

# Pine needle effects on in vivo and in vitro digestibility of crested wheatgrass

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## Abstract

In vitro and in vivo digestion trials with lambs were conducted to determine effects of ponderosa pine needles (PN; *Pinus ponderosa* Laws.) on digestibility of crested wheatgrass (CW; *Agropyron desertorum* [Link] Schultes) hay. Pine needles contained shikimic acid (15–28 mg/g) and several monomeric phenolics (p-hydroxy benzoic acid, caffeic acid, p-coumaric acid, ferulic acid) and flavonoids. Tannin concentration exceeded assay limits (>10%) and terpenes were not found, probably due to the drying procedure. In the in vitro trial, needles were mixed with CW in 10% increments from 0% to 100%. In the in vivo trial, PN were fed to lambs as follows: (1) 0%, (2) 12.5%, (3) 25%, and (4) 50%, with the remainder of the diet as CW. In vitro organic matter digestibility (IVOMD) was regressed on level of PN in the substrate. As the proportion of PN increased, IVOMD declined cubically ( $P < 0.01$ ). The IVOMD values ranged from 54% for 100% CW to 24% for 100% PN. In vivo digestibility of organic matter, neutral detergent fiber and acid detergent fiber declined linearly ( $P < 0.01$ ) as PN were increased from 0% to 50% of the diet. Apparent crude protein digestibility and N retention by lambs declined cubically ( $P = 0.02$  and  $P < 0.01$ , respectively) and urinary N increased cubically ( $P < 0.01$ ) as dietary PN increased from 0% to 50%. We concluded that PN reduce in vitro and in vivo nutrient digestibility, reduced N retention by lambs, and effects were detectable even at low levels.

**Key Words:** *Pinus ponderosa*, plant secondary compounds, sheep toxic effects, poisonous plants, nitrogen retention

Cattle grazing winter range consume variable but sometimes large quantities of ponderosa pine needles (PN; *Pinus ponderosa* Laws.; Pfister and Adams 1991). Consumption of needles from ponderosa pine causes abortion (James et al. 1977), retained placentas, and endometritis in cattle (Stuart et al. 1989). Pine trees grow in much of the western U.S., and pine needle abortion has a severe impact on livestock producers in areas with ponderosa pine (Lacey et al. 1988).

Pine needles contain a number of secondary phytochemicals such as phenolics, terpenes, and tannins (Smith 1964, Hanover 1966). Levels of secondary compounds and forage digestibility are negatively correlated (Jung and Fahey 1981, Akin 1982, Jung 1985); phenolics also negatively impact rumen microbial populations (Akin 1982, Chesson et al. 1982).

Despite the negative effects of pine needles on cattle reproduction and possible negative influences of pine needles on forage digestibility, there is little information available on effects of pine needles on digestion and metabolism of ruminants. The objectives of this study were to determine effects of pine needles on digestibility in vitro and in vivo and on N retention in lambs. Our hypothesis was that pine needles would reduce forage digestibility and N retention.

## Materials and Methods

### Digestibility Trials

In vitro and in vivo digestion trials were conducted to determine effects of PN on digestibility of crested wheatgrass (CW; *Agropyron desertorum* [Link] Schultes) hay. Mature (full seedhead) CW hay was harvested from a 13-ha site 1 km west of Miles City, Mont. Crested wheatgrass was selected because it is often grazed and/or fed as a hay on sites with ponderosa pine where cattle are wintered. Green PN on branches from trees were collected in the spring and fall at a site about 20 km east of Miles City, Mont., and were air dried in a room at 20° C. After drying, PN were stripped by hand from branches. Spring PN were fed with CW to lambs (average weight = 26 kg) in a conventional digestion trial. Treatments were diets of (1) 100% CW, (2) 87.5% CW and 12.5% PN, (3) 75% CW and 25% PN, and (4) 50% CW and 50% PN. Before feeding, CW and PN were ground through a hammermill with a 2.5-cm screen. Sixteen crossbred (primarily Targhee × Suffolk × Hampshire) wether lambs were utilized in the digestion trial. The trial consisted of 2 periods with 8 lambs per period (2 lambs/treatment). Lambs were fed experimental diets for a 12-day adjustment period, followed by a 7-day total collection of feces and urine. Lambs were fed at 0700 and 1600 daily (90% of their ad libitum intake during 12-day adjustment period) during the collection period. Feces and urine were collected daily, and composited by lamb across days and frozen until laboratory analysis. The CW and PN were sampled daily and composited across days and lambs within each period for chemical analysis. In a few instances, there were some orts from PN or CW during the collection period. Orts were collected and composited across days for each lamb.

Dry matter (DM), ash, and N in feed, orts and fecal samples and N content of urine were determined by standard methods (AOAC 1984). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) content were determined in feed and fecal samples by the procedure of Goering and Van Soest (1970).

Intake of DM, ash, N, NDF, and ADF was adjusted for content in orts. Digestion coefficients for DM, organic matter, NDF, ADF, and the apparent digestion coefficient for crude protein were calculated as described by Schneider and Flatt (1975).

Spring- and fall-collected PN were mixed in varying proportions with CW in an in vitro digestion study. The CW for both in vitro trials was a subsample of CW used in the in vivo trial. Before in vitro digestion, PN and CW were ground to pass a 1-mm screen in a Wiley mill. In independent trials (i.e., different dates) using spring or fall PN, substrate was varied from 100% CW to 100% PN in 10% increments. To more precisely define PN effects at low levels, the fall PN, substrate was also varied from 100 CW% to 80% CW by adding PN in 2% increments.

In vitro organic matter digestibility (IVOMD) was determined in triplicate for spring and quadruplicate for fall PN for each PN: CW combination with a modified (Cochran et al. 1986) 2-stage Tilley and Terry (1963) procedure. Rumen fluid inoculum for the in vitro trials was collected through a rumen fistula from a steer maintained on a diet of CW hay. Rumen fluid was collected approximately 4 hours after feeding for both in vitro trials. Rumen fluid was strained through 8 layers of cheese cloth and samples within each trial were handled simultaneously within a single run. Spring and fall in vitro trials were conducted independently on 2 separate dates.

The in vivo trial was analyzed as a randomized complete block by analysis of variance. Dry matter intake was tested as a covariate for each digestion coefficient, urinary N excretion, and N balance. When the covariate was significant, means were adjusted to a common dry matter intake. Treatment means for each digestion coefficient, urinary N excretion and N balance were compared with

orthogonal polynomials (Steel and Torrie 1980). In vitro trials were analyzed by regression techniques. In vitro organic matter digestibility was regressed on level of PN in substrate. Linear, quadratic, and cubic regression coefficients were tested, as was the season × level of PN interaction. Linear, quadratic and cubic regression coefficients were tested for incubations of fall PN in 2% increments.

### Chemical Methods for Pine Needle Analysis Carbohydrates, Acids, and an Alcohol

Pine needle tissue was air dried and ground through a 1-mm screen through a Wiley mill. Ground tissue (500 mg) was extracted in 80/20 methanol/water (20 ml). A 9-ml aliquot was blown dry, taken up in pyridine, and 0.5 mg erythritol, the internal standard, was added. The compounds in solution were derivitized with trimethylchlorosilane and hexamethyldisilazane. A 5  $\mu$ l aliquot of the supernatant was injected into a Hewlett Packard capillary gas chromatograph equipped with a 100% methyl polysiloxane, 25-m column. Nitrogen was the carrier gas at 0.5 ml/min. Injector temperature was 250° C, detector temperature was 260° C, and initial temperature was 100° C for 1 min. Compounds were identified by co-chromatography using known standards. Data for these compounds, as well as all other compounds, were expressed as mg/g dry weight of tissue.

### Monomeric phenolics

Air dried, ground plant tissue (500 mg) was extracted in 8 ml 100% methanol. This was concentrated to 4 ml under N at 46° C. Water was added, the extract was washed with 8 ml hexane, and blown dry at 46° C. Methanol/HCL (0.5 ul) was added to the extract, the extract was heated to 100° C for 30 min, and then 1 ml water was added. This was extracted with 1.5 ml ethyl acetate, dried using Na<sub>2</sub>SO<sub>4</sub>, and the internal standard vanillic acid was added (0.25 mg). This was blown dry as above, derivitized in 100  $\mu$ l dimethylformamide and d100  $\mu$ l bis-trifluoroacetamide plus 1% trimethylchlorosilane, and 5  $\mu$ l were injected in a Hewlett Packard capillary gas chromatograph (GC). The GC was equipped with a Hewlett Packard ultra 2, 5% phenyl/5% methylsilicone column, 25-m long, and ID 0.32 mm. The temperature regime is that described for carbohydrates. Compounds were identified by co-chromatography using known standards.

### Tannins as Analyzed by Astringency

Because the ecologically important property of tannins has been suggested to be astringency (i.e., protein-complexing activity), tannin content was analyzed by the astringency method (Horner et al. 1987). Air dried, ground plant (200 mg) was extracted in 70% acetone. Acetone was removed by passing N over the supernatant at 55° C, filtered through a 0.45  $\mu$ m nylon filter and hemoglobin solution used as the reaction protein. The astringency of the tissue is expressed as that relative to the standard quebracho tannin.

## Results

### Pine Needle Chemistry

Pine needles contained several carbohydrates; galactose was the most prevalent (Table 1). Two acids, shikimic and citric acid, were present in PN tissue (Table 1). An alcohol, inositol, was also found in needles. The spring and fall pine needles had large numerical differences in levels of several carbohydrates and shikimic acid.

We found several monomeric phenolic acids in PN tissue; flavonoids were also present in needles (Table 1). Terpenes were not detected in PN tissue, probably due to volatilization during air-drying plant material. Tannins were present in large quantities (i.e., >10%), exceeding detection limits of the astringency technique used. Fall and spring needles differed quantitatively in levels of several phenolic and flavonoid compounds; the biological signifi-

**Table 1. Carbohydrates, acids, an alcohol, phenolic monomers, tannins, flavonoids, and terpenes (mg/g dry weight) in pine needle (PN) tissue collected during fall and spring in eastern Montana.**

Class of compound/chemical	Spring PN <sup>1</sup>	Fall PN
	mg/g	mg/g
<b>Carbohydrate</b>		
Fructose	8.78	4.29
Galactose	16.30	23.86
Glucose	10.20	4.83
Mannose	0.84	0.42
Sucrose	3.58	9.25
<b>Acids</b>		
Shikimic acid	15.94	27.76
Citric acid	2.43	2.58
<b>Alcohol</b>		
Inositol	1.10	0.61
<b>Monomeric phenolic acids</b>		
p-hydroxy benzoic acid	0.085	0.118
caffeic acid	0.008	0.016
p-coumaric acid	0.082	0.000
ferulic acid	0.049	0.052
gallic acid	0.154	0.493
vanillic acid	0.059	0.058
<b>Flavonoids</b>		
kaempferol	0.025	0.039
naringen	1.429	1.750
quercetin	0.000	0.214
taxifolin	0.128	0.075
<b>Terpenes</b>		
alpha-pinene	absent	absent
beta-pinene	absent	absent
<b>Tannins<sup>2</sup></b>	present	present

<sup>1</sup>Pine needles used in an in vitro and in vivo trial.

<sup>2</sup>Tannin concentration was not quantified because levels exceeded detection limits (>10%) of the method used.

cance of these differences is unknown in the context of our study.

Nutrient composition of CW and PN used during the study is shown in Table 2. Although collected at 2 seasons, nutrient composition of needles was similar.

#### In vivo Trial

Digestion coefficients for the in vivo trial are presented in Table 3. Dry matter intake as a covariate was not significant ( $P > 0.05$ ) for digestibility of any nutrient measured. In vivo digestibility of DM, OM, NDF, and ADF declined linearly ( $P < 0.01$ ) with increasing levels of PN in the diet. However, there was a consistent trend ( $P = 0.09$  to  $P = 0.20$ ) for a cubic relationship between level of PN and digestibility for each of these 4 variables. The cubic relationships

**Table 2. Nutrient composition of crested wheatgrass (CW) and pine needles (PN) used in digestion trials<sup>1</sup>.**

Item	CW	Spring PN <sup>2</sup>	Fall PN
Dry matter (%)	91.7	92.7	92.2
Organic matter (%)	95.0	96.5	97.6
Crude protein (%)	5.9	7.8	6.5
Neutral detergent fiber (%)	70.5	54.8	49.9
Acid detergent fiber (%)	40.6	42.3	36.9

<sup>1</sup>Dry matter basis.

<sup>2</sup>Pine needles used in an in vitro and in vivo trial.

each revealed a large reduction in digestibility between the 0% and 12.5% levels of PN. The digestion coefficients for 12.5% and 25% PN levels were similar, and a large reduction in digestibility occurred as PN proportion was increased from 25 to 50%. Apparent digestibility of crude protein was markedly different at all levels of PN (Table 3). A cubic relationship ( $P = 0.02$ ) occurred between dietary PN proportion and crude protein digestibility. Apparent protein digestibility was reduced by more than 90% as PN were increased from 0% to 50% of the diet.

Dry matter intake significantly affected urinary N excretion and N retention by lambs; therefore, urinary N and N retention were adjusted to a common level of intake. Dry matter intake was 17.0, 16.6, 16.0, and 12.0 g/kg of body weight for diets containing 0%, 12.5%, 25%, and 50% PN, respectively. The relationship between N excreted in the urine and dietary PN percentage was cubic ( $P = 0.02$ ). Excretion of N in the urine declined slightly as PN percentage was increased from 0% to 25%. A large increase in urinary N excretion occurred between the 25% and 50% levels of PN.

Nitrogen retention was negative for lambs on all treatments (Table 3). The relationship between N retained and level of PN consumption was cubic ( $P < 0.01$ ). Nitrogen retention declined slightly between the 0% and 12.5% levels of PN, then increased slightly as PN were increased from 12.5% to 25% of the diet. A large reduction in N retention occurred between the 25% and 50% PN levels.

#### In vitro Trial

A cubic relationship occurred between IVOMD and level of PN for fall and spring PN in 10% increments from 0% to 100% of substrate. The season  $\times$  level of PN interaction was not significant, nor were regression coefficients different for fall and spring PN. Thus, a single cubic ( $P < 0.01$ ;  $R^2 = 0.99$ ) regression equation represented the relationship between level of PN and IVOMD for both spring and fall PN (Fig. 1). The IVOMD declined rapidly as PN increased from 0% to 50% and then declined at a much slower rate from 50% to 100% PN.

The IVOMD declined linearly ( $P < 0.01$ ;  $R^2 = 0.74$ ) as PN replaced CW substrate in 2% increments from 0% to 20%. The

**Table 3. Effects of pine needles on digestibility of dry matter (DM), organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF) and crude protein (CP), and on urinary N excretion (UN) and N retained (NR) by lambs.**

Variable <sup>1</sup>	Percent of pine needles in the diet <sup>2</sup>				Significance of orthogonal polynomial <sup>3</sup>			
	0	12.5	25	50	L	Q	C	EMS <sup>4</sup>
DM (%)	58.7	50.6	51.0	41.8	$P < 0.01$	$P > 0.2$	$P = 0.09$	16.6
OM (%)	60.6	52.7	52.5	43.7	$P < 0.01$	$P > 0.2$	$P = 0.14$	17.6
NDF (%)	58.8	49.4	44.4	31.0	$P < 0.01$	$P > 0.2$	$P = 0.20$	15.7
ADF (%)	52.4	39.8	37.3	24.5	$P < 0.01$	$P > 0.2$	$P = 0.20$	40.5
CP (%)	43.9	22.9	30.6	3.9	$P < 0.01$	$P < 0.01$	$P = 0.02$	83.6
UN (g/day)	2.8	2.5	2.3	8.4	$P < 0.01$	$P < 0.01$	$P = 0.02$	0.42
NR (g/day)	-0.1	-1.3	-1.0	-7.7	$P < 0.01$	$P < 0.01$	$P < 0.01$	0.30

<sup>1</sup>Digestibility coefficients are on a dry matter basis.

<sup>2</sup>Remainder of diet was crested wheatgrass hay.

<sup>3</sup>L = linear, Q = quadratic, C = cubic, N = 4.

<sup>4</sup>EMS = Error mean squares.

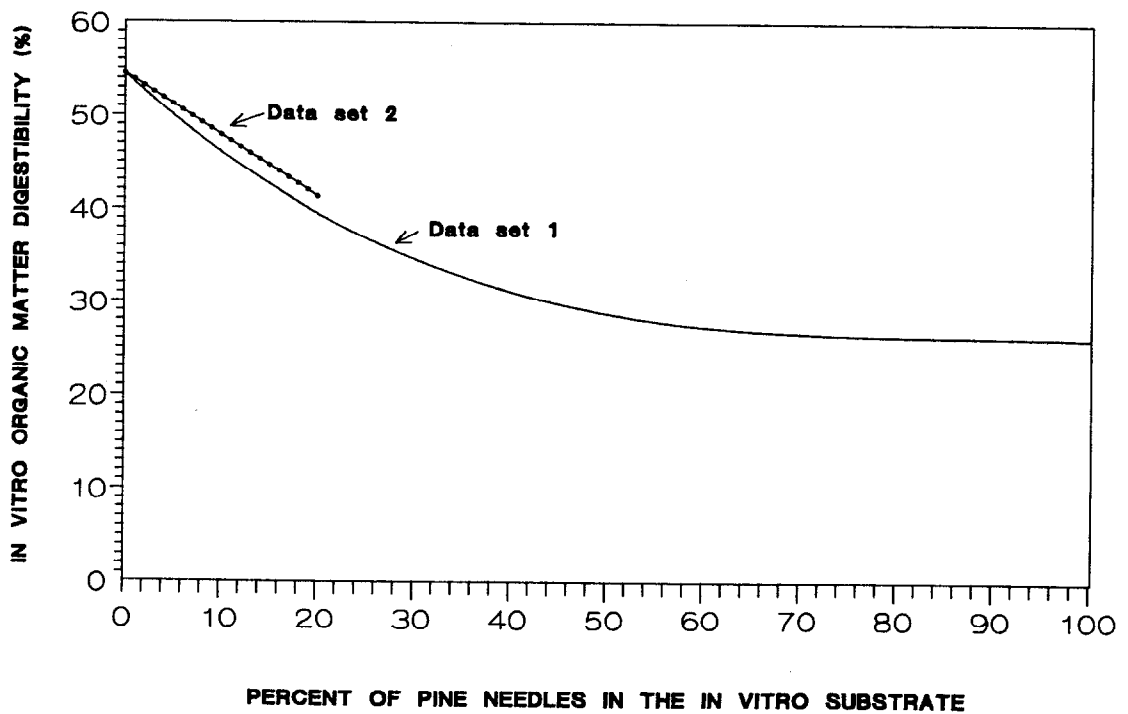


Fig. 1. Effects of increasing level of pine needles on in vitro organic matter digestibility of a crested wheatgrass hay-based substrate. In Data set 1, pine needles varied from 0% to 100% of the in vitro substrate in 10% increments (regression:  $Y = 54.5 - .944X + 1.06 \times 10^{-2} X^2 - 4.00 \times 10^{-5} X^3$ ,  $R^2 = .99$ ). In Data set 2, pine needles varied from 0% to 20% of the in vitro substrate in 2% increments (regression:  $Y = 54.5 - .660X$ ,  $R^2 = .74$ ).

inhibitory effect of PN on digestibility added in smaller increments produced a similar relationship as when PN were added in larger amounts (Fig. 1).

### Discussion

Pine needles contained a number of secondary phytochemicals that have been shown to depress forage digestibility. For example, Jung (1985) and Cherney et al. (1989) found that benzoic, cinnamic (e.g., p-coumaric and ferulic acid), and caffeic acids depressed digestion of cellulose. Flavonol glycosides (e.g., kaempferol) are also of interest (Strack et al. 1988) because these compounds may limit degradation of plant material in ruminants (Huang et al. 1986). The predominant acid in PN tissue, shikimic acid, is a major precursor of cinnamic acid and lignin in plant tissue (Goodwin and Mercer 1972).

There are numerous published reports that indicate tannins have negative impacts on digestibility (Kumar and Singh 1984). The high level of tannins found in our study may have inhibited microbial breakdown of plant cell wall (Cooper and Owen-Smith 1985). However, it appears unlikely that dietary tannins inhibit digestion through inhibition of digestive enzymes (Bernays et al. 1989). Under in vivo conditions, dietary tannins are generally not accessible to digestive enzymes (Butler et al. 1984, Mehansho et al. 1983). Tannins have been shown to be much more deleterious under in vitro conditions (Blytt et al. 1988).

Isolation of crude extracts and individual compounds and testing their effects in an in vitro system will be required in future work to determine which compound(s) is most inhibiting to forage digestion.

The reduction of digestibility observed for dietary components examined in this study is consistent with findings for other plant species containing various phytochemicals (Barry and Manley 1986, Jung 1985, Palo 1985, Burritt et al. 1984). Organic matter digestibility declined as PN percentage increased with both in vitro and in vivo digestion. Surprisingly, the impacts of PN on digestibil-

ity traits were observed at as little as 2% PN in vitro and had a marked effect at 12.5% of the diet with lambs.

The effects of PN were greater for in vitro than for in vivo digestion, and the difference became greater as level of PN increased. Our findings suggest that toxic effects of PN metabolism accumulate at higher levels in in vitro compared to in vivo systems, accentuating PN effects in vitro.

Although PN reduced digestibility of all nutrients in the in vivo trial, the most striking effect was on digestibility of crude protein. The negative effects of PN on digestibility are at least partially due to effects of phenolics or other secondary compounds on rumen microbial populations (Wiedmeier, unpublished data); other studies have noted that low molecular weight phenolics limit microbial degradation of structural carbohydrates (Akin 1982, Chesson et al. 1982).

We found a large inhibition of protein digestibility with PN inclusion in diets. Research at our laboratory (Pfister, unpublished data) with cattle consuming pine needles has shown that PN levels about 15% of the diet reduce ammonia-N concentrations below levels adequate for ruminal function, which would further reduce forage digestibility (Slyter et al. 1979, Ørskov 1982).

Nitrogen retention was negative for all levels of PN because of low levels of intake and concentrations of crude protein in the diet. The high negative N balance at the 50% level of dietary PN is explained by the 3.9% apparent crude protein digestibility and the 8.4 g N/day excreted in the urine. At some point between the 25% and 50% level of PN, increasing the level of dietary PN increases N passing through the feces and urine and profoundly reduces N retention. Ingestion of browse material commonly results in poor N retention in ruminant livestock. Holechek et al. (1990) fed 6 shrub diets varying in phenol content to goats, and found that all diets produced a negative N balance.

From relationships between PN consumption and digestibility coefficients and N retention observed in this study, we conclude that PN at low levels adversely affect in vitro and in vivo forage digestibility and N retention. Because of the adverse effects of PN

on digestibility and N retention, PN may have negative impacts on the nutritional status of cattle (i.e., loss of live weight), in addition to abortifacient effects.

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