

Forage Legume Seeds Submitted to *in Vitro* and *in Situ* Fermentation Techniques

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ABSTRACT

The present study was carried out to evaluate the survival rate of four tropical legume seeds submitted to different periods of ruminal incubation simulated by *in vitro* and *in situ* fermentation. The species studied were butterfly pea, stylosanthes, archer and perennial soybean. Four Holstein dairy cows fistulated in rumen were used in the *in situ* stage. A completely randomized design was used, in a 4 × 6 factorial arrangement (four species, six treatment periods) in the *in vitro* stage, with four repetitions. A completely randomized block design, arranged in a 4 × 6 factorial scheme (four species × six incubation periods), was used in the *in situ* stage, with four repetitions. The seeds (50 units of only one species at each repetition) were placed in erlenmeyer flask (250 mL) and were put in a 10 × 15 cm nylon bag in the *in vitro* stage. The times of incubation were: 6, 12, 24, 48, 96 and 144 hours and the time zero was estimated in the laboratory. The results demonstrate that, when submitted to ruminal incubation, due to their hard and impermeable teguments, legume seeds presented high resistance potential, and, thus, greater chances of germinating after passage through the gastrointestinal tract of bovines, when defecated in pastures. However, stylosanthes are not recommended for insertion in bovine feeding for this purpose, because they do not resist ruminal digestion.

Keywords: Germination; Ruminal Liquid; Fabaceae Seeds

1. Introduction

The use of legumes is an alternative to decrease erosion and restore and maintain soil fertility of Brazilian pastures. The benefits of inclusion of legumes in pastures are unquestionable. Unfortunately, however, such procedure is rarely adopted in livestock production system. Moreover, in Brazil, there are just a few studies aiming at developing technology for legume seed dispersion by introducing seeds in the diet of cattle, goats, sheep and/or horses [1-3]. On the other hand, many studies using the collection of manure from cattle grazing reported that many plant species are able to survive passage through the digestive tract of ruminants [4,5]. Until now, however, Ecology and Animal Science handle the dispersal of leg-

ume seeds differently. But it is a relevant issue for both fields and is related to the dynamics of plants in Brazilian pastures.

The advantages of using the *in vitro* technique to determine the survival of seeds after ruminal digestion are the speed, physical-chemical uniformity of the fermentation microenvironment and the fact that it is not necessary to maintain fistulated animals. Most *in vitro* methods, however, may fail because they do not use correctly the inoculum, buffers or the equipment that ensures the conditions of pH, anaerobiosis, number of micro-organisms and nutrients that are essential for their performance [6]. The main disadvantage of the *in vitro* method is that it does not reproduce the *in vivo* environment perfectly. However, this disadvantage can be reversed when the objective of the assay is to determine the survival/germina-

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tion of seeds after ruminal digestion, since the *in vitro* conditions may be controlled so as to prevent physical-chemical fluctuations in the environment.

Thus, this study aimed to analyze the effects of ruminal fermentation in seeds of four tropical forage legumes, using the *in vitro* and *in situ* fermentation technique on the survival/germination of seeds.

2. Methods

The experiments were conducted at the Laboratory of Animal Science and Animal Nutrition and at the Laboratory of Plant Science of the UENF, in Campos dos Goytacazes—RJ. The following species were used: *Clitoria ternatea*, *Stylosanthes spp.* cv. Campo Grande, *Neonotonia wightii* and *Macrotyloma axillare*.

The *in vitro* assay was arranged in a completely randomized design, with 4 × 6 factorial scheme (four species × six fermentation times) and four replications. The seeds of the four species (50 seeds of only one species per replicate) were placed together with rumen fluid obtained via ruminal fistula plus a buffer to simulate digestion in the reticulum—rumen and omasum at a temperature of 39°C and pH of 6.9. The rumen fluid was collected before morning feeding via rumen cannula, using a vacuum pump and a Kitassato, with capacity of 2000 mL, always kept in waterbath at 39°C. The inocula were prepared according to [7], modified according to [8]. The seeds were placed in 250 mL erlenmeyer containing 10 mL of ruminal fluid and 40 mL of buffer solution, at a temperature of 39°C. The fermentation times were: 6, 12, 24, 48, 96 and 144 hours, and the time zero was performed in the laboratory. CO₂ was inoculated in about 30 seconds before and after the addition of ruminal fluid. At the end of the fermentation periods, the jars were drained and the seeds were washed in the same jar, five to six times, with distilled water. Then, the seeds were submitted to germination test. The seeds were placed in a BOD germinator (12 h of light) at 25°C, where they remained for 10 days, using four replicates of 50 seeds per replication. The seeds were placed for germination in rolled germitest paper moistened with distilled water at the proportion of 2.5 times the paper weight. The percentages of germination of each species were determined at each fermentation time.

The *in situ* assay used crossbred cattle (Holstein × Zebu), live weight of 500 kg, with rumen cannula, housed in individual pens. The diet consisted of chopped elephant grass, mineral salt and water ad libitum. The total daily amount of food was provided at two different times, 8 am and 6 pm. The amount offered was adjusted according to the leavings observed every day. The leavings were controlled so that they would be 10% of the amount offered on the previous day, so as to ensure voluntary intake of animals. The assay was arranged in a

randomized block design, with four species and four replications. The equivalent to 50 seeds of only one type was placed in each 10 × 15 cm nylon bag per replication. The incubation times were: 6, 12, 24, 48, 96 and 144 hours, and the time zero was performed in the laboratory. At incubation, the bags were tied with nylon thread and attached to a 540-g stainless steel cylindrical rod, which was secured by an iron chain with length of 50 cm, in the cannula, during the incubation period. After “*in situ*” incubation, the bags were washed with distilled water, and the seeds were submitted to the germination test. The seeds were placed in BOD germinator (12 h of light) at 25°C, where they remained for 10 days. The percentages of germination of each species were determined at each incubation time. After testing the germination of seeds, a high percentage of hard seeds was observed, except for stylosanthes. Thus, scarification of the hard seeds was carried out with water sandpaper number 100, until grooves were observed in the integument of the seeds to break dormancy. The germination test was performed after scarification. The seeds were placed in BOD germinator at 25°C, with 12 hours of light.

The results for germination were given in percentage, and were subjected to the analysis of variance, using the Tukey test at 5% significance for the comparison of the averages. Then, a regression analysis was performed. All values were transformed into $\arcsin \sqrt{x/100}$ for the analysis of variance.

3. Results and Discussion

Regression Non-linear regression analysis allowed the achievement of the equations that describe the behavior of germination percentage (GP%) and hardness percentage (%HP) of the seeds submitted to different periods of *in vitro* fermentation with rumen fluid and artificial saliva (**Figures 1 and 2**) and the percentage of germination (GP%) and percentage of hardness (%HP) of the seeds subjected to different ruminal *in situ* incubation period (**Figures 3 and 4**).

As for the percentage of germination, the seeds presented little increase as fermentation period increased, until 48 hours. After this period, the value decreased slightly. However, the seeds of stylosanthes behaved differently from the others and rapidly reduced the number of germinated seeds, precisely because of the number of dead seeds observed in the germination test. This behavior can be explained by the fact that the integument of stylosanthes is not very resistant, since recommendation for breaking dormancy requires immersion in concentrated sulfuric acid for a maximum of 10 minutes [9]. [10] studied the process of *Phyllanthus emblica* seed dispersal by ruminants in tropical forests and observed deers (*Axis deer*) regurgitating intact seeds of *Phyllanthus emblica* after retaining them in their rumen for 7 - 27 hours, and

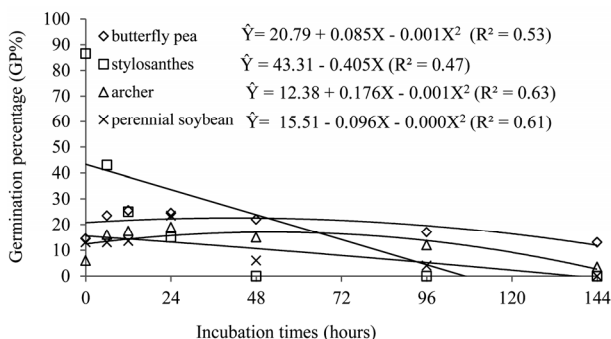


Figure 1. Regression for germination percentage of hard seeds of butterfly pea, stylosanthes, perennial soybean and archer, due to periods of *in vitro* fermentation.

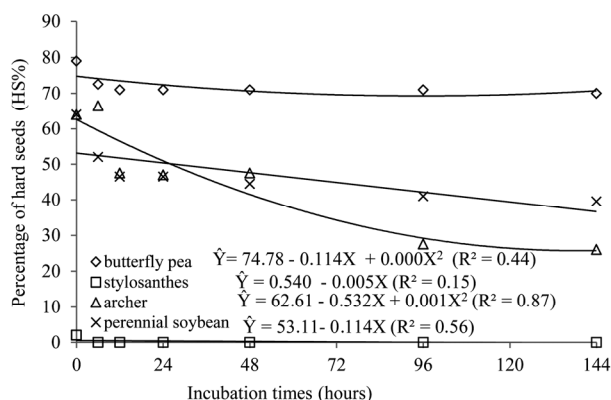


Figure 2. Regression for hardness percentage seeds of butterfly pea, stylosanthes, perennial soybean and archer, due to periods of *in vitro* fermentation.

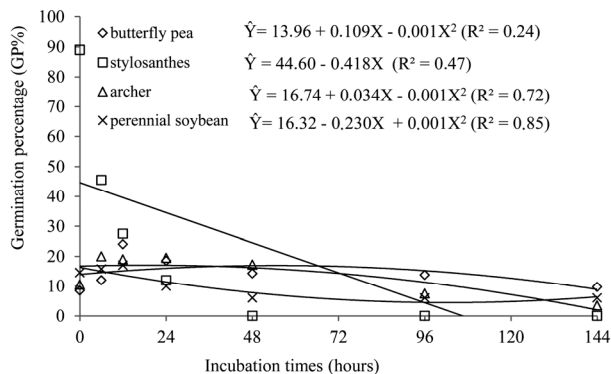


Figure 3. Regression for the germination percentage of seeds of butterfly pea, stylosanthes, perennial soybean and archer, due to ruminal incubation periods (*in situ*).

that a significant fraction (22%) of the seeds germinate, although the percentage of original germination of the seeds consumed is 72%, which suggests that size and hardness of seeds ensure their regurgitation, instead of being defecated.

The response of hard seeds was similar for all species. In other words, the number of hard seeds decreased as time of ruminal incubation increased, although the slopes

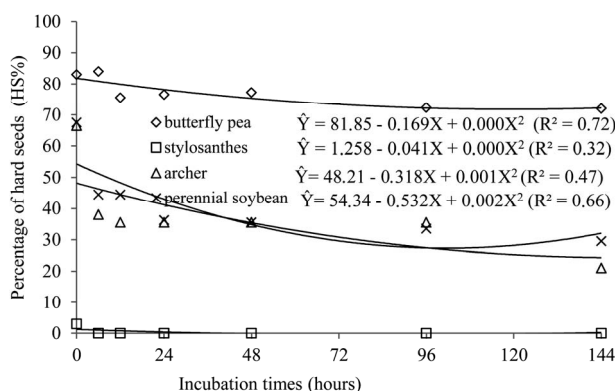


Figure 4. Regression for the hardness percentage seeds of butterfly pea, stylosanthes, perennial soybean and archer, due to ruminal incubation periods (*in situ*).

of the curves were different, i.e. more or less accentuated, in relation to one another. However, the seeds of the species archer, butterfly pea and perennial soybean presented high resistance and survival rates, exactly due to the hardness of their teguments.

In a study on the germination of scarified and non-scarified *Enterolobium cyclocarpum* seeds subjected to *in vitro* fermentation technique using rumen fluid (bovine) and cecal inoculum (horse), [11]. observed that, after 24 hours of exposure to *in vitro* fermentation, 90% of the scarified seeds died, while all non-scarified seeds germinated in the germination test. This behavior suggests that scarification of the seeds promoted by microbiota, temperature and immersion in rumen fluid has little effect on the permeability of the tegument of seeds of butterfly pea, perennial soybean and archer, while the seeds of stylosanthes suffer lethal damage when passing through rumen, regardless of length of stay [12]. Investigated the germination of seeds incubated in the rumen of sheep and goats and observed high germination rate when the tegumenting was eroded, which occurs when seeds are incubated in the rumen (32% within 48 hours of ruminal incubation, while the control reached 12%). [13] studied the survival of seeds of 25 species of plants introduced to the feeding of deer (*Dama dama* L) and found that, out of the 25 species of plants whose seeds had been ingested by the animals, 24 survived and germinated in their faeces (0.5% to 42% of the ingested seeds) and 50% of all the seeds were recovered within the first 25 h, at a 13 to 38-h range.

Legume seeds have physical latency due to the presence of a hard and impermeable tegument. Thus, they need to be scarified to germinate [14]. In Salta, a province of the Chaco region, in Argentina, [15] examined the germination of *Caesalpinia paraguariensis* after passage through the digestive tract of cattle, and observed that seed germination is low and germination percentage does not increase, because the viable seeds are dormant

due to their strong impermeable tegument. For [16], species with impermeable teguments present higher percentages of germination after passage through the digestive tract of cattle, although no significant difference can be noticed in germination, survival or speed rates. The hypothesis that latency is broken when seeds pass through the digestive tract of animals is widely accepted, but some results are conflicting [14,17-22]. It can be observed in **Figures 5** and **6**, which show no dormancy breaking in most seeds subjected to either *in vitro* or *in situ* fermentation.

However, these results suggest that most seeds survive and may germinate. It is clear that seeds introduced into the diet of these animals that survive rumen degradation and remain dormant may germinate in the feces of cattle in the field.

4. Conclusions

Ruminal digestion, simulated by *in vitro* and *in situ* fermentation techniques, showed that seeds with resistant teguments have high potential to be introduced into the diet of cattle, for ruminal digestion poses low risk, mainly to the seeds of butterfly pea (cunhã), perennial soybeans and archer.

In this study, stylosanthes should not be inserted in the

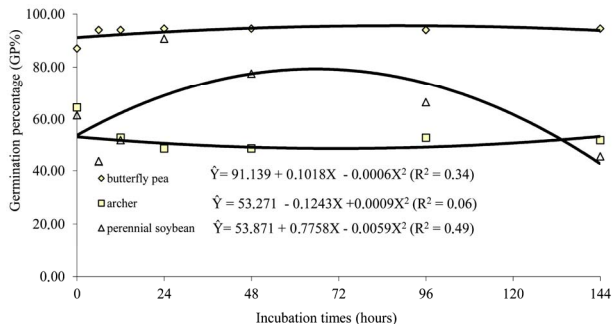


Figure 5. Regression for the percentage of germination of “hard” seeds of butterfly pea, perennial soybean and archer, due to periods of *in vitro* fermentation and after scarification.

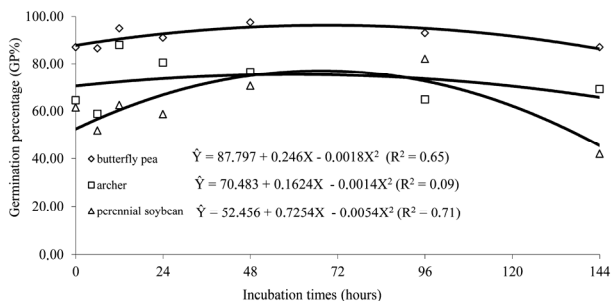


Figure 6. Regression for the percentage of germination of “hard” seeds of butterfly pea, perennial soybean and archer, due to periods of incubation *in situ*.

feeding of cattle aiming to be introduced in the pastures precisely because it is sensitive to the ruminal digestion stage. Further studies should be conducted for better knowledge about these results and for assessing the possible introduction of legumes through bovines into pastures.

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