AN ABSTRACT OF THE THESIS OF

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in CROP SCIENCE presented on October 19, 1977

Title: STANDARDIZATION OF THE TYROSINASE TEST FOR IDENTIFICATION OF WHEAT VARIETIES

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Abstract approved: _____
Don F. Grabe

This study was undertaken to standardize and evaluate the tyrosinase test as a variety identification procedure for wheat. The potential value of the tyrosinase test lies in its ability to quantify the colored products, rather than relying on visual estimation as in the phenol test.

The specific objectives of this study were to: (a) standardize the methodology of the tyrosinase test, (b) determine the effect of various non-genetic characteristics on the results of the test, (c) determine the ability of the tyrosinase test to distinguish Pacific Northwest wheat varieties, and (d) compare the relative merits of the tyrosinase and phenol tests as variety identification methods for wheat.

The test parameters evaluated were soaking period, temperature, pH of tyrosine solution, concentration of tyrosine solution, and

shaking effect on enzyme reaction.

The following procedures are suggested for conducting the tyrosinase test on wheat seed:

For the bulk-seed method soak 2 g of seeds in 10 ml of .1% tyrosine solution in sodium citrate buffer .11 M, pH 8, for 2 hours at 35 C. For the single-seed method, soak the seed in 5 ml of .1% tyrosine solution in sodium citrate buffer .11 M, pH 8, in a shaking water bath for 17 hours at 35 C. Then read the absorbance of the resulting solution at 470 nm against the substrate solution.

Tyrosinase activity of WS-1 and Yamhill wheat seeds declined with increasing degrees of deterioration induced by artificial aging.

Vitavax treatment caused a reduction in color development or enzyme activity PCNB (Pentachloronitrobenzene) depressed color development in WS-1 but not in Yamhill. HCB (Hexachlorobenzene) had no effect on either variety.

Yamhill seeds produced in Pendleton developed a darker colored product or had more enzyme or higher enzyme activity than seeds produced in Moro, while Hyslop seeds from the two locations produced similar colored product or were comparable in enzyme activity.

Tyrosinase activity of seeds of Hyslop variety was not affected by seed protein contents of 9, 11, and 13%, indicating that total seed protein does not affect pericarp enzyme (tyrosinase) quantity or activity.

Larger seeds possessed greater tyrosinase activity than small seeds because of more pericarp tissue. Variation in enzyme activity also occurred in seeds of similar size within a variety.

Comparisons with the phenol test showed that the bulk-seed tyrosinase test probably will differentiate more varieties than the phenol test and it is less affected by fungicide seed treatments. Both tests are affected by seed deterioration and production area. Both tests are relatively quick and can be completed in approximately the same amount of time.

The phenol test is superior to individual-seed tyrosinase test for detecting varietal mixtures. Seeds within a variety differ in tyrosinase activity because of variations in seed size and other unknown factors.

The bulk-seed test may be useful in differentiating between varieties belonging to the same phenol color group. Such a quantitative measurement may also be useful in describing varieties being entered under the Plant Variety Protection Act. The tyrosinase test may also provide more accurate results on fungicide treated seed.

Standardization of the Tyrosinase Test for Identification of Wheat Varieties

by

Marlene de Matos

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1978

APPROVED:

Redacted for Privacy

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Date thesis is presented October 19, 1977

Typed by Lyndalu Sikes for Marlene de Matos

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to all those who have aided me during my research and preparation of this manuscript.

A special thanks to Dr. Don F. Grabe, my major professor, for his guidance, encouragement and patience throughout my program.

AGIPLAN for the financial support of my graduate program.

Brazilian Government (State of Minas Gerais) for co-subsidizing my studies at O.S.U.

Dr. Te May Ching for advice and for making her equipment available for my use.

The Oregon State University Seed Laboratory staff for providing assistance and facilities whenever necessary.

Finally a special thanks to my family, for their love and encouragement while I was completing this degree.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

STANDARDIZATION OF THE TYROSINASE TEST FOR IDENTIFICATION OF WHEAT VARIETIES

INTRODUCTION

The phenol test for identification of wheat varieties is based on the activity of phenol oxidase enzymes in the pericarp of the seed. To perform the test, seeds are placed on filter paper saturated with phenol solution. After a period of time the seeds develop a brown coloring indicative of the variety. Interpretation of the tests involves placing the seeds into one of five or six groups of varieties based on intensity of the brown coloration.

Although the phenol test has been in common use for many years, it has certain limitations that restrict its usefulness. Analysts frequently have difficulty in classifying varieties into the five color groups, leading to lack of uniformity of test results. Many varieties stain similarly so positive identification is usually not possible. Fungicide seed treatment, age of the seed and the location where the seed is produced all may influence the enzyme reaction and coloring of the seed. Furthermore, many analysts find phenol to be unpleasant to work with.

Because of the above limitations of the phenol test, efforts have been made to find more suitable substrates and techniques. The tyrosine is a phenolic compound and it can be used as substrate

by phenol oxidases. When this specific substrate is used, the test is called tyrosinase test. For this test seeds are placed in tyrosine solution and the soluble colored product after a period is measured in a colorimeter. Quinone, that is the first reaction product, can be quantified in solution by its absorbance at 470 nm. These quinones are easily complexed to insoluble compounds of brown color and that results in brown precipitate observed in the solution of long term incubation in this test. As the test was developed by Blakeney (9), 50 seeds, or a standard weight of seeds, are placed in a 50-ml beaker and 10 ml of .1% tyrosine solution at pH 5.3 is added. The color change of the solution is observed after 1 and 3 hours at room temperature. The shades of brown color are rated on a scale of 0 to 5. The test may be made quantitative by filtering off the solution and reading the absorbance of the filtrate at 470 nm in a spectrophotometer.

This is a promising test for wheat variety identification, but the techniques need to be standardized and the effects of non-genetic factors on the enzyme reaction need to be determined.

The objectives of this study were to:

(a) standardize the methodology of the tyrosinase test,

(b) determine the effects of various non-genetic seed characteristics on the accuracy of the test,

(c) determine the ability of the tyrosinase test to distinguish Pacific Northwest wheat varieties, and

(d) compare the relative merits of the tyrosinase and phenol tests as variety identification methods for wheat.

LITERATURE REVIEW

Tyrosinase

Enzymatic oxidation of mono- and d-phenolic compounds has been known to biochemists since the report of Bertrand in 1894 (7). Hayaishi (20) and Hayashi and Nozaki (21) classified d-phenolase as an oxidase, and monophenolase or cresolase as oxygenase. These enzymes are also known under several other names such as phenolase, phenol oxidase, catechol oxidase, tyrosinase and cresolase (6).

The presence of tyrosinase in wheat and wheat products has been well established (25). In 1907, Bertrand and Muttermilch (8) first detected the presence of tyrosinase in bran. Amounts present in sound wheat flours appear to be quite low (41) with the bran containing most of the tyrosinase activity (41, 29, 39, 30).

Wide variation of tyrosinase activity among wheat varieties has been reported (30, 41). The enzyme reaction is described as the oxidation of tyrosine to complex brown pigments (37, 16). In animals tyrosinase is responsible for the formation of the skin pigment melanin. In plants it is the cause of browning in damaged fruit tissue (9). High levels of tyrosinase are present in the grain coat of dwarf wheats and cause darkening in whole-wheat dough and chapatties (1, 2).

Enzymes catalyzing the oxidation of phenols occur widely in plant tissues and have been extensively studied (31, 33, 38, 19), but the diversity of their properties and complexities of the reactions catalyzed have made assays and their interpretation uncertain.

The two principal types of phenol oxidase or tyrosinase can best be defined in terms of the substrates used for their detection, for example, catechol for catecholase or polyphenolase, and p-cresol or phenol for cresolase or monophenolase (40). In each case the first reaction product is believed to be highly reactive equinone (31).

The Phenol Test for Variety Verification

The use of phenol color reaction for the identification of genuineness of different varieties of seed is a standard method in seed testing practice. It has been found particularly useful for varietal identification in paddy (32) and in wheat (24, 18). The Association of Official Seed Analysts has published a standardized phenol method for testing wheat seed for varietal purity (44).

Biochemical studies of the phenol color reaction have shown that it involves the enzyme phenol oxidase using phenol as a substrate (18). Joshi and Banerjee (23) determined that the site of the reaction is in the pericarp, a copper containing enzyme such as tyrosinase is involved in the reaction, and the character is

monogenically controlled, the allele for melanin pigment formation (black color) being dominant. Kruger (25) reported the polyphenol oxidase activity forming early in kernel development and decreasing with kernel maturation.

Dhesi and Desormeaux (17) and McKee (26) reported that difficulties have been experienced in the interpretation of the phenol color reaction when testing treated seeds. Age and origin of material has some influence on the phenol reaction (42). Wagner (43) reported that seeds from different harvests vary in their reaction to phenol and that classification may therefore become difficult.

Although the phenol test has been known for many years, it is unsuitable for rapid field screening because it requires long soaking and development periods (27). Furthermore it is at times difficult to decide on the degree of darkening, and phenol is an unpleasant chemical to work with (9).

Wrigley (46) stated that the toxic nature of the phenol and the possibility of skin allergy due to prolonged contact is disadvantageous and that the solution to these problems lies in the use of other reagents, less toxic than phenol.

Development of the Tyrosinase Test

Several exacting biochemical methods exist for the

determination of tyrosinase (9). Many are based on the oxidation of catechol in the presence of ascorbic acid which reduces the oxidized product back to catechol. After a time interval the residual ascorbic acid in a reaction mixture is measured (29, 30).

Abrol and co-workers (3) published a color test using tyrosine as the substrate. Although titled a rapid test, Blackeney (9) called it unsuitable for the following reasons: the substrate used is unbuffered, much of the tyrosine is insoluble at the concentration recommended, the peeling of grain coat is tedious, and the spots of reagent tend to dry out before color can develop.

Blakeney (9) reports on the development of the current tyrosinase test, beginning with Miller and Dawson (28) and more recently Horowitz et al. (22) and Abrol et al. (3). These researchers have used spectrophotometric methods using 3, 4 dihydroxy phenylalanine as the substrate. All these methods are long and tedious and unsuitable for large scale screening (9).

Milner and Gould (30) investigated variables affecting tyrosinase reaction in wheat and other grains. They found that in wheat, a pH of 4 in phosphate and 5.2 in citrate buffers was optimum, with citrate the most favorable for maximum activity. Catechol pyrogallol, p-cresol, phenol and gallic acid all acted as substrates, in decreasing order of rate of oxidation, but tyrosine itself was not

tested. The temperature optimum for the enzyme reaction was not established.

Bodine et al. (12) reported the temperature range from 55 C to 70 C produces maximum activity of tyrosinase from the egg of grasshoppers while exposures to higher temperatures resulted in inactivation. Csala (15), studying the mechanism of phenol reaction in cereals, measured the enzyme reaction as a function of time and temperature. He found that near 0 C the coloring was slow, taking about 1-1/2 days. At 20 to 22 C the coloring took place within 3 hours, at 30 to 40 within 1-1/2 hours, at 50 to 60 C it took from 2 to 2-1/2 hours, at 70 C from 2 to 2-1/2 hours and at 80 C it occurred within 3 hours. In all cases there were differences characteristic of the varieties. Full inhibition occurred at 90 to 95 C. This study compliments the findings of Blakeney (9) who reported room temperature satisfactory for the tyrosinase test though temperatures up to 40 C gave increased rates of color development. Above 50 C color development in the solution is inhibited, though the grains still discolor.

A relatively wide range of pH is cited by several workers. An optimum pH of 7.0 was observed for eggplant polyphenol oxidase (35), McFarlin cranberry polyphenol oxidase (13) and bean leaf polyphenol oxidase (34). In a more recent report, Wong et al. (45) separated the polyphenol oxidase of peaches into four components

having pH optima of 6.8, 6.5, 7.2 and 7.0. From these data the pH optima of various polyphenol oxidase systems appear to be near neutrality, contrary to the findings of Blakeney (9), Smith and Stotz (40) and Milner (29) who reported a pH range of 5.1 - 5.5, 6 and 5.2 respectively, as optimum for wheat polyphenol oxidase.

To overcome the disadvantages of the phenol color test, Blakeney (10) has developed a new color test using tyrosine instead of phenol. The tyrosinase test consists of soaking 50 seeds (or 1-1/2 g) in 10 ml of a dilute buffered solution, the tyrosinase activity of a sample being directly proportional to the amount of color formed. After 1 hour the level of tyrosinase can be distinguished, and is obvious after 3 hours. On long standing the color precipitates but by this time the bran coat has developed color. In the field, color development in the solution may be judged on a five-point visual scale. In the laboratory the test is made by measuring the color intensity in a spectrophotometer (11).

MATERIALS AND METHODS

Seed Lots

Seeds of the wheat varieties used in this work were produced in experimental plots at Hyslop Crop Science Field Laboratory, Corvallis, Oregon, unless noted otherwise. The seed lots are described in Table 1.

Optimum Conditions for Conducting the Tyrosinase Test on a Bulk-Seed Basis

Basic Test Procedures

The test procedures developed by Blakeney (9) served as the starting point for evaluating test parameters. Certain procedures were held constant throughout the evaluation studies: (a) 2 g seeds were placed in 50-ml beakers in a controlled-temperature chamber or water bath; (b) 10 ml substrate of tyrosine solution in sodium citrate buffer (0.11 M) were added; (c) transmittance of the solution was read at 470 nm in a B&L Spectronic 20; (d) a blank reference of the substrate solution was used to set the colorimeter at 100% T; (e) before making a transmittance reading the solutions were mixed by shaking the test tube three times; (f) transmittance readings were transformed to absorbance by the formula $A = \log\frac{1}{Tf}$, where A = absorbance and Tf = fractional transmittance (4); (g) each

Table 1. Description of wheat seed lots.

Lot no.	Variety	Class	Additional comments
1	Adams	soft, white, spring	
2	Brevor	soft, white, winter	
3	Daws	soft, white, winter	
4	Druchamp	soft, white, winter	
5	Fielder	soft, white, spring	
6	Gaines	soft, white, winter	
7	Henry	hard, red, spring	
8	Hyslop	soft, white, winter	Produced in Pendleton, OR
9	Hyslop	soft, white, winter	Produced in Moro, OR
10	Hyslop	soft, white, winter	9% protein
11	Hyslop	soft, white, winter	11% protein
12	Hyslop	soft, white, winter	13% protein
13	Idaed 59	soft, white, spring	
14	McDermid	soft, white, winter	
15	Marfed	soft, white, spring	
16	Nugaines	soft, white, winter	
17	Paha	soft, white, winter	
18	Profit 75	hard, red, spring	
19	Stephens	soft, white, winter	
20	Wandell	durum spring	
21	Wared	hard, red, spring	
22	WS-1	hard, white, spring	
23	WS-1	hard, white, spring	
24	WS-3	durum spring	
25	Yamhill	soft, white, winter	
26	Yamhill	soft, white, winter	Produced in Pendleton, OR
27	Yamhill	soft, white, winter	Produced in Moro, OR

test consisted of 12 observations; and (h) tests were conducted with WS-1 (lot 22), a variety relatively high in tyrosinase activity and Yamhill (lot 25), a variety relatively low in tyrosinase activity.

The test parameters evaluated were soaking period, temperature, pH of tyrosine solution, concentration of substrate solution, and shaking during the soaking period. All other conditions were held constant while the parameter in question was being evaluated.

Criteria for evaluating each of the test conditions included accuracy, quickness, degree of varietal discrimination obtained, statistical significance, and availability of equipment in seed testing laboratories.

Evaluation Parameters

Soaking period. Seeds of WS-1 and Yamhill were soaked for 1, 2, 3, 6, 9, 12 and 15 hours in a .1% solution of tyrosine, pH 5.3, at 25 C.

Temperature. Seeds were soaked at 15 and 35 C for 6 hours in a .1% solution of tyrosine, pH 5.3.

pH of tyrosine solution. Seeds were soaked in .1% tyrosine solution with pH of 5, 6, 7 and 8 for 6 hours at 35 C. The standard procedure for preparation of a .1% tyrosine solution was modified as follows (T. M. Ching, personal communication): 1 g tyrosine and 21.48 g citric acid were dissolved in 600 ml water. This

solution was divided between four beakers, 150 ml in each beaker. The solution in each beaker was titrated with 1N NaOH to obtain the desired pHs of 5, 6, 7 and 8. Sufficient water was added to a total of 250 ml solution in each beaker.

Soaking period at pH 8. Seeds were soaked for 1, 2, 3, 4 and 5 hours in a .1% solution of tyrosine, pH 8, at 35 C.

Concentration of tyrosine solution. Seeds were soaked in solutions of .01, .025, .05 and .1% tyrosine, pH 8, for 2 hours at 35 C.

Shaking during soaking period. Seeds were soaked for 2 hours at 35 C in a .1% tyrosine solution, pH 8. One set of beakers was placed in a shaking water bath and another set was placed in a non-shaking water bath. There were eight observations per treatment instead of the usual 12.

Optimum Conditions for Conducting the Tyrosinase Test on a Single-Seed Basis

Basic Test Procedures

Procedures developed for bulk seed samples served as the starting point for evaluating test parameters for single seeds. One seed was placed in a 10-ml test tube in a water bath at 35 C. Five ml of .1% tyrosine solution, pH 8, were added. Twenty seeds were used for each evaluation.

Tests were conducted with WS-1 (lot 22) and Yamhill (lot 25) varieties.

Evaluation Parameters

Soaking period. Seeds were soaked for 5 and 17 hours.

Shaking during soaking period. Seeds were soaked for 5 and 17 hours. One set of test tubes was placed in a shaking water bath and another set was placed in non-shaking water bath.

Effect of Non-Genetic Seed Characteristics on Tyrosinase Activity

Basic Tyrosinase Test Procedures

Two grams of seed were soaked for 2 hours at 35 C in a .1% tyrosine solution, in sodium citrate (0.11 M), pH 8.

Seed Characteristics

Artificial aging. One-hundred-gram lots of WS-1 (lot 22) and Yamhill (lot 25) were adjusted to 15% moisture and stored in closed jars at 35 C. Small quantities of seed were removed from the jars at 2-day intervals for 20 days, air-dried, and tested for viability and tyrosinase activity. There were four observations per treatment.

Fungicide seed treatments. Twenty-gram lots of WS-1 (lot 22) and Yamhill (lot 25) were treated with PCNB,[2/] HCB,[3/] Vitavax[4/] and Vitavax 200[5/]. The fungicides were applied by shaking the seeds in a jar containing 1 ml of fungicide in 30 ml of water until the seeds were uniformly covered. The seeds were then tested for tyrosinase activity. Each test was conducted with eight observations. The possible contribution of the fungicide dye to the colorimeter reading was tested by reading the transmittance when drops of the fungicides were added to test tubes of tyrosine solution.

Production area. Hyslop and Yamhill wheat produced in Pendleton and Moro, Oregon (lots 8, 9, 26 and 27) were tested for tyrosinase activity. There were eight observations per treatment.

Total Protein content. Hyslop wheat containing 9, 11 and 13% protein (lots 10, 11 and 12) was tested for tyrosinase activity. There were eight observations per treatment.

Seed size. Seeds of Wandell (lot 20) were divided into large, medium and small size catagories with hand screens. Large seeds were those held by an 8/64 x 3/4" screen, medium seeds were those that passed through an 8/64 x 3/4" screen but were held by a 6/64 x

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2/ Pentachloronitrobenzene, active ingredient pentachloronitrobenzene

3/ Hexachlorobenzene, active ingredient hexachlorobenzene

4/ Vitavax, active ingredient carboxin 75%, F. Uniroyal

5/ Vitavax 200, active ingredient carboxin 17%, thiram 17%, F. Uniroyal

3/4" screen, and small seeds were those that passed through a 5-1/2/64 x 3/4" screen.

Individual seeds were then weighed. Ten seeds of each size were tested individually for tyrosinase activity by soaking 15 hours at 35 C in a shaking water bath.

In a second study of seed size effects, 20 seeds of Wandell (lot 20) were selected, individually weighed, and tested for tyrosinase activity by soaking 15 hours at 35 C in a shaking water bath.

Comparison with Phenol Test Results

For comparison purposes, phenol tests were also conducted on the seed lots representing different levels of artificial aging, fungicide treatment, and production area.

The procedures followed for the phenol test were those outlined by Walls (44). Two replicates of 100 seeds were placed in a container and covered with scalding water for 10 minutes. Then, these seeds were flushed with tap water and the excess water was removed from the surface of the seeds.

Two layers of filter paper were placed in a container and moistened with a 1% phenol solution. The seeds were placed palea side down on the layer of filter paper and the containers were covered.

Observations were made at 2 hours. Seed coat colors were evaluated according to the catagories: ivory, fawn, light brown, brown and dark brown.

Tyrosinase Activity of Pacific Northwest Wheat Varieties

After optimum testing procedures were established, the tyrosinase activity of 20 Pacific Northwest wheat varieties was determined, both on a bulk and single-seed basis.

For bulk-seed tests, 2 g of seeds were soaked 2 hours at 35 C in 10 ml of .1% tyrosine solution, pH 8. There were four observations per variety.

For single-seed tests, single seeds were soaked 17 hours at 35 C in 5 ml of .1% tyrosine solution, pH 8, with shaking. Twenty seeds were tested for each variety.

Statistical Analysis

Data were analyzed by Analysis of Variance according to standard procedures.

The t test at 5% and 1% levels of significance was used to test differences between two means.

The test criteria used for comparison of more than two means were the Least Significant Difference (LSD) or the Honestly Significant Difference (HSD) at the 5% probability level.

RESULTS AND DISCUSSION

Optimum Conditions for Conducting the Tyrosinase Test on a Bulk-Seed Basis

Evaluation Parameters

Soaking period. When the tyrosinase test was conducted by Blakeney's method (9), absorbance readings increased as soaking time was extended up to 12 hours (Figure 1). At 15 hours, absorbance readings decreased, an indication of precipitation occurring in the solution, which Blakeney also found.

The discrimination between varieties increased after 3 hours, suggesting that a period longer than Blakeney's 1 and 3 hours would be beneficial. Since it is important to complete the test in a single working day, 6 hours was selected as the optimum soaking period when the test conditions are as specified by Blakeney. Also, as Csala (15) has pointed out, the enzymatic reaction is completed in a relatively short time and color changes continue to occur with extended soaking due to non-enzymatic processes.

Temperature. Using a 6-hour soaking period, the effect of temperature on the tyrosinase reaction was next determined (Figure 2). Discrimination between variaties increased as the temperature was increased from 15 C to 25 C to 35 C. Although other studies

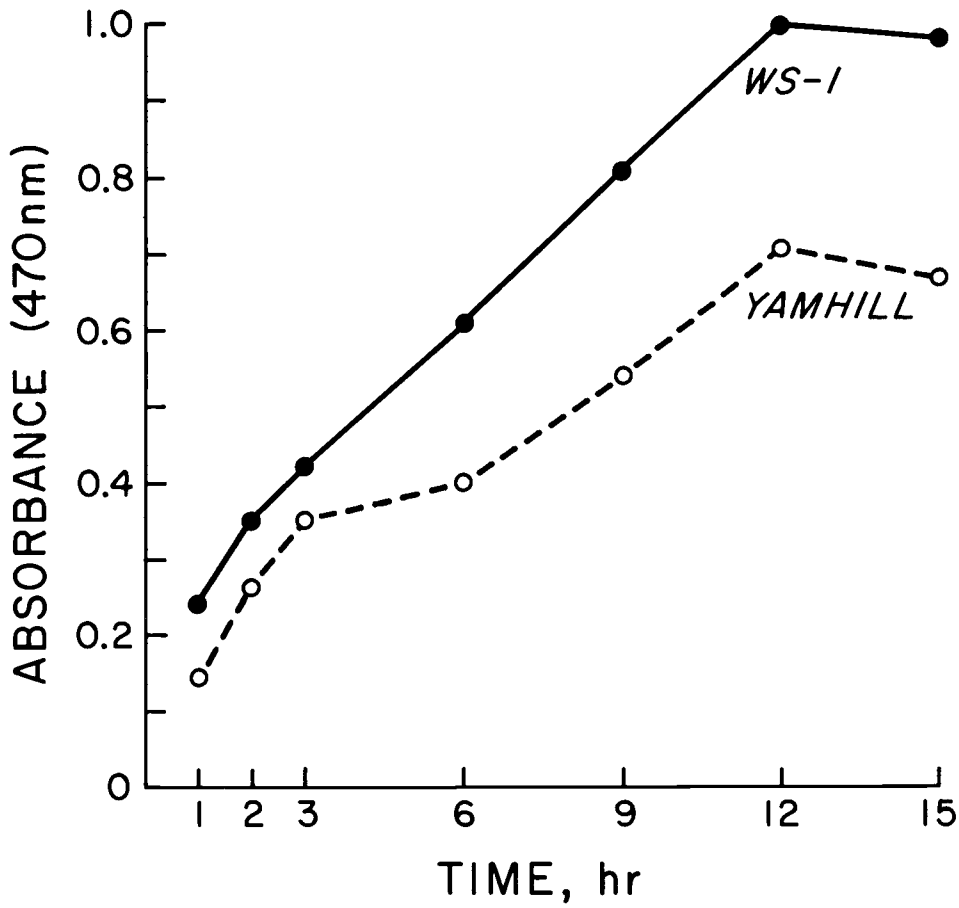

Figure 1. Effect of soaking time on tyrosinase activity of WS-1 and Yamhill wheat seed. Two g seed soaked in .1% tyrosine solution with pH 5.3 at 25 C.

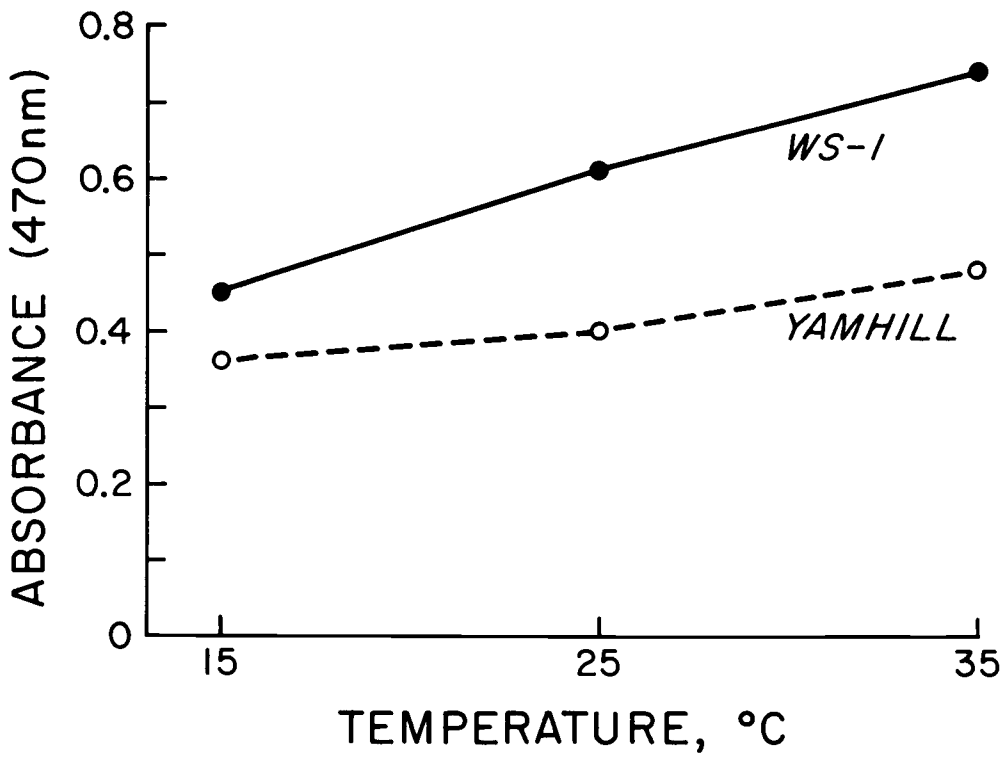

Figure 2. Effect of temperature on tyrosinase activity of WS-1 and Yamhill wheat seed. Two g seed soaked 6 hr in .1% tyrosine solution with pH 5.3.

(9, 12, 15) have shown the reaction rate to increase up to 40 C, 35 C was chosen as a convenient temperature for seed testing laboratory use. This temperature can be maintained in a good seed germinator and purchase of additional equipment is unnecessary.

pH of tyrosine solution. Next, the effect of pH level on the reaction after 6 hours at 35 C was determined. The greatest discrimination between the test varieties occurred at pH 8 (Figure 3) and this pH was chosen for all subsequent tests.

Several workers (13, 34, 35, 45) have found the pH optima for various polyphenol oxidase systems to be near neutrality, while others (9, 29, 40) have reported pH optima of 5.1 to 6 for wheat polyphenol oxidase.

Soaking period at pH 8. Since the reaction occurs several times faster at pH 8 than at pH 5, the optimum soaking period was restudied at pH 8 to determine if the test period could be shortened. As shown in Figure 4, little change in discrimination between varieties occurred after 2 hours; therefore 2 hours was chosen as the optimum soaking period for test purposes.

Concentration of the tyrosine solution. As shown in Figure 5, the degree of discrimination between varieties remained approximately the same at solution concentrations of .025% and above. However, the absorbance reading increased with each increase in concentration up to .1%. Higher concentrations cannot be used as

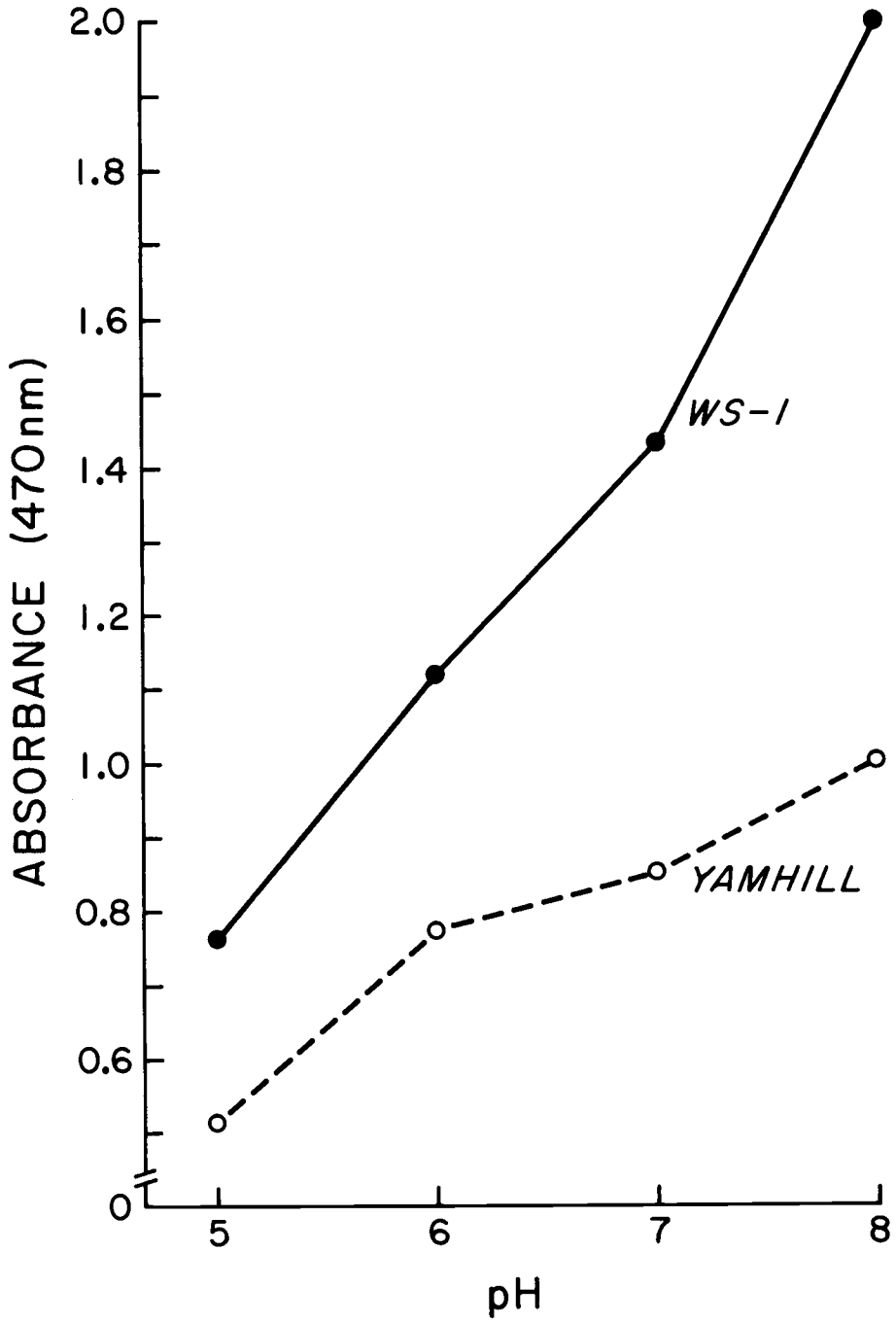

Figure 3. Effect of pH on tyrosinase activity of WS-1 and Yamhill wheat seed. Two g seed soaked 6 hr in .1% tyrosine solution at 35 C.

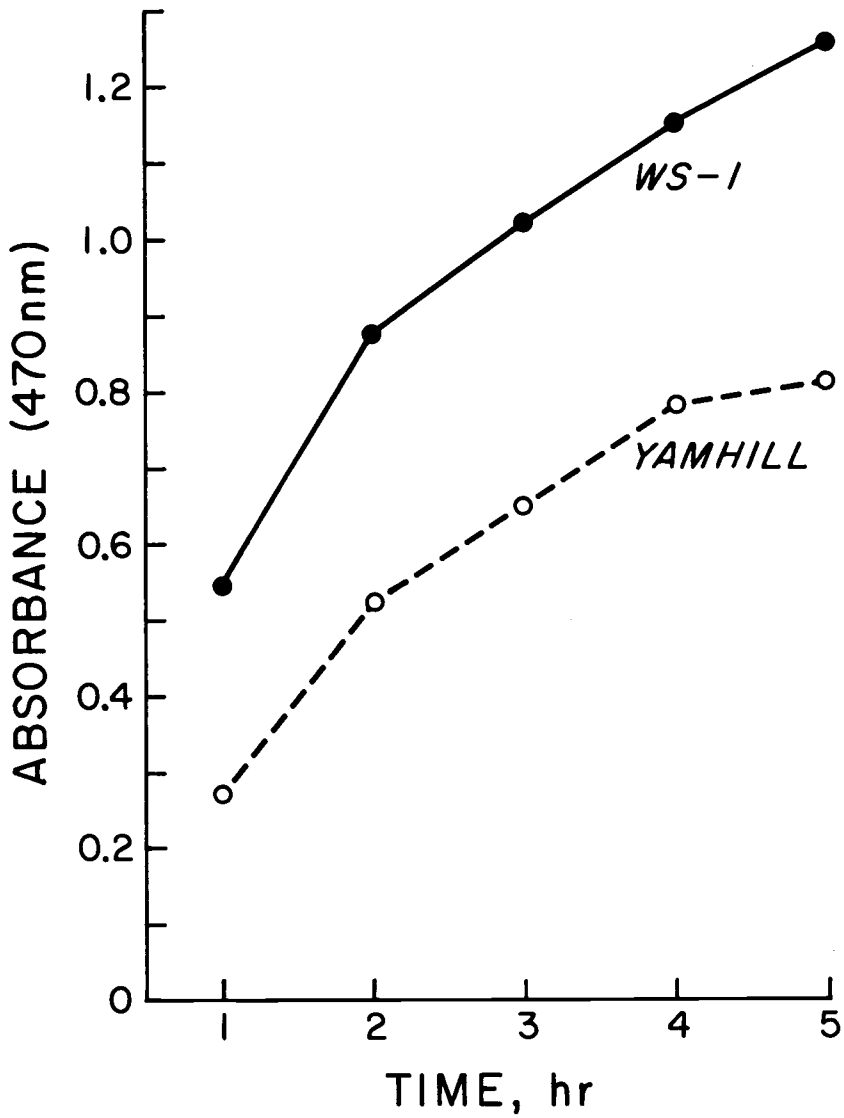

Figure 4. Effect of soaking time on tyrosinase activity of WS-1 and Yamhill wheat seed. Two g soaked in .1% tyrosine solution with pH 8 at 35 C.

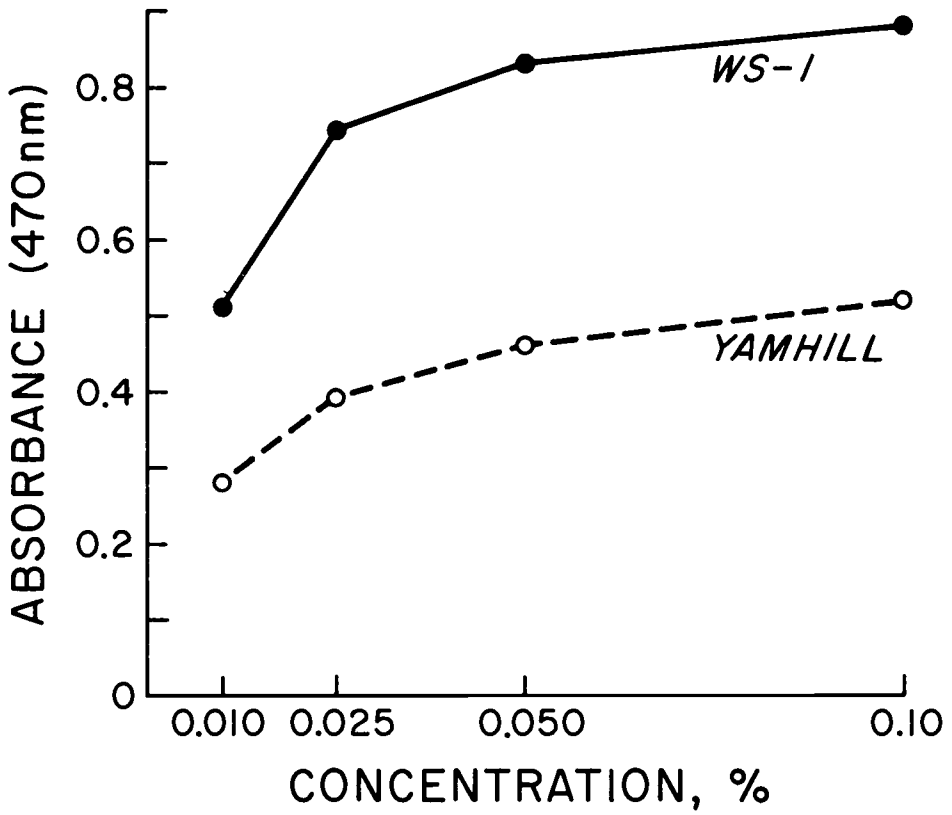

Figure 5. Effect of substrate concentration on tyrosinase activity of WS-1 and Yamhill wheat seed. Two g seed soaked 2 hr in tyrosine solution with pH 8 at 35 C.

the solution is supersaturated at .1%. An optimum concentration of .1% as suggested by Blakeney (9) was affirmed in this test.

Shaking during soaking period. Continuous agitation of the beakers caused a small increase in the absorbance readings (Figure 6). This increase was not considered important enough to recommend the use of a shaking water bath for routine testing.

Optimum Conditions for Conducting the Tyrosinase Test on a Single-Seed Basis

Seeds must be examined separately to determine the percentage of varietal purity in a seed lot. The procedures established for testing bulk samples of seeds did not produce sufficient discrimination on a single seed basis. Therefore tests were conducted with individual seeds to determine the effects of longer soaking periods and shaking during the reaction period. As shown in Figure 7, both the color intensity and discrimination between varieties was increased by soaking 17 hours in a shaking water bath. These two procedures are recommended for testing individual seeds.

Effect of Non-Genetic Seed Characteristics on Tyrosinase Activity

For a varietal verification test to be reliable, the results must not be influenced by factors to which the seeds are subjected

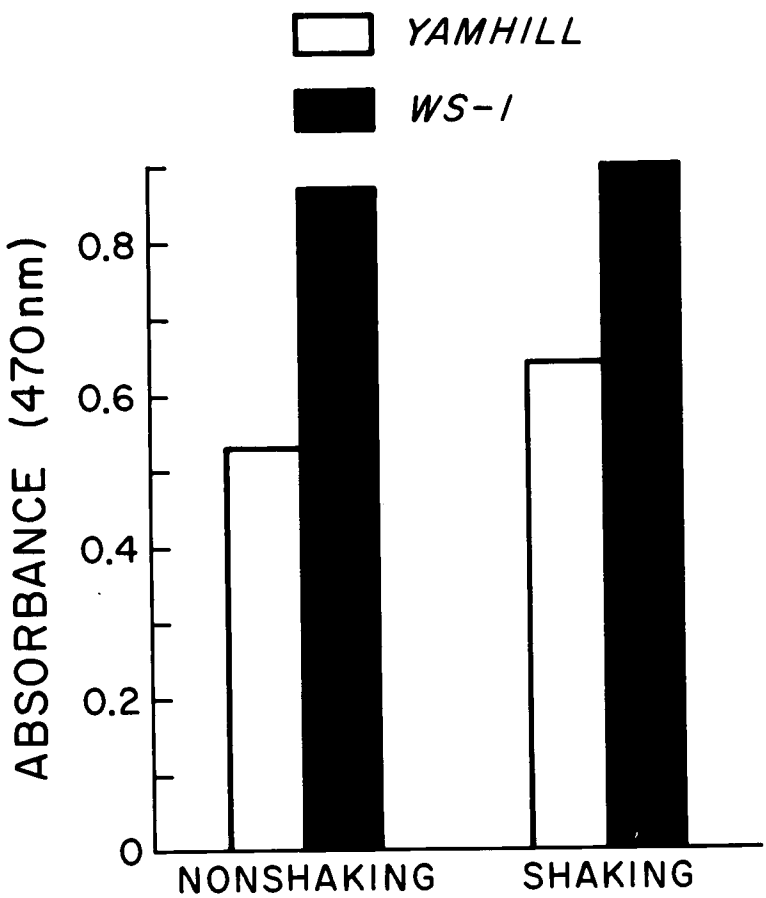

Figure 6. Effect of shaking on tyrosinase activity of WS-1 and Yamhill wheat seed. Two g seed soaked 2 hr in .1% tyrosine solution with pH 8 at 35 C.

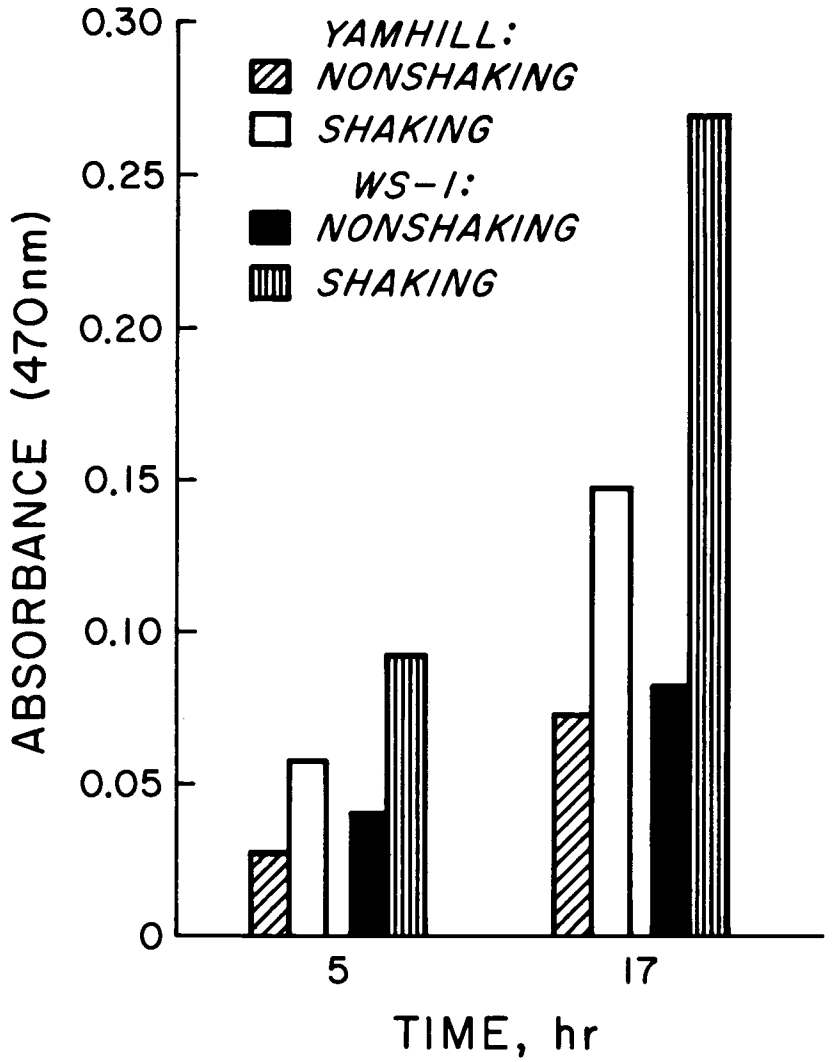

Figure 7. Effect of soaking time and shaking on tyrosinase activity of WS-1 and Yamhill wheat seed. Single seed soaked in .1% tyrosine solution with pH 8 at 35 C.

during production and marketing. Therefore a series of tests were conducted to determine the effect of seed size, deterioration, production area, fungicide seed treatment and seed protein content on tyrosinase reaction. Comparisons were also made of the effects of these factors on the phenol test.

Artificial Aging

Seeds of WS-1 and Yamhill were artificially aged to determine the effect of seed deterioration on tyrosinase activity.

As seen in Table 2, tyrosinase activity declined with increasing stages of deterioration. Phenol tests conducted on seeds aged for 20 days (Table 3) showed the same relationship, with a definite shift toward a lighter color reaction than the non-deteriorated seeds.

A decrease in tyrosinase activity as seeds deteriorate is to be expected since the activity of many other enzymes decline as seeds age (36).

Fungicide Seed Treatment

Fungicides, because of the dyes they contain, may obscure the phenol staining reaction (17, 26). As shown in Table 4, HCB and PCNB tended to make the seeds appear darker. Vitavax, a systemic fungicide, prevented any color development, rendering

Table 2. Effect of artificial aging on germination and tyrosinase activity of WS-1 and Yamhill wheat seed.

Storage Period	WS-1		Yamhill	
	Germination	Tyrosinase activity	Germination	Tyrosinase activity
days	%	absorbance	%	absorbance
0	97	.882	90	.532
2	98	.732	82	.484
4	89	.779	67	.408
6	91	.752	51	.429
8	85	.736	44	.419
10	76	.762	31	.409
12	66	.720	20	.407
14	54	.722	15	.396
16	37	.637	13	.399
18	29	.612	8	.348
20	13	.679	4	.349

Tyrosinase activity

LSD_{05} for WS-1 = .025

LSD_{05} for Yamhill = .032

Correlation of germination and tyrosinase activity

r for WS-1 = .78**

r for Yamhill = .89**

** significant at 1% level of probability.

Table 3. Effect of artificial aging on phenol reaction of WS-1 and Yamhill wheat seed.

Phenol Color Reaction	WS-1		Yamhill	
	Check	Aged Seed	Check	Aged Seed
	- % -			
Ivory				25
Fawn		45	26	53
Light Brown	15	55	74	2
Brown	85			
Dark Brown				

Table 4. Effect of fungicide treatment on the phenol reaction of WS-1 and Yamhill wheat seed.

Phenol Color Reaction	WS-1					Yamhill				
	Untreated	PCNB	HCB	Vitavax	Vitavax 200	Untreated	PCNB	HCB	Vitavax	Vitavax 200
	%									
Ivory				100	100				100	100
Fawn						39	38	14		
Light Brown	5	22	5			61	54	77		
Brown	95	78	95				8	9		
Dark Brown		6								

the phenol test useless on Vitavax-treated seed. Vitavax 200, which contains Arasan, reacted similarly.

Vitavax treatment also caused a reduction in color development in the tyrosinase test (Table 5) but not to the extent that occurred in phenol. PCNB appeared to depress color development in WS-1 but not in Yamhill. HCB had little or no effect on either variety.

The contribution of fungicide and dyes to the absorbance readings were: Vitavax .012; Vitavax 200, .012; PCNB, 0 and HCB, .021.

Production Area

Seeds produced in different locations may vary in their phenol reaction (42). This is also shown in Table 6 where seeds of Yamhill produced in Pendleton produced a lighter color reaction. In tyrosinase tests, Yamhill seed produced in Pendleton produced a darker color reaction than seed grown in Moro, while Hyslop from the two locations produced similar reactions (Figure 8).

Protein Content

The tyrosinase activity of Hyslop wheat was similar in seed lots with 9, 11, and 13% protein (Table 7).

Table 5. Effect of fungicide treatment on the tyrosinase activity of WS-1 and Yamhill wheat seed.

Fungicide	Tyrosinase Activity	
	WS-1	Yamhill
	- - - - absorbance - - - -	
Untreated	.822	.504
PCNB	.764	.493
HCB	.818	.484
Vitavax	.785	.342
Vitavax 200	.770	.282

LSD_{05} for WS-1 = .038

LSD_{05} for Yamhill = .031

Table 6. Effect of production area on the phenol reaction of Yamhill and Hyslop wheat seed.

Phenol Color Reaction	Yamhill		Hyslop	
	Moro	Pendleton	Moro	Pendleton
	- - - - - - - - - - - - - % - - - - - - - - - - - - -			
Ivory				
Fawn		5		
Light Brown	9	10	15	14
Brown	91	85	85	86
Dark Brown				

Table 7. Effect of total protein content on the tyrosinase activity of Hyslop wheat seed.

Protein Content	Tyrosinase Activity
%	Absorbance
9	.51
11	.51
13	.49

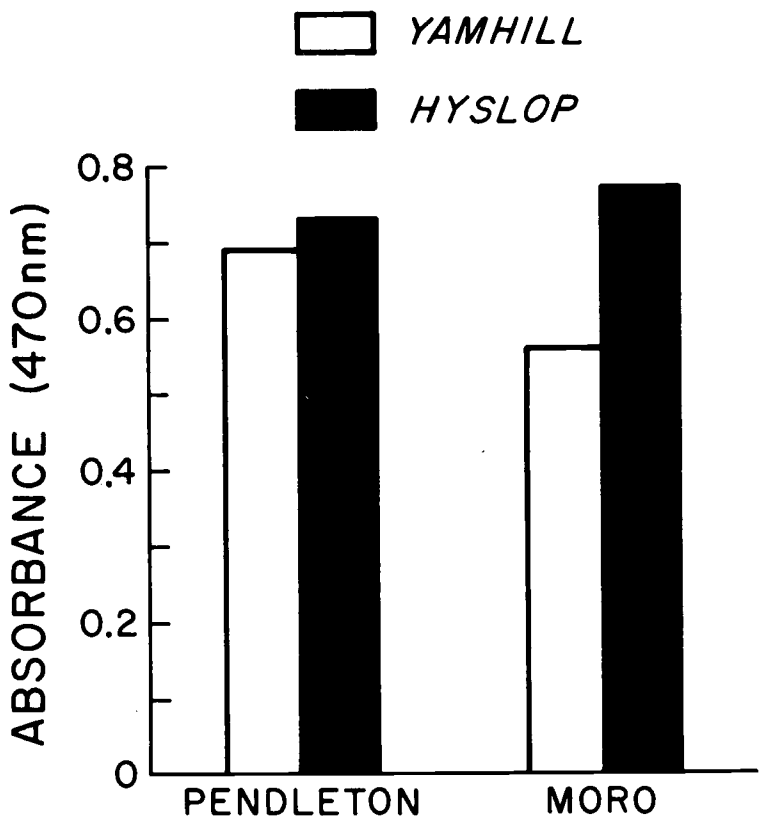

Figure 8. Effect of production area on tyrosinase activity of Yamhill and Hyslop wheat seed. Two g seed soaked 2 hr in .1% tyrosine solution with pH 8 at 35 C.

Seed Size

This experiment was designed to test the hypothesis that larger seeds, having more pericarp area, would have greater tyrosinase activity (23). That this is true is shown in Tables 8 and 9. When Wandell wheat was sized with screens, the average absorbance reading was .063 for large seed, .051 for intermediate seed, and .014 for small seed (Table 8). However, considerable variation in absorbance readings still occurred in seeds of similar size. Both the seed-to-seed variation and seed size effects must be considered when conducting the tyrosinase test on an individual seed basis.

When 20 seeds were selected to represent a continuum of sizes from very small to very large, the correlation between seed size and tyrosinase activity was .741 (Table 9). Thus seed size is clearly responsible for part of the variation in absorbance readings between individual seeds within a seed lot.

Crisp and Wrigley (14) reported that seed size affected the amount of colored material extracted per seed in the phenol test.

Table 8. Relationship of seed size and weight to tyrosinase activity of Wandell wheat seed.

Seed Number	Seed Size					
	Large		Intermediate		Small	
	Seed Weight	Tyrosinase Activity	Seed Weight	Tyrosinase Activity	Seed Weight	Tyrosinase Activity
	mg	absorbance	mg	absorbance	mg	absorbance
1	52	.046	17	.029	9	.016
2	52	.092	19	.028	10	.009
3	56	.052	19	.043	10	.011
4	56	.029	20	.080	11	.009
5	58	.073	21	.051	11	.018
6	59	.102	21	.043	12	.014
7	59	.040	24	.053	12	.004
8	59	.066	25	.052	13	.032
9	60	.047	26	.060	14	.009
10	71	.086	26	.072	15	.018
Mean	58.2	.063	21.8	.051	11.7	.014
S. D.	5.3	.024	3.2	.017	1.9	.008

Table 9. Relationship of seed weight to tyrosinase activity of Wandell wheat seed.

Seed Weight	Tyrosinase Activity
mg	absorbance
8	.018
9	.013
15	.034
20	.029
22	.022
24	.022
27	.036
29	.063
32	.036
35	.055
36	.061
37	.032
44	.039
47	.034
51	.053
52	.050
52	.078
55	.039
69	.076
73	.161

r = .741**

** significant at 1% level of probability

Tyrosinase Activity of Pacific Northwest Wheat Varieties

Bulk-Seed Method

The ability of the tyrosinase test to differentiate between varieties was determined on 16 white varieties and 4 red varieties. Absorbance readings on bulk samples of the white varieties ranged from .080 to .804, a reaction range allowing for differentiation between numerous varieties (Figure 9). A wide range of absorbance readings also occurred in the red varieties (Figure 10).

Maguire et al. (27) classified the Pacific Northwest winter and spring wheat varieties in 5 groups using the phenol test. Additional classifications could be made by use of the tyrosinase test.

Single-Seed Method

The ability of the tyrosinase test to differentiate between varieties on a single-seed basis was determined on 16 white varieties and 4 red varieties. The absorbance mean for 20 single seeds for each white variety ranged from .039 to .156, allowing for differentiation between numerous varieties (Figure 11). A wide range of absorbance readings also occurred in the red varieties (Figure 12).

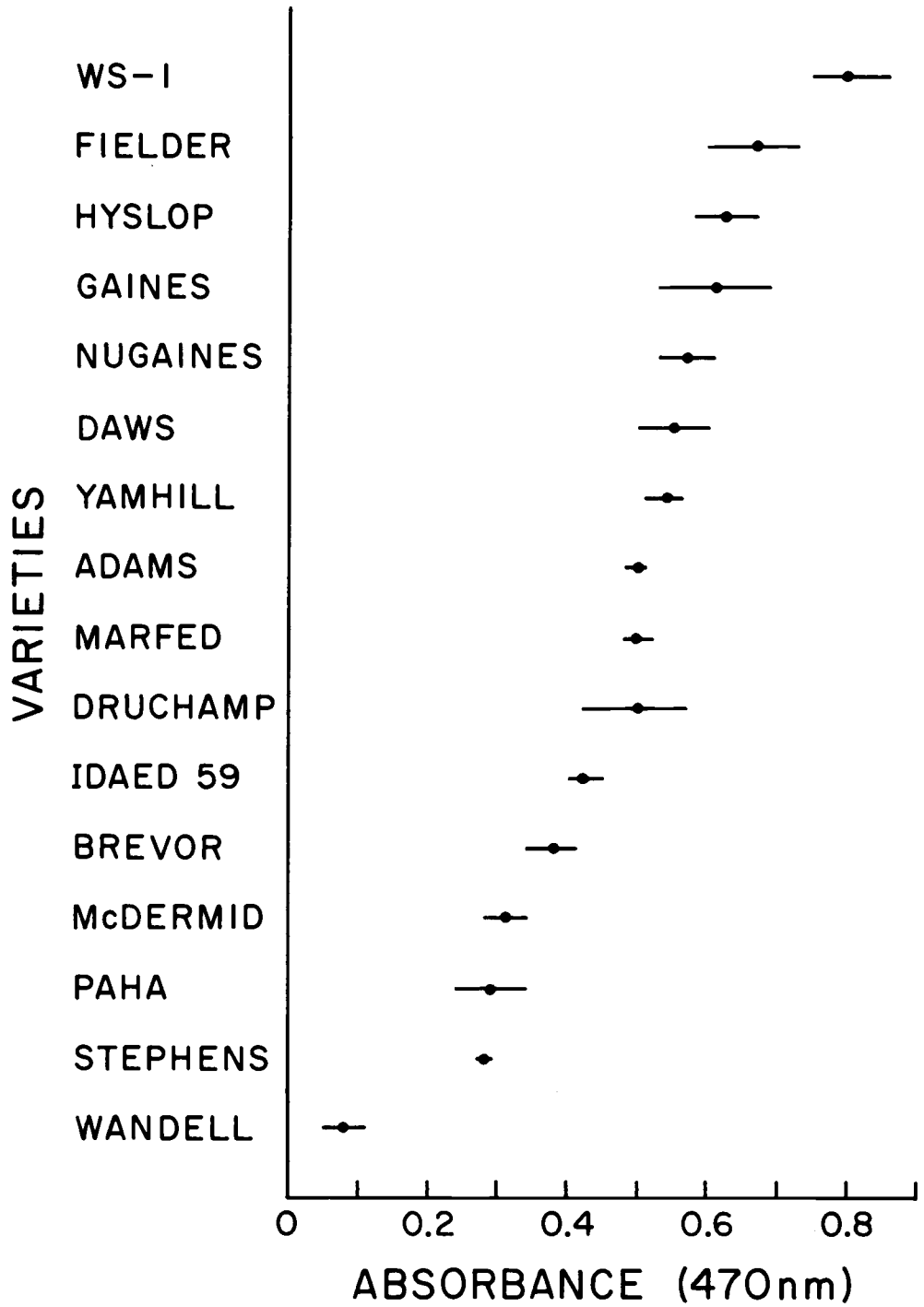

Figure 9. Mean and confidence limit (5%) of tyrosinase activity of Pacific Northwest white wheat varieties. Two g seeds soaked 2 hr in .1% tyrosine solution with pH 8 at 35 C.

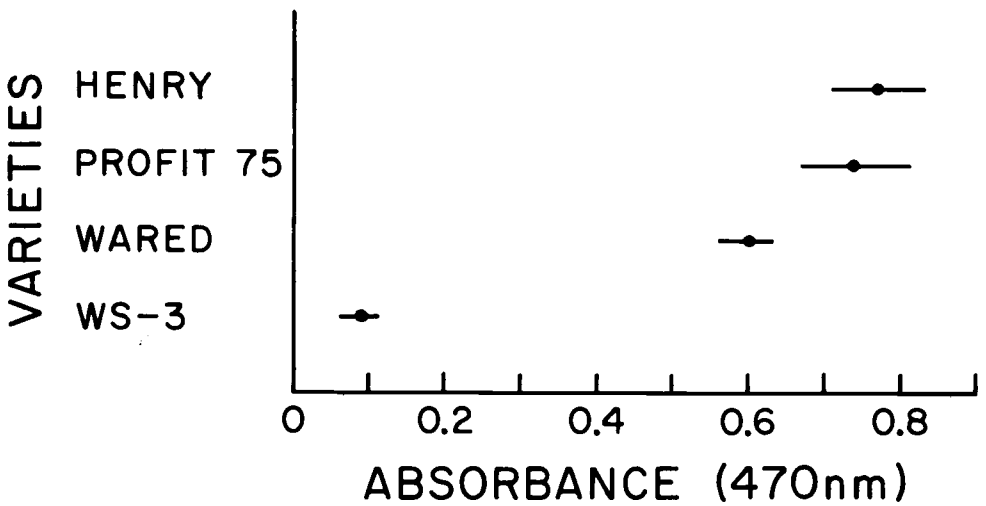

Figure 10. Mean and confidence limit (5%) of tyrosinase activity of Pacific Northwest red wheat varieties. Two g seeds soaked 2 hr in .1% tyrosine solution with pH 8 at 35 C.

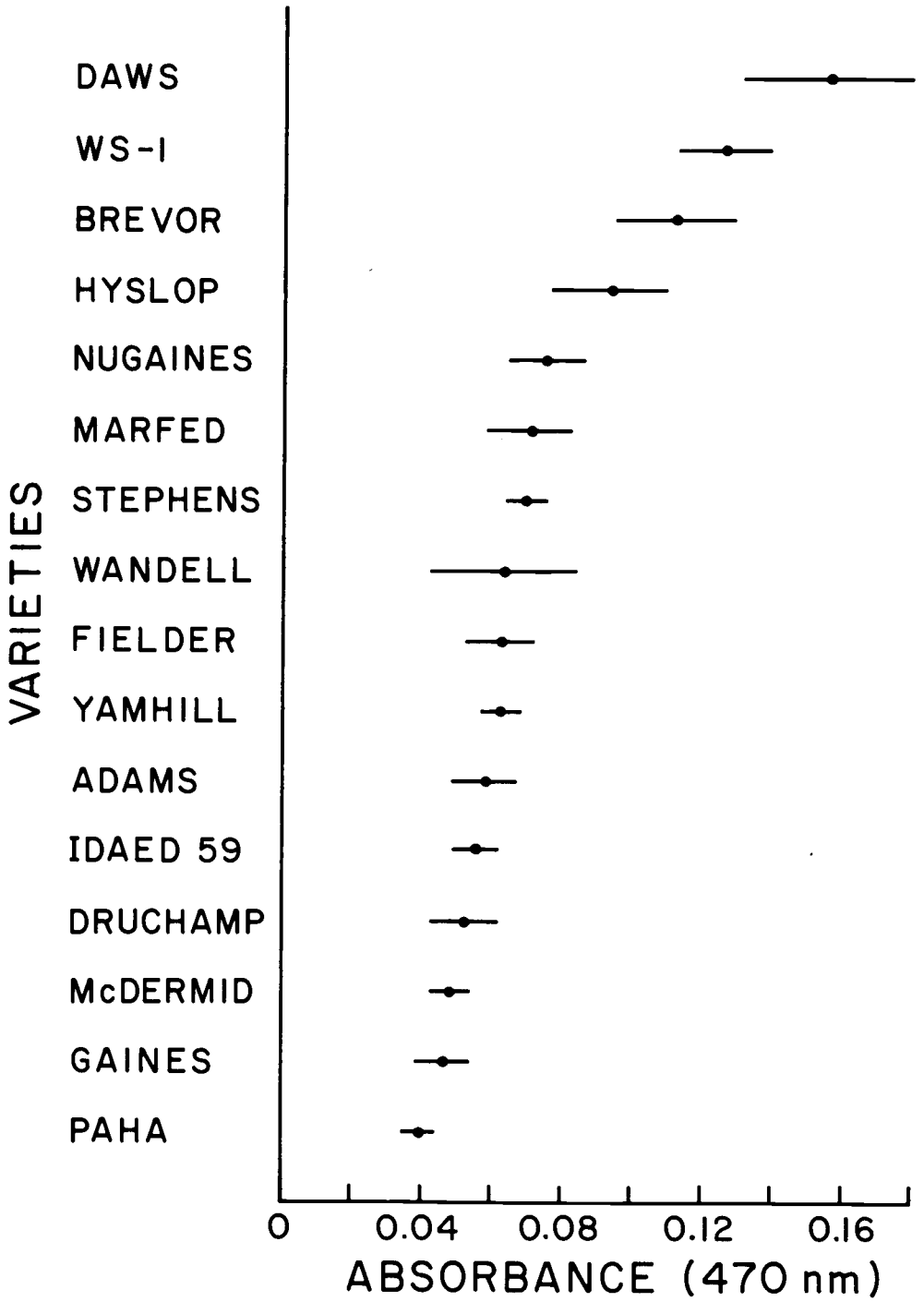

Figure 11. Mean and confidence limit (5%) of tyrosinase activity of Pacific Northwest white wheat varieties. Single seed soaked 17 hr in .1% tyrosine solution with pH 8 at 35 C, with shaking.

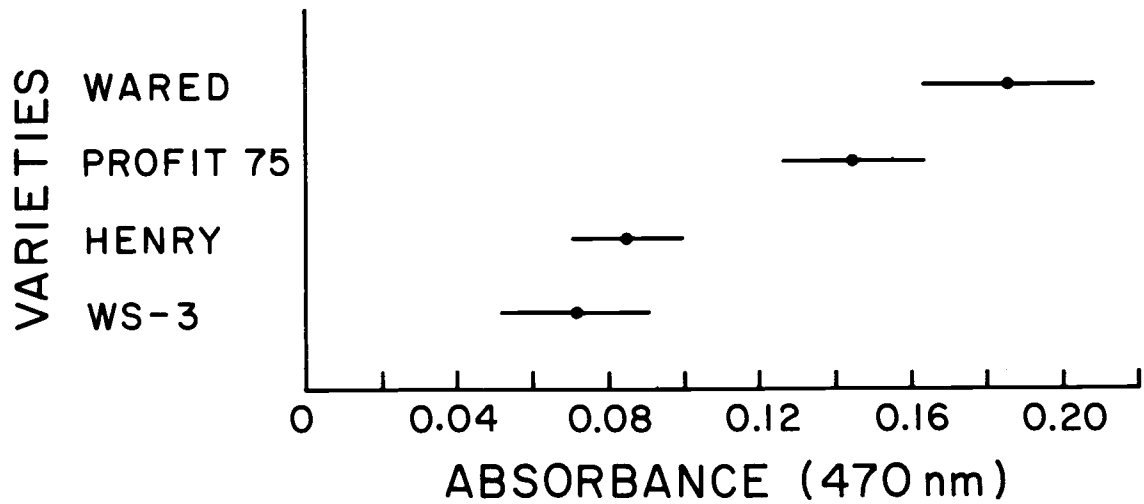

Figure 12. Mean and confidence limit (5%) of tyrosinase activity of Pacific Northwest red wheat varieties. Single seed soaked 17 hr in .1% tyrosine solution with pH 8 at 35 C, with shaking.

The bulk-seed method and single-seed method ranked varieties differently in regard to tyrosinase activity (Table 10). One possible reason for this difference is that the absorbance from single seeds is affected by seed size variations between varieties (Table 8 and 9), while the weight of seed used in the bulk method remained constant between varieties. Another factor may have been the different seed: solution ratios in the two methods. In the bulk method, the ratio of approximately 0.2 ml tyrosine solution to one seed may have limited the reaction in some varieties. In the single seed method, one seed reacted with 5 ml solution, which, together with shaking, resulted in much higher absorbance readings. Seed: solution ratios should be evaluated further in the bulk-seed method.

Table 10. Differential ranking of wheat varieties by bulk and single-seed tyrosinase tests.

Variety	No. of Seeds in 2 g	Bulk-Seed Method		Single-Seed Method	
		Rank	Absorbance[1/]	Rank	Absorbance
WS-1	50	1	.0161	4	.1257
Henry	55	2	.0140	7	.0850
Fielder	50	3	.0133	13	.0624
Hyslop	48	4	.0130	6	.0934
Daws	43	5	.0128	2	.1558
Yamhill	42	5	.0128	14	.0619
Profit 75	59	7	.0125	3	.1445
Gaines	49	8	.0124	19	.0462
Wared	54	9	.0111	1	.1860
Druchamp	45	10	.0110	17	.0516
Nugaines	54	11	.0106	8	.0750
Adams	51	12	.0097	15	.0583
Brevor	44	13	.0085	5	.1115
Marfed	59	13	.0085	10	.0698
McDermid	41	15	.0075	18	.0484
Idaed 59	57	16	.0074	16	.0549
Stephens	38	17	.0073	11	.0693
Paha	46	18	.0062	20	.0393
WS-3	43	19	.0021	9	.0707
Wandell	44	20	.0018	12	.0631

[1/] $\text{absorbance/seed} = \frac{\text{absorbance reading for 2 g}}{\text{no. of seeds in 2 g}}$

GENERAL DISCUSSION

On the basis of these studies, the following procedures are suggested for conducting the tyrosinase test on wheat seed:

Bulk-seed method:

1. From the pure seed, weigh four replicates of 2 g seed.
2. Place the seeds in 50-ml beakers and add 10 ml of .1% tyrosine solution in 0.11 M sodium citrate buffer.[6/]
3. Stir the mixture until seeds are completely submerged.
4. Place the beakers in a controlled temperature chamber or water bath at 35 C.
5. After 2 hours, stir and pour all the solutions into 1/2" colorimeter tubes.
6. Shake the test tube three times before making the absorbance reading.
7. Make the absorbance reading at 470 nm.

Single-seed method:

1. Obtain 20 seeds from the pure seed.
2. Place each seed in a 10-ml test tube and add 5 ml of .1% tyrosine solution in 0.11 M sodium citrate buffer.[6/]

6/ Prepare the .1% tyrosine solution with pH 8 as follows: Dissolve 1 g of tyrosine and 21.48 g of citric acid in 600 ml of distilled water. Titrate the solution with 1N NaOH to pH 8. Add distilled water to 1000 ml.

3. Stir the mixture until the seed is completely submerged.
4. Place the tubes in a shaking water bath at 35 C.
5. After 17 hours, stir and pour the solutions into 1/2" colorimeter tubes.
6. Shake the test tube three times before making the absorbance reading.
7. Make the absorbance reading at 470 nm.

The phenol test for wheat variety differentiation has been used by seed testing laboratories for many years because it is a quick and convenient test and does not involve special laboratory equipment. The percentage of varietal mixture can be determined if the color characteristics of the varieties are significantly different. However, visual classification of varieties into the five color groups is often difficult, leading to lack of uniformity of results among analysts.

The potential value of the tyrosinase test lies in its ability to quantify the color reactions, rather than relying on visual determinations. Such quantification should also theoretically lead more than the five separations possible with the phenol test. This was shown to be true when the bulk-seed method was used. When the individual seed method is used, however, difficulty arises because of the variation in color caused by seed size and other unknown factors. This variation is great enough that the tyrosinase test can

not be recommended over the phenol test for routine examination of varietal characteristics of individual seeds.

The tyrosinase test may be useful in differentiating between varieties within a phenol color group or between adjacent groups, particularly when varietal mixtures are not suspected. Such a quantitative measurement may also be useful in describing varieties being entered under the Plant Variety Protection Act. The tyrosinase test is also less affected by fungicide seed treatments, particularly Vitavax which renders the phenol test useless.

Varietal reactions are affected by seed deterioration and production area, regardless of which test is used. These factors, plus seed treatment effects, must be taken into consideration when interpreting the results of either test.

Seed coat color has some effect on phenol color readings, with darker seed coats tending to cause the seed to appear darker in the phenol test. Seed coat color is not a factor in the tyrosinase test, so the two tests sometimes rank varieties differently in regard to relative darkness of color.

Both tests are relatively quick and can be completed in approximately the same amount of time.

Undiluted phenol can cause burning if accidentally spilled on the skin, while tyrosine does not.

SUMMARY AND CONCLUSIONS

Optimum conditions for conducting the tyrosinase test by the bulk-seed method were a soaking period of 2 hours, a temperature of 35 C, and a tyrosine concentration of .1% in sodium citrate buffer 0.11 M, pH 8. The absorbance of the resulting solution is read at 470 nm against the substrate solution. Optimum conditions for the single-seed method were similar except that a soaking period of 17 hours in a shaking water bath was necessary.

Tyrosinase activity of WS-1 and Yamhill wheat seeds declined with increasing degrees of deterioration induced by artificial aging.

Vitavax treatment caused a reduction in color development or enzyme activity PCNB (Pentachloronitrobenzene) depressed color development in WS-1 but not in Yamhill. HCB (Hexachlorobenzene) had no effect on either variety.

Yamhill seeds produced in Pendleton developed a darker colored product or had more enzyme, or higher enzyme activity than seeds produced in Moro, while Hyslop seeds from the two locations produced similar colored product or were comparable in enzyme activity.

Tyrosinase activity of seeds of Hyslop variety was not affected by seed protein contents of 9, 11, and 13% indicating that total seed protein does not affect pericarp enzyme (tyrosinase) quantity or activity.

Larger seeds possessed greater tyrosinase activity than small seeds because of more pericarp tissue. Variation in enzyme activity also occurred in seeds of similar size within a variety.

Comparisons with the phenol test showed that the bulk-seed tyrosinase test probably will differentiate more varieties than the phenol test and it is less affected by fungicide seed treatments. Both tests are affected by seed deterioration and production area. Both tests are relatively quick and can be completed in approximately the same amount of time.

The phenol test is superior to the individual-seed tyrosinase test for detecting varietal mixtures. Seeds within a variety differ in tyrosinase activity because of variations in seed size and other unknown factors.

The bulk-seed tyrosinase test may be useful in differentiating between varieties belonging to the same phenol color group. Such a quantitative measurement may also be useful in describing varieties being entered under the Plant Variety Protection Act. The tyrosinase test may also provide more accurate results on fungicide treated seed.

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APPENDIX

Appendix Table 1. t test for effect of soaking time on tyrosinase activity of WS-1 and Yamhill wheat seed.

Time (hour)	t value
1	21.20**
2	12.11**
3	3.00**
6	7.32**
9	6.95**
12	8.63**
15	5.66**

Appendix Table 2. t test for effect of temperature on tyrosinase activity of WS-1 and Yamhill wheat seed.

Temperature (^{o}C)	t value
15	3.74**
25	7.32**
35	24.29**

Appendix Table 3. t test for effect of pH on tyrosinase activity of WS-1 and Yamhill wheat seed.

pH	t value
5	16.60**
6	13.36**
7	17.28**
8	9.07**

** Significant difference at 1% level of probability

Appendix Table 4. t test for effect of soaking time at pH 8 on tyrosinase activity of WS-1 and Yamhill wheat seed.

Time (hour)	t value
1	51.03**
2	33.41**
3	22.14**
4	3.14**
5	12.20

Appendix Table 5. t test for effect of substrate concentration on tyrosinase activity of WS-1 and Yamhill wheat seeds.

Concentration (%)	t value
.10	33.41**
.05	37.48**
.025	31.28**
.010	38.21**

Appendix Table 6. t test for effect of shaking on tyrosinase activity of 2 g of WS-1 and Yamhill wheat seed.

Varieties	t value
WS-1	3.36**
Yamhill	4.82**

** Significant difference at 1% level of probability

Appendix Table 7. t test for effect of soaking time and shaking on tyrosinase activity of WS-1 and Yamhill wheat seed. Single-seed tyrosinase test.

Testing Conditions	t value
5 hours, shaking	- 4.73**
5 hours, nonshaking	- 2.68*
17 hours, shaking	- 9.10**
17 hours, nonshaking	- 1.43 ns

Appendix Table 8. t test for effect of production area on tyrosinase activity of Yamhill and Hyslop wheat seed.

Varieties	t value
Yamhill	6.78**
Hyslop	-1.43 ns

Appendix Table 9. Analysis of variance for effect of total protein content on the tyrosinase activity of Hyslop wheat seeds.

SV	SS	DF	MS	F	SIG
Protein	.0032	2	.0016	2.6667	NS
Error	.0128	21	.0006		
Total	.0160	23			

* significant difference at 5% level
** significant difference at 1% level
NS not significant at the 5% level of probability

Appendix Table 10. Analysis of variance for tyrosinase test of the Pacific Northwest white wheat varieties on a bulk-seed basis.

SV	SS	DF	MS	F	SIG
Varieties	1.90	15	.12	155.70	**
Error	.03	48	.0008		
Total	1.93	63			

HSD_{05} = .071

Appendix Table 11. Analysis of variance for tyrosinase test of the Pacific Northwest red wheat varieties on a bulk-seed basis.

SV	SS	DF	MS	F	SIG
Varieties	1.19	3	.39	368.85	**
Error	.01	12	.001		
Total	1.20	15			

HSD_{05} = .066

Appendix Table 12. Analysis of variance for tyrosinase test of the Pacific Northwest white wheat varieties on a single-seed basis.

SV	SS	DF	MS	F	**
Varieties	.30	15	.02	28.26	**
Error	.21	304	.0007		
Total	.51	319			

HSD_{05} = .029

** Significant difference at 1% level

Appendix Table 13. Analysis of variance for tyrosinase test of the Pacific Northwest red wheat varieties on single-seed basis.

SV	SS	DF	MS	F	SIG
Varieties	.17	3	.05	34.21	**
Error	.12	76	.001		
Total	.29	79			

HSD_{05} = .027