Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminants, D.E Akin, L.G. Ljungdahl, J.R. Wilson, P.J. Harris, Eds.,Elsevier, New York, 1990 pp.173-182

Phenolic-Carbohydrate Complexes in Plant Cell Walls and Their Effect on Lignocellulose Utilization

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INTRODUCTION

The phenolic components of plant cell walls, including polymers (core lignin) and low-molecular weight monomers (non-core lignin), have been implicated as factors which limit forage fiber digestibility by ruminants (24). Concentration and composition of these lignin fractions is variable among plant parts, species and stage of physiological maturity (6,18). Negative correlations for concentration and composition of lignin fractions with forage digestibility have been reported by numerous workers (16,32,37). A recent review of literature pertaining to the distribution of lignins and their possible effects on forage fiber digestion is available (24).

While there is extensive literature on the phenolic/lignin concentration of forage cell walls, descriptions of structural characteristics are limited. Ford and Elliott (13) have suggested that such structural features as cross-linkage of cell wall polymers are of greater importance to controlling ruminal degradation of fiber than simple concentration. Also, evidence for the inhibitory properties of cell wall phenolics/lignin are limited primarily to correlation types of analyses (16,32). Information on specific structural features of cell wall lignification and mechanistic explanations of how phenolics/lignin impede forage fiber degradation are lacking. The objective of this review will be to discuss current knowledge and future directions of research regarding phenolic/lignin structural features of forage cell walls and mechanisms by which lignification limits cell wall polysaccharide degradability by ruminal microorganisms.

ASSOCIATION OF PHENOLIC ACIDS WITH CELL WALL POLYMERS

The association of non-core lignin phenolic acids, notably *p*-coumaric and ferulic acids, with polysaccharides in grasses has been extensively

investigated. Oligomeric compounds containing ferulic acid, arabinose and xylose were released by partial enzymatic degradation of isolated cell walls (30,45). Ferulic acid is invariably connected by an ester bond to the C-5 hydroxyl of arabinofuranoside in arabinoxylans. Structural analysis identified the compounds as *O*-[5-*O*-(trans-feruloyl)-α-L-arabinofuranosyl]-(1-->3)-*O*-β-D-xylopyranoside (FAX) and *O*-[5-*O*-(trans-feruloyl)-a-L-arabinofuranosyl]-(1-->3)-*O*-β-D-xylopyranosyl-(1-->4)-D-xylopyranoside (FAXX). Mueller-Harvey et al. (33) isolated the analogous *p*-coumaric ester, PAXX, from barley (*Hordeum vulgare*) and estimated that one in every 15 arabinose units was esterified with ferulic acid residues and one in 31 with *p*-coumaric acid.

In dicots the esterification of ferulic acid to cell wall components is much less common. Low concentrations of esterified non-core lignin phenolics, approximately 10% of that found in grasses, have been identified in legumes (26,35). Feruloylated polysaccharides have been identified in spinach (*Spinacia oleracia*) (14) and sugar beets (*Beta saccharifera*) (38). In both cases the ferulic acid appears to be esterified to neutral sugar side groups (arabinose and galactose) of pectins. The pectins are unique in that the ferulic acid is bound to regions of the polygalacturonans with a high degree of neutral sugar substitution, "hairy regions".

Phenolic acids have also been shown to be bound to core lignin. Ester-linked *p*-coumaric acid was first identified in a lignin preparation from wheat (*Triticum aestivum*) straw (44). Higuchi's group reported the presence of *p*-coumaric esters in grasses (43) and carried out a detailed study on *p*-coumaric esters in bamboo (*Bambusa tulda*) lignins (34). The resistance of *p*-coumaric esters in bamboo to methanolysis led them to suggest that *p*-coumaric acid was linked to the γ-position of the core lignin side chain rather than the α-position.

Himmelsbach and Barton (20) identified phenolic esters in both warm season and cool season grasses by ^{13}C NMR. In an extension of the pioneering work on ^{13}C NMR of lignins, Nimz et al. (36) examined milled cell wall lignins from maize (*Zea mays*), bamboo and wheat straw, and found evidence for both esterified and etherified *p*-coumaric acid, supporting earlier work (12) suggesting the presence of etherified structures. Monties group established the presence of ether linkages between phenolic acids and core lignin fractions from wheat straw (40). After complete hydrolysis of esters with NaOH, a further release of phenolic acids was quantified by acidolysis, a technique traditionally used to cleave ether bonds in lignin itself. ^{13}C NMR spectra indicated the presence of both etherified and esterified phenolic acids, although the former only appeared in fractions treated with alkali. A study of phenolic acids in normal rice (*Oryza sativa*) and a mutant brittle stem rice cultivar showed higher levels of esterified *p*-coumaric acid and markedly lower etherified ferulic acid in the mutant (42). Model studies (41) confirmed that phenolic acids could add to the α-position of quinone methides (intermediates formed during lignification) but no evidence for the regio-chemistry of phenolic acids on core lignin was presented.

Core lignin itself is generally assumed to be covalently bound to polysaccharides, but evidence mainly arises from work on woods. Das et al. (10) presented evidence that 4-O-methyl-D-glucuronic acid side chains of jute (*Chorchurus capsularis*) xylan were involved in ester linkages to lignin. The difunctional phenolic acids present in legumes and grasses, but not in woods, offer a cross-linking mechanism for associating lignin with polysaccharides (4), shown schematically in Figure 1. The specific points of attachment of phenolic acid esters and ethers to the core lignin polymer remain unidentified. Iiyama et al. (21) looked for evidence of phenolic acids bridging lignin and carbohydrates. Their data support the existence of such structures, but the regiochemical relationship to core lignin remains unaddressed. Extensive phenolic acid ester and ether data from fractionation of wheat internodes indicated that etherification of phenolic acids is an early event in plant maturation (21). The location of alkali-extractable *p*-coumaric and ferulic acids in rice has been studied by He and Terashima (19) using autoradiographic techniques. It was found that phenolic acids were present in all types of cell walls and increased with the lignification process. They also reported the presence of sinapic acid (sinapinic acid), the third cinnamic acid in the lignin biosynthetic pathway which has received little attention to date, and suggested its connection to lignin.

MECHANISMS OF DEGRADATION INHIBITION

Free *p*-coumaric and ferulic acids have been shown to inhibit cellulose degradation by rumen bacteria and fungi (1,3). However, concentrations of free phenolic acids in forages are very low (26,35). Recent work by Hartley and Akin (17) demonstrated that synthetic (methyl and *t*-butyl) esters of *p*-coumaric acid were more inhibitory to forage degradation than the free acid. While these synthetic esters have not been identified in forages, soluble esters of *p*-coumaric and ferulic acids have been found (7). Concentrations of these cytoplasmic phenolic esters have probably been severely overestimated due to pre-extraction with neutral detergent as a method of isolating cell wall bound phenolics (28). Phenolic-carbohydrate esters are released from the forage cell wall during ruminal fermentation (23). In sheep (*Ovis aries*), ruminal concentrations of soluble *p*-coumaric acid esters have ranged from 1.10 to 7.92 μM on immature alfalfa (*Medicago sativa*) and mature tall fescue (*Festuca arundinacea*) hay diets, respectively (26). Soluble ferulic acid ester concentrations in the rumen of sheep on mature alfalfa and immature tall fescue hay diets were 0.15 to 5.15 μM, respectively (26). These observed ruminal concentrations are much lower than the 5 mM concentrations at which free phenolic acids and synthetic esters have been shown to inhibit cellulose degradation (1,17). While micro-environments in the rumen may contain higher concentrations of these soluble phenolic esters than measured in total rumen contents, a thousand-fold increase would appear unlikely and these phenolic-carbohydrate complexes must still be demonstrated to inhibit ruminal fiber degradation.

Inhibition of cell wall polysaccharide fermentation by forage cell wall phenolics/lignin has not been proven. While there is a great deal of correlation-based evidence for such inhibition (24), a mechanistic cause and effect relationship has not been demonstrated. Recent data, where plant maturity and anatomical fractions were controlled, indicated that core lignin concentration was not correlated with fiber degradability (29). In this study, concentrations of non-core lignin phenolics and core lignin composition were related to grass cell wall degradation (Table 1). It appears that core lignin concentration is strongly related to forage maturity, but variation in cell wall degradability within a maturity stage may be more a function of lignin structural features.

Synthetic models where *p*-coumaric and ferulic acids were esterified to neutral detergent fiber of grasses, isolated celluloses, and hemicelluloses have shown that esterification of phenolics to polysaccharides, at concentrations which mimic levels found in forage cell walls, will depress degradability of the carbohydrate fraction (Fig. 2) (5,23,27,39). A core lignin polymer model, polyeugenol, only depressed cellulose degradation by a fungal cellulase and rumen microorganisms when the lignin model was bound to the cellulose (Table 2) (15,25). Free polyeugenol did not affect cellulose degradation. While synthetic cell wall models have been criticized as being crude and poorly characterized representations of the plant cell wall (5), it does appear that linkage of phenolics/lignin to cell wall polysaccharides is a requirement for these cell wall components to limit microbial polysaccharide degradation.

Table 1. Best single variable for predicting stem neutral detergent *in vitro* fiber degradability of warm-season grasses. Sign in parenthesis shows direction of variable's effect[a]

	Maturity			
Species[b]	Combined	Vegetative	Boot	Heading
Switchgrass	FA(+)***	FA(-)***	NS	NS
Big bluestem	LIG(-)**	PSYAL(-)*	NBO(+)*	NBO(+)***
Combined	LIG(-)***	FA(+)***	FA(+)**	NBO(+)***

[a]From Jung and Vogel (29). Abbreviations are esterified ferulic acid (FA); core lignin concentration (LIG); yield of aldehydes by nitrobenzene oxidation of core lignin (NBO); molar proportion of syringaldehyde in the nitrobenzene oxidation productions (PSYAL). Regresssions were significant (*,**,***) at $p < 0.10$, 0.05 and 0.01, respectively.
[b]Switchgrass (*Panicum virigatum*), Big bluestem (*Andropogon gerardi*).

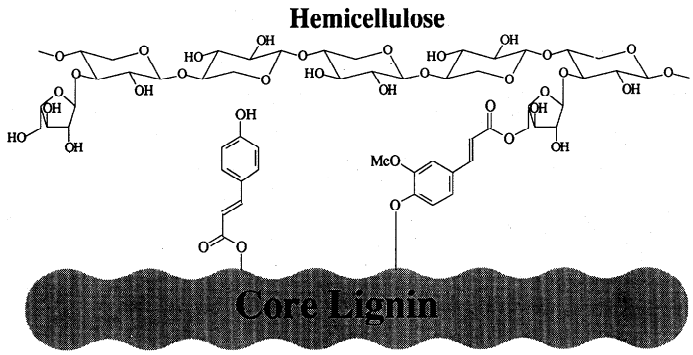

Figure 1. Schematic structure of cross-linkage of hemicellulose to core lignin via an ester-ether ferulic acid linked moiety. *p*-Coumaric acid is shown as an ester of core lignin.

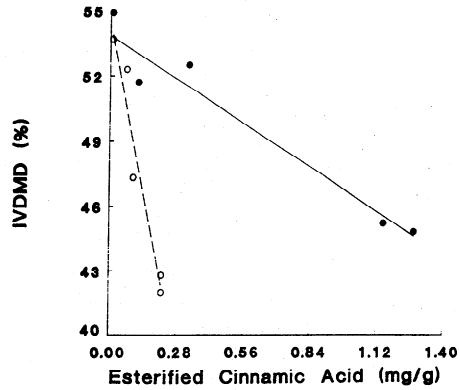

Figure 2. Effect of esterification of *p*-coumaric (•) and ferulic (o) acids to A-linear hemicellulose, isolated from oatspelts xylan, on *in vitro* dry matter disappearnace (IVDMD). Modified from Jung (22).

Based upon changes in bacterial growth rates and profiles of fermentation end-products, it appears that free phenolic acids and soluble synthetic phenolic esters are inhibitory to rumen bacteria (17,46). Esters of benzoic acids and aldehydes isolated from *in vitro* ruminal forage fermentations have been shown to cause similar shifts in volatile fatty acid end-products to those observed with free acids (23). It also has been demonstrated that free phenolics will interfere with attachment of *Fibrobacter succinogenes* to cellulose (46). In contrast, no changes in fermentation end-products were observed with *p*-coumaric and ferulic acids artificially esterified to cellulose (27). These data suggest that soluble phenolics, free or as carbohydrate esters, inhibit ruminal fiber fermentation by disruption of bacterial metabolism whereas phenolics/lignin bound to the cell wall restrict access to polysaccharide for enzymatic hydrolysis. Given the high concentrations of cell wall bound phenolics/lignin, the latter mechanism is probably of greater importance to limiting forage fiber utilization.

Table 2. Effect of a synthetic core lignin model, polyeugenol, on *in vitro* cellulose degradation

Treatment[a]	Degradation (%)[b]
Untreated control	16.3*
Treated control	8.5†
1.1% bound polyeugenol	4.4‡
3.6% bound polyeugenol	0.4§
10% free polyeugonol + treated control	8.8†
SEM	1.3

[a]Treated control cellulose was subjected to polyeugenol synthesis protocol, but no eugenol was added to reaction. Bound polyeugenol was the polymer synthesized in the presence of cellulose which could not be removed by water or acetone washing.
[b]Means not sharing a common superscript are different ($p < 0.05$).

Morrison (31) demonstrated that core lignin concentration had a greater effect on hemicellulose degradability in grasses than seen for cellulose degradation. After various methods of chemical delignification of alfalfa, smooth bromegrass (*Bromus inermis*) and maize stems, 72 h *in vitro* ruminal degradabilities of arabinose, xylose and glucose were related differently to components of lignification (H.G. Jung and F.R. Valdez, unpublished data). Arabinose degradation was negatively correlated with esterified ferulic acid concentration of the cell wall, while xylose degradability was negatively correlated with core lignin concentration. Core lignin concentration and total concentration of esterified non-core lignin phenolics were included as secondary variables for the prediction of arabinose and xylose degradabilities, respectively. Glucose degradation from cellulose in these forages was not correlated to any measure of lignification. These relationships of lignification with degradability of various cell wall polysaccharide components might be expected because ferulic acid is esterified to arabinose, which is bound to xylan, and core lignin is bound to xylan via arabinose-phenolic acid ester-ether bridges and other linkages (8). Cellulose apparently is not covalently linked to any other cell wall polymer. These data suggest that degradation of the carbohydrate components of the cell wall will be controlled by different phenolic/lignin components depending on which structural linkages are present.

Microbial degradation of cell walls from forages is variable among plant parts and cellular tissues within different plant parts (2). Chesson et al. (9) claimed that all neutral sugar components of the cell wall were removed at roughly equivalent rates from isolated cell walls from mesophyll, epidermis or fibre cells of perennial (*Lolium perenne*) and Italian (*L. multiflorum*) ryegrass leaves. However, the various cell types had different cell wall compositions, and rates and extents of polysaccharide degradability. These

data have led Chesson (8) to propose that the differences observed in rate and extent of degradation for different cell wall neutral sugars in forages (31) are a function of differences in composition and degradability of the various cell wall types. Proportion of lignified cellular tissues in the forage versus non-lignified tissues is the critical determinant of forage fiber utilization in this model, as all polysaccharide components are equally degradable within a specific tissue's cell wall (8). This hypothesis has not been adequately tested, especially for variation in cell wall structure associated with physiological maturation. However, in smooth bromegrass selected for high and low *in vitro* dry matter disappearance (IVDMD), the high IVDMD genotypes did have greater cross-sectional area percentages of more readily degradable tissues (11).

CONCLUSIONS

Improvement in utilization of lignocellulose from forages can be accomplished through post-harvest treatment or genetic improvement of forages to increase cell wall degradability. Both of these approaches, but especially genetic manipulation, would be more productive if more accurate and detailed models of cell wall structure existed. This knowledge, plus information on the mechanisms of microbial cell wall degradation, would allow intelligent choices to be made for selection criteria in forage breeding programs. For example, would greater progress be achieved for improved ruminal cell wall degradability if forages were selected for lower concentrations of core lignin, esterified non-core lignin, or ester-ether ferulic acid bridges between hemicellulose and core lignin? Current knowledge does not provide a clear choice.

Research in the future must emphasize structural as opposed to compositional analysis. Care will be required to identify and segregate forage species, plant parts, and maturity differences. Developmental processes in cell wall lignification, and how these control overall cell wall structure, must be characterized. The importance of cell wall chemistry vs. proportion of cellular tissue types in determining forage cell wall degradability will need to be addressed as to their significance for genetic manipulation. The mechanisms and sequence of polysaccharide hydrolysis from forage cell walls by ruminal microorganisms must be understood in detail if we are to identify those components of cell wall structure which must be altered to maximize improvement in lignocellulose utilization. Simple correlation analysis of phenolic/lignin concentrations with fiber degradability of forages will not be sufficient for achieving the goal of substantial advances in forage cell wall digestion by ruminants.

LITERATURE CITED

1. Akin, D. E. 1982. Forage cell wall degradation and *p*-coumaric, ferulic and sinapic acids. Agron. J. 74:424-428.

2. Akin, D. E. 1989. Histological and physical factors affecting digestibility of forages. Agron. J. **81**:17-25.
3. Akin, D. E. and L. L. Rigsby. 1985. Influence of phenolic acids on rumen fungi. Agron. J. **77**:180-182.
4. Bacic, A., P. J. Harris, and B. A. Stone. 1988. Structure and function of plant cell walls, pp. 297-371. *In* The biochemistry of plants, Vol. 14. Academic Press, New York.
5. Bohn, P. J. and S. L. Fales. 1989. Cinnamic acid-carbohydrate esters: an evaluation of a model system. J. Sci. Food Agric. **48**:1-7.
6. Buxton, D. R. and J. B. Russell. 1988. Lignin constituents and cell-wall digestibility of grass and legume stems. Crop Sci. **28**:553-558.
7. Cherney, J. H., K. S. Anliker, K. A. Albrecht, and K. V. Wood. 1989. Soluble phenolic monomers in forage crops. J. Agric. Food Chem. **37**:345-350.
8. Chesson, A. 1988. Lignin-polysaccharide complexes of the plant cell wall and their effect on microbial degradation in the rumen. Anim. Feed Sci. Tech. **21**:219-228.
9. Chesson, A., C. S. Stewart, K. Dalgarno, and T. P. King. 1986. Degradation of isolated grass mesophyll, epidermis and fibre cell walls in the rumen and by cellulolytic rumen bacteria in axenic culture. J. Appl. Bacteriol. **60**:327-336.
10. Das, N. N., S. C. Das, A. S. Dutt and A. Rey. 1981. Lignin-xylan ester linkage in jute fiber. Carbohyr. Res. **94**:73-82.
11. Ehlke, N. J. and M. D. Casler. 1985. Anatomical characteristics of smooth bromegrass clones selected for *in vitro* dry matter digestibility. Crop Sci. **25**:513-517.
12. Erickson, M., G. E. Miksche, and I. Somfai. 1973. Characterization of angiosperm lignins by degradation. II. Monocotyledons. Holzforschung **27**(5):147.
13. Ford, C. W. and R. Elliott. 1987. Biodegradability of mature grass cell walls in relation to chemical composition and rumen microbial activity. J. Agric. Sci. **108**:201-209.
14. Fry, S. C. 1982. Phenolic components of the primary cell wall: Feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. Biochem. J. **203**:493-504.
15. Gressel, J., Y. Vered, S. Bar-Lev, O. Milstein, and H. M. Flowers. 1983. Partial suppression of cellulase action by artificial lignification of cellulose. Plant Sci. Lett. **32**:349-353.
16. Hartley, R. D. 1972. *p*-Coumaric and ferulic acid components of cell walls of ryegrass and their relationships with lignin and digestibility. J. Sci. Food Agric. **23**:1347-1354.
17. Hartley, R. D. and D. E. Akin. 1989. Effect of forage cell wall phenolic acids and derivatives on rumen microflora. J. Sci. Food Agric. **49**:405-411.
18. Hartley, R. D. and J. Haverkamp. 1984. Pyrolysis-mass spectrometry of the phenolic constitutents of plant cell walls. J. Sci. Food Agric. **35**:14-20.

19. He, L. and N. Terashima. 1989. Formation and structure of lignin in monocotyledons. II. Deposition and distribution of phenolic acids and their association with cell wall polymers in rice plants (*Oryza sativa*). Mokuzai Gakkaishi **35**:123-129.
20. Himmelsbach, D. S. and F. E. Barton. 1980. Carbon-13 nuclear magnetic resonance of grass lignins. J. Agric. Food Chem. **28**:1203-1208.
21. Iiyama, K., T. B. T. Lam, and B. A. Stone. 1990. Lignin and phenolic acids in wheat internodes. II. Phenolic acid bridges between polysaccharides and lignin. Phytochemistry **29**:733-737.
22. Jung, H. G. 1988a. Inhibition of hemicellulose fermentation by esterified cinnamic acids. J. Dairy Sci. **71**(Supplement 1):182.
23. Jung, H. G. 1988b. Inhibitory potential of phenolic-carbohydrate complexes released during ruminal fermentation. J. Agric. Food Chem. **36**:782-788.
24. Jung, H. G. 1989. Forage lignins and their effects on fiber digestibility. Agron. J. **81**:33-38.
25. Jung, H. G. 1990. Effects of lignin linkage and hydrogen bonding on cellulose degradation in model systems. J. Anim. Sci., In press.
26. Jung, H. G., G. C. Fahey, Jr., and N. R. Merchen. 1983. Effects of ruminant digestion and metabolism on phenolic monomers of forages. Brit. J. Nutr. **50**:637-651.
27. Jung, H. G. and T. Sahlu. 1986. Depression of cellulose digestion by esterified cinnamic acids. J. Sci. Food Agric. **37**:659-665.
28. Jung, H. G. and S. C. Shalita-Jones. 1990. Variation in the extractability of esterified *p*-coumaric and ferulic acids from forage cell walls. J. Agric. Food Chem. **38**:397-402.
29. Jung, H. G. and K. P. Vogel. 1989. Reevaluation of lignin's role in forage fiber digestibility. Proc. Conf. on Rumen Function **20**:(Abstract #19), Chicago, IL.
30. Kato, A., J-I. Azuma, and T. Koshijima. 1983. A new feruloylated trisaccharide from bagasse. Chem. Lett. 137-140.
31. Morrison, I. M. 1983. The effect of physical and chemical treatments on the degradation of wheat and barley straws by rumen liquor-pepsin and pepsin-cellulase systems. J. Sci. Food Agric. **34**:1323-1329.
32. Mowat, D. N., M. L. Kwain, and J. E. Winch. 1969. Lignification and *in vitro* cell wall digestibility of plant parts. Can. J. Plant Sci. **49**:499-504.
33. Mueller-Harvey, I., R. D. Hartley, P. J. Harris, and E. H. Curzon. 1986. Linkage of *p*-coumaric and feruloyl groups to cell-wall polysaccharides of barley straw. Carbohydr. Res. **148**:71-85.
34. Nakamura, M. and T. Higuchi. 1978. Ester linkages of *p*-coumaric acid in bamboo lignin III. Cellulose Chem. Tech. **12**:209-221.
35. Newby, V. K., R. M. Sablon, R.L.M. Synge, K. V. Casteele, and C. F. Van Sumere. 1980. Free and bound phenolic acids of lucerne (*Medicago sativa* cv Europe). Phytochemistry **19**:651-657.

36. Nimz, H. H., D. Robert, O. Faix, and M. Nemr. 1981. Carbon-13 NMR spectra of lignins. 8. Structural differences between lignins of hardwoods, softwoods, grasses and compression wood. Holzforschung **35**(1):16-26.
37. Reeves, J. B., III. 1985. Lignin composition and *in vitro* digestibility of feeds. J. Anim. Sci. **60**:316-322.
38. Rombouts, F. M. and J. F. Thibault. 1986. Feruloylated pectic substances from sugar-beet pulp. Carbohyd. Res. **154**:177-187.
39. Sawai, A., T. Kondo, and S. Ara. 1983. Inhibitory effects of phenolic acid esters on degradability of forage fibers. J. Soc. Grassl. Sci. **29**:175-179.
40. Scalbert, A., B. Monties, J-Y. Lallemand, E. Guittet, and C. Rolando. 1985. Ether linkage between phenolic acids and lignin fractions from wheat straw. Phytochemistry **24**:1359-1362.
41. Scalbert, A., B. Monties, C. Rolando, and A. Sierra-Escudero. 1986. Formation of ether linkage between phenolic acids and Gramineae lignin: a possible mechanism involving quinone methides. Holzforschung **40**:191-195.
42. Sharma, U., J-M., Brillouet, A. Scalbert, and B. Monties. 1986. Studies on a brittle stem mutant of rice, *Oryza sativa* L.; characterization of lignin fractions, associated phenolic acids and polysaccharides from rice stem. Agronomie **6**:265-271.
43. Shimada, M., T. Fukuzuka, and T. Higuchi. 1971. Ester linkages of *p*-coumaric acid in bamboo and grass lignins. Tappi **54**:72-78.
44. Smith, D.C.C. 1955. Ester groups in lignin. Nature **176**:267-268.
45. Smith, M. M. and R. D. Hartley. 1983. Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in Graminaceous plants. Carbohydr. Res. **118**:65-80.
46. Varel, V. H. and H. G. Jung. 1986. Influence of forage phenolics on ruminal fibrolytic bacteria and *in vitro* fiber degradation. Appl. Environ. Microbiol. **52**:275-280.