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Electrophoresis: Uses in Quality Assurance

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ELECTROPHORESIS: USES IN QUALITY ASSURANCE

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Introduction

The objective and accurate assessment of genetic purity is a key component in successful commercial seed production and constitutes one of the perennial concerns of any quality control program. Large enough proportions of off-type individuals can result in diminished yield and overall agronomic quality; lack of genetic uniformity may affect appearance in the field and seriously undermine the marketability of a product.

The traditional method of evaluating genetic purity - the grow-out tests - relies on phenotypic identification of uncharacteristic types by field comparisons made throughout plant growth and development. More recently, however, it has been possible to assess the purity of a seed sample through laboratory genotyping or "fingerprinting". While laboratory screening for genetic uniformity may never completely replace the need for conventional grow-out tests, in many cases it offers an alternative which is preferable in terms of costs, speed of analysis and accuracy. The purpose of this paper is to briefly describe one of the most commonly used laboratory genotyping techniques - isozyme electrophoresis - and top review applications for quality assurance in commercial maize production.

Background

As Goodman and Stuber (1980) -- pioneers in maize isozyme studies -- have pointed out, most of the useful laboratory procedures in genotyping involve the separation of biological macromolecules, often proteins or DNA, by electorphoretic or chromatographic methods. Once separated, individual macromolecules or patterns of macromolecules can be identified or visualized and then compared among individual plants or between pedigrees.

The term "electrophoresis" refers to the migration of particles in an electric field. When a heterogenous mixture of biological

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molecules, such as proteins, are placed onto a solid matrix (e.g., starch, acrylamide or agarose gel) and a current is applied across the matrix or "gel", the molecules are seived through the gel at different rates dependent on their individual molecular charge, size and/or shape. When the current is turned off, the differential migration distances can be seen as discrete bands - visualized by appropriate stains.

The term "isozyme" was first coined by Markert and Moller (1959) to describe different molecular forms of enzymes with the same substrate specificity. Early questions as to whether the multiple forms of enzymes were due to artifacts produced during sample preparation were finally laid to rest with the development of starch gel electrophoresis (Smithies, 1955) and specific histochemical stains - which demonstrated that enzymes could be visualized directly on starch gels (Hunter and Markert, 1957). These "activity stains" allowed specific staining of only those enzyme which could convert or catalyze a supplied substrate. Markert and Moller (1959) further showed that electrophoretic patterns of the enzyme lactate dehydrogenase (LDH) taken from hearts of sheeps, cows, pigs, mice and rabbits were distinct for each species. Deriving from these two fundamental contributions, i.e. (1) the resolution of isozymes in starch gels via specific stains - providing conclusive evidence of enzyme polymorphism, and (2) the demonstration of isozyme variation between species, there has accumulated a vast amount of literature on isozymes. Most importantly, isozyme studies in plants over the past 15 years have revealed the degree of variability within (allelic isozymes or allozymes) as well as between plant species (Brown and Weir, 1983). (Table 1) gives only a partial list of economically important plants on which isozyme studies have been conducted.

The potential usefulness of isozyme electrophoresis in commercial research and development, foundation and quality assurance programs derive in large part from the following isozyme properties:

(1) In distinguishing between individuals it is preferable to base an analysis as close as possible to the actual genetic code - the DNA "blueprint". There are three distinct advantages to the use of isozymes here: Firstly, as a biochemical marker, isozyme expression is not generally influenced by the environment, unlike the majority of morphological traits upon which grow-out data is based (Brown and Wier, 1983). Secondly, isozyme loci themselves, as a rule, do not influence phenotype; they are neutral with respect to plant performance and appearance. Thirdly, isozymes, as single gene products, rarely exhibit epistatic (multi-gene influence of expression) interactions. Thus, inheritance follows strict mendelian predictions. Tanksley and Rick (1980) point out that theoretically one could have an infinite number of

Table 1. Isozymes in selected crop species.

Maize (*Zea mays*)	Cardy et. al., 1981
Sorghum (*Sorghum bicolor*)	Morden et. al., 1987
Soybean (*Glycine max*)	Cardy and Beversdorf, 1984
Barley (*Hordeum* sp.)	Brown, 1983
Wheat (*Triticum* sp.)	Brody and Mendlinger, 1980
Oats (*Avena* sp.)	Price and Kahler, 1983
Tomato (*Lycopersicon* sp.)	Tanksley, 1979
Rice (*Oryza* sp.)	Second, 1982
Proso Millet (*Panicum miliaceum* L.)	Warwick, 1987
Cotton (*Gossypium* sp.)	Wendel, 1987
Pea (*Pisum sativum*)	Weeden and Marx, 1987
Bean (*Phaseolus* sp.)	Weeden, 1984
Lettuce (*Lactuca sativa* L.)	Kesseli and Michelmore, 1986
Strawberry (*Fraaria* sp.)	Bringhurst et. al., 1981
Peanut (*Arachis hypogaea* L.)	Cherry and Ory, 1973
Conifers	Conkle et. al., 1982
Alfalfa (*Medicago* sp.)	Quiros, 1981
Citrus (*Citrus* sp.)	Torres et. al., 1978

isozymes segregating simultaneously and still be able to determine unambiguously the genotype at every locus for every individual. The number of scorable morphological markers, on the other hand, is limited by inevitable epistatic effects - the reliability of classification does not necessarily increase with the number of morphological traits scored.

(2) Allelic isozyme expression is generally co-dominant. This allows heterozygotes to be distinguished from homozygotes - an advantage shared by few morphological markers. Moreover, this means that there will be no deleterious effects on plant phenotype through recessiveness (Moore and Collins, 1983).

(3) Many isozymes are expressed constitutively throughout the plant. For instance, of the 13 isozymes studied by Goodman and Stuber (1980) in corn coleoptiles, all but two were expressed in immature embryos (Smith, 1984b). The accessibility of these isozyme markers throughout the life of the plant, especially in the developing embryo and early seedling, is of major importance in a quality control application. Instead of a several-month grow out, genetic uniformity can be determined as early as 4-6 weeks following pollination. Another advantage to early determination of genetic purity has been noted by Arus (1983): plant viability may be differentially affected in seeds resulting from outcrosses or sib pollinations. Unless special care is taken in grown outs, the estimation of purity may be biased. Since isozyme analysis takes place at the seed or seedling level, such a problem could be avoided.

(4) In starch gels, isozymes differing in charge, molecular size and/or configuration are sieved through the gel matrix under the influence of an electric current. Visualization of an enzyme in the gel is dependent on its catalytic activity. For this reason, special care must be taken to avoid protein degradation or denaturation. Isozyme differences are detected and scored as motility differences in the gels. Such differences are independent of the function or overall variation of the enzyme in question. The main considerations here are: (i) care must be taken to avoid loss of enzyme activity and artificial staining and (ii) migration of isozymes to the same position on the gel does not preclude variation between them; electorphoretic techniques tend to underestimate genetic variation (Brown and Weir, 1983).

Isozymes In Maize

Of the major crops or, indeed, perhaps of any other plant species, maize has enjoyed the broadest range of studies involving isozymes (Goodman and Stuber, 1983b). There is extensive isozyme variation within corn; the presence of this variation has allowed an objective and precise characterization ("fingerprint") of inbred lines (Goodman and Stuber, 1980; Cardy and Kannenberg, 1982; Stuber and Goodman, 1983; Kahler 1984; Smith et al., 1985a; Smith et al., 1985b), of hybrids (Cardy and Kannenberg, 1982; Smith, 1984a; Smith, 1988), and of exotic and wild germplasm (Goodman and Stuber, 1983a; Smith et al., 1984; Doebley et al., 1984; Doebley et al., 1985).

Stuber and Goodman (1983) have adapted and refined starch gel electrophoresis to define genotypes at 23 genetic loci spread over seven chromosomes (13 isozymes) in 406 inbred lines of corn. A total of 80 alleles were identified in the 406 lines; in a smaller subset of 39 widely-used inbred lines, 36 distinct isozyme genotypes were found (i.e., 92% of the fingerprints were distinct). (Figure 1) illustrates the variation among public inbred lines in several isozyme systems. Similar results were obtained in surveys of corn hybrids: 146 of 155 commercial hybrids in Canada had unique fingerprints (Cardy and Kannennberg, 1982), while 100 of 111 U.S. commercial hybrids were distinct with a mean value of 15 to 23% heterozygous loci per hybrid (Smith, 1984). A 1987 survey of U.S. hybrid maize revealed 56% of proprietary hybrids and 65% of foundation-seed company hybrids had unique isozyme fingerprints (Smith, 1988).

Recent work has expanded the number of scorable isozyme loci in maize to forty (Wendel et al., in press). The chromosomal location of many of these isozymes is known. These are genes coding for isozymes on nearly every chromosome - the isozyme fingerprint reflects a fairly representative sampling of the maize genetic blueprint. (Table 2) lists the isozyme systems currently available for fingerprinting.

The most widely used isozyme techniques are those developed by Cardy et al. (1981, revised 1983) and updated by Stuber et al. (1988). To completely fingerprint a line or hybrid, six or seven gel systems (i.e., starch gels) are run; each gel differing slightly in pH, ionic strength and buffering agent. From each starch gel, five to six thin slices are taken and each slice is stained for a specific enzyme. The composition of each gel system represents a compromise between the conditions required for resolution and activity of each component enzyme. Besides the significant amount of work involved in developing such techniques, these workers have initiated a scoring system for each isozyme loci; moreover, careful classification of each locus has included numerous allelism tests, linkage and gene localization studies.

A.

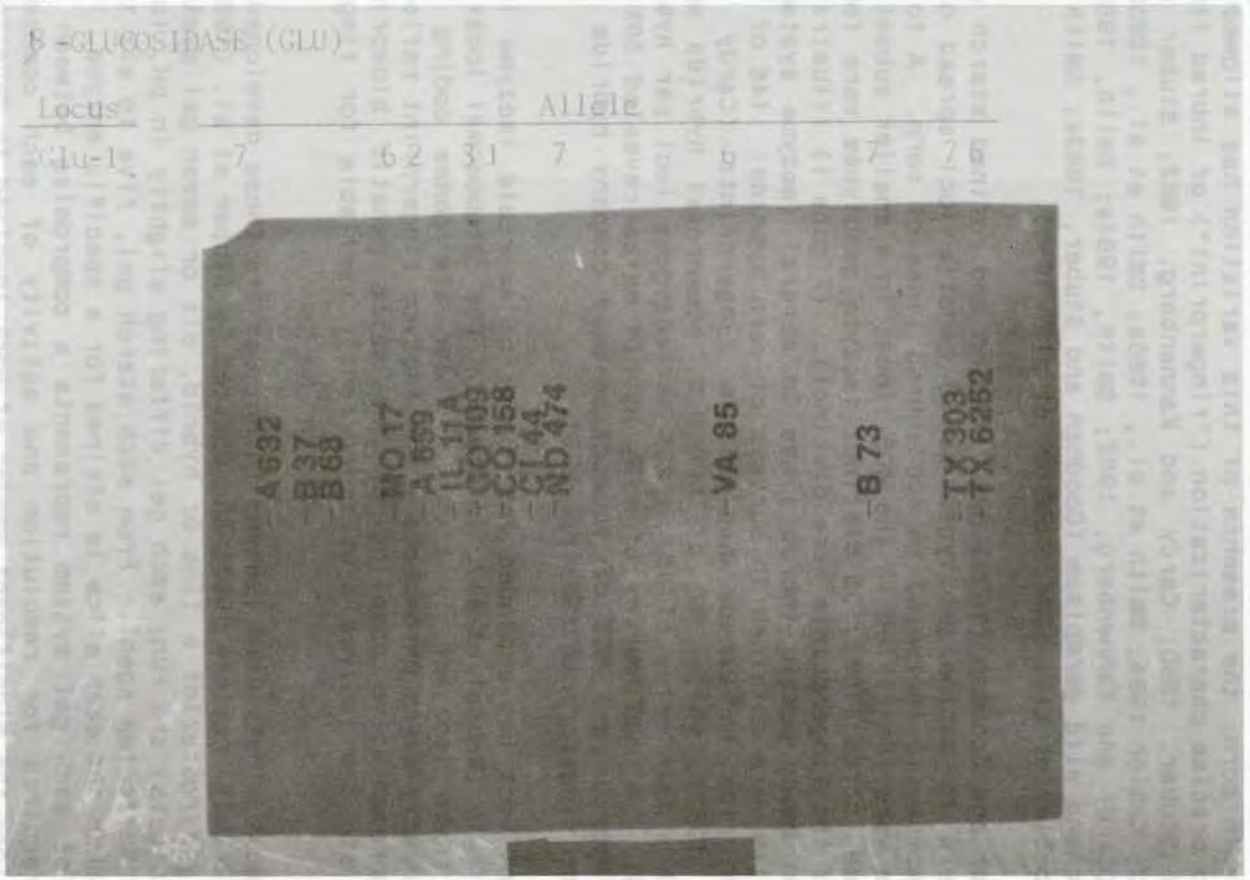

Figure 1. Isozyme variation among maize inbreds. Coleoptiles from a single plant of each inbred were ground; extracts were absorbed onto a thin filter paper wick, loaded (along bottom of gel) and electrophoresed for several hours. Designations are given above each sample lane. "Scores" (A) Banding patterns for B-Glucosidase. (B) Banding patterns for G-Phosogluconate Dehydrogenase.

B.

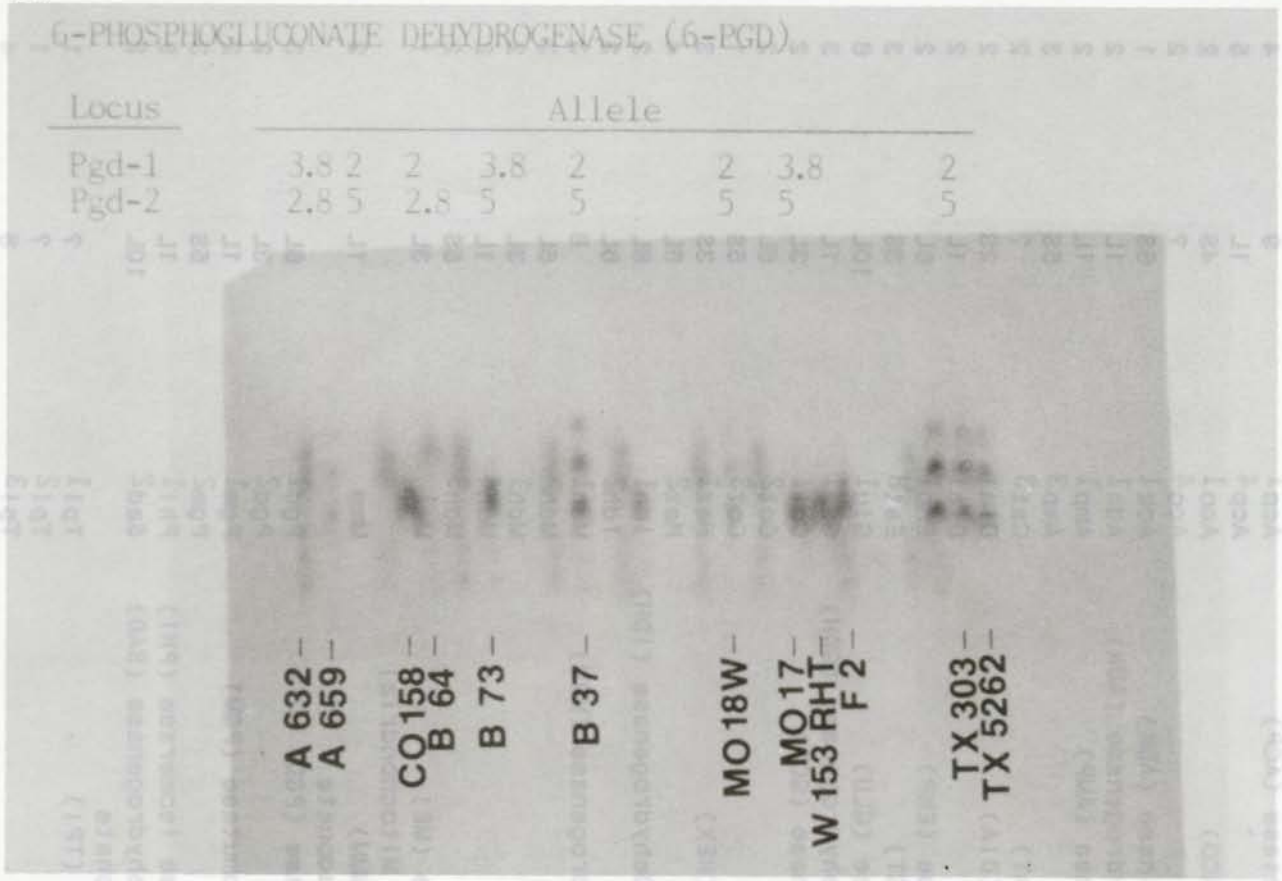

Figure 1. Continued

Table 2. Isozyme systems currently available in maize.

Enzyme Systems	Loci Scored	Chromosome Location	# of Common Alleles
Acid Phosphatase (ACP)	Acp1	9	4
	Acp4	1L	5
Aconitase (ACO)	Aco1	4S	2
	Aco4	?	2
Adenylate Kinase (ADK)	Adk1	6S	1
Alcohol Dehydrogenase (ADH)	Adh1	1L	2
Aminopeptidase (AMP)	Amp1	1L	2
	Amp3	5S	3
Catalase (CAT)	Cat3	?	2
Diaphorase (DIA)	Dia1	2S	2
	Dia2	1L	2
Endopeptidase (ENP)	Enp1	6L	2
Esterase (EST)	Est8	3S	3
B-Glucosidase (GLU)	Glu1	10L	6
Glutamate Dehydrogenase (GDH)	Gdh1	1L	3
Transaminase (GOT)	Got1	3L	2
	Got2	5L	2
	Got3	5S	1
Hexokinase (HEX)	Hex1	3S	3
	Hex2	6L	4
Isocitrate Dehydrogenase (IDH)	Idh1	8L	2
	Idh2	6L	2
Malate Dehydrogenase (MDH)	Mdh1	8	3
	Mdh2	6L	3
	Mdh3	3L	2
	Mdh4	1L	2
	Mdh5	5S	2
Malic Enzyme (ME)	Me1	3L	1
Modifier of Mitochondrial MDH's (MMM)	Mmm	1L	2
6-Phosphogluconate Dehydrogenase (PGD)	Pgd1	6L	3
	Pgd2	3L	2
Phosphoglucomutase (PGM)	Pgm1	1L	2
	Pgm2	5S	3
Phosphohexose Isomerase (PHI)	Phi1	1L	2
Shikimate Dehydrogenase (SAD)	Sad2	10L	2
Triose Phosphate Isomerase (TPI)	Tpi1	?	1
	Tpi2	?	1
	Tpi3	8	4
	Tpi4	3L	2
	Tpi5	?	2

Some of the considerations to be taken into account when scoring or interpreting maize isozyme banding patterns are illustrated in (Figure 2). The allelic difference between the two inbreds is clearly distinguished by position of the bands following electrophoretic migration. In the simplest case, the enzyme itself is comprised of a single unit (monomer) or polypeptide chain. A genetic cross of the two inbreds would result in the hybrid with a dose of each allele or a heterozygote. Both inbred bands or enzyme types would be present in the hybrid. A more complex banding pattern may occur if the enzyme has multiple components - two or more polypeptide units comprising the active enzyme. The case of a dimeric enzyme is illustrated. The hybrid would have both parental bands present plus a new enzyme type comprised of one sub-unit of each parental type. Usually this "hybrid" protein migrates to a point equidistant between the two parent forms. Predictions of banding patterns can be made knowing the number of sub-units in an active enzyme. "Hybrid" bands may also occur between different loci of the same enzyme.

The significant points that emerge from a review of the isozyme research in corn relevant to quality control applications are:

(1) There is extensive isozyme variation in elite U.S. germplasm; a majority of lines and hybrids have unique fingerprints.

(2) Key techniques and procedures for corn isozymes have been worked out in detail and can be readily implemented.

(3) A standard "scoring" system exists for most of the isozymes in maize. By adopting this standard system, it is possible to tap into the extensive existing fingerprint data base.

Applications in Quality Control

Whereas historically, all the description, identification and selection involved in plant breeding and seed production has taken place at the phenotypic level, isozyme electrophoresis techniques have permitted corn researchers their first glimpse at a plant's genotype. As important a development as this is in terms of basic genetic research and plant breeding, perhaps the greatest utility relates directly to quality control activities in foundation seed maintenance and commercial hybrid production. Essentially, isozyme electrophoresis can be used as an instantaneous "grow-out". (Figure 3) illustrates, in a somewhat oversimplified way, the advantages of electrophoresis over conventional grow outs:

(1) With electrophoresis, we are no longer looking at environmental effects on gene expression.

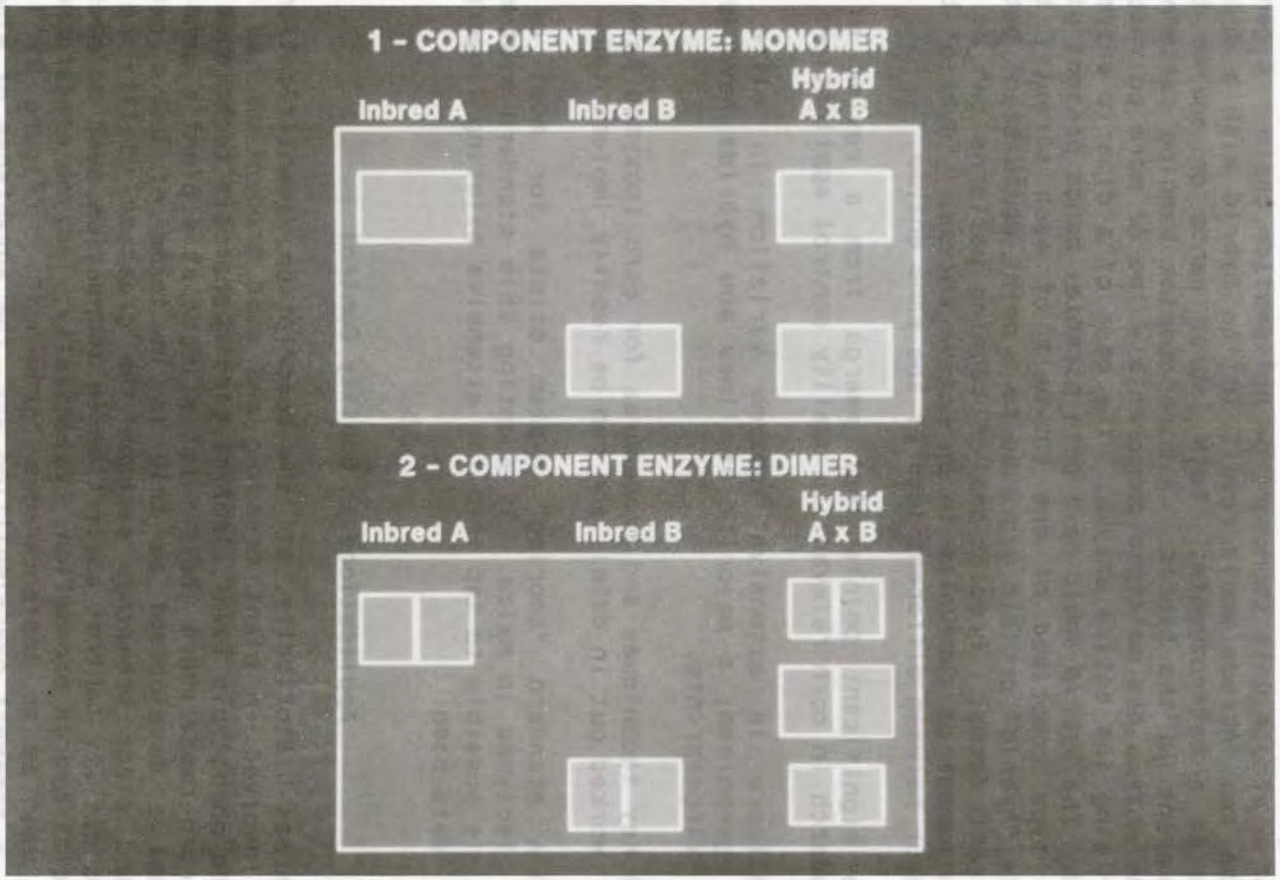

Figure 2. Diagram of electrophoretic patterns for two inbreds and the resulting hybrid given a monomeric (top) or dimeric enzyme.

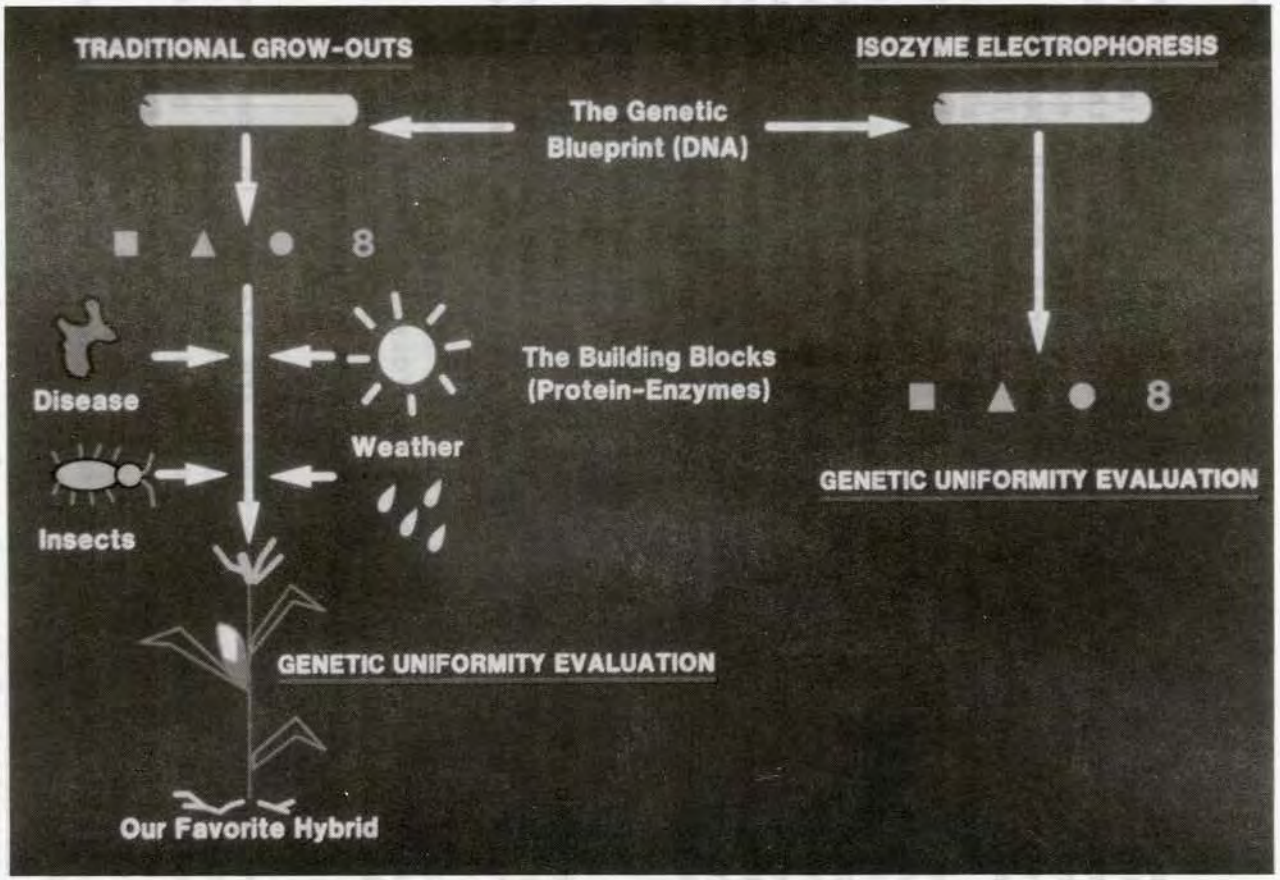

Figure 3. Comparison of genetic purity evaluation techniques: traditional growouts vs. isozyme electrophoresis.

(2) Electrophoresis results are objective; the answer is the same regardless of who reads the trial; field grow outs have an unavoidable subjective component.

(3) Electrophoresis is quicker - analysis is usually made on samples taken from 5-day old plants.

Considering the significant variation at isozymic loci among most maize inbreds, it is safe to predict that most commercial hybrids will be amenable to electrophoresis techniques. Knowing the fingerprints of any two parent lines, it is possible to predict exactly the hybrid fingerprint. A genetically pure hybrid should contain the alleles of each parent: where the parents carry identical alleles, the hybrid will be homozygous; where the parents carry identical alleles, the hybrid will be homozygous; where the parental alleles are different, the hybrid will be heterozygous. (Figure 4) includes a gel photograph showing the hybrid isozyme (malate dehydrogenase) profile flanked by profiles of both parents. Bands present in either parent are all present in the hybrid. (Figure 5) shows the same hybrid, however, one of the "hybrid" individuals lacks the bands contributed by the pollen parent. It also exactly matches the female parent pattern. This individual represents a probable sib pollination. This diagnosis is confirmed by examining several other isozyme systems. In each case, the apparent sib pollination exactly matches the female parent. A second "hybrid" individual is characterized by bands not seen in either parent - a probable out-cross or pollination by "blow-in" or rogue pollen. Note that the out-crossed individual may resemble the female parent in other gels. In these particular cases, the contaminating pollen carried the same allele as the female parent.

Isozyme electrophoresis readily identifies possible sib pollinations. Screening at a single heterozygous locus in any given hybrid should permit identification of all sibs. Out-crosses are less predictable as the source of contamination is often unknown (Smith and Weissinger, 1984).

Although many of the isozymes are present in most tissues throughout the plant's life, for quality control purposes it is often most convenient to sample young coleoptiles (in 5-day old plants germinated in darkness.) Alternatively, immature embryo samples can be taken 4 to 6 weeks following pollination (Smith, 1984b). This may be advantageous from the perspective of making a determination on genetic purity prior to harvest and seed conditioning.

Determination of the number of samples to be run for any given hybrid lot follow statistical guidelines. The probability of detecting 5% contamination either through foreign pollen, improperly detasseled females, rogues or seed mix-ups is better than 95% with a representative sample of 60 kernels (Goodman and Stuber, 1980).

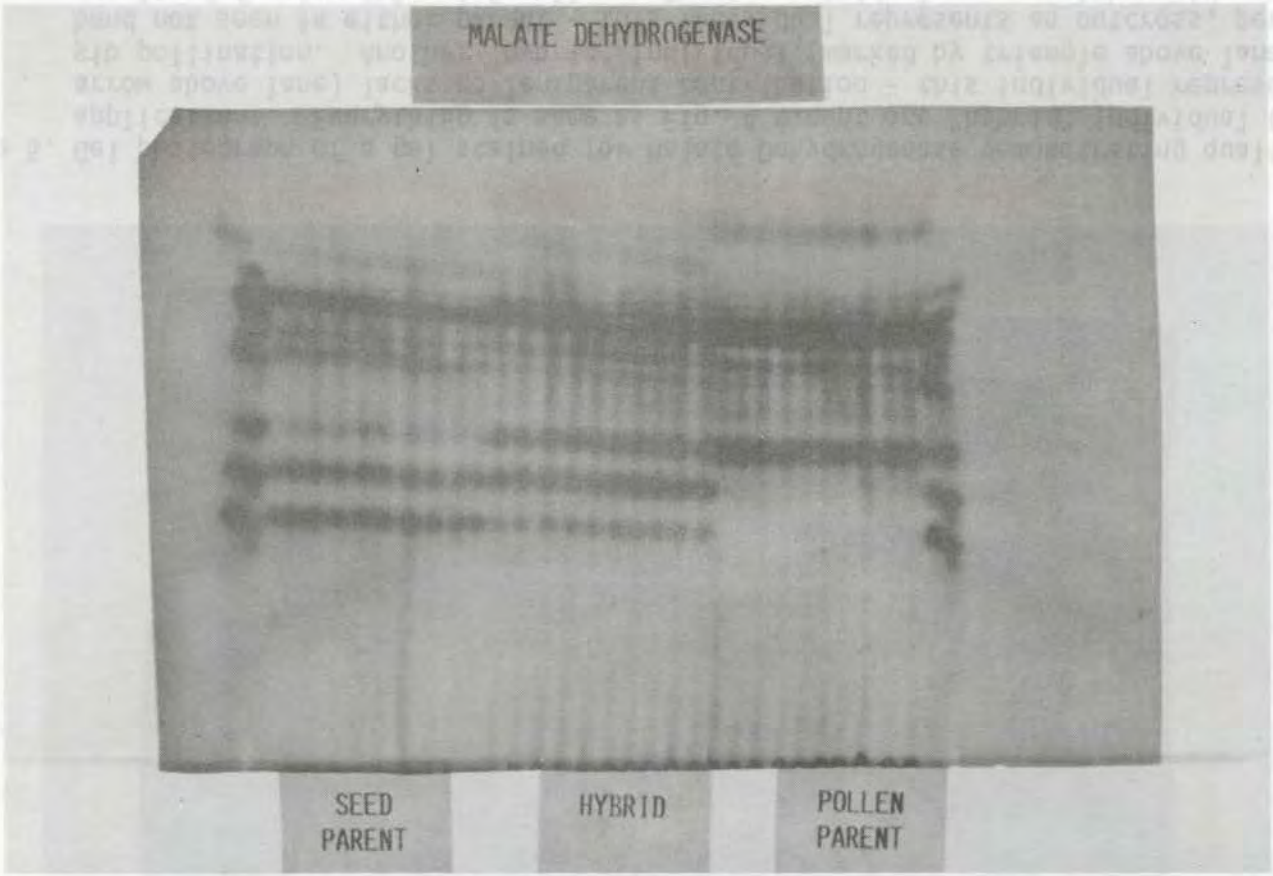

Figure 4. Gel photograph of a gel stained for Malate Dehydrogenase showing respective seed and pollen parent contributions to the hybrid. Ten individual seed parent (right), pollen parent (left) and hybrid (center) coleoptiles were electrophoresed. Note contributions of each parent to the hybrid.

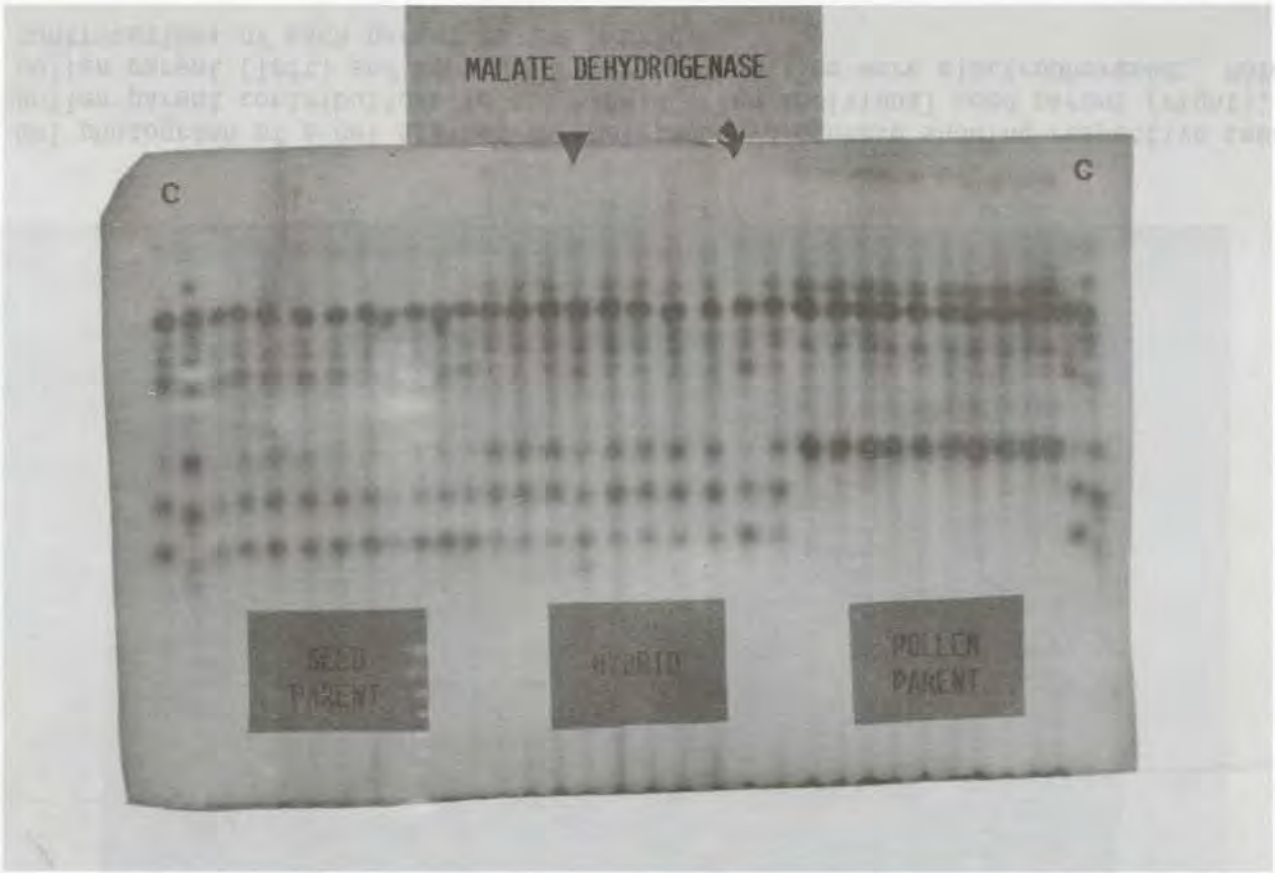

Figure 5. Gel photograph of a gel stained for Malate Dehydrogenase demonstrating quality control applications. Everything is same as Fig. 4 except one "hybrid" individual (marked by arrow above lane) lacks pollen parent contribution - this individual represents a self/sib pollination. Another "hybrid" individual (marked by triangle above lane) has a band not seen in either parent - this individual represents an outcross, perhaps the result of foreign "blow-in" pollen. The two outside lanes on either side are the checks (marked with "C" above lanes) - individuals with known banding patterns.

Foundation activities in inbred lines maintenance and parent seed increases may also benefit substantially from electrophoresis. Once an inbred fingerprint has been established, it can serve as an accurate reference point for long-term line maintenance. In shorter term projects and inbred increases, electrophoresis can be used to detect any contamination in much the same way as it is used to detect outcrosses in commercial hybrid production. Inventory maintenance can also be enhanced: in cases of possible seed lot interchanges, electrophoresis on a few kernels can often establish positive identity. Ideally, any inbreds entering foundation from research breeding programs can be finger printed and added to a company's genotype data base. At this point or even earlier, isozyme analysis can also be used to confirm the degree of inbreeding in a particular line.

Beyond its value as a tool for monitoring genetic purity and uniformity, isozymic methods offer a way to improve production operations. It is often possible to distinguish sources of contamination, for instance whether foreign alleles originated as blow-in from a nearby field or alternatively as volunteer plants in the production field itself. Production methods such as determination of field isolation standards, detasseling, derogueing and seed handling can be evaluated and improved even in cases where skilled supervision is spread thin.

As the commercial seed industry looks to the challenges of the future: changing agricultural markets, increased emphasis on seed quality and genetic purity, ever-rising production costs - often as the price of marketable seed falls, limited numbers of skilled production personnel and increased domestic and foreign competition; it will be important to identify and integrate new and more efficient technology into the tried and proven methods. Often -- and isozyme electrophoresis techniques are an example -- the question is not so much replacement as complementation.

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