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The Use of Isozyme Analysis for Identification of Plant-Pathogenic Fungi

Isozyme analysis is a powerful biochemical technique that has numerous applications in plant pathology. It has long been used by geneticists to study the population genetics of fish, mammals, insects, nematodes, and plants. Mycologists and plant pathologists have adopted the procedure more recently, and it is now being used routinely to settle taxonomic disputes, identify cultures, "fingerprint" patentable fungal lines, analyze genetic variability, trace pathogen spread, follow the segregation of genetic loci, and identify ploidy levels of fungi and other plant pathogens. These topics have been reviewed (9,12,15), and the large number of new publications in this field each year indicates the widespread interest in the subject.

At the Foreign Disease-Weed Science Research (FDWSR) in Frederick, Maryland, we routinely use isozyme analysis to identify and study the genetic makeup of exotic pathogens, often of quarantine significance. In this paper, we discuss the advantages and disadvantages of isozyme analysis over other identification techniques and describe how isozyme studies can be designed to differentiate pathogens.

The Theory of Isozyme Analysis

Isozymes are molecular forms of an enzyme. These forms usually have similar, if not identical, enzymatic properties but slightly different amino acid sequences. Only those isozymes with amino acid compositions of different net charge, or those that result in large differences in the shape of an enzyme, can be differentiated by electrophoresis. This has been estimated to represent about one-third of all the possible isozymes that may be present within a genetic system (9). Isozyme analysis thus provides a conservative estimate of the amount of variation within a genome.

Detectable isozymes arise from three different genetic and biochemical phenomena: 1) different alleles at a single locus (termed "allozymes"), 2) multiple loci coding for a single enzyme, and 3) posttranslational processing and formation of secondary isozymes (6). Recognizable protein banding patterns are frequently associated with each of these situations, thus allowing a genetic interpretation of the data (6,10). When several enzymes are tested, an electrophoretic "fingerprint" reflective of the genes coding for the different enzymes is produced. The identification of an organism is based on the number of genes in common with those of known cultures. With many pathogens, the amount of intraspecific variation determined by isozyme analysis is slight, thus making accurate identification of a species or subspecies possible.

Different electrophoretic techniques, including starch gel electrophoresis, polyacrylamide gel electrophoresis (PAGE), and isoelectric focusing, can be used to separate isozymes. We use starch gel electrophoresis, the oldest and simplest of the procedures. Starch gel electrophoresis is relatively inexpensive, uses fewer toxic chemicals than other methods, and can be used to screen many different enzyme activities simultaneously. The resolution of protein bands in starch gel electrophoresis is not as good as that obtained by PAGE, but a larger number of stains can be used and a larger number of enzymes can be detected from a single sample. Resolution by starch gel electrophoresis is adequate for the detection of allozymes and isozymes coded by different loci as long as a compatible buffer system is used.

We have previously described the procedure we use for electrophoresis (10), and Figure 1 describes the steps. In brief, a suspension of starch is boiled and poured into a rectangular mold to form the gel, which can be several centimeters thick. After the gel cools, a vertical slice is made through the gel close to one of the longer edges. Filter paper wicks that have been dipped into the enzyme sample are placed side by side along one edge of the slit. The two sides of the gel are then squeezed back together and brought into contact with the tray buffer, using cloth wicks. Current is applied for about 2.5–4 hours, depending on the buffer used. Cooling is essential during electrophoresis; otherwise, enzyme degradation will cause very irregular migration patterns. After electrophoresis, the gel can be sliced both vertically and horizontally. A single gel can produce between four and 45 stainable slices, depending on the number of samples and the thickness of the gel. These slices are placed in trays with specific activity stains.

Advantages and Disadvantages of Isozyme Analysis

As with any technique, isozyme analysis has its strengths and weaknesses. When starch gel electrophoresis is used, the technique is relatively inexpensive. At FDWSR, we find the development of fungal identification techniques using isozymes much faster and far less expensive than either immunological tests

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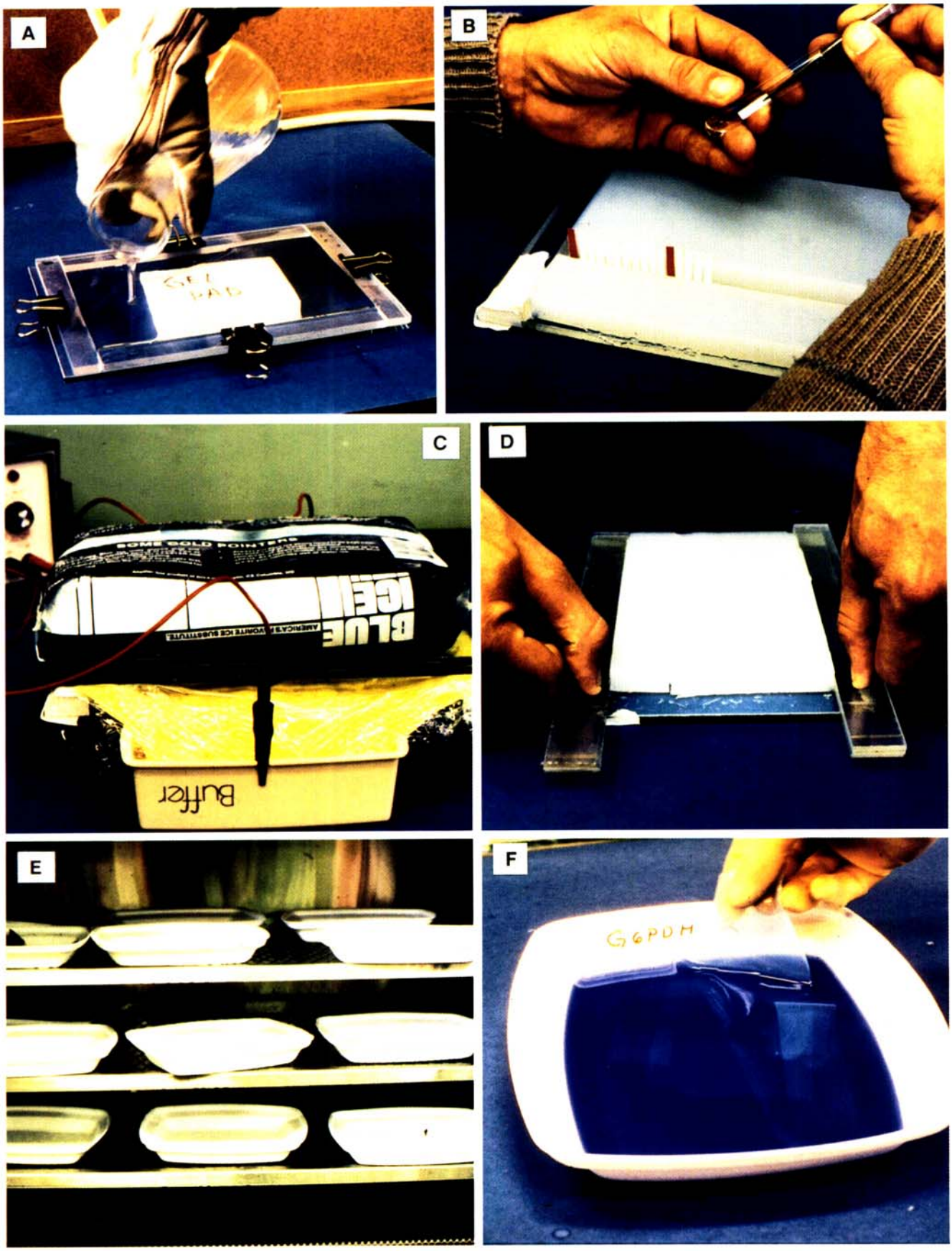

Fig. 1. Specific steps in an isozyme test: (A) A starch gel is poured into a mold. (B) Paper wicks containing enzyme extracts are lined up at the origin of a starch gel, and extraction buffer containing extracted enzymes is drawn into each wick by capillary action. (C) Starch gel loaded with samples during electrophoresis is kept cool by an ice pack resting on two pieces of plate glass stacked on the gel, and a cloth wick is in contact with both the gel and the tray buffer at each end of the gel. (D) Gel is sliced with nylon thread; several slices, each to be stained for a different enzyme, are obtained from each gel. (E) Gel slices are incubated at 37 C to enhance enzymatic activity and appearance of bands. (F) Gel is stained for a specific enzyme.

or tests involving the polymerase chain reaction (PCR) and species-specific DNA primers.

With isozymes, a large number of staining systems can be used, thus allowing the comparison of numerous genetic loci coding for enzymes from many metabolic pathways. This has the advantage of allowing us to draw conclusions about the genetic variability existing within and between species under investigation. Over 100 enzymes have been used in research conducted at FDWSR. In most cases, good resolution and successful genetic interpretations can be obtained with a panel of 15–25 enzymes. Such an isozyme test can be completed within a day. In our experience, isozyme analysis has been particularly powerful for determining relationships at the species or pathovar level.

Unlike restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analyses, which are capable of detecting very small changes in the genome of an organism, isozyme analysis detects only significant differences in enzyme structure. These differences most often are at the species level. For example, we found that only two distinct alleles were present in *Tilletia indica* Mitra (= *Neovossia indica* (Mitra) Mundkur) and *T. barclayana* (Bref.) Sacc. & Syd. in Sacc. for mannose phosphate isomerase and that these could be used for separating the species. Current research at FDWSR in comparing these two species using RFLP and RAPD methods has detected numerous fragment profiles within each species, reflecting the high degree of intraspecific genetic variability. This makes these techniques difficult and developmentally expensive to use for identification purposes, since all possible profiles need to be documented and require significant computer analysis to be useful.

In isozyme analysis, staining systems are usually quite specific and generally detect a single enzyme of known identity on a gel slice. This is in contrast to the numerous bands obtained from a general protein stain. Enzymes coded by different genetic loci usually occur in separate zones of the gel, thus facilitating genetic interpretation. Because of much research relating isozyme banding patterns to the genetics of animals, plants, and microorganisms, it is usually not necessary to conduct additional genetic studies with fungi. For the vast majority of enzymes, only one reasonable interpretation can be made for the banding patterns (10).

The greatest disadvantage of isozyme analysis is that relatively large quantities of an organism must be present, compared with that required for immunological or PCR techniques, in order to extract sufficient enzymes for detection. This usually is not a problem for fungi that can be cultured on artificial media. Obligate pathogens are more of a problem. Considerable effort may be required to obtain sufficient quantities of material to produce distinct bands for obligate parasites. With downy mildews, for example, conidia can be washed directly from the plant surface and concentrated by centrifugation. However, the sporulation may be very sparse and require collection from many plants so that all

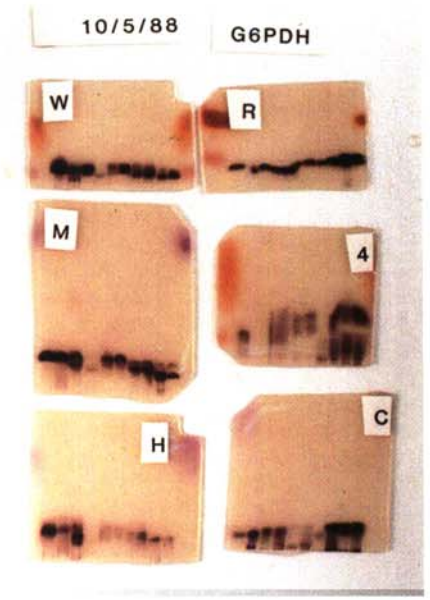

Fig. 2. Results of a typical enzyme screen illustrate the importance of the screen. In this case, the enzyme activity of glucose-6-phosphate dehydrogenase (G6PDH) was examined for 10 isolates of *Colletotrichum* spp. Electrophoresis was carried out in six different buffer systems—W, R, M, 4, H, and C. Buffer R was selected for further studies because it produced the sharpest resolution and clearest polymorphisms.

Fig. 3. (A) Wheat head with grains infected with *Tilletia indica*, the causal agent of Karnal bunt of wheat. (B) Germinated teliospore of *T. indica*. The teliospore, basidium, and resulting basidiospores (primary sporidia) are nearly indistinguishable from those of *T. barclayana*, the causal agent of kernel smut of rice. (C) Starch gel showing a different band of enzyme activity differentiating *T. indica* (higher band) and *T. barclayana* (lower band).

samples are, in fact, populations that may not represent a single genotype. Similar difficulties arise when analyzing urediniospores of some rust fungi.

The amount of material required for isozyme analysis may vary from organism to organism, depending on the quantity of enzyme present in the tissue, the efficiency of the extraction method, and the number of enzymes to be examined. In general, we try to obtain 50–100 mg (wet weight) of mycelium, 50 mg (wet weight) of downy mildew conidia, and 30–50 mg (pregermination weight of dry spores) of germinating rust urediniospores.

Time requirements may be another disadvantage of isozyme analysis. Although the electrophoretic test can be conducted quite rapidly, it often takes several days, or even weeks, to isolate and grow the organisms. For example, the germination of viable teliospores of *T. indica* can require 2 weeks. Continued growth for up to another week is needed to obtain sufficient fungal material for the test. Such time requirements are not acceptable in situations where identification is required within hours, as when grain is being loaded into the holds of ships. Alternate identification procedures, such as immunological or molecular probe techniques, may then be more suitable and should be developed.

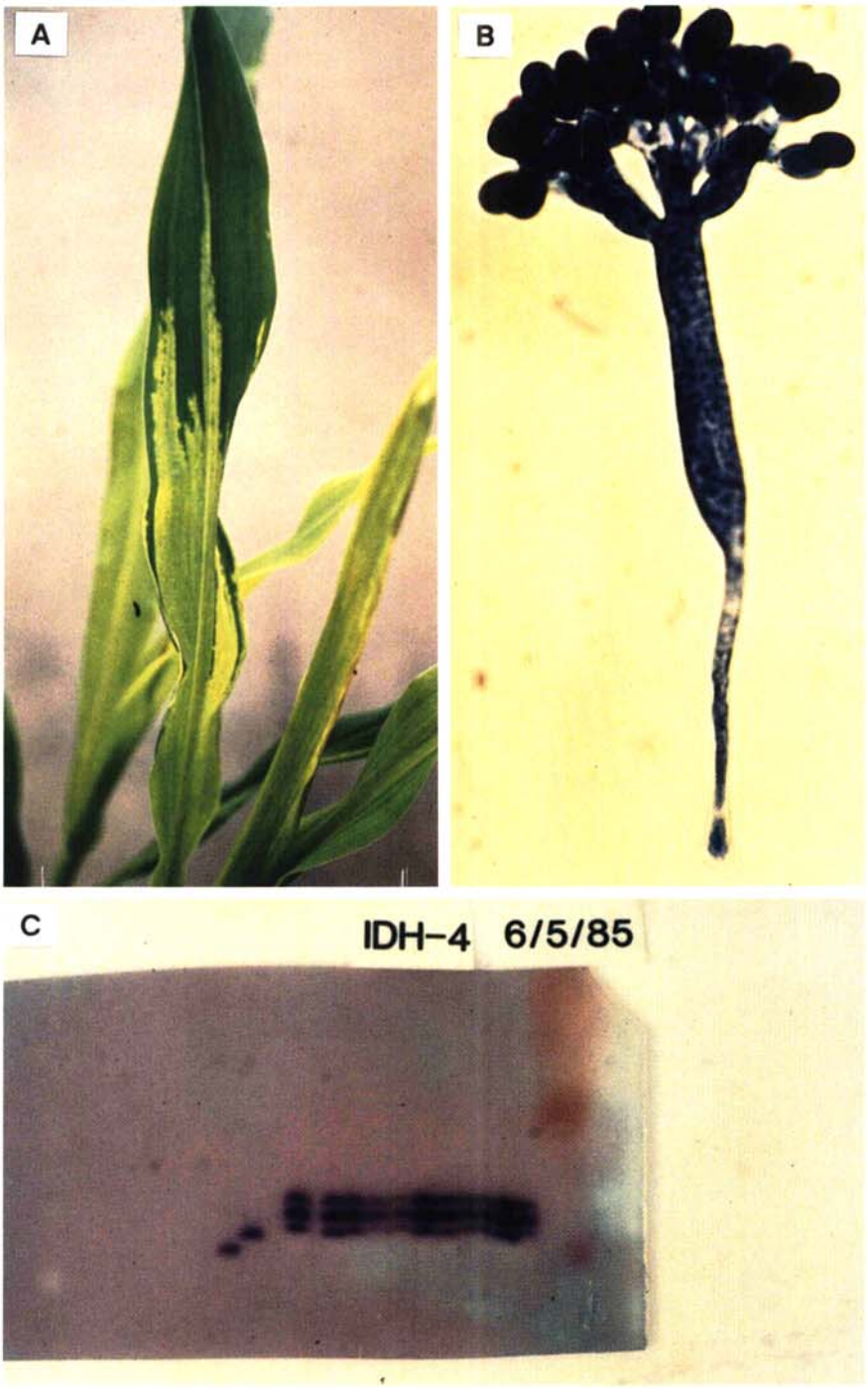

Fig. 4. (A) Symptoms of the downy mildew pathogen *Peronosclerospora sorghi* on maize. (B) Conidiophore and conidia of an isolate of *P. sorghi* from Thailand, which is morphologically indistinguishable from the sorghum strain of the pathogen found in other parts of the world infecting sorghum and maize. (C) Isozyme banding patterns (two bands at far left) for isocitrate dehydrogenase showing differences between the two strains; the organism from Thailand produces the lower band. The coefficient of similarity using 26 enzymes comparing the Thailand strain and typical *P. sorghi* is very low (approximately 0.10), indicating they are not closely related. The three-banded pattern represents isolates of *P. tabacina*, causal agent of blue mold of tobacco. In a comprehensive study comparing isolates of *P. tabacina* from several areas of the world, all isolates for any given enzyme had the same isozyme banding pattern, in contrast to *P. sorghi*.

Developing an Isozyme Test for Identification

At FDWSR, most isozyme projects have two goals. The first is to determine the genetic variability and relatedness within and among species, or groups of isolates of the same species, based on geographic origin (such as states or countries) or on the host from which the pathogen was isolated. Variability and relatedness are measured by comparing the number of isozyme coding alleles in common between individual isolates and groups of isolates, then calculating coefficients of similarity by means of a computer program. The second goal is to identify a number of isozymes and their coding alleles unique to each group that can be used for identification.

The development of an isozyme test for identification can be relatively simple and usually involves a minimal investment of time and money once all appropriate isolates have been collected. In fact, the most time-consuming aspect of the study is often the procurement of pathogen isolates. One should collect as many isolates as feasible, representing the geographic and climatic distribution of the organism. It also is important to include any organisms that may be morphologically or physiologically similar to the pathogen of interest.

Once the isolates have been acquired, an initial screening experiment is conducted to determine which buffer systems should be used for the best electrophoretic separation of each enzyme and to show which enzymes can be extracted in sufficient quantity. The determination of the correct buffer is essential, and the results of the initial screen can save considerable work later. Figure 2 shows the results of a screening experiment in which six different gel buffer systems were used for the same enzyme. Use of an inappropriate buffer can result in the absence of staining (caused by wrong pH), poor resolution, and frequently the inability to correctly assign allele desig-

nations, which leads to false conclusions.

It is important to include in a screening experiment at least one representative isolate from each species or group. For example, when the initial screening was done for a study on strawberry-infecting *Colletotrichum* species, one representative isolate each of *C. fragariae* A.N. Brooks, *C. acutatum* J.H. Simmonds, *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz., and the related species *C. coccodes* (Wallr.) S.J. Hughes were included (3). This assured that the enzymes selected for further study were those most likely to produce interpretable results for separating the species.

The maximum number of samples that we include in a screen is nine. Four to six gel buffers are usually tested, each for the detection and resolution of approximately 65 enzymes. In our laboratory at FDWSR, the test takes 2 days. From an average electrophoretic screen, approximately 20–30 enzymes are identified for inclusion in further tests. The initial screen enables us to select the buffer(s) that give the best results for each specific enzyme. Screens also provide some preliminary data indicating which enzymes exhibit allelic variation and which might be good for separation of the pathogen groups. After the screening experiment is complete, we are ready to conduct the first full-scale comparative analysis.

Although a standard starch gel in our laboratory will accommodate 40 samples, we find it best to limit the comparison to 20 samples, replicated on the right and left sides of the gel (Fig. 1). This becomes useful if an electrophoretic problem develops on one side of the gel. This replication also is helpful in determining whether small differences between two samples are real or are due to artifacts or minor gel distortion. In our studies, the first electrophoretic full-scale test characteristically includes three to five representative isolates from each species or test group. For example, in our initial work to compare isolates of soybean rust from Australasia and the Americas, we compared five isolates from each of the two geographic areas. In a taxonomic study of strawberry-infecting *Colletotrichum* species, we examined five isolates from each of three species. These initial runs allowed us to confirm the results of the isozyme screen and further determine which enzymes were most likely to differentiate species.

The next step is to examine a sufficient number of isolates of each species or group to determine what isozymes and isozyme coding alleles are present. If only a few of the enzymes display differences between groups, one may wish to concentrate on these.

Additional factors may have to be considered in the development of an isozyme identification test. For example, some organisms grow very slowly, even on the best available growth medium. Because rapidly growing cultures are more metabolically active and will provide greater quantities of enzymes, it may be advisable to experiment with different growth media or methods of seeding the medium. In some instances, starting cultures from a suspension of spores or mycelial fragments will provide the proper rapid initial growth rate. At FDWSR, we prefer to have fungal cultures growing for no longer than 7 days prior to harvest from the growth medium. Longer periods can cause artifacts due to molecular changes in enzymes and poor enzyme activity.

Obligate pathogens obviously cannot be grown in artificial growth media. *Peronosclerospora* species that cause downy mildews can be sporulated on the surface of plants and the conidia or sporangia collected for enzyme extraction (14). Urediniospores of rust pathogens can be collected from the surface of host plants, germinated, and extracted (1).

The enzymes that are selected for examination also are an important consideration. Different types of enzymes exhibit different degrees of variation due to the intensity of selection pressure (5,7, 8,16,17). Regulatory enzymes involved with energy metabolism often display less variation than do nonregulatory enzymes, such as esterases and phosphatases. Any study that uses only nonregulatory enzymes probably will detect a disproportionately high level of intraspecific variation and thus be less likely to be a good measure of relatedness or to differentiate species.

When a set of enzymes that can be used for identification has been selected, the technology can be used to analyze large numbers of isolates. Including known isolates as controls on the gel is the most efficient and accurate method for making an identification. For these reference samples, stored mycelia or spores of known fungal cultures maintained in 1.8-ml cryotubes in a liquid nitrogen refrigerator are removed from storage and extracted. Samples stored for 7 years had no appreciable loss of enzymatic activity, and isolates stored in a −80 C ultracold freezer for at least 1 year gave the same results.

In a comprehensive study, the isozymes of many isolates may be com-

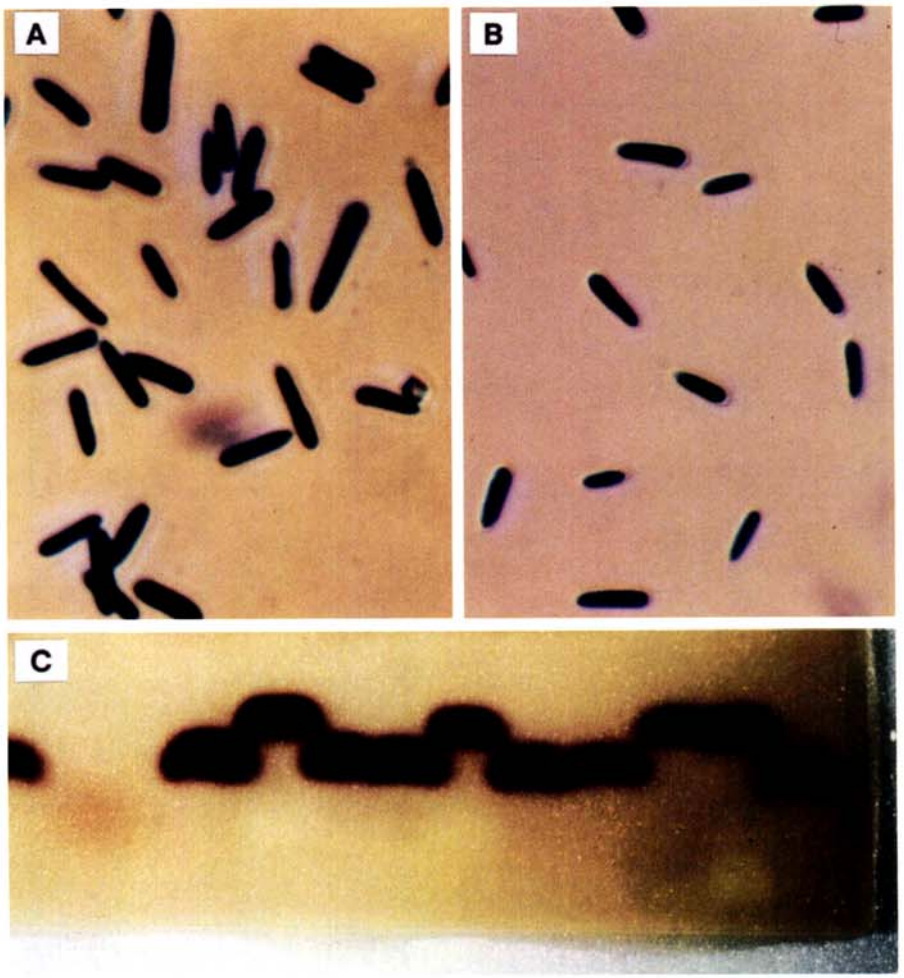

Fig. 5. Conidia of (A) *Colletotrichum fragariae* and (B) *C. acutatum*. (C) Isozyme patterns showing a phenotypic difference between *C. fragariae* (upper band) and *C. acutatum* (lower band) for the enzyme mannitol dehydrogenase.

pared. The bands are scored and interpreted as described elsewhere (12). Coefficients of similarity (CS) values are calculated that measure the degrees of similarities in terms of average numbers of alleles in common within and between species or other groupings of isolates. Isolates of the same species tend to have high CS values, whereas isolates of different species have considerably lower CS values. For example, *T. indica* and *T. barclayana* have intraspecific CS values of 0.83 and 0.85, respectively, whereas the average interspecific CS value of these morphologically very similar and closely related smut pathogens is only 0.04. Analysis of the data can be facilitated by any of a number of computer programs (18). Usually a few isozymes are identified that are unique to the organism of primary interest.

The final value of an isozyme test for identification depends on the speed at which an organism can be isolated and grown (if required) to a suitable mass for extraction and on the reliability of the isozyme banding patterns for identification. In many circumstances, the techniques are highly accurate. Other methods of identification can be used concurrently. When making plant disease regulatory decisions that could affect a major agricultural industry, it obviously is best to use two or more independent techniques to make an identification.

Isozyme Analysis Applications

Isozyme analysis has been used by FDWSR to identify important plant pathogens, many of regulatory concern. One example is *T. indica*, the causal agent of Karnal bunt of wheat. The pathogen does not occur in the United States. The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture regulates wheat partly because of Karnal bunt. One problem in preventing movement of *T. indica* has been the difficulty in distinguishing teliospores of *T. indica* from those of *T. barclayana*, the causal organism of kernel smut of rice. This latter smut pathogen is common in most rice-growing areas of the world, including the United States, and its teliospores sometimes become incorporated as contaminants in wheat or other small grains during harvest, transport, or storage (4). This contamination can cause rejection of a shipment and may even cause the initiation of a costly quarantine if Karnal bunt is perceived to be a risk by the wheat-importing country. We have used isozyme analysis to identify teliospores suspected of being *T. indica* (Fig. 3). This technology has been used in Brazil by Centro Nacional de Pesquisa de Recursos Geneticos e Biotecnologia to identify teliospores of *T. indica* in imported wheat seed (C. Castro, *personal communication*).

We also have used isozyme analysis to determine the relationship of *Septoria* species of quarantine significance that cause leaf and fruit spots on citrus in Australia and the United States (2). Eighteen different species of *Septoria* have been described on citrus. Except for one isolate, isozyme analysis revealed very little genetic variation; the alleles that coded for 23 of 25 enzymes were identical. Isozyme data were compared with morphological observations of herbarium specimens and freshly collected isolates. We concluded that only one species of *Septoria*—*S. citri* Pass.—was present in the main citrus-growing regions of both countries and that it should not be of concern in citrus exports between the two countries.

Taxonomic relationships among other fungal genera have been determined with this technique, often with important implications for disease management and international commerce. For example, we used isozyme analysis to confirm that *Endothia eugeniae* (F.J. Nutman & F.M. Roberts) J. Reid & C. Booth, an opportunistic pathogen of clove, is the same organism as *Cryphonectria cubensis*

(Bruner) C.S. Hodges, the causal agent of eucalyptus canker, an economically important pathogen of eucalyptus (13). The ubiquitous clove can, therefore, serve as a source of inoculum for the eucalyptus canker pathogen in tropical forests. We also found (11) that *Peronosclerospora philippinensis* (Weston) C.G. Shaw, which causes Philippine downy mildew of maize, was probably the same pathogen as *P. sacchari* (T. Miyake) Shirai & K. Hara, the causal agent of sugarcane downy mildew. The relationship of these latter two organisms has been disputed for many years. Their conspecificity was recently confirmed by DNA hybridization (19).

Isozyme analysis sometimes demonstrates that sufficient genetic variation exists within a species to support its division into separate species. This occurred with *Peronosclerospora sorghi* (W. Weston & Uppal) C.G. Shaw on maize in Thailand when compared with the pathogen found infecting both maize and sorghum in other areas of the world (Fig. 4) (11). On average, only about 10% of the alleles, or isozyme bands, were in common between the two organisms. Isozymes of isolates of *Phakopsora pachyrhizi* Sydow, the causal agent of soybean rust, from Australia and Asia were so dissimilar from those of isolates from the New World that separate species also were proposed (1).

Isozyme analysis can lead to the development of a simple technique for identification. One good example is the identification of *Colletotrichum* species that attack strawberry. Three species—*C. gloeosporioides, C. fragariae*, and *C. acutatum*—are generally recognized as common pathogens of strawberry. These fungi are very difficult to identify by morphology alone, and there has been controversy as to whether *C. fragariae* is actually a portion of *C. gloeosporioides*. All three organisms can be distinguished by the electrophoretic pattern of specific enzymes (Fig. 5) (3). The proper, rapid identification of the pathogens (particularly *C. acutatum*) is important for plant disease quarantine regulation and may become important in breeding for disease resistance.

Isozyme data can be very valuable in determining the identity of a fungal pathogen, and analysis of 20 or more isozyme loci usually separates fungal organisms at the species or pathovar level. In instances where the technique appears not to work, the cause may be artificial or inaccurate boundaries of the taxa.

Isozyme analysis is a simple, efficient, and inexpensive technique that can be used to solve many taxonomic and phytopathological problems. It also has practical applications for pathogen detection and identification. We believe that this technique will become even more important for plant pathologists in the years to come.

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Morris R. Bonde

Dr. Bonde graduated with a B.S. degree in botany from the University of Maine in 1967 and from Cornell University with M.S. and Ph.D. degrees in plant pathology in 1969 and 1974, respectively. Since 1973, he has been employed by the USDA-ARS Foreign Disease-Weed Science Research. There he has conducted research to determine the threat of foreign plant pathogens to major U.S. crops. Among the diseases he has conducted research on are Karnal, dwarf, and common bunts of wheat; downy mildews of maize, sorghum, and sugarcane; and soybean rust. Since 1981, Dr. Bonde has conducted research to develop better means of identifying plant pathogens of quarantine significance. He has published extensively on the use of isozyme analysis for identification of fungal pathogens of crops.

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Gary L. Peterson

Mr. Peterson graduated with a B.S. degree from St. Mary's College of Maryland in 1977. Since 1978, he has been employed by the USDA-ARS Foreign Disease-Weed Science Research, where his current position is support scientist. He also has conducted research on Karnal, dwarf, and common bunts of wheat and downy mildews of maize, sorghum, and sugarcane. Since 1981, he has worked on developing better means of identifying plant pathogens of quarantine significance. He has been instrumental in the identification of plant pathogens of national interest affecting international trade.

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