Effects of Polyethylene Glycol (PEG) on *In Vitro* Dry Matter and Nitrogen Digestibility of *Leucaena* Species and Signal Grass (*Brachiaria decumbens*)

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ABSTRACT

The tropical legume *Leucaena* contains condensed tannin (CT) that binds protein and other components of feed. In fact, *Leucaena* reduces digestibility of nutrients and depending on tannin content and astringency. Polyethylene glycol (PEG) has ability to neutralize CT by displacing protein-tannin complexes, as a consequence of CTs interact more strongly with PEG than they do with protein. *In vitro* studies were conducted to investigate the effect of polyethylene glycol (PEG) on *in vitro* digestibility of *Leucaena* species and grass. *In vitro* studies were conducted in two stages as described by Tilley and Terry (1963) and Jones *et al*. (1998).The results indicated that the digestibility vary depending on the nutrients content of forages and its CT content. Low tannin content of forages had a high digestibility of dry matter and nitrogen, and the rate of ammonia-N production. PEG consistently improved the digestibility of nitrogen and to some extent of dry matter, and the rate of ammonia-N production of tannin-containing forages but not non-tannin-containing of grass. The maximum values were 60.6%, 71.2% and 93.6 mg/d for corrected dry matter digestibility, nitrogen digestibility and the rate of ammonia-N production respectively, when PEG was included at rate of 200 mg/g samples of forages. High tannin content of forages required more PEG to neutralize the effect of tannin to the same extent of low tannin content forages. PEG, on the other hand, had no effect on digestibility and the rate of ammonia-N production on non tannin-containing grass.

Key words: in vitro, digestibility, condensed tannin, polyethylene glycol

INTRODUCTION

The tropical legume *Leucaena* contains condensed tannin (CT) concentrations which vary widely with species. Of the 26 species which have been studied, *Leucaena collinsii* has the lowest CT content while *Leucaena pallida* has the highest (Dalzell *et al*., 1998; McNeill *et al*., 1998). Tannins are known to affect the availability of nutrients by formation of soluble and insoluble complexes and their effects on the digestibility of nutrients will vary depending on tannin content and astringency (McNeill *et al*., 1998). *In vitro* studies by Makkar *et al*. (1995) have shown that CT influenced nutrient digestibility, to a great extent as measured by reduced gas production (fermentative activity). These researchers also noted that even at the same levels in feed, different tannins had different degrees of effect.

The use of polyethylene glycol (PEG) to neutralize CT has proved useful in further elucidating the specific nutritional consequences of dietary CT as PEG displaces protein-tannin complexes, as a consequence of CTs interact more strongly with PEG than they do with protein (Mangan, 1988). Palmer and Jones (2000) have shown 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* digestibility of a wide range of *Leucaena* species and a representative grass (*Brachiaria decumbens*) using the two stages digestion technique of Tilley and Terry (1963) and *in vitro* technique using PEG described by Jones *et al*. (1998b).

MATERIALS AND METHODS

Actively growing *Leucaena* species (*Leucaena pallida* K748, *Leucaena leucocephala* cv. Tarramba K636 and KX2 F1 hybrid of *Leucaena pallida* and *Leucaena leucocephala*) and signal grass (*Brachiaria decumbens)* were used as plant sources. Leaf and edible stem

materials were collected from these plants, and immediately frozen with dry-ice in an insulated container. These samples were kept frozen until freeze drying. A mixture of rumen fluid and buffer (1: 3 v/v) was used as an inoculant for incubation. Rumen fluid was collected from permanently rumen fistulated cattle that had been grazing signal grass pastures for ten days. The buffer solution used was based on that described by McDougall (1948). *In vitro* studies were conducted in two stages as described by Tilley and Terry (1963) and Jones *et al*. (1998). The rate of PEG application was 0, 50, 100, 150, 200 and 250 mg/g of sample and the samples size were 5 gram. The *in vitro* digestibility of dry matter (IVDMD) and *in vitro* digestibility of nitrogen (IVND) were estimated by subtracting the DM and N content of the samples before and after incubation, while the rate of ammonia-N production was estimated by the distillation method. The neutral detergent fiber (NDF) fraction of residues was also determined. It is assumed that the NDF differences from one level to the next level of PEG is due to the complexation of PEG and tannin that remained as insoluble fractions to contribute an additional DM fraction of residues after incubation. Subtracting the percentage of increase in NDF on IVDMD values will give a corrected value of IVDMD (CIVDMD).

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 fiber (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} fiber analyzer (ANKOM Technology Corporation, New York, USA). Separation of CT into free, protein bound and fiber bound CT was done as described by Perez-Maldonado (1994). CT was estimated with Butanol-HCl by the method of Dalzell and Kerven (1998).

Statistical Analysis

Data collected for *in vitro* digestibility 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) x 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 AND DISCUSSION

It is clear that the nutritive values of forages differed within the species of legumes and grass. Legumes had a high nitrogen and low fiber content (NDF) and were generally of higher nutritive value than signal grass (Table 1). The nitrogen content of tropical legumes, however, is not the only determinant of their nutritive value. Protein degradability in the rumen and digestibility of by-pass proteins in the small intestine are also important and are related to the tannin type and content in the plant material (Kaitho *et al*., 1998). The tannin content of legumes studied in the current experiment was higher than that suggested by Barry *et al*. (1986) as optimal for ruminants (30-40 g/kg DM).

Effect of Treatments on Dry Matter Digestibility

The reaction between PEG and tannins in the leaf samples reduces the overall *in vitro* digestibility of DM (Table 2), because tannin and PEG form indigestible complexes that cannot be degraded by rumen microorganisms. It is possible that such complexes are also not soluble in the acid-pepsin (Makkar *et al*., 1995). Hence, the inclusion of PEG in incubation mixtures containing tanniniferous forages is likely to result in an underestimation of *in vitro* DMD (McSweeney *et al*., 1999; Palmer and Jones, 2000).

Palmer and Jones (2000) suggested that the amount of PEG have been bound by tanniniferous forage has to be corrected to get the real value of *in vitro* DMD. The present study also found that the NDF content of the residues was increased (Figure 1) when PEG was included in the incubation. *L. pallida* samples resulted in the greatest increase in NDF with increasing PEG and this was presumably due to the fact that *L.*

pallida forage had the highest tannin content (Table 1). Such evidence agrees with the previous studies (Makkar *et al*., 1995; McSweeney *et al*., 1999). Makkar *et al*. (1995) reported that the apparent and true digestibility of tanniniferous feeds was slightly lower when PEG was included, as tannin-PEG complexation increased "apparent" NDF content of the residue.

The present study found a similar trend to that observed by others (Makkar *et al*., 1995; Palmer and Jones, 2000), that is, there are only small to negligible effects of PEG on the DMD of high tannin forages *e.g Pallida*. This result differs to other research which found that the inclusion of PEG or PVP increased *in vitro* gas production of tannin-containing feeds (Makkar *et al*., 1995; Khazaal *et al*., 1996; Getachew *et al*., 2002). However, the true effects of PEG on digestibility in the present study were masked by the formation of insoluble PEG-tannin complexes which are recorded as indigestible NDF residues (Figure 1). When DM digestibilities were corrected for these experimental artifacts, it was found, as shown by others, that the addition of PEG did have a significant and real effect on DM digestibility (Figure 2 B). In the absence of PEG, each forage species differed significantly in *in vitro* digestibility of DM, with signal grass having the highest value, and L. *pallida*, KX2 or *L. leucocephala* having the lowest values (Table 3). This observation may be explained by the different levels of tannin in the forages. Forages with high in tannin content is of lower digestibility, supporting the convention that the digestibility of organic matter, protein and cell walls is inversely related to tannin concentrations in the plant material (Silanikove *et al*., 2001).

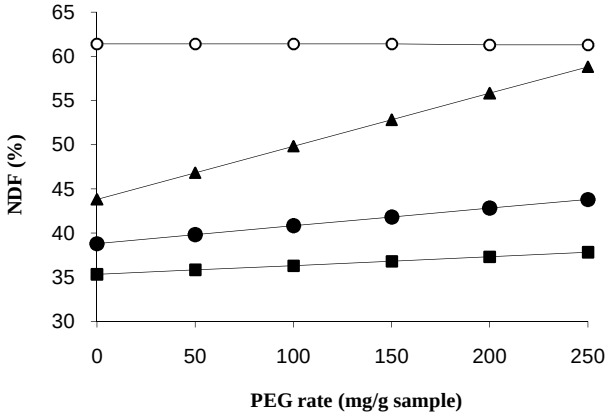

Figure 1. Relationship between neutral detergent fiber (NDF) fraction in the residues of either *L. pallida* (▲), KX2 (●), *L. leucocephala* (■) or signal grass (○) and inclusion rate of polyethylene glycol (PEG) in the mixtures

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*)

	L. pallid	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*
Fiber 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.

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

PEG mg/g sample	IVDMD %	CIVDMD %	IVND %	Ammonia-Nmg/d
0	49.3^{A}	49.3^{A}	30.7^{A}	52.3^{A}
50	52.8^{B}	53.9^{B}	53.8^{B}	78.2^{B}
100	54.9^{C}	57.2^{C}	67.0^{C}	89.1^{C}
150	55.7^{D}	59.1^{D}	68.0^{D}	91.3^{CD}
200	56.1^{D}	60.6^{E}	71.2^{E}	93.3^{D}
250	56.1^{D}	61.7^{F}	69.5^{F}	93.6^{D}
SEM	0.18	0.18	0.24	0.97

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

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

Forages	IVDMD (%)	CIVDMD (%)	IVND (%)	Ammonia-N (mg/d)
L. pallid	41.0^{A}	48.5^{A}	58.4^{A}	95.5^{A}
KX2	51.8^{B}	54.3^{B}	64.6^{B}	98.9^{B}
L. leucocephala	60.0^{C}	61.2^{C}	72.9^{C}	92.4^{C}
Signal grass	63.9^{D}	63.8^{D}	44.2^{D}	45.7^{D}
SEM	0.15	0.15	0.19	0.80

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

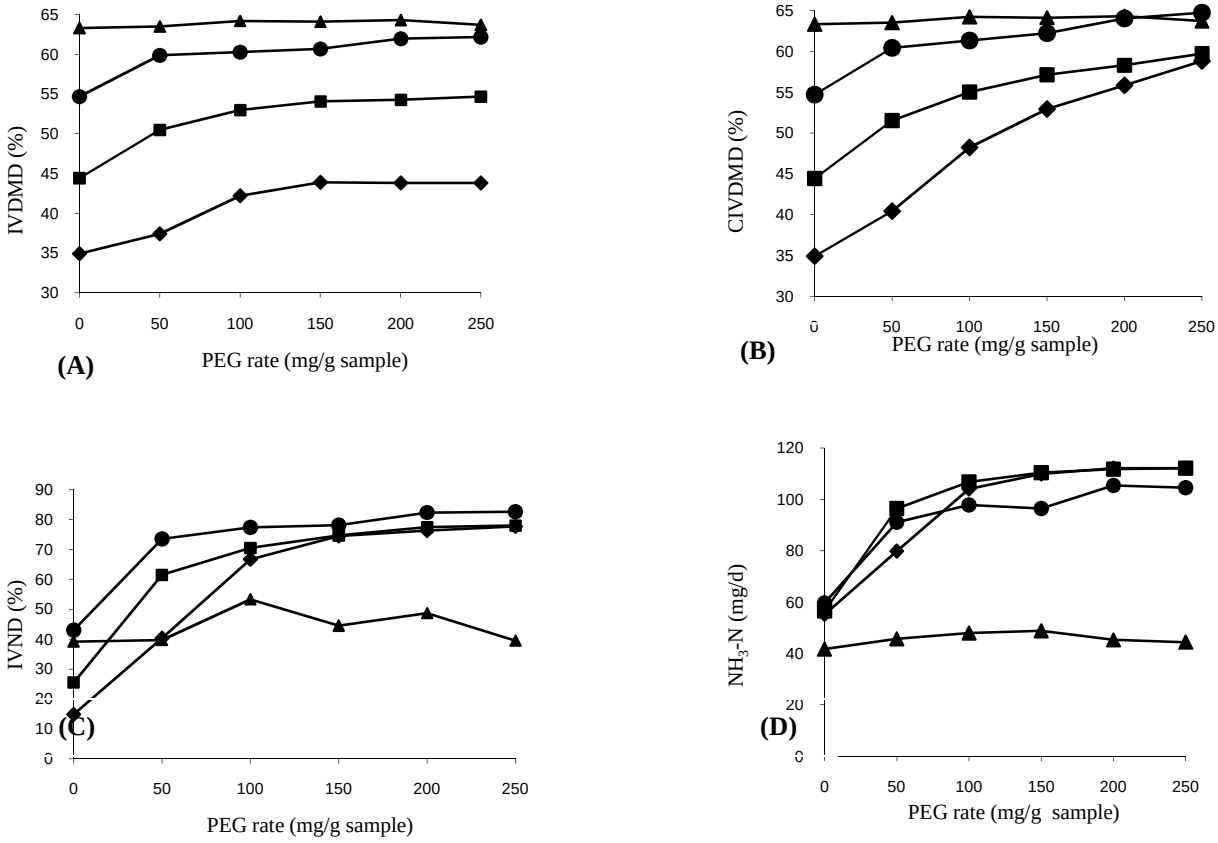

Figure 2. The interaction between inclusion rate of polyethylene glycol (PEG) and types of forages on IVDMD (A), CIVDMD (B), IVND (C) and rate of ammonia-N production (D). The type forages include *Leucaena pallida* (◆), KX2 (■), *Leucaena leucocephala* (●) and signal grass (▲)

Interestingly, at the same level of PEG in the solution, each forages responded differently to PEG effect on IVDMD. The high tannin forages appeared to require more PEG to reduce the negative effects to the same extent as that in low-tannin forages. Signal grass, however, did not show any response in the DMD to the level of PEG applied (Figure 2A, B, C, D) and is consistent with the previous result from *in vitro* studies with non-tanniniferous forages (Makkar *et al*., 1995; Jones and Palmer, 2000). Similarly, PEG had no significant effects on either the *in situ* degradability (Silanikove *et al*., 1996a) or *in vivo* digestibility of wheat straw (Silanikove *et al*., 1996b). There was a small to negligible improvement in DMD from *L. pallida* when PEG concentrations were increased in the solution. This agrees with the previous studies (Jones *et al*., 2000; Jones and Palmer, 2000).

The reduction of DMD is simply because the high CT tannin of *L. pallida* formed insoluble tannin-PEG complexes which were increased as the level of PEG increased. These complexes remained in the solution even after digestion in acid-pepsin and contributed to the indigestible components of dry matter.

Effects of Treatments on Nitrogen Digestibility and Rate of Ammonia-N Production

Increasing levels of PEG steadily increased the IVND of forages (Table 2). 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* (Table 3). 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. Interestingly, the highest value of nitrogen digestibility of *L. leucocephala* did not produce a higher rate of ammonia-N production (Table 3), 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 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 (fiber 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 (Table 3). 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 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.

The digestibility of nitrogen of the *Leucaena* species studied improved as the level of PEG addition was increased (Table 2). 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* 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 in each species.

CONCLUSIONS

The *in vitro* digestibility of *Leucaena* species varied according to their nutrient and CT content. *Leuceana* with a lower CT tended to have higher IVDMD, CIVDMD and IVND. Inclusion of PEG increased IVND and to some extent the IVDMD or CIVDMD. PEG, however, did not have any effect on the dry matter digestibility of signal grass. There was an interaction between the level of PEG and *Leucaena* species; *Leucaena species* with a high CT content required more PEG to neutralize tannins than did species with low tannin content.

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