbon nutrient balance hypothesis	CNBH
Crude protein	CP
Dry matter	DM
Evaporative Light-Scattering Detector	ELSD
Ferric chlorate	$FeCl_3$
Growth-Differentiation Balance hypothesis	GDBH
Hepatic photosensitization syndrome	HPS
Heteronuclear Multiple Quantum Correlation	HMQC
Hertz	Hz
High performance liquid chromatography	HPLC
Hydrochloric acid	HCl
In-vitro dry matter digestibility	IVDMD
Mass Spectral Detector	MSD
Methanol	CH_3OH / MeOH
Nano magnetic resonance	NMR
Neutral detergent fiber	NDF
Near infrared spectroscopy	NIRS
Nitrogen	N
Organic matter	OM
Percent	%
Plants secondary metabolites	PSM
Retention factor	Rf
Response factor	RF
Sulphuric acid	H_2SO_4
Thin layer chromatograph	TLC
Two dimensional	2 D
Ultra-performance liquid chromatograph	UPLC
Ultraviolet	UV

One directional	1 D
Two dimensional	2 D

UNITS

And other	et al.,
Degree celsius	°C
Et cetera	etc.
For example	e. g.
Gram per kilogram	g/kg
Hour	h
Litre	l
Milligram per kilogram	mg / kg
Milligram per litre	mg/L
Milliliter	ml
Microliter	µl
Ton / hectare	t/ha
Centrifugal force	g

CONTENTS

APPENDICES

1.1 Introduction

Brazil been a holder of one of largest herds in the world and these livestock are part of and as well directly and indirectly dependent on these pasturelands. Amongst these pasturelands, Brachiaria species are widely cultivated on 80 million hectare (m ha) (JANK 2014). But with continuous usage the pastures production is getting limited by the soil fertility (RAO et al. 1996). Not only the soil conditions but the abruptly occurring changes in the seasons are also causing a shift in the plant nutritional value and production (WERNER 1994).

As like other plants, the first priority from preventing this situation is the use of the mostly required nutrient “nitrogen”. Nitrogen is the most yield-limiting as well (HUBER; THOMPSON, 2007) but its application also depends on the environment, variety and expected yield. Therefore, in Brachiaria also not a single dose can be recommended to use all time. Along with the nutrient status; the cutting intensity and periods cause a fluctuation in plant production (BORTOLINI et al. 2004) and impose stressful conditions for plants as these are linked to forage regrowth. Because the cutting management plant can easily effect the developmental capacity and production, potential growth and productivity (SKONIESKI et al., 2011).

Although the plant productivity potential and certain traits e.g. height, leaves numbers are already genetically coded but the environment (temperature, luminosity etc.) role cannot be neglected in making timely changes or mutation in the plant’s genetic makeup. As this trend in tropical regions is very obvious influencing the forage production and quality. Obviously the nitrogen enhance the primary metabolism and enhance productivity but in respect of secondary metabolism; the response is not very clear esp. in case of saponin. As N participates in the plants biosynthesis processes (nucleic acids, hormones and chlorophyll (LAVRES; MONTEIRO, 2003).

Although the saponin found in Brachiaria isn’t a byproduct of N- metabolism but still is a component with C- base and N helps or managerial component in dividing the compounds for growth or defense as all factors are interlinked to photosynthesis and carbon or nutrient availability. But a competition between these factors can lead to the final product and the phenotypic appearance of plant. Bryant et al. (1983) suggested that the carbon/nutrient balance of individual plants strongly affects their allocation of resources to primary and secondary metabolites. Fertilization with N not only increase the leaves nutrient concentrations but also stimulates the leaf growth more than photosynthesis response. Thus, the carbohydrate concentrations and the carbon based secondary plant metabolites or defense compounds in leaves decline (MUZIKA; 1993).

But in Brachiaria there seems to have no relation of fertilization with saponin production (LIMA et al., 2009). Therefore, we investigated the effects of nitrogen fertilization upon the phenols, saponin, and primary metabolites as mediated by the sunlight hours, and humidity during seasons.

We have to focus not on nutritional value but mainly on decreasing toxicity as well. Although, the presence of furostanol saponins (protodioscin) in Brachiaria is now alarming for secondary photosensitization in ruminants. Tokarnia et al., 2002; categorized plant poisoning as one of the most lethal (mortality of 800,000 - 1,120,000 cattle/year) and causative of economic losses (160 -240 million US$) to Brazil. According to Riet-Correa at el. (2011) the majority of toxicity cases occurs on *B. decumbens* and its concentration vary with the type of soil, environmental factors (rain, temperature), plant developmental stage and seasons. Various results showed varying results as (FERREIRA et al. 2011) demonstrated the higher protodioscin concentration in young leaves (3.61%) than mature (1.94%) and old (1.01%) ones. As the animal's prefer to pick and consume younger leaves so it enhances the toxicity. According to (CHEEKE 1971); the role of polyphenols in plants are associated with metabolic functions, but has a good cope with saponin in intestine and rumen by making complex structures and compounds; thus decreasing the saponin toxicity. As animals also preferred mixture of various toxins in ecological aspects and natural defense by toxin - toxin neutralization concept (LYMAN T.D.; F.D. PROVENZA; J.J. VILLALBA; 2008).
The environmental conditions and management practices (MARCELINO et al., 2006) for controlling the plant maturity and flowering, stem elongation consequently increase the nutritional value of the forage (SANTOS 2004) and all these factors are interlinked.

But due to the resource allocation between primary and secondary metabolism as alleviated by the nitrogen metabolism; by compensation between C and other nutrients it not only enhance the plant production but favors the secondary metabolites and favors the non-structural carbohydrates. Therefore our objectives was to enhance the *Brachiaria* nutritional value, production and on the other side, to diminish the probable phytotoxic cause in animals.

1.1.1 Hypothesis

We hypothesize that the application of nitrogen fertilizer in *B. decumbens* will boost the growth and will produce greener portions especially leaves with a better nutritional value. Along with the nitrogen the managerial impact of cutting plants according to certain heights will define the more productive strategy with good nutritional value and with minimum concentration of saponin as well, balanced with a higher polyphenols

fractions for minimizing toxicity factor. The seasonal impacts will identify the specific environmental conditions for causing an increase in saponin concentration; responsible for making *B. decumbens* more toxic.

All the experimental factors of nitrogen application, plants cutting heights and seasons will help us in identifying the protodioscin isomers (protoneodioscin and protodioscin) and will also identify if lies any differences in their proportions. Also will elaborate that which fraction is the probable toxicity cause??

1.1.2 Objectives

The general objective of this study was to determine the furostanol saponin production (protodioscin isomers) and to assess the nutritional value of *B. decumbens* and to what extent they get affected by nitrogen, cutting heights and seasons?

1.1.2.1 Specific objectives

a. To determine the seasonal differences in the productive and nutritional value of *B. decumbens*.
b. To determine the effect of seasons and cutting heights on the plant production and nutritional value?
c. To assess the metabolites types, and concentrations as influenced by the experimental factors.
d. Will the nitrogen application behaves according to carbon based secondary metabolites (CBSM) theory and decrease the polyphenols productions?
e. The nitrogen and the cutting heights will follow the growth differentiation balance (GDB) hypothesis to influence the plant to produce more leaves and will improve the *B. decumbens* nutritional value.
f. To evaluate the protodioscin isomers, their concentrations as varied per cutting heights, seasons and nitrogen application.
g. To ascertain information on the differential proportion of protodioscin with protoneodioscin in *B. decumbens*, in respect of and irrespective of all experimental factors.

1.1.3 Properties of *Brachiaria*

The Brazilian cerrado occupies approximately 205 million hectares, in which 40% is occupied by natural pastures and *Brachiaria* occupy over 90 million ha (JANK 2014). It is used extensively due to rapid regrowth and good persistence under frequent defoliation (RIKA et al., 1991). Most of the Brazilian soils are oxisols (acidic and low fertile) has low productive potential (RESCK, 2001), and required fertilization.

Brachiaria species is originally from eastern Africa (KELLER-GREIN et al., 1996) and with C_4 photosynthetic pathway (CLAYTON; RENVOIZE, 1986). The cv. Basilisk was originated in Uganda, access 001058 EMBRAPA, Brazil and was first evaluated by CSIRO, Queensland. It is a perennial grass and belongs to Gramineae family, decumbent nature wise (height - 0.55 – 1.00 m), stoloniferous with leaf blades as 5-20 cm long with common axis of 1.0-8.0 cm long. It can be grown by seeds, broadcasting or planting (GIL et al., 1991), well adapted to humid, areas of rainfall (800 mm), bearing a drought of up to five months (CIAT, 1998). Nevertheless, it grows well on quick-drying, shallow, hillside soils, aggressive, high yielding, responds dramatically to nitrogen application (SHELTON, 2000). *B. decumbens* has some common attributes for enabling them to adapt to low-fertility acid soils:

A. Maintenance of root growth at the expense of shoot growth and responds to low fertility by increasing the root to shoot ratio up to 30% (RAO, et al., 1996).
B. Acquisition and use of both nitrate and ammonium forms of nitrogen through associative fixation cultivated in N-poor soils (REIS et al., 1999).

1.1.4 Effect of soil fertility on forage

Nitrogen; an important nutrient and macro mineral for plants (RAIJ, 1991), to enhance their production by improving the proteins and nucleic acids (MALAVOLTA 2006), manages the photosynthetic processes, plant growth, size of leaves and stems, tillering, ensure better force of regrowth after grazing/cutting by increase in the tissue flow ((DURU; DUCROCQ, 2000; CECATO et al., 1996). The natural and main source of N in the soil is the organic matter, and its availability depends on the mineralization process by microbes, but its uptake varies by the plant species, soil types, and climatic characteristics. However, excess N is bad, its dose must be applied in balance to the nutrients and optimally with the availability of light in pastures. As the carbon flow to the apical meristems is strongly influenced by the absorption and recycling of N (GASTAL et al., 1992).

Brachiaria has higher adaptation capacity to fertilizer, management, and climatic conditions. But still less information is available on the eco-physiological and environmental aspect (FONSECA et al., 2006). The effects of soil on forage crops can be assessed in two aspects: accumulation of minerals in plants and the increase in plant yield, composition, and digestibility. Plants growing in different soils has different characters (REIS, 2000).

Proper nitrogen fertility is essential for plant growth and development by helping in recovery from stresses such as wear, physical injury and damage from pests (BEARD, 2002). However, by applying N in amounts greater than the requirements can increased the above ground growth (CHRISTIANS et al., 1979); and reduces the depth and density of root growth (SCHLOSSBERG; KARNOK, 2001). The decreased root mass ultimately reduces the nutrient uptake (BOWMAN et al., 1998) and water as well (DACOSTA; HUANG, 2006). Therefore, identifying optimal nitrogen levels at establishment (to maximize root and shoot development) and over the longer term. The purpose of this study was to determine the ideal requirements and nitrogen for higher productivity in signal grass.

1.1.5 Chemical composition of forage

With the advancing plant maturity, the concentration of the potentially digestible components e.g. soluble carbohydrates, proteins, and minerals, tends to decrease, while the fibrous portion increases (Acid detergent fiber and Neutral detergent fiber (ADF and NDF) cause a decline in digestibility and consumption (CHERNEY, 2000; GOMES JR., 2000). Studies showed that *Brachiaria* with frequent cuts and at young age tends to be less productive, but of better quality, Zimmer et al. (1988). During the growth season, the dead materials get accumulated by senescence of plants and can be accelerated by drought or frost. The chemical composition of the forages varies amongst species, variety or cultivar, development stage, genetic makeup and environment (REIS, 2000). High-quality forages provide energy, protein, minerals and vitamins to meet the animal's requirements.

Different accessions of *B. decumbens* have been reported to produce 9.5 kg ha^{-1} yr^{-1} in Costa Rica and 11.4 kg ha^{-1} yr^{-1} in Brazil, with 26% of the total biomass produced during the dry season. According to Cabral et al. (2012); N fertilization increases the leaf area especially during rainy season while Menezes et al. (2009) reported the total increase in fodder production. N rates are positively correlated with the recovery of pastures, showing an increase in DM and CP contents and a decrease in NDF and ADF (COSTA et al, 2013). But it is recommended that N fertilizers must be applied in

appropriate doses and sources as inappropriate forms and doses can lead to N leaching and losses (VILLAS BOAS et al., 1999).

1.1.6 Near infrared spectroscopy

According to Batten (1995); analyzing plant samples using NIRS was initiated for many constituents at the same time and at lesser cost (SMIS et al., 2014). It run large batches of pasture for (CP, NDF, ADF, Lignin, and IVDMD) (NORRIS et al. 1976). NIRS calibrations are used for minerals in pasture grasses (BLAKENEY et al. 1994) and other crops also (MCGRATH et al. 1997). Analyzing samples by Near infra-red spectroscopy (NIRS) took 3.0 min per sample, reducing the analysis time by at least 30% compared to the chosen reference method. It analyses the plant structural compounds such as lignin and cellulose (SCHOELYNCK et al., 2010), compounds related to stresses (abiotic and biotic (toxic compounds, metabolites), macronutrients (nitrogen and phosphorus))) also. (DAVIES 1998) reported that NIRS has led to worldwide acceptance in many fields (chemical, pharmacy) and is reported in issues of Journal of Applied Spectroscopy, Journal of Near Infrared Spectroscopy, NIR News, and International NIR Conferences. According to Campo et al., (2013); the NIRS can be used predict water soluble carbohydrates, nonstructural carbohydrates, organic matter, and starch with good accuracy (ALEXANDER, J. S. et al. 1998). Although few studies existed on its validity and potential, but still its importance can't be neglected.

1.1.7 Metabolic pathway and secondary metabolites

Plants carry out many biochemical processes for primary metabolism (PM (sugars, amino acids, and fatty acids) and secondary metabolism (SM (alkaloids, glycosides, flavonoids, etc.)). According to García; Carril, (2009), all the carbon (C), nutrient or nitrogen (N) and produced energy is utilized for the molecules production in PM. However, the plants utilize a significant amount of the C, N and energy by the triggered enzymes for the synthesis of SM; having no roles in normal processes. But they help the plants in ecological functions (protection, attraction, repellent etc. (MAKKAR et al., 1995)). They are produced and stored in specific parts and translocated through the phloem/xylem. (PILLUZA et al. 2014); the literature on SM is huge but conflicting, describing the pros and cons depending on the type, concentration, and chemical structure. Also, very few studies have addressed the SM and primary metabolites intra and interactions in tropical grasses. According to García; Carril, (2009); SM are classified into (terpenes, phenolics, glycosides and alkaloids). SM are largely dependent on the carbon relationship to other nutrients (MUZIKA, 1993;

MÜLLER et al., 2013) and there is a compensation mechanism between the two and if there is any limiting condition; then the plant growth get restricted but the photosynthesis continues and diverts the surplus C to SM. But in the case of *B. decumbens*; nothing can be stated in advance. SM also acts as anti-nutritional factor due to high affinities and forming insoluble complexes with cellulose, starch, pectins; minimizing the feed digestibility.

1.1.8 Temperature

The temperature fluctuates during the seasons and directly influences the plant inner and outer environment (growth rate, senescence etc.) Morison, J.I.L.; Lawlor, D.W. (1999). It is as well described by many researchers that any increase from normal and crucial temperature by 5°C can reduce the plants photosynthetic capacity. Thus decreasing the biomass production and increase the saponin level (GERA et al. 2007). Other important factor is soil temperature which also enhances the saponin in plants (SZAKIEL, A.; PACZKOWSKI, C.; HENRY, M. 2010). All the linked factors to the temperature can influence the intra and inter cellular reactions; as the normally defined temperature for cell functioning is 17-25 °C. (YU, K.; NIRANJANA, M. H.; HAHN E.; PAEK, K. 2005) observed in the hairy root of *panaxginseng* the ginsenoside (saponin) that the light intensity and duration along with temperature target the saponin. While Chan et al., 2010; reported that *Melastoma malabathricum* and strawberry incubated at temperature range of 20 ± 2 °C grow rapidly with a higher flavonoids concentration compare to the 29 ± 2°C. (ZHANG, W.; SEKI, M.; FURUSAKI; 1997), present similar results with higher flavones production at optimal temperature (25°C) has been shown in *Perilla frutescens* and strawberry. As the low temperature below the plants genetically code level exerts stress on cells and decreases the growth. Thus, the low temperatures and stress is directly related to flavonoids production and cause retarded growth in the plant. In the case of strawberry at a 15°C temperature the (13 times) highest flavonoids were obtained as compare to 35°C.

1.1.9 Nutrient Stress

The normal phenomenon of PSM (plant secondary metabolites) is linked to plant stress and nutrient deficiency. Generally under stress, the plants growth gets limited but the photosynthesis continues as normal and the C reservoirs are diverted from for allocation of CBSM (carbon based secondary metabolites (polyphenols); Seigler 1998). Some studies have suggested that nitrogen fertilization interferes with the SM concentration. N is the mostly required nutrient for plant growth, production and

processes (PARIDA A. K.; DAS A. B. 2010). It interacts with the environmental resources as its byproducts are influenced by the plant and soil fertility, taking part in SM (DANESHMAND F.; ARVIN M. J.; KALANTARI K. M. 2010). According to Muzika (1993), N could favor the concentration of nitrogenous compounds in plants, and the excess N is diverted from PM for synthesis of alkaloids. Malinowski et al. (2011) found inconsistent effects of N on concentrations of phenolic compounds in *Triticum aestivum* L. However, Zhang et al., (2012) showed that fertilizer significantly increased the total phenols (TP) by nitrogen in *C. equisetid folia* branches. The soluble sugar or starch concentrations showed both a negative and positive linear correlation with TP, thus the inconsistent relationship between carbohydrates and TP showed that the biosynthetic pathways of different phenols are different. Mudau et al. 2006; stated that the SM biosynthesis can be increased with N, due to the pronounced effect on photosynthesis and accumulation of non-structural carbohydrates but various theories have proved that nutrient poor conditions enhance plants for producing more SM.

While amongst the many related factors as discussed above; analytical method and roughness in analytical methods also affect the saponin contents. (SHIMOYAMADA; 1991) sprouting is highly and positively linked to saponin levels as light intensely effects it in growing plants. Gorski et al. (1984), investigated the effect of plant part and plant age on saponin contents in *Medicago lupulina* and noticed the highest rate during the first 6 germination days. Observing the plant parts, leaves and seeds were having the highest contents compare to the stem and mostly in plants aerial parts as compare to seeds, branches, and petiole.

1.1.10 Environment

The local climate, season (light hours, light intensity, temperature etc.) all affect the SM (MORISON, J.I.L.; LAWLOR, D.W. 1996). Saponins occur in all plant parts and influenced by abiotic factors (SZAKIEL, A; PACZKOWSKI, C.; HENRY, M. 2010). The plants exposed to longer sunlight hours are reported to have higher saponin than unexposed or shorter exposed direct sunlight (LI, T.S.C. et al. 1996). The low nutrients availability in the soil; limits the resource availability and restraining the growth in spite of photosynthetic process and plants allocate the extra C for carbon based secondary metabolites (CBSM). According to growth differentiation balance (GDB) hypothesis, not only the availability of nutrients along with environmental factor influence the SM but also exists a trade-off between growth and different processes.

Similarly, phenyl- alanine enzyme (PAL) (precursor for SM and primary metabolites) can lead to competition. However the SM synthesis may be inhibited due

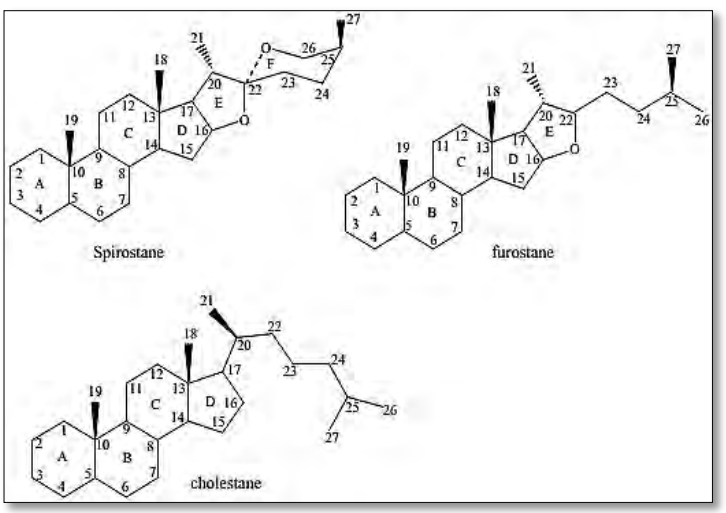
to the resource availability limitation as resources are rapidly incorporated into protein competition model under natural conditions (PEDRAZANI et al. 2013). On the other hand, exists a positive correlation between PAL (phenylalanine lyase enzyme) and CBSM in plants (NAVARRO J.M; FLORES P.; GARRIDO C.; MARTINEZ V. 2006), thus increase the lignification of plants. The data showed an increased PAL activity in N deficient plants, as the plants try to compensate the N deficiency by increasing the PAL activity; ultimately resulting in higher phenolics (LARSON 1988).

1.1.11 Saponins

Saponins are glycosides with a steroidal (C_{27}) or triterpenoidal (C_{30}) nucleus, called sapogenin; with one or more sugar molecules to its side chain (GARCIA; RAILS, 2009). It is divided into three classes by the genin (aglycone/non-sugary) structure, viz, triterpene, steroidal and alkaloidal glycosides, Vasilyeva; Paseshnichenko, 2000 (Figure 1). According to Cheeke (1971); the saponins may have the same core and differs only in the type of main sugar side chain. It has a hydrophilic component (saponin molecule) and the liposoluble type, giving rise to surface tension between molecules and create foaming. The 27 carbon skeleton; is either of 6-rings (spirostane) or 5-rings (furostane).

Steroidal saponins are diverse and is one of largest family (> 100 steroidal sapogenins) with wide range of biological activities. They have three main classes i.e. spirostanol, furostanol and cholestane saponins and are biosynthetically interrelated (Ming and Biao 2006) as presented in Fig. 3 (HOSTETTMANN, K.; MARSTON. 1995)

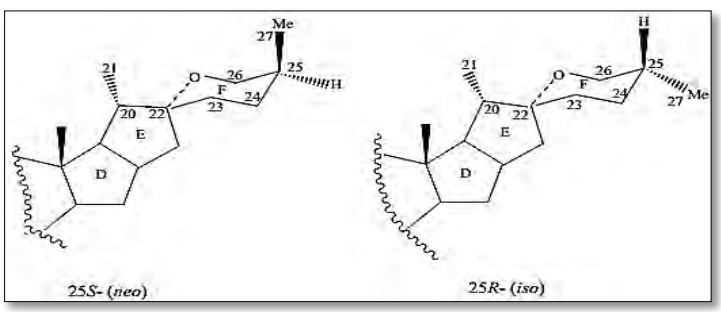
Figure 1. Basic steroidal saponins skeleton and structural differences



(Adapted from steroidal saponin from Tribulus terrestris. Steroids (SU L., et al., 2009)

Generally in the steroidal saponins; the C-21 methyl group is angle wise α-orientated while the C-18 and C-19 angular methyl groups are β-orientated (upper side) with double bonding; and the hydroxyl groups occurs at C-1, C-2, C-5, C-6, and/or C-11 (HOSTETTMANN, K.; HOSTETTMANN, M.; MARSTON, A, 1991, 1995). Spirostanes are characterized by keto spiroketal moiety (E/F rings) and are subdivided into 25*R*- or 25*S*- series (Fig. 2). The C-25 methyl group is axially orientated in 25*R*- (or *iso*- sideway (*protodioscin*)) sapogenins while in 25*S*- (or *neo- upward (protoneodioscin)*) the sapogenins (KINTIA et al. 1987) is equatorially orientated.

Figure 2. Ketospiroketal saponin – Partial structures 25R- and 25S- spirostane



Adopted from Tamar Korkashvili (2006) - Phd thesis.

1.1.11.1 *Brachiaria* and saponin

The presence of saponins in *Brachiaria* is the cause for photosensitization in animals. According to Riet-Correa at el. (2011) the majority of cases are associated with *B. decumbens*, although *B. brizantha* and *B. humidicola* can also cause. Protodioscin was identified in *Brachiaria* species (RIET-CORREA at el., 2011) and its hydrolysis in gastro intestinal tract produces yamogenina, diosgenin, and sapogenins which after being metabolization produce espi-smilagenina and epi-sarsasapogenin. They conjugate with glucuronic acid forming glucuronides and bind calcium ions to form insoluble salts, settling in liver and biliary system as insoluble crystals (RIET-CORREA at el., 2011). This deposition leads to clogging of bile ducts and interferes with the hepatocytes metabolism, preventing excretion of *phylloerythrin* and accumulates in circulation and pass to skin tissues. These photoactive agents (QUINN et al., 2015; LAJIS et al., 1993) upon exposure to the sun (GUPTA; 2012) gets excited causing liver injury, disruption of keratinocytes, tissue destruction (STEGELMEIER 2002), jaundice, skin problems, nervousness, blindness, brownish urine (SMITH, 2000) and finally death.

The saponin concentration varies with the soil type, environmental factors (rain, temperature, and sunlight hours), plant developmental stage and seasons. According to Ferreira et al. (2011), the concentration of protodioscin is widely variable, making it difficult to establish a threshold level for its toxicity. Brum et al. (2009) reported the protodioscin concentrations in the range of 0.5 - 2.1% and 0.8 - 1.9% throughout the growth cycle of *B. brizantha* and *B. decumbens*. The highest concentrations were recorded in seed dropping stage. Conversely, Lima et al. (2012) observed a decrease in the concentration of protodioscin during the growth period, with higher concentrations in plants was recorded up to 60 days of growth with a recording of 2.39% and 3.15% in *B. brizantha* and *B. decumbens*, respectively. Based on varying toxicity rates and protodioscin conc. between *B. decumbens* and *B. brizantha* (FERREIRA et al., 2011; LIMA et al., 2012) suggested to replace *B. decumbens* by *B. brizantha,* to minimize the animal's losses Mustafa et al., (2012). But the toxicity degree depends on animal's resistance, pigmentation and body parts (OLIVEIRA et al., 2013) also. All authors confirm the *B. decumbens* toxicity in recent years in Brazil as shown in Table 1. But further research needed to be conducted for defining the main reason of differences between these similar species toxicity. That's why we focus on the metabolic profile as protodioscin isomer for detecting the differences in conc. and molecular structure.

Table 1. Toxicity occurrence by *Brachiaria* species in Brazil

References	Animal	P %	Morb. (%)	Mort. (%)	Let. (%)
Gracindo et al. 2014	48 Sheep	5.70 ± 2.40 8.50 ± 3.30	4.0	2.0	
Pacheco (2014, un-publish)	80 Sheep		28.75	20.0	69.56
Oliveira et al. 2013	Buffalo	3.24 – 3.54			
Porto et al. 2013	18 Sheep	0.94 ± 0.80	22.2	16.6	75.0
Mustafa et al. 2012	1305 Sheep	0.30 - 2.56	15.4 – 57.0	15.0 - 48.0	29.4 -86.5
Castro et al. 2011	20 Sheep	0.52 - 1.06	50.0	15.0	30.0
Saturnino et al. 2010	24 Sheep		45.8	45.8	100.0
Santos Jr. (2008	21 Sheep	0.77 - 2.37	57.1	42.8	75.0
Mendonça et al. 2008	40 Sheep		37.5	17.5	46.5
Brum et al. 2007	28 Sheep	2.36	25.0	21.4	85.7
Silva et al. 2006	200 Cattle		25.0	25.0	100.0

Adopted from Tatiane G.F. (2014) and modified

P (%) - Protodioscin
Morb. (%) - Morbidity
Mort. (%) - Mortality
Let. (%) - Lethality

These notes have important application in the production system in Brazil since the Brazilian livestock backbone is its pasture. However, the effect of N fertilization on the concentration of these compounds, as well on complex formation and neutralization of the possible toxic effects of the saponin by any alternate factors hasn't been determined. Likewise, research studies that comprise the determination of the amount, type, and possible interactions between these metabolites in tropical grasses are scarce, which limits the exploitation of the potential beneficial effects of phenolics or flavones and saponin interactions in the production systems based on pastures.

1.1.12 Plant Phenolics

Phenolics are synthesized by the two pathways (Shikimic acid and Mevalonic acid pathway) and occur in several forms:

a. Soluble compounds - Extracted with water, methanol or aqueous acetone.
b. Non-extractable forms – Remains as residue after extraction i.e. large molecules (MUELLER-HARVEY et al, 1986).

Flavonoids (C_6-C_3-C_6) falls in the soluble compounds of the polyphenolic group and are derived by phenyl-propanoid pathway (KUMAR; PANDEY, 2013). More than 9,000 compounds are found in plant kingdom (BUER et al., 2010) till now and their chemical nature depends on structure, class, polymerization, etc. (ARORA et al., 2010). In the nature, they exist in glycosidic forms (FORKMAN et al., 1999; EVANS et al., 1996). In plants, due to their diverse chemical structure they perform numerous functions e.g. growth factor (Auxin) (ARORA et al., 2000) stress (Ultraviolet (UV) radiation, heat) and biotic by influencing the plant behavior, signaling molecules (SIMMONDS 2003; SIMMONDS et al., 2001). They give color, fragrance, and taste to fruits, flowers, seeds, making them favorable (KOES et al., 1994).

1.1.12.1 Flavonoids and Environmental Conditions

Plants develop a protective mechanism against the various risks by producing SM; but the type and concentration of SM varies as per (light, UV, temperature, drought, etc.*)*. During the intense UV, light flavonoids get accumulated in the epidermis of leaves and stem thus minimizing the damage to plants from light penetration. However, sometime flavones may transfer their received light energy to or from other molecules; if required; (SISA et al., 2010). The flavonoids mostly occur in the cell sap of younger tissues in Leguminosae, Polygonaceae, Rutaceae in most common types as flavone (flavone, and luteolin), flavonol (quercetin, and fisetin), flavanone (flavanone, and

naringenin), others. Decades of research proved that flavonoids are indicator of plant N status and many equipment are available for measuring the plant nutritional status by leaf chlorophyll content and flavonoids ratio named as NBI (Nitrogen Balance Index).

Observing the flavones concnetrations, (LI et al., 2010) noted the maximum content in roots of *Nyctanthes arbortristis* collected in morning plants under intense light (46.55 mg/g. D.wt.). While the stem collected in the evening under minimal light were having lower flavonoids, proving the impact of light. The Plants of *Nyctanthes arbortristis* grown in light showed better growth as compared to shade plants but the shaded were healthier with large leaves. Total flavonoids were maximum in roots of shade plants (46.55 mg/g.dw) collected in the morning.

1.2 Purpose of the Study

Due to non-availability of detailed studies about the real cause of *Brachiaria* photosensitization throughout various seasons and with or without fertilizer application; also the main reason of variation of the degree of toxicity between *B. decumbens* and *B. brizantha;* all these factors along with other unseen causes enforce us to focus on the root cause. This study will differentiate the components and their degree of variance in the metabolic profile of *B. decumbens* during various seasons and fertilizer application. The hypothesis states that “To which extent, the nitrogen fertilization will cause a shift in metabolic profile of *Brachiaria*?” “Will the seasons make a diversion due to exerted stress on plants metabolism and production of primary and secondary metabolites and will affect *Brachiaria* nutritional value”. “Can the difference in cutting heights will be productive?”

1.3 Scope and Delimitations

This study was chosen with the specific objectives and as the plant metabolites study will basically work on a carbon nutrient balance (CNB) thoery, and at the same time will maintains the allocation cost of defense in the form of saponin. It suggests that plants adapted to the fertile sites respond to their defense by utilizing stored resources for their growth while slower-growing species adapted to infertile soils invests more in carbons based products for their defense (STAMP 2003). On one side fertilizer enhances the mass production but on other it has link with the competition for carbon uptake and balancing with the higher N supply. The predictions and tests have multi views in studies having focus on more than one nutrient for variety of reasons and can lead to various views and confusions.

This study is to summarize the minimal research literature that outlines the *Brachiaria* toxicity. The study was difficult and complexed due to the nature of statistical design (treatments and factors) as well and number of samples also. Many interfering factors can affect the PSM quantities and types and intense stress can cause dramatic changes at cellular levels. As been carried out under a number of factors at the same time in same conditions; the experiment was conducted in greenhouse in pots with limited production. This reserve us for making further analysis and on a large scale as well. Also due to the involvement of many factors; a large number of samples were produced and it makes a complex chain of possibilities for the cause and probable chance of occurrence of a specific component and compound. Statistically, also by the involvement of many factors usually triple interactions (nitrogen, height and season) make it difficult to find the possible cause and reason of error or occurrence and also for justification of possibility of a change. Lastly but not the least; no literature or background was available for making comparisons as per required. In the case of Protoneodioscin; it wasn't easy to define or analyze on the base of a doubt or without carrying a biological test / in-vivo / in-vitro test for confirming the toxicity. However, for protodioscin it been comparably easy due to the availability of a base and studies, but still all literature mainly focus on the human.

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2 CHAPTER 1 - ANALYZING THE NUTRITIONAL VALUE OF *Brachiaria decumbens* UNDER TWO CUTTING HEIGHTS AND NITROGEN FERTILIZATION

ABSTRACT

Brachiaria decumbens stapf. cv. 'Basilisk' has been widely used due to its high productivity, disease resistance, drought resistance and good persistence of the pasture. But most studies focus only on one variable or the other; affecting the plants, but while focusing more variables in plants, the results and literature became limited and not very specific to the plants species as well. So we opt for defining many factors at the same influencing the Brachiaria production. The objectives were to define the best productive time and dose of nitrogen in terms of nutritional value and mass production and that which plant cutting height will be more favorable for obtaining these characters? The experiment was conducted at FZEA-USP during Dec. 13 to Dec. 14; with *B. decumbens* subjected to four nitrogen doses (0, 150, 300 and 450 kg ha^{-1}) and two cutting heights (10 and 20 cm) in the complete randomized block (CRB) experimental design. The results showed that the dry matter yield (DMY) was highly affected by the increase in N levels and seasons. The highest DMY (133.34 g/pot) was observed at 150 kg ha^{-1} N dose in autumn. Within seasons, the lowest DMY was obtained in winter (11.77 g/pot). There was a linear increase in crude protein (CP) with increasing nitrogen fertilization, and the highest CP% was obtained in winter (11.88%) and lowest in autumn (7.78%). The effect of nitrogen application and cutting heights was obvious ($P< 0.05$) during the summer and the 20 cm gives the high CP% (8.34). The highest acid detergent fiber (ADF) (36.12%) and neutral detergent fiber (NDF) (82.80%) were observed in autumn. While, the lowest ADF and NDF (30.88% and 79.60%) in winter and spring, respectively. The In-vitro dry matter digestibility (IVDMD) and in-vitro organic matter digestibility (IVOMD) increased linearly with increasing nitrogen fertilization, and a significant difference occurred between the cutting heights. The higher IVDMD and IVOMD were observed in autumn (67.35% and 61.56%) while the lowest were noted in spring (63.65% and 57.69%), respectively.

Generally, the application of 300 kg ha^{-1} N; increased the CP while reduced the ADF and NDF simultaneously; improving the IVDMD and IVOMD. There was significant differences in the fibrous portions and digestibilities by the seasons as well.

KEYWORDS – Bromatology, *Brachiaria*, Heights, Nitrogen, digestibility, Season.

CAPÍTULO 1

ANALISANDO O VALOR NUTRICIONAL DE *Brachiaria decumbens* SOB DUAS ALTURAS DE CORTE E DA ADUBAÇÃO NITROGENADA

RESUMO

Brachiaria decumbens cv. Stapf. 'Basilisk' tem sido amplamente utilizado devido à sua alta produtividade, resistência às doenças, resistência à seca e boa persistência na pastagem. No entanto, a maioria dos estudos focam apenas em uma variável ou outra. Em se tratando de plantas, apesar do foco ser em mais variáveis nas plantas, os resultados e a literatura tornaram-se limitados e não muito específicos em relação às espécies de plantas também. Por isso, nós optamos por avaliar os diversos fatores que influenciam, ao mesmo tempo, na produção da *Brachiaria*. Os objetivos foran definir a melhor época produtiva e a melhor dose de nitrogênio em termos de valor nutricional e produção em massa e qual a altura de corte da planta será mais favorável para a obtenção destas características? O experimento foi conduzido na FZEA-USP durante 1 ano (Dez. 13 a Dez. 14). Utilizou-se a espécie *B. decumbens*, submetida a quatro doses de N (0, 150, 300 e 450 kg·ha-1) e duas alturas de corte (10 e 20 cm), seguindo o desenho experimental por delineameto inteiramente casualizado (DIC).

Os resultados obtidos demonstraram que a produção de matéria seca foi afetada significativamente pelo aumento nas doses de N e as estações. Sendo o maior produção (133.34 g/vaso) foi observada na epoca de autono com dose de 150 kg·ha^{-1}. Entre as estações, o menor produção de materai seca foi verificado no inverno (11.77 g/vaso). Houve aumento linear nos teores de proteína bruta (PB) com o aumento da adubação nitrogenada, sendo o valor mais elevado obtido no inverno (11,88%) e o menor no outono (7,78%). No verão houve interação entre altura x dose de nitrogênio ($P< 0.05$) para o teor de proteína e a maior concentração foi verificada na altura de 20 cm (8.34%). As concentrações mais elevadas de fibra em detergente ácido (FDA) e fibra em detergente neutro (FDN) foram observadas no outono (36.12 e 82.80%). Enquanto que, os menores valores para FDA e FDN foram observados no inverno e primavera (30.88 e 79.60%) respectivamente. As digestibilidades in-vitro da matéria seca (DIVMS) e matéria orgânica (DIVMO) aumentaram linearmente com o aumento da adubação e ocorreram diferenças significativas entre as alturas de corte. Os maiores valores de DIVMS e DIVMO foram observados no outono (67,35 e 61,56%), enquanto o menor foi observado na primavera (63,65 e 57,69%), respectivamente.

Em geral, a aplicação da dose de 300 kg·ha^{-1} aumentou a produção de proteína bruta, reduzindo simultaneamente FDA e FDN e melhorou (DIVMS) e matéria orgânica (DIVMO) tambem. Houve diferença significativamente nas porções fibrosas e (DIVMS) e matéria orgânica (DIVMO) entre às estações tambem.

PALAVRA-CHAVES – Capim-Braquiária, Altura, Estação, Digestibilidade, Nitrogênio, Bromatologia

Bromatology, *Brachiaria*, Heights, Nitrogen, digestibility, Season.

2.1 Introduction

The genus Urochloa (syn. *Brachiaria)* includes about 100 species (RENVOIZE et al, 1996) and it basically belongs to Africa and was introduced in Brazil in the 1950's as pasture. Since then it has been emerged as a monoculture (LOCH 1977) of the world by covering < 90 million ha (JANK 2014) and major commercial crop (FISHER; KERRIDGE; 1996). It's most prevailing and productive species are *B. decumbens*, *B*. *brizantha,* and B*. humidicola;* due to its long listed benefits and properties (adaptive to soils, resistance against bugs, environment, water logging, drought etc.). Low nutritional status, especially nitrogen (N), is the main limiting factor for Brazilian grass productivity (MYERS; ROBBINS (1991); SPAIN; GUALDRON (1991); RAO et al. 1996) causing a permanent decline in the soil quality and plants nutritional value as well. The process keeps in continuation with every passing by year (BODDEY et al. 2004).

Brachiaria decumbens (Signal grass) alone cover > 50 million hectares (GRACINDO et al., 2014); and has a considerable nutritional value and morphological traits (structure, leaves number and size, and tiller density (BRISKE et al., 1986). All these characters are not only respondent to solar light for photosynthesis but are related to forage production and define the animal's intake (BRISKE, 1991).

Despite the huge literature on *Brachiaria* productivity the basic understanding of the molecular mechanisms underlying N sensing, signaling, responses in pathways, metabolic products, is still lacking. As all the reduction and assimilation pathways linked to N are controlled by the genetic coding of plants by the signaling molecules for controlling the Arabidopsis expression. All B*rachiaria* cultivars are based on wild germplasm, having some agronomic limitations (KELLER-GREIN et al, 1996) but Current breeding schemes for *Brachiaria* seek to exploit apomixis by the development of simple mechanisms (DO VALLE; SAVIDAN, 1996) and will understand the attributes for controlling the genetic level and ploidy level of *Brachiaria* (FERREIRA et al., 2016). The definition of genetic coding in *Brachiaria* will help in delimiting the toxicity by diverting the toxic components towards primary metabolism or phenolic compounds.

Nitrogen; is the main component of amino acid, enzyme biosynthesis, and their quantification (SINCLAIR; VADEZ, 2002). But it also limits the plant capability to cope with the adaptive mechanisms; for growth continuity and developmental aspects (root architecture, leaf (WALCH et al., 2000), seed dormancy and flowering (STITT et al., 2002). Normally fertilized plants tends to increase their photosynthesis thus enhancing the biomass and cope with the increased growth rates and demands. But nitrogen also controls the primary metabolism as all compounds and enzymes serve the basic, well-defined, and essential roles. Any diversion in primary metabolism cause variations in

secondary metabolites as the regulatory mechanisms is extensive. Generally, the plant systems are complex and we have very limited understanding of these mechanisms so we can elaborate them on basic models. Regulation of primary metabolism is substrate inductions, feedback repression and inhibitions, catabolites, and ATP regulation. In each case, regulation is a dynamic process dependent upon the local concentration of effector molecules (SCHEIBLE et al., 2004). But all these factors are related to the seasonal variations, plants age, and plant genetic coding.

According to Sharma (1973) the addition of nitrogen fertilizer increased the plant height and ultimately favors the more production of leaves (AKINTOYE; 1996) as N promotes the plant growth, increases the number of internodes and length of the internodes which results in progressive increase in plant height (GASIM; 2001). Koul ; 1997 also reported the increase in number of leaves per plant and leaf area and resulted in greater plant height, leaf area, number of leaves and stem diameter, fresh and dry forage yield. Leaf to stem ratio was also founded to be increased by N (DUNCAN, 1980).

The nutrient state of plants is evaluated primarily by chemical analysis of leaf tissue, nutrient estimation. Nitrogen application increases the protein content in dry matter (DM). Since proteins are synthesized from amino acids, thus increases in nitrogen supply reduce the soluble carbohydrates and the large accumulation of nitrogen products and proteins cause a dilution in the cell wall fraction, increasing the digestibility indexes (COSTA et al., 2008). Studies indicates that the use of nitrogen fertilizers in grasses, besides increasing the dry matter production (RODRIGUES et al., 2005), also improves the forage quality and crude protein (CP), total digestible nutrients (TDN) contents, and decreased the neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin. Thus overall improve the plant status and digestibility index as well (COSTA et al., 2010).

The experiment studied the influence of cutting interval of 30 days, cutting heights, and nitrogen fertilizer effects on dry matter yield, plant composition and primary metabolic components (crude protein (CP), crude fiber (CF)) as how will they be directed later to the secondary metabolites. The aim was to define some optimum conditions for *B. decumbens* productivity and for limiting the toxicity with the cross involvement of all possible factors. The objective of this experiment was to evaluate the influence of cutting heights and nitrogen fertilizer on dry matter yield and bromatological characteristics of *B. decumbens* during summer, autumn, winter and spring.

Secondary metabolites are more complex in nature and activity and their separation from primary metabolites emphasize their complexity as all the involved precursors in their synthesis are provided by the primary metabolism (STADT 1970).

2.2 Materials and Methods

2.2.1 Local and climate conditions

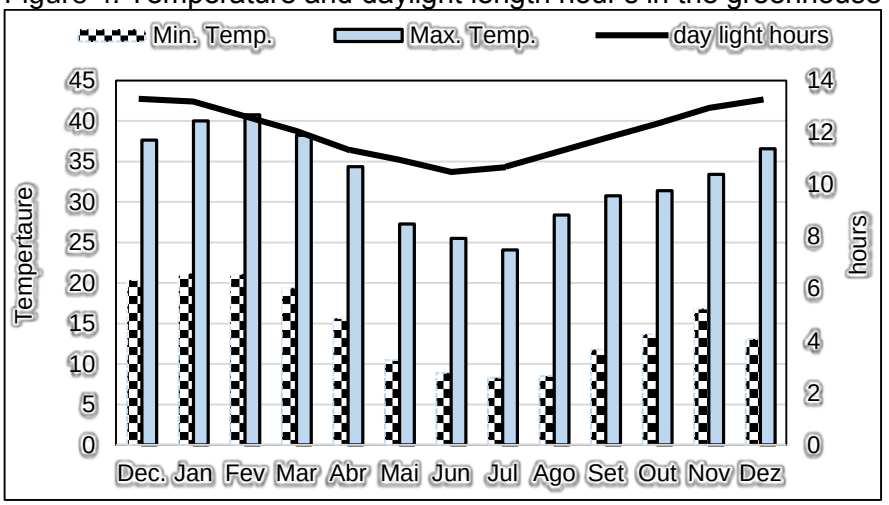
The present research was implemented and conducted in the greenhouse (Fig. 3), of Agrárias sector of Faculty of Animal Science and Food Engineering at University of Sao Paulo, Pirassununga, Sao Paulo, Brazil (21°59'46" S and 47° 25'33", 627 asl.).

Figure 3. Experimental arrangement in greenhouse

Syeda Hussain; PhD (15th Jan. 2014)

The climate of Pirassununga according to köppen system, is CWA (subtropical dry winter humid) with an average temperature > 22 °C in the warmest month and the minimal is < 18 °C. The average annual precipitation is around 1,238 mm and average relative humidity of 73%. The monthly weather data and daylight length (hours) during the evaluation period are in Figure 4:

Figure 4. Temperature and daylight length hour's in the greenhouse



The soil was classified as oxisol and the soil analysis were performed at the Laboratory of soil sciences (FZEA-USP). The average chemical characteristics are presented in Table 2.

Table 2. Soil analysis of the experiment soil

pH	M.O	P (res)	S	K (res)	Ca	Mg	Al	H+Al	SB	T	V
$CaCl_2$	g kg^{-1}	mg/dm_3			$mmol_c/dm_3$						%
6.2	28	126	23	2.7	74	18	0.0	19	94.7	114	83

2.2.2 Treatments and experimental design

The treatments corresponded to all combinations of four nitrogen fertilization rates (corresponding to 0, 150, 300 and 450 N kg ha^{-1} yr^{-1}) and two cutting heights (10 and 20 cm), and were distributed in a complete randomized block design in a 4×2 factorial arrangement, with five replications (pots). In December 2013, eights seeds of *B. decumbens* were sown in plastic pots with capacity for 3 kg of soil. Plants were thinned when reached 5 cm of height, maintaining 5 plants per pot. The pots were watered daily in order to maintain 80% of soil water capacity.

All pots received the equivalent to 288 kg ha^{-1} of K_2O in the form of potassium chloride for raising their cation exchange capacity by 5% and was split applied at each cutting along with nitrogen. Nitrogen was also split-applied in equal fractions after each cut of 30 days, as shown in Table 3.

Table 3. Cutting dates and nitrogen doses of experiment

Activity	Date	Dose N (kg ha^{-1})			
Cuts		**0**	**150 ***	**300 §**	**450 †**
Uniformity	15/01/2014	0	12.5	25	37.5
1st cut	15/02/2014	0	12.5	25	37.5
2nd cut	14/03/2014	0	12.5	25	37.5
3rd cut	13/04/2014	0	12.5	25	37.5
4th cut	14/05/2014	0	12.5	25	37.5
5th cut	15/06/2014	0	12.5	25	37.5
6th cut	13/07/2014	0	12.5	25	37.5
7th cut	14/08/2014	0	12.5	25	37.5
8th cut	15/09/2014	0	12.5	25	37.5
9th cut	08/10/2014	0	12.5	25	37.5
10th cut	11/11/2014	0	12.5	25	37.5
11th cut	10/12/2014	0	12.5	25	37.5

* = 150 kg ha^{-1} Nitrogen equalizes to 0.50 g urea per pot

§ = 300 kg ha^{-1} Nitrogen equalizes to 1.00 g urea per pot

† = 450 kg ha^{-1} Nitrogen equalizes to 1.50 g urea per pot

Plants were harvested with plant scissors after every 30 days at their height specifications (10 and 20 cm) before sunrise and were cutted into 3 cm lenghts portions to stop the occuring biochemical processes. The morphological components were manually separated into leaves, stem + sheath, and dead materials. But as per requirement and keeping in mind the objective of saponin identification as main factor in the leaves as compare to other plant portions; the leaves were selected for further chemical analysis. The leaves were dried in oven below 40°C till complete drying and with a constant weight. Later, the samples were grinded by Wiley Mini Mill at 16 mesh size and stored in black plastic bottles. Chemical analysis and evaluations were conducted from January 2014 to December 2014, and were grouped in the following seasons of the year: summer (Jan. – Mar.); autumn (Apr. – Jun.); winter (Jul. – Sept.); spring (Oct. – Dec.).

2.2.3 Plant chemical analysis

The bromatological characteristics were determined in the leaves of the plant using Near-infrared spectroscopy (NIRS) at EMBRAPA (Sao Carlos). NIRS is very rapid, non-destructive, cheap, repeatable, time-saving, more sample runner in less time than traditional analytical methods (WILLIAMS et al., 1983). The American Society for

Testing and Materials (ASTM) defines the NIR region from 780 to 2526 nm, located between the red band of the visible light and the mid infra-red region. The most prominent absorption bands are due to the light absorbance by the hydrogen bonds functional groups (e.g. –C–H, –S–H, –N–H and –O–H). A sample size (≤ 0.3 g) can be analyzed (CIAVARELLA et al. 2003) easily. For increasing the accuracy a finely ground material and dry sample is recommended.

Plant leaf, shoot or stem material is usually scanned in the reflectance mode and the grounded sample is put in the covered cell, open cell or glass vial or plastic bag. It must be confirmed that reliable results are produced by NIRS by points as:

(i) Developing a calibration using samples by reference methods and representing all expected variations (plant species, tissue, age, status, drying process, grinding size, grinding type, any possible damage, artifacts or traces in samples, moisture content, and temperature etc.).

(ii) Always use the typical samples for developing the calibration with samples having the same size, the same mill due to the influence of the absorbance energy and all available mills should be present in the calibration data.

(iii) The NIRS calibrations being used should be monitored to detect random errors by regular checking through software (lamp voltage, wavelength (BLAKENEY et al. 1995)).

The NIRS provide us the results of dry matter yield (DMY), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), in-vitro dry matter digestibility (IVDMD), and in-vitro organic matter digestibility (IVOMD). For cross checking of results, a random analysis was carried out for all components at the laboratory.

2.3 Statistical analysis

Data were analyzed with the Statistical Analysis System package (SAS Inst., Inc., Cary, NC). Before the actual analysis, data were analyzed for the presence of discrepant information ("outliers") and normality of residuals (Shapiro-Wilk). When the normality assumption was not met, the logarithmic transformation or the square root was required. Data were analyzed according to the Proc Mixed to mixed models in factorial arrangement of treatments by type 4x2, referring to 4 nitrogen fertilizer levels (0, 150, 300 and 450 kg ha^{-1}) and two cutting heights (10 and 20 cm), and seasons (summer, autumn, winter and spring) as the repeated measures (split-plot). For the analysis, among 15 tested different covariance structures that best fit the statistical model was chosen based on the lower value of the information criterion corrected Akaike (AICC) (WANG; GOONEWARDENE, 2004). The model included fixed level of

fertilization effects, cutting heights and season, as well as effects of double and triple interactions between the factors. Seasons of the year were considered repeated measures. Additionally, mean comparisons between the seasons were performed using the adjusted Tukey test and nitrogen fertilization level effect was evaluated with the use of polynomial regression, decomposing the effects of linear, quadratic and the cubic deviation. All test were performed at 5% significance level.

2.4 Results and Discussion

2.4.1 Dry matter yield

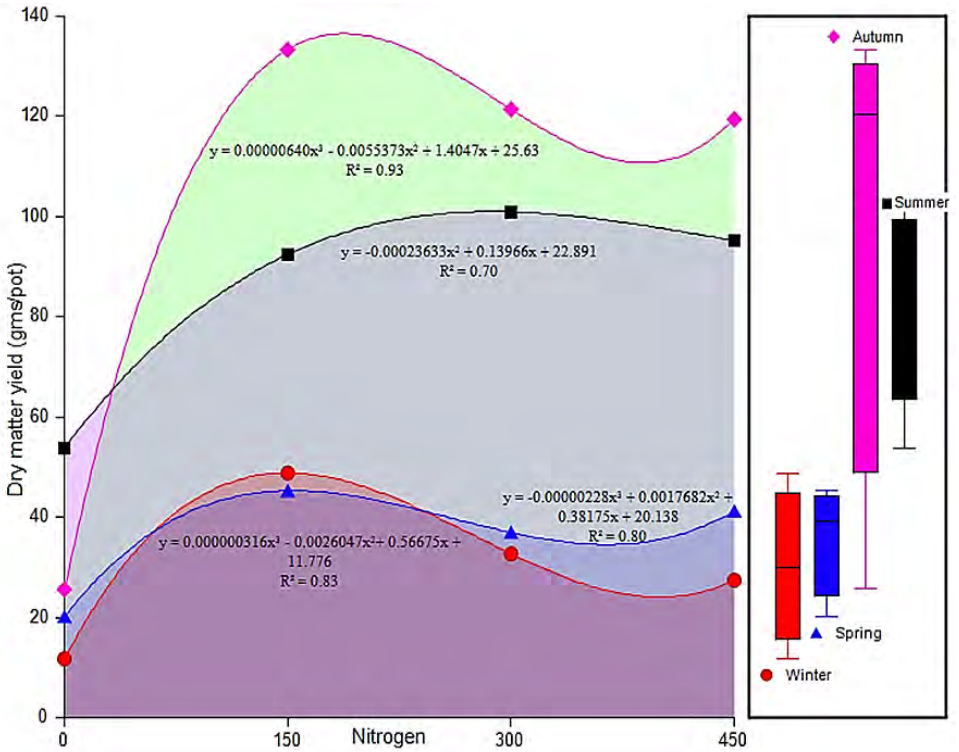
Tropical perennial grasses generally need a large amount of N and responds well to fertilizer (PRIMAVESI et al., 2006). According to Pereira et al. (2010) when there is no other limiting nutrient, nitrogen is probably the most important factor for affecting the growth dynamics by interfering with tissue flows, leaf appearance and elongation rate, tillering and herbage accumulation (SILVA et al., 2009). However, the best results depends on the N fertilizer rates, sources, times, and methods (Hawkseford, 2014).

In our results, the DMY varied with the nitrogen application and seasons and by the interactions (S*N and S*N*H). However, there were no significant effects of cutting heights and interactions of (H*N, S*H, and S*N*H). The DMY increases quadratically with nitrogen application quadratically being 27.84, 79.98, 73.02 and 70.85 g/pot at N doses of 0, 150, 300 and 450 kg ha^{-1} as shown in Table 4. Season of the year have a pronounced effect on growth rates and the average highest DMY (99.97 g/plant pot in) was observed in autumn (eq. 3.97 ton ha^{-1}), probably due to the reason that plants were relatively mature and the number of tillers were maximized as compared to the summer. While the lowest average DMY was observed in winter 30.20 g/plant pot (eq. 1.21 ton ha^{-1}) been three times lesser as to autumn, by the seasonal stresses.

The Moreno 2004 observed on Mombaça and Tanzania cultivars an increase in DMY of 79% and 66% in the summer and 21% and 34% in the winter. But in our exp. the increase in DMY was obvious in autumn as 39.73% compared to the winter (11.99%). Upon the increase in temperatures in late March and April, a maximum number of stems and secondary tillers help the plants to increase the N using efficiency while opposite occurs at a lower temperature in winter. While comparing the two heights; the highest DMY was produced at 10 cm irrespective of seasons (249.18 g/pot eq. 9.26 t/ha), although by adjusting regression the results were non-significant. Also, N contributes to a higher tillering rate and tillers number/area (SILVA et al., 2009) for supporting a high number of leaves (LEMAIRE et al., 1991). SANTOS et al. (2009)

reported similar values in *B. decumbens* cv. Basilisk. However, the increase of forage green mass (summation of leaf blades and stem) is dependent on pasture residual height and the season of grazing or harvesting (SILVA et al., 2009). According to PRIMAVESI et al. (2006); the increase in N dose, accelerates the plant growth rate and DM increasing the forage yield and nutritional capacity.

Figure 5. Dry matter yield mean values under nitrogen during the year in *B. decumbens*



B. decumbens performs better than other *Brachiaria* species in unfertilized conditions (ALVIM et al., 1990) and many researchers obtained 10.3 kg ha^{-1} with application of 75 kg N, but no increase in biomass was recorded when the N dose was doubled, the nitrogen use efficiency being as high as 195 g biomass (shoots + roots) g/N. The CP increased linearly with incremental additions of nitrogen (from 7.2, 10.6 and 13.4%) at N dose of (0, 75 and 150 kg ha^{-1}) respectively. *B. decumbens* can remain productive for many years in the absence of N-fixing legumes or N fertilizer as it has lower P and Ca requirements, than other grasses e.g. *Panicum maximum*, although there are inter-species differences (RAO, et al., 1996).

Table 4 - Least squares means values for Nutritive Value of *B. decumbens* as affected by nitrogen, plant cutting heights and season

Variables	Height (H) (cm)[1]		Nitrogen (N) (kg/ha^{-1})				Mean	SEM[2]	Probability (P) %						
	10	20	0	150	300	450			H	N	H*N	Season (S)	H*S	S*N	N*S*H
DMY (g/pot)[3]	61.56[a]	64.28[a]	27.84	79.98	73.02	70.85	62.92	4.1065	NS	*	NS	*	NS	*	NS
CP % [4]	9.51[a]	8.21[b]	6.49	8.06	11.31	11.59	9.59	0.3106	*	*	NS	*	*	*	*
NDF % [5]	70.94[b]	71.60[a]	72.28	71.92	70.27	70.61	71.31	0.2064	*	*	*	*	NS	*	NS
ADF % [6]	34.75[a]	34.05[b]	37.34	35.82	32.28	32.14	34.43	0.3956	*	*	NS	*	NS	*	NS
IVDMD % [7]	65.53[b]	66.07[a]	62.63	65.37	68.06	66.38	65.60	0.2452	*	*	NS	*	*	*	*
IVOMD % [8]	58.89[b]	59.79[a]	57.97	59.01	60.57	59.78	59.33	0.2525	*	*	NS	*	NS	*	NS

[1] Letters superscript inside the height differs for F test.
[2] Standard error of means
[3] DMY (g/pot) -Dry Matter Yield
[4] CP % -Crude Protein
[5] NDF % -Neutral detergent fiber
[6] ADF % -Acid detergent fiber
[7] IVDMD % -In-vitro digestible dry matter
[8] IVOMD % -In-vitro digestible organic matter
* Significant at the 0.05 probability level
# NS, not significant

In our results, the DMY increases from 0 kg ha^{-1} (111.3 gm/pot) to 150 kg ha^{-1} (319.92 gm/pot), but the yield starts to decline according to a quadratic response with 300 and 450 kg ha^{-1} (292.08 and 283.4 gm/pot). The response above the 150 kg ha^{-1} N seems to be not responsive in terms of nitrogen efficiency and is an investment in increase in cost. Similar reports as observed by Fagundes et al. (2005); obtained a linear response up to 300 kg ha^{-1} yr^{-1}, and the maximum DMY was obtained at 270 kg ha^{-1} yr^{-1} in *B. decumbens* but in our exp. the highest production was at 150 kg ha^{-1} with a linear response from 0 kg ha^{-1}. Bonfim Da Silva; Monteiro (2006), also get a linear response for increase in leaves DM by N in the degraded *Brachiaria* pastures. The yield was 268% lower on average in the plants without fertilizer compared to those fertilized (Figure 5). But Silva et al. (2013) observed almost 151% increase in the DMY at a dose of 300 kg ha^{-1} yr^{-1} in the Marandu pastures compared to the control treatment.

2.4.2 Crude protein (CP)

Crude protein is calculated from the N content, thus, an increase in soil available form of N results in an increase in CP%. The increase in CP (38.6%) is proportionally greater than DMY (27.2%) due to increased protein concentration at high N rates. Normally, the leaves CP is higher than stem as CP is an integral part of enzymes, which are responsible for carbon fixation in photosynthesis, occurs mainly in leaf mesophyll cells (SANTOS et al., 2004; CORSI; NASCIMENTO JR., 1994).

For CP; no significant interactions were found for H*N but the results were significant ($P < 0.0001$) for S*H, S*N and tripled interactions of S*N*H as presented in Table 3. Overall, due to short cutting intervals, the average CP% was around 6.49; just same as Arthington; Brown (2005); that 10-wk regrowth forage gives a decrease of 38% in CP compared to the 4-wk in *bahiagrass, limpograss, bermudagrass* and *Cynodon* spp. The decrease in CP at longer regrowth intervals in warm-season grasses is attributed to the N dilution effects by greater deposition of the cell wall (HADDAD et al., 1999). The two cutting heights tend to decrease CP% (9.51-8.21) from 10 cm to 20 cm. But the CP% increases cubically (6.49, 8.06, 11.39 and 11.59) with N (0, 150, 300 and 450 kg ha^{-1}) as shown in Table 3. Similarly, CP values of 13% in *B. brizantha*. Has been reported by Alves De Brito et al. (2003). Cunha et al. (2012) also reported the 8.3% CP in Brachiaria leaves, superior in quality to the stem CP (3.14%). Season wise, in winter the highest CP% was reported (11.88), the most obvious reason is the slow growth rate which causes the accumulation of nutrients without imparting to the growing plant parts (figure 7). However, the lowest CP was in autumn (7.78) due to the higher temperature which resulted in rapid growth and increased fibrous portions. The season was significant in

combination with height (P<0.009) and nitrogen (P<0.0001). In the summer, the 10 and 20 cm heights give significant differences in CP (4.74, 6.56, 9.58 and 10.70%) and (6.10, 7.71, 9.50 and 10.10%), respectively by the N doses as shown in Figure 6. Being a grass with advantage for faster N intake under fertilized conditions and using at a higher rate (BOBBINK, 2010). In the start, the increase in CP was lesser at low height but the increase by the N was sharp, in a quadratic way (R^2= 0.98) and much quicker. But at 20 cm it behaves in a linear way with not many appreciable results by N application. Santos et al. (2009), evaluating the signal grass under N and deferred, observed linear increase in CP content, with higher concentrations found in green leaves followed by a green stem. At the highest dose of N (120 kg.ha^{-1}), founded the CP content of 8.3% in leaves; the same CP values as observed in our experiment. This guide us for the proper management of fertilization and grazing, and prioritizing the leaves in the animal diet grazing, while decreasing the dead parts and lignified portions (HODGSON, 1990). Euclides et al. 1993; Herling et al. 2000, also reported the value of CP (16.1 and 7.4%) in leaves and stem of forages.

In autumn, significant differences in the cutting heights of plants with N doses (0, 150, 300 and 450 kg ha^{-1}) at 10 cm (5.24, 9.03, 8.73 and 8.95%) and at 20 cm (5.81, 6.47, 8.5 and 9.5%) were observed. The 10 cm height showed a cubic increase in CP while the 20 cm behaves linearly as shown in Figure 8. Teague et al. (1996); also reported a higher CP content of *Trifolium repens* with a decreasing cutting height. In agreement with our results; Herrero et al. (2001) also, reported the CP value of 12% on DM basis in 4-6 weeks age of leaves in *B. brizantha*. Vendramini et al. (2008) reported a linear increase in CP of *Tifton85 bermudagrass* (*Cynodon* sp.) with increasing N fertilization and by the interaction of growth interval*month. This difference may be due to the severity of climatic factors or the normal difference of experimental conditions within greenhouse and field experiments.

According to our experiment, in winter, the highest CP was reported (Figure 7); however (VENDRAMINI et al., 2008); analyzing same species showed a greater CP content in spring. In spring and winter, there were no differences as per the two cutting heights and both behaves in a cubic way on all N doses. However, the increase in CP was very abrupt and immense at cutting height of 20 cm in winter at 300 kg with 200% increase as compared to 150 at 20 cm height and compare to the cutting height production as well. However, at the height of 10 cm, the CP increase was gradual with only 30% increase at 300 kg ha^{-1} and then remains constant at 450 kg ha^{-1} N. In literature the same CP value has been reported by Tinnakorn et al., 1989; that at higher fertility range during regrowth period of 30- 60 days plants showed the 8.10 - 13.87% CP. Simply many authors (CHANDLER et al, 1959) demonstrated the positive effect of nitrogen on

the CP%; probably due to reason of nitrogen reduction to form ammoniac and assimilated the carbon skeletons via cycle GS-GOGAT (glutamic acid and glutamine), which is precursor for various amino acids, used for formation of about 20 (Natural amino acids) for protein formation, in process of "all or none" (MALAVOLTA; MORAES, 2007).

Figure 6. Crude protein % in *B. decumbens* by interaction of nitrogen and heights in summer

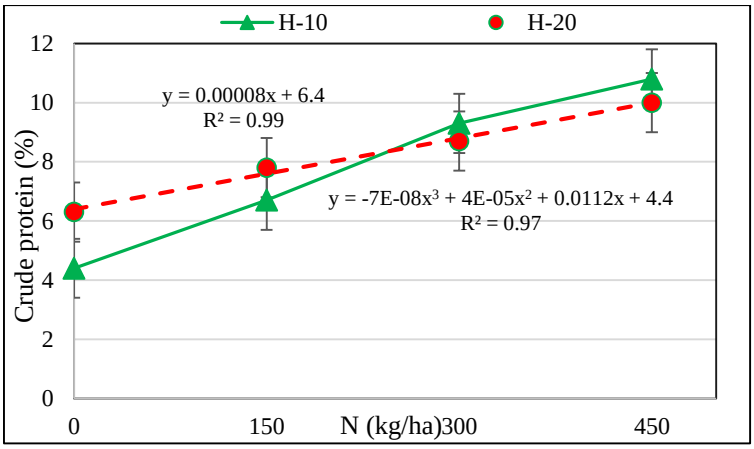


Figure 7. Effect of nitrogen on crude protein % during spring and winter in B. *decumbens*

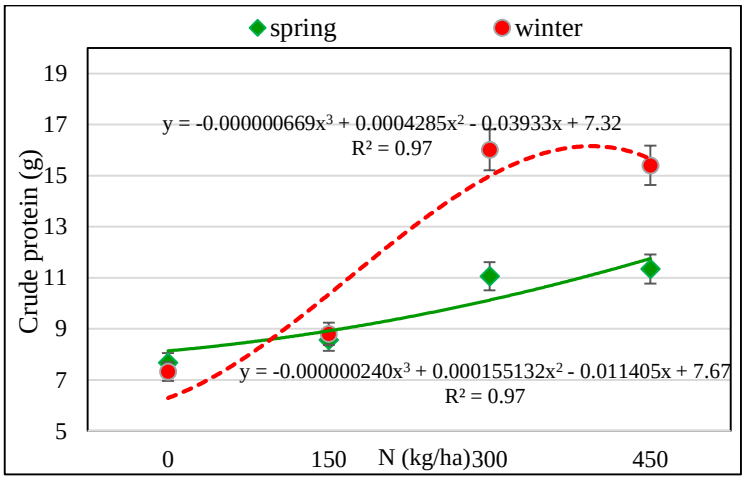
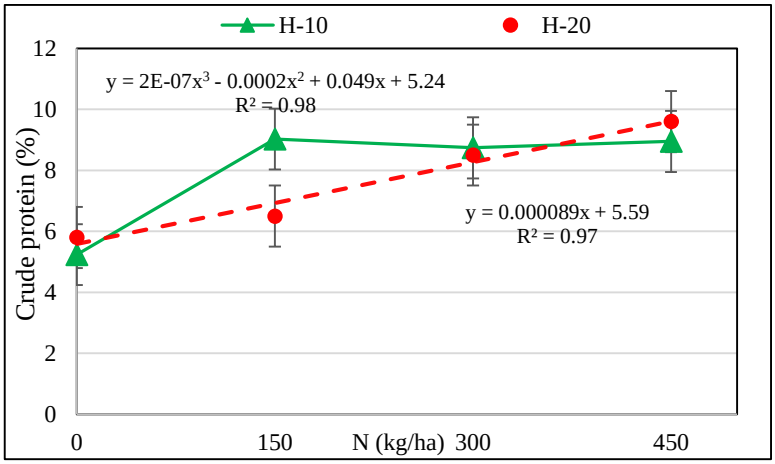


Figure 8. Crude protein % in *B. decumbens* by interaction of nitrogen and heights in autumn



2.4.3 Acid detergent fiber (ADF)

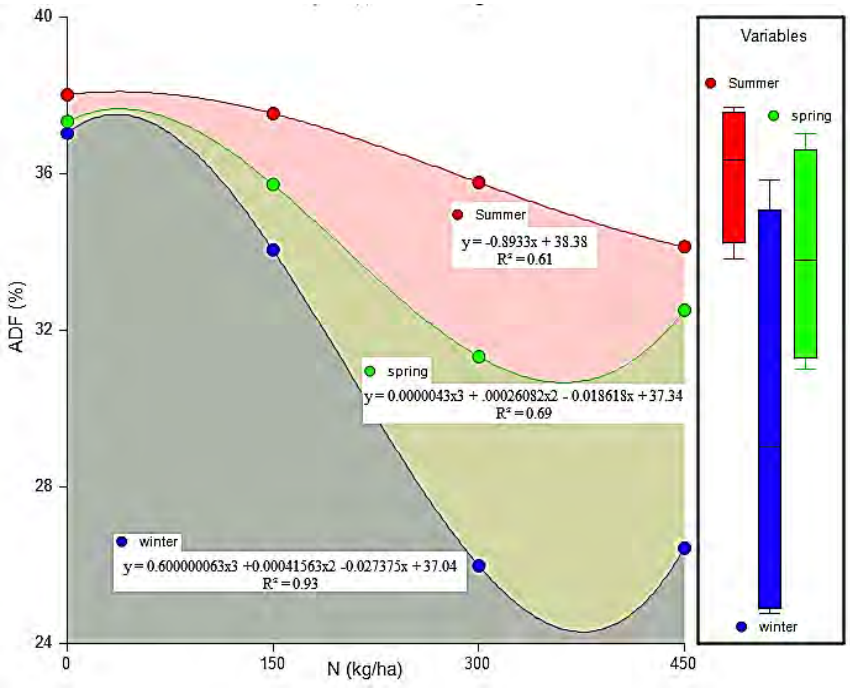
The acid detergent fiber consists of cellulose and lignin and is correlated with the digestibility. Simply as the ADF is higher in the forage, the lower is digestibility (LANA, 2005). According to Van Soest (1994), it is not recommended for not only to refer the nutritional value of plants based on their cellulose, as the digestibility varies completely with the indigestible components, depending on the degree of lignification. The Lignin is not a carbohydrate but an amorphous phenyl polymer presenting structures and is indigestible and inhibitor digestibility of plants.

For ADF; all interactions (H*N, S*H, and S*N*H) were non-significant except for S*N which found to be significant (P<0.0001) as presented in Table 3. But were effected by N fertilization (P<0.0001) and the ADF trend was reducing with seasons as well (P <0.0001). In a way, the ADF showed a bit more flexibility and variation as compared to the NDF in *B. decumbens*. Costa et al., (2007) evaluated the effect of plants age on the chemical composition of *Brachiaria* by cutting at 15, 20, 30 and 60 days. They observed the DM in the range of 16 - 26%, due to lesser cutting intervals as the young plants have more water content. Seasonality showed significant (P<0.0001) differences in the ADF% of signal grass, presenting lowest values in winter (30.88 $\pm$ 0.29) as shown in Figure 9; while the highest was observed at higher temperature (39 ºC) of summer (36.37 $\pm$ 0.29), which probably promotes quick plant maturity and higher lignin fractions. According to Mari (2003), the major changes occurring in the chemical composition of forage plants are due to plants maturity and seasons as the potentially digestible components tends to decrease, the proportion of lignin, cellulose, hemicellulose and other indigestible fractions increase. The interaction of season and fertilizer application significantly

(P<0.0001) effected the ADF%, but the interaction of (nitrogen and height) and (seasons and heights) don't imply any changes. The nitrogen defines a cubic regression effect on the ADF production and with the increasing N doses (0,150, 300 and 450 kg ha^{-1}); ADF% automatically declines (37.34, 35.82, 32.28 and 32.14%).

The 20 cm plant cutting height; gives lesser ADF (34.06 %) while the 10 cm produces more (34.75), but the difference isn't significant. No effects on the ADF% were observed by any experimental factors during the autumn. The effect of N on ADF% in summer was linear while in winter and spring it changes cubically with the lowest value at 300 kg $ha^{-1}$and then increases at 450 kg ha^{-1}. In winter the lowest ADF % of leaves were noticed at 300 kg ha^{-1} (25.99%), making higher its digestibility (Figure 9). According to Bauer et al. (2008), the differences in digestibility between species could be attributed due to lignin, especially. Russo et al., (1998) reported that forage having ADF% around 40% or more, has low intake and digestibility. In the present study, the ADF directly decline with nitrogen doses. Crops having lower ADF content lower than 40%, indicating them as good quality with draft resistance, rapid recovery, the rapid growth of new tillers and hence more yield. Though our results of ADF and NDF% are not different from Sukkasem et al., (2000) that while analyzing the fibrous components of plant didn't been different on manure fertilizer and the noted NDF and ADF was 66.68 and 33.21% respectively. The results are also in accordance with Tinnakorn et al., (1989); that planting grasses in high fertility soil normally have NDF ranging in 60.85 - 69.62%, and ADF in 35.06 - 42.94% during 30- 60 days of regrowth.

Figure 9. Effect of nitrogen on acid detergent fiber % during seasons in *B. decumbens*



2.4.4 Neutral detergent fiber (NDF)

According to Van Soest (1994), the cell wall is cellulose and hemicellulose, more important for rumen metabolism which defines the digestibility of plant. The NDF showed significantly interactions (P<0.0001) for all factors (N, H, S, H*N and S*N) but non-significant for S*H and S*N*H as presented in Table 3. The NDF was effected seasonally and the lowest was reported in spring (69.60) and highest in winter (72.80). The nitrogen cause a cubic effect (R^2 =0.68) on NDF and it decreases (72.28, 71.92, 70.27 and 70.31) by the doses of N (0,150, 300 and 450 kg ha^{-1}). The same range of NDF% (71.95) has been reported by Alves de Brito et al. (2003) in *B. brizantha*.

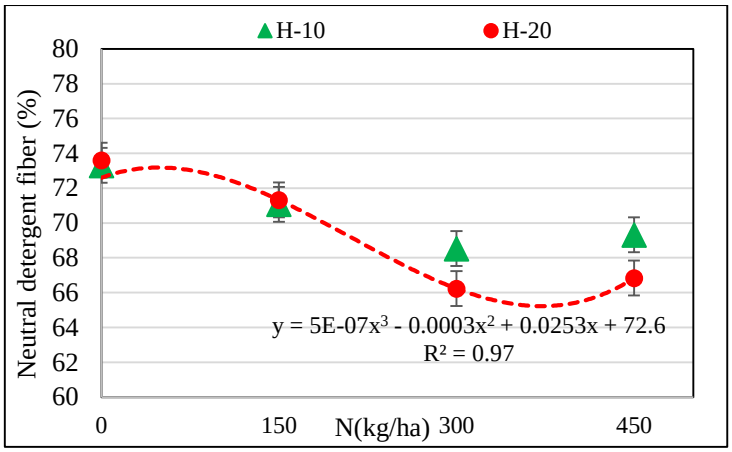
Cutting heights at 20 cm gives the lower NDF (70.94%) while 10 cm produces more (71.60). No effects were observed by any of the two primary experimental factors (N and H) during the autumn, and the 20 cm height cause a decrease in a cubic way by the N as shown in Figure 10, while the 10 cm cutting heights didn't show any change. The NDF % behaves in a quadratic way in summer (R^2 =0.76). Observing the N doses; the 300 kg ha^{-1} N dose gives the optimal NDF production (70.27%) while at 450 kg ha^{-1}, NDF seems to increase again (70.61%). Our results are as Maranhão et al., (2009), that at 225 mg/dm N the NDF was 59% and 61% for the 360 kg ha^{-1}.

Balsalobre et al. (2001) reported that most grasses suffer a decline in the nutritional value with increasing age, resulting in lower leaf/stem ratio and higher lignification. As NDF is correlated with animal's consumption and higher NDF level decrease it (LANA, 2005) above 55-60% (SOEST, 1965, LIMA et al., 2002; NUSSIO et al. 2002). In this study, we found of NDF values of (65-72%) as shown in Table 3. The results of Malafaia et al., (1997) showed the NDF% (80.45) at 60 days age, which prove that depending on plant age it increases. As the increase in N rate cause a decrease in the content of NDF and it behaves cubically in the reduction of NDF. According to Santos et al. (2009), the appearance of more phytomers and stems cause the plant became more rigid and thick to support the plant by the application of nitrogen. For this, the stem will contain a higher percentage of structural support tissue with thicker cell walls which decrease the digestibility (PUTNAM AND OTTMAN, 2013).

Santos et al. (2010) found in deferred *Brachiaria* fertilized with N (120 kg ha^{-1}) produces 82% NDF in the stem but our results are comparatively different in NDF% due to being analyzing the different plant proportions. Also, the higher proportion of NDF may be attributed due to larger growth period while applying a dose of 360 kg ha^{-1}; produces 63% NDF. Justify the authors that high NDF content was due to the growth for 70 days, creating a greater accumulation of lignified tissues. Our results are similar to Santos et al. (2009) founding 62% FDN in leaves of deferred signal grass, fertilized with 120 kg ha^{-}

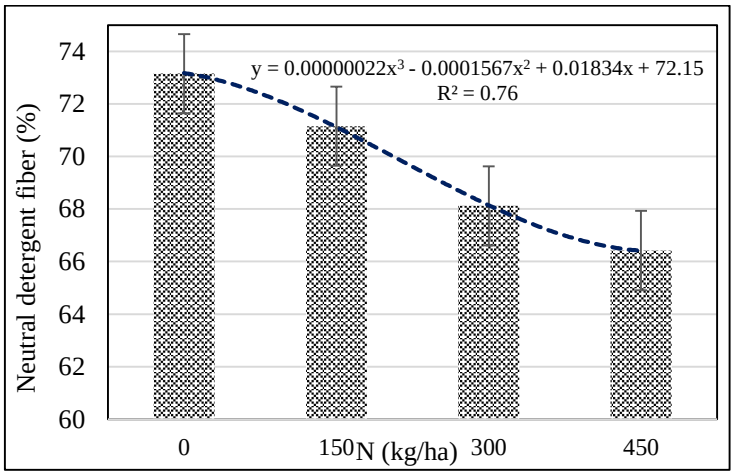
[1] N. In our study we found a content of 66% at the dose of 300 kg ha^{-1}, showing that N increment can improve the digestibility by decreasing the NDF and fibrous portions.

Figure 10. Interaction of nitrogen and heights on neutral detergent fiber % during autumn in *B. decumbens*



In summer, we observed a significant and gradual decline in the NDF% (73 - 66) with N doses (0 - 450 kg ha^{-1}) as shown in Figure 11. The highest decline in NDF occurred at 300 kg ha^{-1}. Similarly, Borghi et al., (2007) evaluated the Marandu palisade grass and obtained NDF of 72% while PARIZ 2011 studied the chemical composition of Marandu and ruziziensis and reported above 60%. Furthermore, Pariz (2010); examined four cultivars of *Brachiaria* intercropped with corn and reported NDF of 66.4 - 74.3 and 70.3 - 78.1 for Mulato and Marandu palisade grass, respectively. It is important to note that during all cuts very lesser variation in NDF was occurred in palisade grass and Xaraes by the seasonal influences (CHIARI 2008).

Figure 11. Effect of nitrogen application on neutral detergent fiber % during summer in *B. decumbens*



2.4.5 In-vitro dry matter digestibility (IVDMD)

According to Bauer et al. (2008), differences in species digestibility could be due to lignin as by the negative correlations between digestibility and cell wall components. IVDMD of grasses generally increases with green leaves, ratio of leaf/stem and CP, whereas strongly decreases with dead parts, age and fiber. For IVDMD significant ($P<0.0001$) interactions were found for (N, H, S, H*N, S*N and S*N*H) while non-significant results were observed for (S*H) (Table 3). In our results; *B. decumbens* IVDMD was higher from Mar. – Jun. (65-68%) as presented in Table 3. According to Lascano and Euclides, (1996); the IVDMD normally lie between 60-70% in young forages and 50-60% in mature ones.

Similarly, average values of IVDMD (62.14%) in *B. decumbens*, has been shown by Santos et al., (2010). While Paciullo et al., (2001) observed the IVDMD of 53.2% under canopy. In the same way, N effected the IVDMD linearly and depending upon the plants cutting heights; the 20 cm gives higher (66.07) digestibility. Similarly, Ruviaro et al., (2012) by evaluating IVDMD in Marandu grass found linear responses by N doses and at 250 kg ha^{-1}; the digestibility was (69%). Only in summer (Figure 13), the cutting heights in interaction with N was significant ($P<0.0135$) but upon observing the differences in both heights; significant results were found for 10 cm (R^2 =0.88) only. Season wise the highest IVDMD was in autumn (67.35%) (Figure 12) by the increase in N. While the lowest was in spring (63.65%) probably as the plant grows and sprouts rapidly due to the climatic factors producing a greater proportion of stem to leaves. This may be imparted to both the temperature, light and plant physiological changes enhancing the photosynthetic process; which ultimately increase the deposition of lignified cell wall; a digestibility barrier in the rumen (FREER et al., 2002). The highest digestibility was recorded at a dose of 300 kg ha^{-1}(68.06 %). However, our results seem bit higher due to the age difference or special structural and degree of lignification differences (HERRERO et al., 2001) that IVDMD can be of 55% at 4-6 weeks age in *B. brizantha* leaves.

Figure 12. Effect of nitrogen on in-vitro dry matter digestibility % in B. decumbens during various seasons

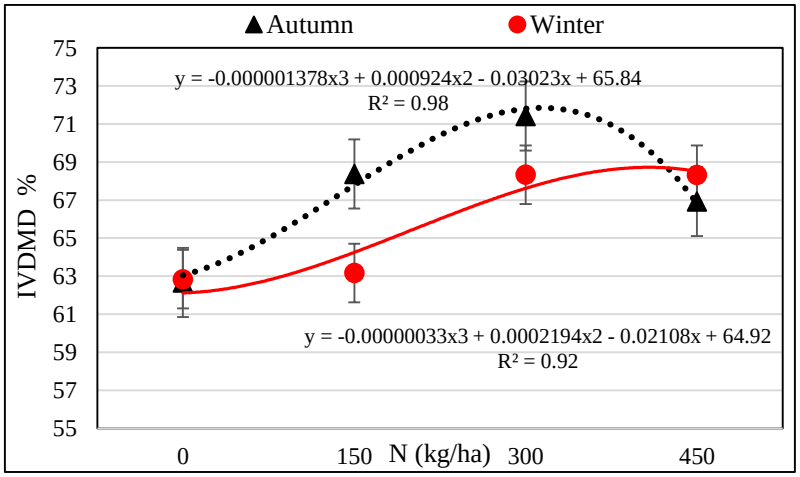
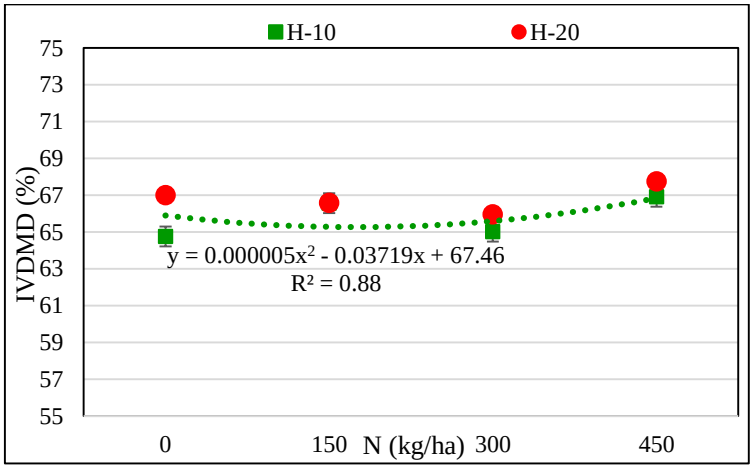


Figure 13. Effect of interaction of season and nitrogen on in-vitro dry matter digestibility % during summer in *B. decumbens*



2.4.6 In-vitro organic matter digestibility (IVOMD)

For IVOMD; non-significant interactions were found for (H*N and S*H and S*N*H) while for (N, H, S and S*N) were significant as presented in Table 3. The season and nitrogen both effected the quality (P<0.0001 and P<0.0045) and jointly (0.0203). N enhance the digestibility by adjusting regression cubically (P<0.0045) as it reduced the fibrous portions and enhances the digestibility. Unlikely to results of Cecato et al. (1996) who found no effect of N fertilizer in Marandu for IVOMD with an average digestibility as 59.7%. (EUCLIDES et al. 1993; HERLING et al. 2000), also reported IVOMD of 61.3 and 56.7% for immature and mature plants digestibility respectively. However, based on this; the fertilized group IVOMD lies (56.11 – 62.72), showing the dependency on plants age and physiological status. However, the change in plants heights changes the digestibility

and at the 20 cm cutting heights, IVOMD was higher (59.79%) (Table 3). While in the temperature range of autumn (25.5-38.0 ºC) the digestibility index was recorded as highest (60.43) and in spring was lowest (57.12). However, all these lies within the range of 50-80% for forages digestibility (COOK et al., 2005). The interaction of height and nitrogen resulted in significant differences only in summer and 10 cm height gives more abrupt response as shown in Figure 14. In autumn, the highest OMD was observed at 300 kg ha^{-1} (61.43%) as shown in Figure 15. As our digestibility results are of higher quality for animals but according to literature, the prostrated plants tillers has a lower digestibility than from erect-growing plants (VAN WIJK; 1980). Universally, increasing cutting frequency reduced fibrous portions and increase CP, soluble sugars and minerals leading to higher organic matter digestibility and nutritive value (RUGGIERI et al., 1994) which proved to be true in our case.

Figure 14. Effect of nitrogen on in-vitro organic matter digestibility % during various seasons in *B. decumbens*

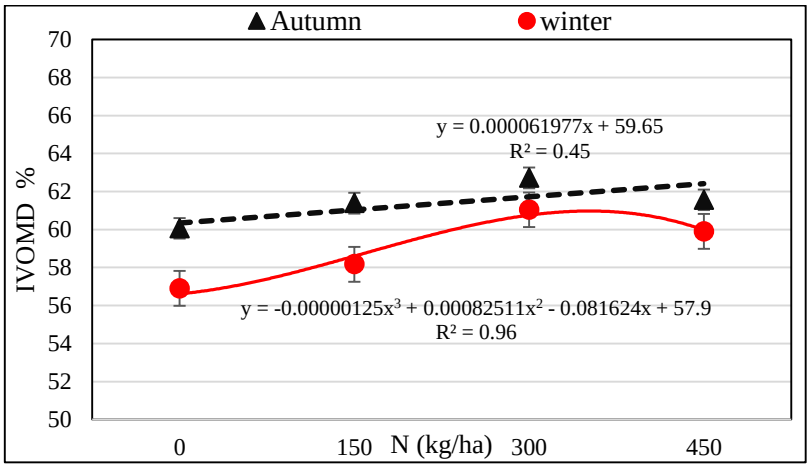
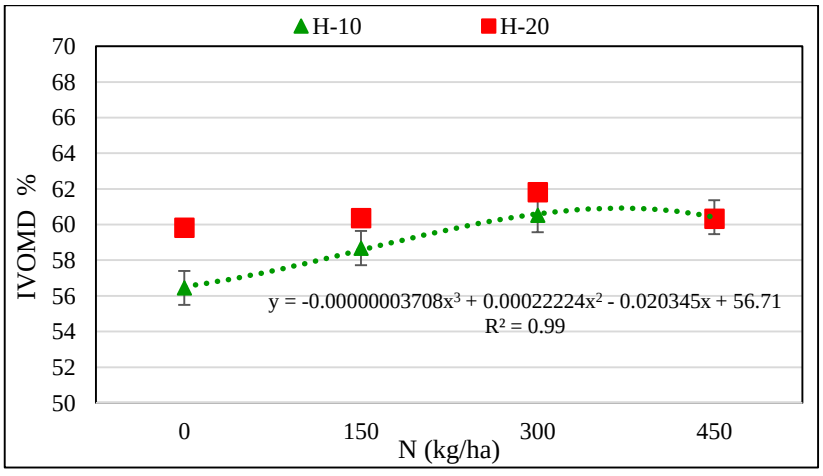


Figure 15. Effect of interaction of season and nitrogen on in-vitro organic matter digestibility % during summer in *B. decumbens*



2.5 Conclusions

In our experiment although no nutritional limitation existed but still N showed the trend as settled by the theory of multiple limitation hypothesis (MLH) that any accompanying aspects (height or seasons) indicates the capacity of growth increase by nitrogen application and were somewhere dependent upon the availability of favorable conditions also.

The highest dry mass production in autumn may be due to the plant full blooming to increase production and also the favorable temperatures maintaining the high production. However, despite the wide variations in yield, nitrogen was consistently having a positive impact improving the protein and decreasing the fibrous portions at doses of 97 mg.dm^{-3}/pot (300 kg ha^{-1}). However, observing the nitrogen use efficiency, the 150 kg/ ha N is supposed to be best as the efficiency of utilizing N was higher compared to the 300 kg/ha N. Season wise the higher dry matter yield was obtained in autumn and summer and highest crude protein in winter, whereas the highest IVDMD (68%) and IVOMD (59%) was reported in autumn. The 10 cm cutting height favors the increase in CP and NDF, while the 20 cm improves the plant quality by an increase in DMY with a decrease in ADF and digestibility indexes.

2.6 Recommendations

Maturity stage affects the DM yield and nutritive composition and the forage must be harvested before reaching full maturity, in our experiment the 30 days cutting interval didn't cause any limits on the plant productivity but probably the rich soil status and conditions limits the plant productivity after 150 kg ha^{-1}. But been a greenhouse experiments needs to be repeated or applied in field for confirmations of results and trends. For cross verification of inter and intra effects of experimental factors; every factor must be evaluated individually for confirmation. Also above 150 kg ha $^{-1}$ N isn't recommended due to not having initrogen use efficeincy as declined by plants and also due to an increase in cost.

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CHAPTER 2 – SEASONAL VARIATION IN THE METABOLIC PROFILE OF BRACHIARIA DECUMBENS UNDER NITROGEN FERTILIZER APPLICATION

ABSTRACT

B. decumbens have been reported as the most phototoxic pasture among *Brachiaria* spp. affecting animals. However, the lack of detailed information and literature on their varying production or chemical profile makes us focus on these properties for further investigation. The study was conducted in the proposal of various plant focused models for proposing the concentration of carbon-based secondary compounds. Thus, the experiment was conducted in complete randomized block design (4×2 factorial) with defining the seasons, nitrogen doses (0, 150, 300 and 450 kg ha^{-1}) and cutting heights (10 and 20 cm) on the metabolic changes in *B. decumbens* cv. Basilisk, at greenhouse of FZEA-USP. The metabolic profile was analyzed on UHPLC/ NMR for the metabolites classes, types, and probable amount. Measurements gave us a total protodioscin isomeric conc. of (34.45 – 51.48 g/g dry weight (d. wt.)) and protodioscin isomers were confirmed by NMR. The structure was elucidated by the use of a combination of 1D (^{1}H, ^{13}C) and 1D NMR technique (COSY-45, edited HSQC, HMBC, H2BC, HSQC-TOCSY, NOESY and $^{1}H,^{1}H$ J) as O-α-L-rhamnopyranosyl-(1!4)-O-β-D- glucopyranosyl- (1!6)-O-β-D-6-O-acetylglucopyranosyl- (1!2)]- β-D-glucopyranosyl medicagen-28-ate.

All the experimental factors effected all the metabolic profile significantly ($P<0.0001$), and behaves cubically by the application of nitrogen. Phenols decreases with application of nitrogen while the flavones increases by nitrogen application. The lower production of phenols and flavones were noted at 20 cm (8.45 and 25.03 mg/g d. wt.). While seasonally the winter produces less phenols and autumn produces minimal flavones (19.65 mg/g d. wt.). The highest phenols were observed in autumn (9.74 mg/g d. wt.) and in spring highest flavones (28.87 mg/g d. wt.) were reported. Observing phenols concentration the trend was seen as per the basic defense theory model; presenting higher concentrations under poor nitrogen situation, while flavones not. Focusing the saponin; seasons always affect their concentrations significantly ($P<0.0001$) and in *B. decumbens* leaves the highest protodioscin isomers concentration was reported in winter (49.21$\pm$6.2 mg/g d. wt.) and the lowest in summer (41.5$\pm$3.2 mg/g d. wt.). While the 300 kg ha^{-1} N produced the highest protodioscin isomers (46.57 mg/g d. wt.). The 10 cm plant cutting height was with higher production (47.47 mg/g d. wt.) as compared to the 20 cm (46.43 mg/g d. wt.). The results showed the significant differential

concentrations of protodioscin (17.63±4.3 to 22.57±2.2 mg/g d. wt.) and protoneodioscin (23.3±1.2 to 31.07±2.9 mg/g d. wt.) in *B. decumbens* per seasons and cutting heights. Hence, the study emphasizes that protoneodioscin could be most probably the basic reason of *B. decumbens* higher toxicity as compared to *B. brizantha.*

KEYWORDS – Metabolites, toxicity, protodioscin, seasons, UPLC*, Brachiaria*

CAPÍTULO 2

VARIAÇÃO SAZONAL NO PERFIL METABÓLICO DE Brachiaria decumbens SOB ADUBAÇÃO NITROGENADA

RESUMO

Entre as espécies de Brachiaria, a espécie relatada como a mais fititóxica para os animais é a espécie B. decumbens, entretanto, a ausência de informações detelhadas na literatura a respeito de seu perfil químico nos objetiva a focar. Neste sentido, este estudo foi conduzindo para verificar a influência das estações do ano, doses de nitrogênio (0, 50, 300 e 450 kg·ha-1) e alturas de cortes (10 e 20 cm) sobre as alterações metabólicas da B. decumbens. O experimento foi conduzindo em casa de vegetação na FZEA-USP em delineamento inteiramente casualizado. O perfil metabólico foi analisado em UHPLC/RMN, analisando-se as classes de metabólitos, tipos e quantidades. As determinações por cromatografia demonstraram teor de saponina (34.45 - 51.48 mg/g d.wt.), confirmado por RMN. A estrutura foi determinada através da combinação de 1D (1H, 13C) e 1D NMR técnica (COSY-45, editado HSQC, HMBC, H2BC, HSQC-TOCSY, NOESY and 1H,1H J) como O-α-L-rhamnopyranosyl-(1!4)-O-β-D- glucopyranosyl.

Todos os fatores experimentais (doses, alturas, e estações) afetaram significativamente o perfil metabólico (P<0.0001) comportando-se de forma cúbica para aplicação de nitrogênio. Para flavonas o menor teor foi verificado no outono (19.65 mg/g·ms) e para os fenóis as maiores concentrações ocorreram nas menores doses de N. Para saponina, houve efeito significativo das estações. O teor de fenóis diminui com a aplicação de nitrogênio, enquanto o teor de flavonas aumenta por aplicação de nitrogênio. A menor produção de fenóis e flavonas foram observadas a 20 cm (8,45 e 25,03 mg/g (matéria seca (ms)). No entanto, de acordo com as estações, no inverno produz-se menos fenóis e no outono menos flavonas (19,65 mg/g ms). Os maiores teores de fenóis foram observados no outono (9,74 mg/g ms) e na primavera os teores de flavonas mais elevados (28,87 mg/g ms) foram relatados materai seca. Observando-se a concentração de fenóis a tendência foi de acordo com o modelo da defesa básica; apresentando maiores concentrações em situação de ausência de nitrogênio, enquanto as flavonas não apresentaram o mesmo quadro. Em se tratando da saponina; as estações sempre afetam as suas concentrações significativamente (P<0,0001) e nas folhas de *B. decumbens* a maior concentração de protodioscina foi relatada no inverno (49,21 + 6,2 mg/g·ms) e a menor no verão (41,5 + 3,2 mg/g ms). Para os 300 kg·ha^{-1} de nitrogênio produziu-se os mais altos valores de protodioscina (46,57 mg/g ms). Os 10

cm de altura da planta foi relacionado com a maior produção de isômeros de protodioscina (47,47 mg/g ms), em comparação com 20 cm (46,43 mg/g ms). Os resultados mostraram as diferenças significativas nas concentrações de protodioscina (17,63+4,30 - 22,57 + 2.20 mg/g·ms) e protoneodioscina (23,30+1,2 - 31,07 + 2,90 mg/g·ms) em *B. decumbens* por estações e alturas de corte.

PALAVRAS-CHAVE - Metabólitos, toxicidade, protodioscina, estações, UPLC, *Brachiaria*

3.1 Introduction

Signal grass (B. decumbens) is the most prominent forage in Brazil due to the high dry matter yield, easy growing, drought resistance, good adaptation to different soils and low maintenance cost (SEIFFERT, 1980; SALAM ABDULLAH 1992; CASTRO et al, 2011). The most widely grown cultivars are *Brachiaria decumbens* and *Brachiaria humidicola* (RIET-CORREA et al., 2011). In Brazil, despite its large impact on livestock production it also cause severe losses by photosensitization in animals e.g. cattle (LEMOS et al., 1996; SOUZA et al., 2010), sheep (RISSI et al., 2007; MUSTAFA et al., 2012), goats (MACÊDO et al., 2008; SILVEIRA et al., 2009), buffalo (OLIVEIRA et al., 2013) and horses (BARBOSA et al., 2006). Analyzing the data; young animals are more susceptible than adults (ALBERNAZ et al., 2010), sheep are more susceptible than cattle while non-pigmented animals are more targeted also.

Brazil; as the largest beef exporter with biggest commercial beef herd (> 200 million heads) (FAO, 2014; PENRITH et al., 2015; TORRES Jr.; NETO; 2012), is almost dependent on $\geq$ 200 million hectares pastures areas (VIGNA et al., 2011) due to easy availability and low production cost (SAMPAIO et al., 2010). *Brachiaria* has covered 85% pasture area by its various species (VIGNA et al., 2011) and *B. decumbens, B. brizantha* are most prominent amongst them with 50 million hectares (JANK 2014) coverage. The most probable reason as de fined by (BARBOSA et al., 2008) is the presence of phytotoxin in these species; making them the largest monocultures in the world by suppressing the nearby plants and canopies.

Apart, being holding large cattle population; Brazil is also facing highest plant poisoning cases; as one of the main three reasons in causing farm animal's death (TOKARNIA et al., 2012; PESSOA et al., 2013; RIET-CORREA et al., 2010) by 113 plant species (RIET-CORREA et al., 2007). The rough estimates of plants toxicity and losses are 160 -240 million US$ (RIET-CORREA; MEDEIROS, 2001; TOKARNIA et al., 2002) with mortality of 800,000 and 1,120,000 cattle/year (TOKARNIA et al., 2012). *B. decumbens* and *B. brizantha*; also amongst the mortality causing plants as highest sporadic outbreaks of photosensitization (LEMOS et al., 1997; BRUM et al., 2007) amongst goats, sheep, llama, buffaloes, deer and cattle across Brazil, Colombia, Australia, Papua New Guinea, Malaysia and Sri Lanka are reported.

Earlier, the hepatogenous photosensitization syndrome and toxicity were correlated to sporidesmin produced by the *Pithomyces chartarum* fungus present in Brachiaria, but later investigation defined the root cause as furostanol saponin (SATURNINO et al., 2010). This lithogenic saponin is actually protodioscin (DRIEMEIER et al., 1999; SANTOS JR., 2008) which after metabolization in the gastrointestinal tract

of animals produce smilagenin, sarsasapogenin epislamigenin, and episarsapogenin (CRUZ et al., 2000; MEAGHER et al., 2001). Afterward, causing lesions by birefringent saponins and sapogenins crystals from *B. decumbens* and *B. brizantha* (TOKARNIA et al., 2012). Both of these species are confirmed for the presence of sapogenol B type, aglycone, pentacyclic derivatives and protodioscin isomers (SANTOS, 2008; ALBERNAZ et al., *2010*; PORTO et al., 2013), but *B. brizantha* is less toxic as compared to *B. decumbens;* due to many suppositions. Although till yet the identified toxic component is the protodioscin isomers but again many contrasting results exists with the need to address the real cause.

Since, the saponin production in plants is correlated to plant's vulnerable status (LEE et al., 2011), environmental stress, age and developmental stage (OLESZEK, 2002), solar radiations, season, grazing, attacks or microbial population (RIET-CORREA et al., 2011). Although, many contrasting results again exist about the protodioscin concentrations; as Castro et al. (2011) and Ferreira et al. (2010); reported 5–10 fold higher saponin concentrations (0.3% to 2.56%) in younger leaves as compared to matured ones. While, others (BRUM et al., 2009) reported higher concentration (1.9 %) during seed fall. (GRACINDO et al., 2014) reported values of protodioscin isomers as 12.2 and 4.5 g kg^{-1} DM; while (BRUM et al. 2009) showed 2.36% and 1.63% in *B. decumbens* and *B. brizantha*. These results amplify the necessity for further detailed investigations to estimate the underlying reasons of toxicity and the base of the differential component for causing the mortality and toxicity cases. As being the largest pasture of Brazil; it is needed to study it further for prevention and control measures against its toxicity. Therefore, we objected in finding saponin or protodioscin isomers concentrations for one-year experimental period with varying conditions for comparison between seasons, plants cutting heights, doses of nitrogen and plant species. But still, the main target of analysis were leaves as all authors confirm the presence of saponin primarily in leaves, followed by the stem.

3.2 Materials and methods

The *B. decumbens* grown in greenhouse, only leaves were chosen for making the chemical analysis.

dried below 40 C and (discussed in chapter 1) were chosen for chemical analysis and determination of metabolic profile. were harvested with plant scissors after every 30 days at their height specifications (10 and 20 cm) before sunrise and were cutted into 3 cm lenghts. The leaves were dried in oven below 40°C till complete drying and been constant with the weight. Later, the samples were grinded by Wiley Mini Mill at 16 mesh size and stored in black plastic bottles. Evaluations were conducted from Jan. 2014 to Dec. 2014, and were grouped in the following seasons of the year: summer (Jan. – Mar.); autumn (Apr. – Jun.); winter (Jul. – Sept.); spring (Oct. – Dec.).

Due to the high number of samples and as shown by literature that saponin and protodioscin components mainly occurs in leaves, therefore, we opted for determination of secondary metabolites only in leaves. The dried samples as discussed in chapter 1 were taken for saponin analysis. Certain preliminary and confirmatory tests were carried out before metabolic analysis on modified scale. The first step was the phytochemical tests as discussed below:

3.3 Statistical analysis

Data were analyzed with the Statistical Analysis System package (SAS Inst., Inc., Cary, NC). Before the actual analysis, data were analyzed for the presence of discrepant information ("outliers") and normality of residuals (Shapiro-Wilk). When the normality assumption was not met, the logarithmic transformation or the square root was required. Data were analyzed according to the Proc Mixed to mixed models in factorial arrangement of treatments by type 4x2, referring to 4 nitrogen fertilizer levels (0, 150, 300 and 450 kg ha^{-1}) and two cutting heights (10 and 20 cm), and seasons (summer, autumn, winter and spring) as the repeated measures (split-plot). For the analysis, among 15 tested different covariance structures that best fit the statistical model was chosen based on the lower value of the information criterion corrected Akaike (AICC) (WANG; GOONEWARDENE, 2004). The model included fixed level of fertilization effects, cutting heights and season, as well as effects of double and triple interactions between the factors. Seasons of the year were considered repeated measures. Additionally, mean comparisons between the seasons were performed using the adjusted Tukey test and nitrogen fertilization level effect was evaluated with the use of

polynomial regression, decomposing the effects of linear, quadratic and the cubic deviation. All test were performed at 5% significance level.

3.3.1 Preliminary tests

3.3.1.1.1 Phenols - Ferric Chloride

A 5% w/v solution of ferric chloride in 90% alcohol is used for the detection of phenols. 1ml each of filtrate is diluted with distilled water and added with two drops of ferric chloride. A transient greenish to black color indicates the presence of phenolics or tannins (figure 16).

3.3.1.1.2 Flavonoids - Lead acetate

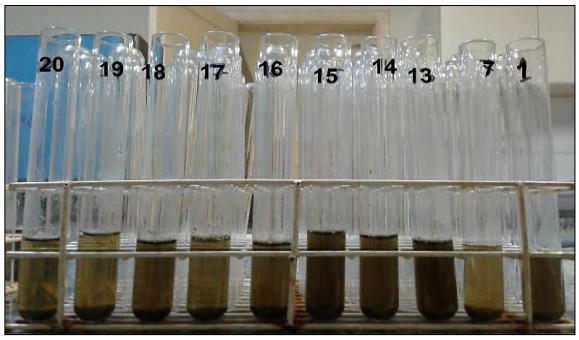
A 25% basic lead acetate solution is used for the detection of flavonoid. A small quantity of the extracts is heated with 10 ml of ethyl acetate in boiling water for 3 minutes. The filtrate was shaken with 1 ml dilute ammonia solution (1%). The layers were allowed to separate and appearance of a yellow color indicates the presence of the flavonoid.

3.3.1.1.3 Saponins - Foaming index (FI)

The FI was determined according to the method described in for medicinal plant materials (WHO, 1989). 1 gm powdered sample was taken in 500 ml flask containing 100 ml boiling water and was boiled for 30 minutes. After boiling, the decoction was cooled and filtered through Whatman No. 42 filter paper into a volumetric flask, and sufficient distilled water was added till completing a volume of 100 ml. The decoction was poured by pipette into 10 test tubes (150 ×16mm) by starting from 1 ml till 10 ml in the 10th tube and making volume in each tube till 10 ml. The tubes were shaken horizontally for 15 seconds and allowed to stand for 15 minutes (figure 17). Afterward, the foam height was measured and results were assessed as follows:

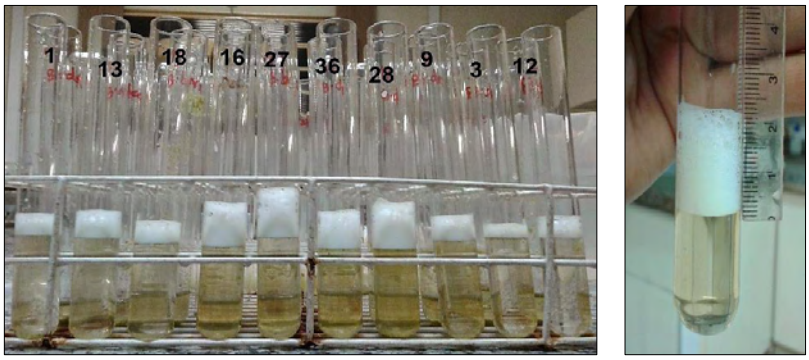
1. If the foam height is < 1 cm, then FI is less than 100.
2. If the foam is > 1 cm, the used decoction sample volume in this tube is used to determine the FI*.

Figure 16. Phenolics in *B. decumbens* leaves



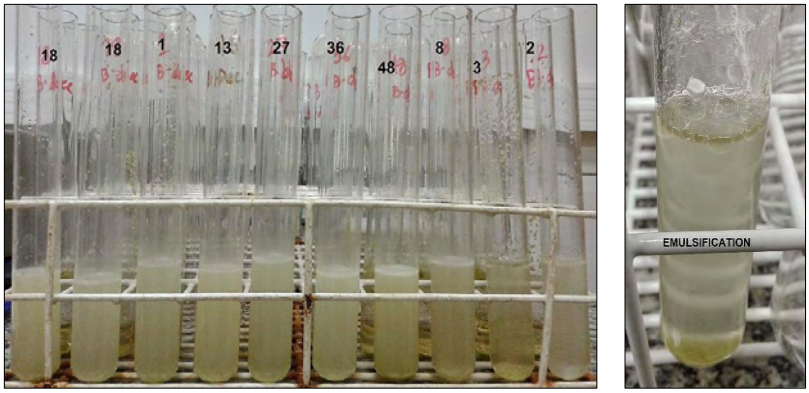
Syeda Hussain; (15th April, 2015)

Figure 17. Foaming index in *B. decumbens* leaves



Syeda Hussain; (27th April, 2015)

Figure 18. Emulsification in *B. decumbens* leaves



Syeda Hussain; (13th May, 2015)

* = If that specific tube is 1st or 2nd decoction tube then further solutions will be obtained in a similar way for more precise results.

If still the foam height is > 1 cm in every tube, then FI is over 1000 and we need to prepare new series of decoction dilution. The FI is calculated as follows:

Foaming Index = 1000/a

Where:

a = The decoction volume (ml) used for preparing the dilution in the tube where 1 cm foaming height is observed.

3.3.1.1.4 Frothing

A 2.5 ml filtrate was diluted to 10 ml with distilled water and shaken vigorously for 2 minutes (frothing indicated the presence of saponin in the filtrate) (figure 17).

3.3.1.1.5 Emulsification

2 drops of olive oil was added to the solution obtained from diluting 2.5 ml filtrate to 10 ml with distilled water and shake it vigorously for few minutes (Fig .18).

3.3.1.1.6 Results

The results of preliminary tests showed (table 5) that there are no flavonoids in samples of *B. decumbens* and plenty of saponins and phenols. Therefore, we opt to proceed for further analysis.

Table 5 - Phytochemical screening of *B. decumbens* leaves

Phytochemical tests	**Results**
Flavonoids	-
Saponins (Foaming index)	+++
Phenolics	+++
Emulsification	++

Key: +++ = Abundant, + = Trace, - = Absent

3.3.2 Chemical analysis

3.3.2.1 Secondary metabolites analysis

The metabolic profile of *Brachiaria* were carried out for identification and quantification of all required metabolites (figure 8) at the institute of soil sciences and plant cultivation (IUNG, Poland); at Laboratory of Biochemistry and plant cultivation. Normally the isolation and determination process of getting pure saponins or glycosides are laborious, time consuming, sensitive and challenging and the structural elucidation is based on:

- Aglycone structure
- Composition and sequence of the sugar in the carbohydrate moiety
- Location of linkages between monosaccharide units

- Location of the carbohydrate moiety on the aglycone

Different qualitative and quantitative tests (physicochemical, and chemical (thin-layer chromatography (TLC), gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), liquid chromatography-mass spectrometry (LC-MS) and nano magnetic resonance spectroscopy (^{1}H and ^{13}C (1D, 2D and 3D)), are widely used for structural elucidation of saponins.

As saponin are polar components and can make easily compounds with other polar components, therefore a suitable extraction method must be applied for their separation. Normally for saponin the commonly used solutions are water / ethanol and the latter is removed by distillation. It is quite impossible to obtain a single saponin from a crude mixture therefore must be separated preliminary by the flash or chromatographic techniques on silica gel with mobile phase of $CHCl_3$-MeOH and Sephadex LH-20. The HPLC/ UPLC with C_{18} columns (normal and reverse phase) also defines artifacts in samples (enzymatic and water hydrolysis, methylation, etc.). However, the classical sugar and permethylated sugar identification is suggested on GC/MS (GUCLU-USTUNDAG; MAZZA, 2007) for their junction points. The carbonyl group (steroidal saponin) absorbs UV light (280-300 nm) and ethylenic double bond appears at 195-198 nm which gives us the functional groups and molecules stereochemistry. The Spirostane derivatives saponins display absorptions between 1350-875 cm^{-1} and the absorptions intensities around 920-950 cm^{-1} and 900-884 cm^{-1} gives use the choice of 25-R or 25-S compounds. In 25-R configuration, the 2nd absorption is more intense while in the 25-S the 1st is intense. NMR spectroscopy (^{1}H and ^{13}C) gives the exact structural dimensions, molecular weight for the intact saponins and the first investigated spectra was steroidal sapogenins by Eggert; Djerassi (1973). The step wise process is shown in figure 19.

3.3.2.2 Extraction by Accelerated Solvent Extractor - 200

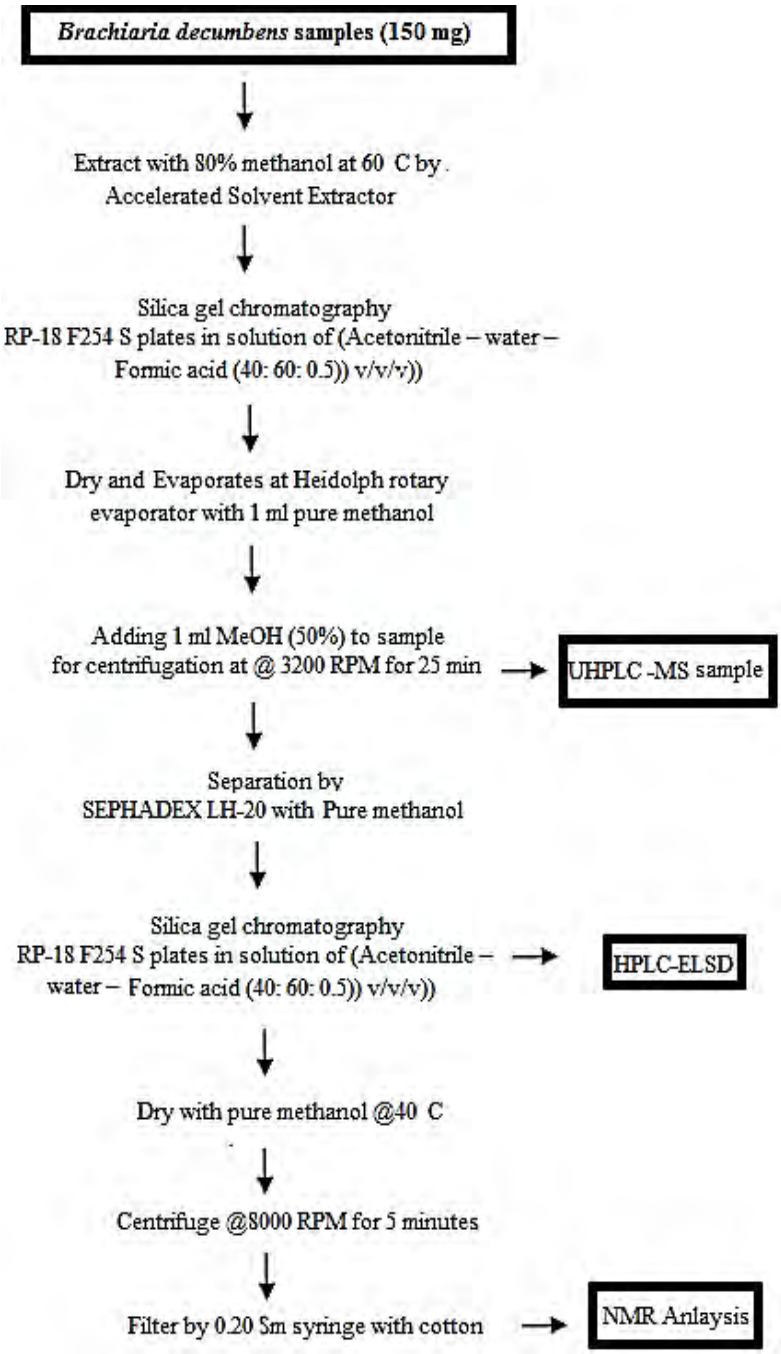
A homogenized sample of 150 mg was taken in metallic cells for extraction on Dionex ASE 200 extractor (Dionex Corp., Sunnyvale, CA, USA). Samples were mixed with diatomaceous earth, 1 g of Li Chroprep RP-18 (40 – 63 µm) (Merck, Darmstadt, Germany) with cellulose filter and were processed with 80% methanol by pressure of (>1000 PSI) and nitrogen supply (100 PSI) at 60 ºC was and collected in Teflon-lined caps (Pierce, Rockford, IL, USA). Each sample took around 21 minutes.

3.3.2.3 Thin-layer Chromatography (TLC)

TLC was carried out with Silica Gel 60 RP-18 F_{254} S plates (Merck, Darmstadt, Germany) using (20 ml) solution of CH_3CN–H_2O-CH_2O_2 (Acetonitrile – water – Formic

acid (40: 60: 0.5)) v/v/v)) as the developing solvent. Spots were visualized by spraying with a solution of (10 ml conc. H_2SO_4 to 90 ml methanol) followed by heating at 100-115 ^oC and were observed under 254 nm and 366 nm UV light. Positively reacting spots were luminescent against dark TLC plate background.

Figure 19. Analytical scheme of secondary metabolites in *Brachiaria decumbens*

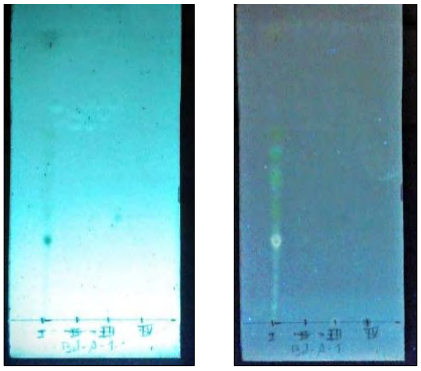


3.3.2.4 Evaporation and drying

The extracts in round bottom flask with 1 ml pure methanol were evaporated (upon foaming; 0.1 ml 50% butanol) at 40 °C and the dried extract was washed with 1

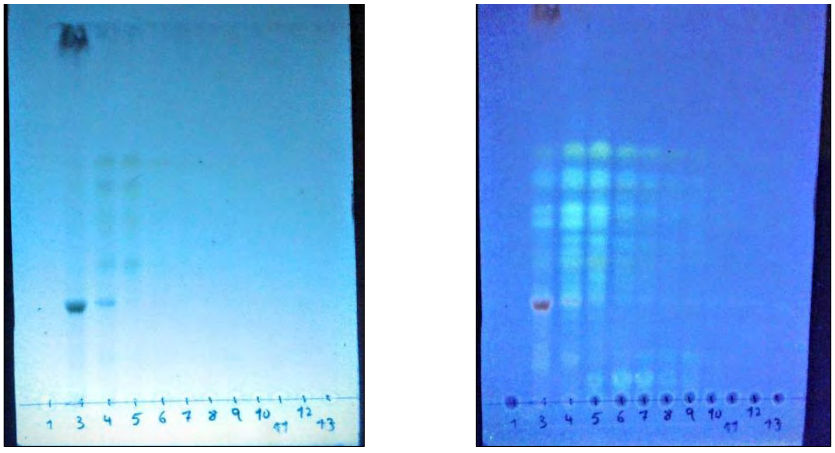
ml (50%) MeOH and deionized water (Milli-Q system; Millipore). Later, was centrifuged for 25 min @ 3200 RPM for separating the residue and MeOH. The solvents for TLC films (figure 20) were made by milli-Q water and MeOH (20%, 80%, and 100%) by Seppak. The water component showed the complete solubility of *B. decumbens* (1st sample) by depicting the colored band under UV. TLC fractions permit the identification of compounds by the approximate Rf (0.3) values (Table 7). Hence, the pure water was chosen for separation on SEPHADEX LH-20 column, eluted with pure MeOH and 13 extracts was collected as shown in figure 21, which were further separated in dextran solution for separation of molecular components and collect them in different test tubes.

Figure 20. TLC RP-18, Sephadex Fractions showing *B. decumbens* polarity in pure water



Syeda Hussain; (27th Sept., 2015)

Figure 21. TLC RP-18, Sephadex Fractions of *B. decumbens* in milli-water



Syeda Hussain; (31st Sept., 2015)

The 2nd tube showed traces of saponin (protodioscin) by a crystalline orangish color due to high polarity and was confirmed by TLC. While at 6th tubes; a transparent bright color showed the sugar component by a bluish or greenish spots as indicators of flavones and phenols in *B. decumbens*. Therefore, we chose the 2nd - 5th tubes for

separation of protodioscin isomers by NMR. The R_f values are shown in Table 6 as initial step.

Table 6 - Overview of the TLC from SEPHADEX and Seppak

Fraction	Solvent system	R_f	Colour	Component
1		0.25	Yellowish /	
2	Acetonitrile : Water :	0.24	orange	Saponin
3	Formic Acid (4:6:1%)	0.86		Phenols /
4	20 ml	0.91	Bluish green	Flavones

3.3.2.5 UHPLC-HR-QTOF-MS

UHPLC analysis was performed with charged aerosol detector, interfaced with high-resolution quadrupole MS. *Brachiaria* metabolome chromatographic separation were performed on Acquity UPLC BEH C_{18} column and data is attached in Annex 1 and Annex 2.

3.3.2.6 UPLC Quantitative Analysis

*S*amples were analyzed by Waters UPLC™ coupled to Waters TQ Detector in ion monitoring, positive electrospray ion mode at 25 °C with a flow rate of 0.4 mL/min for 30 min. Statistical analyses were performed for normality and fitted to Gaussian distribution, two-way / one-way ANOVA analysis was at $p < 0.05$.

3.3.2.7 Detection of protodioscin by HPLC-ELSD

The sample was injected in HPLC-ELSD at (20 µl, 40 µl and 60 µl) at 30 °C oven temperature with 3ml /minute flow rate with nitrogen pressure (60 PSI) and gas pressure as >1000 PSI in Atlantis T3 column. Mobile phases (A and B) were checked for best possible detection and the obtained spectra have been annexed as Annex 3.

Table 7 - Least squares means for metabolites production (mg/g d. wt.) in leaves of *B. decumbens* as affected by nitrogen fertilization, cutting heights and season

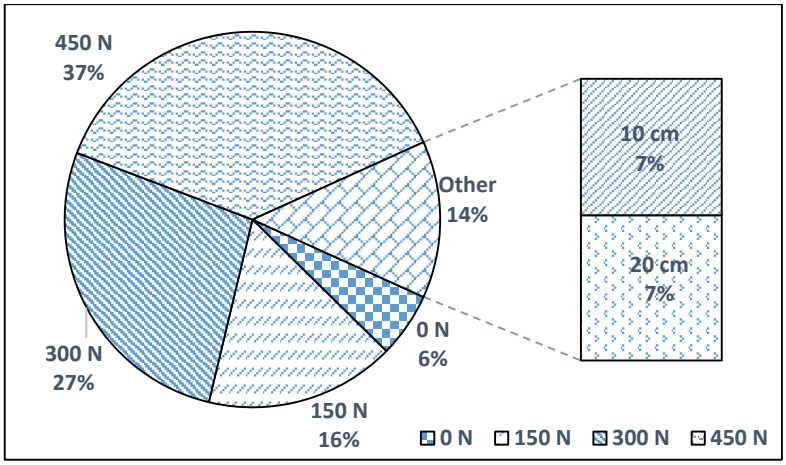
Variable mg g^{-1} (d. wt.)	Height (H) (cm) [1]		Nitrogen (N) (kg ha^{-1})				Mean	SEM[2]	Probability %						
	10	20	0	150	300	450			H	N	H*N	Seasons (S)	H*S	S*N	N*S*H
Ph [3]	8.95[a]	8.45[b]	9.13	8.26	9.12	8.29	8.7	0.1976	*	*	*	*	*	*	*
Fl [4]	27.67[a]	25.03[b]	25.08	27.20	26.39	26.72	26.35	0.4339	*	*	*	*	*	*	*
PDI [5]	47.47[a]	46.43[b]	43.49	45.20	46.57	46.31	45.13	4.88	*	*	*	*	*	*	*

[1] Letters superscript inside the height differs for F test.
[2] Standard error of means
[3] Ph – Phenols
[4] Fl – Flavones
[5] PDI – Protodioscin isomers
* Significant at the 0.05 probability level
# NS, not significant

3.4 Results and Discussion

Many studies have investigated the effects of high nitrogen fertilization on the vegetative and plant primary metabolism but no study hasn't been carried out on the response of CBSM or secondary metabolites to the increasing nitrogen fertilization or seasons or plant cutting heights in *B. decumbens*. Therefore, the objective of this study was to examine the effects of all these factors on the SM (total saponins (Protodioscin Isomers), total flavones (TF) and total phenolic (TP)). We observed; that nitrogen proved to be an influencing factor in causing an increase in flavones and saponin; while phenols act according to the theory of CBSM (shown in figure 22) being highest in without nitrogen fertilizer treatments. While as per plant cutting heights 10 cm plants were having higher secondary metabolites concentrations. But as per score plants; no differences were observed. It is assumed that plants producing N-based defensive compounds increases with increase in N > C; while plants producing higher C>N based SM increases when C is in excess of growth. But for producing any types of metabolites; N-rich metabolites and enzymes are the base.

Figure 22. Variation in concentrations of secondary metabolites by the nitrogen application and as per cutting heights



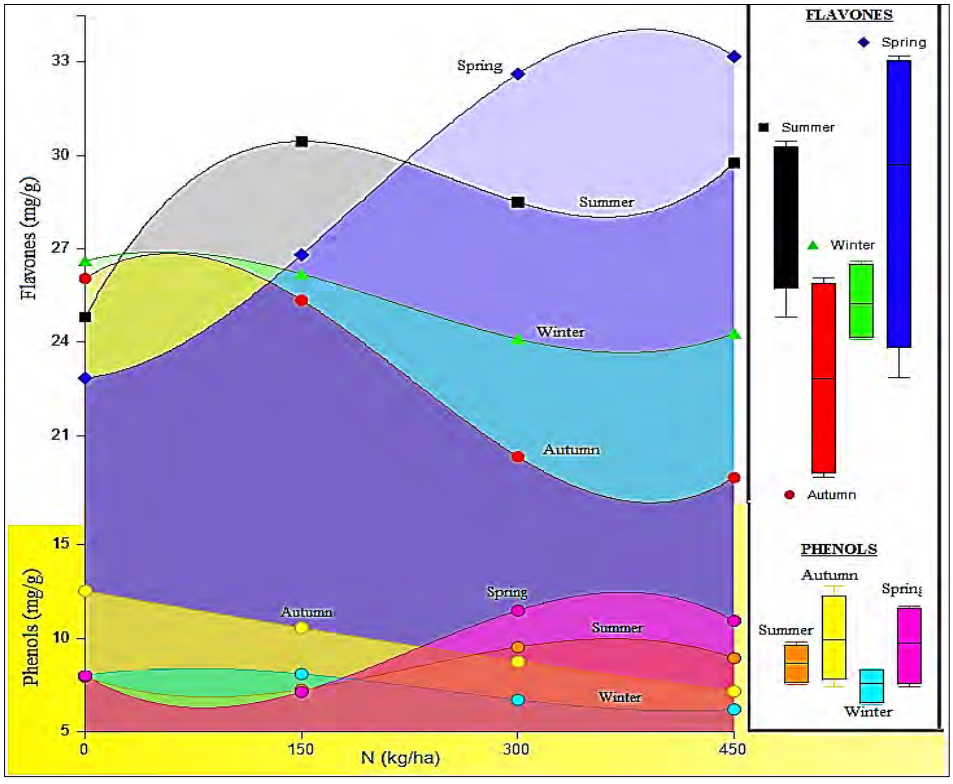
3.4.1 Total phenols and total flavones

As per our tentative analysis, 06 types of phenolic acids, 01 *C*-glicosyl-*O*-glycosil flavone, and 09 *C*-glicosyl Flavones were found in *B. decumbens leaves*. Overall, all the experimental factors (N, H, S, N*S, N*H, S*H, and N*S*H) were having significant effects (P<0.0001) on the production of all specified metabolites (mg/g d.wt.) in the leaves of *B. decumbens*. Nitrogen had a significant (P ≤ 0.0001) impact on the TP and TF concentrations as expressed by cubic adjustment of regression through their production. However, the observed regression in TF and TP were in inverse relation with each other as can be seen in Table 7, as the increasing trend was observed with increasing N in flavones while similarly the phenols decreased with the same dose of N.

The plant cutting heights were also significantly different as per 10 and 20 cm and the highest concentration were noted at 10 cm. Ibrahim et al., 2011 reported that as the nitrogen was increased from 0–270 kg ha^{-1}; the concentrations of TP and TF in the leaves of *Labisia pumila Blume* were decreased showing a negative relationship with nitrogen. Also the plants accumulates more TP and TF in their leaves as compared to the stems and roots. In the leaves, the TF decreased with increasing N fertilization from 90, 180 and 270 kg ha^{-1} by 42, 43 and 57%, respectively, as compare to control group. The similar trend was observed in the leaves TP and the lowest production (0.427 mg gallic acid/g d.wt.) were noticed at 270 kg ha^{-1}N compare to 0 kg ha^{-1}(1.01 mg gallic acid/g d. wt.).

The increase in TF and TP by nitrogen fertilization has been reported by KORICHEVA et al. (2004) and FELGINES et al. (2000) But in our results; the increase in TP under limited N fertilization might be due to higher production of non-structural carbohydrates but the higher TF may attribute towards the growth rate of plants being a competitor for flavones in *B. decumbens* (Figure 23).

Figure 23. The effect of the season and nitrogen fertilizer on total phenols and total flavones concentration (mg/g (d. wt.)



Another possibility for higher flavones may be the higher production of carbon-based primary metabolites (sugar, sucrose content) as shown in chromatography by covering 81.7 and 22% of total peak area when compares to the Protodioscin isomers peak. The higher sucrose content can be a possible reason for increased total flavonoids because they further

diverted by enzymes into more C- based metabolites. Flavonoids prioritized by plant for protection due to sunlight and UV rays. A similar conclusion was also derived by the GUO et al. (2008) that increase in the production of plant SM was due to increase in the production of sucrose as tested on broccoli and onion. But lesser studies exists for dual comparison of phenols and saponin in forages for a quick go through.

Observing the TP; amongst comparison between the two cutting heights; the highest concentration (8.95 mg g^{-1} d. wt.) occurred at 10 cm and at 0 kg ha^{-1} (9.13 mg g^{-1} d. wt.) (figure 23) ruling out the CBSM theory. While season wise the lowest were reported in winter and spring (6.20 and 11.49 mg g^{-1} d. wt.); respectively. In both TF and TP, the highest data variations occurred in spring and minimal in winter (Figure 23).

Table 8. Total flavones concentrations (mg/g d. wt.) in B. decumbens leaves at 10 cm and 20 cm height under nitrogen doses during the seasons

	10 cm		20 cm	
Nitrogen (kg ha^{-1})	Flavones	Regression	Flavones	Regression
		Summer		
0	27.1	$y = -0.0000005156x^3 + 0.0002163x^2 + 0.00504x + 27.07$ $R^2 = 0.99$	22.5	$y = 0.000000669x^3 - 0.0000804x^2 + 0.001019x + 22.52$ $R^2 = 0.82$
150	34.2		26.6	
300	31.1		25.8	
450	29.3		30.2	
		Autumn		
0	26.4	$y = 0.000000711x^3 – 0.00045348x^2 + 0.0056463x + 26.73$ $R^2 = 0.99$	25.7	$y = 0.000000568x^3 - 0.0008022x^2 + 0.00208x + 25.7$ $R^2 = 0.95$
150	28.8		21.9	
300	19.9		20.8	
450	22.8		16.4	
		Winter		
0	26.7	$y = 0.0000001161x^3 – 0.0001739x^2 + 0.0016368x + 26.41$ $R^2 = 0.93$	26.5	$y = 0.0000003465x^3 – 0.00019087x^2 + 0.0017803x + 26.49$ $R^2 = 0.95$
150	26.9		25.5	
300	24.4		23.8	
450	23.3		25.3	
		Spring		
0	24.4	$y = -0.0000008693x^3 + 0.00052848x^2 – 0.0047879x + 24.42$ $R^2 = 0.99$	21.3	$y = -0.000000667x^3 + 0.00043043x^2 – 0.0038777x + 21.29$ $R^2 = 0.1$
150	28.5		25.1	
300	34.5		30.8	
450	34.1		32.3	

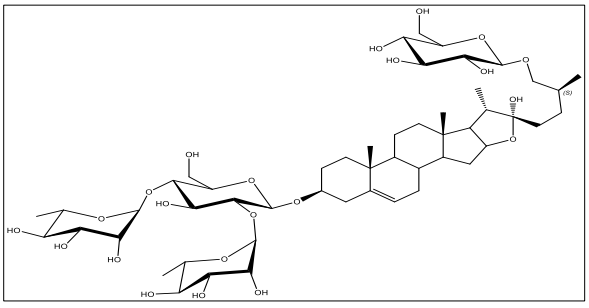
For flavones; the average highest TF concentration (34.5 mg/g d. wt.) in the *B. decumbens* leaves occurred at a dose of 450 kg ha^{-1}N in spring while the lowest was reported in autumn at 450 kg ha^{-1}N (19.65 mg/ g d. wt.) as table 7. (SALAHI et al., 2006) also reported that increase in plant spacing and density increases the flavonoid (1.97 g/ m^2) yield. As the plant height is a genetic trait and is influenced by the environmental factors and with the increase in plant growth period the plant height increases. But this factors enhance the plant density and indirectly the flavones Goldani et al., (2007). Probably that's why in spring when Brachiaria growth was faster and more sprouting tillers appears; as to other seasons the higher growth rate encourages higher plant heights and density; thus, increase the TF and TP. The given theory of CBSM production under nutrient stress on plants proved to be true only in autumn and winter; specifically, they decrease at a dose of 450 kg ha^{-1} N. As the higher stress peaks copes with temperature minimizing the plant growth rate (auxin) and hence diverting the PAL enzyme for production of TF. The highest noticed TF as per cutting heights in *B. decumbens* were (27.67 mg/ g d. wt.) and the lowest were noticed in 20 cm in autumn as (21.19 mg/g d. wt.) table 7. Overall, with an increase in nitrogen the flavones increases and with cutting heights they decrease.

3.4.2 Saponin

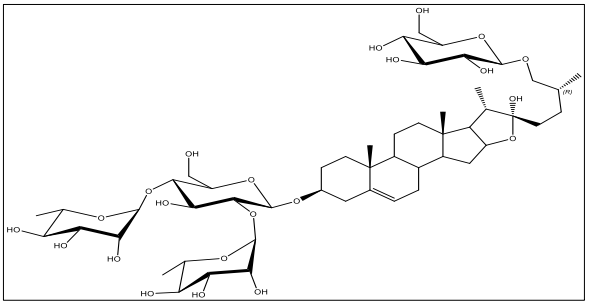
While saponin being the focus of experiment was analysed by the UPLC and (^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY) in *B. decumbens* leaves and four saponins were identified. The 03 steroidal furostanic saponin found were (*O*-hexosyl-Protodioscin or isomer, Protoneodioscin, Protodioscin, and Acetyl-Protodioscin) and the 01 steroidal spirostanic saponins found was (Deoxyhexosyl-Hexosyl-Hexosyl-3-*O*-spirostane) as shown in Figure 24. The found steroid was Cholest-5-en-22-one, 16-[[2-O-acetyl-3-O-[2-O-[(2E)-1-oxo-3-phenyl-2-propenyl] -β-D-xylopyranosyl] -α-L-arabinopyranosyl]oxy]-3,17-dihydroxy-, (3β,16β) or OSW 3, indicating the joint pathway of melonic acid shared by saponins, glycosides, and sterols for their synthesis.

Figure 24. Spirostanol and Furostanic saponins in leaves of *B. decumbens*

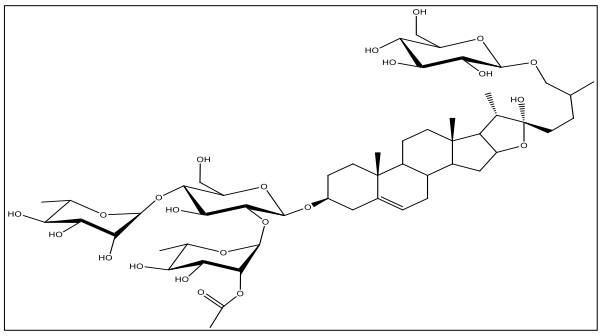
Protoneodioscin (25S-)



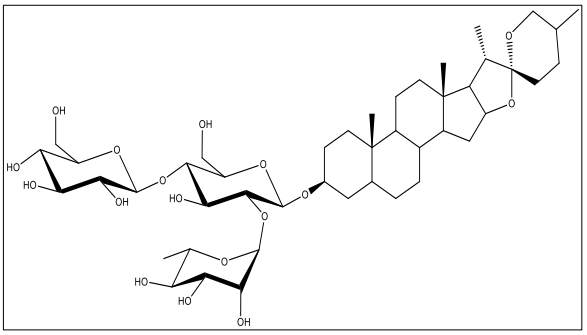
Protodioscin (25R-)



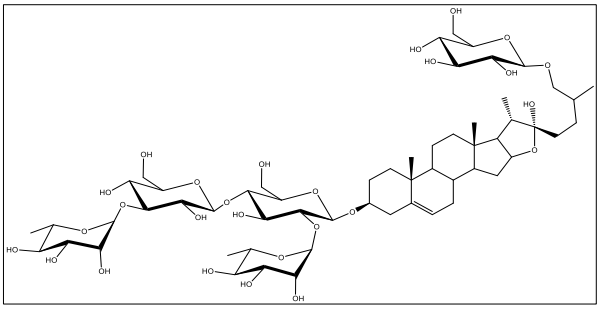
($C_{53}H_{86}O_{23}$-) Acetyl-Protodioscin



Deoxyhexosyl-Hexosyl-Hexosyl-3-*O*-spirostane ($C_{45}H_{74}O_{17}$)



O-hexosyl-protodioscin or isomer



The similar information on the steroidal saponins containing sapogenin, 3-spirostanols in the rumen of effected sheep; grazing on *Brachiaria* are reported by Salam Abdullah et al., 1992; Lajis et al., 1993; describe the saponin type as episarsasapogenin and epismilagenin. Zhang et al., (2002) also reported the presence of diosgenin [(25R)-spirost-5-en-3ß-ol] in *B. decumbens*, causing secondary photosensitization in Malaysia. Similarly, Pires et al. (2002) also identified four steroidal saponins (3ß-methoxy-lanost-9 (11)-ene, 3-O-ß-D-glucopyranosyl-24 (S)-ethyl-22 E-dihydrocholesterol, 3-O-ß-D glucopyra-nosyl-24(R)- ethyl-22 E-dihydrocholesterol and 3-O-{α-L-rhamnopyranosyl-(1→4)-[-L-rhamnopyranosyl (1-2)]-D-glucopyranosyl}-25 (S)-spirost-5-en-3-ol) and three sapogenins (diosgenin, dioscin and yamogenin) from aerial parts of *B. decumbens*. That types of steroidal saponin were also reported in *Brachiaria* species (RIET-CORREA et al., 2011) and in Panicum virgatum L. (LEE et al., 2009). In our results, the Protodioscin isomeric concentration (mg/g d.wt.) in *B. decumbens* leaves were affected significantly ($P<0.0001$) by all the primary and secondary factors (N, S, H, S*H, N*H, S*N, S*N*H) of the experiment (table 7).

The N slightly effected the saponin concentration increasing cubically with increasing doses at 0, 150 and 300 kg ha^{-1} N (43.49, 45.23 and 46.57 (mg/g d. wt.)) while at 450 kg ha^{-1}; it decreases towards 46.31 mg/g d. wt. Similar results have been presented by Massad et al., 2012 that saponins were highest with higher plant growth rate, competition, and high N fertilizer. While the lowest occurs without fertilizer and when the growth rate was slower as well as in Figure 25. Seasonally the highest concentration was noticed in winter (49.21$\pm$6.2 mg/g

d. wt) and lowest was in summer (41.85±3.2 mg/g d. wt). Plant cutting height wise the 10 cm gives over all a higher concentration (47.47 mg/g d. wt).Therefore as the nutritional quantity of *B. decumbens* is also high and palatable so it encourages animals to eat more even if fed alone; as the high level of macronutrients increases the intake of foods high in toxins (WANG; PROVENZA 1996). That's why the Brushtail possums (Trichosurus vulpecula) having the both metabolites (phenolics and terpenes) in a favorable quantity are eaten and preferred by animals compare to the diets containing only one of these toxins (DEARING; CORK, 1999).

Seeing the data; it is obvious that higher saponin was found in winter but the highest fluctuation in saponin quantity was found in spring and summer (figure 25) where the production was comparatively lower. All seasons showed the highest concentration of saponin at 300 kg ha^{-1} N while at 450 kg ha^{-1} it declined (table 7). Brachiaria; followed the growth-differentiation balance hypothesis (GDBH) in all seasons except spring using all plant reserves for growth without C/N/nutrient deficiency (MASSAD et al., 2012) and distributes them as per modelled and required. Analyzing data on the basis of plant cutting heights the 20 cm produces more saponin as compare to the 10 cm; contrast to the GDB hypothesis (Figure 26). Also, the highest data variation occurred at 20 cm plants height (41-53 mg/g d. wt.). But overall, the production was higher (47.47 mg/g d. wt) by the 10 cm cut.

3.4.3 Protodioscin isomers (mg/g (d.wt.)

The Protodioscin ($C_{51}H_{84}O_{22}$) has a molecular weight of 1049.199 g/mol with the power of 13 H as donor and 22 as acceptor and polar surface area (1048.54 g/mol). While the protoneodioscin has same molecular formula but with the molecular weight of 1049.21 g/mol. As per objectives, we analyzed the total saponin and Protodioscin (Pd) concentrations (mg/g d. wt.) in leaves of *B. decumbens*. But later, as the detailed analysis showed the probable cause of differentiation in the two species of *B. decumbens* and *B. brizantha* as the *Protoneodioscin (Pnd*). However, as their concentration cannot be measured along all the large number of samples; therefore we will discuss our samples on the base of Protodioscin isomers (Pdi (Pd + Pnd)).

All the experimental factors (N, H, S, N*H, S*H, N*S and N*H*S) significantly ($P < 0.0001$) effected the Protodioscin production (table 7). Generally the highest Pdi concentration was noted in winter at dose of 300 kg ha^{-1} N as shown in figure 25, followed by 450 kg ha^{-1} N. Height wise the highest production was recorded in 20 cm (figure 26). However, (Lima et al. 2009) found no link between nitrogen fertilizer and saponin concentrations (Protodioscin isomers) in *B. decumbens leaves* and hence grouped them on base of species and found that the saponin levels was quite higher ($p < 0.05$) in *B. decumbens* than *B. brizantha*. Also, the highest saponin concentration (3.15%) was at 60 days in *B. decumbens* which decreased by 70% at 120 days and lowest (1.06%) at 300 days. While we observe opposite results in our experiment, this may be due to the greenhouse effects or the

accumulating senescence materials in the continuity of experiment. Our results showed the highest concentrations at 210 days (4.65%) and lowest at 60 days (4.34%) but still there not seems as much differences during the plant developmental stages. Castro et al. (2011) also found higher levels of in Brachiaria spp. when plant were younger as compared to the older. The concentration of 0.3% to 2.56% is sufficient to cause hepatogenous photosensitization in sheep and in our experiment it was twice of the crucial defined levels for toxicity. While Porto et al., 2013 reported the highest protodioscin concentration (2.2%) in *B. decumbens* plant at 45 days during the 60 days experimental period. However, our results were quite higher as were reported in *B. decumbens* leaves and normally the saponin are found higher in leaves > stems> roots. While (GRACINDO et al. 2014) found the protodioscin concentrations in the pastures of *B. decumbens* (3.3 to 12.2 g kg^{-1} DM) and in *B. brizantha* was (2.8 to 9.1 g kg^{-1} DM) during the 90 days experiment. The differential value of protodioscin from our exp. may be cause of the effect of harsh environmental conditions in field and by the analysis of whole plant. While (TATIANE et al., 2014) reported protodioscin concentrations of 0.87 – 2.58 % in young leaves of *B. brizantha* and with proceeding age of leaves the concentration decreases to 1.16 – 2.53 %, and been lowest in old leaves (0.98 – 2.07 %).

Figure 25. Protodioscin isomers concentration (mg/g d.wt.) around seasons as by the nitrogen fertilizer

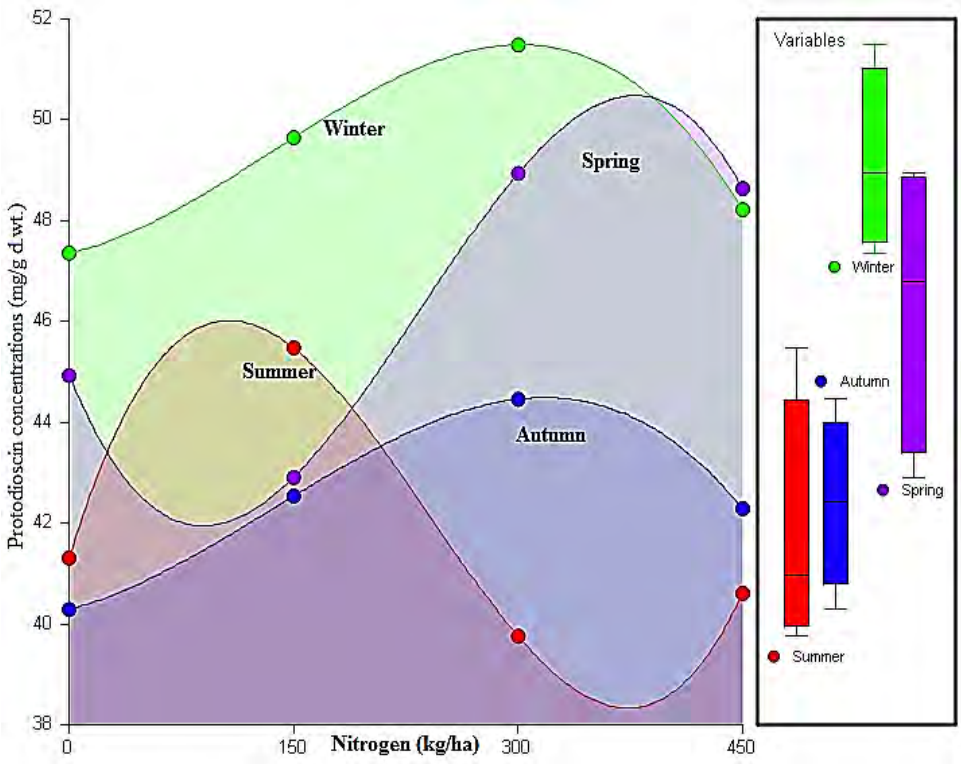
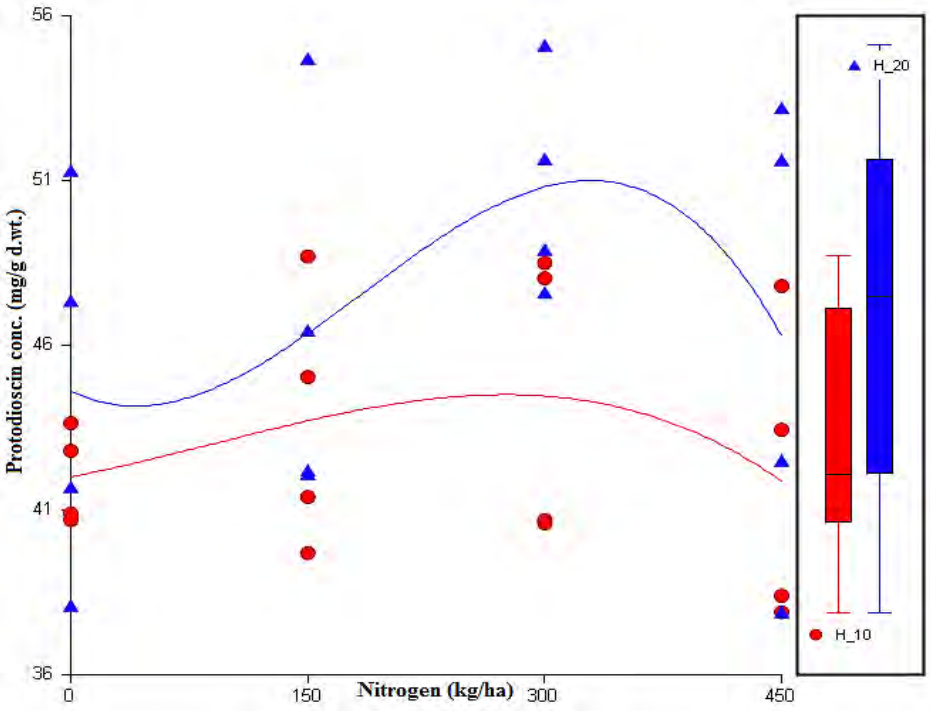
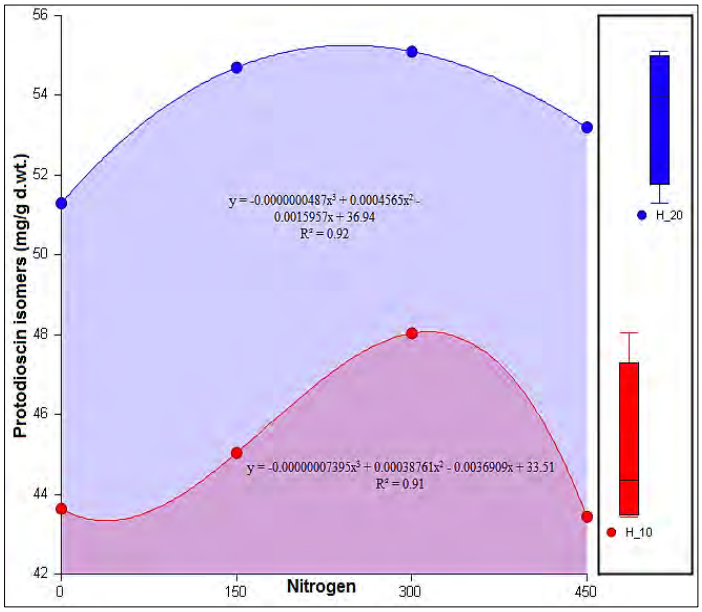


Figure 26. Comparison of Protodioscin isomers concentration (mg/g d.wt.) in the two cutting heights around the seasons under nitrogen fertilizer



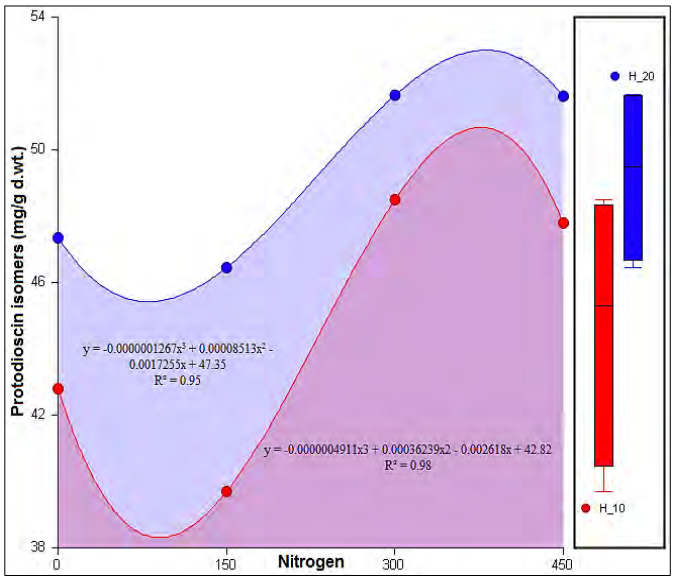
The highest Protodioscin concentrations were observed in winter during all the experimental period; showing that being the plants in stress produce more SM for its defense and survivability as proposed by the defense theories. Observing the Protodioscin isomers (Protoneodioscin and Protodioscin) concentrations (mg/g d.wt.) in winter; the both plant cutting heights (10 cm and 20 cm) express a large variation and significant differences in the mean values as 44.95 vs. 53.60. It adjusted cubically (R^2–0.92 vs 0.91) by application of N doses. But according to Brum et al., (2009) the furostanol-like steroidal saponins were higher (0.5-2.1%) when *B. brizantha* and *B. decumbens* pastures were in the maturity phase; indicating their toxicity. In our exp., the highest observed concentration was noted (55.10) at 20 cm and at dose of 300 kg ha^{-1} N; same as other seasons. While the lowest (43.44) was noted at both heights of 10 cm at dose of 450 kg ha^{-1} N; same as autumn and summer. The Protodioscin isomers concentration were almost uniform at 20 cm cutting height (51.30 – 55.10) while comparatively a higher variance was noticed at the 10 cm (43.44 – 48.04 mg/g d.wt.) as shown in Figure 27. Following the same of other seasons the highest concentration was noticed at both heights by 150 kg ha^{-1} N (48.04 and 55.10) showing that N fully enhance the saponin production. As Ferreira 2011 showed a relationship of mature leaves with temperature (r=0.97) and it totally fits our data that preceding age. Yang et al., 2003; confirmed the complex relation and interference on the saponin concentration.

Figure 27. Interaction of Nitrogen and cutting heights on Protodioscin isomers concentrations (mg/g d.wt.) in winter



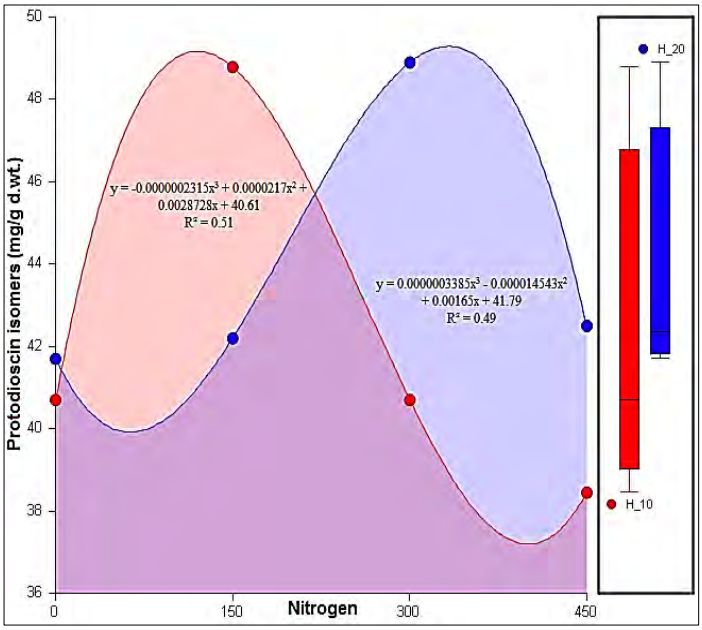
The Protodioscin isomers (25R- and 25S-) concentrations (mg/g d. wt.) in spring was reported as being the second highest in experimental period. Our results are in accordance with MEAGHER et al. (1996) that higher quantities of diosgenin and yamogenin sapogenins in *B. decumbens* were noted when plants were younger. The both plant cutting heights (10 cm and 20 cm) express a large variation and significant differences in the mean values as 44.71 vs. 49.35 with adjustment of regression cubically (R–0.98 vs 0.95) by application of N doses. The highest observed concentration was noted at dose of 300 kg ha^{-1} N (51.65) at 20 cm. Being the same trend that 300 kg ha^{-1} N presents the highest Protodioscin production, ignoring the rules that plants under flourished situation produce more components of defensive mechanism. While the lowest (39.70) was noted at 10 cm height at dose of 150 kg ha^{-1} N. The differentiation within the Protodioscin isomers concentration were almost uniform at both heights of 10 cm and 20 cm as shown in Figure 28. The 10 cm plants showed a great variance in data while the 20 cm was being persistent by producing the same range of Protodioscin throughout all seasons. Following the same of other seasons the highest concentration was noticed at both heights by 150 kg ha^{-1} N (48.50 and 51.65). This emphasize that although saponin molecules hasn't any N molecule and is not a biosynthesis product of shikimic acid pathway but still probably as the plants starts to produce higher greener and younger parts therefore at this stage it is vulnerable to attack by insects and animals and being as natural defense strategy it produces more saponin.

Figure 28. Interaction of Nitrogen and cutting heights on Protodioscin isomers concentrations (mg/g d.wt.) in spring



In summer, the lowest Protodioscin concentration (mg/ g d. wt.) in the *B. decumbens* leaves was observed. This can be in agreement with the hypothesis that as the plants can utilize all the available carbon and nitrogen by synchronization for its growth. Hence, no surplus C and N is available for production of CBSM and hence the Protodioscin concentration was lowered. Seeing the isomers concentration in summer; both plant cutting heights don't give any significant differences in mean values amongst the 10 cm and 20 cm (42.01 and 41.32 mg/g d. wt.) and both behave cubically with doses of nitrogen as applied. However the highest observed conc. in summer was noted (48.9) in 20 cm at a dose of 300 kg ha^{-1} N, and the lowest was noted at the height of 10 cm at a dose of 450 kg ha^{-1} N (38.4) as presented in Figure 29. Overall, there was a higher variance in the concentration of Protodioscin isomers at the 10 cm but also the highest concentration was noted at 10 cm by 150 kg ha^{-1} N while in 20 cm the highest was at 300 kg ha^{-1} N. Ferreira et al. (2011) studied the Protodioscin production in *B. brizantha* by effects of maturity level, sunshine hours, temperature and relative humidity and found the higher concentrations in young leaves (3.61±1.12%) > mature (1.94±0.97%) and minimal in old leaves (1.01±0.79%). Our results don't coincide it as the saponin has a direct relation (r=0.9) with sunshine hours and although they are higher in summer but our results seems to be influenced by the minimal temperatures and stress during winter or Carbon saturation in greenhouse.

Figure 29. Interaction of Nitrogen and cutting heights on Protodioscin isomers concentrations (mg/g d.wt.) in summer



Analyzing the Protodioscin isomers concentration in autumn; both plants cutting heights (10 cm and 20 cm) express a large variation and significant differences in the mean values as 42.3 vs. 41.5 mg/g d.wt. with the adjustment of R cubically (0.99 vs 0.94) by application of N. However the highest observed conc. in autumn was noted (47.6) at 20 cm and at a dose of 300 kg ha^{-1} N; just like summer. While the lowest (37.9) was noted coincidently at both heights of 10 cm and 20 cm at dose of 450 kg ha^{-1} N. The 10 cm cutting height present a rhythm with less fluctuation in production while a higher variance in the concentration was noticed at the 20 cm cutting height (37.9-47.6) as shown in Figure 30. In 10 cm the highest conc. was noticed at 150 kg ha^{-1} N while in 20 cm the highest was at 300 kg ha^{-1} N. The production of Protodioscin isomers in autumn followed almost the same path as in summer.

Figure 30. Interaction of Nitrogen and cutting heights on Protodioscin isomers concentrations (mg/g d.wt.) in autumn

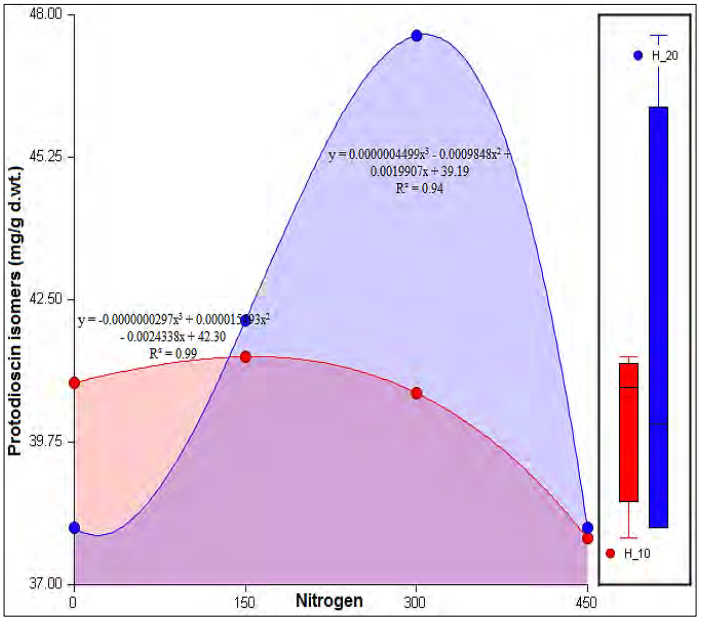
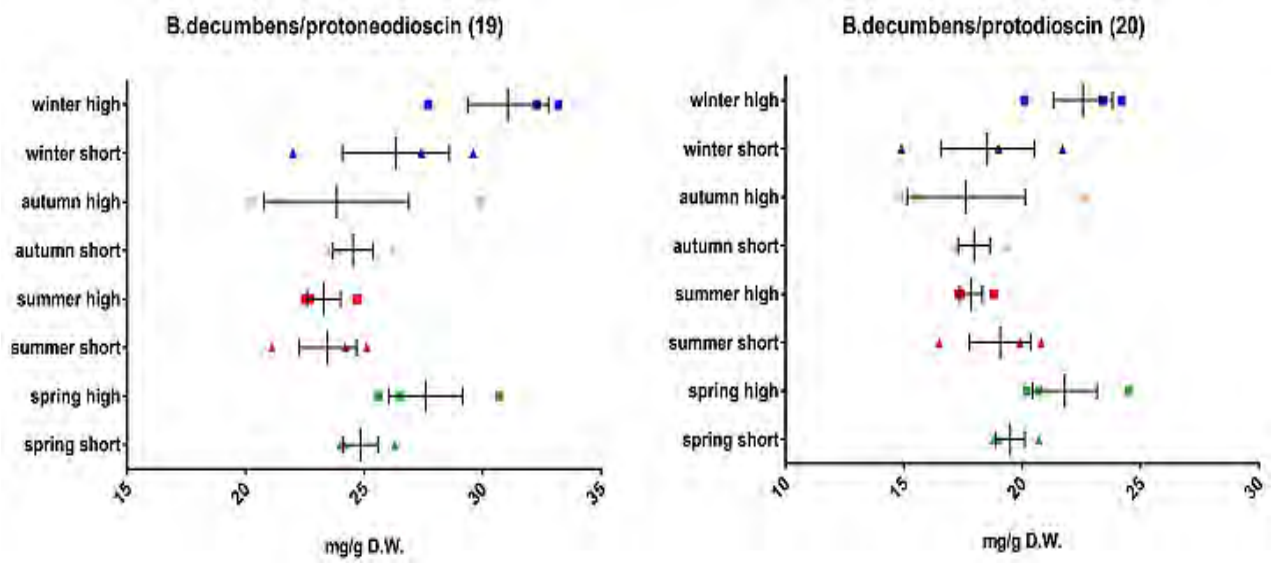


Figure 31; presents the differential concentrations of protoneodioscin and protodioscin in the leaves of *B. decumbens*; irrespective of nitrogen and no significant differences were noticed amongst them and per seasons also. The control treatment was analyzed on the basis of heights amongst the seasons and species. However, it remains same within the species but found significant differences between species for the two components. As per the results showed the highest concentrations were noticed in winter (53.60 ± 5.10 d. wt.) at 210 days, and the lowest was reported in summer (19.07±2.3 mg/g d. wt.) 60-90 days. Similar to our results, Brum et al. (2009) evaluated saponins and found higher levels of Protodioscin in Brachiaria during seed fall. The reported saponin in *B. decumbens* 0.8% to 1.9% were lower than *B. brizantha* (0.53% to 2.1%). Specifically, the protoneodioscin concentration was higher by almost 10 mg from protodioscin among all seasons. But generally protoneodioscin concentrations lies between 23 to 31 mg/g d. wt., while the protodioscin production was 17 to 21 mg/g d. wt. Not a single season was observed that protoneodioscin production was exceeded by protodioscin. Based on the evaluation of protodioscin; CASTRO et al., 2011 reported that concentrations of 0.30% - 2.56% in *Brachiaria* don't cause any hepatotoxicity in sheep. Moreira et al., 2009; BRUM et al., 2007, also reported no cases and signs of photosensitization in cattle grazing on *Brachiaria* of age between 300 and 360 days when Protodioscin conc. was 1.09% and 1.63%; respectively. Therefore, the main probable toxicity problem is considered as protoneodioscin, not actually the protodioscin.

Figure 31. Aligned dot graphs of protoneodioscin and protodioscin concentrations around seasons in leaves of *B. decumbens*



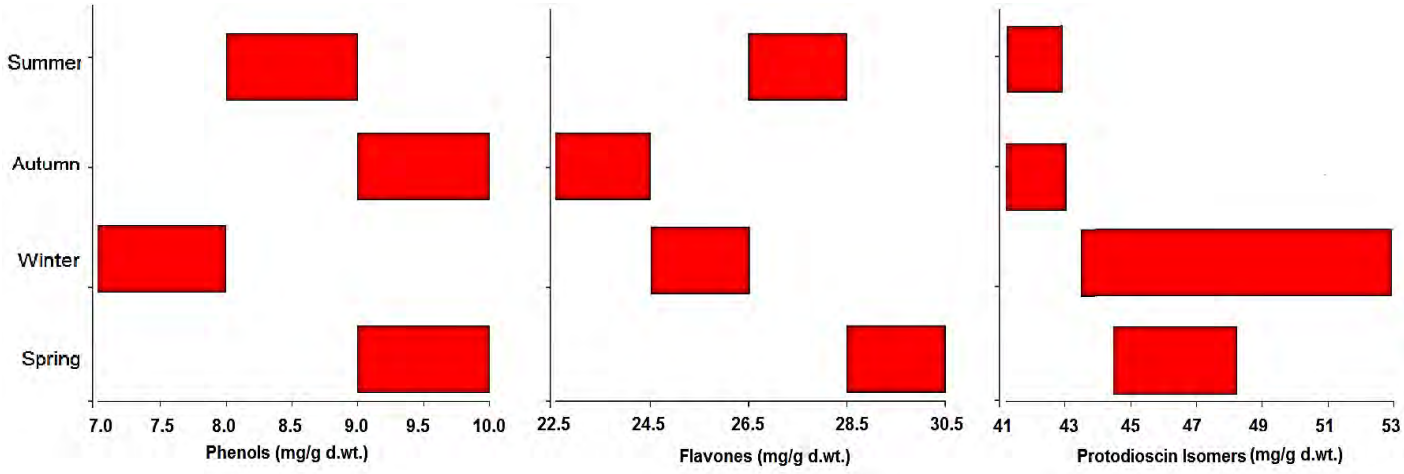
3.5 Conclusions

By the results the seasonal production of SM in *B. decumbens* varied totally as per plant cutting heights, nitrogen application. Observing the basic experimental conditions; the N

fertilization increased the saponin and flavones concentration but decreased the phenols. The highest saponin were reported at 300 kg ha^{-1}. Also the winter and spring favors the higher protodioscin and protoneodioscin concentrations, due to the stress and appearance of new leaves. The protodioscin isomers were confirmed for a great variation in conc. and along with age (210 days) and proved that the most probable toxic component is protoneodioscin.

The higher phenolic concentrations occurred in un-fertilized plants as per CBSM theory. Also, lesser types with higher concentrations of flavones were found, giving birth to the idea that having multi-toxin in animal's feed / pasture can minimize the toxicity as described by the (FREELAND et al., 1985). Thus, the presence of lesser phenols, flavones, c-glycosil flavonoids and the higher proportion of the probable toxic protoneodioscin concentrations entitle *B. decumbens* as more toxic; by absence of neutralizing effect by other SM (DEARING; CORK, 1999). The case also seems to be fit in the model that the expression of one defense component results in an associated and simultaneous decrease in the expression of a second defense (KORICHEVA et al. 2004) and saponin concentration depress the polyphenols. The figure 31 showing that when saponins were higher the expression of phenolics appeared lesser; especially in the season of winter and autumn; the relation of the saponin and phenolic were inverse.

Figure 30. Comparison of phenols, flavones and Protodioscin isomers concentration (mg/g d. wt.) around seasons



3.6 Recommendations

Further study of *B. decumbens* profile is needed; either by in vivo experiments with supplying pure protodioscin isomers or analyzing the pure compounds in in-vitro experiments to check against liver cells being the biological activity target (personal communication with Dr. Andy J. Perez).

Various managerial strategies can be applied to minimize the toxicity; like regular grazing or harvesting within 30 days, avoiding to put young animals of less than 1 year age, or

non-pigmented. Animals should be kept for longer periods or animals rearing on *Brachiaria* pastures from one generation to another; has shown greater resistance as compared to newly adopted animals (RIET-CORREA et al., 2011). Animals on *B. decumbens* pasture can be supplemented with plants containing SM to minimize its toxicity specifically with phenols or tannins (Provenza and Pfister 2013) by the chelating effects of both compounds in the gastrointestinal tract (FREELAND 1985). The long term, most permanent and effective solution is the genetic modifications of *B. decumbens*.

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3.7 References

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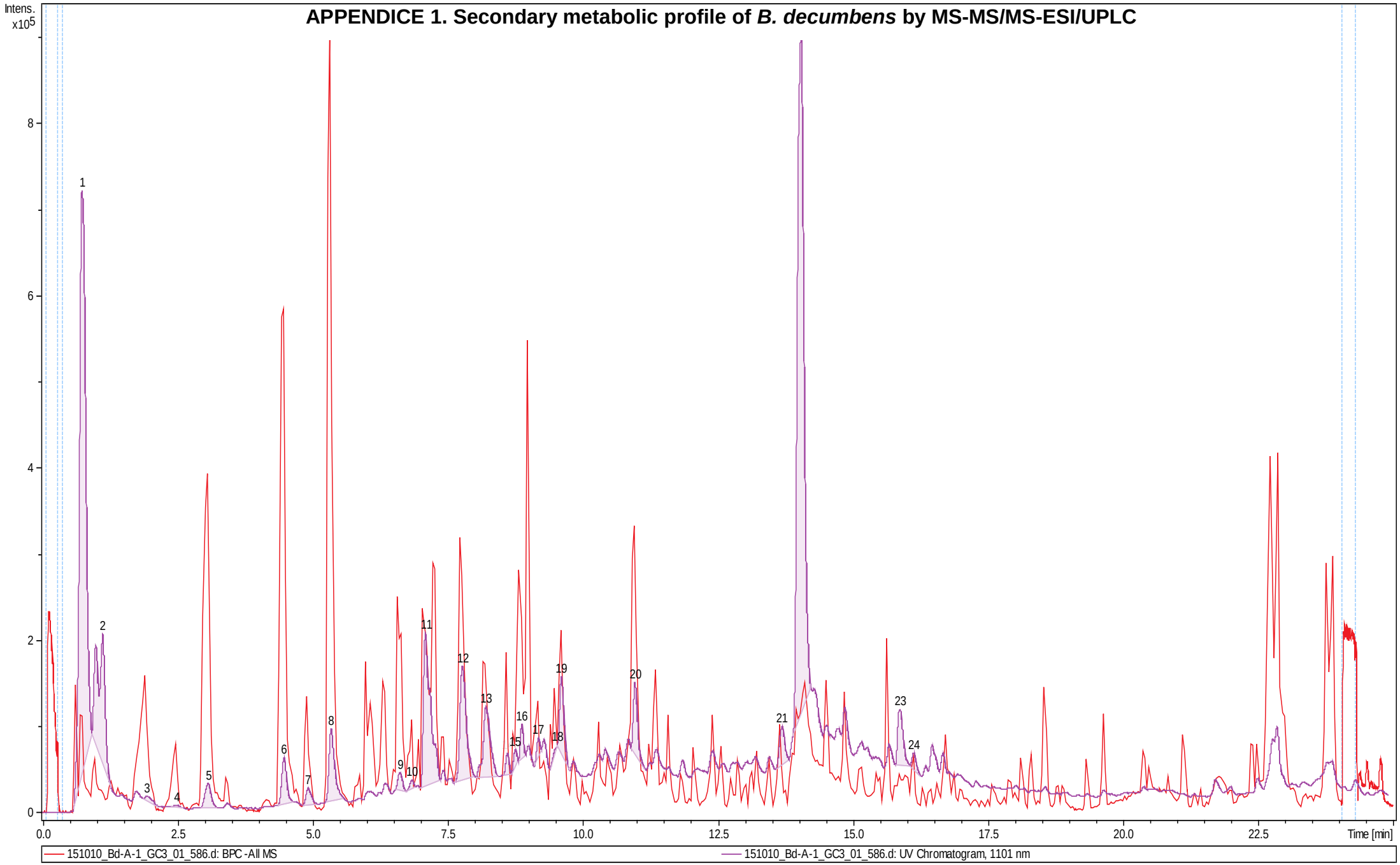
APPENDICE 1. Secondary metabolic profile of B. decumbens by MS-MS/MS-ESI/UPLC
Intens.
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6
4
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7
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9
10
11
12
13
15
16
17
18
19
20
21
23
24
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
Time [min]
151010_Bd-A-1_GC3_01_586.d: BPC -All MS
151010_Bd-A-1_GC3_01_586.d: UV Chromatogram, 1101 nm

APPENDICE 2. Secondary metabolic profile of *B. decumbens* by MS-MS/MS-ESI/UPLC

#	Rt	Q-TOF-MS/MS	Mol. Form.	Tentative Identification
1	0.8	207, 191, 165	$C_{12}H_{22}O_{11}$	Sucrose
2	1.1	341, 179, 154, 111	$C_{18}H_{26}O_{16}$	-
3	1.9	152, 108	$C_{13}H_{16}O_9$	*O*-galloyl-2,6-anhydromannitol or *O*-galloyl-1,5-nahydroglucitol
4	2.5	207, 193, 178, 149, 134	$C_{17}H_{20}O_{10}$	5-Hydroxyferuloylquinic acid
5	3.1	191(-162), 179, 135	$C_{16}H_{18}O_9$	Isomer of Chlorogenic acid
6	4.5	191(-146), 173, 163, 119	$C_{16}H_{18}O_8$	Coumaroylquinic acid
7	4.9	707(2M-H), 353, 191	$C_{16}H_{18}O_9$	Chlorogenic acid
8	5.3	193, 134	$C_{17}H_{20}O_9$	Feruloylquinic acid
9	6.7	205, 190, 153, 138	$C_{20}H_{32}O_{10}$	Unidentified
10	6.9	267, 249, 237(-176), 207, 189, 163, 145, 119	$C_{18}H_{22}O_{11}$	Hexuronic acid, 1-[(2*E*)-3-(2,4,5-trimethoxyphenyl)-2-propenoate]
11	7.1	561(-18), 519(-60), 489(-90), 459(-120), 399[(A+114)-H], 369[(A+84)-H]	$C_{26}H_{28}O_{15}$	Carlinoside (6,8-di-*C*-glycosyl Luteolin)
12	7.8	473(-90), 443(-120), 383[(A+114)-H], 353[(A+84)-H]	$C_{26}H_{28}O_{14}$	Schaftoside (6,8-di-*C*-glycosyl Apigenin
13	8.2	489(-60), 459(-90), 429(-120), 399[(A+114)-H], 369[(A+84)-H]	$C_{25}H_{26}O_{14}$	6,8-di-*C*-pentosylluteolin
14	8.6	473(-60), 443(-90), 413(-120), 383[(A+114)-H], 353[(A+84)-H]	$C_{25}H_{26}O_{13}$	6,8-di-*C*-pentosylapigenin Isomer1
15	8.8	431(-146), 413[-(146-18)], 341[-(146-90)], 311[-(146-120)], 293(A+24), 282(A+13)	$C_{27}H_{30}O_{14}$	vitexin 2''-α-alfa-L-rhamonoside

16	9	515(-18), 473(-60), 443(-90), 383(A+114), 353(A+84)	$C_{25}H_{26}O_{13}$	6,8-di-*C*-pentosylapigenin Isomers
17	9.2	545(-18), 503(-60), 473(-90), 443(-120), 383[(A+114)-H]	$C_{26}H_{28}O_{14}$	Isoschaftoside (6,8-di-*C*-glycosyl Apigenin
18	9.4	357(-60), 327(-90), 297(-120)	$C_{20}H_{18}O_{10}$	6 or 8-*C*-monoglycosylluteolin
19	9.6	473(-90), 459(104), 429(-134), 399[(A+114)-H], 369[(A+84)-H]	$C_{26}H_{28}O_{14}$	Unidentified *C*-glycosylluteolin
20	11.0	429(-146), 385, 367, 325, 311, 298, 285	$C_{27}H_{28}O_{14}$	Cassiaoccidentalin B
21	13.7	1047(-162), 901(-146), 755(-146), 415[-(2x162+16)]	$C_{57}H_{94}O_{27}$	*O*-hexosyl-Protodioscin or isomer
22	14.1	1047(-46), 901(-146), 755(-146), 593(-162), 431(-162)	$C_{51}H_{84}O_{22}$	Protoneodioscin Protodioscin
23	15.9	1047(-42), 901(-146), 755(-146), 593(-162), 431(-162)	$C_{53}H_{86}O_{23}$	Acetyl-Protodioscin
24	16.1	739(-146), 577(-162), 415(-162)	$C_{45}H_{74}O_{17}$	Deoxyhexosyl-Hexosyl-Hexosyl-3-*O*-spirostane
25	22.8	867(-46), 721(-146), 575(-146)	$C_{48}H_{68}O_{14}$	Cholest-5-en-22-one, 16-[[2-O-acetyl-3-O-[2-O-[(2E)-1-oxo-3-phenyl-2-propenyl]-β-D-xylopyranosyl]-α-L-arabinopyranosyl]oxy]-3,17-dihydroxy-, (3β,16β) or OSW 3

APPENDICE 3. HR-QTOF-MS and MS/MS spectra of Protoneodioscin in leaves of *B. decumbens*