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Aomar Ait Amer Meziane for the degree of Master of Science

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Title: LABORATORY METHODS FOR WHEAT VARIETY IDENTIFICATION

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There is an increasing need for development of laboratory identification procedures for purposes of plant variety protection, seed certification, quality control and consumer protection. In the past there were fewer commercial varieties and field testing methods based on morphological differences were used satisfactorily. These tests require several months to complete and are subsequently of limited practical use. With the large number of current varieties, visual differences are much smaller and the task of identification is increasingly difficult.

This study was initiated to investigate various morphological, chemical and biochemical characteristics of 25 varieties grown in the Pacific Northwest.

The five tests described in this study provide the basis for a laboratory identification system for these varieties. The coleoptile color test was of limited value for these varieties, since only Golden exhibited a purple coleoptile while the remaining varieties were green.

The seedling pubescence test separated the varieties into two groups: slightly pubescent and pubescent. However, the variation of the pubescence density caused by environmental conditions limits its practical use.

The phenol test separated the varieties into three distinct groups: Light Brown, Brown, and Mixed. Four varieties, Barbee, Daws, Druchamp, and Luke, exhibited a mixed phenol reaction. This mixture was characteristic of these varieties since they were uniform for the other characters.

Esterase, catalase, acid phosphatase and glutamic oxaloacetic transaminase were not useful for separating the varieties since all of them developed similar banding patterns for each enzyme. Peroxidase electrophoresis separated the varieties into two distinct groups on the basis of the presence or absence of a single band.

Gliadin electrophoresis was the most discriminating test, distinguishing all the varieties except for those genetically closely related. These included varieties within three variety groups: Gaines and Nugaines; Fielder and Fieldwin; and Hyslop, Hill, McDermid, and Stephens. The phenol test provided a useful separation of the group of four varieties which were uniform for gliadins. Hill and Hyslop developed a brown phenol stain while McDermid and Stephens exhibited a light brown stain. Further identification might be possible by other means, such as pathogen

resistance, isoelectric focusing and other chemical tests.

In practice, unknown samples should be compared to authentic, standard samples for final identification.

LABORATORY METHODS FOR WHEAT VARIETY IDENTIFICATION

by

Aomar Ait Amer Meziane

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Professor of Crop Science in charge of major

Redacted for Privacy

Head of Department of Crop Science

Redacted for Privacy

Dean of Graduate School

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Typed by Gloria M. Foster for Aomar Ait-Amer Meziane

DEDICATION

To my wife and my children, Mehdi and Leila, for their love, patience, and support throughout my graduate studies.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDIX TABLES

LABORATORY METHODS FOR WHEAT VARIETY IDENTIFICATION

INTRODUCTION

There is an increasing need for development of laboratory variety identification methods for purposes of plant variety protection, seed certification, quality control and consumer consumption. In the past, the number of varieties grown commercially was limited in number and their identification by visual observations was relatively easy. As the number of varieties grew, visual differences became smaller and the task of identification became increasingly difficult.

Field testing methods, based on morphological characters of the plants, provide a valuable means of distinguishing differences among the varieties grown, but they often require too much time and consequently have limited practical use. Because of the slowness of field testing methods, a number of laboratory procedures have been developed for characterizing varieties. For wheat, these include greenhouse and growth chamber tests and chemical methods such as the phenol test. Unfortunately, such tests usually serve only to separate varieties into a few groups, each group containing several varieties.

More recently, a biochemical technique, known as electrophoresis, has been developed and offered as an additional tool for the purpose of separating and identifying crop varieties. For wheat variety identification, the electrophoresis technique has been

primarily performed on gliadin proteins extracted from the seed and the results of the analysis are obtained in a relatively short period of time (less than 48 hrs). The electrophoretic pattern developed by a given variety is distinctive and not affected by the environmental conditions under which the variety is grown.

Identification of the wheat varieties grown in the Pacific Northwest is, to date, primarily based on the phenol color reaction and morphological characters of the plants, neither of which will positively identify the varieties. Thus, the present study was initiated:

1) to adapt the electrophoretic technique to identification of soft white wheat varieties grown in the Pacific Northwest, and
2) to develop an identification key for these varieties by using various morphological, chemical and biochemical characteristics.

LITERATURE REVIEW

Genuineness of cultivar is tested by means of heritable characters of seeds, seedlings, plants and/or stands. Morphological, chemical and biochemical characters have been used to a great extent by several workers. The present study was conducted on wheat varieties; therefore, the literature review will concentrate mainly on wheat variety identification.

Morphological Characters

Harrington (1932) distinguished Canadian wheat cultivars on the basis of coleoptile color. One group of varieties had a purple coleoptile while the other group was characterized by a green coleoptile.

Korpinen (1964) and Deshi, et al. (1969) reported similar results in their respective studies. The seed samples were planted and placed under controlled conditions and high light intensity. Deshi, et al. reported that cultivars with purple coleoptile used in their studies also developed purple anthocyanin in the leaves when the seedlings were raised in nutrient deficient conditions under high light intensity in controlled environment.

Using another method, i.e., without covering the seeds with sand, Dimon and Viron (1975) found similar results to those obtained by Dhesi, et al. Harrington (1932) reported that seedling hairiness was very useful in separating two varieties (Reward from Marquis). They noticed, however, that in many cases, the

differences between varieties were too small and variations in hairiness were too large to make accurate identification. Korpinen (1964) separated wheat varieties into two groups on the basis of the presence or absence of hairs on the sheath and blade of the first leaf.

In addition to other grain and seedling characters, Nittler (1973), Ferns, et al. (1975), Ulvinen, et al. (1974) and others have used coleoptile color and seedling pubescence in their variety identification studies.

Chemical Characters

Phenol

The phenol test was discovered accidentally by Pieper (1922, cited by Friedberg, 1933). That year, a farmer who disinfected bunt-infected wheat seeds with a chemical (Betanal) containing a phenolic compound, noticed that the treated grains developed a dark color reaction. Based on this observation, Pieper tested some wheat varieties which, after soaking in distilled water and then in a 1% solution of Betanal, developed different color patterns. The phenol was found particularly useful for varietal identification in wheat (Snell and Pfuhl, 1930; Esbo, 1945; Korpinen, 1964).

Technique. Hermann (1924) cited by Friedberg (1933), improved the method initiated by Pieper. He used a blotter as a medium and a 0.1% solution of phenolic acid. This compound was shown to be more active than Betanal. Pfhul (1927) simplified the method by soaking the grains in a tube containing a 1% phenol solution for 4

hrs. Later, Snell and Pfhul (1930) described testing methods and published the color reactions of the German wheats. Friedberg (1933) soaked cereal grains in distilled water for 16 hrs. He then removed and dried the grains on a blotter for 40 min. Afterward, he placed the grains in a tube containing 5 cc of 1% solution of phenolic acid for 4 hrs. Finally, he removed the grains from the solution and dried them on a blotter. After 3 to 4 hrs, the color reactions clearly appeared.

Other methods have been proposed. The Association of Official Seed Analysts (AOSA) (Walls, 1965) and the International Seed Testing Association (ISTA) (1966) standardized the methodology of phenol testing for wheat variety identification. This method has been adopted and is still in use in most seed laboratories. The procedure is briefly as follows: four replicates of 100 seeds each are used. The seeds are soaked in distilled water for 16 hrs, then flushed with tap water and the excess water removed from the surface of the seeds. The seeds are then placed on two layers of filter paper in a container moistened with a 1% phenol solution. After 4 hrs of incubation, the color reaction developed by the seed is recorded. The AOSA has proposed an alternative method for the test. Instead of soaking the seeds in distilled water for 16 hrs, the dry seeds are placed in a container and covered with scalding water. The seeds are then let to steep for 10 min.

Wrigley and McCausland (1975) proposed a rapid method of phenol testing which consisted essentially of reducing the 16-hr soaking period by using boiling water. The seeds were left in this solution

for no longer than 3 to 5 min, then drained. In 1977, the same authors proposed a modified procedure which they called "rapid phenol tests for wheat varieties." The reagents used were a 1% phenol solution and an ammoniacal phenol. One g of phenol was dissolved in water; 1 ml concentrated ammonia solution (25%) was added and the mixture was diluted to 100 ml with distilled water. The seeds were soaked in a 1% phenol solution for 3 to 15 min. The grains were then drained and spread on absorbent paper wetted with ammonia-phenol solution in a covered container. The rate of coloration was then recorded after 10 and 20 min at 40°C or after 15 and 30 min at 20°C. They found that this rapid test gave the same color reactions as did the conventional method.

Because the color reactions of the phenol test described above are qualitatively evaluated, Crisp and Wrigley (1974) proposed a quantitative extension of the phenol test. After grains have reacted with phenol according to the established qualitative procedure, the extent of browning was determined by boiling the grains for 20 min in an alkali buffer and by estimating the brown coloration of the extract photometrically. Results were expressed as the phenol ratio. This was calculated by dividing the absorbence of this extract by the absorbence of similarly prepared extracts of grains untreated with phenol.

Maguire, Steen, and Grzelak (1975), in their studies of classification of Pacific Northwest wheat cultivars, used a combination of the phenol coloration obtained through the established procedure and an evaluation by manometric methods of the phenol oxidase activity.

The enzymatic activity was measured by soaking 50 seeds per variety for 4 hrs in a deionized water at 40°C. Seeds were then removed, rinsed and placed in Warburg flasks that contained 0.5 ml of 1% phenol solution. After equilibration at 25°C, phenol was added to flasks and measurements were taken each 30 min for 4 hrs. The rate of O_2 consumption per hr was then determined.

Banerjee and Chandra (1977) developed a new detailed evaluation method. They closely studied the reaction over time and noticed that a topographical color pattern developed on the seed, starting at the brush end of the grain. They proposed an alternate code for evaluation. For example, if a seed developed a coloration only on one-fourth of its surface while the remaining part remained unchanged, they would record it as 3/4-, 1/4++. The arithmetic signs indicated the color intensity. They also suggested a method for chemical determination of enzymatic activity. The enzyme involved in the reaction was extracted and its activity measured in vitro. They observed recognizable differences in optical density between cultivars. They modified the enzymatic activity by using as a substrate several compounds (monophenols, polyphenols) with varied concentrations from 0.001% to 4.0%. The coloration varied in degree from compound to compound and was very much dependent on the concentrations. Since it was known that copper is present in tyrosinase, they thought that traces of Cu^{++} would stimulate the reaction. They soaked the seeds in $CuSO_4$ solutions at different concentrations (.001% to 4%). A slight stimulation was observed at .001% and concentration of 2%

were inhibitory. At 4% concentration, 4 days were needed for color development. They used several other metallic ions such as Ag^{++}, Hg^{++}, K^{+}, Na^{+}, Fe^{3+}, and so on. Most of them were inhibitory. They concluded that "only Na^{+} could bring out selective and critical effect on the enzyme so as to be able to differentiate two originally brown cultivars." It was then possible to subdivide primary color groups into secondary ones.

The results of the conventional phenol test are qualitatively evaluated. The varieties tested are grouped on the basis of their color reaction. Friedberg (1933) and Wrigley, et al. (1975) proposed five color classes, namely (1) no color, (2) light brown, (3) brown, (4) dark brown, and (5) mixed coloration. The color categories proposed by the AOSA (Wallas, 1965) are (1) ivory, (2) fawn, (3) light brown, (4) brown, and (5) black brown. The ISTA (1966) uses this grouping: (1) black, (2) dark brown, (3) brown, (4) light brown, and (5) negative.

Mechanism. Friedberg (1933), Joshi and Banerjee (1970), Csala (1972), Elekes (1980) and others determined that the site of the reaction was the seed coat, i.e., the pericarp.

Hermann (1924), cited by Csala (1972), suggested that the phenol reaction in wheat was caused by a chemical reaction of unidentified nitrogen compounds in the presence of metal and oxygen. According to Tuzson (1933), cited by Csala (1972) and Elekes (1980), aldehydes formed in the course of primary soaking react with phenol. Voss (1936) and Esbo (1945), cited by Elekes (1980), attributed the color

of an enzymatic reaction, namely to tyrosinase. Mionzynski (1938), cited by Joshi and Banerjee (1970), explained that the trait was due to one or two genetic factors in winter wheat, the dark color being dominant.

Joshi, Banerjee and Naik (1969) determined that there was always a low tyrosinase activity when no phenol reaction took place. Joshi and Banerjee (1970) carried out an experiment to study the genetics of phenol color reaction in Emmer wheats. They found that when seeds were boiled in water for 15 min and then treated with phenol, no color resulted, indicating thereby that the reaction was enzymic. To study the nature of participating enzymes, they soaked the seeds in 0.1% sodium diethyldithiocarbamate solutions for 16 hrs prior to phenol treatment. They observed that no color developed even in those where normally dark black coloration is seen. This established clearly that the reaction oxidizing phenol to melanin (dark color developed in the seed coat) via orthoquinone and hydrozyguinone was catalyzed by a copper-containing polyphenol oxidase, i.e., tyrosinase with the possible participation of other phenolic substances present in the seeds and also of amino acids such as tyrosine and phenylalanine.

For the inheritance studies of phenol reaction, Joshi and Banerjee (1970) and Wrigley and Shepherd (1974) indicated that the polyphenol oxidase enzyme which was responsible for the development of melanin pigment was monogenetically controlled, the allele for melanin pigment being dominant.

Csala (1972) concluded from his studies that the mechanism of phenol reaction may differ for wheat and barley. The mechanism was influenced by enzymic as well as non-enzymic processes. In the case of barley, the role of enzymes appeared to be most important. In wheat, chemical reaction seemed to be more important.

Simon and Viron (1975) have also adopted the theory of an enzymatic reaction. In their quick process, the dry seeds were placed directly in a 1% phenol solution which resulted in a more determined discoloration than that of the traditional methods. Elekes (1980), in his studies, demonstrated that color formation was not an enzymatic process since the phenol reaction occurred over a wide range of pH (1-14) and on seeds fixed or destroyed in different ways. He showed that the reaction will also occur with other reagents than phenol derivatives, such as flavanoid compounds, including ammonium silver nitrate, ferric chloride solutions, basic lead acetate, sulfanic acid, and alcoholic sodium hydrozide. He pointed out, however, that the differences of color intensity are much smaller than those of phenol. His work confirmed that the site of reaction was the seed coat, i.e., the pericarp where cells died off on the ripened seed. Within the live enzyme-active cells of the aleurone, discoloration did not occur. Microscopic examinations revealed that the dried cell structure will discolor while the cell wall will do so only to a small extent during the reaction. He then concluded that the phenol reaction of wheat is a flavanoid reaction, "since flavanoids are dissolved in the living cells and

cell liquids, and, at least partly, they are ready to be absorbed through the cell wall."

Elekes noticed that the epicarp and endocarp responded differently to phenol reaction, depending on varieties. Endocarp developed a medium to dark brown color on each phenol-positive variety tested. In the epicarp, it was the opposite in that discoloration did not occur in several varieties.

Friedberg (1933), Csala (1972), and others reported that the phenol did not react in the seed as a solution (per se), but rather it was the vapor from the solution that produces the color reaction.

The experiments described above show obvious contradictory results and further studies are required to clearly determine the mechanism of the phenol color reaction.

Stability of the test. Kruger (1976) reported that polyphenol oxidase was formed early in kernel development and decreased with kernel maturation. A large part of the polyphenol oxidase in the immature wheat kernel was present in the endosperm.

Clancy, Ciha and Maguire (1982) conducted an experiment to determine at what stage of seed development the phenol test can be used for cultivar identification. Several varieties were chosen for their study and harvests were made at 3- to 4-day intervals from 7-10 days post anthesis to full seed maturity. They concluded that the phenol test could be used for identification purposes once the seed reached the hard dough stage and the chlorophyll content had declined. When chlorophyll content of the chaff was high, very

little stain in green wheat seed was produced by phenol. The authors thought that the chlorophyll in the seed was perhaps masking the brown coloration produced by the phenol reaction.

Friedberg (1933), Csala (1972), Wrigley and Shepherd (1973), Simon and Viron (1975), and Banerjee and Chandra (1977) reported that the phenol reaction is not affected by cultural practices during crop growth.

Csala (1972) and Elekes (1980) demonstrated that the reaction takes place over a wide range of pH (1-14). In the range of pH from 3 to 9, coloring was uniform, while at pH 1 and 11, coloration took place in an uneven, spotted manner.

Csala (1972), Banerjee and Chandra (1977), and Payne and Koszykowski (1982) demonstrated that the power of the phenol test to distinguish cultivars was not improved by using concentrations of phenol other than 1%. Concentrations varying from .25% to 10% were evaluated. Csala and Payne and Koszykowski reported that seeds treated with phenol concentrations lower than 1.0% stained lighter than those treated with concentrations of 1.0 and higher. Csala reported that 5 to 10% solutions resulted in spotted coloring.

The effects of temperature on the test were studied by Csala (1972) and Payne and Koszykowski (1982). Csala reported that at temperatures near 0°C, the coloration was slow (1½ days); at 20-22°C and at 80°C, coloration took place within 3 hrs. At 30-40°C, the staining reaction was completed within 1½ hrs. Payne and Koszykowski (1982) studied the effect of temperature on soaking and

testing periods. They reported that seeds of cultivars that normally stained lightly gave uniform test results regardless of the temperature. However, seeds of cultivars that normally stain darker were darkest after the 4-hr test when soaked and tested at 30°C. These seeds stained lightest when soaked and stained at 5°C.

The seed soak time (Payne and Koszykowski, 1982) did not appear to be critical, but it should be kept constant for all samples and the staining time properly adjusted. The placement of seeds and the source and type of phenol were found to have little or no influence on the testing procedure.

Dhesi and Desormaux (1972), McKee (1973), and Payne and Koszykowski (1982) reported that difficulties have been experienced in the interpretation of the phenol color reaction when testing treated seeds. It has been suggested that seeds treated with fungicides should be rinsed in methanol to remove the fungicide prior to soaking. An exception is seed treated with Vitavax (Payne and Koszykowski). Even after rinsing in methanol, seeds treated with Vitavax took considerably longer to stain than untreated seeds of the same variety.

When evaluating phenol test results, variation in the phenol staining color among seed lots of the same variety should be taken into account. Simon and Viron (1975) reported that the color pattern was not strictly homogeneous within some varieties. Thus, they suggested to consider two varieties as being different only if they varied by at least one group.

Payne and Koszykowski (1982) pointed out the variation in color patterns among seed lots of the same variety. They suggested that seeds be classified as durum, white or red wheat prior to conducting the phenol test and varieties in each wheat class should be recorded as staining either light or dark. This would greatly reduce the chance of misinterpreting variations among seed lots of the same cultivar.

All authors agree that phenol is an unpleasant chemical to work with. In addition, Wrigley and McCausland (1975) underlined the toxic nature of the phenol. Solid or concentrated solutions of phenol are corrosive and burn the skin; thus, they should be handled with care.

Biochemical Characters

Electrophoresis

Electrophoresis, also called ionophoresis, has been found useful for analyzing and separating mixture of proteins or isoenzymes on an agar medium over which an electric field is established. The proteins arrange themselves within this media according to their polar nature. The original method known as free, or moving boundary, electrophoresis was first developed by A. Tiselius in Sweden in the 1930's (Lehninger, 1981). Since then, other methods have been developed. These are: zone electrophoresis, disc electrophoresis, and isoelectric focusing, also called electrofocusing. Electrophoresis is used for various purposes. In

agriculture, one of the most important areas in which electrophoresis (starch or polyacrilamide gels) is applied is variety identification. For example, Larsen (1967) used it as a tool to detect differences among varieties of soybean, as did Gorman and Kiang (1977) and Blogg and Imrie (1982). Wehner, Duich and Watschke (1976) and Spoor and Hay (1979) separated Kentucky bluegrass varieties. Basha (1979) applied electrophoresis to detect differences in peanut cultivars. Identification of subterranean clover cultivars on the basis of electrophoresis was studied by Dalling, Trunoff and Swinden (1979). Many other examples of such studies could be listed. To date, the most extensive use of electrophoresis as a tool for variety identification has been in wheat. Based on the solubility characteristics, Osborne (1907) has identified 5 fractions of wheat flour proteins: (a) a freely water soluble and heat coagulable albumin; (b) a globuline, soluble in dilute salt solution; (c) one or more proteose present in very small quantities; (d) a gliadin, insoluble in neutral aqueous solutions, but distinguished from all others by its ready solubility in 70% alcohol; and (e) a glutenin having the basic composition similar to gliadin, soluble in very dilute acid and alkaline solutions, but insoluble in alcohol or neutral aqueous solutions. Osborne identified gliadin as being the most important of the five proteins.

Technique. Prior to 1959, electrophoretic studies were performed under less than ideal conditions because of limited solubility

of gluten proteins. Jones, et al. (1959) overcame most of these constraints by developing the aluminum lactate acid buffer system with low ionic strength and low pH (3.1). The same year, Holmes and Briggs (1959) found that the aluminum lactate buffer was particularly effective for the study of the wheat gluten. Smithies (1955) investigated forms of electrophoresis other than moving boundary electrophoresis. His results showed that zone electrophoresis gave superior resolution of proteins with starch gel as the supporting medium.

Woychik, Boundy and Dimler (1961) were among the first to adapt starch gel electrophoresis in aluminum lactate buffer with a pH of 3.1 to the native wheat gliadin. For gel preparation, they used potato starch but because of the variability among different batches, they varied the acid concentration and time of hydrolysis at 40°C to obtain a product that would form suitable gels at concentrations of 14-18%. The buffers used were aluminum lactate, pH 3.1; veronal, pH 7.9; and sodium glycinate, pH 9.7; all of 0.05 ionic strength. When urea was used, its concentration was 3M in the buffer. They prepared the gels by heating 18 g of starch in 100 ml of buffer over a free flame with continuous swirling. After the solution had come to a boil, the flask containing the gel was left to stand 4 to 5 min to allow entrapped air bubbles to rise to the surface. The gel was then poured into plastic trays, and was allowed to set for at least 3 hrs before use. To apply the protein sample to the gel, they soaked a filter paper strip in a solution of

gluten protein in buffer and then inserted it into a transverse cut in the gel. An electric field of approximately 8v/cm was applied for 24 hrs. The migration chamber was cooled with circulating tap water. After electrophoresis, the gel was sectioned horizontally with a wire like a cheese slicer. The gel slices were then immersed for 10 min in a staining solution consisting of 0.1% solution of amido Black 10-B in 5% acetic acid. Excess dye was removed by repeated washing with 5% acetic acid.

Lee (1968) developed a method for extracting, with aqueous 2 M urea, all the gluten protein in a form that is capable of penetrating the gel without reduction. He compared these extracts to those made with aqueous acetic acid in terms of behavior on starch gel electrophoresis using 0.017 M aluminum lactate buffer, pH 3.1. The results obtained did not show any obvious difference between the two methods of extraction, judged by the proteins which migrated into the gel. There was, however, a significant difference in the amount of stained material remaining in the starting slot. From this, it was concluded that extraction with urea brought protein into solution in a form of relatively low molecular weight and, from the pattern obtained by gel electrophoresis, it appeared to be gliadin, whereas acetic acid extracts contained both gliadin and glutenin. The method of extraction developed, i.e., extraction by aqueous urea, has shown that it dissolved all the gluten as gliadin and the high molecular weight glutenin was probably disaggregated by urea.

Elton and Ewart (1962) studied various buffer systems to be used in starch gel electrophoresis for characterization of wheat proteins. They found that both sodium phosphate and sodium lactate buffers did not give good results. Sodium chloroacetate and aluminum lactate chloroacetate buffers gave results similar to aluminum lactate buffer, but the latter appeared to be the best.

To compare wheat varieties, Doekes (1968) used starch gel electrophoresis of grain proteins. He, like others, used the aluminum lactate buffer system mentioned above, and a sodium pyrophosphate buffer which were used with and without addition of 3 M urea. For the gel preparation, he used 13 g hydrolyzed starch which was suspended in 100 ml buffer. A concentration of 3 M urea was added to the gel solution after it had completely gelatinized. Air bubbles in the mixtures were removed by using a water vacuum. One hour after the gelatinized starch solution was poured off and cooled to room temperature, the gels were covered with PVC-film to prevent drying and kept at 3°C for at least 4 days before they were used. The slots for the extract solutions were made at one end at 4 cm from the origin of the gel, by suction through a small flattened piece of steel tubing. Connection between the buffer and the gel was assured by using a filter paper. Electrophoresis was carried at 13v/cm with a standard current of 30 mA. The apparatus was kept at a temperature of 3°C and air was circulated. The cutting operation was similar to that used by Syochik, et al. (1961). The gel slices were stained in a solution of methanol-acetic acid-water (5:5:1) with 0.5 g of water-soluble nigrosine and

0.25 g of amidoschwartz. Duration of staining varied between 10 and 30 min. Destaining was done by rinsing the gels with 5% acetic acid and after about 4 hrs, the background contrasted sufficiently with the protein bands.

Wrigley and Shepherd (1974), Wrigley and Baxter (1974), Wrigley and McCausland (1975, 1977), Autran and Bourdet (1973, 1975), Wrigley (1976), Bonnafe (1977), Almgard and Clapham (1977), Bushuk and Zillman (1978, 1979), Ducros and Wrigley (1979), and Qualset and Wrigley (1979) all adopted the method of starch gel electrophoresis in aluminum lactate buffer to the gliadin protein to fingerprint the different wheat varieties grown in their respective countries. The starch concentration, the buffer, the gel preparation, the cutting and slicing of the gel, and the staining procedures were basically identical. There were, however, some modifications in the extraction method and the application of the extracts on the gel.

Since gliadin is poorly soluble in water, Wrigley, et al. (1975, 1977) used mainly a 1 M urea extraction solution. The extraction procedure involved crushing the grain, mixing it with four to six times its weight of solvent, and allowing it to stand about 1 hr. The mixture was then centrifuged to get a clear solution to be used for electrophoresis. The extract solution was applied with a microsyringe in the slots previously made in the gel.

To extract the gliadin, Autran and Bourdet (1975) and Almgard and Clapham (1977) used a 60% ethanol solution or a 25%

glycolmonochlorohydrine solution. One g of crushed grain (or flour) was mixed with 3 ml of solvent. The mixture was then centrifuged and the clear solution was applied to the gel. When using one grain, the extraction was made with 150 µls without centrifugation. The extract solutions were applied to the starch gel by dipping small Whatman paper wicks in the solution and then placing these wicks in the gel. An electric current of 8 volts/cm was then applied for 4 hrs and 45 min.

Bonnafe (1977) used chloro-2-ethanol as the extraction buffer. A maximum of 150 µl of the solution was added to a finely ground grain. The mixture was then allowed to stand overnight. To apply the extract solution, he dipped a 10 x 5 mm Whatman paper strip in the supernatant which was then placed in the sample slots cut in the gel with a scalpel. The duration of the run and the current intensity were the same as those used by Autran and Bourdet (1975).

Wrigley, et al. and Autran and Bourdet preferred to use starch gel instead of polyacrylamide gel because of the apparent superior resolving power of the former.

Buschuk and Zillman (1978), in their wheat cultivar identification studies, used polyacrylamide gel as the support medium. They improved its resolving power and demonstrated that it was much easier to prepare reproducible, highly uniform polyacrylamide gels than starch gels. The buffer used was aluminum lactate. The gliadin extraction was done by mixing a ground grain with three times its weight of 70% aqueous ethanol. The mixture was allowed

to stand for 1 hr at room temperature and then centrifuged for 10 min at 20,000 rpm. A current of 72-74 mA was maintained throughout the electrophoretic run. For the staining of the gliadins, they used Coomassie Brilliant Blue dissolved in 12% trichloroacetic acid. The staining duration was 48 hrs.

Shewry, et al. (1978) used sodium dodecyl-sulphate polyacrylamide gel electrophoresis of gliadin to distinguish between wheat varieties which cannot be differentiated by starch gel electrophoresis.

Interpretation of gliadin bands. Doekes (1968), after a study of a number of densitograms, concluded that all the samples of one variety examined had the same protein components, each holding its specific position among the others accurately. Among the varieties studied, he observed five main groups on the densitograms. Each main group consisted of subgroups comprising varieties with very similar gliadin patterns. He studied the effect of N fertilization on the electrophoretic pattern and concluded that N dressing had no demonstrable influence on the electrophoregram, irrespective of the amount added or the stage of growth at which it was added.

Autran and Bourdet (1975) identified 17 to 25 bands, depending on the variety. They have shown that the electrophoretic heterogeneity of the gliadin fraction of wheat grain in starch gel, was totally independent of growing conditions and thus constituted a valid varietal identification character. They supplied a general table of variety identification based solely on the analysis of

the electrophoretic diagrams of gliadins. They pointed out, however, that they could not differentiate among the French winter wheats which were very closely related. These varieties had very similar gliadin patterns. Gubareva, et al. (1981) also found that closely related varieties have identical gliadin spectra.

Wrigley and McCausland (1975, 1977) found that three bands which developed on the stained gel were present for all varieties and these were useful points of reference for comparing patterns among varieties. In some cases, they observed several different patterns for one variety and they believed this was apparently due to the presence of two or more biotypes within a variety, each biotype having the same general characteristics typical of the variety. Their studies showed that electrophoretic separation of wheat gliadins produced a pattern of lines that is characteristic for the variety, without regard to conditions under which the grain was grown. Many varieties developed a unique electrophoretic pattern which made it possible to distinguish unambiguously each of them from all others. However, in some instances, the same pattern was developed by several varieties. Such electrophoretically indistinguishable varieties were closely related and of the same quality type. The same observations were made by Bushuk and Zillman (1978) using polyacrylamide gel as a support medium.

Almgard and Clapham (1977) distinguished seven different gliadin patterns. Swedish winter and spring wheat varieties had different patterns. They studied the environmental effects on

patterns and no inter-year differences were detected in the gliadin patterns. They also confirmed that closely-related varieties were indistinguishable by their gliadin patterns.

From their studies, Wrigley and Qualset (1979) reported that the combined migration of all the gliadin proteins reflected not only the genetic composition and thus the variety, but they could also provide additional information such as pedigree. The pattern was constant for a particular genotype. The proportions and positions of the zones were not altered by growing conditions or by the protein content of the grain.

Ducros and Wrigley (1979) reported that the electrophoresis of the endosperm proteins has shown the greatest potential for positively distinguishing between varieties, except for those cultivars which are closely related to each other. They studied the pattern variation due to nongenetic factors and concluded that the environmental factors did not alter the electrophoretic pattern for a particular genotype. One exception was that there were significant differences in the relative intensities of many gliadin bands in the electrophoregrams for grain from wheat plants grown in sand culture under conditions of extreme sulphur deficiency. However, such differences were not observed in an examination of field-grown grain from soil known to be deficient in sulphur. Moreover, constant patterns were obtained from the same variety of wheat despite fumigation of the grain with methyl bromide or phosphine, dusting of the grain with various fungicides and heat drying of the grain under

conditions severe enough to destroy baking quality.

Almgard and Clapham (1979) confirmed that gliadin patterns are distinctive varietal characters sufficiently independent of seed sample to be used with confidence for cultivar identification. The cultivar "Drabant" showed the same pattern over 10 locations. Drabant seeds with 14.4% nitrogen content showed the same pattern as seeds with 12.1%.

Nomenclature. A systematic nomenclature of the electrophoregram bands is required for an accurate identification of wheat varieties. Woychik, et al. (1961) distinguished four groups of bands and designated them as α, β, γ, and ω on the basis of four distinct peaks in starch gel electrophoresis. Within each group, the bands were identified by numbers, with the band having the highest mobility being assigned number one.

Coulson and Sim (1964) used an automatic recording double beam reflectance densitomer with an integrator to produce a graphical scan of the starch gel strips. Gubareva, et al. (1975), cited by Bushuk and Zillman (1979), applied this system. The composite gliadin electrophoregram was given the formula α, 1 to 7; β, 1 to 5; γ, 1 to 5; and ω, 1 to 12. The numbers representing bands that stained intensively were underlined, those stained lightly were shown in parenthesis, and doublets were indicated by two dots above the number.

Autran and Bourdet (1975) reported a modified nomenclature for identifying gliadin bands in the electrophoregram. The range of

mobilities for all the gliadins was from .21 to 1.00. The relative mobilities were calculated using a standard band common to all varieties studied. This band was located in the γ group and an arbitrary mobility of .65 assigned to it. The intensity of the stained bands was recorded by using the following scale: 0, tr, +, ++, +++.

Bushuk and Zillman (1978) proposed another nomenclature which is a modification of that proposed by Autran and Bourdet. Because the γ-gliadin band was difficult to locate on the polyaylamide gel, they selected one of the major bands of the Canadian wheat cultivar, "Marquis," as a reference, and an arbitrary relative mobility of 0.50 was assigned. All other bands were identified by a mobility relative to this reference.

Identification by isoenzyme electrophoresis

Wheat cultivars can also be identified to some extent by the electrophoresis of some enzymes. Menke, et al. (1973) reported that esterase and peroxidase enzyme electrophoresis made possible the distinction among some cultivars. The basic procedures for gel preparation were basically the same as for gliadin electrophoresis. Other buffers and staining procedures determined for each enzyme were used. They found that each variety had a unique pattern of isozymes. The inheritance of isozymes was examined and it was concluded that they are generally inherited as major genes. Groups of certain varieties were identified as having similar zymograms. These

varieties could not be distinguished even when different zymograms were combined. In regard to the environmental effects, results from comparison of three varieties grown at two locations for 2 years did not show significant differences that could be attributed to environmental effects.

Auriau, et al. (1976) examined the enzymic (β amylase, α-esterase, peroxidase and acid phosphatase) composition of 39 wheats, Triticum aestivum, on a polyacrilamide gel electrophoresis. They concluded that the growing conditions did not affect patterns obtained. They identified 6 peroxidase bands, four of which were very similar among all varieties, but according to the presence or absence of b or c bands, the bread wheat varieties were classified into two groups.

They identified 3 to 6 acid phosphatase bands and distinguished 7 distinct wheat variety groups. All of the wheat cultivars studied showed the same zymogram patterns for esterase enzymes.

Almgard and Clapham (1977) reported that there were no absolute distinctions among cultivars on the basis of esterase isozymes. The acid phosphatase patterns were identical for spring and winter cultivars as were the peroxidase patterns in spring wheat cultivars. Several other enzymes were studied, including catalase, superoxide dismutase, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, 6 phosphogluconate dehydrogenase, glucose-phosphate isomerase, glutamate-oxaloacetate transaminase and hexokinase, but no useful variation between cultivars was observed.

Konarev, et al. (1981) reported that in most cases, enzymelectrophoresis distinguished only groups, but not individual cultivars.

MATERIALS AND METHODS

Variety and Seed Source

Twenty-five soft white wheat varieties were obtained from the following sources:

a. Dirkwin, Fielder, Fieldwin, Hyslop, McDermid, Stephens, Gaines, Nugaines, Yamhill and Hill were obtained from the Oregon State University Foundation Seed project. All varieties were Breeder or Foundation seed categories.

b. Urquie, Walladay, Daws and Luke were obtained from the Washington State Crop Improvement Association, Inc. All were Foundation seed category.

c. Twin was provided by Dr. D. Sunderman, U.S. Department of Agriculture, associated with the University of Idaho, Aberdeen, Idaho.

d. Faro, Moro and Paha were provided by the Washington State University cereal project.

e. Sprague, Jacmar, White Holland, Barbee, Raeder, Wanser, and Golden were obtained from the Oregon State University cereal project.

The varieties included in (c), (d) and (e) were presumably of Foundation seed category.

The varieties and their respective pedigrees (Zeven, et al., 1976) are listed in Table 1, on the basis of their genetic relationships.

Table 1. Pedigrees of 25 varieties used in study.

Habit	Variety	Pedigree
W	Hyslop	Nord Desprez/2* Pullman-sel 101 (CI 13438)
W	McDermid	Nord Desprez/2* Pullman-sel 101
W	Stephens	Nord Desprez/Pullman-sel 101
W	Hill	Hyslop/Yamhill
W	Yamhill	Heine/Alba
W	Gaines	Norin 10/Brevor//Orfed/Brevor sib/3/Burt
W	Nugaines	Sib of Gaines
S	Urquie	Gaines/Marfed sel 68-3
W	Raeder	Gaines/PI 178383/CI 13431
W	Sprague	PI 18268/Gaines
S	Twin	Norin 10/Brevor 113* Lemhi 53/2/Lemhi 62/ 4/Lemhi 53* 5/3/Lee* 7//Chinese/Aeum
S	Dirkwin	Twin/Triple Dirk
S	Fielder	Yaktana 54A*4/Norin 10/Brevor/3/2* Yaqui 50/4/Norin 10/Brevor//Baart/Onas
S	Fieldwin	Yaktana 54A*4/Norin 10/Brevor/3/2* Yaqui 50/4/Norin 10/Brevor//Baart/Onas
W	Paha	Suweon 92/4* Omar†
W	Moro	PI 178383/2* Omar
W	Faro	Suweon 92/3* Omar//Moro, OR 7147
W	Jacmar	Farmer selection from Omar
W	Luke	PI 178383/Burt//Pullman sel 101 (CI 13438)
W	Walladay	Spring habit derivative of Luke possibly from natural cross
W	Barbee	Omar/1834 - sel - 3//PI 178383/CI 13431
W	Wanser	Burt/Itana
W	White Holland	Wilhelmina = Spijk/Squarehead
W	Golden	Selected from Goldcoin, also called forty-fold‡
W	Daws	CI 14484//CI 13645/PI 178383
†W	Omar	Elgin 19/Elmar
‡W	Goldcoin	CI 4156, probably a descendant of Redchaff or Redchaff bald.

In order to check the varietal purity of these cultivars and to obtain seed from different sources and locations, these varieties were grown at Hyslop Farm during the 1981-82 growing season. Four-foot rows of each were sown on October 28, 1981. They were harvested on August 4.

The morphological characteristics observed on the plants of each variety were compared to those described (if available) by the breeders (Appendix Table 1). Mixtures were observed in some seed plots which were rogued properly to ensure the highest possible purity.

Methods

The variety identification study included morphological, chemical, and biochemical characteristics. These were coleoptile color, seedling pubescence, phenol, and gliadin and enzyme electrophoresis.

Coleoptile Color

The test was performed according to the procedure proposed by Dhesi, et al. (1969) and described below: two 15 x 23 cm filter papers soaked with distilled water were placed in a 16 x 24 cm plastic box. One hundred seeds were placed on the medium with a vacuum counter. The seeds were then covered with moist sand to a depth of 1 cm.

The plastic boxes were covered and placed in a dark germinator at 20°C. After 36 hours, the plastic boxes were transferred to a

growth chamber at 20°C. Continuous illumination was provided by eight fluorescent tubes and four incandescent bulbs which together provided 360 microeinsteins per square meter per second ($\mu E\ m^{-2}s$). Assessment of coleoptile color was made 5 days after emergence. The test was replicated four times for each of the 25 varieties.

A second procedure reported by Simon and Viron (1975) was also followed to verify the results of the first method. Seeds were placed in plastic boxes as described above but were not covered with moist sand. The plastic boxes were placed directly in the growth chamber at 20°C with continuous illumination as noted above. The moisture of the medium was maintained by a filter paper serving as a bridge between the plastic box containing the seed sample and a plastic box full of water. Assessment of coleoptile color was made 5 days later. A diluted solution of HCl was added to the seedlings to intensify the coleoptile color.

Seedling Pubescence

Four replications of 25 seeds from each variety were grown in soil in 10-cm pots in a greenhouse under controlled environment. The temperature was 21°C and 12 hours daylength. Natural light was supplemented with VHO fluorescent lights. Evaluation of seedling pubescence was made 3 weeks after planting dates.

After 3 weeks, observations of pubescence on the blade, sheath and auricles of the first and the second leaf were made by means

of a dissecting microscope with 20 to 50x magnification. Micro-photographs of the pubescence were also taken.

Phenol Test

Four replicate samples of 100 seeds each were taken from each variety. Samples of some varieties were treated with fungicides. A second test was performed with untreated seeds from the experimental plots. The phenol test was performed according to the procedure detailed in the AOSA Handbook No. 28 (Walls, 1965).

(1) Seeds were soaked in distilled water for 16 hours, then flushed with tap water and the excess water removed from the surface of the seeds by blotting them on a filter paper. Treated seeds were rinsed in methanol to remove the fungicide prior to soaking.

(2) Two layers of filter paper were placed in a container (plastic box) and moistened with 1% phenol solution. One hundred seeds were randomly placed on the two layers of filter paper. The container was then closed.

(3) After 4 hours of incubation at room temperature, the number of seeds in each color category was recorded. Two color categories were determined, light brown and brown.

The phenol solution was freshly prepared by mixing 5 grams of phenol (C_6H_5OH) loose crystals (analytical reagent grade) in 500 ml of distilled water.

Gliadin Electrophoresis

The electrophoresis technique used in this study is a combination of methods reported by Menke and Singh (1973), Wrigley and McCausland (1975, 1977), Autran and Bourdet (1975) and Conkle, et al. (1981).

Apparatus. The apparatus used was homemade, based on the design proposed by Conkle, et al. (1981). The base of this flat gel electrophoresis unit measures 22 x 16 cm. An electrode buffer tray of 22 x 4 x 4 cm is located at each end of the unit. The capacity of each tray is 325 ml. To provide electrical connection, a pure carbon welding rod of 5 mm x 305 mm is inserted into each electrode tray and secured with No. 00 rubber stoppers.

The gel mold consisted of 0.5 cm thick plastic rods secured to a 22 x 16 cm single strength glass plate with rubber bands. With this mold, a gel slab measuring 18 x 16 x 0.5 cm was obtained. This slab is capable of containing up to 30 samples. During electrophoresis, pieces of nylon-reinforced cellulose kitchen sponge were used, soaked in the electrode solution, to provide contact between the electrodes and the ends of the gel. The grinding block was made of a 11.5 x 11.5 x 1.2 cm plexiglass plate into which 6 x 6 wells were drilled, each a depth of .8 cm. Separation between 2 wells was 1 cm.

The electric current was supplied by a regulated high voltage power supply, "Heath Zenith" Model 2717, capable of supplying up to 400 volts and 150 milliamps.

Reagents. Reagents used for buffers, staining and destaining solutions are listed in Appendix Table 2 .

Gel Preparation. Thirty-six g of urea and 36 g of hydrolyzed starch (Connaught Laboratories, Toronto, Canada) were poured into a 1000-ml vacuum flask. Two hundred seventy-five ml aluminum lactate buffer were added. The mixture was shaken properly to disperse the urea and starch. This mixture was then heated with vigorous shaking over a strong bunsen flame. The heating process continued until the solution became thick and viscous. The bubbles within the starch were pulled off by vacuum while the mixture was still hot. When the starch solution appeared clear and poured freely, the mixture was poured rapidly into the molds. If bubbles remained on the surface of the gel, they could be removed easily with an eye dropper while the starch solution was hot. The mold containing the gel was then covered with a plastic plate and let to set overnight at room temperature. For easy removal, the plastic plate was coated with paraffin oil.

Extraction of Sample.

Reagent: A solution of 1 M urea (6 g in distilled water diluted to 100 ml) was used for extraction. This solution was prepared fresh each day because of the possibility that cyanate, formed during storage, might react with the protein (Wrigley and McCausland, 1975).

Procedure: One grain from each variety was finely ground with a small mortar and pestle and then transferred into separate

wells on the grinding block. A 1 M urea solution was added to each sample. The amount of solution added was four times the weight of the grain. The mixture was thoroughly mixed with a glass rod and was left to stand for 1 hour.

Sample Application: After the gel had set, the plastic cover was removed carefully. Excess starch was removed from the outer parts of the gel mold with a scalpel. The sample positions were made by cutting the gel horizontally at 4 cm from one end. On the other end, 2 cm are left to provide space for the sponge contact. The actual migration length available was 10 cm. With a one-sided razor blade held vertically, the gel slab was cut along a straight-edged piece of plastic held in line with the origin marks on the sides of mold bars (4 cm from the end). Rubber bands holding the mold bars were then removed and the smaller section of the gel was separated slightly to allow insertion of the wicks.

The extracts in the wells were absorbed by 5 x 10 mm Whatman chromatography paper wicks. The saturated wicks were then removed from the wells with tweezers. The wicks were lightly blotted on a clean section of absorbent tissue and then placed on the fresh-cut edge of the larger gel slice, making sure that the bottom of the saturated wick touched the glass of the gel mold.

A food coloring dye was used as a marker. Filter paper wicks saturated with the food coloring were placed at each side of the gel at 10 cm from the origin of the gel. This was because the

food coloring dye moves toward the positive end while the protein extracts move toward the negative end.

When all wicks were in place, the smaller section of the gel slice was pushed back against the wicks on the larger gel section. To provide a good contact between the two sections of the gel, a glass rod was placed between the mold bar and the edge of the smaller gel slab. The gel mold bar and the rubber bands were then replaced. To remove air gaps and set the gel against the wicks, the gel near the wicks was pressed lightly. The gel was finally covered with a plastic wrap (Handi-Wrap).

Gel and Tray Setup: The loaded gel was placed on the electrode tray. The origin of the gel was positioned near the positive electrode. One end of a sponge was placed in each electrode tray and the other end was placed on the edge of the gel so as to cover about 1.5 cm of the surface. The sponges were saturated with the aluminum lactate buffer and pressed against the gel with a rubber band to make a thorough contact. The electrode trays were then filled with the buffer.

Electrophoresis: Once ready, the setup described above was placed in the refrigerator at 5°C. The carbon rods were then connected to the power supply, the positive wire to the electrode near the sample slots and the negative wire to the electrode at the other end.

The power supply was turned on and adjusted to 200 volts. At this setting, the current was 75 milliamps. After 15 min, the

power supply was turned off, and the wicks were lifted out of the gel with a tweezer. When all wicks were removed, the rejoined gel slices were examined to make sure that there was good contact between the surfaces. The unit was then reconnected to the power supply and the electrophoresis was continued until the red dye reached the origin of the gel. This was normally about 5 1/2 hours.

Removal and Staining of the Gel: At the end of an electrophoresis run, the power was turned off, the wires disconnected, and the gel and tray setup removed from the refrigerator. The plastic wrap was removed and the sponges folded back into the electrode trays. The section of the gel beyond the 10-cm front near the negative end was trimmed and discarded as was the short slab of the gel on the positive electrode side. A small corner from the gel slab on one side was cut off to mark the position of sample No. 1.

To slice the gel, three 2-mm thick plastic strip spacers were placed on each side of the gel slab. The gel was then sliced by running a well-stretched thin music wire along the plastic spacers. After each slice, one spacer was removed from each side and the process was repeated with the remaining spacers. Four slices were obtained from each gel. The upper and the lower slices were discarded.

The gel slices were lifted off, placed into a shallow tray for staining and covered with the nigrosin staining reagent.

Two slices were left to stain overnight at room temperature. Destaining was done the following day as follows: the stain was carefully poured off, the stained gel slices were rinsed several times with water and destained by soaking them for several hours in the destaining solution.

After the destaining period, the banding patterns were recorded and the gels were stored for several weeks in the destaining solution or in a plastic wrap. Caution was taken to avoid the drying of the gels.

Evaluation of Band Patterns: A band present in all the variety patterns was used as a point of reference to compare the patterns developed by the varieties. The relative mobility of this common band was calculated on the basis of the actual migration length of 10 cm. The relative mobilities of the other bands were calculated, taking as point of reference the relative mobility of the common band. Relative mobilities were calculated by dividing the actual mobility of a given band by the mobility of the reference band. Based on these mobilities, diagrams were drawn for every variety.

Pictures of the stained gels were also taken using black-and-white Panatomic-X Kodak film.

Enzyme Electrophoresis

Varietal differences in peroxidase, esterase, acid phosphatase, catalase and glutamate oxaloacetate transaminase enzymes were

evaluated in this study.

The procedures for enzyme electrophoresis were basically the same as for gliadin electrophoresis except for the buffers used for gel preparation and for staining reagents. Seedlings instead of seeds were used for the enzyme extraction. The buffer systems and the reagents used were those reported by Conkle, et al. (1981). These are listed in Appendix Table 2 .

Extraction. Seedlings were harvested when 7 to 10 days old by excising the shoot and the root. The tissues obtained from a single plant were crushed thoroughly in the wells of the grinding block. Two drops of the extraction buffer were added to each well to obtain the crude extract. The banding patterns were diagrammed by the same procedures described for the gliadin electrophoregrams.

RESULTS AND DISCUSSION

Coleoptile Color

Of the 25 varieties included in this study, Golden developed a purple coleoptile, while the other 24 varieties were green. Seedlings within each variety were uniform for this character. In Golden, pigmentation became visible within 24 hours after emergence. Both methods of testing, i.e., covering seed with a layer of sand or placing them on filter paper without covering them, gave equally satisfactory evaluation of the purple pigmentation. The intensity of the purple coloring was enhanced by keeping the seedlings under slight moisture stress.

It was sometimes difficult to detect the faint purple coloring of some seedlings. However, addition of a dilute solution of hydrochloric acid enhanced the coloring sufficiently to allow evaluation of the coloring with confidence.

Coleoptile color was of limited value as a diagnostic character for identifying the commonly grown white wheat varieties in the Pacific Northwest. However, researchers have reported that many wheat varieties have purple coleoptiles and coleoptile color is a useful character for characterizing those varieties. The test is easy to perform, does not require highly trained personnel, and the results are obtained in a relatively short time period (6 to 7 days).

Seedling Pubescence

It was possible to classify wheat varieties into groups based on the presence or absence of hairs on the sheath and the blade of the first leaf. Varieties varied in regard to density of the pubescence, but this character was influenced greatly by environmental conditions. The density of hairiness within a variety was also not constant. Thus, to avoid any confusion and/or misinterpretation, the varieties were classified in two groups according to the presence or relative absence of hairiness, without regard to its density (Table 2). Examination of the second and third leaves did not provide further useful information as their pubescence characteristics were similar to those of the first leaf.

It is important to use relatively high magnification to evaluate seedling pubescence. With a hand lens (10x magnification), hairs on seedlings of those varieties that were slightly pubescent were not readily seen and would have been evaluated as being smooth. However, when the same seedlings were observed through a stereoscopic microscope at 30 to 50x magnification, hairiness was visible. This test is also easy to perform, but requires a relatively longer time period than the coleoptile color test. It is also important to grow the varieties under similar environmental conditions to avoid any misinterpretation.

Phenol Color Reaction

The phenol test has been used to classify wheat varieties into

Table 2. Seedling pubescence of 25 white wheat varieties.

Pubescent to very pubescent	Slightly pubescent
Daws	Barbee
Dirkwin	Faro
Druchamp	Gaines
Fielder	Golden
Fieldwin	Jacmar
Hill	Moro
Hyslop	Nugaines
Luke	Sprague
McDermid	
Paha	
Raeder	
Stephens	
Twin	
Urquie	
Walladay	
White Holland	
Yamhill	

five color groups. As specified in AOSA Handbook No. 28 (Walls, 1965), these are: "I - Ivory", "II - Fawn", "III - Light Brown", "IV - Brown", and "V - Brown-Black." In this study, it was observed that there was some variation in the darkness of phenol staining among seed lots of the same cultivars. This variation was also reported by Payne and Koszykowski (1981) and others. Because of this variation, there is an element of risk of error involved in grouping wheat varieties into as many as five groups. To reduce the chance of misinterpreting variation among seed lots of the same cultivar as cultivar differences, only two color groups were designated. Seeds were recorded as staining either light or dark (Table 3). The light brown appears to be a combination of "II - Fawn" and "III Light Brown" and the brown color appears to be "IV - Brown" and "V - Brown-Black" in the AOSA classification. A third group of four varieties contained a mixture of light brown and brown seeds. The percentage of each color differed among varieties and appeared to be a characteristic feature of those varieties.

AOSA Handbook No. 28 specifies that seeds should be placed palea side down on the filter paper. Preliminary tests comparing this method with random positioning of the seed revealed that there was no difference in seed staining due to the positioning of the seeds. Therefore, subsequent tests were performed without regard to their placement.

Some of the seed samples were treated with unknown fungicides.

Table 3. Phenol color reactions of 25 white wheat varieties.

Phenol color reaction†		
Light brown	Brown	Mixed‡
Dirkwin	Fielder	Barbee (55% LB, 45% B)
Faro	Fieldwin	Daws (60% LB, 40% B)
Golden	Gaines	Druchamp (85% LB, 15% B)
McDermid	Hill	Luke (90% LB, 10% B)
Moro	Hyslop	
Paha	Jacmar	
Stephens	Nugaines	
Twin	Raeder	
White Holland	Sprague	
	Urquie	
	Walladay	
	Yamhill	

†Light brown combines the "II-Fawn" and the "III-Light Brown" of the AOSA classification.

Brown combines the "IV-Brown" and the "V-Brown Black" of the AOSA classification.

‡LB = light brown

B = brown

Those seeds were rinsed with methanol to remove the fungicide prior to the 16-hr soak period. The methanol rinse appeared to remove most of the fungicide and the resulting seeds usually stained satisfactorily with phenol. The sample of the variety Faro was an exception, however, in that seeds remained unstained even when they were rinsed thoroughly with methanol prior to the soaking period. When the phenol test was performed on non-treated seeds of this variety, they developed a light color stain after the 4 hrs of incubation. The cultivar Faro probably was treated with Vitavax, since this chemical has been reported to delay phenol staining even after rinsing treated seeds in methanol (Payne and Koszykowski, 1981).

The phenol test is very easy to perform with large numbers of seeds, and the results are obtained in a short period of time (20 hrs). This period could be shorter if the test is performed by the rapid hot water soak method proposed by Wrigley and McCausland (1975, 1977).

Gliadin Electrophoresis

Electrophoresis tests were conducted with 40 seeds of each variety to determine the degree of homogeneity among seeds within varieties for gliadin banding patterns. In each case, only one banding pattern was obtained per variety. Following this work, single-seed extracts of each variety were run on the same gel. These tests were repeated several times, with similar banding patterns obtained each time. Photographs and diagrams of the

stained gels of single seeds are shown in Figure 1. Numerous differences were found among varieties in number, position and staining intensity of the protein bands.

The band with the highest mobility (cross-hatched on the diagram) that was common to all varieties was chosen as a reference band and the relative mobility of each band was calculated in relation to this reference band. Bands at relative mobilities of .09, .30, and .32 were also common to all varieties.

Over 20 bands were identified on the starch gel. The most distinctive bands (6-13), located between .05 and .50 relative mobilities (on the upper part of the gel near the positive side) were diagrammed. Several poorly resolved bands located beyond .50 relative mobility appeared to be common to all varieties and were not useful in distinguishing among them, so are not shown on the diagrams.

Wrigley and Shepherd (1974), using a combination of gel isoelectric focusing and starch gel electrophoresis, identified over 40 bands for the bread wheat gliadins, and concluded that the apparently single bands separated by starch gel electrophoresis were made up of several components.

The electrophoretic pattern is analogous to a fingerprint. It is distinctive, characteristic of the variety and the same irrespective of the environmental conditions under which the variety is grown. Starch gel electrophoresis of gliadins distinguished differences among almost all varieties since 20 different patterns

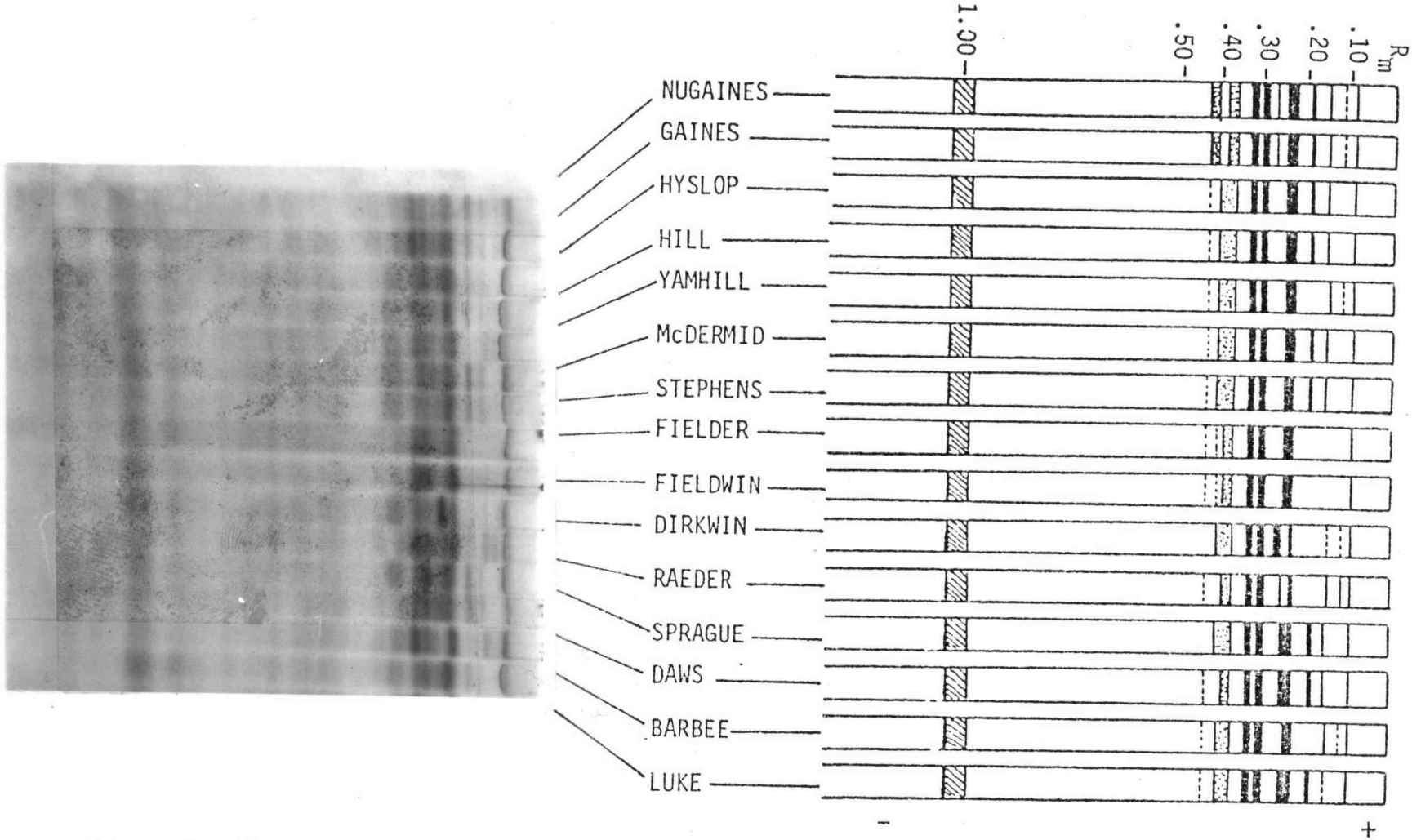
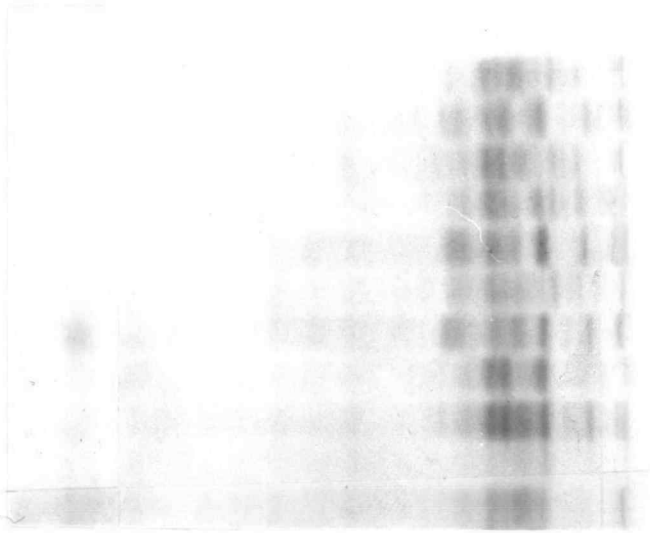


Figure 1. Photographs and diagrams of gliadin electrophoregrams of 25 white wheat varieties. Dashed lines and dotted areas on the diagrams represent light stained bands, narrow solid lines indicate medium stained bands, and wide solid lines indicate dark stained bands. Cross-hatched band represents the reference band.



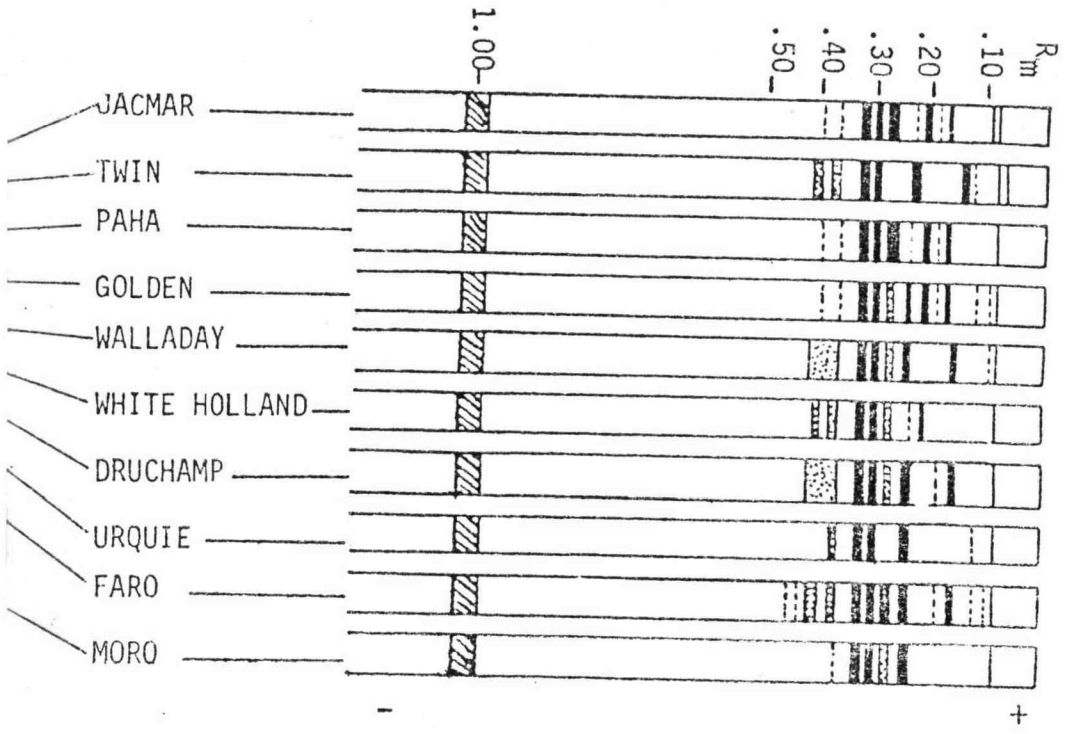


Figure 1. (continued)

were obtained. The distinction between varieties was based essentially on the presence or absence of a given band at a given relative mobility. The most useful bands are located between .10 and .30, and between .32 and .50 relative mobility zones. For example, Nugaines had 10 bands while Hyslop exhibited only 8. The second and sixth bands, located at .12 and .27 relative mobilities in Nugaines, were absent in Hyslop. Fielder and Fieldwin did not develop any bands in the zone between .10 and .24 relative mobilities which makes these two varieties very distinct from the others. Distinction among other varieties is made similarly on the basis of the presence or absence of one or more bands at given relative mobilities on the gliadin electrophoregrams.

The majority of the varieties used in this study are, to a certain extent, related to each other, with Pullman-Sel. 101, PI 178383, Norin 10 and Brevor extensively used as parents. Some closely related varieties developed identical electrophoretic patterns. These were Gaines and Nugaines; Hyslop, Hill, McDermid, and Stephens; and Fielder and Fieldwin. The pedigrees (Table 1) show that Nugaines is a sib of Gaines and Fielder has the same pedigree as Fieldwin (Yaktana 54A*4/Norin 10/Brevor/3/2*Yaqui 50/4/Norin 10/Brevor//Baart/Onas). Hyslop, McDermid and Stephens have the same parents, i.e., Nord Depres/Pullman Sel. 101, with the major difference being the number of backcrosses during their development.

The similarity of banding patterns developed by very closely

related varieties was also observed by Wrigley and McCausland (1975); Autran and Bourdet (1975) and several other workers.

Environmental effects. The environmental conditions under which the varieties were grown did not affect the electrophoretic patterns. Seed lots used for the first set of runs were obtained from varieties grown at various locations in the Pacific Northwest. The second set of runs was performed on seed samples obtained from the varieties grown at Hyslop Farm and no difference in patterns was observed. Fungicide treatments also did not affect the electrophoretic patterns. These observations confirm those of Qualset and Wrigley (1979), Zillman and Bushuk (1979), Ducros and Wrigley (1979), and several other workers.

Relative mobilities. Bushuk and Zillman (1978), Autran and Bourdet (1975) and others demonstrated that all samples of one variety examined had the same gliadin protein components, each holding its specific position among the others. Each component was referred to by means of a number which represents its running speed. This is called electrophoretic mobility. This mobility depends largely on the voltage applied during the run. However, the mobility may vary somewhat from gel to gel because of non-uniformity of gels, gel temperature during electrophoresis, and variations in gliadin extractions. Since it is extremely difficult to precisely control and reproduce experimental conditions, the relative mobilities of the gliadin components were determined

rather than the absolute mobilities. The relative mobilities of the components within each variety were consistently identical from gel to gel. The relative mobilities of the gliadin bands of the 25 varieties are shown in Appendix Table 3.

Band intensities. Band intensities were quantified subjectively by visual comparison of the bands on the photographs and on the several gels stored in the staining solution. Staining intensities varied from very faint to very intense (Appendix Table 3).

The most accurate way to quantify band intensities would be by densitometer readings but, because of the lack of a densitometer, this was not done in this study. However, band intensities are of minor importance compared to the total number of bands and band mobilities, because the band intensities depend on the protein content of the grain sample (Zillman and Bushuk, 1979).

Band intensities were also affected by starch quality. When a high-grade starch was used for making the gels, faint bands were seen that were not evident in gels made from low-grade starch.

It can be concluded that gliadin electrophoresis is a valuable tool for wheat variety identification. In most cases, banding patterns developed by a variety were specific to it and the banding patterns were totally independent of environmental conditions. This technique allows performance of a variety identification test in a short period of time (less than 48 hrs), and is relatively easy to perform. The main limitation of electrophoresis

is that only a limited number of grains can usually be tested per sample because of the expense and labor involved.

Enzyme Electrophoresis

All the wheat varieties in this study developed identical enzyme electrophoregrams, or zymograms, for esterase, acid phosphatase, catalase, and glutamate oxaloacetate transaminase. Studies of some of these enzymes by Auriau, et al. (1976), Almgard and Clapham (1977) and others, revealed that the wheat varieties examined in their studies also had similar zymograms.

Peroxidase enzyme electrophoresis was of some value for varietal identification. It allowed classification of the 25 wheat varieties into two distinct groups on the basis of the presence or absence of the band with a relative mobility of .87 (Figures 2 and 3). The diagram and photograph show only the bands developed on the cathodal side of the gel. The poorly resolved bands which appeared to be identical to all the varieties were not diagrammed. The bands developed on the anodal side of the gel did not provide additional information, since they were all the same.

As in gliadin electrophoresis, enzyme electrophoresis was not affected by environmental factors. This is confirmed by Almgard and Clapham (1977) and several other workers. Enzyme electrophoresis provides additional useful information in separating varieties. It is also relatively easy to perform. Its limitation is similar to that of gliadin electrophoresis.

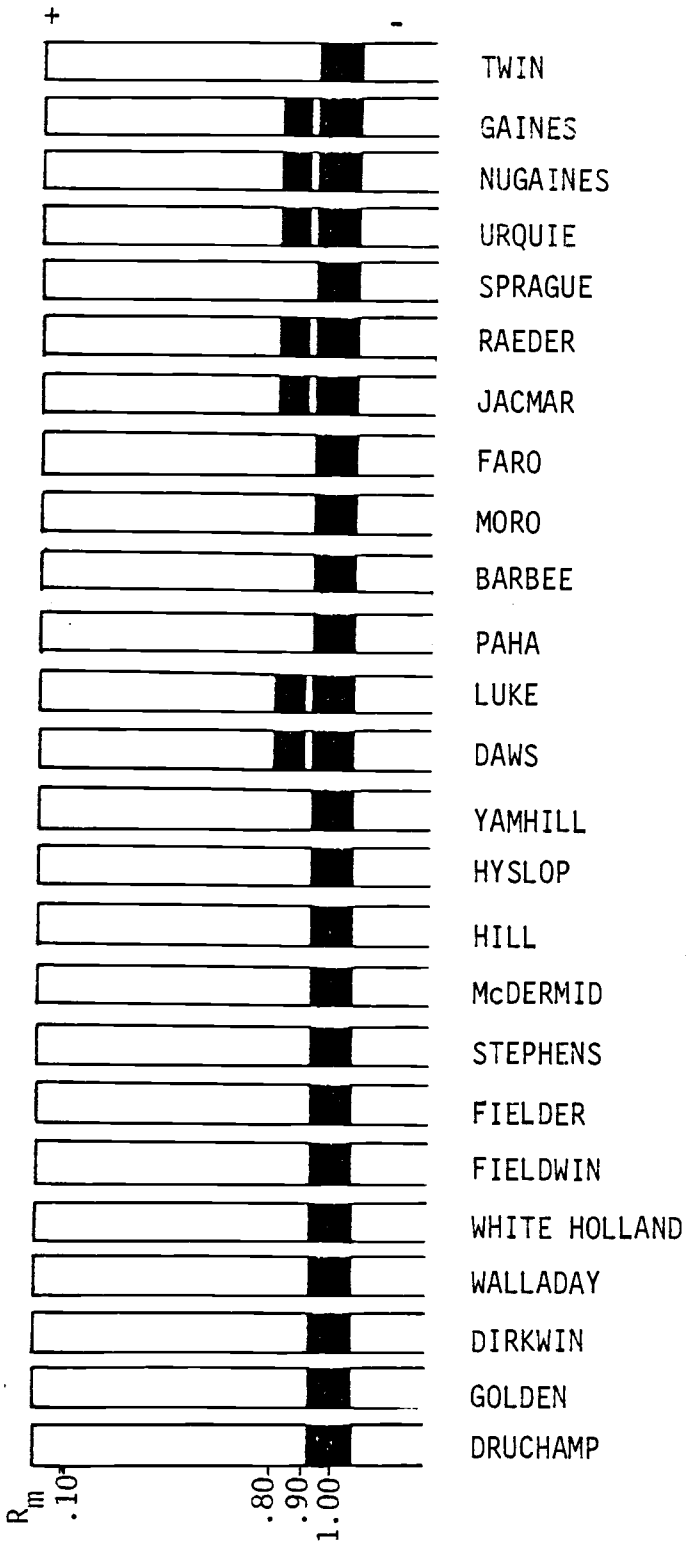


Figure 2. Peroxidase electrophoregram of 25 white wheat varieties.

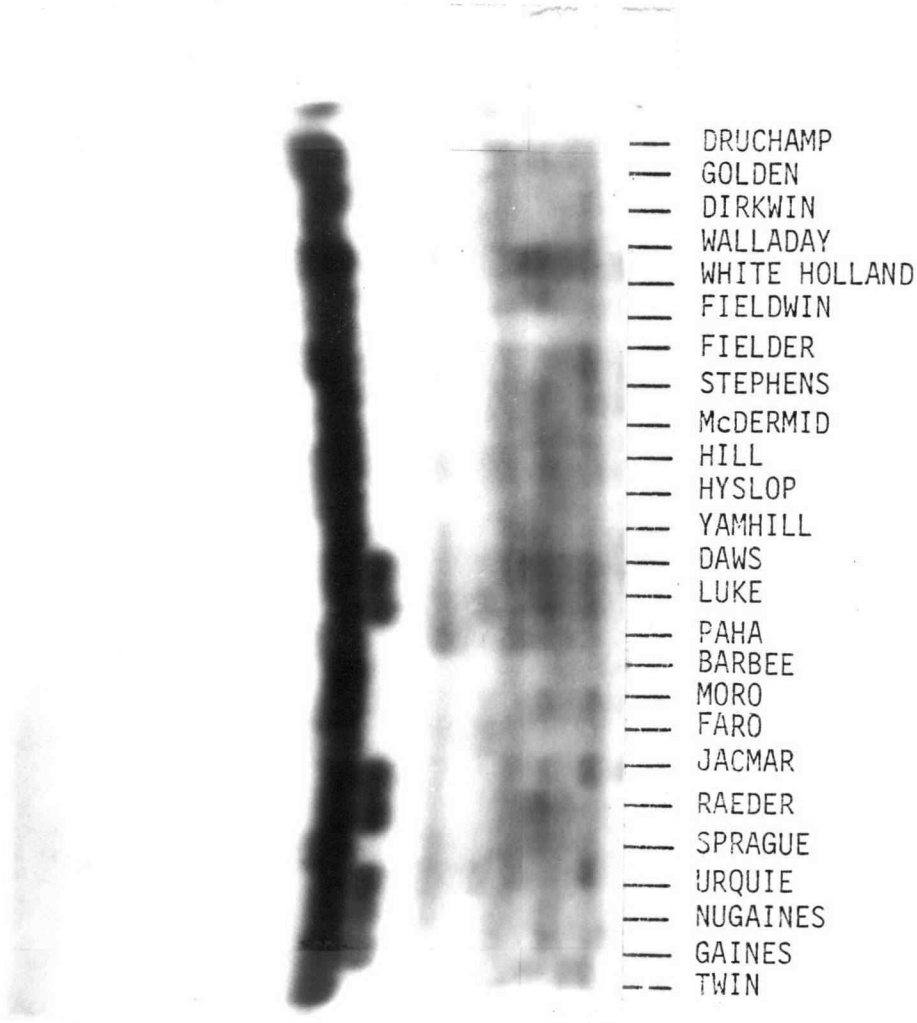


Figure 3. Photograph of peroxidase electrophoregram of 25 white wheat varieties.

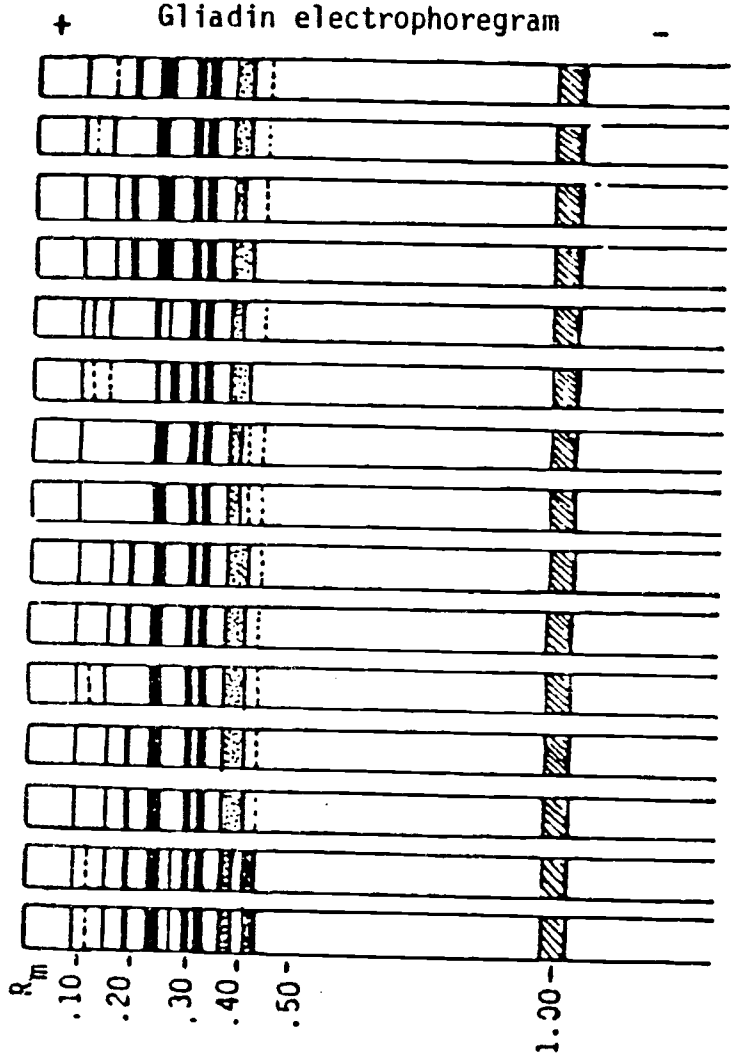
GENERAL DISCUSSION

Of the five diagnostic characters evaluated for their usefulness in identifying soft white wheat varieties, gliadin electrophoresis was the most discriminating and differentiated all varieties except those that were closely related. The varietal characteristics are summarized in Figure 4.

Coleoptile color, seedling pubescence, phenol color reaction, and peroxidase electrophoresis each classified the varieties into two groups. If these four tests are combined, additional classification can be obtained. In most cases, these tests did not provide further differentiation of varieties than that provided by gliadin electrophoresis alone. One exception was in the case of Hill, Hyslop, McDermid, and Stephens. These four closely related varieties possessed the same gliadin banding patterns, whereas the phenol test separated them into two pairs of varieties.

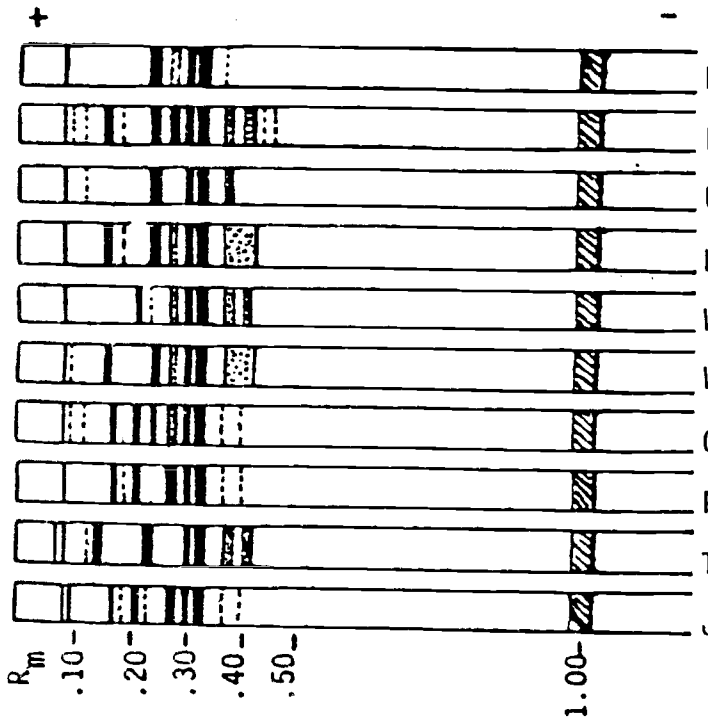
All five characters would be useful in characterizing varieties for entry in the Plant Variety Protection system. Also, in testing for varietal identity, there is a value in basing the identification on more than one character.

The applicability of the various tests also depends on the purpose for which they are intended. When a seedlot is suspected of being mislabeled as to variety, mislabeling can be proven if the seedlot differs from an authentic check sample in any of these five characters. In this case, it is not necessary to determine the actual varietal identity of the seedlot.



	Peroxidase†	Phenol	Coleoptile color	Seedling‡ pubescence
LUKE	a	Mixed	Green	+
BARBEE	b	Mixed	Green	-
DAWS	a	Mixed	Green	+
SPRAGUE	b	Brown	Green	-
RAEDER	a	Brown	Green	+
DIRKWIN	b	Light brown	Green	+
FIELDWIN	b	Brown	Green	+
FIELDER	b	Brown	Green	+
STEPHENS	b	Light brown	Green	+
McDERMID	b	Light brown	Green	+
YAMHILL	b	Brown	Green	+
HILL	b	Brown	Green	+
HYSLOP	b	Brown	Green	+
GAINES	a	Brown	Green	-
NUGAINES	a	Brown	Green	-

Figure 4. Summary of identification characters of 25 white wheat varieties.



	Peroxidase†	Phenol	Coleoptile color	Seedling pubescence‡
MORO	b	Light brown	Green	-
FARO	b	Light brown	Green	-
URQUIE	a	Brown	Green	+
DRUCHAMP	b	Mixed	Green	+
WHITE HOLLAND	b	Light brown	Green	+
WALLADAY	b	Brown	Green	+
GOLDEN	b	Light brown	Purple	-
PAHA	b	Light brown	Green	+
TWIN	b	Light brown	Green	+
JACMAR	a	Brown	Green	-

Figure 4. (continued)

†Peroxidase: a = varieties having the band at .87 relative mobility.
b = varieties which do not have band at .87 relative mobility.

‡Seedling pubescence: + = pubescent to very pubescent.
- = slightly pubescent.

A different approach is needed for positive varietal identification of a seedlot. In many cases, it can be argued that it is never possible to positively identify a variety with 100% accuracy, since there is always a chance of another variety in existence with the same test characteristics. In practice, however, there is a relatively small number of soft white wheat varieties grown in the Pacific Northwest, and gliadin electrophoresis, in combination with the other four tests, plus available production records, will provide positive varietal identification with a high probability of being correct.

Similar conclusions may be made concerning the adaptability of the five tests for detecting varietal mixtures. In this case, it is usually necessary only to be able to detect mixtures without positively identifying them. Thus,the choice of test is made on the basis of cost, time, ease of performing the test, skill of the technician, availability of equipment, the purpose for which it is intented, as well as available information on differentiating characteristics of the varieties.

Additional possibilities exist for further research on differentiating the closely related varieties. Since these varieties frequently differ in disease resistance, pathological tests hold considerable promise. Isoelectric focusing techniques and additional chemical tests should also be investigated.

SUMMARY AND CONCLUSIONS

The five tests described in this study provide the basis for laboratory identification of 25 varieties grown in the Pacific Northwest. The coleoptile color test was of limited value for these varieties since only Golden exhibited a purple coleoptile while the remaining varieties were green.

The seedling pubescence test separated the varieties into two groups: slightly pubescent and pubescent. However, the variation of the pubescence density caused by environmental conditions limits its practical use.

The phenol test separated the varieties into three distinct groups: Light Brown, Brown, and Mixed. Four varieties, Barbee, Daws, Druchamp, and Luke, exhibited a mixed phenol reaction. This mixture was a characteristic of these varieties since they were uniform for the other characters.

Esterase, catalase, acid phosphatase and glutamic oxaloacetic transaminase electrophoresis were not useful for separating the varieties since all of them developed similar banding patterns for each enzyme. Peroxidase electrophoresis separated the varieties into two distinct groups on the basis of the presence or absence of a single band.

Gliadin electrophoresis was the most discriminating test, distinguishing all the varieties except for those genetically closely related. These included varieties within three variety

groups: Gaines and Nugaines; Fielder and Fieldwin; and Hyslop, Hill, McDermid, and Stephens. The phenol test provided a useful separation of the group of four varieties which were uniform for gliadins. Hill and Hyslop developed a brown phenol stain while McDrmid and Stephens exhibited a light brown stain. Further identification might be possible by other means such as pathogen resistance, isoelectric focusing and other chemical tests.

In practice, unknown samples should be compared to authentic, standard samples for final identification.

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APPENDICES

Appendix Table 1. Agronomic characteristics of white wheat varieties grown in Oregon.

Variety	Originating or releasing agency	Year of release	Growth habit	Market class	Head shape	Glumes	Awns
Barbee	ARS & WSU	1976	Winter	White Club	Club	Brown	Awned
Daws	ARS & WSU	1976	Winter	Soft White		White	Awned
Druchamp	Burlingham-Meeker	1949	Winter	Soft White	Oblong	White	Awnleted
Faro	OSU	1976	Winter	White Club	Club	Brown	Awnleted
Fielder	ARS & Idaho	1974	Spring	Soft White	Oblong	White	Awned
Gaines	ARS & WSU	1961	Winter	Soft White	Oblong	White	Awned
Golden	OSC	1930	Winter	Soft White	Clavate	Brown	Awnleted
Hyslop	OSU	1971	Winter	Soft White	Oblong	White	Awned
Luke	ARS & WSU	1970	Winter	Soft White	Oblong	White	Awned
McDermid	OSU	1974	Winter	Soft White	Fusiform	White	Awned
Moro	OSU	1965	Winter	White Club	Club	Brown	Awnleted
Nugaines	ARS & WSU	1965	Winter	Soft White	Oblong	White	Awned
Paha	ARS & WSU	1970	Winter	White Club	Club	Brown	Awnleted
Raeder	ARS & WSU	1976	Winter	Soft White		Red	Awned
Sprague	WSU	1973	Winter	Soft White		Variable	Awned
Stephens	OSU	1977	Winter	Soft White	Fusiform	White	Awned
Twin	ARS & Idaho	1971	Spring	Soft White	Clavate	White	Awnless
Urquie	ARS & WSU		Faculative	Soft White		White	Awned
Yamhill	OSU	1969	Winter	Soft White	Fusiform	White	Awnleted

Appendix Table 1. (continued)

Variety	Height	Straw strength index†	Emergence index†	Winter hardiness index†	Maturity	Test weight index†	Disease reactions†	
							Common bunt	Stripe rust
Barbee	Semi-dwarf	8	5	5	Mid-season	5	6	6
Daws	Semi-dwarf	8	4	8	Mid-season	7	8	8
Druchamp	Short	6			Late		1	8
Faro	Semi-dwarf		6	5	Mid-season		7	4
Fielder	Semi-dwarf	6		-	Mid-season	7		
Gaines	Semi-dwarf	8	5	6	Mid-season	7	6	5
Golden	Tall	4					1	4
Hyslop	Semi-dwarf	8	7	5	Mid-season	7	6	8
Luke	Semi-dwarf	6	6	5	Late	7	7	6
McDermid	Semi-dwarf	8	7	6	Mid-season	7	6	7
Moro	Tall	3	8	5	Mid-season	5	6	7
Nugaines	Semi-dwarf	8	5	6	Mid-season	8	6	6
Paha	Short	8	6	4	Mid-season	6	6	4
Raeder	Semi-dwarf	7	5	7	Mid-season	7		
Sprague	Semi-dwarf	4	7	7	Early	8	7	
Stephens	Semi-dwarf	8		5	Mid-season	6	5	
Urquie	Semi-dwarf	7		2	Late	6		
Yamhill	Tall	8	7	5	Mid-season	6	1	7

†1 = poor; 5 = adequate; 10 = superior

Source: Unpublished preliminary summary presented at Oregon State University Wheat Seminars, January 31, February 4, 1977.

Appendix Table 2. Buffer formulations and staining processes.

Gliadins

Gel and Tray Buffer

Aluminum lactate buffer	pH 3.1
aluminum lactate	5 g
distilled water	
concentrated lactic acid	

Dissolve 5 g of aluminum lactate in 900 ml of distilled water. Add concentrated lactic acid to obtain pH 3.1. Dilute the solution to 1000 ml with distilled water.

Staining Solution

nigrosin aqueous	5 mg
acetic acid	20 ml
distilled water	180 ml

Destaining Solution

methanol	4 parts
distilled water	5 parts
acetic acid	1 part

Enzymes

Esterase, peroxidase, catalase and acid phosphatase buffer systems:

Gel buffer	Tray buffer
Lithium borate pH 8.3	Tris citrate pH 8.3
LIOH (monohydrate) 1.2 g	Trizma base, 6.2 g
Boric acid 11.89 g	Citric acid 1.46 g
Distilled water 1.0 l	Distilled water 1 l
Used as is	Used as follows: 75 ml of tray buffer were added to 675 ml of gel buffer.

Appendix Table 2 (continued)

Staining buffers

ACPH acetate buffer

pH: 4.0	sodium acetate	2.43 g
	glacial acetic acid	4.7 ml
	1 m $Mgcl_2$	5.0 ml
	distilled water	1.0 l

CAT buffer

pH: 6.4	sodium acetate, monobasic	18.5 g
	sodium acetate, dibasic	17.9 g
	distilled water	1.0 l

EST buffer

pH: 6.4	sodium phosphate, monobasic	13.9 g
	sodium phosphate, dibasic	5.3 g
	distilled water	1.0 l

PER buffer

pH: 5.6	sodium acetate	5.74 g
	glacial acetic acid	1.2 ml
	distilled water	1.0 l

Staining Processes

Acid phosphatase: components

naphtyl acid phosphate	100 mg
fast garnet GBC salt	100 mg

These two components are mixed with 80 ml of the ACPH acetate buffer. The gels were developed at room temperature.

Esterase: components

Fast blue RR salt	80 mg
1% naphtyl acetate	1 ml

These components were added to the warm esterase buffers. The gels were then soaked in this mixture and incubated at 37°C.

Appendix Table 2. (continued)

Catalase: components

2% potassium iodine (KI)	100 ml
0.03% solution of H_2O_2	100 ml

The gels were refrigerated at 5°C in the catalase buffer for 30 minutes. The buffer was drained off and soaked in 2% KI for 2 minutes. The KI was drained off and the gel was rinsed in water. H_2O_2 solution was then added to the gel.

Peroxidase: components

50 mg of 3-amino-9 ethyl carbazole were added to 5 ml of N,N dimethylformamide. One ml of 3% H_2O_2 was then added. These components were then added to 75 ml of peroxidase buffer to which 2 ml of 0.1 m of $CaCl_2$ were previously added. The gels were soaked in this solution and developed at room temperature.

For all these enzymes, cathodal and anodal slices of the gel were stained.

Glutamate Oxaloacetate Transaminase Buffer Systems

Gel buffer	Tray buffer
Sodium borate pH 8.0	Tri-citrate pH 8.8
Boric acid 18.55 g	Trizma base 12.11 g
NaOh 2.0 g	0.2 m atricacid solution
Distilled water 1.0 l	Distilled water 1.0 l
Used as is	The chemicals were dissolved and tritrated to pH 8.8 with atric acid solution.
	Used as is.

Appendix Table 2 . (continued)

Staining Process

The stain buffer was the 0.2 M phosphate buffer also used for extraction.

Stain components:

0.5%	pyridoxal 5-phosphate	0.8 ml
3.0%	bovine albumin solution	1.6 ml
0.2 m	L-aspartic acid	6.8 ml
	α-ketoglutarate solution	2 ml
	120 mg of Fast Blue BB salt in 8 ml distilled water.	

The first four components were combined with the buffer. The Fast Blue BB salt solution was added to the mixture just before the staining process. The gels were soaked and developed at room temperature. Both cathodal and anodal slices were stained.

Extraction:

sodium phosphate monabasic	3.84 g
sodium phosphate dibasic	23.86 g
distilled water	1.0 l

Appendix Table 3. Staining intensity and relative mobilities of gliadin electrophoretic bands of 25 white wheat varieties.

Relative mobility	Variety														
	Nugaines	Gaines	Hyslop	Hill	Yamhill	McDermid	Stephens	Fielder	Fieldwin	Dirkwin	Raeder	Sprague	Daws	Barbee	Luke
	Staining intensity[†]														
.07															
.09	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
.10										Tr	+			Tr	
.12	Tr	Tr			Tr										
.13										Tr	+			+	
.15	+	+	+	+	+	+	+					+	+		Tr
.16															
.18	++	++	++	++		++	++					++	++		++
.19															
.20															
.23										++	++				
.24	++	++	++	++	++	++	++	++	++			++	++	++	++
.26											+				
.27										+++					
.30	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
.32	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
.36															
.38	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
.40															
.41								Tr	Tr						
.42	Tr	Tr													
.43			Tr	Tr	Tr	Tr	Tr	Tr	Tr		Tr		Tr	Tr	Tr
.44															

Appendix Table 3. (continued)

Relative mobility	Variety									
	Jacmar	Twin	Paha	Golden	Walladay	White Holland	Druchamp	Urquie	Faro	Moro
	Staining intensity[†]									
.07		+								
.09	+	+	+	+	+	+	+	+	+	+
.10	+			Tr	Tr				Tr	
.12		Tr		Tr				Tr	Tr	
.13		+								
.15					+		+		+	
.16	+		+	+						
.18							Tr		Tr	
.19	Tr		Tr	Tr						
.20	+		+	+		+				
.23	Tr	+	Tr	+	++	Tr	++	++	++	+
.24										
.26										
.27	++		+	Tr	Tr	Tr	Tr		++	Tr
.30	++	++	++	++	++	++	++	++	++	++
.32	++	++	++	++	++	++	++	++	++	++
.36	Tr	Tr	Tr	Tr		Tr		Tr	Tr	Tr
.38	Tr		Tr	Tr	Tr	Tr	Tr		Tr	
.40		Tr								
.41									Tr	
.42										
.43										
.44									Tr	

[†]Tr = trace + = light stain ++ = medium stain +++ = dark stain