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Effect of germination, seed abrasion and seed size on polyphenol oxidase assay activity in wheat

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Abstract

Polyphenol oxidase (PPO) is one of the major enzymes responsible for browning of wheat food products. Wheat cultivars differ in PPO activity and plant breeders wish to select germplasm and cultivars with low PPO activities. Prior to harvest, seeds may be exposed to conditions conducive to preharvest sprouting and mechanical damage may occur during harvesting and subsequent handling. Sprouting, mechanical damage and seed size may affect PPO assay activity and therefore impinge upon the breeder's ability to accurately select low PPO genotypes. The effects of incipient germination, mechanical abrasion and seed size on PPO assay activity were investigated using the cultivars 'Klasic' (hard white spring, high PPO), 'Penawawa' (soft white spring, high PPO) and 'ID377s' (hard white spring, low PPO). The influence of embryo vs. distal portions of the seed on PPO assay activity was also examined. PPO activity was assayed with constant mixing using L-3,4-dihydroxyphenyl alanine (L-DOPA) substrate at pH 6.5 at room temperature; absorbance of the reaction solution was measured at 475 nm. There was no change in PPO assay activity for seeds imbibed in water for up to 8 h. PPO assay activity increased in seeds imbibed from 8 to 16 h, and then gradually declined with increasing imbibing time. PPO assay activity initially increased as a result of mechanical abrasion but then gradually declined with increasing abrasion time. Large seeds had a higher total PPO assay activity than small seeds, but the difference in activity was not proportional to seed weight. There was only a slight difference in PPO assay activity between half-seeds with or without the embryo. Overall, PPO assay activity in dry seeds was stable and exposure of seeds to injury and moisture did not make any significant change in PPO assay activity among the cultivars or their relative rankings.

Key words: *Triticum aestivum* — abrasion — preharvest sprouting — polyphenol oxidase activity — assay

Polyphenol oxidase (PPO, E.C. 1.14.18.1) is an enzyme that is widely distributed in plants, including wheat (Lee and Whitaker 1995). PPO converts a variety of phenolic substrates to dark-coloured polyphenols (melanins). Although PPO is beneficial in producing the dark colour associated with foods such as prunes, dark raisins and teas, it generally reduces food quality as a result of these same discoloration (browning) reactions. In wheat, PPO is responsible for discoloration of noodles, chapattis and Middle East flat breads (Kruger et al. 1994, Morris et al. 2000). A large portion of PPO activity is located in the endosperm of immature seeds. As the kernel ripens, the level of PPO activity in the endosperm decreases, whereas the level of PPO activity in the outer layers and embryo increases (Kruger 1976, Marsh and Galliard 1986). There is substantial variation in PPO activity among wheat cultivars (Lamkin et al. 1981, Park et al. 1997, Anderson and Morris 2001) and recombinant inbred populations (Demeke et al. 2001).

PPO activity in wheat samples has commonly been measured using either the oxygen consumed (oxygen electrode method; Marsh and Galliard 1986) or the production of coloured products (spectrophotometric method). Substrates have included tyrosine (Bernier and Howes 1994, McCaig et al. 1999), catechol (Kruger et al. 1994, McCaig et al. 1999) and L-3,4-dihydroxyphenyl alanine (L-DOPA) (Morris et al. 1998, Anderson and Morris 2001). Anderson and Morris (2001) compared current PPO assays for wheat (Bernier and Howes 1994, Kruger et al. 1994, McCaig et al. 1999) and improved upon each by buffering the solution at pH 6.5, providing continual mixing and employing L-DOPA as substrate. The assay was amenable to 1–10 seeds per reaction.

Prior to harvest, wheat seeds may be exposed to preharvest sprouting conditions. Imbibition after physiological maturity can cause seeds to germinate prematurely leading to a serious reduction in quality. PPO activity has been shown to increase during preharvest sprouting (Edwards et al. 1989, Kruger and Hatcher 1993, Kruger et al. 1996). A two- to three-fold increase in *o*-diphenolase activity was reported in germinating wheat seeds (Taneja and Sachar 1974a). Mechanical damage to seeds might also cause variation in PPO assay activity, especially since about 50% of PPO activity leaches from seeds in solution (Kruger et al. 1994, McCaig et al. 1999) and most PPO activity of mature seeds is associated with the bran (Hatcher and Kruger 1993). However, there are no reports on the effect of surface mechanical damage on wheat seed PPO assay activity. PPO activity reportedly varied with seed size, with higher activity in large seeds than in small seeds (Baik et al. 1994). The objective of this study was to investigate possible confounding effects that would preclude breeders from obtaining accurate PPO measurements on wheat germplasm and cultivars. The effects included incipient germination, mechanical damage (abrasion), seed size and embryo vs. distal portion of the seed. Three wheat cultivars that encompass much of the range in PPO assay activity encountered in the Pacific Northwest of the USA were used.

Materials and Methods

Cultivars and PPO assay: Seeds of 'Klasic' (hard white spring, high PPO), 'Penawawa' (soft white spring, high PPO) and 'ID377s' (hard white spring, low PPO) wheat, *Triticum aestivum* L., cultivars were used throughout the experiment. 'Klasic' and 'Penawawa' seed were obtained from field plots grown at Pullman, WA, USA, and Blackfoot, ID, USA, respectively. 'ID377s' 'breeder' class seed was obtained from Dr Edward Souza. The PPO L-DOPA assay was performed according to Morris et al. (1998) and Anderson and Morris (2001) using 5 mM L-DOPA in 50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 6.5. A 1.5-ml aliquot of the solution was added to a 2-ml microcentrifuge tube containing five seeds. The tubes were rotated for 1 h at room temperature (c. 20°C) to allow the reaction to take place. Absorbance was measured on a 1.0-ml aliquot at 475 nm using a Shimadzu Biospec-1601 spectrophotometer (Shimadzu Corporation, Columbia, MD, USA) against a solvent blank. The L-DOPA solution was made fresh daily.

Incipient germination: Seeds of the three cultivars were imbibed in distilled water for 0, 4, 8, 16, 24, 36 and 48 h. After imbibing, seeds were dried at 27 ± 2°C in a forced-air oven for 48 h before the L-DOPA assay. Three replications were used for each treatment.

Abrasion of seeds: Seeds were abraded with a Tangential Abrasive Dehulling Device (Venables Machine Works Ltd, Saskatoon, SK, Canada) with a grit size of 80. The abrasion treatments lasted 0, 5, 10, 20, 30, 40, 50, 100 and 200 s. Four five-seed replications were used for each treatment and the entire abrasion study was performed twice (a total of eight replications). Seeds were weighed afterward to determine the amount of material removed by the abrasion treatment. After incubation in the L-DOPA solution, the samples were centrifuged for 1 min in a microcentrifuge ($8500 \times g$) to sediment particulates that caused turbidity of the reaction solution and therefore interfered with the spectrophotometer absorbance measurement.

Seed size: Large and small seeds were visually selected from each lot of the three cultivars. All seeds were physiologically mature and plump (i.e. not immature or shrivelled), and represented minor portions of each lot. Individual five-seed aliquots were weighed and, as necessary, seeds were replaced with larger or smaller seeds to obtain the desired final five-seed weight of 0.23 and 0.12 g for large and small seeds, respectively. The average seed weight of the three lots was about 0.19 g per five-seed aliquot. Three replications were used for each treatment.

Embryo vs. distal portions of seed: Seeds were cut in half (half-seed with embryo portion and distal portion without embryo) and a half-seed weight of 0.09 g (for five seeds) was used for each of five replications for the three cultivars. After allowing the reaction to proceed, the tubes were centrifuged as described for the abrasion study, prior to measuring absorbance at 475 nm.

Statistical analysis: Analysis of variance (ANOVA) was conducted with the SAS General Linear Models Procedure (SAS Institute, Cary, NC, USA).

Results

Effect of incipient germination on PPO assay activity

Seeds were imbibed up to 48 h and dried to simulate preharvest sprouting conditions. There was no change in PPO assay activity for seeds imbibed up to 8 h for all three cultivars (Fig. 1). Between 8 and 16 h, PPO assay activity increased slightly in the high PPO cultivars but not in 'ID377s'. Rankings and relative separation of the cultivars remained distinct. From 24 h for 'ID377s' and from 16 h for 'Penawawa' and 'Klasic', PPO levels decreased in a manner roughly proportional to the level of PPO in the dry control seed such that by 48 h all three cultivars showed similar low levels of PPO assay activity. Radicle emergence was observed after 24 h of imbibition, and shoots and roots were visible for all three cultivars after 36 h of imbibition.

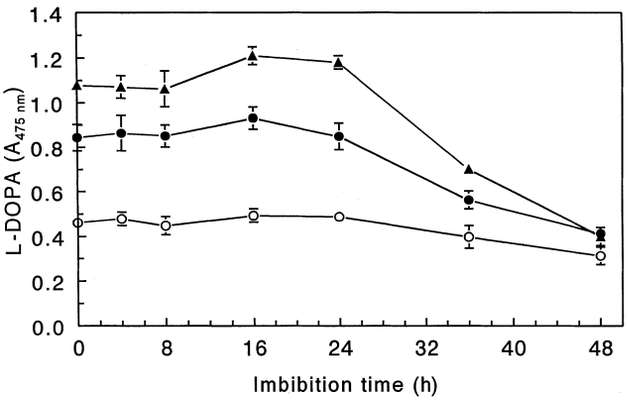

Fig. 1: Relationship between incipient germination of wheat seeds and polyphenol oxidase (PPO) activity measured using L-DOPA substrate. Seed of three wheat cultivars, 'Penawawa' (▲), 'Klasic' (●) and 'ID377s' (○) were imbibed in water at room temperature for the times indicated, air dried and assayed in a whole-seed PPO assay. Standard deviations are from three replications

Effect of abrasion on PPO assay activity

Seeds were abraded for 0–200 s to determine the effect of mechanical damage on PPO assay activity. The abrasion dehulling device uniformly removed parts of the pericarp (bran) and embryo. There was a progressive, linear reduction in seed weight with increasing abrasion time (Fig. 2). Abrading 'ID377s' an additional 200 s (total 400 s) further reduced the weight of the seeds to 7.42 g. There was slightly greater weight loss in the soft wheat cultivar 'Penawawa' compared with the hard wheat cultivars 'Klasic' and 'ID377s'.

From 0 to about 10 or 20 s abrasion, PPO assay activity increased slightly for the three cultivars (Fig. 3). Maximum average PPO assay activity ranged from 1.16 AU (absorbance units) for 'Penawawa' to 0.53 AU for 'ID377s'. These PPO

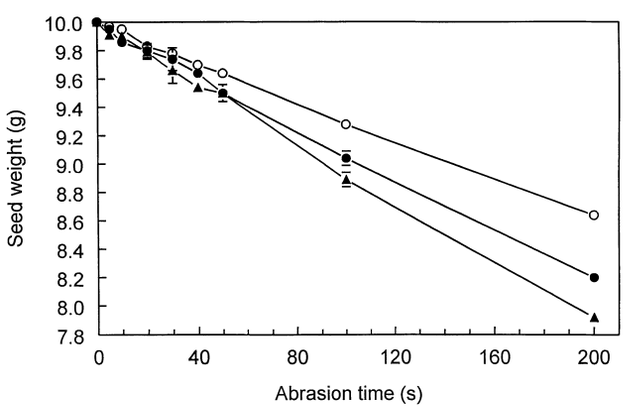

Fig. 2: Reduction in wheat seed weight resulting from mechanical surface abrasion. Seeds of three wheat cultivars, 'Penawawa' (▲), 'Klasic' (●) and 'ID377s' (○) were subjected to surface abrasion in a tangential abrasive dehulling device for the times indicated. Standard deviations were from eight replications

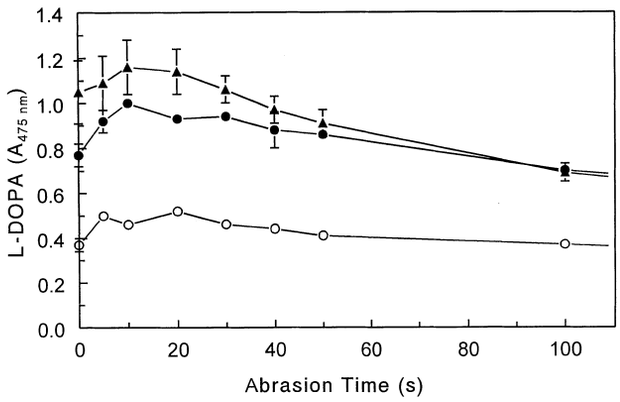

Fig. 3: Relationship between mechanical surface abrasion of wheat seeds and polyphenol oxidase (PPO) activity measured using L-DOPA substrate. Seed of three wheat cultivars, 'Penawawa' (▲), 'Klasic' (●) and 'ID377s' (○) were subjected to surface abrasion in a tangential abrasive dehulling device (see Fig. 2) for the times indicated, and assayed in a whole-seed PPO assay. Standard deviations are from eight replications

activities were approximately 110–142% of the control, unabraded values. From these maxima, PPO assay activity gradually decreased with increasing abrasion time. The ranking of the three cultivars remained the same until 50 s, when approximately 4–5% of the seed weight had been removed. Between 50 and 100 s the ranking of 'Penawawa' and 'Klasic' switched, but neither of these high PPO cultivars changed rank with 'ID377s', the low PPO cultivar. Surface scratches were noticeable at 10–20 s abrasion for all three cultivars. Serious damage to the embryo and pericarp became evident at 100 s abrasion.

Effect of seed size on PPO assay activity

Large and small seeds were visually selected from each lot of the three cultivars, a constant weight of five seeds was taken from each category (0.12 g for small seeds and 0.23 g for large seeds) and PPO activity was measured using the L-DOPA assay. ANOVA showed a highly significant effect of cultivar and seed size on L-DOPA activity for the 5-seed assay (Table 1). However, the magnitude of the cultivar contribution was much larger than was seed size. The interaction was not significant. When L-DOPA assay activity was calculated on a per gram basis, there was a significant cultivar–seed size interaction ($MS = 3.00$, $P = 0.02$). However, it was relatively small and again the major variation was contributed by cultivar differences ($MS = 76.17$, $P < 0.0001$). The ANOVA models accounted for 95% and 97% of the total variation (R^2), five-seed and per gram bases, respectively.

Table 1: Analysis of variance for the effect of wheat seed size on polyphenol oxidase (PPO) activity determined by a whole-seed L-DOPA assay[1]

Source	d.f.	Mean square	F-value	P-value
Cultivar	2	0.964	118.99	< 0.0001
Seed size	1	0.137	16.85	0.0015
Interaction	2	0.004	0.53	0.60
Error	12	0.008		

[1] A constant five-seed weight of 0.23 g and 0.12 g was used for large and small seeds, respectively, for all three cultivars.

Table 2: Effect of wheat seed size on polyphenol oxidase (PPO) activity determined by a whole-seed L-DOPA assay[1]

	Seed size	
Cultivar	Large	Small
'Klasic'	0.921	0.720
'Penawawa'	1.257	1.048
'ID377s'	0.410	0.298
$LSD_{(0.05)}$[2]	0.160	

[1] A constant five-seed weight of 0.23 g and 0.12 g was used for large and small seeds, respectively, for all three cultivars. PPO assay activity = A_{475} of L-DOPA reaction.
[2] This LSD is appropriate for pairwise comparisons between any cultivar and seed size combination.

Overall, five-seed samples of the large seeds always produced significantly higher absolute L-DOPA values than small seeds (Table 2). However, when calculated on a per gram basis, the small seeds of 'Klasic' and 'Penawawa' showed higher PPO assay activities than their large-seed counterparts; the difference for 'ID377s' was not significant ($LSD_{(0.05)} = 0.90$ AU). Most importantly, regardless of seed size, the five-seed samples maintained the relative rankings of the three cultivars. For example, even when comparing the small seed sample of 'Klasic' (0.720 AU) with the large seed sample of 'ID377s' (0.410 AU) the two cultivars are clearly differentiated (Table 2).

Comparison of embryo vs. distal (non-embryo) portions of the seed on PPO assay activity

The procedure of cutting seeds in half transversely and comparing their PPO assay activity was conducted for two primary reasons. First, cutting the seed in half could represent the effect of broken seeds or seeds with severe mechanical damage. Second, comparing embryo half-seeds with the distal portion and with whole-seed controls could represent situations where the distal portion is removed and used for isolation and analysis of high molecular weight glutenin subunits (or other, similar small-scale single-seed genotype assays).

ANOVA revealed a highly significant effect of cultivar on PPO assay activity of half seeds using five embryo or distal half seeds (Table 3). The presence or absence of the embryo had a significant but relatively negligible effect. The cultivar–embryo vs. distal half-seed interaction term was not significant. When L-DOPA assay activity was calculated on a per gram of seed basis, very similar results were obtained, namely: a highly significant cultivar effect, a very minor embryo vs. distal half seed effect and a non-significant interaction term.

Comparison of the half-seed results (Table 4) with the whole-seed data in Table 2 indicated there was no marked

Table 3: Analysis of variance for the effect of wheat embryo half vs. distal half seed on polyphenol oxidase (PPO) activity determined by a L-DOPA assay[1]

Source	d.f.	Mean square	F-value	P-value
Cultivar	2	0.617	151.05	< 0.0001
Seed half	1	0.024	5.91	0.023
Interaction	2	0.004	1.06	0.36
Error	29	0.004		

[1] A constant five-half seed weight of 0.09 g was used for both embryo and distal portions for all three cultivars.

Table 4: Effect of wheat embryo half vs. distal half seed on polyphenol oxidase (PPO) activity determined by an L-DOPA assay[1]

	Seed half	
Cultivar	Embryo	Distal
'Klasic'	0.544	0.553
'Penawawa'	0.669	0.758
'ID377s'	0.190	0.261
$LSD_{(0.05)}$[2]		0.083

[1] A constant five-half seed weight of 0.09 g was used for both embryo and distal portions. PPO assay activity = A_{475} of L-DOPA reaction.
[2] This LSD is appropriate for pairwise comparisons between any cultivar and seed half combination.

effect of cutting the seeds in half. On a per gram basis, the half seeds tended to be more similar to the small vs. the large seeds. Regardless of embryo or distal portion, the relative rankings and mean separation of the cultivars was very similar to those obtained using intact, whole seeds. The low PPO assay activity cultivar, 'ID377s', exhibited only about one-half to one-third the activity of the high PPO cultivars, 'Penawawa' and 'Klasic'.

Discussion

Measuring PPO activity and selecting for low PPO germplasm in early generations has become an important objective in wheat breeding programs in the USA, Canada and Australia. PPO assay activity is predictive of discoloration in Asian noodles and other wheat foods (see review in Anderson and Morris 2001). Our research has produced a small-scale objective noodle colour method (Morris et al. 2000) and an improved PPO assay which uses L-DOPA as substrate and other modifications (Morris et al. 1998, Anderson and Morris 2001). Accurate assessment of PPO levels requires knowing what possible sources of variation may confound or preclude such measures. In this context the following were examined (all of these could potentially bias the test): incipient germination such as would be encountered with preharvest sprouting conditions; mechanical damage (surface abrasion) such as would be encountered during threshing or subsequent grain handling; and seed size. In addition, embryo and distal portions of seeds were compared since this simulates broken or severely damaged seeds and it is sometimes desirable to remove the distal portion for genotype analysis (such as high molecular weight glutenin subunits or granule-bound starch synthase isozymes).

Incipient sprouting of wheat seeds increases α-amylase and other enzyme activities, and this in turn will have a negative consequence on the quality of wheat products (Kruger et al. 1987, Edwards et al. 1989). According to Edwards et al. (1989) the relative increase in PPO activity in weather-damaged grain was about twice that in sound wheat samples. However, out of six enzymes investigated by Edwards et al. (1989), changes in flour properties and product defects were related more to levels of flour protease and α-amylase than to the enzymes catalase, peroxidase, phenol oxidase and lipoxygenase. There was an increase in PPO assay activity for seeds that imbibed water for 8–16 h in our study, but then there was a general decline in PPO assay activity after 16 h. Excised coleoptiles and roots have been shown to have high PPO activity (Taneja and Sachar 1974a), but seeds that imbibe water for a short period of time may not show significant change in PPO assay activity, as our results indicate.

In the present study, there was an increase in PPO assay activity for slightly abraded seeds, and then there was a continuous decline in PPO assay activity with increasing abrasion time. PPO is localized in the bran (Kruger 1976, Marsh and Galliard 1986) and it makes sense to observe a decline in PPO assay activity with more extensive removal of bran at the longer abrasion times. It appears that PPO is stable in dry seeds and the slight damage encountered during harvesting and threshing may not make substantial increases or decreases in PPO assay activity.

Large seed-to-seed variation in PPO assay activity has been reported for wheat seeds using catechol and tyrosine as substrates (McCaig et al. 1999). Baik et al. (1994) also reported that large seeds have higher PPO activity than small seeds. In the study by Baik et al. (1994) seeds larger than 2.8 mm had much higher PPO activity than 2.1 mm seeds. Our results agree with the above studies in that large seeds had higher PPO activities than small seeds, but the increase in PPO activity in large seeds was not proportional to seed size. The small seeds, which weighed only about half that of the large seeds and were a minor portion of all seeds, had, on average, more than 75% of the PPO assay activity expressed by the large seeds. Other physical factors such as seed surface area may relate more directly to PPO assay activity. Based on ANOVA, cultivar and seed size had significant effects on PPO assay activity, but the cultivar effect was proportionally much greater.

The wheat embryo and scutellum are reportedly rich in phenolics such as ferulic, coumaric, sinapic and syringic acids (Walker and McCallum 1992), and thus removal of embryo may affect end-product discoloration by reducing endogenous substrates. In the present study, where L-DOPA was supplied as an exogenous substrate there was a slight increase in PPO assay activity in non-embryo portions of seeds but, overall, there was no significant difference between embryo and non-embryo portions based on ANOVA. This result indicates that the embryo *per se* does not influence PPO enzyme assay activity in the dry state. It has been reported that *o*-diphenolase activity is present in all parts of the seedling, whereas monophenolase activity is confined to embryoless endosperm (Taneja and Sachar 1974b). However, monophenolase activity was not tested for in our study.

There was no dramatic change in PPO assay activity as a result of incipient germination, mechanical abrasion, seed size or the embryo vs. distal half of the seed, and the three cultivars retained their relative, distinct rankings in all treatments. Surface abrasion did result in an increase in PPO assay activity, but the extensive small scratches over the surface of the seed was considered to exceed any level of abrasion commonly encountered during normal breeding procedures. The L-DOPA assay used here (Anderson and Morris 2001) is robust, little affected by the potentially confounding factors examined in this study and, consequently, is effective in differentiating seed PPO activity among wheat cultivars.

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