ELECTROPHORESIS SEPARATION OF GENERAL PROTEIN AND ISOENZYMES OF BLACK CHERRY SEED (PRUNUS SEROTINA EHRH.)

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In addition to seed source studies, biosystematic investigations which describe patterns of variation through comparative anatomy, morphology, biochemistry or related disciplines, provide valuable data on the geographic variation of a given species. In 1966, a comprehensive study of black cherry (Prunus serotina Ehrh.) was initiated at West Virginia University. Initially, seeds from 33 widely separated areas throughout the black cherry range were collected, studied, and planted. Geographic differences in seed characteristics were found (Cech and Kitzmiller, 1968). Several outplantings have been made for future analyses.

As part of this continuing study, a limited biochemical investigation was made on dormant black cherry seeds. Variations in electrophoretic patterns of general protein and selected enzymes were studied as to their possible relationship with geographic origin. The primary analytical method in this study was polyacrylamide gel, "disc" electrophoresis, which utilizes small gel columns as the support media for sample protein migration and separation. This method, and other techniques of zone electrophoresis have been utilized in many studies of biochemical variations in plant and animal tissues.

Clements (1965) was able to demonstrate unique and reproducible patterns for ovary protein extractions of varieties of apples, pears, oranges, lemons, avocado, and bananas. Significant differences in esterase patterns of certain pea varieties were found by Frankel and Garber (1965). In comparative electrophoretic studies of the seed proteins from certain species of *Brassica* and *Sinapis*, Vaughn and Waite (1967) found evidence which supported the established taxonomy of these species. A later study (Vaughn and Waite, 1967) of certain amphidiploid species of *Brassica* revealed that the hybrids had bands from "both suspected parents," but a few new protein systems were thought to have been evolved as a result of hybridization.

The methods of gel electrophoresis lend themselves readily to many types of research in higher plants (Brewbaker, 1968). Even though the number of plant enzymes that may be easily detected by such methods are limited, genetic polymorphisms of these enzymes are sufficient for comprehensive studies of angiosperm systematics, morphogenetics, and physiology.

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METHODS AND PROCEDURES

This study was divided into three phases:

I. The testing, modification or development of satisfactory techniques.

II. A comparative study of materials from the same general geographic area.

III. A comparative study of material from selected sources throughout the natural range of the species.

This paper will present information on phases I and II. The analysis of phase III data has not been completed.

PHASE I

A preliminary consideration was the need for a method for removing the hard coat of black cherry seed. A seed splitter was fabricated from a tubing cutter with a movable cutting wheel. With this device, the seed coats could be split easily and quickly, without damaging the soft interior.

After removing the coats, the embryo and endosperm were ground into a fine powder and an appropriate solvent was used to extract the soluble protein. Various solvents were tested; aqueous buffers were most satisfactory. Of these, a 0.2 M (pH 8.5) tris borate buffer gave the best results and was utilized for all subsequent extractions. A minimum extraction time of 12 hours was used. Seeds and extracts were stored at 5°C.

Brewbaker's (1968) use of refinement procedures in the preparation of soft tissues of root, leaf and endosperm afforded little advantage. Nevertheless, in an attempt to obtain gels of higher resolution, gel filtration (Bio-gel), high speed centrifugation, ion exchange chromatography, sucrose density gradients (ultracentrifuge at 100,000 x G) and salt precipitation were tested. High speed centrifugation (25,000 x G for 15 minutes) was found to be a simple and effective purification method and was incorporated in the extraction procedure.

The filtered extracts were assayed for protein content against a bovine serum albumin standard using the assay procedure of Lowry et al. (1951). Each extract was adjusted to a protein content of 3.3 mgms/ml with a 3 M sucrose solution.

General protein and enzyme systems (acid phosphatase, amylase, catalase, esterase, leucine amino peptidase, and peroxidase) were investigated. Amylase and catalase studies were discontinued due to staining difficulties.

The electrophoretic chamber was manufactured by Buchler Instruments Inc. under the trade name "Polyanalyst." It is a compact, 12 tube instrument with a water jacket around the lower buffer chamber for temperature regulation. A jacket was added around the upper chamber so that the entire apparatus could be cooled during a run. Electrical current was supplied by a model EC 454 D.C. power supply (E.C. Apparatus Corp.).

Anionic buffer and gel systems were used, with the protein migrating down the gel columns toward the positive electrode. The columns were contained inside standard wall glass tubes with an 8 mm outside diameter and a length of 95 mm. With minor modifications, gels were prepared using the procedures developed by Davis (1964). Acrylamide contents of 5%-15% and various gel formulations were tested. A 7% acrylamide content was found to be most satisfactory. Four different gel formulations were used in the study, the specific formulation depending on the type of material to be elucidated. This was also the determining factor for the type and composition of the buffer(s) used.

The same gel and buffer systems were used for general protein and peroxidase. Different systems were used for acid phosphatase, esterase, and leucine amino peptidase. In some instances, modification of formulations found in the literature improved the pattern resolution. Gel and buffer references with the modifications for this study are presented (Table 1).

Table 1.--Gels & Buffers: References and Modification

Material to be stained	Formulation References	Modification of Ref. Formulations
General Protein and Peroxidase	Gels..Davis(1964)	Running gel - 1 part Stock Soln. (A); 1 part Stock Soln. (C); 2 parts small pore solution #2
		Stacking gel - Sub. 3 M sucrose soln. for Stock Soln. (F)
	Buffers..Polyanalyst Instruction Manual (Anonymous, 1966)	None
Acid Phosphatase	Buffer & Gel: Williams & Reichfeld (1964)	None
Esterase Leucine Amino Peptidase	Buffer & Gels: Brewbaker (1968)	Gels: Reduce ammonium persulfate solution from 10% to 2.5%

Except for general protein gels, the protein samples were applied directly to the top of the 7% running or separation gel. In the case of general protein, a 2.5%, light polymerized stacking gel was layered on top of the running gel before application of the sample. The protein loading procedure for all runs was carried out after the gel tubes were in place and the upper buffer solution added. Standardized samples *were* carefully layered at the gel-buffer interface. Although a serological pipette may be used, a semi-automatic pipetting device (Biopette) was preferred. This device delivers a precise amount of liquid under pressure. A protein load of 330 micrograms per tube was used for general protein gels and 660 micrograms for the enzyme gels. These heavy concentrations were necessary to elucidate the weaker bands.

A standard time of 150 minutes was used. For the first 30 minutes, a reduced amperage (1.25 ma/gel) was applied to allow the protein to migrate into the gel without creating convection currents in the upper buffer. The amperage was then increased to 3.5 ma/gel in runs using tris-glycine or barbituric acid buffer systems and to 2.5 ma/gel for those with tris-borate systems. After the 30 minute adjustment, constant voltage was maintained. Voltage/amperage relationships varied between different buffer and gel systems. Slight differences were detected between runs utilizing the same systems.

Upon completion of a run, the gels were immediately removed from the tubes for staining. Although various removal procedures have been described involving hypodermic needles, water pressure, etc., a thin, stainless steel wire, mounted on one arm of a U shaped frame was found to be most effective in extracting the gels without damage.

With the U frame submerged in a shallow pan of water, the loose end of the wire is carefully threaded through the tube between the gel and the tube wall. With the tube submerged and the wire taut, the tube is rotated against the wire. This frees the gel from the tube wall so that it slips out as the wire is withdrawn.

For all enzyme staining, Brewbaker's formulations and procedures were followed without modification (1968). For general protein, the staining method of Chramback et al (1967) was used. This method employs coomassie brilliant blue as a stain and has certain advantages over the napthol blue black methods. An important advantage is its sensitivity to concentrations of protein. The weak bands in a pattern may develop more clearly without the stronger bands overstaining. Also, destaining is unnecessary, thus eliminating the possibility of "washing out" the weaker bands. However, the method was found to have at least one serious disadvantage. Gels stored in the recommended solution of 10% trichloracetic acid tend to shrink, making delayed evaluations difficult and reevaluation inaccurate after long storage periods (10-12 weeks).

Initially, a recording/integrating densitometer was used for evaluating the stained gels. However, this instrument proved to be unsatisfactory in

defining weaker bands, and the gels were therefore plotted and evaluated visually. Qualitative characteristics of the patterns and the relative electrophoretic mobility of the various bands were of primary interest. Quantitative evaluations were limited to the width and color intensity of the various bands.

PHASE II

In addition to general protein, four enzyme systems were studied: acid phosphatase, leucine amino peptidase, peroxidase, and esterase. Eight trees from a single West Virginia source were used for all systems, but four additional trees were tested for general protein. Two of these trees were from a distinctly separated stand, located so as to eliminate the possibility of cross pollination with other trees in the study.

Two lots of six seeds were selected from each tree. The number of seeds per lot was determined by material requirements for extraction, protein content assay, and the various electrophoretic runs required to investigate all systems. Strict isolation was maintained between seed lots during extraction procedures.

A sample of each extract was loaded into single gel tubes. Twelve gels, representing twelve different seed lots (6 trees), were used in a single electrophoretic run. Two such runs were made for general protein and each enzyme system. This procedure gave two independent observations per seed lot, and a total of four observations per tree for each system.

The methods employed in this study yielded results with little or no variation between replications. However, strict control had to be maintained over all system components and conditions. Slight variations in gel or buffer characteristics had serious effects on the resulting patterns.

Yue (1969) found that the protein concentration was very important for satisfactory isoenzyme elucidation of glutamic dehydrogenase. Concentrations above the optimum range produced blurry patterns, while weaker bands could not be detected if the concentration was too low. Similar results were obtained during this study. Due to the limitations of the methods employed, the possibility that some variation in gel patterns may be a result of differences in protein concentration rather than true variations in protein composition should be recognized.

Fuzzy or blurred banding was characteristic of acid phosphatase gel patterns at all concentrations tested. However, apparent variations within and between trees were confined to quantitative characteristics. The same Pattern of one rapid and two slow moving bands was found in all gels (Figure 1). Leucine amino peptidase patterns displayed a similar lack of qualitative variation, both within and between trees. Two narrow, slow moving bands formed the normal pattern of this source (Figure 1).

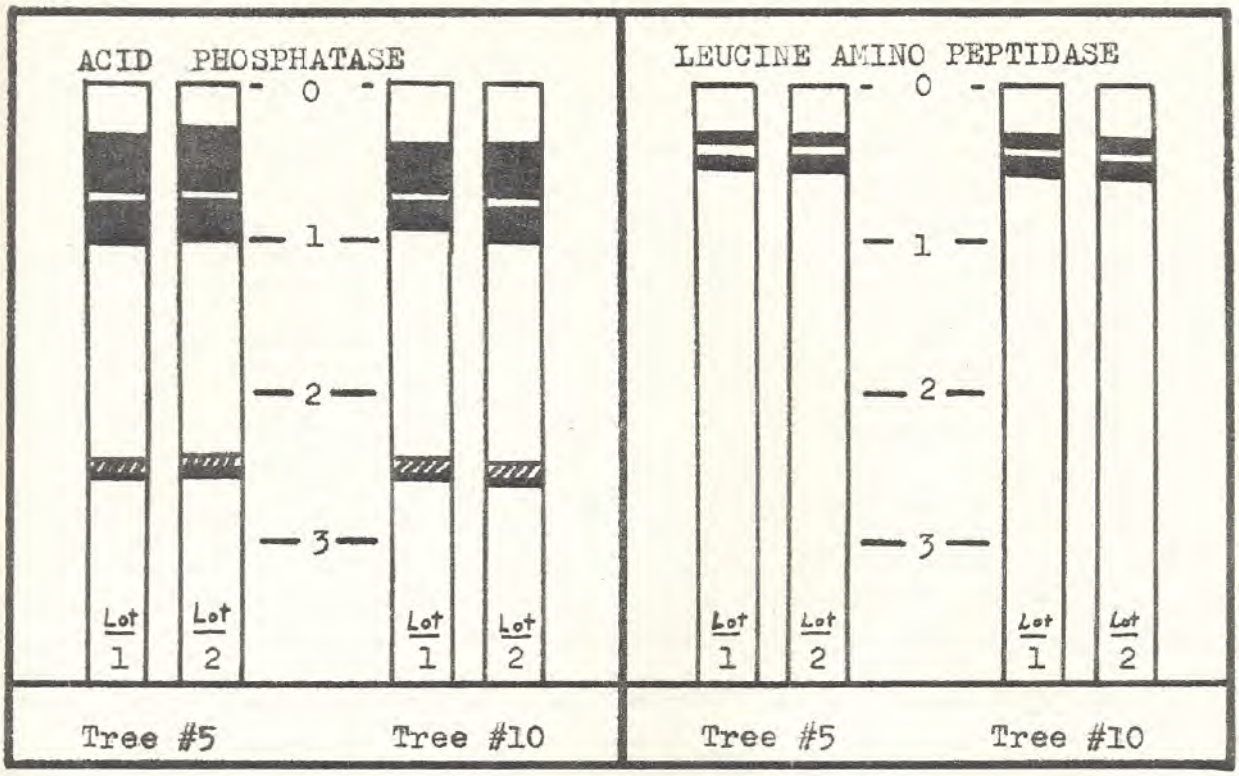

FIGURE #1

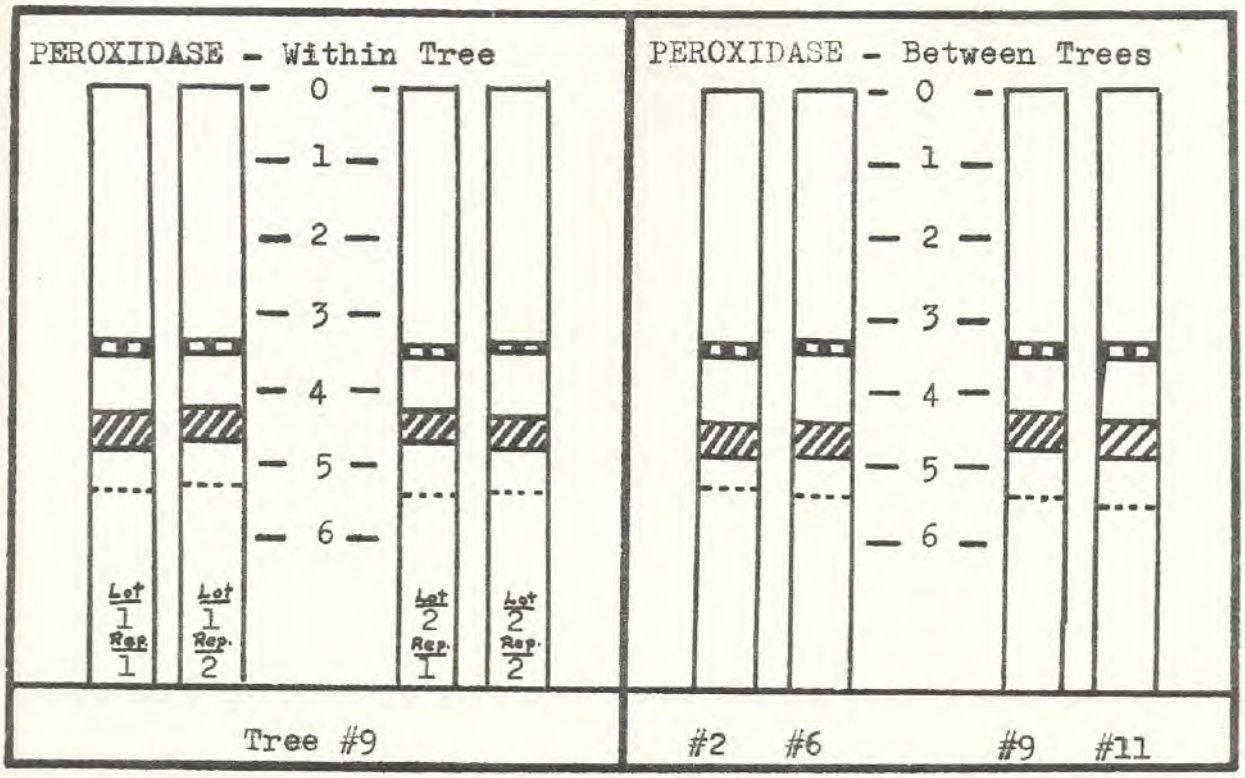

FIGURE #2

Within individual trees, peroxidase gels exhibited a high degree of pattern uniformity. Between trees, only slight variations in apparent quantitative characteristics were evident. The typical banding pattern consisted of two rapidly moving bands, one of which migrated immediately behind the buffer front dye marker and one band of medium mobility. No qualitative pattern differences were detected (Figure 2).

Isoenzyme patterns for esterase were much more variable. Although variations within a tree were normally limited to a single band, multiple banding differences between trees were evident. The basic pattern for this enzyme consisted of one medium width, slow moving band, followed by two narrow bands of slightly lower mobility. A number (0-4) of very slow, narrow bands provided most of the qualitative variability. Some apparent quantitative differences were noted, especially in the fastest moving band (Figure 3).

Due to their non specific and more complex nature, general protein gel patterns displayed more variability than any of the enzyme systems. While differences within trees were confined to one or two bands, differences of three or more bands were normally detected between trees. However, certain bands were found to be present in almost all of the gels, so than an apparent basic pattern for the source was delineated (Figure 4).

Since different gels and buffers were used to elucidate the different enzyme systems, a direct comparison with the general protein pattern was possible with peroxidase only. The fastest moving peroxidase band has the same r.f. as the fastest moving protein band (band 14 in Figure 4). The slowest moving peroxidase band appears to have the same mobility as general protein band 13, and band 13 was not detected on all gels. No general protein band was detected in the area of the middle peroxidase band.

CONCLUSIONS

It appears that there is a high degree of uniformity in acid phosphatase, leucine amino peptidase and peroxidase systems in trees of the West Virginia seed source.

Although esterase and general protein gels displayed numerous qualitative pattern variations, there appears to be a basic set of bands for each of these systems. These basic patterns were found to be stable in most trees tested.

On the basis of pattern resolution, peroxidase, esterase, and general protein were found to be the most promising systems for further study.

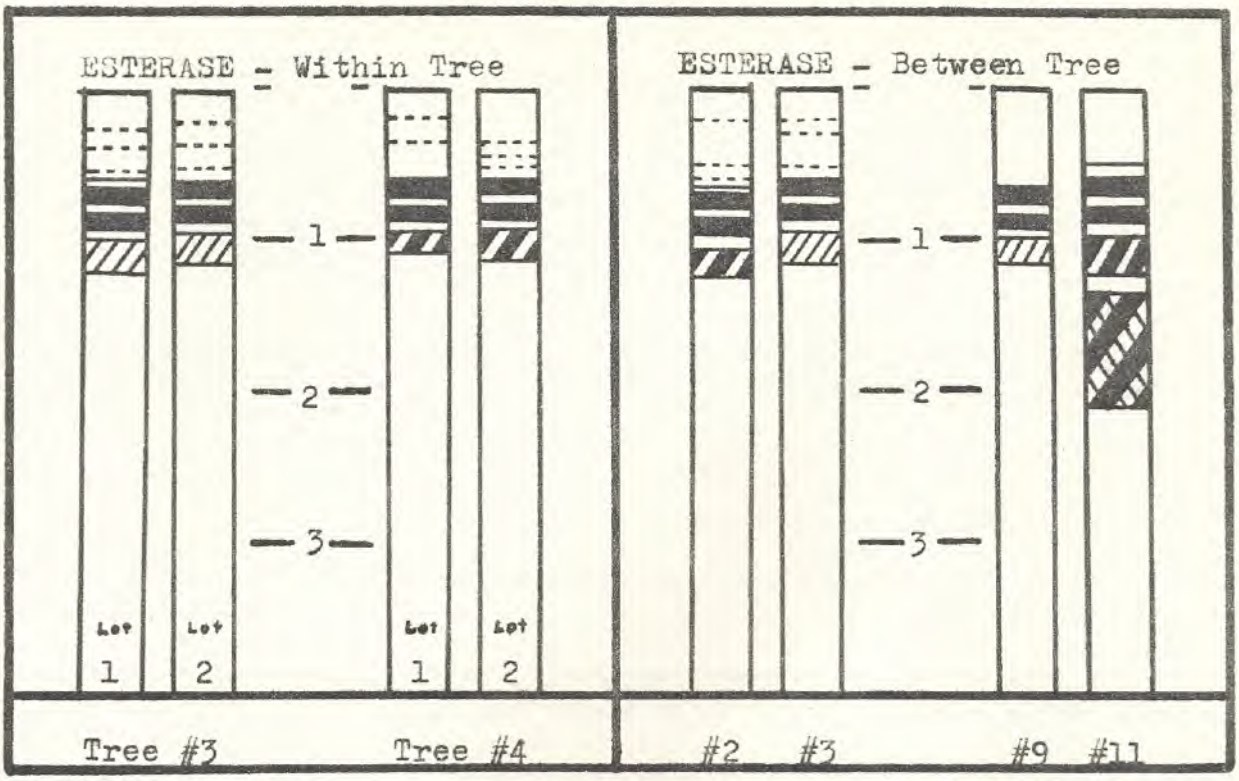

FIGURE #3

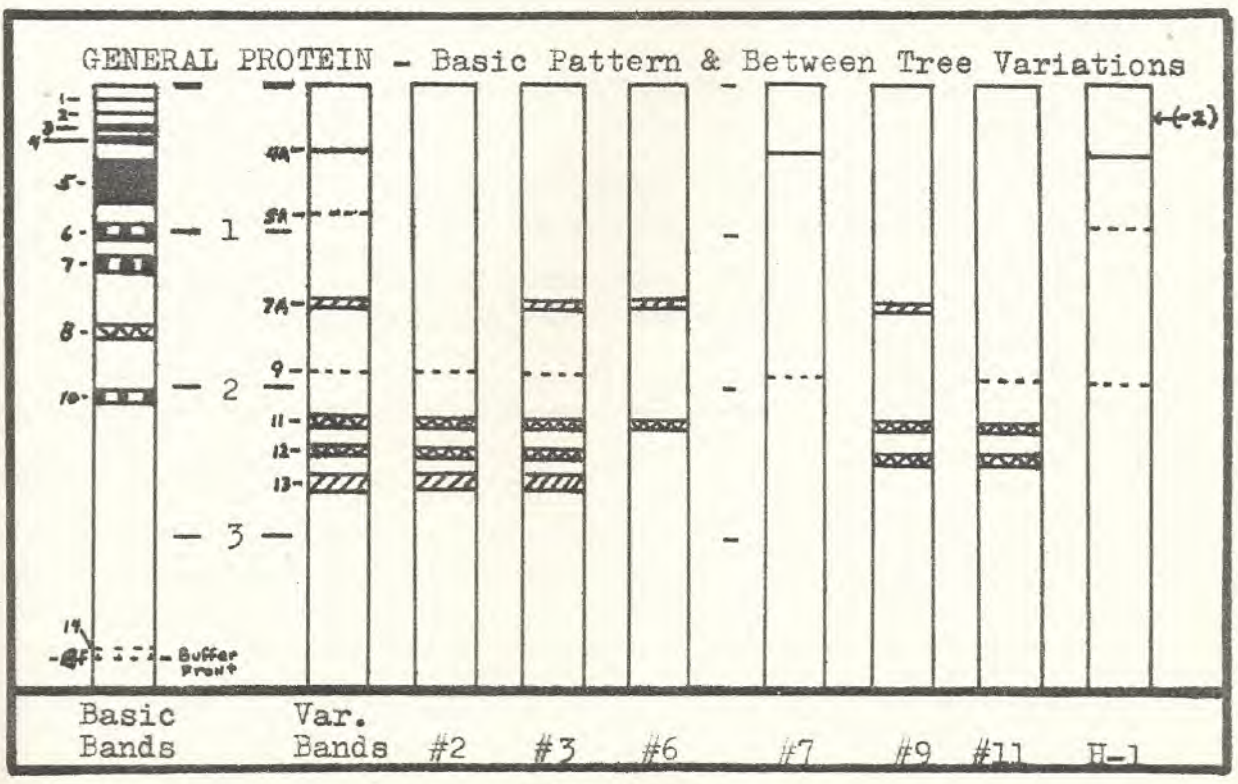

FIGURE #4

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