American Journal of Plant Sciences, 2015, 6, 2317-2328
Published Online September 2015 in SciRes. http://www.scirp.org/journal/ajps
http://dx.doi.org/10.4236/ajps.2015.614235

Germination and Vigor of Fodder *Fabacceae* Seeds Submitted to *in Vitro* and *in Situ* Incubation

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Received 8 April 2015; accepted 12 September 2015; published 15 September 2015

Abstract

The current study was developed with the purpose to evaluate the germination and the vigor of *Kudzu* (*Pueraria phaseoloides*), *Leucaena* (*Leucaena leucocephala*) and *Calopogônio* seeds (*Calopogonium mucunoides*) submitted to 0, 6, 12, 24, 48, 72 and 96 hours of testing to natural (*in situ* incubation) and simulated conditions (*in vitro* incubation) from the ruminal environment of bovines. For each period of *in situ* and *in vitro* incubation, in each kind of seed, the percentage of normal plants was evaluated, as well as abnormal plants, hard seeds, soaked seeds and dead seeds, besides the index of germination speed (IGS). The results were submitted to the Duncan test at a 5% probability. Higher percentages of normal plants were verified in the *Kudzu* and in the *Leucaena* kinds, when the seeds were submitted to *in situ* incubation, as well as the IGS for the three forage species. The *Calopogônio* seeds did not turn out to be susceptible of use in the *in situ* and *in vitro* incubations. The *in vitro* incubation revealed to be more harmful to the seeds of the three species used.

Keywords

***Pueraria phaseoloides*, *Leucaena leucocephala*, *Calopogonium mucunoides*, Digestibility, Fermentation**

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How to cite this paper: Lima, R.V., Vieira, H.D., da Silva, T.O., da Silva Rocha, N. and Deminicis, B.B. (2015) Germination and Vigor of Fodder Fabacceae Seeds Submitted to *in Vitro* and *in Situ* Incubation *American Journal of Plant Sciences*, **6**, 2317-2328. http://dx.doi.org/10.4236/ajps.2015.614235

1. Introduction

The adoption of *Fabacceae* species in pastures in Brazil is still not very common, but there is an increasing interest for the practice by private initiatives, although. One of the big handicaps found is the phase of establishment of the plants in the field [1].

For the introduction of forage *Fabacceae* species in pastures already established with the *Poacceae* kind, the rural producers have, as an alternative, the rational dispersion of the seeds using the animals as spreading agents [2] [3]. In natural conditions of pasture, the seed germination is controlled by factors such as climate, light, temperature and soil conditions, for example pH and humidity. Beside these factors, the germination can still suffer influence of grazing animals, which, by ingesting the seeds, affect the germination positively or negatively.

The dormancy of the *Fabacceae* seeds is an hereditary characteristic, related to the palisade cell layer, which possesses thick walls recovered externally by a waxy cuticular layer [4]. In the species of this family, the seed dormancy is caused by a physical block represented by the resistant and waterproof integument which, by preventing the water transit and the gas exchange, prevents the seed from becoming wet and the oxygenation of the embryo as well. These seeds, denominated hard seeds, achieve great longevity and any procedure that prevents the integument break, making them absorb water, promotes their germination and emergence of plants generally vigorous [5].

The passage of the seeds by the gastrointestinal tract of the ruminant animals can provoke alterations in their dormancy, in percentage, germination speed and in the initial growth of the plant. Besides that, there is the seed exposure to the attack of microorganisms present in the rumen, pH variation and temperature. The rumen is an anaerobic environment, with an average temperature of 39˚C, pH varying between 5.5 and 7.0, where there is a balance of several microorganisms digesting carbohydrates, proteins and lipids ingested by the animal. Furthermore, the rumen size and the retention period influence the capacity of the *in situ* digestion [6]-[9].

The *in situ* digestibility can be simulated in *in vitro* procedures that recreate the rumen and the abomasum conditions. The gravimetric digestibility test consists of two stages, with the first one to recreate the rumen conditions and the second to recreate the abomasum conditions. In digestibility studies, the *in situ* results obtained were always more precise when compared to the methods available in laboratory (*in vitro*). The advent of the *in situ* technique, using nylon packs in bovine animals fistulated in the rumen, permitted a quick and simple evaluation of the material degradation in function of the incubation time [10] [11].

There are several sources of variation that can interfere in the *in vitro* incubation performance and these are variations in the microbial population (diet of the donor animal; differences between animals; handling of the ruminal liquid), variations due to the sample process (granulometry and weight), differences attributed to the environment (ruminal liquid volume in relation to the buffer solution; buffer and nutritive environment adopted) as well as variations in the procedures (fermentation time, laboratory errors) [12].

With this information in hands, this study was developed with the aim to evaluate the germination and the vigor of seeds from three different *Fabacceae* species with forage characteristic after the seed exposure during different periods of testing in natural (*in situ* incubation) and simulated conditions (*in vitro* incubation) from the ruminal environment.

2. Material and Methods

This study was conducted in two individual steps and, in order to perform both of them, homogeneous plots of seeds were used from each one of the three *Fabacceae* species with forage characteristic: *Kudzu* (*Pueraria phaseoloides*), *Leucaena* (*Leucaena leucocephala*) and *Calopogônio* (*Calopogonium mucunoides*).

Primarily, the *in situ* incubation was performed, which consisted in the incubation during 0, 6, 12, 24, 48, 72 and 96 hours of testing in the rumen for the vigor and the seed germination evaluation. For this incubation performance, a half-breed ox was used (Dutch × Zebu), with a live weight of 1030 kg, provided with ruminal fistula and placed in individual stall. The diet was constituted of tifton-grass hay, wheat bran concentrate, mineral salt and water at will, initiated 15 days before the test performance, being this the period of the animal adaptation to the diet and to experimental conditions. The total quantity of the food daily offered was divided in two times, at 8 a.m. and at 6 p.m., and the quantity offered was adjusted in function of the leftovers observed daily (10% of the quantity offered). The leftovers, normally rejected, were controlled to guarantee the animal voluntary consumption.

For the seed incubation in the rumen, special nylon packs were used. Inside the packs, 20 grams from seeds of each kind were put and they were stuck to iron chains of 50 cm length. After such procedure, the *in situ* incubation of all the nylon packs was performed, being removed according to the respective incubation hours.

After the removal, the packs were immediately immersed in a bucket with water at ambient temperature to cease the action of ruminal microorganisms. Next, they were taken to the laboratory and germination and vigor tests were performed, according to [13].

The *in vitro* incubation was performed in accordance with the methodology described by [14] in order to simulate, in laboratory, the condition of the ruminal environment and also to verify the seed vigor and germination after 0, 6, 12, 24, 48, 72 and 96 hours of incubation. For the ruminal liquid collect, the animals were fed daily during 15 days with 2 kg corn bran concentrate to maximize the microbial production in the rumen. Fifty seeds were used in each repetition and the incubation solutions were prepared according to the seed weight from each kind.

For each *in situ* and *in vitro* incubation period, in each legume kind, normal plants (%), abnormal plants (%), hard seeds (%), soaked seeds (%), dead seeds (%) and the index of germination speed (IGS) were evaluated [15].

The IGS is calculated from the data on daily count of germinated seeds and has as an objective to establish differences in the seed germination speed. In this experiment, the counts were performed up to the 10^{th} day after the test preparation.

Each type of incubation was analyzed separately. For both, the experimental delineation was entirely randomized, in factorial scheme 3 × 7 (three *Fabacceae* and seven incubation periods), with four repetitions. The results obtained were submitted to Duncan test at a 5% probability, once the regression model to the variables analyzed was not adjusted.

3. Results and Discussion

The variance analysis showed that the variables analyzed suffer significant effects from the *in situ* incubation periods as well as from the *in vitro* incubation periods.

In **Figure 1(a)** it is possible to observe that, for the *Kudzu* kind, the bigger formation of normal plants (53%) occurred with 12 hours of permanence from the seeds in bovine rumen, although a significant difference in relation to the control (39.5%) and 6 hours (43%) of *in situ* incubation was not evidenced. The increase in the formation of normal plants up to 12 hours of *in situ* incubation, followed by their fall in the next times, evidenced for the *Kudzu* type, reinforce the fact that the stay of the seeds in the bovine rumen makes a chemical scarification on their integument, provoking fissures which allow higher water absorbency and consequently higher germination. The seed viability, still maintained in these conditions, allowed them to form normal plants. But the seed permanence of this kind for longer periods of time in the ruminal conditions, as for instance, from 24 to 96 hours, leads to a higher loss of their viability with the fall in the percentage of the normal plants.

Reference [16] evaluating the germination and the vigor of *Kudzu*, *Leucaena* and *Calopogônio* seeds after the passage through the gastrointestinal tract (GIT) from bovines, observed that *Kudzu* seeds are less susceptible to the damages caused by the chewing and degradation in the GIT, once a higher emerged plant number in bovine feces was formed, as well as higher root lengths and aerial part and higher weight of natural and dry mass from the plants when compared to other species.

For the *Leucaena* seeds, a significant percentage of normal plants were observed in the control treatment (28%), being possible to verify a gradual fall in the number of natural plants as the incubation period increased and, with 96 hours, the percentage of normal plants (4%) was significantly reduced in relation to the control. The *Leucaena* seeds demonstrated an intermediate resistance in the viability loss between the 3 species studied, a fact which can be related to the integument hardness of the kind and its resistance to stand the natural conditions from the rumen and still form normal plants after incubation for long periods of time. Demonstrating the damages caused by the rumen, a significant increase on the abnormal *Leucaena* plants after 12 hours of incubation was observed (**Figure 1(b)**).

The *Calopogônio* seeds, on the other hand, formed normal plants only until 24 hours of seed permanence in the rumen (5%), after this period there was a significant fall and practically normal plants were not formed. The damages suffered by the *Capologônio* seeds were so harmful that, different from the other two species, even the abnormal plant number was significantly reduced after 12 hours incubation. The *Calopogônio* seeds plot used in

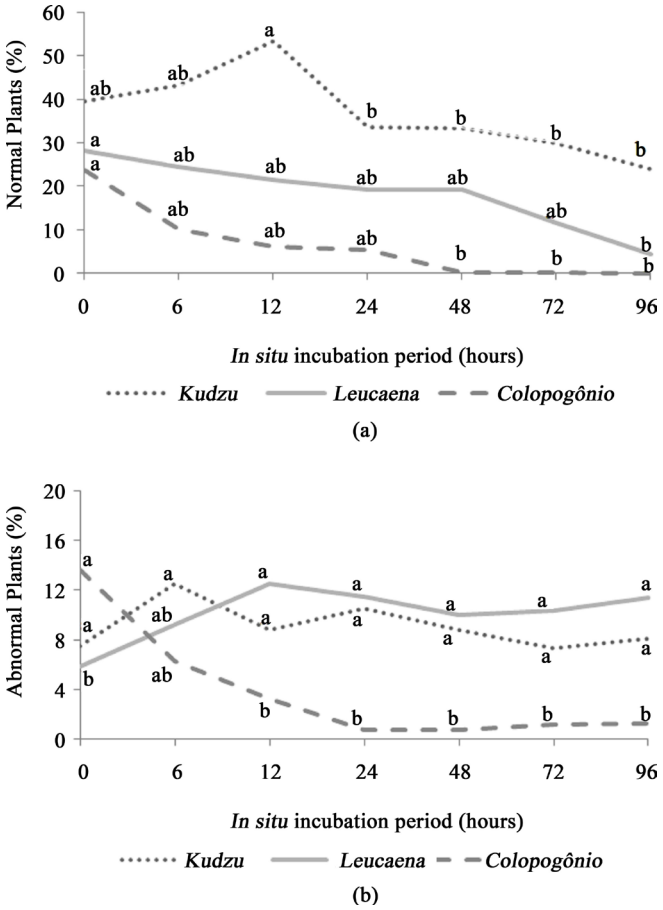

Figure 1. Normal plants (%) (a) and abnormal plants (%) (b) from *Kudzu*, *Leucaena* and *Calopogônio* seeds submitted to *in situ* incubation during 0, 6, 12, 24, 48, 72 and 96 hours.

this study had seeds of permeable integument and, therefore, their exposure to the incubation techniques led to a severe viability loss. For this same plot, the fall in normal plant formation, as well as abnormal plants, is directly related to the elevation in the percentage from dead seeds in the course of the hours of incubation, showing that excessive exposure time of *Calopogônio* seeds to the ruminal condition culminates on their death.

Reference [3] tested *Cunhã*, Perennial Soybean, *Macrotiloma* and Stylosanthes seeds in *in vitro* incubation and verified an increase in the percentage of seed germination percentage from *Cunhã*, Perennial Soybean, and *Macrotiloma* kind with the increase of the incubation period up to 48 hours. The Stylosanthes seeds had the germination percentage quickly reduced associated to a high dead seed percentage.

Reference [17] evaluating the effects of the ruminal degradation (*in situ* incubation) over the germination of C*unhã*, Stylosanthes, *Kudzu* and *Macrotiloma* seeds, verified that, for the Stylosanthes seeds, the permanence in the rumen made the germination reduce principally in the periods from 12 to 96 hours. For the *Cunhã* seeds, their permanence in a ruminal environment promoted an increment in the germination and, among the species evaluated, the *Kudzu* was the one that presented higher germination percentage.

Seeds of species that present impermeable integuments have bigger chances of surviving after the passage through the bovine gastrointestinal tract, even if significant difference in the survival or germination speed is not observed [18].

Reference [19] verified an increase in the percentage of germination from *Helianthemum apennium* seeds, which the increment was from 12% to 32% when they were incubated during 48 hours in sheep rumen, and from 12% to 25.6% germination when incubated during 24 hours in goat rumen. The authors assigned this germination increase to the fact that the ruminal liquid contains proteolytic and cellulolytic enzymes that promote the scarification of the seed integument.

The percentage on hard seeds for the *Kudzu* kind was similar between 0, 6 and 12 hours of ruminal incubation

(34%, 33% and 28%, respectively), decreasing significantly after that in relation to the control. Similar results were found by Demenicis (2009), who reported a reduction of the number on hard seeds from *Cunhã*, *Macrotiloma* and Perennial Soybean species as their ruminal incubation time increased. For the *Leucaena* kind, the *in situ* incubation did not affect significantly the seeds and the hard seed percentage was similar in the course of the incubation periods. With the *Calopogônio* seeds, it was possible to observe, right in the control treatment (0 hour incubation), that the seeds used did not present high integument hardness degree and this fact can be related to the seed plot used, as well as their storage, although they showed to be viable in preliminary tests performed before the incubations (**Figure 2(a)**).

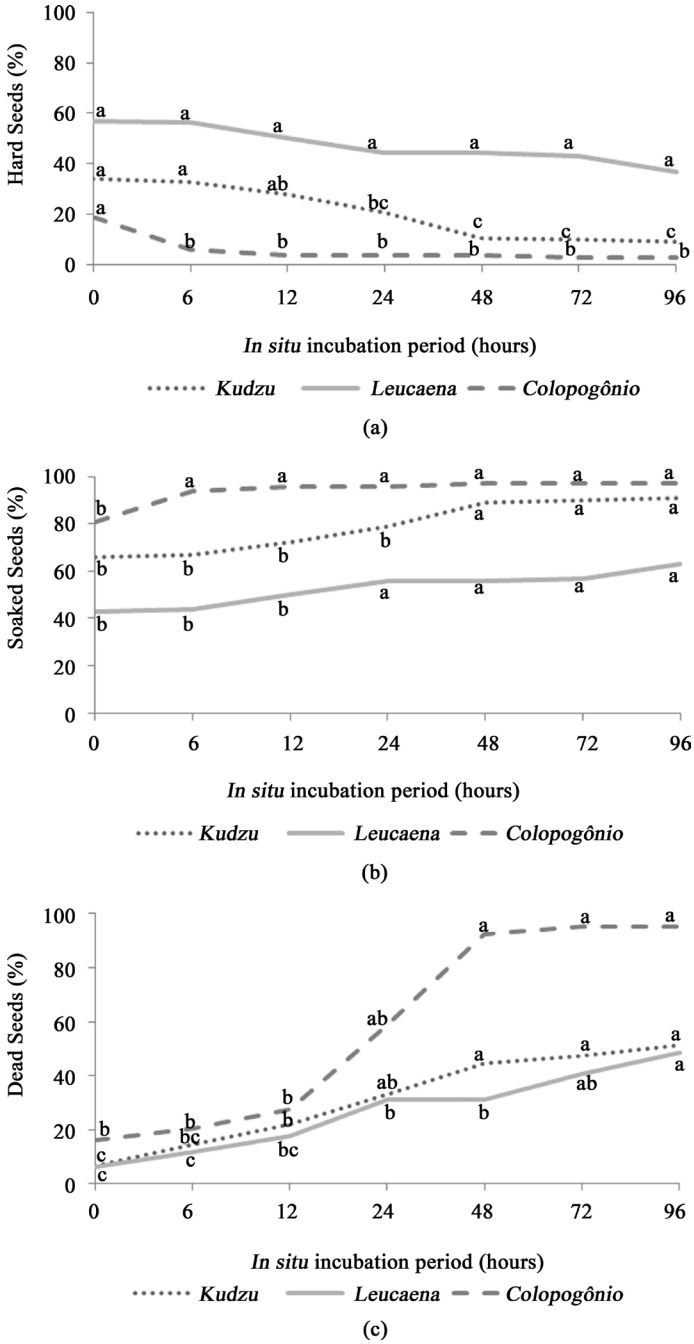

Figure 2. Hard seeds (%) (a), soaked seeds (%) (b) and dead seeds (%) (c) from *Kudzu*, *Leucaena* and *Calopogônio* type found during 0, 6, 12, 24, 48, 72 and 96 hours of *in situ* incubation.

Still in **Figure 2(a)**, 37% of hard *Leucaena* seeds, 9% of hard *Kudzu* seeds and only 3% of hard *Calopogônio* seeds were observed at the end of the 96 hours incubation, which demonstrates a higher integument hardness of the *Leucaena* seeds, suggesting that only the *in situ* incubation is not enough to overcome the dormancy of the seeds, but there is the perspective that the passage of the seeds through all the digestive tract of the bovine promotes a better cease of the dormancy, resulting in higher germination percentage.

In **Figure 2(b)**, it is possible to observe that the percentage of soaked seeds varied significantly between the *in situ* incubation periods for the three forage species. For the *Kudzu* seeds, significantly higher percentages were observed after 48 hours of *in situ* incubation and, for the *Leucaena* seeds, the percentage of soaked seeds was significantly higher after 24 hours of incubation, showing that the seeds of these two species revealed to be more resistant to the ruminal incubation.

Cunhã and *Macrotiloma* seeds also presented high resistance and survival rate when they were submitted to the *in situ* incubation due to the integument impermeability. The seed scarification provoked by the micro-biota, the temperature and the immersion in ruminal liquid caused slight effect in the integument permeability, which guaranteed the seed viability [3] [17].

The *Calopogônio* seeds, on the other hand, revealed to be very soaked with only 6 hours of *in situ* incubation, with percentages significantly higher after this period up to 96 hours in relation to the control (**Figure 2(b)**). This suggests that the ruminal condition (microorganisms and temperature action) has contributed for the integument scarification from these seeds, which were believed to be already permeable. Reference [17] assigned the reduction of the integument hardness to the period of exposure in the rumen and the acceleration on the seed death to the more permeable integument.

Reference [20] related high genetic variability in 195 accesses of *Calopogônio* seeds scattered through all the Brazilian territory. They suggest that this kind presents a reproductive mixed system with autogamy predominance and which can be considered in enhancing programs for several characteristics. Reference [21] also studied the genetic diversity from forage species and, among them, there is the *Calopogonium mucunoides* and these authors also reported the high genetic variability found among the accesses. Maybe, the seed integument hardness also varies in function of the genetic diversity found and, because of that, the seed plot is very heterogeneous when it comes to the seed integument hardness.

Figure 2(c) shows the dead seed percentage for the three *Fabacceae* species in different periods of *in situ* incubation. For the *Calopogônio* kind, an increase of dead seeds percentage is observed after 24 hours of incubation and a significant increase after 48 hours incubation. Meanwhile, for the *Kudzu* kind, a significant dead seed increase was observed after 12 hours. For the *Leucaena* kind, percentages significantly higher of dead seeds were verified after 24 hours of *in situ* incubation in relation to the control, although in percentages way lower than for the *Calopogônio* kind, evidencing a higher seed resistance due to higher integument hardness.

Figure 2(a) and **Figure 2(c)** help to explain the seed behavior exposed to the action from the ruminal environment, where the conditions found make the hard seed percentage decrease and the dead sed percentage increase with the course of their exposure time to the *in situ* incubation, indicating that the seeds with a more permeable integument die and the seeds which are more impermeable become permeable. For the *Sumaúma* kind, 90% of the *in situ* seed degradation occurred with 12 hours of ruminal incubation [22].

In **Figure 3**, it was observed that for the *Kudzu* type, the higher IGS was verified at 6 hours of ruminal incubation (IGS = 13), without presenting significant difference up to 96 hours. For the *Leucaena* seeds, a higher IGS was observed with 24 hours of ruminal incubation of the seeds (IGS = 5.38), being significantly equal for the incubation at 6, 12 and 48 hours. For the *Calopogônio* seeds, the higher IGS observed was in the control treatment (0 hour) (IGS = 18.75), in other words, when the seeds from this kind were exposed to ruminal environment, it was possible to observe their vigor loss after 6 hours of *in situ* incubation, evidenced by the significant fall of the germination speed.

Reference [17] found higher IGS values for the *Cunhã*, *Kudzu*, Stylosanthes and *Macrotiloma* seeds varying between 0 and 12 hours on *in situ* incubation and, for all the species, the author verified a speed reduction rate with the increase of the incubation period.

Reference [23], when evaluating the ruminal liquid effects in the *Vicia angustifolia* seed germination, observed that the bigger seeds of this kind germinated more quickly than the smaller ones and assigned such fact to the higher nutritional reserves of the bigger seeds.

Figures 4-6 illustrate the results found with the *in vitro* incubation of the seeds, a technique which allows to simulate, in laboratory, the ruminal environment.

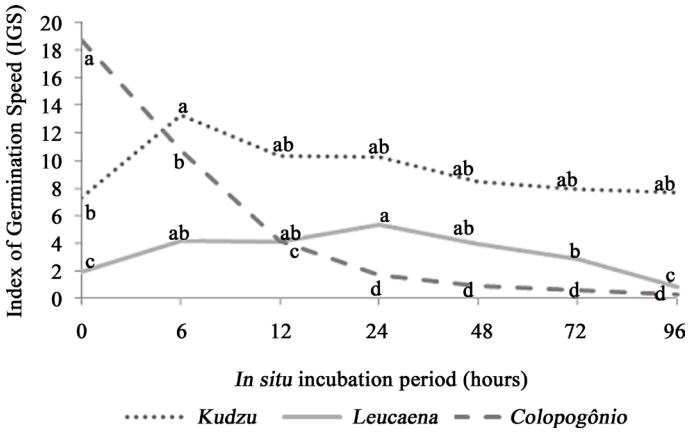

Figure 3. Index of Germination Speed (IGS) from *Kudzu*, *Leucaena* and *Calopogônio* species submitted to *in situ* incubation during 0, 6, 12, 24, 48, 72 and 96 hours.

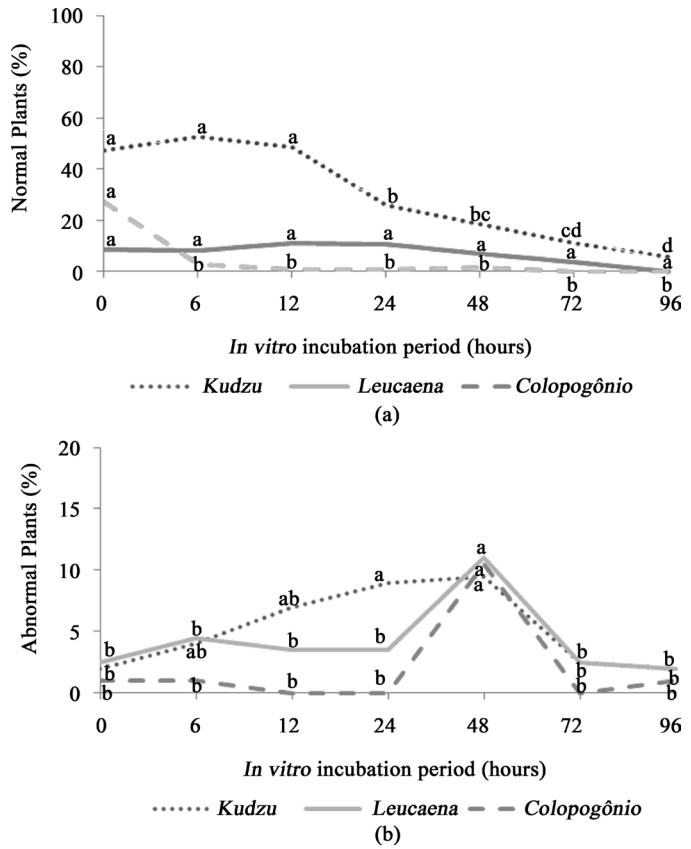

Figure 4. Normal plants (%) (a) and abnormal plants (%) (b) from *Kudzu*, *Leucaena* and *Calopogônio* seeds, undergone to the technique of *in vitro* incubation during 0, 6, 12, 24, 48, 72 and 96 hours.

For the *Kudzu* kind, the normal plant percentage did not present significant difference at 0, 6 and 12 hours of incubation (47.5%, 52.5% and 48.5%, respectively) and at the other hours there was a progressive fall in the formation of normal plants. Here, it is also possible to observe that the prolonged seed exposure to simulated ruminal conditions tends to provide their vigor loss, with a decrease in the formation of the normal plant number. The *Leucaena* seeds presented similar behavior to this variable in all incubation periods, not presenting significant difference among them, and this behavior can be explained by the bigger resistance that this kind of seed present and the *in vitro* incubation was not sufficient for the number of normal plants to suffer significant altera-

tions. As for the *Calopogônio* seeds, they formed normal plants with significant higher percentage in the control treatment (0 hour) (27%), with significant fall after 6 hours of incubation (**Figure 4(a)**), different to what occurred in the *in situ* incubation, in which the formation of normal plants suffered significant fall only with 48 hours of incubation. The storage time of the lot could have interfered in the response of the *Calopogônio* seeds during the *in vitro* incubation, which was performed after the i*n situ* incubation, allied to the fact that they already had a more permeable integument, evidenced by the fall in the *in vitro* incubation, they lost the vigor quickly, evidenced by the fall in the formation of normal plants with only 6 hours of *in vitro* incubation.

Reference [1] submitted *Cunhã*, Stylosanthes, *Macrotiloma* and Perennial Soybean seeds to the technique of the *in vitro* acid-enzymatic digestion and observed that *Cunhã*, *Macrotiloma* and Perennial Soybean species presented small germination addition as the permanence time of the seeds in the hydrochloric acid + pepsin increased, contrary to Stylosanthes seeds, which had a reduced germination percentage, as a result of the increase in the dead seed percentage that the authors observed in the germination test.

Reference [23] studying the seed germination from *Vicia angustifolia* incubated *in vitro* in ruminal liquid, observed a significant reduction of the seed germination from these species, with a percentage close to zero.

In **Figure 4(b)**, the abnormal plant percentage from these seeds after *in vitro* incubation is observed. Percentages significantly higher of *Kudzu* abnormal plants were observed at 24 and 48 hours (9% and 9.5%, respectively) in relation to the control, without presenting significant difference of 6 and 12 hours of incubation (4% and 7%, respectively), though. Percentages significantly higher of abnormal plants from *Leucaena* and *Calopogônio* species (11% and 10.5%, respectively) can be observed when the seeds were incubated for 48 hours.

For the three Fabacceae species, an increase in the abnormal plant formation up to 48 hours of *in vitro* incubation is observed. Such behavior indicates that the seeds have their viability reduced as the time of incubation increased and this viability loss is indicated by the increase in the formation of abnormal plants, with a peak at 48 hours of incubation. Thenceforth, the normal plant number decreased and the dead seed number increased for the three species (**Figure 4(b)** and **Figure 5(c)**).

Figure 5(a) shows that, among the three *Fabacceae* species, the one that presented higher percentage of hard seeds, initially, was the *Leucaena* kind (67%), followed by the *Kudzu* kind (45.5%) and by the *Calopogônio* kind (7%) and it also presented that, as the time of *in vitro* incubation went by, this percentage suffered a gradual fall for the *Kudzu* and *Leucaena* species, evidencing the scarification provoked in the integument when the seeds were exposed to the *in vitro* incubation, according to what happened in the *in situ* incubation. For the *Calopogônio* seeds, this scarification was not confirmed, because the seeds used already had low integument hardness, a fact verified by the percentage significantly higher of normal plants of those seeds which were not submitted to incubation (0 hour) (**Figure 4(a)**) and/or by the high percentage of seeds soaked in the control of **Figure 5(b)**.

Similar results were found by [1] with seeds of the *Cunhã*, Stylosanthes, *Macrotiloma* and Perennial Soybean type, of which the reductions in the percentage of hard seeds happened as the length of stay in the acid + pepsin increased, although the Stylosanthes type suffered accentuated reduction right in the first hours of incubation. These authors also report that the seeds of *Cunhã*, *Macrotiloma* and Perennial Soybean kind presented high resistance and survival of their seeds due to the integument hardness inherent to these species and assigned these results to the small effect that the scarification acid-enzymatic provoked over the permeability of the integument of these three species.

The results obtained with the seeds of Stylosanthes by [1] are in accordance to those found for the *Calopogônio* seeds in this study, in which they also suffered damages that culminated on the death of an elevated percentage of seeds due to the absence or low percentage of seeds with integument hardness.

It is verified, in **Figure 5(b)**, that the intact seeds of *Calopogônio* (0 hour of *in vitro* incubation) already revealed to be very soaked, and this behavior did not alter during the time of incubation, without presenting significant difference among them. For the *Kudzu* kind, higher percentages of soaked seeds were verified after 6 hours of *in vitro* incubation. For the *Leucaena* kind, higher percentages of soaked seeds were observed only after 48 hours of *in vitro* incubation.

The percentage of dead seeds increased significantly after 48 hours for the *Kudzu* and *Leucaena* kinds and 24 hours for the *Calopogônio* kind. *Kudzu* and *Leucaena* seeds resist for longer time when exposed to the simulated *in vitro* conditions, but, the prolonged permanence of the three species of seeds in these conditions decrease gradually the viability and the germination, culminating with their death (**Figure 5(c)**).

As for the IGS, the *Kudzu* seeds presented indexes significantly higher at 6 and 12 hours of *in vitro* incubation

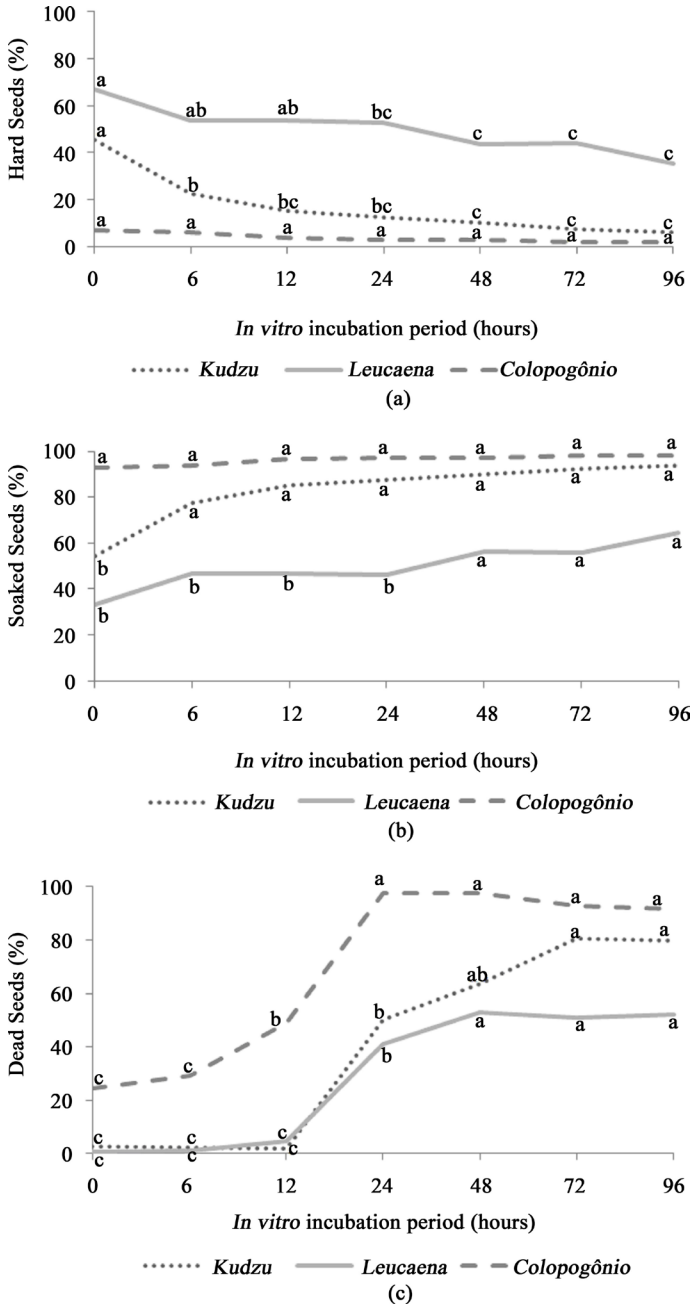

Figure 5. Hard seeds (%) (a), soaked seeds (%) (b) and dead seeds (%) (c) of the *Kudzu*, *Leucaena* and *Calopogônio* kinds founds during 0, 6, 12, 24, 48, 72 and 96 hours of *in vitro* incubation.

(IGS = 49.3 and 47.4, respectively), in other words, the seeds exposed to such conditions for 6 and 12 hours germinated at a higher speed than those exposed to the other periods of incubation and those that kept themselves intact. It is believed that the intact seeds germinated at a lower speed due to the integument that was found still very impermeable before the exposure to the incubation and, when the exposure was extended to periods higher than 12 hours, the viability of the seeds had already been affected and, thereby, the protrusion of the radicle took more time to happen. For the *Leucaena* seeds, such index did not differ significantly among the *in vitro* incubation periods, in other words, this type of incubation did not affect the speed that the *Leucaena* seeds germinated and, for the *Calopogônio* seeds, their exposure to *in vitro* incubation was harmful, once the higher index was observed for the intact seeds (IGS = 19.5) (0 *in vitro* incubation time) (**Figure 6**).

On **Table 1**, the response of the forage seeds in relation to the formation of normal and abnormal plants and index of germination speed (IGS) is verified, undergone to *in situ* and *in vitro* incubation, independently of the time they remained exposed.

Percentages significantly higher of normal plants were observed for the *Kudzu* kind (36.71%), as well as for the *Leucaena* kind (18.36%), undergone to the *in situ* incubation. For the *Calopogônio* kind, the percentages of normal plants did not differ significantly between the two types of incubation, despite the reduction in the *in vitro* incubation. This indicates that the *in vitro* incubation is more harmful, which can be caused by the exposure of the seeds to a direct action of the acids used in this technique, which does not happen in the *in situ* incubation (**Table 1**).

For the variable "abnormal plants", percentages significantly higher were observed for the *Kudzu* seeds (9.07%) and *Calopogônio* seeds (3.86%), when exposed to the *in situ* incubation. For the *Leucaena* seeds, this variable did not present significant difference between the two types of incubation. The reduction of the normal and abnormal plants matches the increase of dead seeds in the *Kuzdu* and *Calopogônio* species, indicating once more that the action of the *in vitro* incubation was more harmful for the seeds than the *in vivo* and that the methodology needs adjusts to approximate the effect of the *in vitro* incubation to the *in vivo* incubation (**Table 1** and **Table 2**).

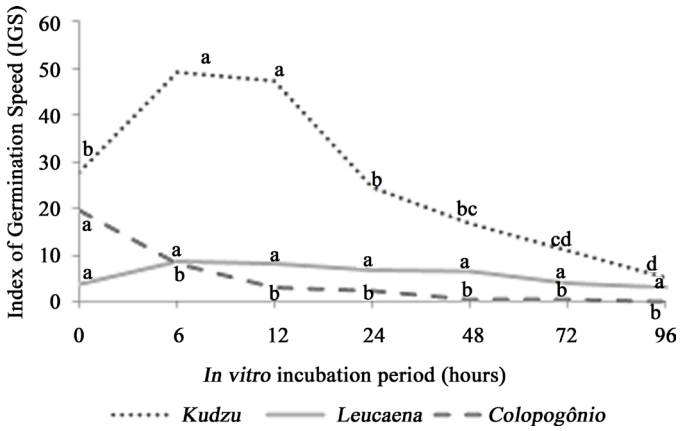

Figure 6. Index of Germination Speed (IGS) of *Kudzu*, *Leucaena* and *Calopogônio* seeds undergone to the technique of *in vitro* incubation during 0, 6, 12, 24, 48, 72 and 96 hours.

Table 1. Normal plants (%), abnormal plants (%) and index of germination speed of *Kudzu*, *Leucaena* and *Calopogônio* species after techniques of *in situ* and *in vitro* incubation of the seeds.

Species	Normal Plants (%)		Abnormal Plants (%)		IGS	
	In situ	*In vitro*	*In situ*	*In vitro*	*In situ*	*In vitro*
Kudzu	36.71A	29.93B	9.07A	5.14B	9.32A	7.02B
Leucaena	18.36A	6.93B	6.00A	4.21A	3.33A	1.47B
Calopogônio	6.54A	4.57A	3.86A	1.93B	5.31A	1.23B

For each level, the averages followed in the lines by the same letter do not differ statistically among them by the Duncan test at a 5% probability level.

Table 2. Hard seeds (%), soaked seeds (%) and dead seeds (%) of *Kudzu*, *Leucaena* and *Calopogônio* species, resulted from the techniques of *in situ* and *in vitro* incubation of the seeds.

Species	Hard Seeds (%)		Soaked Seeds (%)		Dead Seeds (%)	
	In situ	*In vitro*	*In situ*	*In vitro*	*In situ*	*In vitro*
Kudzu	21.00A	17.00A	79.00A	83.00A	31.41B	40.08A
Leucaena	47.00A	50.00A	53.00A	50.00A	26.67A	29.00A
Calopogônio	6.00A	3.78A	94.00A	96.22A	57.99B	68.86A

For each level, the averages followed in the lines by the same letter do not differ statistically among them by the Duncan test at a 5% probability level.

The IGS also revealed to be significantly higher for the seeds of the three forage species when undergone to the *in situ* incubation. In other words, the seeds germinated more quickly when exposed to the natural conditions and less harmful of the bovine rumen when compared to the simulated conditions in laboratory (*in vitro* incubation). Numerically, this index was higher for the *Kudzu* seeds in both the incubation techniques, showing that the seeds of this kind germinated more quickly in relation to the others two species, and it is suggested, thus, that the *Kudzu* seeds presented better vigor in relation to the *Leucaena* and *Calopogônio* seeds in the conditions of performance of this experiment (**Table 1**).

Table 2 shows the percentages of hard seeds, soaked seeds and dead seeds after the exposure of the forage seeds to the *in situ* and *in vitro* incubation, independently of the length of time they remained exposed.

For the three forage species, the percentage of hard seeds did not present significant difference between the incubation techniques, although numerically, the *Leucaena* kind has been the one that presented hard seeds at the end of the germination test in the two incubation techniques. It is probable that both the techniques have provoked a similar effect of scarification in the seeds and, thus, they did not present significant differences between them (**Table 2**).

A similar behavior for both incubation techniques was also verified in the percentages of soaked seeds for the three forage species, which also did not present significant difference between the incubation techniques, which can also be related to the analogous effect of scarification that they have presented in the seeds of the three species (**Table 2**).

The percentage of dead seeds was significantly higher for the *Kudzu* and *Calopogônio* seeds undergone to the *in vitro* incubation. Dead *Leucaena* seeds did not present significant difference between the two types of incubation techniques. Apparently the *in vitro* incubation has a scarification action similar to the *in situ* incubation, however, when permeable seeds are exposed to the two incubation, the action of the *in vitro* incubation is more harmful to the seeds (**Table 2**).

4. Conclusions

Kudzu and *Leucaena* seeds originated higher percentages of normal plants in the *in situ* incubation and they can be recommended for such technique.

A higher IGS was observed for the seeds of the three forage species in the *in situ* incubation.

The *Calopogônio* seeds used in this study were not revealed to be adequate to use in the *in situ* and *in vitro* incubation techniques.

The *in vitro* incubation resulted in higher percentages of dead seeds of *Kudzu* and *Calopogônio* species.

The *in vitro* incubation technique revealed to be more harmful to the seeds of the three species used in this experiment.

Clarification

The proceedings were approved and carried out according to Protocol 207, registered in the Ethics and Animal Use Committee of UENF.

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