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Abstract

Oats (*Avena sativa* L.) have received significant attention for their positive and consistent health benefits when consumed as a whole grain food, attributed in part to mixed-linkage (1-3,1-4)-β-d-glucan (referred to as β-glucan). Unfortunately, the standard enzymatic method of measurement for oat β-glucan is costly and does not provide the high-throughput capability needed for plant breeding in which thousands of samples are measured over a short period of time. The objective of this research was to test a microenzymatic approach for high-throughput phenotyping of oat β-glucan. Fifty North American elite lines were chosen to span the range of possible values encountered in elite oats. Pearson and Spearman correlations (*r*) ranged from 0.81 to 0.86 between the two methods. Although the microenzymatic method did contain bias compared with the results for the standard streamlined method, this bias did not substantially decrease its ability to determine β-glucan content. In addition to a substantial decrease in cost, the microenzymatic approach took as little as 6% of the time compared with the streamlined method. Therefore, the microenzymatic method for β-glucan evaluation is an alternative method that can enhance high-throughput phenotyping in oat breeding programs.

Disciplines

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Comments

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Microenzymatic Evaluation of Oat (*Avena sativa* L.) β-Glucan for High-Throughput Phenotyping

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ABSTRACT

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Oats (*Avena sativa* L.) have received significant attention for their positive and consistent health benefits when consumed as a whole grain food, attributed in part to mixed-linkage (1-3,1-4)-β-D-glucan (referred to as β-glucan). Unfortunately, the standard enzymatic method of measurement for oat β-glucan is costly and does not provide the high-throughput capability needed for plant breeding in which thousands of samples are measured over a short period of time. The objective of this research was to test a microenzymatic approach for high-throughput phenotyping of oat β-glucan. Fifty North American elite lines were chosen to span the range of possible values encountered in elite oats. Pearson and Spearman correlations (*r*) ranged from 0.81 to 0.86 between the two methods. Although the microenzymatic method did contain bias compared with the results for the standard streamlined method, this bias did not substantially decrease its ability to determine β-glucan content. In addition to a substantial decrease in cost, the microenzymatic approach took as little as 6% of the time compared with the streamlined method. Therefore, the microenzymatic method for β-glucan evaluation is an alternative method that can enhance high-throughput phenotyping in oat breeding programs.

Mixed-linkage (1-3,1-4)-β-D-glucan (referred to as β-glucan) is a major hemicellulose in cereal grain endosperm that makes up about 70% of the cell walls in oats and barley (Carpita 1996). The importance of β-glucan is most well known in oats (*Avena sativa* L.) and barley (*Hordeum vulgare* L.), for which breeders carry out selection in the positive and negative directions, respectively. Increased oat β-glucan has been a major target for breeding operations because of its positive and consistent health implications when oats are consumed as a whole grain. Because of the major health claims associated with consumption of oat β-glucan, it has been a major target for oat breeding programs around the world. Various research studies have attempted to uncover the genetics behind oat β-glucan by identification of genome locations associated with the trait (Chernyshova et al 2007; Newell et al 2012; Asoro et al 2013a) and application of processes to increase efficiency of selection for the trait (Asoro et al 2013b). From a health standpoint, β-glucan has been demonstrated to improve health with respect to blood pressure (Keenan et al 2002), diabetes (Jenkins et al 2002), cholesterol (Braaten et al 1994), and immune response (Estrada et al 1997), all of which are important given the increases in human health cases related to diet over the past few decades.

Various approaches have been developed for evaluation of β-glucan in cereals, including calcofluor-, antibody-, and enzymatic-based methods. The calcofluor approach relies on an increase in fluorescence of the dye calcofluor when it forms a complex with β-glucan (Manzanares and Sendra 1996). The antibody-based method relies on monoclonal antibodies with cross-reactivity to β-glucan applied in an enzyme-linked immunosorbent assay for routine application (Rampitsch et al 2003). More recently, evaluation of oat β-glucan is most often done enzymatically following an approach first described by McCleary and Glennie-Holmes (1985). All of these methods are routinely used for evaluation of β-glucan, but none provide utility for high-throughput capacity requirements. The enzymatic evaluation of oat β-glucan was also applied successfully to other end uses, including in the brewing industry (McCleary and Nurthen 1986). Since then the enzymatic approach has been modified (McCleary and Codd 1991) and sold as a streamlined mixed-linkage β-glucan assay kit (Megazyme International, Bray, Ireland) that allows higher throughput capacity. The streamlined mixed-linkage β-glucan kit has been widely accepted as the method of choice across disciplines; thus, recent work on methodology of β-glucan is limited at best. Although this method works well with respect to accuracy and precision, its throughput when thousands of samples need to be evaluated over a short time frame render it ineffective for breeding applications. For plant breeding, where selections are made based purely on phenotypic rank, the importance of precision outweighs that of accuracy, leaving speed the major hindrance of the streamlined method for β-glucan evaluation in the breeding context. Various studies have demonstrated the effects of high β-glucan lines compared with typical β-glucan lines on human health (Kim and White 2009, 2012). These studies exhibit both the effectiveness of the streamlined β-glucan kit and the positive value of selection for increased β-glucan lines. The speed of the evaluation process for β-glucan concentration is of great importance for breeding, given that the potential gain in β-glucan concentration is proportional to the number of lines evaluated. Thus, a method of evaluation that can maintain precision and enable high-throughput phenotyping would be valuable for breeding for increased β-glucan in oats. The objectives of this research are to 1) evaluate a microenzymatic method to measure oat β-glucan, 2) compare the microenzymatic method with the standard streamlined method, and 3) determine the repeatability and error of the two methods.

MATERIALS AND METHODS

Microenzymatic β-Glucan Assay. Megazyme's mixed-linkage β-glucan kit (AACC International Approved Method 32-23.01; AOAC Method 995.16; EBC Methods 3.11.1, 4.16.1 and 8.11.1; ICC Standard Method No. 166) was used with modification to the streamlined method at 1/10 of scale. Approximately 0.5–3 g of groats were ground in 15 mL polycarbonate grinding vials with two stainless steel grinding balls (OPS Diagnostics, Lebanon, NJ, U.S.A.) for 3 min at maximum speed. A Talboys high-throughput

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homogenizer (Henry Troemner LLC, Thorofare, NJ, U.S.A.) was used for grinding. Flour sample (8–12 mg) was weighed into 1.2 mL strip tubes arranged in a 96-well plate. Each sample was wetted with 20 μL of aqueous ethanol (50% v/v) to aid dispersion, followed by addition of 400 μL of sodium phosphate buffer (20mM, pH 6.5). The contents were stirred on a vortex mixer until they were well dispersed in solution. The plate was then placed in a boiling water bath (100°C) and incubated for 15 s and immediately stirred on a vortex mixer until in solution. This incubation and stirring was repeated for a total of three times. Following three incubation periods of 15 s at 100°C, the plate was incubated at 50°C for 5 min. Lichenase (20 μL, 1 U) was added to each sample and stirred on a vortex mixer until in solution and incubated at 50°C for 1 h. The plate was removed from the incubator every 10–15 min and mixed on a vortex mixer. Following the 1 h incubation, 500 μL of sodium acetate buffer (200mM, pH 4.0) was added and stirred on a vortex mixer. The plate was allowed to equilibrate at room temperature for 5 min and centrifuged at 1,000 × *g* for 10 min.

Each sample (10 μL) was then dispensed into two 96-well plate reader plates. To the first plate (the measurement reaction), 10 μL of β-glucosidase (0.02 U) in sodium acetate buffer (10mM, pH 4.0) was added to each well. To the second plate (the reaction blank), 10 μL of sodium acetate buffer (50mM, pH 4.0) was added to each sample. For both plates, the measurement reaction and reaction blank were incubated at 50°C for 10 min. Glucose oxidase–peroxidase (GOPOD) reagent (300 μL) was added to each well and incubated at 50°C for 20 min. For each measurement reaction plate, one well with 10 μL of D-glucose standard (1.0 mg/mL) was included with 300 μL of GOPOD reagent. Finally, the absorbance of the measurement reaction and reaction blank plates was determined at 510 nm with a plate reader.

The percent β-glucan on a wet weight basis was determined by the following equation:

$$\beta\text{-glucan (\%)} = \Delta A \times F \times 94 \times (1/1{,}000) \times (100/W) \times (162/180)$$

where ΔA is the absorbance of the reaction minus the absorbance of the reaction blank, F = 10 μg/absorbance of 10 μg of D-glucose, and W is the weight of the sample (as is moisture basis).

It is important to point out that the percent β-glucan was only calculated on a wet weight basis, ignoring moisture content, for the microenzymatic method.

Streamlined β-Glucan Assay. The streamlined β-glucan assay was implemented in accordance with Megazyme's mixed-linkage β-glucan streamlined method. The percent moisture content was calculated for the streamlined method, but only the percent β-glucan on a wet weight basis (ignoring moisture content) was used for comparison with the microenzymatic method. This was done so that even comparisons could be made between the two methods. The β-glucan content on a dry and wet weight basis was highly correlated (0.999), and percent moisture content for the samples analyzed covered a range of only 2% moisture.

Genetic Material. Oat lines (444) were grown in 2009 in Ames, Iowa, at the Iowa State University Agronomy Farm. β-Glucan evaluations were initially conducted as part of a large-scale genomewide association study (Asoro set al 2013a). Best linear unbiased predictions (BLUPs) for each line were used to choose a smaller group of 50 lines that spanned the range of β-glucan values commonly encountered in elite oats. The BLUPs (plus intercept) for the 444 lines ranged from 1.9 to 7.2% β-glucan and consisted of elite material from the United States and Canada. The 50 lines evaluated in this study were chosen by sorting the lines for percent β-glucan and sampling the lines at even intervals with respect to β-glucan, excluding outliers at the tails of the distribution. Thus, the range of BLUPs for the lines resulted in a nearly uniform distribution from 2.4 to 6.3% β-glucan. A sample (approximately 10 g) of grain harvested from each line was split into two samples; the first sample was evaluated with Megazyme's streamlined method, and the second was used for the microenzymatic procedure, as indicated earlier. Only the first field replicate was used in the assays for this study; this was important to ensure that differences in β-glucan were not simply because of field effects but were in fact inherent to the lines themselves.

Statistical Analysis. Each sample was evaluated once with the Megazyme streamlined method and twice with the microenzymatic method for which all β-glucan comparisons were evaluated on a wet weight basis. Results for the streamlined method are referred to as S1. An additional 20 random samples were evaluated with the streamlined method and are referred to as S2. Only 20 random samples were included in S2 because of the long time required for the streamlined analysis. The microenzymatic method was applied as a completely randomized design with two replicates in which plates were considered replicates. In total, testing for a significant correlation between the two methods was done at three levels. The first two correlation tests were between each microenzymatic replicate, or plate, and the streamlined method. These tests will be referred to as P1:S1 and P2:S1 for the first and second plates, respectively. Each plate included a D-glucose standard, and for P1 and P2 the percent β-glucan was calculated by using the D-glucose absorbance for each plate individually. The third correlation test was between the least squares (LS) means for the microenzymatic method across plates and the streamlined method; this is referred to as LS:S1. All models were analyzed with R computer software (R Project for Statistical Computing, Vienna, Austria, www.R-project.org) using the AOV and LM functions. The fixed-effects model used for ANOVA was y = mean + plate + line + error where y is the response, plate is the effect of plate, and line is the line effect. The mean D-glucose standard across plates was used to calculate the response values for LS, because differences between plates should be accounted for in the plate effect. Testing for significant correlation, both Pearson and Spearman, between the microenzymatic method and the streamlined method was completed in R with the COR.TEST function for P1:S1, P2:S1, and LS:S1.

An alternative approach that explores the relative bias when comparing measurement methods was also implemented. First explained by Altman and Bland (1983) and later reviewed by Ludbrook (2002), the method of differences evaluates two types of bias, fixed and proportional. Fixed bias is because of the change in mean value across all measurements and can be thought of as accuracy. Proportional bias is because of differences across measurements correlated to the level of analyte and is related to precision. The method plots, for each paired measurement, the mean between methods versus the difference. If the mean for all points is significantly different from zero, it is an indication of fixed bias. On the other hand, if the slope of the regression is significantly different from zero, it is concluded that there is proportional bias. The method of differences was applied to three comparisons: P1:S1, P2:S1, and LS:S1.

Repeatability is relevant to comparing methods because the repeatability of two methods of measurement limits the amount of agreement that is possible (Bland and Altman 2010). Therefore, the repeatability for each method was calculated. The mixed-effects model was y = mean + replicate + line + error, where y is the response, replicate is the fixed effect of replicate, and line is the random line effect. The model was implemented twice, once for the microenzymatic method and once for the streamlined method. For the microenzymatic method, P1 and P2 were used as the first and second replicates, respectively. For the streamlined method, 20 of the lines were chosen randomly and analyzed for a second replicate, referred to as S2. Thus, the sample sizes for the two models were 50 and 20 for the microenzymatic and streamlined methods, respectively. The repeatability, or intraclass correlation, was calculated from the variance estimates for the random effects such that repeatability =

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Var(line)/[Var(line) + Var(error)]. The error variance is also reported. Mixed-effects models were implemented in the LME4 package within the R software. Repeatability and error variance for these two comparisons are referred to as P1:P2 and S1:S2 for the microenzymatic and streamlined methods, respectively. In addition, the Pearson and Spearman correlations were tested for significance for P1:P2 and S1:S2. All data is reported in Table I, including the lines tested and β-glucan values for both the microenzymatic and streamlined methods.

RESULTS

Results for all β-glucan evaluations are presented in Table I, including values for each line with regard to P1, P2, LS, S1, and S2, along with standard deviations. For the microenzymatic method, P1, P2, and LS were 2.85–6.93, 3.08–6.98, and 2.97–6.96% β-glucan, respectively. For the streamlined method, the β-glucan concentration was 3.03–8.31 and 3.58–6.35% β-glucan for S1 and S2, respectively. Overall, values for the microenzymatic method

TABLE I
Data for the Evaluation, Including the Arbitrary Line Names, Entry (Variety) Names, and Results for the Microenzymatic and Streamlined Methods[a]

		Microenzymatic Method			Streamlined Method					
		Wet Weight (% BG)			Wet Weight (% BG)		Wet Weight (%BG SD)		Moisture Content (%)	
Line	Entry	P1	P2	LS	S1	S2	S1 SD	S2 SD	S1	S1 SD
AM001	05RAT22	4.55	4.41	4.48	5.66	5.34	0.27	0.10	9.75	0.09
AM005	87Ab5632	5.19	5.06	5.12	7.02	6.10	0.23	0.18	9.69	0.01
AM008	97Trp77	4.24	4.57	4.41	4.43	NA	0.29	NA	10.33	0.18
AM020	Andrew CIav4170	5.02	5.35	5.19	6.10	5.73	0.14	0.04	10.25	0.08
AM043	HiFi PI633006	6.85	6.50	6.67	7.44	NA	0.28	NA	9.95	0.08
AM049	IA00059-9-1	3.84	4.28	4.06	4.30	4.42	0.23	0.35	10.15	0.09
AM057	IA02130-2-2	5.40	5.60	5.50	6.49	6.35	0.42	0.06	9.96	0.01
AM059	IA03144-7	5.37	5.69	5.53	6.09	NA	0.12	NA	9.66	0.07
AM060	IA03146-6	6.18	6.17	6.17	8.31	NA	0.67	NA	9.51	0.03
AM065	IA91462-4-1-6	5.48	5.28	5.38	5.81	NA	0.10	NA	10.04	0.08
AM067	IA91524-1-5-1	5.53	5.31	5.42	7.15	NA	0.91	NA	8.94	0.17
AM069	IA93359-3	5.02	4.92	4.97	5.64	NA	0.26	NA	9.60	0.02
AM072	IA94190-10-1	5.64	5.60	5.62	5.68	5.99	0.20	0.48	8.62	0.19
AM073	IA95029-3-2	5.34	5.30	5.32	5.85	5.61	0.17	0.05	9.11	0.01
AM074	IA95111	6.93	6.98	6.96	7.49	NA	0.14	NA	10.49	0.11
AM075	IA95148-3-5	5.85	5.37	5.61	6.63	NA	0.20	NA	9.37	0.09
AM078	IA95258	6.38	6.89	6.64	7.80	NA	0.19	NA	9.60	0.11
AM083	IA99072-2	4.51	4.50	4.51	4.86	NA	0.16	NA	10.61	0.13
AM093	IL00-205	4.12	4.50	4.31	4.62	4.77	0.15	0.06	10.49	0.17
AM114	IL91-9023	4.70	5.40	5.05	5.87	NA	0.02	NA	10.42	0.02
AM127	IL95-8217	4.85	5.13	4.99	5.85	5.40	0.25	0.29	9.65	0.18
AM133	IL97-6202	5.24	5.76	5.50	5.88	6.18	0.00	0.25	8.94	0.04
AM153	LAO-793-NZ-055	4.86	4.54	4.70	5.86	NA	0.57	NA	9.20	0.04
AM160	Maida	3.72	3.79	3.75	4.33	NA	0.22	NA	9.37	0.16
AM163	MN00226	5.25	5.45	5.35	5.71	4.92	0.10	0.43	10.08	0.14
AM171	MN02231	4.32	4.27	4.30	5.10	4.88	0.32	0.18	9.97	0.08
AM185	MN93272	3.66	3.34	3.50	4.94	NA	0.02	NA	9.25	0.06
AM188	MN94238	4.57	4.72	4.65	5.91	NA	0.06	NA	8.97	0.08
AM193	MN96220	5.52	5.44	5.48	5.49	NA	0.33	NA	9.79	0.16
AM195	MN97139	3.31	3.26	3.28	3.92	3.58	0.28	0.14	9.61	0.14
AM198	MN97234	3.75	4.26	4.01	4.65	4.62	0.02	0.22	10.11	0.27
AM211	MO8715	5.10	4.92	5.01	5.33	NA	0.03	NA	10.13	0.11
AM231	ND030349	4.01	4.94	4.48	5.84	NA	0.04	NA	9.66	0.03
AM237	ND930376	4.31	4.71	4.51	6.74	NA	0.18	NA	9.28	0.26
AM239	ND931475	4.66	4.70	4.68	5.60	5.37	0.13	0.03	10.45	0.11
AM249	ND970651	4.23	3.92	4.07	5.14	4.66	0.36	0.02	9.90	0.13
AM255	ND990118	3.42	4.19	3.81	5.14	NA	0.47	NA	9.89	0.00
AM269	OA1029-21	4.91	4.48	4.69	4.69	4.93	0.20	0.14	9.71	0.14
AM311	OT378	4.62	4.87	4.75	4.06	NA	0.75	NA	8.91	0.11
AM355	SA98824	4.69	4.97	4.83	5.57	5.41	0.08	0.16	9.49	0.04
AM367	SD011197	2.85	3.08	2.97	3.03	NA	0.12	NA	10.23	0.07
AM385	SD041016	3.20	3.33	3.27	3.89	NA	0.05	NA	9.11	0.03
AM404	SD97575-38-154	3.62	4.12	3.87	3.77	NA	0.56	NA	9.25	0.06
AM405	SD97575-5-29	4.12	4.47	4.29	4.94	NA	0.14	NA	9.66	0.06
AM416	Spurs PI638523	4.02	3.84	3.93	4.34	NA	0.18	NA	10.49	0.01
AM424	W00403	4.46	4.95	4.71	5.07	5.10	0.09	0.18	9.77	0.09
AM429	W96391	4.18	4.23	4.21	4.58	NA	0.14	NA	10.52	0.06
AM430	W99040	3.96	3.95	3.96	4.54	NA	0.07	NA	8.89	0.17
AM442	WIX8718-1	4.02	4.14	4.08	4.39	4.62	0.34	0.01	9.52	0.16
AM443	WIX8787-2	5.27	5.09	5.18	6.22	NA	0.47	NA	9.24	0.04
Minimum	…	2.85	3.08	2.97	3.03	3.58	0.00	0.01	8.62	0.00
Maximum	…	6.93	6.98	6.96	8.31	6.35	0.91	0.48	10.61	0.27
Mean	…	4.70	4.81	4.75	5.48	5.20	0.23	0.17	9.71	0.10

[a] BG = β-glucan; P1 and P2 refer to the first and second replicates of the microenzymatic method; LS refers to the least squares means for P1 and P2; and S1 and S2 refer to the first and second replicates of the streamlined method. Percent BG on a wet basis for the microenzymatic and streamlined methods was used as the primary attribute for comparison. Additional information for the streamlined method includes the percent BG standard deviations (% BG SD) on a wet weight basis and the means and standard deviations for the percent moisture content of the streamlined method.

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were smaller than those for the streamlined method of evaluation. Testing for significant correlations between the two methods was done at three levels: P1:S1, P2:S1, and LS:S1. Pearson correlations were highly significant ($P \leq 1 \times 10^{-13}$) at all levels and ranged from 0.84 to 0.86 (Table II, Fig. 1). As expected, LS:S1 showed a higher level of correlation than did P1:S1 and P2:S1, most likely because of reduced measurement error in the microenzymatic method resulting from replication. Although the high Pearson correlation between methods is important, it is the Spearman correlation that gives information about changes in selections in plant breeding that are based on phenotypic rank. Spearman correlations were also highly significant ($P \leq 1 \times 10^{-12}$) and ranged from 0.81 to 0.83 for P1:S1, P2:S1, and LS:S1, respectively (Table II).

Because the method of correlation has been described as a poor approach for comparing methods of measurement (Ludbrook 2002), the method of differences was also implemented. Unlike exploring correlation, the method of differences attempts to delineate fixed and proportional bias associated with the method of measurement. For all of the comparisons, the method of differences identified both fixed and proportional bias (Fig. 2). For all three comparisons, the means of the differences and the slopes of the regression lines were significantly greater than zero. A closer look at the proportional bias shows that there is a larger bias at higher levels of β-glucan content. This bias can be seen by the positive relationship between the mean and difference of pairwise measurements. Repeatability, calculated from the variance of the random effects for the two linear models, was similar between the two methods. Repeatability for the streamlined (S1:S2) and microenzymatic (P1:P2) methods was 0.93 and 0.87, respectively. These values indicate that the repeatability is high when measured for each of the methods. More importantly, it is crucial that the methods have similar repeatability, because if poor repeatability existed in one of the methods it would be expected to cause a low level of agreement between them. The error variance associated with the two methods was also similar, with values of 0.06 and 0.05 for S1:S2 and P1:P2, respectively. The within-method comparisons for the Pearson and Spearman correlations were similar but greater for P1:P2 than for S1:S2. Table II shows a full summary of results for all comparisons for Pearson and Spearman correlations, repeatability, and error.

TABLE II
Summary of Results for Five Methods of Comparison[a]

Method	Comparison				
	P1:S1	**P2:S1**	**LS:S1**	**P1:P2**	**S1:S2**
Sample size (*n*)	50	50	50	50	20
Pearson correlation	0.85	0.84	0.86	0.94	0.90
P	6.22×10^{-15}	1.68×10^{-14}	1.33×10^{-15}	2.20×10^{-16}	7.88×10^{-8}
95% confidence interval	0.75–0.91	0.73–0.91	0.77–0.92	0.88–0.96	0.75–0.96
Spearman correlation	0.81	0.83	0.83	0.92	0.90
P	7.48×10^{-13}	1.34×10^{-13}	1.23×10^{-13}	2.20×10^{-16}	5.81×10^{-8}
95% confidence interval	0.69–0.89	0.72–0.90	0.72–0.90	0.86–0.95	0.76–0.96
Y intercept	…	…	…	–0.02	0.10
Slope	…	…	…	0.98	1.00
Standard error of slope	…	…	…	0.05	0.12
R^2	…	…	…	0.87	0.81
Repeatability[b]	…	…	…	0.87	0.93
Error[c]	…	…	…	0.05	0.06

[a] P1 and P2 refer to the first and second replicates of the microenzymatic method; LS refers to the least squares means for P1 and P2; and S1 and S2 refer to the first and second replicates of the streamlined method. Results for Pearson and Spearman correlations with *P* values and 95% confidence intervals are provided for analyses conducted in regard to correlation analyses. For regression models P1:P2 and S1:S2, the *y* intercept, slope, standard error of the slope, coefficient of determination (R^2), repeatability, and error variance are included.
[b] Repeatability, or intraclass correlation, is calculated as the proportion of line variance to all sources of variation.
[c] Error = respective error variances.

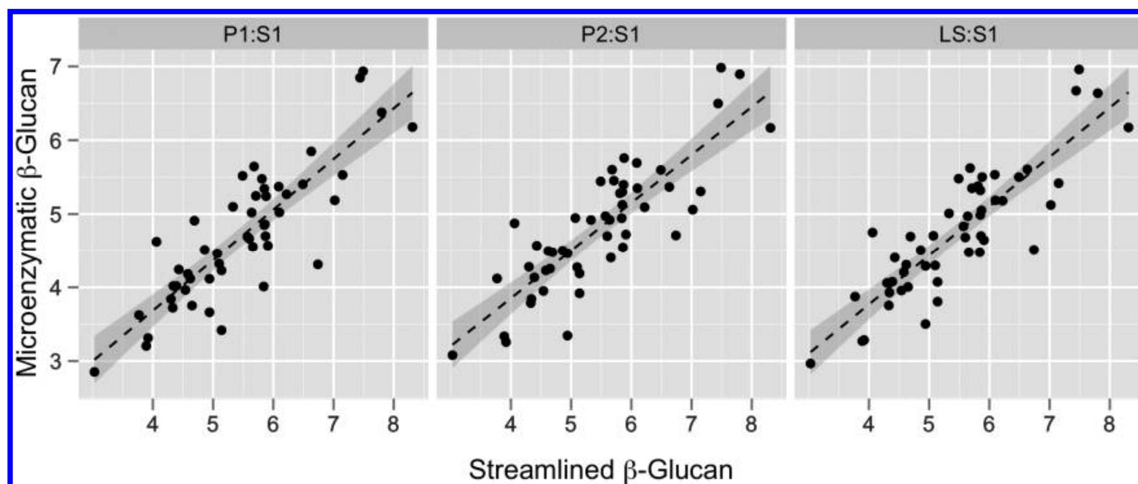

Fig. 1. β-Glucan values enzymatically determined with the streamlined method versus the microenzymatic method for P1:S1, P2:S1, and LS:S1 with 95% confidence bands. Pearson correlations for the three comparisons were significant with values of 0.85, 0.84, and 0.86, respectively. Spearman correlations for all three approaches were also significant with values of 0.81, 0.83, and 0.83, respectively. P1 and P2 refer to the first and second replicates of the microenzymatic method; LS refers to the least squares means for P1 and P2; and S1 refers to the first replicate of the streamlined method.

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DISCUSSION

Before adopting a new method of measurement, it is important to characterize its behavior across samples that will likely be encountered. We have presented a method for the evaluation of β-glucan for high-throughput phenotyping with high correlation to the standard method. In addition to evaluating the microenzymatic method based purely on the Pearson correlation, evaluations were also used to characterize change of rank and bias. Specific to plant breeding, in which lines are chosen based on phenotypic rank, the Spearman correlation was implemented to address this issue. The Spearman correlation was lower than the Pearson, indicating that rank changes had occurred between the two methods. The method of differences was used to characterize the bias introduced when using the microenzymatic method compared with the standard. Results demonstrated that both fixed and proportional biases were present in the microenzymatic approach. Lastly, linear models were used to characterize the repeatability and error for the two methods. The relatively high and consistent repeatability across methods indicated that they performed well with respect to evaluations on the same sample.

We do recognize that the streamlined method produced larger β-glucan values than the microenzymatic approach. This increase could be explained by the less precise grinding method used for the microenzymatic method wherein it does not require flow through a screen to maintain consistency across samples. Hence, the smaller β-glucan values for the microenzymatic approach could be a result of larger flour size that is not easily broken down by the enzymes. However, it is difficult to speculate why this difference occurred in the present study. Regardless of the mechanism for this difference, the microenzymatic approach does allow, based on monetary and time resources, evaluation of a large number of samples otherwise not attainable by the streamlined method.

A major factor for deciding the usefulness of a method specifically for high-throughput phenotyping is the relative cost and time requirement compared with the standard approach. Based on only the cost of the kit itself, the microenzymatic procedure is 1/10 the cost of the streamlined method, excluding the additional gain in cost for user-supplied reagents that include sodium phosphate and sodium acetate buffers. The microenzymatic method is most remarkable with respect to its time requirement. For grinding, including cleaning of the grinding equipment, the streamlined method takes 7 min per sample. The streamlined assay takes approximately 15 min per sample, assuming 10 can be done within 2.5 h. The microenzymatic method takes 45 and 15 min for grinding and cleaning, respectively, for 100 samples, taking approximately 0.6 min per sample. After grinding, for the assay itself the microenzymatic method takes 3.5 h for two plates (192 samples) or 4 h for four plates (384 samples). Thus, the microenzymatic method takes in the range of 0.6–1.1 min per sample. After grinding, the β-glucan assays take approximately 0.7 and 15 min per sample for the microenzymatic and streamlined methods, respectively. Considering the grinding and assay procedures together, the microenzymatic method takes approximately 1.3–1.8 min per sample, whereas the streamlined method takes 22 min per sample. Thus, the microenzymatic procedure takes 6–8% of the time required to implement the streamlined method. Approximately 68 and 800 samples could be ground in one day for the streamlined and microenzymatic methods, respectively. For the assay itself, a maximum of approximately 32 and 800 samples could be evaluated in one day, respectively. The milling procedure used for the microenzymatic method could be used for the streamlined method to save time; however, a bottleneck in the number of samples that can be assayed at one time would remain. Although the microenzymatic method does have some bias, from a practical perspective it performs quite well. The time and cost savings of the microenzymatic method allows for greater levels of replication than the streamlined method, potentially increasing the precision of the method. The relative cost and time requirement enable the breeder to make selections faster by reducing the cost per evaluation. Most importantly, the improvement in cost and time does not substantially decrease the method's ability to determine β-glucan content.

CONCLUSIONS

A modified method for the evaluation of oat β-glucan has been proposed. The field of plant breeding requires methods suitable for evaluation of thousands of samples in the short time between field seasons. The currently accepted streamlined method of evaluation for β-glucan does not deliver the throughput requirements needed to run a plant breeding program efficiently. The microenzymatic method is 1/10 in cost of the standard streamlined approach and allows rapid evaluation of oat β-glucan in a 96-well plate. Application of a procedure such as this reduces cost and time for evaluation and thus increases the number of samples that can be evaluated for a given season. Further, it decreases the turnaround time in laboratory evaluation, facilitating logistics for applications in which selections must occur rapidly. Although the approach was found to have bias, the correlation between the two

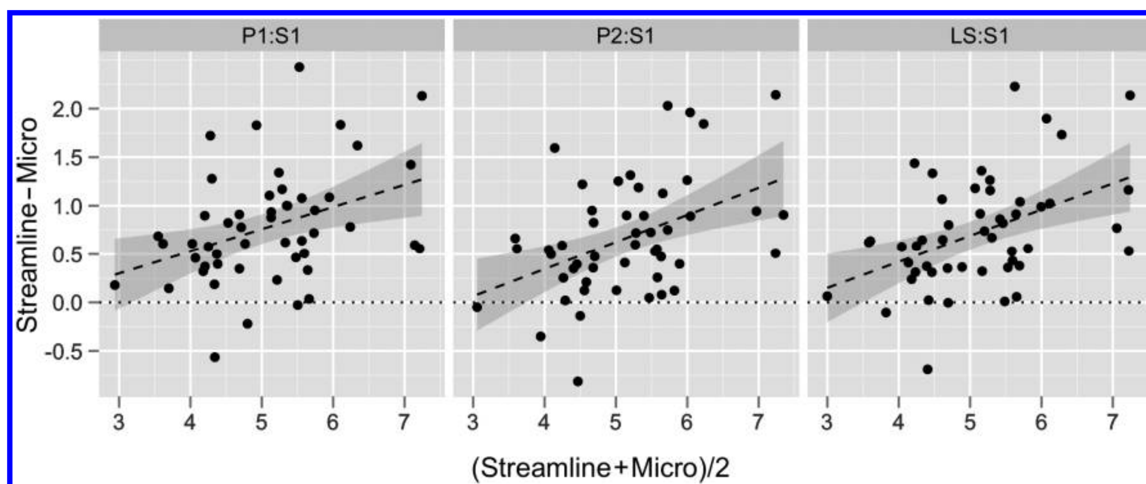

Fig. 2. Results for the method of differences to determine the types of bias in the microenzymatic method compared with the streamlined approach with 95% confidence bands. P1:S1, P2:S1, and LS:S1 all show fixed and proportional bias, represented by the mean of values significantly greater than zero and the slope of the regression significantly greater than zero, respectively. P1 and P2 refer to the first and second replicates of the microenzymatic method; LS refers to the least squares means for P1 and P2; and S1 refers to the first replicate of the streamlined method.

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methods was high. Additionally, the error for the two methods was comparable, and the repeatability was larger for the proposed microenzymatic method. Thus, the microenzymatic procedure offers an alternative method for evaluation of oat β-glucan to meet high-throughput requirements within applied oat breeding programs.

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