Do NOT REMOVE

PACIFIC SOUTHWEST
Forest and Range
Experiment Station

FOREST SERVICE
U.S.DEPARTMENT OF AGRICULTURE
P.O. BOX 245, BERKELEY, CALIFORNIA 94701

ANALYZING GENETIC DIVERSITY IN CONIFERS...
isozyme resolution by starch gel electrophoresis

M. Thompson Conkle

USDA Forest Service
Research Note PSW-264
1972

RECEIVED
SEP 27 1972
PSW EXPT. STATION
INSTITUTE OF FOREST GENETICS

Abstract: Enzymes in forest tree materials can be resolved by starch gel electrophoresis. A gel slab is prepared in a mold assembled from glass and plastic. Wicks containing an aqueous extract of macerated plant material are inserted in the gel and processed. The gel is sliced, stained, examined, and photographed. Isozyme bands produced by differential migration of enzymes indicate genetic segregation and recombination. This technique is familiar in other uses, but the procedure described here is specifically adapted to conifers.

Oxford: 174.7:160.22–015.28:U537.363.
Retrieval Terms: coniferae; natural variation; genetic diversity; chemical composition; enzyme analysis; electrophoresis; techniques and procedures.

The resolution of enzymes from the aqueous extract of macerated plant material is a precise tool with wide application in the genetic analysis of forest trees. In this procedure, proteins separated by electrophoresis are stained to reveal specific classes of enzymes. Genetic diversity is identified when the stained isozyme bands show migration at different rates. Interpretation of the isozyme patterns reveals segregation and recombination in families according to simple expectations of Mendelian inheritance.

Genetic theory holds that nuclear genes contain molecular codes responsible for the precise sequence of amino acids in specific proteins. Slight differences between nuclear codes bring about altered sequences within the protein, and in some instances, a resulting change in the net electrostatic charge on the protein. Thus, in a diploid organism, the products from different loci and allelic products from the same loci can carry charge differences. During electrophoresis a direct electric current is applied to gel medium containing plant extracts. The current, in conjunction with the protein's net charge, produces differential electrophoretic mobility.

Electrophoresis is an old and well-known process for protein separation. Of two standard techniques—disc and gel electrophoresis—the gel technique is most practical for genetic studies of forest trees. The essentials of the technique were described by Smithies in 1955.[1] It consists of casting the support medium in the form of a slab. Paper wicks containing sample extracts are inserted into the slab and a current applied. Gels can be formulated from several materials, but starch and acrylamide are most commonly used.

Proteins in pine pollen were investigated through electrophoresis as early as 1964.[2] Later, soluble proteins from female gametophytes and embryos of two pine species and a spruce were resolved.[3] In 1966, population geneticists became aware of the

potential of enzyme separation techniques,[4] and demonstrated their applicability to analysis of natural populations.[5] Since then, enzyme inheritance has been described in numerous organisms. Researchers in Professor R. W. Allard's laboratory at the University of California, Davis, have developed and adapted procedures which give unequivocal separations of isozyme bands and allow for the genetic assay of large numbers of individuals.[6]

This note describes some procedures proven to be applicable to pines[7] so that other geneticists working with conifers can bypass trivial problems of technique.

STARCH GEL TECHNIQUE

Good references for starch gel procedure include those by Smithies,[1] Shaw and Koen[8] and Brewbaker *et al.*[9] Scandalios[10] described a formulation for both gel preparations and enzyme stains. These formulations are appropriate for conifer enzyme separations. Kristjansson[11] relates in essential detail the procedures followed at the University of California, Davis, which are appropriate for pine.

Gel Preparation

Fresh gels should be prepared on the day of an electrophoresis run. For 12 percent gels, add 84 g. of hydrolyzed starch to 700 ml. of gel buffer solution (630 ml. tris-citric buffer, pH 8.3, 0.2 M, and 70 ml. lithium-borate buffer, pH 8.3, 0.2 M).[10] Heat 500 ml. of the buffer to a boil and add it rapidly to the starch, which has been suspended in the remaining 200 ml. of buffer. During addition and for a short period thereafter, swirl the flask vigorously 60 times or for 20 seconds. Immediately degas the viscous mass by applying a vacuum, slowly at first, as the gel tends to fill completely with air bubbles. Allow degassing to continue for about 1 minute. Disconnect the vacuum and pour medium directly into the prepared gel molds. The quantity of medium fills two molds.

Molds should be assembled before the gel is prepared. They consist of a plate glass bottom, plastic spacer held in place with paper clips, and a plate glass top *(fig. 1)*. Both glass plates are 16.5 cm. by 25 cm. Attach clips to the bottom plate, two clips per edge. The spacers are 9 mm. thick and 1.2 cm. wide; the long ones are 21.5 cm., and those on each end are 16.5 cm., the same dimension as the glass plate. Enough medium is poured in each mold to reach the inner margins of the spacers. Bring the top plate down on the gel, starting from one end and progressing slowly in much the same manner that a cover slip is placed on a microscope slide. For the best consistency, cool the gels at room temperature for 2 to 3 hours. They can be cooled more rapidly by refrigeration.

Preparation of Plant Material

The presence or absence of specific enzyme bands in the processed gel depends on both the physiological development of the plant and the plant part that is sampled.[10,12] Procedures will be described here for studying enzymes from conifers at germination, but the technique can be extended to various other materials.

Seeds of hard pines are stratified 30 days on moist filter paper at 3°C. and germinated at room temperature. An optimum period for alcohol dehydrogenase and leucine aminopeptidase analysis is the developmental stage in which the radicle of the embryo extends about 2 to 5 mm. beyond the seed coat. The embryo can be separated from the megagametophyte and both materials assayed. Place each material to be examined in a separate microsize disposable weigh tray *(fig. 2)*. For materials of small mass, add one drop of gel buffer solution. Then thoroughly macerate the plant material with a plastic rod. Pick up the aquaeous phase from the macerated fraction on paper wicks (0.4 by 1 cm.) cut from chromatography paper. Wicks should be thoroughly moistened but excess liquid should be blotted away. Place the prepared wicks in petri dishes and refrigerate them until all wicks are prepared.

Electrophoresis

Remove the top glass and clips from the prepared gel. Cut the gels once across their width at a distance of 5 cm. from one end. Separate the gel at the cut and insert the prepared wicks *(fig. 3)*. Wicks can be inserted with as little as 1 mm. between them; 20 such wicks can be accommodated across a single gel. The wick size and spacing can be varied according to the needs of a particular experiment. Once wicks are inserted, place the end slice against the wicks, and cover the gels with wicks with plastic wrap.

To rig the gels with electrode connections *(fig. 4)*, place trays containing solutions (lithium-borate buffer, pH 8.3, 0.2 M) at either end of the gel. Place thin cellulose sponges saturated with electrode buffer in the buffer tray and lap them over each end of the gel. The plastic wrap should be turned back at each end and lapped under the sponge a short distance to give an even contact of sponge with gel surface. The wrap is lapped back over the top of the sponge and held in place with clips along the outer edge. Use platinum

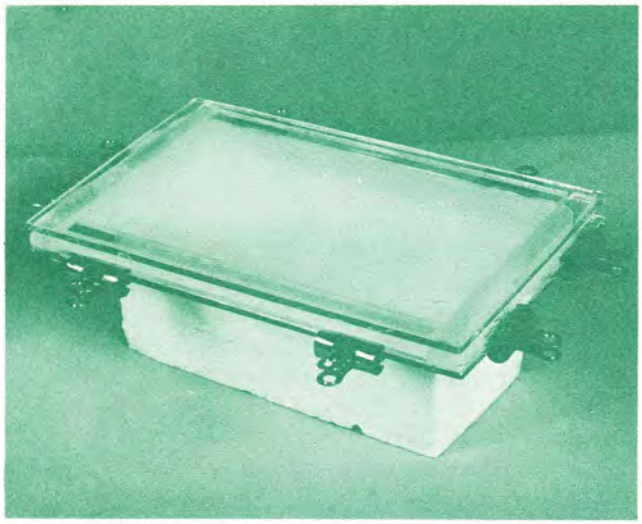

Figure 1—Mold with starch gel in place. Top and bottom pieces are of plate glass, separated by plastic spacers on all four sides. Clips hold the spacers in place. After the gel is poured, the top plate is laid over it.

Figure 3—Placement of saturated wicks on cut gel surface. Gel, with wicks, is covered with plastic wrap.

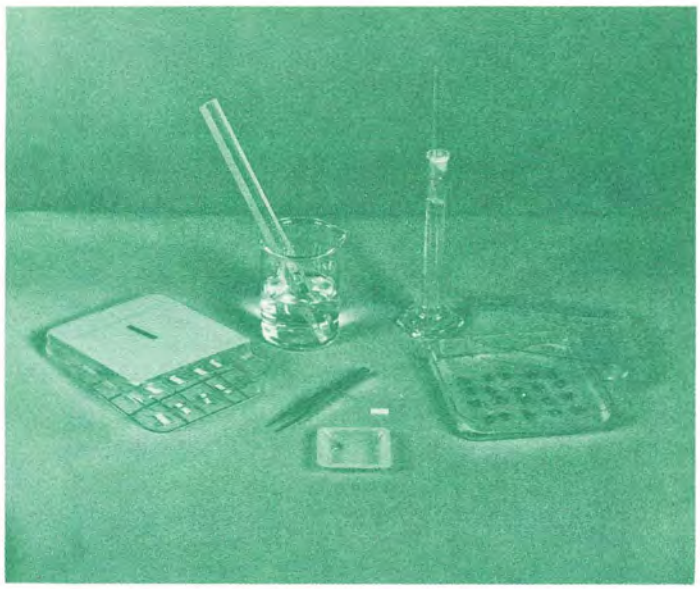

Figure 2—Materials for wick preparation. Paper wicks are saturated with the liquid fraction from plant material macerated with a plastic rod. Each material is prepared in a separate disposable weigh tray.

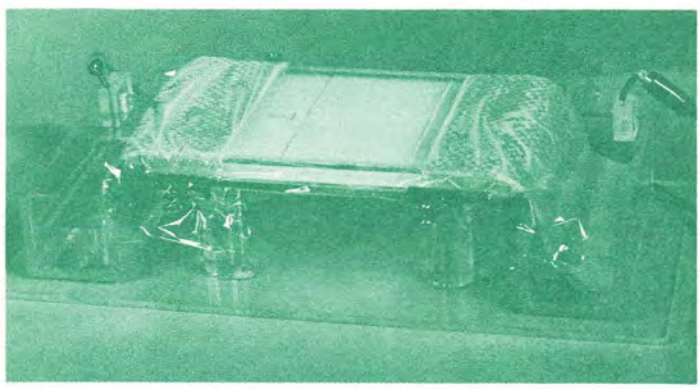

Figure 4—Gel electrode connections and solutions in place. Thin cellulose sponges lap over ends of gel and extend into trays of buffer solution. Platinum electrodes lead from the power source to the solution trays. The plastic wrap covers the gel and the sponges, and is clipped to the outer edge of the bottom plate.

electrodes to connect power sources and electrode tray solution.

Two precautions are taken to prevent heat build-up, which can result in uneven movement of fronts in the gel or denaturation of proteins. First, cover gels with a thin cellulose sponge and the sponge with crushed ice. Second, place the entire gel system in an electrically grounded refrigerator set to its coldest setting.

Various power sources have been used in this system, and all have given satisfactory results. I have used four power supplies, each delivering 400 v. d.c. with 100 ma. to process four gels. The negative pole from the power supply is connected to the electrode adjacent to the wick end of the gel slab. The positive pole is attached to the alternate electrode. Power supplies are located on top of the refrigerator and connecting wires to the electrodes bypass the door gasket. A small fan in the refrigerator, located near

the freezing compartment, distributes cold air during a gel run.

For the gel run supply power initially for 12 minutes. Then open the gels and remove the wicks. Replace the gels and again supply power. The procedure of removing wicks is designed to give sufficient time for the proteins to move into the gel with the ultimate purpose of attaining close contact between gels for the duration of the run. Allow the visible migrating front in the gel to progress to a distance of 8 cm. beyond the origin (point of wick insertion). This front, recognized as a slight depression and somewhat clearer area in the gel, reaches the 8 cm. mark after about 2 hours. During the run, examine gels periodically to determine that good contact is maintained between gels at the cut. Correct any separations.

Figure 5—Gel being sliced before staining. A piece of fishing line is used as a cutter to produce five layers.

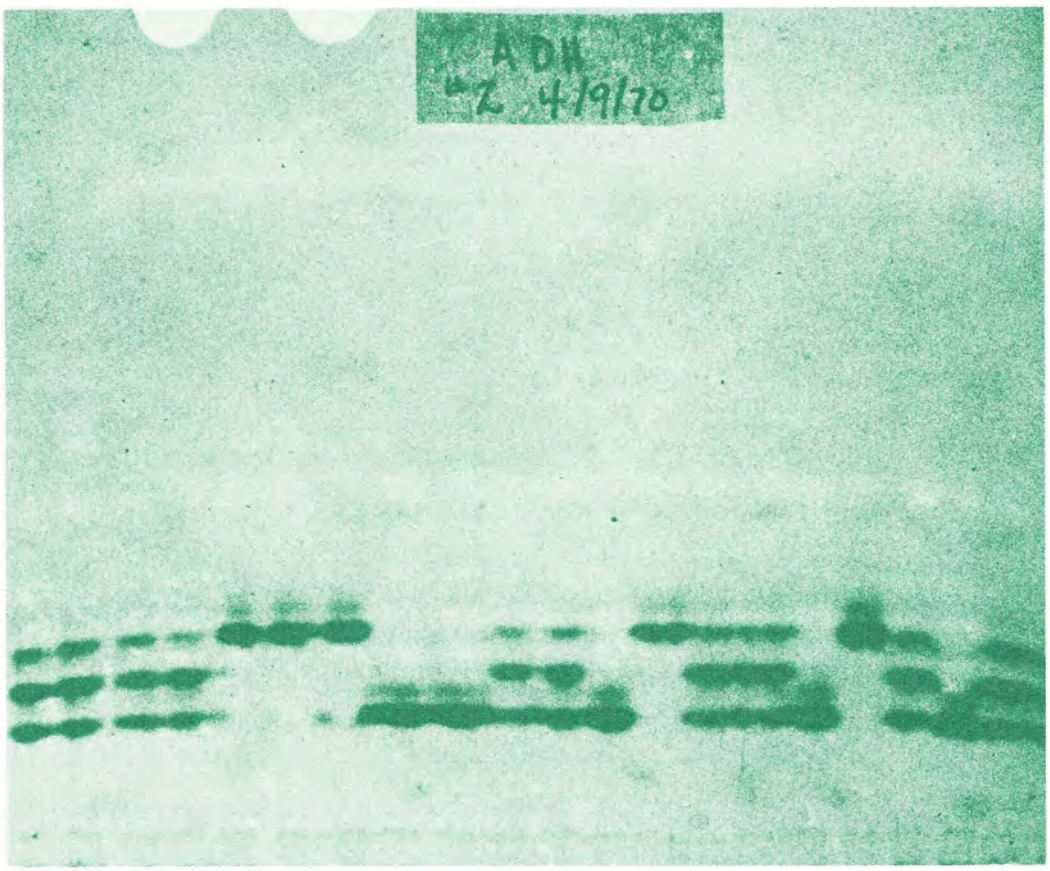

Figure 6—Gel stained for alcohol dehydrogenase from twenty embryos of a selfed knobcone pine. From *left* to *right,* the first four embryos are triple banded; the next three embryos have fast migrating bands; then comes two embryos with slow phenotypes, and two with triple bands. Phenotypic segregation is five embryos with fast bands; 10 with triple bands; five with slow bands. The genotypic inference is that the phenotypes are determined by five homozygotes for fast, 10 heterozygotes, and five homozygotes for slow.

When the front reaches 8 cm., turn off the power and remove from the refrigerator. Dismantle each gel system to its bottom glass plate with accompanying gel slab. The gel is thick and can be sliced into several layers for staining *(fig. 5)* by using the cheese cutter principle. Place four spacers each slightly less than 2 mm. thick, on either side of the gel. With spacers held in place by clips, pull a 4 lb.-test monofilament fishing line across the top of the spacers. Remove a spacer from each side and repeat the procedure until the slab is sliced four times (five slices). Each slice is then stained for a specific class of enzyme.

Staining

The stain solutions are prepared in open trays while gels are being electrophoresed. A single slice from each gel is added to a solution. Staining processes are designed to yield directly visible results. Some stains allow the gels to be preserved for a relatively long period of time, but other stains are unstable, and the gels must be examined immediately. A back-lighted table is helpful in examining the stained gels.

RECORDING DATA

By using the procedures outlined, one person can process four gels (80 samples) in an 8-hour-day. For ease of identification of all materials, each gel is handled as a unit. Gels are numbered 1 through 4. Data sheets with the date, gel number, and spaces numbered 1 through 20 are used to identify each individual sample. Samples are identified by gel and numbered position across the gel. When stained, gels are nicked to code for their number. The type of stain and the data of the run are recorded and attached to each gel *(fig. 6)*. Gels are photographed by using film for color transparencies. Both the gels and photographs serve as records from which relative migratory rates of the various isozymes can be determined.

NOTES

[1]Smithies, O. *Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults.* Biochem. J. 61: 629-641. 1955.

[2]Bingham, W. E., S. L. Krugman, and E. F. Estermann. *Acrylamide electrophoresis of pine pollen proteins.* Nature 202: 923-924. 1964.

[3]Durzan, D. J. *Disc electrophoresis of soluble protein in the female gametophyte and embryo of conifer seeds.* Can. J. Bot. 44: 359-360. 1966.

[4]Hubby, J. L., and R. C. Lewontin. *A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at different loci in Drosophila pseudoobscura.* Genetics 54: 577-594. 1966.

[5]Lewontin, R. C., and J. L. Hubby. *A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of Drosophila pseudoobscura.* Genetics 54: 595-609. 1966.

[6]I am indebted to A. L. Kahler of Dr. R. W. Allard's laboratory for his help in adapting starch gel procedures to pine material.

[7]Conkle, M. Thompson. *Inheritance of alcohol dehydrogenase and leucine aminopeptidase isozymes in knobcone pine.* For. Sci. 17: 190-194. 1971.

[8]Shaw, Charles R., and Ann L. Koen. *Starch gel zone electrophoresis of enzymes. In, Chromatographic and electrophoretic techniques.* I. Smith, Ed., vol. 2, 2nd ed. Zone electrophoresis. p. 325-364. 1968. New York: Interscience Publishers.

[9]Brewbaker, James L., Mahesh D. Upadhya, Yrjö Mukinen, and Timothy MacDonald. *Isoenzyme polymorphism in flowering plants. III. Gel electrophoretic methods and applications.* Physiol. Plant. 21: 930-940. 1968.

[10]Scandalios, John G. *Genetic control of multiple molecular forms of enzymes in plants: A review.* Biochem. Genet. 3: 37-79. 1969.

[11]Kristjansson, F. K. *Genetic control of two pre-albumins in pigs.* Genetics 48: 1059-1063. 1963.

[12]Conkle, M. Thompson. *Isozyme specificity during germination and early growth of knobcone pine.* For. Sci. 17(4): 494-498, illus. 1971.

The Author

M. THOMPSON CONKLE is a geneticist doing research on genetics of western conifers, with headquarters in Berkeley, Calif. A forestry graduate of Michigan State University, he also holds an M.S. degree in genetics from North Carolina State University (1962). He joined the Station's research staff in 1965.

The Forest Service of the U.S. Department of Agriculture

. . . Conducts forest and range research at more than 75 locations from Puerto Rico to Alaska and Hawaii.

. . . Participates with all State forestry agencies in cooperative programs to protect and improve the Nation's 395 million acres of State, local, and private forest lands.

. . . Manages and protects the 187-million-acre National Forest System for sustained yield of its many products and services.

The Pacific Southwest Forest and Range Experiment Station

represents the research branch of the Forest Service in California and Hawaii.

☆ U.S. Government Printing Office 794-416/3703