PROCEDURES FOR THE EFFECTIVE COMPARISON OF RED AND WHITE WINTER WHEAT

Benjamin Meyerink, Brian Kontz, Ryan Geraets, and Fedora Sutton
Department of Plant Science
South Dakota State University
Brookings, SD 57007

ABSTRACT

Wheat seed color is often associated with a variety of properties, such as flour production and pre-harvest sprouting. Therefore, it is important for wheat breeders to have the capability of performing simple procedures to easily distinguish white wheat seeds from red wheat seeds. Three processes commonly used for seed color differentiation are: treating whole seeds with NaOH to extract bound phenolics, staining seeds with vanillin-HCl to detect proanthocyanidins, and extracting free phenolics from bran using cold 80% EtOH. In this study we evaluated and compared these three techniques and identified beneficial protocol modifications, specifically time and temperature incubation requirements. In order to quantify results, spectrophotometric analysis was performed to obtain UV-Vis spectra for comparing free and bound phenolics. In conclusion, we determined that all three tests effectively distinguish the true color of wheat kernels, but the level of clarification is the most pronounced using the NaOH procedure. In addition, the NaOH protocol generates sufficiently interpretable results from boiling water bath incubation times greater than or equal to ten minutes.

Keywords

NaOH treatment, vanillin-HCl, bran extract, seed color

INTRODUCTION

Simple procedures for discriminating red and white wheat (Triticum aestivum) seed coat color are useful to farmers and wheat breeders for numerous reasons. It is said that red wheat has a much higher resistance to pre-harvest sprouting than white wheat, which can in turn reduce grain quality (McCaig and DePauw 1992). However, white wheat lines with greater tolerance to pre-harvest sprouting, such as Clark's Cream, have been developed (Upadhyay, Morris, and Paulsen 1987). Dependent on milling techniques, hard white wheat has the potential to produce greater amounts of flour than hard red wheat and many cultures around the world prefer its characteristics for food production (Matus-Càdiz et al. 2008).

Three techniques that can be used for seed color determination are: NaOH treatment of whole seeds with *n*-butanol extraction of bound phenolics, vanil-

lin-HCl staining, and extraction of free phenolics using cold 80% EtOH. The NaOH protocol for seed coat color determination is a simple procedure that effectively differentiates red and white wheat grain (Matus-Càdiz et al. 2008). In NaOH, red grains typically become brownish-orange while white grains turn straw yellow. Vanillin-HCl staining is widely accepted for its use in identifying proanthocyanidins, a type of flavonoid (Aastrup et al. 1984). The vanillin-HCl stain displays red upon binding to subunits of proanthocyanidins, which are more abundant in the seed coat of red wheat seeds. Free phenolics can be extracted by mixing bran with cold 80% EtOH (Matus-Càdiz et al. 2008). In order to further analyze seed coat color, spectrophotometric analysis was performed on extracts from the NaOH treatment and cold 80% EtOH treatments in order to determine presence of particular flavonoids in seed coats. All three procedures were performed in order to determine the positive and negative aspects of each.

METHODS

***Plant Material*—**Seeds from hard red winter wheat cv. 'Winoka' (Wells et al. 1969) and a mutant-derived hard white winter wheat (Kenefick 2009) were harvested, threshed, and stored at 5 °C before use.

***Whole Seed NaOH Treatment and N-Butanol Extraction*—**Three replicates of nine seeds from both the red-grained wheat (RW) and white-grained wheat (WW) lines were placed in separate tubes containing 2 mL of 1.25 M NaOH, with caps loosely threaded, and incubated for 15 min in a 90-100°C water bath. Due to solidification of the extract upon cooling, light vortexing was used on some samples to allow for easier separation of seeds from the extract. The extract was placed in new micro-centrifuge tubes, and 6 N HCl (1/4 vol) was used to lower the pH to 3.0. *N*-butanol (1/3 vol) was then added to each sample, vortexed, and centrifuged at 20,000g for 10 min. Seeds were transferred to filter paper to dry for visual inspection. In order to quantify results, liquid from each sample underwent *n*-butanol extraction and was analyzed spectrophotometrically in order to verify seed color (Figure 4).

In order to improve the efficiency of the NaOH treatment, a 90-100 °C hot water bath and a 70 °C shaking incubator were compared; time requirements for both were also evaluated. A 2 mL volume of 1.25 M NaOH was added to eight tubes containing ten WW seeds and eight tubes containing ten RW seeds, and samples were placed in either a hot water bath or a shaking incubator (Shake 'N' Bake Hybridization Oven from Boekel Scientific) for time periods of 2.5, 5, 10, and 20 min. After treatment the remaining extracts were transferred to new 2.0 mL micro-centrifuge tubes, while seeds were left to dry on filter paper for visual inspection. Analysis of incubation requirements was strictly limited to observation of seed color after heating.

***Bran Flavonoid Extraction*—**To obtain bran from RW and WW, seeds were ground separately using the second coarsest setting on a Hamilton Beach *Custom Grind* coffee grinder. Bran was collected by sifting the ground seeds through a 1 µm wire. Bran (50 mg) was placed in each tube and 250 µl of cold 80% EtOH

was added. Samples were shaken for 10 min at room temperature and then centrifuged at 4°C for 5 min at 20,000g.

Vanillin Staining (Aastrup et al. 1984, Peterson, Peterson, and Melville 2008)—A 0.65 M vanillin, 6 N HCl solution was prepared prior to seed staining and stored at 5°C. When preparing the vanillin-HCl solution, it was observed that a significant color change of the original solution occurred when it was prepared more than 48 hours before use. Therefore, we prepared a 0.65 M vanillin stock and mixed it with 6 N HCl immediately before staining. Five seeds from each line were placed in 1 mL of vanillin-HCl and left to sit at room temperature for 15 min. Seeds were removed from stain and observed using an Olympus SZX16 Microscope.

UV-Vis Spectrophotometric Analysis—After completion of the *n*-butanol extraction from seeds treated with NaOH, the top organic layer was removed and analyzed using the UV-Vis module on a NanoDrop ND-1000 Spectrophotometer. A scan of 220 nm to 640 nm was obtained. After completion of the cold 80% EtOH bran extraction, aliquots of each supernatant fraction were removed and analyzed using the UV-Vis module, again obtaining a scan of 220 nm to 640 nm.

RESULTS AND DISCUSSION

Visual Discrimination of Seed Color—RW and WW seed coat color were easily distinguishable when treated with NaOH. RW grains appeared to have a dark brown tint while WW grains were characterized by their straw yellow color (Figure 1). These color differences also were seen when using other hard winter wheat lines and when using wheat lines that were crosses of red-grained and white-grained wheat (personal communication). Thus, it appears that the NaOH treatment is sufficient for farmers and scientists alike to use in the discrimination of hard winter wheat seed color.

Staining with the vanillin-HCl solution effectively differentiated RW and WW seed color. The color differences among seeds after treatment were not as visually discernable as those resulting from NaOH treatment; therefore, the vanillin-HCl seeds were microscopically analyzed. The analysis of seeds after staining typically requires microscopic examination in order to obtain a clearer contrast in color differences. Microscopically, WW seeds appeared golden-brown while RW seeds appeared reddish-orange after staining (Figure 2).

Evaluation of Incubation Time and Temperature Requirements for NaOH Treatment—The most efficient method for extracting bound phenolics with NaOH was determined by comparing samples treated either in a 90-100°C water bath (95°C)

Figure 1. RW and WW seeds after NaOH treatment.

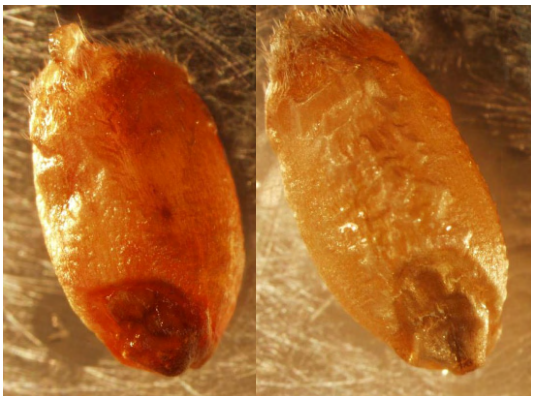

Figure 2. Grains after treatment with the vanillin-HCl solution. RW seed (left) displayed reddish-orange color while WW seed (right) displayed golden-brown color.

or a 70°C shaking incubator for various heating durations up to 20 min. The visual differences between samples that underwent contrasting heating techniques as part of the NaOH treatment were noticed within minutes of heating. Overall, placing samples in a 95°C water bath provided greater visual contrast between red and white seeds than did the use of a shaking incubator.

Seeds that were heated for longer incubation periods generated more colorful extractions. As can be seen in Figure 3, the darkest extract resulted from the 20 min 95°C water bath; however, the 10 min sample also produced significant differences between RW and WW. Therefore, one could save time by placing samples in a 95°C water bath for 10 min instead of 20 min and still obtain acceptable results. Both the 10 min and 20 min hot water incubation generated significantly better results than extracts from solutions that either underwent 70°C incubation and shaking, regardless of time, or a 95°C water bath for less than 10 min.

Spectrophotometric Distinction of Extracts—A UV-Vis module was used for spectrophotometric analysis of extracts between the wavelengths 220 nm and 640 nm. The UV-Vis spectra of cold 80% EtOH bran extracts had peaks in both the Band I and Band II regions with maximum absorption at 325 nm and 274 nm, respectively. However, the NaOH whole seed *n*-butanol extracts displayed only one peak in the Band I region at 322 nm (Figure 4). The single peaks displayed by the extracts from the RW and WW whole seeds were higher in absorbance than those of the bran extracts. The significance of the Band I and Band II regions is explained in Table 1. The spectra for the WW from both the

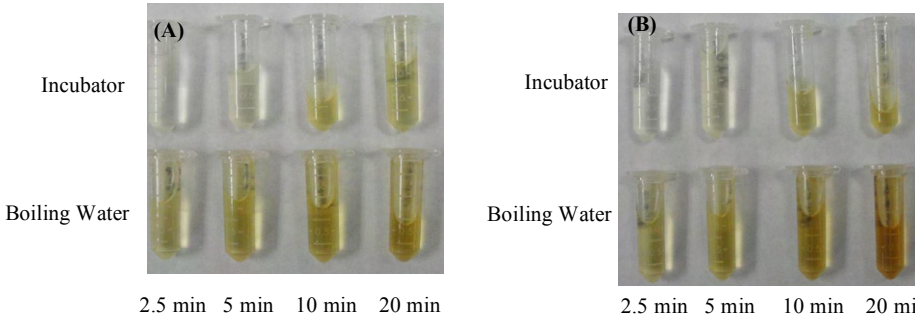

Figure 3. (A) Color comparison of liquid extract from WW seeds treated with NaOH. (B) Color comparison of liquid extract from RW seeds treated with NaOH.

bran extract and seed extract were higher in absorbance than those of the RW within each treatment due to higher flavonoid content in the WW (Table 1). The spectrophotometric data can be compared with Table 1 in order to determine the quantity of specific flavonoids which can be used for downstream application.

Vanillin-HCl staining may be the quickest and simplest method for determining seed coat color for those who possess a microscope, while those without a microscope could use the NaOH treatment for easy visual analysis. Using a 95°C water bath to heat grains in NaOH for ten minutes increases the efficiency and still generates feasible results. The cold 80% EtOH treatment would be feasible only for those with a spectrophotometer; however, it is useful for obtaining absorbance readings that can differentiate flavonoids that are expressed in RW and WW. It is not certain if there is damage to the wheat germ from the use of either the NaOH treatment or vanillin-HCl staining; in the future this effect could be analyzed. Overall, the three procedures proved sufficient for the successful discrimination of red and white kernels.

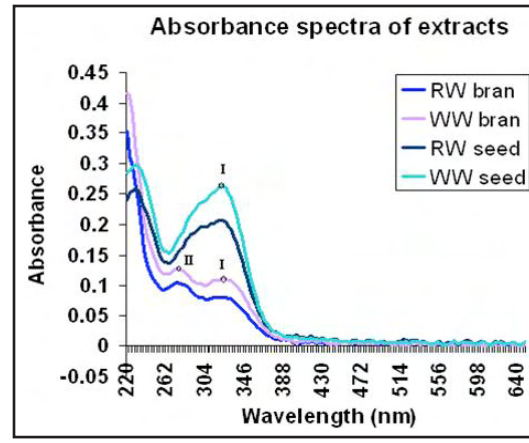

Figure 4. UV-Vis spectra of extracts from bran and seeds of both RW and WW.

Table 1. Interpretation of flavonoids causing expression in Band I and Band II regions (Prati et al. 2007).

	Absorption maxima (nm)	
Flavonoid	**Band II**	**Band I**
Flavones, biflavones	250-280	310-350
Isoflavones	245-275	310-330
Flavonols	250-280	350-385
Flavanones	275-295	310-330

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LITERATURE CITED

Aastrup, S., O. Henrick, and K. Erdal. 1984. Location of the proanthocyanidins in the barley grain. Carlsberg Res. Commun. 49: 105-109.

Kenefick, D. Personal interview. August 2009.
Matus-Càdiz, M.A., T.E. Daskalchuk, B. Verma, D. Puttick, R.N. Chibbar, G.R. Gray, C.E. Perron, R.T. Tyler, and P. Hucl. 2008. Phenolic compounds contribute to dark bran pigmentation in hard white wheat. Journal of Agricultural and Food Chemistry 56: 1644-1653.
McCaig, T, and R. DePauw. 1992. Breeding for preharvest sprouting tolerance in white-seed-coat spring wheat. Crop Science Society of America 32: 19-23.
Peterson, R, C Peterson, and L Melville. 2008. Teaching plant anatomy through creative laboratory exercises. Ottawa, Ontario: NRC Press. 133 pp.
Prati, S., B. Baravelli, D. Fabbri, C. Schwarzinger, V. Brandolini, A. Maietti, P. Tedeschi, S. Benvenuti, M. Macchia, I. Marotti, A. Bonetti, P. Catizone, and G. Dinelli, 2007. Composition and content of seed flavonoids in
forage and grain legume crops. Journal of Separation Science 30: 491-501.
Upadhyay, M.P., C. F. Morris, and G. M. Paulsen. 2008. Characterization of preharvest sprouting resistance in Clark's Cream white winter wheat. Euphytica 38.1: 85-92.
Wells, D.G., C.L. Lay, G.W. Buchenau, V.A. Johnson, and K.F. Finney. 1969. Registration of
Winoka wheat. Crop Science 9(9): 526.