# An Improved Whole-Seed Assay for Screening Wheat Germplasm for Polyphenol Oxidase Activity

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## ABSTRACT

Polyphenol oxidase (PPO) causes darkening and discoloration of wheat (*Triticum aestivum* L.) foods such as noodles. Consequently, a simple, nondestructive, quantitative assay for determining PPO on one to a few wheat seeds could identify superior germplasm and eliminate inferior breeding lines, thus greatly assisting the development of wheat cultivars with superior noodle color. We sought to (i) examine current PPO whole-seed assays and develop an improved assay that would facilitate rapid, efficient evaluation of wheat breeding lines and cultivars, be amenable to single seeds, and not adversely affect seed viability; (ii) use the assay to evaluate a large collection of wheat germplasm with the aim of identifying lines with very low PPO levels for crossing; and (iii) gain additional information on the location of PPO gene(s). Phenol, L-tyrosine, catechol, methyl catechol, 3,4 dihydroxyphenyalanine (L-DOPA), and caffeic acid were evaluated as potential substrates. Kinetic studies indicated that L-DOPA and catechol at pH 6.5 produced the greatest enzyme activity. L-DOPA did not reduce seed viability, whereas catechol is reportedly toxic to seeds. A standard assay [1.5 mL of 10 m$M$ L-DOPA in 50 m$M$ 3-($N$-morpholino) propane sulfonic acid (MOPS) buffer, pH 6.5, with 3 to 5 seeds constantly rotated in a 2-mL microcentrifuge tube for 0.5 or 1 h at room temperature] was used to screen 1953 germplasm accessions grown in a common environment. Lines with low levels of PPO (i.e., 10% of the population) were identified; 24 of 66 lines displayed low PPO when evaluated under a second environment. Lastly, chromosome 2D was identified as a location of PPO gene(s) based on 'Langdon' durum/'Chinese Spring' D-genome substitution lines, and homoeologous group 2 nullisomic/tetrasomic stocks of Chinese Spring. The L-DOPA standard assay described here provides a robust and efficient method of evaluating germplasm and cultivars for PPO.

THE COUNTRIES OF EASTERN ASIA are major wheat users, with as much as 50% of the flour consumed in the form of noodles (Miskelly and Gore, 1991). Two of the leading types of noodles in these markets are termed white salted and yellow alkaline. Color is a key quality trait for both of these types of noodles, and can vary dramatically among different wheat cultivars and grain lots (Morris et al., 2000). Consequently, any means of predicting noodle color from grain is highly desirable. Noodle color is conveniently and routinely measured using the Commission Internationale de l'Eclairage 1976 international color measurement system (Wyzecki and Stiles, 1982) triaxial color space, L*, a*, b*, where L* is the black–white, a* is the red–green, and b* is the yellow–blue axes. Higher L* values, described as greater whiteness or brightness, are considered especially important for consumer acceptance (Moss, 1971; Miskelly, 1984, 1996), while low L* values indicate undesirable discoloration or darkening of noodles.

There is a need for a simple, quantitative assay for determining the potential noodle color or discoloration characteristics of wheat germplasm. A test that is nondestructive to the wheat seed, maintains viability, and which requires as few as one seed would be particularly advantageous for permitting plant propagation from individual seeds or lines early in breeding programs. Identifying superior germplasm and eliminating inferior breeding lines would greatly assist the development of wheat cultivars with superior noodle color.

Darkening and discoloration of noodles, especially raw alkaline, has been associated primarily with the activity of PPO (Kruger et al., 1992, 1994a,b; Baik et al., 1994, 1995; Crosbie et al., 1996; Miskelly, 1996). The presence of PPO has been studied in wheat grain since at least 1907, when tyrosinase was detected in bran (Bertrand and Muttermilch, 1907, cited in Lamkin et al., 1981). PPO activity is usually greatest in immature seeds, and may result from as many as 12 isozymes (Taneja et al., 1974; Kruger, 1976). In higher plants, PPO is a copper-containing metalloprotein that catalyzes the hydroxylation of *o*-monophenols to *o*-diphenols (E.C. 1.14.18.1; monophenol monooxygenase, tyrosinase, or cresolase) and the oxidation of *o*-dihydroxyphenols to *o*-quinones (E.C. 1.10.3.2; diphenol oxygen oxidoreductase, diphenol oxidase, or catecholase) (see review by Steffens et al., 1994). Because of their electrophillic nature, quinones undergo secondary reactions, such as autooxidation and polymerization with amino acid groups of cellular proteins. These secondary reactions of quinones are likely responsible for the brown and black pigmentation associated with reduced noodle quality.

In addition to the current interest in the relationship between PPO and Asian noodle color, the reaction of whole wheat seeds with phenol (Csala, 1972; Fraser and Gfeller, 1936; Joshi and Banerjee, 1969; Maguire et al., 1975; Walls, 1965; Wrigley, 1976), tyrosine (Mahoney and Ramsay, 1992), and catechol (Milner and Gould, 1951) has been used to characterize cultivars and test grain lots for purity. Polyphenol oxidase also plays a role in darkening and discoloration of flat breads (Y. Abrol and co-workers, see Tikoo et al., 1973; Singh and Sheoran, 1972; and Faridi, 1988), pan bread (McCallum and Walker, 1990), and steamed breads (Dexter et al., 1984). Phenol and tyrosine were used to study the genetics of PPO in diploid, tetraploid, hexaploid and synthetic hexaploid wheats (Bhowal et al., 1969; Fraser

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and Gfeller, 1936; Joshi and Banerjee, 1969, 1970; Joshi et al., 1969; Jimenez and Dubcovsky, 1999; Mares and Panozzo, 1999). Lamkin et al. (1981) used various phenolic substrates to study differences among wheat cultivars and U.S. market classes.

More recently, and due largely to the need to select wheat breeding lines with superior noodle color potential, small-scale whole seed assays for PPO have been studied (Bernier and Howes, 1994; Kruger et al., 1994b; Morris et al., 1998; McCaig et al., 1999). Bernier and Howes (1994) adapted the assay of Mahoney and Ramsay (1992), which used tyrosine at pH 9.0. Five individual wheat seeds of each cultivar were placed singly in wells of a microtiter plate and incubated in 0.2 mL of substrate solution for 2.5 h at 37°C. An aliquot was transferred at the end of the assay to a second microtiter plate for measuring absorbance. Kruger et al. (1994b) chose catechol as the substrate, as it resulted in considerably greater color production compared with tyrosine and phenol, and it paralleled the use of catechol in previous ground-sample oxygen-electrode assays (Marsh and Galliard, 1986). The test used five seeds per reaction and a 16-h steeping step before addition of substrate solution. At the end of the 30-min reaction at 37°C, an aliquot was transferred to a microtiter plate for measuring absorbance. McCaig et al. (1999) studied the application of the Bernier and Howes (1994) and Kruger et al. (1994b) methods to large breeding populations. Using a group of 13 wheat and 2 triticale (×*Triticosecale rimpaui* Wittm.) genotypes, the two methods produced results which were well-correlated $r^2 = 0.85$); however, the tyrosine test was more variable. Further, because of solubility limitations, tyrosine was used at 10 m$M$ versus 90 m$M$ for catechol, and the reaction time for tyrosine was five times longer. However, tyrosine reportedly did not affect seed germination, whereas catechol concentrations down to 30 m$M$ reduced germination by at least 20%; 90 m$M$ catechol reduced germination by more than 80%.

We report in detail the development of a new and improved, nondestructive method for assessing PPO in whole seeds. An initial report (Morris et al., 1998) outlined the procedure which uses L-DOPA, pH 6.5, at room temperature, requires no steeping step, and measures the reaction at 475 nm after as little as 30 min. The method routinely uses two five-seed replicates, but is amenable to single seeds. The L-DOPA substrate does not reduce germination, and seeds may be planted directly after assay.

## MATERIALS AND METHODS
### Seed Stocks

Three sets of seed stocks were used in the study. The first set was comprised of four wheat cultivars, 'Eltan' (soft white winter), 'ID377s' and 'Klasic' (hard white spring), and 'Penawawa' (soft white spring), and used for the assay development. The second set was a large ($n = 1953$), group of wheat germplasm accessions obtained from Dr. Harold Bockelman at the USDA National Small Grains Collection, Aberdeen, ID. According to the Germplasm Resources Information Network (GRIN), all are *T. aestivum*. These accessions were grown in

single rows in Aberdeen and harvested in 1997 (H. Bockelman, 1998, personal communication). This entire set was evaluated for PPO using two five-seed repeated measures and our L-DOPA standard assay (5 m$M$ L-DOPA and 1-h reaction time). In the fall of 1998, ≈10% ($n ≈ 200$) of the accessions with the lowest L-DOPA values were hand-planted in single rows at Barmore Farm, Washington State Univ., Pullman, WA. Of these, only about 15 survived an especially severe low temperature episode. In the spring of 1999, 19 of the 20 accessions with the lowest L-DOPA values (1% of the original population, Table 1) and an additional 36 lines selected at random from the group of accessions ranging in rank from 21 to 200 (i.e., drawn from the next 9% of the original population) were replanted by hand alongside the surviving fall-planted accessions for a total of 70 lines. In both years, each accession was sown at a 10-cm within-row plant spacing, with 30 cm between rows, and grown using common cultural practices and supplemental irrigation. From each row reaching maturity, three individual plants were harvested at random and the seeds analyzed for L-DOPA activity in duplicate using our standard assay (described herein). Accessions that did not head and mature due to spring sowing were abandoned.

The third set of experimental materials was comprised of wheat cytogenetic stocks. Chinese Spring D-genome substitution lines in Langdon durum (*T. turgidum* L.) were a generous gift of Dr. Leonard Joppa (Joppa and Williams, 1988). Three replications were conducted, each on a different day. Substitution line 4D for Langdon 4A was not initially available and had only two replicates. Each assay used three seeds, 10 m$M$ L-DOPA and 2-h incubation. Chinese Spring nullisomic/tetrasomic lines for homoeologous group 2 chromosomes were provided by the Wheat Genetic Resources Center, Manhattan, KS. Two to three plants each of the original Chinese Spring nullisomic/tetrasomic lines were grown in a glasshouse, harvested and used to sow single-row field plots. The seed from these field plots, harvested in 1995, was stored at −20°C until used in the L-DOPA assay. Chinese Spring nullisomic/tetrasomic lines were assayed using 4 replications, 5 seeds each, and 5 m$M$ L-DOPA for 1 h.

### Assay Development

Initially, three seeds were placed in standard 2-mL microcentrifuge tubes containing 1.5 mL of phenolic substrate made up in 50 m$M$ MOPS buffer (pH 6.5). The phenolic substrates tested were: 3,4-dihydroxycinnamic acid (caffeic acid), 3,4-dihydroxybenzene (catechol), L-DOPA, 3,4-dihydroxytoluene (4-methylcatechol), phenol, and L-tyrosine. All substrates were adjusted to 10 m$M$ with the exception of phenol, which was diluted to a 2 g L$^{-1}$ solution. Because of solubility constraints, tyrosine assays were conducted at pH 8.5 using 50 m$M$ Tris [tris(hydroxyethyl)aminomethane]. The tubes were then incubated at room temperature with rotation (Labquake, model no. 415110, "rotisserie" mode, Barnstead/Thermolyne, Dubuque, IA)[1] for 0 to 2 h. Following incubation, solutions were removed from the tubes and the change in absorbance was compared with a substrate only control. For phenol and catechol reactions, change in absorbance was recorded at 410 nm and for all other reactions change in absorbance was recorded at 475 nm. One unit of PPO activity was defined as a change of 0.001 absorbance unit/min/mL. All reactions were conducted at room temperature (about 20°C).

**Table 1. Lines exhibiting the lowest L-DOPA values (best 1%) from screening a set of $n$ = 1953 wheat germplasm accessions grown at Aberdeen, ID, and harvested in 1997; and the lowest L-DOPA and SD values of three individual plants harvested at Barmore Farm in Pullman, WA in 1999.**

| Accession† | Accession number | Origin | L-DOPA 1997 crop | L-DOPA 1999 crop‡ |
|---|---|---|---|---|
| | | | $A_{475}$ | |
| MG27070 | PI 469014 | Greece | 0.013 | 0.075 (0.008) |
| Aggia | PI 534333 | Ethiopia | 0.038 | 0.127 (0.019) |
| CNNS 2B (XII) | PI 542457 | USA, NE | 0.048 | 0.016 (0.038) |
| Sindi | PI 479799 | Ethiopia | 0.048 | 0.074 (0.012) |
| Haynes Bluestem | PI 286057 | USA, ND | 0.051 | 0.096 (0.085) |
| MG27965 | PI 469078 | Greece | 0.055 | 0.069 (0.022) |
| Sonora | PI 45404 | South Africa | 0.065 | 0.048 (0.010) |
| MG27978 | PI 469090 | Greece | 0.066 | 0.073 (0.008) |
| Blue Norco | PI 542465 | USA, OR | 0.071 | 0.140 (0.008) |
| 153-M-M-3 | PI 170907 | South Africa | 0.072 | 0.083 (0.098) |
| Swift | PI 591925 | Australia, NSW | 0.074 | 0.362 (0.103) |
| 85SEB230ST | PI 574195 | USA, MT | 0.083 | 0.295 (0.081) |
| SST333 | PI 591941 | South Africa | 0.088 | 0.510 (0.018) |
| Arnautka 1963P | CItr 1493 | USA, ND | 0.089 | 0.089 (0.021) |
| Hermosillo 77 | PI 591792 | Mexico | 0.092 | – |
| SST124 | PI 591942 | South Africa | 0.093 | 0.466 (0.028) |
| NW100A | PI 429704 | Nepal | 0.093 | 0.078 (0.044) |
| Mei Xiu Huang | PI 525072 | China | 0.093 | – |
| MG27055 | PI 469001 | Greece | 0.093 | 0.257 (0.044) |
| Carifen | PI 583682 | Chile | 0.097 | – |

† Line designation follows that of the Germplasm Resources Information Network of the National Plant Germplasm System.
‡ Lowest value of three individual plants harvested at random. The SD among the three plants is in parenthesis.

### Germination Assay

Seeds of Klasic and ID377s were incubated in 5 m$M$ L-DOPA solution or water for 1 h, then placed on moistened blotter paper in 10-cm diameter polystyrene petri dishes and incubated in the dark. Three replicate plates of each cultivar and treatment contained 50 seeds each. Germination counts were made after 4 and 7 d.

## RESULTS AND DISCUSSION

### Assay Development

Preliminary studies indicated that auto-oxidation of some dihydroxyphenolic substrates occurred rapidly and therefore precluded accurate PPO measurements. The non-enzymatic kinetics of this phenomenon were more pronounced at pH values above 7.0. Figure 1 shows a comparison of auto-oxidation for both monophenolic and dihydroxyphenolic substrates at pH 6.5, 7.5 and 8.5. The monophenolic substrates phenol (2 g L$^{-1}$) and 10 m$M$ L-tyrosine (pH 8.5 only) exhibited virtually no auto-oxidation across a 21-h time period. Of the dihydroxyphenolic substrates, 10 m$M$ L-DOPA showed the greatest levels of auto-oxidation across a 21-h time period at pH 8.5. At pH 8.5, both methyl catechol and L-DOPA began to change color immediately, and continued darkening across a 21-h period. Methyl catechol was the only substrate that showed significant auto-oxidation below a pH value of 7.0. 3,4 Dihydroxyphenyalanine solutions below pH 7.0 were relatively stable across a 21-h time period, and were similar to those observed for 10 m$M$ catechol. Auto-oxidation also was observed with 10 m$M$ solutions of the dihydroxyphenolic substrates chlorogenic acid and caffeic acid (data not shown). Of all the dihydroxyphenolic substrates tested, 10 m$M$ catechol was the most stable at pH values above 7.0, although auto-oxidation was still 10 times higher at pH 8.5 compared with pH 6.5 ($A_{475}$ = 0.34 vs. 0.03, respectively) after 21 h. Thus,

to reduce auto-oxidative effects in our seed assays, all solutions (with the exception of tyrosine assays) were maintained at pH 6.5. Tyrosine has very limited solubility at neutral pH, and normally is dissolved and used at pH 9.0 (Bernier and Howes, 1994; Marsh and Galliard, 1986). Even at pH 9.0, dissolution of 10 m$M$ tyrosine was difficult. Similarly, caffeic acid also demonstrated poor solubility at pH 6.5.

In prior observations using the phenol seed test (Walls, 1965; Wrigley, 1976), seeds completely immersed in solution reacted little or very slowly, suggesting that free oxygen was required for color development. This observation caused us to question the use of microtiter plates (Bernier and Howes, 1994) where a boundary layer could develop around individual seeds immersed in solution. An additional concern related to the possible boundary layer effect was diffusion of PPO into the solution (McCaig et al., 1999) and substrate to PPO remaining associated with the seed (Kruger et al., 1994b). For these reasons, we sought a means of mechanically mixing the assay vessel to provide free oxygen. These concerns were addressed by attaching standard 2-mL microcentrifuge tubes containing 1.5 mL of solution and the remaining volume (0.5 mL) air to a rotating shaft (Labquake). As the tube rotates, the air bubble effectively mixes the tube contents and the seed(s) are free to move about. Kinetic studies showed that the oxygen in the assay tube was sufficient for the PPO reaction (data not shown). McCaig et al. (1999) found that the method of Kruger et al. (1994b) was affected by mixing and air volume. Vortexing produced a reaction rate 40% greater than when the tubes were inverted only twice. Advantages of the mixer used here (Labquake) were that the tubes were rotated at a constant rate (8 rpm) for the entire duration of the test, and the mixer can accommodate about 40 tubes.

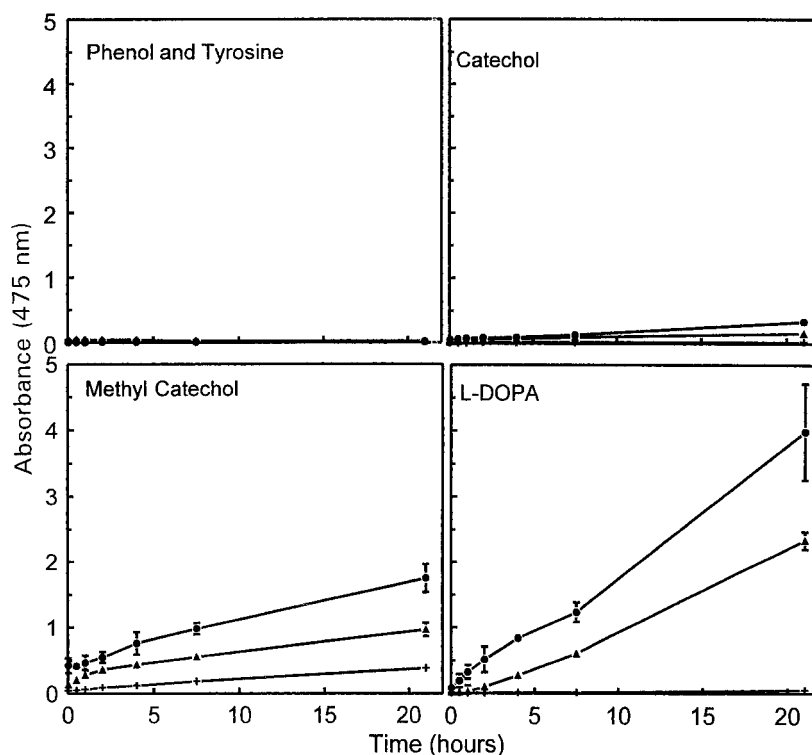Regarding the number of seeds, we sought to develop an assay that was adaptable to very small samples, in

**Fig. 1. pH-Dependent auto-oxidation of monophenolic and dihydroxyphenolic substrate solutions across time. Solutions of 2 g L$^{-1}$ phenol, and 10 m$M$ L-tyrosine, catechol, methyl catechol, and 3,4 dihydroxyphenylalanine (L-DOPA) were prepared in 50 m$M$ 3-($N$-morpholino) propane sulfonic acid (MOPS) buffer (pH 6.5) or 50 m$M$ Tris buffer (pH 7.5 and 8.5). L-tyrosine was prepared at pH 8.5 only. The solutions were allowed to stand at room temperature, and absorbance at 475 nm was measured at the time points indicated. pH levels are indicated as: 6.5 (—+—), 7.5 (—▲—), and 8.5 (—●—). Absorbance values are the average of two replicates conducted on separate days and measured at 475 nm at the time points indicated. Error bars indicate one standard deviation above and one below the mean value. Where no error bars are apparent, they are less than the size of the symbol, on the order of SD <0.1 absorbance unit.**

the 1- to 10-seed range. Both Bernier and Howes (1994) and Kruger et al. (1994b) recognized this feature and used five seeds, either in individual microtiter plate wells (Bernier and Howes, 1994) or together in a tube (Kruger et al., 1994b). Additional studies indicated that our assay conditions were amenable to this number of seeds with only slight modification (for example, increasing or re-
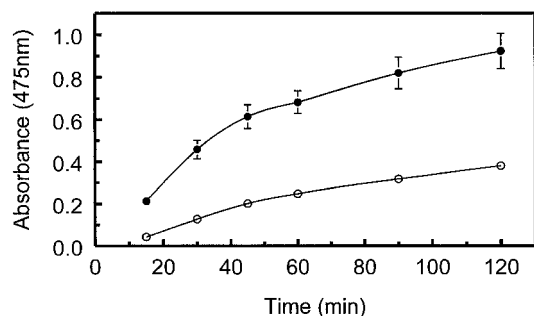


**Fig. 2. Change in absorbance across time for a wheat cultivar with high (Penawawa —●—) or low (ID377s, —○—) seed polyphenol oxidase levels. The assay used three seeds incubated with 1.5 mL of 50 m$M$ 3-($N$-morpholino) propane sulfonic acid buffer (pH 6.5) containing 10 m$M$ 3,4 dihydroxyphenylalanine (L-DOPA) in 2-mL microcentrifuge tubes with constant rotation. Absorbance values are the average of three replicate assays measured at 475 nm at the time points indicated. Error bars indicate one standard deviation above and one below the mean value. Where no error bars are apparent, they are less than the size of the symbol, on the order of SD <0.008 absorbance units.**

ducing assay time with fewer or more seeds, respectively). For our initial studies, we chose three seeds as a means of both reducing the number of seeds used while minimizing the effect of individual seeds on the result, that is, that the assay is an "average" of the three seeds. Figure 2 shows a time course for a "high" (Penawawa) and "low" (ID377s) PPO cultivar. These cultivars represent much of the range in PPO commonly encountered among breeding lines. For our standard assay, 30 min was selected as a relatively short time period that gave suitable absorbance values without significant substrate auto-oxidation or loss of zero-order kinetics. One- and two-hour assay times were also satisfactory for discriminating lines, and were used in other parts of this research.

Having established some of the assay parameters and limitations, we sought next to compare several commonly-used phenolic substrates. Figure 3 shows the results of evaluating six different phenolic substrates. Four wheat cultivars were used that were known a priori to encompass a substantial range of PPO activity that is present in U.S. Pacific Northwest cultivars. The dihydroxyphenolic substrates L-DOPA and catechol gave the greatest and similar color developments. Both ranked the four cultivars in the same order. Using L-DOPA as substrate, the wheat cultivars Penawawa and Klasic exhibited approximately two to three times more PPO activity (103 and 106 units g$^{-1}$, respectively) than ID377s
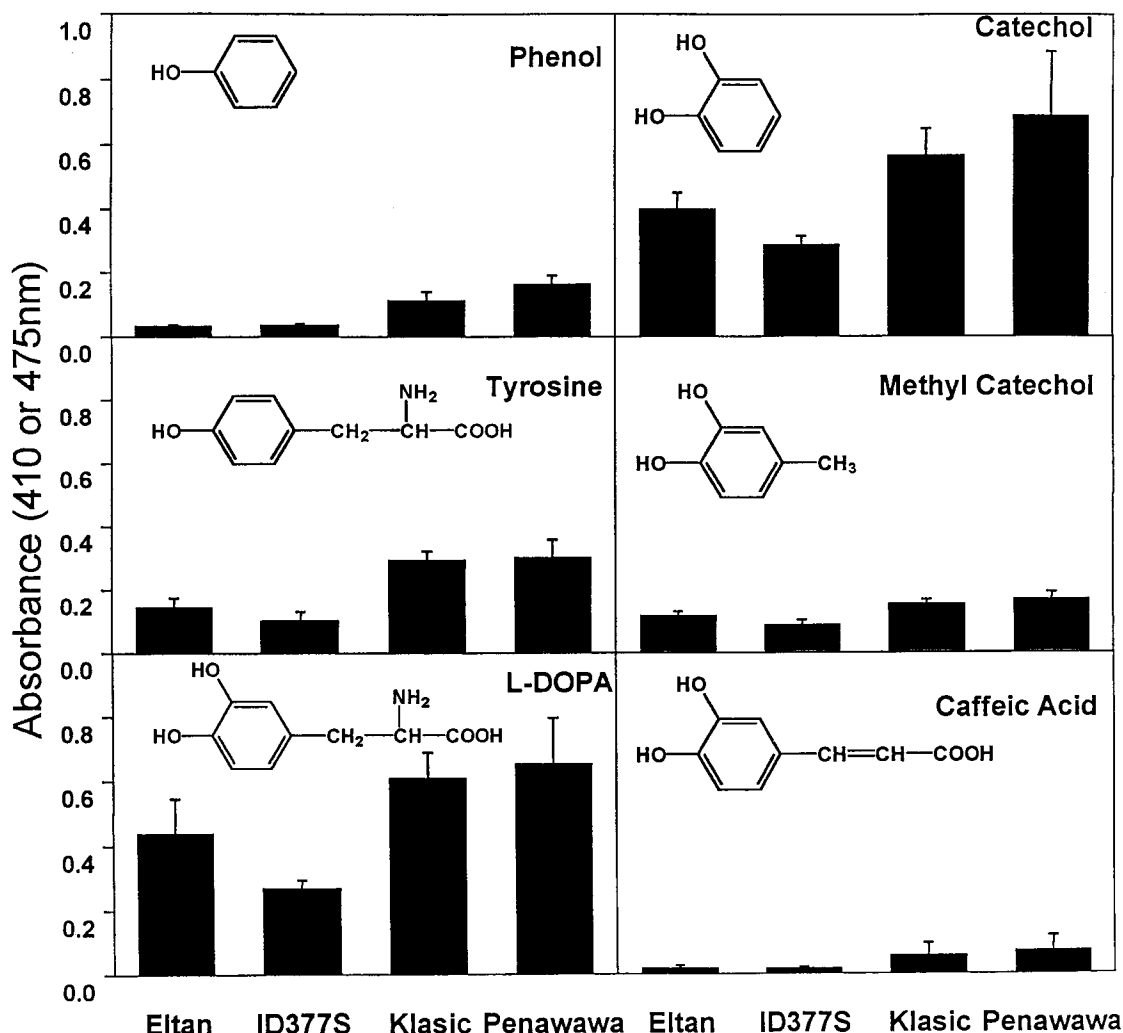
**Fig. 3. Relative polyphenol oxidase activity of seeds obtained from four wheat cultivars using the six different substrates indicated. The ordinate shows the change in absorbance at 410 nm (phenol and catechol) or 475 nm [L-tyrosine, methyl catechol, 3,4 dihydroxyphenylalanine (L-DOPA), and caffeic acid]. Catechol, methyl catechol, L-DOPA and caffeic acid reactions were conducted at 10 m$M$ substrate in 50 m$M$ 3-($N$-morpholino) propane sulfonic acid (MOPS) (pH 6.5); phenol at 2 g L$^{-1}$ and 50 m$M$ MOPS (pH 6.5); and tyrosine at 10 m$M$ in 50 m$M$ Tris (pH 8.5). Absorbance readings are the average of nine replicate standard assays per cultivar (three seeds per assay). Error bars represent one SD above the mean.**

and Eltan (38 and 61 units g$^{-1}$, respectively). These data correlate well with $L^*$ brightness values of raw alkaline noodle sheets from the same cultivars (Morris et al., 2000). Caffeic acid produced the least reaction product among the substrates tested. Methyl catechol, and the monophenolic substrates phenol and tyrosine showed an intermediate reaction rate, and generally ranked the four cultivars in a similar order as L-DOPA and catechol (Eltan and ID377s produced similar phenol reactions). Our kinetics data indicated that the seeds were more reactive to the dihydroxyphenol substrates, an effect especially evident when comparing the absorbance values for the monophenol, phenol, and the dihydroxyphenol, catechol. However, our results additionally suggest that side chain substitutions are also an important factor in substrate reactivity. Of the substrates tested, those containing an alanine side chain appear to be favored for both monophenolic substrates (L-tyrosine) and dihydroxyphenolic substrates (L-DOPA).

Since it is often highly desirable to assay one or more seeds and directly propagate desirable individuals, the effect of substrate on seed viability was investigated. When using catechol, Kruger et al. (1994b) indicated that the supernatant had to be withdrawn from the seeds and then an aliquot mixed with the substrate, thus requiring additional manipulations. McCaig et al. (1999) reported that as low as 30 m$M$ catechol reduced germination by 20%, and that all tyrosinase activity and 50% catecholase activity remained with the seed. Thus, it appears that an assay that uses the steep water only (seeds removed) because of the toxicity of the substrate to germination will not measure all of the endogenous PPO activity. Our L-DOPA assay had no significant effect on wheat seed viability. Seeds of Klasic and ID377s incubated in L-DOPA for 1 h and then germinated at room temperature reached ≈96% germination after 4 d, essentially the same as control seeds (incubated in water). After 7 d, germination (defined as radicle and
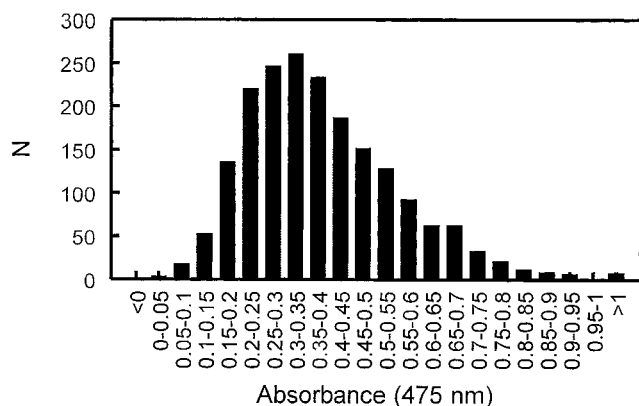
Fig. 4. Frequency histogram of the whole-seed polyphenol oxidase 3,4 dihydroxyphenylalanine (L-DOPA) values of 1,953 wheat germplasm grown under a common environment in Aberdeen, ID, and harvested in 1997.
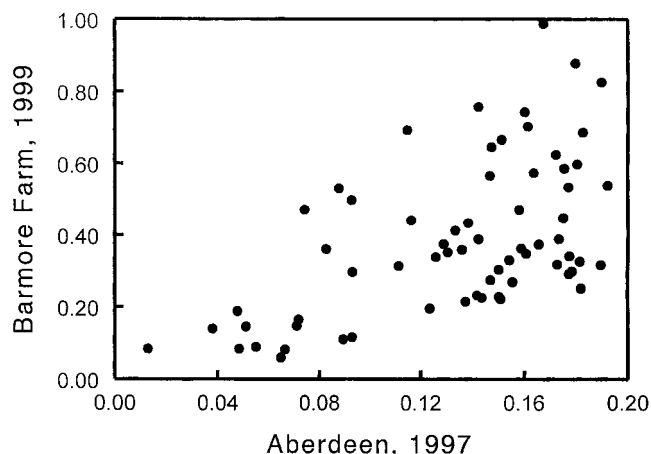


Fig. 5. Relationship between 3,4 dihydroxyphenylalanine (L-DOPA) values of low polyphenol oxidase (PPO) germplasm lines grown under two environments. The set includes 17 of 20 lines representing the best 1%, and 49 lines randomly drawn from the next 9% of the original population of 1953 lines.

coleoptile emergence) was 98.7 ± 1.2% for both Klasic and ID377s controls, and 99.3 ± 1.2% for both Klasic and ID377s L-DOPA treatments.

After reviewing these results, we chose L-DOPA as our standard assay substrate for the following reasons: (i) tyrosine and caffeic acid showed poor solubility and poor-to-intermediate kinetics, (ii) phenol is toxic, a protein denaturant, requires the use of a fume hood, and showed poor kinetics, (iii) methyl catechol auto-oxidized at pH 6.5 and showed only intermediate kinetics, and (iv) the germination of seeds incubated in catechol was reportedly reduced. Our results indicate that L-DOPA showed excellent kinetics, good solubility, and underwent minimal auto-oxidation at pH 6.5. Additionally, L-DOPA had no significant effect on seed viability.

## Screening Wheat Germplasm

Having established our standard assay conditions, we applied the method to the evaluation of a large collection of wheat germplasm. Our objective was to identify low PPO accessions, since high PPO activity is associated with darkening of Asian noodles (Baik et al., 1994, 1995; Crosbie et al., 1996; Kruger et al., 1992, 1994a,b; Miskelly, 1996).

A large set of 1953 germplasm accessions grown under a uniform environment so as to minimize potential confounding of PPO results with environmental variation was assayed using two five-seed repeated measures per accession (Fig. 4). L-DOPA values for the population were skewed to the higher absorbance values. Simple descriptive statistics for the population were mean 0.385 AU (absorbance units), median 0.359AU, skewness 0.874, and kurtosis 1.044. The Shapiro-Wilk statistic (Proc Univariate) of 0.958 was highly significant ($P < 0.0001$), indicating a significant departure from normality. The average standard deviation between the two five-seed repeated measures was 0.057 AU. Recent research has shown that incipient sprouting, abrasion, and seed size have no marked effect on the L-DOPA assay used here (Demeke et al., 2001).

The lowest 1% ($n = 20$) of this population on the basis

of the initial PPO screening are presented in Table 1. L-DOPA values from the Aberdeen environment ranged from 0.013 AU for 'MG27070' to 0.097 AU for 'Carifen'. MG27070, 'MG27965' and 'MG27055' are all listed by GRIN as originating from central Greece (39°N, 22°E). 'CNNS 2B (XII)' is cross-referenced in GRIN with 'CNNS 2D', which are 'Cheyenne' chromosome 2B and 2D substitutions, respectively, in Chinese Spring. These lines carry the D2 and D4 dwarf clump alleles. Further, GRIN lists 'Blue Norco' as an addition line carrying a gene for blue aleurone located on a pair of *Agropyron* spp. chromosomes. '153-M-M-3' is listed as being derived from a cross with *Agropyron*.

Although our initial aim was to reevaluate the 10% of the original population with the lowest PPO values, severe winter injury eliminated all but ≈15 of the lowest 200 accessions. Consequently, the following spring, 19 of the lowest 20 ("best" 1%) and a random selection of 36 germplasm of the next 9% of the original population were replanted for a total of 70 lines. Of these, 'Hermosillo 77', 'Mei Xiu Huang', and Carifen were lost from the lowest 1%, and one line from the next 9% was lost. The plants that produced seed were harvested and subjected to a second round of L-DOPA evaluation (Fig. 5).

Figure 5 presents the association between the initial L-DOPA value obtained from the National Small Grains Collection in Aberdeen, and the mean of three individual plants grown at Barmore Farm and harvested in 1999. Germplasm lines include 17 from the lowest 1% and 49 random selections from the following 9% of the original population. The simple linear correlation between environments was $r = 0.56$ ($P < 0.0001$). Approximately 12 of the 17 lowest 1% lines retained very low L-DOPA values ($\leq 0.2$ AU), whereas the remaining five lines exhibited substantially higher values. Table 1 provides the lowest L-DOPA values of three individual plants assayed from the 17 lowest lines (best 1%) obtained from the Barmore Farm environment and the SD

**Table 2. Additional 10 germplasm accessions exhibiting the lowest L-DOPA values (mean of three plants) after rescreening 49 additional germplasm accessions randomly selected from the next 9% of an original set of $n = 1953$ wheat germplasm accessions. L-DOPA values are the mean and lowest of three individual plants harvested in Pullman, WA in 1999.**

| Accession† | Accession number | Origin | L-DOPA mean | L-DOPA lowest† |
|---|---|---|---|---|
| | | | $A_{475}$ | |
| Elgin (bunt resist. 1) | CItr 12680 | USA, WA | 0.196 | 0.181 |
| Katunga | PI 591096 | Australia, VIC | 0.215 | 0.164 |
| 83SEB23SN | PI 574172 | USA, MT | 0.223 | 0.190 |
| Opata 85 | PI 591776 | Mexico | 0.226 | 0.200 |
| 459-X/115 | PI 345460 | Boznia & Herzegovina | 0.229 | 0.206 |
| Papago 86 | PI 591782 | Mexico | 0.232 | 0.183 |
| Zonk 1 | PI 283861 | India | 0.251 | 0.224 |
| Ivanovskaja 12 | PI 591853 | Russian Federation | 0.269 | 0.213 |
| Beltskaya 7 | PI 591972 | Moldova | 0.274 | 0.256 |
| Bormans 339 | PI 340681 | The Netherlands | 0.291 | 0.268 |

† Line designation follows that of the Germplasm Resources Information Network of the National Plant Germplasm System.
‡ Lowest value of three individual plants harvested at random.

among the three plants assayed. 'SST333' and 'SST124' exhibited the greatest differences between the two environments, while '153-M-M-3' and 'Swift' were the most variable (highest SD). Additional lines drawn from the next 9% of the original population that exhibited low L-DOPA values from the Barmore Farm environment are listed in Table 2. Generally, these lines averaged (mean of three plants) about 0.2 to 0.3 AU with single best plant only about 0.02 to 0.07 AU less than the mean.

Retesting lines drawn from the lowest 10% and produced under a second environment confirmed the very low PPO activity of 24 of 66 lines. Until heritability estimates can be determined and sources of variance more fully characterized, we suggest basing germplasm selection on a minimum of two concurrent or two sequential testing environments. One of our objectives in evaluating this germplasm was to identify sources of very low PPO activity for crossing with adapted lines. Lines with particularly low L-DOPA values are presented in Tables 1 and 2. Although the genetic make-up of these lines was not determined cytologically, some may be misclassified *T. durum* L. Dr. Stephen Fox of Saskatoon, SK, grew most of the lowest 1% of the original germplasm accessions in 1999 and corroborated further the low L-DOPA values among them (2000, personal communication). Clearly, application of the assay may vary depending on the specific objective. For example, to simply identify very low PPO lines in unadapted or poorly adapted germplasm, one or two environments, probably sequential with heavy selection pressure after the first (similar to the screening conducted here) may be appropriate. However, when screening early generation breeding lines with the aim of advancing promising material, a more appropriate strategy may be to only eliminate the high PPO lines, since data would likely be generated on a single, multi-seed sample. In other words, the L-DOPA assay would be used as a quick *negative* selection tool. With this strategy, populations could be efficiently improved for color potential, and the erroneous rejection of the null hypothesis (where $H_0$ = the value of the experimental line is not different from the population mean) (Type I error) is less serious. At the Western Wheat Quality Laboratory, later breeding generations receive a full alkaline noodle color analysis (Morris et al., 2000) to confirm their relative noodle color potential.

## Chromosome Location of L-DOPA Activity

Durum cultivar Langdon with D-genome homoeologous substitutions of Chinese Spring wheat chromosomes (Joppa and Williams, 1988) indicated that gene(s) associated with increased L-DOPA activity were located on Chinese Spring 2D (Fig. 6). Substitution of Langdon chromosome 2A with Chinese Spring 2D increased L-DOPA activity six times more than the mean value of the other homoeologous A-genome substitution lines. Similarly, substitution of Langdon chromosome 2B with Chinese Spring 2D increased L-DOPA activity six times more than the mean value of the other B-genome substitution lines. Jimenez and Dubcovsky (1999), using the same genetic stocks and the tyrosine method of Bernier and Howes (1994), observed essentially the same result.

The finding that Chinese Spring chromosome 2D carries PPO gene(s) was explored further using homoeolo-
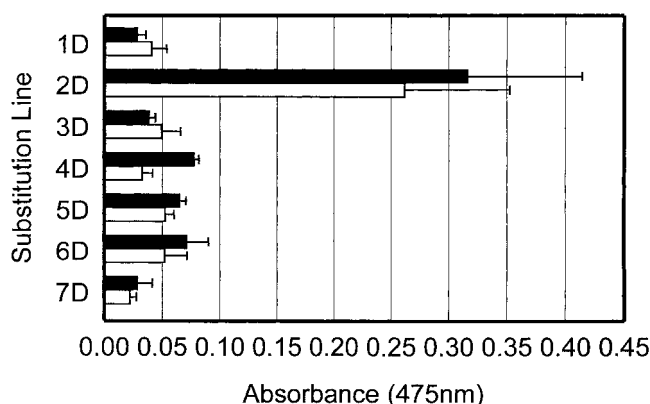


Fig. 6. 3,4 Dihydroxyphenylalanine (L-DOPA) values obtained from chromosome substitution lines of Langdon durum and Chinese Spring. Three replicates of three seeds each from each of the substitution lines indicated were incubated for 2 h in 1.5 mL of 10 m*M* L-DOPA in 50 m*M* 3-(*N*-morpholino) propane sulfonic acid (MOPS) (pH 6.5). Solid bars are Chinese Spring D-genome for A-genome substitutions, open bars are Chinese Spring D-genome for B-genome substitutions. Error bars represent one SD above the mean.
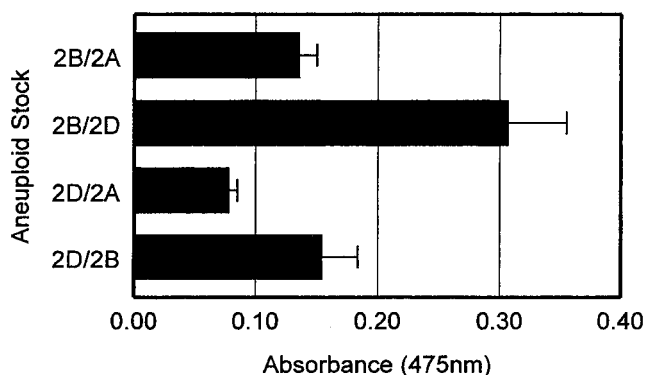
**Fig. 7. 3,4 Dihydroxyphenylalanine (L-DOPA) values of nullisomic/tetrasomic lines in Chinese Spring wheat. Absorbance values were obtained by incubating four replicates of five seeds each of each line for 1 h in 50 m$M$ 3-($N$-morpholino) propane sulfonic acid (MOPS) (pH 6.5) containing 5 m$M$ L-DOPA. Error bars represent one SD above the mean.**

gous group 2 nullisomic/tetrasomic lines of Chinese Spring (Fig. 7). Lines maintained as monosomic/tetrasomic (i.e., mono-2A/tetra-2B and mono-2A/tetra-2D) were not included. Substitution of the 2D chromosome (nullisomic-2D) with 2A (tetrasomic-2A) decreased L-DOPA activity to about one-half the activity observed in nullisomic-2B/tetrasomic-2A. Conversely, doubling the 2D-complement (nullisomic-2B/tetrasomic-2D) increased L-DOPA more than two times relative to the nullisomic-2B/tetrasomic-2A line. However, the nullisomic-2D/tetrasomic-2B exhibited a similar level of PPO activity compared with nullisomic-2D/tetrasomic-2B. Jimenez and Dubcovsky (1999) similarly observed that nullisomic-2B/tetrasomic-2D lines produced a greater color change than the nullisomic-2D/tetrasomic-2A and nullisomic-2D/tetrasomic-2B. However, they found a slightly higher level of PPO in the nullisomic-2D/tetrasomic-2A compared with the nullisomic-2D/tetrasomic-2B. We found just the reverse in these latter two lines. It should be noted that they used the monophenol tyrosine in their research.

Other research has similarly implicated group 2 chromosome(s) in PPO activity and/or suggested relatively simple genetics. Fraser and Gfeller (1936) suggested a single gene controlled the variation in phenol reaction observed among the progeny of 'Red Fife' and 'Garnet'. Bhowal et al. (1969) conducted a limited survey of diploid A-, B-, and D-genome Triticeae taxa, and found that all the A-genome species produced a black phenol seed color reaction, whereas all the B- and D-genome species produced a white phenol reaction. Joshi and Banerjee (1969, 1970) examined progeny from crosses involving eight different tetraploid wheats and concluded that the phenol reaction of seeds was controlled by a single dominant gene with three alleles (which conferred white, brown, and black phenotype). The lack of phenol reaction was later shown to result from lack of enzyme activity measured using tyrosine and oxygen consumption (Joshi et al., 1969). Souza et al. (1998) used three sets of recombinant inbred lines and found in two sets one gene, and in the third, two genes segregating for PPO. In the 'NY6432-18' × 'Clark's Cream' set, PPO activity was significantly linked to the marker, *Xcdo373*.

*Xcdo373* is linked with whole-seed L-tyrosine activity on chromosome 2AL, but not with L-DOPA activity (C. Morris, T. Demeke, and A. Campbell, unpublished data, 2000). Lastly, our data from the germplasm screening identified CNNS 2B (XII), which is described as a Cheyenne chromosome 2B substitution in Chinese Spring. This line had very low L-DOPA activity (Table 1) and may possess null alleles from Cheyenne (2B) and Chinese Spring (2A). However, Jimenez and Dubcovsky (1999) included the equivalent genotype in their study, and its reaction with tyrosine was not different from the majority of the other substitutions. This discrepancy may point to differences in genes or alleles for monophenols vs. diphenols.

These results provide strong evidence for the role of homoeologous Group 2 chromosomes as the site of an orthologous series of PPO genes in wheat. We deduce that Chinese Spring carries a null allele at the 2A locus, but carries a functional allele at the 2D locus. Furthermore, much of the variation observed for PPO may be attributable to one or two major genes, and multiple alleles conferring intermediate levels of enzyme activity or substrate specificity may exist. Consequently, the potential utility of the L-DOPA assay described here to reduce PPO in breeding populations seems high.

## CONCLUSION

Our improved assay for screening wheat seeds for PPO activity is similar in nature and objective to those described by Kruger et al. (1994b), Bernier and Howes (1994), and McCaig et al. (1999). However, our assay differs in that the reactions can be completed in as little as 30 min, does not require a microtiter plate reader, and uses L-DOPA as the substrate. Our objective in developing this assay was to provide a fast, nondestructive, predictive test to identify germplasm and early-generation breeding lines with low PPO activity, and therefore the potential to produce Asian noodles and other foods with superior color. Regarding assay parameters, pH 6.5 (50 m$M$ MOPS) was chosen, as it eliminated most auto-oxidation which occurred at higher pH levels; L-DOPA was chosen as substrate, as it was freely soluble at 10 m$M$, showed high reaction kinetics, and minimally affected seed viability; and a combination of 1.5 mL of solution with 0.5 mL of air in 2-mL microcentrifuge tubes with constant mixing for 0.5 to 2 h at room temperature eliminated boundary layer concerns and provided linear zero-order kinetics. The assay is amenable to single seed evaluations, with 3 to 5 seeds being the preferred number.

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