



SHORT NOTE [NOTA CORTA]

NUMBER OF COWS AND BAGS NEEDED TO ESTIMATE *in situ* DRY MATTER DEGRADATION OF KINGGRASS (*Pennisetum purpureum*) LEAVES

[NÚMERO DE VACAS Y DE BOLSAS NECESARIO PARA ESTIMAR LA DEGRADACIÓN *in situ* DE HOJAS DE KINGGRASS (*Pennisetum purpureum*)]

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SUMMARY

The number of bags to be incubated per sample, and the number of fistulated cows needed to estimate *in situ* ruminal degradation of Kinggrass (*Pennisetum purpureum*) dry matter (DMD %) were determined. Three rumen-fistulated cows and 10 bags per incubation time (3, 6, 9, 12, 24, 48 and 72 h) were used. Five grams of dried leaves were weighed per bag. Variance components for cows (V_c) and bags (V_b) per incubation time were estimated and used to calculate the standard error (SE) of mean DMD. The adequate number of bags to be used was the one that produced a value of 1 % (SE of the study). Precision increased as the number of cows increased, with mean SE of 1.66, 1.18 and 0.96 % for one, two and three cows, respectively. Precision remained more or less constant for incubation times of 3, 6, 9, 12 and 48 h, with respective values of 0.93, 0.81, 1.05, 0.92 and 1.08 %. The SE values doubled at 24 (2.15) and 72 (1.92) h. The EE diminished as the number of bags increased, being the values higher for one cow than for two or three cows, but these differences were slight when 10 bags were used. The use of two cows seems reasonable, since the increase in precision from one to two cows was greater than from two to three cows. Three bags were the minimum necessary to estimate the SE value of 1%.

Key words: *In situ* degradation; dry matter; number of cows; number of bags; Kinggrass.

RESUMEN

Se determinó el número necesario de bolsas a incubar por muestra y de vacas fistuladas para estimar la degradación ruminal *in situ* de la materia seca (DMS %) de kinggrass (*Pennisetum purpureum*). Se usaron tres vacas fistuladas al rumen y 10 bolsas por tiempo de incubación (3, 6, 9, 12, 24, 48 y 72 h). Se pesaron 5 g/bolsa de forraje seco. Se estimaron los componentes de varianza para vacas (V_v) y bolsas dentro de vaca (V_b) por tiempo de incubación, que fueron usados para calcular el error estándar (EE) de la DMS promedio. El número de bolsas adecuado fue aquel que produjo un valor de 1 % (EE del experimento). La precisión incrementó al aumentar el número de vacas; el EE promedio fue 1.66, 1.18 y 0.96 % para una, dos y tres vacas, respectivamente. La precisión fue más o menos constante para los tiempos de incubación de 3, 6, 9, 12 y 48 h, con valores respectivos de 0.93, 0.81, 1.05, 0.92 y 1.08 %. Los EE se duplicaron a las 24 (2.15) y 72 h (1.92). Los EE disminuyeron al aumentar el número de bolsas, siendo mayores los valores para una vaca, que para dos o tres vacas, pero tales diferencias fueron ligeras cuando se emplearon 10 bolsas. Parece razonable usar dos vacas, pues el aumento en precisión al pasar de una a dos fue mayor que de dos a tres. Tres bolsas fue el número mínimo necesario para estimar el EE de 1 %.

Palabras clave: Degradación *in situ*; materia seca; número de vacas; número de bolsas; kinggrass.

INTRODUCTION

The *in situ* or *in sacco* ruminal digestion technique was developed in the late 1930s by Quin *et al.* (1938), but it started to be used by nutritional researchers when Ørskov's team at the IGER used it to study

ruminal protein degradation and also as a screening test for by-pass protein in feeds (Ørskov, 2000).

In spite of the wide use of the *in situ* technique in tropical regions, very seldom researchers on these parts of the world conduct and publish methodological

studies designed to test the various sources of variation of the procedure. Only recently has a Brazilian team started to study the effect of bags made of different textile materials on ruminal degradation profiles of forages, finding out that degradation profiles of dry matter and neutral detergent fiber must not be evaluated using Ankom F57 and non-woven textile bags, as these underestimate the degradation rate due to constraints regarding exchange between bags content and rumen environment (Valente *et al.*, 2011).

Three major sources of variation in rumen dry matter (DM) disappearance data are: between animal variation, between period (day) variation within the same animal, and between bag variation (van der Koelen *et al.*, 1992). The number of animals and bags that should be used is of particular concern, as these two factors determine the cost of running a sample. As compared to cattle, sheep is less expensive, but the number of samples ran at the same time is reduced and, obviously, for studying nutrition of cattle it is preferable to use cattle instead of sheep (Nocek, 1988).

Several reports have addressed the number of animals and bags to be used (Mehrez and Ørskov, 1977; Lindberg, 1985; Michalet-Doreau and Ould-Bah, 1992; van der Koelen *et al.*, 1992; Vanzant *et al.*, 1998). In general, the use of a minimum of two cows and three bags per cow has been recommended. However, these recommendations have been generated for temperate cattle or sheep and for temperate feeds, which quality is higher and less variable than that of C4 grasses, the main source of nutrients for cattle in the tropics. Therefore, it is necessary to generate information on this topic in cattle and grasses found in tropical regions. Thus, the objective of the study was to determine the minimum number of cows and bags needed to run the *in situ* procedure in Kinggrass (*Pennisetum purpureum*) leaves grown in a low fertility soil under hot and humid conditions of Veracruz, Mexico, in order to generate recommendations to researchers in tropical regions.

MATERIALS AND METHODS

Forage material

Kinggrass (*Pennisetum purpureum*) harvested at 12 weeks of regrowth was used. Three 2 m x 5 m plots were harvested at 20 cm cutting height from December 21 to 23, 2009. Leaf blades were separated from the plant and then dried in an oven at 62 °C for 72 h. The dried material was ground in a Wiley mill model 4, using a 2 mm sieve, and stored in plastic bags at room temperature until analyzed for *in situ* dry matter degradation. The crude protein (CP) content of leaves was 9.8 %, with values for neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin of 75.8, 55.7 and 12.1 %, respectively.

In situ digestion procedure

The *in situ* DM degradation (DMD, %; Ørskov and Mc Donald, 1979) procedure was conducted as follows. Three 6 year-old rumen-fistulated crossbred cows with 630 ± 55 kg live weight were used. The cows were fistulated at one year of age and had been used several times for *in situ* measurements; since they had never been exposed to bulls or artificial insemination, they were barren during the experiment. The rumen fistulas remained closed with commercial 10 cm diameter cannulas with removable stopper (Bar Diamond, Parma ID, USA). During the trial, the animals grazed continuously a mixed grass pasture (*Paspalum* spp., *Axonopus* spp., *Brachiaria* spp.) with approximately 3000 kg/ha of DM, 50 % leaves and 9 % CP at the beginning of the trial. In order to have a large, very active and uniform ruminal bacterial population, the cows were supplemented with 2 kg/head/day of a commercial concentrate with 14 % CP, from five days before the beginning of DMD run until finishing on day eight.

Commercial 10 cm x 20 cm N-free white polyester monofilament bags with 53 ± 10 µ pore size (Bar Diamond, Parma ID, USA) were used. Bags were numbered with indelible black ink marker, dried to constant weight and then between 4.9999 to 5.0001 g of dried forage sample weighed into each bag, which was closed tightly with a rubber band and lightly wetted with water at room temperature, before being introduced into a 24.0 cm x 38.5 cm net-fabric bag, that was suspended in the ventral sac of the rumen. To secure it in place and to reach it easily, it was held in with a 60-cm length, 1.25-cm diameter plastic cord attached to the net bag at one end and fixed to the cannulae with a wooden handle left in the outer side of the stopper at the other end.

The field work of the experiment took place from January 4 to 11, 2010. The cows were fed concentrate during the eight days the trial lasted, being the first five days the adaptation period. Degradation of leaves was made in a single run of 70 dacron bags per cow, that took place from January 9 to 11, 2010. Samples corresponding to 72 h were put into the rumen first, then those corresponding to 48 h, and so on. All bags were withdrawn at the same time and introduced in cold water (≈ 1 °C) to halt microbial enzymatic activity. The net bags with the polyester bags containing sample inside were rinsed several times in a commercial 3 kg-capacity washing machine, until water came out clean. Then, individual bags were hung out for 6 h to drip-drain excess water. After that, they were dried to constant weight at 62 °C. Dry matter degradation was calculated by difference: ((Initial DM - Final DM)/Initial DM)*100 and expressed as percent units.

Statistical analyses

Dry matter degradation data were fitted to the model $y = a + b(1 - e^{-ct})$, where: 'y', is the DM degraded at time 't', 'a' is the highly soluble DM when $t = 0$ (%), 'b' is the insoluble but slowly degradable DM (%), 'a + b' is the extent of degradation (%), 'c' is the degradation rate of b (%/h) and 't' is incubation time in rumen (h) (Ørskov and Mc Donald, 1979). The model was fitted by non linear regression with the software GraphPad Prism version 5.03 for Windows © (Prism 5, 2009).

The DM disappearance data were grouped according to incubation time in rumen (3, 6, 9, 12, 24, 48 and 72 h), forming seven groups, each with 30 values produced from three cows by 10 bags. The data per each incubation time was analyzed with the model: $Y_{i(j)} = \mu + V_j + B(V)_{i(j)}$, where: $Y_{i(j)}$ is the i^{th} bag incubated in the j^{th} cow, μ is the general mean, V_j is the variation due to the j^{th} cow, and $B(V)_{i(j)}$ is the variation attributable to the i^{th} bag incubated within the j^{th} cow, or residual variation. Following the approach of Mehrez and Ørskov (1977), variance components of cows and bags were used to calculate the variance of the mean per combination of number of cows (c) and number of bags (b), with the equation: $(V_b + (b \cdot V_c)) / (b \cdot c)$, where: V_b and V_c are the variances attributed to bags and cows, respectively. Variance components were estimated with PROC VARCOMP of SAS (SAS, 2010) using the default option MIVQUE0. The nested model was considered appropriate for the data, thus negative variance components, if present, were treated as if they were zero (SAS, 2010). Van der Koelen *et al.* (1992) used a variance value of 0.5 % as criterion to select an appropriate combination of number of cows and bags. We used the same approach, but instead of variance, the mean standard error calculated from all data (1 %), was visually compared against the SE obtained for the number of bags curves, to find what number of animals and number of bags combination would produce a SE with a value of 1 %.

RESULTS

Ruminal DMD of Kinggrass leaves followed the expected course (Figure 1). The model fitted the data well, with a R^2 value close to the unit and a standard error of estimate (Sy.t) value around 2 % *in situ* DMD.

The estimated variance components are presented in Table 1. The range in values for between cow variations went from -1.11 % at 9 h of incubation, to 5.79 % at 24 h of incubation, whilst that for between bags within cows were 3.87 % and 17.84 % at 3 h and 72 h of incubation. Variance components for between cows were smaller than those due to between bags.

Variance components increased with incubation time (Table 1).

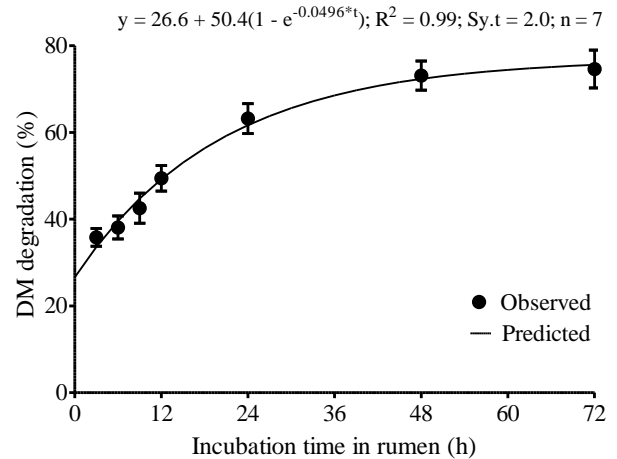


Figure 1. Dry matter degradation curve of Kinggrass (*Pennisetum purpureum*) leaves. Closed circles represent observed means and vertical bars represent standard deviations.

Table 1. Variance components for between cows and between bags of *in situ* dry matter disappearance of Kinggrass (*Pennisetum purpureum*) leaves.

Incubation time (h)	Variance components (%)	
	Between cows (V_c)	Between bags (V_b)
3	0.46	3.87
6	-0.62	7.43
9	-1.11	12.90
12	-0.71	9.13
24	5.79	7.93
48	-0.83	11.92
72	1.73	17.84

Standard errors of DMD, as a function of the number of bags when one, two or three cows were used, are shown in Figure 2. Precision increased as the number of cows increased, with mean SE of 1.66, 1.18 and 0.96 % for one, two and three cows, respectively. So, a larger gain in precision occurred when two or three cows were used instead of one, but less was gained by using three cows over two. As the number of bags increased, the difference in SE values for one, two or three cows became smaller. Nevertheless, the magnitude of such difference depended on time of incubation. Precision remained more or less constant for incubation times of 3, 6, 9, 12 and 48 h, with respective mean SE values of 0.93, 0.81, 1.05, 0.92 and 1.08 %, and doubled for 24 (2.15) and 72 (1.92) h.

DISCUSSION

In general, variance components found in this study were larger than those reported for sheep (Mehrez and Ørskov, 1977) and cows (Michelet-Doreau and Ould-Bah, 1992; van der Koelen *et al.*, 1992).

Even though the SE of the mean increased with incubation time, it did so slightly at 3 to 12 h and 48 h and strongly at 24 h and 72 h (Figure 2). This is in

disagreement with literature. Hopson *et al.* (1963) used five replications of duplicate bags per period of time in each of four sheep (n = 40), put all bags at the same time, and withdrew them at 6 h intervals from 6 to 42 h of incubation. They found that variation coefficients diminished exponentially with incubation time. Both, different ruminant species and opposite withdrawal of bags, might have produced a reverse order in variance values in comparison with our results.

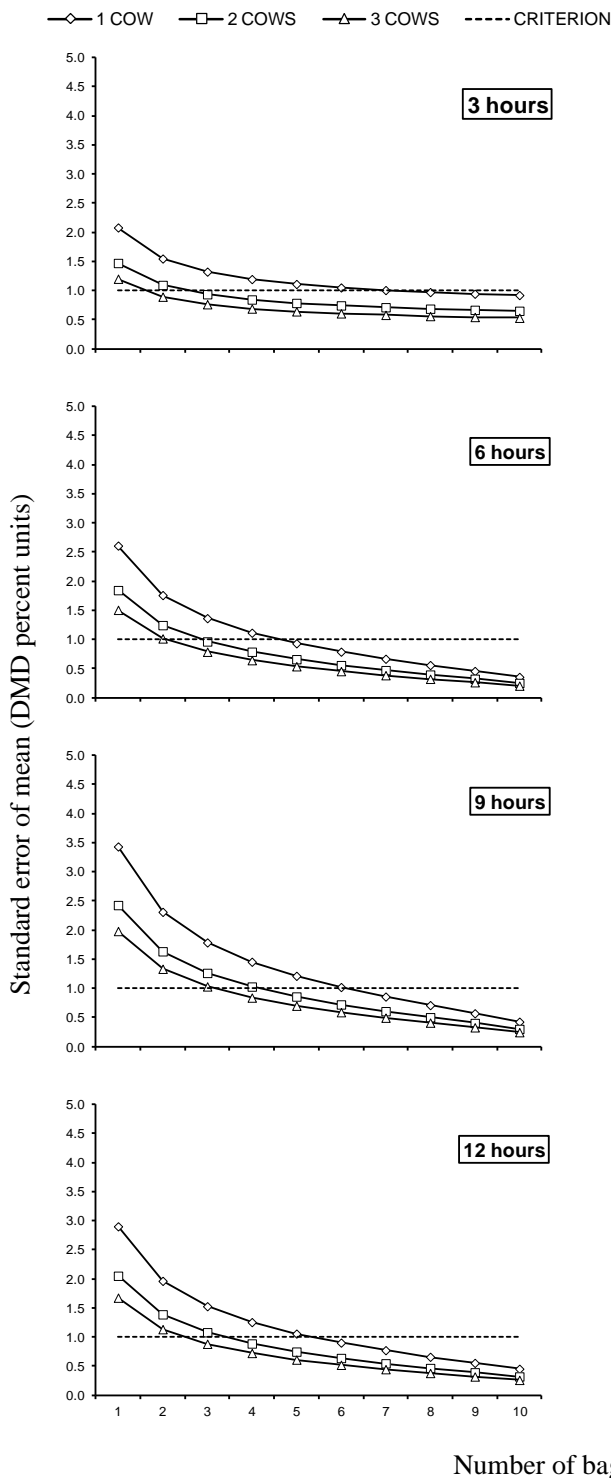


Figure 2. Standard error of mean dry matter degradation of kinggrass (*Pennisetum purpureum*) leaves, for different combinations of number of cows and number of bags incubated in the rumen per cow, according to incubation time. The horizontal dotted line is the 1.0% S.E. value used as criterion to select minimum number of bags for each number of cows.

Nocek (1988) gave general guidelines to standardize the *in situ* DMD procedure, but he gave no suggestions on the number of bags and cows to use. On the other hand, Vanzant *et al.* (1998) quoted values from various sources that ranged from two to four cows and one to eight bags per feed, and after a thorough review of the literature, also gave general guidelines for replications *i. e.* > 2 cows, > 1 bag and > 2 days. In the present case, it was clear that marginal increments in precision were larger when number of cows increased from one to two cows than from two to three cows, and therefore, the use of two over three cows is practical and less expensive. However, the present trial showed that variability due to bags was larger than that usually quoted in literature. Furthermore, SE trends depended on incubation time (Figure 2).

Table 2 shows the number of animals by bag replication combinations that produced a SE of 1 %. At incubation time of 3 h, such a SE would be obtained with seven, three and one bags when one, two or three cows were used, but at incubation times of 24 and 72 h many more than 10 bags would have been necessary to meet that criterion, mostly due to the much larger SEs produced by those times.

Table 2. Combination of number of cows and bags necessary to produce standard errors with values of 1.0 % or 2.0%, according to rumen incubation time of leaves.

Incubation time (h)	Number of cows		
	One	Two	Three
Number of bags to obtain SE = 1.0%			
3	7	3	1
6	5	3	2
9	6	4	3
12	5	3	1
24	>10	>10	>10
48	6	4	3
72	>10	>10	>10
Number of bags to obtain SE = 2.0%			
3	1	1	1
6	2	1	1
9	3	2	1
12	2	1	1
24	> 10	4	1
48	3	2	1
72	8	3	2

If the results at 24 and 72 h are left out, the average number of bags for one, two and three cows would be six, three and two, respectively. It is necessary to point out that in order to include 24 and 72 h results, a larger variability should be accepted. Therefore, if a mean SE of 2 % is used as criterion, the best combination for

the number of bags at incubation times of 3 to 48 h would be obtained with one bag only, using three cows. An ideal situation would be that these values would apply to all incubation times. In this particular case, the number of bags varied between one and four when two cows were used, and between one to more than 10 with a single cow (Table 2). Therefore, if a SE = 2 % is accepted, three bags would be a reasonable choice to run the procedure.

Practicality of the *in situ* technique must comprise precision and cost; the latter depends on the cost of cow maintenance and purchase of bags. As shown above, a similar precision can be reached with different combinations of number of animals and number of bags (van der Koelen *et al.*, 1992). The following cost comparison is given in American dollars. A fistulated cow costs \$ 1.27/cow/day during an *in situ* run, and commercial bags cost \$ 0.65 a piece. So, if three cows and two bags were used, total cost for cow maintenance would be \$ 1.27/cow * 3 cows * 8 days = \$ 30.48, and that of bags: 6 bags * \$ 0.65 = \$ 3.90 for a total of \$ 34.38. Using two cows and three bags per cow reduces the cost by 25.8 % to \$ 24.22. This small reduction is not trivial matter in developing countries always short in research funding. Therefore, our data indicate that the use of two cows and three bags per cow fits well within the general guidelines given by Vanzant *et al.* (1998).

CONCLUSION

In the case of leaf material of tropical grasses, the use of two cows and three bags per incubated sample is suggested. This result is similar to previous findings by researchers working with temperate grasses and feeds with European cattle, then, it would be fair to say that their recommendations are applicable to trials run under tropical conditions. Nevertheless, the present study also points out to the need for collaborative research aimed at studying the different sources of variation of the *in situ* technique under hot and humid conditions of the Mexican tropics, in order to have standardized procedures for the different laboratories that so far have been working with this useful technique.

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