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U.S. Dairy Forage Research Center 2003 Research Report

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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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It is a pleasure to bring you these summaries of research conducted over the past year at the U.S. Dairy Forage Research Center (USDFRC). 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, and chemists, who all work together to increase the efficiency of forage production and utilization by dairy farmers. At present, we have seventeen scientists: fourteen 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 Heathcliffe Riday, Research Geneticist (Plants) in January 2004. Dr. Riday received a B.S. degree in Conservation Biology from Brigham Young University in 1997, and M.S. and Ph.D. degrees in Plant Breeding from Iowa State University in 2001 and 2003, respectively. His thesis topic was "Heterosis in a Broad Range of Alfalfa Germplasm." Dr. Riday came to us from the Raymond F. Baker Center for Plant Breeding, Agronomy Department, at Iowa State University. He brings training and knowledge in breeding and genetics to lead our legume breeding effort. His presence completes our staff to support our newest CRIS project: "Designing Forage Plants with Enhanced Value for Dairy Production, Profitability, and Sustainability." Our newest staff member in June 2004 was Mary Beth Hall, Research Dairy Scientist. Dr. Hall filled the vacancy created by Larry Satter's retirement. She brings seven years of experience as a Dairy Scientist at the University of Florida. She received a B.S. degree in Animal Science from Cornell University in 1982, a M.S. degree in Animal Science from Virginia Polytechnic Institute and State University in 1983, and a Ph.D. degree in Animal Science from Cornell University in 1996. Dr. Hall's Ph.D. thesis topic was "Neutral Detergent-Soluble Fiber: Analysis, Variation in Feedstuffs and Ruminal Fermentation Characteristics." Between receipt of her M.S. degree and initiating her Ph.D. studies, Dr. Hall worked as a Sales Feed Representative and a County Agricultural Agent for Cornell Cooperative Extension. In 1996, she joined the Department of Animal Science at the University of Florida on an extension/ research appointment, being promoted to Associate Professor in 2002. She brings experience in dairy nutrition and management with emphasis in laboratory methods for non-fiber carbohydrates, as well as an interest to study how dairy cows can increase forage digestibility under various non-fiber carbohydrate plant characteristics and supplements.

The FY 04 budget provided funding to support two more USDFRC scientists–a Research Dairy Scientist and a Research Agronomist/Soil Scientist–to be housed at the Agricultural Research Station, University of Wisconsin-Madison at Marshfield, WI. These positions are part of a plan to add a new working location for USDFRC in Marshfield. This facility will be called the Institute for Environmentally Integrated Dairy Management, and will house six USDA-ARS scientists, as well as four USDA-Natural Resources Conservation Service (NRCS) specialists. This plan is a collaborative effort between USDA-ARS, USDA-NRCS, the University of Wisconsin, and the National Farm Medicine Center of the Marshfield Medical Clinic. Research at the Institute will be conducted to improve nutrient management, minimize emissions, and control pathogens at all stages in the dairy production system. Research objectives of the unit are: 1) improved nutrient management and manure handling, storage, and application strategies; 2) reduced degradation of air, water, and soil quality by manure; 3) controlled pathogen transmission between livestock, wildlife, humans, and the environment; and 4) education, outreach, and technical assistance. FY 03 and 04 funding has been appropriated to construct animal housing and manure containment facilities.

We continue to recruit for a Research Agricultural Engineer and a Research Agricultural Scientist (Dairy Systems Specialist).

I want to thank scientists, support staff, students, visiting scientists, and stakeholders for making 2003-2004 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

Forage Yield of Smooth Bromegrass Collections From Rural Cemeteries

M.D. Casler

Introduction

Smooth bromegrass (*Bromus inermis* Leyss.) is an important forage grass in much of temperate North America, used primarily for infrequent hay harvests, soil conservation, or other situations that are characterized by relatively low levels of management. It is preferentially adapted to hay management and favored by infrequent cutting, relatively high cutting heights, and high nitrogen fertility. Smooth bromegrass is not well adapted to frequent defoliation. Unlike many other cool-season forage grasses, forage production of smooth bromegrass is not stimulated by defoliation, regardless of the growth stage. Smooth bromegrass stands decline under rotational grazing, an effect that is magnified by increasingly intensive grazing.

Rural cemeteries are a source of smooth bromegrass germplasm that likely occurs as remnants of plantings from the 1930s. Many rural cemeteries of the North Central USA are characterized by a Kentucky bluegrass (*Poa pratensis* L.) sod that is well maintained by members of a local church or cemetery association. Smooth bromegrass often survives in both the cemetery sod and the area surrounding the cemetery. In many cases, the fence or border population of smooth bromegrass is unmanaged, creating two visually distinct habitats for smooth bromegrass: a frequently mowed sod and an uncut fence or border area. There is clear phenotypic divergence between fence and sod populations, suggesting the possibility that natural selection may be responsible for a degree of genetic differentiation between them. Natural selection pressures may have created considerable differentiation between fence and sod smooth bromegrass populations since the 1930s. Because sod plants can only reproduce by rhizomes, natural selection pressure in the sod habitat would favor genotypes with a greater tolerance for frequent defoliation and greater long-term survivorship. The objective of this study was to quantify and describe the responses of paired fence and sod populations to differential harvest management.

Methods

The study consisted of 60 smooth bromegrass populations representing fence and sod habitats of 30 rural cemeteries. The populations were planted at Ames, IA and Arlington, WI in 2001 and evaluated for forage yield under three harvest managements at each location: four, five, or six harvests over the 2-year period. The first harvest was made at flowering for the 4-harvest management, at heading for the 5-harvest management, and at jointing for the 6-harvest management. Nitrogen fertilizer was applied at a total rate of 224 kg/ha (200 lb/A) to each trial, split in equal applications among the harvests.

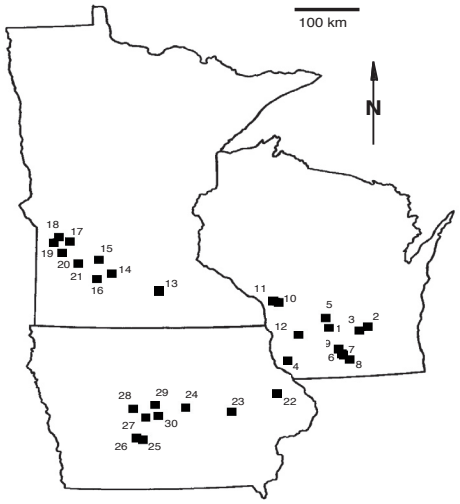

Figure 1. Geographic distribution of 30 rural cemeteries.

Results and Discussion

Total forage yield declined with increasing harvest frequency, averaging 9.81, 7.39, and 5.30 Mg/ha for the 4-, 5-, and 6-harvest managements. As forage yield declines, regrowth percentage increased, averaging 22.6, 33.7, and 49.4% for the 4-, 5-, and 6-harvest managements. Fence populations had slightly higher forage yield than sod populations (7.70 vs. 7.30 Mg/ha), but they did not differ in regrowth percentage.

Fence and sod population did not decline in forage yield at an equal rate as harvest frequency increased, as illustrated by a representative sample of eight cemetery sites shown in Figure 2. For seven of these cemeteries, the fence population had a greater decline in forage yield with increasing harvest frequency. Forage yield was higher for the fence population at an infrequent harvest management, but the two populations became gradually more similar or reversed in ranking as the harvest frequency increased. This was most striking for Site 6 which was near Belleville, WI (-1.4 vs. -1.8 Mg/ha for each extra harvest). This response was also notable for Sites 15 and 18 (Olivia and Watson, MN) for which the fence and sod populations declined by -2.5 vs. -1.6 Mg/ha for each extra harvest. Site 3 (near East Bristol, WI) seemed to be an anomaly in which the fence population declined less rapidly than the sod population (-1.9 vs. –2.5 Mg/ha for each extra harvest).

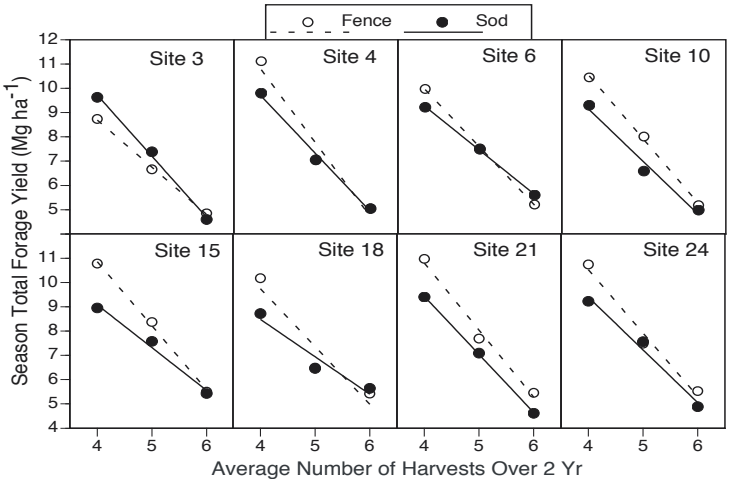

Figure 2. Linear regressions of mean season total forage yield, over two locations and 2 years, on average number of harvests over the 2-year period for fence and sod populations of smooth bromegrass collected from eight rural cemeteries.

Site 10 (near Four Corners, WI) may hold some of the most valuable germplasm, because the sod population declined less in forage yield than the fence population, but also increased more in regrowth percentage than the fence population with increasing harvest frequency (14.5 vs. 7.6 % per harvest). This was the only cemetery in which the sod population had a more favorable response than the fence population for both forage yield and regrowth percentage. Several sod population had more favorable responses to increased harvest frequency compared to the commercial cultivars for both forage yield and regrowth percentage.

Conclusions

The plants collected from these cemeteries have proven to be very interesting and potentially valuable for improving the regrowth potential, tolerance to frequent defoliation, and potential grazing tolerance of smooth bromegrass. It should be possible, through selection and breeding, to incorporate plants from some of the most favorable populations into commercial germplasm for development of new cultivars that have greater regrowth potential than current cultivars.

Molecular Marker Variation in Smooth Bromegrass Collections From Rural Cemeteries

M.D. Casler, H. Maierhofer, and C. Stendal

Introduction

Smooth bromegrass (*Bromus inermis* Leyss.) is an important forage grass in much of temperate North America, used primarily for infrequent hay harvests, soil conservation, or other situations that are characterized by relatively low levels of management. Rural cemeteries appear to be a source of smooth bromegrass germplasm with potentially greater tolerance for frequent defoliation and greater long-term survivorship. Populations of plants selected from some cemetery sods appear to have superior regrowth potential and tolerance to frequent harvesting compared to fence populations that are never mowed. However, the origin of these populations is unknown and it is not clear that fence and sod populations have the same origins at each cemetery. The objective of this study was to use molecular markers to study genetic diversity within and among smooth bromegrass plants collected from six rural cemeteries.

Methods

The study consisted of 12 smooth bromegrass populations representing six rural cemeteries in Iowa, Minnesota, and Wisconsin. Random amplified polymorphic DNA (RAPD) markers were used to measure genetic diversity within and among populations. A total of 82 bands on five RAPD primers were scored as polymorphic, demonstrating differences in presence or absence of the band in different plants. Categorical data (presence = 1, absence = 0) were used to create an analysis of molecular variance (AMOVA) of all plants, a multidimensional scale analysis of all plants, and a cluster analysis of the 12 populations.

Results and Discussion

The AMOVA revealed that 8.0% of the molecular variance could be attributed to cemeteries and 8.4% of the molecular variance could be attributed to habitat within cemeteries. Thus, 83.6% of all molecular variance exists within populations. This value is similar to that expected for most forage grasses, because they are made up of highly heterogeneous and heterozygous individuals. There were no marker profiles that were repeated in more than one individual plant in this study, indicating that there were no clones or multiple samples of a single genotype. Because many of the original plant samples came from locations within 1-2 m of each other, this is strong evidence that reproduction and recruitment of new plants in these two habitats occurs exclusively by seed and not by rhizomes or tillering. New genotypes may colonize the sod habitat when it is disturbed, creating open ground and an opportunity for a seedling to grow and establish itself. It is unlikely that new genotypes occur in the fence habitat, due to the dense sward and intense competition against new seedlings.

The cluster analysis showed that there was no pattern to the variability among the six cemeteries. The genetic composition of these populations showed little or no relationship to habitat or geographic location of the cemetery. These populations appear to have different origins, perhaps resulting from different establishment dates, different sources of seed, differential management of the cemetery, or different mechanisms of conversion from a smooth bromegrass dominated sod to a Kentucky bluegrass (*Poa pratensis* L.) dominated sod.

Of the six cemeteries, three showed almost complete differentiation between fence and sod habitats, while three showed almost no differentiation between habitats (Figure 1). The AMOVAs revealed Sites 3, 14, and 26 to have relatively high migration rates of 4.3-5.0 seeds/generation. For these three sites, it appears that seeds produced on the fence population fall onto the ground or are carried there by birds, and many of these seeds become established plants. Fence and sod populations at these sites are thus maintained in a perpetual state of homogeneity and any selection that occurs in the sod habitat is not sufficiently strong to create genetic differentiation between the two populations.

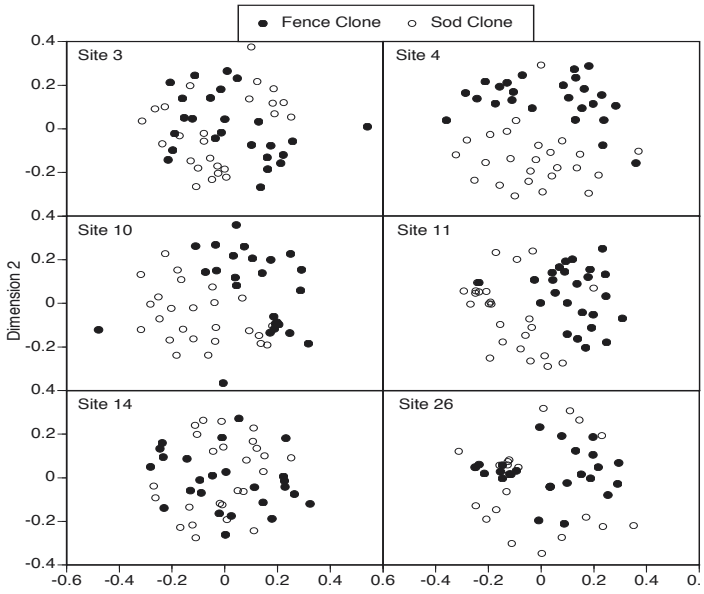

Figure 1. Scatterplot of the first two multidimensional scales of the RAPD marker data matrix for 292 smooth bromegrass plants representing six rural cemeteries, coded by site number.

For Sites 4, 10, and 11, migration rates were relatively low (2.3 seeds/generation) and the fence and sod populations were almost completely differentiated. Such a level of differentiation could not occur simply as a result of different sources of introduction, because of the extremely large within-population variability of this species (see above). Rather, such differentiation can only arise from differential selection between habitats, creating genetic divergence between the two populations. Most of the selection pressure likely occurs in the sod habitat, due to the stresses associated with frequent mowing. The migration rate for these cemeteries is too small to offset the large selection pressure on the sod habitat, and can only maintain a small degree of genetic similarity between habitats. This is shown by a small frequency of plants in both the fence and sod habitats that are similar to plants from the alternate habitat.

Conclusions

Rural cemeteries in the north central USA are a potential source of breeding materials for improving smooth bromegrass. For some cemeteries, smooth bromegrass plants are genetically homogeneous between the fence and sod habitats, indicating that there has not been any selection for tolerance to frequent mowing in the sod. In these cemeteries, the sod population is being maintained by a trade-off between mortality of existing plants and frequent establishment of new genotypes. For other cemeteries, there is clear evidence for differential selection between fence and sod habitats, most likely a result of selection for tolerance to frequent mowing in the sod. The sod population of these cemeteries is likely being maintained by long-term survivorship of highly persistent clones, with occasional additions of new genotypes by seedling recruitment. These cemeteries may contain the most valuable germplasm for improving tolerance to frequent harvest or grazing in smooth bromegrass.

Molecular Markers Reveal a Metapopulation in Switchgrass Prairie Remnants

C. Stendal, L. Kapich, M.D. Casler

Introduction

Switchgrass is a warm-season, perennial grass native to North American tallgrass prairies and is broadly adapted to the central and eastern United States. The movement of germplasm throughout this area creates a situation where local gene pools could be contaminated by the “foreign” genes of planted cultivars or of other native populations from different regions. The objectives of this research were to identify potential genetic differences between cultivars and native populations and to identify spatial variation among native populations.

Methods

This study analyzed 46 populations collected from prairie remnants in the northern USA, from Minnesota to New York and 11 cultivars that originate from the Central Great Plains to West Virginia (Figure 1). Each population averaged about 15 plants. The DNA samples were analyzed by scoring a total of 117 polymorphic bands using random amplified polymorphic DNA (RAPD) markers. Marker frequencies were calculated for each band and population, which were then used to compute a distance matrix among all 57 populations.

Figure 1. Map of the north central USA, showing the location of prairie-remnant sites that provided switchgrass germplasm.

Spatial variation was investigated using six statistical approaches. For the 46 prairie-remnant populations, Moran's I (autocorrelation of marker distances with geographic distances) and the Mantel test of matrix correlation between marker and geographic distances were computed (Sokal and Oden, 1978). All 57 populations were clustered, using UPGMA (unweighted pair-group method of averages), and identified by their state of origin. Analysis of molecular variance (AMOVA; Excoffier et al., 1992) was computed to test differences in marker variation for cultivars versus prairie-remnant populations and hardiness zones of prairie-remnant collection sites. Individual markers were analyzed by regression of marker frequency on latitude and longitude and by analysis of variance for state-to-state differences in marker frequency.

Results and Discussion

The autocorrelation with geographic distance gave a correlogram that showed a slight reduction in correlation relative to geographic distance. However, despite the significant trend and significant autocorrelation for sites less than 5 km distant, this correlation was too low to be of practical importance. The Mantel test of matrix correlation between molecular and geographic distance matrices revealed no significant correlation between the two. If there were spatial variation to be detected, the population pairs that were geographically proximal should be genotypically similar; however, the data shows the geographically proximal pairs were genotypically very different from each other. The cluster dendrogram revealed no pattern between the normalized genotypic distances and the population origin, as might be expected when spatial variation is present (Figure. 2). Very few populations clustered close together and most of those were geographically distant from each other.

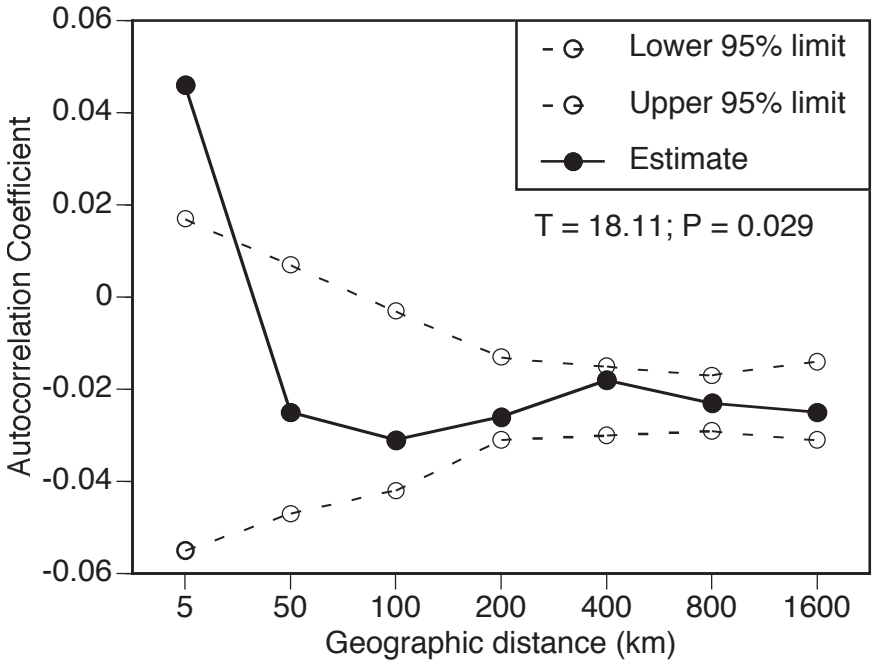

Figure 2. Autocorrelogram, showing the decline in RAPD marker correlation between paired prairie remnants as a function of geographic distance.

An analysis of molecular variance among cultivars and native populations as well as between and within the populations within these groups showed the majority of the genetic variance comes from within groups (Table 1). Cultivars versus prairie-remnant populations were similar in RAPD marker profiles, as indicated by the small amount of variability attributed to this difference. The prairie-remnant populations grouped by the hardiness zones of their origin were similar in RAPD marker profiles, indicated by the small amount of variability attributed to this difference. A regression of marker frequencies on latitude and on longitude of the population collection sites resulted in no detectable relationships. These marker frequencies were also analyzed for state-to-state variation, but nothing above expected background levels of variation were found.

Table 1. Analysis of molecular variance for RAPD markers evaluated on 818 switchgrass plants originating from 11 cultivars or 46 prairie-remnant collection sites.

Source of variation †	df	Sum of squares	Variance component	Percentage
Cultivars vs. Prairie remnants	1	210.9	0.306	0.7
Prairie remnants				
USDA Hardiness zone origins	3	400.1	0.048	0.1
Sites/Hardiness zones	42	4906.2	7.236	16.8
Plants within locations	607	9050.3	14.910	34.7
Cultivars				
Among cultivars	10	1044.6	6.069	14.1
Plants within cultivars	154	2220.8	14.421	33.5
Total	817	17832.9	42.990	

Conclusions

All of the results in this study suggest that there is no spatial variability among these switchgrass populations collected from prairie remnants. Phenotypic variation for cold tolerance, heat tolerance, and photoperiodism has led to large adaptive differences among switchgrass collections from different hardiness zones. However, our RAPD marker data suggests that these populations are part of a large

metapopulation with most variability arising from population divergence that does not relate to their geographic location. Population divergence may arise from restricted population sizes due to habitat loss or destruction followed by isolation from other remnant prairie sites. Our sample represents a single "snapshot" in the evolution of switchgrass in its native prairie habitat. Destruction and loss of this ecosystem is a relatively recent phenomenon in the evolution of this species and the lack of spatial variability suggests that insufficient time has passed for spatial variability to manifest itself. Migration of switchgrass populations between regions, whether cultivars or prairie-remnant populations, will not likely lead to the swamping or erosion of local gene pools. Utilizing cultivars or natural populations of switchgrass for conservation or restoration projects in regions different from their origin presents little danger of significant contamination of the local gene pool.

Breeding Timothy for Tolerance to Frequent Harvesting or Grazing

M.D. Casler

Introduction

Timothy (*Phleum pratense* L.) cultivars are well adapted to hay management practices based on relatively infrequent harvests. Relatively few timothy cultivars developed or commercialized in North America were bred to withstand frequent defoliation. Timothy cultivars show considerable variation for persistence in mixture with alfalfa under hay management or under management intensive rotational grazing in pure or mixed stands. Most timothy cultivars ranked low in persistence compared to cultivars of other species in both studies. Hay-type timothy plants should be erect, tall, and early heading, with long, wide leaf blades. Conversely, timothy plants that are most persistent under long-term frequent defoliation are relatively prostrate and late heading. The purpose of this study was to determine the relationship between performance under frequent and infrequent harvest of timothy cultivars and selections developed for use in pasture systems.

Methods

Timothy plants were selected from old turfs in southern Wisconsin, including lawns, golf courses, and cemeteries. Additional timothy plants were selected for forage yield from superior forage germplasm. All plants were intercrossed within their own groups to form seeded families that could be planted into small plots to simulate timothy swards. Plots were planted in 1999 and managed for two hay cuts in 2000 and 2001 or four hay cuts in 2002 and 2003. Forage yield, survival, and regrowth heading were determined in each year.

Results and Discussion

The relationship between forage yield under frequent harvest and forage yield under infrequent harvest was strongly positive with a genetic correlation of 0.88 ± 0.07 for forage selections and 0.73 ± 0.47 for turf collections (Figure 1). There was no difference in average forage yield between forage selections and turf collections under either harvest management. Forage selections had 9.4% higher forage yield under infrequent harvest and 4.9% higher forage yield under frequent harvest than the timothy cultivars, indicating that considerable progress was made in improving forage yield of this forage-type timothy population. Forage selections were higher than the cultivars in survival (88 vs. 82% for infrequent harvest and 81 vs. 77% for frequent harvest) and in resistance to leafspot (4.8 vs. 5.0 leafspot score).

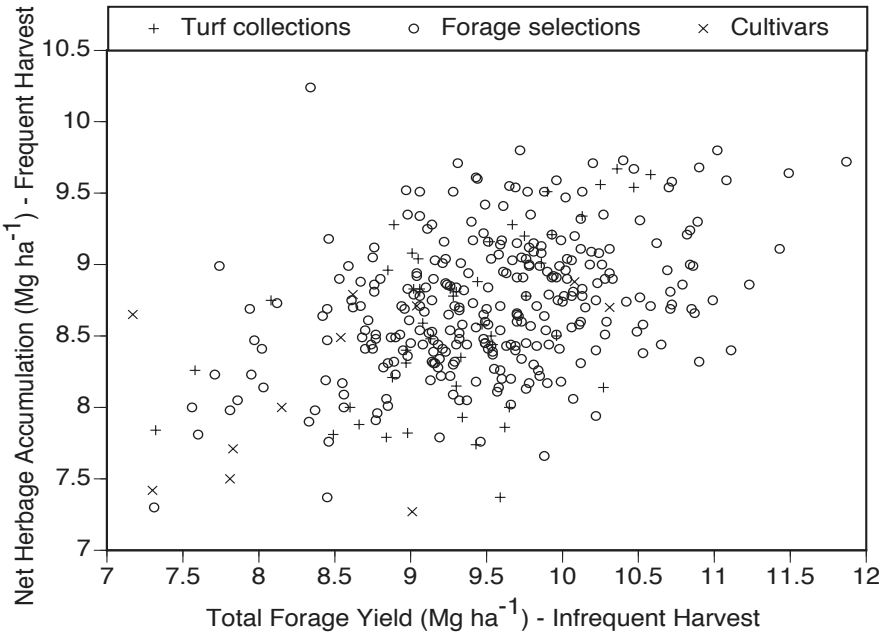

Figure 1. Plot showing the relationship between forage yield under frequent harvest and under infrequent harvest for 352 timothy cultivars and breeding lines.

A high frequency of heads in the second harvest is one problem with turf collections under both managements. Turf collections had an average of 57 heads per m^2, while forage selections had an average of 40 heads per m^2 ($P<0.01$). Although the difference between forage and turf was consistent for both harvest managements, the differences among the various types of turf were highly inconsistent: collections from golf courses and cemeteries had the highest regrowth heading under infrequent harvest (80 and 88 heads per m^2 vs. 48 heads per m^2 for lawns), while collections from lawns had the highest regrowth heading under frequent harvest (78 heads per m^2 vs. 19 and 22 heads per m^2 for golf courses and cemeteries).

Timothy cultivars from North America had 18% higher forage yield under infrequent harvest (9.32 vs. 7.87 Mg/ha) and 7% higher forage yield under frequent harvest (8.55 vs. 7.96 Mg/ha) than cultivars from overseas. European cultivars averaged 9-10% higher in forage yield than Japanese cultivars for the two harvest managements. North American timothy cultivars also had superior survival under infrequent harvest (87 vs. 74%) and superior leafspot resistance (4.6 vs. 5.6 leafspot score) compared to cultivars from overseas. These results indicate that cultivar recommendations for timothy should be limited to cultivars bred and evaluated in North America and should avoid cultivars developed overseas, particularly in milder climates or environments that are dissimilar to timothy growing regions of North America.

Conclusions

The timothy breeding program appears to be moving in the proper direction. The results of our first cycle of selection indicate improvement in forage yield potential under both frequent and infrequent harvest managements, suggesting that we can develop a multi-purpose timothy for hay, silage, and grazing. Comparisons among cultivars revealed a distinct trend toward superior performance of cultivars bred and evaluated in North America, indicating that cultivar recommendations should be limited to that group.

Why has Reed Canarygrass Changed From Benign Native to Aggressive Invader?

M.D. Casler

Introduction

Reed canarygrass (*Phalaris arundinacea* L.) is a very unique grass, more so than many of us imagine. It is the only cool-season grass that is native to North America and heavily utilized in agriculture. It is this use in agriculture that has resulted in reed canarygrass becoming one of the most hated and highly vilified members of the grass family. The purpose of this report is to describe the circumstances that have reduced this native grass to the status of "invasive species". While there is no debate over the invasive properties of reed canarygrass, a myth has been perpetuated over the cause of this invasiveness.

Native status

Reed canarygrass is native to North America, and can currently be found from coast to coast and from Oklahoma to central Alaska. Reed canarygrass was documented in the western USA as early as 20 years after the initial Lewis and Clark expedition of 1805, verified from preserved herbarium samples found in several herbaria and museums in the USA. Numerous samples of this species were collected and preserved from unsettled and remote areas near settlements long before extensive Euro American settlement and agriculturalization of the region.

Prior to settlement and agriculturalization of North America, reed canarygrass was found largely in bogs, lake shores, river banks, sloughs, fens, and wet meadows. Its lack of prominence in early descriptions of North American flora suggests that, in the 19th century, it was not yet considered to be an invasive species. Reed canarygrass can be readily propagated by rhizomes, axillary buds on stem nodes, or seeds. Its seeds shatter and fall to the ground immediately upon ripening and can float on surface waters, making it very amenable to widespread propagation. Reed canarygrass has no major insect or disease pests that affect plant longevity or survival. It is highly tolerant of a wide range of environmental conditions and stresses, including heat, drought, and waterlogging (anoxia).

Unlike the important European grasses that were imported into North America in the 17th through 19th centuries, there are no records of reed canarygrass being introduced into North American agriculture until 1905. This supports the evidence for native status of reed canarygrass, albeit circumstantially, because the introduction of cool-season European grasses was so well documented with dates, names, and locations. More importantly, it further suggests that reed canarygrass was not considered an invasive species during the early settlement of North America. Early farmers likely recognized this grass as a productive wetland grass that was highly unpalatable, and sometimes toxic, for grazing livestock and highly inaccessible for hay production due to its wetland habitat, opinions that exist to this day.

Cultivation

Reed canarygrass was first cultivated in North America likely about the 1830s in the northeast USA and eastern Canada. At this time, cultivation consisted of harvesting seed from native stands and planting the species in disturbed areas, largely for reclamation of peatlands and marshes. Despite widespread propagation of commercial seed, these seed lots representing natural populations of reed canarygrass were not improved by selection or breeding. Agronomic research on reed canarygrass began sometime around 1920, with most efforts focused on comparing it to other perennial grasses, defining management systems, and improving seed production and harvesting methodology.

In 1914, reed canarygrass was still considered to be of minor importance to agriculture, barely warranting mention in textbooks of forage crops. The use of reed canarygrass in agriculture likely increased in the 1930s, as farmers and extension personnel were desperate for perennials that could withstand the severe drought that plagued the midwestern USA in the early 1930s. Reed canarygrass is one of the most drought tolerant cool-season grasses adapted to eastern North America. The ability to propagate reed canarygrass by seed, sod, or stem cuttings was an additional advantage, partly responsible for the increased use of this grass in conservation programs. The results of these efforts can still be seen today in the abundance of reed canarygrass along roadsides, streambanks, and conservation strips within and between tilled fields.

Germplasm and Breeding

The first evidence for selection and breeding research on reed canarygrass occurred with the development of 'Iowa Phalaris' between 1921 and 1930 (Wilkins and Hughes, 1932). Reed canarygrass breeding efforts intensified in the late 1940s and have occurred almost continuously in Iowa, Minnesota, Wisconsin, and/or Ontario since the 1940s. Approximately 15 to 20 cultivars have been developed since the 1940s. Until 1972, these cultivars represented no more than two cycles of sexual reproduction removed from wild populations and could not be phenotypically distinguished from them. In 1972, this changed with the first release of the low alkaloid cultivars, six of which have been developed, commercialized, and distributed in many temperate regions of North America.

Alkaloids of reed canarygrass can be grouped into three families based partly on chemistry and partly on their effect on livestock. Hordenine appears to have little effect on livestock performance and has largely been ignored by plant breeders. Gramine has a large effect on palatability and feed intake by grazing livestock, reducing weight gains over long-term grazing of high-gramine reed canarygrass. Tryptamines and β-carbolines are highly unpalatable and highly toxic to grazing livestock, severely reducing feed intake, and causing numerous pathological problems during long-term grazing of reed canarygrass that contains alkaloids in these families.

The first modified-alkaloid cultivar was Vantage, which was bred to have moderate levels of gramine, but was free of tryptamines and β-carbolines. Vantage was shown to have a significant effect on improving health and weight gains of grazing livestock. Further improvements were made with subsequent cultivars that were free of tryptamines and β-carbolines, but also possessed lower levels of gramine than Vantage. Because of their profound effect on livestock performance and the greater value of reed canarygrass as a pasture crop than a hay crop, these six modified-alkaloid cultivars have nearly overtaken the reed canarygrass seed market in North America. As a result of these successes, European breeders began to develop low-alkaloid reed canarygrasses in the early 1980s.

The plants utilized in breeding these new reed canarygrass cultivars were derived directly from wild reed canarygrass populations that originated in both North America and Europe. Large populations of individual plants were screened for presence/absence of tryptamine and for concentration of gramine. Plants with the desired alkaloid profile were intercrossed to create the new cultivars. Thus, these low-alkaloid cultivars also represent germplasm that is no more than two or three sexual cycles removed from wild germplasm. Thus, apart from their alkaloid profiles, these new reed canarygrass cultivars are genetically and phenotypically identical to wild reed canarygrass. The pedigree of most reed canarygrass cultivars consists of a mixture of selections from old pastures, which presumably traces to seed harvested from native stands, and European introductions. Plant breeders, with the most practiced and acute eye for phenotypic detail cannot discern a typical cultivar from a typical wild collection of reed canarygrass.

Invasive Status of Reed Canarygrass

Gradually, during the last half of the 20th century, reed canarygrass became recognized as an invasive species, because it was found in wetlands where it had never been observed and it typically became the dominant species within 5-6 years. Following World War II, three things happened in agriculture that had profound effects on the use, culture, and invasiveness of reed canarygrass: (1) an increase in efforts to develop, commercialize, and distribute improved reed canarygrass cultivars for use in agriculture, (2) an increased frequency and intensity of tillage and the soil erosion associated with tillage and row cropping, and (3) an increased use of nitrogen-based fertilizers for a wide array of row, pasture, and hay crops.

The coincidence of these events with the introduction and utilization of European germplasm and the increase in reed canarygrass breeding activity led to the development of a hypothesis that has become dogma among many in the conservation and natural areas community. Some authors have speculated that selection for vegetative vigor in breeding programs and hybridization of native genotypes with European cultivars has resulted in a hybrid form of reed canarygrass that has an invasive capability that was not present in native genotypes. This hypothesis has been cited in numerous papers and has been elevated to de facto scientific dogma. This "fact" is now frequently cited as the reason that reed canarygrass has become invasive only in the latter half of the 20th century, easily reconciling the presence of relatively benign native genotypes with highly aggressive, invasive, introduced genotypes. This has occurred despite any evidence that native and introduced genotypes differ for any trait that influences invasiveness. Indeed, they do not. Only two changes, with any potential effect on invasiveness, have occurred to reed canarygrass through breeding and/or introduction of European germplasm: (1) the modification of alkaloid profiles, which might reduce the ability of this species to resist insect herbivory, and (2) a delay in seed abcission in two cultivars, a trait that reduces seed loss and escape from seed orchards. Both of these traits, if they have any effect at all, would act to reduce the invasive properties of these cultivars.

Reed canarygrass cultivars do not exhibit hybrid vigor to any extent that would affect their invasiveness. Populations of reed canarygrass, whether native or cultivated, consist of a large number of highly heterogeneous and heterozygous individuals. Most reed canarygrass is tetraploid, adding to the potential heterogeneity and heterozygosity among individuals, compared to a diploid species. Hybrid vigor in perennial grasses with similar genetic structure to reed canarygrass is relatively low and of little consequence in explaining differences in vigor of individual plants or populations of plants. Due to the phenotypic buffering that occurs within these populations, hybrid vigor cannot logically be the cause of a sudden change in aggressiveness of reed canarygrass or any other perennial grass. Invasiveness of reed canarygrass is not likely the exclusive domain of European germplasm or native x European hybrids.

Recent studies on the ecology of reed canarygrass, conducted by several prominent researchers, has shown that sedimentation and nutrient loading of wetlands has a profound effect on the ability of reed canarygrass to become established by seed or rhizome segments. Sedimentation provides a rich and moist environment in which seed can readily germinate and seedlings have relatively little competition. Because reed canarygrass seeds can float on surface waters, heavy rainfall or flooding can carry both seeds and sediments into wetlands. Sedimentation can cover existing vegetation, particularly over long periods of time, reducing its ability to survive and compete against the invading reed canarygrass seedlings. Nutrients, also carried in flood waters, feed reed canarygrass seedlings and adult plants, which show dramatic increases in growth and competitive ability with increased fertility.

Conclusions

There is strong evidence that agriculturization of the North American landscape is largely responsible for the invasiveness of reed canarygrass, providing an efficient mechanism for movement of seed, sediment, and nutrients into wetlands where this species had not previously occurred. The introduction of European germplasm in reed canarygrass breeding and the development of new cultivars by selection may be a factor partially contributing to the increases in invasiveness of reed canarygrass in the latter half of the 20th century. Although there is no evidence to support this hypothesis, it cannot be entirely refuted simply due to lack of supporting evidence. A comprehensive survey of molecular markers in European germplasm, North American cultivars (both modern and historic), and germplasm from natural areas (including both recent invasions and long-term stands) should be undertaken to provide a scientific test of this hypothesis. Such a survey should be sufficiently robust to account for the large amount of within-population genetic variability within this species.

Dinitrogen Fixation by Soybean and Alfalfa in the Mississippi River Basin

M.P. Russelle and A.S. Birr

Introduction

Significant improvements in crop yield and animal production have resulted from nitrogen (N) use on farms, but environmental degradation has also occurred in some areas. For example, agriculture has been cited as a major source of N that contributes to hypoxia in the Gulf of Mexico. Although other sources of N have been well characterized in large ecosystem studies, the contribution of legume crops to the N cycle has not. Furthermore, the potential role of legumes to reduce excess N is not widely recognized. Legumes fix less N from the atmosphere when they take up more N from the soil, fertilizer, or manure. This situation means that farmers may be able to reduce the amount of N coming into their farms by using livestock manure on legumes, like soybean and alfalfa. To know how much manure could be applied, we needed to estimate typical amounts of N fixation by these crops.

Methods

We estimated symbiotic N_2 fixation as the difference between harvested N in each crop and the supply of inorganic N to the crops. Reported crop yield by county and reported or estimated N (or protein) concentration in harvested beans or forage provided the basis for calculating harvested N. Nitrogen supply from sources other than N_2 were limited to soil organic matter mineralization and deposition of atmospheric N (background wet deposition amounts measured in the National Atmospheric Deposition Program/National Trends Network plus estimated redeposition of locally-derived ammonia from manure, fertilizers, and plant senescence). We used estimates of N supply from soil organic matter mineralization and atmospheric N deposition that were available for 7-digit hydrologic unit codes from some ARS researchers at the National Soil Tilth Laboratory in Iowa. We assumed a net 80% efficiency in plant uptake and assimilation into harvested tissues from these sources, and assumed that the remainder was utilized in nonharvested plant residues, immobilized by soil microorganisms, and lost by various pathways. County-level yields were obtained from the National Agricultural Statistics Service. We estimated haylage production of alfalfa for states where those yields are acreages are not reported.

Results and Discussion

Our analysis shows that total alfalfa production (hay plus haylage) in the Mississippi River Basin likely exceeds reported alfalfa dry hay production by 20% and that land area devoted to alfalfa production totals about 6.4 million ha, about 11% larger than reported for alfalfa in the Census of Agriculture for 1997. This is an important finding that should be verified in new surveys, because it means that estimates of alfalfa production based on dry hay alone are too low.

Soybean is the most widely grown grain legume in the Basin, occupying about 23 million hectares. Soybean production has increased ten-fold in the USA since 1950, so its effect on N cycling in the basin has increased substantially over the past three decades. Total soybean production averaged 147 kg N ha^{-1}, about 57% (84 kg N ha^{-1}) of which was derived from symbiotic N_2 fixation. There was high variation in estimated N_2 fixation rate across the 570 watersheds in the basin where soybean was grown, ranging from 0 to 185 kg N ha^{-1} (Figure. 1). The fraction of bean N derived from symbiotic N_2 fixation ranged from 0 to 96%. Hay and haylage harvested from alfalfa fields in the Mississippi River Basin contained an average of 193 kg N ha^{-1}, of which 79% (152 kg N ha^{-1}) was derived from symbiotic N_2 fixation. These figures vary across the 787 watersheds where alfalfa was grown in the Basin, with N_2 fixation ranging from 43 kg N ha^{-1} to 471 kg N ha^{-1} (29 to 99% of crop N derived from the atmosphere).

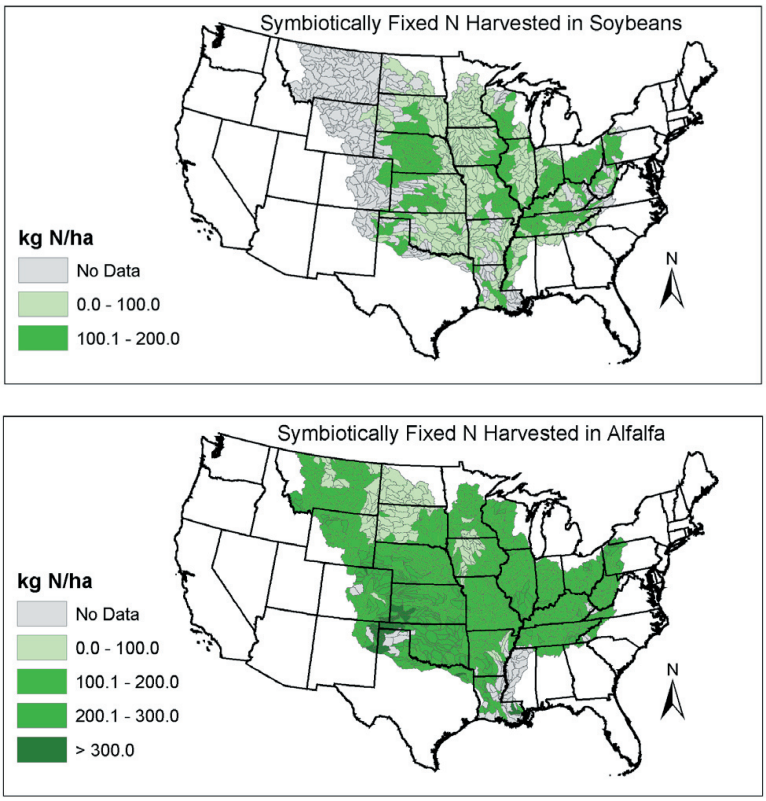

Figure 1. Maps showing the estimated amount of N_2 fixation by soybean and alfalfa in the Mississippi River Basin.

The estimated amount of symbiotically fixed N in soybean is about 1.9 billion kg and in alfalfa forage is about 1.0 billion kg, totaling nearly twice the estimated 1.5 billion kg of N delivered annually by

the Mississippi River to the Gulf of Mexico. This is the first time such estimates have been made for a large area like the Mississippi River Basin. The results make it clear that soybean and alfalfa could be managed to reduce the N input in the Basin, which may help minimize agriculture's role in nutrient loss to the river and Gulf.

Conclusion

On farms or local areas where soybean and alfalfa are fed to livestock, livestock producers have access to considerable 'N buffering power'. That is, by applying manure to these legumes, farmers may be able to reduce a portion of the excess amount of reactive N in the region caused by N_2 fixation. Soybean and alfalfa can utilize N and other nutrients in manure applied either before seeding or after alfalfa harvests. The rate of manure application must be appropriate for the conditions to avoid over-application of both N (considering N requirements of the crop, other N supplies, and likely N losses) and other nutrients, especially phosphorus. To avoid excessive soil P accumulation, which can threaten surface water quality, manure application rate should be determined by P removal capacity of the crop. Based on data we received from forage testing laboratories, mean P concentration in alfalfa forage in the Mississippi River Basin is about 3 g kg^{-1}. Therefore, P removal in harvested alfalfa is usually no greater than about 40 kg P ha^{-1}. Soybean usually contains about 6 g P kg^{-1} beans, yielding an average of about 13 kg P ha^{-1}. Farmers should calculate manure application rates based on P removal by crops and, in those fields near surface water, should also monitor soil test P levels to reduce the risk of P runoff.

Cloning, Molecular Characterization, and Expression Analysis of Several Red Clover cDNAs

M.L. Sullivan and S.L. Thoma

Introduction

Red clover (*Trifolium pratense* L.) is an important and versatile forage legume. In agriculture, red clover is probably the second most important forage legume after alfalfa in the United States, and in some parts of the world, such as Scandinavia, it is the most widely used forage legume. There has also been recent interest in the use of red clover-derived supplements in human medicine including the treatment of post-menopausal symptoms and prostate cancer because of its high content of phytoestrogens and isoflavanoid compounds. Despite the importance of red clover in agriculture and its potential utility as a source of pharmaceutical compounds, relatively little work has been carried out on red clover at the molecular (gene) level. To begin gathering information regarding red clover genes, the extent of sequence similarity they share with those of other plant species, and their patterns of expression, we set up a college laboratory class project in which students analyzed random clones from a red clover leaf cDNA library.

Methods

Undergraduate students from Edgewood College (Madison, WI) isolated and sequenced random cDNA clones from a red clover leaf cDNA library using well-established methodologies. DNA sequences were deposited in the National Center for Biotechnology Information's public domain GenBank database. Gene sequences were compared to those in several online databases, including Genbank, TIGR MtGI

(a database of sequences from the model legume *Medicago trucatula*), and TAIR databases (a database of the entire genome of the model flowering plant *Arabidopsis thaliana*) using BLAST and other sequence analysis software. Expression analyses via RNA blotting and nucleic acid hybridization were also carried out using standard procedures.

Results and Discussion

Ten full-length clones were sequenced and gene identifications were made by BLAST searches of the Genbank, TAIR, and TIGR MtGI databases (Table 1). Of the ten genes analyzed in this study, nine encoded chloroplast proteins. The cDNAs included genes encoding actin; several proteins involved in photosynthesis including PsaH, PsbR, PsbX, early light-induced protein (ELIP), ferridoxin, chlorophyll a/b binding protein; fructose-bisphosphate aldolase; chloroplastic superoxide dismutase; and GTP-binding protein typA., The red clover genes showed 57-85% (median=73%) and 87-95% (median=92%) identity with their *A. thaliana* and *M. trucatula* counterparts, respectively.

Table1. Characteristics of several cloned red clover genes.

Clone	Genbank Accession	Encoded Protein	Nucleotide Sequence Identity (%)		Chlorplast Localized
			A. thaliana	*M. trucatula*	
MM2	AY372368	Actin	84	95	N
RL1	AY340641	10.2 kDa Photosystem I protein (PsaH)	73	92	Y
MM9	AY348557	10 kDa Photosystem II protein (PsbR)	72	92	Y
CM1	AY340642	UV-repressible protein (PsbX)	61	88	Y
PN2	AY340640	Early Light-induced protein (ELIP)	57	91	Y
PN1	AY340639	Ferredoxin I (Fed)	64	87	Y
JS1	AY430082	Chlorophyll a/b binding protein (Cab)	77	93	Y
ME2	AY430081	Fructose-bisphosphate aldolase	75	92	Y
RT2	AY434497	Chloroplstic superoxide dismutase (SOD)	73	91	Y
RT1	AY445630	GTP-binding protein typA (GTP-BP)	74	93	Y

Not surprisingly, RNA blotting experiments indicate the genes show a wide range of expression patterns, both in terms of the levels of gene expression and tissues in which the genes are expressed (Figure 1 and data not shown). Several of the photosynthetic genes, including chlorophyll a/b binding protein had especially high expression, particularly in green tissues, whereas actin and superoxide dismutase had relatively low expression levels.

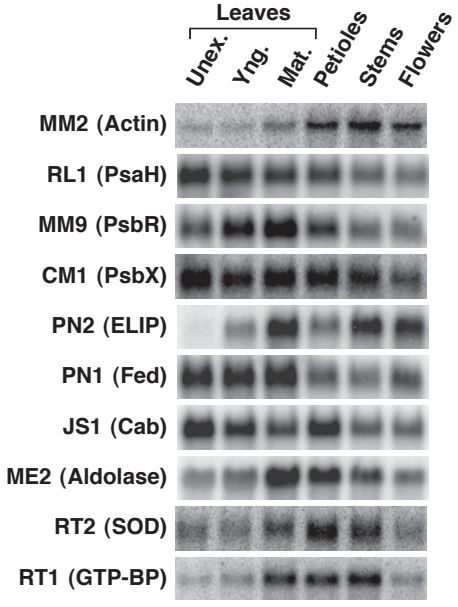

Figure 1. Expression patterns of several red clover genes. Red clover RNA from unexpanded (Unex.), young (Yng.), and mature (Mat.) leaves; mature petioles; stems; and flowers was fractionated on denaturing agarose gels and transferred to nylon membranes. The resulting RNA blots were used in hybridization experiments with ^{32}P-labeled DNA probes corresponding to the indicated red clover clones and imaged using a phosphoimager. The gene product name of each clone is indicated in parenthesis

Conclusions

The high level of sequence identity between red clover and *M. trucatula* genes suggests that emerging *M. trucatula* genomics tools, such as gene microarrays, may be useful in the analysis of red clover gene expression. Additionally, with their differing levels and patterns of expression, the isolated red clover cDNAs are proving to be useful controls in other gene expression studies in both red clover and alfalfa.

Cloning and Characterization of Polyphenol Oxidase Genes From Red Clover

M.L. Sullivan, R.D. Hatfield, and S.L. Thoma

Introduction

Excessive proteolysis of ensiled forages can result in both economic losses to farmers and negative impacts on the environment due to poor utilization of non-true protein nitrogen (ammonia, amino acids, and small peptides) by ruminant animals. Proteolytic losses in alfalfa (*Medicago sativa*) are especially high. In contrast, red clover (*Trifolium pratense*), a forage of protein content similar to alfalfa has been found to have up to 90% less proteolysis than alfalfa during ensiling. Several experimental observations suggest that the reduced proteolysis seen in ensiled red clover is due to the presence of the enzyme polyphenol oxidase (PPO) and *o*-diphenol PPO substrates. In an effort to understand the role of PPO in inhibition of post-harvest proteolysis, we have been using both biochemical and molecular approaches. Here we report the cloning and characterization of genes encoding PPO in red clover.

Methods

Using sequence data from conserved regions of previous cloned PPO genes, we designed degenerate oligonucleotide primers and used them for polymerase chain reaction (PCR) of reverse transcribed mRNA from red clover leaves. One of the resulting PCR products was used to screen a red clover leaf cDNA library for full-length PPO clones using well-established procedures. The isolated clones were sequenced using standard methodologies, and the sequences were deposited in the National Center for Biotechnology Information's public domain GenBank database. The sequences were also analyzed using appropriate sequence analysis software. We analyzed expression of red clover PPO genes using both RNA blotting with gene-specific ^{32}P-labeled probes, and immunoblotting using an anti-red clover PPO antibody we developed and transgenic alfalfa expressing individual red clover PPO genes (described in an accompanying report).

Results and Discussion

Three unique PPO cDNAs (which we have designated PPO1, PPO2, and PPO3) were identified and sequenced following screening of a red clover leaf cDNA library with a PCR-derived PPO probe (Table 1). Sequence comparison among the three red clover PPO clones indicates they are 84-89% identical at the nucleotide level (encoded proteins are 80-83% identical). Analyses of the predicted protein sequences indicate the presence of conserved copper binding motifs characteristic of PPO enzymes and cleavable signals to localize the proteins to the lumen of the chloroplast thylakoid (Figure 1). Such targeting would result in processing to mature proteins of 504-522 amino acids in length. Additional

experiments have confirmed processing of the PPO1 gene product in red clover to the predicted mature form (data not shown).

Table 1. Characteristics of red clover PPO genes.

Gene	Genbank Accession	Protein Length[a]	Expression Pattern
red clover PPO1	AY017302	605/511	leaf
red clover PPO2	AY017303	623/522	flower, petiole
red clover PPO3	AY017304	599/504	leaf, flower

[a]Length in amino acids, predicted precursor/predicted mature.

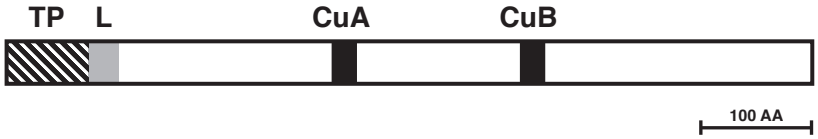

Figure 1. Schematic representation of PPO protein showing predicted chloroplast transit peptide (TP), lumenal targeting sequence (L), and conserved copper binding motifs (CuA and CuB).

Expression of the various red clover PPO genes was examined by RNA blotting and hybridization using gene-specific probes (Figure 2). PPO1 is most highly expressed in unexpanded and young leaves, PPO2 is expressed most highly in flowers and petioles, and PPO3 is expressed in both leaves (albeit to a lower level than PPO1) and flowers. Although leaf expression for PPO2 is not apparent in the RNA blot, recovery of the cDNA from a leaf library suggests it is expressed in leaves, albeit to a low level. Using antiserum raised against red clover PPO1 protein, we found that the individual red clover PPOs expressed in transgenic alfalfa are distinguishable by mobility on SDS-PAGE protein gels (Figure 2). Immunoblot analysis of red clover leaf and flower extracts show that PPO1 and PPO3 are most highly expressed in leaves whereas PPO2 is expressed most highly in flowers. These findings are consistent with the RNA blot analysis.

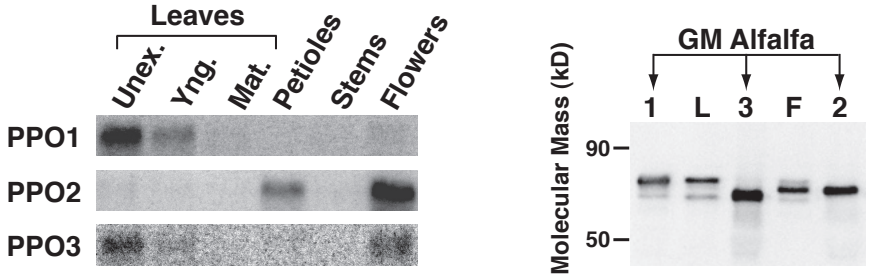

Figure 2. Red clover PPO genes show tissue-specific expression. (Left) Blots of RNA isolated from unexpanded (Unex.), young (Yng.) and mature (Mat.) leaves; petioles; stems; and flowers of red clover were hybridized with gene-specific probes as indicated. (Right) An immunoblot of extracts of red clover leaves (L) and flowers (F) or leaves from genetically modified (GM) alfalfa expressing PPO1, PPO2, and PPO3 as indicated was probed with anti-PPO1 antiserum.

Conclusions

Here we identified three unique red clover PPO genes that represent at least two genetic loci. Analyses of expression of these PPO genes at both the RNA and protein levels indicate the PPO1 gene product is the major PPO present in red clover leaves. This study has also provided materials for continued study of PPO biochemistry and the role PPO plays in inhibition of post-harvest proteolysis.

Expression of Active Red Clover Polyphenol Oxidase in *Escherichia coli* and Transgenic Alfalfa (*Medicago sativa*)

M.L. Sullivan, R.D. Hatfield, and D.A. Samac

Introduction

Ensiled red clover experiences considerably less proteolysis than many other forage crops, including alfalfa. Several experimental observations suggest that red clover's lower extent of post-harvest proteolysis is due to the presence of polyphenol oxidase (PPO), an enzyme associated with post-harvest browning in plant tissues, and *o*-diphenol PPO substrates. Interestingly, alfalfa leaves and stems have little if any polyphenol oxidase activity or *o*-diphenols. We have cloned three unique PPO genes from red clover. To further characterize the PPO enzymes encoded by these genes, we have expressed them in a bacterial system, *Escherichia coli*, and a plant system, alfalfa (*Medicago sativa*).

Methods

Standard cloning methods were used to make expression constructs for *E. coli* and alfalfa. For *E. coli* expression, a portion of the red clover PPO1 coding region lacking chloroplast targeting sequences was cloned behind a strong inducible promoter in the pET28 expression vector. The resulting plasmid was transformed into BL21(DE3)RIL *E. coli* and PPO was induced by standard protocols. For alfalfa expression, the entire coding region (including chloroplast targeting sequences) from red clover genes PPO1, PPO2, or PPO3 was cloned behind a strong constitutive promoter in the pILTAB357 binary vector. The resulting plasmid was transformed into the regenerable alfalfa line RSY27 via *Agrobacterium*-mediated transformation. Expression of PPO in *E. coli* was assessed by SDS polyacrylamide gel electrophoresis and a quantitative PPO activity assay. Expression of PPO in alfalfa was assessed by both extract browning following addition of an *o*-diphenol substrate and by a quantitative activity assay.

Results and Discussion

When expressed in *E. coli*, 85-90% of the PPO protein was insoluble and accumulated in inclusion bodies (Figure 1). The insoluble protein was purified by preparative SDS-PAGE (Figure 1) with an approximate yield of 5 mg protein per liter of culture and the resulting protein was used for antibody production in a rabbit. Results of immunoblotting experiments with red clover and transgenic alfalfa expressing red clover PPO genes are detailed in an accompanying report. Immunoblotting of soluble fractions from *E. coli* expressing the red clover PPO1 protein indicate 10-15% of the PPO is soluble with migration indistinguishable from that present in the insoluble fraction (data not shown). Further, compared to extracts of control *E. coli* containing the pET28a vector only, extracts of *E. coli* expressing PPO1 protein contain low but significant and reproducible levels of PPO activity as determined by a quantitative PPO assay using a caffeic acid substrate ($4.0X10^{-2}$ versus $2.4X10^{-4}$ μmol/min/mg protein for PPO1-expressing and control *E. coli* extracts, respectively). This result indicates that at least some fraction of PPO produced in *E. coli* is correctly folded to produce soluble active protein. To our knowledge, this is the first report of expression of enzymatically active PPO in a bacterial system.

Given that only a relatively small fraction of PPO protein expressed in *E. coli* is active, it might be that expression in a plant and targeting to a plastid are required for optimal folding and activity. We decided to express the red clover PPO genes in alfalfa because we have been unable to detect endogenous PPO activity in alfalfa leaves, and because alfalfa appears to lack *o*-diphenols, the prime substrates for PPO. We reasoned that this lack of endogenous PPO and substrates would facilitate our analyses of the red

clover gene products. Several independent transformants were generated for each PPO gene, as well as control plants transformed with the pILTAB357 vector only. When an *o*-diphenol substrate, caffeic acid, was added to extracts of alfalfa expressing the red clover PPO transgenes, browning was apparent within two to fifteen minutes. (Figure 2) Of several independent tranformants analyzed (4 for PPO1, >15 each for PPO2 and PPO3), extracts of alfalfa expressing PPO1 and PPO3 generally showed the most rapid browning. No browning was seen without the exogenously added substrate, and no browning occurred in extracts of control alfalfa transformed with the pILTAB357 vector alone, even after a 24-hour incubation in the presence of caffeic acid. We also carried out quantitative measurements of PPO activity using caffeic acid as substrate (Table 1). Consistent with the browning results, extracts of transgenic alfalfa expressing any of the red clover PPO genes had enzymatic activity above that of the control alfalfa extract, with that of PPO1-alfalfa extracts approaching activities measured in red clover leaf extracts (0.57 to 6.74 μmol/min/mg protein). Interestingly, red clover PPO activity was not only stable in alfalfa extracts, the measured activity increased five- to fifty-fold upon a two- to seven-day incubation at 30°C. No activity increases were detected in extracts of control alfalfa, and red clover extracts exhibited a total loss in activity upon a comparable 30°C incubation.

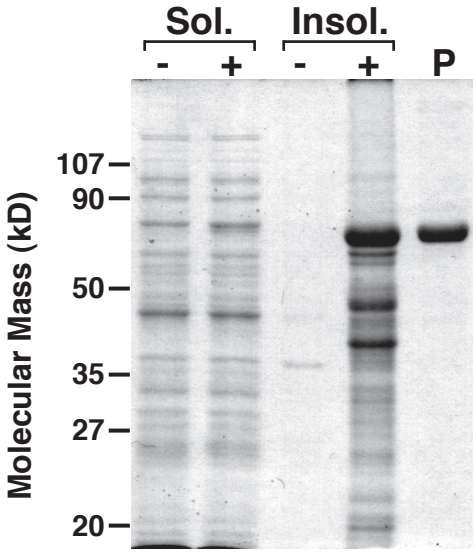

Figure 1. Expression and purification of the red clover PPO1 gene product in *E. coli*. *E. coli* expressing the red clover PPO1 protein (+) was fractionated into soluble (Sol.) and insoluble (Insol.) fractions. Portions of the soluble (5 μg protein, equivalent to approximately 50 μl induced culture) and insoluble (equivalent to approximately 100 μl of induced culture) fractions were resolved on a 10% SDS-PAGE gel and stained to detect protein. *E. coli* transformed with the pET28 vector served as a negative control (-). A sample of the insoluble red clover PPO1 protein purified by preparative SDS-PAGE and used to immunize a rabbit (P, approximately 2 μg) was also run on the gel.

Table 1. PPO activity of red clover and transgenic alfalfa extracts using caffeic acid substrate.

Extract Source	PPO Activity (μmol/min/mg protein)	
	Fresh Extract	Incubated Extract[a]
Red Clover	6.74	0.01
Control Alfalfa	0.02	0.01
PPO1-Alfalfa	0.59	3.49
PPO2-Alfalfa	0.03	0.26
PPO3-Alfalfa	0.07	3.33

[a]Extracts were incubated two to seven days at 30°C. Maximum activity is reported.

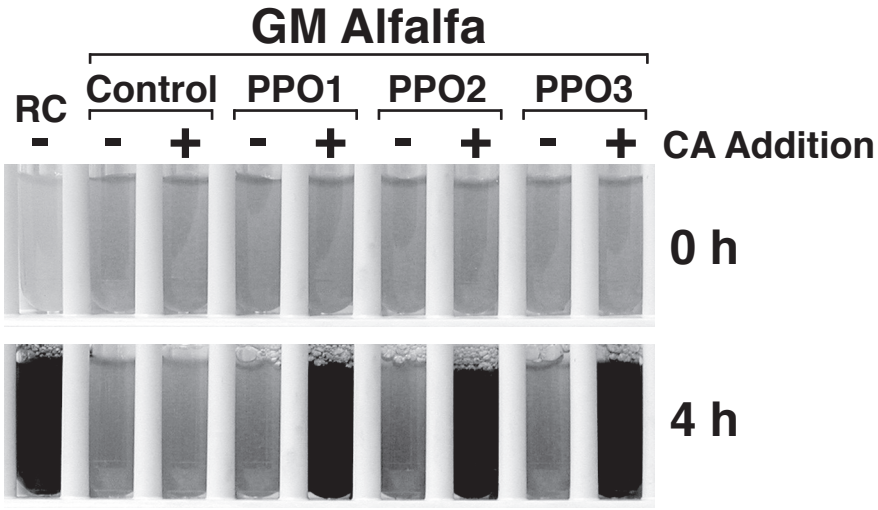

Figure 2. Leaf extracts of transgenic (GM) alfalfa expressing red clover PPO1, PPO2, or PPO3 were incubated at room temperature with (+) or without (-) exogenously added PPO substrate (caffeic acid, CA, 3 mM final concentration). Leaf extracts of red clover (RC) and alfalfa transformed with the pILTAB vector only (Control) served as positive and negative controls, respectively. Browning was apparent within two to fifteen minutes for transgenic alfalfa expressing red clover PPO genes. The control alfalfa extract showed no apparent browning, even after 24 hours.

Conclusions

Expression of red clover PPO1 in *E. coli* resulted in production of mostly insoluble protein, which was used to produce antiserum recognizing red clover PPO proteins. These antibodies are proving to be useful tools in analyzing the red clover PPO system. Besides the insoluble protein produced, a small amount of *E. coli*-expressed PPO was enzymatically active, suggesting that with modifications, bacterial systems could produce large amounts of active enzyme for further studies. Expression of PPO1, PPO2, and PPO3 in transgenic alfalfa resulted in the production of active PPO enzyme, with, in some cases, activities approaching those detected in red clover leaf extracts. Transgenic PPO-expressing alfalfa plants will be valuable tools in further characterizing the role of PPO in inhibition of post-harvest proteolysis, as well as detailed characterizations of PPO biochemistry, structure, and *in planta* function.

FORAGE HANDLING, PRESERVATION AND STORAGE

Alternate Covering System for Bunker Silos

R.E. Muck

Introduction

The most common covering on bunker and pile silos is 150 - 220 μm thick polyethylene plastic held in place by used tires. The plastic may be black or white. Typical practice in recent years at our research farm has been to use 220 μm white plastic because it is easier to handle on warm and/or breezy days and produces less surface spoilage than 150 μm black plastic. The handling of used tires has also been improved by using a combination of half tires at the edges and down the middle of the bunker and tire sidewalls in between. These are easier to handle than full tires and do not accumulate water. Even so, the farm crew does not look forward to bunker covering. An alternative system has been developed in Italy that uses a new plastic formulation (45 μm thick but more impermeable to oxygen), plastic on the walls as well as the top, a reusable woven plastic tarp to protect the plastic, and sand bags only at the edges to hold the plastic and tarp in place. We are in the process of comparing the two systems on bunker silos at our research farm.

Methods

The new covering system (Silostop) was compared to our standard system of 220 μm white plastic and tires on two bunker silos, one filled with second and third cutting alfalfa and the other with corn. Schematics of the two systems are shown in Fig. 1. Each bunker was split in half, front to back. One covering system was used on the back half and the other on the front half. Immediately before covering, core samples were taken on the top of the silo at 0-15, 15-30, 30-45 and 45-60 cm depth and at six locations (four at 60 cm from the wall and two in the middle) for each half. When the silos were opened for feeding, samples were again taken at similar locations in each half by cutting holes in the plastic and taking cores at each of the four depths. The holes were sealed with plastic tape immediately after sampling.

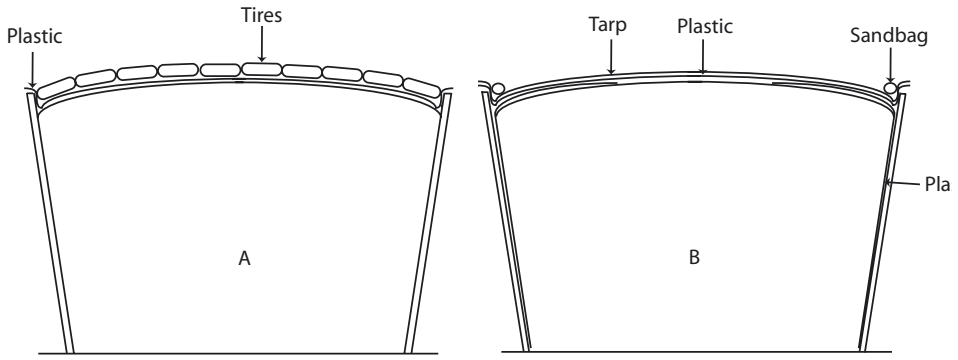

Figure 1. Diagrams of bunker covering systems. A) Conventional plastic weighted with used tires, B) Silostop system – plastic sheets on side walls folded over crop, plastic top sheet above side sheets, reusable tarp on top of plastic, sandbags at walls to anchor plastic and tarp.

Results and Discussion

The bunker with alfalfa was initially filled with second cutting in July and covered. On August 3 it was opened to add third cutting. It was resealed on August 7 and then opened for feeding the following February. Dry matter (DM) concentrations and pH values averaged for all locations are shown in Table 1. After less than one-month storage, the surface samples (0-15 cm) under the white plastic had higher pHs than those under the Silostop system, particularly near the wall. Differences in pH were even greater in February, extending down 30 cm. This indicates more spoilage under the white plastic. This is also supported by the reduced DM concentrations in the surface layer under the white plastic in February. We will be analyzing the ash content of these samples to make an estimate of DM losses in these surface layers.

Table 1. DM concentrations and pH values of cores taken immediately after filling and at opening in alfalfa silage. Second (7/11) and third (8/7) cuttings were ensiled in the same bunker silo.

		7/11	8/3		8/7	2/14	
Plastic	Depth, cm	DM, %	DM, %	pH	DM, %	DM, %	pH
White	0-15	35.8	34.3	5.36	38.8	31.7	7.18
	15-30	39.7	37.1	4.86	38.4	38.6	6.21
	30-45	45.2	44.4	4.86	40.5	41.3	4.90
	45-60	43.5	41.9	4.84	35.7	34.9	5.02
SiloStop	0-15	35.3	38.2	4.77	39.9	37.3	5.50
	15-30	40.0	42.7	4.91	41.3	40.7	5.42
	30-45	38.7	37.0	4.79	40.2	40.3	5.17
	45-60	34.6	34.2	4.76	42.6	41.4	4.92

Differences between the two plastic systems were less evident in the corn silage bunker (Table 2). The pH values under the white plastic were slightly higher. This appeared to be due to higher acetic acid concentrations in the silage beneath the white plastic. This suggests more oxygen exposure under the white plastic. However, the short duration between filling and emptying along with cooler fall temperatures probably contributed to smaller differences between the two systems.

Table 2. DM concentrations and pH values of cores taken immediately after filling and at opening in corn silage.

		9/26	11/3	
Plastic	Depth, cm	DM, %	DM, %	pH
White	0-15	41.5	38.8	4.32
	15-30	40.2	40.5	4.10
	30-45	40.1	40.5	4.00
	45-60	36.2	37.5	4.04
SiloStop	0-15	42.8	40.0	4.31
	15-30	42.3	40.9	3.99
	30-45	43.6	42.3	3.93
	45-60	44.6	43.3	3.94

Conclusions

The Silostop system produced noticeably less spoilage on the top of a bunker silo in alfalfa silage, particularly near the walls. Differences were not significant in corn silage most likely due to the short duration of that trial. Further analyses on these trials along with additional trials will be done to ascertain differences in losses between the two systems. Such determinations are crucial to evaluating the Silostop system, which is more expensive than standard systems.

Inoculant Effects on Ensiling and *in vitro* Gas Production in Alfalfa Silage

R.E. Muck, I. Filya and F.E. Contreras-Govea

Introduction

Inoculants (additives supplying principally lactic acid bacteria) are the most common additives used in making silage in the United States. Most inoculants contain homofermentative lactic acid bacteria that shift fermentation toward lactic acid production, reduce pH, improve dry matter (DM) recovery from the silo and improve animal performance (milk production or rate of gain). While the effects of inoculants on fermentation and DM recovery are understood, the effects on animal performance are often greater than expected from the shifts in fermentation. *In vitro* analyses may help uncover how inoculants may affect rumen fermentation and ultimately dairy cattle performance. Previously we found that inoculant bacteria can survive in rumen fluid. In these trials, we studied how inoculation of alfalfa silage affected *in vitro* gas production.

Methods

Alfalfa was ensiled in two trials [first (48% DM) and second cutting (39% DM)] in 2003. In both trials, alfalfa was harvested with standard field equipment (mower-conditioner, forage harvester) without inoculation. The chopped alfalfa was brought back to the laboratory and ensiled in 1-l and 500-ml glass Weck jars in first and second cutting, respectively, at a density of 500 g/l. Each trial had fifteen treatments (uninoculated control, 14 inoculants), four silos per treatment. Eight inoculants were commercial products; the others were single strains provided by two companies. All inoculants were applied at 10^6 colony-forming units (cfu)/g crop (not label rates) to help insure domination of fermentation. Silages were stored for a minimum of 30 d at room temperature (~22°C). Silages were analyzed for pH, DM concentration and fermentation products. A portion of silage was wet ground in a Büchi mixer. *In vitro* gas production was measured on 1-g samples of the wet-ground silage in 160-ml Wheaton bottles. One replicate *in vitro* analysis was performed shortly after grinding. The remaining ground sample was frozen, and two other replicate *in vitro* analyses were performed using the frozen samples. *In vitro* analysis was carried out at 39°C, and gas pressure was measured at 3, 6, 9, 24, 48 and 96 h. At 96 h, the bottles were opened, and pH was measured. The *in vitro* samples were centrifuged and the centrate frozen for later fermentation product analysis.

Table 1. The pH values of the silages at opening for first and second cutting alfalfa ranked from highest to lowest.

First Cutting		Second Cutting	
Treatment	pH	Treatment	pH
E. faecium C	5.144	Biotal Buchneri	4.651
Control	5.081	11A44	4.642
Biotal Buchneri	4.899	*E. faecium* C	4.470
11A44	4.825	*L. pentosus*	4.464
L. pentosus	4.657	*P. pentosaceus* A	4.463
E. faecium Q	4.578	*P. pentosaceus* E	4.459
P. pentosaceus E	4.577	*E. faecium* Q	4.445
P. pentosaceus A	4.569	*L. plantarum*	4.425
Biotal Plus	4.511	Control	4.422
Ecosile	4.507	Biotal Plus	4.418
1174	4.497	Biomate	4.399
L. plantarum	4.462	Ecosile	4.397
Biomate	4.429	1174	4.336
H/M Plus	4.377	H/M Plus	4.318
Biomax 5	4.335	Biomax 5	4.287

Results and Discussion

The average pH values for the silages ranked from highest to lowest for both trials are presented in Table 1. In first cutting, the natural lactic acid bacterial population at ensiling was 1.5 x 10^5 cfu/g, and all inoculants except *E. faecium* C reduced pH relative to that of the control silage. The commercial homofermentative inoculants produced the largest reductions in pH whereas the two commercial heterofermentative (*Lactobacillus buchneri*) inoculants (Biotal Buchneri and 11A44) produced the smallest reductions as expected.

In second cutting, the natural population (2.7 x 10^7 cfu/g) at ensiling was more than 10 times higher than the inoculant application rates, providing a stiff challenge. The commercial homofermentative inoculants were the only treatments producing lower pH values than the control. The highest pHs were the commercial *L. buchneri* inoculants.

Our expectations were that inoculants would improve *in vitro* DM digestibility, resulting in increased gas production. Surprisingly inoculants had either no effect on 96-h gas production per unit DM or gas production was reduced (Figs. 1, 2). This did not appear related to silage pH as indicated in the figures. The buffers in the *in vitro* system were such that the pH values of the *in vitro* fluid at the end of an analysis run did not differ across treatments. Thus, the low pH of some of the inoculated silages did not reduce the pH of the *in vitro* fluid, potentially explaining the reduced gas production.

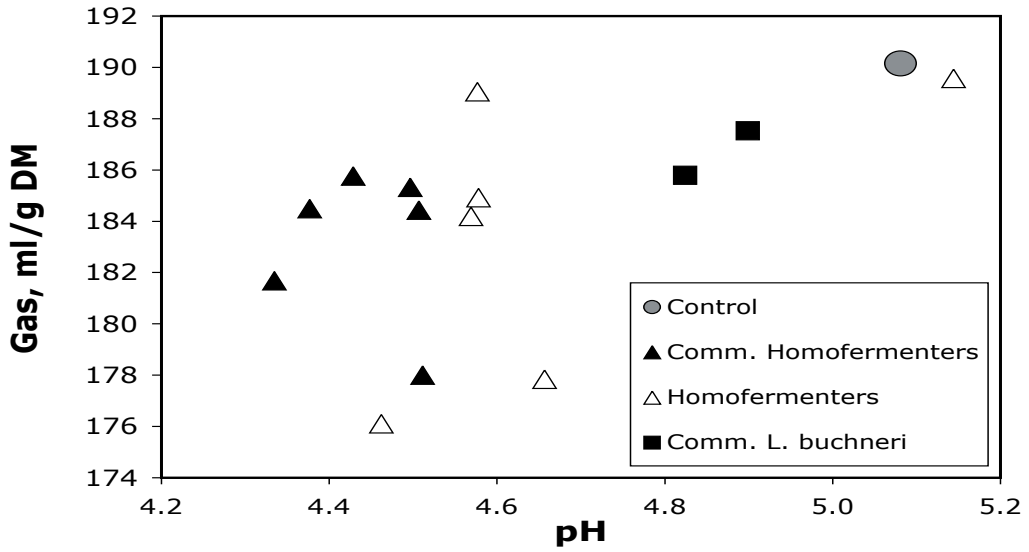

Figure 1. *In vitro* gas production from first cutting alfalfa silages as correlated to silage pH and separated by treatment group.

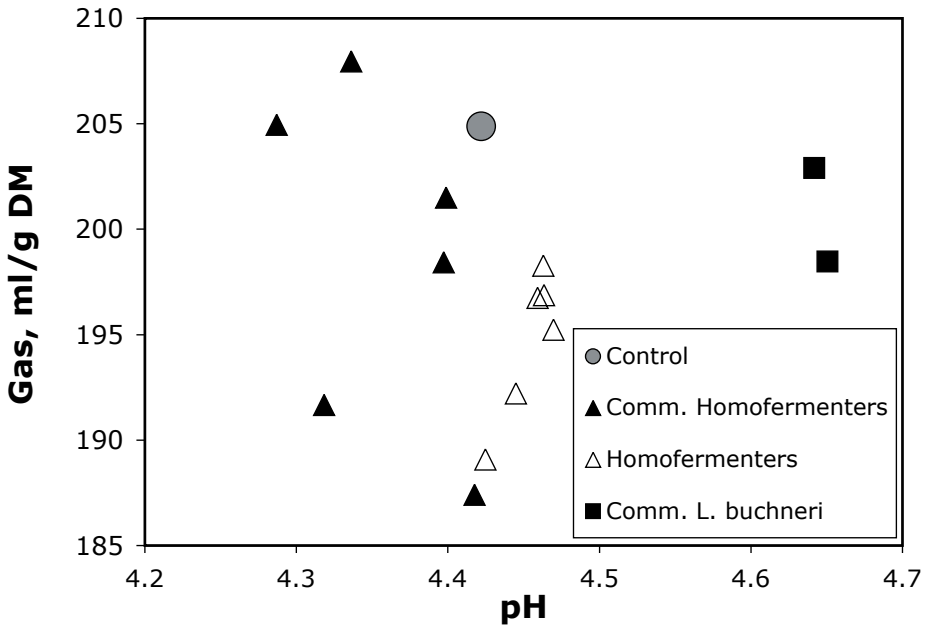

Figure 2. *In vitro* gas production from second cutting Alfalfa silages as correlated to silage pH and separated by treatment group.

Conclusions

These results indicate that inoculation of alfalfa at ensiling is affecting rumen fermentation but in a manner different from expectations. Currently we are analyzing the fermentation products from these *in vitro* assays as well as performing more standard tests of *in vitro* DM and fiber digestibility on these silages to help explain our results. Together, these results may begin to explain how silage inoculants are beneficial to dairy cattle.

PLANT CHEMISTRY/BIOCHEMISTRY

Tissue Development and Degradability of Corn Internode Cell Walls

H.G. Jung

Introduction

It is a well established fact that the ruminal degradability of forage cell walls declines during the maturation process. Deposition of lignin in cell walls is commonly regarded as the primary cause of this decrease in cell wall degradability. However, simply measuring lignin concentration of ground forage samples obscures the fact that forages are composed of many tissue types and that all tissues do not develop in the same manner. As a result, the various tissues in forages differ in their susceptibility to ruminal degradation. In order to most effectively target genetic improvement of forage degradability, particularly when using molecular techniques, it is critical that these differences in tissues are understood. The author previously showed that in alfalfa it is the proliferation of xylem tissue during stem development that accounts for almost all of the increase in lignin and decline in degradability associated with maturation. The current study was conducted to provide similar detailed information on tissue development and degradability in grasses using corn stems.

Materials and Methods

Three corn hybrids of divergent pedigrees (A632 x A619, A679 x FR481, and Mycogen 2677) were grown in replicate plots at St. Paul, MN for two years. The fourth elongated, aboveground stem internode was sampled at 10 stages of development beginning when this internode was approximately 1-cm long (late June). Additional samples were collected 2, 4, 8, 12, 19, 26, 40, 68, and 96 d after the initial internode collection. This sampling plan provided a profile of cell wall development for a specific internode, from early internode elongation and cell growth through secondary cell wall deposition and maturity. Four internodes from each hybrid and plot were immediately preserved in 50% ethanol. Thin sections (100 μm in thickness) were made using a sliding-type microtome for microscopic evaluation of tissues. To visualize the presence of lignin in tissues, some sections were stained with phloroglucinol. This histochemical stain turns lignified tissues a reddish color. Other replicate sections were mounted on slides with double-sided tape and incubated in a rumen fluid/buffer solution for 24- and 96-h, after which degradation of tissues was monitored relative to non-degraded replicate sections.

Results and Discussion

Figure 1 illustrates the pattern of tissue development and in vitro ruminal degradability for the rind region of the fourth above-ground, elongating stem internode of corn hybrid A632 x A619. Similar patterns were observed for all three corn hybrids examined. Young, elongating internodes collected at the second harvest date (Fig. 1a) had very thin cell walls and only the protoxylem tissue stained red with phloroglucinol for the presence of lignin. Sclerenchyma tissue at harvest two did not stain for lignin and had thin walls. Other than a small increase in cell diameter, internode tissues collected at harvest four (Fig. 1b) appeared similar to harvest two tissues. By the sixth harvest (Fig. 1c), sclerenchyma tissue had developed thick walls that stained intensely for the presence of lignin. The epidermis and some of the parenchyma in the rind area also stained red for lignin. By the eighth harvest date (Fig.

1d), all tissues stained for the presence of lignin except for phloem and some parenchyma immediately under the epidermis. Phloem tissue was not lignified at any harvest. Parenchyma and sclerenchyma in the rind developed thicker walls by harvest 10 (Fig. 1e). Sclerenchyma and parenchyma in the pith region of the internode followed the same pattern, although neither tissue developed as thick walls as in the rind, and a ring of parenchyma cells immediately adjacent to the pith vascular bundles never lignified (data not shown).

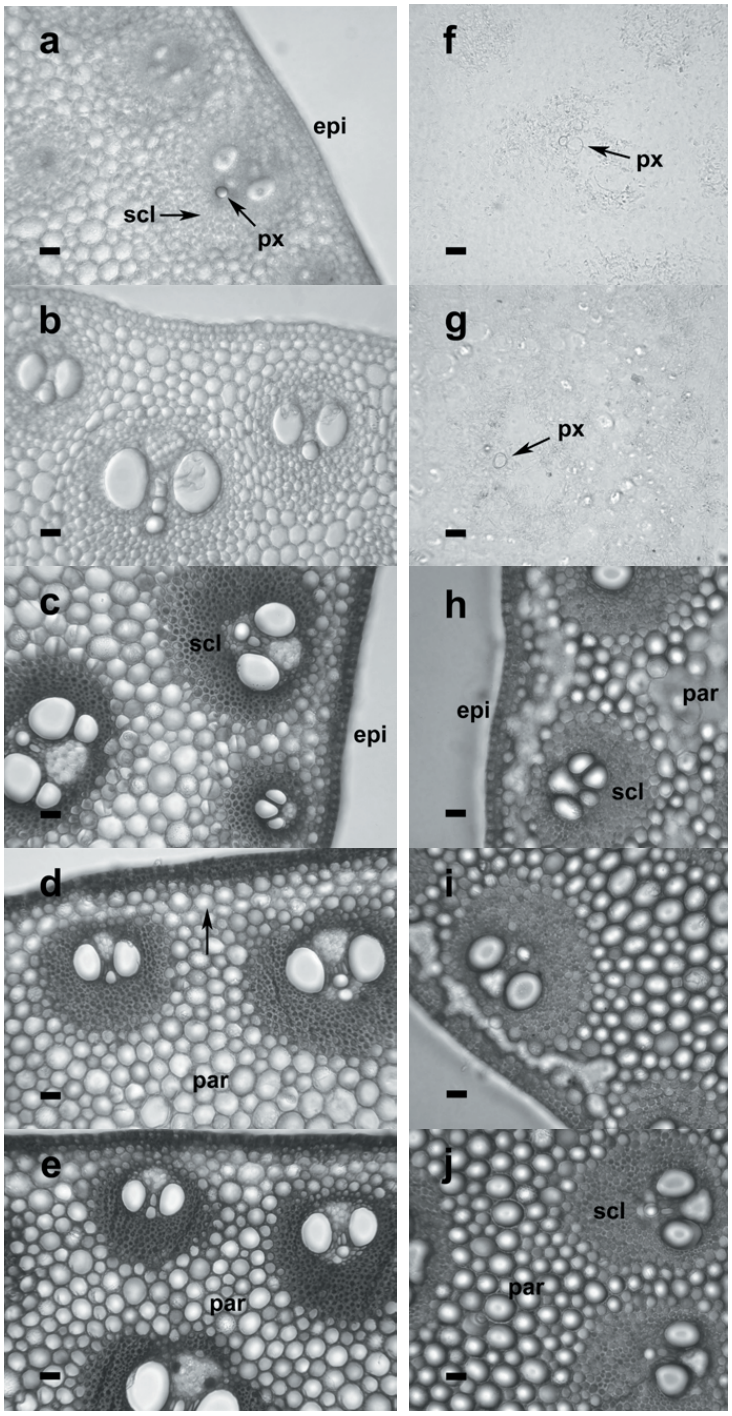

Figure 1. Thin sections taken from the fourth above-ground, stem internode of corn hybrid A632 x A619 at harvest dates two (a & f), four (b & g), six, (c & h), eight (d & i), and ten (e & j). Lignification of tissues was visualized using the phloroglucinol stain on control sections (a though e). Degradation of tissues after 96-h in vitro incubations with rumen fluid are shown in plates f through j. Epidermis (epi), sclerenchyma (scl), protoxylem (px), and parenchyma (par). Bar is 100 μm in length.

All internode tissues from harvests two and four, except for the protoxylem, were completely degradable after 96-h of exposure to rumen microorganisms (Fig. 1f & 1g). By the sixth harvest (Fig. 1h) the sclerenchyma, epidermis, and portions of the parenchyma were only partially degradable after 96 h. The thick sclerenchyma walls were reduced to thin cell wall residues due to extensive, but incomplete, degradation. The proportion of the parenchyma tissue that was incompletely degraded increased for internodes from the eighth and tenth harvests (Fig. 1i & 1j). The non-lignified phloem tissue always remained completely degradable. Similar degradation patterns were observed for sections exposed to ruminal degradation for 24 h, although the extent of degradation was less.

Conclusion

Corn stem tissues differ in their developmental pattern and susceptibility to ruminal degradation. This result is similar to previous observations with alfalfa, although the grass and legume species differ dramatically in how their tissues mature. Because all corn tissues lignify during maturation, other than phloem, targeting of genetic reductions in lignin may not need to be as tissue specific as in alfalfa. However, sclerenchyma and parenchyma in the rind region appear to contain much thicker walls than in the pith. Also, rind parenchyma lignifies earlier than this tissue in the pith. These observations suggest that genetic modification should perhaps be directed at the rind tissues in preference to the pith. The validity of this hypothesis will need to be tested using molecular biology and tissue specific promoters.

Ferulate Deposition in Developing Corn Internode Cell Walls

H.G. Jung

Introduction

Past work by the USDFRC's Cell Wall Group has shown that lignin/polysaccharide cross-linking by ferulates limits the degradability of grass cell walls by rumen microorganisms. Ferulate esters appear to act as nucleation sites where lignification begins. Using internode position on a young corn stem to provide a maturity profile, it was found that maximum ferulate cross-link concentration is reached much earlier than maximum cell wall and lignin concentrations in the developmental process. This result, plus the microscopic observation that the original primary wall of lignified grasses remains completely non-degradable even when the heavily lignified secondary wall has been degraded, led the author to propose that ferulate cross-links are only deposited in primary cell walls during the cell growth phase, and not during secondary wall thickening. This report describes an experiment conducted to test this hypothesis.

Materials and Methods

Three unrelated corn hybrids (A632 x A619, A679 x FR481, and Mycogen 2677) were grown in replicated field trials at St. Paul, MN for two years. The fourth elongated, aboveground stem internode was sampled at 10 stages of development beginning when this internode was approximately 1-cm long (late June). Additional samples were collected 2, 4, 8, 12, 19, 26, 40, 68, and 96 d after the initial internode collection. This sampling plan was adopted to provide a complete profile of cell wall

development of a single specific internode, from early internode elongation and cell growth through secondary cell wall deposition and maturity. The sampled internodes were ground to pass a 1-mm screen in a cyclone-type mill. Corn internodes were analyzed for cell wall concentration and composition using the Uppsala Dietary Fiber Method to provide information on polysaccharide composition (glucose, xylose, arabinose, galactose, mannose, and uronic acids) and Klason lignin concentration. Ester- and ether-linked ferulic acid was determined by low- and high-temperature NaOH treatment, respectively, of the ground internode samples. Data were adjusted to a "per internode" basis using the weights of the sampled internodes to allow evaluation of total deposition of cell wall components during internode growth and maturation. Ether-linked ferulate represented the cross-link between lignin and the cell wall polysaccharides, although it is known that several other non-recoverable ferulate cross-linking structures occur.

Results and Discussion

Growth in length and cross-sectional area of the fourth elongated, aboveground internode reached its maximum between the fifth and sixth sampling dates (Fig. 1a). The accumulation of organic matter in the internode continued until the ninth sampling date in early September, with a loss of organic matter occurring by the end of September. This decline in organic matter accumulation was assumed to result from sucrose translocation to the ear. Cell wall deposition reached a maximum in early August and remained constant thereafter (Fig. 1b). The major secondary cell wall components (glucose, xylose, and Klason lignin) all showed the same pattern of accumulation as did total cell wall (Fig. 1c). If the hypothesis that ferulates are only deposited in the primary wall were correct, then the pattern of ferulate ester and ether deposition should have shown a maximum accumulation near the end of internode elongation, with no addition of ferulates during secondary wall thickening later. However, the pattern of ferulate deposition matched the accumulation profile of all the major cell wall components associated with secondary wall development (Fig. 1d). Clearly ferulates continued to be deposited throughout cell wall maturation, including deposition of secondary wall components. The data indicate that ester-linked ferulates were deposited in the secondary wall and approximately 50% of the ferulates became cross-linked to lignin.

Conclusion

Contrary to the author's previous hypothesis, ferulates are deposited in both primary and secondary walls of grasses with at least two-thirds of the total ferulates found in the secondary wall. This observation raises the question of why the primary wall of lignified tissues is non-degradable while the secondary wall with similar ferulate cross-linking is extensively degraded.

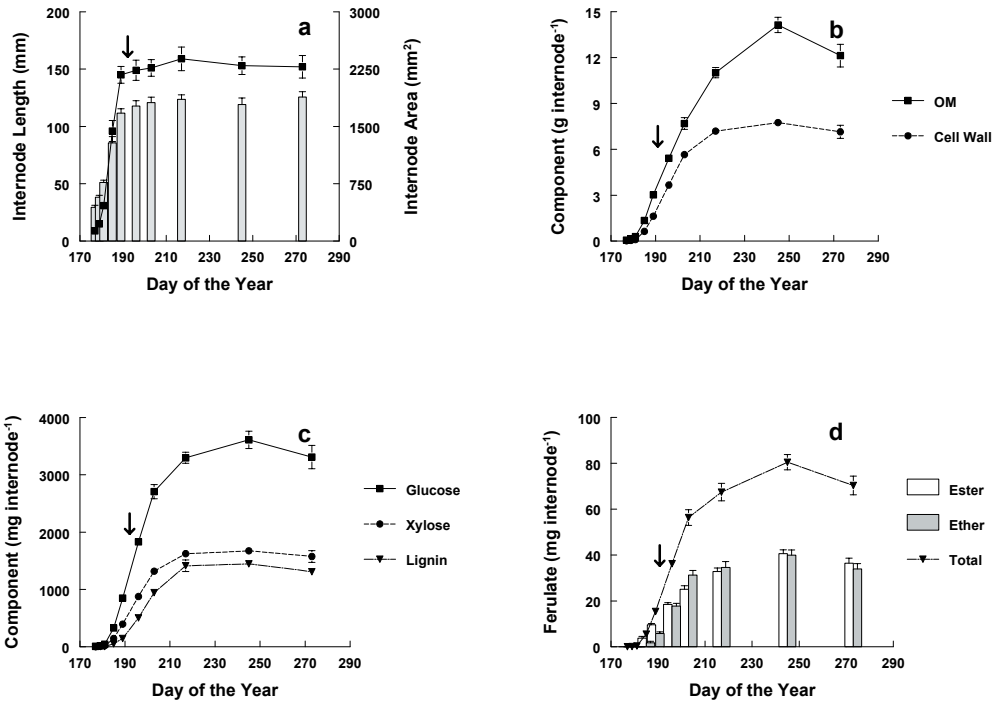

Figure 1. Length (line) and cross-sectional area (bar) of the fourth elongated, above-ground internode of corn sampled throughout development (a). Accumulation of organic matter (OM) and cell wall material (b); and patterns of cell wall glucose, xylose, and Klason lignin (c); and ester- and ether-linked, and total ferulates (d) are during development. Data are averaged across three hybrids and two years. The arrows indicate the point during development when it was assumed that growth of all cells in the internode had reached completion and all cell wall development had shifted to secondary wall deposition. Error bars represent one standard error of the mean. Absence of error bars indicates that the standard error interval was smaller than the size of the data symbol.

Polyphenol Oxidase (PPO) and *o*-Diphenols Inhibit Post-Harvest Proteolysis in Red Clover and Alfalfa

M.L. Sullivan and R.D. Hatfield

Introduction

Dairy cows do not utilize forage protein efficiently, resulting in excess nitrogen excretion. This problem is amplified for forages stored as silage, since during the ensiling process forage proteins are partially degraded by plant proteases and the released amino acids and peptides are readily degraded by rumen microbes. The nitrogen is excreted as urea and is no longer available to the animal. Proteolytic losses in alfalfa (*Medicago sativa*) are especially high. In contrast, red clover (*Trifolium pratense*), a forage of protein content similar to alfalfa has up to 90% less proteolysis than alfalfa when ensiled. This reduced level of proteolysis is not due to inherent differences in protease activities present in the tissues of these two forages. Rather, several observations suggest that reduced post-harvest proteolysis in red clover is due to the enzyme polyphenol oxidase (PPO) and *o*-diphenol PPO substrates present in red clover leaves. Until recently, it has been difficult to rule out the involvement of other mechanisms, such as specific protease inhibitors, in inhibition of post-harvest proteolysis in red clover. Using transgenic alfalfa expressing a red clover PPO gene, we are now able to establish the central role of PPO and *o*-diphenols in post-harvest proteolytic inhibition in red clover.

Methods

Clarified extracts of leaves of red clover, transgenic alfalfa expressing the red clover PPO1 gene (PPO-Alfalfa), and control alfalfa not expressing a PPO transgene (Control-Alfalfa) were made in 50 mM MES, pH 6.5. Where indicated, clover extracts were desalted using a Sephadex G-25 spin column equilibrated with 50 mM MES, pH 6.5, to remove low molecular weight molecules (i.e. *o*-diphenols). Extract protein concentrations were measured by Bradford assay using a BSA standard and were adjusted to 2 mg/ml with buffer. Where indicated, the *o*-diphenol caffeic acid (CA) was added to a final concentration of 3 mM. Extracts were incubated at 37ºC for four hours, trichloroacetic acid (TCA) added to 5%, and release of free amino acids (a measure of proteolytic activity) into the TCA-soluble supernatant was determined by ninhydrin assay.

Results

Little proteolyis is seen in red clover extracts (Figure 1A). Removal of low molecular weight factors from the extract by gel filtration, however, results in extensive proteolysis during the four hour time period analyzed (Figure 1B). Assuming an average protein molecular weight of 50,000 Daltons, approximately 8% of the amino acids initially present in protein are released into the TCA supernatant by proteolysis during the four-hour incubation. This may be an underestimation of the extent of proteolyisis taking place, since our assay will only measure release of free amino acids and very short peptides. Proteolytic events that result in larger peptides (i.e. endoproteolytic cleavages) would not be detected. Addition of 3 mM caffeic acid to the extract reduced proteolysis to an extent comparable to the non-desalted red clover extract (80-85% reduction compared to no caffeic acid addition, compare Figure 1C to 1B). These results indicate involvement of a low molecular weight molecule in red clover proteolytic inhibition, and that this molecule is likely an *o*-diphenol.

These results were extended using transgenic alfalfa expressing a red clover PPO gene (PPO-Alfalfa) and control alfalfa lacking a PPO transgene. Considerable proteolysis is seen in leaf extracts of control alfalfa (Figure 1D), with 7-8% of the amino acids initially present in protein being released into the

TCA supernatant during the four-hour incubation. Addition of caffeic acid had no significant effect on proteolysis in the control alfalfa extracts (Figure 1E). In the absence of added caffeic acid, extracts of PPO-Alfalfa showed an extent of proteolysis similar to that seen for the control alfalfa (Figure 1F). Addition of caffeic acid to PPO-Alfalfa extract resulted in an almost 80% reduction in proteolysis (Figure 1G), and effect similar in magnitude as that seen for red clover. These results demonstrate that the combination of PPO and *o*-diphenols can significantly inhibit in vitro proteolysis in alfalfa extracts, and is likely the major mechanism of post-harvest proteolytic inhibition in red clover.

Conclusions

Our results indicate that the post-harvest proteolytic inhibition seen in red clover is the result of the action of the enzyme polyphenol oxidase (PPO) on *o*-diphenols and that this natural system of protein protection can be made to function in alfalfa and possibly other forage crops. Future studies using both red clover and PPO-alfalfa should help to elucidate the mechanisms of PPO-mediated proteolytic inhibition.

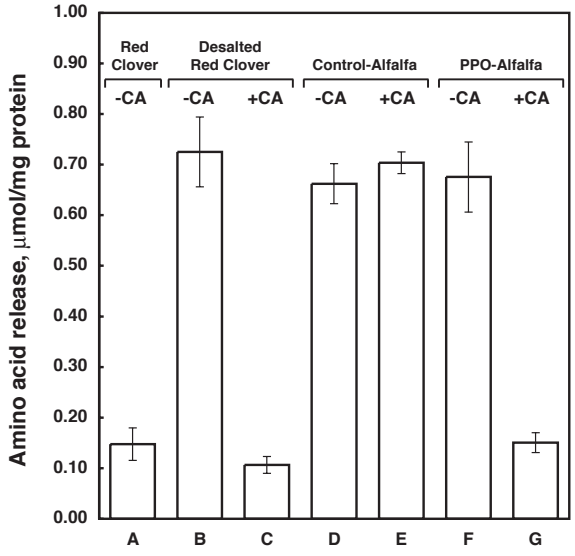

Figure 1. Proteolysis in extracts of red clover and transgenic alfalfa was measured and expressed as release of free amino acids (in μmol) per mg of extract protein in a four-hour period. Caffeic acid (CA) was added as indicated. Data are the average of three experiments using independent tissue samples. Error bars represent standard error.

Hydroxycinnamates in Cell Walls of Diverse Corn Germplasm

Ann Chaptman and Ron Hatfield

Introduction:

Grasses contain significant amounts of hydroxycinnamates, mainly ferulic (FA)and *p*-coumaric acids (*p*CA), primarily ester linked within the cell wall matrices. Ferulates are incorporated into the cell wall ester linked to arabinosyl side chains of arabinoxylans. They can become covalently linked (radical mediated coupling reactions) to each other and to lignin during the lignification process to form cross-links between xylans and lignin. Although *p*CA can also be ester linked to arabinoxylans, they are more frequently attached to lignin. It has been clearly shown that *p*CA attachment to lignin is predominately to the C-9 hydroxyl of monolignols. Field corn contains relatively high amounts of *p*-coumarylation on its lignin with levels of 15-20%. The amount of *p*CA esterified within cell walls can be quite variable depending upon the type of grass. Temperate (C3) grasses tend to have lower amounts of *p*CA then warm season grasses (C4). There is little information concerning the levels of *p*CA within different

corn germplasm and the distribution between arabinoxylans and lignin. This study was undertaken to investigate the levels of *p*CA in diverse corn germplasm to determine if variability for this trait exists and if it is primarily associated with lignin within the cell walls.

Methods

Three replicates of the following varieties of *Zea mays* germplasm were grown in the greenhouse under supplemental light (14h); B73xMo17 field corn, Rainbow corn, Robust White popcorn, Silver Queen sweetcorn, and Teosinte (subspeices mexicana). All plant*s* were harvested at tassel emergence. The 9 and 10th internodes and roots (thoroughly washed) were collected and freeze dried.

Dried samples (stems and roots) were ground via. a Udy Mill (1mm) and cell walls isolated from total dry matter. Cell wall samples were analyzed for neutral sugar composition, total uronosyls, lignin, lignin composition, and hyroxycinnamates using established methods. Only the results of hydroxycinnamate and lignin analyses will be presented in this report.

Results and Discussion

Analyses of cell walls isolated from corn stems and roots indicated that general characteristics were similar among the different types of corn (Table 1). Total hydroxycinnamates accounted for between 2.6 and 4.3% of the total cell wall. The distribution of hydroxycinnamates between *p*CA and FA was heavily shifted to *p*CA as one might expect from previous studies involving warm season grasses. Levels of *p*CA ranged from 0.0138 g/g CW to 0.0331 g/gCW in the different tissues and germplasm (Table 2). There were no significant differences between tissues (roots versus stems) when comparing *p*CA levels. The one exception was the teosinte that had much lower levels of *p*CA in the root samples. Ferulates on the other hand showed markedly higher levels of total FA in the root tissues compared to the rind. It is not clear if all of the FA in roots is attached to arabinoxylans, but the amount of arabinose in this tissue was higher and the ratio of arabinose to xylose was at least twice that of the rind tissues indicating potentially more highly substituted arabinoxylans in roots.

Table 1. General cell wall characteristics of rind and root tissues isolated from different corn germplasm.

		g/g CW			
Germplasm	Tissue	Total NS[1]	Total UA[1]	Lignin	HCA[1]
Siver Queen	rind	0.7826	0.0305	0.1603	0.0267
Rainbow		0.7601	0.0340	0.1798	0.0262
Popcorn		0.7320	0.0304	0.1994	0.0383
B73xMo17		0.7603	0.0238	0.1768	0.0391
Teosinte		0.7215	0.0307	0.2119	0.0359
Siver Queen	roots	0.7089	0.0330	0.2236	0.0345
Rainbow		0.7231	0.0336	0.2140	0.0293
Popcorn		0.7048	0.0289	0.2268	0.0395
B73xMo17		0.7251	0.0340	0.1977	0.0432
Teosinte		0.7141	0.0342	0.2214	0.0303

[1] Total NS= total neutral sugars from cell wall analysis, Total UA= total uronic acids, HCA= hydroxycinnamates.

Table 2. Hydroxycinnamates released from cell walls isolated from different types of corn germplasm. Cell wall samples were treated with 2M NaOH at room temperature to release all ester-linked hydroxycinnamates. Analysis by GC-FID as sylated derivatives.

		g/g CW	
Germplasm	Tissue	pCA	FA
Silver Queen	Rind	0.0207	0.0050
Rainbow		0.0208	0.0044
Popcorn		0.0295	0.0053
B73xMo17		0.0331	0.0068
Teosinte		0.0298	0.0048
Silver Queen	Root	0.2240	0.0071
Rainbow		0.0212	0.0072
Popcorn		0.0273	0.0010
B73xMo17		0.0330	0.0057
Teosinte		0.0138	0.0084

To determine if *p*-coumarates were primarily attached to lignin in the different corn germplasm, cell wall samples were treated with a mild acid hydrolysis (0.1N TFA, 100 °C for 1h) to release arabinosyl side chains from the arabinoxylans. This treatment effectively hydrolyzes the glycosyl linkage attaching arabinose to xylan, but does not hydrolyze the hydroxycinnamate ester linkage. Analysis by gas chromatography-mass spectrometry of the released components revealed no detectable *p*CA-Ara, only arabinose and FA-Ara (data not shown). This would indicate that nearly all *p*CA is attached to lignin in corn.

These results indicate that diverse corn germplasm have variable amounts of hydroxycinnamates in the cell walls of rind and root tissues. It would appear that virtually all of the *p*CA (within detectable limits) is attached to lignin and there is not a strong correlation between the quantity of lignin and the amount of *p*CA. This may be due to the generally higher apparent levels of Klason lignin in the root samples versus the stems (Table 1) that may not be a true reflection of the actual lignin in the walls due to other compounds such as suberin that would be counted as lignin.

Isolation and Identification of a Ferulic Acid Dehydrotrimer from Saponified Maize Bran Insoluble Fiber

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Introduction

Cross-linking of plant cell walls via ferulate dehydrodimerization reactions is now well established. Ferulates acylate various polysaccharides, notably arabinoxylans in grasses and in cereal grain dietary fiber, particularly in the insoluble fiber fraction. Ferulate dehydrodimerization is therefore a mechanism for linking two polysaccharide chains, providing structural integrity to the cell wall, but inhibiting fiber degradability. Being phenolic entities capable of radical coupling reactions, ferulates and their dehydrodimers may also be incorporated into lignin polymers, furthering the cross-linking of the wall, and further limiting degradability of the polysaccharides.

The dehydrodimerization reaction is via radical coupling. As such, the reaction is somewhat combinatorial in nature, much like the radical coupling of monolignols to each other and to lignins. A range of dehydrodiferulates can be found in a variety of materials. The dehydrodiferulates are

characterized by the new bond formed in the radical coupling reaction between the two ferulates (at their 4–O-, 5- or 8-carbons) as 5–5- 8–8-, 8–5-, 8–O–4-, and 4–O–5-coupled dehydrodimers. Characterization of the cross-linking of walls via ferulate has therefore become considerably more interesting but more complex than it was before 1994, when only the 5–5-coupled dehydrodimer **2**, Fig. 1, was known.

We have recently provided the first detailed characterization of releasable diferulates from a range of cereal grains. Cereal grains are one of the most important food groups. Their fiber properties and even some of their health benefits can likely be attributed to the nature of their cell wall polymers and their chemical architecture. We recently also discovered disinapates and sinapate-ferulate cross-products in wild rice, rice, wheat and other grains, implicating sinapates in similar polysaccharide cross-linking reactions in the wall.

The idea that ferulates might continue their radical polymerization reactions and form higher oligomers in the wall has long been discussed, but rarely in the literature. Certainly, dehydrogenation oligomers and polymers can be made in vitro. However, ferulates encumbered by their direct attachment to an extensive polysaccharide chain were thought unlikely to be able to approach two other such ferulates. A possible exception is at the ends of chains or if there is rather extreme clustering of ferulates on polysaccharide chains such that dimerization (cross-linking two polysaccharide chains) might create a dehydrodiferulate in sufficient proximity to another ferulate on one of the chains that a further coupling reaction could occur. This dehydrotrimer formation would still only cross-link two polysaccharide chains, not three. In view of the finding reported in this paper, such possibilities should be examined through the use of molecular modeling.

In this paper, we report on the isolation and structural identification of a ferulate dehydrotrimer from maize bran insoluble fiber, and discuss its implications for cell wall cross-linking in cereal grains.

Methods

Alkaline hydrolysis and extraction of Maize fiber. Insoluble maize fiber (4 x 5g) was weighed into four screw-cap tubes (200 mL). Saponification with NaOH (2 M, 100 mL, degassed with N_2) was carried out under nitrogen and protected from light. The mixture was acidified (pH <2) with concentrated HCl (~19 mL). Liberated phenolic acids were extracted into diethyl ether (40 mL, three times). The volume of the combined ether extracts was reduced to 50 mL (rotary evaporation) and extracted with $NaHCO_3$ solution (5%, 25 mL, three times). The combined aqueous layers were acidified (pH <2) with concentrated HCl and phenolic acids were extracted into diethyl ether (30 mL, three times). The combined ether extracts were dried over $NaSO_4$ and evaporated to dryness. The residue was further dried under a stream of nitrogen and finally redissolved in 10 mL MeOH/H_2O 50/50 (v/v).

Separation of phenolic acids using Sephadex LH-20 chromatography. Following conditioning of the Sephadex LH-20 column (83 x 2 cm) with 0.5 mM aqueous trifluoroacetic acid (TFA)/MeOH 95/5 (v/v) the sample was applied. A four step elution was performed: 1) elution with 0.5 mM TFA/MeOH 95/5 (v/v) for 73 h, flow rate: 1.5 mL (L-6000 pump, Merck/Hitachi, Darmstadt, Germany); 2) elution with 0.5 mM TFA/MeOH 50/50 (v/v) for 53 h, flow rate: 1.0 mL; 3) elution with 0.5 mM TFA/MeOH 40/60 (v/v) for 64 h, flow rate: 1.0 mL; 4) rinsing step with 100% MeOH. An UV-detector equipped with a preparative flow cell (L-7400, Merck/Hitachi, Darmstadt, Germany) was used for the detection at 325 nm. Fractions were collected every 18 min. The ferulic acid trimer 1 (Fig. 1) eluted in the third elution step (0.5 mM TFA/MeOH 40/60) between 45 h and 53.5 h.

Structural identification (NMR and MS). Trimer **1**, Fig. 1, was firmly identified by its mass spectra under negative and positive ion conditions, and by the usual diagnostic range of NMR experiments.

Results and Discussion

Dehydrotriferulate Isolation. Our group in Hamburg has recently developed a method for isolating milligram quantities of diferulates from maize bran as an alternative to laborious chemical synthesis. The method involves the alkaline hydrolysis of ester-bound compounds, a clean-up using liquid-liquid partitioning and separation of the liberated phenolic acids (monomeric, dimeric and trimeric ferulic acids and other phenolic acids). A key step in the separation procedure is Sephadex LH-20 chromatography using organic solvents. Using Sephadex LH-20 as stationary phase, fractionation is based not only on molecular weight differences but also on secondary interactions between the stationary phase and the phenolic compounds. Trimer **1** (Fig. 1) elutes within the third elution step using methanol (60%) and 0.5 mM TFA (40%) as eluant. Analytical HPLC shows that the trimer is quite pure (>95%, based on peak areas at 325 nm and 210 nm, respectively) following the Sephadex LH-20 chromatography. Using the method described, 10 mg of trimer **1** were isolated.

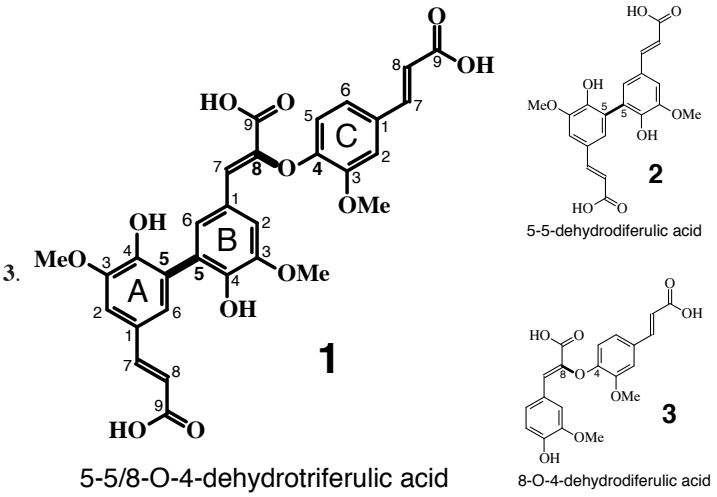

Figure 1. Structures of the new dehydrotriferulic acid **1**, along with the two component dehydrodiferulic acids **2** and **3**.

Structural identification. The UV-spectrum acquired via analytical HPLC with photodiode-array-detection has characteristics of ferulate structures. The mass-spectrum indicated trimeric ferulate structures. Negative-ion MS produced an $(M\text{-}H)^-$ of m/z 577 suggesting a molecular weight of 578. Positive-ion MS gave a high-mass M+23 of m/z 601 $(M+Na)^+$ and M+39 of m/z 617 $(M+K)^+$, suggesting the same molecular weight. Several potential ferulate dehydrotrimers have that mass. Interpretation of the MS proved to be consistent with the final structure more unambiguously elucidated from the NMR data below. The NMR spectra provided diagnostic evidence for the 5–5/8–O–4-dehydrotrimer **1**. Proton NMR revealed three methoxyls and enough aromatic protons to suggest three aromatic rings confirming the product as a trimer. There were two trans-cinnamic acid sidechains. Long-range ^{13}C–^{1}H correlations via the HMBC experiment showed that one (A) was attached to a 5-linked guaiacyl ring (with aromatic protons only on the 2- and 6-carbons), whereas one (C) was linked to a normal (4–O-linked) guaiacyl unit (with protons on 2-, 5- and 6-carbons). A singlet proton at 7.36 ppm appeared to indicate an 8–O–4-linked structure for the third unit (B); the HMBC spectrum revealed that this sidechain was on a 5-linked guaiacyl ring. Full analysis of the usual array of 1D and 2D NMR experiments (^{1}H, ^{13}C, DEPT, COSY, TOCSY, ^{13}C–^{1}H HSQC, HMBC) provided assignments fully consistent with structure **1**. The structural assignment is unambiguous since no other isomers of this compound match the data.

Implications for Cell Wall Cross-linking. The isolation of this trimer provides concrete evidence for at least one ferulate dehydrotrimer, confirming the evidence from Fry et al. for ferulate oligomers. Unfortunately, with this structure it is not possible to tell the order in which the coupling reactions occurred; i.e. was the compound formed by 8–O–4-coupling of two ferulates followed by coupling at the product's 5-position with the third ferulate at its 5-position, or did the 5–5-coupling occur first. In an in vitro reaction, we could make some predictions since ferulate addition to a 5–5-dehydrodimer favors coupling of the ferulate monomer at its 8-position with the 5–5-dehydrodimer at one of the 4–O-positions and eventually forming a dibenzodioxocin structure. However, the constraints of having all ferulate moieties attached to large polysaccharide chains allows either possibility.

The fact that this first ferulate dehydrotrimer should contain a 5–5-coupled dehydrodimeric unit suggests to us that Hatfield's observation is very likely, i.e. that the 5–5-coupled dehydrodiferulate is actually formed as an intramolecular product and both ferulate units are therefore tethered to the same polysaccharide chain. Cross-coupling (at one of its 8-positions) with a ferulate (at its 4-O-position) on a second polysaccharide chain would then create the dehydrotrimer and cross-link the two polysaccharide chains. Thus, we surmise that, because of the involvement of a 5–5-dehydrodiferulate unit, the appearance of this novel dehydrotriferulate in cereal grain insoluble dietary fiber need not implicate the cross-linking of three polysaccharide chains. Had the trimer involved units other than the 5–5-dehydrodimer (i.e. had it been an 8–5/8–8, 8–5/8–O–4, 8–O–4/8–8, 8–O–4/8–O–4, or 8–5/8–5 dehydrotrimer), the option that ferulates were capable of cross-linking three polysaccharide chains would have to be more seriously considered. In fact, since each of these other linkage types typically exceeds the level of 5–5-dehydrodiferulate, although the 5-5-dimer is surprisingly prevalent in maize bran, the very fact that we were able to isolate such a substantial amount of the 5–5-based trimer likely indicates that its formation is predicated on the special intramolecular nature of the 5–5-dehydrodimer.

Conclusions

The first ferulate dehydrotrimer has been isolated and identified from maize cereal grain. As it stands, it is most likely that it still represents cross-linking of only two polysaccharide chains. Nevertheless, its isolation and identification is important as it extends the role of ferulates in cell wall cross-linking, and provides circumstantial evidence that previous modeling observations may be correct, i.e. that 5–5-dehydrodiferulates may form intramolecularly. The race will now be on to see if other dehydrotrimers or indeed higher oligomers can be isolated, and to define their roles in cross-linking the cell wall.

Signatures of Cinnamyl Alcohol Dehydrogenase Deficiency in Poplar Lignins

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Introduction

Angiosperm lignins are composed mainly of guaiacyl (**G**) and syringyl (**S**) units linked by labile ether bonds and/or resistant carbon-carbon linkages. The formation of coniferyl and sinapyl alcohols, the immediate precursors of **G** and **S** lignin units, requires the reduction of coniferaldehyde and sinapaldehyde. For many years, this enzymatic step has been thought to be catalyzed by an enzyme with broad specificity, cinnamyl alcohol:$NADP^+$ dehydrogenase (CAD, EC 1.1.1.195), capable of reducing both hydroxycinnamaldehydes. Various CAD isoforms involved in monolignol biosynthesis

have been implicated in a variety of species. The role of CAD in the formation of sinapyl alcohol has been recently revised by the discovery, in *Populus tremuloides*, of a novel enzyme, sinapyl alcohol dehydrogenase (SAD), which was suggested to be specifically involved in the reduction of sinapaldehyde. This hypothesis has lent support to a model in which coniferaldehyde is channeled to coniferyl alcohol and sinapyl alcohol via two metabolic pathways.

In the last few years, down-regulating the various enzymes of the lignin biosynthetic pathway by genetic transformation has proven to be an efficient way to appraise their specific roles in controlling the lignification process. This was made possible by in-depth structural analysis which revealed the structural peculiarities of the transformed lignins. The effect of CAD deficiency on angiosperm lignins has been investigated in brown-midrib sorghum, maize and Arabidopsis mutants, and in poplar, tobacco and alfalfa transgenic lines.

In order to clarify the respective role of CAD enzyme in poplar, we made a systematic and in-depth structural investigation of lignin in a series of transgenic poplar lines specifically down-regulated for CAD activity to a variable extent. We have recently identified thioacidolysis-derived syringyl indene derivatives which are diagnostic for the incorporation of sinapaldehyde into angiosperm lignins. By GC-MS monitoring of these indene derivatives, we demonstrate herein that the incorporation of sinapaldehyde into poplar lignins increases concomitantly with the CAD deficiency level.

Results and Discussion

A series of primary transformants deficient in CAD activity was produced in the poplar clone Ogy (*Populus deltoides* x *Populus nigra)* by the antisense strategy. The Klason lignin content of the poplar lines with CAD activity ranging between 40 and 100% of the control level was found close to 20% of the extract-free dry wood. In contrast, this value was reduced to 17-18% in all lines with severely down-regulated CAD activity (residual CAD activity lower than 10% of the control level). This result shows that down-regulating CAD activity in angiosperms provides a way to moderately reduce the plant lignin content. Such a moderate decrease is necessary when the objective is to simultaneously preserve the field performances of the plants and to obtain lignocellulosic material more susceptible to both industrial kraft pulping and cellulase hydrolysis.

Thioacidolysis was used to explore the specific structural traits of lignins in CAD-deficient poplar wood. This analytical degradation mainly provides thioethylated phenylpropanoid compounds **1G** and **1S** from conventional β–O–4-ethers **L1** in lignins (Fig. 1). These lignin-derived monomers, recovered as a pair of *erythro*/*threo*-isomers (chromatographic pairs **1G** and **1S** in Fig. 2), were released in substantially lower amounts from lignins of the poplar lines displaying a severe CAD deficiency. This result indicates that reduction in lignin levels in plants with down-regulated CAD activity is associated with a decrease in lignin units only involved in β–O–4-bonds, which are the parent structures of the main thioacidolysis monomers (structures **L1** in Fig. 1). This decrease was found to be much greater in **S**-units than in **G**-units.

Relative to the control, the thioacidolysis reaction mixture recovered from CAD-deficient poplar samples not only provided about 60% less conventional **1S** compounds, but also contained two new isomeric syringyl indene monomers (**2Sa** and **2Sb** in Figs. 1 and 2). While the conventional β–O–4-ethers **L1** in lignins yield the main **1G** and **1S** monomers, we recently established that the source of the indene derivatives **2Sa** and **2Sb** are the sinapaldehyde 8–O–4-coupled units (structures **L2** in Fig. 1). In the guaiacyl series, only one indene isomer **2Gb** (Figs. 1 and 2) could be observed as a trace

component. While these indene compounds could be satisfyingly determined on the GC-MS trace obtained from a milled wood lignin fraction isolated from a CAD-deficient poplar (Fig. 2A), these peaks were obscured by peaks from hemicellulose-derived products (Fig. 2B, peaks annotated with an asterisk) when thioacidolysis was run on the corresponding extract-free wood. However, the **2S** and **2G** indene isomers could be monitored, without any interference from other compounds, on selected-ion chromatograms reconstructed at *m/z* 384 and 354, which respectively correspond to the base peaks of their trimethylsilylated derivatives. By so doing, we could determine that the levels of the indene syringyl compounds **2S**, relative to conventional syringyl monomers **1S**, increased together with the degree of CAD deficiency (Fig. 3). Moreover, this increase was observed before any wood phenotype (red coloration of the xylem) could be seen or before any other lignin structural alteration could be detected (eg line ASCAD8,3 with 44% residual CAD activity, Fig. 3). In poplar lines with residual CAD activity lower than 10% of the control level (Fig. 3 and other data not shown), these isomers averaged 5-8% of the conventional **S**-monomers while recovered in trace amounts in the lines with CAD activity ranging between 40 and 100% of the control level. These indene compounds can thereby be considered as a sensitive signature of CAD deficiency that can be used by researchers to monitor the CAD deficiency level in transgenic angiosperms, from a few milligrams of cell walls.

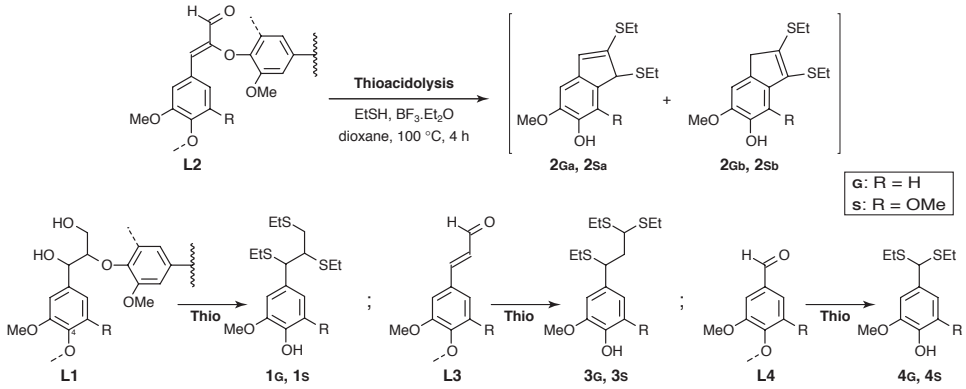

Figure 1. Structures in lignins and their main thioacidolysis products. Conventional β–O–4-ethers **L1** in lignins yield the conventional thioacidolysis monomers **1G** and **1S** (as isomeric pairs, see Fig. 2). Hydroxycinnamaldehydes 8–O–4-coupled into lignins **L2** yield the diagnostic indene markers **2G** and **2S**. Hydroxycinnamaldehyde end-groups **L3** produce the dithioketal products **3G** and **3S**, whereas hydroxybenzaldehyde end-groups **L4** produce the dithioketals **4G** and **4S**. For all compounds, **G** is for R = H and **S** is for R = OMe.

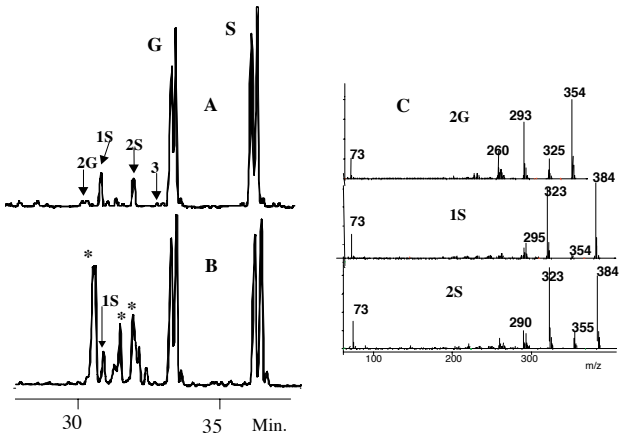

Figure 2. Partial GC-MS trace showing the separation of the main thioacidolysis monomers **1G** and **1S** (analyzed as their TMS derivatives) recovered from A) a soluble lignin fraction isolated from a CAD deficient poplar wood and from B) the corresponding extract-free wood. Peaks labeled **1G** and **1S** correspond to the *erythro*/*threo* pairs of isomers **G**–CHR–CHR–CH_2R and **S**-CHR–CHR–CH_2R (R = –S–CH_2–CH_3), respectively. Peaks labeled **2Sa** and **2Sb** are two syringyl indene isomers specifically released from sinapaldehyde 8–O–4-linked units and peak **2Gb** is the guaiacyl analogue of **2Sb**. Peaks **3G** and **3S** correspond to the dithioketal derivatives released from coniferaldehyde and sinapaldehyde end-groups and are recovered in low and trace amounts, respectively. On the B trace, peaks with asterisks are degradation products released from hemicellulosic components. The determination of compounds **2S** and **2G,** which is not possible from the total ion chromatograms due to peak overlap, is therefore carried out on selected-ion chromatograms reconstructed at *m/z* 384 and 354. Compound structures are given in Fig. 1.

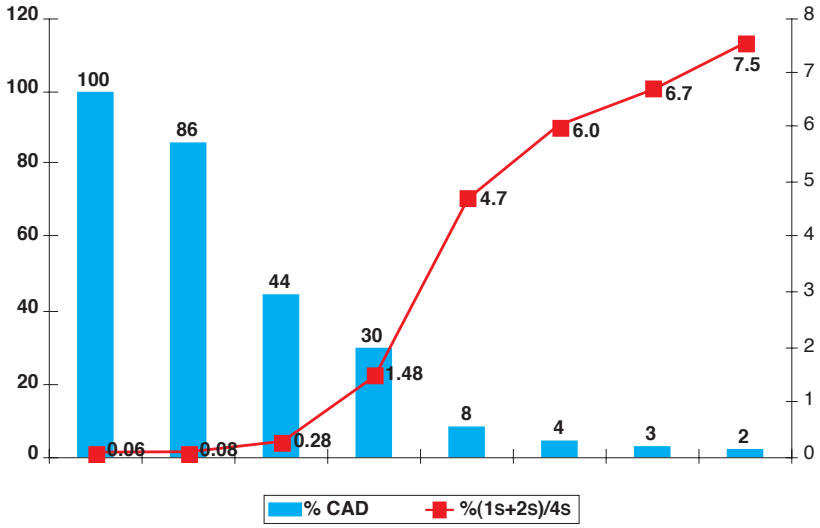

Figure 3. Relative levels of the thioacidolysis markers to conventional monomeric products [%(**2Sa**+**2Sb**)/**1S**] depends on the residual CAD activity level (100% = control level). The sample with 44% residual activity does not show any red xylem phenotype. Samples with <10% activity are different OGY70ASCAD lines.

These results provide evidence that down-regulating CAD activity in poplar specifically causes the incorporation of sinapaldehyde into lignins. This incorporation primarily occurs through 8–O–4-cross-coupling, analogously with the conventional monomer, sinapyl alcohol. The incorporation of sinapaldehyde as end-groups (structure **L3** with R = OMe, Fig. 1) happens only to a negligible extent, as evidenced by the trace amount of the dithioketal derivative **3S** (Fig. 1). In contrast, this dithioketal derivative and its analogue methylated at C4 were recovered as the main monomers (50% to 80% recovery yield) from sinapaldehyde and from 3,4,5-trimethoxy-cinnamaldehyde that was used as a representative of sinapaldehyde end-groups.

While CAD deficiency induced the substantial incorporation of sinapaldehyde through 8–O–4-coupling in poplar lignins, the level of coniferaldehyde was not substantially increased, whatever its bonding mode. In the CAD deficient lines and relative to the control, the marker compound **2G** released from 8–O–4-linked coniferaldehyde units **L2** (Fig. 2) was observed to occur in much lower amount than the analogues **2S**. The product **3G** originating from coniferaldehyde end-groups was recovered in similar amount in the transgenic and control lines. As these end-groups characteristically stain with phloroglucinol-HCl, the phloroglucinol-HCl staining reaction proved to be ineffective to discriminate CAD-deficient and control lines; the reagent does not stain the hydroxycinnamaldehydes incorporated into 8–O–4-structures. In addition to the thioacidolysis compounds released from *p*-hydroxycinnamaldehyde units **L2** and **L3**, we estimated the relative importance of a dithioketal derivative **4S** (Fig. 1). This syringaldehyde-derived marker was found to be released in higher amount from the CAD deficient lines, with recovery yields that approximate that of the sinapaldehyde-derived markers. The yield for the vanillin dithioketal derivative **4G** was not substantially increased by CAD deficiency (data not shown). As a model dimer for 8–O–4-linked sinapaldehyde almost quantitatively yielded the indene derivatives **2S** and only trace amount of **4S,** this syringaldehyde dithioketal most likely originates from syringaldehyde end-groups (structure **L4** in Fig. 1, with R = OMe). Syringaldehyde might originate from the oxidative degradation of accumulated sinapaldehyde monomers under the conditions prevailing during lignin polymerization. This aldehyde would be incorporated into the lignin polymer as end-groups, a hypothesis supported by the recovery of deuterated syringyl C_6C_1 derivatives from the thioacidolysis of $NaBD_4$-reduced CAD-deficient samples (data not shown).

Overall, the data indicate that the CAD down-regulation event obtained herein in poplars more specifically impacts the formation of conventional **S**-lignin units while that of conventional **G**-lignin units is affected to a much lower extent. That mainly sinapaldehyde incorporates into the lignins of CAD-deficient poplars suggests that the recently identified sinapyl alcohol dehydrogenase (SAD), which is structurally distinct from the CAD enzyme targeted herein, does not play any substantial role in constitutive lignification in poplar. The specific incorporation of sinapaldehyde into the lignins of CAD-deficient poplars may be the consequence of the F5H and COMT enzyme activities that channel coniferaldehyde to sinapaldehyde. From the coniferaldehyde pool which might transiently increase as a consequence of CAD deficiency, these F5H and COMT activities would lead to unusually high levels of sinapaldehyde. This sinapaldehyde could be stored and/or transported to the lignifying cell walls, possible as the glucoside, in a similar way as the corresponding alcohol. It could be the substrate of peroxidases and thereby incorporated into lignins. Another hypothesis to account for the preferred incorporation of sinapaldehyde over coniferaldehyde might be its higher oxidizability. The analysis of soluble phenolic compounds could be of considerable interest in order to determine whether transgene expression might affect the pool of phenolic metabolites.

Conclusions

Thioacidolysis-derived indene marker compounds are valuable for ascertaining plant responses to various levels of CAD down-regulation. Beside these additional sinapaldehyde-derived units and relative to the control samples, lignins in CAD-deficient poplar lines had less conventional **S**-units and β–O–4-bonds and more free phenolic groups. We found that almost half of the polymer fraction in the most deficient lines could be solubilized in alkali at room temperature. This unusual behavior suggests that lignins in CAD-deficient poplars occur as small, alkali-leachable lignin domains.

What Makes a Good Monolignol Substitute?

(Commentary)

John Ralph

Among the more intriguing components in the cell wall are the lignin polymers. Their formation, occurring after the polysaccharides are laid down in the wall, provides structural integrity to those lignified cell walls, facilitates water transport, and provides defensive functions. But what makes the polymers enigmatic is their mode of formation. Unlike the polysaccharides and proteins, no exact chemical "sequence" of units is dictated by the cell. Although there is considerable control by the cell over aspects of the structure by the supply of the various lignin monomers to the wall and the supply and control of oxidants (in the peroxidase-H_2O_2 system), the assembly of the polymer is a combinatorial process under the whim of simple chemical control, i.e. governed by normal chemical concerns such as the concentrations of reactants, their natural coupling and cross-coupling propensities, the matrix, and the physical conditions during the polymerization. The process is not under the control of enzymes, for example, and no two lignin molecules need have the same structure. This theory has been recently challenged, and even a text book has pronounced that lignification is a process under absolute structural control, but this challenge is wholly without merit and can be simply dismissed.

In addition to providing a flexible system for the plant to respond to various stresses, such a polymerization mode provides unparalleled opportunities to re-engineer this component of the wall. Nature herself has explored many variations on the theme already. Certain plants use monomers that

most texts would not consider to be lignin monomers; it is widely assumed that lignins derive from only three monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols. And nature of course experiments with genetic engineering. Mutations sporadically appear in which "crucial" lignin-biosynthetic-pathway genes are knocked out. Some such plants have made substitutions for the monolignols whose biosynthesis has been negatively impacted.

The process of lignification has been well implemented. In what remains best described as a process of radical coupling of phenols, the monolignols are rather ideal monomers. Like most other chemical polymerizations, the primary polymerization reaction involves the coupling of a monomer with the growing polymer (Fig. 1a); monomer-monomer reactions certainly occur but are less significant in the overall scheme. Polymer-polymer (or oligomer-oligomer) coupling reactions play a significant role in lignification, creating branching points in the polymer. The radical coupling process is what allows the polymerization to be somewhat combinatorial. The monolignols are potentially capable of coupling at various sites (β-, 4–O-, 1-carbons for coniferyl and sinapyl alcohols, and additionally the 5-carbon for coniferyl alcohol, but almost overwhelmingly react at their β-position with phenolic ends on the growing polymer. This conveys a certain linearity to the polymer. The phenolic end can couple at its 4–O- and, less frequently, 1-positions, and/or (in the case of guaiacyl units only) the 5-position. In many ways it is the favored β–O–4-coupling of a monolignol with the growing polymer that characterizes lignins, but it is the other reactions including β–5- and β–1-coupling, as well as 5–5- and 4–O–5-coupling between oligomers, that defines them.

So what kinds of monomers can substitute for the primary monolignols? From studies to date, only those that are capable of undergoing analogous β–O–4-coupling with the growing polymer seem to be successful. This may be the major requirement, although it still provides considerable variability, as will be seen below. Plants which have available, and apparently attempt to utilize, monomers that can not undergo this type of coupling reaction fare more poorly, presumably because their lignin substitutes do not adequately exhibit the properties required of them.

A pertinent example is in a viable but unhealthy CAD-deficient mutant pine. CAD is the last enzyme on the monolignol biosynthetic pathway, reducing coniferaldehyde to coniferyl alcohol in softwoods. Coniferaldehyde might be anticipated to build up in this plant, and evidence is that it does. On paper, coniferaldehyde appears to be a good candidate for a lignin monomer. It doesn't have the alcohol group, but it has the requisite conjugated double bond of the cinnamyl unit, and it was early on shown that it undergoes the same range of monomer-monomer coupling reactions as coniferyl alcohol, forming its own versions of β–O–4-, β–5-, and β–β-dehydrodimers. It could even make a dehydrogenation polymer. The drawback, as was not revealed until later studies on angiosperms, is that coniferaldehyde simply will not chemically β–O–4-cross-couple with phenolic guaiacyl units in the polymer. It is purely a chemistry problem; it will undergo such coupling with suitably unsaturated units, but with a normal guaiacyl unit, the reactants are not sufficiently chemically compatible to react either *in vitro* or, apparently, *in vivo*. Since the CAD-deficient pine still makes some coniferyl alcohol, the polymer needs to incorporate monomers compatible with a normal guaiacyl lignin. Coniferaldehyde simply does not comply. It therefore becomes relegated to homo-coupling reactions and cross-coupling reactions solely with the monolignol coniferyl alcohol, and is therefore left adorning only the periphery of the lignin molecule (as end-groups) and not significantly incorporating into the polymer chain. Interestingly, this pine also produces, for unknown reasons, high levels of dihydroconiferyl alcohol. It also finds itself in the lignin. Obviously dihydroconiferyl alcohol cannot couple at its β-position — there is no way to get the required single electron density to the β-position without the presence of the double bond. It therefore is also limited to a set of reactions that do not incorporate it into the lignin chain, again relegating it to the periphery of the structure. Nevertheless, it is found in the lignin at striking

levels due to its ability to still 5- and 4–O-couple with guaiacyl units, as well as with the monolignol, coniferyl alcohol. This plant then appears to have attempted to augment its polymer by substituting coniferaldehyde and dihydroconiferyl alcohol monomers for some of the coniferyl alcohol. It remains viable, but not vigorous.

The above example would be unfulfilling if it were not for the fact that coniferaldehyde is a well-behaved monomer in angiosperms. How can this be? Again, it is simple chemistry. Angiosperms have guaiacyl/syringyl lignins (deriving from both coniferyl and sinapyl alcohols). Coniferaldehyde readily undergoes β–O–4-cross-coupling reactions with syringyl end-groups. As it turns out, sinapaldehyde readily undergoes cross-coupling with either guaiacyl or syringyl end-groups. As a result, both coniferaldehyde and sinapaldehyde couple in a similar way as the monolignols do, and can therefore incorporate integrally into the body of the polymer in CAD-deficient angiosperms. These details are revealed by NMR studies on the lignins, and the release of thioacidolysis marker compounds specifically from these hydroxycinnamaldehyde-β–O–4-linked units. The plants appear to grow essentially normally, but the hydroxycinnamaldehydes may not be quite perfect monomers. It appears that coupling to the new type of conjugated β–O–4-phenolic end-units that these create (see Fig. 1c), the next step in the polymerization, becomes difficult. The result is that many of the incorporated coniferaldehyde/sinapaldehyde groups remain as free-phenolic end-units, limiting the degree of polymerization of the lignin. In addition to the property changes caused by the structural changes, these lower molecular weight lignins are presumably less ideal for the plant. An interesting side-benefit however is that the lignins are much more easily broken down and removed in chemical pulping, so plants with limited CAD-deficiency are being pursued for their enhanced pulping potential.

Beyond the (partial) substitution of the hydroxycinnamaldehydes for their hydroxycinnamyl alcohol monolignol analogs in CAD-deficient angiosperms is a particularly successful substitution in the case of COMT-deficiency. COMT is a methyl transferase enzyme necessary for the biosynthesis of sinapyl alcohol and ultimately syringyl groups in lignins. It operates primarily at the aldehyde level, i.e. with 5-hydroxyconiferaldehyde as its substrate. Knock-out mutants are essentially or totally devoid of syringyl components, and COMT down-regulation will reduce the syringyl content. As CAD-deficient angiosperms incorporated the immediate CAD precursor (the hydroxycinnamaldehydes), COMT-deficient plants must deal with the un-methylated 5-hydroxyconiferaldehyde precursor. Apparently CAD is able to reduce this aldehyde as it is 5-hydroxyconiferyl alcohol, not the aldehyde, that is exported to the wall and incorporated into lignins. 5-Hydroxyconiferyl alcohol has all the makings of an ideal monolignol substitute. It beautifully β–O–4-couples with guaiacyl, syringyl, or new 5-hydroxy-guaiacyl phenolic endgroups, integrating into the polymer as would a primary monolignol (Fig. 1d). The lignins' structures become strikingly different however. The presence of the extra phenolic OH, the 5-OH, drastically affects the post-coupling reactions. Novel benzodioxane units are formed in the polymer as a result of incorporating 5-hydroxyconiferyl alcohol, at striking levels (essentially replacing the syringyl units in the control plants). The plant does not seem to mind; COMT-deficient plants appear to grow essentially normally. In this case, the severe structural changes are a serious detriment to chemical pulping. Despite still being β-ethers, these units will not efficiently cleave under pulping conditions, as do the syringyl units which they displace. However, COMT-deficient plants appear to be more digestible. A possible reason is that, by providing a rapid alternative internal pathway for rearomatizing the quinone methide intermediate, these units cannot cross-link with polysaccharides in the wall (via addition to quinone methides). Lignin-polysaccharide cross-linking has been shown to have a significant effect on cell wall digestibility.

Another class of successful lignin precursors are the variously acylated monolignols implicated in an assortment of plants. Sinapyl acetate has been demonstrated to be a monomer in kenaf bast fiber

and palm lignification, and presumably at low levels in other hardwoods. Sinapyl *p*-hydroxybenzoate is similarly a monomer in palm, aspen, poplar and willow lignification. And sinapyl *p*-coumarate (as well as lower levels of coniferyl *p*-coumarate) analogously contribute to lignification in maize and, presumably, in all grasses. What benefits the plant receives from such lignin acylation are little understood. *p*-Hydroxybenzoate and *p*-coumarate are excellent substrates for many of the plant (and fungal) peroxidases which only slowly oxidize sinapyl alcohol. And, since they form less stable radicals, they readily undergo radical transfer with, for example, sinapyl alcohol, generating the sinapyl alcohol radical required for its incorporation into the polymer. Their roles as radical transfer agents make them a sort of catalyst for lignification, especially with respect to sinapyl alcohol monomers. Despite being phenolic, they do not undergo coupling reactions and are found adorning lignins as pendant free-phenolic entities. In model reactions, it is not until other phenolics in the system are depleted that these components couple. Their presence in free-phenolic form provides evidence that the cell limits radical concentrations. These phenolic esters then may have a role in facilitating the oxidation of sinapyl alcohol and oligomer substrates. The role of the acetylated sinapyl alcohol in kenaf is even less understood. Over 50% of the monolignols in the kenaf bast fiber are acetylated. Presumably the resultant polymer is more hydrophobic than normal lignins, so the acetylation may be associated with drought tolerance.

Whatever the reasons for these acylated components, the three types of acylated monolignols (acetate, *p*-hydroxybenzoate, and *p*-coumarate) are now well implicated as authentic lignin precursors in their respective plants. It is just becoming apparent by in vitro studies that the acylation does not significantly affect the course of the coupling reactions (Fig. 1b). That means that the acylated monolignols also behave nicely as lignin monomers, most importantly undergoing the β–O–4-coupling reactions that incorporate them into the chain of the polymer. Acylated monolignols alter the structure of the lignins by more than just adorning them with pendant groups, however. This is because the γ-OH group functions in some post-coupling reactions, internally trapping the quinone methide following β–β-coupling, for example (not shown). With the γ-OH group acylated, such internal reactions are no longer possible, and the quinone methide must be rearomatized by trapping an external nucleophile, usually water, and forming quite different products in the lignin as a result. Additionally, the stereochemistry of water attack on the quinone methide intermediate following β–O–4-coupling is altered. The lignins therefore differ form "normal" lignins in both substantial and subtle ways.

Conclusions

A small range of monomers are now known to substitute for the conventional monolignols in various natural and transgenic plants. Monolignol substitution appears to be most successful when the novel monomer behaves, in its chemical radical coupling and cross-coupling reactions, like a normal monolignol. Most important is the β–O–4-coupling reaction with the phenolic end of the growing polymer to extend the polymer chain. The post-coupling reactions that may be altered by the different functionality on the monomer seem to have less effect. Thus massive changes in the lignin structure occur when 5-hydroxyconiferyl alcohol substitutes for sinapyl alcohol, for example — the coupling reactions are analogous, but post-coupling steps produce novel benzodioxane structures that drastically change the lignin. Observations that plants with monolignol substitution and profoundly altered lignin structure can fare well supports the heretical tongue-in-cheek idea expressed at a conference some time back that the actual structure of lignins is not that important to the functioning of the plant. The plant requires certain properties and functionality of its lignins, but expends no resources dictating those properties by exactly stipulating its primary structure. Such biosynthetic malleability functions well for the plant, but also provides significant opportunities for engineering the polymer. Already it

has been demonstrated that natural and industrial processes ranging from ruminant digestibility to chemical pulping can be both positively and negatively impacted by alterations to lignin composition and structure. It is also apparent that phenolic components from beyond the monolignol pathway itself may be incorporated into lignins if they have compatible reaction chemistry and are transportable to the wall. Future work should reveal opportunities beyond the interesting deviations achieved by up- and down-regulating genes on the monolignol pathway to date.

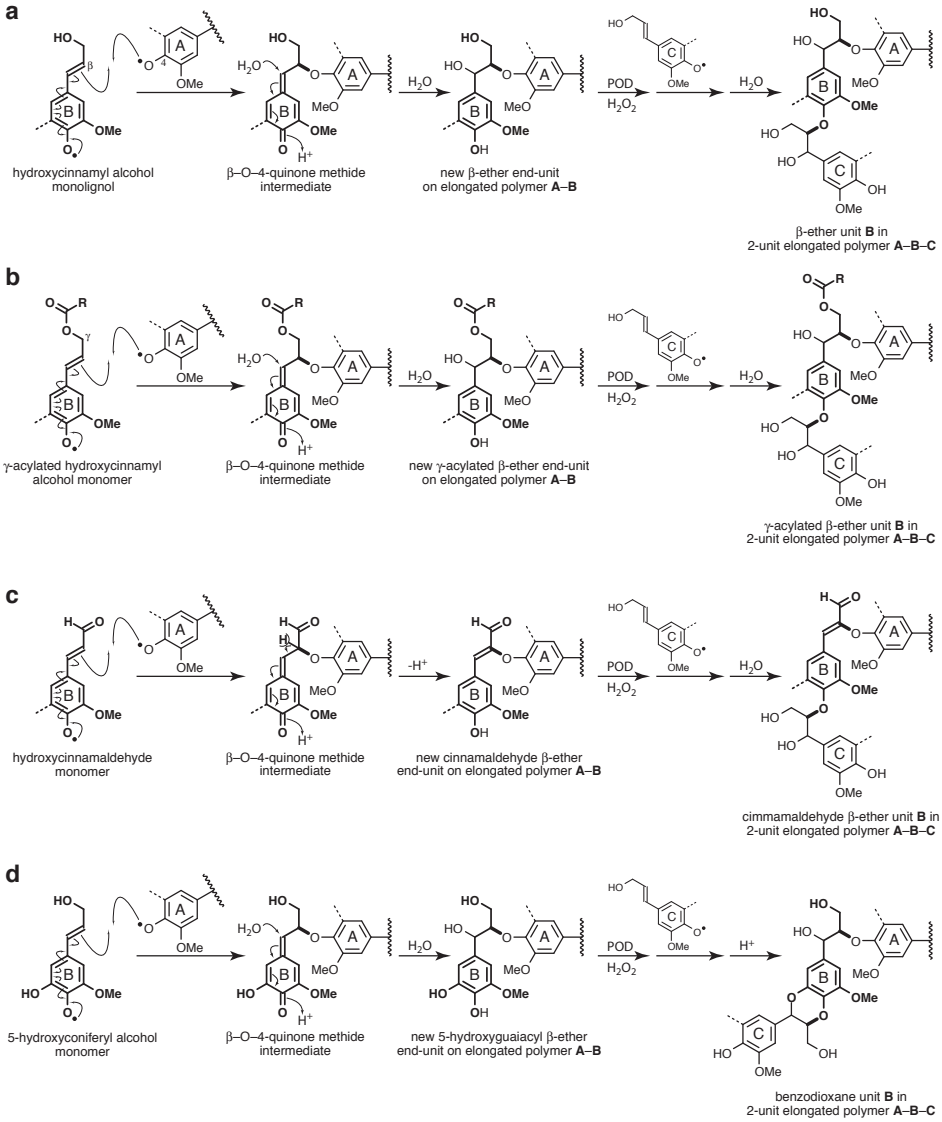

Fig. 1. Differences in cross-coupling and post-coupling reactions for various well-suited "monomers" incorporated into lignification. Illustration is for the major β–O–4-coupling only. a) Normal hydroxycinnamyl alcohol radicals **B** cross-couple with the phenolic end of the growing polymer **A**, mainly by β–O–4-coupling, to produce an intermediate quinone methide which rearomatizes by nucleophilic water addition to produce the elongated lignin chain **A**–**B**. The subsequent chain elongation via a further monolignol radical **C** etherifies the unit created by the prior monomer **B** addition, producing the 2-unit-elongated polymer unit **A**–**B**–**C**. b) Various γ-acylated monolignols (*p*-coumarate, *p*-hydroxybenzoate, and acetate) cross-couple equally well producing analogous products but with the β-ether unit **B** γ-acylated in the lignin polymer unit **A**–**B**–**C**. c) Hydroxycinnamaldehydes **B** may also cross-couple with the phenolic end of the growing polymer **A**, again mainly by β–O–4-coupling, to produce an intermediate quinone methide again, but one which rearomatizes by loss of the acidic β-proton, producing an unsaturated cinnamaldehyde-β–O–4-linked **B** end-unit. Incorporation further into the polymer by etherification is analogous to a). The unsaturated aldehyde units **B** give rise to unique thioacidolysis markers. d) 5-Hydroxyconiferyl alcohol monomer **A** also cross-couples with the phenolic end of the growing polymer **A**, mainly by β–O–4-coupling, to produce an intermediate quinone methide as usual which rearomatizes normally by nucleophilic water addition to produce the elongated lignin chain **A**–**B** bearing a novel 5-hydroxyguaiacyl phenolic end-unit. The subsequent chain elongation via a further monolignol radical **C** coupling β–O–4 to the new phenolic end of **A**–**B**, but this time the rearomatization of the quinone methide (not shown) is via internal attack of the 5-OH producing novel benzodioxane units **B**–**C** in the 2-unit-elongated polymer unit **A**–**B**–**C**. 5-Hydroxyconiferyl alcohol incorporation produces a lignin with a structure that deviates significantly from the "normal" lignin. The bolded bonds are the ones formed in the

RUMEN MICROBIOLOGY

Effect of pH on Bovicin HC5, a Bacteriocin from *Streptococcus bovis* HC5

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Introduction

Cattle are often fed antibiotics to improve feed efficiency. However, in recent years there has been an increased perception that the use of any antibiotic in animal feed leads to proliferation of antibiotic-resistant pathogens, and some groups have called for a widespread ban of antibiotics as growth promotants. Many Gram-positive bacteria produce bacteriocins, and these peptides are not considered classical antibiotics. Some ruminal bacteria produce bacteriocins, and they have been proposed as an alternative to antibiotics currently used in animal feed.

Streptococcus bovis is a rapidly growing, Gram-positive ruminal bacterium, and early work indicated that some strains produce bacteriocins. Approximately 50% of *S. bovis* strains isolated from the rumen had antimicrobial activity, but some strains were distinctly more active. *S. bovis* HC5 was the best strain, and its bacteriocin (bovicin HC5) had a broad spectrum of activity. Bovicin HC5 inhibited the methane production of mixed ruminal bacteria, and ammonia production by the amino acid-fermenting ruminal bacterium, *Clostridium aminophilum*. These results supported the idea that bacteriocins have the potential to be used as ruminal additives.

Many bacteriocins have higher activity under low pH conditions, but this effect has not been well defined. Acidic pH can affect peptide stability, bacteriocin release from the cell surface, post-translational modifications or membrane activity. Previous continuous culture experiments indicated that production of bovicin HC5 by *S. bovis* HC5 was maximal at pH 5.5, but the effect of pH on its activity per se was not determined. The following experiments were designed to assess the effect of pH on the activity of bovicin HC5. Because HC5 causes the efflux of potassium from *S. bovis* JB1, we used potassium depletion as an index of activity.

Methods

S. bovis JB1 and HC5 were routinely grown under O_2-free CO_2. Growth was monitored via changes in optical density (1 cm cuvette, 600 nm, Gilford 260 spectrophotometer). Bovicin HC5 was liberated from *S. bovis* HC5 by a procedure involving acidic NaCl. The preparation was assayed for antibacterial activity by serially diluting the extract in distilled water (2-fold increments), and placing each dilution (10 μl) on a lawn of *S. bovis* JB1 (approximately 10^5 cfu ml^{-1}). *S. bovis* JB1 cultures were harvested anaerobically and treated with semi-purified bovicin HC5. The cell suspensions were centrifuged through silicone oil and the tubes were frozen. Once the liquid above the silicone was solid, cell pellets were removed with dog nail clippers. Cell pellets were digested in 3 N HNO_3. The intracellular potassium concentration was determined by flame photometry. Cells were assayed for ATP using the firefly luciferine-luciferase.

Results and Discussion

The bacteriocin, bovicin HC5, catalyzed potassium efflux from *Streptococcus bovis* JB1, and this activity was highly pH-dependent. When the pH was near neutral, glucose energized cells were not affected by bovicin HC5, but the intracellular steady state concentration of potassium decreased at acidic pH values. The idea that pH was affecting bovicin HC5 activity was supported by the observation that acidic pH also enhanced the efflux of potassium from non-energized cells that had been loaded with potassium. The relationship between bovicin HC5 concentration and potassium depletion was a saturation function, and cooperativity plots indicated that the binding of one bovicin molecule to the cell membrane facilitated the binding of another. Under normal feeding conditions, the rumen is not highly acidic, but silage fermentations can have pH values as low as 4.0. R.E Muck and his colleagues previously noted that *S. bovis* had potential as a silage inoculant, but they did not test the ability of their *S. bovis* strains to produce bacteriocins. Our work indicates that *S. bovis* HC5 can inhibit detrimental silage bacteria.

Conclusion

The bacteriocin of *S. bovis* is much more active at low pH, and this effect would enhance its ability to serve as a silage inoculant.

Effect of a Bacteriocin (bovicin HC5) on *Clostridium sporogenes* MD1, a Bacterium That has the Ability to Degrade Amino Acids in Ensiled Plant Materials

Michael D. Flythe and James B. Russell

Introduction

Researchers have developed silage inoculants to augment the natural microflora of plant materials. These inoculants are typically lactobacilli, but R.E. Muck and his colleagues noted that the ruminal bacterium *Streptococcus bovis* might also be useful. *S. bovis* "grew faster than any of the commercial species tested and resulted in the most homolactic fermentation," but bacteriocin production was not determined. Recent work indicated that many *S. bovis* strains from the bovine rumen produced bacteriocins, and the strain with the greatest activity was designated as HC5. Later work revealed that the bacteriocin of *S. bovis* HC5 (bovicin HC5) inhibited a variety of Gram-positive bacteria, but its effect on silage clostridia was not determined. The following experiments sought to: 1) isolate clostridia from fresh plant materials and silages, 2) examine the ability of these bacteria to ferment amino acids at low pH, and 3) determine their susceptibility to bovicin HC5.

Methods

Fresh, immature finely chopped alfalfa plants, freshly chopped corn, corn silage and alfalfa haylage samples (1 g) were added to tubes containing an anaerobic amino acid mediium. The tubes were subjected to a heat shock (80˚ C, 20 min). Once the tubes had cooled to room temperature, the sub-samples (1 ml) were serially diluted (10-fold increments) into sterile basal medium. The dilutions were spread on plates and incubated at 39˚ C. Large colonies appeared after approximately 48 h. All of the colonies had the same morphology and all of the isolates were rod-shaped bacteria with spores.

The bacteria were grown in continuous culture and pH was adjusted by adding HCl to the culture vessel via a pH-controlled peristaltic pump. Ammonia was assayed by a colorimetric method. The 16S rRNA gene region was amplified and the sequences were subjected to an NBLAST. A phylogram was created using the neighbor-joining function of Clustal X. Cultures were assayed for toxins by Dr. R. H. Whitlock (University of Pennsylvania, Kennett Square, PA) using a mouse protection bio-assay that has been previously described. Fermentation acids in cell-free supernatant samples were analyzed by high-performance liquid chromatography.

Results and Discussion

Fresh plant materials can be fermented and preserved as silage for cattle, but clostridia that deaminate amino acids increase pH. If the pH of the silage rises, spoilage microorganisms proliferate, and undesirable products accumulate. Rod-shaped, anaerobic bacteria with spores were isolated from fresh alfalfa, fresh corn, and silages. Strain MD1 had the highest specific activity of amino acid deamination, and it was most closely related to *Clostridium botulinum* A and B. However, MD1 did not produce a toxin. Opinion 69 of the Judicial Commission of the International Committee on Systematic Bacteriology states that the *C. botulinum* designation should be reserved for toxin producing strains, and *C. sporogenes* should be reserved for "non-toxigenic strains." Based on these results, MD1 was classified as *Clostridium sporogenes*.

Washed cell suspensions of *C. sporogenes* MD1 had specific activities as great as 690 nmol ammonia mg protein^{-1} min^{-1}, and this rate did not decrease until the pH was less than 4.5. Batch cultures of *C. sporogenes* MD1 did not initiate growth if the initial pH was less than 5.0, but continuous cultures (0.1 h^{-1} dilution rate) persisted until the pH of culture vessel was 4.6. When *C. sporogenes* MD1 was co-cultured with a bacteriocin producing *Streptococcus bovis* strain (HC5), ammonia production was greatly reduced. The ability of *S. bovis* HC5 to inhibit MD1 was pH-dependent. When the pH was 5.5 or less, MD1 could no longer be detected. Further work will be needed to assess the impact of bacteriocin producing bacteria on silage fermentations. However, all of our amino acid fermenting isolates were inhibited by bovicin HC5.

C botulinum is not typically found in properly prepared silages; but those contaminated with animal carcasses (e.g. dead birds) often have *C. botulinum* type C. The impact of *S. bovis* HC5 on *C. botulinum* was not determined, but it should be noted that *C. sporogenes* MD1 is very closely related to *C. botulinum* type A and B. If *S. bovis* HC5 inhibits *C. botulinum*, the use of *S. bovis* HC5 as a silage inoculant could have yet another positive role.

Conclusions

Bacteriocin-producing bacteria may be used to improve silage quality.

Characterization of the Glycocalyx of the Ruminal Cellulolytic Bacterium *Ruminococcus albus*

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Introduction

Ruminococcus albus is a ruminal bacterium capable of fermenting cellulose and hemicelluloses to ethanol, acetic acid, H_2 and CO_2. Like most anaerobic cellulolytic bacteria, this species presents most of its cellulase enzyme activity on the cell surface to maximize contact with the insoluble cellulose substrate. *R. albus* adheres tenaciously to the cellulose via a glycocalyx (extracellular polymeric substance, EPS), and glycocalyx synthesis appears to be a critical early step in cellulose degradation. Moreover, we have previously shown that the residue of cellulosic fermentations (containing bacterial cells, glycocalyx, and partially fermented cellulosic substrate) may have utility as an adhesive for plywood manufacture. Despite these interesting properties, the composition and structure of the glycocalyx is largely unknown. We used a combination of electron microscopy and chemical analysis to characterize the glycocalyx of this organism.

Methods

R. albus 7 was grown in a modified Dehority medium under CO_2, in 160 ml serum vials or a 40 liter glass carboy. Whatman #3 filter disks and Sigmacell 50 microcrystalline cellulose powder were used as substrate for electron microscopy and chemical analysis experiments, respectively. After 30 to 96 h incubation, the residual solids (cells plus glycocalyx plus unfermented substrate) were collected by decantation and centrifugation. Samples were prepared for electron microscopy by Au sputter coating under vacuum, with or without prior critical point drying under CO_2, and samples were visualized with a Leo VP1600 scanning electron microscope. Chemical analyses were performed on residues before or after boiling for 1 h in neutral detergent or acid-detergent solution, or by extraction with 0.1 *N* NaOH for 1 h at 70 °C. The residues were hydrolyzed to monosaccharides with trifluoroacetic acid (TFA) at various concentrations (0.1 to 2.0 *N*) at 120 °C for 90 min, prior to separation and quantitation of the neutral sugars by ion chromatography, or by gas chromatography as their alditol acetates. Carbohydrates were determined by the phenol/sulfuric method, and uronic acids were determined by the phenol/boric acid/sulfuric acid method. Proteins determined by Coomassie blue binding.

Results and Discussion

During early stages of growth, cultures displayed preferential degradation of cellulose fibers within crevices along the fiber axis, rather than at the cut ends of fibers (Fig. 1A). Later growth was accompanied by formation of thin cellular extensions that adhered to cellulose, and which formed a ramifying network that interconnected individual cells to one another and to the unraveling cellulose microfibrils (Fig. 1B). Extraction of whole cultures (grown for 30 or 48 h) with 0.1 *N* NaOH released carbohydrate and protein in a ratio of 1:5. Treatment of adherent cells with a detergent solution at pH 7 removed almost all of the cells without removing the network of adhering noncellular material (Fig. 2). Subsequent treatment of this residue (cellulose plus glycocalyx, C+G) with 2 *N* trifluoroacetic acid (120 °C, 90 min) resulted in complete removal of the glycocalyx, and the released sugars were primarily Glc, along with substantial amount of Xyl and Man (two sugars not typically associated with bacterial glycocalyces). Only traces of Gal (the primary sugar associated with the cellulosome organelle of the phylogenetically related bacterium, *Clostridium thermocellum*) were detected. Separate treatment with increasing concentrations of TFA (0.1 to 2.0 *N*) increased the molar proportion of Glc and decreased

the molar proportion of Xyl (Fig.2), suggesting that a significant proportion of the Glc was derived from partially degraded cellulose, which is less amenable to hydrolysis than are heteropolysaccharides such as bacterial EPS.

Separate extraction of C+G with 0.1 *N* NaOH resulted in removal of both carbohydrate and protein in a ratio of ~10:1, suggesting that the EPS contained little protein. Calculations, based on the amount of carbohydrate and protein removed by these treatments and on the known energy requirements for biosynthesis of cellular components, indicate that the energetic demands of EPS synthesis for this organism depend on the extent of incorporation of partially degraded cellulose into the EPS. However, in all cases this represents only a small fraction (2 to 4%) of the anabolic ATP expenditure of the culture.

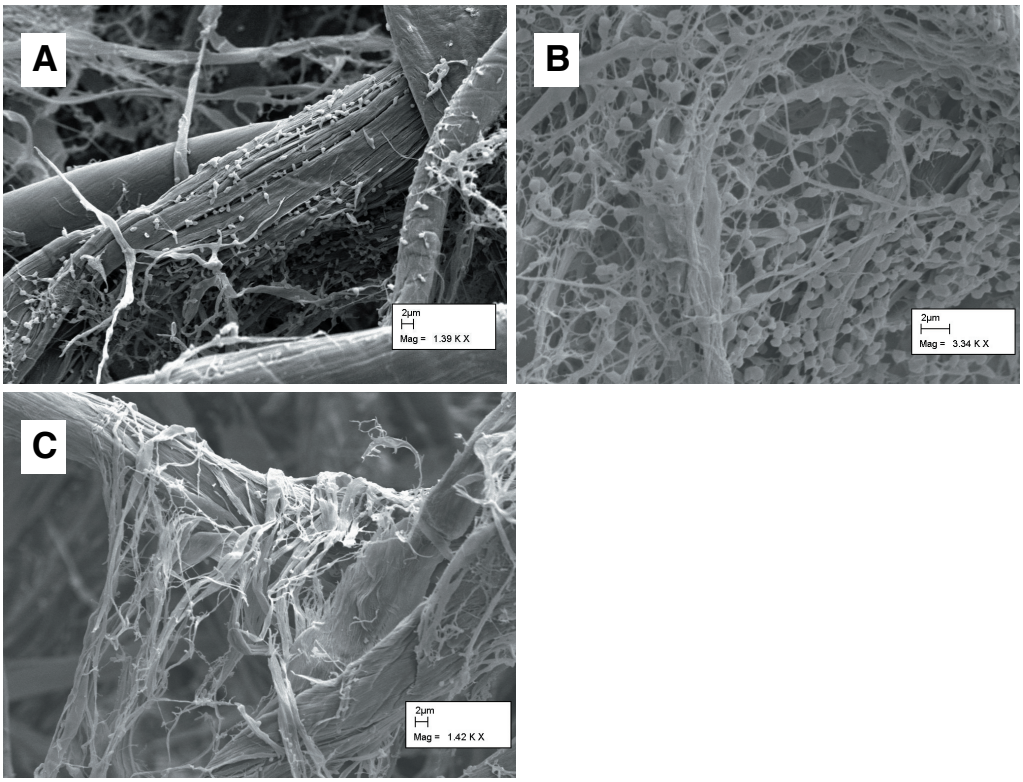


Figure 1. Scanning electron micrographs of *R. albus* 7 cells and associated glycocalyx adhering to cellulose fibers. A) Young cultures (24 h) showing preferential adherence in fiber crevices. B) Older cultures (48h) showing greater coverage of the fibers and formation of a ramifying network of cells, glycocalyx and partially-degraded cellulose. C) Residual cellulose, with adherent glycocalyx following removal of bacterial cells by boiling in neutral detergent solution.

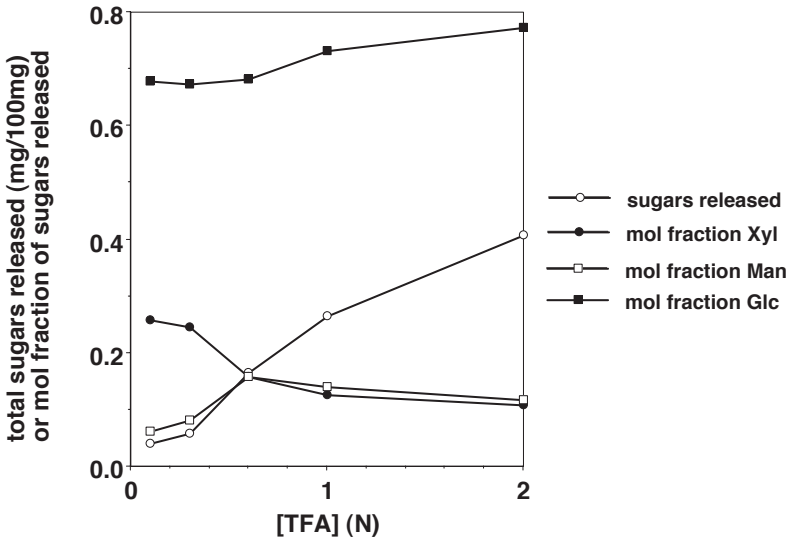


Figure 2. Release of sugars associated with the *R. albus* 7 glycocalyx by different concentrations of TFA (120 °C, 90 min). The cellulose fermentation residue was first boiled in neutral detergent solution to remove bacterial cells.

FORAGE QUALITY

Impact of the Bt Transgene on Lignin and Forage Quality of Corn Silage

H.G. Jung and C. C. Sheaffer

Introduction

Commercially available transgenic corn hybrids provide producers with herbicide tolerance and insect resistance. Insertion of the *cry*1 Ab gene for an insecticidal protein from *Bacillus thuringiensis* (Bt) into corn has resulted in hybrids resistant against European corn borer attack and has reduced insecticide application. These Bt hybrids accounted for approximately 29% of the corn acreage planted in the USA in 2003. While virtually all studies of transgenic corn have indicated no difference in nutritional quality, a report in 2001 indicated that nine Bt corn hybrids contained 33 to 97% more lignin in the bottom of the stem than isogenic conventional hybrids. If validated, this finding could be of concern because lignin is known to reduce the availability of cell wall polysaccharides for rumen digestion and the possibility of increased lignin concentrations occurring in Bt corn hybrids raises concerns about the feeding value of silage made from these genetically modified hybrids. Our objective was to determine if *cry*1 Ab transgenic corn hybrids contain more lignin than non-transgenic corn when grown in diverse field environments, and whether the trait affected plant yield and other indicators of feeding value such as in vitro ruminal digestibility, fiber, starch, and crude protein concentration. Because different procedures for measuring lignin content are known to provide different estimates, we compared the acid detergent, Klason, and acetyl bromide methods.

Materials and Methods

Twelve commercial corn hybrids adapted to Minnesota were planted in replicated trials at four locations. The hybrids included three incorporating the MON810 *cry*1 Ab transgene event (35R58, DKC44-42, DKC53-32) and their respective genetic controls (35R57, DK440, DK537); and three hybrids with the Bt11 *cry*1 Ab transgenic event (N2555Bt, N3030Bt, N45-A6) and their respective controls (N2555, N3030, N45-T5). Field plots were not treated with insecticide to control European corn borer. Fifteen randomly selected plants of normal appearance were harvested from the interior of each plot by cutting at ground level. Ten plants were chopped through a garden-style chipper/shredder, bulked and weighed, and a 1 kg subsample retained for analysis as whole plant samples. The fourth elongated, above-ground internode was excised from the stalks of the remaining five plants by cutting through the stem nodes. These internodes were bulked by plot. Prior to chopping, the stems of three maize stalks were split-open lengthwise and scored for how many internodes contained tunnels associated with European corn borer damage. Dried samples were analyzed for protein and starch by NIRS. Neutral (NDF) and acid detergent fiber, acid detergent lignin (ADL), Klason lignin, acetyl bromide lignin, and 24- and 96-h in vitro ruminal NDF digestibility were determined by wet chemistry.

Results and Discussion

The non-Bt corn hybrids showed signs of European corn borer damage (23 to 35% of plants sampled) whereas the Bt hybrids showed no damage, except for N2555Bt where one plant had some possible

stalk tunneling. Of the non-Bt plants that had stalk tunneling, one to four internodes in the stem were affected with a mean of 1.5 internodes/plant having tunnels. However, neither the presence of the Bt transgene or European corn borer damage affected corn silage yield because only small, inconsistent differences were detected between non-Bt/Bt hybrid pairs. This same pattern of no consistent impact of the Bt transgene was observed for whole corn plant protein, starch and fiber concentrations, and in vitro NDF digestibility (Table 1). For both whole corn plants and the lower stem internode, there was no trend for either increased or decreased lignin concentration in the Bt hybrids compared with their respective non-Bt hybrids (Table 2). This lack of a transgene effect on lignin concentration was observed for all three methods of lignin analysis. As expected, Klason lignin concentrations were greater than ADL values for the same samples, with acetyl bromide results being slightly lower than Klason lignin concentrations.

Conclusion

Contrary to previous reports that corn hybrids expressing a transgene for production of the *Bacillus thuringiensis* Cry1 Ab protein have significantly greater concentrations of lignin than normal hybrids, a multi-environment field study in Minnesota of six commercial non-Bt/Bt hybrid pairs did not detect Cry1 Ab-related differences in lignin concentration of lower stem internodes or whole plants. All forage quality and yield data support the conclusion that presence of the *cry*1 Ab gene does not alter the chemical composition or ruminal fiber digestibility of corn. Producers can use corn hybrids containing the Bt trait to reduce the costs and environmental consequences of pesticide application for European corn borer control without impacting forage quality or yield.

Table 1. Hybrid means across experimental locations for whole corn plant chemical composition and in vitro ruminal fiber digestibility.

Hybrid	Bt event	Crude protein	Starch	NDF[†]	ADF[‡]	In vitro digestibility 24-h	In vitro digestibility 96-h
		g/kg dry matter				g/kg	
35R57	None	75	324	424	225	363	560
35R58	MON810	76	317	437	234	352	559
DK440	None	73	347	440	231	358	567
DKC44-42	MON810	72	341	437	228	356	581
DK537	None	74	331	441	235	358	579
DKC53-32	MON810	75	328	437	233	367	581
N2555	None	77	325	431	229	352	551
N2555Bt	Bt11	76	325	436	232	349	547
N3030	None	76	328	435	227	350	567
N3030Bt	Bt11	73	333	431	227	354	561
N45T5	None	74	306	456	246	329	555
N45-A6	Bt11	74	306	459	247	357	549
SE		1	9	9	6	8	6
$LSD_{0.05}$		2	24	NS[§]	NS	NS	17

[†] NDF, neutral detergent fiber.
[‡] ADF, acid detergent fiber.
[§] NS, non-significant ($P > 0.05$).

Table 2. Mean lignin concentrations (across locations) for whole plants and the fourth elongated, above-ground internodes of corn hybrids as determined by three lignin analysis methods.

Hybrid	Bt event	Whole plant			Internode		
		Acid detergent	Klason	Acetyl bromide[†]	Acid detergent	Klason	Acetyl bromide
		g/kg dry matter					
35R57	None	22	107	98	67	153	142
35R58	MON810	23	108	100	66	148	143
DK440	None	22	106	102	69	156	144
DKC44-42	MON810	20	106	101	63	149	145
DK537	None	20	104	100	57	139	137
DKC53-32	MON810	19	103	103	60	143	141
N2555	None	23	108	106	64	145	139
N2555Bt	Bt11	23	116	101	66	147	143
N3030	None	22	107	102	62	150	141
N3030Bt	Bt11	21	106	99	65	155	142
N45T5	None	23	112	105	69	145	147
N45-A6	Bt11	23	110	103	66	149	146
SE		9	3	2	1	3	2
$LSD_{0.05}$		3	NS[‡]	NS	4	8	5

[†] An isolated maize stover lignin was used as the standard.
[‡] NS, non-significant (P > 0.05).

The Use of in Vitro Ruminal Gas Production as a Surrogate Measurement of Biomass Fermentability for Bioenergy Crops

P.J. Weimer, T.L. Springer, B.S. Dien and M.D. Casler

Introduction

Forages and other biomass materials have the potential to serve as feedstocks for production of fuels and industrial chemicals. A variety of biomass fermentation systems are under investigation worldwide, including simultaneous saccharification and fermentation (SSF) using fungal cellulases and *Saccharomyces* yeast, and direct microbial fermentations using anaerobic bacteria that produce their own fibrolytic enzymes and ferment the resulting hydrolytic intermediates to ethanol and other products. Assessing the potential of individual biomass samples to serve as feedstocks for these fermentations has been hampered by the lack of a facile screening method that can handle large numbers of biomass samples. We here report the use of an in vitro ruminal fermentation of biomass materials, coupled with measurement of fermentative gas production, as a rapid screen for substrate fermentability, and we compare the results to those obtained with a conventional SSF fermentation of biomass to ethanol.

Methods

Several cultivars of Eastern gamagrass (*Tripsacum dactyloides* L.), big bluestem (*Andropogon gerardii*) and sand bluestem (*A. hallii*) were grown in replicated field plots at up to nine locations from Texas to New York to Florida during 2001. Forages were cut from one to four times, depending on amount of growth. Air-dried, Wiley-milled (1 mm) samples (n=432 for gamagrass, n=168 for bluestem) were dispensed in 100 mg amounts into volume-calibrated serum vials (~60 ml) and suspended in 7 ml of reduced Goering/Van Soest buffer under a CO_2 gas phase. Vials were warmed to 39 °C and then inoculated with 3.0 ml of mixed ruminal inoculum, composited from two Holstein cows and diluted with an equal volume of buffer. Vials were sealed with butyl stoppers and aluminum crimp seals. Gas

pressure was measured at 0, 24, and 96 h, using a SenSym digital gas pressure gauge. Gas production (in ml) was calculated from the pressure reading, headspace volume, and a pressure/volume standard curve. Gas production values were determined from duplicate analyses in different fermentation runs, and were corrected for gas produced in inoculated blank vials that lacked biomass substrate. To determine if the in vitro gas production method can serve as a useful alternative to SSF fermentations, a subset of samples (n=12 for each species) was selected to span the entire range of in vitro ruminal fermentability of each species. These samples were subjected to in vitro fermentation with or without prior autoclaving treatment, and gas production was compared to SSF fermentations of the autoclaved material. SSF was conducted in sealed serum vials containing 1 g sample, 20 ml of TYE medium, 15 fpu of cellulase/beta-glucosidase, and *S. cerevesiae* Y-2034 inoculum (initial OD_{600}=0.50). After 7 d incubation at 32 °C and 100 rpm, ethanol production was measured by gas chromatography.

Results and Discussion

In vitro ruminal gas production (IVG) for Eastern gamagrass ranged from 66.89 to 184.56 ml gas/g added DM after 24 h, and 138.39 to 262.35 ml gas/g added DM after 96h of fermentation, respectively. For the bluestems the range was 65.65 to 184.56, and 146.93 to 284.79 ml gas/g added DM after 24h and 96h, respectively. Analysis of variance of cultivars grown at multiple locations revealed that both cultivar and growth environment strongly influenced fermentability of Eastern gamagrass (Table 1) and big bluestem (data not shown), and significant cultivar x environment interactions were noted as well. In general, effects were more pronounced for gas production than for nongaseous fermentation products (VFA production and final culture pH), though effects on the latter were often highly significant as well.

Using a subset of samples selected to span the complete range of gas production, IVG was shown to be reasonably well-correlated with ethanol production by SSF (Table 2). Surprisingly, ethanol production by SSF after 7d was more highly correlated with IVG production after 24h than with IVG production after 96h suggesting that the fungel enzyme treatment is less effective at degrading the complete set of available linkages in non-pretreated forage. Autoclaving of the substrates prior to fermentation had little effect on gas production, although slightly better correlations between IVG production and ethanol production by SSF were obtained for non-autoclaved material subjected to in vitro ruminal fermentation, despite the fact that the SSF experiments required autoclaving of the samples prior to addition of enzyme or yeast.

Table 1. Effect of cultivar and growth environment for Eastern gamagrass on gas production, pH, and VFA production. Five cultivars were grown in replicated field plots at six locations, with one to four harvests per location, (total n=299) during 2001.

Probability of a greater F value

	Main Effects			Interactions			
Variable	Cult	Loc	Harv	C*L	C*H	L*H	C*L*H
24h Gas	<0.001	<0.001	<0.001	0.006	0.067	<0.001	0.024
96h Gas	<0.001	<0.001	<0.001	0.074	0.064	<0.001	0.203
Normalized 24h Gas	<0.001	<0.001	<0.001	0.010	0.008	<0.001	0.028
Normalized 96h Gas	<0.001	<0.001	<0.001	0.109	0.050	<0.001	0.145
pH	0.001	<0.001	0.164	0.372	0.814	0.004	0.175
Acetate molar ratio	0.316	<0.001	0.007	0.346	0.362	0.001	0.862
Propionate molar ratio	0.144	<0.001	0.001	0.382	0.519	<0.001	0.841
Butyrate molar ratio	0.059	0.030	0.088	0.097	0.961	0.002	0.068
Ac/Pro molar ratio	0.060	<0.001	<0.001	0.419	0.319	<0.001	0.795

Table 2. Coefficients of determination (r^2) for in vitro ruminal gas (IVG) production at 24 and 96 h, and ethanol production by SSF after 7 d incubation in Eastern gamagrass and bluestem samples. Samples were autoclaved or not prior to fermentation, as indicated.

Eastern Gamagrass:	Not autoclaved		Autoclaved	
	IVG 24h	IVG 96h	IVG 24h	IVG 96h
Not autoclaved IVG 96h	0.763	--	--	--
Autoclaved IVG 24h	0.826	0.792	--	--
Autoclaved IVG 96h	0.629	0.938	0.780	--
Autoclaved SSF 7d	0.865	0.675	0.787	0.576

Big Bluestem/Sand bluestem:	Not autoclaved		Autoclaved	
	IVG 24h	IVG 96h	IVG 24h	IVG 96h
Not autoclaved IVG 96h	0.939	--	--	--
Autoclaved IVG 24h	0.964	0.958	--	--
Autoclaved IVG 96h	0.870	0.964	0.927	--
Autoclaved SSF 7d	0.738	0.584	0.671	0.588

Conclusions

Fermentation of unsterilized biomass materials using an in vitro ruminal inoculum and a 24h incubation time, with subsequent measurement of fermentative gas production with a pressure transducer, provides an acceptable surrogate measurement of biomass fermentability. This method thus has the potential to serve as a primary screen to narrow the number of samples for analysis by the more time-consuming and labor-intensive SSF method.

Modifications of the NDF Methods May Explain Variation Among Laboratory Results

D. R. Mertens

Introduction

In 2002 and 2003, it was observed that a significant portion of the laboratories participating in the National Forage Testing Association's proficiency evaluation program reported high values for amylase-treated neutral detergent fiber (aNDF) for several of the alfalfa hays. In addition, several other samples of alfalfa were sent to the USDFRC that had large discrepancies between laboratories in aNDF. Because variation among laboratories in dry matter, crude protein, and acid detergent fiber for these materials were within acceptable bounds, it was concluded that heterogeneity among samples could not explain the variation in aNDF results. The objective of this research was to investigate the effects of extraction technique and the use of heat-stable alpha-amylase and sodium sulfite on NDF to determine if a specific modification of the procedure could explain the deviations from the AOAC Int. Official Method 2002.04 for aNDF.

Materials and Methods

Ten alfalfa hays, including six with previously reported discrepancies for NDF results when analyzed by different laboratories, were used to determine if differences in NDF methodologies could be the source

of variation among laboratory results. A corn silage in-house standard material was also included. Each material was analyzed for NDF in a 2X4 factorial design using two extraction techniques (refuxing in beakers with filtration in crucibles [CR] or extraction in F57[1] filter bags using the ANKOM 200 Fiber Analyzer[1] system [FB]) and four modifications of the NDF procedure (with heat-stable alpha-amylase and sodium sulfite [+A+S], without heat-stable alpha-amylase and with sodium sulfite [-A+S], with heat-stable alpha-amylase and without sodium sulfite [+A-S], or without heat-stable alpha-amylase and sodium sulfite [-A-S]). Each analysis was done in duplicate in different analytical batches. The four modifications were a complete 2X2 factorial design involving the use of amylase or sulfite.

Results and Discussion

For the crucible extraction technique, treatment +A+S corresponded to AOAC Int. Official Method 2002.04 for aNDF , treatment -A+S corresponded to the original NDF method of Goering and Van Soest (1970) and treatment +A-S corresponded to the neutral detergent residue (NDR) method developed by Robertson and Van Soest (1980). Treatment -A-S was included to complete the possible permutations of amylase and sulfite use in the neutral detergent method and assess the independent effects of these two reagents, although it has not been used as a routine techniques for feed analysis. Because it corresponds to the AOAC Official Method, treatment +A+S was used as the reference method for comparisons.

There were several significant interactions among extraction techniques, method modifications and materials that made it difficult to interpret main effects across all combinations of treatments. Therefore, average results for all materials using each of the modifications were presented separately for CR and FB extraction techniques (Table 1). When the crucible technique was used to analyze alfalfa materials, not using amylase with or without using sodium sulfite resulted in a non-significant decrease in NDF of about 0.3 %-units. Previous comparisons typically indicated that not using amylase to determine NDF in alfalfa resulted in a slight increase in values of about the same magnitude. For the corn silage sample, using amylase resulted in a significant 1.0 %-unit decrease in NDF values. Not using sulfite when determining NDF using the CR technique, resulted in a significant 1.8 %-unit increase in fiber values regardless of whether or not amylase was used. There was no interaction between the use of amylase and sulfite with the CR technique.

Table 1. Effect of using (+) or not using (-) sodium sulfite (Sulfite) or heat-stable alpha-amylase (Amylase) on percentage of neutral detergent fiber in 10 alfalfa hays when extracted in beakers and filtered in crucibles (Crucible technique) or extracted in filter bags (Filter Bag technique).

	Crucible technique			Filter Bag technique		
Reagent Used	+ Sulfite	- Sulfite	Sulfite Difference	+ Sulfite	- Sulfite	Sulfite difference
+ Amylase	34.97	36.74	+1.78	34.33	36.52	+2.19
- Amylase	34.65	36.48	+1.83	34.90	37.72	+2.82
Amylase difference	-0.32	-0.27		+0.57	+1.19	

The effects of using amylase or sulfite in the determination of NDF in alfalfa were larger for the FB extraction technique compared to the CR technique (Table 1). For the FB technique, not using amylase increased NDF values significantly and the effect was even larger when sulfite was not used. Not using sulfite also increased fiber values by more than 2.0 %-units and the effect of sulfite was greater

for the FB compared to the CR technique. When neither amylase nor sulfite are used with the FB extraction technique, the values for NDF (37.72%) are much greater than those for the aNDF method using CR (34.97%). Thus, differences in NDF methodology may explain some of the variation among laboratories. However, the effects of extraction technique and modifications related to amylase and sulfite were similar in magnitude between the six alfalfa materials with large and the four alfalfas with small discrepancies among laboratories.

Conclusions

It is evident that the use of sodium sulfite, and to a lesser extent the use of heat-stable alpha-amylase, is crucial to determination of NDF in alfalfa hays. This is especially true for the filter bag extraction technique. Some, but not all, of the differences in aNDF analyses of alfalfa materials among laboratories may be due to modifications in NDF methodology.

Comparison of In Vitro Digestibility Continues After 24 Hours of Fermentation

D. R. Mertens

Introduction

Measurement of in vitro neutral detergent fiber digestibility (IVNDFD, % of NDF) using the traditional technique is laborious because undigested residues must be transferred manually between vessels several times during extraction. The Ankom Daisy II[1] system offers a semi-automated alternative because materials are fermented and extracted in a filter bag. One preliminary study indicated that Ankom in situ (IS) digestion bags used in Daisy II rotating-jar incubators produced 24-h IVNDFD for corn silage similar to the traditional flask method, but Ankom F57[1] filter bags used for fiber analysis yielded lower results. In a second study, IS bags provided 24-h IVNDFD similar to the traditional flask system for corn silage but not alfalfa hay. The objective of this research was to compare our traditional flask in vitro technique to the Daisy II system in which various types of filter bags were used.

Materials and Methods

In vitro techniques were compared using seven materials: two alfalfa silages (35.3 or 40.8% aNDF), two corn silages (40.0 and 41.1% aNDF), two grass hays (52.7 or 65.4% aNDF) and soybean hulls (64.0% aNDF). All materials were ground in a Wiley cutter mill with a 1-mm screen. In the flask in vitro system, .5 g of test sample was fermented in 125 ml Erlenmeyer flasks that contained 50 ml of inoculum and buffer, which were connected to a gas manifold that was kept under approximately 1 mm of carbon dioxide pressure during fermentation. In the Daisy rotating jar system, .5 g of test material was heat-sealed in 5X5 cm Dacron IS, F57, or F58 filter bags. The F57 and F58 filter bags were washed with acetone and dried before samples were placed in them. Each of the seven materials in each of the three bag types were placed (with a blank bag of each bag type) into each of the four jars in four Daisy II incubators. In addition to the 24 bags, each jar contained 2000 ml of inoculum and buffer. The inoculum and buffer mixture was 60% Georing and Van Soest buffer (1970), 10% strained rumen fluid, and 10% strained buffer extract of ruminal solids (squeezed ruminal contents blended with buffer in a ratio of 1g to 2 ml, respectively). Test samples were placed in fermentation vessels,

buffer was added, fermentation flasks or jars were warmed to 39° C, purged with carbon dioxide and reduced with a mixture of sulfide and cysteine before ruminal inoculum was added.

Fermentations were replicated in two runs scheduled one week apart. Inocula were prepared by combining strained ruminal fluid and buffer-extracted ruminal solids from four lactating cows fed rations containing alfalfa silage and corn silage mixed with concentrates. Two batches of inocula were used in each run and half of the duplicate samples in flasks and half of the jars within each Daisy incubator were inoculated with each batch. Because long-term fermentations approach a plateau that minimizes differences among substrates or techniques a 24-h fermentation time was chosen. Residues in flasks were transferred to beakers, refluxed in boiling neutral detergent solution with sulfite for 60 min before filtration and washing in Gooch crucibles (amylase in the first wash). After fermentation, bags were washed twice in a washing machine using the rinse spin cycle (5 min. agitation, 2 min. empty, + 7 min. spin). Bags were then extracted with neutral detergent containing amylase and sulfite in a pressurized Ankom 200 Fiber Analyzer[1]. The aNDF concentration of each material was determined using crucibles (AOAC 2002.04) and used to calculate the IVNDFD of all test samples (flasks and filter bags).

Results and Discussion

Our Daisy II incubators had been extensively modified previously to reduce variation among and within incubators. Modifications consisted of adding insulation to the door and all sides of the incubator, adding an additional fan to mix and circulate heated air within each unit more thoroughly, and setting individual controllers to the temperature necessary to obtain a temperature of 39° C in each unit. Analysis indicated no significant variation in the IVNDFD or in vitro dry matter true digestibility (IVDMTD) among jar locations within incubators. Variation among Daisy incubators also was non-significant.

There was a significant material by technique interaction, which indicated that differences among techniques were not consistent across materials. Therefore, in vitro techniques were compared for each material (Table 1). Although absolute differences among techniques were smaller, the statistical significance of differences for IVDMTD and IVNDFD were the similar, and only IVNDFD will be presented. For all materials, the IS bags generated the highest and F58 bags generated the lowest estimates of IVNDFD. Determinations of IVNDFD were not statistically different between flasks and F57 bags for one alfalfa silage, one grass hay and soybean hulls. However IVNDFD generated using flask and F57 bags differed for the other materials and in each case the values for F57 bags were lower.

Table 1. The 24-h in vitro neutral detergent fiber digestibilities (% of aNDF) of selected materials when fermented in flasks or in the Daisy II system using Ankom in situ (IS) Dacron bags, F57 filter bags, or F58 filter bags.[1]

Material	IS bags	Flasks	F57 bags	F58 bags
Alfalfa silage (40.8% aNDF)	58.6^{a}	45.8^{b}	40.5^{c}	36.4^{c}
Alfalfa silage (35.3% aNDF)	61.2^{a}	45.9^{b}	44.9^{b}	42.7^{b}
Corn silage (40.0% aNDF)	72.2^{a}	64.0^{b}	44.1^{c}	26.9^{d}
Corn silage (41.1% aNDF)	63.4^{a}	54.1^{b}	39.3^{c}	25.2^{d}
Grass hay (65.4% aNDF)	52.6^{a}	42.8^{b}	39.4^{b}	22.5^{c}
Grass hay (53.7% aNDF)	62.1^{a}	56.3^{b}	45.8^{c}	38.5^{d}
Soybean hulls (64.0% aNDF)	89.6^{a}	73.2^{b}	68.8^{b}	44.2^{c}

[1] IVNDFD within a row with different superscripts are statistically different P<.05.

Conclusions

In situ Dacron bags and F58 filter bags did not generate IVNDFD similar to the flask method for any material. The F57 filter bags gave similar IVNDFD results for some materials, but not others. Additional research is needed to determine if these discrepancies can be eliminated and if the Ankom Daisy II[1] system can be used to measure digestion kinetics comparable to the traditional flask system. For example, Jung (personal communication) indicated that pretreatment of corn silage with amylase before fermentation with ruminal fluid reduced variation among replicates and increased IVNDFD using the Daisy system.

Using Different Grinder Mills and Screen Apertures to Prepare Samples Affects Fiber Analysis

D. R. Mertens

Introduction

The type of grinder and screen aperture for grinding of samples for aNDF analysis was specified for AOAC Official Method 2002.04 to improve the reproducibility of results among laboratories. The specified grind, cutter mill with a 1-mm screen or equivalent, was a compromise between particle loss, extraction effectiveness and filtration ease. Grinding more finely improves extraction effectiveness, but results in particle losses through fritted disk crucibles and filter bags or clogging of pores, which increases filtration difficulties. Coarser grinding impedes extraction of soluble material from the large particles, resulting in higher fiber values. Preliminary research indicated that abrasion mills result in smaller mean particle size (MPS) and lower NDF results than cutter mills when using the same screen aperture. The objective of this research was to determine the effect of grinding on aNDF determined by crucible or filter bag techniques and to compare the effects of abrasion or cutter mill grinding on MPS and aNDF.

Materials and Methods

Eight forages (high and low fiber sources of alfalfa hay, alfalfa silage, corn silage and grass hay), two grains (corn and oats), two protein sources (soybean meal and cottonseed meal) and two by-product feeds (distiller's grains and soybean hulls) were each ground using a UDY Corp.[1] abrasion mill using a 1 (U1.0) or 2-mm (U2.0) screen or a Wiley[1] cutter mill using a 0.5 (W0.5), 1 (W1.0), or 2-mm (W2.0) screen. All forages were ground previously through a cutter mill with a 16-mm screen so they could be thoroughly mixed and sub-sampled before the final grinding. Particles were separated by shaking for 10 min using a Ro-Tap shaker through a series of sieves with apertures of 3.50, 2.36, 1.70, 1.18, 0.85, 0.60, 0.45, 0.212, 0.150, 0.108 and 0.075 mm plus pan. Mean particle size was calculated assuming a log-normal distribution of particles retained on each sieve. Log size was converted to linear dimensions.

Amylase-treated NDF was determined for each sample grind by refuxing in beakers with filtration in crucibles (CR) or extraction in F57[1] filter bags using the ANKOM 200 Fiber Analyzer[1] system (FB). For the CR technique, .5 g of test sample was extracted in 50 ml of neutral detergent solution with sodium sulfite. Hot plates were calibrated to heat 50 ml of room temperature water to boiling in 5 min. The first dose of amylase was added after 5 min on the hot plates and samples were extracted at boiling

for 60 min. Residues were filtered in Gooch crucibles and a second dose of amylase was added during the first water soak. Residues were washed by two additional soakings in boiling water for 5 min. each. Residues were then extracted twice in acetone for 5 min., dried and weighed.

For the FB technique, the pressurized extraction chamber was verified to heat 2000 ml of room temperature water to 100° C in 15 min. (controller reading was also verified using a reference thermometer). Sodium sulfite was dissolved in room temperature neutral detergent solution and amylase was added and mixed before pouring into the extraction chamber. The chamber was sealed and heated to 100° C within 15 min and extracted under pressure for an additional 60 min (ending pressure > 12 psi). Residues were rinsed four times with 2000 ml of 80-90° C water with lid sealed and heat turned on (first two rinses contained amylase). Extracted FB were pressed between absorbent paper to remove excess water and soaked twice for 5 min. in about 500 ml of acetone with gentle shaking.

Results and Discussion

There were significant linear relationships between the MPS of each grinding treatment and the W1.0 grind; however, for each grinding treatment the MPS of materials differed. In general, forages had smaller MPS for each grind than concentrates (Table 1) and there was an inverse relationship between the MPS and aNDF concentration of materials. Perhaps fibrous materials pass out of mills more slowly, thereby resulting in smaller MPS.

Table 1. Mean particle size (mm) for concentrate and forage materials processed by selected grinding treatments.

Grind treatment	Concentrates	Forages	All materials
UDY abrasion mill, 1.0-mm	189	143	171
UDY abrasion mill, 2.0-mm	244	183	215
Wiley cutter mill, 0.5-mm	243	162	203
Wiley cutter mill, 1.0-mm	326	212	267
Wiley cutter mill, 2.0-mm	428	302	362

Although there were material by grinding treatment and material by fiber technique interactions, there was no grinding treatment by fiber technique interaction and results in Table 2 are the average of CR and FB techniques. Across all materials and grinding treatments, FB averaged 1.1 %-units lower in aNDF than CR, but this difference ranged from +1.6 %-units for distillers grains to –2.7 %-units of aNDF for one of the corn silages, and this variation explains the material by fiber technique interaction.

Table 2. Average of aNDF determined by the crucible and filter bag techniques for concentrate and forage materials processed by selected grinding treatments and differences in aNDF from cutter mill 1-mm for the current and previous experiments.[1]
[1] IVNDFD within a row with different superscripts are statistically different P<.05.

Grind treatment	Concentrates	Forages	All	Difference	Previous
UDY abrasion mill, 1.0-mm	24.5	39.7^{a}	32.9^{b}	-0.9	-1.1
UDY abrasion mill, 2.0-mm	24.7	40.9^{bc}	33.7^{c}	-0.1	n.d.
Wiley cutter mill, 0.5-mm	24.3	40.0^{ab}	32.3^{a}	-1.5	-2.0
Wiley cutter mill, 1.0-mm	24.7	41.1^{c}	33.8^{c}	0.0	0.0
Wiley cutter mill, 2.0-mm	24.9	41.0^{c}	33.9^{c}	+0.1	+1.1

The U1.0 and W0.5 grinding treatments resulted in lower aNDF concentrations across all materials and this effect was more significant for forages than concentrates (Table 2). It also appears that U2.0 and W1.0 generate similar aNDF results even though the MPS for U2.0 is less than that for W1.0. Although the aNDF results for W1.0 and W2.0 were not different in this study, previous trials in our laboratory and observations of other researchers indicated that W2.0 typically results in higher aNDF compared to W1.0.

Conclusions

Both grinder mill and the aperture of the screen in the mill affect the MPS and aNDF of the prepared sample. Using the same screen aperture, abrasion mills result in smaller MPS and lower aNDF than cutter mills. The effect of grinding treatment was similar for the CR and FB techniques for measuring aNDF. It appears that a U2.0 grind generates equivalent results to the W1.0 grind specified for aNDF, but that U1.0 grinds will result in significantly lower values.

FEED UTILIZATION BY CATTLE

Effect of Level of Dietary Crude Protein on Performance and Nitrogen Excretion in Lactating Dairy Cows

J. J. Olmos Colmenero and G. A. Broderick

Introduction

Optimizing protein flow to the small intestine to meet the requirement of the dairy cow is crucial to increasing efficiency of conversion of dietary N into milk protein. Overfeeding CP reduces profit margins because of the relatively high cost of protein supplements. As dietary CP increases, most of the amino acids (AA) resulting from ruminal proteolysis are deaminated by the ruminal microorganisms rather than being used for protein synthesis. Moreover, excessive ammonia resulting from AA deamination is absorbed and eventually excreted as urea in the urine. Hence, overfeeding CP will result in excessive production of urinary N, the most polluting form of excreted N. The objective of this study was to determine the dietary CP level necessary to optimize milk production with minimal N wastage for dairy cows fed typical Midwestern diets.

Material and Methods

Forty lactating Holstein cows (10 with ruminal cannulas) were blocked by DIM into 8 groups of 5 and randomly assigned to treatments in an incomplete 5 x 5 Latin square. A single basal diet with 25% DM from each of alfalfa silage and corn silage was fed; CP contents of the five diets, A through E, were adjusted in steps of approximately 1.5 percentage units from 13.5% to 19.4% CP by replacing high moisture corn with solvent soybean meal (Table 1). Each experimental period lasted 28 d--14 d for adaptation and 14 d for data collection. Feeds were analyzed weekly for CP and DM to adjust to the desired CP contents as a proportion of dietary DM. Apparent digestibility was estimated from spot fecal samples using indigestible ADF as an internal marker; urinary excretion was estimated from spot urine samples using creatinine as volume marker. Rumen metabolites were determined at 0, 4, 8, 12, 16, 20, 24 hours after feeding, and microbial CP flow was estimated from urinary allantoin excretion. Data were analyzed using the Proc Mixed procedures of SAS.

Results and Discussion

Content of NDF and ADF was similar across diets, indicating that they were of high energy and typical for diets fed to early lactation cows (Table 1). The target of 1.5% increase in CP with each increment of SBM supplementation was essentially achieved in this trial. Dry matter intake did not change in response to dietary CP. However, yield of both milk and protein (Table 2) increased with the first two increments of dietary CP ($P < 0.05$) and were greatest at 16.5% CP. Microbial CP flow (estimated from urinary allantoin; Table 4) also was maximal at 16.5% CP, indicating that metabolizable protein supply from ruminal synthesis plus dietary escape was adequate at that CP level. Milk and protein yield actually appeared to decline beyond 16.5% CP. We speculate that this resulted because of the energy cost to excrete the additional N load at 18.0 and 19.4% CP and the fact that higher energy high moisture corn was replaced by lower energy soybean meal when the unneeded CP was added to the diet. Fat yield, BW change and Milk/DM intake did not differ, although there was numerically greater fat yield on the three highest levels of dietary CP (Table 2).

Milk urea (Table 2), ruminal ammonia, isobutyrate and isovalerate (Table 3), as well as blood urea, urinary urea N and total urinary N excretion (Table 4) all increased linearly with dietary CP content ($P < 0.05$). Elevated MUN is a good indicator of wastage of dietary CP. High concentrations of ruminal ammonia and branch-chain VFA are related to excessive protein degradation and amino acid deamination. Fecal N excretion was largely unaffected by dietary CP because it was not different between the lowest CP diet and the three highest CP diets. Fecal N is the form that remains relatively stable after excretion. Total urinary N excretion increased by a factor of 2.3 over the five diets, going from 111 g/d at 13.5% CP to 255 g/d at 19.4% CP. The opposite trend occurred for apparent N efficiency (milk N secreted divided by feed N consumed), which fell from 37% on the 13.5% CP diet to 31% on the 16.5% CP diet. Efficiency declined a further 5 percentage units when going from 16.5% CP, the diet giving optimal production, to 19.4% CP. Nearly all of the extra N consumed was excreted in the urine, the form that causes the most problems in the environment.

Conclusions

Optimal production of milk and protein was obtained when cows were fed 16.5% CP. Adding greater amounts of CP above this level by replacing dietary high moisture corn with SBM did not improve, or may have even decreased, levels of production. Moreover, increasing dietary CP greatly increased N excretion in the form of environmentally labile urinary N.

Table 1. Composition of diets.

	Diet				
Ingredient	A	B	C	D	E
	----------% of DM----------				
Alfalfa silage	25.0	25.0	25.0	25.0	25.0
Corn silage	25.0	25.0	25.0	25.0	25.0
Rolled high moisture shelled corn	44.0	40.6	37.2	33.8	30.4
48% Soybean meal	2.40	5.80	9.20	12.6	16.0
Roasted soybeans	2.50	2.50	2.50	2.50	2.50
Bicarbonate	0.60	0.60	0.60	0.60	0.60
Minerals & vitamins	0.50	0.50	0.50	0.50	0.50
	----------Chemical composition----------				
DM, %	52.3	52.9	52.9	52.9	53.2
OM, % of DM	94.2	94.3	94.1	93.8	94.1
CP, % of DM	13.5	15.0	16.5	17.9	19.4
NDF, % of DM	24.9	24.5	24.4	24.7	25.1
ADF, % of DM	15.0	14.5	14.9	14.9	15.2

Table 2. Effect of dietary crude protein on intake, milk production, and milk composition

	Diet							
	A	B	C	D	E	SE[1]	Linear	Quad
DMI, kg/d	22.2	21.9	22.7	22.0	22.7	0.45	0.21	0.71
Milk Production, kg/d	36.3[b]	37.2[ab]	38.3[a]	36.6[b]	37.0[ab]	0.87	0.60	0.11
Weight gain,	0.49	0.46	0.70	0.57	0.64	0.16	0.21	0.72
Composition and yield								
Fat yield, kg/d	1.14	1.20	1.24	1.23	1.24	0.06	0.06	0.30
Protein yield, kg/d	1.10[b]	1.15[ab]	1.18[a]	1.13[ab]	1.15[ab]	0.03	0.21	0.10
MUN, mg/dL	7.71[d]	8.50[d]	11.2[c]	13.0[b]	15.6[a]	0.61	<0.01	0.13
Milk/DMI	1.71	1.71	1.72	1.70	1.72	0.038	0.87	0.99
Milk N/N Intake	0.37[a]	0.34[b]	0.31[c]	0.28[d]	0.25[e]	0.01	<0.01	0.58

[a,b,c,d]Means in rows with no common superscripts are different ($P < 0.05$)

Table 3. Effect of dietary crude protein on digestibility and rumen metabolites.

	Diet							
	A	B	C	D	E	SE[1]	Linear	Quad
DM Digestibility, %	71.2[c]	74.6[a]	74.0[a]	72.5[b]	72.3[bc]	0.63	0.79	<0.01
OM Digestibility, %	72.1[c]	75.5[a]	75.0[a]	73.6[b]	73.5[b]	0.59	0.47	<0.01
NDF Digestibility, %	45.8[c]	51.2[a]	49.5[ab]	48.0[b]	48.7[b]	0.95	0.18	<0.01
Rumen metabolites								
Total VFA, mM	78.0	83.0	84.7	84.1	84.3	4.77	0.17	0.34
Acetate, mM	45.1[b]	48.4[ab]	49.0[ab]	49.9[ab]	50.9[a]	2.87	0.03	0.56
Propionate, mM	18.5	20.1	20.9	19.0	18.5	1.32	0.76	0.06
Butyrate, mM	9.70	9.51	9.89	10.6	10.1	0.70	0.23	0.82
Isobutyrate, mM	1.03[b]	1.10[ab]	1.13[ab]	1.17[a]	1.20[a]	0.06	<0.01	0.56
Valerate, mM	2.12	2.06	2.15	1.88	1.76	0.23	0.07	0.48
Isovalerate, mM	1.54[b]	1.70[ab]	1.71[ab]	1.74[a]	1.74[a]	0.10	0.04	0.23
Ammonia, mM	4.34[c]	5.49[b]	6.54[b]	9.08[a]	9.14[a]	0.54	<0.01	0.34

[a,b,c,d]Means in rows with no common superscripts are different (P < 0.05)

[1]SE = Standard error of the differences of the least square means

Table 4. Effect of dietary crude protein on blood urea, N excretion, and microbial CP flow

	Diet							
	A	B	C	D	E	SE[1]	Linear	Quad
Blood urea, mM	3.79[e]	4.91[d]	6.08[c]	7.61[b]	8.68[a]	0.29	<0.01	0.97
Urine volume excretion, L/d	17.3[bc]	15.4[c]	17.9[b]	19.3[ab]	21.7[a]	1.22	<0.01	0.03
Urinary urea-N excretion, g/d	63.2[e]	91.0[d]	128[c]	174[b]	208[a]	6.60	<0.01	0.43
Total urinary-N excretion, g/d	111[e]	141[d]	180[c]	213[b]	255[a]	7.82	<0.01	0.50
Fecal N excretion, g/d	190[a]	173[b]	191[a]	188[ab]	199[a]	8.57	0.06	0.14
Microbial CP Flow, g/d	993[b]	1082[ab]	1144[a]	1127[a]	1144[a]	66.6	0.02	0.21

[a,b,c,d]Means in rows with no common superscripts are different (P < 0.05)

[1]SE = Standard error of the differences of the least square means

Effect of Dietary Rumen-Degraded Protein on Nutrient Digestion and Excretion in Lactating Dairy Cows

S. M. Reynal and G. A. Broderick

Introduction

Rumen-degraded protein (RDP) fed in excess of the requirement for maximal microbial yield is largely converted to urea and excreted in the urine. Once excreted, urea can be rapidly degraded to ammonia and lost to the environment through volatilization, contributing to atmospheric and hydrospheric pollution. On the other hand, feeding RDP levels below requirements can compromise microbial protein production, ruminal digestion, and energy and protein availability to the cow. The objectives of this study were to 1) study the effects of the level of dietary RDP on production, N metabolism and nutrient digestion, and excretion in lactating dairy cows; and 2) determine the level of dietary RDP required to optimize milk production and N efficiency.

Materials and Methods

Twenty-eight (8 with ruminal cannulas) lactating Holstein cows averaging 72 DIM were blocked by DIM into seven 4 x 4 Latin squares. Cows were randomly assigned within squares to balanced diet sequences. Four TMR were formulated to contain 50% of dietary DM from forage (3/4 corn silage and 1/4 alfalfa silage) and 50% from concentrate (Table 1). The proportions of high-moisture shelled corn, solvent soybean meal (SSBM), lignin-sulfonate soybean meal (LSBM; SoyPass; Ligno-Tech, Rothchild, WI) and urea in the concentrate were adjusted to provide similar levels of CP coming from ingredients other than urea and energy levels but varying RDP levels across diets. Dietary RDP levels predicted using the NRC (2001) model were 12.5, 10.9, 9.2, and 7.7% of DM for diets A, B, C, and D, respectively. However, RDP levels observed in vivo were 13.2, 12.3, 11.7, and 10.6, respectively. Each experimental period lasted 28 d and consisted of an 18-d adaptation period and a 10-d sample collection period. Digesta flow leaving the rumen was quantified in the 8 ruminally cannulated cows using the omasal sampling technique. Spot fecal and urine samples were collected from all the cows. Urinary and fecal excretions were estimated using, respectively, creatinine and indigestible ADF as internal markers. Microbial protein flow from the rumen was quantified using $^{15}NH_3$ as external marker and also estimated from the urinary excretion of allantoin plus uric acid. Statistical analysis was done using Proc Mixed in SAS; differences among least square means were reported only if F-test for treatment was significant at $\alpha \leq 0.05$.

Results and Discussion

The present diet formulation allowed us to study the effects of dietary RDP level without confounding effects of RDP and RUP source, and energy concentration and source. Dietary RDP level had no effect on DMI or yields of milk, FCM, milk fat and SNF, but had a linear effect on milk protein content and a quadratic effect on milk protein yield, with the quadratic maximum at 12.3% RDP (10.9% by NRC prediction; Table 2). Quadratic effects of RDP level were also observed for the amount of OM apparently and truly digested in the rumen (data not shown), with quadratic maxima occurring at RDP concentrations of, respectively, 11.9 and 12.2% (10.1 and 10.4% by NRC prediction). True digestibility of CP in the rumen decreased linearly as LSBM replaced SSBM in diets A to D, reflecting the lower ruminal degradability of LSBM. Because the main differences among diets fed in our experiment derived from the replacement of SSBM with LSBM, similar total tract digestibilities of CP among treatments indicated that both SBM supplements had similar RUP digestibilities (Table 3). Microbial protein flow from the rumen decreased linearly with decreasing levels of dietary RDP and, therefore,

it was not possible to determine an RDP optimum for microbial growth. Although the use of purine derivatives excretion underestimated the microbial NAN flows by 120 to 170 g/d across treatments, it accurately defined the direction of the changes induced by treatments.

Table 1. Composition of diets.

Item[1]	Diet A	B	C	D
Ingredients		(% of DM)		
Corn silage	37.1	37.1	37.1	37.1
Alfalfa silage	12.7	12.7	12.7	12.7
Rolled HMSC	32.4	32.1	31.9	31.7
Solvent SBM	16.43	10.95	5.48	–
Ligno-sulfonate treated SBM	–	5.87	11.74	17.61
Urea	0.50	0.33	0.17	–
Vitamins and minerals[2]	0.90	0.90	0.90	0.90
Nutrient content of diets				
Crude protein, % of DM	18.8	18.3	17.7	17.2
Non-urea N, % of DM	2.77	2.77	2.75	2.75
NDF, % of DM	28	28	28	30
ADF, % of DM	16	16	15	15
RDP,[3] % of DM	12.5	10.9	9.2	7.7
RUP,[3] % of DM	6.3	7.4	8.5	9.5
NE_L,[3] Mcal/kg of DM	1.56	1.55	1.55	1.55
NFC,[3] % of DM	49.2	49.9	50.6	51.4

[1]HMSC = High moisture shelled corn; NFC = Nonfiber carbohydrates; RDP = rumen-degraded protein; RUP = rumen-undegraded protein; SBM = soybean meal.
[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, 6640 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E.
[3]Predicted by the NRC (2001) model.

Table 2. Effect of dietary rumen-degraded protein (RDP) on milk production and ruminal digestion.

Item	Diet A	B	C	D	SE	$P > F^1$ RDP	L	Q
Production (all 28 cows)								
DMI, kg/d	25.1	25.7	25.7	25.4	0.6	NS	NS	NS
Milk yield, kg/d	42.3	42.8	42.4	41.5	0.9	NS	NS	NS
3.5% FCM, kg/d	38.4	40.0	40.2	39.4	1.3	NS	NS	NS
Milk fat, %	3.21	3.36	3.28	3.40	0.15	NS	NS	NS
Milk fat, kg/d	1.23	1.31	1.33	1.32	0.07	NS	NS	NS
Milk protein, %	3.14[a]	3.14[a]	3.07[b]	3.04[b]	0.05	< 0.01	< 0.01	NS
Milk protein, kg/d	1.30[a]	1.34[a]	1.30[a]	1.23[b]	0.03	0.02	0.02	0.03
MUN,[2] mg/dl	15.9[a]	15.6[a]	13.6[b]	12.8[b]	0.5	< 0.01	< 0.01	NS
Intake and ruminal digestion of nutrients (8 ruminally cannulated cows)								
DM intake, kg/d	23.6[a]	23.7[a]	24.4[a]	22.4[b]	1.0	0.01	NS	0.02
True digestibility, %								
OM	65.2	64.9	65.8	65.0	1.0	NS	NS	NS
Crude protein	68.8[a]	66.3[a]	65.7[a]	61.5[b]	1.6	< 0.01	< 0.01	NS
Apparent digestibility, %								
NDF	50.3	48.5	49.3	49.6	2.3	NS	NS	NS
ADF	51.6	52.0	46.8	49.3	2.4	0.07	0.08	NS

[a,b,c]Least square means within the same row without a common superscript differ ($P<0.05$).
[1]Probability of significant effects of RDP or of linear (L) or quadratic (Q) effects of RDP level.
[2]Milk urea-N determined using a colorimetric assay (Broderick and Clayton; J. Dairy Sci. 80:2964- 2971, 1997).

Table 3. Effect of dietary rumen-degraded protein (RDP) on intake and omasal N flows (8 ruminally cannulated cows), N excretion and efficiency, and total tract digestion (all 28 cows).

	Diet					$P > F^2$		
Item[1]	A	B	C	D	SE	RDP	L	Q
Intake and omasal N flows (8 ruminally cannulated cows)								
N intake, g/d	715[a]	701[a]	690[a]	619[b]	26	< 0.01	< 0.01	0.06
RDP supply, g/d	3076[a]	2918[a]	2839[a]	2403[b]	143	< 0.01	< 0.01	NS
% of DMI	13.2[a]	12.3[ab]	11.7[b]	10.6[c]	0.4	< 0.01	< 0.01	NS
Flow at omasal canal								
NAN, g/d	684[a]	664[a]	653[ab]	615[b]	26	0.03	< 0.01	NS
RUP, g/d	1370	1459	1470	1469	77	NS	NS	NS
% of DMI	5.79	6.18	6.04	6.61	0.28	NS	0.07	NS
NANMN, g/d	209	226	229	233	13	NS	NS	NS
% of total NAN	30.4[c]	34.2[b]	34.9[ab]	37.8[a]	1.3	< 0.01	< 0.01	NS
Microbial NAN								
Total, g/d	470[a]	438[b]	425[b]	384[c]	18	< 0.01	< 0.01	NS
FAB, g/d	199[a]	189[a]	191[a]	167[b]	10	0.05	0.04	NS
PAB, g/d	271[a]	249[b]	233[bc]	217[c]	14	< 0.01	< 0.01	NS
% of total NAN	69.6[a]	65.8[b]	65.1[bc]	62.1[c]	1.3	< 0.01	< 0.01	NS
Microbial efficiency								
g NAN/kg OMTDR	32.3[a]	30.1[b]	28.1[c]	28.0[c]	0.8	< 0.01	< 0.01	NS
Nutrient digestion and N efficiency (all 28 cows)								
Urinary excretion								
L/d	22.3[a]	19.9[ab]	19.7[ab]	17.9[b]	1.1	0.03	< 0.01	NS
N, g/d	295[a]	293[a]	237[b]	239[b]	13	< 0.01	< 0.01	NS
Microbial NAN,[3] g/d	349[a]	318[a]	256[b]	264[b]	16	< 0.01	< 0.01	0.09
Fecal excretion								
DM, kg/d	7.93[a]	7.41[ab]	7.02[b]	6.70[b]	0.34	0.03	< 0.01	NS
OM, kg/d	7.27[a]	6.76[ab]	6.39[b]	6.12[b]	0.32	0.03	< 0.01	NS
N, g/d	222	220	219	197	10	NS	0.06	NS
Total N excretion, g/d	517[a]	514[a]	456[b]	437[b]	18	< 0.01	< 0.01	NS
% of N intake	73.4[a]	72.1[ab]	66.7[bc]	66.2[c]	2.3	0.04	< 0.01	NS
Total tract apparent digestibility, %								
DM	66.4[b]	69.8[a]	71.2[a]	72.1[a]	1.7	< 0.01	< 0.01	NS
OM	67.6[b]	70.9[a]	72.3[a]	73.0[a]	1.2	<0.01	< 0.01	NS
NDF	40.2[c]	46.4[b]	49.7[ab]	53.1[a]	2.1	<0.01	< 0.01	NS
N	68.5	69.4	68.2	70.2	1.3	NS	NS	NS
N efficiency								
milk N, % of N intake	29.6	29.5	30.4	30.4	0.9	NS	NS	NS
kg milk:kg N excreted	84.5[b]	87.2[b]	94.3[a]	99.8[a]	3.9	< 0.01	< 0.01	NS

[1]FAB = Fluid-associated bacteria; NAN = nonammonia N; NANMN = nonammonia non-microbial N; NS = non-significant; OMTDR = OM truly digested in the rumen; PAB = particle-associated bacteria; RUP = rumen-undegraded protein.
[2]Probability of significant effects of RDP or of linear (L) or quadratic (Q) effects of RDP level.
[3]Estimated from urinary allantoin excretion using equation from Vagnoni et al. (J. Dairy Sci. 80:1695-1702, 1997) and measured N:purines ratio in bacteria isolated from omasal digesta.

Decreasing dietary RDP from 13.2 to 11.7% of DM (12.5 to 9.2% by NRC prediction) for diets A to C, respectively, resulted in reduced excretion of urinary N (from 295 to 237 g/d), total N (from 517 to 456 g/d), and total N as percent of N intake (from 73.4 to 66.7%) without affecting yield of milk and FCM. Although cows fed diet B had higher milk protein content and yield than those fed diet C, the 40-g per day increase in milk protein yield was at the expense of a 58-g per day increase in total N excretion, which depressed N efficiency from 94.3 to 87.2 kg of milk per kg of N excreted. Moreover, almost all the increased N excretion was in the form of urinary urea, with the most negative effects on environmental pollution. However, reducing RDP from 11.7 to 10.6% (9.2 to 7.7% by NRC prediction) resulted in a small reduction in N excretion (19 g N/d) but a significant reduction in milk protein yield (70 g/d). Therefore, the recommended level of dietary RDP will depend on the criteria used to define optimum N utilization efficiency (i.e., the dietary CP required to maximize milk protein yield may not match that required to minimize N losses to the environment). If such criteria took into account

environmental impact from dairy operations and current regulations on whole-farm N balance, then the recommended level of RDP would be 11.7% (9.2% by NRC prediction) of DM because N losses were significantly reduced with minimal reduction in milk production.

Despite the relatively high level of CP required to optimize N utilization in the present trial, increasing evidence shows that milk yield and N efficiency of high producing dairy cows can be maximized at dietary CP contents substantially lower than 17.7% of dietary DM (corresponding to diet C). In the studies of Olmos Colmenero and Broderick (J. Dairy Sci. 86(Suppl. 1): 273, 2003) and Broderick (J. Dairy Sci. 86:1370-1381, 2003), maximum milk production was achieved at dietary CP levels of 16.5 and 16.7% of DM, respectively. Furthermore, by balancing AA through selection of highly digestible RUP sources and by Met supplementation, N excretion could be minimized while improving milk production. Using this approach, Noftsger and St. Pierre (J. Dairy Sci. 86:958-969, 2003) were able to decrease dietary CP from 18 to 17% of DM while maximizing milk (46.6 kg/d) and milk protein (1.65 kg/d) yields.

Conclusion

Optimum N utilization efficiency (maximal milk production with minimal N loss) was not attained at either maximal microbial protein yield or minimal N excretion, but rather at a balance point between dietary protein degradation and microbial protein synthesis in the rumen.

Comparison Between Total Purines and ^{15}N as Markers for Quantifying Microbial Protein Flow From the Rumen of Dairy Cows

S. M. Reynal, G. A. Broderick, and C. Bearzi

Introduction

In order to reduce the environmental impact of dairy operations, rations should be formulated to optimize N utilization by dairy cows. Ration formulation models are needed that accurately predict the amino acid (AA) requirement for each physiological state as well as the AA supply to the intestines when feeding different combinations and qualities of feeds. Because high quality microbial protein synthesized in the rumen accounts for the majority of the total AA flow to the small intestine, accurate measurement of microbial protein flow is essential for the development of prediction models. One of the most important sources of error in the determination of microbial flows in vivo is associated with microbial marker analysis. Although total purines (TP) and the stable N isotope ^{15}N have been widely used as internal and external microbial markers, respectively, comparisons between the 2 are lacking. The objective of this study was to compare the precision and accuracy of in vivo measurements of microbial flow obtained using either TP or ^{15}N as markers.

Materials and Methods

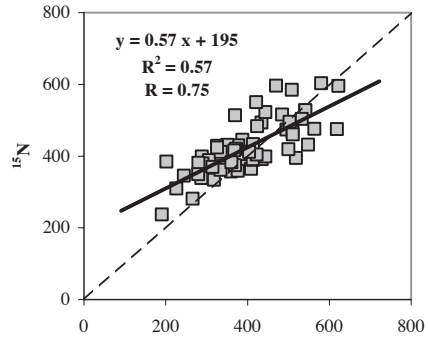
Eight ruminally cannulated lactating Holstein cows that were part of a larger 4 x 4 Latin square trial studying the effects of dietary rumen-degraded protein (RDP) level on production and N utilization, were used to quantify flows of microbial protein in omasal digesta using TP and ^{15}N as microbial

markers. Diets were formulated from corn silage, alfalfa silage, high moisture corn, solvent soybean meal (SSBM), lignin-sulfonate treated soybean meal (LSBM; SoyPass; Ligno-Tech, Rothchild, WI), urea, plus vitamins and minerals to provide similar levels of CP coming from ingredients other than urea. Dietary SSBM, LSBM, and urea were adjusted to achieve NRC-predicted RDP levels of 12.5, 10.9, 9.2, and 7.7% of the DM in diets A, B, C and D, respectively. Diets are described in detail in Table 1 of the companion summary (Reynal and Broderick, 2003 USDFRC Res. Sum.). A solution containing 10% atom excess $^{15}NH_4SO_4$ was infused into the rumen at a constant rate of 182 mg of ^{15}N per cow per day during the last 7 days of each period. Using the omasal sampling technique, spot samples were collected from the omasal canal 6 times daily at 1-h intervals during the last 4 days of the infusion to represent two 12-h feeding cycles over 2 days each. Fluid-associated bacteria (FAB) and particle-associated bacteria (PAB) were isolated from omasal subsamples by differential centrifugation. Flows of omasal digesta were determined for both the fluid phase (FP) and the small particle plus large particle phase (PP). Samples of FAB, PAB, FP and PP were analyzed for NAN, TP (adenine + guanine) by an HPLC method, and for ^{15}N enrichment by isotope ratio mass spectrometry. Microbial protein flow was calculated from N:purine ratios and from ^{15}N enrichments in FAB, PAB, FP and PP samples, using the daily flows of FP and PP digesta at the omasal canal.

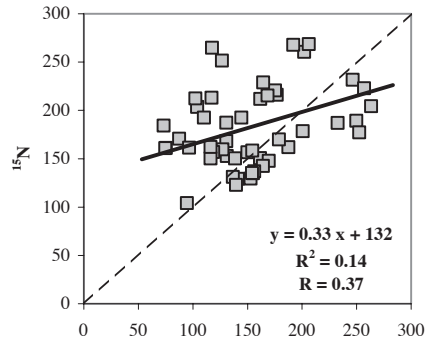
Results and Discussion

Coefficients of variation (CV) between replicates of FAB, PAB, FP, and PP for TP analysis were 3.5, 3.1, 3.3, and 3.5%, respectively. However, the CV between replicates of FAB, PAB, FP, and PP for ^{15}N analysis were 0.12, 0.04, 0.03, and 0.06%, respectively. Therefore, ^{15}N determinations had much greater precision than TP analyses. The N:purine ratios and ^{15}N enrichments for FAB, PAB, FP, and PP were within the ranges reported in the literature. Recoveries of adenine and guanine added as yeast-RNA to samples of FAB, PAB, FP, and PP were, respectively, 100 and 101%, 98 and 100%, 103 and 101%, and 99 and 98%. Because all purines flowing out of the rumen were assumed to be of microbial origin only (based on literature reports of rapid and extensive ruminal degradation of dietary purines), the guanine:adenine ratios in FAB and PAB should have been similar to those of their corresponding omasal FP and PP. Although this was the case for PP and PAB, the guanine:adenine ratio in omasal FP was 4.5 times higher than the ratio found in FAB. The complete recoveries of added adenine and guanine and the low CV between replicates indicated that discrepancies between FP and FAB were not due to analytical error. This suggested that either adenine of microbial origin was partially degraded in FP or substantial amounts of dietary guanine escaped degradation. Moreover, when microbial N flows measured using TP were regressed against those measured using ^{15}N as the microbial marker (Figure 1), there was good agreement between markers for total microbial (R = 0.75; Figure 1A) and PAB flows (R = 0.76; Figure 1C). However, the correlation between markers for FAB flow was poor (R = 0.37; Figure 1B). Therefore, flows of bacteria associated with the fluid phase of digesta may be biased when calculated using TP as microbial marker.

A.



B.



C.

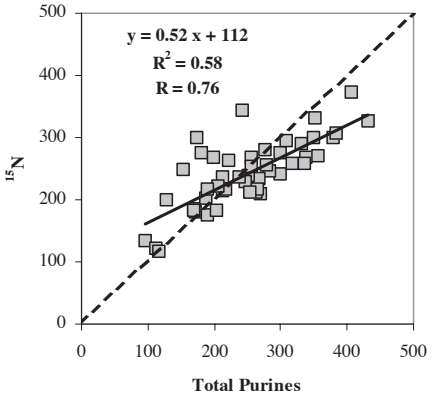


Figure 1. Regressions between ruminal microbial flows measured using total purines and ^{15}N for: A.) total microbial flow; B.) FAB flow; and C.) PAB flow.

Except for the linear effect of dietary RDP on total purine concentration in FAB, none of the variables measured using TP were affected by diets (Tables 1 and 2). However, when ^{15}N was used as the microbial marker, dietary RDP had linear and quadratic effects on ^{15}N-enrichment of FAB, PAB, FP and PP, with the highest enrichment corresponding to the lowest dietary RDP level (diet D; Table 1). Moreover, the flows of FAB, PAB and total microbial NAN at the omasal canal, as well as the efficiency of microbial growth, decreased linearly with decreasing levels of dietary RDP (Table 2). Thus, statistical power was substantially improved when ^{15}N was used as the microbial marker, compared to the use of TP. This was evident when accounting for ruminal escape of dietary protein. Intake of RUP increased as the highly degradable protein supplements SSBM and urea were gradually replaced with the more resistant LSBM. However, when TP were used as microbial marker, NAN flow from dietary origin (as percent of total NAN flow) decreased linearly from diets A to D (Table 2). On the contrary, when ^{15}N was used as microbial marker, dietary NAN flows increased linearly from diet A to D as expected. The use of ^{15}N as an external marker for measuring microbial protein flow from the rumen of dairy cows resulted in more precise and biologically meaningful results than when TP were used as an internal microbial marker.

Conclusion

Although there is no standard marker with which to assess the accuracy of results obtained using these two markers, the findings from this trial suggest that, compared to TP, use of ^{15}N yielded more precise and accurate measurement of microbial flow from the rumen.

Table 1. Effect of dietary rumen-degraded protein (RDP) on nonammonia N (NAN) and purine concentrations, N:purine ratios, and ^{15}N enrichment of microbes and omasal digesta.

	Diet					$P > F^2$		
Item[1]	A	B	C	D	SE	RDP	L	Q
NAN, % of DM								
FAB	7.75[a]	7.78[a]	7.30[ab]	6.98[b]	0.19	< 0.01	< 0.01	0.45
PAB	7.99[ab]	8.15[a]	7.87[bc]	7.72[c]	0.09	< 0.01	< 0.01	0.04
FP	4.46[a]	4.32[ab]	4.16[b]	3.91[c]	0.17	< 0.01	< 0.01	0.41
PP	4.40[b]	4.64[a]	4.51[ab]	4.60[a]	0.08	0.05	0.16	0.27
Total Purines, µmol/g								
FAB	80.5[ab]	82.6[a]	76.1[bc]	71.8[c]	2.8	0.01	< 0.01	0.26
PAB	78.9	82.7	76.6	71.6	4.4	0.23	0.12	0.34
FP	30.9	31.4	31.9	30.5	1.3	0.86	0.86	0.53
PP	23.4	24.0	22.5	21.7	1.1	0.11	0.07	0.47
N:total purine ratio								
FAB	0.98	0.96	0.97	1.00	0.04	0.80	0.48	0.44
PAB	1.06	1.03	1.07	1.14	0.05	0.27	0.14	0.31
^{15}N atom % enrichment								
FAB	0.037[b]	0.037[b]	0.040[b]	0.051[a]	0.003	< 0.01	< 0.01	< 0.01
PAB	0.032[c]	0.032[c]	0.036[b]	0.045[a]	0.002	< 0.01	< 0.01	< 0.01
FP	0.032[c]	0.032[c]	0.036[b]	0.045[a]	0.002	< 0.01	< 0.01	< 0.01
PP	0.018[b]	0.017[b]	0.018[b]	0.022[a]	0.001	< 0.01	< 0.01	< 0.01

[a,b,c]Least square means within the same row without a common superscript differ ($P < 0.05$).
[1]FAB = Fluid-associated bacteria; FP = fluid phase; PAB = particle-associated bacteria; PP = small particle plus large particle phase.
[2]Probability of a significant effect of RDP or of a linear (L) or quadratic (Q) effect of RDP level in the diet.

Table 2. Microbial nonammonia N (NAN) flows at the omasal canal measured using either total purines or ^{15}N as microbial markers.

	Diet					$P > F^2$		
Item[1]	A	B	C	D	SE	RDP	L	Q
Total Purines								
Microbial NAN flow								
FAB, g/d	149	149	155	151	11	0.95	0.78	0.75
PAB, g/d	253	255	249	244	20	0.92	0.81	0.72
Total, g/d	406	405	404	395	26	0.92	0.80	0.69
% of NAN flow[3]	59.4	60.2	62.0	63.6	2.5	0.21	0.04	0.90
NANMN flow								
% of NAN flow[3]	40.6	39.8	38.0	36.4	2.5	0.21	0.04	0.90
Microbial efficiency,								
g N/kg OMTDR	27.8	27.6	26.9	28.4	1.2	0.66	0.66	0.43
^{15}N								
Microbial NAN flows								
FAB, g/d	199[a]	189[a]	191[a]	167[b]	10	0.05	0.04	0.45
PAB, g/d	271[a]	249[b]	233[bc]	217[c]	14	< 0.01	< 0.01	0.80
Total, g/d	470[a]	438[b]	425[b]	384[c]	18	< 0.01	< 0.01	0.71
% of NAN flow[3]	69.6[a]	65.8[b]	65.1[bc]	62.1[c]	1.3	< 0.01	< 0.01	0.85
NANMN flow								
% of NAN flow[3]	30.4[c]	34.2[b]	34.9[ab]	37.8[a]	1.3	< 0.01	< 0.01	0.85
Microbial efficiency,								
g N/kg OMTDR	32.3[a]	30.1[b]	28.1[c]	28.0[c]	0.8	< 0.01	< 0.01	0.28

[a,b,c]Least square means within the same row without a common superscript differ ($P<0.05$).
[1]FAB = Fluid-associated bacteria; FP = fluid phase; NANMN = nonammonia non-microbial nitrogen; OMTDR = OM truly digested in the rumen; PAB = particle-associated bacteria; PP = small particle plus large particle phase.
[2]Probability of a significant effect of rumen-degraded protein (RDP) or of a linear (L) or quadratic (Q) effect of RDP level in the diet.
[3]As percent of total NAN flow at the omasal canal.

Effects of Different Dietary Ratios of Alfalfa and Corn Silage on Milk Production of Lactating Dairy Cows

A.F. Brito and G.A. Broderick

Introduction

Feed protein that escapes ruminal degradation, microbial protein synthesized in the rumen, and endogenous proteins are the sources of metabolizable protein used by lactating cows for milk production. Microbial protein has a good balance of amino acids (AA) and its production should be maximized in the rumen. Diets based on alfalfa silage (AS) are high in both CP and rumen-degraded protein (RDP) but deficient in rumen-undegraded protein (RUP). Corn silage (CS) is low in CP but a good source of fermentable energy due to its high starch content, making it complementary to AS. Replacing AS with CS will necessitate feeding more protein concentrate such as solvent soybean meal (SSBM). Protein in SSBM is more resistant to ruminal degradation than that in AS and, consequently, will supply more RUP to the cow. In addition, these two distinct sources of RDP (AS and SSBM) likely will support differing amounts of microbial protein formation. Thus, the objectives of this study were to evaluate the effects of different dietary ratios of AS to CS (AS:CS) on milk yield, ruminal metabolites, and N metabolism of high producing dairy cows.

Material and Methods

Twenty-eight multiparous Holstein cows (8 with ruminal cannulae) were randomly assigned to seven 4 × 4 replicated Latin squares with 28-d periods. Diets contained (% of DM) the following proportions of AS:CS, 50:0 (diet A), 37:13 (diet B), 23:27 (diet C), or 10:40 (diet D) as is presented in Table 1. Production data were collected during the last 2-wk of each period. At the end of each period, ruminal samples were collected from the 8 cannulated cows over a 24 h period. Spot samples of urine and feces were collected from all cows at the end of each period; urine volume was estimated from creatinine while total tract apparent digestibility was estimated using indigestible ADF as internal marker. Blood samples were collected 4 h after feeding from the coccygeal artery or vein of each cow and later analyzed for urea. Statistical analyses were done using proc mixed in SAS; differences between least squares means were declared only if the F-test for dietary treatment was significant ($\alpha \leq 0.05$).

Table 1. Composition of diets.

Item	Diets			
	A	B	C	D
AS:CS ratio	50:0	37:13	23:27	10:40
	----------% of DM----------			
Alfalfa silage	50.6	37.2	23.7	10.2
Corn silage	…	13.3	26.7	40.0
Rolled high moisture shelled corn	43.3	39.1	34.7	30.5
Solvent soybean meal	2.95	7.25	11.7	16.1
Energy booster	1.98	1.99	1.99	1.99
Sodium bicarbonate	0.74	0.74	0.74	0.74
Salt	0.20	0.20	0.20	0.20
Dicalcium phosphate	0.20	0.20	0.20	0.20
Vitamin premix and trace minerals	0.10	0.10	0.10	0.10
Chemical composition				
Crude protein	17.2	16.9	16.6	16.2
Ash	8.32	7.57	6.82	6.06
NDF	23.3	23.5	23.8	24.1
ADF	16.1	15.1	14.1	13.1

Results and Discussion

Dietary CP content decreased slightly when CS replaced AS whereas dietary ADF had a more pronounced decrease, from 16.1% on diet A to 13.1% on diet D (Table 1), reflecting the higher ADF content of AS compared with CS (data not shown). Neutral detergent fiber was similar across diets and averaged 23.7%.

Dry matter intake (DMI) decreased linearly from diet A to D (Table 2). Cows fed diets A and B had the highest DMI, cows fed diet D were lowest, and intake on diet C was intermediate. A significant quadratic effect also was observed for DMI. Milk yield did not differ among diets A, B and C but was lower on diet D (Table 2). Both linear and quadratic effects were significant for milk yield. Milk fat content and yield decreased linearly from diet A to diet D, being highest on diet A, intermediate on B and C, and lowest on D. The linear decrease in milk fat percentage may be related to low dietary fiber: ADF decreased when CS replaced AS and was below NRC (2001) recommendations for all diets. Therefore, it may be speculated that reducing effective fiber in going from diet A to D led to reduced rumination time, lower salivation, and depressed ruminal buffering capacity. Although ruminal pH did not differ across diets over the 24-h feeding cycle (Table 3), it is possible that pH fell below critical levels for longer periods when CS replaced AS. Literature reports have shown that lower ruminal pH may induce formation of *trans*-fatty acids that depress incorporation of dietary fat. We also observed a linear decrease in ruminal acetate when CS replaced AS (Table 3); acetate is a major precursor for milk fat synthesis.

Table 2. Effects of different alfalfa:corn silage (AS:CS) ratios on production of lactation dairy cows.

Item	Diets					Significance (P-value)[1]		
	A	B	C	D				
AS:CS ratio	50:0	37:13	23:27	10:40	SED[2]	AS:CS	L	Q
DMI, kg/d	26.8[a]	26.5[a]	25.4[b]	23.7[c]	0.44	< 0.01	< 0.01	0.04
N intake, g/d	744[a]	719[b]	675[c]	617[d]	12.3	< 0.01	< 0.01	0.06
Milk yield, kg/d	41.5[a]	42.0[a]	41.5[a]	39.5[b]	0.86	0.03	0.02	0.04
3.5% FCM, kg/d	43.3[a]	42.7[ab]	40.5[bc]	38.7[c]	1.29	< 0.01	< 0.01	0.53
Milk fat, %	3.81[a]	3.58[ab]	3.38[bc]	3.34[c]	0.12	< 0.01	< 0.01	0.27
Milk fat, kg/d	1.56[a]	1.51[ab]	1.40[bc]	1.33[c]	0.06	< 0.01	< 0.01	0.97
Milk protein, %	3.07[b]	3.13[ab]	3.14[ab]	3.17[a]	0.04	0.05	< 0.01	0.77
Milk protein, kg/d	1.26	1.32	1.30	1.25	0.03	0.13	0.73	0.02
Lactose, %	4.88	4.85	4.83	4.84	0.03	0.32	0.12	0.30
Lactose, kg/d	2.02	2.06	2.02	1.93	0.06	0.11	0.07	0.10
SNF, %	8.88	8.90	8.89	8.94	0.05	0.53	0.21	0.65
SNF, kg/d	3.66	3.77	3.70	3.55	0.09	0.13	0.18	0.05
Milk/DMI	1.53[b]	1.58[b]	1.64[a]	1.67[a]	0.03	< 0.01	< 0.01	0.77
Milk NPN, mg/dl	33.6[ab]	32.5[b]	32.5[b]	33.8[a]	0.61	0.04	0.88	< 0.01
MUN, mg/dl	13.8	13.9	14.1	14.4	0.47	0.60	0.18	0.86
MUN, % of milk NPN	41.3	43.2	44.0	42.5	1.78	0.47	0.40	0.19
BUN, mg/dl	18.4[ab]	17.1[b]	18.6[a]	19.0[a]	0.72	0.05	0.16	0.10
BW change, kg/d	0.92	0.90	1.08	1.03	0.19	0.71	0.37	0.92

[1]L = linear effect; Q = quadratic effect.
[2]SED = standard error of the least square means difference.
[a,b,c,d]Means in the same row with different superscripts differ ($P \leq 0.05$).

Milk protein content increased linearly with increasing SSBM in the diet (from A to D), possibly due to increased RUP supply; diets A and D were different while diets C and D were intermediate. Yield of milk protein did not differ by diet but there was a significant quadratic effect, indicating optimal milk protein yield at intermediate AS:CS ratios. Milk NPN was highest on diet D, intermediate on diet A, and lowest on diets B and C. We did not expect a significant increase in milk NPN on diet D (compared to diets B and C), nor did we expect higher BUN on diets C and D. However, dietary CP content is

the major factor influencing MUN and BUN and all diets were similar in CP. Milk urea N content and proportion of milk NPN did not differ, averaging 14.1 mg/dl and 42.8% across diets.

Apparent total tract digestibility of DM, OM, and hemicellulose did not differ across diets; however, apparent total tract digestibility of NDF and ADF decreased linearly from diets A to D (Table 3). Significant quadratic effects also were observed for NDF and ADF digestibility. The linear decrease in fiber digestibility may be related to depression of cellulolytic bacteria with increasing dietary CS, plus the linear decrease in ruminal acetate, suggesting that replacing AS with CS may have inhibited fiber digestion. As expected, ruminal ammonia and total free AA decreased linearly with decreasing AS, probably due to reduced dietary NPN and RDP. Ruminal propionate, butyrate, and total VFA did not differ across diets and averaged 30.2, 15.4, and 135 mM (Table 3). On the other hand, ruminal acetate, isobutyrate, and acetate:propionate ratio decreased while valerate increased linearly with increasing dietary CS.

Table 3. Effects of different alfalfa:corn silage (AS:CS) ratios on total tract apparent digestibility and on ruminal metabolism in lactating dairy cows.

	Diets					Significance (P-value)[1]		
Item	A	B	C	D				
AS:CS ratio	50:0	37:13	23:27	10:40	SED[2]	AS:CS	L	Q
Apparent digestibility, %								
DM	65.2	65.5	67.0	65.8	0.82	0.14	0.20	0.19
OM	65.8	66.2	67.7	66.9	0.84	0.13	0.08	0.30
NDF	38.9^{a}	37.2^{ab}	36.1^{b}	31.2^{c}	1.11	< 0.01	< 0.01	0.04
ADF	41.3^{a}	40.1^{a}	38.8^{b}	33.5^{c}	0.91	< 0.01	< 0.01	< 0.01
Hemicellulose	23.7	22.1	25.3	22.2	1.67	0.20	0.91	0.55
N	63.0^{b}	63.6^{b}	66.2^{a}	66.0^{a}	1.20	0.01	< 0.01	0.61
Ruminal metabolism								
pH	6.29	6.31	6.30	6.31	0.05	0.99	0.77	0.90
Ammonia, mM	7.52^{a}	7.14^{ab}	6.23^{b}	4.42^{c}	0.66	< 0.01	< 0.01	0.13
Total free AA, mM	4.20^{a}	3.87^{a}	4.07^{a}	2.57^{b}	0.50	< 0.01	< 0.01	0.11
Acetate, mM	88.6^{a}	84.8^{ab}	79.6^{bc}	74.0^{c}	3.46	< 0.01	< 0.01	0.73
Propionate, mM	29.2	29.7	30.3	31.5	2.09	0.73	0.27	0.82
Butyrate, mM	16.3	15.3	15.1	14.7	1.05	0.48	0.14	0.71
Isobutyrate, mM	1.92^{a}	1.82^{ab}	1.71^{b}	1.73^{b}	0.08	0.05	0.01	0.31
Valerate, mM	2.82^{b}	2.64^{b}	2.58^{b}	3.36^{a}	0.24	0.01	0.06	0.01
Isovalerate, mM	2.79	2.61	2.65	2.70	0.17	0.75	0.67	0.36
Total VFA, mM	142	137	132	128	6.04	0.13	0.02	0.93
Acetate:Propionate	3.22^{a}	2.95^{b}	2.84^{b}	2.53^{c}	0.14	< 0.01	< 0.01	0.88

[1]L = linear effect; Q = quadratic effect.
[2]SED = standard error of the least square means difference.
[a,b,c]Means in the same row with different superscripts differ ($P \leq 0.05$).

As expected urinary excretion of urea N and total N, as well as fecal N and total N excretion, decreased linearly when CS replaced AS; diets A and B were significantly higher than diets C and D (Table 4). Therefore, N efficiency expressed either, as milk N/N intake or as kg of milk per kg of N excreted, increased linearly when the proportion of dietary AS was reduced.

Table 4. Effects of different alfalfa:corn silage (AS:CS) ratios on nitrogen metabolism of lactating dairy cows.

Item	Diets					Significance (P-value)[1]		
AS:CS ratio	A 50:0	B 37:13	C 23:27	D 10:40	SED[2]	AS:CS	L	Q
Urinary excretion								
Total N, g/d	217[a]	215[a]	201[b]	188[b]	7	< 0.01	< 0.01	0.28
Urea N, g/d	183[a]	165[b]	158[b]	157[b]	7.3	< 0.01	< 0.01	0.11
Total volume, L/d	29.1[a]	24.4[b]	21.5[c]	17.6[d]	1.5	< 0.01	< 0.01	0.68
Creatinine, mmol/d	172	172	173	173	1	0.73	0.39	0.96
Allantoin, mmol/d	367	386	392	383	14	0.29	0.20	0.17
Fecal excretion								
N, g/d	275[a]	263[a]	230[b]	211[b]	10	< 0.01	< 0.01	0.62
Total N excretion								
g/d	491[a]	479[a]	431[b]	400[c]	12	< 0.01	< 0.01	0.31
% of N intake	66.5	66.6	64.1	65.3	1.4	0.27	0.22	0.54
N efficiency								
Milk N, % of N intake	26.5[d]	28.5[c]	30.0[b]	31.7[a]	0.5	< 0.01	< 0.01	0.70
kg of milk: kg of N excreted	85.7[b]	89.7[b]	98.9[a]	101.0[a]	2.9	< 0.01	< 0.01	0.58

[1]L = linear effect; Q = quadratic effect.
[2]SED = standard error of the least square means difference.
[a,b,c,d]Means in the same row with different superscripts differ ($P \leq 0.05$).

Conclusions

Replacing dietary AS with CS reduced N excretion in urine and feces. Diets with AS:CS ratios of 37:13 (B) and 23:27 (C) maintained high animal performance and reduced N excretion and feeding these proportions of alfalfa and corn silages were optimal and complementary. Based on this study, we would not recommend diets with either AS or CS as the only forage because of respectively, environmental concerns and poor animal performance.

Effects of Different Protein Supplements on the Production of Lactating Dairy Cows

A. F. Brito and G. A. Broderick

Introduction

Ammonia is a major N source for synthesis of microbial protein and is essential for the growth of several species of ruminal bacteria. According to the CNCPS model, ruminal bacteria fermenting structural carbohydrates use only ammonia while those fermenting nonstructural carbohydrates use peptides and amino acids (AA) as N sources. However, several studies established clearly that AA and peptides increased microbial growth and fiber digestion. Because protein supplements may increase ruminal concentration of peptides and AA, an increase in microbial protein synthesis may be expected with feeding true protein supplements instead of urea. Therefore, the objective of this trial was to compare three true protein supplements, solvent soybean meal (SSBM), cottonseed meal (CSM), and canola meal (CM), as sources of RDP for protein formation in the rumen and RUP for lactating dairy cows. Effects of these different RDP sources on animal production and ruminal metabolism also were investigated.

Material and Methods

Sixteen multiparous and 8 primiparous Holstein cows (8 with ruminal cannulae) were blocked by parity and DIM and randomly assigned to six 4 × 4 Latin squares and fed diets containing (DM basis) 20.7% alfalfa silage and 35.1% corn silage (Table 1). The following protein supplements were added to make the diets isonitrogenous: urea (diet A), SSBM (diet B), CSM (diet C), or CM (diet D). Production data were collected during the last 2-wk of each period. At the end of each period, ruminal samples were collected from the 8 cannulated cows over a 24 h period. Spot samples of urine were also collected at the end of each period from all cows; urine volume was estimated from creatinine. Cobalt-EDTA, $YbCl_2$, and $^{15}NH_4SO_4$ were infused continuously into the rumen for 6 d and digesta leaving the rumen was taken using the omasal sampling technique; omasal true digesta were reconstituted using a triple-marker approach. Bacterial samples also were collected and separated by differential centrifugation to give fluid-associated bacteria (FAB) and particle-associated bacteria (PAB). Statistical analyses were done using proc mixed in SAS; differences between least squares means were declared only if the F-test from treatment was significant ($\alpha \leq 0.05$).

Table 1. Composition of diets.

	Diets[1]			
Item	A Urea	B SSBM	C CSM	D CM
	----------% of DM----------			
Alfalfa silage	20.7	20.7	20.7	20.8
Corn silage	35.0	35.1	35.1	35.0
Rolled high moisture shelled corn	40.7	30.5	28.5	26.5
Urea	1.9	…	…	…
Solvent soybean meal	…	12.1	…	…
Cottonseed meal	…	…	14.1	…
Canola meal	…	…	…	16.1
Limestone	0.5	0.5	0.5	0.5
Sodium bicarbonate	0.5	0.5	0.5	0.5
Salt	0.3	0.3	0.3	0.3
Dicalcium phosphate	0.1	0.1	0.1	0.1
Vitamin premix and trace minerals	0.2	0.2	0.2	0.2
Chemical composition				
Crude protein	16.5	16.5	16.6	16.6
Ash	6.8	7.5	7.8	8.0
NDF	22.7	22.8	24.6	25.5
ADF	12.6	12.9	14.2	14.9
ADIN, % of total N	1.1	1.1	1.8	2.6
NE_L,[2] Mcal/kg of DM	1.56	1.58	1.55	1.56

[1]CM = Canola meal; CSM = cottonseed meal; SSBM = solvent soybean meal.
[2]Computed by discounting dietary energy based on actual DM intakes (NRC, 2001).

Results and Discussion

The CP contents were very similar averaging 16.6% across diets (Table 1). However, both NDF and ADF progressively increased from diet A (urea) to diet D (CM), reflecting the differential NDF and ADF contents among SSBM, CSM and CM (data not shown). Production data are presented in Table 2. Cows fed diet A (urea) had the lowest DM intake and intake of cows fed diet C (CSM) was intermediate compared with that on diets B (SSBM) and D (CM). Yield of milk and milk components and feed efficiency (milk yield/DMI) were much lower on urea compared to that on diets supplemented with true protein, probably due to both lower DM intake and lower RUP supply. Cows fed true protein supplements had similar performance. Exceptions to this trend were yields of fat and protein, which

were lower on CSM than on CM, with that on SSBM being intermediate. It is possible that greater fat mobilization in cows fed diet A (cows on this diet gained only 0.6 kg/d) compared to that of cows fed the other diets (average weight gain = 1.2 kg/d) accounted for the higher fat test observed on that diet.

Table 2. Effects of protein supplements on production of lactating dairy cows.

	Diets[1]					
Item	A Urea	B SSBM	C CSM	D CM	SED[2]	$P > F^3$
DM intake, kg/d	22.1[c]	24.2[b]	24.7[ab]	24.9[a]	0.4	< 0.01
N intake, g/d	574[c]	639[b]	655[ab]	662[a]	11	< 0.01
Milk, kg/d	32.9[b]	40.0[a]	40.5[a]	41.1[a]	1.1	< 0.01
3.5% FCM, kg/d	30.6[c]	37.1[ab]	36.7[b]	38.8[a]	1.1	< 0.01
Fat, %	3.26[a]	3.09[ab]	2.94[b]	3.14[ab]	0.15	0.23
Fat, kg/d	1.01[c]	1.22[ab]	1.18[b]	1.29[a]	0.05	< 0.01
Protein, %	2.91[b]	3.15[a]	2.97[b]	3.12[a]	0.05	< 0.01
Protein, kg/d	0.92[c]	1.23[ab]	1.18[b]	1.27[a]	0.03	< 0.01
Lactose, %	4.88	4.95	4.98	4.87	0.05	0.14
Lactose, kg/d	1.56[b]	1.96[a]	1.99[a]	2.01[a]	0.06	< 0.01
SNF, %	8.70[b]	8.98[a]	8.84[ab]	8.89[a]	0.07	< 0.01
SNF, kg/d	2.76[b]	3.54[a]	3.53[a]	3.65[a]	0.10	< 0.01
Milk/DM intake	1.51[b]	1.67[a]	1.65[a]	1.66[a]	0.05	< 0.01
Body weight gain, kg/d	0.58[b]	1.23[a]	1.00[a]	1.25[a]	0.15	< 0.01

[1]CM = Canola meal; CSM = cottonseed meal; SSBM = solvent soybean meal.
[2]SED = standard error of the least squares means difference.
[3]Probability of a significant effect of diet.
[a,b,c]Means with different superscripts differ ($P < 0.05$).
with different superscripts differ ($P < 0.05$).

Ruminal pH did not differ and averaged 6.54 across diets (Table 3). As expected, ruminal ammonia was highest in the diet supplemented with urea compared to the diets supplemented with the true proteins. Urea probably was degraded to ammonia too rapidly to be efficiently captured by the ruminal microbes. Ammonia will be absorbed at the rumen and intestine, converted to urea in the liver and, although it can be recycled to the rumen, most will be excreted in the urine and contribute to environmental pollution. This accounts for the greater urinary urea excretion on diet A (Table 3). Total free AA were higher on diet B (SSBM), intermediate on diet A (urea), and lower on diets C (CSM) and D (CM). No differences were observed for ruminal concentrations of VFA measured in this trial (Table 3), except for isobutyrate. Isobutyrate is formed from AA catabolism, which explains why it was greater on the diets containing true protein. Among these 3 diets, that supplemented with CSM gave the lowest urinary urea excretion. However, cows fed diet C also had lower N efficiency (Table 3). It is speculated that the poorer AA balance of RUP from CSM reduced milk protein secretion.

Table 3. Effects of protein supplements on ruminal metabolism, urinary excretion, and N efficiency of lactating dairy cows.

	Diets[1]					
Item	A Urea	B SSBM	C CSM	D CM	SED[2]	P > F[3]
Ruminal metabolism						
pH	6.59	6.53	6.52	6.51	0.06	0.60
Ammonia, mM	7.76[a]	5.92[b]	5.65[b]	5.85[b]	0.52	< 0.01
Total free AA, mM	8.08[ab]	9.02[a]	7.70[b]	7.11[b]	0.62	0.02
Acetate, mM	53.1	55.7	56.3	54.7	1.6	0.21
Propionate, mM	19.9	21.6	19.0	20.6	1.22	0.20
Butyrate, mM	10.8	10.1	10.1	9.82	0.61	0.44
Isobutyrate, mM	1.06[b]	1.20[a]	1.15[ab]	1.17[a]	0.05	0.04
Valerate, mM	2.04	1.95	1.77	1.83	0.16	0.33
Isovalerate, mM	1.85	1.97	1.91	1.79	0.09	0.28
Total VFA, mM	88.7	92.1	90.3	89.9	2.8	0.67
Acetate:Propionate	2.79	2.69	3.03	2.71	0.14	0.09
Urinary excretion						
Urea-N, g/d	165[a]	110[b]	94.6[c]	108[b]	5	< 0.01
Total volume, L/d	20.0[ab]	18.3[b]	21.3[a]	18.1[b]	1.2	0.04
N efficiency						
Milk N, % of N intake	24.9[c]	30.4[a]	28.5[b]	30.2[a]	0.9	< 0.01

[1]CM = Canola meal; CSM = cottonseed meal; SSBM = solvent soybean meal.
[2]SED = standard error of the least squares means difference.
[3]Probability of a significant effect of diet.
[a,b,c]Means with different superscripts differ ($P < 0.05$).

Table 4. Effects of protein supplements on intake and omasal nutrient flows in lactating dairy cows (8 ruminal cannulated cows only).

	Diets[1]					
Item[2]	A Urea	B SSBM	C CSM	D CM	SED[3]	$P > F$[4]
DM intake, kg/d	21.4[b]	23.6[a]	24.0[a]	24.7[a]	1.0	0.02
DM flow, kg/d	13.0[b]	15.1[a]	16.2[a]	16.1[a]	0.7	< 0.01
OM intake, kg/d	20.0[b]	21.8[a]	22.2[a]	22.8[a]	0.9	0.03
OM flow, kg/d	10.7[b]	12.4[a]	13.4[a]	13.3[a]	0.6	< 0.01
OMADR, %	46.7[a]	43.2[b]	39.6[c]	41.4[bc]	1.1	< 0.01
OMTDR, %	67.4[a]	64.4[b]	61.4[c]	62.1[c]	1.1	< 0.01
FAB-NAN flow, g/d	173[b]	194[ab]	180[b]	205[a]	11	0.04
PAB-NAN flow, g/d	199[b]	235[a]	249[a]	234[a]	16	0.03
Total bacterial-NAN, g/d	372[b]	429[a]	428[a]	439[a]	21	0.02
Bacterial efficiency, g/kg OMTDR	27.7[b]	30.6[a]	31.5[a]	31.2[a]	0.8	< 0.01
Ammonia-N, g/d	12.0[a]	10.2[b]	9.30[b]	11.9[a]	0.7	< 0.01
Free AA-N, g/d	48.9[b]	61.3[a]	53.1[ab]	57.0[ab]	4.1	0.04

[1]CM = Canola meal; CSM = cottonseed meal; SSBM = solvent soybean meal.
[2]FAB-NAN = Fluid-associated bacterial nonammonia N; NAN = nonammonia N; OMADR = OM apparently digested in the rumen; OMTDR = OM truly digested in the rumen, PAB-NAN = particle-associated bacterial nonammonia N.
[3]SED = standard error of the least squares means difference.
[4]Probability of a significant effect of diet.
[a,b,c]Means with different superscripts differ ($P < 0.05$).

Ruminally cannulated cows fed diet A (urea) had lower intake and omasal flow of DM and OM compared to cows fed diets supplemented with true protein (Table 4). However, OM apparently digested in the rumen (OMADR) and OM truly digested in the rumen (OMTDR) were highest in cows supplemented with urea. Lower intake, and the resulting slower passage rates, likely account for the greater extents of digestion. Efficiency and total yield of ruminal bacterial protein was greater on the diets supplemented with true protein; total yields of nonammonia N averaged 50 g/d more than on the urea diet. Because diet C (CSM) yielded the same amount of bacterial protein as diets B (SSBM) and D (CM), it is suggested that the somewhat poorer protein and fat yield on CSM may be due to a lower contribution or poorer AA pattern of RUP.

Conclusions

Feeding diets supplemented with true protein gave better lactation performance than feeding a diet supplemented with urea. Cows fed CM yielded more fat and protein than cows fed CSM, with yields on SSBM being intermediate, indicating that CM would serve as a more effective protein supplement for dairy cows fed diets based on alfalfa and corn silage. Similar microbial protein yields on SSBM, CSM and CM suggested that better performance on CM may result from better AA pattern, greater RUP content, or both.

MANURE NUTRIENT MANAGEMENT

A Novel Approach to Regulate Nitrogen Mineralization in Soil

K. Kumar, C.J. Rosen, and M.P. Russelle

Introduction

One of the most difficult aspects of nitrogen management on farms is predicting the amount of nitrogen that will become available from organic sources, such as soil organic matter, manure, and plant residues. Farmers often need to apply manure or incorporate plant residues in autumn, far ahead of the time the next crop will require the nitrogen that will be released from these organic materials. If nitrogen release occurs quickly, much of it could be lost by leaching to ground water or movement of gases to the atmosphere. In order to reduce the risk of negative environmental impacts, nitrogen release needs to match the plant nitrogen requirements. The only methods available in the past to alter nitrogen mineralization rate of these organic materials has been through tillage management or residue placement. We tested a new approach to this problem by using protease inhibitors, which regulate a crucial step in ammonia release from proteins, as amendments to soil and organic residues.

Methods

Soil amended with alfalfa residues and non-amended soil was incubated in the laboratory with and without different protease inhibitors. Soil was extracted periodically with 2*M* KCl and inorganic nitrogen (ammonium and nitrate) was determined. Release of inorganic nitrogen was taken to be as a measure of nitrogen (N) mineralization (Equation 1).

Net N mineralization = inorganic N at end of incubation – inorganic N at start of incubation (1)

In a second greenhouse experiment, 2 kg of Hubbard loamy sand was amended with alfalfa residues supplying N at the equivalent of 150 kg N ha^{-1} in pots, and was then planted with corn. The six protease inhibitor treatments applied were: (i) complete inhibitor (CI); (ii) Aprotinin; (iii) Leupeptin; (iv) EDTA; (v) CI at day 1 and another dose on day 15; and (vi) control without protease inhibitor. Some of these inhibitors are used to treat patients suffering from viral infections. Each treatment was replicated six times. Corn from three replicates was harvested after 30 days and the rest after 45 days of growth.

Results and Discussion

In a short-term incubation, some protease inhibitors were more efficient in reducing nitrogen mineralization than others, and there were interactions between soils and protease inhibitors. This means that the amount of protease inhibitor required will vary with the situation (soil and organic matter addition, but maybe also weather and other variables).

The effects of protease inhibitors in reducing net N mineralization were also seen after 50 d of incubation (Figure 1a). The complete protease inhibitor cocktail was most efficient in reducing net N mineralization in both the control soil and soil amended with alfalfa. The reduction in net N

mineralization for alfalfa-amended soils was not as great as in the non-amended control soil. Alfalfa residues probably increased protease activity in soil, reducing the effectiveness of a single dose of inhibitor to decrease the protease activity. However, a second dose of inhibitor at 25 d reduced N mineralization during the next 25 d (Figure 1b).

Corn was stunted under EDTA treatment, probably due to deficiency of metal ions. Protease inhibitors significantly reduced corn N uptake at the first harvest. The treatments that included addition of two doses of CI and Aprotinin showed lowest N uptake. There were no differences, however, among control and protease inhibitor treatments for the corn crop harvested after 45 days, except in the EDTA treatment where corn growth was stunted.

We tested a precise, accurate, and simple assay for protease enzyme activity in soil by the use of QuantiCleave Fluorescent Protease Assay Kit. This method is rapid compared to the traditional method as we can measure protease activity at the same time on multiple samples using 96 well plates. The protease activity of 10 different soils using this method highly correlated with the traditional methods using proteins as a substrate.

Conclusion

Protease inhibitors appear to have promise as a new means to regulate nitrogen release from soil organic matter and other organic materials. More research is needed to determine the amounts needed under different situations. Currently, protease inhibitors are too expensive to use agriculturally, but this may change as technology improves. This discovery may lead to methods for on-farm control of nitrogen supply to crops, which will reduce both the amount of commercial fertilizer needed and the loss of nitrogen to water and the air.

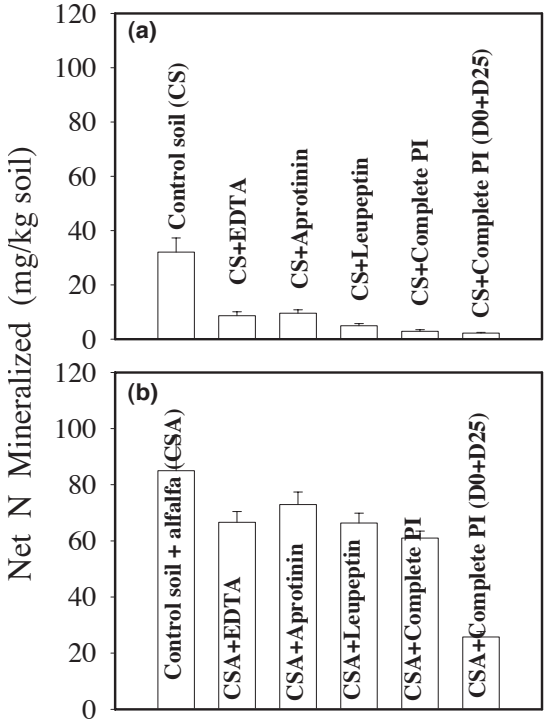


Figure 1. Effect of protease inhibitors on nitrogen mineralization during a 50-d incubation of (a) soil and (b) soil amended with alfalfa herbage (11.2 Mg dry mass ha^{-1} or 150 kg N ha^{-1}).

Exploring the Use of Animal Density Standards for Nutrient Management Policy on Wisconsin Dairy Farms

H. Saam, J.M. Powell, D.B. Jackson-Smith, W.L. Bland, and J.L. Posner

Introduction

As demands for more controlled manure management heighten, policy makers seek indicators to assess the environmental impacts of livestock production and to subsequently direct manure management policy. Herd size indicators are currently used to target federal manure management policy. While recognizing the increased level of management needed to safely store and land apply very large amounts of manure, size-based indicators have been criticized for holding large livestock operations solely responsible for the majority of agriculture's pollution problems. The issue of whether large farms contribute disproportionately to pollution problems is increasingly unclear. It is often suggested, for example, that the economies of size, more modern technologies and potentially higher management skills associated with large-scale operations may actually put large farms at a decreased potential to pollute in relation to smaller, outdated facilities. An alternative to herd size or location-based indicators for targeting nutrient management policy is animal density, expressed in terms of animals per unit area of cropland. Animal density is increasingly being used in Europe and in certain parts of the U.S. The strength of using animal density as a regulatory standard lies in its ability to provide a straightforward, relatively easy to calculate indicator of a farm's nutrient balancing potential. By characterizing the relationship of animal numbers (and the manure they produce) to the available cropland area for manure utilization, animal density addresses the core movement of nutrients within the farm nutrient cycle. Without adequate cropland on which to recycle manure nutrients, farms of all size have an increased potential for nutrient loss. The objective of this study was to investigate the implications of using alternative definitions of animal density standards to target nutrient management policy on Wisconsin dairy farms. Different conceptualizations of the land base available for manure application were developed and used to (1) predict the ability of Wisconsin dairy farmers to balance manure production with adequate cropland for recycling manure nutrients, (2) explore the relationship between dairy herd size, herd size expansion, and animal density levels, and (3) contrast cropland area potentially available for manure application with actual reported manure spreading areas.

Materials and Methods

In the late-winter and early-spring of 1999, a state-wide random survey of approximately 1,600 representative Wisconsin dairy farmers was conducted. Data on dairy herd size, livestock inventories, and crop production were used to calculate animal densities (animal:land ratios). The animal component of the animal:land ratio (ALR) was calculated using animal unit (AU) equivalency factors, where 1 AU equals 454 kg animal live weight. Live weights of 635 and 410 kg were assumed for cows (both lactating and non-lactating) and heifers, respectively. The land area available for manure application was computed for each farm based on reported crop areas for the 1998 cropping season. Three different ALRs were calculated for each farm: (1) animal units:total cropland (ACLR), (2) animal units:tilled cropland (ATLR), and (3) animal units:manured cropland (AMLR). Low, medium, and high animal density categories were delineated based on the amount of N and P in manure and the average removal of these nutrients by a typical dairy cropping system.

Low animal density: Dairy operations having less than 1.85 AU/ha would have sufficient cropland to recycle all manure P. However, crop N needs would not be met through manure applications alone. These operations should not face a significant degree of difficulty in implementing either N- or P-based manure management standards.

Medium animal density: Dairy operations having between 1.85 and 3.70 AU/ha have enough manure to meet crop N requirements, but manure P would exceed crop P requirements. The ability of these operations to comply with nutrient management standards would greatly depend on whether a N- or P-based standard is in place.

High animal density: Even with the best manure management strategies, it would be nearly impossible for farms with animal densities greater than 3.70 AU/ha to apply manure to meet, and not exceed, crop N and P requirements.

Note: These animal densities, or estimates of a farm's nutrient balancing potential, do not account for nutrient additions from legume N, commercial fertilizer, and existing soil nutrient levels.

Results and Discussion

ACLRs indicate that most (95%) Wisconsin dairy farmers have sufficient cropland for meeting N-based manure application standards, and 63% have sufficient cropland for meeting P-based standards. Under ATLR calculations, about 79% of farmers have sufficient cropland for meeting, and not exceeding crop N requirements. However, 75% of farms produce manure in excess of crop P requirements. Under AMLR calculations, about 71% of Wisconsin dairy farms apparently apply manure in excess of crop N and P requirements.

Having sufficient cropland for agronomic manure application rates is a key aspect of nutrient management planning. Without an adequate land base on which to recycle manure nutrients livestock operations of all sizes have an increased risk of over-applying manure and polluting the environment. Our analyses suggest that the distribution of animal density categories is somewhat similar on dairy farms having herd size of less than 200 cows (Table 1). Dairy farms having more than 200 cows, however, have greater percentages of medium and high density farms, and fewer low density farms compared to other herd size categories.

Table 1. Nutrient balancing potential by herd size on Wisconsin dairy farms

		Herd size category (AU)					
		1 to 34 (n=65)	35 to 69 (n=242)	70 to 104 (n=235)	105-139 (n=88)	140-279 (n=98)	280+ (n=27)
Animal:land ratio	Animal density	% of farms within herdsize class					
ACLR							
	Low	55.4	60.7	68.1	60.2	73.5	25.9
	Medium	36.9	35.1	29.3	34.1	22.4	55.6
	High	7.7	4.2	2.6	5.7	4.1	18.5
	Total	*100*	*100*	*100*	*100*	*100*	*100*
ATLR							
	Low	23.1	22.7	25.5	26.1	32.7	11.1
	Medium	40.0	51.2	61.3	52.3	52.0	55.6
	High	36.9	26.1	13.2	21.6	15.3	33.3
	Total	*100*	*100*	*100*	*100*	*100*	*100*

Differences between ALR calculations based on total (ACLR) and manured (AMLR) cropland indicate the "manure gap", i.e. the amount of cropland where manure can be potentially applied but is not (Figure 1). On dairy operations having up to 200 cows, only about 25 to 30% of the total cropland receives manure (Figure 2). Farms having more than 200 cows use almost twice the amount of available cropland to apply manure.

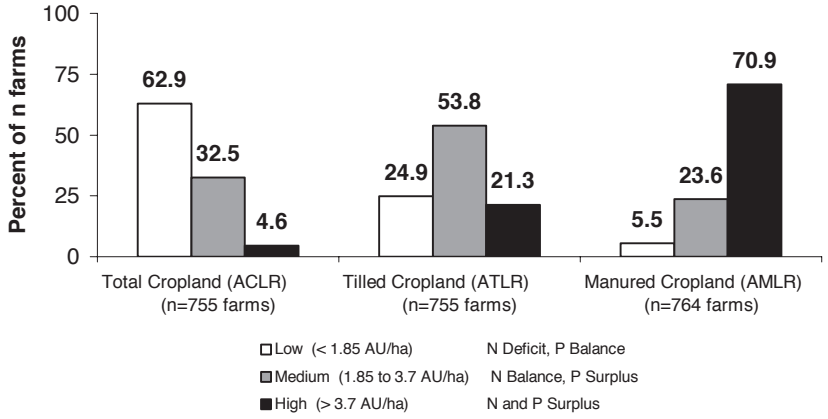


Figure 1. Animal density by cropland category for Wisconsin dairy farms

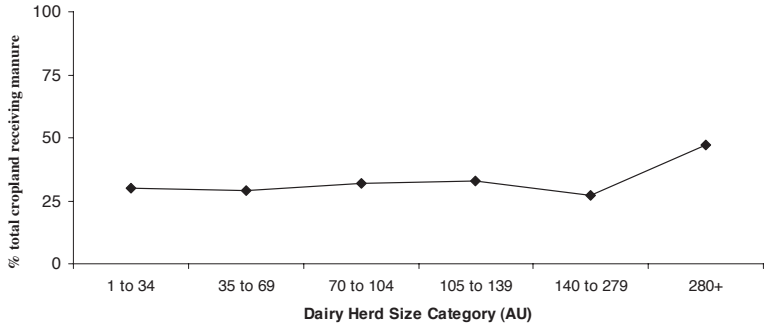


Figure 2. Percent of total cropland receiving manure by dairy herd size category

There are great regional differences in the percentage of total cropland that receives manure. The greatest contrast is between the northeastern and southwestern regions of the state. While dairy farmers in the Northeast have the lowest animal:land ratios based on total cropland (ATCR), they have the highest average animal-land ratios based on actual manure spreading behavior (AMLR). The inverse was true for the southwestern region. This pattern is explained by the fact that an average of 44% of total cropland is utilized for manure application in the Southwest, compared to only about 23% of total cropland in the Northeast. There are several important differences between these two regions that

might affect manure spreading behavior. These include differences in soil texture, land tenure, and development pressures. The southwestern region is characterized by more coarsely textured silt loam soils that have relatively high permeability and drier field conditions in the spring and fall. By contrast, the northeast is characterized by more finely textured and less permeable clayey and red loam soils. As a result, farms in the Southwest may have a wider "manure spreading window", or number of days that soil conditions are favorable for manure spreading. Farmers in the Southwest may therefore be able to access a larger proportion of their operated cropland acreage over a greater period of time than farms situated in the Northeast. Differences in land tenure, or the percentage of operated land that is owned, may also explain the regional differences in manure spreading behaviors.

Among all farms in our sample, as the percentage of operated land that is rented increases, the proportion of cropland used to spread manure decreases. Meanwhile, farmers in the Southwest tend to rent smaller areas and thus own a greater proportion of their total operated land area than farmers in the Northeast. In general, the greater areas of rented land found on farms in the northeastern region may contribute to a decreased percentage of cropland that receives manure. Moreover, the travel distance between where the animals are housed (and where the manure is produced) and the location of rented land parcels can greatly affect whether or not rented land receives manure. Spreading to distant, rented fields may be very time and energy consuming, and hence not an economically viable option. Evidence from several on-farm follow-up visits suggest that farms in the Northeast are more likely to be renting fields that are more distant from the home farm and more difficult to access without transporting manure on heavily traveled commuter roads. This could create additional disincentives for farmers in this region to spread on their rented cropland, compared to areas with lower development pressure.

Conclusions

While there are various approaches to developing manure management policy, an animal density indicator may be particularly appropriate for Wisconsin's dairy industry. Most farms continue to integrate crop and livestock production and have the potential for on-farm recycling of manure nutrients. From a policy perspective, unlike water quality-based indicators that are difficult to measure and attribute to farm practices, animal density can be accurately assessed on each farm at low costs.

Comparison of Estimates of First-Year Dairy Manure N Availability or Recovery Using ^{15}N and Other Techniques

G.R. Muñoz, K.A. Kelling, J.M. Powell and P.E. Speth

Introduction

Over the past 10 to 15 yr, there has been an increasing effort to improve the use of manure as a crop nutrient source for both environmental and economical reasons. Optimal manure N use that ensures adequate crop nutrition while avoiding pollution requires accurate and reliable estimates of manure N availability or recovery by the crop during the growing season. Two commonly used methods are (1) the "fertilizer equivalent" approach to determine manure N availability to crops, and (2) measurement of apparent N recovery by the "difference method". These indirect methods are highly variable. The objective of this research was to compare estimates of dairy manure N availability or recovery by corn using direct (^{15}N labeled manure) and indirect (fertilizer equivalent and difference method) techniques in a field study.

Materials and Methods

A field trial was conducted from 1998 to 2000 at the West Madison Agricultural Research Station in Madison, Wis. (45° 05' N, 89° 31' W) on a Plano silt loam (fine-silty, mixed, mesic, Typic Argiudolls). Treatments were five levels of fertilizer N (45, 90, 135, 179 and 224 kg ha^{-1}, applied as NH_4NO_3), two manure rates (estimated to provide approximately 90 and 180 kg available N ha^{-1} to corn in the first year following application), and a control receiving neither fertilizer N nor manure. The original goal of this trial was to test the effect of manure application rates and intervals over a longer period, but this report focuses on first-year N availability, or N uptake only in plots receiving manure for the first time. The plots for FE and Diff Meth determinations were 10.6 x 6 m, separated by 1.5 m alleys and contained eight corn rows. For the ^{15}N experiment, microplots of 1.5 x 2.3 m containing three corn rows were established within each of the low manure rate plots. Fertilizer and manure were applied about 5 d before planting. The field was disked twice within 3 to 20 h after manure application. All plots were planted to corn. Corn above-ground tissue (henceforth referred to as 'whole-plant') was harvested at approximately physiological maturity by cutting 10 adjacent plants from one row in 1998 and 1999, and 15 plants from three rows (five from each) in 2000. After sampling, the remaining plants were removed from the field. The site was chisel plowed each fall.

Manure N availability or recovery was estimated using three methods: (1) fertilizer equivalence (FE) compared crop yield or N uptake in the manure treatments with responses obtained from inorganic N fertilizer. For each year, whole plant nitrogen uptake (WPNU) were regressed against fertilizer N rate. To solve for FE, data from manured plots were entered into the regression curves, and the fertilizer rate that would have produced the same response (the FE) was interpolated; (2) the difference method (Diff Meth) assumes that all crop N uptake in the amended (manure or fertilizer) plots in excess of that taken up by the control was the result of the treatment; and (3) ^{15}N recovered in above-ground corn tissue at physiological maturity using the equation:

$$^{15}N\ recov\ \% = \frac{P(c-d)}{f(a-b)} \times 100$$

In this equation, P = total crop N, f = total manure N, a = atom % ^{15}N in the manure applied, b = atom % ^{15}N in the control manure (0.377 in 1998, and 0.366 in 1999 and 2000), c = atom % ^{15}N in the treated crop, d = atom % ^{15}N in the control crop (0.366).

Results and Discussion

At the low manure rate, N availability estimates obtained in 2000 were higher than in 1998 ($p = 0.045$) and 1999 ($p = 0.040$). This was possibly due to a gradual lowering of the soil N supply and a reduced influence of the previous alfalfa crop. As a result, corn likely had to rely more heavily on additional N inputs. Control plot responses decreased with time and greater responses to additional N were obtained in the manured and fertilized plots.

Regressions for WPNU against fertilizer rate, and the corresponding FE calculations were made by year because of the great across-year variation observed in crop performance. As an example of FE calculations, in 1998, the average whole-plant N uptake was 275 kg ha^{-1} (Fig. 1) at the low manure rate (194 kg total N ha^{-1}). According to the regression line, a fertilizer rate of 60 kg ha^{-1} would have resulted in the same crop response: this is the FE for this manure rate.

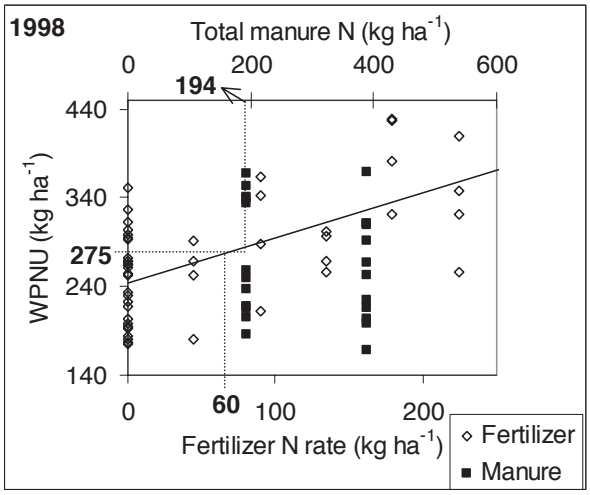


Fig.1. Corn whole-plant N uptake (WPNU) at various fertilizer and manure N rates after initial manure applications, 1998. Solid lines represent the regression ($p < 0.001$) of whole-plant N uptake against fertilizer N rate (primary X-axis). Secondary X-axis shows manure N rate. Dashed lines and bold numbers illustrate the calculation of fertilizer equivalents.

By relating the apparent manure N recovery to apparent recovery from a similar fertilizer rate, the relative effectiveness of manure can be calculated. Relative effectiveness of manure N at the low manure rate ranged from 24 to 61% with an average of 32%. This means that manure N was approximately 32% as effective as a similar rate of fertilizer N in increasing crop N uptake. Consistently lower, although not significant ($p = 0.14$) estimates were obtained at the high manure rate, which ranged from 4 to 27%, with a mean of 15%. These results were a consequence of only small increases in crop response after doubling the manure rate.

First-year recoveries of ^{15}N in whole-plant ranged from 10 to 22%, with an average across years of 14% (Table 1). In our experiment, ^{15}N recovery in 1998 (10%) was significantly lower than in 1999 (17%, $p = 0.052$) and 2000 (22%, $p = 0.011$). One possible reason for this trend might be the effect of the previous alfalfa crop and high initial soil fertility. Another influencing factor was likely reduced NH_3 volatilization in 1999 and 2000. In 1998, manure was incorporated about 20 h after being applied, whereas in 1999 and 2000, incorporation was done within 3 h. Several researchers have shown manure N volatilization losses can be very high in the first few hours after application.

Table 1. Estimates of first-year manure N availability and recovery using various methods, for the low manure rate, 1998–2000.

		N recovery				N availability			
		^{15}N recovery		App recov†		Rel effec†		FE (WPNU)	
Year	n‡	mean	range	mean	range	mean	range	mean	range
		%							
1998	12	10	4 to 15	15	-31 to 62	24	-51 to 100	31	-60 to 124
1999	8	17	8 to 26	18	9 to 31	28	15 to 49	43	10 to 148
2000	4	22	7 to 42	17	-4 to 43	61	-14 to 156	68	-10 to 142
Mean§		**14**		**16**		**32**		**41**	

†According to the Diff Meth.
‡Number of observations.
§Across years, weighed by number of observations.

Table 1 shows the N availability and recovery estimates according to the FE, difference and ^{15}N methods for each year. The ranges were usually quite large, especially for the FE and Diff Meth. However, in order to compare the indices, we averaged them. Only the low manure rate was considered, since this was the only level of application common to all three methods.

Estimates of N recovery were not significantly affected by method or year. However, ranges for ^{15}N recovery were somewhat narrower than for the Diff Meth, particularly in 1998. More importantly, several of the N recoveries, as computed by the Diff Meth were negative (more commonly in 1998), meaning that crop N uptake in control plots exceeded those in manured plots. If during 1998 native N levels were high due to the previous alfalfa crops, then it is reasonable that no extra N was needed.

A comparison of manure N availability estimates using Rel Eff and FE showed that the two methods did not statistically differ, and year did not have an effect either. Nevertheless, it is noteworthy that in 1999 N availability estimates using the FE approach were 50% higher than estimates for Rel Eff. In this year, an exponential function was chosen to describe the relationship between WPNU and fertilizer N. This model presents a steeper slope for low N rates, which asymptotically approaches zero for higher N rates. As a result, higher crop responses were assigned proportionally much higher FE values than even slightly lower responses. Given the wide range of crop N uptake values observed for manure treatments, just a few high responses can result in an apparently disproportionately high FE estimation. If the eight observations for WPNU for the low rate in 1999 were averaged and entered into the response curve as one value, the FE would be considerably lower, resulting in a N availability of 24% instead of 43%, much closer to the Diff Meth value. This illustrates the strong influence the mathematical function chosen has on the results for the FE approach. This is perhaps a major limitation of the method, which requires that one select a specific function to describe the crop behavior. That function might change from season to season and for different crop responses.

The Diff Meth only compares crop responses in manured plots to those obtained in control plots. This approach has limited applicability in extreme situations, such as when the soil is either high or severely deficient in available N. The ^{15}N method does not require calibration curves or controls (the "control" is ^{15}N natural abundance) and should be, therefore, a more precise and direct estimate of manure N recovery. However, this method does not allow for N availability estimates *per se* unless ^{15}N-enriched fertilizer treatments are included.

In spite of the apparent lower accuracy of the Diff Meth, it provided virtually the same average

estimate of manure N recovery estimates as the ^{15}N method. This might suggest that, at least for our experimental conditions, the Diff Meth could be the most cost-effective approach for determining manure N recovery. However, considering the breadth of the N recovery ranges, sometimes going from negative to more than 100%, it is somewhat surprising that it has worked out so well.

Conclusion

Manure N recovery measurements by the ^{15}N method are invariably more consistent and reliable than estimates using fertilizer N equivalence or the difference methods. However, the ^{15}N method is costly and involves much more work, from experiment setup to sample analyses.

Ammonia Production and Loss from Dairy Farms

J.M. Powell and L.D. Satter

Introduction

Over the past several years, environmental policy related to animal agriculture has focused on land application of manure, especially how to stop or reverse soil phosphorus build up, runoff, and the subsequent pollution of lakes, streams and other surface water bodies. Policy is now is aimed at reducing air emissions from animal agriculture. For dairy, ammonia emissions is the main air quality concern. Under the federal Consolidated Emissions Reporting Rule, each state will be required by 2004 to report ammonia gas emissions to the US Environmental Protection Agency. These estimates will be used in air quality regulations to control the air-borne particulates and haze that affect many regions of the US. The following report was comprised as an non-technical overview of the environmental impacts of ammonia and management practices that can reduce ammonia production and loss from dairy farms.

Environmental Impacts of Ammonia

The environmental impact of ammonia can be broken down into two parts: (1) how much ammonia-based compounds is actually deposited into different ecosystems, and (2) once deposited, how does the nitrogen (N) cycle within each ecosystem respond to this input. Some emitted ammonia is deposited not too far from its source (e.g., barn, lagoon, fields where manure has been applied). In the Midwest and Northeastern parts of the U.S. where most dairy farms grow their own crops, some 20 to 30% of the emitted ammonia can be deposited on adjacent cropland. When deposited in natural ecosystems, ammonia contributes to ecosystem fertilization, acidification, and eutrophication. This N input can cause dramatic shifts in the vegetation, enhancing grass growth and fire hazards in some areas. Emitted ammonia also combines with acidic compounds in the upper atmosphere to form particulates. These particulates have been related to haze in urban areas, and also have been attributed to a variety of adverse health effects, including premature mortality, chronic bronchitis, asthma, and hospital admissions.

How Ammonia is Formed and Lost

Ammonia losses from dairy operations begin to occur immediately after N excretion in urine and feces, and continue through manure handling, storage and land application. Only 20 to 30% of the N

(protein) fed to dairy cows is converted into milk. The remaining N is excreted about equally in urine and feces. About three-fourths of the N in urine is in the form of urea. Urease enzymes, which are present in feces and soil, rapidly convert urea to ammonium. Ammonium can be transformed quickly into ammonia. Feces contain little or no urea. For this reason urinary N is much more vulnerable to ammonia volatilization than is fecal N.

Figure 1 is a graphic illustration of ammonia N losses from a free stall barn, slurry manure system using an uncovered earthen pit for manure storage. Four different systems for field spreading manure are illustrated. Nitrogen flow through the system from feed through the cow, manure, field application of manure, and eventually back to feed is portrayed.

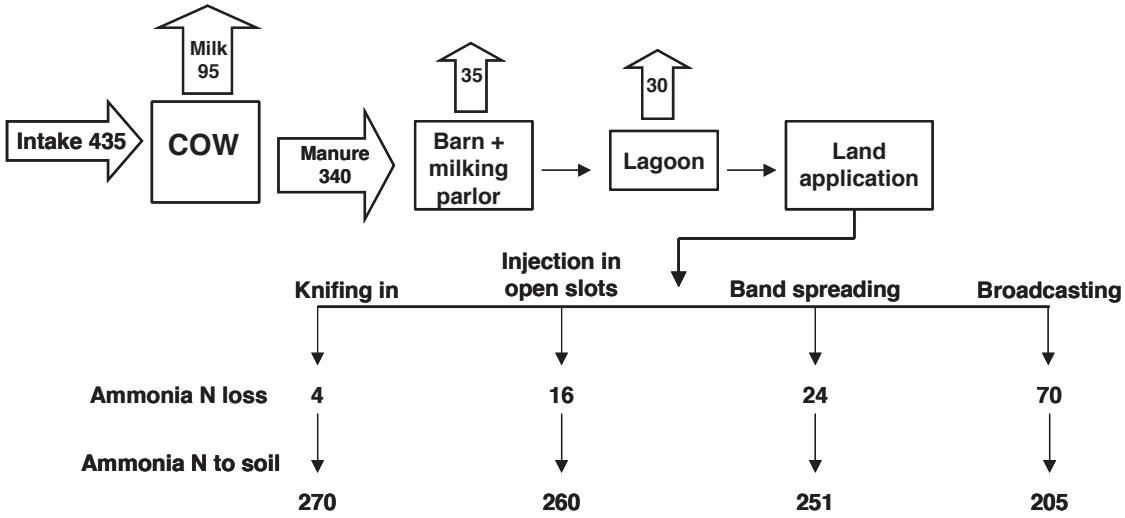


Figure 1. Model of nitrogen (lbs.) inputs, outputs and cycling for a typical lactating dairy cow.
Footnote: The numbers represent one cow, producing 18,400 lbs. milk per year containing 3.2% crude protein. The cow is lactating during 10 months of the year, and dry for two months. She consumes 15,920 lbs. feed dry matter per year containing 17.5% crude protein while lactating and 13% protein while dry. To calculate nitrogen flows for the entire mature herd, multiply the numerical values in Figure 1 by the number of lactating cows plus dry mature cows in the herd.

While this model of N flow through a dairy operation could be considered indicative of what may be expected under average conditions in a confinement-based feeding operation in the Midwest and Northeast part of the U.S., many factors affect actual loss, such as farm size and animal density (cow number per unit land area); animal diet impacts on milk production and relative excretion of N in urine and feces; housing type; manure collection and storage; soil type and land application practices; and weather (short-term) and climate (long-term).

The Protein We Feed Dairy Cows Affects Ammonia Loss

Knowing the amount of N that dairy cows excrete in urine and feces is the first approximation of how much N is vulnerable to ammonia loss. Nitrogen excretion by dairy cows via urine and therefore the amount of manure N susceptible to loss is highly influenced by the amount and type of protein fed. As the amount of protein in feed exceeds what is required, relatively less of the N goes into milk and more goes directly into urine production. Diet formulation to eliminate excess protein usually reduces feed cost, and it is one of the most effective tools for reducing emission into the atmosphere of nitrogen

containing compounds from dairy farms. For example, if 17.5% dietary protein currently represents an industry average for lactating cows, carefully formulated diets containing 16.0-16.2% crude protein, which meets requirements for the lactating cow and still provides a reasonable margin of safety, would reduce N excretion in urine by about 20%.

Ammonia Loss From Housing

Ammonia emissions from housing range from 20 to 55% of the N excreted by a dairy cow. The main factors that affect this value are housing and bedding type, frequency of manure removal, ventilation and temperature. Lowest ammonia loss occurs on farms that scrape and remove manure daily for the farm, and highest losses occur in outside areas where no manure is collected.

Dairy farms in Midwest and Northeast have one or more of the following housing and manure collection systems:

1) Confinement free stall systems. Free stall systems are common on large dairy farms. Cows are under roof and are free to move between stalls. Sand or mattresses covered with a minimum of bedding are used in free stalls. Slurry manure is scraped two or three times per day from the concrete alleys, and is typically hauled daily for field application or stored in an earthen pit that is emptied two or more times per year. A few larger dairies separate manure solids from the slurry. The solids may be composted, or the liquid either irrigated onto fields, or used to flush alley manure. Ammonia loss from free stall barn floors are greatest during the summer and lowest in winter.

2) Stanchion or tie stall barn. Stanchion or tie stall barns are most common on dairy farms having 100-125 cows or less. Cows are confined to a stall, and manure is collected in a gutter behind the cows. Moderate to large amounts of straw, wood shavings, or crop residue are used for bedding. The manure mixed with bedding is typically removed with a gutter cleaner twice daily, and field applied daily or stored for later field application. Cows may have access to a small exercise lot, or may be allowed access to a pasture to graze for part of the day.

3) Loose housing and manure pack. This system is found on smaller dairies. Cows are housed in an open shed, and provided large amounts of bedding material to absorb moisture. The bedded pack may build up to 3 feet in depth before being emptied once or twice per year. This system requires less capital, and often is used in conjunction with grazing.

4) Grazing with no housing. This system is more common on smaller than larger dairy farms. During that time of the year when weather permits, some dairy operators bring cows into a building only to be milked, and possibly to be offered some supplemental feed. The rest of the time cows are on pasture, and most of the urine and feces is deposited in the paddock. Ammonia loss from pasture is generally proportional to stocking rates and the time spent, and therefore, the amount of urine and feces deposited, in these locations. The spatial location of manure deposition and soil, weather and climatic conditions all affect the actual rate and extent of ammonia loss from these areas.

Ammonia Loss During Manure Storage

Solid and semi-solid dairy manure is stored in piles on concrete or earthen pads. Slurry is stored in a concrete or lined lagoon or storage tank, or mixed with flush water in earthen or lined lagoons that may be covered with biological material (e.g., straw), impermeable material (e.g., synthetic polymers) or left uncovered. Ammonia loss from manure storage depends on the structure used. Lowest N losses

occur on farms that conserve urine N in underground pits and in bedded packs. Very large amounts of ammonia are emitted from anaerobic lagoons, especially during agitation before manure tankers are filled for field application.

Ammonia Emissions During Land Application of Manure

Ammonia losses during field application of manure are usually expressed as a percentage of total ammoniacal N (TAN: ammonium-N plus ammonia-N). TAN is the portion of manure N that is readily susceptible to loss, and available to plants. Ammonia losses can range from close to 100% of TAN for surface application with optimal conditions for volatilization, to only a few percent when manure is injected or incorporated immediately into the soil. Ammonia loss from land-applied dairy manure can vary tremendously depending on environmental conditions (temperature, wind speed, rainfall); application method (degree of application, zone of application, timing); manure characteristics (pH, dry matter and ammonium contents); and soil conditions (soil moisture and other properties, residue cover).

Recommendation on how to apply manure to fields must consider the complete chain of events that affect manure N cycling in soils. For example, if manure is injected or incorporated into soil to minimize ammonia loss, this may increase the risk of nitrate loss.

Impact of Ammonia Loss on Plant Availability of Manure N

Ammonia loss is important because it is a direct loss of plant available N to the farmer. The loss of ammonia also reduces the ratio of N to phosphorus (N:P) in manure. This increases the risk of manure P applications in excess of crop needs. Many dairy farms have soil test P levels that already exceed agronomic recommendations. The runoff of P from these fields and subsequent pollution of lakes, streams and other surface waters is a concern.

Reducing ammonia losses from dairy farms and making greater use of conserved manure N may quickly make economic sense. Natural gas accounts for 75-90% of the cost of making anhydrous ammonia. As the price of natural gas continues to skyrocket, the fertilizer N value of manure, and therefore the conservation on ammonia N will become more important.

Approaches to Reduce Ammonia Emissions From Dairy Farms

Substantial reductions in ammonia loss from dairy operations can be achieved by feeding less protein to the cow, by reducing in-barn losses, by covering manure storage, and by incorporation of manure in the field (Table 1). The following steps, in descending order of potential benefit, can be a guide for action.

1. Remove excess protein from the cow's diet. This normally saves on cost of feed.
2. Incorporate manure in the field. However, this strategy needs to consider potential tradeoffs in situations where nitrate leaching may be a concern.
3. Cover the manure storage. When organic bedding such as straw is used in free stall barns, a crust will form on the surface of the slurry pit. This reduces ammonia N losses and odors. Excessive agitation during unloading of the slurry from storage should be avoided.
4. For new construction, floors that divert urine away from feces can reduce ammonia emissions. Slatted floors facilitate this, but there is still considerable loss of ammonia from the surface of the slatted floor.

Implementation of steps 1-3 above could potentially ammonia N loss to the atmosphere from about 115 to 30-40 lbs/cow/yr, a 65-70% reduction. This means additional 70-80 lbs. N per cow would be available annually for application to field crops.

Table 1. Impact of improved management practices on reductions in ammonia emission
* of the nitrogen entering that component

Management practice	Mechanism for decrease ammonia loss	Decrease in ammonia loss (%)
Remove excess and/or feed balanced dietary protein	Decrease N output in urine	10 to 15
Cover manure storage	Decrease ammonia escape	20 to 30*
Incorporate or inject manure	Reduce ammonia production and loss	30 to 50*

Apparent Manure Collection on Wisconsin Dairy Farms

J.M. Powell, D.F. McCrory, H. Saam, and D.B. Jackson-Smith

Introduction

With economic pressures due to high input and low product prices, and recent and impending passage of government regulations pertaining to non-point pollution and gaseous emissions from animal agriculture, improved management of the nutrients contained in feed, fertilizer and manure will become increasingly critical to the long-term viability of the U.S. dairy industry. The development of sound nutrient management strategies needs to consider not only the biophysical factors (e.g. soils, weather) that affect nutrient use and loss, but also the socio-economic conditions that affect farmers' behavior and technology adoption. Farmers involvement in the policy formulation process is needed to identify the real barriers and opportunities for improving nutrient use on dairy farms. The "On Farmers' Ground"(OFG) project was initiated in 2002 to expand our understanding of (1) the biophysical and socioeconomic obstacles typical dairy farmers face in managing agricultural nutrients, and (2) the innovative solutions farmers may have already developed to meet these challenges. This report summarizes initial findings of the OFG project with a focus on manure collection.

Materials and Methods

The sampling framework for the OFG project was drawn from the 750 dairy farm respondents to the 1999 Wisconsin Farm Poll. The OFG sample drew a stratified random sample of fifty-four dairy farms from the subset of 260 poll respondents who were located in prominent dairy counties of the state. The sample was stratified to include equal numbers (n=18) of operations that had low, medium, and high animal density (dairy cows per acre of cropland). Moreover, the sub samples were stratified to differentiate three broad biophysical regions thought to represent distinctive challenges to nutrient management on dairy farms: (1) the hilly, southwest region; (2) the undulating south-central region; and (3) the relatively flat, northeast region. The southwest (SW) region is characterized by more coarsely textured silt loam soils that have relatively higher permeability and drier field conditions in the spring

and fall than other regions. By contrast the northeast (NE) has more finely textured and less permeable clayey and red loam soils, and the south-central (SC) region has physical characteristics somewhat intermediate to those of the SW and NE. By making these regional distinctions we hypothesized that farms in the SW may have a wider "manure-spreading window", or days soil conditions are favorable for manure spreading compared to farms situated in flatter, heavier-textured and perhaps more waterlogged soils of the NE. In addition, there is a notable higher level of non-farm development pressures in the NE region of the state, which may limit farmers' access to some of their land base.

The research team visited each farm in the fall of 2002 and a structured survey was used to collect information on farm characteristics and practices, including livestock, crops, and the use of feeds, fertilizer, manure, and basic operation characteristics (e.g., ownership, management, labor, scale, use of key technologies). Information about the time spent by lactating cows, non-lactating cows and heifers in various locations (buildings, barnyards, pastures, exercise lots, etc.) were gathered on each study farm throughout one year. These data were used to calculate the "apparent manure collection" rate (AMC, % total manure collected). Our preliminary analysis relates only to the lactating portion of the dairy herd.

Results and Discussion

Any evaluation of a farm's ability to effectively recycle manure nutrients must make an assumption as to how much of the total manure produced by the dairy herd is actually collected, and therefore must be spread on cropland. Based on our initial results, most Wisconsin dairy farms do not collect all manure. For example, only approximately 22% of the OFG dairy farms manage to collect all manure from lactating cows (Table 1). The highest percentage (32%) of farms that collect all manure occurs in the SC region and the lowest in the SW (12% of all farms), where pastures have a particular importance than other regions. AMC for farms that partially collect manure were similar across regions with a range of 40 to 88, and a mean of 61% for the SW; 44 to 86, and 65% for the SC; and 45 to 92, and 66% for the NE. Across the state, highest AMC occur in the winter (96%) and spring (74%). Approximately one-half of the manure produced by lactating cows in the summer remains in outside areas.

Table 1. Apparent manure collection on Wisconsin dairy farms

	Region			OFG Weighted average
	Southwest	South-central	Northeast	
Percent of Lactating Cow Manure collected:				
Fall	49.7	69.8	59.9	58.3
Winter	94.7	97.6	95.8	95.9
Spring	68.5	67.6	89.8	74.2
Summer	43.0	56.4	53.9	49.6
ANNUAL BASIS	65.6	77.3	75.6	72.1
Farms that collect all manure (% of all farms)	12.5	31.6	27.8	22.1
AMC for farms that partially collect manure (% of manure produced)	61.3	66.0	66.2	64.2
Farms that allow access to outside areas (% of all farms)	87.5	68.4	72.2	77.9
For Farms Allowing Access to Outside Areas:				
Reasons for access to outside areas				
Exercise, health	86.7	90.0	77.0	82
To Graze	33.3	20.0	15.4	24.9
Less manure in the barn	20.0	20.0	23.1	17.9
Heat detection	0.0	0.0	23.1	15.4
Types of outside area used				
Pasture	86.7	16.7	23.1	45.4
Partially vegetated feedlot	13.3	41.7	38.5	30.2
Un-vegetated barnyard	0.0	41.7	38.5	24.4
Mean surface areas (acres)				
Pasture	19.2	21.1	13.3	15.9
Partially vegetated feedlot	2.6	4.1	3.1	1.9
Un-vegetated barnyard	n/a	2.0	1.2	1.2
Percent of farms that collect manure from outside areas	13.3	7.7	15.4	13.4
Percent of farms that rotate outside areas	6.7	0.0	38.5	16.9

Manure collection on Wisconsin dairy farms can be divided into two broad groups: those that collect all manure and those that only partially collect manure (Figure 1). While there are farms of all sizes, from 40 to 480 cows, that manage to collect all manure, the smallest farms apparently have the most difficulty collecting manure. For farms having less than 85 cows there is a significant ($p<0.05$) relationship between apparent manure collection (y, % of total manure production) and herd size (x, number of lactating cows) according to the relationship
$y = 0.334x + 46.6$ (Rsq. = 0.27).

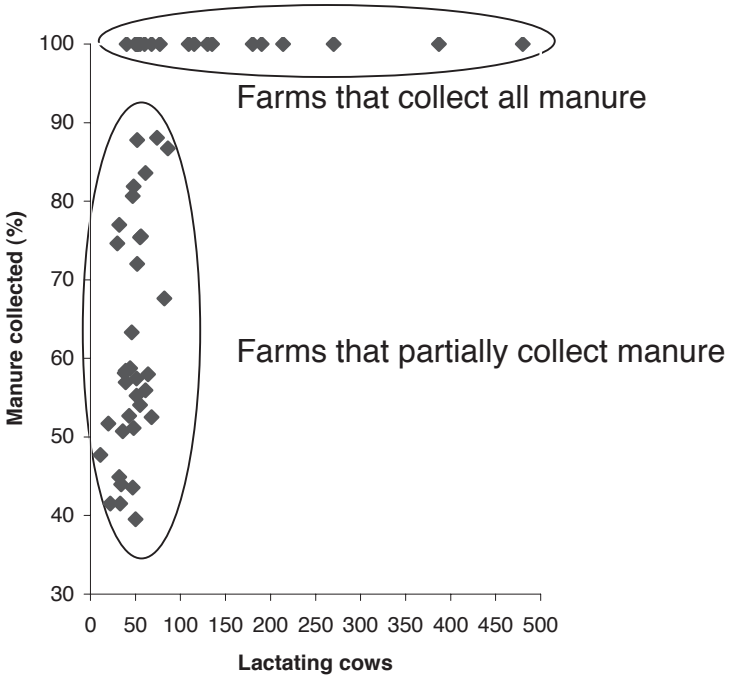


Figure 1. Relationship between lactating cow numbers and amount of manure collected.

While there is a growing trend in Wisconsin toward year-round confinement of dairy cows, the majority of dairy farmers (78%) allow lactating cows some access to outside areas. Three general types of outside areas have been identified: un-vegetated barnyards, partially vegetated areas where livestock are fed, and pastures. Our preliminary measurements show that barnyards and outside feeding areas are usually between 1 to 3.5 acres (Table 1). Based on our AMC calculations, these small areas may be accumulating some 30-40% of the annual manure produced on dairy farms.

Approximately 40% of all farms in the SC and NE regions provide lactating dairy cows access to barnyards or partially vegetated feedlots. No farms in the SW reported time spent by lactating cows in barnyards. Access to pastures is much more important in the SW (87% of all farms provide access to pastures) than in the SC (23%) or the NE (27%). Mean pasture areas are 18 acres in the SW, 21 acres in the SC and 14 acres in the NE.

One of the major objectives of the OFG project is to solicit feedback from farmers on reasons for certain practices. Farmers offered several reasons why they allow their lactating cows access to outside areas. These include:

“I let my cows outside because of general health reasons. I feel it’s better for their legs and they have less hock problems. It keeps my vet bills low.”

“Letting my cows out on solid ground, in the sunshine, keeps them drier and cooler. I think it keeps them more comfortable.”

“I like my cows outside where I can keep an eye on them and keep track of when they are bred.”

While the leading reason for allowing cows access to outdoor areas is for exercise or improved health, other factors may influence the type of outside area used and time the dairy herd is allowed access to these areas. The next phase of OFG will seek farmer insight into factors such as investments in capital,

labor and time that may influence herd and manure management and the use of outside areas. Whereas there appears to be significant manure buildup in many outside areas, some farmers appear to manage these areas by either removing manure and/or rotating these areas with crops or forage. For example, in the NE where 40% of the dairy farms reported use of outside feeding areas, 14% of the farms haul some manure off these outside areas and 40% reported that they rotate these areas with crops and/or forage (Table 1). No farms in the SC and only 7% of the farms in the SW reported manure collection from these outside areas. Few farms in the SW (13%) and the SC (8%) reported that they rotate outside areas.

While follow-up visits will be used to better understand farmer management of outside areas, several farmers have already reported on the benefits of rotating cows between different outside areas and in and out of crop production.

"I have four different areas I use for exercise lots. I keep one covered with grass and let the cows out there when it rains. The main thing is to keep them dry and clean. I started rotating lots ten years ago and have seen my somatic cell count drop dramatically. It's good for my cows' udder health."

"I have a couple different areas close to the barn that I let the cows out on. The one I'm using now has been there for a year. Every two years or so I plow it up and plant it to corn or seed it down."

"The cows put the manure out there. I don't have to haul it. I just plow it (exercise area) up every few years, reseed it, and move the cows to a new area."

Conclusions

- Many dairy farms do not collect all manure;
- Lowest manure collection is on farms having small and medium herd size, especially in the SW region of the state; and
- Outside areas may have much higher manure loading rates than cropland, which may have lower manure loading rates than previously thought.

Manure management issues on dairy farms that have small and medium herd size may require a particular focus. Most dairy farms of this size have been owned and operated by a single-family since their establishment generations ago. Many farmsteads were originally built close to streams or springs. Given their close proximity to surface water and increasing animal densities and manure production, many of these operations may have to meet regulatory nutrient management standards. Although typical dairy herds in Wisconsin are too small to be considered a large CAFO (>700 mature dairy cows), many dairy farms in the state may be designated as a medium-sized CAFO (200-699 mature cows) if manure or waste water is directed towards surface water and/or animals come into contact with surface water, or a small CAFO (<200 mature cows) if they are found to be a significant contributor of pollutants to surface waters.

U.S. DAIRY FORAGE RESEARCH CENTER
ANNUAL DAIRY OPERATIONS REPORT
JANUARY 2004 (for 2003)

JILL A. DAVIDSON - HERD MANAGER

HERD STATISTICS			CHANGE FROM PREVIOUS YEAR
Herd Inventory			
Milking cows		304	+ 13
Dry cows		53	+ 0
average cow age	45 months		- 4
percent first lactation	43 %		+ 5
percent second lactation	27 %		- 5
percent third lactation	17 %		- 2
percent greater than third	13 %		+ 2
Herd replacements		346	+ 15
Total		**703**	**+ 28**
Rumen fistulated cows		24	- 1
Herd Performance			
Cows calved		371	+ 16
Heifer calves born live		187	+ 18
Heifer calves born dead		14	+ 5
Bull calves born live		155	- 8
Bull calves born dead		20	+ 6
Heifer calves died < 1 year old		3 (1.5%)	- 2
Number of sets of twins		21	
DHIA rolling herd average			
milk		24,518 lbs.	+1362
protein		728 lbs.	+26
fat		929 lbs.	+2
Milk sold in 2003		7,912,512 lbs.	+434,526
Average Mailbox Milk Price/cwt		$12.03	-0.05
Heifer calves sold		4	- 3
Bull calves sold		153	- 10
Cows Sold			
Cows culled for:			
Reproduction Problems		55	+ 0
Poor Production		19	+ 3
Poor Feet and Legs		25	+ 15
Mastitis		14	+ 3
Other		31	+ 3
Cattle Sales Revenue		87,399	+ 7557
Herd Reproduction			
Average days open		140	- 14
Average calving interval		13.6 months	- 0.2
Average services per conception		2.4	- 0.2
Average age at first calving		25 months	+ 1

During the past year, the dairy herd personnel included Merilyn Marti, Dan Wendt, Marsha Key, Mark Hintz, Deidre Kannenberg, Kelly Green, Rich Campbell, Kurt Pickar, Ken Lazzara, Frank Klemm, John Biech, Bob Breunig, Dick Hager, John Howery, Jim Lukken, Greg Schultz, Mike Wilson, Tim Schott, and Mark Breitenbach. The operation had a very successful year with a 24,500 pound DHIA rolling herd average along with steady production of milk components. Milk production per cow has steadily increased to compensate for the increased culling rate. During the past year, cows and heifers committed to research experiments ranged from 4 to 144 at any given time. The scope of research projects continued to increase with focuses on nitrogen efficiency in rumen, forage utilization, and environmental impacts of dairy systems. Gains were made in our reproductive efficiency to support the increased culling rate and to provide additional replacement heifers. Facility updates included additional calf hutches, additional gutter grates in tie-stall barns, the addition of computerized data collection feeding system, and new headlocks in the heifer barn. Continued efforts will be made to improve cow performance and health, cow comfort, greater labor efficiency with an increase in our research efforts. Additional renovations targeted for the upcoming year include modifications tie-stalls for ammonia emission research, replacement of tie-stall cow mattresses, and renovations of heifer and free-stall barns to improve cow comfort and feed accessibility.

The farm continues to increase involvement in state and multi-state functions hosting many national and international visitors, meetings, and veterinary, technical school, and undergraduate students throughout the year. Pat Fedewa was a Michigan State University Ag Tech student employed during the summer break as an intern. Two additional undergraduate students will have intern experiences at the research farm in 2004. The continued development of the intern programs are a priority for the next year. Other future targets and goals include the development of long-term farmstead plans for the center, improved milk production, heifer growth, reduction of involuntary culling, and reproductive performance, and plans for updating the facilities for greater cow comfort and improved labor efficiencies.

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