Effect of growing location and variety on key properties of proso millet (*Panicum miliaceum*) grown as a double crop

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ABSTRACT

Despite positive agronomic and nutritional characteristics, millets are underutilized for food use in the Western hemisphere. Little is known about the end-use quality characteristics of available proso millet varieties, nor their adaptation to and performance in double crop situations in northern states. Therefore, the objective of this work was to evaluate several proso millet varieties grown in two locations for composition as well as attributes that influence processing, nutritional quality, and physiological benefits. Proso millet varieties were similar in chemical composition (total starch, protein, lipid, dietary fiber, and ash content), but were notably different in amylose to amylopectin ratios (ranging from 7.8 – 34.8% amylose). Amylose content markedly affected the pasting profile, especially for the variety with the lowest amylose content. Varieties also differed in carotenoids and hydroxycinnamic acids content as well as in antioxidant activity. Slowly digestible starch represented the major starch fraction in cooked flour, and protein digestibility was reduced to less than 50% after cooking. Overall, growing location did not have a great impact on chemical and functional characteristic, while some varietal differences were noted. The basic information provided regarding composition and functionality differences among various millet varieties, will aid in the identification of potential food applications.

INTRODUCTION

Millet species used for human consumption include pearl millet (*Pennisetum glaucum*), finger millet (*Eleusine coracana*), kodo millet (*Paspalum setaceum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italic*), little millet (*Panicum sumatrense*), and barnyard millet (*Echinochloa utilis*). Millets have traditionally been grown and consumed as a staple food in several African and Asian arid and semiarid tropical regions. Millets are short season grasses that have excellent adaptability to a wide range of climate conditions, can grow under drought conditions,, and generally have good resistance to pests and diseases (Saleh et al. 2013). At the present time, millet grains are not placed as a single important commodity in the North American and European food basket. However, the feasibility of millet production is currently being explored in Western countries, as climate change requires grain producers to find alternatives to traditional grains (Ko et al. 2012).

From a nutritional standpoint, whole-grain millets are superior to major cereals, including rice, corn and wheat, being rich sources of dietary fiber, phytochemicals, and micro-nutrients (Saleh et al. 2013). Moreover, the low glycemic index of millet-based foods (Devi et al. 2014; Ren et al. 2016), make them an ideal diet choice for diabetics (Kam et al. 2016). Finally, because millets do not contain gluten-forming proteins, they can be consumed by people with celiac disease (Taylor and Emmambux 2008).

Among the different millet varieties, proso millet (also known as true millet, common millet, hog millet, or yellow hog) is the only millet grown as a grain crop in the US, with main production in the states of Nebraska, Colorado, and South Dakota, where it is often employed as a rotational crop with winter wheat (Graybosch and Baltensperger 2009). Proso millet's short

40 growing period, low water requirements and its positive effect on wheat, corn, and sorghum
41 yield make its cultivation desirable from an agricultural standpoint (Lyon and Baltensperger
42 1995). To the best of our knowledge, millet has not been evaluated as a double crop in other
43 northern US states such as Minnesota and North Dakota, leading states in the production of
44 corn, wheat and soybeans. Part of our motivation to evaluate proso millet production in
45 Minnesota is in response to a potential future need for a second crop to follow winter annual
46 oilseeds such as camelina (*Camelina sativa* L.) and field pennycress (*Thlaspi arvense* L.). New,
47 comprehensive research programs on these and other cover and perennial crops have been
48 initiated at the University of Minnesota as part of the Forever Green Initiative
49 (forevergreen.umn.edu). Camelina and Field pennycress are harvested in June in Minnesota,
50 ideal for planting of proso millet in late June/early July.

51 Various traditional millet-based foods and beverages, such as porridge, fermented and
52 non-fermented flat breads, popping meals, and beer (Baltensperger and Cai 2004; Taylor 2004)
53 are consumed in many Asian and African countries. In the United States, proso millet is mostly
54 used as animal feed and bird seed. However, its excellent nutritional properties make it a
55 potential resource for food diversification (Cho et al. 2010). Proso millets' agronomic attributes
56 make it a good candidate to diversity cropping systems in the Upper Midwest.

57 Identification of nutritional and functional features of US grown millet must be undertaken
58 as a prerequisite for the development of millet-based products in the U.S. market. Therefore,
59 the purpose of this study was to evaluate the chemical, nutritional and functional properties of
60 proso millet flours from six varieties grown at two locations in Minnesota.

MATERIALS AND METHODS

Samples. Seed of six varieties of proso millet [Dawn (Nelson et al. 1976), Earlybird (Baltensberger et al. 1995), Horizon (Baltensberger et al. 2004), Snowbird (Robinson et al. 1973), Sunrise (Baltensberger et al. 1997), and Sunup (Nelson et al. 1990)] were graciously provided by Dr. Dipak Santra and sown at University of Minnesota Research and Outreach Center farms at Lamberton and Waseca, MN, USA. The Lamberton location was seeded June 15, 2015 onto a prepared seedbed that was fallow in spring 2015 and the 2014 crop was soybean. Four 1 m rows were harvested October 1, 2015 for the purposes of calculating grain yield and the remainder of the plot was harvested to provide additional grain for compositional analyses. The Waseca location was seeded July 1, 2015 as 7-row plots measuring 6 m long and 1.5 m wide. The entire plot was combine harvested October 9, 2015. Yield was recorded and seed test weight was measured following a standard method (AACC 55-10.01, 1999). Seeds were kindly decorticated by Bunge Limited (St. Louis, MO, USA) with a Satake TM05 laboratory mill (Satake, Houston, Texas, USA). The endosperm fraction was then aspirated using a Grain-Man model 63-115-60-vs (Grain Machine Corporation, Miami, FL, USA) to further separate the endosperm from the pericarp and germ fraction. Decortication yield was determined based on the 300 g total weight versus the fraction weights after decortication. The endosperm fraction was ground (particle size ≤ 0.25 mm) using a Cyclone Sample Mill (UD Corporation, Boulder, CO) and the flour was stored a 4° C before further analysis. A commercial decorticated white proso flour (Bunge Limited, St. Louis, MO, USA) was used as control.

Reagents. All chemicals were of reagent grade or higher. High performance liquid chromatography (HPLC) grade solvents, pepsin (3,200 - 4,500 U/mg protein), trypsin (10,000

N_α-benzoyl-L-arginine ethyl ester U/mg protein), carotenoid and hydroxycinnamic acid standards, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox, sodium carbonate, gallic acid, and Folin-Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (St. Lois, MO). Test kits for total starch, resistant starch, dietary fiber, amylose to amylopectin ratios, and glucose oxidase/peroxidase reagent for the glucose assay were purchased from Megazyme (Wicklow, Ireland). Other reagent grade chemicals were purchased from Sigma-Aldrich, and Thermo Fisher Scientific (Waltham, MA).

Chemical Composition. All analyses were carried out at least in duplicate, unless otherwise noted. A TruSpec N (Leco 165 Corporation, St. Joseph, MI) was used to measure protein content according to the Dumas method of analysis (AACC method 46-30.01). Ash was determined via dry ashing (AACC method 08-01.01), fat following Mojonnier (AACC method 30-10.01), and dietary fiber by means of an enzymatic-gravimetric method (AACC method 32-07.01). Moisture content was determined using an infrared moisture analyzer, MB45 (Ohaus, Parsippany, NJ). Total starch content and resistant starch were measured according to AACC method 76-13.01 and AACC method 32-40.01, respectively, utilizing Megazyme test kits. Amylose to amylopectin ratio was measured following the ConA precipitation procedure, also using a Megazyme test kit.

Carotenoids. Carotenoids were extracted and analyzed in duplicate following a method developed for carotenoids in grains (Abdel-Aal et al. 2007). Two main carotenoids, all-*trans* lutein and all-*trans* zeaxanthin, were quantified, using external standards, in extracts obtained from 0.5 g millet flour extracted with a total of 5 mL of water-saturated butanol. One mL aliquots were dried under nitrogen, reconstituted in 200 μL of water-saturated butanol, and centrifuged at 16,100 x g for 10 min. The supernatant was collected and analyzed by HPLC using

105 a Shimadzu system (Shimadzu Scientific Instruments, Columbia, MD) consisting of a SPD-M20A
106 PDA detector, CBM-20A communication bus module, CTO-20A column oven and two LC-20AT
107 pumps. After injecting 50 µL of extract onto a Prontosil C_{30} column (250 x 4.5 mm, 5 µm),
108 separation was performed at 35° C with a binary mobile phase system (phase A: 66/33/1
109 methanol/methyl *tert*-butylether/water; phase B: 90/10 methyl *tert*-butyl ether/methanol). The
110 elution was performed using the following gradient: 0-9 min, 0-40% B; 9-12 min, 40-90% B; 12-
111 15, hold 90% B; 15-20 min, 90-0% B; 20-25min, hold 0% B. The flow rate was 1 mL/min and
112 quantification was performed at 450 nm against external standards of lutein and zeaxanthin (>
113 98% purity).
114 **Hydroxycinnamic acids.** *Trans*-ferulic acid and *trans-para* coumaric acid were identified as the
115 main hydroxycinnamic acids in millet, and were quantified as the sum of esterified and non-
116 esterified compounds based on the extraction method reported by Vaidyanathan and Bunzel
117 (2012). Duplicates were extracted from each sample and analyzed on a Shimadzu system similar
118 to that used for the analysis of carotenoids. The HPLC separation method was based on
119 Dobberstein and Bunzel (2010) with the following modifications: a Phenomenex (Torrance, CA)
120 Luna phenylhexyl column (250 x 4.6 mm, 3.5 µm) was used with a binary gradient of 1 mM
121 trifluoroacetic acid (phase A) and 0.1 mM trifluoroacetic acid in 90/10 acetonitrile/water (phase
122 B). External standard curves were employed.
123 ***In vitro* antioxidant activity.** The Folin-Ciocalteu (FC) and the DPPH assay were performed on
124 two types of phytochemical extracts, 80% methanol extract and an alkaline hydrolysate. Freely
125 extractable millet phytochemicals were obtained by extracting twice 250 mg of millet flour with
126 5 mL of 80% methanol for 30 min at room temperature, followed by centrifugation at 1500 x g

for 5 min. Pooled supernatants were concentrated under a gentle stream of nitrogen. Two consecutive extractions, each with 5 mL of a 1:1 mixture of diethyl ether/ethyl acetate for 30 s, were performed, and the organic layers were pooled and evaporated to dryness under nitrogen. Prior to the *in vitro* antioxidant assays, the residue was reconstituted in 250 μL of 50% aqueous methanol.

After obtaining the freely extractable millet phytochemicals, the residual flour was defatted twice with 5 mL of acetone, and then dried under nitrogen. This was followed by an extraction procedure analogous to the alkaline extraction method for esterified hydroxycinnamic acids (Vaidyanathan and Bunzel 2012). Prior to performing the antioxidant assays, the residue (referred to as "alkaline hydrolysate") was reconstituted in 500 μL of 50% aqueous methanol.

The DPPH assay was conducted with reagent ratios as described previously (Ndolo and Beta 2013), using ferulic acid (100-700 μM) in methanol as a reference to express results as ferulic acid equivalents (FAE). The FC method was performed as described by Dewanto et al. (2002) using a gallic acid standard curve (30-300 μg/mL) to express results as gallic acid equivalents (GAE). Extracts obtained with 80% methanol were filtered through 0.45 μm syringe filters before recording absorbance in order to obtain clear solutions. This was not necessary for the alkaline hydrolysates.

Pasting properties. Pasting properties were measured, in duplicate, on a Micro-Viscoamylograph device (MVAG; C. W. Brabender Instruments, South Hackensack, NJ) using a ratio of 10 g flour to 100 mL water, with a correction to a moisture level of 14%, a speed of 250 rpm, and a temperature rate of 7.5 °C/min. The following temperature profile was applied:

heating from 30°C to 95°C, holding at 95°C for 5 min, cooling from 95°C to 30°C, and holding at 30°C for 1 min.

***In vitro* digestibility.** For protein and starch *in vitro* digestibility determination, millet flour was cooked in the MVAG device under the conditions reported above for the determination of pasting properties. The use of the MVAG device allowed for cooking the millet flours under controlled conditions (e.g. stirring, heating rate). Samples from two independent cooking treatments were collected at the peak viscosity (previously determined through the pasting curves), and gelatinized slurries were immediately transferred to petri dishes, and immediately immersed in liquid nitrogen, to block amylose reorganization, thereby preventing starch retrogradation. The samples were then lyophilized and ground (particle size less than 0.5 mm) with a mortar and pestle prior to use.

In vitro starch digestibility. The *in vitro* starch digestibility of the cooked samples was measured following the method developed by Englyst et al. (1992), with modifications reported by Annor et al. (2013), and a reduced sample size (0.3 g). Glucose released from starch hydrolysis was quantified using the D-glucose assay as outlined by Megazyme (Wicklow, Ireland). Available starch for enzymatic digestion was classified into rapidly digestible starch (RDS) and slowly digestible starch (SDS), where RDS = glucose released at 20 min × 0.9; and SDS = (glucose released at 120 min – glucose released at 20 min) × 0.9. RDS and SDS values are reported as percentage of available starch, i.e. the sum of RDS+SDS. The test was carried out in duplicate on two independently cooked samples.

Resistant Starch. Resistant starch (RS) content was measured in duplicate using a Megazyme test kit according to the AACC method 32-40.01.

In vitro Protein digestibility. Lyophilized cooked millet samples were subjected, in duplicate, to sequential pepsin and trypsin digestion based on the procedure reported by Shastry and John (1991) and Mokrane et al. (2010). For the digestion with pepsin, a 100 mg of sample was incubated for 2 hours at 37 °C with 5 mL of a pH 2 phosphate buffer (0.1 M) containing pepsin (2mg/mL). Digestion with pepsin was followed by another 2-hour digestion, after adjusting the pH to 7.6 and adding 5 mL of pH 7.6 phosphate buffer containing trypsin (1mg/mL). Digests were centrifuged (2,000 x g, 10 min), supernatants were collected, and pellets (undigested residues) were washed twice with 1 mL of the same buffer followed by centrifugation. Blanks were prepared using the same buffers but with no enzymes added. The amount of digested protein was measured by quantifying nitrogen in lyophilized residual pellets on a TruSpec N following the Dumas method.

Statistical analysis. One-way analysis of variance (ANOVA) was performed using R 3.1.0 (R Core Team, 2015). Differences among the means were evaluated using Tukey-Kramer Honest Significant Difference (HSD) mean comparison test ($P < 0.05$). The effect of location was assessed via a 2-sided t-test after testing for homogeneity of variances. In case of inhomogeneity, a Welch's t-test was used. The t-tests, as well as correlations and regression analysis, were performed using Excel 2010.

RESULTS AND DISCUSSION

Seed characteristics. The growing location had a significant effect on kernel characteristics (Table 1), with all varieties grown at Lamberton exhibiting higher yield, test weight, and decortication yield than those cultivated at Waseca. Considering varietal differences, Dawn grown in Lamberton exhibited the lowest yield, whereas Earlybird showed the lowest test

192 weight regardless of the growing location. Among the tested varieties, Dawn is the oldest and is
193 in fact a parent of many of the more recent proso varieties grown in the Midwest, which were
194 developed to produce higher yields (Lyon et al. 2008). Differences in kernel characteristics (i.e.
195 test weight) among varieties affect the milling process (i.e. decortication yield). In general, test
196 weight correlated positively with the decortication yield ($r = 0.77$; $p = 0.0033$). The decortication
197 yield of our proso millet varieties grown in Lamberton was similar to that reported for pearl
198 millet (Obilana and Manyasa 2002). In particular, Horizon exhibited the highest decortication
199 yield, similar to the value reported by Anderson (2014). Although decortication of millet grains
200 was found to reduce contents of certain nutrients such as fiber and minerals (Saleh et al. 2013),
201 this operation is strategic for improving millets' edible and sensory properties and for
202 enhancing the appearance of millet-based food products.
203 **Chemical composition.** Starch was the major grain constituent and did not greatly differ among
204 the samples (Table II). The impact of growing location on starch content was significant for only
205 Dawn and Earlybird grown in Lamberton. Amylose to amylopectin ratios, however, varied
206 considerably among the samples, an observation consistent with literature findings that
207 illustrated the diversity of the germplasm in proso millet (Zhang et al. 2014). Previously
208 reported amylose contents for proso millet starches span a relatively wide range with some
209 studies reporting values ranging from ca. 27-34% of total starch (Annor et al. 2014; Zhang et al.
210 2014), and others reporting less than 20% of total starch (Kumari and Thayumanavan 1998,
211 Wen et al. 2014). White proso, Sunrise from both locations, and Dawn from Waseca had the
212 highest amylose contents, which were significantly different from those of most other varieties.
213 While most varieties had greater than 20% amylose, Earlybird from both locations had less than

11%, significantly lower than that of all other varieties. Growing location had no significant impact on the amylose to amylopectin ratio.

The protein content range of the samples is in agreement with other reports (Jones et al. 1970; Ravindran 1992; Kalinova and Moudry 2006; Bagdi et al. 2011). The protein content significantly ($P < 0.01$) varied among samples, but did not vary based on location. On the other hand, fat content differed by variety as well as by location. Lamberton-grown samples had higher fat levels than samples grown in Waseca, except for Sunrise. No significant differences in total, soluble, and insoluble dietary fiber were noted. While there were few statistical differences in ash content, the extent of variation was as expected (Obilana and Manyasa 2002). Finally, for moisture content, the location alone had a significant impact. As is commonly known, moisture content is influenced by pre- and post-harvest conditions.

Carotenoids. Minnesota-grown proso millets contained two main carotenoids, lutein and zeaxanthin, in agreement with other work on proso millet (Zhang et al. 2014) and other grains such as emmer and einkorn, which contain more carotenoids than common wheat (Abdel-Aal et al. 2007). Lutein content was higher than zeaxanthin in all samples (Fig. 1). The lutein content is within the range of previously reported values by Zhang et al. (2014), who however found zeaxanthin as the dominant carotenoid (around 16 μg/g). In our commercial control, white proso, only lutein was detected, yet in a lower amount than the lowest standard used (3 μg/g). Kim et al. (2006) reported higher amount of lutein than zeaxanthin in white proso, but the reported lutein contents were about one magnitude lower than our value. These lower values for white proso are unsurprising given that carotenoids are responsible for the yellow color of

numerous plants, including cereal grains such as millet, corn and durum wheat (Abdel-Aal et al. 2013).

Most notably, Earlybird from both growing locations contained significantly ($P < 0.05$) more lutein and zeaxanthin than all other varieties. The observed values are within the range reported for proso millet grown in India (2.5-5.2 μg/g) (Asharani et al. 2010). Proso millet had higher carotenoid levels than other species, including little millet and foxtail millet, and equal to or higher than finger millet (Asharani et al. 2010). While some researchers found zeaxanthin as the main carotenoid in foxtail millet (Liu et al. 2015; Zhang and Liu 2015), others reported higher lutein levels (Shen et al. 2015; Yano et al. 2016). Shen et al. (2015) used an analytical procedure similar to ours, and their reported lutein and zeaxanthin contents are in agreement with the range we determined for Minnesota-grown proso millet. Additionally, differences among the results of various studies could be related to differences in the color of the millet kernels. Proso millet kernels vary in color from white cream, yellow, orange, red, brown to black (Taylor and Emmambux 2008). Shen et al. (2015) and Yano et al. (2016) stated that yellow foxtail was analyzed, whereas (Liu et al. (2015) and Zhang and Liu (2015) did not indicate the color. Yellow millet has been reported to contain lutein as the main carotenoid, whereas red millet contains more zeaxanthin (Howitt and Pogson 2006).

Among the cereal grains, corn as well as einkorn and emmer are known for high carotenoid contents (Abdel-Aal et al. 2007). The millet varieties analyzed in this study contained higher lutein and zeaxanthin levels than einkorn (7.41 μg lutein/g of flour and 0.94 μg zeaxanthin/g of four) and emmer (5.53 μg lutein/g of flour and 0.71 zeaxanthin/g of four). However our samples had lower levels than corn (21.92 μg lutein/g of flour and 10.91 μg

zeaxanthin/g of four) (Abdel-Aal et al. 2007). Accordingly, proso millet can be considered a better source for lutein and zeaxanthin than most other cereal grains. These carotenoids may have several physiological benefits. The most established effect is on visual function, as these two carotenoids are the two pigments present in eye tissue (Johnson 2014). This is of special importance as diabetics often suffer from retinopathy, impairing vision and quality of life (Murillo and Fernandez 2016). Observational studies suggest that lutein is associated with cardiometabolic health, including a reduced risk for cardiovascular disease, stroke and lower occurrence of the metabolic syndrome (Leermakers et al. 2016), and that lutein and zeaxanthin are among the carotenoids that lower the levels of markers for inflammation and oxidative stress (Cocate et al. 2015). The effects of carotenoids are hypothesized to be related to their role as antioxidants, which includes quenching of highly reactive singlet oxygen (Fiedor and Burda 2014), and extends to modulation of antioxidant enzymes (Hozawa et al. 2007) as well as transcription factors that regulate inflammation pathways (Cocate et al. 2015; Murillo and Fernandez 2016). However, carotenoids can be degraded during processing (Shen et al. 2015), thus further work is needed to evaluate carotenoid levels in millet-based food products.

Hydroxycinnamic acids*.* Pre-trials evaluating the presence of phenolic acids revealed very low contents in free phenolic acids, thus only total phenolic acids (free and esterified collectively) were quantified in the extracts obtained following alkaline hydrolysis. This observation was attributed mostly to the effect of decortication, which is known to reduce the content of phenolic phytochemicals (Taylor and Duodu 2015). Only two phenolic acids, the hydroxycinnamic acids *trans*-ferulic and *trans*-*p*-coumaric acid, were present in quantifiable amounts. These two compounds are known to be the main phenolic acids in millet (Dykes and

279 Rooney 2006). Contents of *trans*-ferulic acid among all varieties were around one magnitude
280 higher than those of *trans*-*p*-coumaric acid (Table III). Our values for *trans*-ferulic acid and
281 trans-*p*-coumaric acid are in the range previously reported for decorticated proso millet (Zhang
282 et al. 2014; Pradeep and Sreerama 2015). The presence of other phenolic acids, namely caffeic,
283 sinapic, syringic and chlorogenic acid, was also reported by Zhang et al. (2014) and Pradeep and
284 Sreerama (2015). However, these compounds were not detected in our samples. Pradeep and
285 Sreerama (2015) analyzed whole proso, whereas Zhang et al. (2014) analyzed decorticated
286 samples. Both groups of researchers tested different varieties than the ones used in our study,
287 which may explain differences that are not only attributed to decortication. While little work is
288 done on genomic influences with regard to phenolic acids in millet, studies on *Triticum* species
289 have shown that variety is one of the influencing factors on phenolic acid contents (Li et al.
290 2008; Gawlik-Dziki et al. 2012; Martini et al. 2015). Not every variety contains the same
291 phenolic acids at detectable levels (Moore et al. 2005). Similarly, t*rans*-ferulic and *trans*-*p*-
292 coumaric acid were found as the main phenolic acids in millets other than proso, mostly in the
293 esterified form (Chandrasekara and Shahidi 2011a; Gabaza et al. 2016). When whole proso
294 millet was compared to other whole millets, esterified *trans*-*p*- coumaric acid levels were higher
295 than esterified *trans*-ferulic acid levels (789.6 vs 245.3 μg/g). Higher *trans*-*p*-coumaric acid than
296 *trans*-ferulic acid contents were also observed for little, finger and foxtail, but not for kodo
297 millet (Chandrasekara and Shahidi 2011a).
298 ***In vitro* antioxidant activity.** Overall, there were few differences among the samples, and
299 neither variety nor growing location exerted remarkable effects. No single variety was found to
300 be superior to others in either assay (Table III). The activity of alkaline hydrolysates consistently

and as determined by both assays, DPPH and the FC assay, exceeded that of the 80% methanol extracts. This observation is in line with previous research on decorticated proso (Zhang et al. 2014). The higher activity exerted by the alkaline hydrolysate is likely due to its high content in ferulic acid, which correlated weakly (r=0.4), but significantly (p=0.044) with the DPPH assay results. Alkaline hydrolysis frees esterified phenolic acids resulting in higher content of ferulic acid in the alkaline hydrolysate than in the 80% methanol extract.

DPPH assay. Growing location did not have a significant impact on DPPH scavenging activity, except for Dawn's 80% methanol extract. In general, the observed DPPH scavenging activity of our investigated proso millet varieties were similar to those of other small millets (barnyard and foxtail) (Pradeep and Sreerama 2015), yet lower than other millet species (finger, kodo, and pearl) (Chandrasekara and Shahidi 2011a; Chandrasekara et al. 2012). Reported DPPH scavenging activity were mostly those of whole millet (Chandrasekara and Shahidi 2011b; Kim et al. 2012; Pasha et al. 2015; Kim et al. 2010), while our values were for decorticated samples. Decorticated millet is more palatable due to reduced bitterness, and potentially has longer shelf life due to removal of lipids during the process. However, decortication reduces DPPH scavenging activity of proso and other millets (Chandrasekara et al. 2012). Additionally, the antioxidant activity among millet species varied with the different *in vitro* antioxidant assays used. For example, one of the finger millet varieties investigated by Chandrasekara and Shahidi (2011a) had the highest DPPH scavenging activity among the tested millet varieties, yet had the lowest singlet oxygen scavenging ability (Chandrasekara and Shahidi 2011a). Therefore, the proso millet varieties analyzed in our study may display distinct differences in other *in vitro* antioxidant assays.

Folin-Ciocalteu (FC) assay. Although often referred to as total phenolic assay, the FC assay is considered an antioxidant assay that directly measures reducing compounds (Everette et al. 2010). The antioxidant activity of the alkaline hydrolysates in the FC assay significantly ($r=0.745$, $p<0.001$) correlated with their DPPH scavenging activity, which was not the case for the 80% methanol extracts. This indicates that the constituents of the alkaline hydrolysates (mostly ferulic acid in this case) have high reducing as well as DPPH scavenging activity, unlike those of the 80% methanol extract. Other authors have also observed a correlation between DPPH assay results and those of the FC assay when analyzing different millet species (Ragaee et al. 2006; Chandrasekara and Shahidi 2011a) or comparing pearl millet to other grains (Ragaee et al. 2006). However, another study fractionated a sorghum extract by chromatography and tested these fractions as well as the original aqueous methanol extract in the DPPH and FC assay (Kamatha et al. 2004). No correlation was found between the two assays. This finding indicates that different phytochemicals can differ in their response in the two assays and thus the most active constituent of an extract in the DPPH assay may not be the most active constituent in the FC assay. The response in the FC assay is based on an electron transfer mechanism (Huang et al. 2005), which is not limited to phenolic compounds (Everette et al. 2010; Tyl and Bunzel 2012). Thus, the difference in composition of the 80% methanol extract and alkaline hydrolysate may influence the results, in addition to possible synergistic effects.

Our values for GAE from both extracts are lower than those reported for 80% methanol extracts from whole grain millet (the authors did not report the millet species), which ranged from 275-305 μg GAE/g (Pasha et al. 2015). Millet decortication is known to cause a lower response in the FC assay (Chandrasekara et al. 2012). When antioxidant activity of various millet species was

compared, extracts obtained from whole proso with 70% acetone exhibited higher activity in the FC assay than alkaline hydrolysates (Chandrasekara and Shahidi 2011a). The same was true for extracts of 70% acetone from other small millets (finger and little millet) but not for kodo millet. As the 70% acetone extract contained less *trans*-ferulic and *trans*-*p*-coumaric acid than the alkaline hydrolysates, other components of this extract and possibly synergistic interactions among them were likely responsible for its higher response.

Higher GAE values and DPPH scavenging activity were reported for whole pearl millet than for whole barley, wheat and rye (Ragaee et al. 2006). However, systematic studies comparing the antioxidant activities and phytochemical contents in refined grains including proso and other millet varieties are, to the best of our knowledge, lacking. While the consumption of whole grains is associated with numerous health benefits, millets decortication may be an essential step to promote its consumption, as discussed above. Our results show that antioxidants were still present after decortication, and that even decorticated proso millet could contribute to overall antioxidant intake from grains.

Pasting properties. The pasting profiles of the proso millet flours are shown in Supplementary Fig. S1, while the related indices are presented in Table IV. Proso millet samples showed lower peak viscosity, breakdown, final viscosity and setback values compared to other proso varieties (Lorenz and Hinze 1976) and other millets (McDonough et al. 2000). Differences from previously reported values could be attributed to differences in starch structure, amylose/amylopectin ratio, and/or cooking conditions (i.e. starch:water ratio, heating/cooling rate, etc).

366 White proso significantly ($P < 0.05$) differed from Minnesota grown varieties in several
367 pasting parameters. White proso had higher pasting temperature, peak temperature, final
368 viscosity, and setback values compared to most Minnesota-grown proso millets. Different proso
369 millet varieties showed similar pasting profiles, with the exception of Earlybird from both
370 growing locations, which had the lowest peak temperature, final viscosity, and setback (Table
371 IV). The pasting properties of this variety are likely related to starch composition and in
372 particular the amylose/amylopectin ratio (Table II). The low amylose content of Earlybird
373 accounts for the lowest tendency to retrograde and form a gel during cooling. A positive
374 correlation between amylose content and retrogradation has been reported (Kim et al. 2012;
375 Wu et al. 2014). Additionally, for our set of samples, amylose content was positively correlated
376 with setback (r=0.88, $P < 0.01$) and final viscosity (r= 0.84, $P < 0.01$). When gelatinized starch
377 paste is subjected to cooling, the extent of increase in viscosity is mainly governed by the rapid
378 re-association of linear amylose chains via formation of a gel matrix.
379 No significant differences in breakdown viscosity among the different varieties was
380 observed. This index, measuring paste stability during the holding phase at 95°C, provides
381 information on rigidity or fragility of the swollen starch granules, and it is an indication of the
382 degree of molecular organization (Kumari and Thayumanavan 1998). Proso millet varieties
383 analyzed in this study showed similar starch granule resistance to thermal and mechanical
384 stresses, likely suggesting a similar behavior during processing (i.e. cooking).
385 Growing location did not affect pasting profiles of the different proso millet varieties,
386 with the exception of the pasting temperature of Dawn. Dawn grown in Lamberton had higher

pasting temperature than that grown in Waseca. Although the location effect is statistically significant for Dawn millet, the difference in the pasting temperature does not seem impactful.

Pasting properties are important in determining starch functionality during processing. While pasting temperature provides an indication of the minimum temperature required to cook the flour, viscosity at 95°C measures the viscosity of the hot paste, final viscosity indicates the ability of flour to form a viscous paste, and setback measures retrogradation tendency upon cooling of the cooked paste. Samples other than Earlybird, with higher hot and cold viscosity values, would be well suited for food applications that require stable thickening after heat treatment, such as soups, sauces, or puddings. However, for samples more capable of forming a firm gel after cooling (i.e. white proso), their high degree of retrogradation makes them undesirable for shelf-stable sauces and baked goods, as they could be more prone to precipitation, water separation, and staling. Varieties with higher final viscosity are more prone to both gelatinization and retrogradation, which makes them suitable for gluten-free dried pasta production without the use of additives (Marti and Pagani 2013). Among our samples, only Earlybird would likely not be well suited for this application, as it had low setbacks and final viscosity. However, other factors such as the protein profile, and use of additional ingredients such as hydrocolloids, may also influence product properties. In future studies, investigation of other factors that may influence the final product quality is warranted.

***In vitro* starch digestibility.** Despite the growing interest in millet's nutritive value and potential health benefits in recent years, these aspects have not been fully studied and utilized (Zhu 2014; Annor et al. 2017). In the current work, starch digestibility of cooked millet was performed using a well-established *in vitro* assay, which allowed the determination of

409 nutritionally important starch fractions, RDS and SDS, which are related to *in vivo* postprandial
410 glycemic responses for certain foods (Ren et al. 2016).

411 The SDS represented the major starch fraction for all samples (Table V), indicating that
412 these samples may have favorable properties in terms of glycemic response and insulin
413 demand, which have been shown to be related to the RDS:SDS ratio (Garsetti et al. 2005). No
414 significant difference was found in SDS contents of the Lamberton-grown varieties. Among the
415 Waseca-grown varieties, Dawn had significantly higher SDS levels than Earlybird and Sunup.
416 Only Snowbird was significantly affected by growing location ($p=0.034$). *In vivo* studies would
417 need to be conducted to evaluate if such differences in SDS content affect glycemic response of
418 millet-based foods.

419 The obtained RDS and SDS values were in the range to those reported in literature
420 (Annor et al. 2015), with few exceptions. Factors which influence starch digestibility include
421 differences in millet species and variety, sample preparation (flour vs whole grain; whole grain
422 vs decorticated grains; (Bora 2013), and cooking procedure (Roopa and Premavalli 2008).

423 The RS levels of the samples were evaluated separately, and all cooked samples had less
424 than 2% RS (data not shown). Earlybird grown in both locations had essentially no resistant
425 starch. Work performed on other grains suggests that low RS % may be related to its low
426 content of amylose (Table II), and thus low level of retrogradation. For instance, higher amylose
427 in rice resulted in the formation of more resistant starch after processing when compared to
428 rice having low or intermediate amylose content (Sagum and Arcot 2000).

429 ***In vitro* protein digestibility.** Similar to sorghum proteins, millet proteins are known to be less
430 digestible than the proteins in most other grains used as staple foods (Mertz et al. 1984). The

protein digestibility (Fig. 2) in cooked samples was low (<40%) for all varieties and all growing locations. Using pancreatin (which is an enzyme mix that can digest starch) instead of trypsin did not improve protein digestibility (data not shown), indicating that protein-starch interactions may not have been the reason for the low digestibility. Dawn, Horizon and Sunrise samples had the highest, whereas Earlybird, Snowbird and Sunup samples had the lowest protein digestibility, but the varietal as well as the location effect was minor compared to the effect of cooking. When raw millet flours were subject to the same digestion procedure as the cooked millet, higher percentages (between 55 and 80%; data not shown) of protein digestibility were observed. The decrease in protein digestibility upon cooking has been attributed to the formation of protein aggregates based on hydrophobic interactions (Gulati et al. 2017). This low protein digestibility is a potential limiting factor for the promotion of millet utilization in foods. Further work is therefore needed to find processing conditions that do not result in such distinct protein digestibility loss.

CONCLUSIONS

The compositional, functional and nutritional characterization of millet varieties is strategic for guiding breeding programs in selecting varieties with promising features for food use and assessing genotype x environment interactions. Regardless of growing location, proso millet varieties did not differ in starch content, their main constituent. The differences observed in protein, lipid, and ash among varieties, and between growing locations, were small in magnitude and thus require further assessment of their importance. For instance, differences in fat content may lead to different stability over storage. As for proteins, their content may not influence millet's functionality as much as the protein profile (i.e. the protein components and

453 molecular structure), and protein interactions during processing, which needs to be assessed in
454 subsequent studies. As for minor constituents, lutein and zeaxanthin were the dominant
455 carotenoids, and *trans*-ferulic and *trans*-*p*-coumaric were the main hydroxycinnamic acids. The
456 comparatively higher levels of carotenoids than in many other cereal grains, as well as the
457 presence of hydroxycinnamic acids, and the antioxidant activity in the DPPH and FC assay make
458 proso millet varieties a compelling grain in the market place. Further research is needed to
459 evaluate the antioxidant activity of millet-based food products, determine contents of
460 carotenoids and phenolics after processing, and compare proso millet to other refined flours.
461 Pasting properties were influenced by variety, and, to a lesser extent, growing location.
462 Differences in amylose content resulted in different pasting parameters. As a consequence,
463 amylose contents can be used as a selection criterion for a particular food application. The low
464 amounts of amylose observed for Earlybird would make it a good choice for applications where
465 retrogradation needs to be prevented, as in bread applications. The other proso millet varieties
466 would be better suited for products that require higher cold paste viscosity or starch gel-
467 forming abilities, desired for applications such as gluten-free dried pasta. Additionally, since SDS
468 was the main starch fraction, regardless of variety and growing location, proso millets may be
469 an attractive option for consumers who wish to lower their post-prandial glucose spike. The
470 market for gluten-free options is still growing (USDA, 2017), and millets could be established as
471 an ancient grain with a favorable nutrient composition. Expanding the market for millet beyond
472 bird seeds and animal feed, however, still requires optimization across the whole processing
473 chain. While farmers may consider yield, lodging and fit in a cropping system when selecting a
474 proso variety, determining potential for end use is crucial for an economic benefit. This work

475 provides basic information regarding composition and functionality differences among various
476 millet varieties. Further work is needed to evaluate the use of these varieties in different food
477 applications.

478 **ACKNOWLEDGMENTS**

479 The authors would like to acknowledge Brian Anderson from Bunge Limited (White Plains, NY)
480 for his help with decortication and milling of the millet grains, Erica Gardner for her help with
481 experimental work, and the Minnesota Department of Agriculture for funding the project.
482 Finally, we are grateful to the late Dr. Koushik Seetharaman for initiating the project.

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Figure captions

Fig. 1. Lutein (A) and zeaxanthin (B) content in Minnesota-grown proso millets. Error bars represent standard errors (n=2). Lowercase letters indicate significant differences among Lamberton-grown varieties, and uppercase letters represent differences among Waseca-grown varieties according to the Tukey-Kramer HSD means comparison test ($P < 0.05$). Presence of an asterisk denotes a significant difference between the two locations within one variety.

Fig. 2. Protein digestibility in cooked millet samples. Error bars represent standard errors (n=2). Lowercase letters indicate significant differences among Lamberton-grown samples, and uppercase letters represent differences among Waseca-grown samples according to the Tukey-Kramer HSD means comparison test ($P \leq 0.05$). Presence of an asterisk denotes a significant difference between the two locations within one variety.

Fig. S1. Pasting profiles of millet varieties grown in different locations. (A) Lamberton; (B) Waseca.

Table I. Kernel characteristics of proso millet varieties grown in 2015 in two Minnesota locations.

	Yield (kg/ha)		Test Weight (lb/bu)		Decortication Yield (%)	
	Lamberton	Waseca	Lamberton	Waseca	Lamberton	Waseca
Dawn	1776^{b,*}	1280^{A}	71.7^{a,*}	68.3^{AB}	74.6	68.0
Earlybird	3536^{a,*}	960^{A}	69.7^{b,*}	65.5^{B}	71.7	69.2
Horizon	3247^{a,*}	1629^{A}	72.4^{a,*}	69.2^{A}	75.5	70.0
Snowbird	3551^{a,*}	1555^{A}	72.5^{a,*}	68.5^{A}	72.7	68.4
Sunrise	3749^{a,*}	1639^{A}	72.9^{a,*}	69.2^{A}	73.3	69.0
Sunup	3042^{ab,*}	1531^{A}	72.4^{a,*}	68.8^{A}	70.7	69.0

Means (n=4) followed by lowercase letters indicate significant differences among Lamberton-grown varieties, whereas uppercase letters represent differences among Waseca-grown varieties according to the Tukey-Kramer HSD means comparison test ($P \leq 0.05$). Presence of an asterisk denotes a significant difference between the two locations within one variety.

Table II. Chemical composition of millet varieties grown in Minnesota at two locations.

	Starch g/100g flour (db)		Amylose g/100g starch (db)		Protein g/100g flour (db)		Fat g/100g flour (db)		Insoluble fiber g/100g flour (db)		Soluble fiber g/100g flour (db)		Ash g/100g flour (db)		Moisture g/100g flour (wb)	
	Lamber-ton	**Waseca**	**Lamber-ton**	**Waseca**	**Lamber-ton**	**Waseca**	**Lamber-ton**	**Waseca**	**Lamber-ton**	**Waseca**	**Lamber-ton**	**Waseca**	**Lamber-ton**	**Waseca**	**Lamber-ton**	**Waseca**
Dawn	73.05^{b*}	77.13^{A}	23.2^{c}	31.0^{ABC}	12.98^{a*}	10.93^{A}	3.27^{ab*}	2.31^{BC}	2.83^{a}	2.19^{A}	0.45^{a*}	1.12^{A}	1.23^{a*}	0.99^{B}	10.80^{a*}	8.66^{B}
Earlybird	72.80^{b*}	75.95^{A}	7.8^{d}	10.2^{D}	11.60^{ab}	11.66^{A}	3.66^{a*}	2.97^{A}	1.08^{a}	1.51^{A}	0.56^{a}	0.79^{A}	1.17^{ab*}	1.07^{A}	10.73^{a*}	8.45^{B}
Horizon	75.88^{a}	78.37^{A}	25.1^{c}	26.0^{BC}	10.92^{bc}	9.82^{A}	3.39^{ab*}	2.82^{AB}	1.59^{a}	1.40^{A}	0.87^{a}	0.86^{A}	1.17^{ab}	0.87^{C}	11.50^{a*}	8.66^{B}
Snowbird	80.14^{a}	75.42^{A}	28.9^{bc}	25.6^{C}	9.88^{c}	10.11^{A}	2.37^{c*}	1.84^{C}	1.01^{a}	0.82^{A}	0.85^{a}	0.86^{A}	1.02^{abc*}	0.84^{C}	11.23^{a*}	8.60^{B}
Sunrise	76.94^{a}	75.31^{A}	31.7^{ab}	35.7^{AB}	10.86^{bc}	11.29^{A}	1.99^{c*}	2.25^{C}	2.13^{a*}	0.89^{A}	0.94^{a}	0.43^{A}	0.86^{abc}	0.90^{C}	10.78^{a*}	9.01^{B}
Sunup	74.17^{a}	74.47^{A}	22.9^{c}	24.4^{C}	10.31^{bc}	10.02^{A}	3.02^{b}	3.16^{A}	1.04^{a}	1.68^{A}	0.71^{a}	0.73^{A}	0.78^{bc}	0.91^{C}	11.12^{a*}	9.17^{AB}
White proso	$76.77^{a,A}$		$36.5^{a,A}$		$11.4^{abc,A}$		$2.93^{b,AB}$		$1.57^{a,A}$		$0.35^{a,A}$		$0.68^{c,D}$		$9.87^{b,A}$	

Means (n=2 for starch, protein, insoluble and soluble fiber, and ash; n=3 for fat; n=4 for amylose) followed by lowercase letters indicate significant differences among Lamberton-grown varieties, whereas uppercase letters represent differences among Waseca-grown varieties according to the Tukey-Kramer HSD means comparison test ($P < 0.05$). Presence of an asterisk denotes a significant difference between the two locations within one variety.

Table III. *In vitro* antioxidant activity and hydroxycinnamic acid content of millet flour extracts.

Variety	***p*-coumaric acid μg/g flour (d.b.)**		**Ferulic acid μg/g flour (d.b.)**		**Ferulic acid equivalents in the DPPH assay μmol ferulic acid/g flour (d.b.)**				**Gallic acid equivalents in the Folin-Ciocalteu assay μg gallic acid/g flour (d.b.)**			
	(Alkaline hydrolysate)		(Alkaline hydrolysate)		80% methanol extract		Alkaline hydrolysate		80% methanol extract		Alkaline hydrolysate	
	Lamberton	Waseca	Lamberton	Waseca	Lamberton	Waseca	Lamberton	Waseca	Lamberton	Waseca	Lamberton	Waseca
Dawn	10.88^{b}	8.36^{B}	190^{a}	166^{AB}	0.100^{ab*}	0.160^{AB}	0.628^{a}	0.558^{A}	57.4^{a}	77.7^{A}	163.8^{a}	159.9^{AB}
Earlybird	25.23^{a}	21.96^{A}	188^{a}	196^{A}	0.127^{a}	0.201^{A}	0.495^{ab}	0.560^{A}	58.0^{a}	57.5^{AB}	150.8^{a*}	170.3^{A}
Horizon	9.67^{bc}	6.08^{CD}	177^{abc}	176^{AB}	0.146^{a}	0.142^{AB}	0.528^{ab}	0.455^{AB}	38.3^{ab}	39.2^{C}	153.9^{a}	157.1^{AB}
Snowbird	7.18^{cd*}	3.36^{E}	181^{ab}	192^{AB}	0.105^{ab}	0.102^{BC}	0.480^{b}	0.444^{AB}	36.2^{ab}	41.2^{BC}	147.3^{a*}	133.9^{B}
Sunrise	9.33^{bc*}	6.84^{BC}	165^{bc*}	126^{B}	0.117^{ab}	0.145^{AB}	0.532^{ab}	0.376^{B}	30.8^{b}	39.9^{C}	151.9^{a}	139.6^{B}
Sunup	6.49^{cd}	6.83^{BC}	192^{a}	164^{AB}	0.089^{ab}	0.167^{AB}	0.607^{ab}	0.551^{A}	47.1^{ab}	52.2^{BC}	158.3^{a}	161.9^{AB}
White proso	$4.42^{d,DE}$		$159^{c,AB}$		$0.052^{b,C}$		$0.497^{ab,AB}$		$37.6^{ab,C}$		$147.9^{a,AB}$	

Means (n=2) followed by lowercase letters indicate significant differences among Lamberton-grown varieties, whereas uppercase letters represent differences among Waseca-grown varieties according to the Tukey-Kramer HSD means comparison test ($P \leq 0.05$). Presence of an asterisk denotes a significant difference between the two locations within one variety.

Table IV. Pasting properties of proso millet varieties grown in Minnesota at two locations.

	Pasting Temperature (°C)		Peak Viscosity (BU)		Peak Temperature (°C)		Breakdown (BU)		Final Viscosity (BU)		Setback (BU)	
	Lamberton	**Waseca**	**Lamberton**	**Waseca**	**Lamberton**	**Waseca**	**Lamberton**	**Waseca**	**Lamberton**	**Waseca**	**Lamberton**	**Waseca**
Dawn	$74.5^{ab,*}$	73.0^{B}	213.0^{a}	197.5^{A}	90.2^{ab}	89.7^{AB}	71.0^{ab}	68.0^{A}	426.5^{a}	365.5^{B}	276.5^{ab}	237.0^{B}
Earlybird	72.7^{b}	73.1^{B}	209.0^{a}	191.0^{A}	78.7^{c}	78.7^{C}	74.0^{ab}	66.5^{A}	269.0^{c}	259.5^{C}	134.0^{c}	135.0^{C}
Horizon	73.6^{b}	73.1^{B}	224.5^{a}	206.0^{A}	89.2^{ab}	90.1^{AB}	79.0^{a}	68.0^{A}	413.0^{ab}	388.0^{B}	267.5^{ab}	250.5^{B}
Snowbird	74.0^{b}	73.2^{B}	238.5^{a}	193.0^{A}	88.3^{b}	89.6^{AB}	89.5^{a}	63.0^{A}	428.0^{a}	366.0^{B}	279.0^{ab}	236.0^{B}
Sunrise	73.3^{b}	73.0^{B}	$217.0^{a,*}$	201.0^{A}	88.5^{b}	89.3^{B}	76.0^{ab}	69.0^{A}	409.0^{ab}	378.0^{B}	268.0^{ab}	246.0^{B}
Sunup	73.7^{b}	73.7^{B}	203.0^{a}	194.0^{A}	88.9^{b}	90.2^{AB}	69.5^{ab}	55.5^{AB}	353.5^{b}	384.0^{B}	220.0^{b}	245.5^{B}
White proso	$76.2^{a,A}$		$226.5^{a,A}$		$91.3^{a,A}$		$47.0^{b,B}$		$507.5^{a,A}$		$328.0^{a,A}$	

Pasting temperature: temperature of initial viscosity increase; maximum viscosity: maximum viscosity achieved during the heating cycle; peak temperature: temperature at maximum viscosity; breakdown: index of viscosity decrease during the holding period, corresponding to peak viscosity minus the viscosity after the holding period at 95°C; final viscosity: viscosity achieved at the end of the test at 30°C; setback: index of the viscosity increase during cooling corresponding to the difference between final viscosity and the viscosity reached after the first holding period at 95°C . Means (n=2) followed by lowercase letters indicate significant differences among Lamberton-grown varieties, whereas uppercase letters represent differences among Waseca-grown varieties according to the Tukey-Kramer HSD means comparison test ($P < 0.05$). Presence of an asterisk denotes a significant difference between the two locations within one variety.

Table V. Starch digestibility fractions in cooked proso millet.

	Rapidly digestible starch (g/100g available starch)		**Slowly digestible starch** (g/100 g available starch)	
	Lamberton	Waseca	Lamberton	Waseca
Dawn	45.6^{a}	36.9^{B}	54.4^{a}	63.1^{A}
Earlybird	45.7^{a}	51.8^{A}	54.3^{a}	48.2^{B}
Horizon	36.8^{a}	40.2^{AB}	63.2^{a}	59.8^{AB}
Snowbird	43.3^{a*}	49.0^{AB}	56.8^{a*}	51.0^{AB}
Sunrise	46.8^{a}	46.7^{AB}	53.2^{a}	53.3^{AB}
Sunup	45.9^{a}	52.2^{A}	54.1^{a}	47.8^{B}
White proso	$44.0^{a,AB}$		$56.0^{a,AB}$	

Means (n=3) followed by lowercase letters indicate significant differences among Lamberton-grown varieties, whereas uppercase letters represent differences among Waseca-grown varieties according to the Tukey-Kramer HSD means comparison test ($P < 0.05$). Presence of an asterisk denotes a significant difference between the two locations within one variety.