Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences

Volume 12 Article 1

Fall 2011

Discovery: The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences - Volume 12 2011

Several Authors

DISCOVERY

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UofA
UNIVERSITY OF ARKANSAS
DIVISION OF AGRICULTURE

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DALE BUMPERS COLLEGE
OF AGRICULTURAL, FOOD & LIFE SCIENCES

DISCOVERY

The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences

Vol. 12 **Fall 2011**

Contents

Undergraduate Research Articles

DISCOVERY is published annually by the Dale Bumpers College of Agricultural, Food and Life Sciences.
http://bumperscollege.uark.edu/

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Cover: A research project by animal science student Whitney Downum documented significant benefits from calfhood vaccination for bovine viral diarrhea virus. Protocols are evolving for this relatively new concept. (Photo by Bruce Fritz, courtesy USDA Agricultural Research Service).

Michael Vayda

Value-Added Learning

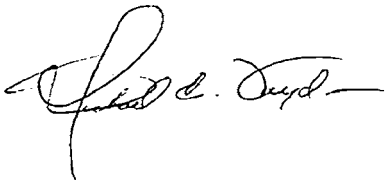
The *DISCOVERY* undergraduate journal is one of the ways Dale Bumpers College of Agricultural, Food and Life Sciences encourages students to engage in value-added learning experiences beyond the classroom. The student authors are reporting on the results of research projects they have conducted with faculty mentors.

The *DISCOVERY* journal provides a reporting outlet for our student scholars and scientists. It does not supersede publication elsewhere, but it does provide a forum for students and faculty to share their results and findings in a citable publication.

We encourage student research by awarding undergraduate research grants, and our students have been very competitive for research and travel grants awarded by the Honors College and the Arkansas Department of Higher Education.

Many undergraduate research projects are designed to meet the requirements of an honors thesis in the Bumper College Honors Program, which enables our students to enrich their educational experience and provide a very tangible service to society in the process.

We are proud to present these articles as examples of the work of our undergraduate students. I heartily congratulate the student authors and extend thanks to their faculty mentors and to the editors who reviewed their manuscripts.



Michael Vayda, Dean and
Associate Vice President–Academic Programs

Factors that contribute to turbidity on the West Fork of the White River in Arkansas

Chris Cotton[*] *and Brian Haggard*[†]

ABSTRACT

The West Fork of the White River (WFWR) exceeds the water quality standard for turbidity (10 NTU) set by the Arkansas Department of Environmental Quality and, since 1998, the river has been on Arkansas's 303 (d) list of impaired water bodies unsuitable for aquatic life because of turbidity exceedances. To understand the factors that could be related to turbidity, total suspended solids (TSS), total inorganic suspended solids (TISS), total volatile suspended solids (TVSS), sestonic chlorophyll-a (Chl-a) concentrations, and turbidity were measured on three sample dates from nine sites on the WFWR. As the site location changed in the downstream direction, turbidity values generally increased from less than 5 NTU (nephelometric turbidity units) at upstream sites to greater than 10 NTU on average at the two most downstream sites. A similar trend was observed in TSS, TISS, TVSS and sestonic chlorophyll-a concentrations, and regression analysis showed that TISS and TVSS were significantly related to turbidity across the WFWR. The multiple regression analysis for all collected data showed that TISS alone accounted for 73% of the variation in turbidity values. Where the turbidity exceeded 10 NTU, there were select soil series (Enders-Allegheny complex and Sloan, Razort, Taloka, and Pickwick silt loams) in the riparian zone that were not present in the upstream soils matrix. The reaches of the WFWR which had both elevated turbidity values and the selected soils composed on 6% (2.2 km) of the river length, and suggested that properties of those soil series should be investigated further as a contributing factor to increased turbidity at downstream sites of the WFWR.

* Chris Cotton is a 2011 graduate with a major in Environmental, Soil, and Water Science.

† Brian Haggard is a faculty mentor, director of the Arkansas Water Resource Center and a professor in the department of Biological and Agricultural Engineering.

MEET THE STUDENT-AUTHOR

Chris Cotton

I am originally from Vicksburg, Miss., although I have called Russellville, Ark., my home since the age of two. I graduated in May 2011 from the University of Arkansas with a Bachelor of Science in Environmental, Soil, and Water Science. While growing up in Arkansas, I developed an appreciation for the pristine scenery of the natural state and came to understand the importance of upholding the integrity of the state's natural resources. During the past four years of my academic career in the Dale Bumpers College of Agricultural, Food and Life Sciences, I have been involved in various college and department associations including various officer positions within the undergraduate Crop, Soil, and Environmental Sciences Club. I also had the opportunity to participate in the Bumpers College honors program and a student exchange program during the summer of 2010 with Brazilian Institutes in Botucatu, Spain, and Londrina, Paraná, Brazil. During the summer of 2011, I will begin preparing for a two-year commitment for the position as a high school general sciences teacher and Teach For America corps member in Kansas City, Mo.

I would like to thank Dr. Brian Haggard for his extensive help and guidance in researching and preparing my honors thesis. With the additional assistance of Dr. Wolf, Dr. Miller, and faculty and staff of the crop, soil, and environmental sciences department, I was able to successfully complete and defend my honors thesis. The research and revisions of such a project will provide a useful experience for future tasks and challenges I will face along my career path.

INTRODUCTION

The Arkansas Department of Environmental Quality (ADEQ) is required by Section 303 (d) of the Clean Water Act to identify waters which do not meet applicable water quality standards (ADEQ, 2002). The intent of water quality standards is to protect the designated beneficial uses of the states' waters, and these beneficial uses defined in Arkansas include agricultural and industrial water supply, recreation, public water supply, and aquatic life (Haggard and Scott, 2010). A variety of factors can influence whether or not designated beneficial uses are met. Two of the most common factors as defined by ADEQ (reviewed in Rogers, 2010) and nationally (U.S. EPA, 2010) are sedimentation and turbidity.

Turbidity is defined as a cloudy condition in water due to organic matter and suspended silts and clays transported from land into the water column. Soil erosion from stream banks, riparian areas, and the landscape in a watershed can contribute to increased turbidity levels in rivers. The sources of sediment include agricultural land, land undergoing urban development, and as a result of stream bank erosion, as well as natural transport. Turbidity can also result from organic matter production within the water column of streams and rivers (U.S. EPA, 1999).

The focus of this study was the West Fork of the White River (WFWR) in Northwest Arkansas. The objectives were (1) to determine, from collected data, where turbidity on the WFWR becomes pronounced, (2) to obtain chemical, physical, and biological data from samples collected at nine access locations and analyzed at the Arkansas Water Resources Center (AWRC) Water Quality Lab (WQL), (3), to quantify the percent river miles for each soil series present on the stream banks of the WFWR and (4) to identify factors related to the measured turbidity. This study will help watershed managers further understand the factors contributing to turbidity at WFWR, and then assist in developing and targeting remedial actions to reduce stream bank and riparian erosion.

MATERIALS AND METHODS

Site Description. The study site was the West Fork of the White River which has had a recorded decline in total fish species since the 1960s with an increase in tolerant species and a decrease in sensitive species (Formica et al., 2004). The 54-km impaired segment of the WFWR is south of Fayetteville and empties into the White River, which is a major tributary to Beaver Lake. Beaver Lake is the drinking water supply for 300,000-plus residents of Washington and Benton Counties, Ark.

Land Use and Soil Surveying. Land use distribution of the WFWR watershed area is 65% forested, 23% agriculture (mainly pasture), and 12% urban use. To better understand the composition and landscape patterns of the WFWR watershed, a state-issued soil survey for Washington County was examined (Harper et al., 1969), and a spreadsheet was compiled to record the percent and description of the soil series that were found adjacent to the river.

Water Sampling and Analysis. Water samples were collected from nine access points along the WFWR during base flow conditions in April, September, and October 2010 (Fig. 1, Table 1). At each site, an alpha type sampler was dropped from the center of each bridge on the downstream side. Once the sampler was full, water was transferred to a field-rinsed, 1-L high-density polyethylene (HDPE) bottle. Water samples were analyzed for turbidity, total suspended solids (TSS), total inorganic suspended solids (TISS), total volatile suspended solids (TVSS), and sestonic chlorophyll-a (Chl-a).

Turbidity. To determine turbidity, a WTW Turb 550 turbidimeter was used to obtain a nephelometric measurement (NTU) for samples from each site. The 1-L raw sample was vigorously shaken to mimic natural stream conditions for turbidity analysis, and a 5-mL sample was placed into a clean cuvette. The cuvette was then inserted and aligned into the optical well of the turbidimeter, and a reading for NTU was recorded. The value demonstrated the relative cloudiness of the sampled water.

Total Suspended Solids. A well-mixed sample from each site was taken from the 1-L bottles and filtered through a weighed standard glass-fiber filter (pore size = 0.7 μm). The sample (25 ml) for each site was filtered using a vacuum apparatus and was washed three times with 10 ml of distilled water. The filter was then carefully removed and transferred to an aluminum dish and dried in an oven at 103-105 °C for 1 h. After drying, the filter plus the dried residue was weighed and the total suspended solids concentration was calculated by using the following equation:

$$\text{TSS, mg/L} = \frac{(\textit{weight of filter and weight of residue, mg} - \textit{weight of filter, mg})}{\textit{sample volume, L}}$$

Total Volatile Suspended Solids. TVSS provided an estimation of the amount of suspended organic material in the sample, where the residue obtained from the TSS was ignited in this procedure to determine the amount of volatile solids in the sample. Following determination of TSS, the filter with dried residue was ignited in a muffle furnace at 550 °C. The cooled filter disk weight was recorded and used in the following equation:

$$\text{TVSS, mg/L} = \frac{(\textit{weight of filter and residue before ignition, mg} - \textit{weight after ignition, mg})}{\textit{sample volume, L}}$$

Total Inorganic Suspended Solids. To calculate the inorganic portion of the sampled solids collected in the TSS method, the concentration for the volatile solids was subtracted from the concentration of the total solids.

Sestonic Chlorophyll-a. The U.S. EPA standard method 446 (Arar, 1997) was used to estimate concentration of Chl-a in the samples, and provided the relative abundance of algal cells in the water samples (Aminot and Rey, 2000). A 500-ml sample was vacuum filtered onto a glass-fiber filter. The filter was macerated and placed in 5 ml of 90% acetone aqueous solution to extract chlorophyll from the algal cells. The extract was centrifuged, placed in a cuvette, and analyzed using a *Beckman-Coulter* DU 720 spectrophotometer (Miami, Fla.) which measured the absorbance of the samples at wavelengths of 750, 664, 647,and 630 nm. The trichromatic method followed used Jeffery and Humphrey's equation (Jeffery and Humphrey, 1975 as cited in Arar, 1997) to determine Chl-a concentrations as follows (Arar, 1997):

$$\text{Chl-a, } \mu g/L = (11.85 * (E664 - E750) - 1.54 * (E647 - E750) - 0.08 * (E630 - E750)) * Ve/L * Vf$$

Where:

E# = absorbency at specified wavelength
L = Cuvette light-path in cm
Ve = Extraction volume in ml
Vf = Filtered volume in L

Statistical Analysis. To statistically analyze the concentrations of TSS, TVSS, TISS, and sestonic chlorophyll-a in relation to the increased NTU values observed in the WFWR, linear regression analyses were conducted for monthly data. From these analyses, the following information was collected for each parameter for the data points of each month: slope, intercept, R^2, and P-value The formula used in the regression was:

$$NTU = constant + (B_0 * X)$$

Where *NTU* is turbidity units, B_0 is the regression coefficient or slope, and *X* represents the independent variable including TSS, TISS, TVSS and sestonic chlorophyll-a. A multiple linear regression of turbidity was also conducted for data across all sampling months using multiple variables, including TISS and TVSS. The following formula was used:

$$NTU = constant + (B_1 * TISS) + (B_2 * TVSS)$$

Where *NTU* is turbidity units, B_1 and B_2 are regression coefficients, and *TISS* and *TVSS* are the dependent factors.

RESULTS AND DISCUSSION

Turbidity. On average, turbidity values was least at the upstream sites 1-7, with mean values less than 10 NTU

(Fig. 2). Turbidity generally increased in the downstream direction of the West Fork of the White River. Turbidity over the sampling period was greatest at site 8 at river km 31, averaging over 15 NTU during base flow. This monitoring program showed that the standard of 10 NTU was exceeded at sampling sites 8 and 9 from river km 31-36. These sampling sites comprise 5 kilometers or just fewer than 17% of the sampled river length.

The average TSS concentrations were less at the sites upstream (site 1-7) compared with that measured at the downstream sites 8 and 9 (Fig. 3). Average TSS was least at site 2 at river km 8 (average of 2.4 mg/L); whereas, the greatest average was 13.2 mg/L at site 8 at river km 31. The trends in both turbidity and TSS concentrations were similar across the WFWR, increasing in the downstream direction. The TSS is comprised of TISS and TVSS and differences determine if the suspended solids causing turbidity in the stream were from inorganic or organic sources. Mean TISS concentrations ranged from 1.6 mg/L at site 2 to 10.9 mg/L at site 8. The mean TVSS concentrations were less compared to those observed for TISS, ranging from 0.6 mg/L at sites 3 and 5 to 2.3 mg/L at site 8 (Fig. 3).

The lowest average concentration for sestonic chlorophyll-a was 1.2 µg/L at site 3, and the greatest average recorded was at site 9 with a concentration of 6.9 µg/L (Fig. 4). These data showed general concentration increase for all measured parameters in the samples collected further downstream (relative to upstream), and turbidity at the two most downstream sites was of most concern relative to exceeding water quality standards.

Regression Analysis. The TSS and TISS regressions against turbidity showed the greatest R^2 values for each individual month, suggesting that the inorganic portion of the suspended material explained most of the variation in turbidity across the WFWR (data not shown). However, TVSS concentrations were also an important determinant in the variability of turbidity across these sites when all data were combined for the regression analysis. Sestonic chlorophyll-a concentrations were not significantly related to turbidity measurements during each of the three months nor were concentrations significantly related to turbidity when all data were pooled together ($P > 0.05$).

The multiple regression analysis suggested that TISS and TVSS accounted for 85% of the variability in turbidity across these sampling sites and dates at the WFWR. It was noted that both B_1 and B_2 held positive values, thus it can be further concluded that increases in either TISS or TVSS resulted in increased turbidity. To further confirm this, the R^2 values showed that TISS explained 73% of the variation in turbidity, and TVSS explained an additional 12% (data not shown). Therefore, the multiple regression analyses suggested that TISS accounted for the majority of variability in turbidity in the water column.

Soils. To provide further detail concerning the inorganic materials possibly causing turbidity in the WFWR, riparian soil series along the river were evaluated. The dominant soil series throughout the WFWR was Cleora fine sandy loam, occupying 66.4% of the measured riparian zone, with a slight erosion hazard and low runoff potential; 13 other soil series comprise the remaining 33.6% of the land area. The sites of major concern for high turbidity levels were downstream from site 7, representing the last 11 km of the sampled section of the WFWR. As turbidity increased downstream, the presence of select soil series (including Enders-Allegheny complex, Sloan, Razort, Taloka, and Pickwick silt loams) also increased (data not shown).

The erosivity hazard index for the soils at these sites were highest for the Pickwick silt loam which constituted 0.305 km between sites 8 and 9. The Taloka silt loams, covering 0.25 km between sites 8 and 9, have a moderate erosion hazard. The Razort silt loam which is present for 0.32 km between sites 7 to 8, had a moderate runoff potential, but only a slight erosion hazard. The presence of these soils potentially could be related to the increased downstream turbidity and inorganic material in the WFWR due to the higher erosivity hazards and runoff potentials compared to those of the dominant Cleora fine sandy loam series.

From this study, we observed that turbidity measurements increased as the WFWR near Fayetteville flowed downstream. The measured turbidity was significantly related to TSS concentrations, and more specifically to concentrations of TISS and TVSS. Regression analyses showed that TISS had a greater influence on turbidity over the three sampling dates. These findings were consistent with results found in previous studies conducted on the WFWR in 2004 and 2006 which determined that TSS concentrations were mostly inorganic in composition (Formica et al., 2004). Additionally, logarithmic regression equations relating turbidity to TSS concentrations were developed (U.S. EPA, 2006). As turbidity and TISS concentrations in the water column increased downstream, there was an observed occurrence of select soil series (Enders-Allgeheny complex, Sloan silt loam, Razort loam, Taloka silt loam, and Pickwick silt loam) which were characterized as having moderate to high erosion hazards and runoff potentials.

In 2004, a study was conducted for the Arkansas Natural Resource Commission on the siltation and turbidity of the WFWR (Formica et al., 2004). These researchers found that stream bank erosion was estimated to contribute 13,962 Mg annually of suspended sediment to the WFWR, accounting for 66% of the annual TSS load. Additionally, turbidity and TSS data were collected over two years and a regression analysis showed that TSS explained over 75% of the variability in turbidity with a slope of 1.202. In the

current study, regression values were consistent during the six month sampling period with a slope of 0.915 and a R^2 value of 0.781. This observation indicates that TSS explains the majority (78%) of the variability in turbidity both spatially (upstream and downstream) and temporally (across different studies).

A similar study of the Illinois River near Fayetteville was conducted from 2005-2007 by the U.S. Geological Survey to quantify the percentages of organic and inorganic materials in the water column (Galloway, 2008). It was noted that there was significant relation between TSS concentrations and turbidity measurements in the Illinois River. However, the Illinois River had mean TVSS concentrations (3.1 mg/L) that were three times greater than those found in the current study of the WFWR (TVSS = 1.03 mg/L). However, mean sestonic Chl-a concentrations were relatively similar between the Illinois River (3.1 µg/L) and the WFWR (2.9 µg/L). In both systems, the inorganic material was the largest part of TSS measured in the water column, given that the mean values of TISS were 4 mg/L on the Illinois River and 5.2 mg/L on the WFWR (Galloway, 2008).

Formica et al. (2004) used a soil survey to map potential areas of concern for sediment loss due to agricultural pasture land use in the WFWR watershed. It was concluded that soils with a slope of 8-16% in the Enders association were responsible for 65% of the sediment loss from riparian pasture land at the WFWR. This is consistent with the riparian soils information gathered in the current study which indicated that increased percentages of Enders-Allegheny soil series had increased records of turbidity and TSS. Further evaluation of the select soil series (Enders-Allegheny complex and Sloan, Razort, Taloka, and Pickwick silt loams) present upstream from sites with the greatest turbidity measurements could provide an understanding of which soils contribute more silts and clays into the water column.

The current study suggests that select soil series at downstream sites (Enders-Allegheny complex and Sloan, Razort, Taloka, and Pickwick silt loams) may contribute to the elevated turbidity at the WFWR. Prior to the current study, the Watershed Conservation Resource Center (WCRC) restored and redesigned the fluvial channel and banks near site 1 on the WFWR and concluded that riparian vegetation, bank stabilization, and constructed floodplains reduced sediment loads by 96% at this site (WCRC, 2011). Conservation management practices such as riparian buffer strips and vegetation can help reduce soil erosion by decreasing surface water velocity and depositing suspended sediments (Henley et al., 2000). Efforts by the WCRC provide an effective model for stream bank restoration on the WFWR, and soils found downstream could benefit from similar management (Fig. 5).While this conservation effort reduced sedimentation at an upstream site, data in the current study provided that turbidity limits were still being exceeded downstream which may further support the hypothesis that the soil series composition has an effect on the turbidity values on the WFWR.

CONCLUSIONS

With the results from this study, we provided an analysis of factors in the WFWR that can affect turbidity and water quality. We examined water quality conditions from three samples at nine sites along 36 km of the WFWR over a six month period. It was concluded that only the two furthest downstream sites had conditions that exceeded turbidity standards (10 NTU). In general, the turbidity increased as the location of the sampling site changed in the downstream direction. To examine factors that could possibly be causing the high turbidity, TSS, TISS, TVSS, and Chl–a were measured from the samples, and all parameters tested show an increase in concentrations as the sample site location moved in the downstream direction. Linear regression analyses showed that TISS and TVSS concentrations were significantly related to changes in turbidity. A multiple linear regression showed that TISS and TVSS together explained 85% of the variation in turbidity, and 73% of the variance in turbidity was influenced by TISS alone. Thus, inorganic materials comprised the majority of suspended solids in the water column. At downstream sites that exceeded the water quality standard (>10 NTU), there were select soils (Enders-Allegheny complex and Sloan, Razort, Taloka, and Pickwick silt loams) in the riparian zone which were not as prevalent at the upstream sites. The segments of the WFWR which were found to exceed turbidity and had the select soils represents only 2.2 km (6%) of the studied stream reach. Past restoration efforts on the WFWR showed results of lowered turbidity upstream, although the downstream sites exceeded the turbidity limit in the current study. The observed soil types which were identified at those sampling sites with higher turbidity values should be investigated further for physical factors that attribute to suspended solids so that further data can be collected and contribute in the progress towards a management plan for lowering turbidity in the WFWR.

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Table 1. Site description of sampling locations on the West Fork of the White River

Site No.	Description	Distance (km) from site 1	Latitude, degrees	Longitude, degrees	Elevation (m)
1	Brentwood Mt. Rd.	0	35.85545	-94.109650	462
2	Woolsey Rd.	8	35.88368	-94.165782	451
3	West Fork Main	14	35.92812	-94.184479	427
4	Dye Creek Rd.	16	35.94138	-94.186562	404
5	Greenland Main	19	35.98113	-94.173714	389
6	Wilson Hollow	22	36.01485	-94.142342	370
7	Black Oak Rd.	25	36.01897	-94.123222	367
8	Dead Horse Mt. Rd.	31	36.05080	-94.118584	361
9	Harvey Dowell Rd.	36	36.05382	-94.083092	354

Fig. 1. Sampling sites on the West Fork of the White River southeast of Fayetteville, Ark. (Image courtesy of GoogleEarth 2011).

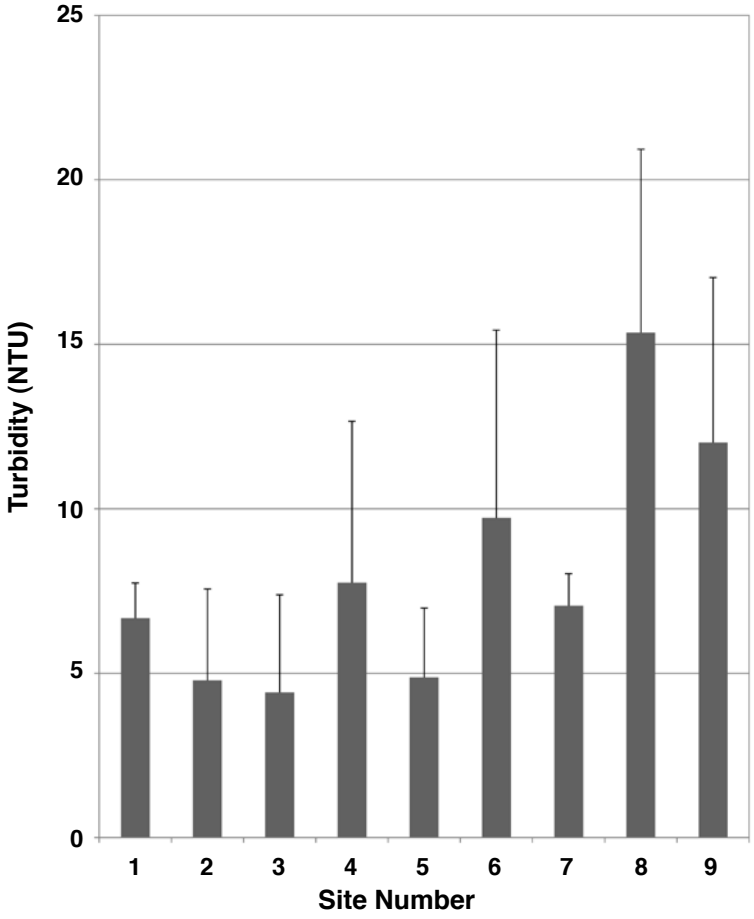

Fig. 2. Average turbidity (NTU) at the West Fork of the White River and the error bars are corresponding standard deviation amongst samples at each site (1-9) across three sampling dates. See Table 1 for site descriptions.

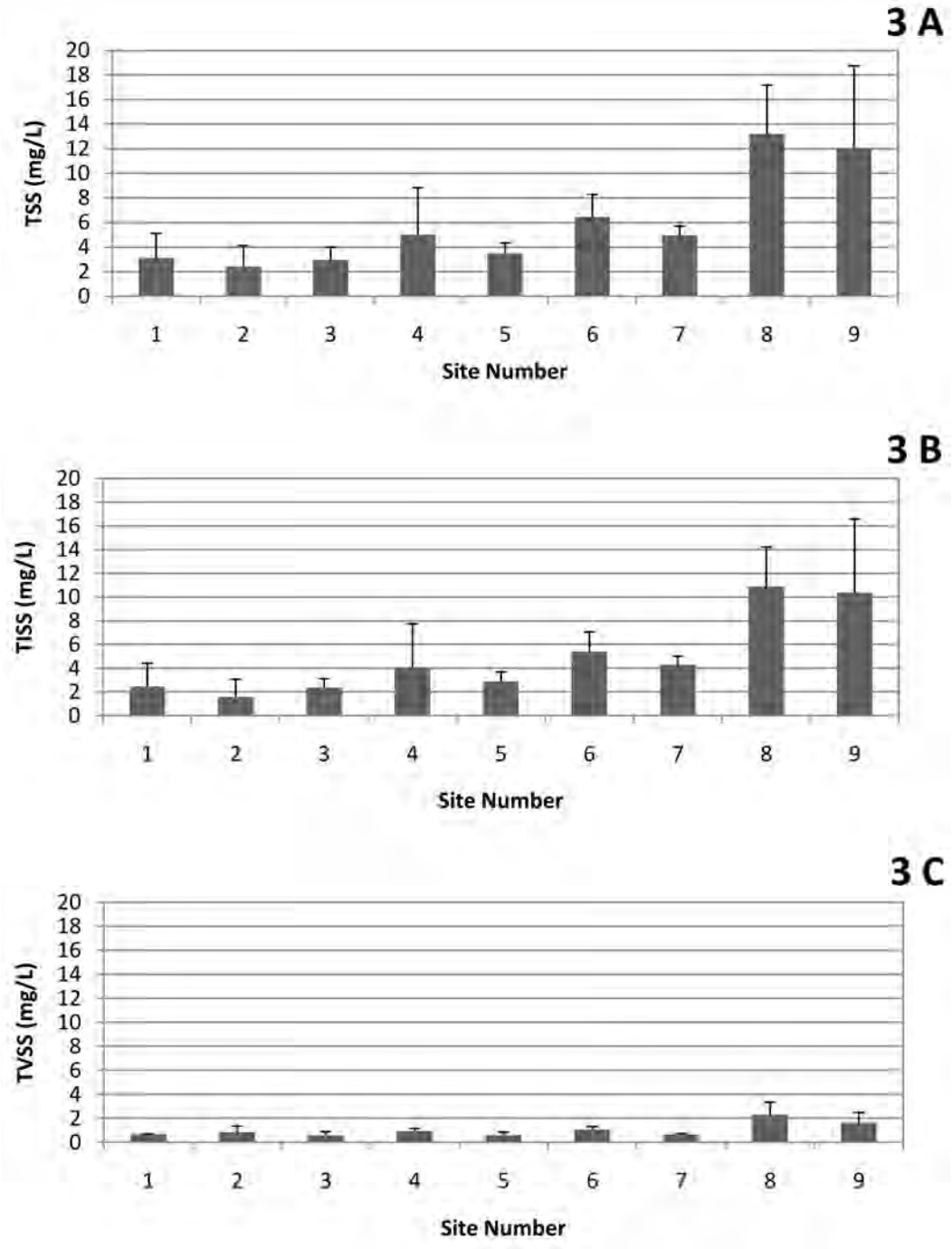

Fig. 3. A. Total suspended solids (TSS), **B.** total inorganic suspended solids (TISS), and **C.** total volatile suspended solids (TVSS) at the West Fork of the White River and the error bars are corresponding standard deviation amongst samples at each of nine sites averaged across the three sampling dates. See Table 1 for site descriptions.

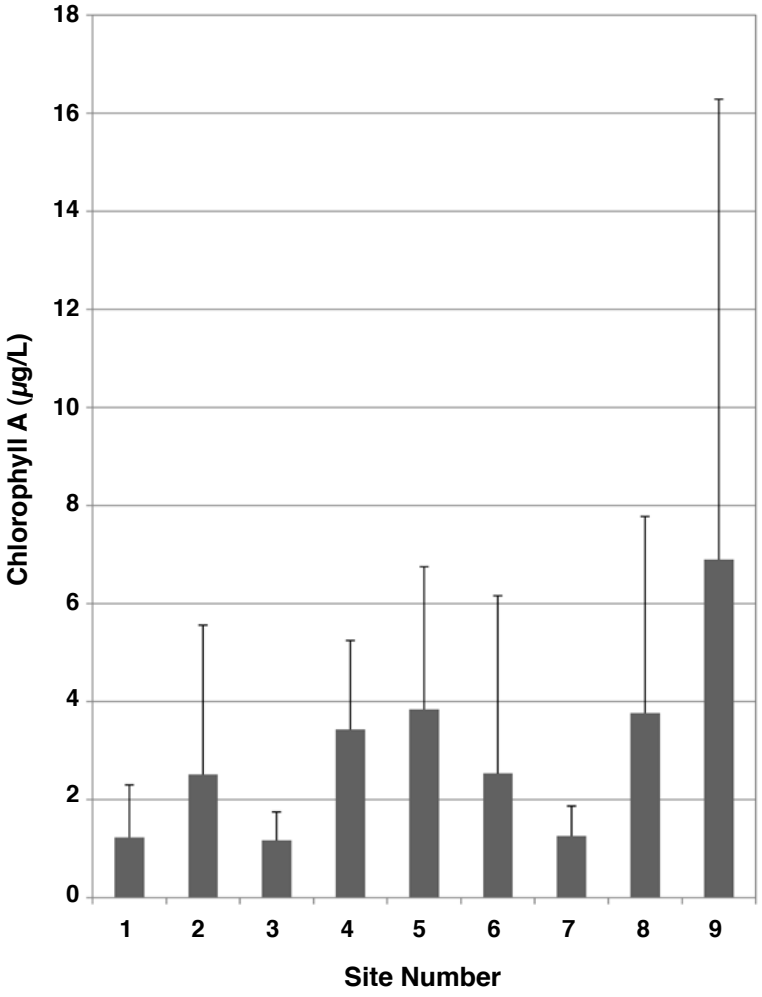

Fig. 4. Average sestonic chlorophyll a concentrations at the West Fork of the White River, and the error bars are corresponding standard deviation amongst samples at each of nine sites averaged across the three sampling dates. See Table 1 for site descriptions.

Fig. 5. West Fork of the White River at Dead Horse Mountain Road (site 8) with visible stream bank erosion and turbid water conditions along a Razort-Sloan-Enders soils complex.

Calf pre-weaning traits and immunoglobulin response to bovine viral diarrhea virus vaccination

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ABSTRACT

Calfhood vaccination for bovine viral diarrhea virus (BVDV) is a relatively new concept, and protocols are evolving. Our objective was to determine effects of BVDV type I vaccination protocol, calf behavior (chute score, and chute exit velocity), and gender on calf gain and immunoglobulin (Ig) response. Crossbred calves (n = 64) were randomly allotted to one of two vaccination protocols. In protocol 1, calves were vaccinated at 60 d of age (d 0) and at weaning (d 147). Calves assigned to protocol 2 were vaccinated against BVDV type I at 21 d prior to (d 126) and at weaning (d 147). Blood samples were collected from half of the calves in each protocol group on d 0 (60 days of age), d 21, d 126 (21 days prior to weaning), and d 147 (at weaning); serum was harvested and Ig titers were determined. Titers for BVDV type I were transformed (log base 2) and analyzed using a mixed model procedure. Calves vaccinated at d 0 and weaning had larger ($P < 0.0001$) titers than calves vaccinated at d 126 and weaning (7.5 ± 0.36 and 5.1 ± 0.36, respectively). Mean BVDV titers were larger ($P < 0.0001$) on d 147 when compared with d 126, d 21, and d 0 (8.3 ± 0.39, 5.1 ± 0.40, 5.9 ± 0.39 and 5.7 ± 0.39, respectively). A treatment × day interaction ($P < 0.0001$) also affected BVDV titers. However, BVDV titers were not affected ($P > 0.05$) by calf gender, chute score, or chute exit velocity. Weaning weight and pre-weaning average daily gain (ADG) were not related to BVDV type I titers. This study indicated that vaccinating beef calves against BVDV was effective in triggering an Ig response. Furthermore, our results suggest that calves should be vaccinated against BVDV type I at 60 d of age for greater disease resistance.

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MEET THE STUDENT-AUTHOR

Whitney Downum

I am from Springdale, Ark., and graduated from Springdale High School in 2007. I started my college career at Northwest Arkansas Community College and graduated with an Associate of Science degree in 2009. After graduation, I transferred to the University of Arkansas with a double major in animal science and poultry science with a pre-veterinarian emphasis. I have a double minor in equine science and agricultural business. I am a member of Alpha Zeta, Pre-Vet, and Mortar Board.

During the spring of 2010, I started working with Dr. A. Hayden Brown, Jr. and Dr. Jeremy Powell on a research project concerning calf temperament and immunoglobulin response. Through this research, I have gained valuable experiences. The research that we conducted has allowed me to compete at the 2010 American Society of Animal Science Southern Section in Corpus Christi, Texas, and at the Gamma Sigma Delta competition at the University of Arkansas.

I plan on applying to a veterinary school in the fall of 2011. I hope to pursue my career choice as a large animal veterinarian.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an immunosuppressive, single-stranded, enveloped RNA virus (Ridpath, 2002; Glew and Howard, 2001). It is classified in the *Pestivirus* genus of the family *Flaviviridae* (Bolin and Ridpath, 1998). Since BVDV is an RNA virus it can mutate rapidly, which is why there are several strains, and more to develop (Ridpath, 2002). An immunosuppressive virus causes an infection that weakens the immune system, which then leads to secondary infections from other pathogens.

Bovine viral diarrhea virus has an economic impact not only on the U.S. beef industry, but worldwide (Baker, 1995; Houe, 1999). Larson et al., 2002 reported a loss of $15.33 to $20.16 per cow, which impacted the cow-calf segment of the industry. Individual herd outbreaks have estimated losses of a few thousand up to one hundred thousand dollars depending on the herd, with estimated losses at the national level ranging between $10 and $40 million dollars per million calvings (Houe, 2003).

Bovine viral diarrhea virus infects a high percentage of cattle on the national level, and can cause a number of clinical diseases ranging from subclinical infection to acute fatal mucosal diseases (MD) (Baker, 1995; Houe, 1995) and various reproductive problems. A BVDV infection during gestation can cause infertility, abortions, stillbirths, abnormalities, weak calves and development of BVDV persistently infected (PI) calves (Van Campen et al., 2000). If the cow is infected with BVDV between 42 to 120 days of the gestational period, the calves may become persistently infected, which means they will persistently shed the virus and keep the herd infected (Fulton et al., 2005, Van Campen et al., 2000) and cause the greatest impact in the feedlot or replacement heifers (Ridpath, 2002). Fetuses infected with the virus after 125 days of gestation usually are not PI calves.

Excitable temperament has been shown to negatively affect the individual's immune system (Fell et al., 1999) and the cattle industry (Curley et al., 2006). Excitable temperament can compromise immune function making it difficult for the animal to produce a sufficient response when challenged with disease causing organisms (Oliphint et al., 2006). Calves with desirable temperament have greater response to the vaccine (Oliphint et al., 2006). Calfhood vaccinations have increased subsequent growth and decreased subsequent morbidity (Oliphint et al., 2006). Average daily gain (ADG), feed conversion, morbidity, fertility, and beef quality has been shown to be related to temperament (Hoppe et al., 2010). Stimulating a calf's immune system while it still has a high maternal antibody can be beneficial, since the calves will change from maternally derived immunity to long-lasting acquired immunity without experiencing a period of vulnerability before a vaccine can induce protection (Endsley et al., 2003).

Calfhood vaccination for BVDV is a relatively new concept; additional research seems appropriate. The purpose

of this study was to determine the effects of treatment, calf age, and age of dam on immunoglobulin (Ig) response to BVDV vaccination and to determine the relationship of Ig response with chute behavior score (CS), chute exit speed (CES), weaning weight, and pre-weaning ADG in crossbred beef calves.

MATERIALS AND METHODS

Crossbred calves (n = 64) were Angus sired, born in the spring, and weaned in the fall. The cows were Angus based, but not straightbred. The group of calves tested averaged 60 days of age and was located at the Savoy research unit at the start of the trial. The calves were stratified by date of birth, gender (heifers or steers [castrated at birth]), and age of cow then assigned randomly to one of two treatment groups resulting in 32 calves in each treatment. All calves were tested using Pyramid 5 vaccine (Boehringer-Ingelheim Vetmedica, St. Joseph, Mo.) which includes BHV, BVDV (Type I and II), PI3, and BRSV. All calves were tested for PI BVDV using the AC-ELISA procedure (CattleStats, Oklahoma City, Okla.) at branding.

Calf temperament or chute score (CS) was evaluated on a scale of 1-5 where 1 is extremely docile and 5 is berserk frenzy. Calves were then assigned a score of 1-5 to designate behaviors using the following five-point temperament rating system, similar to that which Grandin (1998) used: 1–calm, no movement, extremely docile; 2–restless shifting, slightly nervous; 3–squirming, continuous shaking of chute, down on foreknees; 4–rearing, twisting, continuous violent struggle, back and forward movement; and 5–berserk frenzy.

Serum from jugular samples taken on d 0 (60 d of age), d 21, d 126 (21 d prior to weaning), and d 147 (at weaning) from half of the calves in each group (approximately 16 calves) was harvested for determination of Ig response (Table 1). Blood was collected via jugular venipuncture in an evacuated tube. Serum was sent to Iowa State University Veterinary Diagnostic Laboratory (Iowa State University, Ames, Iowa) for measurement of Ig response using viral neutralization.

Chute exit velocity (CEV) was calculated as velocity = distance (m)/time (s). The CEV of the calf was measured electronically (Polaris Wireless Timer; FarmTek, Inc.; Wylie, Texas) over a 1.8-m distance beginning 1.8 m in front of the head gate. To trigger the starting and stopping of the timer, infrared sensors were used. Chute exit velocity was calculated on d 147 of the trial.

Calves were weighed on d 0, d 21, d126, and d 147 of the trial to determine pre-weaning ADG, and weaning weight was taken on d 147 of the trial.

Statistical Analysis. Titer data were transformed to log base 2 ($\log_2$). Data were analyzed using mixed model procedures of SAS (SAS Inst. Inc., Cary, N.C.). Fixed effects were treatment, sex, and date. Calf was a random effect. Chute score (CS), chute exit velocity, weaning weight, and pre-weaning ADG were analyzed using CORR procedures of SAS. Means were separated with PDIFF option of SAS.

RESULTS AND DISCUSSION

The least square means of CS and CEV by treatments did not have a significant change. In treatment 1, calves had an average CS of 1.60 m/s ± 0.171 and a CEV of 2.26 m/s ± 0.241; while calves in treatment 2 had an average CS of 1.63 m/s ± 0.171 and a CEV of 2.69 m/s ± 0.254. The least square means of CS and CEV by sex did not have a significant change. Heifers had an average CS of 1.53 m/s ± 0.172 and a CEV of 2.31 m/s ± 0.251. While steers had an average CS of 1.70 m/s ± 0.178 and a CEV of 2.64 m/s ± 0.255. A treatment × day interaction ($P < 0.0001$) was found for mean $\log_2$ of BVDV Type I titer. Mean $\log_2$ of BVDV Type I titer were not affected ($P > 0.05$) by sex, CEV, CS, age of dam, weaning weight, and pre-weaning ADG.

Calves in treatment 1 were vaccinated against BVDV using Pyramid 5 vaccine on d 0 and d147, while calves in treatment 2 were vaccinated on d 126 and d 147 of the trial. On d 0 and d 21 of the trial, both treatments showed high maternal antibody titers. On d 126, calves in treatment 1, which had previously been vaccinated, continued to show high serum titers, while treatment 2 titers declined at that time. On d 147 of the trial, both treatments showed an increase in high serum titers (Table 2). Vaccination of beef calves with modified-live virus vaccine at 67 days of age was effective in an immunological response (Kirkpatrick et al., 2008).

Vaccinating beef calves at 60 d of age (d 0 of the study) against BVDV was effective in triggering an Ig response. Bovine viral diarrhea virus vaccination had no effect on other production parameters that were studied. Calves should be vaccinated against BVDV Type I at 60 d of age for greater disease resistance.

ACKNOWLEDGEMENTS

I would like to thank Dr. Brown for his encouragement, advice, and support in this project. Thanks to Dr. Powell for his help and explanation of the project. Gratitude is also extended to the Sugar Hills and Dale Bumpers College of Agricultural, Food and Life Sciences for their financial funding of my project.

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Table 1. Experimental design of treatments, vaccination times, and blood collection times (n = 16) during the 147-d study.

	60 days of age[z]		21 days prior to weaning	Weaning
Treatment	**d 0**	**d 21**	**d 126**	**d 147**
	May 6	**May 27**	**September 9**	**September 30**
1	Vaccinated for BVDV (Pyramid 5) Blood collected for titer response	Blood collected for titer response	Blood collected for titer response	Vaccinated for BVDV (Pyramid 5) Blood collected for titer response
2	Blood collected for titer response	Blood collected for titer response	Vaccinated for BVDV (Pyramid 5) Blood collected for titer response	Vaccinated for BVDV (Pyramid 5) Blood collected for titer response

[z] Day (d) 0 = 60 days of age.

Table 2. Least square means of log 2 type I titer for treatment x day interaction.

Treatment	Day[z]	Mean Log2 BVDV type I titer
1	0	4.95 ± 0.56d[y]
	21	6.19 ± 0.56bc
	126	9.30 ± 0.56a
	147	9.45 ± 0.55a
2	0	6.48 ± 0.55bc
	21	5.69 ± 0.55ad
	126	0.99 ± 0.56e
	147	7.11 ± 0.56b

[z] Day (d) 0 = 60 days of age.
[y] Means with different letters differ ($P < 0.05$).

Comparison of growing media for container grown plants

Paul Harris[*], *David E. Longer*[†], *Derrick Oosterhuis*[§], *and Dimitra Loka*[‡]

ABSTRACT

Greenhouse and growth chamber experiments are conducted worldwide in efforts to produce solutions that would increase yields of agronomic crops. However, the results of those experiments vary due to the many growth media being used. An experiment was conducted in the fall of 2010 to identify a broadly acceptable growth media that would produce uniform stands and optimum results in greenhouse and growth chamber settings. A total of six growth media were tested on cotton (*Gossypium hirsutum*) at the Arkansas Agricutural Research and Extension Center's Altheimer Lab in Fayetteville. The plants grown in each medium were harvested six weeks after planting and the measurements performed included plant height, plant dry matter, leaf area, and nutrient analysis. The results indicated that a positive, significant difference ($P < 0.05$) existed between "Sunshine" mix (MIX1) and the other media. Plants grown in MIX1 experienced greater plant height, dry matter, leaf area, and also experienced higher leaf tissue levels of N, P, and S. "Sunshine" (Mix1) is a readily available growth medium that produces optimum plant growth and uniform results in growth chamber and greenhouse experiments.

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MEET THE STUDENT-AUTHOR

Paul Harris

I am a native of Kennett, Mo., and a 2006 graduate of Kennett High School. After graduation, I began my education as an undergraduate student at the University of Arkansas. After three years of pursuing a degree in chemistry, I came to the realization that this particular area of study was not for me. Coming from a strong agricultural background with a passion for the agricultural industry, I made the decision to change my major to crop management in the department of crop, soil and environmental sciences (CSES), with a minor in pest management. After only a few classes, I realized that switching to agriculture was the right decision.

In the fall of 2010, I was given the opportunity to conduct experimental research under the direction of Dr. Derrick Oosterhuis, distinguished professor of cotton physiology and Dr. David Longer, professor of agronomy in the CSES department. In the spring of 2011, I was given the opportunity to conduct additional undergraduate research under the guidance of Dr. Jason Norsworthy an associate professor of weed science in the CSES department. Also, in 2011 I was selected for the Who's Who Among Students in American Universities and Colleges. I plan to graduate from the University of Arkansas in the fall of 2011, and I will pursue my career in agriculture with my wife Bethany, and her family at Wildy Family Farms in Manila, Ark.

I would like to thank Dr. Derrick Oosterhuis and Dr. David Longer for their guidance and assistance with this project and their ongoing support for my education.

INTRODUCTION

With the world's population on a steady increase, pressure has been placed on crop scientists from around the world. To date, much research has been directed to help keep up with the high demand for food. Whether the research focus is testing new cultivars with improved genetics, chemical treatments, or growth techniques, many experiments are performed in controlled environmental chambers. By controlling all environmental factors (temperature, water, humidity, nutrients, etc.) it is fairly simple to determine whether a particular treatment is affecting a plant's growth. However, although experimenters worldwide are able to set environmental factors at a constant value, the potting media often varies, making meaningful comparisons difficult. Corporate and academic researchers prefer a certain growth media that performs best in their laboratory. For example, agricultural researchers at Texas A&M University perform experiments using fritted clay as the growth medium. Researchers at Utah State University as well as the NASA research lab at the Kennedy Space Center use calcined clay, while University of Arkansas CSES personnel use a peat moss based media called "Sunshine" marketed by Sun Gro Horticulture Canada Ltd. (pers. comm. with Dr. D. Oosterhuis). With the growth medium varying across the world, problems arise when the results are analyzed because nearly identical experiments can vary and are unable to be compared due to the differing mediums used for plant growth. Soilless cultures, often used in greenhouse experiments, will present a different range of physical and hydrological properties compared with natural and agricultural soils (Casadesus et al., 2007); this may explain why soilless substrates have experienced a rapid expansion over the last decade (Raviv, et al., 2002). Thus, the desired uniformity between container cultures and between container and field cultures will continue to elude researchers if a universally reliable and accepted medium is not developed.

Growing media differ in many ways such as nutrient availability, water holding capacity, pH, bulk density, etc., and they all determine how certain plants grow in certain medium. Clays, for instance, are made up of very fine particles which decrease the pore size, available soil water and oxygen while increasing the pore space (Brady and Weil, 2009). The very fine particles of clay, according to Asli and Neumann (2009), may accumulate at the external root surfaces of transpiring plants, thereby reducing root hydraulic conductivity and plant availability of external water sources. Clayey soils are notoriously difficult to manage. The window of opportunity between too wet and sticky

(gummy, adhesive) and dry and hard is short compared to loamy soils (Popp et al., 2003). However, expanded clays (natural clays heated at 1050 °C) contain large amounts of air because the porosity is increased after heating, and physical characteristics of the clays are unchanged after 5 years of intensive cropping (Raviv, et al., 2002), indicating sufficient soil consistency. Also, fritted clay, often referred to as "kitty litter," is a material that has been found suitable for growing experimental plants because it holds 31% by volume of plant-available water which is excellent for plant growth purposes (Van Bavel et al., 1978).

Sand cultures are often the opposite of clays in regards to agricultural soil physical and chemical properties. Sand particles are smaller than 2 mm but larger than 0.05 mm and primarily consist of quartz which means sands generally contain fewer plant nutrients (Brady and Weil, 2009). Because of sand's inability to hold water or nutrients, it is normally not the medium of choice by most agricultural researchers. However, sands can be used as a component of various growth media mixtures (Raviv et al., 2002). When working with a either a drought-tolerant plant or one sensitive to large amounts of water, a sand based-culture is the medium of choice because of the large particle size and resultant large pore spaces, which help to make water management easier.

Soilless media, sometimes called artificial soils, offer the plant several advantages. They are readily available, easy to handle, and produce uniform plant growth from year to year (Boodley and Sheldrake, 1977). Peat-based media usually contain large amounts of nutrients and other minerals supportive of plant growth and are known for high water-holding capacities. Peat mixes also contain methane-oxidizing bacteria that reduce methane emissions to the atmosphere and supply carbon dioxide for photosynthesis (Szafranek-Nakonieczna and Bennicelli, 2010). A different type of "soilless" media commonly used in laboratory experiments would be hydroponic solutions that date back to the mid 18th century (Jones Jr. and Benton, 1982). Hydroponics can be broadly defined as the practice of growing plants in a mineral nutrient solution without the presence of soil.

With the increasing demand for new crop growth technology, higher yields, and wise use of resources, comes the increasing demand for accurate and uniform agricultural experimental designs and comparable results. For convenience and cost savings, many experiments have been and will continue to be carried out in a climate-controlled setting and will involve container-grown plants. Those plants will be grown in media that vary in chemical and physical properties and will produce data that will also vary, no matter how similar the experiment. It is important that a growth medium not restrict plant growth to an artificially low level and bias the experimental results. Therefore, the objectives of this research were to define both the benefits and the disadvantages of various growth media and to find a broadly adaptable growth medium that will produce optimum and meaningful results while producing uniform stands, across media, in the greenhouse or growth chamber setting.

MATERIALS AND METHODS

Planting Materials and Growth Conditions. The experiment was conducted at the Arkansas Agricultural Research and Extension Center's Altheimer Laboratory, University of Arkansas, Fayetteville. The plant species chosen for this trial was cotton (*Gossypium hirsutum L.*), cultivar Stoneville 5288B2R. Six different plant media were chosen and included a Calloway silt loam soil from Marianna, Arkansas (SOIL and SOILH – Hoagland's solution added), sand with added nutrients (SAND), fritted clay (FC), calcined clay (CC), and two different peat moss-based media named "Sunshine Mix 1" (MIX1) and "Sunshine NB1" (NB1). Each medium was analyzed at the University of Arkansas Fayetteville Soil Testing Laboratory where the following chemical properties were measured: (1) Mehlich-3-extractable nutrients; (2) pH and electrical conductivity (EC); (3) total N and C. Mehlich-3-extractable P, K, Ca, Mg, Na, S, Fe, Mn, Zn, Cu and B were analyzed by SPECTRO CIROS ICP using a 1:10 soil to extractant (wt/vol.) ratio (Table 1). The pH was measured using a 1:2 soil to water (wt/vol.) ratio (Table 1).

Each treatment was replicated 10 times and placed in 1.5-L pots. Each pot was filled with a certain media approximately 2.5 cm from the upper rim of the pot. Five cotton seeds were planted in each pot about 2.5 cm below the surface of the media. All treatments were subjected to 14-hour photoperiods at a constant humidity of 60%. The plants were watered daily using 200 mL of deionized water per pot. At two weeks, the plants were thinned to one plant per pot. After thinning, the daily watering schedule consisted of watering on alternate days with 150 mL Hoagland's solution per pot and a onetime rinse with deionized water. Also, half of the pots containing field soil were watered daily with 200 mL deionized water only. Hoagland's solution was not added to these five pots so that observations could be taken on how the representative field soil would compare to the other media under more field related circumstances. This treatment, designated as "SOIL" was set as the experimental control.

Measurements and Data Analysis. At six weeks after planting, plant height (cm), the number of nodes, leaf area (cm^2), plant dry weight (g), leaf symptoms of deficiencies/toxicities and nutrient analysis of the leaves were determined. Plant height was measured from the base of the plant at the soil surface to the apical meristem. Leaf

area was calculated using a LICOR Leaf Area Analyzer (LI-COR Biosciences, Lincoln, Neb.). Harvested plant biomass was placed in a forced-air oven at 60 °C for 72 h to remove moisture and then weighed to determine plant dry weight. Leaf tissue nutrient analysis was performed by the University of Arkansas-Fayetteville Soil Testing Laboratory. A statistical analysis of the six growth media treatments, with 10 replications, was conducted using JMP software, version 9.1 (SAS Institute; Cary, N.C.). Analysis of variance and conventional LSD ($\alpha = 0.05$) post hoc analysis were used to compare significance between mean values. The main effects of growth media on plant growth factors were separated by LSD comparisons of the treatment means at the ($P < 0.05$) level.

RESULTS AND DISCUSSION

Plant Height. Significant treatment differences existed in plant height (Table 2). The greatest average plant height was found in MIX 1 and averaged 29.55 cm. In addition, MIX 1 contained the highest concentrations of nutrients (Table 1) while still existing in the cotton-preferred pH range of 6-7 (Table 1). The FC medium also consisted of relatively high levels of most nutrients while resulting in the smallest average plant height. Short plant height may have been due to the pH level of 8.68, which is considered by most to be too high for cotton. Also, the electrical conductivity level of 22,200 umhos/cm (Table 1) indicates high salinity. Growth and yield of cotton are severely inhibited in salinity levels higher than 10,000 umhos/cm at the germination and emergence stages (Ashraf, 2002). In regards to the two field-based soil media referred to as SOIL and SOILH, addition of Hoagland's solution (SOILH) significantly increased plant height by nearly 2.5 cm (Table 2).

Nodes Per Plant. In comparison with plant height, the number of nodes per plant experienced a similar ranking, however with fewer significant differences among media (Table 2). The MIX 1 medium contained plants with the greatest plant node number, averaging 9.3 nodes per plant. The NB1 medium and the two field soil media (SOIL and SOILH) had 7.5 nodes per plant average and were not significantly different from one another. The fewest nodes per plant was 5.3 and occurred when using FC growth medium.

Leaf Area. In addition to the greatest leaf dry matter (Table 3), the MIX1 treatment had the highest leaf area with an average of 730.5 cm² (Table 2). This is likely due to the higher levels of nutrients contained in MIX1 in comparison with the other types of media (Table 1). The next largest average leaf area was 470.05 cm² belonging to the plants in SOILH; however, leaf area was not significantly different than NB1 with an average leaf area of 454.29 cm². Similar to previous data, leaf area was significantly smaller in FC than for plants growing in other media (Table 2).

Plant Dry Matter. The MIX 1 medium and the field soil that received Hoagland's (SOILH) had the largest total plant dry matter respectively and were not significantly different from one another (Table 3). Plants in SOILH and MIX 1 were significantly different from the plants in other mixes in terms of stem dry matter, but they were not significantly different from each other. Plants in SOILH and MIX1 were significantly different in leaf dry matter. The two clay media, FC and CC, exhibited the lowest total dry matter (Table 3).

Nutrient Analysis. The data (Table 4) showed that the greatest uptake and retention of N occurred with MIX 1 and CC and were not significantly different from one another. The MIX 1 medium also featured the highest levels of P. The water retention capacity of these two media may have contributed to higher plant nutrient uptake because water films in the soil support nutrient transport from soil solids to plant roots. The plants in MIX1 and CC media contained the highest percent N with no significant difference (Table 4). As expected, SOIL contained the lowest percent N due to the absence of Hoagland's solution. Plants grown in MIX1 possessed the highest percentage of both P and S and were not significantly different from NB1; and both were significantly higher than all other mixes except CC in terms of S levels. The FC medium contained a significantly lower percent P than the other media, but the cause for this is unknown. It should be noted that FC had an extremely high value for electrical conductivity, but no cause and effect relationship seemed apparent. The CC plants possessed the highest percentages of Mg and K and these were significantly greater than the nutrient levels found in the other media (Table 4).

In addition to macronutrients, micronutrient analysis was also performed (Table 5). Not only did plants grown in CC contain the highest concentrations of Mg and K, but they also contained the largest amounts of Fe, Mn, and B. However, CC plants contained the lowest amounts of Cu and a relatively low amount of Zn compared to MIX1 plants, which contained at least 32% more Zn than the other media. As expected from the soil test, plants grown in FC contained the largest amount of Na. The high Na concentration may be one reason why plants were negatively affected by the FC treatment in terms of plant height, plant dry matter, and leaf area. High amounts of Na in media solution can be detrimental to plants. The damage of salinity to plants is mainly caused by Na ion accumulation which alters ion transfer across cell membranes and can be toxic to non-halophytes (Wu et al., 2004).

Overall, leaf-based nutrient analysis of the plants grown in MIX1 points to a positive treatment influence on the plants nutrient uptake and the plant growth parameters tested. Plants grown in MIX1 were significantly greater in leaf dry matter than plants grown in the other media. In

stem dry matter, the MIX 1 grown plants were not significantly different than plants grown in SOILH, but greater than all the other growth mediums. Total plant dry matter was highest in the MIX1 and SOILH media, which did not differ from one another (Table 3). In addition to MIX1, NB1 ranked second in percent P, K and S which could result from peat-based media water holding capacities, nutrient retention and reduced leaching. Due to the water-holding capacity and rewetting ability of calcined clay, CC plants contained high percentages of nutrients. Rewetting ability refers to how rapidly a root medium absorbs water, and thus reaches its potential for maximum available water-holding capacity, with minimal leaching (Argo and Fisher, 2007). Calcined clay or vermiculite can be added to a root medium to increase rewetting because both absorb and distribute water independently of their moisture content prior to water being applied (Argo and Fisher, 2007). However, the physical plant growth properties of CC showed negative, significant differences when compared with MIX1 or SOILH. In addition to the plant growth properties found in CC, FC also produced plants that were significantly lower in height, dry matter, and leaf area.

Our research showed that MIX1 produced plants that attained greater aboveground biomass and leaf area along with improved plant nutrient uptake as evidenced by improved levels of overall plant nutrition (Fig. 1). Also, peat-based media such as MIX1 may require less water than other media because of the retentive nature of peat, but we did not test this. All other media, besides MIX1, demonstrated some level of restricted plant growth. For future research involving plant growth media, it is important to understand that limiting the growth of the plants because of the growth media used, could possibly mask any positive effects caused by the treatment. The MIX1 medium is an economical, available medium that is ideal for producing uniform cotton seedlings in greenhouse and growth chamber settings. Additional research should focus on similar studies conducted with other plant species.

ACKNOWLEDGEMENTS

I would like to extend my appreciation to Dr. David E. Longer, Professor of Agronomy, University of Arkansas-Fayetteville and Dr. Derrick Oosterhuis, Distinguished Professor of Cotton Physiology, University of Arkansas-Fayetteville for the financial support and opportunity to perform this project. Also, I would like to thank Dimitra Loka, a graduate student under the tutelage of Dr. Oosterhuis, who assisted in maintaining this experiment as well as performing statistical analysis of the data at the experiment's conclusion.

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Table 1. Standard soil test analysis of plant nutrient levels found in selected plant growth media. pH levels and electrical conductivity levels are also presented.

Media Types[a]	pH	EC	mg/kg									
		(μmhos/cm)	P	K	Ca	Mg	S	Na	Fe	Mn	Zn	Cu	B
FC	8.68	22200	7.5	754	7389	823	527.6	4253.5	132.8	26.2	6.5	1.1	1.9
CC	6.52	81	40.5	839	449	372	25.7	47.6	113.3	5.5	1.5	0.4	2.2
SAND	10.24	128	<0.6	26	405	26	16	15	70.6	7.8	0.9	0.3	0.2
MIX1	6.58	582	309.7	1232	8188	3078	729	308.2	247.2	21	11.7	2.5	1.4
NB1	7.02	312	8.9	209	7654	3209	835.2	206.2	203.4	9.4	3.9	1	0.4
SOIL	7.1	142	74.5	248	1310	313	24.9	23.5	259.7	138	2.2	2.1	0.3

[a]Abbreviations for media types are: FC (Fritted clay), CC (Calcined clay), SAND (sand with nutrients added), MIX 1 ("Sunshine" commercial peat mix), NB1 (commercial peat mix), SOIL (Calloway silt loam soil), and SOIL H (Calloway silt loam soil with Hoagland's nutrient solution).

Table 2. Mean physical properties of cotton plants grown in different plant growth media.

Media[1]	Plant Height (cm)	# of Nodes	Leaf Area (cm²)
MIX1	29.55 a[2]	9.33 a	730.5 a
NB1	25.96 b	7.66 b	454.29 b
SOILH	22.42 c	7.8 b	470.05 b
SOIL	19.94 d	7.2 bc	290.64 c
SAND	19.63 d	6.5 cd	277.44 c
CC	18.15 de	6.33 d	251.66 c
FC	16.96 e	5.33 e	200.95 d

[1] Abbreviations for media types are: MIX 1 ("Sunshine" commercial peat mix), NB1 (commercial peat mix), SOIL H (Calloway silt loam soil with Hoagland's nutrient solution), SOIL (Calloway silt loam soil), SAND (sand with nutrients added), CC (Calcined clay), FC (Fritted clay).
[2] Means in the same column with the same letter are not significantly different at the 0.05 alpha level.

Table 3. Mean plant dry matter components of cotton plants grown in different plant growth media.

Media[1]	Leaf Dry Matter (g)	Stem Dry Matter (g)	Total Dry Matter (g)
MIX1	2.99 a[2]	1.53 a	4.52 a
NB1	2.16 c	1.22 b	3.38 b
SOILH	2.51 b	1.62 a	4.13 a
SOIL	1.80 d	1.13 b	2.93 b
SAND	1.42 e	0.81 c	2.23 c
CC	1.14 e	0.56 d	1.70 d
FC	1.29 e	0.52 d	1.81 cd

[1] Abbreviations for media types are: MIX 1 ("Sunshine" commercial peat mix), NB1 (commercial peat mix), SOIL H (Calloway silt loam soil with Hoagland's nutrient solution), SOIL (Calloway silt loam soil), SAND (sand with nutrients added), CC (Calcined clay), FC (Fritted clay).
[2] Means in the same column with the same letter are not significantly different at the 0.05 alpha level.

Table 4. Plant macronutrient leaf tissue analyses from plants grown in different plant growth media.

Media[1]	N	P	K	Ca	Mg	S
MIX1	5.63 a[2]	0.81 a	3.34 b	3.17 bc	1.18 b	1.18 a
NB1	4.51 b	0.63 b	3.29 b	3.15 bc	1.30 b	1.12 ab
SOILH	4.62 b	0.34 c	2.15 c	2.65 d	0.70 c	0.83 c
SOIL	2.57 c	0.22 d	1.49 d	2.61 d	0.59 c	0.38 d
SAND	4.25 b	0.20 d	2.99 b	3.43 b	0.67 c	0.83 c
CC	5.50 a	0.34 c	4.38 a	2.89 cd	1.58 a	1.04 b
FC	4.39 b	0.13 e	1.78 cd	4.17 a	0.57 c	0.85 c

[1] Abbreviations for media types are: MIX 1 ("Sunshine" commercial peat mix), NB1 (commercial peat mix), SOIL H (Calloway silt loam soil with Hoagland's nutrient solution), SOIL (Calloway silt loam soil), SAND (sand with nutrients added), CC (Calcined clay), FC (Fritted clay).

[2] Means in the same column with the same letter are not significantly different at the 0.05 alpha level.

Table 5. Plant micronutrient tissue analysis from plants grown in different plant growth media.

Media[1]	Na	Fe	Mn	Zn	Cu	B
MIX1	374 c[2]	105 c	6 e	51.8 a	2.9 c	59.5 c
NB1	545 b	128 b	4 e	20.6 c	1.1 e	42.6 de
SOILH	312 c	97 c	19 e	20.1 c	4.9 b	36.5 e
SOIL	189 d	62 d	51 d	11.6 d	3.6 c	47.6 d
SAND	582 b	106 c	162 b	35.3 b	5.7 a	62.8 c
FC	795 a	99 c	86 c	6.5 e	2.6 d	72.7 b
CC	755 a	183 a	266 a	12.8 d	1.1 e	97.6 a

[1] Abbreviations for media types are: MIX 1 ("Sunshine" commercial peat mix), NB1 (commercial peat mix), SOIL H (Calloway silt loam soil with Hoagland's nutrient solution), SOIL (Calloway silt loam soil), AND (sand with nutrients added), CC (Calcined clay), FC (Fritted clay).

[2] Means in the same column with the same letter are not significantly different at the 0.05 alpha level.

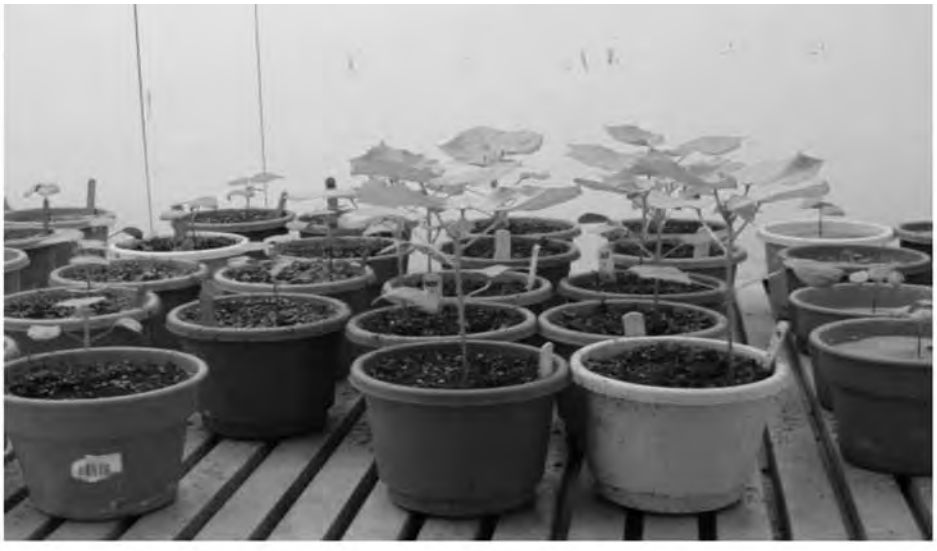

Fig. 1. The difference of physical properties and appearance of plants grown in NB1 (left) and MIX1 (right).

Effects of tall fescue and lactate dehydrogenase genetic polymorphisms on dairy heifer growth and immune function

Rachel Henry[*]*, Marites Sales*[†]*, and Charles Rosenkrans, Jr.*[§]

ABSTRACT

Objectives of this project were to evaluate polymorphisms in upstream elements of the lactate dehydrogenase B (LDHB) gene in crossbred dairy heifers (n = 27) and their effects on immune function and heifer growth when grazing endophyte-infected tall fescue. Two cultivars of tall fescue were utilized: Kentucky 31 (KY31), a wild-type endophyte-infected tall fescue, and HiMag 4 (HiMag), a domesticated non-toxic endophyte-infected tall fescue. Crossbred dairy heifers (Holstein × Jersey) were stratified by weight and randomly allotted to forage. The LDHB gene codes for one subunit of lactate dehydrogenase (LDH), an enzyme that catalyzes pyruvate to lactate and back to pyruvate. Forward primer used for amplification was 5'-ACACACCAGCAGCATCTCAG-3' and reverse primer was 5'- GATAAGGGCTGCACGAAGAC-3'. The amplicon size for this LDHB primer set was 457 base pairs. Sequenced amplicons were aligned with Clustal2W for polymorphism detection and genotype assignment. Heifers that had a heterozygous genotype and grazed HiMag were heavier when compared with other heifer groups. Number of red blood cells and hemoglobin concentrations for heifers grazing KY31 were greater when compared to heifers grazing HiMag. Distribution of white blood cells was affected by LDHB genotype. Two dairy heifer management tools, stockpiled tall fescue and LDHB genotyping, were assessed in this study, both of which impacted heifer growth and immune function as assessed by blood cell differentials.

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MEET THE STUDENT-AUTHOR

Rachel Henry

I grew up in Fort Smith, Ark., and graduated from Southside High School in 2007. My B.S. degree in animal science with a minor in equine science allowed me to fulfill the pre-veterinary program requirements. This fall I begin my studies at Louisiana State University Veterinary School. I have enjoyed my time at the University of Arkansas, becoming involved in clubs such as the Pre-Veterinary Club, Alpha Zeta, and Sigma Alpha. Through an exchange program set up by the Dale Bumpers College, I was able to complete an internship at the Royal School of Veterinary Studies in Scotland and gained valuable research experience at the same time.

This particular research experience could not have been achieved without the help and guidance of Dr. Charles Rosenkrans, Dr. Tes Sales, Dr. Elizabeth Kegley, and Dr. Jeremy Powell.

INTRODUCTION

Milk, cheese, and other dairy products are important sources of nutrition for people around the world. Despite record-breaking high milk prices, dairy profitability has been limited due to high feed prices; therefore, it is important to consider alternative feed options. One such option is tall fescue (*Lolium arundinaceum* (Schreb.), *Darbyshire*), a cool season grass commonly found in the Midwestern and Southeastern United States (Bouton, 2000). Success of tall fescue is due in no small part to its symbiotic relationship with an endophytic fungus (*Neotyphodium coenophialum*), which produces alkaloids to protect the plant from pests, stress, drought, and heat (Glenn et al., 1996). Ergot alkaloids increased animal respiration rates and rectal temperatures, decreased animal weight gain, and caused rough hair coats (Nihsen et al., 2004). One management practice to reduce the effects of grazing toxic tall fescue is to stockpile tall fescue in the fall and graze it during the winter months instead of feeding hay. By exposing the toxic fescue to the weather, the ergot alkaloids produced by the endophytic fungus are degraded naturally (Kallenbach et al., 2003).

Lactate dehydrogenase (LDH) activity is associated with cattle profitability (Looper et al., 2002), and polymorphisms in the LDH promoter are associated with serum LDH activity (Maekawa et al., 2002). Stress in general, and ergot alkaloids specifically, results in elevated LDH activity in serum. However, physiological and genetic relationships among LDH polymorphisms, LDH activity, and heifer growth and health have not been established. The objective of this research was to evaluate the polymorphic nature of the promoter/enhancer regions of the LDHB gene in dairy heifers and the relationship among LDHB genetic polymorphisms, immune function, and heifer growth while grazing stockpiled tall fescue.

MATERIALS AND METHODS

Animals. Cattle used in the experiment were Holstein-Jersey crossbred dairy heifers (n = 27) approximately nine months of age. Before the experiment, they were dewormed with ivermectin in the fall, and had grazed mixed grass paddocks or were fed similar hay at the Arkansas Agricultural Research and Extension Center located in Fayetteville. The trial began on December 6 and proceeded through February 28 for a total of 84 days.

Heifers were stratified by weight into eight tall fescue pastures. Tall fescue cultivars were Kentucky 31 (KY31; n = 4 pastures) and HiMag 4 (HiMag; n = 4 pastures). Cultivar KY31 is a wild-type endophyte-infected tall fescue and HiMag is a non-toxic endophyte-infected tall fescue (Nihsen et al., 2004). Based on visual evaluation (step point analysis), the KY31 stands were 81% tall fescue and the HiMag stands consisted of 80% tall fescue at the initiation of stockpiling. All heifers were fed a corn-based supple-

ment at approximately 0.8% body weight (BW) daily prorated for feeding five days each week. Automated watering devices and tanks were utilized to allow free access to water. Blood and animal weights were collected on days 0, 28, 56, and 84.

Digital scales were used to determine individual heifer body weight. Scales were calibrated before each weigh date and tare was established in between each heifer. On each weigh date, all heifers were gathered and weighed in random order. Heifers were sorted into their original pasture groups and returned to their original pasture after all heifers were weighed.

Pasture Design. Eight pastures (1.62 hectares each) were used in the trial. Pastures were separated by high-tensile electric fence, and each individual pasture was subdivided into thirds with poly-wire electric fence. During days 0-28 heifers were allowed to graze the first third of the pasture, on day 28 the poly-wire fence was moved to allow the grazing of an additional one-third of the pasture, and on day 56 the poly-wire fence was removed. Since no back fence was used, the cattle had access to all previous pasture sections as the trial progressed.

DNA Preparation, Amplification, and Sequencing. Blood was collected from the cattle through jugular venipuncture, into tubes containing ethylenediaminetetraacetic acid (EDTA; Vacutainer, Becton-Dickson, Rutherford, N.J.). The whole blood was centrifuged at 700 × *g* for 25 min and the plasma decanted and stored at -20 °C. Buffy coats were removed and stored at -80 °C until thawed for DNA extraction. Genomic DNA extraction was accomplished using the Purgene Genomic Purification Kit (Qiagen, Valencia, Calif.). The extracted DNA was stored at -20 °C until used for amplification.

Specific DNA primers for bovine LDHB were designed based on the GenBank accession number for human LDHB (NW 001495085) and cattle genome build 1.1 (Bovine Genome Database, Georgetown University, Washington D.C.). Primer design was accomplished using Primer 3 software (Center of Genome Research at the Whitehead Institute for Biomedical Research, Cambridge, Mass.). Cattle LDHB primers for the promoter region were: LDHB BovinePro forward: 5'-ACACACCAGCAGCATCTCAG-3' and LDHB Bovine 36 reverse: 5'-GATAAGGGCTGCACGAAGAC-3'. The amplicon size was 457 base pairs.

Extracted DNA was thawed and mixed with PCR water and the contents of the Biolase Red DNA Polymerase kit (Bioline, Randolph, Mass.) in concentrations of 74 µL of PCR water, 10 µL of 10 × NH_4 buffer, 3 µL of $MgCl_2$, 2 µL of LDH forward primer, 2 µL of LDH reverse primer, 2 µL of Red Taq, a protein dye, and 5 µL of DNA. The total amount of 100 µL was split into two tubes of 50 µL each to improve amplification when loaded into the PCR instrument.

Amplification of the promoter sequences by polymerase chain reaction (PCR) was performed using a PTC-100 Peltier Thermal Cycler (Bio-Rad, Hercules, Calif.). The amplification protocol began with 2 min heating at 98 °C followed by 35 cycles of 94 °C for 30 s, 1 min at 55 °C, and 1 min at 68 °C, and a final elongation step held for 10 min at 68 °C. Amplification products were visualized using gel electrophoresis (1.2% agarose gels in 1.0 × Tris/Borate/EDTA (TBE)) and stained with ethidium bromide.

Amplicons were verified using the gel electrophoresis and purified with QIAquick PCR Purification Kit (Qiagen, Valencia, Calif.). Concentration of the purified amplicons was determined with a DNYA Quant Fluorometer (Hoefer, San Francisco, Calif.) followed by sequencing at University of Arkansas DNA Resource Center. Submitted samples were subjected to both forward and reverse sequencing using the Applied Biosystems Big Dye Chemistry on an ABI 3130xl (Applied Biosystems, Foster City, Calif.). The resulting sequences were analyzed using ClustalW2 program (European Bioinformatics Institute, Cambridge, UK), which provided multiple alignments and identified nucleotide polymorphisms.

Blood Sample Analysis. Accutrend™ Lactate Analyzer (Roche Diagnostics, Alameda, Calif.) was used to determine lactate concentrations (mmol/L) in whole blood. Plasma protein concentrations, and lactate dehydrogenase activity (forward (LDHf) and reverse (LDHr)) were determined using modified colorimetric kinetic assays on a Spectra Max 250 (CV within 10%; Molecular Devices, Sunnyvale, Calif.). Forward reaction of LDH was determined using reagents β-nicotinamide adenine dinucleotide (0.0194 mmol/L; Sigma, Saint Louis, Mo.) and pyruvic acid (16.2 mmol/L; Sigma). Samples were analyzed at 340 nm four times one minute apart.

Reverse reaction of LDH was determined using reagents NAD^+, Free Acid (7 mmol/L; Calbiochem, San Diego, Calif.) and L-lactic acid lithium salt (50 mmol/L; Tokyo Kasei Kogyo Co., Tokyo, Japan). Samples were analyzed at 340 nm three times 30 s apart. When catalyzing the oxidation of lactate to pyruvate, LDH simultaneously reduces NAD^+ to NADH (Katz and Sahlin, 2002). The rate of reduction of NAD^+, measured by determining the rate of increase in absorbance at 340 nm, was directly proportional to LDH activity. Lactate dehydrogenase activity was expressed using the International Unit (IU), which is the amount of enzyme that catalyzes the transformation of one µmole of substrate per minute under defined conditions.

Total protein concentrations were determined using Pierce commercial BCA Protein Assay Kit (#23225; Thermo Fisher Scientific Inc., Rockford, Ill.). Both LDHf and LDHr were corrected for plasma protein concentration and were expressed as international units per milligram of plasma protein. Blood cell differential counts were determined within

four hours of collection using a Cell-Dyne 3500 hematology analyzer (Abbott Laboratories, Abbott Park, Ill.).

Statistical Analysis. Data were analyzed using analysis of variance with pasture as the whole plot experimental unit, and heifer weights, blood cell distributions, and enzyme activities as repeated measures. Pasture within cultivar was considered a random affect. Effects of cultivar, LDHB genotype (heterozygous (Del/In), and homozygous insertion (In/In)), days on trial, and their interactions were determined on dependent variables. When F-tests were significant ($P < 0.05$) means were separated using multiple *t*-tests, while *P*-values between 0.1 and 0.05 were defined as a tendency.

RESULTS AND DISCUSSION

Distribution of heifer LDHB genotype was the same ($P = 0.98$) across the two tall fescue cultivars (Table 1). Heifer weight increased ($P < 0.001$) with days on trial (207, 236, 260, 277 ± 5 kg, respectively, for d 0, 28, 56, and 84). In addition, weight was affected by an interaction ($P < 0.01$) between LDHB genotype and cultivar (Fig. 1). Heifers with heterozygous (Del/In) genotypes grazing HiMag were heavier than all other heifer groups.

Both LDHf activity (pyruvate to lactate) and LDHr activity (lactate to pyruvate) fluctuated ($P < 0.001$) over time, and plateaued at days 56 and 84 (Fig. 2). Heifer LDHB genotype tended ($P = 0.1$) to affect LDHf activity (12.2 vs. 11.7 IU/mg ± 0.29, respectively, for Del/In and In/In). In addition, LDHr activity was affected ($P < 0.05$) by LDHB genotype. Heifers that were In/In had lower ($P < 0.05$) plasma LDHr activity than heifers that were heterozygous (3.0 vs. 3.2 IU/mg, respectively). Heifer plasma lactate concentrations increased ($P < 0.01$) with days on trial (Table 2), and were affected ($P < 0.05$) by an interaction between LDHB genotype and cultivar (Fig. 2).

Concentration of red blood cells (RBC), whole blood hemoglobin content, hematocrit percent, and concentration of platelets were affected ($P < 0.05$) by days on trial (Table 2). Red blood cell concentrations were affected ($P < 0.01$) by an interaction between LDHB genotype and tall fescue cultivar; however, based on diagnostic lab values, all heifers had RBC concentrations within "normal" range (Fig. 3). Heifers grazing KY31 had higher ($P < 0.05$) whole blood hemoglobin concentrations than heifers grazing HiMag (12.4 vs. 11.3 g/dL ± 0.2), and In/In heifers had higher ($P < 0.05$) whole blood hemoglobin concentrations than Del/In heifers. Hematocrit percentages were larger ($P < 0.05$) in heifers grazing KY31 when compared with heifers grazing HiMag pastures (35.4% vs. 32.2%).

White blood cell (lymphocytes, neutrophils, basophils, eosinophils, and monocytes) concentrations were affected by the number of days on trial (Table 2). An interaction between tall fescue cultivar and LDHB genotype affected ($P < 0.01$) white blood cell concentrations (Fig. 4). Lymphocyte concentrations were affected ($P < 0.01$) by an interaction between tall fescue cultivar and LDHB genotype (Fig. 5). The concentration of monocytes was affected by LDHB genotype; specifically, In/In heifers had a larger ($P < 0.01$) concentration of monocytes than Del/In heifers (1.0 vs. 0.85 ± 0.06 × 10^4/μL). The In/In heifers had more ($P < 0.05$) basophils than Del/In heifers (0.15 vs. 0.12 ± 0.01 × 10^4/μL). Percentage of basophils also was affected ($P < 0.05$) by an interaction between tall fescue cultivar and days on trial (Fig. 6).

Heifers grazing HiMag that were heterozygous had the greatest weight gain. Lactate concentrations appeared to be inversely related to weight gain, with In/In heifers grazing HiMag having the highest lactate concentrations and heterozygous heifers grazing HiMag the lowest lactate concentration. This supports other findings related to high blood lactate concentrations, such as decreased reproductive performance and profitability in beef cattle (Looper et al., 2008). An increase in lactate concentrations in cattle has been associated with nutritionally induced muscular dystrophy (Kursa, 1975). These findings suggest that the more stress to which the cow is subjected requires the cow to use more energy.

Erythrocyte and hemoglobin concentrations were affected by LDHB genotype and tall fescue cultivar. All blood cell and component concentrations were within the accepted "normal" range. Higher RBC and hemoglobin concentrations for homozygous heifers grazing KY31 are consistent with results from other studies. A three-year study was conducted to determine the long-term effects of grazing toxic tall fescue on steers—one result of which was a higher RBC concentration in steers grazing toxic fescue (Oliver et al., 2000). An interaction between genotype and cultivar was noted on RBC concentration in this study, but the reason for the interaction is still unclear and warrants further study. A similar interaction was found between genotype and cultivar on white blood cell concentrations, but there were no overarching tendencies found.

Cow health and reproduction are acutely related to dairy herd profitability. Based on blood cell differential counts, genotyping for LDHB may be related to dairy cow health; although, that linkage will need additional testing for definitive relationships. Stockpiling tall fescue resulted in acceptable dairy heifer gain and immune function based on white blood cell distributions. However, many questions remain pertaining to the use of stockpiled toxic tall fescue as a management tool for developing dairy heifers. Specifically, whether or not ergot alkaloids would be stored in the fat of the heifer only to be released later, causing health issues for her, or deposited in her milk and result in a human health issue?

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Table 1. Distribution of heifers by LDHB genotype on tall fescue cultivars.

LDHB Genotype	HiMag (n)	KY31 (n)
Heterozygous (DI)	6	7
Homozygous insertion (II)	7	7

Table 2. Effects of trial days on heifer weights and blood characteristics.

	Day of Trial				Pooled
Trait[a]	0	28	56	84	SE
Body wt, kg	212 d[b]	241 c	265 b	283 a	4.9
LDHf, IU/mg	11.3 b	10.8 b	13.2 a	12.5 a	0.3
LDHr, IU/mg	2.8 b	2.8 b	3.5 a	3.4 a	0.1
Lactate, mM	2.6 b	3.3 a	3.4 a	3.4 a	0.2
Protein, mg/ml	83 a	87 a	69 b	70 b	1.8
RBC, # x $10^6/\mu$L	8.0 c	8.4 bc	8.7 ab	9.1 a	0.21
Hemoglobin, g/dL	10.5 d	11.8 c	12.3 b	12.7 a	0.2
Hematocrit, %	30.3 c	32.6 b	34.6 a	36.2 a	0.77
Platelets, k/uL	561 a	513 a	396 b	512 a	34
WBC, # x $10^4/\mu$L	8.9 b	11.0 a	11.3 a	8.3 b	0.49
Lymphocytes,# x $10^4/\mu$L	5.9 c	7.4 b	9.6 a	4.7 d	0.5
Neutrophils, # x $10^4/\mu$L	1.6 c	2.0 b	0.5 d	2.6 a	0.18
Eosninophils, # x $10^4/\mu$L	0.04 c	0.48 a	0.26 b	0.17 bc	0.06
Monocytes, # x $10^4/\mu$L	1.3 a	1.0 b	0.8 c	1.0 bc	0.07

[a]Items were determined on the stated trial days. Heifer (n = 27) body weight, white blood cell (WBC) and red blood cell (RBC) counts in whole blood. Serum concentrations of lactate, protein, and lactate dehydrogenase (LDH) activites are presented. The LDH activities forward (LDHf) and reverse (LDHr) were corrected for serum protein concentration.
[b]Means in a row followed by the same letter are not significantly different at $P < 0.05$.

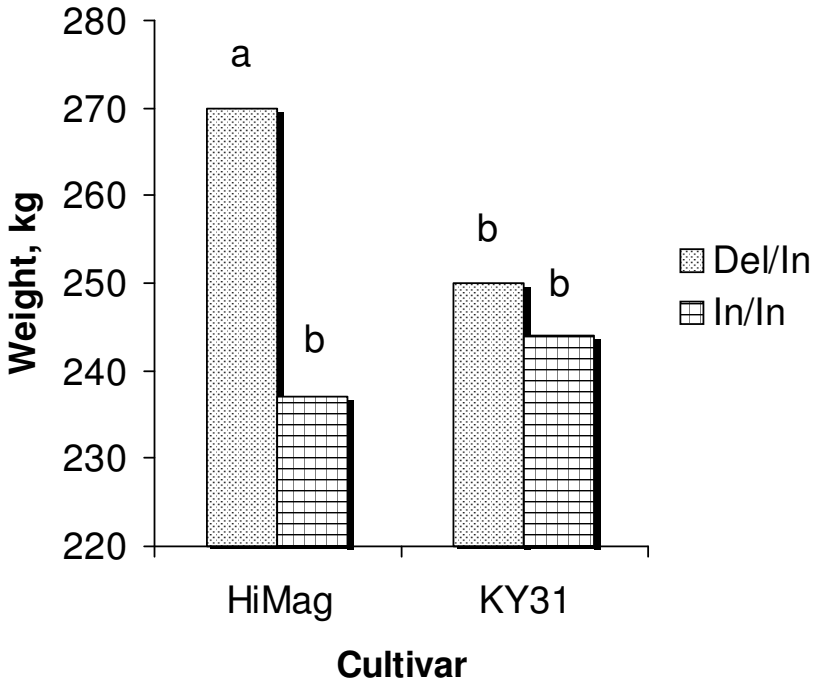

Fig. 1. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on heifer weight. Values in columns without a common superscript letter differ ($P < 0.01$).

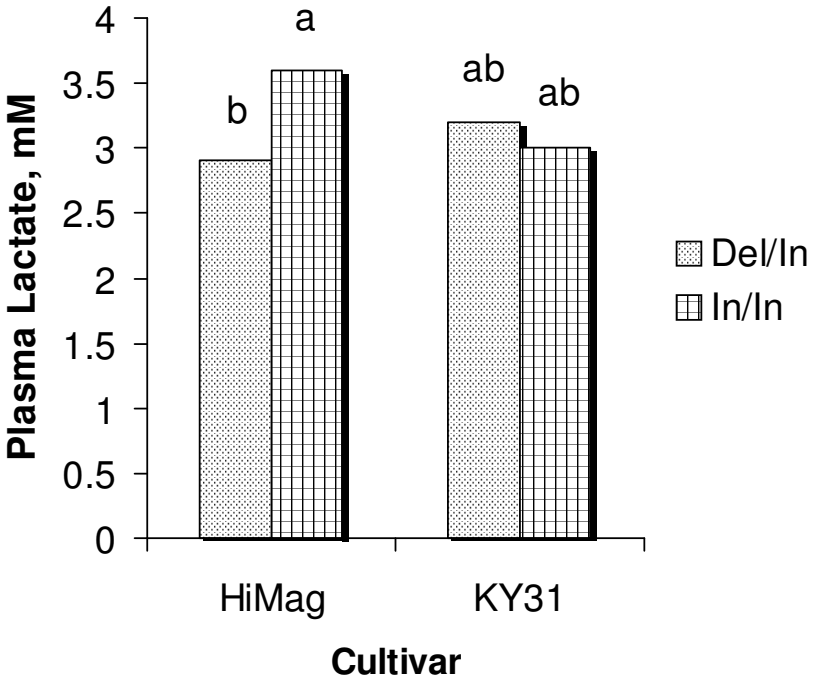

Fig. 2. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on plasma lactate concentrations. Values in columns without a common superscript letter differ ($P < 0.01$).

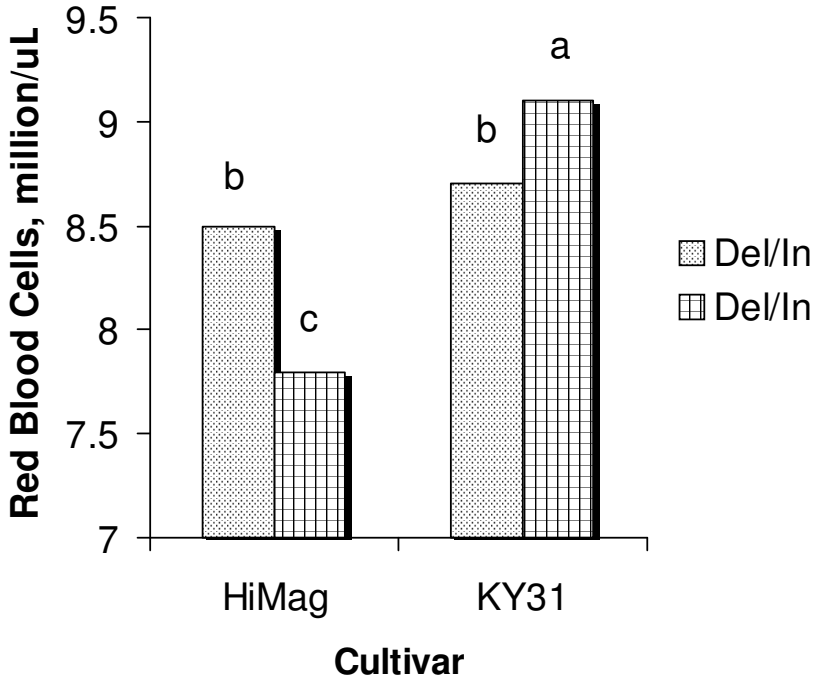

Fig. 3. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on circulating red blood cell concentrations. Values in columns without a common superscript letter differ ($P < 0.01$).

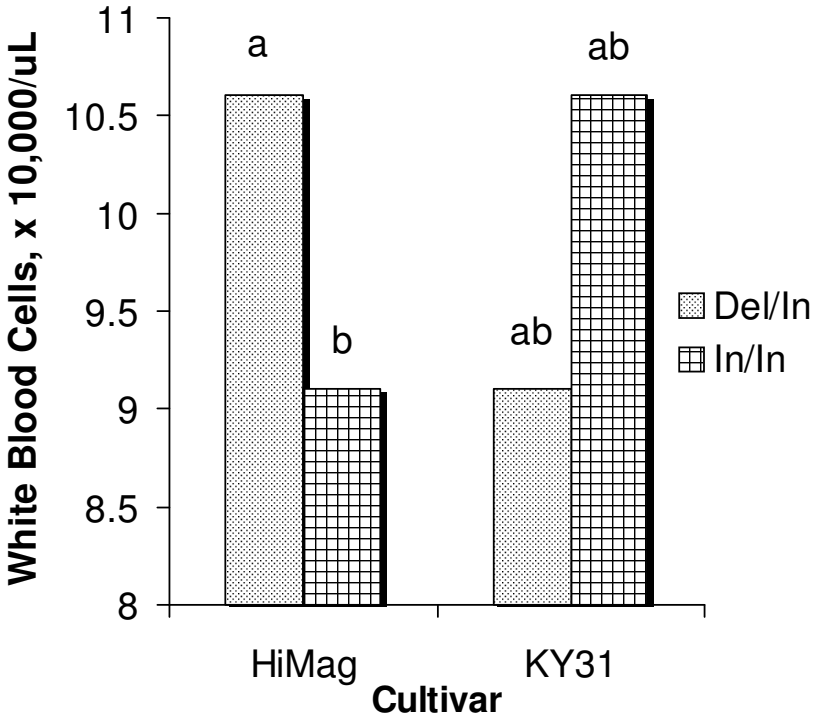

Fig. 4. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on white blood cell concentrations. Values in columns without a common superscript letter differ ($P < 0.01$).

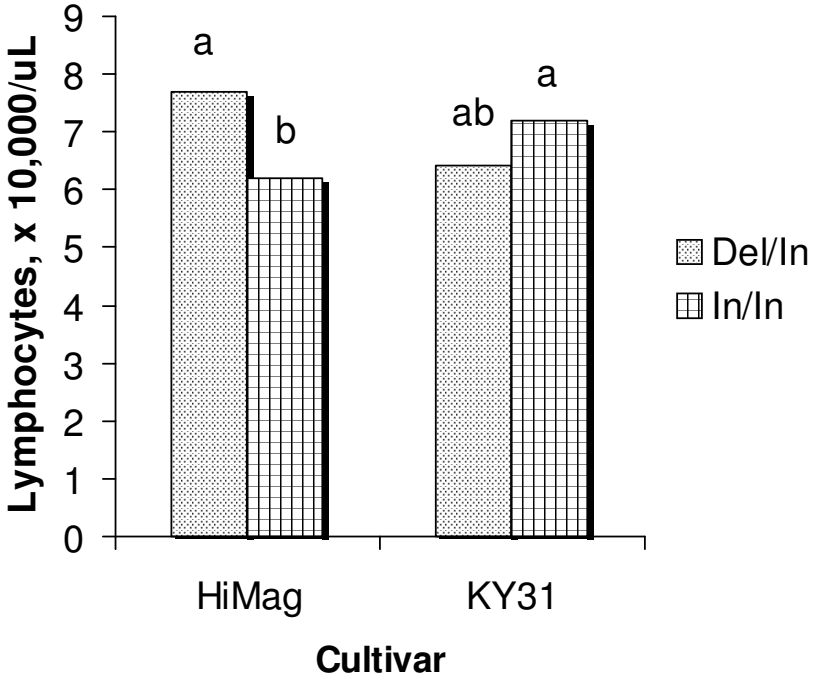

Fig. 5. Interactive effects of LDHB genotype (Del/In or In/In) and tall fescue cultivar (KY31 or HiMag) on lymphocyte percentages. Values in columns without a common superscript letter differ ($P < 0.01$).

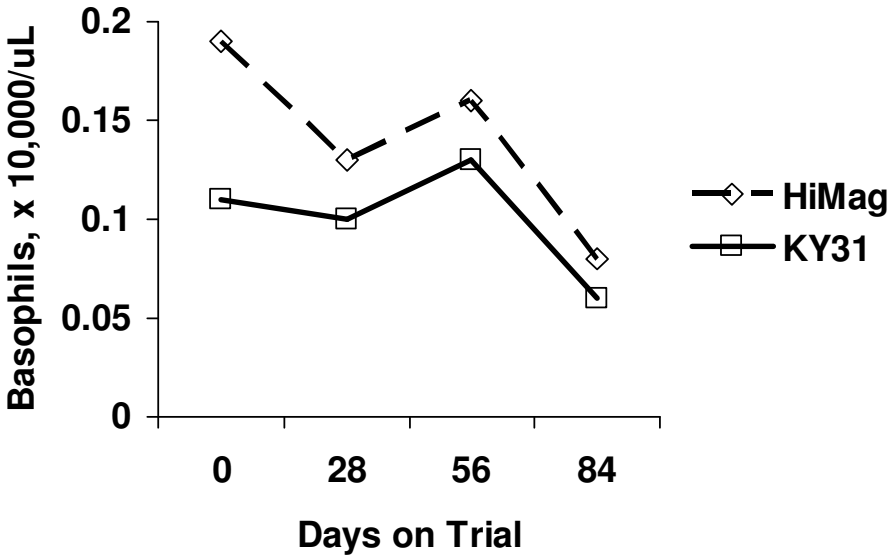

Fig. 6. Effects of time and tall fescue cultivar (KY31 or HiMag) on the percentage of basophils.

Molecular and phenotypic comparisons of salt effects on soybean cultivars with differential chloride uptake capacities

Sharon Faye Holifield[*], *Fernando Ledesma Rodriguez*[†], *Richard D. Cartwright*[§], *Pengyin Chen*[‡], *and Kenneth L. Korth*[**]

ABSTRACT

Genetic manipulation of crop plants, through breeding or transgenic approaches, for enhanced tolerance to abiotic stress holds great promise for improving yields and promoting new methods for sustainable agriculture. This study examines the potential role that genes of the soybean, *Glycine max* L., encoding elongation factor-1 alpha (*EF-1*α) and glyoxalase I (*GlxI*) might play in response to salt stress. Previous reports have suggested a possible function for both *GlxI* and *EF-1*α in conferring enhanced salt tolerance in other plant species. In addition to other possible mechanisms, salt tolerance in soybeans can be regulated by plant uptake and transport of chloride ions. Soybean lines that transfer chloride to their foliage from the soil are termed "includers" and are considered to be more susceptible to salt stress than their counterparts, "excluders" that do not transport chloride into their leaves. We used chloride "includers", cv. Clark and Dare, and "excluders", cv. Lee68 and S100, to compare gene expression responses and plant susceptibility to chloride salts. Mineral analysis of Clark and Lee68 cultivars by inductively coupled plasma mass spectrometry was performed to verify the differences in chloride uptake. In an optimized greenhouse screening procedure, the excluder cv. Lee68 demonstrated fewer visual symptoms of salt stress when treated with the same salt concentrations as the includer, cv. Clark. RNA blots showed the soybean genes encoding *EF-1*α and I *GlxI* were equally induced in both includers and excluders following treatment with NaCl or $CaCl_2$. Although transcript levels for *EF-1*α and *GlxI* are induced by salt treatments, transcript profiles do not differ between salt-tolerant and susceptible soybean cultivars. This suggests that the cultivars respond to salt stress in similar ways, but that these genes are not responsible for the differential phenotypes.

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MEET THE STUDENT-AUTHOR

Sharon Faye Holifield

After I graduated from Mountain Home High School in 2007, I began my studies in Environmental, Soil, and Water Science at the University of Arkansas.

In my sophomore year, I took Biotechnology in Agriculture, taught by Dr. Ken Korth and immediately became interested in the work in his laboratory. Since working in Dr. Korth's lab, I have received awards from both the American Society of Plant Biologists and the Student Undergraduate Research Fellowship. I am also a member of the Arkansas Native Plant Society and the university's Crop, Soil, and Environmental Sciences Club.

I have enjoyed studying new techniques that improve the way that we use and take care of our environment. I will begin working on my master's degree in crop, soil and environmental sciences at the University of Arkansas in the summer of 2011.

INTRODUCTION

Salinity is an ever-present threat to crop yields, especially in countries where irrigation is an essential aid to agriculture. As global population increases, farmers will be forced to make use of marginal lands not suitable for today's food crops. The increase in land use for food production will also have a major impact on the environment and wildlife. Genetic manipulation of crop plants, whether through breeding or transgenic approaches, for enhanced abiotic stress tolerance holds great promise for improving yields in marginal lands and promoting new methods for sustainable agriculture.

Soybean, *Glycine max* L., is a legume that is an important staple crop grown worldwide, and susceptibility to abiotic stresses can be a factor in limiting overall production. One approach to generating plant lines that are more resistant to environmental stresses depends on understanding the plant's natural means of tolerance to stressful conditions. There are several possible mechanisms of salt tolerance in soybeans, including metabolic pathways and gene products that respond to environmental stresses (Phang et al., 2008). Salt sensitivity in soybean is often correlated with accumulation of chloride in the foliar tissues. Prevention of chloride transport from soil via the roots to foliar tissues has been shown to play a major role in salt tolerance (Luo et al., 2005; Valencia et al., 2008). Cultivars that have been characterized previously as either chloride includers or chloride excluders, which differ in systemic transport of the chloride ion, can be useful tools in breeding programs and in the study of mechanisms of salt tolerance (Table 1). Chloride includers transport the chloride ion from saline waters into leaves where it accumulates and damages the foliar tissue. Excluders take up chloride in roots, but the ion is not transported to foliar tissues (Figs. 1 and 2).

It is likely that multiple independent stress response pathways, and the genes that control them, are involved in plant strategies to cope with salt stress. In addition to chloride toxicity as a major factor in soybean, salt sensitivity in the wild species *G. soja* can be due to sodium accumulation in foliar tissues (Luo et al., 2005).

One approach to identifying genes that are employed by the plant to combat abiotic stresses is to seek out genes expressed at higher levels during a controlled stress treatment. In an attempt to identify such stress-induced genes, we searched an online soybean expressed sequence tag (EST) database for potential salt-induced genes and found ESTs encoding a putative *G. max* elongation factor-1 alpha (*GmElf1-*α) protein and a gene encoding a putative glyoxalase I (*GmGlxI*). Elongation factor-1 alpha (*EF-1*α), an essential component of the eukaryotic translational apparatus, is a GTP-binding protein that catalyses the binding of aminoacyl-transfer RNAs to the ribosome. It also may protect cellular proteins under salt-stress conditions in plants and yeasts by promoting the correct refolding of unfolded proteins *in vivo* (Shin et al., 2009). The glyoxalase system is comprised of two enzymes, glyoxalase I and II, with reduced glutathione as a catalyst (Fig. 3). Methylglyoxal, the primary physiological substrate for the glyoxalase-I reaction, is a potent cytotoxic compound that is produced as a byproduct of glycolysis, and can also be de-

rived from fatty acid metabolism or threonine degradation. Transgenic approaches with *GmEf-1α* and *GlxI* have been used previously to show that overexpression of these gene products can confer enhanced salt tolerance in *Arabidopsis* and tobacco plants, respectively (Shin et al., 2009; Singla-Pareek et al., 2003).

To gain a better understanding of the potential role of these genes in salt tolerance, one objective of these studies was to test the responsiveness of *GmEf-1α* and *GlxI* gene expression following salt treatments in soybean. We measured mRNA accumulation for each gene in cultivars characterized as either chloride includers or excluders. A second objective was to determine the potential role for salt components other than chloride for their impact on salt damage in soybean. To that end, we assessed phenotypic responses to salt treatments in four cultivars and assayed mineral accumulation in foliar tissues.

MATERIALS AND METHODS

Plant Materials and Growth Conditions. Soybean plants were grown in a greenhouse at the Rosen Center for Alternative Pest Control at the University of Arkansas, Fayetteville. The greenhouse was kept between 20 °C and 25 °C. Soybeans were grown under a 14-hour light and 10-hour dark cycle. Soybeans were germinated in a small-celled seed tray and then chosen for uniformity after emergence, five days after planting. The soybeans were then transplanted to 4-inch square pots, two plants per pot, and filled with sandy loam soil. Soybeans were grown without salt treatment for three weeks, and the initial salt treatment occurred after emergence of the first trifoliate leaf. The pots were placed in a shallow basin and flooded daily from below with one of four solutions: water, 80 mM NaCl, 40 mM $CaCl_2$,or 120mM NaCl. Soybeans in all groups were treated with Miracle Gro fertilizer every three days as per the manufacturer's instructions. For phenotypic scoring and mineral analysis, the plants were treated with one of three solutions: water, 80 mM NaCl or 40 mM $CaCl_2$; thus, equivalent amounts of chloride were provided in each salt treatment. For transcript measurements and glyoxalase assays, plants were treated with either water or 120 mM NaCl. The pots with soybean plants were flooded daily for one hour with water (control) or salt solution at around noon. After eight days of treatment, the soybeans were visually scored, foliar chlorophyll levels measured, and foliar tissue was collected for further analysis.

Plant Screening. Visual symptoms were recorded based on a scale of 1-6. The scale was defined as follows: 1 for healthy plant with no chlorosis, 2 for 25% of leaf chlorosis, 3 for 50% of leaf chlorosis, 4 for 75% of leaf chlorosis, 5 for 100% chlorosis, and 6 for complete leaf necrosis and plant death (Tamura and Chen, 2009).

Foliar chlorophyll levels were measured using a Minolta SPAD Chlorophyll Meter. The SPAD readings were taken from the 2nd trifoliate of each plant. Three readings were taken from the terminal leaflet, avoiding areas of necrosis, and then averaged to get the reading for the plant. Plants with a visual rating of >5 could not be accurately measured with the SPAD meter.

Mineral Analysis. Total Ca^{++}, Na^{+}, and other minerals were measured by digesting dried and ground leaf tissue that passed through a 1.18-mm mesh sieve in concentrated nitric acid and hydrogen peroxide followed by Inductively Coupled Plasma–Mass Spectrometry (ICP-MS) analysis (Plank, 1992). All minerals except for chloride were analyzed using a Spectro Arcos ICP (Inductively Coupled Plasma Spectrophotometer, SPECTRO Analytical Instruments, Mahway, N.J. 07430). Chloride was extracted with deionized water and content of the leaves was measured using a digital chloridometer.

Glyoxolase Enzyme Activity. Extraction and analysis of glyoxalase I activity was performed according to the procedure of Ramaswamy et al. (1983). Terminal leaflets of soybean leaves at the V3 stage, from 1-month-old plants, were collected and immediately placed on ice. Tissue was homogenized in 2.0 ml of extraction solution at 4 °C. The extract was collected in 2-ml microcentrifuge tubes and the tissue was pelleted by centrifugation at 10,000 × g for 20 min. Supernatant(20 µl) was added to a quartz cuvette containing 1.0 ml of freshly prepared enzyme assay buffer and formation of thioester product was quantified by monitoring absorbance at 240 nm. This measured the activity of glyoxolase I enzyme in the tissue that could catalyze the formation of S-D-lactoylglutathione from methylglyoxal and reduced glutathione at 25 °C. Total protein concentration of extracts was determined with a BioRad Protein Assay kit (Bio-Rad Laboratories, Hercules, Calif.).

Gene Expression. Expressed sequence tags (EST) for *GmEf-1α* and *GlxI* were identified from the Dana Farber Cancer Institute *Glycine max* Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=soybean) and these sequences were used for oligonucleotide primer design. The sequence used for *GlxI* primer design is based on a cDNA that has been confirmed as encoding an active *GlxI* enzyme (Skipsey et al., 2000).

The primers used for amplification of *GmEf-1α* (Genbank accession AK246053) were:

EF-1αF 5'-TCTGTTTCTCCCTCACTCTGATCCAC-3' and

EF-1αR 5'-ACTCCACATACGAGCAAAAGACCCA-3'.

The primers used for amplification of GlxI (Genbank accession AJ010423) were:

GlxIF 5'-TCTGTTTCTCCCTCACTCTGATCCAC-3' and

GlxIR 5'- ACTCCACATACGAGCAAAAGACCCA-3'.

Gene expression was measured by RNA gel blots. Probes were prepared from cDNA via reverse-transcription polymerase chain reaction. RNA was extracted from the plant tissue using TriReagent (Molecular Research Center, Inc., Cincinnati, Ohio) according to manufacturer's instructions, and separated on denaturing formaldehyde gels (Sambrook et al., 1989). Flowers did not develop on Lee68 so we were unable to collect RNA for this tissue. Following transfer to nylon membranes, RNA was hybridized with ^{32}P-labeled probes as indicated (Figs. 7 and 8). Hybridization conditions were according to Church and Gilbert (1984) and signal was measured with a phosphorimager. Tissues assayed were the roots, shoots, leaves and flowers.

Data Analysis. Data for visual foliar plant health, glyoxolase I activity, SPAD chlorophyll levels, and mineral concentrations were subjected to an analysis of variance. Honest significant difference (Tukey) was used to compare means between each cultivar and each salt treatment, separately ($P = 0.05$). All statistical analysis was done using JMP version 9.0.0 (SAS Institute, Cary, N.C.) and graphs were constructed using GraphPad version 5.03 (GraphPad Software, Inc., La Jolla, Calif.) showing the statistical mean of each group and the standard error of the mean.

RESULTS AND DISCUSSION

Soybean Foliar Health. To confirm the phenotypic response of differential cultivars of soybean, plants were treated with salt solutions, and a visual scoring system (Tamura and Chen, 2009) was used to assess salt damage. Statistical analysis of the visual scoring showed a significantly greater reduction in plant foliar health in the Clark cultivar compared to Lee 68 when subjected to an 8-day treatment of 40 mM $CaCl_2$ or 80 mM NaCl (Fig. 4). These salt concentrations were chosen based on levels shown to be effective in previous studies (e.g., Valencia et al., 2008) and so that equivalent levels of chloride were applied in each case. Treatment with NaCl or $CaCl_2$ had severe effects on cv. Clark, whereas $CaCl_2$ treatment caused more damage than NaCl to the excluder Lee68. Although NaCl treatment did not lead to significantly different foliar ratings in Lee68 as compared with water-treated controls, the plants treated with salt solutions were visibly smaller (data not shown).

In addition to visual scoring, we measured chlorophyll content as a potential means to assess salt damage. There was a significantly lower level of chlorophyll in salt-treated Clark plants as compared to water-treated controls (Fig. 5). In addition, chlorophyll levels in Clark were lower than in Lee 68 following NaCl treatments. As in the visual scoring results, treatment with $CaCl_2$ had a slightly, but not significantly, greater negative impact on Lee68 than did NaCl, as compared to water-treated controls. These measurements were taken with a handheld Minolta SPAD meter (Konica Minolta Sensing, Inc., Osaka, Japan). This instrument is very simple to use and provides a rapid and non-destructive reading of chlorophyll content, taking just seconds to gather a single reading. Given that the trend of these data agree well with the visual ratings (Fig. 4), the measurement of chlorophyll with a SPAD meter could potentially provide an easy alternative method for screening soybean plants for salt tolerance. Decreased chlorophyll levels in response to salt stress have been shown to occur in other plant systems (Robinson et al., 1983). This method provides a quantitative output and avoids human bias that might impact visual ratings.

Mineral Accumulation in Salt-treated Plants. Measurement of leaf chloride levels confirms the differential uptake by the cvs. Clark (includer) and Lee 68 (excluder). Regardless of whether chloride was supplied as the sodium or calcium salt, Clark accumulated substantially higher levels of the chloride than did Lee 68 (Fig. 6a). In the includer Clark, chloride accumulated to higher levels when supplied to the plant as NaCl rather than $CaCl_2$. Not surprisingly, plants treated with $CaCl_2$ accumulated higher levels of Ca^{++}; however, we also noted that Ca^{++} accumulation was higher in cv. Clark, a chloride includer, than in the excluder Lee 68 (Fig. 6c). The same trend, i.e., higher sodium accumulation in the chloride includer, was observed in NaCl treatments (Fig. 6b). Taken together, the data suggest a possible parallel uptake of the cations with chloride when applied in high-salt conditions.

Transcript Analysis In Salt-Treated Leaves And Soybean Tissues. Transcript levels for genes encoding both the glyoxalase I and elongation factor-1 alpha (Fig. 7) were induced by NaCl treatment. This stress-induced molecular response appears to have been equivalent in both includer and excluder cultivars, suggesting that both types of soybean respond to salt stress with activation of defense mechanisms. Both includers and excluders accumulate higher levels of Na^+ and Cl^- after salt treatments, and even though excluder lines to not exhibit severe phenotypic damage, based on transcript induction they are also activating molecular defense responses. In addition, since both lines showed no difference in *GmEf1a* and *GlxI* expression in response to salt stress, it is unlikely that the different salt-tolerance phenotypes are due to the expression of either of these two genes.

Accumulation of transcripts encoding *GlxI* in plants has been shown previously to increase in response to salt treatments. In tomato, *GlxI* transcripts increased after 72 h of salt treatment (Espartero et al., 1995), and the orthologous gene was induced in wheat following infection with the fungal pathogen *Fusarium graminearum* or treatment with either $ZnCl_2$ or NaCl (Lin et al., 2010). These observations, along with reports of transgenic overexpression of *GlxI* in plants conferring enhanced salt tolerance (Singla-Parek et al., 2006; Veena et al., 1999), strongly suggest that this gene plays an important role in helping plants cope with salt stress. This

role of *GlxI* in salt tolerance might be widespread among organisms, as even the yeast *S. cerevisiae* responds to salt treatments and osmotic stress with enhanced *GlxI* transcripts and enzyme activity (Inoue et al., 1998). Induced transcription of gene family members of *GmEf-1α* has not been widely reported. Transcripts encoding *GmEf-1α* and the protein itself have been shown to accumulate in response to wounding in potato (Morelli et al., 1994), and expression levels of the gene in transgenic *Arabidopsis* correlate with salt tolerance (Shin et al., 2009). Therefore, this gene also likely plays a conserved role in stress responses in plants.

Transcripts for *GmEF-1α* are expressed throughout the plant but in different levels in various tissues (Fig. 8). Young leaves have the highest transcript levels, consistent with a role for *EF-1α* in growth and cell division. The mature leaves had the lowest transcript levels among tissues tested. Overall, there were no differences in transcript levels between the cultivars, suggesting that constitutive expression of *EF-1α* is not the cause of the differing tolerances to saline environments.

Glyoxalase Enzyme Assay. A glyoxalase-I enzyme assay of Lee68 and Clark under salt-stressed and control treatments was completed in order to further evaluate the relationship of the transcript level production, protein activity, and the phenotypic salt-stress tolerance. The enzyme analysis showed that although an increase may be detected on a transcript level, the *GlxI* activity in salt-treated soybeans was not significantly affected (Fig. 9).

This is in contrast with up-regulation of *GlxI* specific activity with a 20-fold increase noted in tomato plants that also correlated with an up-regulation of *GlxI* transcripts (Espartero et al., 1995). In contrast, tobacco cells stressed by the addition of NaCl demonstrated a decrease in the total amount of *GlxI* protein (Hoque et al., 2008). These contrasting studies suggest that the different species of plants (e.g., soybean, tomato, and tobacco) combat the negative effects of salt stress differently in relation to the glyoxolase pathway. The differences in change between the transcript level and the amount of protein activity in the soybean varieties could be due to post-transcriptional regulation within the plant cell.

CONCLUSIONS

We have further optimized a rapid and effective screening method for salt tolerance in soybean. Although transcript levels for *EF-1α* and *GlxI* are induced by salt treatments, transcript profiles do not differ between salt-tolerant and susceptible soybean cultivars. This suggests that the cultivars respond to salt stress in similar ways, but that these genes are not responsible for the differential phenotypes. As expected, the chloride-includer cultivar accumulated higher levels of chloride. Under the respective high-salt conditions, the chloride-includer also transported more Ca^{++} and Na^{+}, suggesting a possible parallel mechanism for transport of these elements with chloride.

ACKNOWLEDGEMENTS

We thank Lacy D. Nelson, Fariba Rafiei Boroujeni, Wayra Navia-Giné and Nancy Wolf for technical assistance. Sharon F. Holifield was generously supported by a Summer Undergraduate Research Fellowship from the American Society of Plant Biologists, and by a SURF grant from the Arkansas Department of Higher Education. Additional research support was provided by the Arkansas Soybean Promotion Board and the NSF-EPSCoR-funded P3 Center.

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Table 1. Cultivars of soybean (*Glycine max*) with known sensitivites to salt and chloride-uptake capacities used in study.

Cultivar	Reaction to Salt	Classification
Lee 68	Tolerant	Cl-excluder
S-100	Tolerant	Cl-excluder
Dare	Sensitive	Cl-includer
Clark	Sensitive	Cl-includer

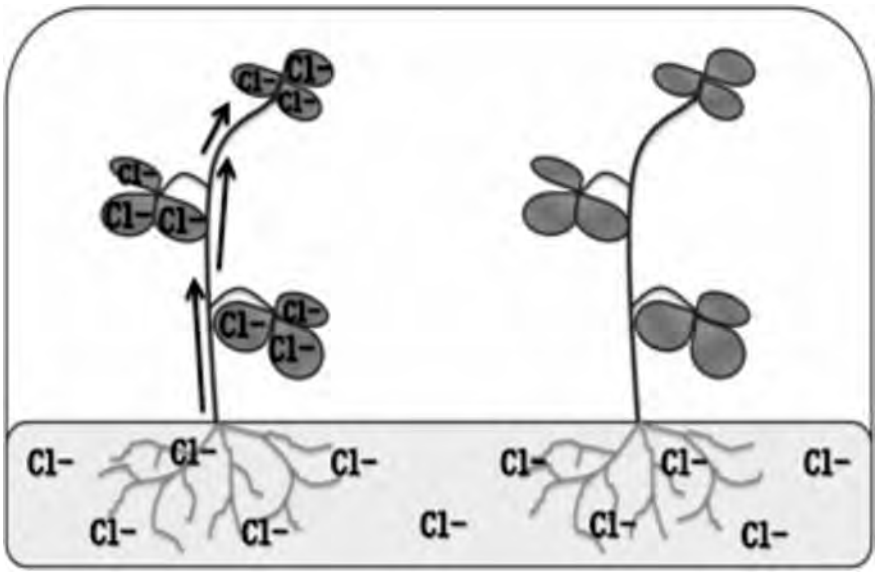

Fig. 1. The differential transport of chloride to foliar tissues in soybean by includers (left) and excluders (right).

Fig. 2. Soybean chloride includers exhibited severe damage after salt treatments. Soybeans were started from seed and flooded daily with 120 mM NaCl for eight days after emergence of the first trifoliate. An includer cultivar, Dare (left), and an excluder, Lee 68 (right) showed dramatically different phenotypes following 8 days of treatment with 120 mM NaCl.

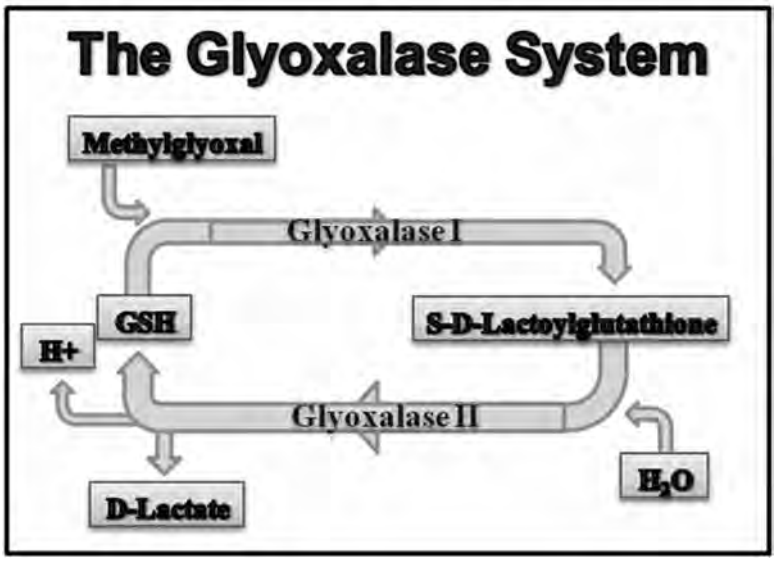

Fig. 3. The glyoxalase pathway is a ubiquitous detoxification pathway in prokaryotes and eukaryotes. Diagram of the glyoxalase system which includes glyoxalase I (*GlxI*) and glyoxalase II. *GlxI* converts methylglyoxal and reduced glutathione (GSH) to the product S-D-lactoylglutathione; *GlxII* forms D-lactate and glutathione.

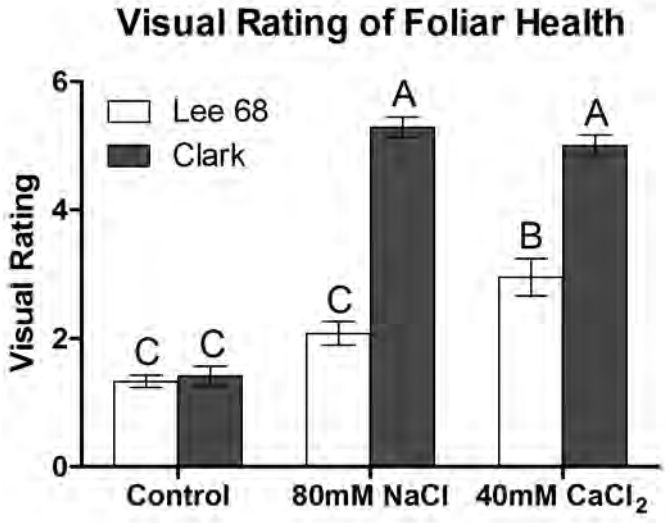

Fig. 4. Visual scoring of salt effects confirms tolerance of chloride excluders. After 8 days of salt treatments with 80 mM NaCl or 40 mM $CaCl_2$, soybean foliar health was rated on a scale of 1 (healthy plant with no chlorosis or necrosis) to 6 (complete necrosis). Similar letters indicate no significant difference. Error bars indicate standard error of the mean.

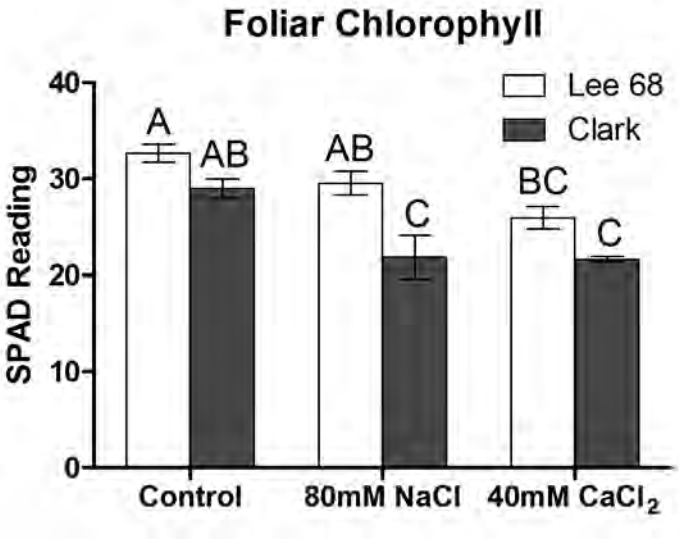

Fig. 5. Salt treatments lower chlorophyll levels in a chloride includer. Chlorophyll levels were determined with a Minolta SPAD 502 chlorophyll meter after 8 days of salt treatment. Similar letters indicate no significant difference. Error bars indicate standard error of the mean.

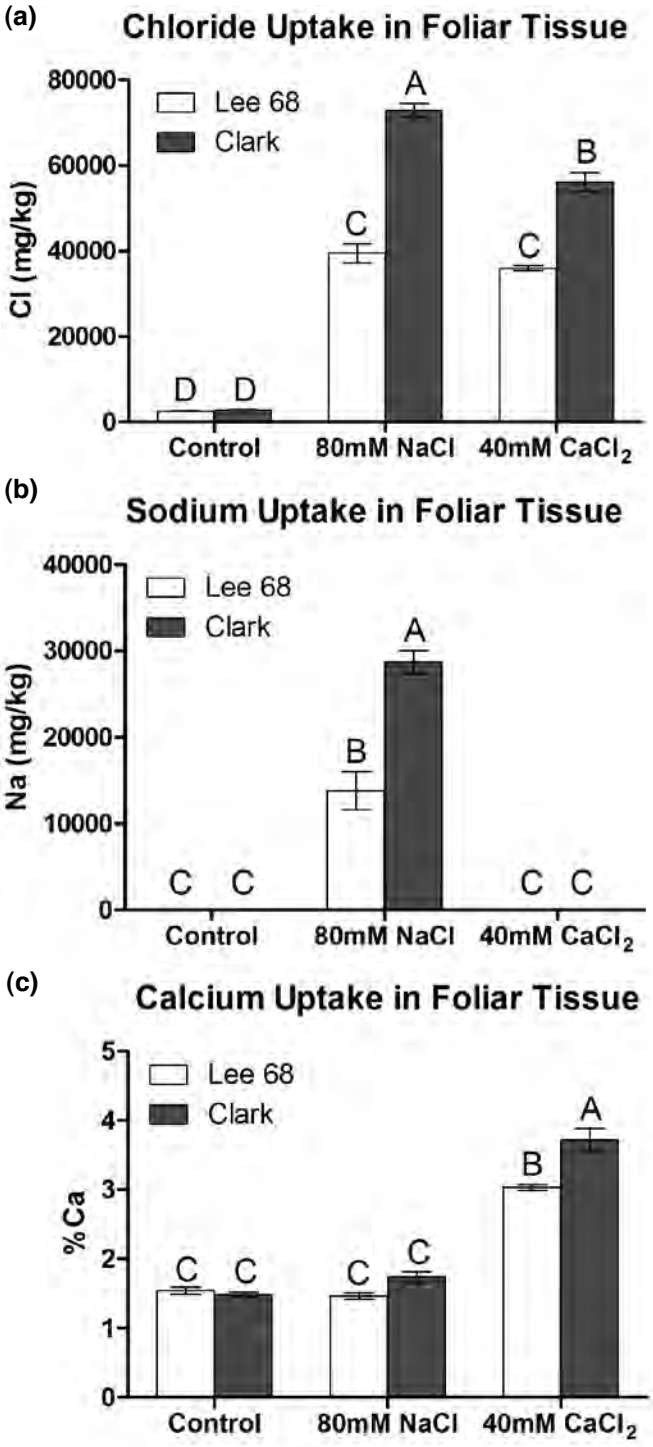

Fig. 6. Mineral levels differ in salt-treated Clark (includer) or Lee68 (excluder) cultivars. Soybeans were treated with 80 mM NaCl or 40 mM $CaCl_2$ for 8 days and then mineral concentrations were determined after foliar tissue was ground and analyzed. a) chloride uptake was greater in Clark than in Lee 68, verifying the includer and excluder classifications; b) sodium accumulated to significantly greater quantities in Clark than in Lee 68; c) calcium levels are highest in the $CaCl_2$-treated chloride includer.

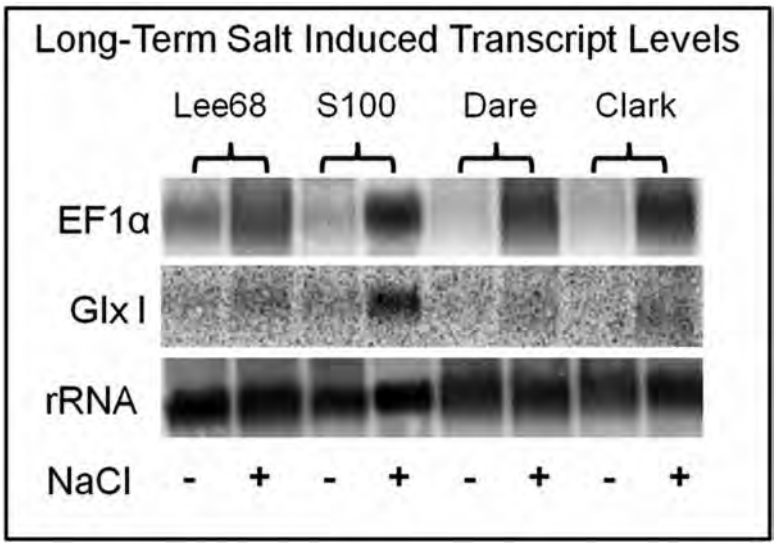

Fig. 7. Salt treatment increases transcript levels of both elongation factor-1 alpha (*EF-1*α) and glyoxalase I (*Glxl*) in both includers (Dare and Clark) and excluders (Lee68 and S100). The soybeans were treated with either water (-) or 80 mM NaCl (+) for 20 days before tissues were analyzed, and RNA probes were applied as indicated. rRNA probes were used as a control for measuring transcript levels.

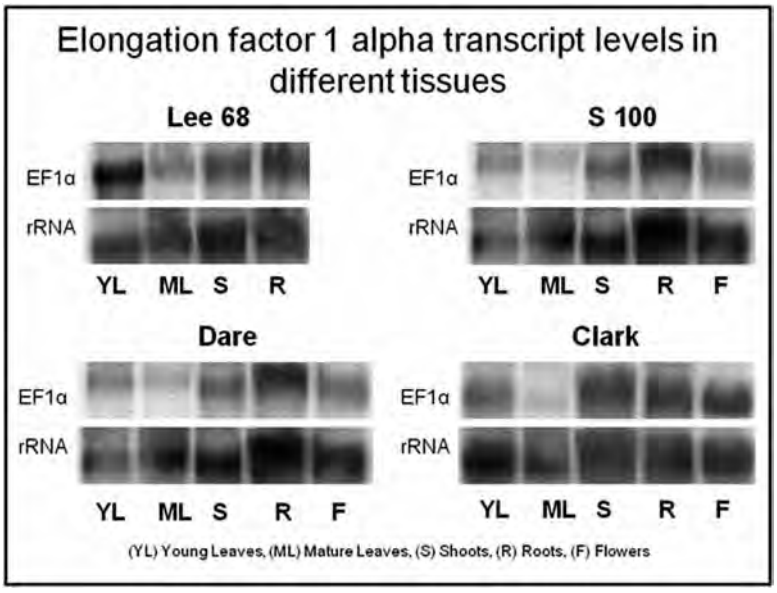

Fig. 8. Elongation factor 1 alpha (*EF-1*α) was expressed throughout the plant at different levels. Tissues tested were young leaves (YL,) mature leaves (ML,) stem (S,) roots (R,) and flowers (F). The transcript levels were lowest in the mature leaves in all soybean varieties. There were no noticeable differences in transcript levels between the excluders (Lee 68 and S 100) and the includers (Dare and Clark). Flowers did not form on Lee68 during the duration of this experiment. rRNA probes were used as a control for measuring transcript levels.

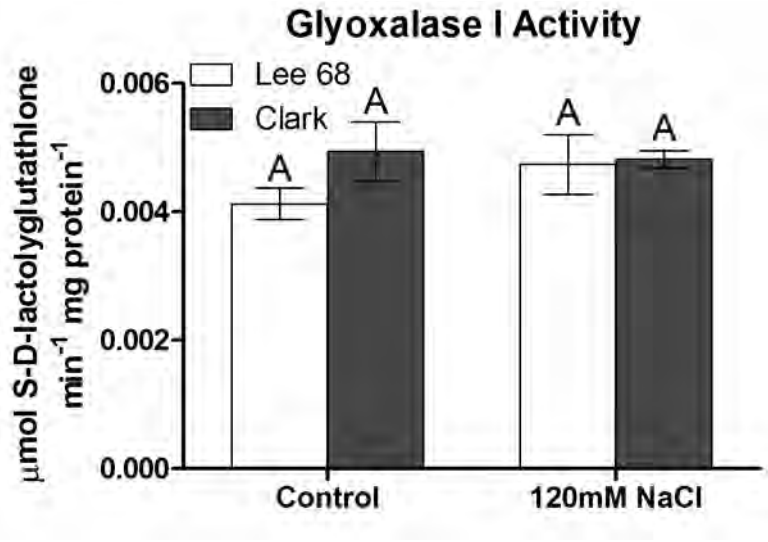

Fig. 9. Glyoxalase I activity is not affected by NaCl treatment. The amount of glyoxolase I enzyme activity in the tissue that could catalyze the formation of S-D-lactoylglutathione from methylglyoxal and reduced glutathione at 25 ºC was measured from tissue collected at day 8 of 120 mM NaCl treatment. Values were not significantly different. Error bars indicate standard error of the mean.

A novel gene silencing vector for plant genomics and biotechnology

Dustin K. Hoover[*]*, Scott J. Nicholson*[†]*, and Vibha Srivastava*[§]

ABSTRACT

Gene silencing is a process of suppressing activity of specific genes by producing "interfering" RNA encoded by foreign genes. This process serves as the principle of genetic modification in plants and animals, which is an important tool in genomics and biotechnology, allowing scientists to manipulate organisms to better meet human demands. New approaches of gene silencing may enable improvements on current practices of genetic modification, and broaden the application and impact of gene silencing in biotechnology. Recently, a novel vector design consisting of the transcription of short gene fragments lacking transcription termination signals was demonstrated to be effective in partial silencing of two separate genes in the model plant, *Arabidopsis thaliana*. To test the efficacy of this unterminated transgene technique on a broader range of genes in *A. thaliana*, a DNA vector to clone gene fragments was required. The objective of the present study was to design a silencing vector for rapid cloning of gene fragments and test its utility on new genes. Here, we report the successful construction of a simple transgene vector, pSJN15A, for cloning gene fragments, then plant transformation upon *Agrobacterium* infection. The pSJN15A vector was designed for direct cloning of gene fragments obtained by polymerase chain reaction. Transcription of gene fragments is directed by read-through activity of a hygromycin resistance gene promoter. The pSJN15A vector was used to develop silencing vectors against four new *Arabidopsis* genes. Thus, pSJN15A serves as an important DNA resource for testing the efficacy of silencing mediated by the transcription of gene fragments in various dicotyledonous plant species.

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§ Vibha Srivastava, the faculty mentor, is an associate professor in the Department of Crop, Soil and Environmental Sciences, and Department of Horticulture.

MEET THE STUDENT-AUTHOR

Dustin Hoover

I grew up in Conway, Ark., and graduated from Conway High School in 2007. That fall, I began to pursue a biology degree at the University of Arkansas, where I soon became a member of Alpha Epsilon Delta and Sigma Alpha Epsilon Fraternity. Over the duration of my attendance here, I have enjoyed attending football and baseball games and have developed an appreciation of the U of A student on-campus community.

With the tremendous help of Dr. Vibha Srivastava and Scott Nicholson (department of crop, soil and environmental sciences) I conducted an undergraduate honors research project funded by the University of Arkansas Honors College. I have also worked in Dr. Srivastava's lab as a technician. After earning my bachelor's degree, I will be attending medical school at the University of Arkansas for Medical Sciences. I would like to thank all my family and friends for their continued support.

INTRODUCTION

The field of biotechnology holds new importance for the growing human population in a global economy. Biotech applications will allow for the improvement of crops dedicated for food, feed, and bioenergy. One example of such applications is gene silencing. The cellular process of gene silencing has been used to develop molecular tools necessary to change the appearances and functions of organisms that humans depend upon for food, energy, and natural resources. By selectively eliminating or suppressing the expression of even one gene, scientists can produce favorable changes within an organism to better suit the diverse demands of societies around the world (Wang and Waterhouse, 2002). Gene silencing is also considered an upcoming platform for drug development and medical applications (Bernards, 2006). The research of this project investigates the possibility of partially silencing the expression of a gene, instead of completely abolishing expression. This new application would serve as a useful alternative tool to engineer new traits in plants for the future and current demands of food and energy.

Some of the first observations of successful gene silencing occurred when scientists introduced copies of a native gene sequence into petunias using a powerful promoter. What the scientists did not know at the time was that they were observing the effects of gene silencing, later termed as RNA interference (RNAi). The RNAi process begins with double-stranded RNA (dsRNA), which is processed into 21-24 nucleotide short interfering RNA duplexes (siRNA). These siRNA help guide a RNA-Induced Silencing Complex (RISC) to direct the degradation of homologous mRNA sequences (Baulcombe and Hamilton, 1999). Once these mRNA sequences are eliminated, the genetic information cannot be translated or expressed (Meister and Tuschl, 2004). Since the RNAi process occurs after the endogenous DNA has been transcribed, RNAi is also referred to as post transcriptional gene silencing (PTGS) (Vaucheret et al. 2001). Post transcriptional gene silencing via RNAi has previously been initiated through various methods. Studies have shown that injection of dsRNA in the form of sense and antisense RNA strands can lead to successful silencing in insects and nematodes (Fire et al., 1998). The most common silencing methods utilize a transgene coding for the expression of a "hair-pin loop" of DNA, a self-complementary strand that is processed into siRNA (Meister and Tuschl, 2004). These loop sequences arise after their sequences are incorporated into the organism's genome, along with a strong promoter sequence. The promoter ensures expression of the sequence and its consequential effects. The silencing demonstrated by hair-pin loops is long-term, as the vector continues to express the fold-back structure generating dsRNA (Fire et al., 1998). Expression of hairpin DNA structures (also called an inverted-repeat structure) is the most popular approach for inducing gene silencing in plants. However, such transgene constructs usually generate extreme levels of gene silencing leading to complete elimination of the target mRNA (knock-out).

For some applications, partial silencing (gene knockdown) of the target gene is a more desirable outcome. Therefore, alternative approaches must be explored. Recent research demonstrates the effectiveness of a single transgene construct, which lacks a transcription termination signal, as a partial silencer (Nicholson and Srivastava, 2006). This transgene construct is referred to as an "unterminated" construct hereafter. The study reported effective silencing of the Phytochrome A (*PHYA*) and Phytochrome B (*PHYB*) genes in *Arabidopsis* lines transformed with the unterminated *PHYA* or *PHYB* constructs. The silenced lines exhibited characteristic mutant phenotypes indicating successful modification of the target gene activity (Nicholson and Srivastava, 2009). Additionally, the findings indicated that unterminated transgenes do not produce a complete knockout of the targeted gene, but instead produce moderate to severe phenotypic suppression among primary transformants (Srivastava, pers. comm.).

The objective of our research was to develop a reliable model with which to apply this new transgene construct and then test the efficiency of the approach on several genes in *Arabidopsis*. The development and testing of an original model entailed: 1) construction of plasmids containing truncated *Arabidopsis* genes of interest; 2) verification of correct plasmid sequence; and 3) analysis of the silencing efficiency of the plasmid through introduction to Arabidopsis thaliana individuals.

MATERIALS AND METHODS

The DNA Vector Construction. The plasmid pPZP200 was used to construct the silencing vectors (Hajdukiewicz et al., 1994). This plasmid includes sequences for spectinomycin resistance and a T-DNA region. The T-DNA region is the section of DNA that would be incorporated into the plant's genome upon *Agrobacterium* infection. The pPZP200 plasmid was modified to include, within its T-DNA region, a hygromycin resistance gene driven by a 35S promoter and terminated by a *nos3*' transcription terminator. The pPZP200 plasmid was cut with restriction enzyme *XbaI* while suspended in autoclaved deionized water and the appropriate buffer in a 1.5-mL eppendorf tube. Incubation time was 1.5 hours. Temperature was set at 37 °C. The tubes were mechanically rotated while incubation took place. The 35S promoter sequence was extracted from pHPT using the same *XbaI* enzyme, then ligated into pPZP200 at the *XbaI* site. DNA fragments were ligated in a 1.5-mL eppendorf tube overnight at 16 °C using T4 DNA Ligase in autoclaved deionized water and the appropriate buffer. The resulting plasmid was named "pSJN15A" (Fig. 1).

To ensure ligation occurred as desired, pSJN15A was checked for correctness twice using restriction enzymes. In the first test, *EcoRI* was used to cut the plasmid in two places. These cuts would produce fragments of 0.75 and 8.0-kilobases in a correct ligation, while an incorrect ligation would yield fragments of 1.25 and 7.5-kilobases. The second trial test was based on cutting pSJN15A with *HindIII* and *Eco*RV. The expected fragments for correct ligation here were 1.65 and 7.1-kilobases, with 0.35 and 8.4-kilobase fragments representing an incorrect ligation. Directly downstream of the *XbaI* site within this plasmid, there is a *HindIII* restriction site where transgene fragments lacking a polyadenylated termination sequence were inserted to complete the plasmid as a silencing vector.

Truncated sequences of *Arabidopsis* genes were created for the purpose of testing the silencing efficiency of unterminated transgenes. DNA primer pairs were designed for the PCR amplification of 500-bp fragments of genes *Var*2, *ClV3*, *BRI1*, and *TTG* from native *Arabidopsis* genomic DNA, excluding the polyadenylation termination sequence in each case. These particular genes were chosen as viable targets to test the transgene due to their observable phenotypic influence on development. In designing the primers, a *Hind*III restriction site was added to both forward (5') and reverse (3') primer sequences. These sites ensured proper ligation of each transgene into pSJN15A at the *Hind*III site. For successful vector construction, no transgene sequence could contain additional *Hind*III restriction sites, as both pSJN15A and the transgene were each cut with *Hind*III prior to ligation.

Escherichia Coli Transformation. The synthesized vectors were used to transform separate cultures of *E. coli* (DH5α). Successfully transformed bacteria were selected on LB broth with 100 mg/L spectinomycin (Sigma-Aldrich, St. Louis, Mo.). The individual colonies were cultured in LB broth. The plasmids were then extracted from *E. coli* using the standard procedure. Briefly, the bacteria were centrifuged in microcentrifuge at full speed and the supernatant was drained off. The bacteria were then resuspended in a suspension buffer, utilizing a vortex to homogenize. A lysis buffer was added to break bacterial cells and release plasmid molecules, and then a high salt solution was added to precipitate cell debris. The resulting mixture was centrifuged and the supernatant was transferred to a new tube, leaving the debris behind. The plasmid was precipitated using 95% ethanol, inverted several times, and centrifuged. The ethanol was poured off and the DNA pellet was washed with 70% ethanol and air dried before adding 50 µL of autoclaved deionized water to dissolve the DNA pellet.

Arabidopsis Transformation. Vectors were then transferred to *Agrobacterium tumefaciens* strain GV3101 through electroporation. After spectinomycin selection of successful transformants, these bacteria were used in a floral dip transformation of *Arabidopsis* (Clough and Bent, 1998). Cultures (500 mL) of transformed *Agrobacterium*

were made for each plasmid, which were centrifuged at 6000 rpm for 20 minutes to pellet bacterial cells. The supernatant was discarded. The pellet was resuspended in 5% sucrose solution. Silwet (0.2 mL) of was mixed into each solution. One pot containing several *Arabidopsis* plants, with flowers having recently opened, was dipped for a few seconds into each solution. After dipping, each pot was placed on its side, covered with aluminum foil, and left for 24 hours. The next day, the plants were moved back to the light. Once the dipped plants were mature, seeds were collected. Demonstration of hygromycin resistance served as the selection marker for the successful *Arabidopsis* transformation. Therefore, primary transformant (T1) seeds were selected on Murashige Skoog (MS) media containing hygromycin (25 mg/L). The hygromycin resistant individuals were transferred to soil and allowed to mature.

Effectiveness of the construct was determined through evaluation of the rate of mutant phenotypes among the transformed T1 lines. Phenotypes of transformants containing the transgene were compared to phenotypes of wild type *Arabidopsis* individuals, which served as the control. In the ongoing project, RT-PCR and qRT-PCR will be done to analyze the rate of mRNA degradation among the putative silenced lines.

RESULTS AND DISCUSSION

Molecular Strategy. Previously, researchers reported that transcription of gene fragments lacking proper transcription termination signal (*3*' untranslated region), induces silencing of complementary genes within *Arabidopsis* genome (Luo and Chen, 2007; Nicholson and Srivastava, 2009). The production of truncated transcripts lacking polyadenylation signal (polyA) was the basis of silencing observed in these studies. Such polyA(-) transcripts are converted to double-stranded RNA (dsRNA) mediated by plant RNA Dependent RNA Polymerase 6 (RDR6) (Luo and Chen, 2007), which is degraded by plant RNA silencing machinery to generate short-interferring RNA (siRNA). The siRNA anneal with complementary mRNA to divert it from translation machinery to RNA degradation machinery. These two studies also found that transcription initiated by cauliflower mosaic virus 35S RNA promoter (35S promoter) is not completely terminated by the termination signal of the nopaline synthase gene (*nos3*'). This read-through activity of the 35S promoter generates RNA encoded by DNA sequence downstream of *nos3*'. Based on this, we designed a simple silencing vector in which 35S promoter of the selectable marker gene (hygromycin resistance), an essential component of the vector, drove read-through transcription of gene fragments cloned into unique restriction sites of the vector. As no *nos3*' or other transcription terminator sequences would be present downstream of the cloned fragments, the transcription would be improperly terminated. As a result, the unpolyadenylated RNA that would be synthesized are potent inducers of gene silencing.

Development of Silencing Vector. The pSJN15A cloning vector was successfully created and used to generate silencing vectors against 4 new *Arabidopsis* genes. The pSJN15A vector contains the following properties:

(a) A plasmid backbone of pPZP200 that contains a spectinomycin resistance gene for selecting bacterial strains transformed with this plasmid. This gene is important for mobilizing pSJN15A into *Agrobacterium tumefaciens* for subsequent plant transformations.

(b) A T-DNA region containing a hygromycin resistance gene. The T-DNA is transferred from bacteria into plant genome, thus hygromycin resistance is utilized for selecting transformed plant cells/seedlings.

(c) A unique restriction site, *Hind*III, downstream of the hygromycin resistance gene. Thus, a PCR fragment flanked by *Hind*III sites can be cloned into pSJN15A, and transcribed by the read-through activity of the 35S promoter of the hygromycin resistance gene.

It should be noted that the direction of the inserted transgene proved to be irrelevant, as transcription of both sense and antisense sequences would result in efficient silencing in transformed individuals. We recommend that at least 400 bps of gene fragment be cloned for efficient silencing by pSJN15A, as fragments smaller than 400 bps display significantly lower rate of silencing (Nicholson and Srivastava, pers. comm.).

Induction of Gene Silencing in Arabidopsis. The silencing vectors containing fragments of following genes were developed: *VARIEGATED 2 (Var2)*, *CLAVATA 3 (ClV3)*, *BRASSINOSTEROID INSENSITIVE 1 (BRI1)*, and *TRANSPARENT TESTA GLABRA 1 (TTG)*. These vectors were used to transform *Arabidopsis* through floral dip method. The resulting transgenic lines (T1 lines) were identified by plating seeds on hygromycin containing MS media. The seedlings were either directly evaluated or transferred to soil for phenotypic evaluation at the later stages of plant development. Loss of function (null mutation) of *Var2* generates white sectors on cotyledons and leaves of the seedlings, *CLV3* null mutation generates increased number of stamens and carpel in flowers, *BRI1* null mutation generates a range of phenotypes including reduced fertility, and *TTG* null mutation abolishes trichomes on stem base and leaves (TAIR, Arabidopsis Information Resource, www.Arabidopsis.org). Efficiency of silencing in T1 lines was assessed by the appearance of the respective mutant phenotype in the transgenic lines (Table 1). Appearance of white sectors on 77% of hygromycin resistant *Var2* seedlings grown on germination media, indicated gene silencing of *Var2* gene. Similarly, *BRI1* plants

displayed curled leaves and very low fertility phenotype. Approximately 50% of *BRI1* plants displayed severe phenotype characterized by high rate of sterility in plants. Silencing phenotypes in *CLV3* and *TTG* transformants were not apparent (Table 1), indicating that manifestation of *CLV3* and *TTG* mutant phenotypes requires severe suppression of these genes.

In the ongoing work, real-time PCR approach will be taken to study the level of steady-state mRNA in these transgenic lines. This analysis will reveal the quantitative strength of silencing induced by "unterminated" gene fragments.

CONCLUSIONS

The pSJN15A plasmid that was successfully constructed in this research demonstrated the potential of a new model, "unterminated" transgene, as a competent tool in future gene silencing applications. The model allows for simple insertion of a truncated gene sequence at a specific site, with no need for an additional promoter nor any regard for sequence direction, to form a complete vector. This design has application in gene silencing in various plant species.

ACKNOWLEDGEMENTS

I would like to thank Drs. Vibha Srivastava and Scott Nicholson for allowing me to assist them in the study of gene silencing. I would also like to thank Aydin Akbubak, Souman Nandy, Gulab Rangani, and Jamie Thomas for all of their assistance and patience throughout the duration of my research. Financial support for this project was provided through a University of Arkansas Honors College Research Grant.

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Table 1. Efficiency of unterminated transgene mediated silencing.

Gene[a]	Mutant phenotype[b]	Total number of transformed lines analyzed	Number of lines found to display mutant phenotype	Silencing Efficiency[c] (%)
Var2	Bleached cotyledons	35	27	77
CLV3	Altered floral organs	15	1	0.06
BRI1	Reduced fertility	9	5	55
TTG	No trichomes	18	0	0

[a] *Var2* = VARIEGATED 2; *CLV3* = CLAVATA 3; *BRI1* = BRASSINOSTEROID INSENSITIVE 1; *TTG* = TRANSPARENT TESTA GLABRA 1.
[b] From www.Arabidopsis.org.
[c] Percent lines displaying mutant phenotype.

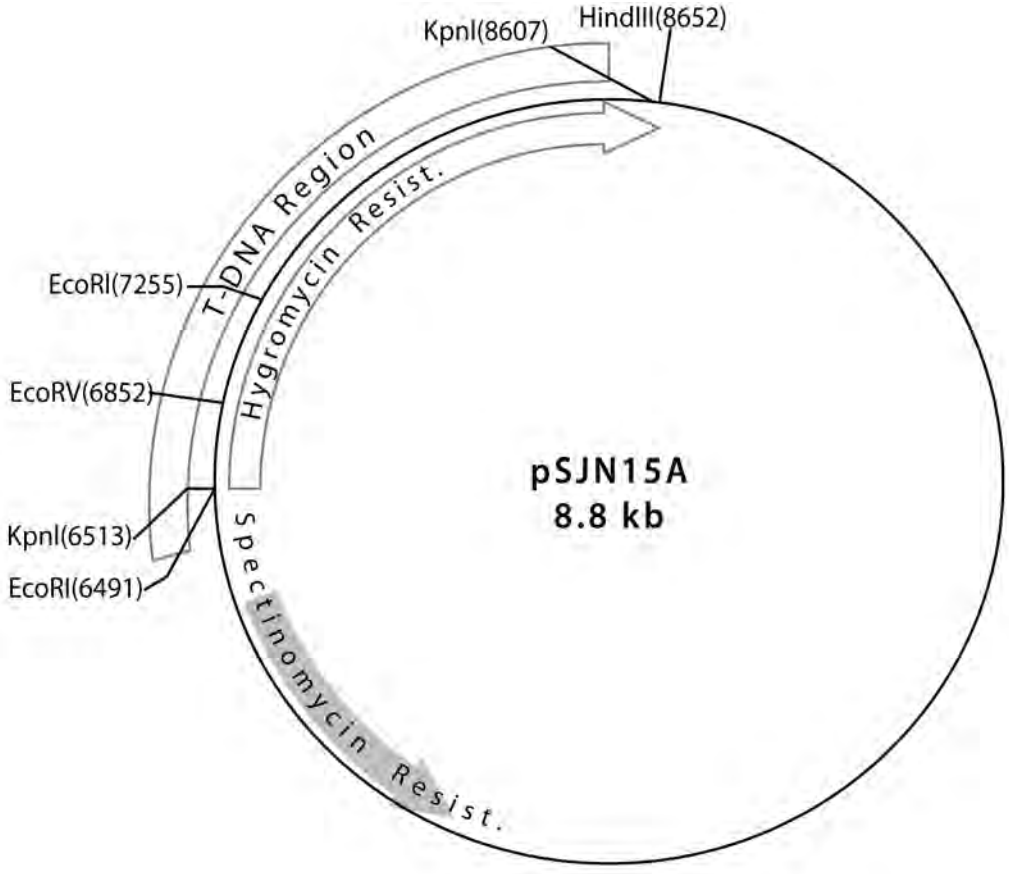

Fig. 1. Design of pSJN15A. The T-DNA region contains a hygromycin resistance gene, which consists of a 35S promoter driving hygromycin phosphotransferase gene (*HPT*), terminated by *nos3'* terminator. The *HindIII* site is the unique cloning site for introducing gene fragments. Note that the absence of *nos3'* after the cloning sites allows for the unterminated transcription of gene fragments. The plasmid contains a spectinomycin resistance gene in the backbone, which serves as a bacterial transformation selection gene.

Antioxidant and antihypertensive activities of rice bran peptides

Janika N. Hull[*]*, Arvind Kannan*[†]*, and Navam S. Hettiarachchy*[§]

ABSTRACT

Protein isolates and peptide fractions from food sources (cereal grains), have been shown to exert bioactive properties including antiobesity, anticancer, antiangiogenic, etc. One such food source is rice bran, which is an underutilized co-product of rough rice milling. It contains 90% of the nutrients and nutraceuticals of value to health, including high quality protein. The high quality protein is a potential source to generate peptides that can reduce hypertension and oxidative stress, both being important risk factors for cardiovascular diseases. The objective of this study was to extract peptide hydrolysates from heat stabilized defatted rice bran by enzymatic hydrolysis, evaluate the hydrolysates for gastrointestinal (GI) resistance, fractionate the GI-resistant hydrolysates by ultrafiltration to obtain >50 and 10-50 kDa fractions, and determine antihypertensive and antioxidant activities in the fractions. For antihypertension activity, angiotension-1 converting enzyme (ACE) assay, and for antioxidant activity, the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay was conducted. We report that the ACE-I inhibition activity values for the unfractionated and unhydrolyzed (control), and fractions of >50 kDa, and 10-50 kDa were 6% (control), 78%, and 55%, respectively, clearly denoting antihypertensive activity for the peptide fractions. When tested for antioxidant activity, the >50 kDa fraction decreased from an initial DPPH of 95.48 to 78.99 mg/g, while the 10-50 kDa fraction decreased from an initial 110.35 to 76.53 mg/g, depicting reduction of radical-induced oxidant stress. The results demonstrated that the high molecular sized peptide hydrolysate fractions (>50 and 10-50 kDa) from rice bran bear antihypertensive and antioxidant properties and could possibly find a place as a health beneficial nutraceutical ingredient in food applications.

[*] Janika Hull is a 2011 graduate of Alabama A&M University with a major in food science and a minor in amimal science. This paper is based on research conducted with the protein chemistry group at the University of Arkansas during her summer internship in 2010 funded through the Masterfoods USA, IFT Foundation.

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[§] Navam S. Hettiarachchy is a faculty mentor and a professor in the Department of Food Science.

MEET THE STUDENT-AUTHOR

Janika Hull

I am from Cuba, Ala. I attended Sumter County High school where I graduated valedictorian in May 2007. I began my college career in the fall of 2007 at Alabama A&M University on a Presidential Scholarship. I am majoring in food science with a minor in animal science.

I am involved in various activities in the food science department at Alabama A&M University such as Phi Tau Sigma Honor Society, Food Science Club, and I am also a part of the Institute of Food Technology. I have served as public relations officer and the junior class representative on the executive board of the Food Science Club.

I was awarded an internship through Masterfoods USA, IFT foundation to conduct exciting and challenging research in the protein chemistry group at the University of Arkansas in the summer of 2010. I plan to get my M.S. degree in nutritional biochemistry. I would like to thank Dr. Hettiarachchy, Dr. Kannan, and everyone that is a part of the protein chemistry group in the department of food science for all their advice, help, and guidance.

INTRODUCTION

Rice is the cereal grain with the second highest worldwide production of over 550 million tons annually. It is considered a staple food for a major part of the world. Asian countries like China and India top the overall production of rice, followed by the United States. Approximately 85% of the rice consumed in the United States is grown in the United States (USA Rice Federation, 2009). Rice is grown globally, but the bran and germ—the most nutritious and versatile parts of the rice kernel—traditionally have been underutilized as food sources.

Rice bran is a by-product of the rice milling process. Rice bran contains high quality proteins, which have unique nutritional value and properties (Tang et al., 2002). Rice bran is a hypoallergenic food ingredient and has been reported to have anti-cancer activity (Kannan et al., 2008, 2009).

Rice bran has become popular because of the complex number of nutrients contained within the bran. It contains phytosterols, polysaccharides, beta-sitosterol, fiber, Vitamin E complex and a large complement of B vitamins; including B15, a vital antioxidant. Rice bran also contains Coenzyme Q10, omega-3 and omega-6 fatty acids and oleic acid also (Good Nutrition Center, 2008). Antioxidants, which may also be found in rice bran, are nutrients that fight off free radicals in our bodies. These free radicals cause premature aging and many of the degenerative diseases of old age.

Hypertension (high blood pressure) affects 73 million adults in the United States alone (Crosta, 2009). Hypertension occurs when the blood pressure in arteries is too high. When this occurs, the heart has to work harder and this can lead to heart attack, stroke, heart failure, aneurysm, or renal failure. Rice bran fractions have been shown to possess the ability to inhibit angiotensin I-converting enzyme (ACE-I) which has the ability to control blood pressure (Bernstein, 2006). Although this has been found, not much work has been done with rice bran to characterize antihypertensive and antioxidant protein components.

Bioactive compounds are chemical compounds, such as vitamins, minerals, and electrolytes, that produce biological activity in the body. They possess the ability to impart health benefits or reduce the risk of disease. Bioactive compounds are being studied in nutrition and medicine for disease fighting properties. The positive health benefits of these bioactive compounds have been shown to come from plants and animals. It has been found that vegetarianism is on the rise; therefore, more plants are being pursued for bioactive compounds. Cereal grains and their components are being investigated for the presence of these components.

Hence the overall objective of this study was to utilize protein components for potential bioactive properties from a natural co-product such as rice bran. The specific objectives were 1) to produce peptides from heat-stabilized defatted rice bran (HDRB) by specific food-grade enzymatic

hydrolysis; 2) to produce gastrointestinal (GI)-resistant peptides; 3) to fractionate the peptides to obtain high molecular size (10-50, and >50 kDa) fractions; and 4) to evaluate them for antioxidant and antihypertensive activities.

MATERIALS AND METHODS

Heat-stabilized defatted rice bran was obtained from Riceland Foods (Stuttgart, Ark.), and the Romicon ultrafiltration system was purchased from Koch Membrane Systems, (St. Louis, Mo.). The food-grade alcalase enzyme from a bacterial strain was purchased from Novozyme (Cambridge, Mass.). All other chemicals were of reagent grade, and were obtained from BDH Chemicals (St. Louis, Mo.).

Preparation of Peptide Hydrolysates from Heat-Stabilized Defatted Rice Bran. Heat-stabilized defatted rice bran was ground and passed through a 60-mesh sieve. Fifty grams of HDRB was suspended in a total volume of 500 mL with deionized water. The mixture was homogenized (VWR Adaptable Homogenizer-VDI 25) on a setting of 3 for 3 min. The pH was then adjusted to 8.0 using 1N NaOH. Alcalase was added at 3.5 AU at 55 °C for 1 h in a shaker bath. The enzyme was inactivated at 85 °C for 5 min. The mixture was then centrifuged 10,000 x g for 15 min. The hydrolysate was separated as the supernatant.

Treatment of Peptide Hydrolysate with Simulated Gastric Juices. Sodium chloride (0.2 g) and 0.7 mL of concentrated hydrochloric acid were added to 100 mL of the hydrolysate. The combination was stirred for 30 min. The pH was adjusted to 2.0 using 3N HCl. Pepsin was added at 0.32 g/100 mL and the solution was allowed to shake incubate at 37 °C for 1 h. To inactivate the enzyme, the pH was adjusted to 7.2. The mixture was centrifuged at 3000 g for 20 min to obtain soluble peptide hydrolysates in the supernatant.

Simulated Intestinal Juice Preparation. Potassium phosphate monobasic (0.68 g) was prepared and stirred for 30 min. The pH of the solution was adjusted to 8.0, and the mixture was maintained at 37 °C. Pancreatin (Sigma-Aldrich, St. Louis, Mo.) at a final concentration of 0.1% was added and stirred. The simulated gastric juice treated hydrolysate was dissolved in this simulated intestinal juice and allowed to incubate at 37 °C with constant shaking. After 120 min, the enzyme was inactivated by heating at 85 °C for 10 min. The reaction mixture was centrifuged at 3000 g for 20 min to obtain soluble peptide hydrolysate in the supernatant. The hydrolysate was stored at 4 °C.

Fractionation of Gastrointestinal (GI)-Resistant Peptide Hydrolysate by Ultrafiltration. Fractionation was carried out with a Romicon ultrafiltration system (Koch Membrane Systems, St. Louis, Mo.) equipped with 2.54-cm diameter hollow-fiber polysulfone membrane cartridges. The filtered soluble GI-resistant peptide hydrolysate was run through sequential ultrafiltration columns with membrane cartridges having nominal molecular weight cutoffs (MWCO) of 50 and 10 kDa. The resulting retentates from each of the MWCO were freeze dried and stored at 4 °C until used for bioactivity assays.

ACE-1 Inhibition Activity. Fifty µL of the rice bran sample was added to 50 µL ACE-I (25 mU/mL). The mixture was then added to 150 µL H-H-L (Hippuryl-L-Histidyl-L-Leucine) in 0.1 M sodium borate buffer-pH 8.3 plus 0.5 M NaCl. The solution was incubated for 30 min at 37 °C, followed by 1N HCL addition at 250 µL along with 1 mL of ethyl acetate. The suspension was centrifuged for 10 min at 3,000 x g, and 0.75 mL was collected from the upper layer. The collected sample was evaporated at 95 °C for 30 min. After evaporation, 1 mL of deionized water was added to the residue. Absorbance of the sample was read at 228 nm on a spectrophotometer and percent inhibition was calculated using the equation:

$$\% \text{ Inhibition} = \left[\frac{1 - (\text{Abs}_{\text{peptide}} - \text{Abs}_{\text{blank}})}{(\text{Abs}_{\text{positive}} - \text{Abs}_{\text{blank}})}\right] \times 100$$

DPPH Assay to Evaluate Antioxidant Activity. One hundred microliters of the rice bran samples were added into 1.0 mL of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) solution. The decrease in absorbance (mg/g of DPPH utilized) was determined at 515 nm using a spectrophotometer after 120 min, using the equation:

$$\text{Antioxidant activity} = \frac{\text{Abs}_{\text{control-sample}}}{\text{Abs}_{\text{control}}}$$

Data Analysis. All values obtained were from triplicate analyses with standard error, and significance of $P < 0.05$. Controls were unfractionated/unhydrolysed rice bran; positive control for antihypertensive study was Captopril at 25 µM.

RESULTS AND DISCUSSION

ACE-I Inhibition. The rice bran hydrolysates were obtained by the use of the alcalase enzyme treatment and treated with the gastrointestinal enzymes, pepsin and pancreatin, to obtain GI-resistant peptides. These GI-resistant peptides were fractioned into 10-50 kDa and >50 kDa by ultrafiltration and characterized for ACE-1 inhibitory activities.

Angiotensin II is a potent constrictor of blood vessels. It is derived from Angiotensin I by the action of an enzyme Angiotensin convertor enzyme-I (ACE-I). There are drugs available that prevent this enzyme's activity in order to prevent formation of Angiotensin II (Weir and Dzau, 2000).

We evaluated the ability of high molecular sized (>50 and 10-50 kDa) peptide fractions to inhibit the ACE-I enzyme for a possible antihypertensive effect. We report that the ACE-I inhibition activity values for the unfractionated and unhydrolyzed (control), and fractions of >50 kDa, and 10-50 kDa were 6% (control), 78% for the >50 kDa fraction, and 55% for the 10-50 kDa fraction, respectively (Fig. 1). When compared to the control, the rice bran extract showed significantly higher activity in ACE-I inhibition, indicating a possible antihypertensive activity for the peptide fractions. Ardiansyah et al. (2006) fractioned rice bran using Driselase and ethanol, and found that there were no significant differences between the two fractions compared to controls. No other study has evaluated these bioactive effects using GI-resistant peptides that were fractionated based on molecular sizes. In our study, the >50 kDa fraction had a significantly higher percent inhibition compared to the 10-50 kDa fraction. This study is the first to characterize rice bran extract based on molecular size for antihypertensive and antioxidant effects.

There are commercially available drugs for antihypertension such as Captopril and Enalapril, but these drugs have been reported to have adverse side effects (Ardiansyah et al., 2006). It is important to research rice bran for antihypertensive characteristics because rice bran is a natural food source having the potential to combat high blood pressure without side effects and inexpensively. A study done on ACE-I inhibition for soy protein suggested the potential for production of peptides with ACE-inhibitory activity upon physiological digestion of soy protein (Lo et al., 2006); and as with our study, a simulated digestion was used. Such studies, in complement with ours, can add value to natural food sources and co-products that can provide alternative potential as therapeutic agents for chronic diseases.

Antioxidant Activity. The DPPH radical assay is extensively used when assessing the radical scavenging activity of several natural compounds. The 2,2-Diphenyl-1-Picrylhydrazyl radical is scavenged by antioxidants through the donation of an electron. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. Radical scavenging activity increases with increasing percentage of the free radical inhibition (Brand-Williams et al., 1995).

In our study we assessed the radical scavenging activity of the peptide fractions, demonstrating the amount (in mg/g) of remaining DPPH after reaction over time. We observed a decrease in DPPH levels, signifying reduction in free radical formation in the presence of peptide fractions (Fig. 2). It should be noted that both high molecular sized fractions (>50 and 10-50 kDa) caused an initial drop in DPPH levels in the first 30 min, and then remained until 90 min. We find a further reduction in DPPH level in the 10-50 kDa fraction from 90 to 120 min possibly signifying a delayed response. Nevertheless, both fractions were able to reduce the DPPH levels and maintain the observed effect for up to 90 min, signifying antioxidant property. The control slightly increased from 49.41 but later decreased back to 49.41, the >50 kDa fraction decreased from an initial DPPH of 95.48 to 78.99 mg/g after 120 min, and the 10-50 kDa fraction decreased steadily from an initial of 110.35 to 76.53mg/g.

Free radicals can cause many undesirable reactions leading to tissue damage. Lipids, proteins and DNA can all be victims of free radicals. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation. Recently much focus has been cast on natural antioxidants that serve to eliminate or reduce the risk of developing oxidative stress rather than synthetic ones, owing to reduced potential side effects and affordability. Several antioxidants have emerged from natural sources but very few from co-products. In our study, we not only establish anti-hypertensive action for peptides from rice bran but also suggest possible antioxidant activity too. Common peptides present in these fractions may function as key players in the reduction of chronic symptoms or manifestations occurring with cardiovascular diseases. In comparison to other natural cereal grains, such as corn, wheat, oat, and rice, the antioxidant levels were relatively high: corn had 87%, wheat had 90%, oat had 58% and rice had 71% (Horax et al., 2005, Horax, 2009). Rice bran had a higher antioxidant activity than rice and oat, but lower activity when compared to corn and wheat. Rice proteins are also hypoallergenic and hence are widely preferred over proteins derived from other sources. Thus it will be appropriate and valuable to characterize and use peptides derived from rice bran that show potential as antihypertensive and antioxidant agents.

In conclusion, the ACE-I inhibitions for the fractions were higher when compared to the negative control. The results show that the rice bran fractions (especially the >50 kDa fraction) have the ability to reduce high blood pressure. Antioxidant activity of rice bran extracts using DPPH resulted in a decrease in mg/g DPPH during a 120-minute time period. The decrease in DPPH signifies antioxidant activity promoting reduction in free radicals. These results show great promise and potential for rice bran being utilized as a nutraceutical to aid management of chronic illnesses like hypertension and oxidative stress.

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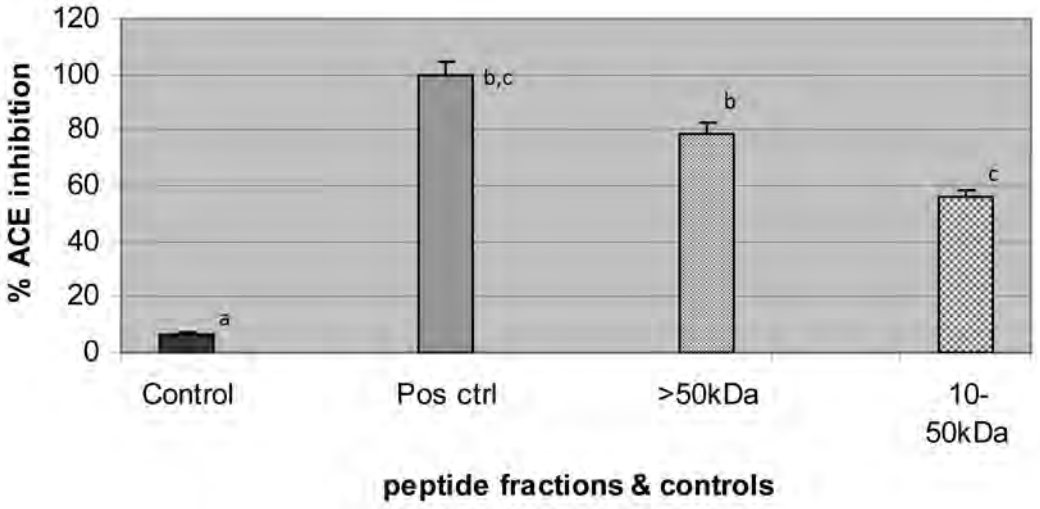

Fig. 1. ACE inhibitory activity of rice bran peptide hydrolysate fractions. (ACE-I) Angiotensin I Converting Enzyme inhibition was assayed using HHL (Hippuryl-LHistidyl-L-Leucine) substrate in presence and absence of the peptide fractions. All values are means of three replications ± Standard Deviation. Control: unfractionated and unhydrolyzed rice bran; pos ctrl; Captopril 25 μM.

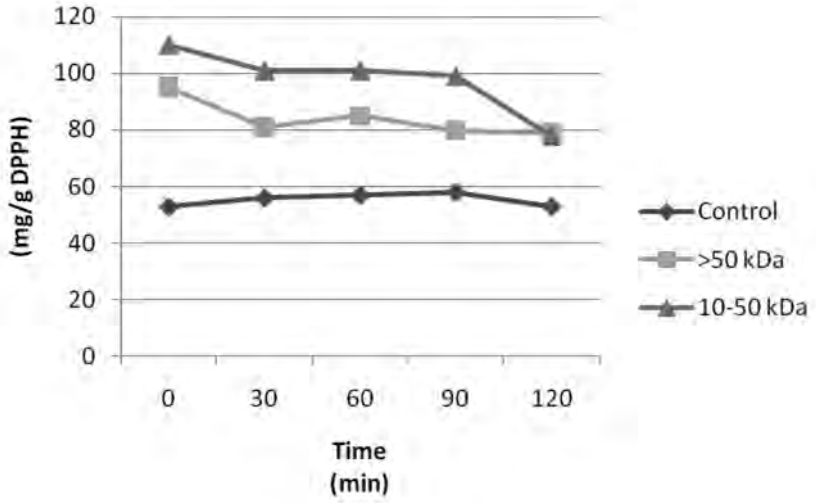

Fig. 2. Antioxidant activity of rice bran peptide hydrolysate fractions. Antioxidant activity was assayed using the DPPH (2,2-Diphenyl-1-Picrylhydrazyl) assay. All values are means of three replications with standard error (too minimal to observe). Control: unfractionated and unhydrolyzed rice bran.

Storage effects of gel encapsulation on stability of chokeberry monomeric anthocyanins, procyanidins, color density, and percent polymeric color

Mary C. Kordsmeier and Luke R. Howard†*

ABSTRACT

Chokeberries (*Aronia melanocarpa*) are an antioxidant-rich plant product due to their high content of polyphenols, especially anthocyanins and procyanidins. These polyphenols have been shown to provide protection against coronary heart disease, stroke, and lung cancer, as well as against oxidative stress, the main cause behind chronic diseases promoted by free radicals. The objective of this study was to determine the storage effects of gelatin encapsulation on monomeric anthocyanins, procyanidins, color density, and percent polymeric color of three gummy candies of different strengths formulated with a base of 25.4% chokeberry concentrate, 47.6% sucrose, 1.3% Splenda, and 0.025% potassium sorbate. The gum strengths varied by percentages of gelatin and water in the formulations, with 19.1:6.6, 17.8:7.9, and 16.5:9.2 ratios used to produce soft, medium, and hard strength gummies, respectively. Total monomeric anthocyanins, total procyanidins, color density, and percent polymeric color of the gummies were determined 1 day post-processing and after 2, 4, and 6 months of storage at refrigerated and room temperatures. Storage for 6 months at room temperature resulted in dramatic losses of monomeric anthocyanins (80-82%), total procyanidins (48-54%), and color density (76-80%). Anthocyanin losses during storage coincided with marked increases in percent polymeric color values indicating that anthocyanins and procyanidins underwent condensation reactions to form polymers. Refrigerated storage ameliorated losses of monomeric anthocyanins (61-65%), total procyanidins (17-22%), and color density (60-67%) over 6 months of storage compared to samples stored at ambient temperature. Refrigerated storage also ameliorated the increase in polymeric color values observed in samples stored at room temperature indicating condensation reactions responsible for polymer formation were retarded. Gum strength did not have a significant effect on retention of anthocyanins and procyanidins.

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MEET THE STUDENT-AUTHOR

Mary Kordsmeier

I graduated from Conway High School in May 2007. In the fall of 2007, I started as a freshman at the University of Arkansas after receiving the Chancellor's Scholarship and the Arkansas Governor's Distinguished Scholarship. Since my first food science class, I have really enjoyed my time learning and working in the department. I have been very active in the food science field, serving as president of the Food Science Club, student representative for the Ozark Institute of Food Technologists Board, and as a member of the Institute of Food Technologists Student Association. I have gotten to work in both the functional foods lab and the rice processing program, where I have greatly strengthened my research experience. I have also had the honor of receiving the Outstanding Senior Student Award in the food science department.

With the tremendous support and guidance of my honors mentor, Dr. Luke Howard, and his laboratory team, I have been able to complete my thesis project, which was funded by a Student Undergraduate Research Fellowship and the Dale Bumpers College of Agricultural, Food, and Life Sciences Undergraduate Research Fellowship. I graduated with my B.S. degree in food science and a minor in animal science in May 2011. In the summer after graduation, I plan to start my M.S. degree in food science at the U of A, continuing to work under Dr. Howard. I have gained a lot of experience and knowledge during my undergraduate career that I will carry with me through the rest of my life.

INTRODUCTION

The interest in plant products rich in health-protecting bioactive substances such as polyphenols is increasing (Borowska et al., 2009). Polyphenols are secondary metabolites of plants, which contain more than one aromatic ring carrying one or more hydroxyl groups. Fruits, especially berries, contain high levels of polyphenols, particularly one class of polyphenols called flavonoids (anthocyanins, flavonols, flavan-3-ols, and proanthocyanidins) (Howard and Hager, 2002). The chokeberry (*Aronia melanocarpa*) is a rich source of these polyphenols, especially anthocyanins, which account for >50% of the total polyphenols in chokeberry. Chokeberry anthocyanins are a mixture of cyanidin glycosides: 3-galactoside, 3-glucoside, 3-arabinoside, 3-xyloside, of which cyanidin-3-galactoside is predominant (Oszmiański and Wojdylo, 2005). These compounds are not only responsible for the pigments found in berries (Cabrita et al., 2000), but they have also been shown to exhibit antioxidant, antimicrobial, anti-inflammatory, and vasodilatory functions (Kähkönen et al., 2001). Due to limited shelf life and season availability, fresh berries are commonly distributed and consumed in processed forms including jams, jellies, juices, canned, and dehydrated products. Unfortunately, anthocyanins in processed berry products are unstable during storage, due in large part to condensation reactions involving anthocyanins and procyanidins, resulting in the formation of large molecular weight polymeric pigments (Brownmiller et al., 2008; 2009).

Storage temperature plays a key role in anthocyanin stability and antioxidant capacity. Anthocyanin degradation in model solutions doubled with a temperature increase from 10 to 23 °C over 60 days (Cabrita et al., 2000), and the antioxidant content of strawberry jams was reported to be more stable at 4 °C than 20 °C (Wicklund et al., 2005). Visible light is also harmful to anthocyanins and increases their rate of degradation (Jackman and Smith, 1996). Anthocyanin losses in stored blueberry products were accompanied by increased polymeric color values, suggesting that anthocyanins underwent polymerization reactions with procyanidins to form anthocyanin-procyanidin polymers (Brownmiller et al., 2008).

Gel encapsulation is a promising technology that may be useful in stabilizing berry polyphenols during storage. Improved anthocyanin stability has been reported in several studies through the use of natural polymers including pullulan (Gradinaru et al., 2003), cyclodextrin (Mourtzinos et al., 2008), and (1→3,1→4)-β-$_D$-glucan (Xiong et al., 2006). Maier et al. (2009) studied the effects of gelatin and pectin encapsulation on polyphenols in grape pomace extracts. They reported considerable losses in total phenolics

in the gelatin gels, which they attributed to thermal treatment of the gels, while pectin gels had greater anthocyanin retention possibly due to a stabilizing effect of pectin during processing. In addition to improved polyphenol stability, gel encapsulation may provide controlled release of bioactive polyphenols in the human body, and be a useful platform for clinical feeding trials. The objective of this study was to determine the effects of gelatin encapsulation on the stability of chokeberry anthocyanins during six months dark storage at 4 °C and 23 °C.

MATERIALS AND METHODS

Preparation of Gummy Candies. Chokeberry concentrate (70° brix, or 70 g sugars per 100 g solution) was obtained from Mae's Health & Wellness (Omaha, Neb.). Three formulations of chokeberry gummy candies were developed to obtain textures of soft, medium, and hard by varying the gelatin-to-water ratio in the formulation (Table 1).

For the base, sugar was added to chokeberry concentrate and the mixture was boiled on a hot plate until the sugar was completely dissolved. Splenda® and potassium sorbate were then added to the mixture. The mixture was constantly stirred while boiling on the hot plate until the ingredients were completely dissolved. For each gummy, the required amount of Knox gelatine® (Kraft Foods, Inc., Northfield, Ill.) was dissolved in the appropriate amount of water at 40 °C. The melted gelatine mixture was added to the boiling chokeberry mixture. After stirring the mixture was poured into gummy molds (Cakes 'N Things, Inc., Gridley, Ill.) greased with olive oil cooking spray. The gummies were allowed to equilibrate to room temperature, and then were placed in a -20 °C freezer for 10 min. The gummies were placed into Ziploc® bags with half stored in the dark at 4 °C and half stored in the dark at 23 °C. The gummies were sampled in triplicate after 1 day and at 2, 4, and 6 months of storage.

Extraction of Anthocyanins. Anthocyanins in the chokeberry concentrate were extracted by the method of Cho et al. (2004) with modifications. The gummies were soaked in 20 mL of methanol/water/formic acid (60:37:3 v/v/v) for 15 min. The gummy-solvent mixture was homogenized using a Euro Turax T19 Tissuemizer (Tekmar-Dohrman Corp., Mason, Ohio). The samples were filtered through Miracloth (Calbiochem, La Jolla, Calif.), and the filtrates adjusted to a final volume of 100 mL with the extraction solvent.

Monomeric Anthocyanins Analysis by UV-Visible Spectroscopy. Monomeric anthocyanins were analyzed using the pH-differential method of Giusti and Wrolstad (2001). Absorbance values of diluted extracts were measured by a diode array spectrophotometer (Hewlett Packard, Palo Alto, Calif.). The absorbance of the diluted sample (A) was calculated as follows:

$$A = (A_{510\,nm} - A_{700\,nm})_{pH\,1.0} - (A_{510\,nm} - A_{700\,nm})_{pH\,4.5}$$

The total monomeric anthocyanin pigment concentration (mg/kg gummy) in the original sample was calculated using the following formula:

$$\text{Monomeric anthocyanin pigment} = \frac{\left(\begin{array}{l}A \times \text{molecular weight} \times \text{dilution factor} \times \\ \text{extraction volume} \times 1000\end{array}\right)}{(\text{molar absorptivity} \times 1000 \times \text{gummy weight})}$$

Cyanidin-3-glucoside, with a molar absorptivity of 26900 (Jurd and Asen, 1966), and molecular weight of 467.2 (Giusti and Wrolstad, 2001) was used as standard, with results expressed as mg of cyanidin 3-glucoside equivalents/kg gummy. The dilution factor used was 10.

Procyanidin Analysis. Procyanidins (PACs) were analyzed using the 4-dimethylaminocinnamaldehyde (DMAC) colorimetric method of Prior et al. (2010) with one modification. The method uses acetone/water/acetic acid (75:24.5:0.5 v/v/v) to extract PAC, but in order to liquefy gelatin in the gummies, extractions were done using methanol/water/formic acid (60:37:3 v/v/v). The concentrations of PACs in the sample extract were measured using a BioTek Synergy HT microplate reader (Winooski, Vt.), using catechin as standard. The total PAC concentration (mg PAC/kg gummy) in the original sample was calculated using the following formula:

$$\text{Total PACs} = \frac{\left(\begin{array}{l}\text{concentration of PACs in sample extract} \times \\ \text{dilution factor} \times \text{extraction volume} \times 1000\end{array}\right)}{(1000 \times \text{gummy weight})}$$

Color Density and Polymeric Color Analysis. Color density and percent polymeric color were analyzed using the method of Giusti and Wrolstad (2001). Color density was calculated using the control sample according to the following formula:

$$\text{Color density} = [(A_{420\,nm} - A_{700\,nm}) + (A_{510\,nm} - A_{700\,nm})] \times \text{dilution factor}$$

Polymeric color was calculated using the bisulfite-bleached sample as follows:

$$\text{Polymeric color} = [(A_{420\,nm} - A_{700\,nm}) + (A_{510\,nm} - A_{700\,nm})] \times \text{dilution factor}$$

Percent polymeric color was calculated using the following formula:

$$\%\ \text{Polymeric color} = (\text{polymeric color}/\text{color density}) \times 100$$

Color density, polymeric color, and % polymeric color were expressed as unitless numerical values.

Statistical Analysis. Effects of gel strength, storage temperature, and storage time on total anthocyanins, total PAC,

color density, and polymeric color were analyzed by analysis of variance (ANOVA) using JMP® software (SAS Inst. Inc., Cary, N.C.). Significant differences ($P < 0.05$) between means were determined by Student's *t*-test. Pairwise correlations between total anthocyanins, total PAC, color density and polymeric color were also determined using JMP® software.

RESULTS AND DISCUSSION

Monomeric Anthocyanins. Monomeric anthocyanin content was affected by the interaction between storage temperature and storage time ($P < 0.0001$) (Fig. 2) and the interaction between gum strength and storage time ($P = 0.05$) (Fig. 1). Hard gummies had higher retention of total monomeric anthocyanins than medium or soft gummies 1 day after processing. Hard gummies also had higher total monomeric anthocyanins than soft gummies at 4 months storage (Fig. 1).

For both storage temperatures, total monomeric anthocyanins decreased over storage time (Fig. 2). This finding was consistent with a previous study on stability of black currant anthocyanins encapsulated in glucan gel, where refrigerated storage resulted in greater retention of anthocyanins (Xiong et al., 2006). There was a clear temperature effect after 2 months of storage, with greater total monomeric anthocyanin retention in refrigerated gummies at months 2, 4, and 6 compared to those stored at room temperature at each respective sampling point. Total monomeric anthocyanin retentions were not affected by gum strength, with retention values of 46%, 47%, and 46% in soft, medium, and hard gummies, respectively, stored for 4 months at room temperature (data not shown). Anthocyanin retention continued to decrease over storage in gummies stored at room temperature, with retention values of 18%, 20%, and 19% in soft, medium, and hard gummies, respectively, after 6 months of storage (data not shown). With gummies stored in refrigeration, anthocyanin retention was much higher during the first 4 months of storage. Monomeric color loss at 4 months was minimal for refrigerated gummies of all three strengths, with retentions of 87%, 87%, and 84% for soft, medium, and hard gummies, respectively (data not shown). After 6 months of storage at refrigerated temperature, monomeric color retention values decreased to 38%, 39%, and 35% for soft, medium, and hard gummies, respectively. These values are lower than previously reported values for a study on storage effects on anthocyanin retention of grape pomace extracts encapsulated by gelatin, which found retentions of 72% and 80% for gels stored for 24 weeks in the dark at 20 °C and 6 °C, respectively (Maier et al., 2009). This discrepancy may be attributed to differences in fruit structure, type and localization of anthocyanins, and gel type.

Monomeric anthocyanins were found to be more stable over time when stored at refrigerated temperatures. This is consistent with previous studies with aqueous solutions of anthocyanin 3-glucosides as well as strawberry jam, where anthocyanin stability was found to increase with decreased storage temperature (Cabrita et al., 2000; García-Viguera et al., 1999). Anthocyanin degradation in the gummies during storage may have been due to two different reactions. During heating at pH 3.5, a hydrolysis reaction occurs in anthocyanins, whereby the compounds are converted to chalcones, which are aromatic ketones, and then further cleaved to form phenolic acids and aldehydes (Sadilova et al., 2007). This type of degradation may have occurred during thermal processing of the gummies. Another more plausible mechanism involves condensation reactions of anthocyanins with other phenolic compounds, including flavan-3-ols or polyflavan-3-ols, which may occur through direct anthocyanin-tannin reactions (Reed et al., 2005). It was hypothesized that hard gummies would better retain anthocyanins due to limited amount of water in the gummies needed for direct anthocyanin-tannin condensation reactions to occur. However, our results indicated that increased gum strength did not affect anthocyanin retention, suggesting anthocyanins and procyanidins were in close proximity to react regardless of varying moisture content in the gummies.

Total Procyanidins. Total procyanidin (PAC) content was affected by the interaction between storage temperature and time ($P < 0.0001$). Month 4 values were considered outliers due to temperature abuse that caused degradation of procyanidins in the month 4 extracts.

For gummies stored at room temperature, there was a significant loss in procyanidins from 2 to 6 months, with retention dropping to 48% after 6 months storage (Fig. 3). Procyanidin content was significantly greater in gummies stored in refrigeration, with 82% retention at 6 months. These findings indicate room temperature storage was detrimental to procyanidins, which are consistent with a study by Brownmiller et al. (2009) who reported total procyanidin retentions of 11%, 7%, and 22% in blueberry juices, purees, and canned samples after 6 months storage at room temperature. Procyanidins are known to be sensitive to polymerization reactions at higher temperatures (Spanos and Wrolstad, 1990). It is likely that procyanidins reacted with anthocyanins to form polymers, thereby decreasing both monomeric anthocyanin and procyanidin content in the gummies over time. This reaction appeared to be retarded when samples were stored refrigerated.

Color Density and Percent Polymeric Color. Color density was affected an interaction between storage treatment, gum strength, and storage time ($P = 0.02$). Hard gummies had higher color density than soft and medium gummies, with average values of 3.9, 3.5, and 3.5 in hard, medium, and soft gummies, respectively (data not shown). This was expected as monomeric color and color density are highly correlated ($r = 0.94$). Hard gummies stored under refrigeration had significantly higher color density than medium and soft gum-

mies the day after processing. Refrigerated hard gummies also had higher color density than medium gummies stored under refrigeration after 2 months of storage (Fig. 4). This suggests that at lower temperatures, the increased gelatin concentration in the hard gummy may have helped retain some color density. This may be due to the water content of the gummies, as hard gummies contained less water than medium and soft gummies. The water may have caused a dilution effect in the spectrophotometric method, resulting in higher anthocyanin content and color density values in hard gummies at month 0.

As with anthocyanins, there was a clear storage time effect on color density, which decreased over the 6 months of storage for all gum strengths (Fig. 4). After 6 months of storage, color density was lowest in gummies stored at room temperature compared to those stored under refrigeration, indicating color density was more stable at lower temperatures.

Percent polymeric color was measured to determine the amount of polymerized anthocyanins resistant to bleaching with sodium metabisulfite (Fig. 5). A higher percent polymeric color indicates the formation of anthocyanin-procyanidin polymers. Polymeric color was affected by the interaction of storage temperature and time ($P < 0.0001$). The increase in polymeric color was accompanied by a decrease in total procyanidin content ($r = -0.62$), monomeric anthocyanins ($r = -0.78$), and color density ($r = -0.68$), which agrees with findings of previous studies on black raspberry products, processed blackberry products, and processed blueberry products stored for 6 months at room temperature (Hager et al., 2008a; Hager et al., 2008b; Brownmiller et al., 2009). While correlations in this study were only moderately strong, the results of this study agreed with expected trends. Hard, medium, and soft gummies had approximately 20% polymeric color values one day after processing, but differences due to storage temperature were apparent over storage time. The polymeric color of gummies stored at room temperature increased to 35%, 43%, and 47% after 2, 4, and 6 months of storage, respectively. Gummies stored refrigerated had much lower polymeric color values, with values only increasing to 25%, 26%, and 26% after 2, 4, and 6 months of storage, respectively. These results indicate that gummies stored at room temperature had lower anthocyanin retentions as a result of the formation of anthocyanin-procyanidin polymers as is suggested by higher percent polymeric color values.

These results are not surprising, as it has been shown that a sandwich structure between anthocyanins and polyphenols can be easily formed and have high stability at lower temperatures. At higher temperatures, though, the irreversible polymer-like structure of these two compounds forms, which is brown in color, increasing percent polymeric color (Kunsági-Máté et al., 2011). It is not known whether these polymers are readily absorbed *in vivo* to provide antioxidant benefits, as they are most likely large molecular weight compounds. However, it has been suggested that enzymes in the gastrointestinal tract can degrade phenolic compounds into more readily absorbed metabolites, which could provide health benefits upon absorption (Selma et al., 2009). Because the gummies contained sugar, it is possible that the Maillard Browning reaction could have taken place, with the products formed also contributing to increased polymeric color values.

SUMMARY

Anthocyanins and procyanidins were more susceptible to polymerization in gummies stored at room temperature than under refrigeration. Encapsulation by gelatin in hard gummies did not affect anthocyanin and procyanidin degradation over storage time. More research is needed to determine the bioavailability of the anthocyanin-procyanidin polymers formed and to identify treatments to prevent their formation. Chokeberry-containing products should be stored at refrigerated temperatures to better retain beneficial polyphenols.

ACKNOWLEDGEMENTS

Financial support for this project was provided by an Arkansas Student Undergraduate Research Fellowship and a University of Arkansas Dale Bumpers College of Agricultural, Food and Life Sciences Undergraduate Research Grant. I would personally like to thank Dr. Luke Howard, Cindi Brownmiller, Brittany White, and Jeana Boucher for all of their help and guidance over the last couple of years. I could not have completed my thesis without you!

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Table 1. Formulations for soft, medium, and hard gummies.

	Gum Strength					
	Soft		Medium		Hard	
Ingredient	Amount	%	Amount	%	Amount	%
Aronia	100 mL	25.397	100 mL	25.397	100 mL	25.397
Sugar	187.5 g	47.619	187.5 g	47.619	187.5 g	47.619
Splenda	5 g	1.270	5 g	1.270	5 g	1.270
Potassium sorbate	0.0985 g	0.025	0.0985 g	0.025	0.0985 g	0.025
Water	75.175 mL	19.092	69.9 mL	17.752	64.9 mL	16.483
Gelatin	25.975 g	6.597	31.25 g	7.937	36.25 g	9.206
Total		100		100		100

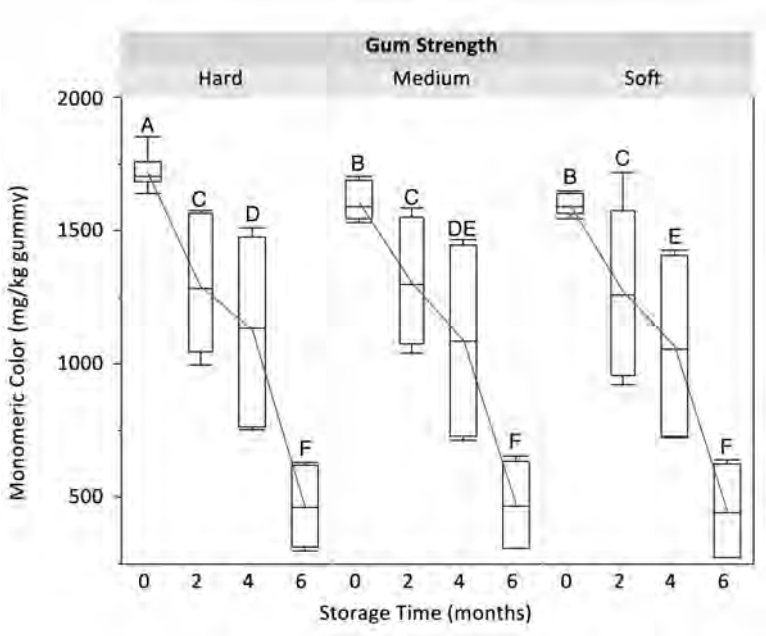

Fig. 1. Total monomeric anthocyanin content of chokeberry gummies of various gum strengths over storage time of 6 months. Letters indicate significant differences in total monomeric anthocyanin content among treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.

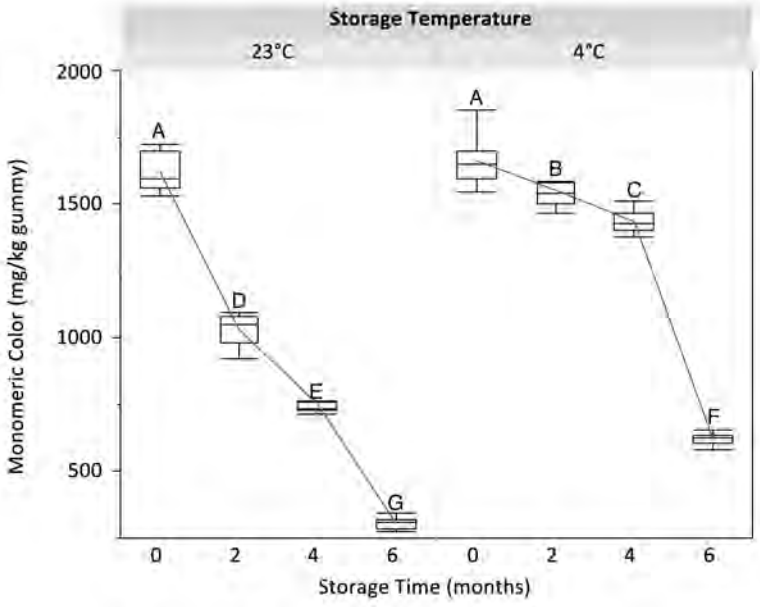

Fig. 2. Total monomeric anthocyanin content of chokeberry gummies stored at refrigerated and room temperatures for 6 months. Letters indicate significant differences in total monomeric anthocyanin content among treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.

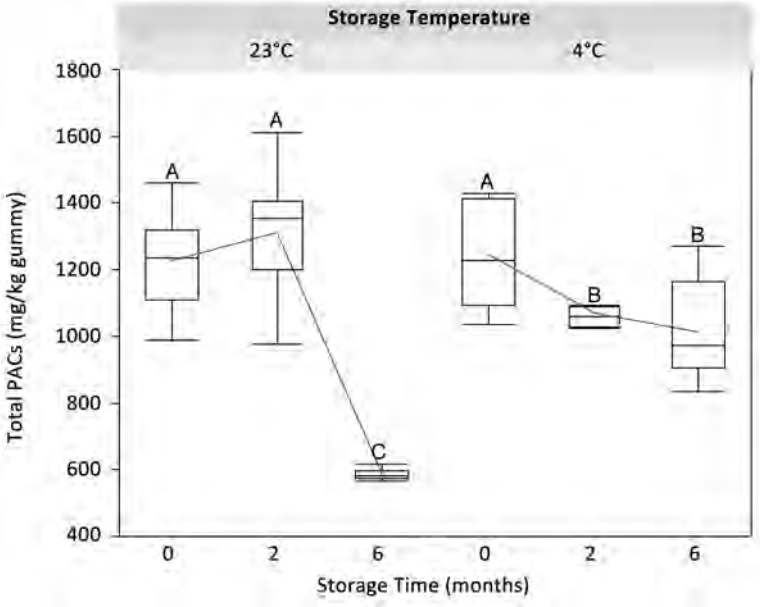

Fig. 3. Total procyanidins in chokeberry gummies stored at refrigerated and room temperatures at 0, 2, and 6 months. Letters indicate significant differences in total procyanidin content among treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.

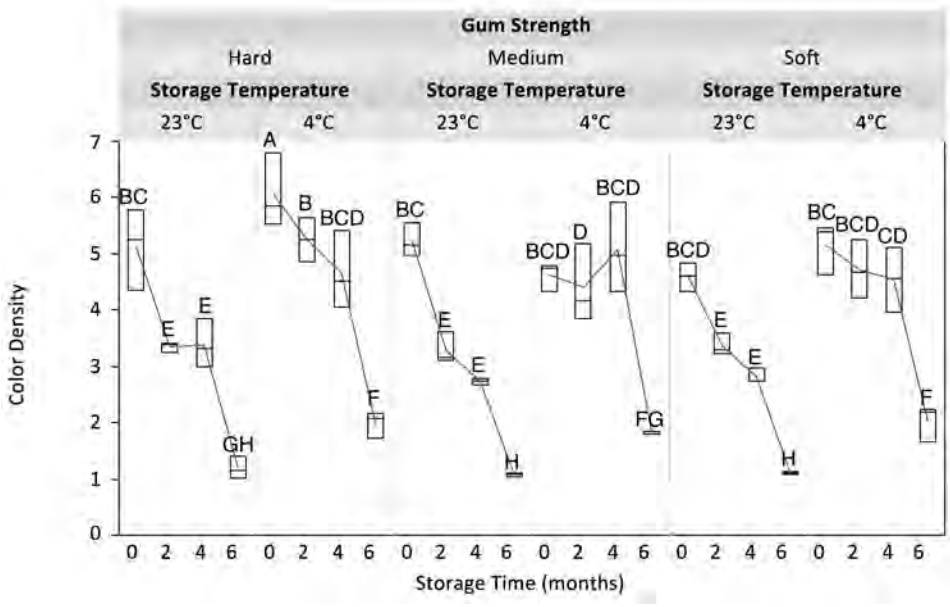

Fig. 4. Color density values for hard, medium, and soft gummies at various storage times and temperatures. Letters indicate significant differences in color density among treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.

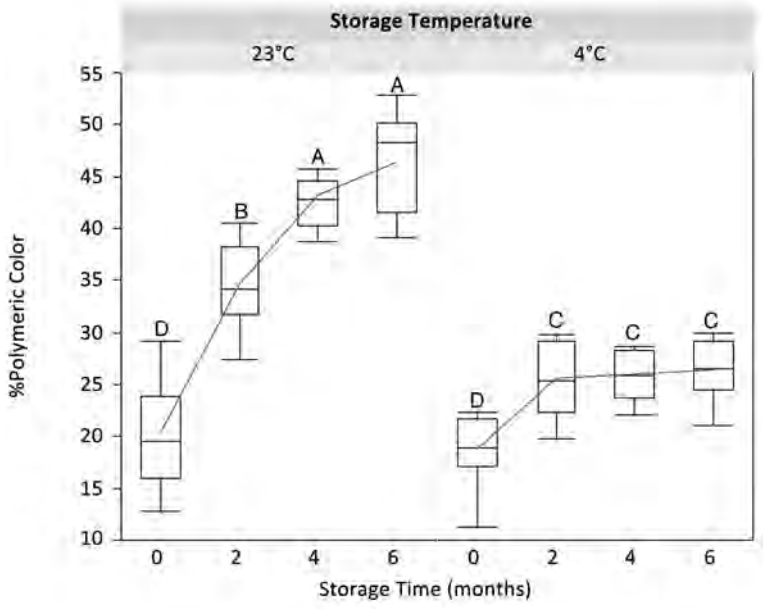

Fig. 5. Percent polymeric color values of chokeberry gummies at various storage times and temperatures. Letters indicate significant differences in percent polymeric color among storage treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.

Properties of gluten-free pasta prepared from rice and different starches

Alexandria W. Mertz and Ya-Jane Wang†*

ABSTRACT

Rice is one of the few cereal products that does not contain gluten. However, the absence of gluten poses problems in the structure of and cooking quality of rice pasta. The objective of this project was to investigate the addition of starch on the physicochemical properties and cooking quality of rice-based pasta. Rice-based pasta was prepared from parboiled long-grain rice flour with the addition of 25% cooked starch from different sources. The color and pasting properties of the ground pasta flour were measured by a chroma meter and a Rapid Visco Analyser, respectively. The pasta was cooked to the optimum cooking time to evaluate its hardness, stickiness, adhesiveness and resilience by using texture profile analysis with a texture analyzer. The water absorption and cooking loss of pasta were determined by weight difference. Overall the addition of starch improved the processibility of rice pasta and enabled the b* value (yellow color) of the uncooked rice pasta to be close to that of the semolina control. The pasting properties of rice pasta containing common corn starch were close to those of the semolina control. Upon cooking, the rice flour control had higher water absorption and greater cooking loss than the semolina control. The addition of starch decreased both the water absorption and the cooking loss relative to the rice flour control. The effect of starch addition on the textural properties of rice-based pasta varied with the type of starch. The addition of waxy starch or tapioca starch resulted in rice pasta with an increase in hardness and stickiness, whereas the addition of common corn, Hylon V, or Hylon VII resulted in rice pasta with a decrease in hardness, stickiness, and adhesiveness. This project demonstrates that the addition of starch significantly changes the color, pasting, cooking and texture properties of rice pasta, and these changes are governed by the type of starch incorporated into the rice pasta. Gluten-free rice pasta can be prepared with properties similar to the semolina pasta by incorporating starches.

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MEET THE STUDENT-AUTHOR

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I am a senior majoring in food science and minoring in both music and general foods and nutrition. I am originally from St. Charles, Mo. I graduated from St. Charles High School in 2008 and enrolled at the University of Arkansas that fall. I was awarded the Division of Agriculture Land Grant Scholarship, Department of Music Scholarship, and several others. During my stint at the university I have participated in Associated Student Government, Emerging Leaders, LeaderShape, Food Science Club, and the University Symphonic Orchestra. I am currently a member of Alpha Zeta Honors Fraternity.

I would like to thank Dr. Wang, my faculty sponsor, for guiding me through this project and the research process. I would also like to thank Curtis Luckett and Emily Arijaje for teaching me how to use various equipment during this project.

INTRODUCTION

Pasta is an essential staple found in many different cultures across the world. Traditionally pasta is made by mixing durum wheat flour and water. Durum wheat flour has been proven to deliver the highest quality pasta, which is attributed to the gluten that is present in wheat. Gluten is a structural protein that gives pasta cooking stability and firmness or "bite" (al dente). Gluten is composed of glutenin, which gives dough elasticity, and gliadin, which is responsible for making the dough extensible (Sissons et al., 2007).

Unfortunately, many people are affected by celiac disease, which is a genetic disorder resulting in gluten intolerance. Since gluten is found in the majority of cereal products (wheat, barley, and rye), people who suffer from celiac disease have a small selection of grain-based products in which they can consume. The only treatment for celiac disease is to maintain a strictly gluten-free diet. The absence of grains and cereals in a diet can lead to nutrient deficiencies in iron, calcium, folate, and select fat-soluble vitamins. In the United States, approximately one out of every 250 to 500 people is suffering from this disease. In Europe approximately one out of every 200 to 300 people is suffering from celiac disease. The occurrences of celiac disease in Asia and Africa are rare (Sozer, 2009).

Rice is one of the few cereal products that does not contain gluten, i.e. hypoallergenic, and therefore, is a safe food for people with celiac disease to consume. Although it is mainly consumed as whole-grain form, rice can be processed into flour to make gluten-free pasta. However, the absence of gluten poses a few problems in the structure of the pasta and pasta cooking quality. The gliadin and glutenin in wheat gluten form a "network" structure when water is added to wheat dough, which traps the water in the dough and prevents the dissolution of the pasta during cooking. The absence of gluten makes it difficult to achieve a cohesive dough structure, and consequently paste made of rice flour has a different and undesired textural quality.

Starches are widely used in the bakery industry to improve texture and appearance of cereal-based foods. The replacement of wheat starch with rice starch at 3-9% levels resulted in gluten-free breads with similar crust hardness but reduced crumb hardness (Gallagher et al., 2002). Rice flour combined with corn starch and cassava starch were shown to produce high-quality gluten-free bread with good taste and appearance (Sanchez et al., 2002). High amylose starch in the amount of 10-40% was reported to improve cooking tolerance and firmness in retorted pasta and tortilla products (Miller et al., 1986). Because limited work has been done on the improvement of rice-based pasta quality by the incorporation of starch, the objective of this project was to investigate the influences of starch from different botanical sources on the cooking quality of rice-based, gluten-free pasta in an attempt to improve the texture of pasta made from rice flour.

MATERIALS AND METHODS

Rice was provided by the University of Arkansas Rice Processing Program. Starch samples of different sources, including waxy corn, common corn, high amylose corn (50% amylose, Hylon V, and 70% amylose, Hylon VII), potato, and tapioca, were obtained from National Starch LLC (Bridgewater, N.J.). Commercial semolina flour was purchased from a local store to prepare semolina control pasta.

Preparation of Parboiled Rice. Because the slightly darker color of parboiled rice resembles pasta, parboiled rice was used in this study. Long-grain rice was parboiled by soaking 250 g of rough rice in 800 mL of deionized water in a shaker water bath (OLS200, Grant Instruments, Cambridge, UK) at 65 °C for 16 h (Newton and Wang, 2011). Afterwards, soaked rice was removed and autoclaved in an autoclave (Tuttnauer Brinkman, Westbury, N.Y.) at 120 °C and 15 psi for 40 min. The autoclaved rice was placed in a sealed ziplock bag at room temperature for 24 h and then dried at 50 °C for 3 h. The rice sample was dehulled using a Satake dehusker (THU-35, Satake Corp., Hiroshima, Japan), and then milled in a friction mill (MCgill Miller, Brookshire, Texas) for 30 s. The milled rice was seperated into head rice and broken kernels using a double-tray sizing machine (GrainMan Machinery, Miami, Fla.). The head rice was ground into flour for pasta preparation with a cyclone sample mill (UDY Corp., Ft. Collins, Colo.) fitted with a 0.25-mm mesh sieve.

Preparation of Pasta. For the semolina or rice flour control, 75 g of commercial semolina flour or parboiled long-grain rice flour was combined with 65 g of deionized water to form dough. The dough was rolled into a sheet about 1 mm thick using a pasta maker (Imperia Trading S.r.I., C.So Susa, Italy) and then into pasta strands using the fettuccini noodle extrusion attachment. The pasta strands (10 mm in length, 1 mm in width and 0.1 mm in thickness) were placed on a stainless steel metal test tube rack and dried at 90 °C for 1 h. The process was repeated by combining 56 g of rice flour with 19 g of different types of starch to produce different pasta samples. Half of the deionized water was combined with the starch and cooked on a hot plate until the starch was gelatinized, and the other half of the water and the flour were added to form dough (Miller et al., 1986).

Physicochemical Properties of Pasta. The moisture content of pasta was determined by drying 2 g of ground pasta in an oven at 130 °C for 2 h. The color of dried, uncooked or cooked, ground pasta was measured using a Minolta Chroma Meter (CR-100, Osaka, Japan) by recording L^*, a^*, and b^* values. The L^* value describes the lightness of the product with a range from 0 (black) to 100 (white). The a^* value describes the color of the product in terms of red (positive) to green (negative). The b^* value describes the color of the product in terms of yellow (positive) to blue (negative). The pasting properties of ground pasta were determined using a Rapid ViscoAnalyser (RVA, Newport Scientific Pty. Ltd, Warriewood NSW, Australia) operated at 160 rpm according to Approved Method 61-02 (AACC, 2000) with modification. The slurry was prepared by mixing 3.0 g of ground pasta (12% moisture basis) with 25.0 mL of deionized water in a canister (10 % w/w). The slurry was heated from 50 °C to 95 °C at 3 °C/min, held at 95 °C for 10 min, cooled to 50 °C at 3 °C/min, and held at 50 °C for 10 min.

Cooking and Textural Properties of Pasta. Six strands of pasta were weighed, placed in 600 mL of deionized water in a 1000-mL beaker, and cooked in a boiling water bath. The optimum cooking times for the semolina control and rice flour control were 12 and 7 min, respectively. The optimum cooking time for the pasta made with starch was 5 min. The optimum cooking time was defined as the minimum time needed to completely gelatinize starch in the pasta until the disappearance of the white core by visual inspection. The cooked pasta strands were drained and weighed. The water absorption of pasta was calculated as the weight difference and expressed as a percentage of the original sample weight (as is) before cooking. Cooking loss was measured by evaporating the cooking water to dryness in a 100 °C oven and expressed as a percentage of the original sample (as is) (AACC, 2000).

Textural properties of cooked pasta, including hardness, stickiness, adhesiveness, and resilience, were measured using texture profile analysis (TPA) with a TA-XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, N.Y.) after cooking the noodles for the optimum time. The TA-XT2 Texture Analyzer was calibrated with a load cell of 5 kg. A 35-mm cylindrical probe was used to compress a single strand of pasta. Pasta strands were cut into 0.5 cm length before they were compressed. The attachment compressed the pasta strand at a constant rate of 1 mm/s to 70% of the original thickness of the pasta (Petitot et al., 2010).

Data Analysis. The experiment was performed in duplicate and each property was measured at least in duplicate to calculate its mean value and standard deviation.

RESULTS AND DISCUSSION

We noted that it was difficult to sheet the pasta prepared from long-grain rice flour without the addition of starch. The addition of starch to the long-grain rice flour made the dough more pliable and thus easy to sheet. The color properties of uncooked, ground pasta samples are presented in Table 1. Although different values were observed among different pasta samples, the addition of

starch resulted in decreased L* value (slightly darker), and increased a* (more reddish) and b* values (more yellowish) for uncooked rice pasta when compared with the rice flour control. Overall the addition of starch enabled the b* value (yellow color) of the uncooked rice pasta to be close to that of the semolina control, indicating the benefit of adding starch to rice besides improving the processibility. The color of cooked pasta samples was also compared after the cooked pasta was dried and ground (Table 2). The addition of starch generally decreased the L* value (slightly darker) of the rice pasta, but the changes in the a* and b* values of rice pasta containing starch were affected by starch type. For example, rice pasta containing potato starch had lower a* (less reddish) and b* (less yellowish) values, whereas rice pasta containing waxy corn starch had higher a* (more reddish) and b* (more yellowish) values when compared with the rice flour control.

The pasting profiles of pasta samples are presented in Fig. 1. The overall pasting profile of the rice flour control was much higher than the semolina control, presumably the higher protein content in semolina flour restricted starch swelling. The rice flour control had very high peak viscosity (2705 cP), breakdown (the difference between peak and trough viscosity) (1340 cP), and final viscosity (2973 cP), whereas the semolina control has little breakdown (101 cP) and low peak (1203 cP) and final (2313 cP) viscosities. Because parboiled rice was cooked during the parboiling process, the rice flour control displayed a higher initial viscosity than the semolina control. The pasting properties of rice pasta containing starch varied with the starch source. The pasting properties of the pasta sample containing common corn starch were similar to those of the semolina control in terms of peak, breakdown, and final viscosities. For rice pasta containing waxy corn, potato, or tapioca starch, it exhibited a higher peak viscosity and a larger breakdown when compared with the semolina control. On the other hand, the rice pasta containing Hylon V or Hylon VII had a very low overall pasting profile, which was attributed to its high amylose content. These results indicate that the pasting profile of rice flour can be modified for specific applications by the addition of an appropriate starch.

When cooked to their respective optimum cooking times, the rice flour control exhibited a slightly higher water uptake and a greater cooking loss compared with the semolina control (Table 3). The greater cooking loss of the rice flour control is not desirable for commercial production because the starchy water is often disposed at a cost as wastewater. The addition of starch not only decreased the water absorption but also greatly decreased the cooking loss of the rice flour control. The addition of potato starch decreased the cooking loss relative to the rice flour control. Both water absorption and cooking loss of rice pasta containing potato starch were close to those of the semolina control.

The textural properties of cooked pasta samples are listed in Table 4. Using texture profile analysis (TPA), the pasta prepared from the rice flour control was softer (lower hardness value), less sticky (lower stickiness value), less adhesive (lower adhesiveness value), and less resilient (lower resilience value) than the semolina control. The addition of starch had different impacts on the textural properties of rice pasta. The incorporation of waxy corn starch or tapioca starch to the rice flour resulted in pasta with significantly harder, stickier, more adhesive, and more resilient texture. In contrast, common corn starch and high amylose corn starch (Hylon V and VII) produced pasta of softer, less sticky, less adhesive, and less resilient texture. Overall, rice pasta containing potato starch had the hardness more close to that of the semolina flour control.

The results of this study indicate that different starches can be combined to improve the texture and pasting and cooking properties of rice-based pasta to have properties similar to the semolina pasta. The use of waxy corn starch or tapioca starch could reduce the cooking loss and increase the hardness, area of peak force, stickiness, adhesiveness, and resilience of rice pasta; whereas the opposite effects would result by incorporating common corn starch or high amylose corn starch (Hylon V and VII) into rice flour.

In conclusion, this project demonstrates that the addition of starch significantly changes the color, pasting, cooking and textural properties of rice-based pasta, and the changes are governed by the type of starch incorporated into the pasta. Knowing how starch affects the properties of rice pasta will help the improvement and future development of hypoallergenic pasta and cereal products, thus expanding the foods available to those suffering from gluten intolerance.

ACKNOWLEDGEMENTS

I would like to thank Dr. Ya-Jane Wang for all of her support, motivation, and advice during this project. I would also like to thank the University of Arkansas Rice Processing Program for providing the rice used for this project. I would like to express my gratitude to Dr. Jean-Francois Meullenet for demonstrating the use of the texture analyzer. Also, my gratitude is extended to Curtis Luckett and Emily Arijaje for teaching me how to use various equipment during this project.

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Table 1. Color properties of uncooked pasta samples as measured by a chroma meter[1]

Pasta sample	L*[2]	a*	b*
Semolina flour	89.6 ± 0.5	-1.1 ± 0.2	14.4 ± 0.2
Rice flour	90.9 ± 0.4	-0.6 ± 0.2	6.4 ± 0.5
Rice flour + waxy corn starch	87.8 ± 0.3	0.7 ± 0.1	13.5 ± 0.0
Rice flour + common corn starch	84.1 ± 1.7	0.6 ± 0.1	13.1 ± 0.2
Rice flour + Hylon V corn starch	86.3 ± 0.2	0.7 ± 0.3	14.0 ± 0.3
Rice flour + Hylon VII corn starch	88.9 ± 0.3	0.7 ± 0.1	12.9 ± 0.3
Rice flour + potato starch	88.3 ± 0.2	0.7 ± 0.0	12.9 ± 0.2
Rice flour + tapioca starch	88.2 ± 0.4	0.8 ± 0.0	13.0 ± 0.1

[1] Mean of duplicate measurements ± standard deviation.
[2] L* value describes the lightness of a product with values ranging from 0 (black) to 100 (white); a* value describes the color of a product, ranging from red (positive values) to green (negative values); and b* value describes the color of a product, ranging from yellow (positive values) to blue (negative values).

Table 2. Color properties of cooked pasta samples as measured by a chroma meter[1]

Pasta sample	L*[2]	a*	b*
Semolina flour	80.0 ± 1.6	-0.2 ± 0.02	22.3 ± 0.4
Rice flour	81.4 ± 0.2	2.3 ± 0.02	18.7 ± 0.0
Rice flour + waxy corn starch	75.3 ± 0.3	3.4 ± 0.02	21.5 ± 0.0
Rice flour + common corn starch	74.9 ± 0.6	2.2 ± 0.03	18.5 ± 0.2
Rice flour + Hylon V corn starch	78.7 ± 0.1	2.5 ± 0.01	20.2 ± 0.0
Rice flour + Hylon VII corn starch	72.4 ± 0.1	2.5 ± 0.04	19.0 ± 0.1
Rice flour + potato starch	68.9 ± 1.0	1.6 ± 0.04	15.4 ± 0.3
Rice flour + tapioca starch	81.7 ± 0.3	1.2 ± 0.01	17.7 ± 1.3

[1] Mean of five measurements ± standard deviation.
[2] L* value describes the lightness of a product with values ranging from 0 (black) to 100 (white); a* value describes the color of a product, ranging from red (positive values) to green (negative values); and b* value describes the color of a product, ranging from yellow (positive values) to blue (negative values).

Table 3. Cooking properties of pasta samples[1]

Pasta sample	Water Absorption (g/g)	Cooking loss (g/g)
Semolina flour	1.06 ± 0.00	0.04 ± 0.01
Rice flour	1.22 ± 0.04	0.22 ± 0.09
Rice flour + waxy corn starch	0.57 ± 0.01	0.02 ± 0.00
Rice flour + common corn starch	0.75 ± 0.04	0.06 ± 0.02
Rice flour + Hylon V corn starch	0.53 ± 0.05	0.05 ± 0.00
Rice flour + Hylon VII corn starch	0.71 ± 0.00	0.14 ± 0.00
Rice flour + potato starch	1.01 ± 0.05	0.06 ± 0.01
Rice flour + tapioca starch	0.77 ± 0.03	0.04 ± 0.01

[1] Mean of duplicate measurements ± standard deviation.

Table 4. Textural properties of pasta samples[1]

Pasta Sample	Hardness (N)	Stickiness (N)	Adhesiveness (N•sec)	Resilience
Semolina flour	83.5 ± 10.8	-8.2 ± 1.1	-3.8 ± 1.2	-0.10
Rice flour	73.9 ± 17.5	-5.1 ± 1.6	-1.0 ± 0.5	-0.07
Rice flour + waxy corn starch	106.9 ± 7.6	-12.4 ± 1.0	-2.4 ± 1.0	-0.12
Rice flour + common corn starch	59.6 ± 14.2	-4.7 ± 0.2	-0.7 ± 0.0	-0.08
Rice flour + Hylon V corn starch	41.8 ± 6.7	-3.3 ± 1.4	-0.6 ± 0.2	-0.08
Rice Flour + Hylon VII corn starch	56.0 ± 16.6	-2.7 ± 1.9	-0.6 ± 0.2	-0.05
Rice flour + potato starch	98.2 ± 14.6	-4.0 ± 0.9	-0.7 ± 0.2	-0.04
Rice flour + tapioca starch	126.3 ± 8.8	-12.2 ± 0.9	-3.9 ± 0.6	-0.10

[1] Mean of five measurements ± standard deviation.

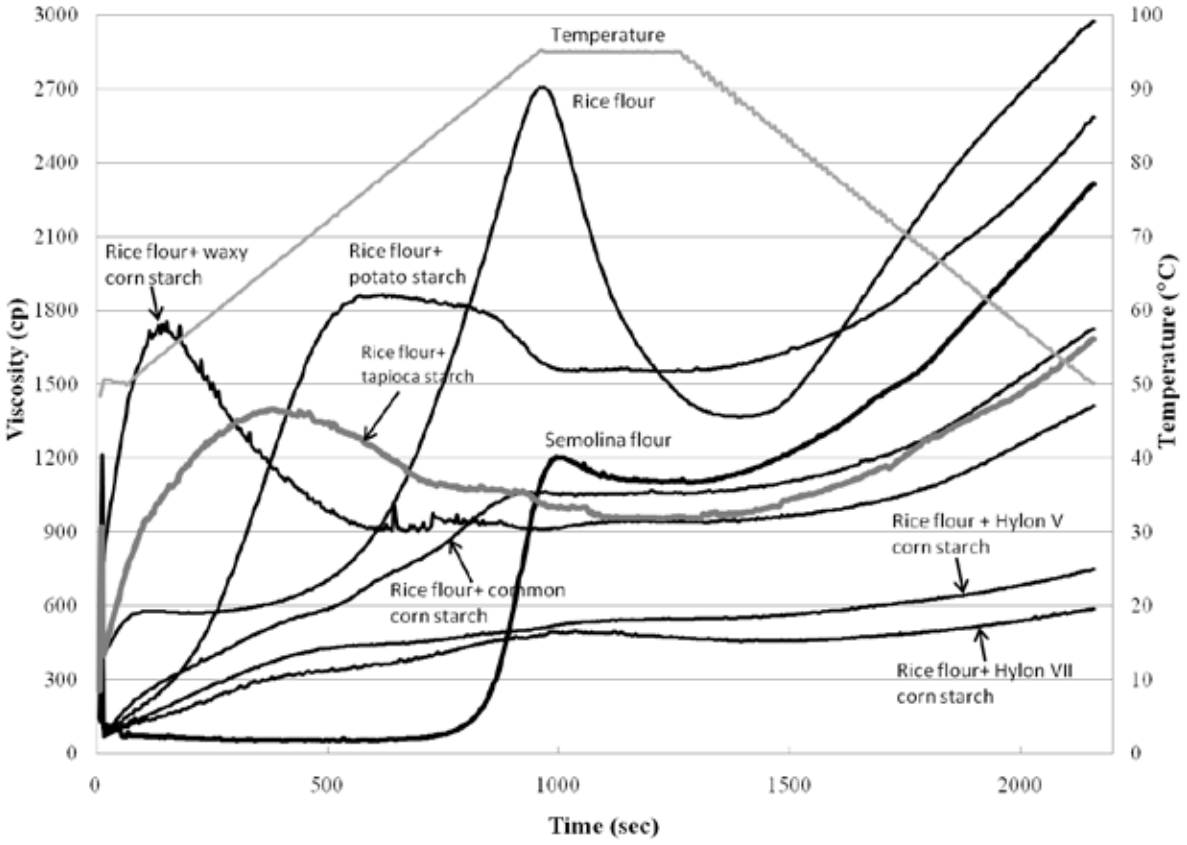

Fig. 1. Pasting properties of pasta samples as measured by a Rapid ViscoAnalyser. Standard deviation is ±50 cp.

Plant growth in soil amended with drilling mud

Satoshi Takaki and Duane C. Wolf†*

ABSTRACT

Extraction of natural gas generates drilling fluid and drilling mud that contain high concentrations of salts. Land application of the fluid and mud can have negative impacts on plant growth and soil properties. The objective of this study was to determine the effects of drilling mud on plant growth, plant chemical concentrations, and soil chemical properties. Sudangrass (*Sorghum sudanense* [Piper] Stapf [Piper]) and bermudagrass (*Cynodon dactylon* L.) were grown in a Roxana loam soil amended with 0%, 5%, or 10% (w/w) drilling mud in a 6-wk greenhouse study. Plant biomass production and concentrations of elements in biomass were determined. Electrical conductivity, pH, and concentrations of extractable and total elements in soil were analyzed. The addition of drilling mud significantly reduced shoot and total biomass production of both plant species and root biomass of bermudagrass. When drilling mud was added to the soil, plant Ca and Mg levels increased. Soil levels of Na, Cl, and the electrical conductivity significantly increased with increased levels of drilling mud application which indicated that salinity was most likely limiting plant growth. Excessive rates of drilling mud application can adversely impact soil properties and reduce plant growth.

* Satoshi Takaki is a 2010 graduate with a major in Environmental, Soil, and Water Science.
† Duane C. Wolf is a faculty mentor and a professor in the Department of Crop, Soil, and Environmental Sciences.

MEET THE STUDENT-AUTHOR

Satoshi Takaki

After I graduated from high school in Japan, I left for the United States and attended the University of Arkansas beginning in the fall 2006 semester. Since I hope to dedicate myself to addressing environmental issues in the future, I completed my B.S. degree in environmental, soil, and water science in December 2010. As an undergraduate I was involved in several research projects, including working with Dr. Thad Scott on a project to evaluate eutrophic urban streams and lakes in the Fayetteville area. The experience allowed me to learn the processes involved in scientific studies, which has been valuable in my subsequent studies. For my honors thesis project, I worked with Dr. Duane Wolf to evaluate the effect of drilling mud from natural gas extraction wells on plants and soil. I successfully conducted the study and completed the honors program. I returned home to Japan in December 2010 and plan to continue my education at the graduate level. Because there are still many Asian countries that have not installed adequate infrastructure for wastewater treatment, I would like to address wastewater treatment and water degradation problems in my future career path.

INTRODUCTION

Natural gas is the third largest energy resource and accounts for 15.6% of the annual global energy consumption (IEA, 2009). Annual production of natural gas increased from 2.1×10^{12} m^3 in 1995 to 2.8×10^{12} m^3 in 2005 (BP, 2006). It is anticipated to increase to 4.9×10^{12} m^3 by 2025 (Balat, 2009).

Extraction of natural gas uses water to lubricate and cool the drilling apparatus, transport formation cuttings to the surface, and seal porous geologic formations (ASME, 2005). The resulting water is known as drilling fluid that is transported to a holding pond where solids settle (Argonne National Laboratory, 2010). The drilling fluid is removed for disposal and the drilling mud remains. Drilling fluid and drilling mud often contain salts (Na and Cl), barite, water-based surfactants or diesel, and montmorillonite (Miller and Pesaran, 1980). The concentrations of soluble salts, trace elements, and the high pH of drilling mud have the potential to reduce plant growth (Nelson et al., 1984).

The objective of this study was to determine the effects of three rates of drilling mud on growth of two plant species, plant chemical concentrations, and chemical properties of a Roxana loam soil following a 6-wk greenhouse study.

MATERIALS AND METHODS

Experimental Set-up. Drilling mud was collected from a depth of 0 to 15 cm from a holding pond from which the drilling fluid had been removed. The soil used for the study was Roxana loam (coarse-silty, mixed, superactive, nonacid, thermic Typic Udifluvents), collected from a depth of 0 to 15 cm at the Vegetable Research Station at Alma, Ark. Drilling mud and Roxana soil were crushed to pass a 2-mm stainless-steel sieve. The drilling mud and soil were analyzed at the Arkansas Agricultural Diagnostics Laboratory (Fayetteville, Ark.) for pH and electrical conductivity (EC) in a slurry of 1:2 soil to water (w/w). The total nitrogen (TN); total carbon (TC); water-extractable Cl; concentrations of Mehlich-3-extractable Ca, Mg, Na, Cu, and B; and the total elemental analysis of Ca, Mg, Na, Cu, and B were also determined. The values of TN and TC were obtained by the Dumas combustion method using vario Max CN Element Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Water-extractable chloride, Mehlich-3-extractable, and total element analyses were conducted using an inductively coupled plasma atomic emission spectrometer (ICP-AES; SPECTRO ARCOS; SPECTRO Analytical Instruments Inc., Mahwah, N.J.; Mehlich, 1984; Donahue, 1992; Table 1). The total elemental analysis used United States Environmental Protection Agency method 3050 B (USEPA, 1996). The Roxana soil contained 12%, 49%, and 39% clay, silt, and sand, respectively, and had a -33 kPa moisture potential of 16% (w/w). Two samples of the drilling mud and soil were analyzed and the drilling mud had mean TN, TC, pH, EC, and Cl values of 0.13%, 3.66%, 8.0, 6.42 dS/m, and 6,425 mg/kg, respectively. The Roxana soil had mean TN, TC, pH, and EC values of 0.05%, 0.57%, 6.3, and 0.05 dS/m, respectively.

Greenhouse Experiment. The study involved three drilling mud rates (0%, 5%, or 10%; w/w), three vegetation treatments (sudangrass (*Sorghum sudanense* (Piper) Stapf), bermudagrass (*Cynodon dactylon* L.), or no plant), and four replications, for a total of 36 individual sample units. Each Conetainer® contained 500 g (dry weight) of Roxana loam soil. The rate of drilling mud amendment was 0.0 g (0%), 25.0 g (5%), or 50.0 g (10%) on a dry-weight basis. Roxana soil and drilling mud were thoroughly mixed and added into the 6.4-cm diameter by 25-cm long Conetainers® (Kirkpatrick et al., 2006). In each sudangrass treatment, five seeds of sudangrass were planted into each pot. The soil was adjusted to a moisture potential of -33 kPa by addition of distilled water.

The Conetainers® were blocked and arranged randomly in a greenhouse and watered daily. After 1 week, sudangrass germinated and was thinned to one plant per Conetainer®, and a 5-cm-sprig of bermudagrass was transplanted into the appropriate Conetainers®. After 3 weeks, each Conetainer® was fertilized with 25 mg NH_4NO_3/kg dry soil (Chapman, 1999).

Shoot and Root Analysis. After 6 weeks, plant shoots and roots were harvested. Shoots were cut at the soil surface, rinsed with distilled water, and dried to a constant weight at 65 °C. After weighing the shoot biomass, the samples were ground to pass a 2-mm stainless-steel sieve (Kirkpatrick et al., 2008). Harvested roots were washed, dried, and weighed.

The concentrations of Ca, Mg, Na, Cu, and B in shoots were analyzed by the ICP-AES method at the Arkansas Agricultural Diagnostics Laboratory (Donahue, 1992).

Soil Analysis. After shoots and roots were harvested, soils were crushed to pass a 2-mm stainless-steel sieve and air-dried. The EC, pH, Cl; the concentrations of Mehlich-3-extractable Ca, Mg, Na, Cu, and B; and the concentrations of total Ca, Mg, Cu, and B were analyzed at the Arkansas Agricultural Diagnostics Laboratory.

Statistical Analysis. The statistical analysis was based on a two-factor factorial design incorporated with four randomized complete blocks (RCB). The two factors were drilling mud and vegetation. The statistical analysis method employed was analysis of variance and multiple comparisons were conducted using least significant difference (LSD) with $\alpha = 0.05$. The analysis was carried out using SAS® version 9.2 (SAS Institute, Inc, Cary, N.C.).

RESULTS AND DISCUSSION

Plant Parameters. Shoot and total plant biomass were reduced at higher drilling mud rates (Table 2). Bermudagrass root biomass was not different among the drilling mud rates, but root biomass of sudangrass was less at higher drilling mud rates (Table 3). Bermudagrass root biomass was smaller than sudangrass at 0% and 5% drilling mud rates. Total bermudagrass biomass was smaller than sudangrass biomass with values of 1.35 g/pot and 3.27 g/pot, respectively.

The concentrations of plant Ca and Mg at 0% drilling mud rate were less than at 5% and 10% which reflected the levels of the nutrients contained in the added drilling mud (Tables 1 and 4). The concentrations of Na and Cu in bermudagrass were greater than sudangrass (data not presented). The concentrations of B in bermudagrass were not different among drilling mud rates, but the concentration in sudangrass at 10% drilling mud rate was greater than 0% and 5% (Table 5). The concentrations of B in bermudagrass were smaller than sudangrass at the same drilling mud rates. The B levels were not sufficient to be toxic to plants (Nable et al., 1997).

Soil Parameters. The EC and the concentrations of extractable Ca, Mg, Na, Cu, and B increased with increased drilling mud rates (Table 6). The soil pH following the 6-wk study was approximately 1 unit greater in soil amended with 5% and 10% drilling mud compared to the 0% drilling mud rate (data not presented). The Cl concentrations in soil from the no plant, bermudagrass, and sudangrass treatments increased with increased drilling mud rates (data not presented).

The increased soil extractable concentrations of Ca, Mg, and Na (Table 6) and Cl due to drilling mud addition resulted in increased soil salinity. Increased soil salinity was measured as greater EC levels at higher mud rates (Table 6). High soil salinity has negative effects on plant growth (Dashti et al., 2009). Others in previous studies of soils amended with drilling fluids and drilling mud have concluded that increased salinity was the major factor that inhibited plant growth (Bauder et al., 2005; Miller and Pesaran, 1980; Miller et al., 1980). The increased salinity was likely the major factor in reducing bermudagrass and sudangrass growth at the 5% and 10% rates of drilling mud addition used in the current study. Soils with soluble salt levels that inhibit plant growth are known as saline soils (Brady and Weil, 2002).

The concentrations of total Ca, Na, Cu, and B in soil were significantly increased with increased drilling mud rates (Table 7). The concentrations of total Mg were not different between 0% and 5% drilling mud rates, but the amount at 10% was greater than at 0% or 5%.

In addition to salt and Na levels, high concentrations of trace elements in soil can inhibit the growth of plants (Athar and Ahmad, 2002). Addition of high levels of trace elements contained in the drilling mud can be a concern. The concentrations of Mehlich-3-extractable and total Cu and B in soil were greater at higher mud rates (Tables 6 and 7). However, the Cu levels in the plants did not increase (data not shown) in response to drilling mud addition which could be related to the increased pH levels that reduce plant availability of Cu (Nelson et al., 1984; Miller and Pesaran, 1980; Miller et al., 1980). Results from the greenhouse study did not indicate

that Cu or B added in the drilling mud amendment reduced bermudagrass or sudangrass growth.

Other Factors. In addition to the parameters reported in the current study, several other factors could reduce plant growth in drilling mud-amended soils. Barite ($BaSO_4$) is used to regulate density and viscosity of drilling fluid and control filtration (ASME, 2005). The drilling mud contains Ba, S, several trace elements, and lubricants and surfactants that would be added to the soil when the drilling mud was soil applied. Determining levels of the above parameters was beyond the scope of the present study. The Arkansas Department of Environmental Quality (ADEQ) Water Division is in charge of land application of drilling fluids and requires a land application permit (ADEQ webmaster, 2009). Land application of drilling mud in Arkansas is not currently allowed and disposal at an appropriate landfill is the common method of drilling mud disposal.

In summary, plant biomass production indicated that the addition of 5% or 10% drilling mud rates increased soil salinity and Na and Cl concentrations to levels that had negative impacts on the growth of bermudagrass and sudangrass. Future research should focus on determining suitable rates of drilling-mud application and applying agronomic management techniques to provide options for disposal of drilling mud and to protect the environment.

ACKNOWLEDGEMENTS

Financial support for this project was provided by an Honors College Undergraduate Research Grant and a Dale Bumpers College of Agricultural, Food and Life Sciences Undergraduate Research Grant. The author expresses appreciation to Ms. Faith Chu and Dr. Edward Gbur for statistical analysis of the data.

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Table 1. Mehlich-3-extractable chemical concentrations of the drilling mud and Roxana soil and total elemental analysis of the drilling mud.

Element	Drilling Mud Extractable	Drilling Mud Total	Roxana Loam Extractable
	mg/kg		
Ca	5,767	17,825	1,372
Mg	513	4,263	326
Na	4,980	5,335	13
Cu	11.0	59.1	2.3
B	4.7	13.4	0.2

Table 2. Influence of three drilling mud rates (w/w) on mean shoot and total biomass production of sudangrass and bermudagrass after the 6-week greenhouse study.

Drilling Mud Rate	Shoot Biomass	Total Biomass
-----%-----	g/pot	
0	2.47 a*	3.62 a
5	1.49 b	2.11 b
10	0.84 c	1.20 c
LSD	0.47	0.70

*Means in a column followed by the same letter are not significantly different at $P = 0.05$.

Table 3. The interaction of three drilling mud rates (w/w) and two vegetation types on root biomass production after the 6-week greenhouse study.

Vegetation	Drilling Mud Rate (w/w) 0%	5%	10%
	g/pot		
Bermudagrass	0.57 c*	0.31 c	0.22 c
Sudangrass	1.72 a	0.92 b	0.51 c

*Means followed by the same letter are not significantly different at $P = 0.05$ (LSD = 0.35).

Table 4. Influence of three drilling mud rates (w/w) on plant element concentrations after the 6-week greenhouse study.

Drilling Mud Rate	Ca	Mg
-----%-----	%	
0	0.39 b*	0.17 b
5	0.60 a	0.23 a
10	0.69 a	0.25 a
LSD	0.15	0.06

*Means in a column followed by the same letter are not significantly different at $P = 0.05$.

Table 5. The interaction of three drilling mud rates (w/w) and two vegetation types on plant boron concentration after the 6-week greenhouse study.

	Drilling Mud Rate		
Vegetation	**0%**	**5%**	**10%**
	---------- mg/kg ----------		
Bermudagrass	2.6 c*	2.6 c	3.7 c
Sudangrass	7.1 b	9.6 b	13.6 a

*Means followed by the same letter are not significantly different at $P = 0.05$ (LSD = 3.0).

Table 6. Influence of three drilling mud rates (w/w) on soil electrical conductivity (EC) and extractable element concentrations after the 6-week greenhouse study.

Drilling Mud Rate	EC	Ca	Mg	Na	Cu	B
----%----	--dS/m--	----------mg/kg----------				
0	0.094 c*	1,166 c	249 c	20 c	2.6 c	0.4 c
5	0.585 b	1,703 b	272 b	316 b	3.7 b	0.8 b
10	1.102 a	2,240 a	307 a	612 a	5.0 a	1.1 a
LSD	0.096	70	10	22	0.3	0.1

*Means in a column followed by the same letter are not significantly different at $P = 0.05$.

Table 7. Influence of three drilling mud rates (w/w) on total soil element concentration after the 6-week greenhouse study.

Drilling Mud Rate	Ca	Mg	Na	Cu	B
-----%-----	----------mg/kg----------				
0	1,631 c*	2,936 b	58 c	7.3 c	6.7 c
5	2,519 b	2,988 b	338 b	9.7 b	7.3 b
10	3,559 a	3,099 a	592 a	13.1 a	8.0 a
LSD	169	65	14	0.4	0.2

*Means in a column followed by the same letter are not significantly different at $P = 0.05$.

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The *Results and Discussion* section presents appropriate data, but not all data, in text, tables, and figures and places the findings in context with other research in the field. The discussion emphasizes new and important aspects of the research and conclusions that follow from them. Include implications and impact of the findings. Relate your findings to observations of other studies. State new hypotheses when warranted, but avoid unqualified statements not supported by your data.

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