EFFECT OF POLYETHYLENE GLYCOL SUPPLEMENTATION ON *IN VITRO* NITROGEN DIGESTIBILITY OF *LEUCAENA SHRUB LEGUM SPECIES* AND SIGNAL GRASS (*Brachiaria decumbens*)

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SUMMARY

An *in vitro* study was conducted to investigate the effect of polyethylene glycol (PEG) supplementation on *in vitro* nitrogen digestibility in shrub species legume and grass. The nitrogen digestibility of varied correspondingly to their nutrient and condensed tannin (CT) content. *Leuceana species* with a lower CT tended to have higher *in vitro* nitrogen digestibility (IVND) and rate of ammonia-N production. PEG consistently increased IVND and the rate of ammonia-N production. The increased values were from 30.7 to 71.2% and 52.3 to 93.6 mg/d for nitrogen digestibility and the rate of ammonia-N production respectively when the inclusion rate of PEG was elevated from 0 to 200 mg/g sample of forages. *Leucaena species* with a high CT content required more PEG to neutralize negative effect of tannins on nitrogen digestibility than did species with low tannin content. PEG, however, had no effect on the nitrogen digestibility of signal grass.

Keywords: In vitro, condensed tannin and PEG

PENGARUH PENAMBAHAN POLYETHYLENE GLYCOL TERHADAP KECERNAAN NITROGEN PADA *SEMAK LEGUM LAMTORO SPECIES* DAN RUMPUT *SIGNAL*

RINGKASAN

Penelitian secara *in vitro* telah dilakukan untuk melihat pengaruh penambahan polyethylene glycol **(**PEG) terhadap kecernaan nitrogen pada legum dan rumput. Hasil menunjukkan bahwa kecernaan bervariasi antara spesies dan tergantung pada kandungan tannin pekat (CT). *Lamtoro spesies* yang rendah kandungan CTnya cenderung mempunyai kecernaan nitrogen dan produksi N ammoniak yang tinggi. PEG meningkatkan kecernaan nitrogen dan produksi N ammoniak pada legume *Lamtoro spesies* dan tidak ada pengaruhnya terhadap rumput. Kecernaan nitrogen meningkat dari 30,7% menjadi 71,2% dan produksi N ammoniak meningkat dari 5,3 mg/hari menjadi 93,6 mg/hari, apabila penambahan PEG mencapai 200 mg/sampel hijauan. *Lamtoro spesies* yang mengandung CT yang tinggi membutuhkan PEG yang lebih banyak untuk menetralisir pengaruh tannin pada level yang sama ketimbang *Lamtoro spesies* yang mempunyai kandungan CT lebih rendah. Akan tetapi, PEG tidak berpengaruh terhadap kecernaan nitrogen pada rumput *signal.*

Kata kunci: In vitro, kandungan tannin dan PEG

Introduction

Over 26 of Leucaena species studied for which *Leucaena colinsii* has the lowest content of condensed tannin (CT) and *Leucaena pallida* has the highest CT content (Dalzell *et al*., 1998). The condensed tannin has been reported to strongly bind protein, and other component of the diet, rendering them are not available for digestion and absorption, which in turn affecting animals performance (Mangan, 1988).

Polyethylene glycol (PEG) is reported to neutralize CT in *in vitro* studies, which increase the potential use of tannin containing-diet to the livestock as CT interact more strongly with PEG than they do with protein (Mangan, 1988). Recently, Palmer and Jones (2000) found that PEG improved the *in vitro* digestibility of nitrogen in *Calliandra* and most other legumes containing tannins. The objective of the present study was to investigate the effect of the level of PEG on the *in vitro* nitrogen digestibility of a wide range of *Leucaena* species and a representative of signal grass (*Brachiaria decumbens*) using the two stages of digestion technique of Tilley and Terry (1963) and *in vitro* technique using PEG described by Jones *et al*. (1998).

Materials and methods

Plant samples

Actively growing *shrub legume Leucaena species* (*Leucaena pallida* K748, *Leucaena leucocephala* cv. Tarramba K636 and KX2 F1 hybrid of *Leucaena pallida* and *Leucaena leucocephala*) and signal grass (*Bracharia decumbens)* were used as plant sources. Leaf and edible stem materials were collected from these legumes and the grass, and immediately frozen with dry-ice in an insulated container. These samples were kept frozen until freeze drying, and then ground to pass a 1 mm screen. All

samples were analyzed for dry matter (DM), organic matter (OM) and nitrogen (N) before and after incubation.

Rumen fluid-buffer mixture

A mixture of rumen fluid and buffer was used as inoculant for incubation. Rumen fluid was collected from permanently rumen fistulated cattle that had been grazing signal grass pastures for ten days. Rumen fluid samples were filtered through two layers of nylon cloth to eliminate coarse particles and the strained rumen fluid was stored in a pre-warmed insulated flask and transferred to the laboratory for further processing. The buffer solution used was based on that described by McDougall (1948). A mixture of rumen fluid -buffer was prepared by mixing one part of rumen fluid with four parts of buffer at 39^{o}C.

Procedures

A half g of plant samples (*Leucaena pallida*, *Leucaena leucocephala* and KX2) and grass were accurately weighed and placed into the 50mL tubes in four replicates within treatments. The rates of PEG application were 0, 50, 100, 150, 200 and 250 mg/g of sample. PEG was dissolved in the water (1.0 mL) and added to 40 mL of rumen fluid-buffer mixture. Five tubes containing the rumen-fluid/buffer mixture only were also included to each batch as a control (no substrate). The tubes were then incubated at 39^{o}C for 72 hours. After incubation of 72 h (stage1), tubes were centrifuged for 10 minute at 2500 g, 5-mL of supernatants were collected and transferred to the 10 mL tube containing 5 mL of 0.2 M HCl for the rate production of ammonia-N analysis. The remaining supernatant was discarded. The residues were washed twice with 10 mL water, centrifuged for 10 min, each time discarding the supernatants. Then, 40 ml of acid pepsin was added to each tube (2 g pepsin in 1 L of 0.1M HCl), and after thorough

vortex mixing, was incubated at 39^{o}C for 24 h (stage 2). Following this procedure, the tubes were centrifuged, supernatants were discarded and residues washed with 10 mL of water, vortexed and then centrifuged. This washing procedure was further repeated twice to totally remove acid pepsin. After washing, the residues were dried at 80^{o}C for 48 h and weighed for dry matter. Then residues were also analyzed for nitrogen, organic matter and neutral detergent fibre (NDF).

Chemical analysis

Dry matter (DM) was calculated as the residue remaining after the samples were dried at 65^{o}C for 48 h, and organic matter (OM) as the loss of sample DM weight after incineration at 550^{o}C for 5 h. The N content of the samples was determined by using a Leco CNS-2000 Combustion Analyzer (Leco Corporation, USA). The rate of ammonia-N production after the first stage of incubation was also analyzed by using the steam distillation and titration method. Neutral detergent fibre (NDF) determinations were based on the method of Van Soest (Van Soest and Wine, 1967), by using the Filter Bag Technique (FBT) in an ANKOM^{220} fibre analyzer (ANKOM Technology Corporation, New York, USA). Separation of CT into free, protein bound and fibre bound CT was done as described by Perez-Maldonado (1994) and quantified with Butanol-HCl by the method of Dalzell and Kerven (1998).

Statistical analysis

Data collected for *in vitro* digestibility of nitrogen and the rate of ammonia-N production were analyzed using analysis of variance to test for the effect of treatments by using GLM of SAS (SAS, 1998). The model used was 4 (forage types) by 6 (levels of PEG applied) factorial design with 4 replicates per plot. The extent of the digestibility was regressed on the level of PEG. The further analysis for the mean values used LSD for the comparison between treatments.

Results

Chemical composition of the samples

The chemical composition of the freeze-dried samples from the Leucaena species and grass is presented in the Table 1. The nitrogen content of the Leucaena forages varied from 29.9 to 34.2 g/kg DM. The total condensed tannin of the Leucaena species ranged from 9.8 to 23.8% DM, of which 92% was present as free tannin. However, CTs were not detected in signal grass. The neutral detergent fibre (NDF) content of signal grass was higher than that for Leucaena species.

Table 1. Chemical composition (g/kg DM) of selected edible fractions of Leucaena species (*L. pallida*, KX2 and *L. leucocephala*) and signal grass (*Bracharia decumbens*)

Component	*L. pallida*	KX2	*L. leucocephala*	Signal grass
OM	945.0	935.7	945.2	896.3
NDF	243.5	278.8	173.1	586.5
N	29.9	32.5	34.2	5.9
CT:				
Free	225.7	169.7	90.1	ND*
Fibre bound	3.7	2.9	2.0	ND
Protein bound	8.2	7.8	5.6	ND
Total	237.6	180.3	97.8	ND

*) ND not detected

Effect of PEG on digestibility and the rate of ammonia-N production

The addition of PEG to the incubation medium significantly ($P<0.0001$) affected the *in vitro* nitrogen digestibility of the legumes studied. The mean values of IVND and rate of ammonia-N production are given in Table 2. IVND all legumes samples increased as PEG levels increased ($P<0.0001$) from 0 to 100 mg/g of the sample, with no further significant changes ($P>0.05$) there after (inclusion rates 150-250 mg/g).

However, inclusion of PEG up to 250 mg/g of sample steadily elevated the values of ammonia-N production very highly significant ($P<0.001$).

Table 2. Mean values for the main effects of increasing levels of PEG on the *in vitro* nitrogen digestibility (IVND) and rate of ammonia-N production from the incubation of different forage types.

PEG mg/g sample	IVND %	Ammonia-N mg/d
0	30.7^{A}	52.3^{A}
50	53.8^{B}	78.2^{B}
100	67.0^{C}	89.1^{C}
150	68.0^{D}	91.3^{CD}
200	71.2^{E}	93.3^{D}
250	69.5^{F}	93.6^{D}
SEM	0.24	0.97

Values within the columns followed by different superscript are significantly different ($P<0.001$).

Effect of forage types on digestibility and the rate of ammonia-N production

Forage and todder types had a significant effect on the mean values of IVND and rate of ammonia-N production ($P<0.001$), which is shown in Table 3. The grass forage resulted in the lowest value of IVND and the shrub legume fodder had the highest value of IVND ($P<0.0001$). The *in vitro* digestibility of KX2 resulted in intermediate values ($P<0.0001$), but had the highest value of ammonia-N production ($P<0.0001$).

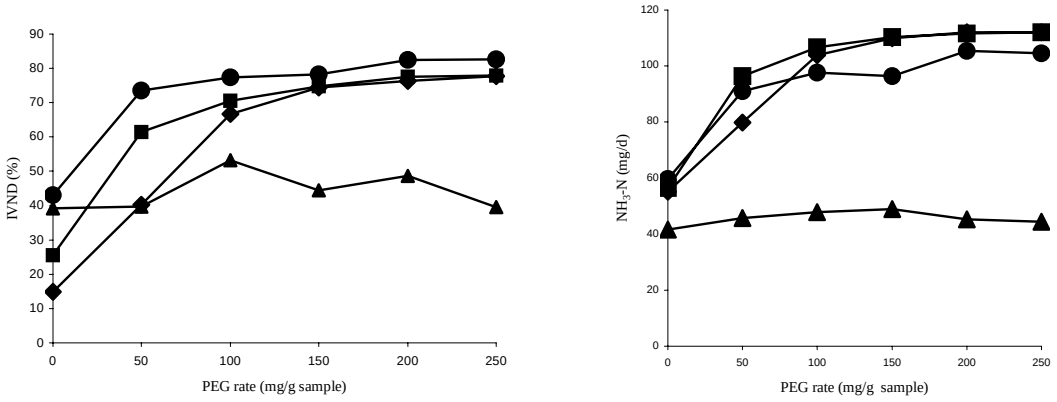
Table 3. Mean values for the main effects of forage types on the *in vitro* nitrogen digestibility (IVND) and rate of ammonia-N production from the incubation of forage types at a common level of polyethylene glycol (PEG) inclusion.

Forages	IVND	Ammonia-N
	%	mg/d
L. pallida	58.4^{A}	95.5^{A}
KX2	64.6^{B}	98.9^{B}
L. leucocephala	72.9^{C}	92.4^{C}
Signal grass	44.2^{D}	45.7^{D}
SEM	0.19	0.80

Values within the column followed by different superscript are significantly different (P<0.001).

Polyethylene glycol (PEG) and forage types interaction on the *in vitro* nitrogen digestibility and the rate of ammonia-N production

An analysis of variance showed that there were significant effects (P<0.001) of forage type, level of PEG applied and the interaction between these two factors. Mean values for IVND and rate of ammonia-N production of the individual forages and their response to the inclusion of PEG are illustrated in Figure 1. The *in vitro* digestibility of tannin-containing forages responded to the inclusion of PEG (P<0.0001), in which digestibility increased as the inclusion rate of PEG increased.



(A) **(B)**

Figure 1. The interaction between inclusion rate of polyethylene glycol (PEG) and types of forages on IVND (A) and rate of ammonia-N production (B). The type fodder include *Leucaena pallida* (◆), KX2 (■), *Leucaena leucocephala* (●) and signal grass (▲)

Discussion

Increasing levels of PEG steadily increased the IVND of forages. The increase of nitrogen digestion was accompanied by an increase in the rate of ammonia-N production, supporting the view of Palmer and Jones (2000) that this technique (*in vitro* N digestibility) provided a better evaluation of the effects of tannins on the nutritive value of tannin-containing feeds and forages. The significant correlation between the level of PEG and digestibility of N and rate of ammonia-N production in tannin-containing forage indicated that the presence of tannins depressed the digestibility of nitrogen and further reduced the ammonia level in the rumen. The linear interrelationship between the level of PEG and improvement of digestibility supports the concept that PEG may replace protein in pre-existing tannin-protein complexes, releasing proteins for further digestion (Mangan, 1988).

The inclusion rates of PEG in the present study were lower (0-250 mg PEG/g sample) than those used by Makkar *et al*. (1995)(2 g PEG/g sample) or Palmer and Jones (2000)(0-1000 mg PEG/g sample), but the levels used here were still higher than the recommended optimum level of PEG (160 mg/g sample) for binding *Leuceana* tannins (Palmer and Jones, 2000). None of these authors actually measured the tannin content of their samples, and the large differences in apparent PEG requirements for tannin neutralisation may simply be related to differences in the tannin contents of the materials assayed. It is therefore not possible to directly compare the efficacy of PEG in the present experiment with their results. In the present case, at the "optimum" level of PEG applied (to achieve about 77% of N digestibility), 1.06, 1.05 and 1.02 mg PEG/mg tannin were required to approximately neutralize tannins from *L. pallida*, KX2 and *L. leucocephala* respectively.

In general, *L. leucocephala* produced the highest value of *in vitro* digestibility of nitrogen, followed by KX2 and *L. pallida*. The superiority of *L. leucocephala* in nitrogen digestibility indicates that *L. leucocephala* would have potentially high nutritive values for animals, with *L. pallida* having the lowest nutritive value, with KX2 being intermediate. This is similar to finding in other *in vitro* studies of plant materials (Jones *et al*., 2000; Jones and Palmer, 2000). Interestingly, the highest value of nitrogen digestibility of *L. leucocephala* did not produce a higher rate of ammonia-N production, suggesting that significant amounts of ammonia N were being incorporated into microbial cells during the period of incubation. However without direct measurements of microbial protein synthesis this explanation must remain speculative.

The superiority of *L. leucocephala* as compared to the others legumes in terms of IVND is likely related to its low tannin levels, particularly in the free form (Dalzell *et al*., 1998). The form in which the tannins exist in the plant material are an important determinant of the extent to which protein digestibility and microbial protein synthesis are affected by plant tannin content. For example, high levels of free tannins are most likely to directly affect protein (nitrogen) digestibility, because free tannins can readily bind to soluble proteins rendering them indigestible (Fondevila *et al*., 2002). The consequences of complexes between tannin and protein (protein bound) or carbohydrate (fibre bound) and decreased digestibility, the microbial population is denied access to essential amino acids and decreased N availability which may lead to restricted growth and depressed fermentative activity (Longland *et al*., 1995). Therefore, the *in vitro* digestibility of *L. leucocephala* in the current study was significantly higher than that of *L. pallida* or KX2. Nevertheless, the level of tannins in a feed alone cannot be used to determine the value of a legume as a protein supplement since McSweeney *et al*. (1999) found a poor correlation between total tannin content and digestibility of dry matter and

nitrogen. Factors such as reactivity, structure, molecular weight and interactions of different secondary compounds in the plant are also important (Barry *et al*., 1986; Waghorn *et al*., 1994). For instance, studies by Kaitho *et al*. (1998) showed that the rumen degradability of protein was 22.9 and 37.7% for *L. pallida* and *L. leucocephala* respectively and the intestinal digestibility of *Leucaena* proteins was low, 45.2 and 46.0% for *L. pallida* and *L. leucocephala* respectively, even though the total soluble tannin of *L. leucocephala* was higher than that of *L. pallida* (Kaitho *et al*., 1998; Garcia *et al*., 1996). The latter effect could be linked with the ability of tannins to bind with feed protein and enzymes, thus reducing their digestibility. Furthermore, the chemical composition of the forages seems to have an effect on the extent of digestibility, where high N content tends to induce greater digestibility (Dalzell *et al*., 1998). The N content of the legumes varied where *L. leucocephala* had the highest value, followed by KX2 and *L. pallida*, leading to high value on its nutrients digestibility. On the other hand, the potential nitrogen digestibility of signal grass was lower as compared to the *Leucaena* species. This is likely due to its low N content and to the high N content associated with NDF (586.5 g/kg DM; Table 1), allowing lower availability of N to the rumen microbes in the signal grass as a consequence of lower N digestibility and thus the rate production of ammonia-N. Mupangwa *et al.* (2003) reported that the variation in protein degradation of tropical legumes was due to the difference in their N content, with a lower N content producing a lower value of protein degradation. Therefore, by considering the chemical composition and values of *in vitro* digestibility, the potential protein sources of legume forage for animal feed could be ranked as follows: *L. leucocephala*>KX2>*L. pallida.*

The digestibility of nitrogen of the Leucaena species studied improved as the level of PEG addition was increased. A similar trend was also recorded for the rate

production of ammonia-N. These observations suggest that the CT of *Leucaena* caused a significant depression on the digestion of nitrogen, diminishing its value as a feed for animals consuming such legumes. However, the extent of improvement in *in vitro* nitrogen digestibility at the same rate of PEG application varied among the legumes, with some legumes requiring more or less PEG to counteract the effects of tannins. For instance, additional PEG at the rate of 50 mg/g sample resulted in 73.6% IVND for *L. leucocephala* compared with 40.3% of IVND for *L. pallida*. If these results were to be translated into practical recommendations, then animals consuming different tannin containing leguminous feeds will require different levels of PEG supplementation to overcome the varying negative effects of the different tannins content in each species.

Conclusions

The *in vitro* nitrogen digestibility of Leucaena species varied according to their nutrient and CT content. *Leuceana* with a lower CT tended to have higher IVND and ammonia-N production and inclusion of PEG increased IVND and ammonia-N production. PEG, however, did not have any effect on *in vitro* nitrogen digestibility of signal grass. There was an interaction between the level of PEG supplementation and *Leucaena* species; *Leucaena species* with a high CT content required more PEG to neutralize tannins than did species with a low tannin content.

References

Barry, T.N., Manley, T.R. and Duncan, S.J. (1986). The role of condensed tannins in the nutritional value of Lotus pedunculatus for sheep. 4. Sites of carbohydrate and protein digestion as influenced by dietary reactive tannin content. British Journal of Nutrition 55:123-137.

Dalzell, S. and Kerven, G.L. (1998). A rapid method for the measurement of Leucaena spp. Proanthocyanidins by the proanthocyanidin (Butanol/HCl) assay. Journal of the Science of Food and Agriculture 78:405-416

Dalzell, S.A., Stewart, J.L., Tolera, A. and McNeill, D.M. (1998). Chemical composition of Leucaena and implication for forage quality. In: H.M. Shelton, R.C. Gutteridge, B.F. Mullen and R.A. Bray (eds). Leucaena-Adaptation, quality and farming systems.Vol. 86 ACIAR. Canberra,pp227-246.

Fondevila, M., Nogueira-Filho, J.C.M. and Barrios-Urdaneta, A. (2002). *In vitro* microbial fermentation and protein utilization of tropical forage legumes grown during dry season. *Animal Feed Science and Technology* 95:1-14.

Garcia, G.W., Ferguson, T.V., Neckles, F.A. and Archibald, K.A.E. (1996). The nutritive value and forage productivity of *Leucaena leucocephala*. *Animal Feed Science and Technology* **60**:29-41.

Jones, R.J. and Palmer, B. (2000). In vitro digestion studies using 14C-labelled polyethylene glycol (PEG) 4000: comparison of six tanniferous shrub legumes and the grass *Panicum maximum*. Animal Feed Science and Technology 85:215-221

Jones, R.J., Meyer, J.H.F., Bechaz, M. and Stoltz, M.A. (2000). An approach to screening potential pasture species for condensed tannin activity. Animal Feed Science and Technology 85:265-277

Jones, R.J., Stoltz, M.A., Meyer, J.H.F., Bechez, F.M. (1998). The effect of rumen fluid storage time on the digestive capacity with five forage browse species. *Tropical Grassland* **32**:270-272

Kaitho, R.J., Umunna, N.N., Nsahalai, I.V., Tamminga, S. and van Bruchem, J. (1998). Nitrogen in browse species. Ruminal degradability and post-ruminal digestibility measured by mobile nylon bag and in vitro technique. Journal of the Science of Food and Agriculture 76:488-498

Longland, A.C., Theodorou, M.K., Sanderson, R., Lister, S.J., Powell, C.J. and Morris, P. (1995). Non-starch polysaccharide composition and *in vitro* fermentability of tropical forage legumes varying in phenolic content. *Animal Feed Science and Technology* **52**:161-177

Makkar, H.P.S., Blummel, M. and Becker, K. (1995). Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in *in vitro* technique. *British Journal of Nutrition* **73**:897-913

Mangan, J.L. (1988). Nutritional effects of tannins in animal feeds. Nutrition Research Review 1:209-231.

McDougall, E.I. (1948). Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochemistry Journal 43:99-109.

McSweeney, C.S., Palmer, B., Bunch, R. and Krause, D.O. (1999). *In vitro* quality assessment of tannin-containing tropical legumes: protein and fibre digestion. *Animal Feed Science and Technology* **82**:227-241

Mupangwa, J.F., Ngongoni, N.T., Topps, J.H., Acamovic, T. and Hamudikuwanda, H. (2003). Rumen degradability and post-ruminal digestion of dry matter, nitrogen and amino acids in three tropical legumes estimated by the mobile nylon bag technique. *Livestock Production Science* **79**:37-46

Palmer, B. and Jones, R.J. (2000). The effect of PEG addition in vitro on dry matter and nitrogen digestibility of Calliandra calothyrsus and Leucaena leucocephala leaf. Animal Feed Science and Technology 85:259-268.

Perez-Maldonado, R.A. (1994). The chemical nature and biological activity of tannins in forage legumes fed to sheep and goats. PhD Thesis. The University of Queensland. Department of Agriculture. Brisbane, Australia

SAS (Statistical Analytical System) (1998). Guide for personal computers, version 7. SAS Institute Incorporation North Caroline, USA.

Tilley, J.M.A. and Terry, R.A. (1963). A two-stage technique for the *in vitro* digestion of forage crops. *Journal of British Grassland Society* **18**:104-111

Van Soest, P.J. and Wine, R.H. (1967). Use of detergents in the analysis of fibrous feed. IV. Determination of the plant cell wall constituents. *Journal of the Association of Official Analytical Chemists* **50**:50-55

Waghorn, G.C., Shelton, I.D., McNabb, W.C. and McCutcheon, S.N. (1994). Effects of condensed tannins in Lotus pendunculatus on its nutritive value for sheep. 2. Nitrogenous aspects. Journal of Agricultural Science (Cambridge) 123:109-119