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Appreciation is expressed to Peggy Carroll for her dedication to the task of typing and assembling this research summary.

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September 2003

TABLE OF CONTENTS

RUMEN MICROBIOLOGY

FORAGE QUALITY

FEED UTILIZATION BY CATTLE

MANURE NUTRIENT MANAGEMENT

FARM/HERD REPORT-WISCONSIN

Preface

It is a pleasure to bring you these summaries of research conducted over the past year at the U.S. Dairy Forage Research Center. The Center's mission is to build a knowledge and technology base for the dairy industry to fully exploit the use of forages in the production of milk. The Center was established in 1980 on the University of Wisconsin-Madison campus in Madison, WI, but is a federal unit of the Agricultural Research Service, U.S. Department of Agriculture (USDA). We employ agricultural engineers, plant and soil scientists, microbiologists, ruminant nutritionists, chemists and an agricultural economist who all work together to increase the efficiency of forage production and utilization by dairy farmers. At present, we have nineteen scientists: sixteen at Madison, two cluster scientists at the University of Minnesota in St. Paul, MN, and one cluster scientist at Cornell University in Ithaca, NY. These scientists hold faculty appointments in university departments and provide supervision for approximately 6-8 graduate students and 4 postdoctoral fellows. We function in close cooperation with the agricultural experiment stations of several states.

The Center's 63-acre research farm is located in Prairie du Sac, WI and has facilities for housing and feeding 320 milking cows and 350 replacement heifers and dry cows. An additional 1,600 acres of adjacent land is utilized by the Center in agreement with the U.S. Department of the Army. In 1999, the U.S. Defense Department declared that the former Badger Army Ammunition Plant (BAAP), adjacent to our research farm, is excess property. The USDA has requested a no-cost transfer of custody of 1,943 acres of this excess federal land so that we can continue our research efforts. We are working with the Ho Chunk Nation, Wisconsin Department of Natural Resources, Sauk County, Sumpter and Merrimac Townships in Sauk County, the GSA, and the Army to develop a unified management strategy for the entire property to facilitate transfer of the land. We are encouraged by the cooperation of all parties to bring about a solution.

Regarding staff updates, we hired Geoffrey E. Brink as Research Agronomist. Geoff comes to us with 18 years experience in USDA-ARS from the Waste Management and Forage Unit at Mississippi State, MS. Geoff received a B.S. degree in Agronomy from The Pennsylvania State University, and M.S. and Ph.D. degrees in Agronomy from the University of Minnesota. He brings an expertise in grazing management, forage utilization, and forage quality. Michael L. Sullivan, Research Molecular Geneticist, also joined us this year. Mike received a B.S. degree in Biochemistry and Molecular Biology from Purdue University and a Ph.D. degree in Cell and Molecular Biology from the University of Wisconsin. He brings experience in molecular biology of proteins and had been working as an ARS post-doc with Ron Hatfield to enhance perennial legumes to use polyphenol oxidase (PPO), present in red clover to protect protein during ensiling.

I am pleased to announce that Jill A. Davidson has been hired by the University of Wisconsin as Herd Manager at our research farm, replacing Leonard L. Strozinski who retired after 22 years in that position. Jill received a B.S. in Animal Science from The Ohio State University, a M.S. in Dairy Science from the University of Florida and a Ph.D. in Dairy Science from Michigan State University. She wanted to be involved in dairy research and also manage a large dairy operation. We are pleased to have Jill on the USDFRC team.

Larry D. Satter retired on July 3, 2003, following a distinguished 39-year career in dairy cattle nutrition. Larry worked for 17 years as a faculty member in the Department of Dairy Science at the

University of Wisconsin and 22 years as a Research Dairy Scientist with USDA-ARS at USDFRC. He served as Director of USDFRC from 1987 to 1998. Larry and his co-workers have conducted research on protein utilization by ruminants, phosphorus requirements of lactating dairy cows, and forage quality. He was recently recognized as a Highly Cited Researcher by ISI, publisher of Current Contents. This work helped usher in new concepts of protein utilization by ruminants. Research from Dr. Satter's group established the criteria for heat processing of soybeans to decrease protein degradation in the rumen. This research resulted in rapid growth in use of heat-processed soybeans as a supplement for lactating dairy cows. More recently he and his colleagues have developed strong evidence in support of reduced supplementation of phosphorus to dairy cows. Larry has received a number of honors, including the American Feed Manufacturer's Association Award for Outstanding Research in Dairy Cattle Nutrition, Distinguished Nutritionist Award by the Distillers Feed Research Council, Outstanding Teacher Award in the College of Agricultural and Life Sciences, Pioneer Hi-Bred Forage Award, ADSA Fellow, Nutrition Professionals Applied Nutrition Award from ADSA, and Award of Honor for contributions to the welfare of ADSA. His retirement plans include spending more time growing hardwood trees on his farm near Madison.

The FY 03 budget provided funding to support the addition of two scientists to DFRC: a Research Geneticist (Forage Plants) and a Research Agricultural Scientist (Dairy Systems Specialist). We are actively recruiting to fill these positions, as well as an Agricultural Engineer position (due to the retirement of Richard Koegel in 2002) and Larry Satter's position as a Research Dairy Scientist.

I want to thank scientists, support staff, students, visiting scientists, and stakeholders for making 2002-2003 a tremendous success. USDFRC stakeholders are currently developing a plan to enhance the dairy forage research capacity to meet the needs of the industry in the 21st century.

This collection of research summaries illustrates the progress that scientists and staff are making in developing information to help dairy farmers utilize their resources more effectively. The research is intended to benefit producers of forage crops, dairy farmers, and the consumers of dairy products.

Sincerely,

Neal P. Martin, Director
U.S. Dairy Forage Research Center

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#Member of Agricultural Research Stations Department in the College of Agricultural and Life Sciences, and supported by the U.S. Dairy Forage Research Center.

Forage Genetics and Production

Contrasting Growth Response of N_2-Fixing and Non-N_2-Fixing Plants to Elevated CO_2 Depends on Soil N Supply

L.D. Tali, M. G. Tjoelker, P. B. Reich, M.P. Russelle

Introduction

Rising atmospheric carbon dioxide (CO_2) concentration and increasing inputs of fixed forms of nitrogen (N) into the global N cycle are predicted to have profound effects on ecosystems. While numerous studies document the response of plants to these factors independently, fewer evaluate the combined effects of elevated CO_2 and increased soil N supply, which likely interact in complex ways and differently at different scales. Plant responses to elevated CO_2 are fundamentally mediated by photosynthesis and a suite of physiological, morphological and growth changes. Typical increases in photosynthetic rates and biomass accumulation in elevated compared to current ambient CO_2 concentrations have ranged between 20 and 50% in crops.

The variation in growth and photosynthetic enhancements under elevated CO_2 may be associated with the differential responses of species to other limiting resources such as nutrients, water, and light. Since available N already limits productivity in most ecosystems, and because tissue N is a major determinant of photosynthesis, low N may reduce potential photosynthetic rate and growth enhancements under elevated CO_2, and thus limit the ability to incorporate additional carbon. Some simulation models suggest that growth responses to elevated CO_2 concentrations are constrained by N limitation, although actual evidence is mixed. Our objective was to consider the role of symbiotic N_2 fixation in the CO_2 response and to evaluate potential interactive plant responses to elevated atmospheric CO_2 and enriched N addition by combining measures of net photosynthesis and whole-plant growth with estimates of N derived from symbiotic N_2 fixation in N_2-fixing and non-fixing species. We used plant species native to the Upper Midwest USA in this experiment.

Methods

Seeds of wild lupine and yarrow were sown in Nymore sand contained in plastic pots. Lupine was inoculated with appropriate rhizobia. Plants were grown in growth chambers programmed to mimic day length at 45˚ N latitude from May 20 until July 30, with light intensity of about 1100 mol m^{-1} s^{-1} and either 365 or 700 mg L^{-1} CO_2 . Nitrogen treatments were calculated so that additions were equivalent to rates of 0, 4, 8 12, 16, or 20 g N m^{-2} yr^{-1} based on a 12-week growing season. Applications were made every third day as a solution of ^{15}N-enriched NH_4NO_3 with ammonium and nitrate equally labeled at 5.7 atom percent ^{15}N. Initial soil pH was 5.3, Bray-extractable P was 61 mg P kg^{-1} soil, and 1*M* ammonium acetate-extractable cations were 51 mg K kg^{-1} soil, 328 mg Ca kg^{-1} soil and 50 mg Mg kg^{-1} soil. Final harvest was at 56 days after sowing when flowering began.

Results and Conclusion

Total plant biomass of lupine increased on average 80% under elevated compared to ambient CO_2 and N additions did not affect biomass at either CO_2 concentration (Fig. 1). In contrast, total plant biomass of yarrow increased faster with CO_2 enrichment under elevated than ambient N, ranging from no difference in low N soil to a 25% increase at the highest levels of N (Fig 1). Across the wide range of N additions, whole plant N concentration in lupine did not change with either CO_2 enrichment or N addition (Fig. 2). In contrast, whole plant N concentration in yarrow decreased 20% in elevated compared to ambient CO_2-grown plants and N concentration increased linerarly with increasing N addition (Fig. 2).

Importantly, the dependence of lupine on N_2 fixation increased with higher CO_2, in response to improved growth potential (Fig. 3), and this increased dependence did not disappear as inorganic N supplies increased, within the rates used here. Variations in the magnitude of this response likely correlate with the availability of other resources such as soil moisture, or levels of other nutrients like P, Mo, or Fe, which are critical for and often limit N_2 fixation. It is clear that the relationship among plant species in mixed communities, such as is found in many native plant communities, grasslands, and pastures, will change, at least in the short term under higher atmospheric CO_2.

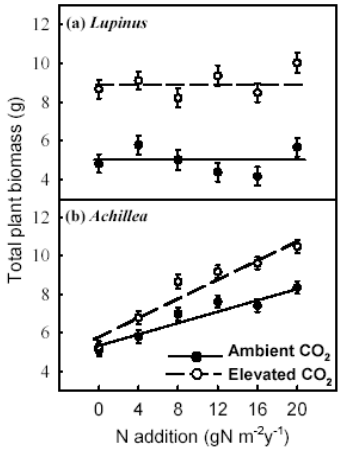


Figure 1. Response of lupine and yarrow plant biomass to increased CO_2 in the atmosphere and to available N.

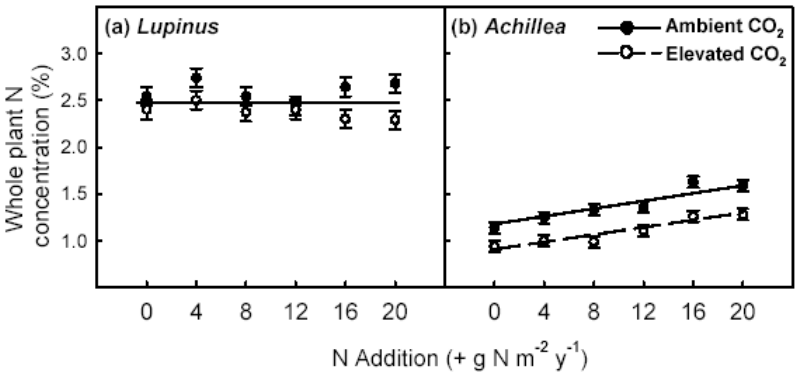


Figure 2. Response of lupine and yarrow plant N concentration to increased CO_2 in the atmosphere and to available N.

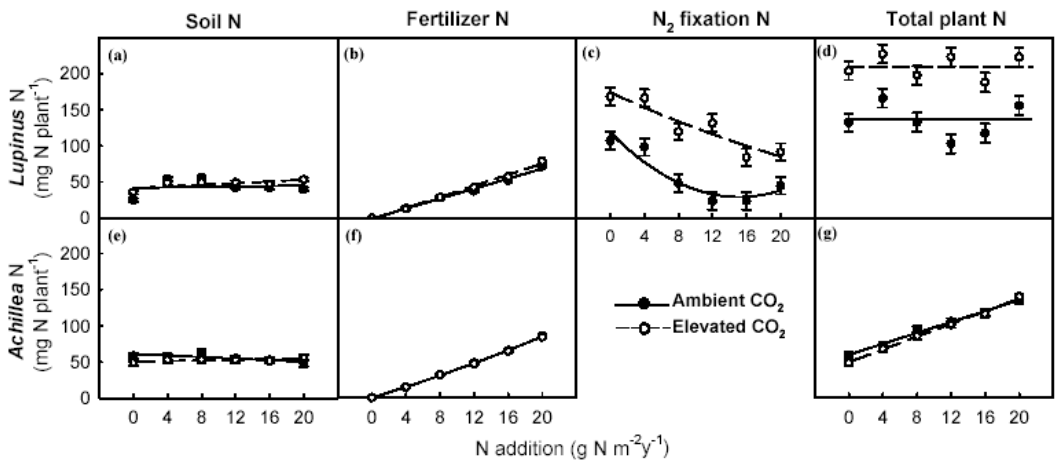


Figure 3. Sources of N in lupine and yarrow under different inorganic N supply and atmospheric CO_2 levels.

Dinitrogen Fixation in Illinois Bundleflower

J. Byun, C.C. Sheaffer, M.P. Russelle, N.J. Ehlke, and D.L. Wyse

Introduction

Legumes have the potential to increase forage production and quality in warm-season pastures and to serve as an ecologically sustainable source of N. Fixed N can be transferred from legumes to companion grasses, reducing the need for application of fertilizer N. Agroecosystems dependent on legume-fixed N rather than fertilizer N tend to have improved N balances and reduced leaching losses. The use of perennial species rather than annual species provides additional benefits of reduced erosion and improved soil nutrient cycling. However, in the northern USA, warm-season grasses are not compatible with alfalfa or other cool-season legumes, due to differing biomass accumulation patterns. On the other hand, many native warm-season legumes are not persistent when grown in mixtures with grasses.

Illinois bundleflower, an herbaceous perennial legume native to the central USA as far north as Minnesota and North Dakota, may fulfill the need for a persistent, high-quality warm-season forage legume. According to preliminary work in Minnesota by Lee DeHaan towards developing varieties of Illinois bundleflower adapted to the north-central USA, northern accessions of the species produce much of their biomass between July and August, when productivity of cool-season grasses and legumes in the region is low. In addition to its value as a forage crop, Illinois bundleflower produces large yields of seeds with high protein concentration. Given the growing interest in Illinois bundleflower as a forage and grain legume, it is important to obtain information on its N_2 fixation capability.

Methods

Experiments were conducted at three Minnesota locations: at Becker on a Hubbard loamy sand, at Rosemount on a Waukegan silt loam, and at Lamberton on a Normania clay loam. Soil fertility was modified according to soil test results at each location. Three accessions of Illinois bundleflower from the University of Minnesota Perennial Native Legume collection were grown. Accessions were selected for winter survival, maturation, height, and seed yield characteristics using the data from DeHaan's evaluation of phenotypic diversity among accessions of Illinois bundleflower from the north-central USA. Wild senna [*Senna hebecarpa*], a warm-season perennial legume native to the northeastern and east-central USA, was grown as the non-N_2-fixing reference plant for use in the ^{15}N methodologies. In Minnesota, it has similar emergence times and biomass accumulation patterns to Illinois bundleflower. Seed was planted at all locations in spring 2000.

We used three methods to estimate N_2 fixation: ^{15}N natural abundance, ^{15}N isotope enrichment, and total N difference. Plants were harvested at maximum aboveground biomass, which occurs at approximately 10% seedpod fill, before significant leaf loss. Harvest dates were mid August to mid September in 2000 and 2001. We also determined nodule occupancy at each location using PCR.
Results and Conclusions

The PCR results revealed that at both Becker and Rosemount, 26 of the 30 isolates per location were derived from a single inoculant strain. In contrast, 13 of the 28 isolates from Lamberton represented multiple indigenous strains, while the others were derived from the single inoculant strain found at Becker and Rosemount. Estimated N_2 fixation was considerably higher at Lamberton than at the other locations, which suggests that the single inoculant strain that was most competitive may not provide optimal N_2 fixation rates.

Herbage yield averaged across accessions ranged from 1.0 Mg ha^{-1} to 3.7 Mg ha^{-1} in yr 1, and 3.0 Mg ha^{-1} to 8.3 Mg ha^{-1} in year 2. Accessions differed in herbage yield, aboveground N yield, and N_2 fixed at certain locations in year 1, but did not differ among the locations in year 2. Estimates of percentage of N derived from the atmosphere (%Ndfa) varied with location but not with accession in either year. Differences in N_2 fixed among accessions in year 1 were therefore due to differences in N yield rather than to %Ndfa. The ^{15}N natural abundance method gave consistently lower estimates of %Ndfa than the ^{15}N enrichment method. In year 1, N_2 fixation estimates ranged from 0 to 30 kg ha^{-1} N (^{15}N natural abundance method), 11 to 43 kg ha^{-1} (^{15}N enrichment method), and 0 to 50 kg ha^{-1} N (total N difference method), and in year 2 these estimates at two locations were 60 to 67 kg ha^{-1} N, 79 to 127 kg ha^{-1} N, and 67 to 142 kg ha^{-1} N, respectively. No N_2 was fixed in year 2 at Rosemount, possibly due to adverse weather or to S and/or Mo deficiency.

We conclude that Illinois bundleflower can produce reasonable herbage yields in southern Minnesota, provided that appropriate rhizobia are present and that nutrient supply does not limit growth.

Strategic Reductions in Nitrate Leaching Using Alfalfa

D.W. Kelley and M.P. Russelle

Introduction

Long-term environmental impacts of agricultural activities on ground and surface water quality are of major concern to society. For example, high levels of nitrate (NO_3^-) loading from the Mississippi River, which drains the bulk of the agricultural area in the central USA, may be a primary contributor to the hypoxic zone appearing annually in the Gulf of Mexico. In addition, human health may be impaired by NO_3^- in drinking water.

Leaching of excess or non-utilized NO_3^- below the plant root zone depends on both soil solution NO_3^- concentration and amount of water percolation. Soil characteristics (texture, structure, depth, organic matter content) affect water percolation rates. Weather, including irrigation events in agricultural settings, interacts with crop water use to affect percolation volumes and timing.

Plant species differ in N requirements and uptake potentials, which moderates the quantity of NO_3^- available for leaching. Annual summer crops such as corn and soybean need to be reestablished yearly, and there is a relatively limited period between rapid crop growth in late spring and senescence in the late summer when available NO_3^- is absorbed by plants. Additionally, their root systems are generally shallow (<1.5 m), limiting access to only the upper portions of the soil solution. Established stands of cool-season perennial species, such as alfalfa, begin to actively absorb water and NO_3^- early in the growing season and continue later in the year than annual summer species. Because their root systems often are deeper and can access soil solution to greater depths and over a longer time than annual crops, these perennials have more opportunity to remove NO_3^- from percolating water. Our objective was to evaluate how conversion of strategically selected land to alfalfa might reduce NO_3^- leaching in Midwestern landscapes. Such conversion might also improve the profitability of dairy farming, due to reduced alfalfa hay prices.

Methods

Model simulations were made using the GLEAMS model (version 3.0) to predict NO_3^- leaching below the root zone under various management and weather conditions. Results were generalized over larger study areas using GIS software. Three study areas were selected in the Midwestern USA from Nebraska to Illinois. These areas are predominantly agricultural, where annual cropping systems, primarily corn-soybean rotation and continuous corn, dominate the landscape. The mean annual precipitation in these transects ranges from 640 mm yr^{-1} in the west (NE), where irrigation is often necessary for optimum yields, to 940 mm yr^{-1} in the east (IL), where rainfed agriculture is typical. Continuous corn and corn-soybean rotations were used in the model as the representative annual crops, and alfalfa was used as the perennial crop. Soil maps from the STATSGO database, at a mapping scale of 1:250,000, were used for extracting soil characteristics. Topology and attribute data were obtained from the USDA-NRCS Soil Survey Division. From these statewide coverages, representative transects averaging about 23,000 km^2 were delineated and displayed for this modeling simulation. Following model calibration with field data from the region, the model was applied to 40-ha sample areas in each soil type identified in each transect using 20 years of weather data (1980-1999) from the closest available station based on quadrant location within the transect.

Results and Discussion

Predicted leaching for this 20-yr precipitation record was highest under continuous corn for soils coarser than fine sandy loam (>27 kg N ha^{-1} yr^{-1}). Predicted leaching was lowest under fine-textured soils (silt loam or finer) for all crops, but lowest overall under alfalfa regardless of soil type (3 to 10 kg N ha^{-1} yr^{-1}). The predicted losses under alfalfa may be exaggerated, because we limited active rooting depth to 1.5 m in these simulations. Critical areas or "hot spots" were apparent in all three areas under annual crops, but were absent under alfalfa for the same conditions; one of the more noticeable areas was in Nebraska under continuous corn on a coarse sandy soil, where predicted losses were 30 to 40 kg N ha^{-1} yr^{-1}. Extensive leaching was predicted in select areas of Iowa and Illinois for annual crops, but with a greater extent under continuous corn, especially in Illinois with its uniformly greater precipitation. There was generally a 20 kg N $ha^{-1}yr^{-1}$ difference in predicted NO_3^- leaching losses between perennial and annual crops in Iowa and Illinois for this precipitation record.

Conversion of selected areas of annual crop in each transect (3% of the area in NE and IA, 13% for IL) to alfalfa resulted in a reduction in predicted NO_3^- leaching of between 1400 and 5600 Mg N yr^{-1} from continuous corn and between 600 and 2700 Mg N yr^{-1} from corn-soybean rotations. This reduction is partly attributable to alfalfa's deeper root system and higher N-removal capacity, and partly to the reduction in external N inputs that accompanies the transition to a crop that requires no commercial fertilizer application. A more aggressive conversion (roughly 23% for IA and IL, and 47% for NE) could reduce the predicted mass of NO_3^- losses by 6100 to 8600 Mg N yr^{-1} from continuous corn and 1300 to 3900 Mg N yr^{-1} from corn-soybean rotations.

Conclusion

Most perennial forages have limited lifespan. The land manager will need to be particularly careful to avoid NO_3^- leaching when establishing a new stand or when rotating to an annual crop. Appropriate legume N credits should be used to reduce subsequent fertilizer N applications or effective diagnostic tests (such as the pre-sidedress soil NO_3^- test) should be employed to determine the need for fertilizer N. This has proven successful in limiting NO_3^- losses during rotation from alfalfa to corn. We do not recommend that perennial forages on these sensitive sites be harvested by grazing, as the preponderance of literature shows that grazing increases the potential for NO_3^- leaching.

Questions arise about how to determine which "hot spots" should be converted to perennial crops and how to achieve the conversion. Clearly, when those soils overlay surficial drinking water aquifers or are tile drained, it is in the public interest to limit NO_3^- leaching. Motivation could be provided by persuasion, peer pressure, a subsidy to protect the public resource (such as the Conservation Security Program provides), or regulation. In any case, our research confirms work by others that the use of simulation models and GIS is a powerful tool to strategically target an environmental protection program.

Forage Handling, Preservation and Storage

The Survival of Silage Inoculant Lactic Acid Bacteria in Rumen Fluid
Z.G. Weinberg, R.E. Muck, and P.J. Weimer

Introduction

Inoculants containing principally lactic acid bacteria (LAB) are used as silage additives in order to improve preservation efficiency. In recent studies, silage inoculation has increased milk production or rate of gain in approximately half the animal trials. However, the cause of improved animal performance is unclear. Some results suggest a possible probiotic effect from inoculant LAB, the mechanism of which is also unclear. One hypothesis is that specific LAB strains interact with rumen microorganisms to enhance rumen functionality and animal performance. For this to occur, LAB ingested by the animals would have to survive in rumen fluid in order to affect rumen microflora. The purpose of the current study was to assess the survival of selected LAB from commercial silage inoculants in rumen fluid.

Methods

Rumen fluid (RF) was collected for each experiment from two fistulated Holstein cows fed on total mixed ration containing 30% alfalfa silage, 30% corn silage, 10% solvent soybean meal, 30% ground shell corn and supplemental vitamins and minerals. The combined RF from the two cows was strained (SRF) through four layers of cheesecloth. The SRF was subdivided into sterile Erlenmeyer flasks, each of which was inoculated with a commercial LAB silage inoculant at 10^7 cfu ml^{-1}. Rumen fluid with no LAB inoculant served as a control. Each flask was further subdivided, and to one half was added sterile 50% (w/v) glucose solution to a final concentration of 5 g l^{-1}. The various treatments were added to sterile serum bottles that were flushed with CO_2 before sealing. The bottles were incubated at 39^0C without shaking. At 6, 12, 24, 48 and 72 h after inoculation two bottles from each treatment were sampled for analysis. The rumen fluid was analyzed for pH and LAB. The 48 and 72 h samples were also analyzed for lactic acid and volatile fatty acids.

Results and Discussion

The inoculants were tested in two experiments: inoculants 1 to 5 in experiment 1 and 6 to 12 in experiment 2. The fresh SRF prior to inoculation contained LAB counts of 6.2 and 5.7 $\log_{10}$ cfu ml^{-1} and pH values of 5.70 and 5.57 for experiments 1 and 2, respectively. Thus, inoculation provided approximately a 10-fold increase in LAB numbers over background levels in the control treatment.

Fig. 1A shows the change in pH of selected treatments during incubation of SRF in Experiment 1. In both experiments, glucose supplementation resulted in lower pH values throughout the incubation period, as compared with no glucose addition. A striking observation is that many of the inoculated

treatments had higher pH values than the respective uninoculated control during the incubation period. Without glucose, 8 of 12 inoculants resembled strain 2 (Fig. 1A), providing a significantly higher pH than the uninoculated treatment at 24 to 72 h. With glucose, 8 of 12 inoculants (not necessarily the same strains) had higher pH values at 24 and 48 h than the control, like strain 2.

Fig. 1B shows the change in LAB of selected treatments with time in the SRF. In the inoculated treatments without glucose addition, there was generally a decrease in LAB counts relative to the inoculation rate (10^7 cfu ml^{-1}) especially in the initial 12 to 24 hours, followed by a recovery at 48 h to near initial values. With glucose, the LAB counts in inoculated treatments declined less in the first 12 h and generally were higher at a given time point than those in corresponding vials without glucose. LAB numbers in the control samples increased, without and with glucose, for the first 24 h and decreased later in each experiment.

Some shifts in fermentation products were observed, but the results were not consistent across the two experiments. In Experiment 1, propionate and butyrate were significantly affected by inoculation, decreasing and increasing, respectively, relative to the controls. In Experiment 2, in which volatile fatty acid (VFA) levels in the control samples were high, inoculation suppressed VFA formation. Inoculation in Experiment 2 increased the molar fraction of propionate and produced a trend for reduced acetate.

Conclusions

The LAB tested were able to survive and in many cases grow in strained rumen fluid. As expected, glucose addition markedly enhanced the survival of the inoculant LAB in the rumen fluid, suggesting that silage inoculant LAB strains can compete effectively with rumen microflora in the presence of exogenous glucose. Many of the LAB inoculant strains were able to buffer pH and shift VFA profiles. This suggests that at least some inoculant LAB strains could have an effect on rumen microbial fermentation. However, more research is needed to determine if this direct effect is the principal means by which silage inoculants improve animal performance.

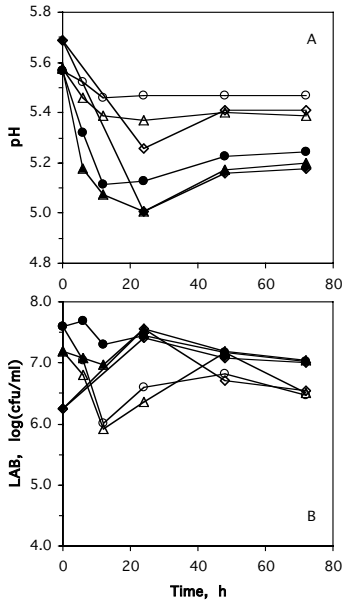


Figure 1. Changes in pH and LAB counts in strained rumen fluid during incubation with inoculants in Experiment 1. ◆Uninoculated; ▲ Inoculant 1; ● Inoculant 2. Open symbols, without glucose; solid symbols, with glucose.

Density and Losses in Pressed Bag Silos

R.E. Muck and B.J. Holmes

Introduction

The pressed bag silo is an increasingly popular method of making silage. It is relatively inexpensive. Storage size varies with the quantity of forage harvested. For farms with expanding herd size, silo capacity can be added with little capital cost. Small diameter bags allow small farms to consider making silage rather than hay. Finally, bag silos make it easy for farmers to inventory and manage silage, e.g., reserving high quality silage for the best animals. While bag silos have been used for more than 20 years, relatively little research has been published on the performance of these silos. Thus, the objectives of this study were to measure densities and losses in bag silos and to determine potential factors affecting both.

Methods

All bag silos made at three University of Wisconsin research farms [Arlington (Arl), Prairie du Sac (PDS), West Madison (WM)] during 2000 and 2001 were monitored. Most of the silages were either alfalfa or whole-plant corn. The primary bagging machines were a 2.4 m Ag-Bag G6000 (AB) at PDS and a 2.7 m Kelly-Ryan DLX (KR) shared between Arl and WM. Occasionally Arl rented a 2.7 m Ag-Bag machine. All loads of forage entering the bags were weighed. While each load was emptied into a bag, a grab sample was taken consisting of a composite of several handfuls. After each load was pressed into the bag, the side of the bag was marked to indicate the distance filled by the load. The load samples were analyzed for moisture content and particle size distribution. At emptying, the weight of all silage removed from a bag was recorded. Any spoiled silage not fed was weighed and specifically identified as such on the emptying log. A grab sample from the face of each silo was taken periodically, one per filling load. Spoiled silage was sampled separately. Samples from emptying were analyzed for moisture, pH and fermentation products. Average densities for the bags were calculated based on weight ensiled, overall length and nominal diameter. Core samples were taken at the face of several bags during emptying to measure density variation across the face.

Results and Discussion

Over the two years, 47 bag silos were made at the three farms. All were filled rapidly with no longer than two days from the start of filling until sealing. The dry matter (DM) contents of the hay crop silages were generally drier than recommended (30 to 40% DM) whereas the corn silages were largely within that range.

Dry matter densities for the 47 bag silos are shown in Figs. 1 and 2. Dry matter density increased with DM content in hay crop silages on average 3.0 kg/m^3-% DM. The effects of DM content on density in corn silage varied by bagging machine. Density increased with DM content with the AB machine whereas density was unaffected by DM with the KR. The DM densities in corn silage were generally lower than those in hay crop silages with the KR. Densities with the AB were generally higher in corn silage, particularly corn silage without kernel processing. Operators affected density. The KR was used at two farms, and one farm consistently averaged higher densities than the other. Densities in hay crop silage with the AB machine at PDS improved the second year after the crew received advice from the manufacturer.

Core samples taken at the face of bags during emptying found considerable variation in density. The outer 30 cm on the top and upper sides had densities on average 40% of those in the center and lower portions, suggesting the need for higher feed out rates than might be anticipated for similar average densities in bunker silos.

Dry matter losses have been calculated on the first year's bags. Average DM losses were 9.5% invisible plus uncollected losses and 6.9% spoilage losses for a total of 16.4% loss. Of the 24 bags, six had severe total losses of more than 25%. The high losses were attributed to either issues of plastic integrity or overly dry silage (>40% DM) being fed out under warm weather. Removing those six bags from the average reduced spoilage and total losses to 2.7% and 11.4%, respectively. Spoilage was primarily at the ends in the remaining bags. Invisible plus uncollected losses did not appear to be affected by feedout temperature or storage time. These losses were elevated at low DM contents and tended to be higher at low feedout rates.

Conclusions

The DM densities in bag silos varied by machine, operator, DM content and crop. The densities observed in this study were somewhat lower than, but within the range of, those observed for bunker silos. However, the wide variation in density across the face plus occasional heating problems in bags fed out at 20 cm/d suggest a minimum feedout rate of 30 cm/d. Our results indicate that the 30 to 40% DM content recommendation for ensiling in bags does result in the lowest losses. In that range, total losses from bag silos can be similar to losses from tower silos. Substantial spoilage losses can occur if bags are not routinely monitored for holes and patched or if overly dry silage (>40% DM) is fed out under warm weather.

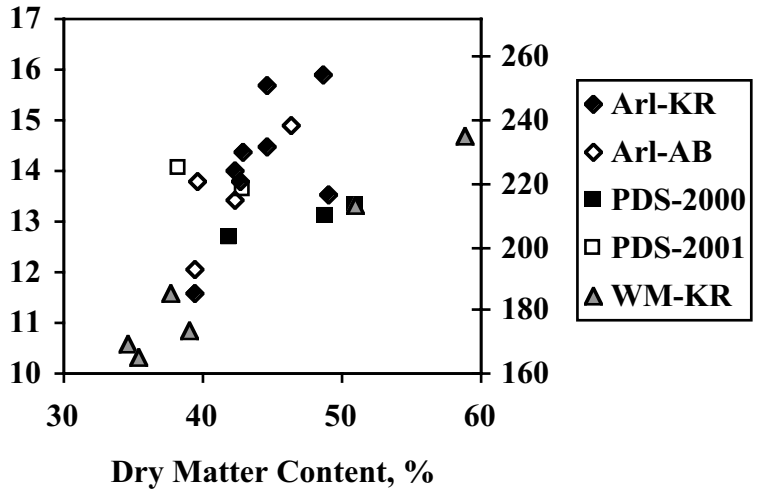


Figure 1. Average dry matter densities (left, lbs/ft^3; right, kg/m^3) in hay crop silages.

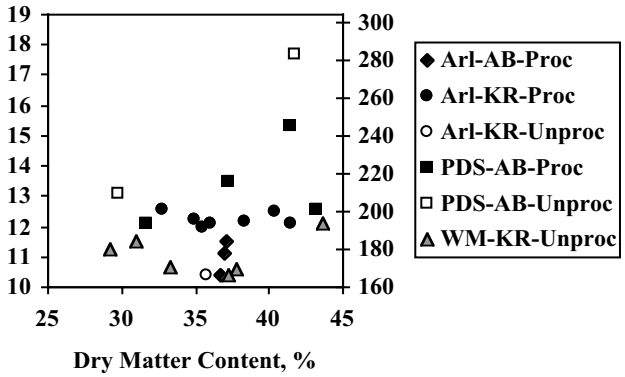


Figure 2. Average dry matter densities (lbs/ft^3, left; kg/m^3, right) in corn silages. Proc - kernel processing; Unproc - not processed.

Factors Influencing Density in Bunker Silos
R.E. Muck, P. Savoie and B.J. Holmes

Introduction

A high silage density is desirable to increase silo storage capacity and reduce silage porosity, thereby reducing oxidation loss and preserving a high feed value. Previous research has shown that dry matter (DM) density in bunker silos is very variable, between 106 and 434 kg/m^3 from a survey of 168 commercial bunker silos in Wisconsin. That study indicated that density was correlated with packing tractor weight, packing time per as fed tonne, layer thickness, DM content, and silage height. However, such correlations between density and a packing practice do not necessarily mean that a particular practice is important for obtaining a high density. A pilot-scale compactor was built and used to test the significance of these packing factors on density.

Methods

A platen press with a 0.58 m long by 0.48 m wide footprint was used to compress chopped forage (alfalfa, grass, whole-plant corn) in a rectangular chamber. The platen was pressed against the forage with a hydraulic cylinder (64 mm dia.), achieving pressures of 20 to 80 kPa. Depending on the trial, layers of chopped forage were laid in non-compacted thicknesses of 0.15, 0.30 or 0.46 m. After each layer was placed in the chamber, the platen was lowered at the designated pressure for times varying between 1 and 10 s. After compaction, the forage was left to relax about 1 min before the next non-compacted layer was added. The compressed and relaxed heights as well as height after adding a new layer were measured to estimate the compressed, relaxed and pre-compression densities, respectively.

For a given trial, an equivalent of six 0.30 m layers were placed in the chamber at a minimum. On a given day, a series of compaction trials were performed by varying only one of the following variables: pressure, layer thickness or time of compaction. The standard conditions were 40 kPa, 0.30 m layer thickness and 5 s hold time. All the forage for a given day was chopped with a commercial forage harvester, usually set to 10 mm theoretical length of cut, and blown in the back of a pickup

truck for transport to the press. Moisture content of the forage was measured to calculate DM density but was not controlled from one experiment to the next.

Results and Discussion

A total of 48 trials were performed (17 alfalfa, 3 alfalfa-grass mixture, 3 grass, 25 corn). Three sets of trials with corn are shown in Figs. 1 to 3. Pressure (Fig. 1) increased density, and the magnitude of the differences between pressures increased with each succeeding layer. Longer times of compaction per layer (Fig. 2) also increased density, but the effect was not linear. Increasing compaction time per layer from 1 to 2 s substantially increased density, but longer compaction times (5, 10 s) produced only small further increases in density. On several sets of trials (data not shown), each layer was compressed for 6 s, but the 6 s was achieved in one of three manners: one 6-s compression, two 3-s compressions or three 2-s compressions. How the 6 s of compression per layer was achieved had no consistent effect on density. Layer thickness (Fig. 3) had a smaller effect on density than expected from the bunker silo density survey.

For each trial, the increase in relaxed density with each succeeding layer fit a logarithmic equation well:

$$r = a + b \ln N$$

where **r** is the dry matter density (kg DM/m^3), **a** is a parameter reflecting the density of the first or uppermost compacted layer, **b** is a parameter reflecting the increase in density with an increasing number of layers, and **N** is the number of 30-cm layers. In hay crop forages, **b** was significantly affected by DM content and pressure whereas **a** was also affected by crop and chop length as determined by stepwise regression. Layer thickness and time were not significant. In corn, time and pressure affected both **a** and **b**. Additionally, processing affected **a** whereas layer thickness affected **b**.

Conclusions

The pilot-scale compactor results indicate that pressure, packing time and layer thickness are important in determining density, but the magnitudes of contributions of these packing factors to density are somewhat different than suggested by our earlier survey of bunker silos. From the pilot-scale trials, pressure appears more important than the other two. However, field-scale trials will be important to confirm the results obtained in the pilot-scale research.

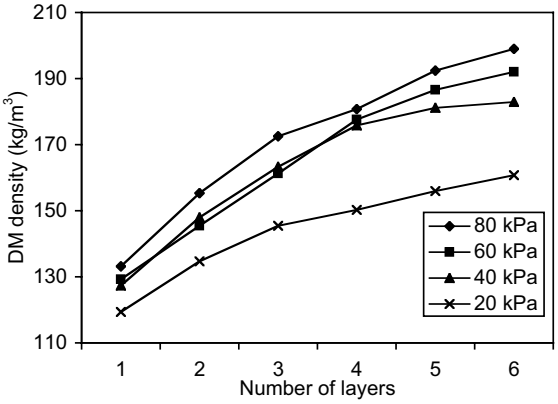


Figure 1. Effect of pressure on relaxed DM densities in corn silage.

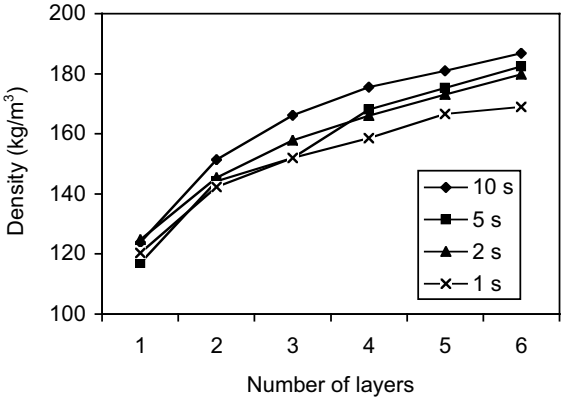


Figure 2. Effect of compaction time per layer on relaxed DM densities in corn silage.

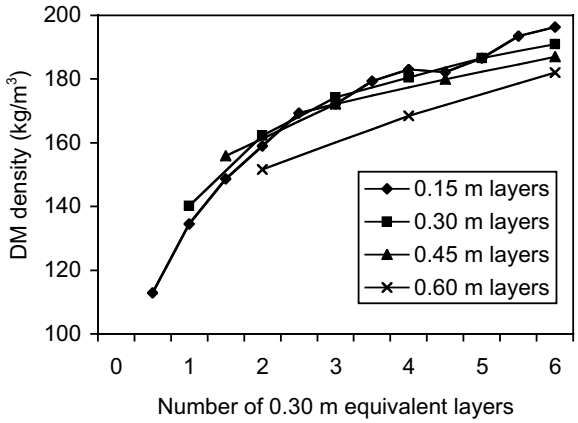


Figure 3. Effect of initial layer thickness on relaxed DM densities in corn silage.

Plant Chemistry/Biochemistry

pH-Dependent Lignin Cross-Linking Predominates Over Lignin Structure in Controlling Maize Cell Wall Degradability

J.H. Grabber, R.D. Hatfield, and J. Ralph

Introduction

The enzymatic degradation of fiber in grasses declines during plant maturation due to progressive lignification of primary and secondary cell walls. In most cases, lignified primary walls are less degradable than secondary walls. Differences in the rate of monolignol secretion into the apoplast and apoplastic pH during lignification may contribute to degradability differences between primary and secondary walls. Rapid "bulk" polymerization, as may occur in primary walls, favors C-C coupling of monolignols into a highly branched polymer. A branched structure may enhance entrapment of structural polysaccharides by lignin, restricting access of hydrolytic enzymes into primary walls. In contrast, slow "end-wise" polymerization, as may occur in secondary walls, favors β–*O*–4 coupling of monolignols into a relatively linear polymer. A linear lignin structure is thought to permit greater access of hydrolytic enzymes into secondary cell walls. Apoplastic pH also influences lignin structure by altering the reactions of quinone methide intermediates formed by β–*O*–4 coupling of monolignols. Acidic conditions (pH <5) favor the reaction quinone methide intermediates with water, uronic acids, or neutral sugars to form relatively linear lignins substituted with α-hydroxyl groups or cross-linked by α-ester and α-ether linkages to cell wall polysaccharides (Fig. 1). Less acidic conditions (pH >5) enhance the reaction of quinone methide intermediates with monolignol hydroxyl groups to form lignins with a highly branched structure. In this study, dehydrogenation polymer-cell wall (DHP-CW) complexes were formed by *in situ* polymerization of coniferyl alcohol into primary walls to evaluate the effect of monolignol polymerization rate and apoplastic pH on lignin formation and cell wall degradability.

Methods

Primary cell walls isolated from suspension cultures of corn (*Zea mays* L.) were suspended in pH 4 or 5.5 buffers and synthetically lignified by gradual "end-wise" or rapid "bulk" polymerization of coniferyl alcohol. During lignification, the activity of cell wall peroxidase was monitored with guaiacol. DHP-CWs were collected on glass-fiber filters and washed thoroughly with water followed by acetone to remove non-incorporated coniferyl alcohol. Degradability was estimated by incubating DHP-CWs with a cocktail of fungal enzymes with pectinase, hemicellulase, and cellulase activities. DHP-CWs and indigestible residues were hydrolyzed with H_2SO_4 to estimate Klason lignin. Enzyme and acid hydrolysates from DHP-CWs and indigestible residues were analyzed for uronic acids and neutral sugars. DHP-CW complexes were saponified with 2 M aq NaOH to release alkali-labile ferulate and diferulates for analysis by GC-FID. Monomeric lignin products released from DHP-CWs by thioacidolysis were identified by GC-MS and quantified by GC-FID.

Results and Discussion

Coniferyl alcohol was more efficiently incorporated into cell walls as end-wise polymers at pH 5.5 (93%) than as bulk polymers formed at either pH (59-68%). End-wise lignification was less efficient at pH 4 (48%) due to inactivation of cell wall peroxidase. Therefore, if lignification of primary walls begins as a bulk polymerization process, it must quickly transition to a more end-wise process if large quantities of wall-bound lignin are to be formed. Extensive lignification of grass cell walls under acidic conditions would also be unlikely unless the abundance or acid tolerance of wall peroxidases far exceeds that of our model system. Thioacidolysis of cell walls revealed that end-wise polymers had 1.8 to 2.6 fold more ether inter-unit linkages and 70% fewer end-groups than bulk polymers (Table 1). Low pH enhanced the formation of ether linkages, particularly for end-wise polymers. End-wise and bulk polymers depressed the enzymatic degradability of cell walls to the same degree, indicating that both types of lignin have similar interactions with structural polysaccharides. In contrast, lowering apoplastic pH from 5.5 to 4.0 during lignification reduced the degradability of cell walls by about 25% (Table 2). Low lignification pH depressed wall degradability only as cell walls become hydrophobic with at least 10% lignin. In another experiment, it was observed that low lignification pH depressed the degradability of DHP-CWs formed with coniferyl alcohol but not with coniferaldehyde (Table 3). Although coniferaldehyde undergoes similar types of coupling reactions as coniferyl alcohol, β–*O*–4 coupling of coniferaldehyde does not permit benzyl ester and ether cross-linking of matrix components *via* nucleophilic addition to quinone-methide intermediates. Cross-links of this type are most readily formed under hydrophobic and acidic lignification conditions; the same conditions that depressed the degradability of DHP-CWs formed with coniferyl alcohol. The release of major neutral sugars and uronic acids by hydrolytic enzymes were uniformly depressed by forming lignins at low pH (Table 4), suggesting that cross-linking *via* quinone methide intermediates involved all types of structural polysaccharides. After exhaustive enzymatic hydrolysis, degradability differences for each cell wall sugar were very small (*ca* 25 mg/g), suggesting that benzyl ester and ether cross-links were only slightly more abundant when walls lignified under acidic conditions. Future work will be directed at characterizing the types and abundance of benzyl ester and ether cross-links formed in DHP-CWs and in naturally lignified plant cell walls.

Conclusion

Overall, our results suggest that lignification pH influences lignin-matrix interactions, perhaps by altering the abundance of benzyl ester and ether cross-links formed *via* lignin quinone methide intermediates. Additional studies are, however, needed to clearly establish the existence of these cross-links in our model system and whether acidic conditions increase their abundance in cell walls. Our results suggest that small variations in benzyl ester and ether cross-linking can have a considerable impact on cell wall degradability. These results further support our contention that cell wall degradability is influenced by the amount of lignin in cell walls and its interactions with matrix components and not by normal variations in lignin composition or structure. The latter may only influence cell wall degradability if they markedly alter the hydrophobicity of lignin or its propensity to form cross-linked structures with other cell wall components.

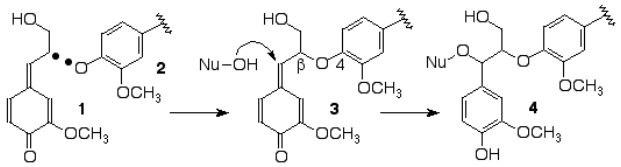


Figure 1. Monolignol **1** and lignin **2** radicals can undergo β-*O*-4 coupling to form quinone methide intermediates **3**. These intermediates are stabilized by the addition of nucleophiles (Nu), such as water, uronic acids, and neutral sugars to form structures **4** which are substituted with α-hydroxyl groups or cross-linked by α-ester and *α*-ether linkages.

Table 1. Klason and mass balance lignin concentrations (mg/g), and guaiacyl (G) units (μmol/g) released by thioacidolysis of DHP-CWs. Values in parentheses indicate percent of yield derived from end groups.

Treatment	Klason lignin	Mass lignin	G yield
Bulk polymerization			
pH 4.0	123.8	76.1	556 (28.8)
pH 5.5	126.4	81.6	537 (26.2)
End-wise polymerization			
pH 4.0	117.3	71.7	1441 (9.5)
pH 5.5	122.8	103.9	949 (8.3)

Table 2. Carbohydrate released (mg/g) from DHP-CWs at a Klason lignin content 123 mg/g after 6 and 72 h of hydrolysis by Celluclast and Viscozyme.

Treatment	6 h	72 h
Bulk polymerization		
pH 4.0	128	385
pH 5.5	172	473
End-wise polymerization		
pH 4.0	128	367
pH 5.5	181	487

Table 3. Carbohydrate released (mg/g) from DHP-CWs at a Klason lignin content of 120 mg/g after 6 and 72 h hydrolysis with Celluclast and Viscozyme.

Treatment	6 h	72 h
Coniferyl alcohol		
pH 4.0	169	504
pH 5.5	259	520
Coniferaldehyde		
pH 4.0	155	395
pH 5.5	161	377

Table 4. Concentrations (mg/g) of arabinose (Arab), galactose (Gal), glucose (Glu), xylose (Xyl), and uronosyls (Uro) in DHP-CWs and their release (%) by fungal enzymes. DHP-CWs were lignified at pH 4.0 or 5.5 to a Klason lignin concentration of 123 mg/g.

	Arab	Gal	Glu	Xyl	Uro
Monosaccharide concentration					
DHP-CW	167.8	74.4	287.4	136.9	91.0
Release of monosaccharides					
6 h hydrolysis					
pH 4.0	19.8	19.1	19.0	10.5	26.5
pH 5.5	23.2	22.6	26.5	15.1	35.3
72 h hydrolysis					
pH 4.0	58.1	58.5	51.6	36.1	80.3
pH 5.5	71.8	73.8	66.1	50.0	82.9
Exhaustive hydrolysis					
pH 4.0	76.2	83.6	87.3	61.1	91.6
pH 5.5	79.6	85.8	90.7	63.6	93.3

A Comparison of Methods For Determining Lignin in Plant Samples

R Fukushima and R.D. Hatfield

Introduction

As forages mature there is generally a decline in digestibility of the fiber fraction (cell wall) that is associated with an increase in lignin. People have tried to use lignin concentration as a means of predicting cell wall and whole plant digestibility. This seems to work reasonably well as long as one is making comparisons within the same plant species. One of the problems when trying to compare published data from a range of plants is the type of lignin assay method used may vary. Not all procedures give you the same results. It has been shown that acid detergent lignin (ADL) methods result in a significant solubilization of lignin from grass cell walls, this is not the case with legumes or other plant samples. We have been interested in refining the acetyl bromide soluble lignin (ABSL) as a method for measuring lignin in forage samples. It is convenient and allows several samples to be run in a single day. It is also applicable to using small sample sizes if tissue size is a problem. We have compared a number of plant samples using the four most popular lignin methods, ADL, ABSL, permanganate lignin (PerL), and Klason lignin (KL). In addition, we have determined the IVDMD and IVCWD of these samples to see how the lignin values generated by these procedures correlate to digestibility.

Methods

Lignin standards for the acetyl bromide method were extracted from the cell walls of a wide range of plants (see Table 1 for a complete list). These same plants were used for the ABSL, ADL, PerL, and KL lignin determinations as well as materials for IVDMD and IVCWD determinations. All analyses were run in duplicate. Statisitical anlaysis was used to correlate lignin method concentration with digestibility characteristics.

Results and Discussion

In order to utilize the ABSL method, extinction coefficients for each type of isolated lignin were generated. Theoretically all lignin should give the same or similar results. As can be seen in Table 1 this is generally true with a couple of exceptions. Even though extraction of lignin from plant cell walls provides a relatively clean preparation of lignin it is still contaminated with small amounts (10-15%) of carbohydrates and proteins. Corrections for these contaminates is dependent upon accurate assessment of what and how much is in the different lignins in order to make adjustments for absolute weights of lignin.

Other methods of lignin determination also have a problem when it comes to determining the absolute amount of lignin. As already mentioned the ADL method can greatly underestimate the total lignin in forage samples, especially grasses. This is due to the partial solubility of lignin/lignin-carbohydrate complexes in the hot detergent solution. This is not as much of a problem for legumes suggesting that the lignin structure of these two different types of plants differs in their physio chemical make up. Permanganate lignins are based upon the ability to oxidize the lignin from a plant cell wall without affecting the cell wall carbohydrates. This method was developed originally for woody species in which the cell wall carbohydrate was predominately cellulose. In forages the cell wall contains significant amounts of other polysaccharides that are quite susceptible to oxidation by

permanganate. For non-woody species this procedure tends to give much more variable results depending upon the cell wall make-up. Klason lignin is one of the oldest procedures and is frequently used for forage samples. The ADL procedure is basically the same except for the hot acid detergent treatment to insure the removal of protein and non-cellulosic polysaccharides before acid solubilization of cellulose. Klason lignin values tend to be over-inflated due to the potential for protein contamination. This is not too much of a problem with samples that are typically low in protein such as stem tissues. The acetyl bromide method does not have the problems of contaminating materials since it is based on absorbance of lignin dissolved in the reaction mixture. However, it is important to start with a sample preparation in which small molecules (simple phenolics compounds) have been removed, i.e., doing cell wall isolation before analysis. Also carbohydrate degradation during the heating step can contribute to some additional non-lignin absorbance.

We have compared several forage samples using these four lignin techniques to determine how each procedure within general groupings of plant samples (Table 2). From the data in Table 2 it is clear that each procedure generates a different lignin value. Depending upon the sample type some procedures given similar results; for example generally Klason lignin and ABSL procedures generated numbers that were similar across all species. It is not possible to say that one type of method gives the true lignin value for a given forage species. We also compared the digestibility of these forage materials (Table 2). Statistical correlation analysis of the lignin data with digestibility data indicated that no method was highly correlated to digestibility, although Klason and ABSL provided the best correlations (Table 3).

Conclusions

It would appear that the acetyl bromide method for determining lignin concentration holds promise as a technique for quickly estimating digestibility in a wide range of plants. Further work with a wider range of forage plants, maturities, and environmental growing conditions is needed in order to determine if this relationship will hold up or be improved

Table 1. Lignin coefficients developed for the acetyl bromide lignin method based on lignins isolated from each type of plant material.

Plant Material Description	MEAN	
Alfalfa FB lower 30	14.23	
Alfalfa FB upper 30	15.99	
Alfalfa PSD	15.30	
Alfalfa Y	15.69	
Bromegrass M1	17.11	
Bromegrass M2	16.73	
Bromegrass M3W	17.40	
Bromegrass Y	17.44	
Cornstalk PA	17.75	
Oat straw leaf	20.10	
Oat straw stem	18.91	
Red clover FB	14.49	
Wheat straw leaf	19.81	
Wheat straw stem	17.54	Overall Mean 17.04 ± 1.80

FB= full bloom, PSD= seed development, Y=young, pre bud stage, M1=pre-boot stage, M2= anthesis, M3W= post anthesis, PA= post anthesis

Table 2. A comparison of lignin values generated from application of the four different lignin methods. All values are given in mg g^{-1} cell wall. Also the in vitro dry matter digestibility (IVDMD) and the in vitro cell wall digestibility (IVCWD) is given for each sample.

	mg g^{-1}											
Plant Material	ADL		KL		PerL		ABSL		IVDMD		IVCWD	
	Avg	± STDev	avg	± STDev	avg	± STDev	avg	± STDev	avg	± STDev	avg	± STDev
Alfalfa FB lower30	92.5	±3.0	144.8	±0.4	157.6	±16.8	134.7	±1.6	418.4	±21.3	240.6	±27.7
Alfalfa FB upper 30	59.3	±0.3	111.4	±0.6	95.3	±10.9	71.4	±4.9	616.6	±37.5	394.9	±6.6
Alfalfa PSD	90.6	±1.4	138.8	±1.0	153.8	±10.5	117.3	±2.3	443.2	±11.4	254.1	±13.8
Alfalfa Y	83.6	±2.0	123.0	±3.1	134.7	±6.7	116.6	±4.3	438.1	± 8.8	320.9	±7.7
Bromegrass M1	30.4	±0.1	100.4	±13.8	64.1	±1.7	127.5	±0.4	451.6	±12.8	298.1	±5.9
Bromegrass M2	36.5	±1.3	109.8	±15.6	69.9	±6.4	144.6	±12.9	401.0	±20.4	252.8	±14.1
Bromegrass M3W	45.6	±1.7	130.1	±0.1	67.1	±1.1	139.1	±5.2	366.3	±18.6	139.9	±10.3
Bromegrass Y	28.5	±1.5	102.2	±5.8	56.0	±0.7	123.4	±0.2	527.2	±24.0	365.1	±21.7
Corn stalk PA	24.8	±0.2	76.7	±7.8	45.2	±13.2	92.0	±2.9	498.2	±0.0	289.4	±7.8
Oat straw leaf	106.9	±2.8	138.1	±1.9	71.3	±8.1	123.5	±0.3	254.5	±10.9	331.0	±22.3
Oat straw stem	83.3	±0.6	171.2	±1.6	111.5	±5.4	186.3	±5.9	124.6	±25.0	92.9	±16.9
Red clover FB	41.7	±1.3	71.3	±1.5	115.6	±8.1	90.4	±1.1	531.0	±10.6	410.5	±28.8
Wheat straw leaf	103.4	±10.5	141.6	±0.1	74.3	±11.6	149.9	±3.4	233.8	±26.6	336.5	±22.3
Wheat straw stem	89.1	±10.8	184.2	±2.1	122.0	±12.9	213.0	±15.0	129.7	±58.6	61.6	±22.2

ADL= acid detergent lignin, KL= Klason lignin, PerL=Permanganate lignin, ABSL= acetyl bromide soluble lignin

Table 3. Correlation analysis of lignin determination method with digestibility.

Digestibiltiy	ADL	KL	PerL	ABSL
IVDMD	-0.44	-0.64	-0.06	-0.76
IVCWD	-0.23	0.74	-0.21	-0.83

Characteristics of Red Clover Polyphenol Oxidase

R.D. Hatfield, K. Frost, and M.L. Sullivan

Introduction

Polyphenol oxidase (PPO) also known as O-diphenol oxidase and catechol oxidase, is widely distributed among higher plants. In the presence of oxygen, PPO catalyzes the oxidation of *o*-diphenols to *o*-quinones which non-enzymatically polymerize with proteins and other phenols to produce brown pigments. This property makes the PPO enzyme an important enzyme in the food industry because it often causes post-harvest browning of fruit and vegetable crops leading to off flavors and loss of nutritional quality. To date, no clear function of PPO has been established within the plant, but the characteristic browning of fruit and vegetable tissues and extracts such as banana, grape, blueberry, sweet potato, and loquat have been studied. The distinctive browning reaction of PPO has also been observed in the forage legume red clover (*Trifolium pratense*). However, unlike many of the fruit and vegetable crops, the browning reaction in red clover has been correlated to a reduction of proteolysis in the silo, a desired outcome for producers. The mechanism for this reduction of proteolysis is still unknown, but it is thought to involve both the PPO enzyme and its substrates. We have been characterizing the chemical and biochemical properties of the red clover PPO enzyme in order to possibly determine its function in the plant as well as to understand the mechanism for the reduced proteolysis in red clover silage.

Methods

Fresh red clover leaves were ground to a fine powder under liquid nitrogen before extraction with 20 mM TRIS buffer (100 mM sodium acetate, 50 mM sodium ascorbate, 5% glycerol, pH 7.9). Partially purified PPO extracts were analyzed to determine the pH optimum, temperature optimum, molecular weight, and substrate specificity. A spectrophotometric assay using 5-thio(2-nitrobenzoate) (TNB) as a chromophore was used to monitor PPO activity. The substrate, caffeic acid, when oxidized by PPO to an *o*-quinone reacts with the sulfhydryl group on TNB forming a caffeoyl sulfhydryl conjugate that has decreased absorbance at 412 nm. The assay solution consisted of 20 μl of 100 mM caffeic acid (dissolved in 95% ethanol), 20 μl of TNB solution, 950 μL of 20 mM TRIS buffer (pH 7.0) and 10 μL of the enzyme solution. One unit of activity was defined as the amount of enzyme that resulted in decreased absorbance of 1 unit min^{-1}. For substrate specificity determinations various *o*-diphenols were substituted for the caffeic acid. A 0.01 M piperazine hexahydrate and 0.01 M gly-gly buffer with pH ranging from 4.5 to 9.0 was used to determine the optimal pH for PPO activity. For temperature optimum 20 mM TRIS buffer (pH 7.0) containing caffeic acid (0.25 mM) was used to measure PPO activity directly by monitoring the absorbance shift of caffeic acid (284 nm) as it reacts with the PPO. Absorbance change was monitored for 5 minutes over a range of temperatures from 10-70 ˚C. Temperature stability was measured under the same conditions except that the PPO enzyme solution was pre-incubated at various temperatures for 1, 2, and 4h prior to adding to the reaction mixture.

Results and Discussion

The red clover PPO was partially purified utilizing a series of chromatographic steps including two size exclusion columns Toyopearl HW-40 and Toyopearl HW-55 followed by anion exchange chromatography Toyopearl 650-DEAE. One of the problems with the red clover system is that there

is such a high level of soluble phenolics (including the *o*-diphenol substrates for PPO) that enzyme activity is quickly lost if the enzyme is not separated from these materials. On a small scale (1-3 mL) this can be accomplished quite rapidly and produce a stable crude extract. Increasing the size of the extract results in a significant loss of total activity; however sufficient amounts were obtained that allowed further purification and characterization of the PPO. We were able to obtain an approximately 1400-fold increase in specific PPO activity reflecting a significant amount of enzyme purification. This resulted in an approximate 4% recovery of total protein from the original extract.

The properties of red clover PPO were similar to those published from other species and are summarized in Table 1. The one interesting observation concerning the PPO properties is the maintenance of good PPO activity (≥ 50%) over a wide range of pH conditions. This would probably aid in the effectiveness of inhibiting proteolytic activity in red clover silage since it would be able to maintain good activity as the pH is dropping and the limitation would only be the availability of oxygen required to produce the *o*-quinones. This may help explain why red clover produces good silage even though the pH has not dropped to a level typically thought to be important to inhibit proteolytic activity.

Of particular interest in characterizing red clover PPO is to determine the effectiveness in utilizing a range of *o*-diphenol substrates. There are two *o*-diphenol compounds that have been identified in red clover, phasic acid and clovamide (Fig. 1). We have not been successful in purifying sufficient amounts of these compounds for in vitro chemical analyses. However, we have been able to monitor both compounds in crude extracts of red clover as the endogenous PPO utilizes them. Phasic acid and clovamide are both rapidly utilized in the crude extracts. Since both are derivatives of caffeic acid (Fig. 1) we investigated a range of other *o*-diphenols to determine the effectiveness as PPO substrates (Table 2). From these results it is clear that caffeic acid and close derivatives of it are the preferred substrates of the red clover PPO.

Conclusions

Red clover polyphenol oxidase is a soluble enzyme with an apparent molecular weight of 68 kD. It has relative good temperature stability and is active over a fairly wide pH range (4.5-8.0). These are important characteristics that may contribute to the sustained activity during the ensiling process for red clover. Red clover PPO can utilize several *o*-dihenols, but has much higher activity with substrates that are caffeic acid derivatives.

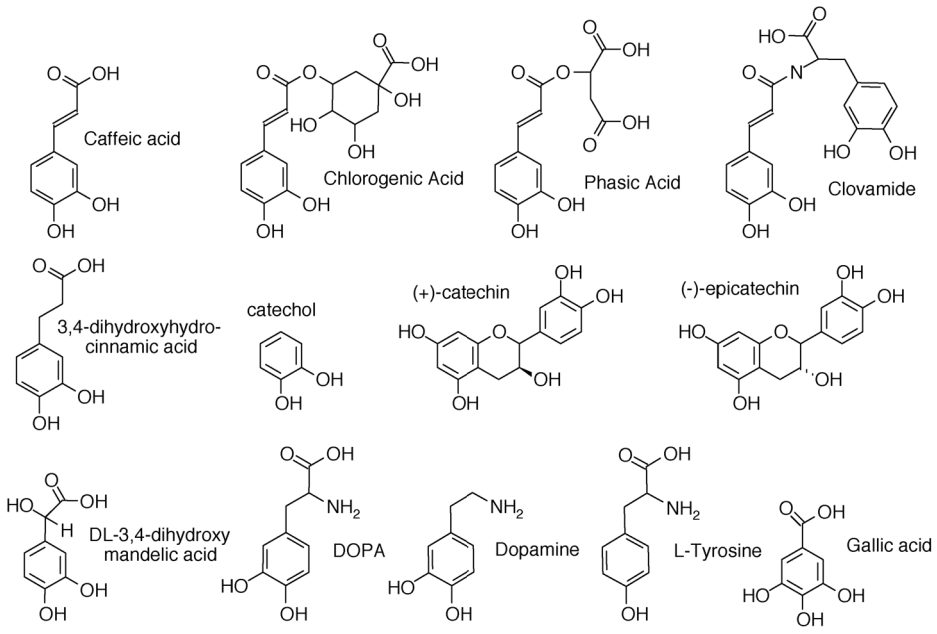


Figure 1. Structural characteristics of O-diphemols tested as possible substrates for the red clover PPO.

Table 1: Properties of the PPO enzyme extracted and partially purified from red clover. All enzyme activities are based upon caffeic acid as the *o*-diphenol substrate.

PPO Characteristic	Value	Comments
Molecular weight	68kD	Based on SDS-PAGE
	71kD	Based upon gel filtration chromatography
pH optimum	6.5-7.0	50% activity fro 4.5 to 8.0 pH
Temperature optimum	25 °C	Broad range 80% activity from 15-35°C
Temperature stability	50 °C	Based on 2h preincubation

Table 2. Relative activities of *o*-dipenol substrates utilized by red clover polyphenol oxidase

Substrate	Relative Activity
caffeic acid	100
phasic acid (estimated)	+90
clovamide (estimated)	+90
chlorogenic acid	96
3,4-dihydroxyhhydrocinnamic acid	28
catechol	24
(-) epicatechin	7
(+) catechin	6
dopamine	6
L-DOPA	3
gallic acid	2
DL-3,4-dihydroxymandelic acid	1
protocatechuic acid	1
tyrosine	0

Non-degradative Dissolution and Acetylation of Ball-milled Plant Cell Walls; High-resolution Solution-state NMR

F. Lu and J. Ralph

Introduction

The ability to dissolve plant cell walls without degradation would provide significantly improved methods for cell wall structural analysis and allow standard solution-state derivatization and reaction chemistries to be more effectively applied.

Researchers have therefore been anxious to develop methods that might allow the entire lignin fraction, and indeed the whole cell wall, to be analyzed by NMR. In the past, we and others have tried to apply the various cellulose solvent systems to the whole cell wall, but with little success. Other dissolution methods are simply too destructive on the components of the wall. Here we describe a solvent system (one of two that we have developed) that fully dissolves finely divided (vibratory ball-milled) cell walls, apparently non-degradatively. Ball-milling is commonly used in lignin isolation procedures. *In situ* acetylation is demonstrated for its value in providing derivatized cell walls that are completely soluble in chloroform. The derivatized cell wall can therefore be analyzed by high-resolution solution-state NMR methods, providing considerable insight into wall structure without the need for polymer fractionation. This work is all on wood cell walls; we make no apologies for developing techniques on the cleaner and more readily available woods first since if it does not work for wood, it won't work for anything. We shall of course be developing these methods for forages in the near future.

Results and Discussion

Cell wall dissolution in DMSO-NMI

A system not previously described as a cell wall or even a cellulose solvent system has the properties required to produce cell wall materials in a form suitable for NMR; it rapidly and completely dissolves the wall, allows for facile derivatization reactions such as acetylation, and can be used to generate products ideal for solution-state NMR. The system is N-methylimidazole in DMSO (DMSO/NMI). Complete dissolution of a variety of ball-milled cell wall materials can be effected in 1-3 h at room temperature. The solutions are homogeneous and clear. N-methylimidazole is an excellent acylation catalyst, so *in situ* acetylation (for example) is readily accomplished. The acetylated cell wall (Ac-CW) is readily isolated, essentially quantitatively, by precipitating into water. It is completely soluble in chloroform or methylene chloride.

NMR of Acetylated Cell Walls

Fig. 1 shows a picture of NMR tubes of Ac-CWs from pine and aspen woods, ca. 150 mg/mL in deuterochloroform ($CDCl_3$); the clarity of the solutions is demonstrated by the background (part of a plotted proton NMR spectrum) showing through the tubes. The solutions are rather viscous at over about 200 mg/mL, but quite mobile at <150 mg/mL. Such concentrations are quite suitable for NMR. However, the lignin component of interest, typically only ~20% of the cell wall, is now considerably more dilute than in samples of comparable total concentration prepared directly from isolated lignins. The 5-fold sensitivity decrease can be offset by utilizing higher-field instruments and/or cryogenically cooled probes.

Although the spectra were expected to be dominated by polysaccharide peaks, the various (acetylated) cellulose, hemicelluloses, and lignin resonances are well dispersed in 2D NMR, allowing substantive interpretation. Here we concentrate on the lignin assignments. Fig. 2 shows 1-bond ^{13}C–^{1}H correlation spectra taken on a 360 MHz spectrometer. The upper plots (a) show the lignin sidechain regions of the Ac-CW spectra and, for comparison and aid in spectral assignment, the same regions from spectra of isolated lignins from similar (but not identical) samples are shown in the lower plots (b).

As seen by the color coding in Fig. 2b1, the diagnostic lignin methoxyl, major β-ether units **A**, substantial phenylcoumaran units **B**, and more minor resinol **C** and dibenzodioxocin **D** units are well resolved in the guaiacyl acetylated milled-wood lignin (Ac-MWL) from pine. The predominantly guaiacyl (4-hydroxy-3-methoxyphenyl) lignins in pine (and softwoods in general) derive from the mono-methoxylated monomer coniferyl (4-hydroxy-3-methoxycinnamyl) alcohol. At least some of the sidechain correlations for all of these structures are also remarkably well resolved in the pine Ac-CW spectrum, Fig. 2a1.

Hardwood lignins, including the poplar lignin shown on the right of Fig. 2, derive more substantially from sinapyl (3,5-dimethoxy-4-hydroxycinnamyl) alcohol and have syringyl/guaiacyl lignins. The additional methoxyl at the aromatic ring 5-position precludes the involvement of syringyl units in 5-linked structures such as the phenylcoumaran **B** and the 5–5-linked moiety of dibenzodioxocins **D**. The lignins are characterized by a higher β-ether **A** content, as well as having more prominent β–β-coupled (resinol) units **C**. Also, cinnamyl alcohol endgroups **X** may be more prominent. The poplar Ac-MWL shows the prominent **A**, and **C** units, and minor amounts of **B** and **X**, Fig. 2b2. Again, these structures and the methoxyl are revealed in the related Aspen Ac-CW spectrum, Fig.

2a2 (although viewing at lower contour levels are needed to clearly see the **B** and **X** units).

The aromatic regions (not shown) are particularly noteworthy in that the aromatic components appear to be entirely due to the lignins as seen by the comparison of the aromatic profile between the Ac-CW and Ac-MWL spectra. Also the anomeric C/H regions for the polysaccharide components are extremely well resolved suggesting that substantive interpretation of these components will be possible. Reduced polysaccharide degree of polymerization (DP) caused by ball-milling is strongly evident. Cursory integration of the cellulose internal anomeric peak to the presumed acetylated α- and β-anomers of the reducing end units (at 88.9/6.22 and 91.5/5.63 ppm) suggest molar ratios of only 20-25:1. This DP estimate of 20-25 is considerably lower than the DP of cellulose reported *in planta*, in the 7-10,000 range, suggesting that ball-milling has cleaved the long cellulose chains hundreds of times. There is no evidence to suggest that the depolymerization is coming from the dissolution processes, but the findings heighten the need to evaluate less severe milling conditions for future work. There are a great variety of ball-milling methods. The steel ball-mill used here is extremely efficient, requiring only 1.5 h. for effecting particle-size reduction. Others use much longer times, and have strongly recommended ball-milling in toluene to avoid lignin structural alteration.

Fig. 3a further illustrates the value of being able to apply solution-state NMR methods. The 2D section is from the same aspen HSQC experiment used in Fig. 2c2, but plotted at much higher contour levels so that only the most intense peaks, essentially just the cellulose and methoxy contours, are seen. The spectrum at this level is almost identical for the pine Ac-CW (not shown). A short 1D ^{13}C experiment is shown on the left projection, with the 1D ^{1}H experiment on the top. Although there is some overlap of the carbons (notably carbons 5 and 3) and the protons (notably proton-1 and one of the 6-protons, as well as the lignin methoxyl with proton-4) in their respective 1D spectra (projections), all six carbon/proton pairs are beautifully resolved in the 2D experiment allowing all of the data to be readily determined. Also shown is a solid-state ^{13}C spectrum of pine wood, Fig. 3b. Although chemical shift comparisons are not valid for this sample compared to the acetylated cell wall in the remainder of the figure, the spectrum is included for comparison of linewidths and resolution. The solid-state spectrum provides additional information regarding the two allomorphic forms of crystalline cellulose, but the greater carbon linewidths (and the extreme linewidths of proton NMR data in solid-state spectra) preclude the acquisition of informative 2D spectra.

Obviously the Ac-CW material can be subjected to the whole range of solution-state NMR methods, including homonuclear correlation experiments such as COSY and TOCSY, and heteronuclear experiments such as the HSQC illustrated in Figs. 2 and 3, and also long-range ^{13}C–^{1}H correlation experiments. Even 3D experiments are perfectly viable, and should provide a way to obtain complete sidechain data for the individual lignin units since each unit is likely to be reasonably well isolated onto its own plane in a 3D TOCSY-HSQC experiment, for example.

Conclusions

Although a total interpretation of all the contours in the congested regions of the spectra is unlikely, it should be obvious that high-resolution solution-state NMR of (acetylated) whole cell wall material will be extremely valuable in assessing the structural components of the wall. The dispersion is sufficient that most lignin structures can readily be identified, and will be potentially quantifiable by

more quantitative versions of HMQC or HSQC spectroscopy. The degree of detail discernable is far higher than can be accomplished by solid-state NMR on solid material, and yet there is every indication that we are looking at the whole cell wall (that has admittedly been degraded by the ball-milling step). Sensitivity reduction caused by utilizing whole cell walls instead of isolated fractions can be largely offset by using cryogenically cooled probes that are becoming common in modern NMR instruments.

For the many valuable samples accumulated from previous studies of lignin-biosynthetic-pathway mutants and transgenics, we shall be applying these new methods to both the whole cell walls and to the residues remaining following the dioxane-water extraction of "milled wood lignins" from polysaccharidases-treated walls — often this material still contains the bulk of the lignin. It should be possible to observe the diagnostic signatures of gene-downregulation that have to date been noted only on the soluble component. For example, COMT-deficient angiosperms (poplar, aspen, corn, arabidopsis, and alfalfa) have been shown to incorporate substantial amounts of the novel monolignol 5-hydroxyconiferyl alcohol into their lignins when the pathway to sinapyl alcohol is down-regulated; this incorporation produces benzodioxane structures in the lignins. The diagnostic α- and β-C/H correlations appear at ~77/5.0 and 76/4.4 ppm, clear regions in the spectra in Fig. 2, suggesting that their detection will be straightforward.

A significant limitation at present is the need to finely grind the material. Fine-grinding, by a method such as vibratory ball-milling, that is already required to isolate lignin fractions, does of course break bonds and therefore reduces polymer DP (as shown here with the cellulose component) and causes structural alteration. We are assessing just how much milling is required. Cryogenic milling to a less fine state also appears to provide material that can be fully dissolved, although material mechanically ground to pass through a 1 mm mesh in a mechanical Wiley mill will not. Although we shall endeavor to find methods that work for less finely ground material, it is already extraordinarily useful to be able to prepare samples of the whole cell wall (and particularly the entire lignin component), without fractionation or partitioning the polymers, in days instead of weeks, for analysis by high-resolution solution-state NMR.

In conclusion, the ability to fully dissolve ball-milled plant cell walls, without further apparent degradation, portends enormous potential for applying high-resolution solution-state NMR to elucidate structural details without having to resort to the laborious isolation methods of the past. It is also expected that the systems will allow the application of standard chemical derivatization and reaction methods, significantly improving the old heterogeneous methods.

Experimental Procedures

Dissolution and Derivatization in DMSO/NMI

The ball-milled cell wall sample (600 mg) was suspended in DMSO (10 mL) and NMI (5 mL) was added. A clear solution formed in 3 h or less, depending on the sample.

Excess acetic anhydride (3 mL) was added and the mixture stirred for 1.5 h. The clear brown solution was transferred into water (2000 mL) and the mixture allowed to stand overnight. The precipitate was recovered by filtration through a nylon membrane (0.2 μm). The product was washed with water (250 mL) and dried under vacuum at room temperature. The weight of acetylated cell walls was typically 137-140% of the weight of the original cell wall.

[Color Figures that are more easily interpretable will be in the pdf version of these Research Summaries available from our web site. The full version of a paper on this subject will be available in the "Full Text Publications" section, directly at: http://www.dfrc.ars.usda.gov/DFRCWebPDFs/pdfIndex.html]

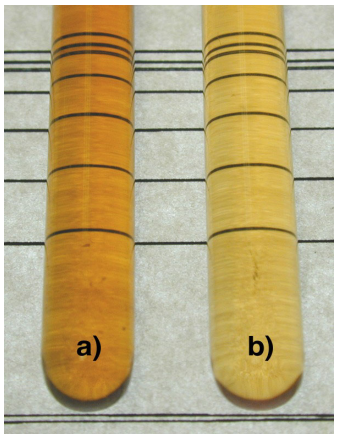


Figure 1. Plant cell walls can be solubilized for NMR. The picture shows NMR tubes containing solutions of acetylated CWs from solvent-extracted ball-milled a) pine wood and b) aspen wood from the DMSO/NMI system. Over 100 mg are dissolved in $CDCl_3$ (0.5 mL) in 5 mm tubes. The homogeneous solutions are viscous, but clear, as evident from the transmitted lines (from a paper-plot placed behind the tubes).

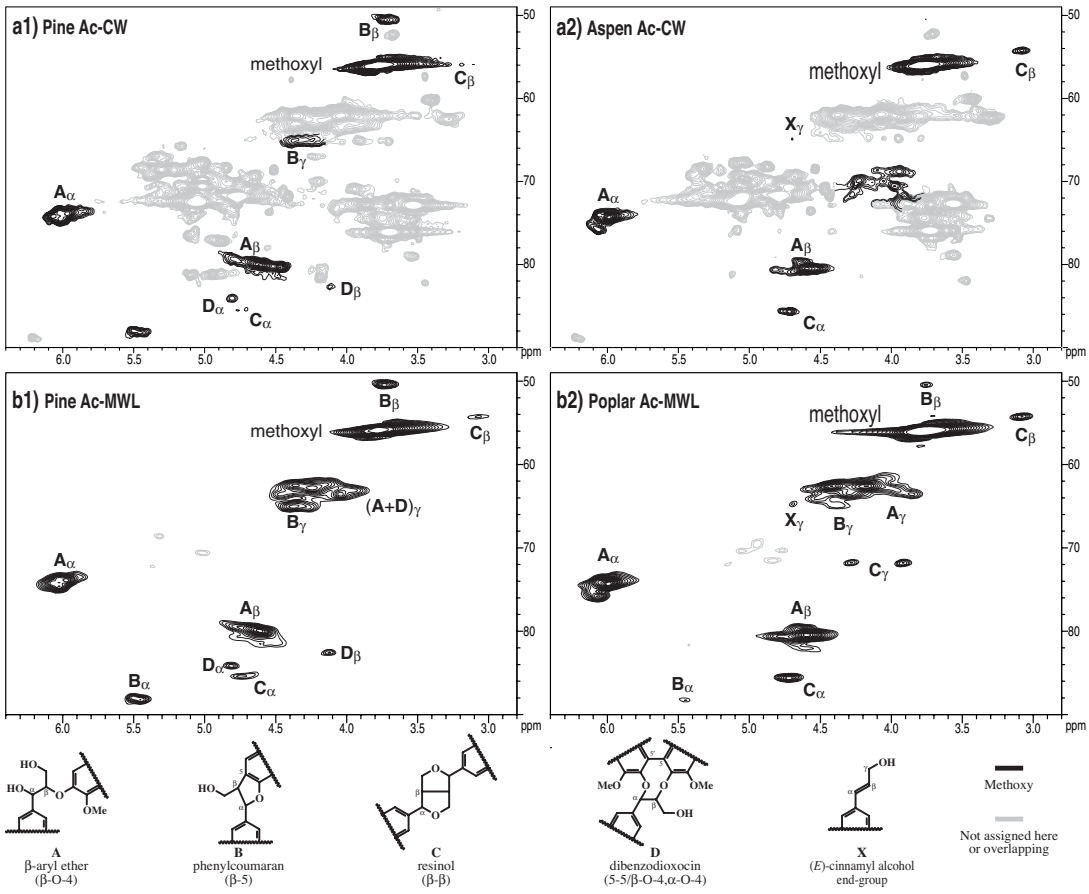


Figure 2. 2D HSQC NMR spectra of acetylated cell walls and isolated lignins. Spectra from samples in $CDCL_3$ show how readily the lignin components can be seen even in complex whole cell wall mixtures of pine (left column, 1) and aspen/poplar (right column, 2). a-b) zoomed-in lignin sidechain region. Note how well resolved at least one correlation is for each major lignin structure. The predominant lignin structures **A**-**D**, **X**, and the methoxyl are highlighted, in CW samples (Figs. a) where they can be clearly defined.

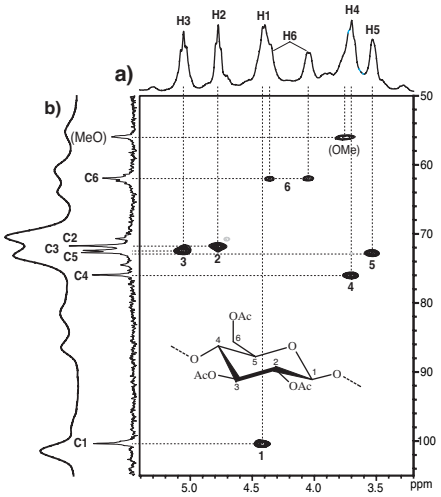


Figure 3. The cellulose component. a) The cellulose (and methoxyl) region of the 2D HSQC spectrum of aspen Ac-CW showing the advantage of solution-state NMR in completely resolving all C/H correlations. b) A solid-state NMR spectrum of pine wood simply for comparison of line widths.

Synthesis of Hydroxycinnamoyl-L-Malic Acids and Identification of 5-Hydroxyferuloyl-L-Malic Acid in COMT-Downregulated *Arabidopsis*.

P. Schatz, C. Lapierre, and J. Ralph.

Introduction

Hydroxycinnamoyl-L-malic acids are natural products that have been isolated from a variety of plants, e.g., sinapoyl-L-malic acid **1a** from red turnip (*Brassica campestris* L.*)* and *Arabidopsis*, phaselic acid **1c** from kidney beans (*Phaseolus vulgaris*) and red clover (*Trifolium pratense*), and *p*-coumaroyl-L-malic acid **1d** and feruloyl-L-malic acid **1e** from radish (*Raphanus sativus*). Our interest in this type of compound has been piqued by the severe reduction in sinapoyl malate **1a** in plants which have been downregulated in a crucial lignin-biosynthetic-pathway enzyme, COMT. Interestingly, when COMT is downregulated in *Arabidopsis*, the novel 5-hydroxyferuloyl-L-malic acid **1b** is observed, paralleling the incorporation of the novel monolignol 5-hydroxyconiferyl alcohol into its lignins.

Since the structural assignments of **1a** and **1b** were based on mass spectral data only, we sought to confirm the assignments by synthesis of the authentic compounds. To date, the only reported synthesis of a cinnamoyl-L-malic acid is the synthesis of phaselic acid **1c** by Scarpati and Oriente. This approach was not amenable for the target molecules.

Methods and Materials

The *t*-butyl ester of L-malic acid **3c** was prepared by a circuitous, yet necessary, route involving protection of the hydroxyl group on malic acid **2**, Fig. 2. L-Malic acid **2** was heated with excess acetyl chloride to yield the acetate anhydride. This was hydrolyzed to 2-acetoxy-L-malic acid **3a** by

stirring overnight with water. The *t*-butyl ester of 2-acetoxy-L-malic acid **3b** was prepared by acid catalyzed addition of isobutylene. Hydrolysis of the acetate group was carried out with aqueous sodium hydroxide to afford di-*t*-butyl L-malate **3c**.

The required substituted cinnamoyl chlorides were prepared starting with appropriately substituted aldehydes, syringaldehyde **4a** and 3,4-dihydroxy-5-methoxybenzaldehyde **4b**. The hydroxyl groups were protected by converting them to acetates **4c** and **4d** using pyridine and acetic anhydride.

The α,β-unsaturated esters, **5a** and **5b**, were prepared from the protected aldehydes using the Wadsworth-Emmons modification of the Wittig reaction (*tert*-butyldiethylphosphonoacetate and NaH as the strong base). Hydrolysis of the *t*-butyl esters with 90% trifluoroacetic acid afforded the acids **5c** and **5d**, which were subsequently converted to the acid chlorides **5e** and **5f** using thionyl chloride.

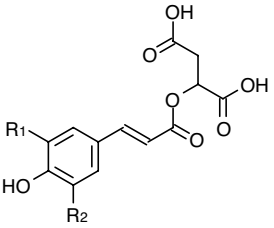
Coupling of the acid chlorides with di-*t*-butyl L-malate **3c** yielded esters **6a** and **6b**. The acetate groups were removed using pyrrolidine to afford **6c** and **6d**. Finally, the *t*-butyl groups were removed using 90% trifluoroacetic acid to yield sinapoyl-L-malic acid **1a** and 5-hydroxyferuloyl-L-malic acid **1b.**

Results and Discussions

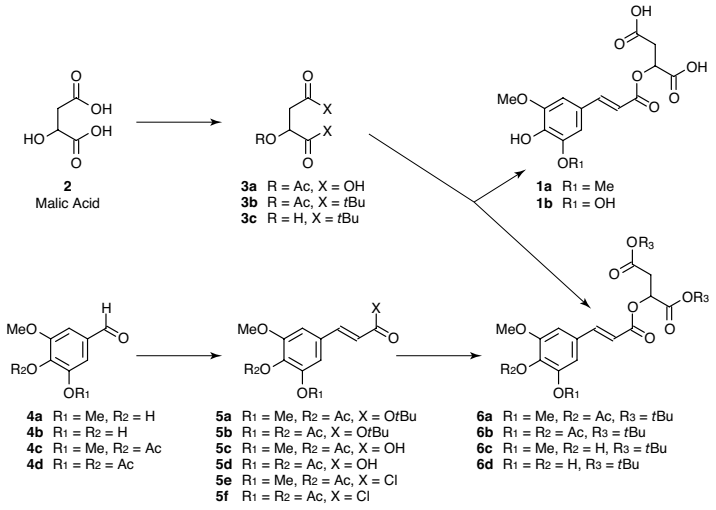
We preferred a synthetic approach which did not require chromatography nor require a Craig apparatus to isolate the product. In the method developed, the acid groups of malic acid were masked as *t*-butyl esters which were hydrolyzed in the last step of the synthesis. Since the hydrophilic groups (hydroxyls and acids) were masked, isolation and purification of the intermediate products was greatly simplified. In the last step, hydrolysis conditions can be controlled to minimize the presence of water and thus facilitate isolation of the very water-soluble final products.

The synthesized sinapoyl malate **1a** and 5-hydroxyferuloyl malate **1b** were found to be the same products as those produced in wild-type and COMT-deficient arabidopsis. COMT is the enzyme that methylates 5-hydroxyconiferaldehyde to sinapaldehyde which is reduced to the monolignol sinapyl alcohol in the monolignol biosynthetic pathway. It has now been well established that COMT-deficient plants utilize a product of incomplete monolignol biosynthesis, 5-hydroxyconiferyl alcohol, as a substitute monomer for sinapyl alcohol for their lignification, producing novel benzodioxane structures in the lignin polymer.

The sinapoyl ester pool is also affected by COMT-downregulation. It seems logical that plants are effectively utilizing 5-hydroxyconiferyl alcohol to make a functional lignin polymer, and these products suggest that other biochemical pathways (e.g. to sinapoyl malate) also have the flexibility to accommodate the substitution of 5-hydroxyferuloyl analogs (although the specificity of the enzymes involved is unknown). Whether the plant is “deliberately” making these sinapate analogs as functional alternatives, or whether they are simply unanticipated products resulting from sloppy transferase enzyme specificity become intriguing issues to ponder for future research. The COMT-deficient Arabidopsis plants are not noticeably more susceptible to UV exposure (sinapoyl malate is a major UV-protecting agent in Arabidopsis) suggesting that the plant is “deliberately” utilizing the 5-hydroxy analog in a similar role.



1a $R_1 = R_2 =$ OMe (sinapoyl malate)
1b R_1 = OMe, R_2 = OH (5-hydroxyferuloyl malate)
1c R_1 = OH, R_2 = H (phaselic acid)
1d $R_1 = R_2 =$ H (*p*-coumaroyl malate)
1e R_1 = OMe, R_2 = H (feruloyl malate)



2 Malic Acid

3a R = Ac, X = OH
3b R = Ac, X = *t*Bu
3c R = H, X = *t*Bu

1a R_1 = Me
1b R_1 = OH

4a R_1 = Me, R_2 = H
4b $R_1 = R_2$ = H
4c R_1 = Me, R_2 = Ac
4d $R_1 = R_2$ = Ac

5a R_1 = Me, R_2 = Ac, X = O*t*Bu
5b $R_1 = R_2$ = Ac, X = O*t*Bu
5c R_1 = Me, R_2 = Ac, X = OH
5d $R_1 = R_2$ = Ac, X = OH
5e R_1 = Me, R_2 = Ac, X = Cl
5f $R_1 = R_2$ = Ac, X = Cl

6a R_1 = Me, R_2 = Ac, R_3 = *t*Bu
6b $R_1 = R_2$ = Ac, R_3 = *t*Bu
6c R_1 = Me, R_2 = H, R_3 = *t*Bu
6d $R_1 = R_2$ = H, R_3 = *t*Bu

Sequencing Around 5-Hydroxyconiferyl Alcohol-Derived Units in COMT-Deficient Lignins
F. Lu, J. Ralph, J.M. Marita, C, Lapierre, and W. Boerjan

Introduction

Lignins are polymeric aromatic constituents in woody plant cell walls. Although they are traditionally considered to be dehydrogenative polymers from three monolignols, *p*-coumaryl alcohol **1P**, coniferyl alcohol **1G**, and sinapyl alcohol **1S**, Fig. 1, they can vary greatly in their composition. Recently there has been considerable interest in genetically modifying lignins with the goal of improving the utilization of lignocellulosics in various agricultural and industrial processes. Studies on mutant and transgenic plants with altered monolignol biosynthesis have suggested that plants have a high level of metabolic plasticity in the formation of lignin. Lignins in angiosperm plants with depressed COMT (caffeic acid *O*-methyltransferase) were found to include significant amounts of 5-hydroxyconiferyl alcohol monomers **15H**, substituting **15H** for the traditional monomer, sinapyl alcohol **1S**. NMR analysis of lignins from poplar deficient in COMT revealed that benzodioxane structures are formed through β–O–4-coupling of a monolignol with 5-hydroxyguaiacyl unit, followed by internal trapping of the resultant quinone methide by the phenolic 5-hydroxyl. When the lignins were subjected to thioacidolysis, a novel 5-hydroxyguaiacyl monomer **2** was found in addition to the normal guaiacyl and syringyl thioacidolysis monomers. Also, a new compound **3G** was found in the dimeric products of the thioacidolysis followed by Raney nickel desulfurization, Fig. 1.

Further study with the lignins using the DFRC method also confirmed the existence of benzodioxane structures in this lignin, with compounds **4**, Fig. 1, being identified. However, the monomeric 5-hydroxyguaiacyl unit could not be detected in the DFRC products of the lignin. These facts suggested that the DFRC does not cleave the benzodioxane structures and might therefore be useful as an analytical tool for determination of benzodioxane structures linked by β–O–4 ethers. Using a modified DFRC procedure, we report here our results that provide further evidence for the existence of benzodioxane structures in lignins of COMT-deficient plants (and therefore that 5-hydroxyconiferyl alcohol is behaving as a monolignol and can be integrated into plant lignins), and demonstrate the usefulness of the DFRC method for determining the details of 5-hydroxyconiferyl alcohol incorporation into lignins.

Experimental Procedures

Materials

Two independent sets of COMT-deficient poplar lignin samples were used in this study; their lignins have been available in greater quantities than from analogously downregulated forage plants which are only now beginning to be analyzed. One is from antisense methods; the other, with even lower COMT-activity, is from sense-suppression (gene-silencing).

Compounds **5**-**6** were synthesized by methods too complex to describe here (Fig. 2).

Modified DFRC procedure for COMT-deficient lignins and cell walls

DFRC: Lignins (8 to 10 mg) or extracted wood cell wall (40 mg) were used. The acetyl bromide treatment and zinc reduction steps were performed as the standard DFRC procedure. After the zinc reduction step the degraded products were methylated as follows (instead of acetylation).

Methylation: the above residue was methylated with iodomethane (50 ml) and cesium carbonate (100 mg) in 3 ml acetonitrile for 30 min. The excess reagents were quenched by addition of 1 ml acetic acid and the product isolated.

Solid phase extraction (**SPE**): The above residue was transferred with 2 ml dichloromethane into a 10 ml pear shape flask, concentrated to less than 50 ml, and loaded with dichloromethane (the total volume should not larger than 150 ml) to a 3 ml pre-conditioned (5/1 cyclohexane/ethyl acetate) normal phase SPE column (LC-Si, Supelco). The monomers were eluted with 9 ml cyclohexane/ethyl acetate (5/1, v/v). Then the dimeric products were eluted with 9 ml cyclohexane/ethyl acetate (1/1.5, v/v). This dimeric fraction was evaporated under reduced pressure.

Hydrolysis: The above dimeric products were dissolved in 3 ml methanol in a 25 ml flask to which 3 ml 1 M potassium carbonate aqueous was added. This mixture was stirred for 1 h. The solution was concentrated to about 3 ml and acidified with 4 ml 1 M aqueous HCl solution and saturated with sodium chloride followed by extraction with dichloromethane (3x10 ml). The combined dichloromethane extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure after filtration.

TMS-derivatization and GC analysis: The above residue was transferred with 2 ml dichloromethane into a 10 ml pear shape flask and internal standard (mono-4-methylated 5–5-diferulic acid) in pyridine 10-15 ml were added. The products were concentrated and transferred into a reacti-vial with 50 ml pyridine and derivatized with 50 ml BSTFA for 30 min at 50 ˚C before 5 ml was injected into the GC.

Results and Discussion

The fact that 5-hydroxyconiferyl acetate has not been found in DFRC degradation products of COMT-deficient poplar lignin implies that all of the 5-hydroxyconiferyl alcohol monomer is incorporated into lignin as benzodioxane structures and these structures totally survive DFRC conditions (without cleavage to monomeric products). This absolute survivability has now been demonstrated with dimeric model compounds (not shown).

When a COMT-deficient polar lignin was degraded by the standard DFRC procedure, the benzodioxane marker compound **4G** was detected by GC-MS. But the absence of the corresponding **4S** raised a question whether the syringyl benzodioxane was not in this lignin or the marker compound **4S** could not get through the GC. With synthetic compound **4S**, we found that **4S** could not survive the GC conditions because of its thermo-lability, but the TMS-derivatized compounds $\mathbf{6S_F}$ and $\mathbf{6S_E}$ (Fig. 2) proved to be amenable to GC quantitative analysis. So a modified DFRC procedure was developed (Fig. 3) to determine the benzodioxane structures. First of all, the normal DFRC allows cleavage of β-ethers and leaves the benzodioxanes intact. After zinc reduction, the phenols released due to β-ether cleavage are methylated and the phenolic acetates originally from free phenols of lignins remain unaffected. So the benzodioxanes with free phenols on G/S units in lignins will be acetylated whereas and the benzodioxanes with etherified phenols on G/S units become methylated. A solid-state extraction step was used to clean up sample and enrich the dimeric products, allowing more accurate GC analysis. The final step in this procedure, base hydrolysis, converts all benzodioxane dimers in DFRC degradation products into compounds **6**. Their TMS-derivatives were analyzed by GC.

The partial FID-GC profiles of the degradation products of four samples by the modified DFRC procedure are shown in Fig. 4. The target compounds **6** were identified by mass spectra and GC retention time comparison with synthetic and authentic model compounds. An internal standard 4-monomethylated 5–5-diferulic acid was added to samples right before TMS-derivatization and GC analysis.

From the results summarized in the Table, it can be seen that guaiacyl benzodioxanes released by DFRC mostly (70-86%) were from the end of lignin molecules and the released syringyl benzodioxanes mostly (67-77%) were from lignins' internal units. By comparing results of MWL and residual lignin after dioxane extraction, some partitioning of benzodioxane structures among lignin fractions occurred during preparation of MWL with dioxane extraction and this partitioning is more significant for the guaiacyl benzodioxanes than for the syringyl ones.

One important aspect to note (that also applies to a lesser degree to the thioacidolysis products) is that the overall yields for benzodioxanes released by DFRC are relatively low compared to results obtained from NMR analysis of the lignins. Since 5-hydroxy-units could also couple with further 5-hydroxyconiferyl alcohol monomers producing benzodioxane chains that are not cleavable by DFRC, trimers, tetramers and higher oligomers of benzodioxane chains may exist in DFRC products and can not be measured by GC. In fact, evidence for such benzodioxane chains has recently been demonstrated in a COMT-deficient alfalfa transgenic. The isolation of those trimers or tetramers will give further evidence that 5-hydroxyconiferyl alcohol is truly a monolignol that is integrated into lignins, particularly of COMT-deficient plants.

Conclusion

In summary, a combination of prior NMR and thioacidolysis data and this DFRC data demonstrates compellingly that 5-hydroxyconiferyl alcohol is incorporating intimately into lignins like the traditional monolignols…

• 5-Hydroxyconiferyl alcohol is cross-coupling (at its b-position) with the phenolic end of growing lignin oligomers, and…

• New monolignols (**1G**, **1S**, and **15H**) can all cross-couple with the newly formed 5-hydroxyguaiacyl end unit, extending the chain in a typical endwise fashion, and…

• Further monolignols will cross-couple with the new end-unit from this latest addition, such that it too becomes further etherified.

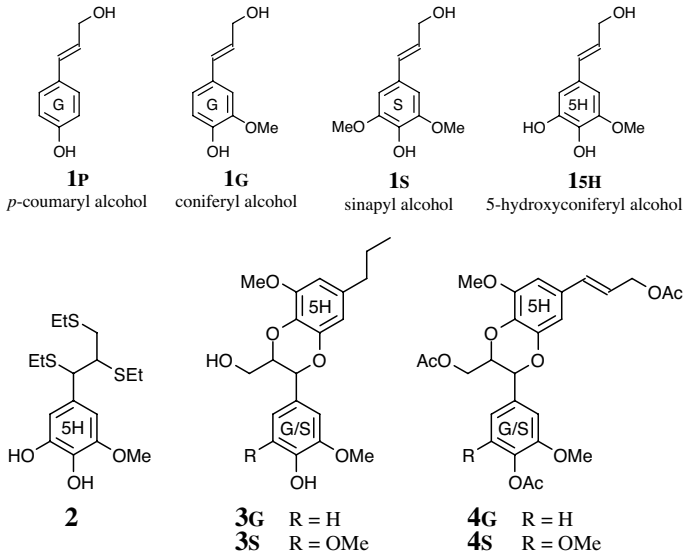


Figure 1. The monolignols **1**, and marker compounds **2**-**4** resulting from 5-hydroxyconiferyl alcohol incorporation into lignins; thioacidolysis monomeric marker **2**, dimers **3**, and DFRC dimeric markers **4**.

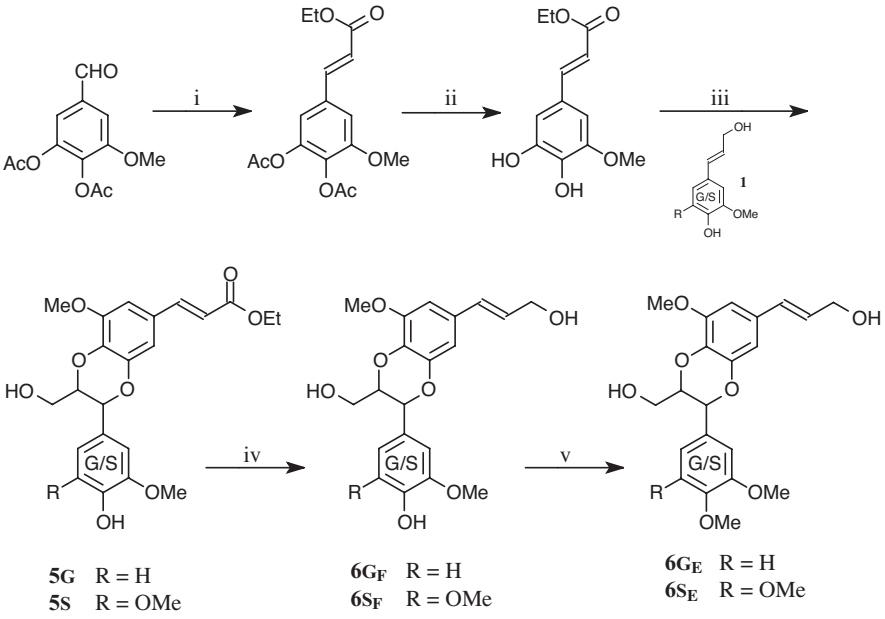


Figure 2. Synthesis of products **5**-**6** required for DFRC methods. i. NaH-triethyl phosphonoacetate,THF; ii. pyrrolidine; iii. Ag_2CO_3, benzene-acetone (5/1, v/v); iv. DIBAL-H, toluene; v. CH_3I-K_2CO_3-acetone.

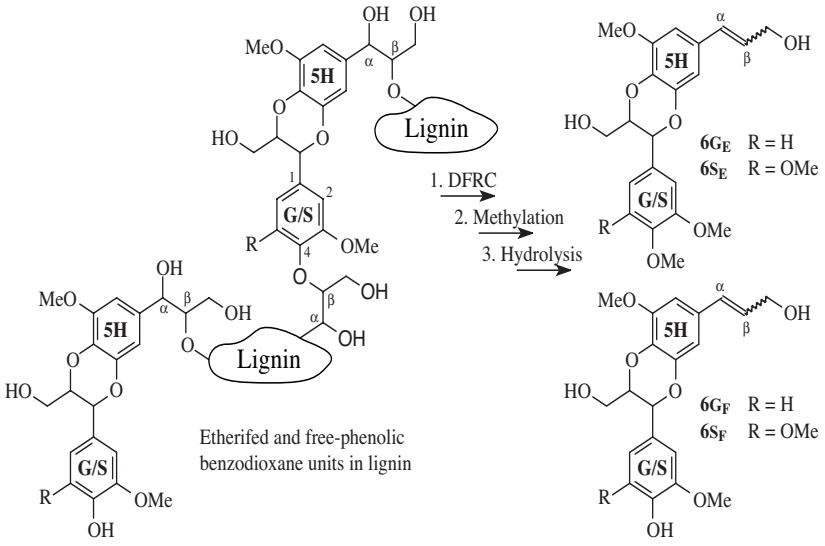


Figure 3. Benzodioxane compounds released from DFRC degradation of a hypothetical lignin containing free-phenolic and etherified benzodioxane units.

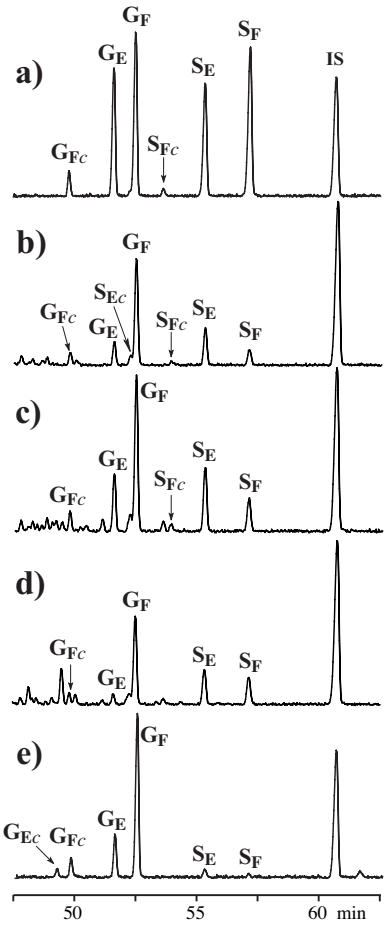


Figure 4. Partial GC-FIDs showing TMS-derivatized benzodioxane products from degradation of COMT-deficient poplar lignins or cell walls by the modified DFRCprocedure.
a) Synthesized models.
b) antisense COMT-deficient poplar lignin.
c) residual lignin after isolation of lignin in b.
d) antisense COMT-deficient poplar cell wall.
e) Gene-silenced COMT-deficient poplar lignin.

Sample Description	Relative Ratios		Overall yields (%lignin sample)		
	GOMe/GOH	SOMe/SOH			
	6GF/6SF	6GE/6SE	G (6GF+6SF)	S (6GE+6SE)	G/S
Lignin (antisense)	20/80	72/28	0.39	0.19	2.05
Residual Lignin (antisense)	30/70	71.5/28.5	--	--	1.33
Lignin (sense)	26.5/73.5	77.3/22.7	0.43	0.02*	21.5*
Cell wall (antisense)	13.5/86.5	66.7/33.3	--	--	0.93

An Improved ^{13}C-Tracer Method for the Study of Lignin Structure and Reactions — Differential 2D $^{13}C/^{1}H$-NMR

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Introduction

Lignin is a structurally heterogeneous polymer formed from several kinds of monolignols by an irreversible, combinatorial radical polymerization. Therefore, it is impossible to degrade lignin quantitatively into monomeric or oligomeric units, and degradation loses information on its 3D macromolecular structure. Accordingly, destructive analyses can provide limited information on the chemical structure of protolignin in the cell wall. Among various non-destructive analytical methods, NMR spectrometry is one of the most powerful techniques that provides direct information on the structure of lignin. In the ^{13}C-NMR spectrum, signals are distributed over a wide range of chemical shifts. However, considerable overlap of the signals sometimes causes difficulty in assignments of weak signals and in quantitative determination of the signal intensities. Two-dimensional spectrometry provides a remarkable improvement in assignment and quantitative determination of the signal intensities. Nevertheless, there is still some overlapping of signals.

Selective ^{13}C-enrichment of a specific carbon in the lignin improves the signal intensity of the enriched carbon in its ^{13}C-NMR spectrum, and the difference spectrum between spectra of ^{13}C-enriched and unenriched lignins allows further improvements in the assignment of the signal and quantitative determination of the intensity. Selective ^{13}C-enrichment is achieved by feeding ^{13}C-enriched lignin biosynthesis precursors to the growing stem of a tree or inner cavity of a plant stalk. The monolignol glucosides have been shown to be the best precursors. The ^{13}C-NMR also gives information on the behavior of the bonds and functional groups during various types of reactions that is difficult to obtain by any other conventional destructive analyses.

This paper demonstrates that exceedingly clean differential spectra can be obtained by 2D NMR for synthetic lignins as a prelude to the application for isolated lignins.

Methods

Preparation of ^{13}C-labeled synthetic lignins.

Coniferins specifically ^{13}C-enriched at the side chain carbons, α, β, γ and at the aromatic ring car-

bons 1, 3, 4 and 5 were synthesized by the procedure of Terashima *et al*., and the solution of the coniferins in phosphate buffer (pH 6.0) was treated with a mixture of three kinds of enzymes, β-glucosidase, glucose oxidase and peroxidase to produce guaiacyl (G) type synthetic lignin (DHP).

Results and Discussion

For a DHP with a hypothetical structure such as shown in Fig. 1, two types of 2D ^{13}C–^{1}H partial spectra (just the sidechain regions) are shown on the left and right columns of Fig. 2. The left column (a-d) has HSQC spectra in which each carbon correlates with its directly attached carbon. The right column (e-h) shows each carbon correlating with all of the protons in the sidechain from that unit. [The spectra are much more readily interpreted in the color version of this figure which is on the CD version of these Research Summaries or on our web site at www.dfrc.wisc.edu]. The unlabeled control shows correlations from all three carbons and their associated protons, whereas the differential spectra from the specifically enriched lignins show only correlations with the enriched carbons, remarkably cleanly. Evidence for the excellent subtraction is the disappearance of the strong methoxyl group in the 2D difference spectra for Cα-Cβ. Coniferin unfortunately appears to be incorporated as an α-O-6 (glucose) ether in these DHPs.

With the labeling and NMR subtraction methods in hand, these techniques (along with a full quantitative analysis of the 1D ^{13}C NMR spectra) will be attempted for actual lignins by Terashima and colleagues.

Acknowledgements

Financial support from the research foundation Stiftelsen Nils och Dorthi Troëdssons Forskningsfond is gratefully acknowledged.

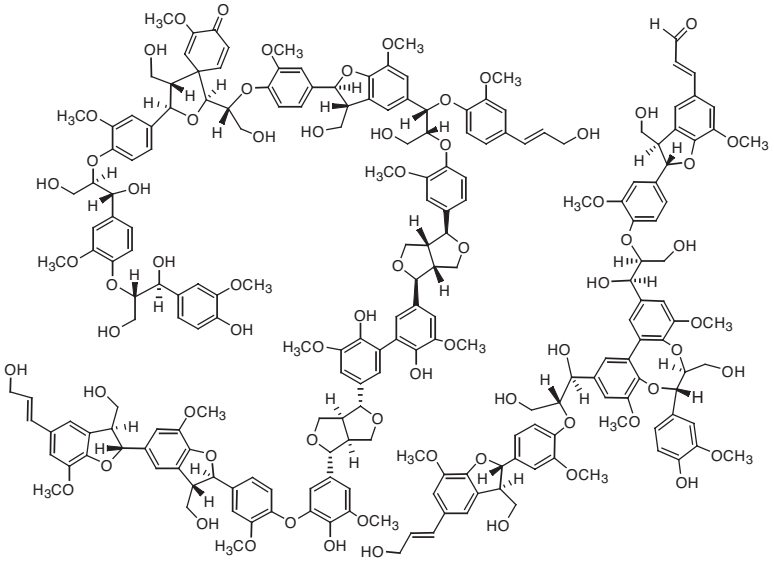


Figure 1. A part of proposed structure for the G-DHP

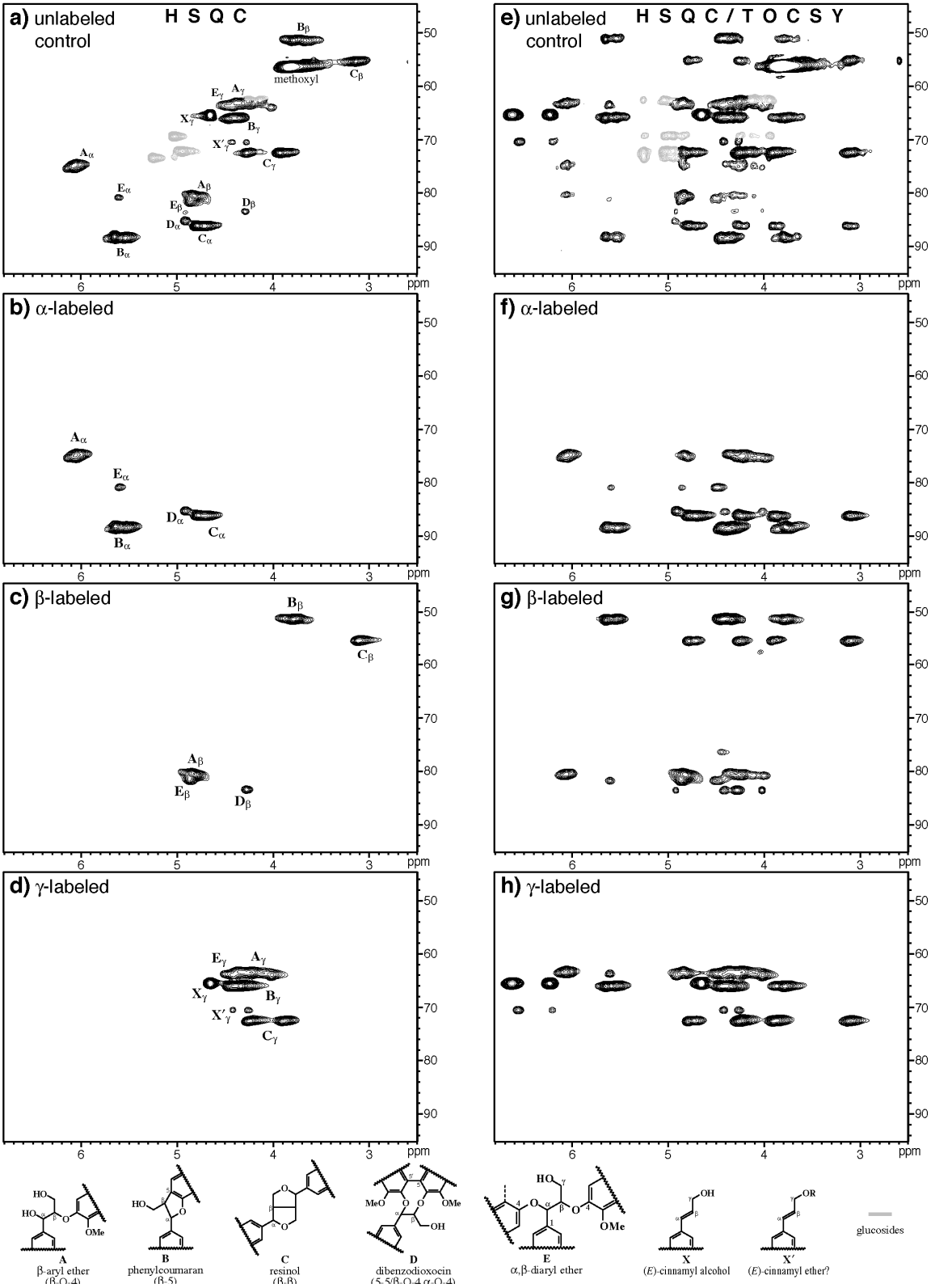


Figure 2. Two-dimensional spectra of acetates of ^{13}C-enriched DHPs. a-d) HSQC spectra. a): unenriched control. b)-d): Difference spectra obtained by subtraction of the spectrum of the unenriched control from the spectra of DHPs specifically ^{13}C-enriched at side-chain carbon, Cα, Cβ, Cα. e-f) Analogous HSQC-TOCSY spectra — see text.

Variations in the Cell Wall Composition of Maize *Brown Midrib* Mutants

J.M. Marita, W. Vermerris, J. Ralph, and R.D. Hatfield

Introduction

There are four *brown midrib* (*bm*) mutants known in maize (*Zea mays* L.). These mutants, *bm1*, *bm2*, *bm3* and *bm4*, are believed to be Mendelian recessives and recognized by reddish-brown vascular tissue in their leaves and stems. The *bm* mutants are of interest because of their potentially higher nutritional value as a forage, presumably because of the lower lignin content and more digestible cell wall structure. The data presented represented a comprehensive analysis of the effects of *bm* mutations on lignin in a single genetic background. There were variations in the *bm* mutational effects compared to results previously published in other genetic backgrounds. This may be attributed to the multiplicity of genes in maize whereby a mutational effect in one isoform is compensated for by other isoforms present and functioning or variable expression of a specific transgene having different impacts depending on the genetic background in which it is being expressed.

Results and Discussion

Nuclear Magnetic Resonance - NMR

Lignin itself is a complex polymer that hardens the cell walls of a plants xylem tissue. The *bm1* mutation has been shown to affect the activity of the enzyme cinnamyl alcohol dehydrogenase (CAD) and the Bm3 gene corresponds to an insertion and a deletion in the caffeic acid *O*-methyltransferase (COMT) gene, both involved in the lignin pathway. The *bm1* and *bm3* mutational effects (elevated aldehyde levels and the presence of benzodioxane units **H** signatures of CAD and COMT-deficiencies) were undeniably present as determined by NMR. The most striking difference discovered by NMR was the significant presence of benzodioxane units in the *bm3* isolated lignin.

Maize Cell Walls (CW)

The CWs from the *bm3* and *bm1-bm2* mutants had a 20% and 10% lower lignin content compared to wild-type, while the *bm1*, *bm2* and *bm4* mutant CWs contained the same amount (Table 1A). The *bm* mutants are generally recognized by a reduction in lignin content; however, the results from numerous studies vary to the same extent as the number of genetic stocks investigated. Consequently, differences in the lignin content of any given *bm* mutation compared to wild-type were quite variable.

The sum of Klason lignins, total neutral sugars and uronic acids accounted for 92% to 95% of the *bm* mutant cell wall. The proportion of individual neutral sugars (mg g^{-1} CW) was generally the same for all CWs; between 47 to 50% cellulose (as determined by glucose) and 22 to 27% xylan (as determined by xylose) with substitution with arabinose varying between 1:8 in the *bm3* mutant and 1:6 in wild-type maize.

The most noticeable changes in cell wall composition were elevated levels of ferulate (FA) monomers in the *bm3* mutant with no change in the amount of cross-linking (as evidenced from etherified-FA levels; Table 2). The only maize samples to show a reduction in their level of cross-linking (between arabinoxylans indicated by total FA dimers) were the *bm1* and *bm1-bm2* mutants. Any attributable mutational effects in the *bm1-bm2* CWs appear to be *bm1* in origin, especially changes in cell wall composition; however, since exact *bm2* mutational effects are not known, other param-

eters than those examined may represent more closely the *bm2* mutational effect. Esterified-*p*Coumarate (pCA) levels were variable among all maize samples with the *bm1*, *bm3* and the double *bm1-bm2* mutants having ~50% less esterified-*p*CA than all other CW maize samples.

Maize Lignin Extract (LE) and Lignin Residue (LR) Fractions
Striking differences between the *bm* mutants were revealed by compositional shifts in their LR and LE fractions. When enzyme digested cell walls are partitioned into the LE and LR fractions, the sum of their Klason lignin values should theoretically add up to the corresponding CW Klason lignin value. However, the sum for all samples, particularly the *bm* mutants, was lower. Differences in the levels of acid soluble components lost during hydrolysis and/or differences in individual carbohydrate profiles may account for such discrepancies.

Parallel reductions in esterified-*p*CA were observed in the same maize LR and LE fractions; every maize sample had lower total esterified-*p*CA (LR+LE) than amounts released from their CW. For example, the *bm1* mutant displayed the greatest reduction at 46% of its CW level (68% of the wild-type level). This parallels the reduction observed in the *bm1* mutant Klason lignin (LR+LE) at 53% of its CW level (51% of the wild-type level).

Previous reports suggested that little *p*CA is esterified to arabinoxylans in maize; therefore, the *p*CA released during low temperature hydrolysis must be predominantly esterified to lignin in the LR and LE fractions. These results suggest that *p*CA-lignin complexes were solubilized and lost in the supernatants during cellulase enzyme treatment. Low temperature alkaline hydrolysis of the enzyme supernatants did reveal substantial amounts of esterified-*p*CA. Furthermore, 0.1 N trifluoroacetic acid (TFA) hydrolysis of the maize CWs released *p*CA-arabinosyl-conjugates, detectable by GC/MS selective ion monitoring. Since only small amounts of *p*CA were found esterified to arabinoxylans in the maize samples, the esterified-*p*CA measured after low temperature hydrolysis from the supernatants clearly indicates that significant amounts of *p*CA-lignin complexes are being solubilized during cellulase enzyme treatment. These results help explain why parallel reductions of *p*CA and lignin were observed in the *bm* mutant LR and LE fractions; specifically, the higher the solubility of *p*CA-lignin complexes in the *bm* mutants, the greater the loss after the cellulase enzyme treatment.

Compositional shifts in total neutral sugars existed between the LR and LE fractions, Table 1B. Cellulose (as measured by glucose) was greater in all the maize LR fractions versus LE fractions. The *bm3* mutant with the greatest degradability (88%) had the lowest residual cellulose (LR+LE glucose; 40 mg g^{-1} CW), whereas the *bm4* mutant with the lowest degradability (71%) had the highest residual cellulose (LR+LE glucose; 134 mg g^{-1} CW). Individual neutral sugars were not partitioned equally among samples implying that the hydrolysis of component polysaccharides was not consistent across *bm* mutants. Equivalent neutral sugars levels (mg g^{-1} CW) were observed among the maize LE fractions suggesting that the composition of the neutral sugars extracted in the soluble lignin fraction among all maize samples is the same regardless of the *bm* mutation. The presence and detection of neutral sugars in the LE fraction supports an interaction between carbohydrates and lignin. A reasonable conclusion since arabinoxylans are known to be cross-linked to lignin by FA.

The reason why neutral sugar components are partitioned predominantly into the LR fraction may be an organizational issue whereby their incorporation into the cell wall is dependent on the respective *bm* mutation being expressed. Other studies of *bm* maize have alluded to these plants lacking some

function controlling lignification. These data would suggest that the function lacking does not control lignification *per se* but may control *elements* leading to lignification such as peroxidase activity or hydrogen peroxide production availability. As a result, the lignin polymer is altered in such a way that incorporation of esterified-*p*CA is reduced and consequently the structure of the lignin polymer altered at some organizational ("non-polymeric") level not detected. Because of a difference in the structural organization of lignin there is less *p*CA incorporation and greater solubility of smaller-sized *p*CA-lignin complexes. Further study into greater solubility versus greater degradability should be of interest.

Experimental Procedures

Lignin isolation and preparation

The stalk sections were cut into 2-3 cm pieces and ground to pass a 2.0 mm screen of a Wiley mill prior to a cyclone mill (1 mm). The ground maize stems were extensively extracted with water, methanol, acetone, and chloroform. The isolated CW's were ball-milled, digested with crude cellulases, and extracted into 96:4 dioxane:H_2O. The dioxane:water fractions were lyophilized and saved as maize lignin extract (LE) and maize lignin residue (LR).

Cell Wall Composition

For all CW, LR, and LE samples, Klason lignin, total uronic acids, total neutral sugars, and phenolic compositions were determined. Klason lignin determinations were the ash-corrected residue remaining after total hydrolysis of CW polysaccharides. Total uronic acids were estimated colorimetrically with glacturonic acid as the calibration standard. Neutral sugars from total CW hydrolysis were determined by high-pressure liquid chromatography. CW (~50 mg), LR (~55 mg), and LE (~30 mg) samples were analyzed for esters and ethers using internal standards, 2-hydroxycinnamic acid (0.1 mg) for monomers and 5-5-diferulic acid monomethyl ether (0.05 mg) for dimers. Derivatives of phenolic acids were separated by gas liquid chromatography.

Table 1A. Cell wall (CW) composition of wild-type and *bm* mutants of maize in A619 background averaged over two replicates. **B.** The sum of dioxane:H_2O residue (LR) and dioxane:H_2O extract (LE) total neutral sugars (TNS) of wild-type and *bm* mutants of maize in A619 background and percent cell wall digested following cellulase enzyme treatment (averaged over two replicates).

A	**A619**	***bm1***	***bm2***	***bm3***	***bm4***	***bm1-bm2***
Klason lignin (mg g^{-1} CW)	130±3	130[a]	131±4	104±2	138±1	118±3
Uronosyls (mg g^{-1} CW)	41±2	49±2	43±2	37±0	41±2	42±0
TNS (mg g^{-1} CW)						
Arabinose	36.7±0.6	34.4±0.1	32.6±0.3	32.7±1.0	34.4±0.6	33.0±0.5
Glucose	479.1v±5.4	471.8±20.3	469.1±4.2	491.2±3.5	485.7±7.1	472.5±9.7
Xylose	226.6±3.2	252.9±0.4	252.0±4.9	262.1±3.2	242.0±0.3	244.9±3.9
Total[b]	755.4	770.1	764.6	796.5	772.2	761.2
Cell Wall Total[c]	927	949	939	937	951	920
B						
TNS – LR (mg g^{-1} CW)						
Arabinose	6.6±0.0	5.0±0.1	6.4±0.0	2.3±0.1	7.4±0.5	5.0±0.0
Glucose	62.4±11.4	67.9±2.9	91.4±1.3	38.0±0.7	131.2±5.4	66.7±1.4
Xylose	56.8±1.0	60.4±2.8	71.5±0.7	26.3±0.9	89.4±4.4	50.2±0.2
Totals[b]	129.4	135.2	171.3	68.0	230.7	124.7
TNS – LE (mg g^{-1} CW)						
Arabinose	1.6±0.1	0.9±0.0	1.4±0.0	1.5±0.0	1.3±0.1	1.1±0.0
Glucose	5.4±0.3	1.9±0.0	2.9±0.2	1.9±0.0	2.9±0.2	4.4±0.1
Xylose	14.0±0.0	8.3±0.1	11.9±1.0	10.8±0.5	10.9±0.6	8.8±0.1
Totals	21.2	11.3	16.3	14.2	15.3	14.4
% degradability[d]	76%	82%	77%	88%	71%	82%

[a] Based on one replicate
[b] Total =∑(fucose+arabinose+rhamnose+galactose+glucose+xylose+mannose)
[c] Cell Wall Total =∑(Klason Lignin+Uronosyls+Total Neutral Sugars)
[d] Degradability is ((1-recovered weight/initial weight) mg g^{-1} CW)

Table 2 Cell wall (CW) phenolic acids released from wild-type and *bm* mutants of maize in A619 background averaged over two replicates.

	A619	*bm1*	*bm2*	*bm3*	*bm4*	*bm1-bi*
Monomers(mg g^{-1} CW)						
esterified-*p*CA	14.07±0.20	8.46±0.02	15.03±0.18	7.90±0.10	16.14±0.03	7.89±0.24
esterified-FA	4.46±0.07	5.09±0.05	5.24±0.24	7.29±0.07	4.67±0.09	4.89±0.01
etherified-FA	2.21±0.05	1.85±0.19	2.69±0.24	2.51±0.23	2.36±0.11	1.78±0.19
% wild-type	100%	84%	122%	114%	107%	81%
FA dimers(mg g^{-1} CW)						
Dimer total	1.85	1.96	1.79	1.87	1.90	1.50
FA Total	8.52	8.90	9.72	11.67	8.93	8.17

Modifications in Lignin of Transgenic Alfalfa Down-Regulated in COMT and CCoAOMT

J.M. Marita, J. Ralph, R.D. Hatfield, D. Guo, F. Chen, R.A. Dixon

Introduction

Alfalfa (Medicago sativa L.) is a leading forage crop. It has high nutritive value because it is rich in protein, minerals, and vitamins, and if harvested prior to flowering can retain a low fiber and high energy content. The intake and digestibility of forage by dairy animals directly affect their production of meat and milk. A reduction in feeding value results from a lower leaf to stem ratio and the deposition of lignin and polysaccharides in stem cell walls during maturation. The present study examined the alterations to alfalfa lignin structure and composition resulting from independent down-regulation of caffeic acid 3-O-methyltransferase (COMT) and caffeoyl Coenzyme A 3-O-methyltransferase (CCoAOMT) two key enzymes in the lignin pathway. Previous work has shown genetically modified alfalfa to have altered lignin composition and improved in situ digestibility. The results of this study reveal new details of the incorporation of novel units in the lignin of COMT-deficient alfalfa including units not previously identified and an increase in the cellulose:lignin ratio in CCoAOMT-deficient alfalfa. This information allows researchers insight into which lignin structural modifications positively impact alfalfa digestibility.

Results and Discussion

COMT-deficient alfalfa

2D NMR experiments allowed structural analysis of the major units in isolated lignin from alfalfa. Quantification of interunit type structures based on measuring volume integrals in the 2D HMQC spectra (8) of wild-type and COMT-deficient alfalfa are presented in Table 1.

Table 1. Subunit ratios derived from volume integrals of contours in $^{13}C–^{1}H$ correlation spectra of alfalfa lignin.

Alfalfa Lignin	Unit Type, Relative Proportion						
	A	**B**	**C**	**D**	**H**	**X**	**Σ(β-ethers)[a]**
Wild-type	81	8	6	1	0	3	83
COMT	44	8	3	4	38	4	85
CCoAOMT	88	5	6	0	<1	2	88

[a] Σ(β-ethers) = **A**+**D**+**H**

A=β-O-4; **B**=β-5; **C**=β-β; **D**=5-5/β-O-4/α-O-4; **H**=benzodioxanes; **X**=cinnamyl alcohol endgroups

Most striking was the presence of novel benzodioxane units **H** (38%) in the COMT-deficient alfalfa at the levels of the normal β-ether units **A** (44%). In the wild-type alfalfa, no benzodioxane units **H** were detected; the lignin was comprised of mainly β-ether units **A** (81%).

Long-range correlation spectra provided valuable insight into which types of units were connected to each other. Specifically, HMBC data revealed the extent of S/G compositional changes in β-aryl ether units **A** in the extractable isolated lignins. The correlations clearly showed that the lignin was significantly syringyl depleted. HMBC spectra, also indicated the presence of new 5-hydroxyguaiacyl-glycerol structures $\mathbf{G_{5H}}$ (Fig. 1). Although glycerol structures are typically rare in lignins, they are clearly unique to the 5-hydroxyguaiacyl units in the COMT-deficient alfalfa. HMBC experiments provided compelling evidence that 5-hydroxyconiferyl alcohol was incorporated intimately into the polymerization process. In the examined isolated lignin, the HMBC spectrum showed that about half of the 5-hydroxyguaicyl units were etherified (into benzodioxane structures) by reacting with coniferyl alcohol monomers and about half by reacting with another 5-hydroxyconiferyl alcohol monomer. The 5-hydroxyconiferyl alcohol $\mathbf{1_{5H}}$ was participating in endwise coupling reactions extending the lignin chain in an analogous manner to the normal monolignols, coniferyl alcohol $\mathbf{1_G}$ and sinapyl alcohol $\mathbf{1_S}$ (Fig. 2). Therefore, it is logical to consider 5-hydroxyconiferyl alcohol as an authentic monolignol in these COMT-deficient transgenics,. These observations strongly support our contention that a non-traditional monomer can be utilized for lignification when biosynthesis of traditional monolignols is interrupted.

Klason lignin and total neutral sugars were determined. COMT-deficient alfalfa resulted in a 10% decrease in Klason lignin content compared to wild-type. This decrease in lignin content, was complimented by a (relative) increase (~10%) in cellulose (as measured by glucose) and an increase (~6%) in xylans (as measured by xylose). Alfalfa leaves provide the most nutrition to the animal and stems a limiting component. Any increase in availability of nutrients tied up in the stem should positively affect the overall benefit of the forage to the animal. Off-site examination of rumen digestibility of alfalfa forage in fistulated steers revealed just that — improved digestibility of forage from COMT down-regulated alfalfa plant.

CCoAOMT-deficient alfalfa

Similar structural and compositional analyses were done on isolated lignin from CCoAOMT-deficient alfalfa. All major structural units were still present in the lignin of the CCoAOMT-deficient alfalfa at similar intensities to wild-type levels (Table 1). The only structural and compositional differences were detected near baseplane levels where **H** (benzodioxane) units became discernable.

Klason lignin and total neutral sugars were also determined. The CCoAOMT-deficient alfalfa re-

sulted in a 21% decrease in lignin content compared to wild-type. Unlike the COMT-deficient alfalfa, the complimentary (relative) increase (~11%) in cellulose (as measured by glucose) and increase (~9%) in xylans (as measured by xylose) were not equivalent to the reduction in lignin content. This corresponded to a 39% increase in cellulose:lignin ratio compared to wild-type. The decrease in *in situ* digestibility reported for CCoAOMT-deficient alfalfa is likely a consequence of the CCoAOMT transgenic cell wall attributes, particularly the polysaccharide:lignin ratio.

In general, COMT deficiency resulted in the incorporation of novel units undetected in the normal wild-type, because the plant apparently utilizes monomers from incomplete monolignol synthesis to augment the production of lignin. It appears that COMT-deficiency in legumes such as alfalfa leads to similar compositional changes as COMT-deficiency in hardwoods such as poplar. The extent of these changes varies but the signature effects are clearly evident. For the first time, there is evidence that the next reaction in the lignification sequence following incorporation of 5-hydroxyconiferyl alcohol can be the coupling with either coniferyl alcohol (or sinapyl alcohol) or another 5-hydroxyconiferyl alcohol monomer. The compositional changes evident in the CCoAOMT-deficient alfalfa potentially enhance the utilization of alfalfa as a major forage crop by increasing the digestibility of its normally poorly digestible stem fraction. The data also further support the bi-functional role of CCoAOMT in the lignin pathway and the possible interactions between the two *O*-methyltransferases, CCoAOMT and COMT.

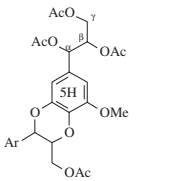
Experimental Procedures

Lignin isolation and preparation

Stem sections were ground to pass a 1.0 mm screen of a cyclone mill. The ground stems were extensively extracted with water, methanol, acetone, and chloroform. The isolated CWs were ball-milled, digested with crude cellulases, and extracted into 96:4 dioxane:H_2O. The soluble dioxane:water fractions were lyophilized before they were dissolved in ~400 μL acetone-d_6 for NMR analysis.

Cell Wall Composition

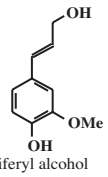
Klason lignin determinations were the ash-corrected residue remaining after total hydrolysis of CW polysaccharides. Total uronic acids were estimated colorimetrically with glacturonic acid as the calibration standard. Neutral sugars from total CW hydrolysis were determined by HPLC.



5-hydroxyguaiacyl glycerol structures

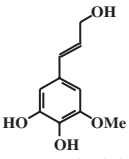
G_{5H}

Figure 1. 5-Hydoxyguaiacyl units with an acetylated glycerol sidechain.



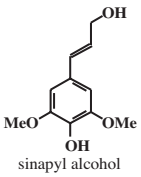
coniferyl alcohol

1_G



5-hydroxyconiferyl alcohol

1_{5H}



sinapyl alcohol

1_S

Figure 2**.** Resulting lignin precursors in the lignin biosynthetic pathway in angiosperms.

Rumen Microbiology

Allisonella histaminiformans and Its Potential Role in Ruminal Histamine Accumulation and Laminitis

J.B. Russell and M.R. Garner

Introduction

Dairy cattle are fed grain supplements to stimulate production, but it has long been recognized that grain can cause problems. When the rate of starch fermentation exceeds the buffering capacity of the rumen, acids accumulate, ruminal pH declines, and in severe cases the animal dies. In less severe cases, the animal survives, but the ruminal wall is ulcerated and often permanently scarred. Because the tissues above the hoof are also affected (laminitis), the animal can suffer temporary or even prolonged lameness (Takahashi and Young, 1981). In the 1940's, Dougherty and his colleagues (Dougherty, 1942) noted that grain feeding also promoted ruminal histamine production. Histamine is a powerful inflammatory agent, and they concluded that there was a direct correlation between "the histamine level of ingesta and the well being of the animal." Histamine is formed from the decarboxylation of the amino acid, histidine, and even a small conversion of histidine to histamine can be toxic (Suber et al. 1970). In the 1950's, Rodwell (1953) isolated histidine decarboxylating lactobacilli from sheep and horses fed grain-based rations. The potential involvement of lactobacilli was consistent with the observation that lactobacilli are highly pH-resistant bacteria that accumulate in the rumen when animals are fed an abundance of cereal grain. However, we were unable to isolate histamine-producing lactobacilli. Our histidine enrichments yielded a new bacterium, *Allisonella histaminiformans* (Garner et al. 2003).

Materials and Methods

Ruminal contents were obtained from dairy cows fed a standard dairy ration. The mixed ruminal bacteria were serially diluted (10-fold increments) into MRS, a medium "selective" for lactobacilli (deMan et al., 1960), that had been supplemented with 50 mM histidine. The histidine enrichments were then transferred successively in an anaerobic, carbonate-based medium (50 mM histidine) without glucose. The histidine enrichments were streaked onto the surface of agar plates (50 mM histidine) and colonies were picked and tested for histamine production. Histamine was assayed by a method that employed thin layer chromatography. Cellular fatty acids were extracted and assayed by gas chromatography. G+C analysis was performed using a spectroscopic DNA-DNA hybridization method. Amino acids were separated on a lithium cation exchange column and detected at 560 nm following ninhydrin post column derivation. Fermentation acids were analyzed by high-performance liquid chromatography. Chromosomal DNA from histamine producing isolates was purified using the FastDNA Spin Kit (Bio101, Vista, CA). Chromosomal DNA was mixed with primers (40 nmol 27F and 1492R), and the 16S rRNA gene region was amplified. By using the neighbor joining method, it was possible to calculate a distance matrix, and the precision of the relationships

improved by generating 1000 trees with a bootstrap method. Sequences from other bacteria were obtained from Genbank and aligned using Clustal X.

Results

When ruminal fluid from dairy cattle was serially diluted in anaerobic MRS medium containing histidine (50 mM), histamine was detected at dilutions as high as 10^{-7}. The histidine enrichments were then transferred successively in an anaerobic, carbonate-based medium (50 mM histidine) without glucose. All of the histamine producing isolates had the same ovoid morphology. The cells stained Gram-negative and were resistant to the ionophore, monensin (25 μM). The doubling time was 110 min. The G+C content was 46.8%. Lysine was the only other amino acid used, but lysine did not allow growth if histidine was absent. Because carbohydrate and organic acid utilization was not detected, it appeared that the isolates used histidine decarboxylation as their sole mechanism of energy derivation. 16s rRNA gene sequencing indicated that the isolates were most closely related to low G+C Gram-positive bacteria, but similarities were ≤ 94%. Because the most closely related bacteria (*Dialister pneumonsintes, Megasphaera elsdenii* and *Selenomonas ruminantium)* did not produce histamine from histidine, we propose that these histamine producing bacteria be assigned to a new genus, *Allisonella,* as *Allisonella histaminiformans* gen. nov., sp. nov. The genus name *Allisonella* honors Milton J. Allison, a prominent rumen microbiologist. Dr. Allison previously isolated *Oxalobacter formigenes,* a ruminal bacterium that decarboxylates oxalate (Allison et al., 1985). The species name is a Latin word that means "forming histamine."

Dairy cows fed a commercial dairy ration had large populations (>10^6 cells per ml) of *A. histaminiformans,* but this bacterium could not be isolated from cattle fed diets consisting only of hay. When stationary phase *A. histaminiformans* MR2 cultures were serially diluted into autoclaved ruminal fluid from cattle fed hay, histamine was not detected at dilutions greater than 10^{-2} even if histidine (50 mM) was added. In contrast, histamine was detected in the 10^{-9} dilution if the autoclaved ruminal fluid was obtained from cattle fed the commercial ration and supplemented with histidine (50 mM). The commercial ration contained large amounts of alfalfa and corn silage, and water-soluble silage extracts stimulated the growth of *A. histaminiformans* MR2 in vitro. Alfalfa silage extract was at least 8-fold more potent than the corn silage extract, and extract from only 5 mg of alfalfa dry matter promoted maximal histamine production in 1 ml of culture medium. Because non-ensiled alfalfa did not stimulate histamine production nearly as much as the alfalfa silage, the factor that stimulates the growth of *A. histaminiformans* appears to be a product of silage fermentation.

Discussion

Histidine decarboxylase, the enzyme that produces histamine, has been studied in great depth and is widely distributed in bacteria, but *A. histaminiformans* is the first histamine producing bacterium that can utilize histidine as its sole source of energy. It had generally been assumed that lactobacilli were responsible for ruminal histamine accumulation. Given the observation that our ruminal enrichments were not stimulated by glucose, it appeared that bacteria other than lactobacilli were responsible for histamine production. Previous workers noted that histamine did not accumulate in the rumen unless the diet had considerable amounts of grain and the pH was acidic. These results are consistent with the observation *A. histaminiformans* is acid resistant bacterium. *A. histaminiformans* could not be isolated from cattle fed hay, and we originally thought that this effect

might be due to pH or an antagonism. However, the observation that small amounts of yeast extract counteracted the "inhibition" in vitro suggested that another factor was involved. Based on the observation that the dairy ration had large amounts of silage, we tested the ability of silage extracts to stimulate growth. Results indicated that silages, and in particular alfalfa silage, stimulated the growth of *A. histaminiformans* in ruminal fluid from a cow fed hay.

Conclusions

Cattle fed dairy rations have A. *histaminiformans*, a bacterium that produces histamine, but its numbers in vivo are clearly diet-dependent. Further work will be needed to define more precisely the chemistry of the growth factor that stimulates *A. histaminiformans*, but the observation that the growth factor can be derived from alfalfa silage could have practical significance. Logue et al. noted that dairy cattle fed grass silage had a significantly greater incidence of laminitis and foot lesions than cattle that were fed non-fermented dry forage. Given the observation that foot problems are a primary reason why dairy cattle are eliminated from the milking herd, any practice that could reduce laminitis would have a positive impact on dairy cattle production.

Degradation of Alfalfa Cell Wall Polysaccharides by Pure Cultures of Five Rumen Bacterial Species

H. G. Jung, P. J. Weimer, and F. M. Engels

Introduction

The rumen contains a diverse assemblage of bacterial species that interact both synergistically and competitively to degrade the cell wall matrix of forages. *Fibrobacter succinogenes*, *Ruminococcus albus*, and *R. flavefaciens* have generally been considered to be the dominant species responsible for cell wall degradation. While all three of these species have been reported to degrade both cellulose and hemicellulose, *F. succinogenes* is unable to utilize the pentose sugars released from hemicellulose. *Butyrivibrio fibrisolvens* is generally considered a poor cellulose degrader but good hemicellulose degrader, and *Lachnospira multiparus* has been suggested to be a specialist for pectin degradation. However, the data for cell wall polysaccharide degradation capabilities of these rumen bacteria are generally based on purified substrates rather than intact forage cell wall matrixes. Differences in affinity of these bacterial species for degradation of specific plant tissues is almost unknown. This study examined the ability of these five rumen species to degrade alfalfa stem tissues and alfalfa cell wall polysaccharides *in situ.*

Materials and Methods

Alfalfa stems were harvested after 21 and 32 d of re-growth from a single alfalfa genotype grown in the field. For quantitative measures of cell wall degradation, stems were freeze-dried and ball-milled. Tissue degradation was assessed using 100 mm thin sections made from 50% ethanol preserved stems. Both ground alfalfa and sections were inoculated with pure strains of *Butyrivibrio fibrisolvens* H17c, *Fibrobacter succinogenes* S85, *Lachnospira multiparus* 40, *Ruminococcus albus* 7, *R. flavefaciens* FD-1, both individually and as a five species mixture. Additional samples were inocu-

lated with rumen fluid. Incubation periods were 6, 24, and 96 h for the sections and 24 and 96 h for the ground alfalfa stems. Sections were evaluated by light microscopy for when degradation of individual tissues began and the extent of degradation achieved. The ground alfalfa stems were analyzed for residual cell wall polysaccharides and degradabilities were calculated. Cellulose was estimated as the cell wall glucose content; hemicellulose as the sum of xylose, mannose, and fucose residues; and pectin as the sum of uronic acids, galactose, arabinose, and rhamnose.

Results and Discussion

Although the alfalfa stem internodes utilized in this study differed in age by 10 d of re-growth, cell wall composition was very similar. The two alfalfa maturities differed primarily in total cell wall concentration and the mature stems were slightly less degradable than the immature stems. For both the ball-milled alfalfa stems and the stem thin sections, increasing time of incubation resulted in increased cell wall degradation, although for the stem sections degradation was virtually complete by 24 h compared to greater degradation observed after 96 h for the ball-milled alfalfa. Averaged across both alfalfa maturity stages and incubation times, *Ruminococcus albus* degraded more cellulose than observed for rumen fluid (62.5 vs. 55.0%), with all other pure cultures and the mixture resulting in less than half the amount of cellulose degradation observed for rumen fluid. Contrary to expectations, *Fibrobacter succinogenes* degraded cellulose poorly (19.2%) and *Lachnospira multiparus* was able to degrade cellulose (19.2%).

Hemicellulose degradation was lower than observed for cellulose, although *R. albus* again exhibited the highest degradation (40.3%), followed by rumen fluid (26.7%). *Butyrivibrio fibrisolvens* was not an especially good hemicellulose degrader (15.6%) and *L. multiparus* was virtually incapable of degrading alfalfa hemicellulose (7.4%). All bacterial species and rumen fluid degraded pectin well. *L. multiparus* was not the best pectin degrader, consistent with reports that this species degrades pectin in intact cell walls much more poorly than it does purified citrus pectin. The microscopic examination of alfalfa stem tissue degradation matched the data from the ball-milled alfalfa (Fig. 1). *R. albus* was clearly able to degrade all tissues as well or better than rumen fluid. *F. succinogenes* and *R. flavefaciens* were able to degrade the non-lignified, thin-walled chlorenchyma and cambial tissues, but did not degrade the thick, non-lignified collenchyma tissue. *B. fibrisolvens* and *L. multiparus* attacked all the non-lignified tissues, but *L. multiparus* did not completely degrade the pectin-rich collenchyma tissue. While most of our results corresponded to literature reports for the degradation characteristics of these five rumen bacterial species, deviations from expectations were observed for several bacteria when grown on intact alfalfa cell wall matrices.

Conclusion

The quantitative measurements of ground alfalfa stem cell wall polysaccharide degradation by various rumen bacterial cultures matched the visual observations for degradation of thin sections from alfalfa stems reasonably well. Differences were noted among bacterial species for which alfalfa stem tissues each species degraded. The ability of bacterial species to degrade in situ cell wall polysaccharides did not always match previous literature for isolated substrates.

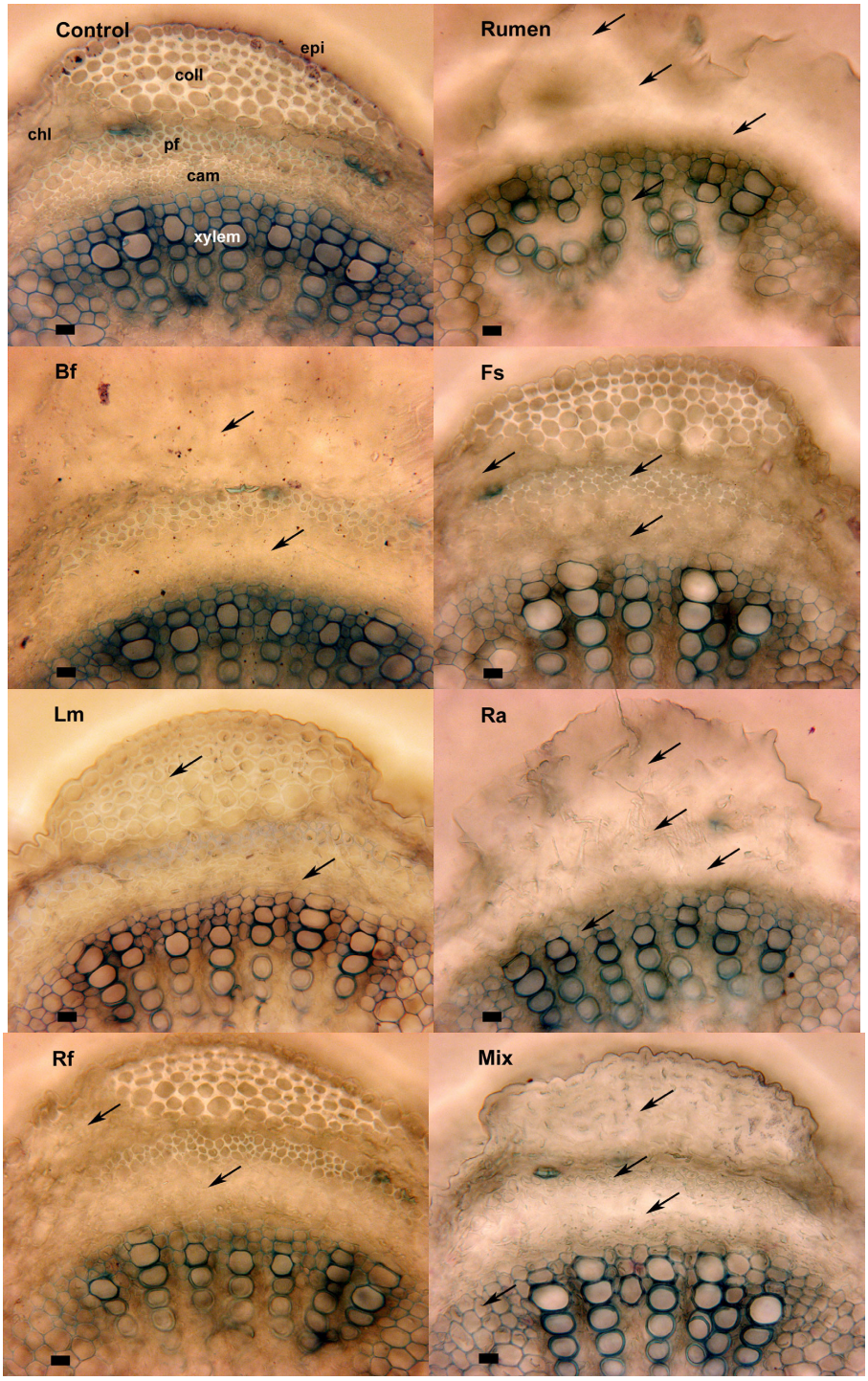


Figure 1. Degradation of immature alfalfa stem tissues after 24-h incubations with rumen fluid or *Butyrivibrio fibrisolvens* H17c (Bf), *Fibrobacter succinogenes* S85 (Fs), *Lachnospira multiparus* 40 (Lm), *Ruminococcus albus* 7 (Ra), *R. flavefaciens* FD-1 (Rf), or a mixture of the five pure cultures (Mix). The alfalfa stem tissues are identified in the Control micrograph (epi = epidermis; coll = collenchyma; chl = chlorenchyma; pf = phloem fibers, cam = cambium and secondary phloem; and xylem = xylem fibers and vessels). Tissues showing signs of degradation are indicated by the arrows. Bar is 20μm.

Albsuin A, a Bacteriocin from *Ruminococcus albus* that Inhibits the Growth of *Ruminococcus Flavefaciens*

J. Chen, D.M. Stevenson, and P.J. Weimer

Introduction

In the rumen, the relative population size of one fiber-digesting bacterial species, *Ruminococcus albus*, has been shown to be considerably greater than the relative population size of two of the other major agents of fiber digestion, *R. flavefaciens* and *Fibrobacter succinogenes*. Several research groups have reported that the predominance of *R. albus* is due in part to its production of a bacteriocin (a protein produced by one bacterial strain that inhibits a closely related strain or species), but such an agent has not been purified from *R. albus*. Bacteriocins are of interest because they may represent a means to control populations of individual bacterial species without the use of antibiotics, which have a broader spectrum of activity and which may have undesirable effects via stimulation of the development of resistance by pathogenic microbes through horizontal gene transfer.

Methods

Ruminococcus albus strain 7 was grown in pure culture under CO_2 in 3.6 L of a modified Dehority medium that contained 3.5 g cellobiose and 1 g of yeast per liter. After 28 h of incubation at 39 °C, culture was passed through a 0.2 μm hollow fiber cartridge filter to remove cells. The resulting permeate was concentrated using a 10 kDa MWCO hollow fiber cartridge, and subjected to a purification scheme that included ammonium sulfate precipitation, Toyopearl HW50F size exclusion chromatography and DEAE 650M anion-exchange chromatography. Bacteriocin activity was assayed by spotting ~50 μL of sample from each stage of the purification onto Petri dishes containing a freshly-poured MDM agar that contained the *R. flavefaciens* test strain, followed by incubation at 39 °C in an anaerobic glovebag. Purification of the bacteriocin was assessed by SDS-PAGE. The purified bacteriocin fraction was recovered from the gel by electrotransfer to a PVDF membrane, and was subjected to N-terminal sequencing at the University of Texas Medical Center - Galveston. Sequence similarities were determined by BLAST search on the website of the National Center for Biotechnology Information.

Results and Discussion

The purification scheme resulted in recovery of a protein fraction that could prevent the growth of all five strains of *R. flavefaciens* tested, but did not affect the growth of *F. succinogenes, Streptococcus bovis, Selenomonas ruminantium*, or *Escherichia coli*. Specific activity of the bacteriocin was 131-fold greater in the fraction than in the original culture supernatant. The fraction contained 36 kDa protein and small amounts of a 45 kDa protein (Fig. 1). Separate elution and testing of the two proteins from an SDS-PAGE gel revealed that the activity resided in the 36 kDa band. The eluted 36 kDa protein, named albusin A, had the N-terminal amino acid sequence SGLDAKGIVSQMKIGWNLGNTLDAXNXKV. This sequence displayed similarity to several endoglucanase enzymes from other *Ruminococcus* and *Clostridium* species (Fig. 2). However, these sequences are not considered to be active-site motifs for endoglucanase enzymes, and the chromatographic fraction containing albusin A had less than 1% of the endoglucanase specific activity of a typical *R. albus* culture supernatant. Moreover, the chromatographic fraction that eluted immediately afterward (peak 3, Fig. 1A), and which contained more of the 45 kDa protein and no 36 kDa

protein (Fig. 1B), displayed five-fold higher endoglucanase activity, suggesting that the endoglucanase activity resided in the 45 kDa protein. Attempts to clone a the gene encoding albusin A into a pGEM-3Z vector, with detection by a degenerate probe based on the N-terminal sequence, were unsuccessful.

Based on its relatively large size (31 kDa), its relatively hydrophilic character, and its bacteriostatic (rather than bacteriocidal) activity, albusin A represent a class III bacteriocin, as defined by Klaenhammer. Unlike the more familiar, membrane-active class I and class II bacteriocins, the mode of action of class III bacteriocins is not known.

Conclusions

Albusin A is the first bacteriocin purified from a ruminal cellulolytic bacterium. Production of this bacteriocin apparently provides one mechanism by which *R. albus* can suppress the growth of *R. flavefaciens* in laboratory culture. Bacteriocin production by *R. albus* may be important in the dominance displayed by this species in the ruminal environment, although more definitive tests of this hypothesis must await purification of the agent in larger quantities that would permit its direct addition to the rumen of test animals.

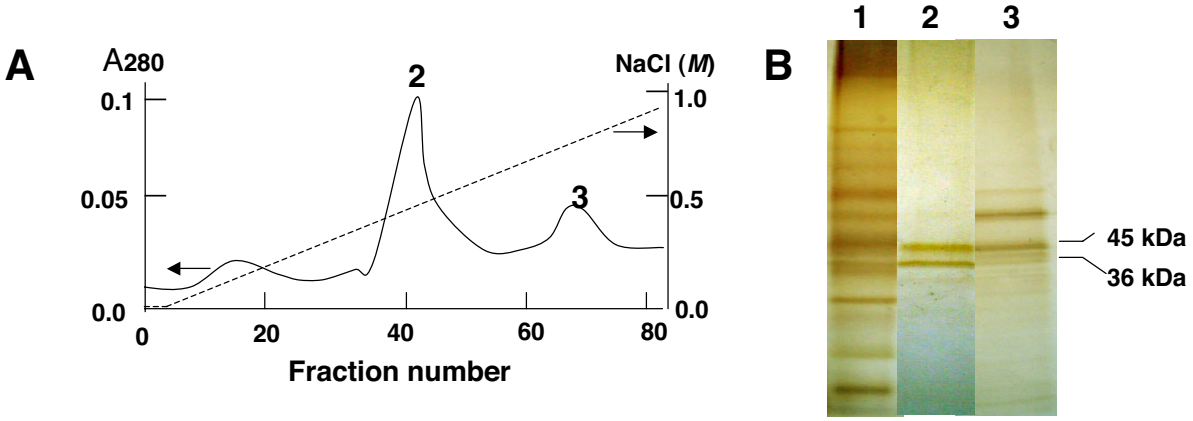


Figure 1. A) DEAE 650M column chromatography yielded a fraction (peak 2) that contained bacteriocin activity, and a fraction (peak 3) that did not contain activity. B) SDS-PAGE gels of peak 2 displayed protein bands of 36 kDa and 45 kDa, while peak 3 contained the 45 kDa band but not the 36 kDa band. The 36 kDa protein (albusin A) contained the inhibitory activity.

Gene product	Total amino acids or MW (kda)		
R. flavefaciens cellulase	455aa	24	hssa..rryadaadntmtafqitenmkvgwnlgntldryaqkanpkdpsk 71
R. flavefaciens EGA	759aa	25	fsfaplddtadaadnimtafqitenmkvgwnlgntldayaqkanpkdpsk 74
C. cellulovorans EG	515aa	25	aftgvrdv.....p.aqqivnemkvgwnlgntmda..... 55
C. cellulovorans EGD	515aa	25	aftgvrdv.....p.aqqivnemkvgwnlgntmda..... 55
C. cellulovorans EGB	441aa	22	aktgirdi.....t.sqqvvkemkvgwnlgntmda..... 52
C. cellulovorans EGB	440aa	22	aktgirdi.....t.sqqvvkemkvgwnlgntmda..... 52
R. albus EG	411aa	22	aktgirdi.....t.sqqvvkemkvgwnlgntmda..... 52
C. acetobutylicum EG5	370aa	21	atvnphdm.....t.sqqivndmkvgwnlgntlda..... 51
C. thermocellum EGE	814aa	37	vmrgmrdi.....s.aidlvkeikigwnlgntlda..... 67
C. longisporum EGA	517aa	21	lsisrnplevqaasmrs.aseivqemgvgwnlgntldakitn..... 63
R. albus EGI	406aa	51	envpvsqthtndtmtvtsakdlvakmtngwnlgntmdataqg..... 64
R. albus F-40 EGIV	50-kDa	1	....lsgl.....d.akgivsqmkiggnlg..... 20
Albusin A (~36-kDa band)	~36-kDa	1	....lsgl.....d.akgivsqmkigwnlgntlda..... 25
Consensus sequence			----------V--MK-GWNLGNT-DA----------

Figure 2. Alignment of the N-terminal sequence of albusin A with sequences of several endoglucanases from Gram-positive bacteria.

Albsuin B, a Second Bacteriocin from *Ruminococcus Albus* that Inhibits the Growth of *Ruminococcus flavefaciens*

J. Chen, D.M. Stevenson, and P.J. Weimer

Introduction

In the rumen, the relative population size of one species, *Ruminococcus albus*, has been shown to be considerably greater than the relative population size of two of the other major agents of fiber digestion*, R. flavefaciens* and *Fibrobacter succinogenes*. The predominance of *R. albus* in both laboratory culture and the rumen appears to be due in part to production of bacteriocins (proteins produced by one bacterial strain that inhibits a closely related strain or species). We have previously purified, from *R. albus* 7, a bacteriocin which we have designated albusin A. Here we report the purification of a second bacteriocin, albusin B, and the sequence of the gene encoding its production.

Methods

Ruminococcus albus strain 7 was grown in pure culture under CO_2 in 10 L of a modified Dehority medium that contained 3 g of cellobiose and 1 g of yeast extract per liter. After 28 h of incubation at 39 °C, cells were removed using a 0.2 μm hollow fiber filtration cartridge. The resulting permeate was passed through a 10 kDa MWCO hollow fiber cartridge, and subjected to a purification scheme that included ammonium sulfate precipitation and size-exclusion chromatography (BioGel P6 column).

Bacteriocin activity was assayed by spotting ~50 *u*L of sample from each stage of the purification onto Petri dishes containing a freshly-poured MDM agar that contained the *R. flavefaciens* test strain, followed by incubation at 39 °C in an anaerobic glovebag Purification of the bacteriocin was assessed by SDS-PAGE. The purified bacteriocin fraction was recovered from the gel by electrotransfer to a nylon membrane, and was subjected to N-terminal sequencing at the University of Texas Medical Center - Galveston. *Hin*cII digests of *R. albus* DNA were cloned into a pGEM vector in *E. coli*, and recombinants selected by blue/white screening. A degenerate probe was used to identify the clone of interest by colony hybridization. DNA sequence upstream of the cloned segment was determined following two stages of PCR gene walking. Sequence similarities were determined by BLAST search on the website of the National Center for Biotechnology Information, and on the unfinished genome of *R. albus 8* at the website of The Institute of Genomic Research .

Results and Discussion

The purification scheme took advantage of the fact that a substantial fraction of the inhibitory activity passed through a 10 kDa hollow fiber filter. The scheme resulted in recovery of a protein fraction that could prevent the growth of *R. flavefaciens* FD-1, but did not affect the growth of *F. succinogenes, Streptococcus bovis, Selenomonas ruminantium*, or *Escherichia coli*. This fraction had a specific activity *against R. flavefaciens* FD-1 that was 220-fold greater than that of the original culture supernatant. Silver staining of SDS-PAGE gels of this fraction revealed a single 31 kDa protein, designated AlbB. A degenerate oligonucleotide probe based on the N-terminal sequence of the purified AlbB was used to identify two putative clones in pGEM3Z vector in *E. coli*. Sequencing of these cloned segments revealed them to be identical but in opposite orientations. Because the clones did not encode for the first nine amino acids of the gene, gene-walking PCR was used to

generate an additional 284 nt of DNA upstream of the cloned segment. The nucleotole sequence of *alb*-B nd its predicted protein Alb B have been deposited in GenBank under accession number AF469209.

The complete *alb*B gene encodes for a preprotein with a cleavage site between Ala46 and Ala47. The mature bacteriocin has a predicted MW of 32,168 Da and a predicted pI of 7.7. BLAST search revealed some similarity between the N-terminal region of AlbB and the carboxy terminal region of LlpA, a 30 kDa bacteriocin produced by a rhizosphere-associated *Pseudomonas* strain. A search of the unfinished *R. albus* 8 genome on the website of The Institute for Genomic Research revealed two homologs to AlbB, each with 73% identity.

Based on its relatively large size (31 kDa), its relatively hydrophilic character, and its bacteriostatic (rather than bacteriocidal) activity, albusin B represent a class III bacteriocin, as defined by Klaenhammer. Unlike the more familiar, membrane-active class I and class II bacteriocins, the mode of action of class III bacteriocins is not known.

Conclusions

Albusin B is the second bacteriocin purified from a ruminal cellulolytic bacterium, and the first for which a gene sequence has been determined. The sequence similarity between AlbB and LlpA suggests that this type of bacteriocin may be widely distributed across phylogenetic groups of procaryotes. Production of AlbB apparently provides yet another means by which *R. albus* can suppress the growth of *R. flavefaciens* in laboratory culture. Bacteriocin production by *R. albus* may be important in the dominance displayed by this species in the ruminal environment, although more definitive tests of this hypothesis must await purification of the agent in larger quantities that would permit its direct addition to the rumen of test animals.

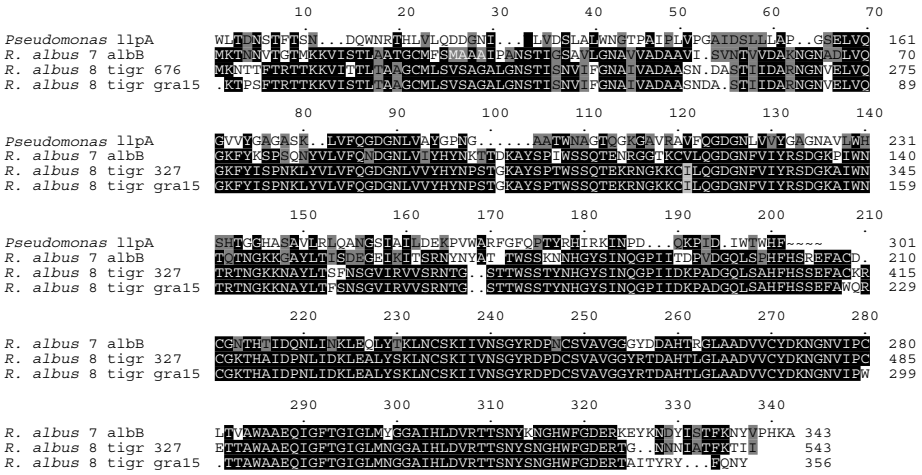


Figure 1. Alignment of the predicted amino acid sequence of the *alb*B gene of *R. albus* 7 with that of the *Pseudomonas llp*A gene (A. Parrett et al., J. Bacteriol. 195:897-908 [2003]) and two contigs from the unfinished genome of *R. albus* 8.

Residues of *Ruminococcus* Cellulose Fermentations as Components of Wood Adhesive Formulations

P.J. Weimer, A.H. Conner, L.F. Lorenz

Introduction

Ruminococcus bacteria are major agents of fiber digestion in the rumen. In pure culture, some ruminococci produce ethanol and other compounds, and thus may be useful for industrial-scale conversion of cellulosic biomass to fuels and chemicals. During the process of degrading cellulosic substrates, these bacteria produce a sticky glycocalyx that allows them to adhere to cellulose, and production of the glycocalyx appears to be a prerequisite of effective fiber degradation. The tenacity with which the bacteria adhere to fiber in an aqueous environment led us to examine the potential utility of these fermentation residues (which contain bacterial cells, their glycocalyx and the remaining cellulosic substrate) as adhesives that can add value to the ethanol production process. Such bio-based adhesives are of potential interest as partial or complete replacements for toxic, petroleum-based phenol-formaldehyde adhesives.

Methods

Ruminococcus albus strain 7, and *R. flavefaciens* strains FD-1 and B34b were grown in pure culture at scales up to 40 liters under CO_2 in a modified Dehority medium that contained 2 to 3.5 g of microcrystalline cellulose, 0.5 g of yeast extract and 25 μ moles of 3-phenylpropionic acid per liter. After 88-108 h of incubation at 39C, the culture liquid was removed by aspiration, and the sticky fermentation residue was recovered by centrifugation. Protein content was determined by the Coomassie blue method following solubilization , and the carbohydrate composition of the glycocalyx was determined by boiling in neutral detergent solution to remove bacterial cells, followed by hydrolysis in 2 *N* TFA at 120 °C for 1.5 h and sugar quantitation by ion chromatography.

Adhesive formulations were prepared with wet fermentation residue (WFR) or lyophilized (freeze-dried) fermentation residue (LFR), either alone or in combination with different proportions of phenol-formaldehyde (PF). For comparison, control PF adhesives were prepared with 8% GLU-X, (a commercial adhesive extender derived from wheat flour) but without fermentation residue. The adhesives were used to prepare three-ply aspen panels (each panel 7" x 7" x 1/8") with the grain of the inner sheet oriented perpendicular to the outer two sheets. Panels were pressed for 8 to 10 min at 180 °C and 1.125 MPa (163 lb/in^2), then cut into 1” x 3” lap shear specimens. The specimens notched with kerfs and subjected to shear strength and wood failure tests under both wet and dry conditions, according to standard testing protocols (American Society for Testing and Materials method PS 1-95).

Results and Discussion

Fermentation residues varied in protein content from 0.4 to 4.2%, depending on the completeness of the original fermentation. The neutral sugar composition of the residues displayed similar ratios (0.71 Glc, 0.18 Xylose, 0.08 Mannose, 0.02 Arabinose, molar basis) independent of bacterial species. The fermentation residues displayed no discernible odor before or after pressing, and the resulting bondlines in the panels were light in color, a desirable end-use property of adhesives.

Fermentation residues from *R. albus* 7 displayed superior adhesive properties to those of the two *R. flavefaciens* strains, most likely because the *R. albus* fermentation proceeded to a greater extent and thus would have had a higher ratio of (glycocalyx plus cells)/(unfermented residue). The LFR formulation displayed shear strength and wood failure values superior to the WFR formulations (Fig. 1). High wood failure rates are desirable, as they indicate that the adhesive is stronger than the wood itself.

Shear strengths of the LFR adhesives approached those of the control PF resins under dry conditions, but displayed undesirably low strength under wet conditions (Fig. 2). By incorporating the *R. albus* LFR into mixed adhesives with PF, acceptable shear strengths and wood failure values could be attained, even when 73% of the formulation was comprised of LFR. As expected, exposure of the panels to wet conditions decreased both shear strength and wood failure, but these values were better than most of those for soy-based protein adhesives..

Conclusions

These data suggest that fermentation residues containing the glycocalyces and bacterial cells may have potential as components of wood adhesives. Additional experiments with more authentic biomass materials (e.g., alfalfa stems), and spanning a range of adhesive proportions and pressing conditions, should permit a more complete analysis of the practicality and economics of producing these residues as a value-added co-product of microbial fermentations.

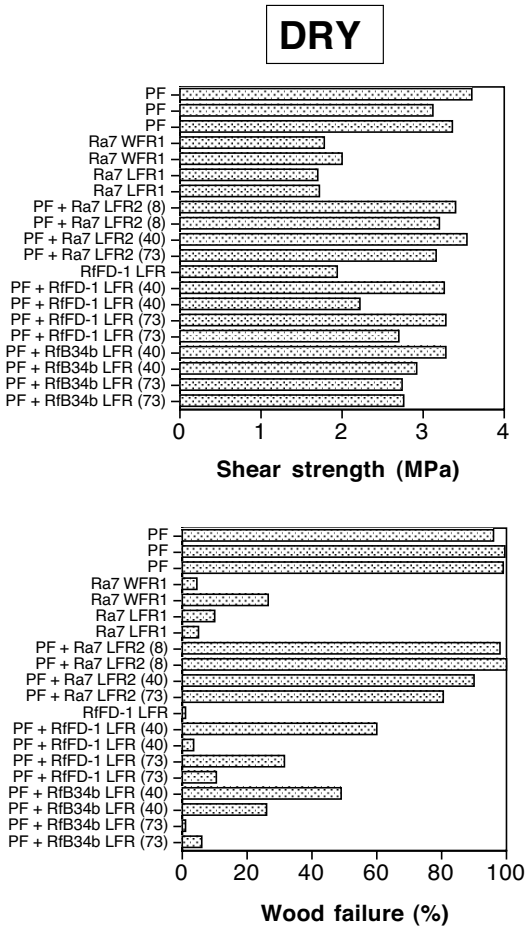


Figure 1. Shear strength (A) and wood failure percentage (B) for 3-ply aspen plywood panels prepared with adhesives described in Table 2, tested under dry conditions. Numbers in parentheses indicate percentage of fermentation residue by weight in the adhesive formulation. Samples having different lower-case letters within treatments differ ($P<0.05$). Pooled standard error for shear strength = 0.58 MPa. Pooled standard error for wood failure = 16.9%.

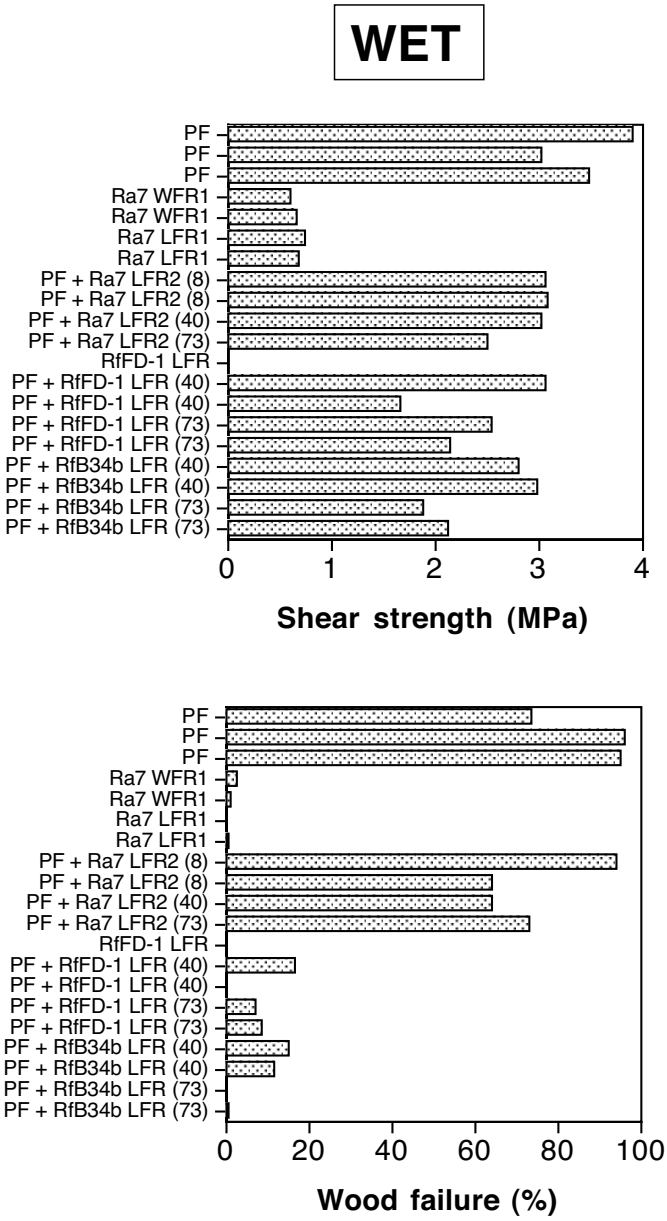


Figure 2. Shear strength (A) and wood failure percentage (B) for 3-ply aspen panels prepared with different adhesives, tested after vacuum/pressure/soak/drying [VPSD] treatment. Numbers in parentheses indicate percentage of fermentation residue by weight in the adhesive formulation. Samples having different lowercase letters within treatments differ ($P<0.05$). Pooled standard error for shear strength = 0.46 MPa. Pooled standard error for wood failure = 16.4%.

Forage Quality

Differences in Fiber and Carbohydrates between Corn Silage and Alfalfa Hay
D.R. Mertens

Introduction

Most of the difference in feeding value between the alfalfa hay (**AH**) and corn silage (**CS**) is related to the nutritional implications of their unique fiber and carbohydrate characteristics in AH and CS. Differences in fiber and carbohydrates not only affect intake potential, but also have significant ramifications on digestibility and ruminal function. Understanding these differences offers the opportunity to formulate dairy rations that take advantage of the attributes of these forages. The objectives of this research were to develop universal relationships between chemical components for AH and CS that could be used to compile a feed information reference database and contrast their carbohydrate characteristics and nutritional properties.

Materials and Methods

Compositions of CS provided in Table 1 were derived from relationships among chemical components in a diverse set of 32 corn silages. Alfalfa hay composition was derived from relationships among chemical components obtained from several published data sets and more than 2000 analyses obtained from ten commercial forage testing laboratories from throughout the U.S. Regression techniques were used to adjust for biases among laboratories and data sets to obtain the universal relationships among chemical components that were consistent among sources.

Acid detergent fiber (**ADF**) was used as the base chemical component for each forage quality and all other composition was related to it or to amylase-treated neutral detergent fiber (**aNDF**) to describe a chemical profile for five qualities of AH and CS. The aNDF method uses both heat-stable amylase and sodium sulfite and was designed to measure insoluble fiber in all types of feeds. It is our best estimate of total dietary fiber for dairy cows and the Association of Official Analytical Chemists, International has approved the aNDF method as Official Method 2002.04 - First Action "Amylase-treated Neutral Detergent Fiber in Feeds Using Refluxing in Beakers or Crucibles." This method has been the reference method for proficiency testing by the National Forage Testing Association since 1994, and results indicate that the 150 participating laboratories routinely have standard deviations among labs for aNDF that are similar to those for ADF.

Nonfibrous carbohydrates (**NFC**) can be estimated by subtracting crude protein (**CP**), fat or ether extract (**EE**) and ash from neutral detergent solubles (**NDS** = 100 – aNDF), i.e. NFC = 100 – aNDF – CP – EE – Ash). Starch was measured after heat or alkali gelatinization and enzymatic hydrolysis to glucose, which was measured colorimetrically. Pectin was determined as the sum of uronic aicds or by solubilization in oxalate solutions.

Results and Discussion

The crude protein, ash, crude fat, fiber, and lignin values in Table 1 agree with those of similar qualities of AH found in the 2001 Dairy NRC. The difference between aNDF and ADF is an estimate of hemicellulose, but for AH, this difference is a slight underestimate because 10 to 20% of the pectin is not extracted by acid detergent. The main NFC in alfalfa is pectin, which is about 40 to 50% of the NFC. In AH, quality is primarily a function of plant maturity. As alfalfa matures the proportions of fiber and lignin increase and the proportions of crude protein and NFC decrease. The negative relationship between maturity and nutritional value are greatest for early spring plant growth.

Ash values are higher and acid detergent lignin values are lower for CS reported in Table 1 than those provided in the 2001 Dairy NRC, but ADF and aNDF values are similar, which suggests that NRC results were obtained using the aNDF method. The predominant NFC in CS is starch and CS contains very little pectin or neutral detergent soluble fiber. Corn silage can vary substantially in chemical composition because grain content is variable and related inversely to fiber concentration. Although immature CS typically have lower grain contents, maturity is not as highly related to nutritional quality in CS as it is in other forages. The inverse relationships between fiber or lignin concentration and maturity often do not hold for CS because it is a mixture of grain and stover. The lignin concentration of corn stover increases and its digestibility declines with maturity; however, as corn matures after silking it generates grain that dilutes the concentration and nutritional impact of the maturing stover often resulting in whole plant digestibilities that vary little with maturity.

Contrasts between alfalfa hay and corn silage. Although the fiber concentrations of AH and CS are very similar at the maturities commonly fed to dairy cows, the non-fiber components and lignin concentrations differ significantly. Like most legumes, alfalfa has much a higher (2-3X) concentration of lignin than grasses such as corn, which indicates that its fiber digestibility will be lower. Because CS has much less protein and ash content (30-50% less) than AH and similar fiber concentration, CS has significantly more NFC, especially when it contains normal or higher proportions of grain. Not only is the concentration of NFC different between AH and CS, but also the composition of the NFC is different. In AH, the major carbohydrates in NFC are pectin and other soluble fibers, but in CS the major NFC is starch. Although pectin is rapidly fermented and is included in the NFC fraction, it ferments in the rumen like other fibrous constituents; its fermentation is sensitive to low ruminal pH and it results in the production of acetic acid. Starch fermentation, on the other hand, is less sensitive to ruminal pH and results in the production of propionic and lactic acids in the rumen.

At similar ADF concentrations, AH is similar in digestibility to CS not because its fiber digestibility is superior, but because its aNDF content is lower (10%-units) and NDS content is higher. Typically the aNDF digestibility of AH is only 75-85% of that of CS. However, the NDS in CS may not have 98% digestibility because starch in whole kernels and large fragments can escape digestion unless it has been effectively processed through rollers with a 1-mm clearance or is thoroughly chewed when fed at 1X maintenance. Of the true DM digestibility in AH, only 19-20% comes from digestible fiber compared to 30-34% for processed CS. In both forages, digestible NDS provides the overwhelming majority of DM digestibility. For processed CS, starch alone provides 28-31% of the true DM digestibility, which is similar to the contribution of digestible fiber in CS.

When 25% processed CS is substituted for very high quality AH in a dairy rations the aNDF and

physically effective NDF in the ration increase about 2%-units, NFC remains constant, and pectin decreases about 3%-units. The increase in aNDF content might result in slightly lower DM intakes, which may also be decreased because the rate of digestion of the NDF in CS is about half of that for high quality AH. Some of the expected decrease in intake may be alleviated by the high palatability and the slightly higher energy density of CS-based rations that contain less lignin than AH-based rations. The impact of the decreased pectin content of the ration containing CS is unclear. If the ration was borderline for peNDF and starch concentration, this small change in pectin concentration might have a significant impact. However, the substitution of CS for AH increases the peNDF content of the ration without significantly changing starch content, which results in a ration that should promote efficient ruminal fermentation and maintain milk fat production. It is expected that the rations containing either 25% high quality alfalfa hay or 25% normal corn silage would obtain similar levels of milk production and overall animal performance. A key requirement is that the corn silage be of high quality with good fermentation and proper management of the silo face.

Conclusions

Alfalfa hay and corn silage can both be excellent forages for dairy rations when managed properly. Each has unique carbohydrate characteristics that may complement one another when used together in rations for dairy cows. The nonfibrous carbohydrates in alfalfa and corn silage are very different. Alfalfa contains a significant proportion of pectin; whereas corn silage NFC is predominantly starch. When corn silage is substituted for alfalfa hay in dairy rations there is a small increase in aNDF and decrease in pectin. It is not expected that these ration differences would result in any difference in animal performance, if the corn silage fermentation quality is high and silo management is acceptable.

Forage/Description	CP[a]	EE[b]	Ash	NFC[c]	Star[d]	Pec[e]	aNDF[f]	ADF[g]	ADL[h]
Alfalfa hays									
Exceptional quality	25.4	2.7	10.4	31.5	3.1	14.2	30.0	24.0	4.53
Very high quality	24.0	2.6	9.9	29.4	2.9	13.2	34.1	27.0	5.38
High quality	22.5	2.5	9.5	27.4	2.7	12.3	38.2	30.0	6.23
Good quality	21.0	2.4	9.1	25.3	2.5	11.4	42.2	33.0	7.08
Fair quality	19.5	2.2	8.7	23.2	2.3	10.5	46.3	36.0	7.93
Corn silages									
Very high grain	8.3	3.2	4.1	48.4	31.1	1.9	36.0	21.0	1.57
High grain	8.6	3.1	4.6	43.2	27.2	1.7	40.5	24.0	1.91
Normal	8.8	3.0	5.1	38.1	23.2	1.5	45.0	27.0	2.25
Low grain	9.0	2.8	5.7	33.0	19.2	1.3	49.5	30.0	2.59
Very low grain	9.3	2.7	6.2	27.8	15.3	1.1	54.0	33.0	2.93

[a] Crude protein
[b] Ether extract or crude fat
[c] Nonfiber carbohydrates calculated by difference (NFC = 100 – aNDF – CP – EE – Ash)
[d] Starch
[e] Pectin, estimated from NFC
[f] Amylase-treated neutral detergent fiber determined with sodium sulfite and amylase
[g] Acid detergent fiber
[h] Acid detergent lignin using 72% sulfuric acid

Table 1. Composition of alfalfa hays and corn silages varying in fiber content.

Feed Utilization by Cattle

Effects of NPN Content of Alfalfa and Red Clover Silages on the Production of Lactating Cows
J. J. Olmos Colmenero, A. F. Brito, G. A. Broderick, S. M. Reyal

Introduction

There is evidence that reducing the NPN content of hay-crop silages will improve protein efficiency in lactating dairy cows. Previous research showed that formic acid treatment of alfalfa silage (AS) reduced NPN and improved milk production (Nagel and Broderick, J. Dairy Sci. 75:140-154, 1992; Broderick and Radloff, 2002 USDFRC Res. Sum.). The lower NPN content of red clover silage (RCS) also has been shown to be related to improved protein efficiency when it replaces AS in the diet (Broderick, 2001 USDFRC Res. Sum.). This study was conducted to compare feeding all of the dietary forage as control AS, AS treated with ammonium tetraformate (ATF; GrasAAT®,HydroAgri, Norway), or as RCS of either early or late maturity. The effect of these forages on milk production and ruminal metabolites was assessed in lactating dairy cows.

Materials and Methods

Alfalfa silage was harvested from third cutting and two RCS were harvested as early or late maturity from second cutting. The late RCS (RCS1) was harvested at about the same maturity as was used in earlier feeding studies; the early RCS (RCS2) was harvested to obtain a forage about equal in CP to the AS. All forages were cut using a conventional mower conditioner, field-wilted to about 40% DM and then chopped at harvest to a theoretical length of 2.9 cm. Control AS and both RCS were ensiled without additives; the formate-treated AS (FAS) was treated with 6.2 L ATF/ton of wet forage while being chopped. Three forages were ensiled in upright concrete stave silos and RCS2 was ensiled in a bag silo. No forage was rained on during harvest. Both AS and RCS1 also were fed in another study (Broderick and Radloff, 2002 USDFRC Res. Sum.). Forage compositions are in Table 1. Twenty-four Holstein cows (eight with ruminal cannulae) averaging 192 DIM were blocked by parity and DIM into six squares of four; cows within squares were randomly assigned to balanced diet sequences in a 4x4 Latin square trial. The TMR were formulated to contain 50% of total DM from one of the four silages. Diet compositions are in Table 2. Experimental periods were 4-wk long (total 16 wk); production data were collected during the last 2-wk of each period. At the end of each period, ruminal samples were collected over the 24-h clock from the eight cannulated cows and spot urine and fecal samples were collected from all cows. Urine volume was estimated from creatinine; apparent nutrient digestibility was estimated using indigestible ADF as internal marker. Statistical analysis was done using proc mixed in SAS; differences between least square means were reported only if the F-test for treatment was significant ($\alpha \leq 0.05$).

Results and Discussion

The silages differed in composition (Table 1). As expected, RCS1 was lower in CP than, and RCS2 more similar to, the two AS. The NDF content of RCS1 was higher than the other three silages. That the two AS and RCS1 were equal, but RCS2 lower, in ADF translated into higher hemicellulose

levels in both RCS. These differences between RCS and AS have been found in most previous studies comparing AS and RCS. The two RCS contained only 56% as much NPN as control AS. Treatment with ATF tended to depress silage pH and added 1.2 percentage units of extra CP from the ammonia N in this compound. Despite the added NPN, FAS had nearly 10% less NPN than control AS, even when expressed as a proportion of total N. Most of the reduction in NPN was due to lower free AA N. Both RCS (particularly RCS1) had high levels of ADIN. In previous trials, RCS averaged (% of total N) 5.1% ADIN versus 3.5% ADIN in AS. High levels of ADIN may depress protein utilization. Elevated ADIN indicated that both of the RCS fed in this trial were not typical and may have over-heated in the silo.

Diets containing AS and FAS had similar compositions; however, the two RCS diets, despite weekly adjustment of soybean meal to equalize N contents, contained about 1 and 2 percentage units less CP (Table 2). Production and nutrient utilization data are summarized in Table 3. Intake of DM was greater on the diet with FAS, intermediate on control AS and RCS1, and lowest on RCS2 (the higher quality RCS). Despite the differences in DMI, weight gain was greater on RCS. Previously, we have observed lower DMI and somewhat greater weight gain when RCS replaced AS in the diet. Milk and SNF yields paralleled DMI. Yield of FCM, fat, and protein all were greater on the two AS versus the two RCS. Although apparent N efficiency was higher and concentrations of milk and blood urea lower on the two RCS, these effects were confounded by the lower N contents of both diets. Other than a reduction in the proportion of milk NPN present as urea, there were no significant effects on production due to formate-treatment of AS in this trial. In our other work feeding the same two silages, we found improved intake and milk yield when FAS replaced control AS (Broderick and Radloff, 2002 USDFRC Res. Sum.). However, mean DMI was nearly 2 kg/d greater in that study. Nutrient digestibility and urinary N excretion were about equal on the two AS diets. However, digestibility of DM, organic matter, NDF, ADF, and hemicellulose all were highest on RCS2 and intermediate on RCS1, reflecting its later maturity. Differences similar to those found between the RCS1 diet and the two AS diets have been observed in all of previous trials comparing these forages where digestibility measurements were made. It is possible that the NEL derived from greater nutrient digestibility (especially on RCS2) were not reflected in improved production because the energy was deposited in body tissue. The lower N digestibilities on RCS probably reflected both the lower CP contents of those diets (Table 2) as well as the high proportions of ADIN (Table 1). Feeding RCS reduced urinary N excretion. It appeared that N utilization was impaired by elevated ADIN levels in the RCS fed in this trial.

Mean metabolite concentrations observed in the rumen when these diets were fed are in Table 4. Ruminal pH, total VFA, and molar proportions of the principal VFA were not altered by diet. Also, no differences resulted from feeding control AS or FAS. Ruminal concentrations of protein degradation products are, of course, influenced by dietary protein intake; however, feeding the two RCS diets reduced ammonia by nearly 50%. Moreover, branched-chain VFA, which are formed from catabolism of branched-chain AA released from protein degradation in the rumen, also were substantially reduced versus feeding the AS diets. Ruminal free AA, which derive partly from dietary protein degradation, were highest on control AS and lowest on RCS1. It was interesting that free AA concentrations were not different between FAS and RCS2. Overall, the pattern of ruminal metabolites indicated that protein degradation was lower on the RCS versus AS in this trial.

Summary and Conclusion

Treating AS with ATF reduced NPN content but its feeding did not result in increased intake or milk yield. Average and high quality RCS both had only 56% as much NPN as control AS; however, both also had elevated ADIN compared to previous trials. Feeding diets containing these RCS increased digestibility of DM and fiber and weight gain but reduced intake and milk production. The RCS diets were lower in CP and gave small increases in N efficiency and reductions in urinary N excretion. Ruminal metabolite patterns also indicated that protein degradation was reduced. The results of this experiment showed no production advantages in lactating dairy cows to formic acid treatment of AS or to feeding of RCS.

Table 1. Composition of alfalfa, formate-treated alfalfa, and red clover silages.[1]

	Silage					
Item	AS	FAS	RCS1	RCS2	SE	*P* > F
DM, %	41.8	42.2	43.4	41.8	0.7	0.85
CP, % of DM	24.8[b]	26.0[a]	18.9[d]	23.5[c]	0.3	< 0.01
Ash, % of DM	11.1[c]	10.9[c]	12.3[b]	12.7[a]	0.1	< 0.01
NDF, % of DM	39.4[b]	39.0[b]	41.4[a]	40.0[b]	0.4	0.04
ADF, % of DM	31.1[a]	30.3[a]	31.2[a]	28.1[b]	0.4	< 0.01
Hemicellulose, % of DM	8.3[c]	8.7[c]	10.2[b]	11.9[a]	0.2	< 0.01
pH	4.96	4.70	4.86	4.94	0.08	0.07
NPN, % of total N	50.0[a]	45.4[b]	27.2[c]	29.0[c]	0.9	< 0.01
NH_3-N, % of total N	4.1[a]	3.4[b]	2.6[c]	3.5[b]	0.1	< 0.01
Free AA-N, % of total N	39.3[a]	30.1[b]	12.6[d]	17.0[c]	0.6	< 0.01
ADIN, % of total N	4.2[c]	4.0[c]	16.8[a]	9.9[b]	0.6	< 0.01

[a,b,c,d]Means in the same row with different superscripts differ ($P < 0.05$).

[1]AS = Alfalfa silage, FAS = ammonium tetraformate treated alfalfa silage, RCS1 and RCS2 = red clover silages no. 1 and no. 2, SE = standard error.

[2]Probability of an effect of silage source.

Table 2. Composition of diets.[1]

Item	Diet[1] AS	FAS	RCS1	RCS2
	% of DM			
Alfalfa silage	50.0	. . .	. . .	. . .
Formate-treated alfalfa silage	. . .	50.0	. . .	. . .
Red clover silage #1	. . .	. . .	50.0	. . .
Red clover silage #2	. . .	. . .	. . .	50.0
Rolled corn silage	10.0	10.0	10.0	10.0
High moisture shelled corn	37.8	37.3	29.3	36.6
Solvent soybean meal	1.8	2.3	10.3	3.0
Salt	0.3	0.3	0.3	0.3
Vitamin-mineral premix[2]	0.1	0.1	0.1	0.1
Chemical composition				
Crude protein	18.9	19.0	16.9	17.9
NDF	32.2	32.0	34.2	33.3
ADF	22.3	22.0	22.2	20.9

[1]AS = alfalfa silage, FAS = ammonium-tetraformate treated alfalfa silage, RCS = red clover silage, CS = corn silage.

[2]Provided (/kg DM) 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E.

Table 3. Effect of feeding forage as control alfalfa silage (AS) or formate-treated alfalfa silage (FAS), or as one of two red clover silages (RCS1 and RCS2), on production of lactating cows.

Item	Diet AS	FAS	RCS1	RCS2	SE	$P > F^1$
DM intake, kg/d	23.3^{ab}	23.7^{a}	22.2^{bc}	21.5^{c}	0.7	0.01
BW gain, kg/d	0.19^{bc}	0.09^{c}	0.66^{a}	0.62^{ab}	0.25	0.03
Milk, kg/d	30.5^{a}	30.8^{a}	29.5^{ab}	28.6^{b}	0.8	0.03
3.5% FCM, kg/d	33.4^{a}	33.6^{a}	31.4^{b}	30.5^{b}	1.0	<0.01
Milk/DMI	1.33	1.31	1.34	1.34	0.03	0.73
Milk N/N intake, %	22.3^{b}	22.4^{b}	24.6^{a}	23.2^{ab}	<0.01	<0.01
Fat %	4.00	4.01	3.88	3.84	0.12	0.32
Fat, kg/d	1.23^{a}	1.25^{a}	1.14^{b}	1.10^{b}	0.04	<0.01
Protein, %	3.24^{b}	3.31^{a}	3.19^{bc}	3.16^{c}	0.04	<0.01
Protein, kg/d	0.99^{a}	1.02^{a}	0.94^{b}	0.90^{b}	0.03	<0.01
Lactose, %	4.81	4.81	4.84	4.80	0.05	0.87
Lactose, kg/d	1.50	1.49	1.44	1.39	0.04	0.06
SNF, %	8.97	9.04	8.93	8.86	0.07	0.12
SNF, kg/d	2.78^{a}	2.79^{a}	2.65^{ab}	2.56^{b}	0.08	0.01
Milk NPN, mg/dl	31.3	33.3	32.6	31.7	1.3	0.32
MUN,[2] mg/dl	19.1^{a}	18.1^{a}	15.3^{b}	14.9^{b}	0.8	<0.01
MUN/milk NPN	62.4^{a}	55.0^{b}	47.3^{c}	48.3^{c}	<0.1	<0.01
Blood urea N, mg/dl	11.6^{a}	11.7^{a}	9.8^{b}	10.1^{b}	0.3	<0.01
Apparent Digestibility, %						
DM	58.7^{c}	57.4^{c}	60.7^{b}	63.9^{a}	1.1	<0.01
Organic matter	59.2^{bc}	57.6^{c}	60.6^{b}	64.1^{a}	1.1	<0.01
NDF	37.2^{c}	36.9^{c}	48.2^{b}	55.0^{a}	1.2	<0.01
ADF	40.2^{c}	40.1^{c}	50.7^{b}	57.3^{a}	1.1	<0.01
Hemicellulose	30.2^{c}	29.8^{c}	43.8^{b}	50.9^{a}	1.7	<0.01
N	55.9^{a}	55.9^{a}	45.5^{c}	50.8^{b}	1.2	<0.01
Excretion, g/d						
Urinary N	209.5^{a}	210.9^{a}	151.3^{b}	158.0^{b}	5.8	<0.01

[a,b,c]LS Means in the same row with different superscripts differ ($P < 0.05$).

[1]Probability of a significant effect of diet.

[2]MUN = Milk urea N.

Table 4. Effect of feeding forage as control alfalfa silage (AS) or formate-treated alfalfa silage (FAS), or as one of two red clover silages (RCS1 and RCS2), on ruminal metabolites.

	Diet					
Item	AS	FAS	RCS1	RCS2	SE	$P > F^1$
pH	6.38	6.41	6.45	6.45	0.05	0.32
NH_3, mg/dl	14.0[a]	12.7[a]	6.5[b]	7.9[b]	1.0	< 0.01
Free AA, mM	3.97[a]	3.44[ab]	2.29[c]	2.85[bc]	0.40	< 0.01
Total VFA, mM	118.2	117.6	117.7	114.6	4.3	0.70
Molar proportions, mol/100 mol						
Acetate	62.9	62.7	64.4	64.1	2.0	0.73
Propionate	18.0	18.0	18.6	18.5	0.8	0.77
Acetate: Propionate	3.56	3.57	3.55	3.54	0.09	0.99
Butyrate	11.1	11.5	10.8	10.7	0.5	0.20
Isobutyrate	1.32[a]	1.23[a]	0.84[b]	0.93[b]	0.05	< 0.01
Isovalerate + 2-methylbutyrate	2.08[a]	1.92[a]	0.97[c]	1.21[b]	0.10	< 0.01
Valerate	1.65	1.61	1.41	1.49	0.13	0.13

[a,b,c]LS Means in the same row with different superscripts differ ($P < 0.05$).

[1]Probability of a significant effect of diet.

Alfalfa Silage Versus Formate-Treated Alfalfa Silage or Red Clover Silage for Lactating Dairy Cows

G.A. Broderick and W.J. Radloff

Introduction

The large amounts of NPN in alfalfa silage (AS) reduce protein efficiency when fed to lactating dairy cows. Red clover silage (RCS) typically has 30 to 40% less NPN, as a proportion of total N, than AS. Research conducted at the Dairy Forage Center found that the enzyme polyphenol oxidase acts in RCS to reduce NPN formation (Jones et al., J. Sci. Food Agric. 67:329-333, 1995) and has shown that protein efficiency is improved when RCS replaces AS (Broderick, 2001 USDFRC Res. Sum.). Although DM efficiency (milk/DMI) has been consistently greater on RCS than AS, depressed DMI on RCS reduced total milk yield in two out of five trials. Formic acid treatments are used extensively in Europe to reduce NPN formation in grass silage. This feeding study was conducted to test the effectiveness of feeding dairy cows forage as control AS or AS treated with ammonium tetraformate (ATF; GrasAAT®, HydroAgri, Norway), or as RCS supplemented with dried molasses to counteract the depressed DMI that occurs with that forage.

Materials and Methods

Alfalfa silage was harvested from third cutting and RCS was harvested from second cutting. Forages were cut using a conventional mower conditioner and field-wilted to about 40% DM. All forages were chopped to a theoretical length of 2.9 cm. Control AS and RCS were ensiled without additives; formate-treated AS (FAS) was treated with 6.2 L ATF/ton of wet forage while being chopped. Forages were ensiled in three upright concrete stave silos and no forage was rained on during harvest. Silage compositions are in Table 1. Fifteen Holstein cows averaging 256 DIM were blocked by parity and DIM into five squares of three; cows within squares were randomly assigned to diet sequences in a 3x3 Latin square trial. The TMR were formulated to contain (DM basis) 40% AS or FAS plus 20% corn silage, or 54% RCS plus 6% dried molasses. Diet compositions are in Table 2. Experimental periods were 4-wk long (total 12-wk); production data were collected during the last 2-wk of each period. Spot fecal samples were collected at the end of each period and apparent nutrient digestibility was estimated using indigestible ADF as internal marker. Statistical analysis was done using proc mixed in SAS; differences between least square means were reported only if the F-test for treatment was significant (≤ 0.05).

Results and Discussion

The silages differed in a number of composition characteristics (Table 1). As expected, RCS was lower in CP than AS. The 24% CP in control AS probably occurred because it was from third cutting. The RCS was slightly higher in NDF but similar in ADF; this translated into 1.5 to 2 percentage units more hemicellulose in RCS than AS. The RCS was much lower in NPN than the AS, with only 56% of that in control AS. Treatment with ATF depressed silage pH but also added ammonia N; the 1.3 percentage units of extra CP in FAS derived from this NPN. Despite the added NPN from ATF, FAS had about 10% less NPN, even when expressed as a proportion of total N. Most of the reduction in NPN was related to reduced levels of free AA. A surprising finding was that ADIN in RCS that was four times that in AS and FAS. In five previous trials, RCS averaged (% of total N) 5.1% ADIN versus 3.5% ADIN in AS. High levels of ADIN will depress protein utilization.

Elevated ADIN indicated that the RCS fed in this trial was not typical and may have over-heated in the silo.

Diets containing AS and FAS had similar compositions; however, the RCS diet contained 1.7 percentage units less CP (Table 2) because it was anticipated that control AS would contain only 21% CP. Production and nutrient utilization results are summarized in Table 3. Intake of DM was greater on the RCS diet versus control AS, indicating that adding dried molasses had the desired effect of countering the depressed feed consumption previously seen with RCS. Although apparent N efficiency was higher and milk urea lower on RCS, the greater intake did not result in improved milk production versus that on the control AS diet. Feeding FAS increased DMI as well as yields of milk, FCM, protein, lactose, and SNF compared to the other two diets. Nutrient digestibility and excretion were similar on the two AS diets. However, digestibility of DM, Organic matter, NDF, ADF, and hemicellulose all were higher on RCS. Improvements of this magnitude were observed in all of the previous trials comparing AS and RCS where digestibility measurements were made. Greater nutrient digestibility was reflected in the lower fecal DM excretion on RCS. Apparent N digestibility was 17% lower on RCS. Although the importance of this depression is confounded by the lower CP content of that diet (Table 2), a difference of this magnitude probably reflected the high proportion of ADIN in RCS (Table 1). Average N efficiency was increased from 23.6% on AS to 27.1% on RCS in five previous trials (Broderick, 2001 USDFRC Res. Sum.). The improvement of only 1-percentage unit versus control AS, and the greater fecal N excretion, suggested that N utilization was impaired by the elevated ADIN in the RCS fed in this trial. Versus both AS, feeding RCS diverted N excretion from urine to feces. Greater nutrient digestibilities and lower NPN content suggested that feeding RCS would result in improved nutrient efficiencies and lower environmental N losses than feeding AS.

The NEL requirements for maintenance, BW gain, and milk output (based on observed fat and SNF content) were used to estimate relative energy contents of the forages (Table 4). The NEL requirements for mean production were higher on FAS and RCS (average 34.8 Mcal/d) versus AS (33.1 Mcal/d). Subtracting the NEL computed to come from the concentrate portion of the diet yielded estimates of NEL supplied by AS and RCS. Per unit DM, the two AS were computed to have 0.73 Mcal/kg, versus 0.89 Mcal/kg for RCS. The absolute values of these estimates are probably low; however, they indicate that RCS contained 20% more NEL than AS, despite the AS having 2 percentage units less NDF (Table 1). Much of the difference in productive energy among the diets was due to greater NEL deposited for weight gain on RCS. Previously, we have observed that cows fed RCS gain more weight, and have a tendency for reduced milk fat content, versus feeding the same amounts of DM from AS.

Summary and Conclusion

Treating AS with ATF reduced NPN content and, when fed with some corn silage, increased intake and milk yield compared to untreated AS. Feeding a diet with all the forage from RCS, but with dried molasses added to stimulate intake, increased DMI but did not alter production. The RCS diet was lower in CP but did not give N efficiency greater than the FAS diet. The RCS fed in this trial was usually high in ADIN, which appeared to have impaired N utilization. Although total N excretion was similar among the three diets, feeding RCS diverted N from urine to feces. The estimated NEL content of RCS was more than 20% greater than that in AS and FAS. The results of this experiment suggested that formic acid treatment of AS lowered silage NPN and improved milk production

in lactating dairy cows. However, high levels of ADIN may have impaired production on RCS in this trial.

Table 1. Composition of alfalfa, formate-treated alfalfa, and red clover silages.[1]

Item	Forage			SE	$P > F^2$
	AS	FAS	RCS		
DM, %	39.8	40.2	42.9	0.7	0.36
CP, % of DM	24.4[b]	25.7[a]	18.1[c]	0.2	< 0.01
Ash, % of DM	11.1[b]	11.0[b]	12.3[a]	0.1	< 0.01
NDF, % of DM	39.6[b]	38.9[b]	41.3[a]	0.4	0.05
ADF, % of DM	31.4	30.3	31.1	0.4	0.29
Hemicellulose, % of DM	8.2[b]	8.7[b]	10.1[a]	0.2	< 0.01
pH	4.97[a]	4.68[b]	4.80[ab]	0.07	< 0.01
NPN, % of total N	49.4[a]	44.7[b]	27.5[c]	1.0	< 0.01
NH_3-N, % of total N	4.2[a]	3.5[b]	2.6[c]	0.14	< 0.01
Free AA-N, % of total N	39.6[a]	30.0[b]	12.7[c]	0.66	< 0.01
ADIN, % of total N	4.2[b]	3.9[b]	16.6[a]	0.6	< 0.01

[a,b,c]LS Means in the same row with different superscripts differ ($P < 0.05$).

[1]AS = Alfalfa silage, FAS = ammonium tetraformate treated alfalfa silage, RCS = red clover silage, SE = standard error.

Table 2. Composition of diets.

Item	Diet[1]		
	AS	FAS	RCS
	% of DM		
Alfalfa silage	40.4	. . .	. . .
Formate-treated alfalfa silage	. . .	40.1	. . .
Red clover silage	. . .	. . .	54.0
Rolled corn silage	20.0	20.1	. . .
Dried molasses	. . .	. . .	5.9
High moisture shelled corn	32.9	33.1	33.4
Solvent soybean meal	6.3	6.3	6.3
Salt	0.3	0.3	0.3
Vitamin-mineral premix[2]	0.1	0.1	0.1
Chemical composition			
Crude protein	18.0	18.0	16.3
Organic matter	92.2	92.2	90.0
NDF	33	33	33
ADF	23	22	22
Total sugars	2.4	2.5	4.6
Non-fiber carbohydrate	39	40	38

[1]AS = Alfalfa silage, FAS = ammonium-tetraformate treated alfalfa silage, RCS = red clover silage.

[2]Provided (/kg DM) 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E.

Table 3. Effect of feeding control alfalfa silage (AS) or formate-treated alfalfa silage (TAS) plus corn silage, or red clover silage (RCS) plus molasses, on production of lactating cows.

Item	Diet			SE	$P > F^1$
	AS	FAS	RCS		
DM intake, kg/d	24.0[b]	25.0[a]	24.9[a]	0.8	< 0.01
BW gain, kg/d	0.31	0.44	0.64	0.16	0.37
Milk, kg/d	27.8[b]	30.2[a]	28.4[b]	1.0	0.01
3.5% FCM, kg/d	30.3[b]	32.4[a]	30.0[b]	1.2	0.04
Milk/DMI	1.16[ab]	1.20[a]	1.15[b]	0.03	0.05
Milk N/N intake, %	21.1b	22.4[a]	22.3[a]	0.4	0.01
Fat, %	4.20[a]	3.96[b]	3.95[b]	0.15	< 0.01
Fat, kg/d	1.14	1.19	1.10	0.06	0.08
Protein, %	3.40[a]	3.43[a]	3.29[b]	0.08	< 0.01
Protein, kg/d	0.93[b]	1.03[a]	0.92[b]	0.04	< 0.01
Lactose, %	4.74	4.78	4.79	0.06	0.21
Lactose, kg/d	1.30[b]	1.44[a]	1.34[b]	0.06	0.01
SNF, %	9.05[ab]	9.12[a]	8.99[b]	0.12	0.02
SNF, kg/d	2.48[b]	2.75[a]	2.52[b]	0.10	< 0.01
Milk urea, mg/dl	17.8[a]	18.0[a]	13.8[b]	0.4	< 0.01
Apparent Digestibility, %					
DM	57.0[b]	56.6[b]	63.3[a]	1.2	< 0.01
Organic matter	58.1[b]	57.4[b]	63.5[a]	1.3	< 0.01
NDF	35.5[b]	35.6[b]	53.2[a]	1.1	< 0.01
ADF	40.6[b]	38.0[b]	55.2[a]	1.7	< 0.01
Hemicellulose	24.3[b]	30.7[b]	49.0[a]	3.6	< 0.01
N	55.8[a]	55.3[a]	46.0[b]	1.4	< 0.01
Excretion, kg/d					
Fecal DM	10.4[a]	10.9[a]	9.1[b]	0.5	< 0.01
Fecal N	0.298[b]	0.318[b]	0.351[a]	0.015	0.01
Urinary N (difference)[2]	0.232[a]	0.231[a]	0.156[b]	0.011	< 0.01

[a,b]LS Means in the same row with different superscripts differ ($P < 0.05$).

[1]Probability of a significant effect of diet.

[2]Urinary N = N Intake - Milk N - Fecal N.

Table 4. Estimated NEL contents of control alfalfa silage (AS), formate-treated alfalfa silage (FAS) and red clover silage (RCS).

Item	AS	FAS	RCS
Total DMI, kg/d	24.0	25.0	24.9
NEL requirement[1]			
Maintenance (668 kg), Mcal/d	10.5	10.5	10.5
BW gain, Mcal/d	1.6	2.3	3.3
Milk yield, Mcal/d	21.0	22.1	20.8
Total requirement, Mcal/d	33.1	34.9	34.6
Non-HCS[2] DMI, kg/d	14.3	15.0	11.5
Non-HCS NEL,[3] Mcal/kg	1.83	1.83	1.98
Non-HCS NEL, Mcal/d	26.1	27.4	22.6
Hay-crop silage NEL, Mcal/d	7.0	7.4	11.9
Hay-crop silage DMI, kg/d	9.7	10.0	13.4
Hay-crop silage NEL, Mcal/kg	0.72	0.74	0.89
Relative NEL, %	100	103	124

[1]Computed based on NRC Nutrient Requirements of Dairy Cattle, 7th Revised Edition (2001):
NEL (Mcal/d) for maintenance = 0.08 x $BW^{0.75}$
NEL (Mcal/d) for gain = 5.12 x BW gain
NEL (Mcal/d) for milk = Milk x (0.0962 x % fat + 0.3512)

[2]Non-HCS = non hay-crop silage ingredients.

[3]Mean NEL contents of DM from Non-HCS at 3X maintenance computed from NRC (2001) tables.

Effect of Level of Rumen Degradable Protein on Milk Yield and N Utilization in Lactating Dairy Cows

S. M. Reynal, G. A. Broderick

Introduction

Feeding ruminal degraded protein (RDP) in excess of the requirement for maximal microbial protein formation is wasted because the extra RDP will be converted to urea N and largely excreted in the urine. Urinary urea is the most labile form of excretory N; it can be rapidly hydrolyzed to ammonia which may volatilized before it has a chance to be incorporated into growing crops. Urinary N may also contribute to pollution of surface and ground water. On the other hand, feeding RDP levels below requirement can compromise microbial protein production, ruminal digestion, and energy and protein availability to the cow. Our objective was to determine the optimum concentration of RDP level in diets fed to lactating dairy cows.

Materials and Methods

Twenty-eight Holstein cows (eight with ruminal cannulas) averaging 192 DIM were blocked by parity and DIM into seven squares of four; cows within squares were randomly assigned to balanced diet sequences in a 4x4 Latin square trial. Four TMR were formulated to contain 50% of dietary DM from forage (three-fourths from corn silage and one-fourth from second cutting alfalfa silage) and 50% from four different concentrate mixes. Concentrations of dietary RDP were varied incrementally from 13.7% to 7.5% of DM by step-wise deletion of urea and replacement of solvent soybean meal with SoyPass¨ (LignoTech, Rothchild, WI), a rumen-protected soybean meal. Diet compositions are in Table 1. Experimental periods were 4-wk long (total 16 wk); production data were collected during the last 2-wk of each period. At the end of each period, ruminal samples were collected over the 24-h clock from the eight cannulated cows and spot urine and fecal samples were collected from all cows. Urinary excretion was estimated from creatinine; apparent nutrient digestibility was estimated using indigestible ADF as internal marker. Statistical analysis was done using proc mixed in SAS; differences between least square means were reported only if the F-test for treatment was significant ($\alpha \leq 0.05$).

Results and Discussion

Dietary CP was not constant because of the stepwise deletion of urea that was not replaced by other sources of N (Table 1). This was necessary to obtain diets that varied from being substantially in excess, to being well below, the NRC (2001) RDP requirement. However, there was little difference over the four diets in NDF, ADF, and computed NEL contents. Thus, the major differences across diets was the decreasing content and rumen balance for RDP, and the increasing content and rumen balance for rumen undegraded protein (RUP) (Table 1). There were no effects on DMI, weight gain, yield of milk and FCM, milk fat and lactose content and yield, or milk SNF content over the ranges of RDP and RUP fed in this trial (Table 2). However, the significant effects of dietary RDP concentration were restricted to milk protein and SNF: there were linear effects on protein content and yield and quadratic effects on protein and SNF yield. As expected, there were linear effects of dietary RDP on both milk and blood urea, with concentrations at the two higher levels being greater than those at the two lower levels. An interesting finding was the quantitative difference between

milk urea determined by infrared analysis (AgSource, Verona, WI) and by colorimetric assay. The pattern of statistical differences was the same for both data sets, but milk urea N determined by colorimetry averaged 2.6 mg/dl greater than milk urea N assayed using infrared analysis.

There were linear declines in urinary volume and urinary excretion of total N and allantoin with decreasing dietary RDP content (Table 2). Based on the similar milk protein yields from 13.7 through 9.5% dietary RDP, urinary total N excretion could be decreased from 288 to 255 g/d without affecting protein production. Urinary allantoin derives from liver purine catabolism and, because absorbed purines originate largely from microbial cells from the rumen, allantoin excretion represents an indirect estimate of ruminal protein synthesis. Yield of microbial nonammonia N (NAN) was estimated by computing ruminal purine flow from urinary allantoin excretion (Vagnoni et al., J. Dairy Sci. 80:1695-1702, 1997) and computing microbial NAN from purine flow (Reynal et al., J. Dairy Sci. 86:1292-1305, 2003). These calculations showed that microbial NAN declined in a step-wise manner, suggesting that an optimum RDP requirement could not be found from this data set. Based on these computations, decreasing RDP from 13.7 to 9.5% reduced microbial NAN flow by 43 g/d. However, the quadratic responses observed for both protein and SNF yield indicated that the optimal dietary RDP concentrations (DM basis) were 11.6% (protein yield) and 10.0% (SNF yield).

Mean metabolite concentrations measured in the rumen when these diets were fed are in Table 3. Ruminal pH, acetate, acetate: propionate ratio, valerate, and branched-chain VFA were not altered by diet. That branched-chain VFA, which are formed from catabolism of branched-chain AA released during protein degradation in the rumen, did not change was surprising in view of the substantial changes in RDP intakes in this trial. As expected, there were linear and quadratic declines in ruminal ammonia, plus a linear decline in free AA, with decreasing RDP. The significant quadratic effect of RDP on ruminal propionate, and the linear and quadratic effects on ruminal butyrate, are difficult to explain. We speculate that the molar proportions of these VFA changed little and the main factor was the quadratic pattern observed in total VFA. Overall, the pattern of ruminal ammonia and total free AA clearly reflected the declining dietary concentrations of RDP.

Summary and Conclusion

Decreasing RDP levels (estimated from NRC, 2001 tables) from 13.7 to 7.5% of dietary DM had significant effects only on milk protein and SNF yield; RDP could be reduced to 9.5%, which reduced urinary N excretion by 33 g/d, without affecting protein production. Concentrations of milk and blood urea, and ruminal ammonia and total free AA, paralleled dietary CP and RDP contents. In this trial, there was a linear decline in microbial NAN flow, estimated from urinary allantoin excretion, as RDP was reduced. However, optimal milk protein yield was predicted from the quadratic response to be 11.6% RDP in dietary DM.

Table 1. Composition of diets.

Item[1]	Dietary RDP, % of DM			
	13.7	11.6	9.5	7.5
	% of DM			
Corn silage	37.1	37.1	37.1	37.1
Alfalfa silage	12.7	12.7	12.7	12.7
Rolled high moisture shelled corn	32.4	32.1	31.9	31.7
Urea	0.50	0.33	0.17	0
Solvent soybean meal	16.43	10.95	5.48	0
SoyPass[1]	0	5.87	11.74	17.61
Sodium bicarbonate	0.45	0.45	0.45	0.45
Dicalcium phosphate	0.10	0.10	0.10	0.10
Salt	0.25	0.25	0.25	0.25
Vitamin-mineral premix[2]	0.10	0.10	0.10	0.10
Chemical composition[3]				
Crude protein	18.8	18.3	17.7	17.2
NDF	27.5	28.3	27.8	30.0
ADF	16.0	16.4	14.6	15.4
NPN, % of total N	56.6	52.5	48.7	41.4
RDP, % DM	13.7	11.6	9.5	7.5
RUP, % DM	4.9	6.5	8.1	9.7
NEL, Mcal/kg	1.56	1.55	1.55	1.55
Non-fiber carbohydrate, % DM	49.2	49.9	50.6	51.4
RDP balance, g/d	960	456	-71	-604
RUP balance, g/d	-347	165	546	598

[1]Obtained from LignoTech, Rothschild, WI.

[2]Provided (/kg DM) 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E.

[3]NEL, rumen degraded protein (RDP), and rumen undegraded protein (RUP) values calculated from NRC (2001) tables based on composition of diets fed.

Table 2. Effect of feeding varying levels of rumen degraded protein (RDP) on production, urinary excretion, and estimated microbial NAN flows in lactating cows.

	Dietary RDP, % of DM					Probabilities[1]	
Item	13.7	11.6	9.5	7.5	SE	Linear	Quadratic
DM intake, kg/d	25.1	25.8	25.7	25.6	0.5	0.34	0.21
BW gain, kg/d	0.51	0.58	0.43	0.52	0.13	0.85	0.98
Milk, kg/d	42.0	42.6	42.1	41.4	1.0	0.43	0.34
3.5% FCM, kg/d	35.2	37.1	38.6	37.1	1.9	0.26	0.23
Milk N/N intake, %	29.5	29.8	30.4	30.2	0.9	0.37	0.78
Fat %	3.21	3.38	3.28	3.41	0.15	0.25	0.82
Fat, kg/d	1.23	1.32	1.33	1.31	0.07	0.22	0.23
Protein, %	3.14[a]	3.13	3.07[b]	3.03[b]	0.05	< 0.01	0.63
Protein, kg/d	1.30[a]	1.33[a]	1.30[a]	1.22[b]	0.03	0.02	0.03
Lactose, %	4.75	4.81	4.78	4.83	0.05	0.16	0.90
Lactose, kg/d	1.98	2.06	2.05	1.97	0.06	0.74	0.06
SNF, %	8.78	8.84	8.78	8.76	0.07	0.57	0.43
SNF, kg/d	3.66[ab]	3.79[a]	3.74[a]	3.56[b]	0.09	0.30	0.03
MUNa,[2] mg/dl	12.8[a]	12.9[a]	11.0[b]	10.9[b]	0.5	< 0.01	0.66
MUNc,[2] mg/dl	15.9[a]	15.6[a]	13.6[b]	12.9[b]	0.5	< 0.01	0.55
BUNc,[2] mg/dl	13.8[a]	14.0[a]	11.8[b]	12.4[b]	0.4	< 0.01	0.66
Urinary excretion							
Volume, L/d	22.7[a]	18.4[bc]	19.4[b]	16.8[c]	1.0	< 0.01	0.27
Total N, g/d	288[a]	285[ab]	255[bc]	235[c]	8	< 0.01	0.43
Allantoin, mmol/d	362[a]	333[b]	305[c]	270[d]	12	< 0.01	0.70
Estimated ruminal flows,[3] g/d							
Total purines	348	310	275	229	15	. . .	. . .
Microbial NAN	355	316	280	234	16	. . .	. . .

[a,b,c]LS Means in the same row with different superscripts differ ($P < 0.05$).

[1]Probability of significant linear and quadratic effects of dietary RDP concentration.

[2]MUNa = Milk urea N determined by infrared analysis by AgSource, Verona, WI; MUNc = milk urea N determined colorimetrically; BUNc = blood urea N determined colorimetrically.

[3]Total purines flows computed from urinary allantoin excretion using the equations of Vagnoni et al. (J. Dairy Sci. 80:1691-1701, 1997). Microbial nonammonia N (NAN) flows computed from purine flows using the equations of Reynal et al. (J. Dairy Sci. 86:1292-1305, 2003).

Table 3. Effect of feeding varying levels of rumen degraded protein (RDP) on ruminal metabolites

	Dietary RDP, % of DM					Probabilities[1]	
Item	13.7	11.6	9.5	7.5	SE	Linear	Quadratic
pH	6.28	6.21	6.18	6.26	0.05	0.67	0.06
NH_3, mg/dl	8.81[a]	8.40[a]	6.20[b]	4.08[c]	0.55	< 0.01	0.04
Free AA, mM	4.89[a]	4.51[ab]	3.79[bc]	3.38[c]	0.27	< 0.01	0.95
Total VFA, mM	91.3[a]	81.0[b]	85.9[ab]	92.0[a]	3.9	0.63	0.03
Acetate, mM	55.0	50.9	52.6	56.1	2.2	0.57	0.07
Propionate, mM	21.7[a]	15.5[b]	18.6[ab]	19.7[a]	1.5	0.66	0.01
Acetate: Propionate	2.56	3.49	2.93	3.11	0.26	0.35	0.16
Butyrate, mM	10.0[b]	10.4[b]	9.7[b]	11.5[a]	0.4	0.02	0.04
Isobutyrate, mM	1.25	1.09	1.21	1.13	0.06	0.27	0.44
Isovalerate, mM + 2-methylbutyrate	1.60	1.67	1.8	1.65	0.09	0.49	0.17
Valerate, mM	1.76	1.63	2.02	1.88	0.25	0.16	0.93

[a,b,c]LS Means in the same row with different superscripts differ ($P < 0.05$).

[1]Probability of significant linear and quadratic effects of dietary RDP concentration.

Comparison of Conventional Linted Cottonseed and Mechanically Delinted Cottonseed as Supplements for Multiparous or Primiparous Lactating Dairy Cows

V. R. Moreira, L.D. Satter, and B. Harding

Introduction

Linted whole cottonseed (**LWCS**) is often regarded as a good source of energy, protein and fiber, and for this reason, cottonseed is commonly used in diets of lactating dairy cows. Cotton lint may be removed and used for other purposes in the industry, such as in the production of absorbent cotton, manufacture of diapers, and felts for mattresses, among others. As a result, mechanically delinted whole cottonseed (**DWCS**) has increased in availability for inclusion in dairy diets. Nonetheless, earlier work raised concern over the possibility that DWCS would result in higher fecal loss of intact seeds when compared to LWCS. The objectiveof the study was to compare performance of lactating cows (primiparous and multiparous) fed linted ("fuzzy") or mechanically delinted whole cottonseed, and to determine if the latter resulted in increased loss of whole undigested seeds in feces.

Materials and Methods

Eighty two cows (41 multiparous and 41 primiparous) were divided into two groups and fed similar diets except for whole cottonseed source, which was either linted or mechanically delinted (Table 1). The experiment was divided into two blocks on time because of barn space and cow availability. Each block lasted 18 weeks, with two weeks of standardization period, and 16 weeks of experimental period. Block I included 54 cows, starting on February 12, 2002, and Block II had 28 cows, beginning on April 19, 2002, equally split between multiparous and primiparous for each period. Cows were fed the same diet, which included both sources of cottonseed, during the first 2 weeks (standardization-covariate period) (Table 1). The assignment of cows to either of the treatments was based on milk yield of the second week of the pre-trial period minus that of the last three days, DIM, and parity. Feed intake and milk yield were measured daily from pre-trial until the end of the experimental period. Body weights were measured on two consecutive days, at the beginning and end of each block. Body condition scores were made by two individuals (1 to 5 scale). Three hundred grams of feces were sampled at week 3 and week 13 of the experimental periods to estimate presence of whole cottonseed. Fecal samples were frozen for at least a week before being passed through a set of three sieves (maximum openings of 10.16, 4.35 and 3.07 mm) to collect excreted intact seeds. Results were expressed as percent of fecal dry matter.

Results and Discussion

Results are presented in Table 2. Source of WCS did not affect dry matter intake and milk yield of primiparous nor multiparous cows. Fat yield fell throughout the experiment similarly for LWCS and DWCS, while protein yield rose. There was no difference between treatments. No treatment effect was detected on body condition score of multiparous cows, although DWCS tended ($P \leq .11$) to increase BCS of primiparous cows (0.23 vs. 0.12). Feeding DWCS significantly increased intact seed excretion by primiparous ($P \leq 0.007$) and multiparous ($P \leq 0.001$) cows. Intact seeds were always retained on the sieve with a maximum opening of 4.35 mm. A large amount of cottonseed hulls were observed on the last screen (3.07 mm) with DWCS. Less than 3% of ingested seeds were excreted in the feces. Mechanically delinted cottonseed still retained approximately 4.7% of lint on the cottonseed, while LWCS used in this trial contained 11.7% of lint. It appears that there was

suffficient residual lint on the mechanically delinted cottonseed so passage of undigested seeds was minimlly affected.

Conclusion

Feeding either linted cottonseed ('fuzzy') or delinted whole cottonseed did not affect performance of dairy cows. The passage of intact seeds into the feces was small with delinted whole cottonseed, 2.5% of consumed seeds appeared in the feces. With linted cottonseed, 1.5% of consumed seeds were in the feces.

Table 1.

Ingredients:	Pre-trial Period	Treatment diets	
		DWCS	LWCS
Corn silage	28.1	28.1	28.1
Alfalfa silage	23.0	23.0	23.0
High moisture shelled corn	27.8	27.8	27.8
Linted whole cottonseed	6.5	0.0	13.0
Delinted whole cottonseed	6.5	13.0	0.0
Soybean meal (48%CP)	1.8	1.8	1.8
Soy-plus®	1.8	1.8	1.8
Blood meal	2.0	2.0	2.0
Salt (NaCl)	0.3	0.3	0.3
Limestone	1.0	1.0	1.0
Dicalcium-phosphate	0.4	0.4	0.4
Sodium bicarbonate	0.7	0.7	0.7
Vitamin-mineral supplement	0.1	0.1	0.1
Chemical Analyses:			
DM (%)	57.5 ± .04	59.2 ± 1.82	59.3 ± 1.76
CP (%DM)	16.5 ± .05	16.3 ± .02	16.2 ± .12
NDF (%DM)	29.8 ± .19	28.9 ± .76	29.2 ± .71
ADF (%DM)	18.6 ± .27	17.6 ± .56	18.4 ± .57
Ether extract (%DM)	5.23 ± .21	5.05 ± .06	5.40 ± .11

Table 2.

	MULTIPAROUS			*P*≤		
	DWCS[1]	LWCS[2]	SEM	Lint	Week	Lint*Week
DMI (kg/d)	23.8	23.1	1.60	0.14	<.001	<.001
Milk Yield (kg/d)	37.4	37.5	0.83	0.91	<.001	0.68
Fat, kg/d	1.17	1.15	0.11	0.84	<.001	0.88
True protein, kg/d	1.06	1.06	0.03	0.87	<.001	0.71
Body weight change (kg/d)	30.2	26.0	9.87	0.76	-	-
BCS change	0.27	0.31	0.05	0.54	-	-
Intact seeds, % fecal DM	1.53	0.72	0.15	<.001	0.92	0.009
	PRIMIPAROUS			*P*≤		
	DWCS[1]	LWCS[2]	SEM	Lint	Week	Lint*Week
DMI (kg/d)	20.5	20.4	0.59	0.83	<.001	<.001
Milk Yield (kg/d)	32.7	32.8	0.78	0.88	<.001	0.96
Fat, kg/d	1.06	1.03	0.06	0.47	<.001	0.38
True protein, kg/d	0.98	0.95	0.02	0.18	0.11	0.81
Body weight change (kg/d)	2.0	-9.6	133.0	0.60	-	-
BCS change	0.22	0.11	0.45	0.11	-	-
Intact seeds, % fecal DM	1.02	0.61	0.10	0.007	0.90	0.65

Effect of Dietary Phosphorus Concentration on Estrous Behavior of Lactating Dairy Cows

H. Lopez, Z. Wu, L.D. Satter and M.C. Wiltbank

Introduction

There is a widespread notion that increasing dietary phosphorus (P) can improve 'strength of heat' in lactating dairy cows. Extremely low dietary P (< than 0.25% of diet DM) can reduce microbial activity in the rumen, which in turn can reduce digestibility of the diet and reduce microbial synthesis of protein. Reduced energy and protein status can definitely interfere with reproductive performance. The objective of this study was to measure the effect of dietary P concentrations of 0.38 (adequate) or 0.48% (excess) on estrous behavior of lactating cows as measured by a radiotelemetric system (Heatwatch).

Material and Methods

Observations on estrous behavior presented in this report were collected during the first year of a two-year trial that analyzed milk production and reproductive performance of dairy cows fed two concentrations of dietary P (Wu and Satter, JDS 83:1052). Cows were fed either a diet that was close to the NRC recommendations (0.38% P=adequate), or a diet that was in excess of the NRC recommendations (0.48% P=excess). Formulation of the TMR was the same for both groups. The low P diet contained no supplemental P, while the high P diet was obtained by adding monosodium phosphate and dicalcium phosphate to the TMR. At calving each cow was randomly assigned to one of the dietary treatments. All animals were housed in a free-stall barn with concrete flooring and fed the TMR ad libitum during the first 34 weeks of lactation (September to May).

Forty-two Holstein cows (n=21 per dietary treatment, including 10 primiparous animals in each group) were used to characterize estrous behavior. Animals were fitted with a radiotelemetric patch and a transmitter on d 40 postpartum. This system was used to collect information on mounting activity related to estrous. Activation of the pressure-sensitive transmitter by the weight of a mounting cow for a minimum of 2 s interrupts a radio-wave transmission generating real time data. Estrous behavior was characterized only by the information collected by the radiotelemetric system. Onset of estrus was identified by the first activation of the transmitter. Duration of estrus was defined as the time interval from the first to last mount recorded during an estrous period, thus excluding an estrus consisting of only one mount.

Visual detection of estrus was performed during the day and while cows were in the holding area prior to milking. Cows were inseminated at the first estrus detected by visual observation after 52 d postpartum following the AM-PM rule. Pregnancy was confirmed by rectal palpation approximately 30 d after insemination.

Results and Discussion

Dietary P had no detectable effect on the length of estrus, the number of mounts per estrus, or the mounting time. Dietary treatment also had no effect (P=0.66) on duration and intensity of estrus (Table 1).

Estrous characteristics in relation to milk production were analyzed. In order to perform this analy-

sis, average milk production for the five days preceding the day of estrus, as identified by radio telemetry, was calculated. This average was used to classify cows as low (< 35.1 kg/d) or high (> 35.1 kg/d) producers. The results are in Table 2.

In lactating dairy cows, the continuous high plane of nutrition needed to meet the requirements of high production appears to increase liver blood flow and the metabolic clearance rate of (P_4) and (E_2) (Sangsritavong et al., J. Dairy Sci. 85:2831). This leads to lower circulating concentrations of steroids in high producing cows with consequent alterations in the normal reproductive processes (Wiltbank et al., JDS 84(Suppl. 1):32). The expression of estrus is induced by high systemic concentrations of E_2 produced by the pre-ovulatory follicle. Thus, the lower peak concentration of E_2 and perhaps a more rapid decrease in E_2 after the onset of estrus could cause cows to display estrus for a shorter time and with less intensity than those displayed by lower producers.

Conclusion

Dietary P concentration has no effect on characteristics of estrus, but milk production had a strong negative correlation with duration of estrus.

Table 1. Characteristics of estrous behavior (mean ± SEM [range]) for cows fed diets containing Low (0.38%) or High (0.48%) P.

Characteristic[1]	Low P (n=37)	%	High P (n=35)	%	P-value
Duration of estrus, h	8.9±1.1		8.6±1.2		0.86
Total mounts, n	7.0±1.2		8.2±1.7		0.57
Total mounting time, s	27.1±4.3		30.8±6.5		0.64
Short duration, low intensity[1]	13	35.1	15	42.8	
Short duration, high intensity[1]	9	24.3	6	17.2	
Long duration, low intensity[1]	12	32.4	9	25.7	
Long duration, high intensity[1]	3	8.2	5	14.3	

[1]Estruses were classified by duration as short (< 8.8 h) or long (≥ 8.8 h). Short estruses were classified as low (< 1.6 m/h) or high (≥ 1.6 m/h) intensity and long estruses as low (< 0.8 m/h) or high (≥ 0.8 m/h) intensity.

Table 2. Characteristics of estrous behavior (mean ± SEM [range]) for low (< 35.1 kg/d) and high (≥ 35.1 kg/d) producing cows.

Characteristic[1]	Low milk (n=33)	%	High milk (n=39)	%	P-value
Average milk production, kg/d	28.0±0.8		40.4±0.8		< 0.0001
Duration of estrus, h	11.1±1.5		6.9±0.8		0.01
Total mounts, n	10.0±2.1		5.5±0.6		0.03
Total mounting time, s	38.9±7.6		20.4±2.6		0.02
Short duration, low intensity[1]	7	21.2	21	53.8	
Short duration, high intensity[1]	10	30.3	5	12.8	
Long duration, low intensity[1]	12	36.4	9	23.1	
Long duration, high intensity[1]	4	12.1	4	10.3	

[1]Estruses were classified by duration as short (< 8.8 h) or long (≥ 8.8 h). Short estruses were classified as low (< 1.6 m/h) or high (≥ 1.6 m/h) intensity and long estruses as low (< 0.8 m/h) or high (≥ 0.8 m/h) intensity.

Effect of Dietary Phosphorus Concentration on Reproductive Performance of Lactating Dairy Cows

H. Lopez, F.D. Kanitz, V.R. Moreira, M.C. Wiltbank and L.D. Satter

Introduction

It is common for dairy producers to increase dietary phosphorus (P) above NRC requirements in an attempt to improve reproductive performance of the herd. The objective of this study was to determine the effect of dietary P concentrations of 0.37% or 0.57% of the TMR (DM basis) on reproductive performance.

Materials and Methods

This study used 267 cows (131 primiparous and 136 multiparous) that were fed either an adequate (0.37%) or an excessive P diet (0.57%) beginning at calving and continuing for 165 days of lactation. Milk weights were recorded at each milking, and blood samples were obtained near day 50 and day 100 post partum for P analysis. Weekly blood samples were used for progesterone (P_4) analysis. Days to first increase in P_4 concentration above 1 mg/ml was determined from the weekly blood samples and used as an indication of first natural ovulation. Cows were fitted with a radiotelemetric transmitter (HeatWatch) at day 50. Cows were bred to natural estrous from day 50 to day 100 and to synchronized estrous after day 100. Weekly ultrasonography was performed from day 50 until the cow was pregnant. Days to first natural estrus were determined from data collected by HeatWatch. A cow was determined to be in anovulatory condition if no new CL was detected during a period of at least three weekly consecutive ultrasound examinations. No treatment was given to anovulatory cows between 50 to 100 days postpartum. After 100 days postpartum anovulatory cows were treated with the Ovsynch protocol.

Results and Discussion

Blood serum P concentrations for the low P diet at day 50 and 100 postpartum were 6.1 and 6.2 mg/dl. Comparable values for the high P diet were 6.8 and 6.9 mg/dl. Treatment effect was highly significant. Milk production, milk composition and body condition score are in Table 1. There were no significant treatment effects.

Characteristics of estrous behavior are in Table 2 and various measurements of reproductive performance are in Table 3. Dietary P had no effect on any of the reproductive measures made.

Characteristics of estrous events for low (<39.5 kg/d) and high (>39.5 kg/d) producing cows is shown in table 4. Level of milk production, in contrast to dietary P content, had a large impact on estrous behavior.

Conclusion

Increasing dietary P above the NRC requirement had no detectable effect on behavioral estrous or any other reproductive measure. In contrast, milk production level had a dramatic impact on estrous behavior.

Table 1. Least squares means for milk production, milk components and body condition score of lactating dairy cows fed diets containing 0.37% or 0.57% dietary P.

	Dietary P content (%)				
	0.37		0.57		
Item	Mean	SE	Mean	SE	P[2]
Number of cows	123		124		
Milk Yield[1] (kg/day)	35.1	0.52	34.9	0.52	NS
3.5% FCM (kg/day)	36.8	0.65	36.9	0.64	NS
Milk Composition Fat (%)	3.92	0.04	3.98	0.04	NS
Protein (%)	2.90	0.02	2.91	0.02	NS

[1]First 165 days of lactation
[2]NS=*P*>0.30

Table 2. Characteristics of estrous behavior for lactating cows fed diets containing 0.37% g or 0.57% g P.

Characteristic[1]	0.37 g P/kg (n=159)	0.57 g P/kg (n=174)	*P*
Duration of estrus, h[2]	8.7±0.5	8.7±0.6	0.99
Total mounts, n	7.5±0.5	7.8±0.5	0.68
Total mounting time, s	25.8±1.8	24.5±1.5	0.59
Average duration of standing events, s	3.4±0.2	3.4±0.2	0.76

[1]Estruses consisting of only one standing event were removed from the analysis.
[2]Number of hours between the first and the last recorded mount of an estrous period

Table 3. Reproductive parameters for lactating cows fed diets containing 0.37% g or 0.57% P.

	0.37 % P	0.57 % P	*P*
Days to first P_4 increase[1]	53 ± 3	53 ± 3	0.97
Days to first natural estrus[2]	68 ± 1.1	67 ± 1.2	0.87
Days to first service	89 ± 2.0	90 ± 2.0	0.87
Conception rate at first AI[3], %	39.4	42.0	0.67
Overall conception rate at 30 d[4], %	34.3	38.0	0.35
Overall conception rate at 60 d, %	29.1	31.8	0.47
Pregnancies lost (30 to 60 d), %	15.2	16.2	0.83
Pregnancies lost after 60 d, %	6.0	5.4	0.87
Days open	112 ± 3.5	116 ± 3.8	0.45
Services/conception[5]	2.9	2.6	0.35
Double ovulation rate, %	19.9	18.4	0.66
Anovulatory condition[6], %	29.9	27.1	0.61

[1]First increase in progesterone concentration >1 ng/ml.
[2]First natural estrus detected by the Heatwatch system between 50 and 100 d.
[3]Number of pregnancies detected at 30 d divided by the number of first services.
[4]Number of pregnancies detected at 30 d divided by the total number of services.
[5]Total number of services divided by the number of pregnancies detected at 30 d.
[6]Cows with no new CL for at least three weekly consecutive ultrasound examinations after d 50.

Table 4. Characteristics of estrous events (mean ± SEM [range]) for low (<39.5 kg/d) and high (>39.5 kg/d) producing cows

Characteristic	Low producers (n[1]=177)	High producers (n=146)	P-value
Average milk production[2], kg/d	33.5 ± 0.3	46.4 ± 0.4	<0.0001
Duration of estrous[3], h	10.9 ± 0.7	6.2 ± 0.5	<0.0001
Total mounts, n	8.8 ± 0.6	6.3 ± 0.4	0.001
Total mounting time, s	28.2 ± 1.9	21.7 ± 1.3	0.007
Average days in milk[4], d	95.8 ± 2.7	90.9 ± 2.8	0.21

[1]Number of estrous events.
[2]Average milk production for the 10 d before the day of estrous.
[3]Number of hours between the first and the last recorded mount of an estrous period.
[4]Days postpartum when information on estrous behavior was collected by radiotelemetry.

Effect of Dietary Protein Content and Alfalfa: Corn Silage Ratios on Nitrogen Excretion and Milk Production of Late Lactation Cows

H.H.B. Santos, S. Lardoux, V.R. Moreira, and L.D. Satter

Introduction

Increasing concerns about ammonia and nitrous oxide emissions from livestock and poultry facilities to the atmosphere are stimulating measures to reduce total nitrogen (N) excretion through diet manipulation, and more specifically, to decrease urinary N. Rapid conversion of urinary urea to ammonia makes the N in urine particularly susceptible to volatile losses. The objective of this study was to evaluate the effect of different dietary protein concentrations and alfalfa: corn silage proportions in the diet on nitrogen distribution between milk, feces, and urine of late lactation cows.

Materials and Methods

Twenty-four cows (12 multiparous and 12 primiparous) were randomly assigned to a 6x6 Latin square design with 14-d periods. Treatments were arranged in a factorial design with two alfalfa silage: corn silage ratios (70:30 and 30:70) and three levels of crude protein (~ 15, ~ 16.5, and ~ 18%). Roasted soybeans and soybean meal replaced high moisture corn to increase dietary protein content. Feed intake was measured daily and analyzed for DM, CP, NDF, ADF, and marker (ytterbium) concentration. Milk yield was recorded daily and sampled at the end of each period. Urine samples were obtained at 4hr-intervals during the last day of each period and 12 rectal fecal samples were obtained during the last 3 days from each cow on every even hour of the 24-hr period. Fecal marker and urinary creatinine concentrations were used to calculate N excretion in feces and urine. Diet ingredients are shown in Table 1.

Results and Discussion

Dry matter intake was slightly lower for the 15% CP treatment than for the 16.5 and 18.0% CP treatments (Table 2). Milk production and milk nitrogen followed a similar pattern. Somewhat surprisingly, milk protein content averaged slightly higher for the high alfalfa diets than for the high corn silage diets. Milk fat (%) was unaffected by diet treatment. Fecal N increased some with an increase in dietary N, but most of the incremental increase in dietary N ended up in urinary N.

Conclusion

The lowest level of dietary protein (~ 15%) caused a slight reduction in milk production, whereas ~ 16.5% crude protein was sufficient to maintain normal milk production for the late lactation cows used in this study. Most of the protein fed in excess of the requirement was excreted in the urine. Results support the NRC (2001) protein recommendation.

Table 1. Diet composition

Diet (% DM)	Diet 1 70/30 AS/CS 14.9% CP	Diet 2 70/30 AS/CS 16.5% CP	Diet 3 70/30 AS/CS 18.1% CP	Diet 4 30/70 AS/CS 15.4% CP	Diet 5 30/70 AS/CS 16.9% CP	Diet 6 30/70 AS/CS 18.5% CP
Normal corn silage	19.50	19.50	19.50	45.50	45.50	45.50
Alfalfa silage early cut	45.50	45.50	45.50	19.50	19.50	19.50
Beet pulp	4.00	4.00	4.00	4.00	4.00	4.00
HMSC	28.80	25.55	21.00	16.50	12.35	9.10
Roasted Soybean	0.00	2.50	7.25	8.00	9.00	9.00
Soybean meal 48	0.00	0.00	0.00	2.75	6.40	9.75
Blood meal	0.70	1.50	1.50	1.50	1.00	1.00
Limestone	0.00	0.00	0.00	1.00	1.00	1.00
Sodium bicarbonate	0.75	0.75	0.75	0.75	0.75	0.75
Dicalcium phosphate	0.65	0.60	0.40	0.40	0.40	0.30
Vit TM Pak	0.10	0.10	0.10	0.10	0.10	0.10
	100.00	100.00	100.00	100.00	100.00	100.00
Forage/concentrate Ratio	65/45	65/45	65/45	65/45	65/45	65/45
Protein supply compared to NRC (2001) requirements						
RUP req'd	904	985	962	970	896	880
RUP supplied	761	950	1121	1048	1198	1304
RDP req'd	1912	1935	2025	1911	1996	1974
RDP supplied	2107	2269	2573	1857	2168	2331

Table 2. Cow performance

Forage ratio	AS:CS (70:30)			AS:CS (30:70)				P <			
Dietary protein, %	14.9	16.5	18.1	15.4	16.9	18.5	SEM	AS vs CS	15 vs 16.5	15 vs 18	16.5 vs 18
DMI, kg/d	19.3	19.5	20.4	18.9	19.9	19.7	0.44	0.31	0.03	0.01	0.21
Milk, kg/d	26.5	27.9	28.4	27.7	28.2	28.0	0.62	0.21	0.01	0.01	0.72
Milk fat, %	3.72	3.84	3.84	3.88	3.83	3.78	0.09	0.29	0.35	0.76	0.52
Milk CP, %	3.09	3.05	2.98	2.99	3.03	3.00	0.04	0.01	0.93	0.01	0.01
N Intake, g/d	460	516	592	465	537	582	11.7	0.38	0.01	0.01	0.01
Milk N, g/d	131	136	136	132	138	136	3.47	0.45	0.01	0.01	0.62
Fecal N, g/d	188	186	212	176	198	204	9.08	0.48	0.03	0.01	0.01
Urine N, g/d	167	193	210	145	184	221	6.33	0.13	0.01	0.01	0.01
Milk N,% NI	28.5	26.4	23.0	28.4	25.7	23.4	0.52	0.64	0.01	0.01	0.01
Recovered N, % NI	106.0	100.1	94.9	98.2	97.1	96.6					

FIBEX-Pretreated Rice Straw as a Feed Ingredient for Lactating Dairy Cows

P.J. Weimer, D.R. Mertens, E. Ponnampalam, B.F. Severin, and B.E. Dale

Introduction

Rice straw is an abundant product of rice production. In northern California, rice straw has traditionally been disposed of by field incineration, but air quality issues and recent changes in state law now allow such burning only on a permit basis in cases where control of plant disease is necessary. Alternate uses have been proposed for the large volumes of rice straw generated, but none have been commercialized. Because of its high ash content and the low digestibility of its organic matter, rice straw is not considered useful as an animal feed without a pretreatment to upgrade its digestibility. One potential pretreatment is the proprietary FIBEX process, which involves continuous treatment of the straw with ammonia under temperature and pressure, with subsequent rapid release of the pressure that causes a "freeze explosion" of the straw fibers. The FIBEX process is thought to enhance digestibility by breaking phenolic-carbohydrate bonds and by increasing the surface area of the substrate. Because essentially all of the ammonia can be recovered, there is no appreciable liquid residue that would represent a disposal problem. The purpose of this study was to determine: a) if FIBEX pretreatment improves the kinetics of digestion of rice straw by mixed ruminal microbes in vitro; and b) if the pretreated rice straw supports milk production when incorporated into dairy rations at a modest level (7% of DM).

Methods

Rice straw was produced at bench scale at the Michigan Biotechnology Institute. Individual samples of rice straw (DM 60-82%) were sprayed with ammonia (0.5-1.0 g NH_3/g DM), placed in a pressure vessel and heated to 87-108 ^oC over the course of 5-10 min, after which the pressure (19-26 atm) was rapidly released. To prepare material in amounts needed for a feeding trial, several tons of rice straw were pretreated (0.3 g NH_3/g,110-132 ^oC, 12.9-15.0 atm) in a Sunds defibrator at the Tennessee Valley Authority pilot plant in Muscle Shoals, AL.

In vitro digestion experiments were conducted in sealed 60 mL vials of precisely-known volume, that contained 100 mg of substrate, 8 mL of cysteine-reduced Goering-Van Soest buffer, 2 mL of ruminal inoculum composited from two cows, and a CO_2 gas phase. Digestion kinetics were determined by automated recording (~ 150 time points) of gas pressure in sealed vials over a 72 h period. After correction for gas produced in sealed blank vials that contained ruminal inoculum but no rice straw, gas production data were fitted to an two-pool exponential model to determine the lag time, first-order rate constant, and extent of gas production for both the rapidly-digesting and slowly-digesting substrate pools.

The feeding trial was conducted with 20 lactating Holstein cows in a switchback design having 21d periods. Control diet © and Rice-straw amended ® diets (35.8% NDF, 25.9% ADF and 18% CP) were formulated to meet NRC requirements, and contained alfalfa hay, corn grain and soybean meal, along with byproduct feeds typically used in northern California, Table 1. Feed samples and orts (collected daily and were composited by cow) were analyzed for NDF and ADF. Milk yield was determined from twice-daily milkings, and milk composition was determined by NIR (Ag-Source, Verona, WI).

Results and Discussion

In vitro fermentations. Gas production from untreated rice straw lagged behind that of inoculated vials lacking substrate, suggesting the presence of a fermentation inhibitor in the rice straw. The slowly digested substrate pool of untreated rice straw was eventually fermented with a lag time of 10.4 h, and a first-order rate constant of 0.035/h, while the slowly-digested pool of the eleven bench-scale pretreated rice straw samples were fermented with a lag time of 6.4-7.4 h and first-order rate constants of 0.50-0.61/h. Total gas yield from the fermentation of untreated and pretreated rice straws were 129.3 and 127.0-191.7 mL/g OM, respectively.

Production trial. One of the R cows stopped eating a few days into the trial and so was removed. The other cows readily ate both rations without visible sorting, and without any display of hyperexcitability that has been reported consuming high levels (50% of DM) of ammonia-treated rice straw. Feeding of ration R resulted in increases ($P<0.05$) in both NDF intake and milk yield that averaged 1.1 and 1.3 kg/d, respectively (Table 2). A decline in milk fat content of 0.3% occurred with ration R, perhaps in response to the small particle size of the rice straw. Despite higher levels of inorganic N in the pretreated rice straw, levels of protein and MUN in the milk did not differ between treatments.

Conclusions

Pretreatment of rice straw by the FIBEX process reduced the lag time prior to the onset of fermentation and substantially increased the rate of digestion of the fiber fraction, and the total amount of substrate fermented. The enhanced digestion in vitro was reflected in an ability of the pretreated rice straw to sustain milk production when added to 7% of DM in a properly balanced ration. The FIBEX process may this provide a means of converting rice straw, a nuisance agricultural residue, to a useful fiber and energy source of lactating dairy cows.

Table 1. Formulated composition of control diet and diet amended with FIBEX-treated rice straw

	Control	Rice straw
Ingredient composition (g/kg DM) [a]		
Alfalfa hay	450	350
Corn grain, dry kernel	183	206
Whole linted cottonseed	120	120
Treated rice straw	--	70
Beet pulp, pelleted	80	80
Corn gluten feed, dehydrated	80	80
Wheat middlings	40	40
Soybean meal, 48% CP solvent	23.5	29.5
Beet molasses, liquid	20	20
Monosodium phosphate	1.0	1.8
Magnesium oxide	1.3	1.6
Salt and vitamin mixture [b]	1.0	1.0
Chemical composition (g/kg DM)		
NDF	358	357
ADF	258	258
CP	180	180
Ash	70	70
Ca	8.4	7.1
Mg	3.5	3.5
P	4.1	4.1

[a] Dry matter content was 89.7% for the control diet and 89.5% for rice straw diet.
[b] TM Vit Pak (Professional Products and Services, Prairie du Sac, WI, USA) contained per g: 229 mg Ca, 56 mg Zn, 46 mg Mn, 22 mg Fe, 12 mg Cu, O.4 mg Co, 0.9 mg I, 0.32 mg Se, 7084 IU vitamin A, 2200 IU vitamin D3, 17.6 IU vitamin E.

Table 2. Feed intake and production of cows fed diets with and without FIBEX-treated rice straw.

	Control	Rice	Pooled S.E.	*P* > F
Intake (kg/d):				
DM	25.0	25.9	2.9	0.151
OM	22.0	22.9	2.1	0.199
NDF	7.0	8.1	0.7	<0.001
ADF	5.2	5.3	0.5	0.349
CP	4.0	4.0	0.1	0.804
Production (kg/d):				
Milk	38.3	39.6	1.6	0.020
Fat	1.47	1.41	0.11	0.062
Protein	1.14	1.19	0.05	0.011
Lactose	1.83	1.86	0.19	0.671
Energy [a] (MJ/d)	114.0	113.6	6.1	0.914
Milk composition (%):				
Fat (%)	3.86	3.55	0.22	<0.001
Protein (%)	2.99	3.00	0.10	0.664
Lactose (%)	4.77	4.82	0.11	0.197
Urea N (mM)	5.11	5.29	0.83	0.626

Corn Silage Maturity and Processing: 1. Effects on Production of Dairy Cows

D.R. Mertens, G. Ferreira, P. Berzaghi, and R.D. Shaver

Introduction

The effect of maturity on the nutritive value of corn silage is unique among forages. The energy value of corn silage typically declines slowly with maturity because the beneficial effects of increased proportion of grain often diminish the detrimental effects of lignification and maturation of the stover. However, the benefit of a high proportion of grain in mature corn silage depends on adequate digestion of the starch. Starch may not be utilized efficiently unless it is adequately ground or chewed. Kernel processors, which pass the harvested corn through rollers with 1-mm clearances, were introduced to crush kernels and insure maximum digestion of starch. At later stages of maturity, corn kernels harden and develop complex matrices that can reduce starch digestion; thus processing corn silage should be more beneficial for mature corn silages. The objective of this experiment was to evaluate the effects of corn silage maturity and processing on intake and lactation performance by cows of different stages of lactation.

Materials and Methods

Forty-eight lactating Holstein cows were blocked by stage of lactation (**mid** or **late**) and parity (1st, 2nd or ≥3rd) and assigned to replicated 4 x 4 Latin squares (28-d periods) with a 2 x 2 factorial arrangement of treatments: early (**E**) or late-maturity (**L**), and processed (**P**) or unprocessed (**U**) corn silages. Mid lactation cows averaged 73 and late lactation cows averaged 455 day in lactation at the beginning of the experiment. The corn hybrid was corn hybrid Dairyland Stealth 1412 and silage dry

matter (**DM**) concentrations were 33.5 and 41.8% for the E and L corn silages, respectively. Corn was processed using a 1-mm roller clearance and the theoretical length of cut (**TLC**) was set at 1.90 and 2.54-cm for unprocessed and processed treatments to reduce the effects of processing on particle size. The amylase-treated NDF (**aNDF**) and starch concentrations of the corn silages were: 36.7, 28.0; 38.1, 26.3; 34.0, 37.3; and 35.4, 35.1% for EU, EP, LU, and LP, respectively. Diets were composed of 70% corn silage and 30% concentrate and averaged 17% CP and 28% NDF.

Results and Discussion

Processing during harvesting crushed kernels effectively as indicated by the percentage of starch >4.75 mm in particle size, which is an index of processing effectiveness. The percentages of starch >4.75 were 58.8 and 42.2 for EU and LU, respectively, which was reduced to 11.9 and 7.7% for processed silages EP and LP, respectively. The reduction in starch >4.75-mm for L compared to E whether processed or not was surprising and may be related to greater brittleness or friability of kernels that were more mature. Although the TLC was increased to compensate for the reduction in particle size due to processing, the lognormal mean particle sizes of processed silages were lower: 4.1, 3.1, 4.1, and 2.7 mm for EU, EP, LU, and LP, respectively. This occurred because cobs and kernels were reduced in size substantially by processing.

Dry matter intake (**DMI**) was greater for mid lactation cows when fed later maturity silages, but processing silages had no effect on intake (Table 1). Neither processing nor maturity of the silage affected milk production of mid lactation cows. Milk fat percentage was higher for mid lactation cows consuming early maturity silages, which resulted in a tendency for 3.5% fat-corrected milk (3.5% FCM) production to also be higher for early maturity silages. Milk protein percentage of mid lactation cows was higher when consuming processed silages.

The DMI of late lactation cows was higher when fed processed silages, primarily to differences between EU and EP. Milk production and composition of late lactation cows was not affected by silage maturity or processing, except that 3.5% FCM was higher for cows fed processed silage. Similar to mid lactation cows, there was a tendency for milk fat percentage to be higher when late lactation cows consumed E silages that were slightly higher in fiber and lower in starch than L silages. The main response of late lactation cows was to increase average daily gain when fed processed silages, although maturity had no effect on the gain of these cows.

Conclusions

Corn silage maturity and processing did not result in large differences in intake and production when fed as 70% of the ration dry matter to cows in mid and late lactation. There was no maturity by processing interaction in cow performance suggesting that processing did not improve the nutritive value of late maturity silage to a greater extent than for early maturity as was postulated. This may be related to the greater friability of corn kernels in the late maturity corn silage as indicated by the smaller proportions of starch that were >4.75-mm in size.

Table 1. Intake and yield of milk and milk components of cows fed diets containing corn silages harvested at two maturity stages with or without kernel processing (least square means).

Cow group	Corn silage[1]					Probability of differences[2]		
Performance	EU	EP	LU	LP	SEM	M	P	M × P
Mid lactation cows								
DM intake, kg/d	21.2	22.0	23.9	23.3	1.61	.042	.911	.372
Production, kg/d								
Milk	32.5	33.6	32.2	33.5	1.47	.880	.327	.941
3.5% FCM	33.2	33.7	32.3	31.7	1.48	.138	.962	.506
Milk composition, %								
Fat	3.62	3.60	3.54	3.28	0.18	.053	.128	.184
Protein	3.03	3.17	3.08	3.11	0.05	.881	.045	.157
Body weight gain, kg/d	0.36	0.57	0.36	0.33	0.13	.367	.463	.339
Late lactation cows								
DM intake, kg/d	18.8	22.8	21.2	21.5	0.95	.431	.033	.019
Production, kg/d								
Milk	13.5	15.1	14.2	14.0	2.29	.738	.252	.145
3.5% FCM	14.5	17.0	15.2	15.2	2.45	.298	.046	.036
Milk composition, %								
Fat	4.33	4.19	4.00	4.07	0.24	.208	.832	.538
Protein	3.83	3.84	3.81	3.75	0.16	.540	.773	.671
Body weight gain, kg/d	0.50	0.97	0.64	0.97	0.12	.679	.025	.614

[1] EU = Early Unprocessed; EP = Early Processed; LU = Late Unprocessed; and LP = Late Processed.

[2] M = Maturity main effect (E vs. L); P = Processing main effect (U vs. P); and M × P = Maturity by Processing interaction.

Corn Silage Maturity and Processing: 2. Effects on Fiber and Starch digestion by Dairy Cows
D.R. Mertens, G. Ferreira, P. Berzaghi, and R.D. Shaver

Introduction

Digestibility of forages is affected not only by their maturity and composition, but also by the level of intake of the animal that consumes them. In general, the digestibility of forages declines with maturity, in part, because more mature plants have more cell walls that are more lignified. Corn silage it unique because the grain that accumulates during maturation is more digestible than cell walls, which often counteracts the lower digestibility of the stover. It is not uncommon for digestible starch to comprise 50 to 70% of the total digestible nutrients in corn silage. Processing corn during harvesting should fracture kernels and maximize starch digestion, especially in mature corn silages that contain starch that is protected by hard seed coats and complex granule matrices. However, few studies have evaluated if processing is more beneficial in mature corn silage (or determined if the level of intake of corn silage has a significant impact on the digestion of corn silage). The objective of this experiment was to evaluate the effects of animal intake and corn silage maturity and processing on the digestion of nutrients by lactating cows.

Materials and Methods

Forty-eight lactating Holstein cows were blocked by stage of lactation (**mid** or **late**) and assigned to replicated 4 x 4 Latin squares with a 2 x 2 factorial arrangement of treatments: early (**E**) or late-maturity (**L**), and processed (**P**) or unprocessed (**U**) corn silages. Mid lactation cows averaged 73 and late lactation cows averaged 455 days in lactation at the beginning of the experiment. Late lactation cows were selected to measure digestibilities at low ad libitum intakes. Silage dry matter (**DM**) concentrations were 33.5 and 41.8% for the E and L corn silages, respectively. Corn was processed using a 1-mm roller clearance and the theoretical length of cut was set at 1.90 and 2.54-cm for unprocessed and processed treatments. Diets were composed of 70% corn silage and 30% concentrate. The rare earth, lanthanum, was used as an external marker to measure digestibility. Fecal grab samples were collected across three consecutive days (starting on day 26 of each 28-d period) at 6-h intervals, shifting two hours at the end of each day so that samples were collected at 2-h intervals during a 24-h feeding cycle.

Results and Discussion

Corn silages contributed about 90% of the amylase-treated NDF (aNDF) and 80% of the starch in their respective rations (Table 1). The aNDF concentration was slightly higher and the starch concentration slightly lower for rations containing processed silage due to a maladjusted processor cover that allowed starch loss during harvesting. Although mature corn silage contained more starch, it was more susceptible to processing so the starch in particles >4.75 mm were very similar within P and U treatments, i.e., 16.7, 3.0, 15.7, and 2.7% for EU, EP, LU, and LP silages, respectively.

Dry matter intake did not differ between cows in mid and late lactation as was expected. Although late lactation cows had a lower nutrient demand for lactation, they tended to gain weight and consume rations in similar amounts to mid lactation cows. Dry matter digestibility (**DMD**) tended to be higher for diets containing E compared to L corn silages (71.4 vs. 69.8%) when consumed by mid lactation cows, but not when consumed by late lactation cows (71.2 vs. 71.4%), Table 2. The

difference in DMD between maturities was due primarily to differences in aNDF digestibilities (**aNDFD**). When mid lactation cows consumed silages, the aNDFD was significantly different (49.2% for E compared to 39.7% for L). The difference in aNDFD was significant, but much lower for late lactation cows (51.2 and 45.7% for E and L, respectively), which may explain the lack of difference in DMD for these cows. When averaged across processing treatments, maturity did not affect starch digestibility (**StarchD**) for either mid or late lactation cows (95.3 and 94.8% for E and L, respectively). For unprocessed silages, the average StarchD for all cows was 92.8 and 91.0% for E and L, respectively.

Processing corn silage improved the DMD for both mid and late lactation cows, averaging 69.0 and 72.9% for U and P, respectively. This response was primarily due to increased StarchD for P compared to U (98.2 vs. 91.9%) for both groups of cows. The aNDFD was also higher for P compared to U, but the difference was significant only for the mid lactation cows.

Conclusions

Both maturity and procession affect the digestibility of corn silages. The early maturity corn silage (DM = 33.5%) was more digestible than the late maturity (DM = 41.8%) and processing increased the digestibilities of dry matter, starch, and to a lesser extent aNDF. However, processing did not improve the digestibilities of mature corn silage to a greater extent than for more immature silages. There was some indication that the effects of maturity and processing on aNDFD were confounded by changes in the starch concentration in the ration. A review of published results suggests a significant negative effect for the ratio of starch to NDF intake on fiber digestibility.

Table 1. Nutrient composition of experimental rations.

	Corn silage used in the rations[1]			
	EU	EP	LU	LP
Dry Matter, %	41.4	41.6	49.9	49.9
Crude protein, % DM	17.5	17.7	17.6	17.6
aNDF, % DM	28.0	28.9	26.1	27.0
Starch, % DM	24.1	22.8	30.6	29.0
aNDF from corn silage, % ration aNDF	91.0	91.2	90.4	90.7
Starch from corn silage, % ration starch	80.7	79.4	84.8	83.9

[1] EU = Early Unprocessed; EP = Early Processed; LU = Late Unprocessed; LP = Late Processed.

Table 2. Nutrient intake and apparent total-tract digestibility by cows in mid lactation fed diets containing corn silages harvested at two maturity stages with or without kernel processor (least square means).

Cow group	Corn Silage[1]					Probability of differences[2]		
Characteristic	EU	EP	LU	LP	SEM	M	P	M × P
Mid lactation								
DM intake, kg/d	21.2	22.0	23.9	23.3	1.61	.042	.911	.372
NDF intake, kg/d	5.3	6.3	5.6	6.0	0.28	.856	.002	.074
Starch intake, kg/d	4.7	4.7	6.9	6.5	0.26	.001	.115	.118
Starch/NDF ratio	0.89	0.74	1.27	1.08	0.03	.001	.001	.335
DMD[3], %	69.4	73.3	68.0	71.6	1.12	.094	.003	.797
NDFD[3], %	47.4	51.0	38.2	41.2	1.85	.001	.053	.943
StarchD[3], %	93.2	97.7	92.0	98.6	0.96	.796	.001	.150
Late lactation								
DM intake, kg/d	18.8	22.8	21.2	21.5	0.95	.431	.033	.019
NDF intake, kg/d	4.9	6.2	4.9	5.4	0.27	.090	.002	.042
Starch intake, kg/d	4.5	4.8	6.0	5.8	0.31	.001	.819	.239
Starch/NDF ratio	0.93	0.79	1.24	1.07	0.03	.001	.001	.707
DMD[3], %	69.1	73.2	69.4	73.4	1.23	.979	.002	.671
NDFD[3], %	51.1	51.2	47.7	43.7	2.50	.010	.181	.196
StarchD[3], %	92.3	98.1	90.1	98.3	0.73	.412	.001	.223

[1] EU = Early Unprocessed; EP = Early Processed; LU = Late Unprocessed; and LP = Late Processed.

[2] M = Maturity main effect (E vs. L); P = Processing main effect (U vs. P); and M × P = Maturity by Processing interaction.

[3] D suffix = digestibility expressed as a percentage of the constituent.

Manure Nutrient Management

Alfalfa Unable to Prevent Groundwater Contamination Under an Abandoned Barnyard on Sandy Soil

M. P. Russelle, N.B. Turyk, B.H. Shaw, J. F.S. Lamb, and B. Pearson

Introduction

There is substantial public concern about water quality. Many point sources of excess N and P are being controlled, yet water quality in many areas of the USA continues to deteriorate. This is especially true in much of central Wisconsin and other areas dominated by sandy soils where ground water contamination has become a serious problem.

Manure addition to soil may result in excessive P accumulation, which can threaten surface water quality and in some instances, ground water quality. Although P is generally considered immobile in soil, significant P leaching may occur after high livestock manure application rates. Increased P loading could be environmentally detrimental in areas where ground water is hydrologically connected with surface water.

Livestock have traditionally been fed in outdoor lots ('barnyards') to minimize production costs. Excessive manure accumulation occurs near feed bunks, hay bales, or watering tanks. Our hypothesis was that alfalfa would protect ground water quality at an abandoned barnyard site in Central Wisconsin, and that special non-N_2-fixing types of alfalfa would be more effective than standard alfalfa.

Methods

The experiment was located in Portage Co., Wisconsin. Dairy young stock had been fed year-round on 4 to 6 ha of Richford loamy sand (0 to 6% slope), which had been nearly devoid of vegetation for about 15 year. Soil and ground water sampling, preliminary piezometer installation, and seeding of alfalfa were initiated in summer of 1998; ground water monitoring well nests were installed in 1999. Two replicate 30 x 60 m plots of non-N_2-fixing alfalfa ('Ineffective Agate') in the other and two of standard N_2-fixing alfalfa ('Agate') were seeded in late July 1998, but stands of one replicate failed. Due to limited seed availability, this replicate was reseeded with 'Ineffective Saranac' and 'Saranac' in August 1999.

Herbage samples were obtained per plot from 1-m^2 sections located in areas of contrasting soil fertility at each of three alfalfa harvests per year. We used the ^{15}N natural abundance technique to estimate symbiotic N_2 fixation by N_2-fixing alfalfa during 2000 and 2001. Soil and ground water were sampled periodically over 3 years and analyzed for inorganic N and P.

Results and Conclusions

At the forage yields produced at this site, we found that standard N_2-fixing alfalfas removed at least 200 kg N ha^{-1} annually from these sandy soils. Nitrogen fixation is a facultative process: when large amounts of inorganic N are available at a site, alfalfa will fix less N_2 from the atmosphere. This research demonstrated how the facultative nature of symbiotic N_2 fixation provides an economic benefit for phytoremediation (Fig. 1). In sites that have heterogeneous accumulations of nitrate, N_2-fixing alfalfa will produce uniformly high yields, yet remove inorganic N efficiently, as long as other factors are optimum for alfalfa growth (water supply, nutrient levels, etc.). Non-N_2-fixing alfalfa did not remove as much total N as N_2-fixing alfalfa at this site, presumably because available N was lost by leching, thereby limiting growth and yield in the non-N_2-fixing plots.

Nitrate leaching to ground water was significant at this site, with all wells exceeding the 10 mg L^{-1} public drinking water standard for NO_3^--N. One example is shown in Fig. 2. Concentrations as high as 80 mg L^{-1} NO_3^--N were found in down-gradient wells in 2001. Both non-point and focused recharge leaching was apparent at this site, highlighting the need for better livestock manure handling and storage on these soils.

Further research to optimize N uptake in remediation sites should focus of companion crops like wheat, barley, or oats planted with alfalfa to provide more first-year uptake of N while alfalfa develops a deep root system, especially in soils having high hydraulic conductivity. The cereal crop can be harvested for silage at the soft dough growth stage to provide feed and to limit competition with alfalfa in midsummer. Farmers who use this forage should be aware of potentially high concentrations of NO_3^- in grass forage, which may be toxic to livestock. Ensiling will reduce excess NO_3^- in the stored forage, whereas conserving the forage as hay will not.

In addition, direct seeding techniques that would not require cultivation should be used where previous soil compaction will not limit root growth. Cultivation destroys near-surface compacted layers, which coincidentally increases both rapid mineralization of organic N and NO_3^- leaching.

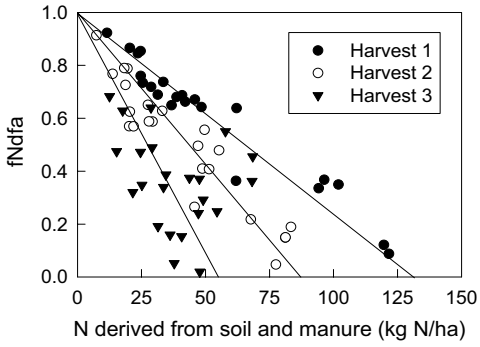


Figure 1. The fraction of N derived from the atmosphere (fNdfa) declined with increasing N derived from the soil and manure.

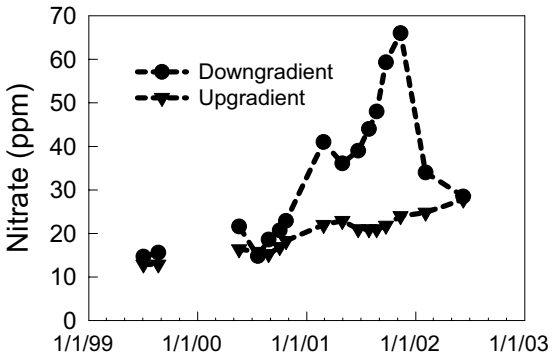


Figure 2. Ground water nitrate increased below the alfalfa plots, regardless of alfalfa type. Slow increases in nitrate in upgradient wells indicate nitrate loading from a new barnyard.

Differential ^{15}N Labeling of Dairy Manure Components for Nitrogen Cycling Studies

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Introduction

Approximately 70 - 80% of the nitrogen (N) consumed by a dairy cow is excreted in urine and feces. Fecal N can be divided into two pools: 1) endogenous N consisting of microbial products and micro-organisms from the rumen, the intestine and the hind gut, and N originating from the digestive tract itself; and 2) undigested feed N. When applied to soil, urine N mineralizes rapidly, followed by fecal endogenous N and fecal undigested feed N. Although undigested feed N in feces may not make a significant contribution to crop N requirements during the year following application, this component likely plays an important role in soil-crop N dynamics over the long-term, and contributes significantly to soil organic matter in manure-amended soils. The objective of this study was to differentially label urine N, fecal endogenous N, and fecal undigested feed N for the purpose of determining their short- and long-term mineralization in soils.

Methods

Two methods were used to differentially enrich dairy urine and fecal N components in ^{15}N. The *forage method* involved labeling alfalfa (*Medicago sativa* L.) hay and corn (*Zea mays* L.) silage then feeding these forages to dairy cows. This technique labels urine N, fecal endogenous N and fecal undigested feed N. The *urea method* involved the direct feeding of ^{15}N-enriched urea to cows with unlabeled forage. This technique labels urine N and fecal endogenous N. No labeled undigested feed N in feces can be expected using the *urea method* since no ^{15}N forage is fed. Two ruminally-fistulated non-lactating cows weighing from 440 to 520 kg were used for each labeling method. Cows were first adapted to a diet consisting of approximately 55% alfalfa hay and 45% corn silage on a dry matter basis (atom % ^{15}N at natural abundance) for 7d. On the last day of the adaptation period, indwelling catheters were inserted into the bladders for urine collection. ^{15}N-enriched forage or urea was fed for 2-3 days. Feces and urine were collected separately at 4 or 8 h intervals for 8 days after the initiation of ^{15}N feeding.

Results and Discussion

In the forage labeling part of this study, highest alfalfa yields and ^{15}N enrichments were generally attained in the first and second harvests (Table 1). Relatively high levels of ^{15}N enrichment were also attained in the third harvest, even though ^{15}N-enriched fertilizer was applied only before the first and second harvests. For the cows fed ^{15}N-enriched forage, the pattern of ^{15}N excretion in urine and feces was similar for both cows during all four years of the study. ^{15}N concentrations in urine and feces increased to a single maximum point and decreased thereafter. Peak ^{15}N concentrations in urine occurred between 20 and 60h after the initiation of feeding ^{15}N-enriched forage (Fig. 1). Peak ^{15}N concentrations in feces occurred between 32 and 72h, or approximately 6 to 32 h after the final offer of ^{15}N-enriched forage (Fig. 2). The pattern of ^{15}N excretion in urine and feces after feeding ^{15}N-enriched urea (data not shown) was very different from the observed pattern of ^{15}N excretion after feeding ^{15}N enriched forage. A single 100g dose of 5 atom% ^{15}N urea fed in 1999 resulted in a single peak of ^{15}N enrichment in urine (approximately 1.25 atom% ^{15}N), which occurred at 8 h, and a single peak of ^{15}N enrichment in feces (approximately 0.75 atom% ^{15}N), which occurred at 32 h. Eight doses of ^{15}N-enriched urea fed at 4 h intervals in 2000 resulted in eight ^{15}N peaks (from 1.25 to 2.15

atom% ^{15}N) in urine. Each peak was recorded within 4 h after feeding ^{15}N-enriched urea. A single ^{15}N enrichment peak in feces occurred (approximately 1.25 atom% ^{15}N) approximately 56 h after the initial offer of ^{15}N-enriched urea. No increases in urinary or fecal ^{15}N concentrations were observed after the 7th or 8th dosing.

Year of forage production	Forage type	Amount fed	Total N content	Atom % ^{15}N excess
		kg DM	g kg^{-1}	
1997	Alfalfa harvest 1	8.1	29.59	3.231
	2	5.4	38.45	3.055
	3	1.7	43.10	1.956
	Corn silage	12.2	8.92	6.436
	Total diet	**27.4**	**22.97**	**3.562**
1998	Alfalfa harvest 1	9.3	23.78	3.643
	2	6.0	33.23	4.761
	3	2.4	26.83	3.125
	Corn silage	11.0	6.52	5.235
	Total diet	**28.7**	**19.39**	**4.188**
1999	Alfalfa harvest 1	9.3	24.98	3.431
	2	10.8	29.84	2.919
	3	5.4	33.52	3.580
	Corn silage	23.2	8.67	4.689
	Total diet	**48.7**	**19.234**	**3.554**
2000	Alfalfa harvest 1	15.2	31.70	1.911
	2	11.9	33.47	3.791
	3	4.2	41.04	1.171
	Corn silage	28.8	9.07	4.624
	Total diet	**60.1**	**21.86**	**2.923**

Table 1. Forage fed to dairy cows for ^{15}N-labeling of manure

The undigested feed N in feces (NDIN) accounted for approximately 20-22% of the total fecal N, or 10% of the total N (urine plus feces) excreted by the cows fed the diets of this study. The homogeneous ^{15}N labeling of fecal N components was evaluated by comparing ^{15}N concentrations in fecal total N and NDIN. Fecal endogenous N [neutral detergent soluble N (NDSN)] was calculated as the difference between total fecal N and NDIN. During all study years, the labeling of NDIN appeared to be slower than NDSN during the period before maximum fecal ^{15}N concentrations were attained (data not shown). The proportionate mixing of feces from periods before and after peak ^{15}N excretions is needed to obtain uniformly labeled feces. The selection of a manure ^{15}N labeling technique would depend on the intended use of the ^{15}N-labeled manure, and associated costs and labor.

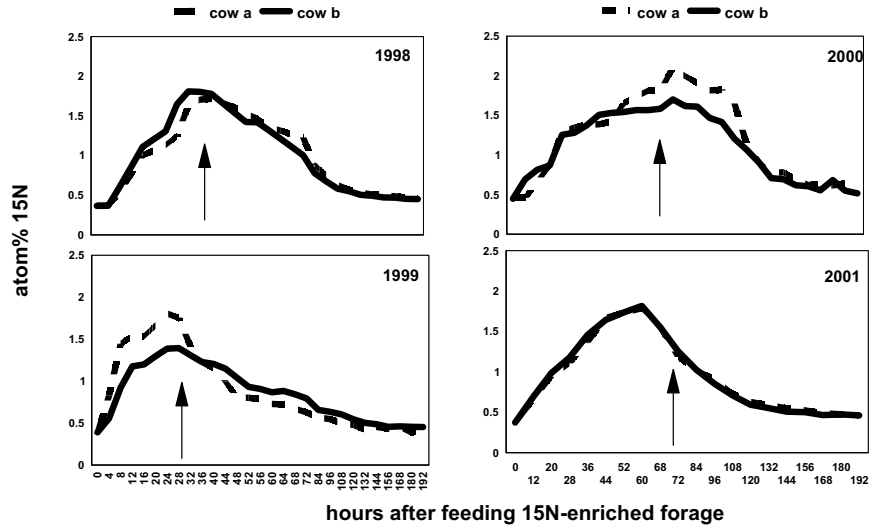

Figure 1. ^{15}N concentration in urine after feeding ^{15}N-enriched forage (base of arrows point to time when last offer of ^{15}N-enriched forage was made).

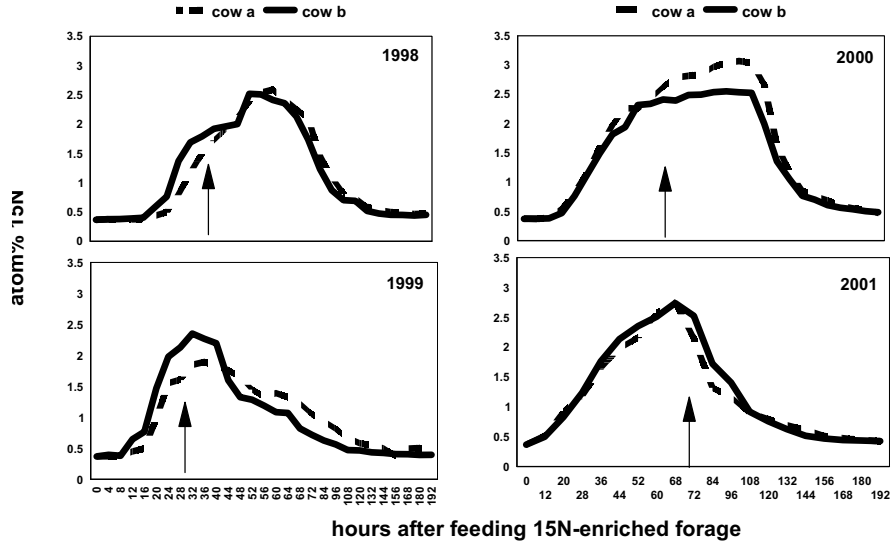

Figure 2. ^{15}N concentration in feces after feeding ^{15}N-enriched forage (base of arrows point to time when last offer of ^{15}N-enriched forage was made).

Of the total ^{15}N used in this study, 22 to 28% was incorporated into feces and urine using the *forage method* verses 64 to 78% using the *urea method* (Table 2). The major difference in ^{15}N use efficiency between the two ^{15}N labeling techniques was the loss of ^{15}N in soil when labeling forage. From 36 to 44% of applied ^{15}N was taken up by alfalfa and 26 to 65% taken up by corn. Lowest ^{15}N uptake by corn occurred in 1998 when high rainfall events following the second and third fertilizer applications likely resulted in ^{15}N leaching losses. Of the total ^{15}N fed, 51 to 64% was recovered in feces and urine from cows fed ^{15}N-enriched forage verses 64 to 78% recovery from cows fed ^{15}N-urea. Higher ^{15}N recovery using the urea method was likely due to urea's rapid incorporation into the rumen

microbial biomass with excess excreted in urine. Un-recovered ^{15}N fed as forage or urea was incorporated into rumen microbial and body tissue.

Conclusions

Manure ^{15}N labeling provides a tool for direct measurement of N flow in various components of the feed-dairy cow-manure-soil/crop continuum. The forage method should be used to label manure for use in long-term N cycling studies so both fecal endogenous and undigested feed N components become ^{15}N-enriched. Uniform labeling of fecal N components can be achieved by the proportionate combination of feces excreted before and after peak ^{15}N excrement levels are attained. The urea method is less laborious and costly and may be used to label manure for short-term studies, for example, to determine crop uptake of manure N during a single cropping season.

Component	1997-98	1998-99		1999-2000		2000-01
	Forage method	Forage method	Urea method	Forage method	Urea method	Forage method
Crop input						
Alfalfa	38.54	53.95		53.95		72.68
Corn silage	13.42	14.41	Not applicable	14.41	Not applicable	19.81
Crop output						
Alfalfa	15.52	19.56		23.86		26.32
Corn silage	7.00	3.75		9.43		12.07
Cow input						
Feed	22.52	24.32	4.85	33.29	15.56	38.39
Cow output						
Feces	5.64	7.05	1.00	9.10	2.73	11.02
Urine	5.97	7.96	2.12	10.15	9.40	13.43
^{15}N Use Efficiency†	40	36	Not	44	Not	36
Alfalfa	52	26	applicable	65	applicable	61
Corn	51	62	Not	58	Not	64
Cow	22	22	applicable	28	applicable	26
Overall			64		78	
			64		78	
Costs‡	$376	$373	$269	$291	$277	$325

Table 2. ^{15}N excess (g) input-output relationships in crop and cow components.
† (^{15}N excess output/^{15}N excess input) x 100 for each component
‡ total cow ^{15}N excess output in feces and urine divided by cost of ^{15}N.
Cost of 10 atom % ammonium sulfate was $1.66 per g, and 5 atom % urea was $4.20 per g.

Evaluation of Dairy Manure ^{15}N Enrichment Methods on Short-Term Crop and Soil Nitrogen Budgets

J.M. Powell, K. Kelling, G. Muñoz and P. Cusick

Introduction

Nitrogenous compounds artificially enriched in ^{15}N have been used extensively to study manure nitrogen (N) cycling in soils. These compounds have been used for (1) post-excretion labeling, which is usually accomplished by adding a ^{15}N-enriched inorganic source, usually ammonium sulfate, to excreta, or (2) labeling feedstuffs, which are then fed to ruminant livestock (Dittert et al., 1998). Post-excretion N labeling is usually used to study ammonium-N cycling in slurry-amended soil. Whereas slurry is the most common manure type on free stall dairy operations having parlor flush systems and lined manure storage pits, semi-solid manure consisting of feces, urine and straw bedding is the most important manure type on small dairy operations (Jackson-Smith et al., 1997).

Two methods for differentially enriching dairy manure N components in ^{15}N have been recently proposed (see **Differential ^{15}N labeling of dairy manure components for nitrogen cycling studies, pp. 90)**. The *forage method* involves the labeling and feeding of ^{15}N-enriched forage to dairy cows to label urine N, fecal endogenous N and fecal undigested feed N (Mason and Frederiksen, 1979). The *urea method* involves feeding ^{15}N-enriched urea directly to dairy cows to label urine N and fecal endogenous N. No fecal undigested feed N is labeled using the *urea method* since no ^{15}N-enriched forage is fed. The objective of this study was to determine corn ^{15}N uptake and the amount and forms of soil ^{15}N in field plots amended with ^{15}N-enriched manure derived from the forage and urea labeling methods.

Methods

In 1999 and 2000 manure derived from the *forage method* and *urea method* were surface applied to a Plano silt loam (fine-silty, mixed, mesic, Typic Argiudolls) in field plots 1.5 m wide x 2.3 m long containing three corn (*Zea mays* L.) rows. Corn was grown for two years after each manure application. In fall 2000, soil samples were taken from each plot to 90-cm depth in 30-cm increments and analyzed for ^{15}N enrichment of soil total N and nitrate N.

Results and Discussion

Corn ^{15}N uptake during the first year after manure application was not significantly affected by method of manure ^{15}N enrichment or year of application (Table 1). Of the total manure ^{15}N applied, 14 to 18% was accounted for in corn harvested the cropping season after manure application. Average residual ^{15}N uptake by corn in 2001 (8% of original manure ^{15}N applied) was significantly greater than residual ^{15}N uptake in 2000 (4%). Total (first year plus second year residual) ^{15}N uptake ranged from 18 to 25% with no significant differences due to manure ^{15}N enrichment method or year of application. Relative manure ^{15}N uptake by corn in our study corresponded well to crop ^{15}N uptake calculated by others (Sørensen and Jensen, 1998; Jensen et al., 1999).

Harvest year	Manure type	1st year ^{15}N uptake	2nd year ^{15}N uptake	Total ^{15}N uptake
		----------% of manure ^{15}N applied----------		
1999	Forage	(6)† 14.0	NA	NA
	Urea	(6) 15.9	NA	NA
2000	Forage	(4) 14.8	(6) 4.3	(6) 18.3
	Urea	(4) 17.5	(6) 4.0	(6) 19.9
2001	Forage	NA	(4) 8.4	(4) 23.2
	Urea	NA	(4) 7.4	(4) 24.9
Mean		15.4	5.6	21.1
p-values				
manure type		.530	.683	.687
Year		.737	.025	.248
type*year		.928	.838	.997

Table 1. ^{15}N uptake by corn the first and second year after application of forage- or urea-labeled dairy manure. (Number in parentheses refers to the number of microplots used in calculation. For example, the 6 forage- and urea-manure plots used in 1999 to calculate 1st year uptake were the same 6 plots used in 2000 to calculate residual uptake).

Soil $^{15}NO_3$–N increases due to manure ^{15}N enrichment method ranged from approximately 2 to 20 kg ha^{-1} (Figure 1). Both the lowest and highest NO_3–N levels were found in plots amended with urea manure (UM) in 1999 and 2000, respectively. Except for plots amended with UM in 2000, most (72 to 98%) NO_3–N was found in the upper 30 cm of soil indicating little nitrate leaching under the conditions of this study. Nitrate-N in the plots amended with UM in 2000 was fairly evenly distributed over the three measured soil depths, perhaps indicating some leaching. Although plots amended with UM in 2000 appeared to have more $^{15}NO_3$–N than plots amended with forage manure (FM), this result was not significant.

Statistical analyses showed that manure type or year of application did not significantly affect soil $^{15}NO_3$–N level. However, plots amended with manure in 2000 had somewhat greater ($p < 0.088$) NO_3–N levels (0 to 90 cm) than plots amended in 1999. This can be attributed to differences in the number of corn crops grown between the time of manure application and soil sampling. Whereas plots amended with manure in 1999 had two corn crops, plots amended in the spring of 2000 had only one corn crop before soil sampling in the fall of 2000. Residual ^{15}N uptake by corn in 2001 averaged 8% (Table 1), or approximately 21 kg ha^{-1} of the manure ^{15}N applied in 2000. Part of this corn ^{15}N uptake in 2001 was likely derived from the 5 to 20 kg ha^{-1} of residual $^{15}NO_3$–N (Fig. 1), as well as from the continuous mineralization of the manure ^{15}N applied in 2000.

There were no significant differences in total soil ^{15}N levels due to manure type or year of application. Total soil ^{15}N levels in plots amended with FM were 123 and 92 kg ha^{-1}, and in plots amended with UM 128 and 142 kg ha^{-1} in 1999 and 2000, respectively (Fig. 2). Depth differences in ^{15}N recovery were statistically significant ($p < 0.001$), with highest recoveries (79%) obtained from the top 0- to 30-cm depth. No differences in ^{15}N recovery were observed between the 30- to 60- (15%) and 60- to 90-cm (6%) depths. This suggests either relatively little downward movement of applied manure N, or that leached N may have moved out of the 0- to 90- layer. Averaged across years, the relative amount of ^{15}N recovered in the 0-30, 30-60 and 60-90 cm soil depths were very similar for each manure type, averaging 32, 6 and 3% in the FM-amended plots and 38, 7 and 3% in the UM-amended plots, respectively (Table 2). On average, 67% of applied manure ^{15}N was accounted for, either in crop uptake (22%) or in the soil (45%). Most of the ^{15}N that could not be accounted for (approximately 30%) was probably lost through ammonia volatilization, and to a lesser extent via

denitrification.

Manure Enrichment Method	Soil Depth (cm) 0-30	30-60	60-90	0-90	Crop†	Total	Unaccounted for
	N recovery (% of applied manure ^{15}N)						
Forage	32 (6.8) ‡	6 (1.3)	3 (0.6)	41 (2.9)	21 (2.7)	62	38
Urea	38 (8.8)	7 (1.6)	3 (1.9)	48 (4.0)	23 (4.9)	71	29

† Total crop N first and second year uptakes for each manure type were averaged for 1999-2000, and 2000-2001 (Table 2). Soils were sampled in fall of 2000. .
‡ Standard errors in parentheses

Table 2. Average total ^{15}N recovery in soil and corn for different dairy manure types in south-central Wisconsin.

Conclusion

A recent analyses of three years data from the main plots of the long-term trial showed that ^{15}N-labeled dairy manure (forage method) provided much more accurate estimates of (1) the contribution of manure N to crop N requirements compared to the "difference method" and "fertilizer equivalent" approach, and (2) effects of manure application on soil NO_3 and total N levels (Muñoz et al., 2002). Our results reported here suggest that the less laborious and costly urea method of labeling the labile dairy manure N components (urine and fecal endogenous N) would be adequate for evaluating short-term N dynamics in manure-amended soils. The contribution of fecal undigested feed N to soil N dynamics in continuously manured soils needs to be assessed before knowing if this manure component needs to be labeled using the forage method to produce manure for use in long-term N cycling studies

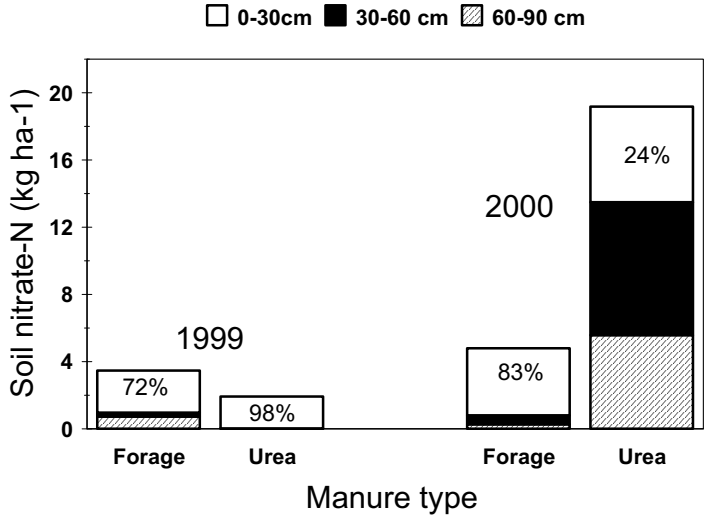

Figure 1. Soil NO_3–N increase over non-manured control plots due to manure type and year of application as estimated by ^{15}N measurements in south-central Wisconsin, 2000. Numbers within each bar represent the percentage of recovered total soil ^{15}N present in the top 30 cm of soil.

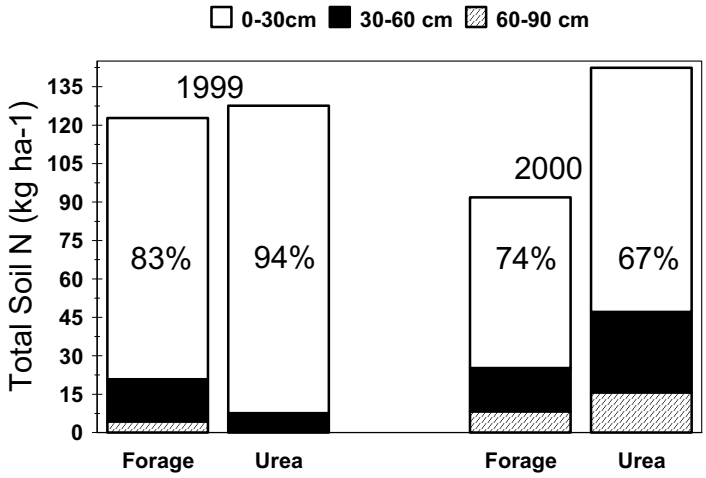

Figure 2. Total soil N increase over non-manured control plots due to manure type and year of application as estimated by ^{15}N measurements in south-central Wisconsin, 2000. Numbers within each bar represent the percentage of recovered total soil ^{15}N present in the top 30 cm of soil. (no significant differences in total soil N increase in the 0-90 cm depth due to manure type, year of application or interactive effects of these two treatments).

On Farmers' Ground: Understanding Nutrient Management on Wisconsin Dairy Farms
J.M. Powell, D. McCrory, H. Saam and Y. Li

In 2001, scientists at the USDFRC and collaborators from the University of Wisconsin-Madison and the Michael Fields Agricultural Institute received a grant from the USDA-CSREES *Initiative for Future Agricultural and Food Systems* to study a range of issues related to nutrient management on dairy farms. A key component of the overall research effort is to better understand how dairy farmers manage agricultural nutrients (the nitrogen and phosphorus contained in feed, fertilizer, and manure) on their farms. The project works closely with 54 dairy farms in Wisconsin over a 12-18 month period in 2002 and 2003.

This on-farm research project is designed to answer questions such as:

* How do management practices used by typical Wisconsin dairy farmers affect the flow of nutrient on their farms?

* What are some innovative solutions that farmers have created to meet their nutrient management challenges?

* What are the most significant obstacles dairy farmers face in managing agricultural nutrients?

Most research to understand and improve nutrient cycling on dairy farms has taken place on experimental farms or other controlled conditions. This research project is designed to improve our understanding of how farmers manage nutrients under more typical production conditions.

Several visits to each farm are being made beginning in the fall of 2002. Dairy farms have been selected from within three regions of distinctive geographic and soil characteristics (Fig. 1), and different stocking densities. Our hope is to understand how farmers living in similar and different biophysical environments, and having different amounts of cropland to grow feed and spread manure, respond to the challenges of managing agricultural nutrients.

What kinds of information are being collected?
On our first visit during the latter months of 2002, we sought to understand the overall farm operation, including information on herd size, composition, management and feeding practices, and on land use and other basic operation characteristics. A map of each farm and field boundaries was made using aerial photographs and discussions with farmers (Fig. 2). These maps have been digitized and serve as the basis for collecting detailed information on cropping patterns, tillage, and manure and commercial fertilizer application practices at the field level.

What will be done with the information?
Several widely used nutrient management computer models will be used to analyze the information we collect. These models have the ability to estimate, for example, whole-farm nutrient balances (in other words, are more nutrients coming onto a farm than leave in the form of milk, meat, and crops?), which individual fields or points on the landscape might be places where nutrients collect and are lost, etc. During each farm visit, we collect samples of feedstuffs, milk, and manure to help calibrate and validate the nutrient flow models.

We will share with farmers the results of the computer-based nutrient management models and what recommendations the models indicate are needed to increase the efficient use of agricultural nutrients. During this project phase, we will be particularly interested in letting the farmers tell us why they make the nutrient management decisions they do, and what opportunities and obstacles might affect any future changes in their nutrient management behavior.

The results of this study should help identify where the greatest gains in nutrient management on dairy farms can be made. Moreover, an expanded understanding of how farmers think about nutrient management, as well as information about the biophysical (soil, crop, weather), financial, labor, and institutional barriers that limit farmer options, will be used to make suggestions for improving the success and minimizing the costs of future nutrient management policies.

Results of the study will be published in ways that can be used by farmers, dairy extension agents, university researchers, crop and feed consultants, policy makers, and others to improve the management of agricultural nutrient on dairy farms.

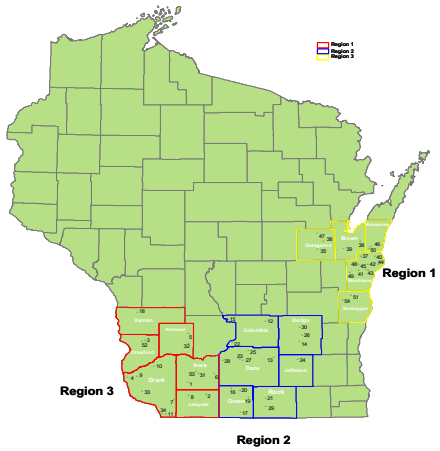

Figure 1. Geographic location of dairy farms participating in "On-Farmers' Ground".

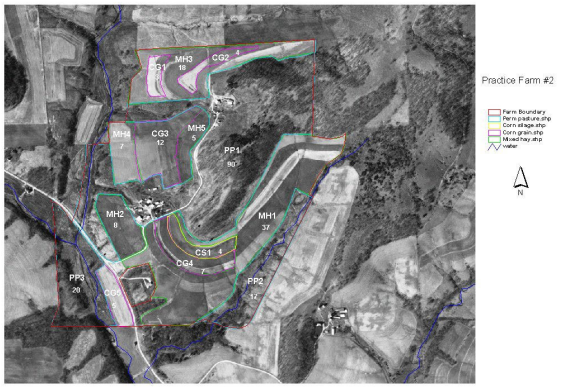

Figure 2. Example of a farm map and its field boundaries for a dairy farm participating in "On-Farmers' Ground".

Nitrogen Availability from Dairy Manure Components

P. Cusick, J.M. Powell, R. Hensler and K. Kelling.

Introduction

Manure nitrogen (N) mineralization in soil determines manure N availability for crop uptake. Soil texture and other controls, such as moisture and temperature create environments where great differences in manure N mineralization can be expected. Understanding the influence of these factors on manure N mineralization is critical to better predicting the amount and rate at which manure N becomes available to agronomic crops. The objective of this study was to determine the N mineralization rate of dairy manure components (feces, urine and bedding) in soils of various textures under different environmental conditions.

Methods

A laboratory incubation study was conducted in which ^{15}N-labeled or unlabeled feces, urine and oat straw bedding were incubated in soil for 168 days. Six soils (Table 1) were selected to represent prominent dairying areas in the state of Wisconsin. Manure amendments (Table 2) were applied at a rate equivalent to 350 kg N ha^{-1} (36% of total applied N derived from feces, 42% from urine and 22% from bedding) into incubation vessels (glass jars containing 250g soil dry wt.). Triplicate vessels per manure treatment plus controls were packed to natural bulk density, kept at 60% water filled pore space and incubated at 11, 18 and 25 C˚.º Vessels were sampled at 0, 14, 21, 42, 84, and 168 days and analyzed for mineralized N (NH4+ and NO3-) and ^{15}N abundance at day 168.

Results and Discussion

Soil type and temperature had significant effects on fecal N mineralization; an interaction between soil type and temperature was found in straw N mineralization; and soil type and temperature did not significantly affect urine N mineralization (Table 3). Uniform urine N mineralization was likely due to the rapid breakdown of most, if not all urine in the soil. Average ^{15}N recovered in the mineralized N fraction in soil amended with labeled urine over all soils and temperatures was 55%. Because it is unlikely N losses occurred during the incubation, the remaining (45%) unaccounted for applied urine N may have exchanged with unlabeled soil N, or may have been immobilized by the soil microorganisms. In a field study conducted in 2001 on a Plano silt loam, it was found that 31% of applied urine ^{15}N was taken up by corn during the cropping season after application (unpublished). The difference between urine N availability in our laboratory incubation (55%) versus urine N uptake in the field (31%) illustrates that urine N may be readily available to crops but some N losses are inevitable.

Fecal N mineralization was significantly higher in the Plano and Symco soils than other soils. It is unclear based on the physical properties of these soils why they mineralized the highest amount of fecal N. Temperature also had a significant effect on the mineralization of fecal N. Highest fecal N mineralization (19%) was found at the highest temperature (25˚C). In the aforementioned field study on the Plano silt loam, 17% of applied fecal N was taken up by corn during the cropping season following application, compared to 19% mineralized in Plano silt loam included in the incubation trial.

Whereas straw N mineralization generally increased in Plano, Symco and Loyal with increasing temperature, straw N mineralization decreased in the Rosholt soil with increasing temperature. Straw N mineralization in the Rozetta soil remained stable for all temperatures. It should be noted that straw N mineralization (17% of applied straw N) was similar to fecal N mineralization (15%) over all soils and temperatures.

Soil type did not affect N mineralization in vessels that received all manure components labeled. Temperature was significant with the highest manure N mineralization occurring at 25˚C. The average amount of manure N mineralized over all soils and temperatures in vessels that received all components labeled was 23%. Average manure N mineralization by adding average individual component mineralization rates was 32% our other research on the Plano silt loam found 14% uptake of manure ^{15}N by corn (*Zea mays* L.) averaged over 3 years of data. Only feces and urine were labeled in this trial and bedding contributions were not considered. University of Wisconsin recommendations for first year N availability of dairy manure are 30% when surface applied and 40% when incorporated. Though this data suggests that recommendations may be high, it should be noted that isotopic studies generally underestimate availability due to ^{15}N exchange with soil organic N pools and the subsequent release of unlabeled soil N. Perhaps estimates of manure N mineralization using ^{15}N should be viewed as a minimum N availability.

Conclusion

Across soil types, 50 to 60% of applied urine N was apparently plant available. Further investigation is needed to understand how soil type affects N availability of the fecal and bedding components of dairy manure.

Acknowledgements

Support for this project was provided by the UW Consortium for Agriculture and Natural Resources, the UW-Madison College of Agricultural and Life Sciences. Appreciation to Jennifer Hegge and Chris Fellner, University of Wisconsin- Stevens Point for their diligent effort in the daily maintenance of this experiment.

Table 1. **Treatment List**

Treatment 1	**^{15}N Feces**, ^{14}N Urine, ^{14}Bedding
Treatment 2	^{14}N Feces, **^{15}N Urine**, ^{14}Bedding
Treatment 3	^{14}N Feces, ^{14}N Urine, **^{15}Bedding**
Treatment 4	**^{15}N Feces, ^{15}N Urine, ^{15}Bedding**
Treatment 5	Control (no manure applied)

Table 2. Initial Soil Properties

Soil Series	Texture	Tot-C	Tot-Org-C	Inorg-C	Bray-P1	Total N	Sand	pH
		%	%	%	mg/kg	%	%	
Loyal	Silt Loam	**2.61**	**2.35**	**0.26**	**42**	**0.212**	**13**	**7.0**
	Fine-loamy, mixed, superactive, frigid Oxyaquic Glossudalfs							
Plano	Silt Loam	**3.55**	**2.56**	**0.99**	**72**	**0.222**	**26**	**7.4**
	Fine-silty, mixed, superactive, mesic Typic Argiudolls							
Rozetta	Silt Loam	**1.82**	**1.23**	**0.59**	**32**	**0.163**	**4**	**6.8**
	Fine-silty, mixed, superactive, mesic Typic Hapludalfs							
Catlin	Silt Loam	**2.86**	**n/a**	**n/a**	**n/a**	**0.173**	**14**	**n/a**
	Fine-silty, mixed, superactive, mesic Oxyaquic Argiudolls							
Symco	Sandy Loam	**0.81**	**0.80**	**0.01**	**35**	**0.087**	**73**	**6.3**
	Fine-loamy, mixed, mesic Aquollic Hapludalfs							
Rosholt	Sandy Loam	**0.87**	**0.83**	**0.04**	**42**	**0.087**	**53**	**5.7**
	Coarse-loamy, mixed, superactive, frigid Haplic Glossudalfs							

Table 3. Mineralized ^{15}N recovered from labeled manure components over various soils and temperatures.

Soil type	Fecal ^{15}N‡				Urine ^{15}N ‡				Straw ^{15}N ‡			
	11ºC	18ºC	25ºC	Soil Avg.	11ºC	18ºC	25ºC	Soil Avg.	11ºC	18ºC	25ºC	Soil Avg.
	----------% ^{15}N Recovered----------											
Loyal	8	8	12	**10**	52	45	69	**55**	12	20	19	**17**
Symco	20	16	27	**21**	49	54	56	**53**	14	16	22	**18**
Plano	13	18	26	**19**	44	60	63	**55**	15	24	25	**22**
Rozetta	12	5	15	**11**	61	61	59	**60**	15	12	16	**14**
Rosholt	15	7	16	**13**	52	46	51	**50**	20	14	15	**16**
Catlin	-----	-----	-----		-----	-----	-----		-----	-----	-----	
Temp Avg.	**14**	**11**	**19**		**51**	**53**	**60**		**15**	**17**	**19**	
	----------Statistical Significance----------											
	Pr>F †		**LSD†**		**Pr>F †**		**LSD†**		**Pr>F †**		**LSD†**	
Soil	0.0001		3.81		0.2467		NS		0.0093		*	
Temp	0.0001		2.95		0.5932		NS		0.0015		*	
S*T	0.0989				0.6240				0.0036			

†See treatment list in Materials and Methods for details.

‡See treatment list in Materials and Methods for details.

*Interaction significant at $p \leq 0.05$

Effects of Manure Handling Systems on Nitrogen Losses from Dairy Farms

V.R. Moreira and L.D. Satter

Introduction

There is growing concern about ammonia and nitrous oxide emissions to the atmosphere from livestock operations, and the potential effect these emissions might have on human health and the environment. Measurements of N loss from livestock operations in Europe suggest that there are variations among management and manure handling systems. In the US, N loss inventories are still lacking for most species, especially dairy cows. Direct measures of volatile N losses from livestock housing and manure storage facilities are often difficult and subject to large error. A new approach to estimating volatile losses was utilized in this study. Since P is not volatilized from manure, the nitrogen to phosphorus ratio (N:P) in manure should reflect volatile N losses The objective of this study was to evaluate the effect of season and manure handling system on nitrogen (**N**) loss from dairy manure utilizing information on manure nutrient content from manure samples submitted to commercial laboratories.

Materials and Methods

The dataset consisted of 1517 manure analyses, unevenly distributed among three commercial laboratories located in MN, PA and WI. Samples deviating more than 2.5x SD (Standard Deviation) from the overall mean were deleted from the original data set, resulting in 1496 manure analyses. Subsets of this larger data set contained information that allowed examination of the effect of different types of management on the N:P ratio in manure. Since dietary N and P content affects the N:P ratio in manure, it was assumed that a large number of manure samples would diminish the impact of variation in individual manure samples due to diet. Phosphorus was used as a marker to compare N disappearance among the different variables, assuming that it would have a recovery of 100%. The ratio between N and P (N/P) was used in this analysis to compare different manure management systems. All samples had the date of sample receipt by the laboratory, and these dates were used to identify season of manure storage. Manure samples were identified as coming from bedded pack (BP), daily haul (DH), or liquid slurry (LS) systems. In addition, some samples had accompanying information indicating type of bedding used. Organic bedding describes those using straw, shavings, sawdust, oat hulls, grass, etc… Inorganic bedding refers to sand, or in some few cases, no bedding. Some liquid slurry samples had information on how the liquid slurry was loaded into the storage systems, i.e., pushed on to the surface of the stored slurry, or pumped into the storage facility from below the slurry surface. Also, some manure samples had information on whether the slurry storage was covered or not.

Results and Discussion

In Fig. 1, no difference was detected between daily haul and liquid slurry (earthen basin and pit) ($P \leq .51$). The interaction between season and storage system ($P \leq .02$) indicated that liquid storage had significantly higher N/P throughout the year, except during the winter, when bedded pack apparently retained more N. The ratio of nitrogen to phosphorus tended to increase ($P \leq .07$) by loading liquid slurry into the storage facility from the bottom (below the surface) instead of pushing slurry on to the surface (Fig. 2). Smaller N:P ratios observed during the summer (interaction $P \leq .16$) could be explained by high ammonia loss from the barn floor before manure went into slurry storage. Table 1

shows the N:P ratio of liquid slurry from covered or uncovered storage facilities. In this population of samples, covering did not reduce N loss. However, only 53 samples were available for this analysis, 12 of which were from covered storage. Season interactions could have had a masking effect on the potential for covers to retain more nitrogen in the manure. Table 2 shows the impact of bedding type on manure characteristics. It has been suggested that organic bedding might be expected to help reduce volatile losses by forming a floating mat or cover on the liquid storage and by providing substrate for conversion of ammonia into microbial N. Nonetheless, no effect of bedding type was noted in this population of manure samples. Overall, manure samples analyzed in the spring resulted in higher N/P than in samples analyzed in the autumn ($P \leq .01$), and tended to be higher than summer samples ($P \leq .12$) (Fig. 3). However, a lower winter N/P in the Minnesota dataset resulted in a significant interaction between laboratory source and season ($P \leq .002$).

Conclusion

The N:P ratio in manure provides one approach for estimating extent of N loss due to manure management practices in a variety of conditions. Liquid slurry storage, the most common method of manure storage in the datasets studied, appeared to be an efficient system for conserving manure nitrogen. Although our analyses did not show statistical differences due to storage cover and type of bedding, it indicated that loading method might have a significant impact on reducing nitrogen losses. The lack of information about diets prevents a reliable estimate of actual N loss in the various data subsets. If we assume an average N:P ratio in dairy manure at the time of excretion of 6.84, and this would be approximately correct when the diet contained about 17.5%CP and .44%P (dry basis), then N:P ratios of 6, 5 and 4 would represent volatile N losses of approximately 12.3, 27.0, and 41.6% of total excreted nitrogen.

Figure 1.

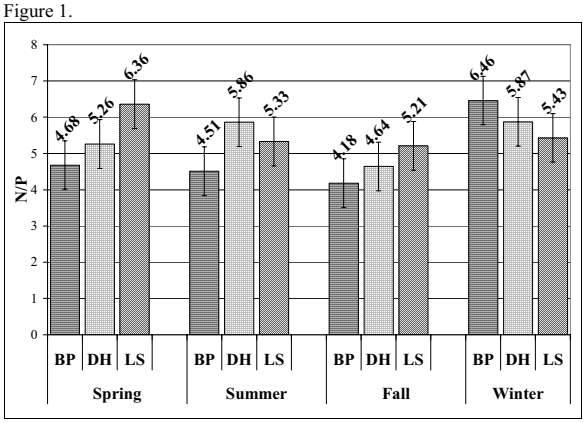

Figure 2.

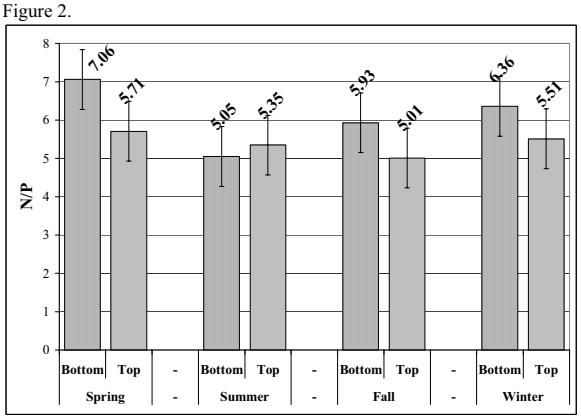

Figure 3.

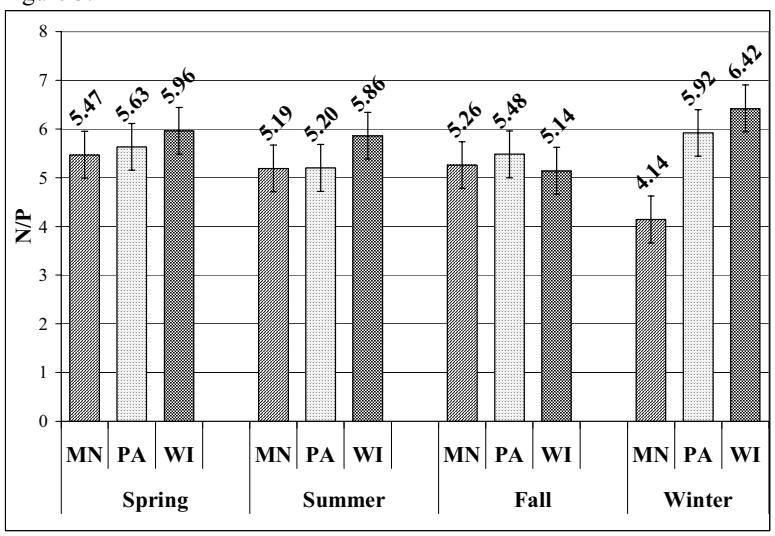

Table 1.

				Main effects			
	C[1]	UC[2]	SEM	Cover	Season	Year	Cvr x Ssn[3]
n	12	41					
DM (%)	10.4	9.74	2.93	0.81	0.87	0.66	0.46
TN (%DM)	5.65	5.21	1.09	0.67	0.96	0.24	0.38
N-NH3 (%DM)	2.56	2.35	0.61	0.71	0.93	0.1	0.49
P (%DM)	0.89	0.82	0.11	0.47	0.18	0.47	0.12
N/P	6.07	6.18	0.71	0.87	0.06	0.19	0.69

[1] C = covered;
[2] UC = uncovered;
[3] Cvr x Ssn = coverage versus season interaction.

Table 2.

				Main effects			
	Inorganic[1]	Organic[2]	SEM	Bedding	Season	Year	Bed x Ssn[3]
N	38	33					
DM	14.20	7.78	2.43	0.05	0.35	0.98	0.62
TN (%DM)	3.66	4.79	0.43	0.06	0.16	0.36	0.93
P (%DM)	0.71	0.82	0.07	0.29	0.005	0.63	0.30
N/P	5.50	6.02	0.49	0.43	0.004	0.72	0.20

[1] Manure samples with accompanying information indicating that sand or no bedding was used. This also included samples where there was no information about bedding.
[2] Manure samples with accompanying information indicating that straw, hay, grass, sawdust, shaving or oat hulls were used for bedding.
[3] Bed x Ssn = bedding versus season interaction.

U.S. Dairy Forage Research Center Annual Field Operations Report
June 2003

R.P. Walgenbach, Management Agronomist & Farm Manager

The 2002 crop year began with warm and dry conditions during April. This was followed by cool and wet weather during early May. Fortunately we had a window to plant legumes, corn and soybeans in a timely manner. The winter was very mild with moderate amounts of snow. The first cutting was started early by spraying two fields with glyposate followed by a harvest and planting of corn and soybeans. The first cutting went well with the exception of one heavy rainfall over a weekend that took us by surprise. The glyposate sprayed fields produced about one ton dry matter per acre, other established fields ranged in yield from 1.8 to 2.4 tons of dry matter per acre for first cutting. Above average rain fell in June but this pattern did not continue in July. Corn and soybeans showed signs of moisture stress but we did get timely rain to help this situation. It seemed that when the crops needed an inch or more of rain they would get º or 1/3 of an inch. The soybean aphids and white mold were not a significant problem this past season. In spite of the dry and hot weather in July we had excellent yields of soybeans.

No turnovers occurred on the field crew this past year. But the only herd manager that has been at the research center announced his retirement. Len Strozinski has served the dairy industry and research center over the last 22 years. I have enjoyed working with Len and appreciate his advise, hard work and friendship over the years. Len's official retirement will actually occur in January of 2003. This will provide a period of overlap between Len and the new herd manager, Dr. Jill Davidson. I am very pleased that Jill has decided to take on the very challenging job of managing our research herd. Jill is originally from Ohio and was raised on a dairy and crop enterprise. She has a BS degree from Ohio State University and a Masters degree in reproductive physiology from Florida State and a PhD in dairy nutrition and management from Michigan State University.

The transfer of crop and pastureland to USDA custody from the Department of Defense is sounding like the proverbial broken record. In spite of my frustrations at the rate of progress I can say that there is progress. A legal survey was completed this summer and the USDA footprint for transfer is becoming a reality. The meetings and discussion concerning the remaining property still continue, as does our involvement in this exciting and unique opportunity. All in all it has been a very successful year at the research farm, which is due in large part to the excellent efforts of our office, field and barn staff. They all have my thanks.

Table 1. 2002 precipitation (ppt)

Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec
ppt inches											
0	.23	2.17	1.02	3.6	3.66	5.55	1.9	3.78	1.92	2.38	0.28	0.32

Table 2. 2002 planting and harvesting dates

		Planting		Harvesting	
Crop	Acres	Start	Finish	Start	Finish
Winter Wheat 02	57.6	-	-	7/15	7/16
Soybeans	331.8	5/7	5/24	9/26	10/30
Corn Grain	264.6	4/26	5/15		
Corn Silage	176.8	-	-	9/3	9/30
Alfalfa-Spring	22.4	4/11	4/12	-	-
Birdsfoot Trefoil	51.2	4/17	4/18	-	-
Red Clover	20.6	4/11	4/11	-	-
Winter Wheat 03	62.0	10/8	10/9		
Birdsfoot Trefoil	10.0	8/19			

Table 3. 2002 forage cutting dates

	Alfalfa-Established			Alfalfa-Spring Seeded		
Crop	Acres	Start	Finish	Acres	Start	Finish
First*	282.5	5/20	5/31	22.4	6/27	-
Second	237.7	6/24	7/1	22.4	7/30	-
Third	237.7	7/18	7/31	22.4	8/27	
Fourth	237.7	8/20	8/27			

	Birdsfoot Trefoil-Spring Seeded		
First	51.2	7/9	7/10
Second	51.2	8/20	-

	Red Clover-Spring Seeded		
First	20.6	7/3	-
Second	20.6	8/15	-

*44.8 acres of alfalfa were sprayed with glyposate and harvested for first cut, 14.3 acres were then planted no-till with corn and 30.5 acres were then no-till planted with soybeans. Corn and soybean acres are reported in these respective crops.

Table 4. 2002 crop yield data

Crop	Acres	Low	High	Mean	Total
	bushels per acre				
Winter Wheat	57.6	—	—	79.8	4,597
Soybeans	331.8	48.9	70.8	60.6	20,119
Corn Grain†	264.6	102.0	187.0	164.2	43,441
	tons DM (as is) per acre				
Corn Silage DM	176.8	5.2	9.6	7.3	1,292
Corn Silage (as is)‡	176.8	14.7	22.0	20.3	3,593
Alfalfa ††	237.7	3.98	6.0	5.0	1,199
Alfalfa Spring Seeded	22.4	—	—	3.2	59
Red Clover	20.6	—	—	2.6	55
Birdsfoot Trefoil	51.2	—	—	2.11	107.88

† 14,084 bushels were harvested and stored as dry shell corn and 29,357 bushels were harvested and stored as high moisture shelled corn. Moisture range at harvest for dry shell corn was 20.0 to 28% and for high moisture shell corn it was 24.6 to 30.8%. Corn grain yields are adjusted to 15.5% moisture.

‡ Corn silage harvest moisture ranged from 54 to 69%

†† Alfalfa yields include hay (28 tons of DM) and silage

U.S. DAIRY FORAGE RESEARCH CENTER
ANNUAL DAIRY OPERATIONS REPORT
JANUARY 2003 (for 2002)

JILL A. DAVIDSON - HERD MANAGER

HERD STATISTICS			**CHANGE FROM PREVIOUS YEAR**
Herd Inventory			
Milking cows		291	-31
Dry cows		53	+6
average cow age	49 months		+5
percent first lactation	38%		-3
percent second lactation	32%		+4
percent third lactation	19%		+2
percent greater than third	11%		-1
Herd replacements		331	+11
Total		**675**	-14
Rumen fistulated cows		25	
Herd Performance			
Cows calved		355	-22
Heifer calves born live		169	+13
Heifer calves born dead		9	-11
Bull calves born live		163	- 7
Bull calves born dead		14	-1
Heifer calves died < 1 year old		5 (2.8%)	0
DHIA rolling herd average			
milk		23,153 lbs.	+735
protein		702 lbs.	+29
fat		927 lbs.	+50
Milk sold in 2002		7,477,986 lbs.	+91,372
Average Mailbox Milk Price/cwt		$12.08	-3.10
Heifer calves sold		7	-12
Bull calves sold		163	-7
Cows Sold			
Cows culled for:			
Reproduction Problems		55	+4
Poor Production		16	+8
Poor Feet and Legs		10	+2
Mastitis		29	+8
Other		28	+8
Cattle Sales Revenue		79,842	-9132
Herd Reproduction			
Average days open		154	+24
Average calving interval		13.8 months	+0.6
Average services per conception		2.7	- 0.2
Average age at first calving		24 months	0

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