# Comparison of Isozyme Polymorphism in Races of *Cochliobolus carbonum*

K. D. Simcox, D. Nickrent, and W. L. Pedersen

Postdoctoral research associate, USDA-ARS, Ohio Agricultural Research and Development Center, Wooster 44691; assistant professor, Department of Plant Biology, Southern Illinois University at Carbondale, Carbondale 62901; associate professor, Department of Plant Pathology, University of Illinois at Urbana-Champaign, Urbana 61801.

We thank Dave Smith for supplying isolates and Morris Levy and Shizung Wu for advice on the use of the NT-SYS program. Research was supported by Hatch project 68-0351 and Illinois Foundation Seeds, Inc.
Accepted for publication 27 February 1992.

## ABSTRACT

Simcox, K. D., Nickrent, D., and Pedersen, W. L. 1992. Comparison of isozyme polymorphism in races of *Cochliobolus carbonum*. Phytopathology 82:621-624.

Differences in isozyme polymorphisms between races of *Cochliobolus carbonum* were compared using starch gel electrophoresis techniques. Of 36 enzymes analyzed, isozyme polymorphisms were detected for aspartate aminotransferase, esterase, glucose-phosphate isomerase, leucine aminopeptidase, and mannitol dehydrogenase. Specific combinations of polymorphisms were found to be diagnostic of race pathotype for the isolates used in this study. Race 3 isolates of *C. carbonum* could be distinguished by the presence or absence of esterase and leucine aminopeptidase polymorphisms, which were not found in race 2 isolates. The extent of isozyme polymorphisms found in *C. carbonum* provides a useful tool to study the genetics and the evolution of a phytopathogenic fungi.

*Cochliobolus carbonum* R. R. Nelson (anamorph, *Bipolaris zeicola* (G. L. Stout) Shoemaker = *Helminthosporium carbonum* Ullstrup), the causal agent of northern corn leaf spot, is composed of distinct pathotypes that exhibit different modes of pathogenicity (11). Race 1 pathotypes produce a pathotoxin, HC-toxin, that is required for successful colonization of maize genotypes homozygous recessive at the *Hm1* locus (23,25,31). Although *C. carbonum* race 2 is the most prevalent pathotype in the United States corn belt, it is a very weak pathogen of maize. Race 2 pathotypes produce small chlorotic lesions on foliar and husk tissue that resemble an incompatible race 1 reaction (1,11). A third race was identified in 1973 that produced symptoms distinct from that of either race 1 or race 2 and was found to have slight differences in conidial morphology (10,21). Race 3 isolates are virulent on a wide variety of maize inbred lines, and virulence is qualitatively inherited (8).

Differences in modes of pathogenicity and in the inheritance of virulence in different races suggest that populations of *C. carbonum* in the United States are genetically diverse. Population studies of *C. carbonum* from different geographical regions of North Carolina identified genetic polymorphisms for traits such as tolerance to cadmium, sensitivity to cycloheximide and carboxin, mating capability, and lesion length (11,16). In areas where isolates of races 2 and 3 coexisted, no evidence of gene flow between different pathotypes was found despite the fact that both mating-type alleles were present in the population (12). The lack of gene flow and the inability to identify the sexual stage in the field (12) suggest that sexual reproduction does not play an important role in generating genetic variability and that new pathotypes arise through stepwise mutations within an asexual clonal lineage. Additional neutral genetic markers will facilitate the understanding of clonal inheritance within populations of *C. carbonum* and provide a means of analyzing the evolution of new pathotypes within *C. carbonum*.

Starch gel electrophoresis (isozyme analysis) and restriction fragment length polymorphisms (RFLPs) have been used successfully to study genetic diversity within populations of phytopathogenic fungi (4,5,13,14,29). They work as taxonomic tools to clarify species distinctions between closely related phytopathogenic fungi with distinct host ranges (2,3,15) and as markers for the genetic analysis of fungal plant pathogens (19,28). The objectives of this research were to evaluate the amount of isozyme polymorphism in *C. carbonum* and to assess its usefulness in comparing genetic diversity among races of the pathogen.



## MATERIALS AND METHODS

**Isolates used in this study.** Isolates of *C. carbonum* were collected from various maize production fields in the United States. The year of isolation, location, and race of each isolate used in this study are listed in Table 1. Race 1 isolates 73-4 and *MAT1* (mating-type locus 1) as well as race 2 isolates 85-5 and *MAT2* (mating-type locus 2) are maintained in the Department of Plant Pathology at the University of Illinois. These isolates are stored on silica gel at 4 C. The additional race 2 and race 3 isolates used in this study were provided by Dave Smith (Dekalb Plant Genetics, Dekalb, IL) and had been stored as dried lesion material at 4 C. Single-spore isolates were obtained from silica gel cultures or from an individual lesion from the dried lesion material at the time of the study. Isolates were maintained as mass-spore cultures on lactose-casein hydrolysate agar (30) at 22 C under 16 h of light (cool-white fluorescent bulbs) alternated with 8 h of dark.

The pathogenicity of each isolate was tested on the appropriate maize inbred by whorl inoculation using a conidial suspension. Inoculum was produced by adding 5 ml of sterile water containing 0.1% Tween 20 to a 14-day-old culture and dislodging the conidia with a rubber policeman. After the conidia concentration was adjusted to 5,000 conidia per milliliter, 100 μl of the conidial suspension was transferred into the whorl of 2-wk-old maize seedlings (approximately three-leaf stage). Inoculated seedlings were incubated overnight in high humidity at 15 C and then maintained in the greenhouse under 16-h light (cool-white fluorescent bulbs)/8-h dark. Temperatures in the greenhouse ranged from 15 to 25 C during the experiment.

**Sample preparation.** Isozyme analysis was performed on crude enzyme extracts prepared from homogenized mycelia grown in liquid culture. Agar plugs taken from the advancing edge of 5-day-old cultures were used to inoculate flasks containing 50 ml of lactose-casein hydrolysate broth. Cultures were incubated at 30 C for 3 days on a rotary shaker. Crude enzyme extracts were prepared by decanting the culture broth and washing the mycelial mat twice with 10 ml of extraction buffer. The extraction buffer was composed of 0.1 M Tris-HCl, pH 7.0; 10 mM KCl; 0.1 mM $MgCl_2$; 1 mM EDTA-$Na_2$; 14 mM β-mercaptoethanol; and

0.1 mM ascorbic acid (15). Excess liquid was squeezed from the mycelial mats, and the mycelium was homogenized in 1.5 ml of ice-cold extraction buffer for 5 min with a Pyrex brand 7-ml Broeck tissue grinder. The homogenate then was transferred to a 13-ml polypropylene tube, sonicated for 1.5 min with a microtip probe (continuous power mode, maximum setting for microtip probe; Branson Sonicator, Danbury, CT), and centrifuged at 12,000 *g* for 30 min. The supernate was decanted and stored at −80 C.

**Starch gel electrophoresis.** Samples were loaded onto 9% (w/v) horizontal starch gels (Sigma Chemical Co., St. Louis, MO) by absorbing mycelial extracts onto 0.5- × 1.5-cm paper wicks cut from Whatman 3M chromatography paper. Sample wicks were loaded onto the gel in a sample slot cut 4 cm from the cathodal edge. Electrophoresis and subsequent enzyme staining were performed as described by Micales et al (18). Enzymes providing repeatable banding patterns, buffers, and electrophoretic conditions used in this study are listed in Table 2. For each isolate listed in Table 1, complete gel sets using two independently prepared mycelia extracts were run at least twice, and each run within a gel set was replicated twice.

**Data analysis.** A relative mobility ($R_f$) value was assigned to each band of enzyme activity detected, regardless of the genetic basis for that band of enzyme activity. The most cathodal band of a standard isolate was assigned an $R_f$ value of 100. Relative mobilities of all other bands were calculated by dividing the distance migrated by 100. For each isolate, presence or absence of a band of enzyme activity for each enzyme used in this study defined an electrophoretic phenotype. Similarities among electrophoretic phenotypes were calculated using Nei and Li's (20) index of genetic similarity for RFLP comparisons ($S_{xy}$), which has the form $S_{xy} = 2n_{xy}/(n_x + n_y)$, where $n_{xy}$ is the number of shared bands and $n_x$ and $n_y$ are the number bands in electrophoretic phenotype $x$ and $y$, respectively. Electrophoretic phenotype similarities were inferred from a cluster analysis of $S_{xy}$ values, employing the unweighted pair group method using the arithmetic averages (UPGMA) option in the Numerical Taxonomy System of Multivariate Statistical Programs (NT-SYS) software package of Rohlf (24).

TABLE 1. Summary of race, location, and year of isolation of *Cochliobolus carbonum* isolates analyzed in this study

| Isolate | Race | Location | Year of isolation |
|---|---|---|---|
| 73-4 | 1 | Urbana, IL | 1973 |
| *MAT1* | 1 | Urbana, IL | 1973 |
| *MAT2* | 2 | Urbana, IL | 1973 |
| 82-1508 | 2 | Redwoods Falls, MN | 1982 |
| 83-1038 | 2 | Mason, MI | 1983 |
| 83-1808 | 2 | Geneva, NY | 1983 |
| 84-2546 | 2 | Fremont, NE | 1984 |
| 84-3201 | 2 | Crawfordsville, IN | 1984 |
| 84-3666 | 2 | Marshall, MO | 1984 |
| 84-4531 | 2 | Mount Olive, NC | 1984 |
| 85-5 | 2 | Urbana, IL | 1985 |
| 85-2215 | 2 | Dayton, IA | 1985 |
| 76-2356 | 3 | Petersburg, VA | 1976 |
| 81-1843 | 3 | Geneva, NY | 1981 |
| 82-4412 | 3 | Warrenton, VA | 1982 |
| 82-4439 | 3 | Warrenton, VA | 1982 |
| 84-3306 | 3 | York, PA | 1984 |
| 84-5027 | 3 | Wythville, VA | 1984 |
| 84-5041 | 3 | Wythville, VA | 1984 |
| 85-1954 | 3 | Marion, OH | 1985 |
| 85-2557 | 3 | York, PA | 1985 |

TABLE 2. Summary of the enzymes analyzed in this study

| Enzyme | EC number | Buffer system[a] |
|---|---|---|
| Aconitase | 4.2.1.3 | CT,TC |
| Adenylate kinase | 2.7.4.3 | CT |
| Aldolase | 4.1.2.13 | CT,LI,TC |
| Aspartate aminotransferase | 2.6.1.1 | TM |
| Catalase | 1.11.1.6 | CT,HC,TC |
| Esterase | 3.1.1.- | CT,TM |
| Fumarase | 4.2.1.2 | CT,TC,TM |
| Glucose dehydrogenase | 1.1.1.47 | CT |
| Glucose-6-phosphate dehydrogenase | 1.1.1.49 | CT,TC |
| Glucose-phosphate isomerase | 5.3.1.9 | CT,TC,TM |
| Glutamate-pyruvate transaminase | 2.6.1.2 | CT,TC |
| Hexose kinase | 2.7.1.1 | CT,TC,TM |
| Leucine aminopeptidase | 2.4.11.- | CT,HC,TM |
| Malate dehydrogenase | 1.1.1.37 | CT,HC,TC |
| Mannitol dehydrogenase | 1.1.1.67 | CT |
| Mannose-6-phosphate isomerase | 5.3.1.8 | CT |
| Menadione reductase | 1.6.99.2 | CT,TC,TM |
| Phosphogluconate dehydrogenase | 1.1.1.44 | CT,TM |
| Phosphoglucomutase | 5.4.2.2 | CT,HC,TC |

[a]CT = amine-citrate, pH 6.1, 200 V, 5 h (7); HC = histidine-citrate, pH 7.0, 100 V, 4 h (27); LI = Tris-citrate lithium-borate, pH 8.1, 200 V, 8 h (26); TC = Tris-citrate, pH 7.5, 200 V, 5 h (27); and TM = Tris-malate EDTA, pH 7.4, 100 V, 5 h (26).

## RESULTS

Enzyme activity was detected in mycelial extracts of *C. carbonum* for 31 out of the 36 enzyme-staining procedures tested. Of the 31 enzymes in which enzyme activity was detected, only 19 enzymes were selected for use in this study (Table 2). These enzymes provided consistently uniform staining and adequate isozyme resolution. Enzyme-staining protocols that resulted in a less-than-desirable degree of staining intensity or uniformity were acid phosphatase (3.1.3.2), alcohol dehydrogenase (3.1.3.1), diaphorase (1.6.4.3), β-glucosidase (3.2.1.21), glutamate dehydrogenase (1.4.1.2), glyceraldehyde-3-phosphate dehydrogenase (1.21.12), glycerol-3-phosphate dehydrogenase (1.1.1.8), isocitrate dehydrogenase (1.1.1.42), lactate dehydrogenase (1.1.1.27), malic enzyme (1.1.1.40), peroxidase (1.11.1.7), and superoxide dismutase (1.15.1.1). Enzyme activities in mycelial extracts of those enzymes listed in Table 2 were stable for longer than 1 yr when stored at −80 C (K. D. Simcox, W. L. Pedersen, and D. Nickrent, *unpublished data*).

The amount of isozyme variation present within isolates of *C. carbonum* was comparable to the amount of variation observed for morphological and other biochemical traits reported in the

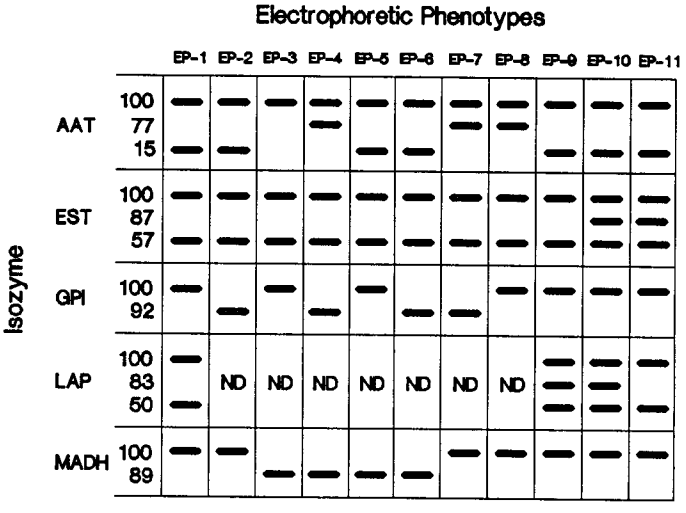


**Fig. 1.** Electrophoretic phenotypes of isozyme polymorphisms identified in *Cochliobolus carbonum*. AAT = aspartate aminotransferase, EST = esterase, GPI = glucose-phosphate isomerase, LAP = leucine aminopeptidase, MADH = mannitol dehydrogenase, and ND = isozyme bands not detected. For each isolate listed in Table 1, two complete gel sets, consisting of independently prepared mycelial extracts, were run using the 19 enzymes listed in Table 2. Each gel set was replicated twice.

literature (11). Of the 19 enzymes tested, five were polymorphic: aspartate aminotransferase (AAT), esterase (EST), glucose-phosphate isomerase (GPI), leucine aminopeptidase (LAP), and mannitol dehydrogenase (MADH) (Fig. 1). The remaining 14 enzymes were found to be monomorphic with the set of *C. carbonum* isolates tested and under the electrophoretic conditions and buffer systems used in this study.

Isolates with isozyme-banding patterns (Fig. 1) in common were grouped into 11 different electrophoretic phenotypes (Table 3). The ability to group isolates that have the same race phenotype by their electrophoretic phenotype is readily apparent even with the small number of isozyme polymorphisms detected in this study. Both race 1 isolates grouped within EP-1, which was not unexpected because both isolates were obtained from the same source. The limited number of race 1 isolates used in this study is a function of the rare occurrence of race 1 isolates in nature (1) and places some restrictions on inferences that can be drawn from this data. The majority of race 3 isolates also were associated with a single phenotype, EP-10, despite the range of years and locations in which the isolates were collected. Of the three isolates that did not group within EP-10, isolate 76-2356 differed only by the absence of the EST-87 band (EP-9), while isolates 85-1954 and 85-2557 (EP-11) differed from EP-10 by the absence of the LAP-83 band.

The association of isolates belonging to the same race with electrophoretic phenotypes differing by only a single step was not observed with race 2 isolates. Seven distinct electrophoretic phenotypes associated with race 2 isolates were identified (Table 3). No distinct polymorphisms were characteristic of race 2, and the isozyme polymorphisms characterizing these electrophoretic phenotypes could be found in one or more race 1 or race 3 isolates. The lack of data for LAP from race 2 isolates was unexpected. No enzyme activity was observed for race 2 isolates in any of the gel sets run. The lack of enzyme staining was treated as missing data when analyzed using the NT-SYS UPGMA program.

The overall similarity among all isolates of *C. carbonum* was approximately 83% (Table 4). Genetic similarity index values were used to generate a phenogram that is shown in Figure 2. The highest average similarity was seen within race 3 and race 1 isolates. Both EP-9 and EP-10 had an average genetic similarity index greater than 0.97, and the overall average genetic similarity with all race 3 isolates was 0.97. Surprisingly, the two race 1 isolates making up EP-1 were more similar to EP-11 than all race 3 isolates taken together. As suggested by the large number of electrophoretic phenotypes containing race 2 isolates, a lower overall average similarity within race 2 (0.93) was seen when compared with that observed between race 1 and race 3 pathotypes (0.97). Two electrophoretic phenotypes within race 2, EP-3 and EP-5, were found to be more similar to races 1 and 3 than to the majority of race 2 isolates.

TABLE 3. A summary of electrophoretic phenotypes of *Cochliobolus carbonum* isolates used in this study

| EP[a] | Isolate | Pathotype | Location | Phenotype frequency |
|---|---|---|---|---|
| EP-1 | 73-4 | 1 | IL | 0.09 |
| | *MAT2* | 1 | IL | |
| EP-2 | 82-1508 | 2 | MN | 0.14 |
| | 83-1808 | 2 | NY | |
| | 84-4531 | 2 | NC | |
| EP-3 | 83-1038 | 2 | MI | 0.05 |
| EP-4 | 84-2546 | 2 | NE | 0.05 |
| EP-5 | 84-3201 | 2 | IN | 0.09 |
| | 84-3666 | 2 | MO | |
| EP-6 | 85-5 | 2 | IL | 0.05 |
| EP-7 | 85-2215 | 2 | IA | 0.05 |
| EP-8 | *MAT1* | 2 | IL | 0.05 |
| EP-9 | 76-2356 | 3 | VA | 0.05 |
| EP-10 | 81-1843 | 3 | NY | 0.29 |
| | 82-4412 | 3 | VA | |
| | 82-4439 | 3 | VA | |
| | 84-3306 | 3 | PA | |
| | 84-5027 | 3 | VA | |
| | 84-5041 | 3 | VA | |
| EP-11 | 85-1954 | 3 | OH | 0.09 |
| | 85-2557 | 3 | PA | |

[a]See Figure 1 for description of isozyme banding pattern for each electrophoretic phenotype (EP).

## DISCUSSION

Isozyme analysis was found to be extremely useful in identifying specific races of *C. carbonum* on the basis of electrophoretic phenotypes (Table 3). Although the extent of genetic diversity in *C. carbonum* was similar to results obtained by Leonard and co-workers (11,12,16), the ability to distinguish or "fingerprint" specific races by their electrophoretic phenotypes was unexpected. Earlier studies using isozyme analysis to estimate the extent of genetic diversity in populations of phytopathogenic fungi detected associations of specific electrophoretic patterns with host specificity, with vegetative compatibility, and with groups of races having similar virulence characteristics (2,3,5,15). To our knowledge this is the first report in which isozyme analysis was used to fingerprint specific races of a pathogen. Similar results using isozyme analysis in *Septosphaeria turcica,* causal agent of northern corn leaf blight, have been obtained by the authors (manuscript in preparation).

The association of virulence phenotypes with electrophoretic phenotypes, combined with the apparent lack of sexual repro-

TABLE 4. Genetic similarity[a] between electrophoretic phenotypes of *Cochliobolus carbonum*

| | Electrophoretic phenotypes[b] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | EP-1 | EP-2 | EP-3 | EP-4 | EP-5 | EP-6 | EP-7 | EP-8 | EP-9 | EP-10 | EP-11 |
| EP-1 | 1.00 | 0.95 | 0.92 | 0.85 | 0.95 | 0.90 | 0.90 | 0.95 | 0.98 | 0.96 | 0.98 |
| EP-2 | | 1.00 | 0.87 | 0.90 | 0.90 | 0.95 | 0.95 | 0.90 | 0.95 | 0.93 | 0.93 |
| EP-3 | | | 1.00 | 0.93 | 0.97 | 0.92 | 0.87 | 0.92 | 0.92 | 0.90 | 0.90 |
| EP-4 | | | | 1.00 | 0.90 | 0.95 | 0.95 | 0.90 | 0.85 | 0.83 | 0.83 |
| EP-5 | | | | | 1.00 | 0.95 | 0.85 | 0.90 | 0.95 | 0.93 | 0.93 |
| EP-6 | | | | | | 1.00 | 0.90 | 0.85 | 0.90 | 0.89 | 0.89 |
| EP-7 | | | | | | | 1.00 | 0.95 | 0.90 | 0.88 | 0.88 |
| EP-8 | | | | | | | | 1.00 | 0.95 | 0.93 | 0.93 |
| EP-9 | | | | | | | | | 1.00 | 0.98 | 0.96 |
| EP-10 | | | | | | | | | | 1.00 | 0.98 |
| EP-11 | | | | | | | | | | | 1.00 |

[a]Genetic similarity determined using the Dice coefficient, $S_{xy} = 2n_{xy}/(n_x + n_y)$ (see 20).
[b]Electrophoretic phenotypes are described in Table 3.

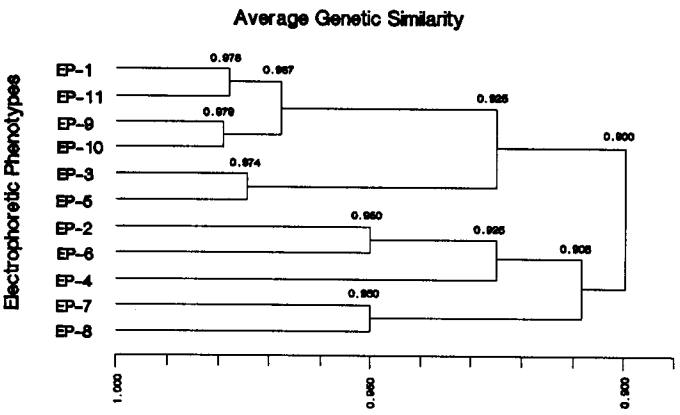


**Fig. 2.** Phenogram of isozyme polymorphism similarities in *Cochliobolus carbonum* produced by the Numerical Taxonomy System of Multivariate Statistical Programs (NT-SYS; 24). Genetic distances between electrophoretic phenotypes indicated in Table 4 were used with the unweighted pair-group method with arithmetic mean (UPGMA) to generate the phenogram.

duction in nature and the restricted gene flow between races (11,12,16), suggests that populations in *C. carbonum* are composed of clonally propagated subpopulations. Variation within a pathotype subpopulation might arise through successive stepwise mutations within a clonal lineage, resulting in electromorph variation within pathotypes (6,29). Similar observations have been made in the rice (*Oryza sativa*)-*Pyricularia grisea* pathosystem, in which specific clonal subpopulations, which have distinct pathotype phenotypes, were identified using a host-specific middle repetitive DNA probe (14). If the *C. carbonum* population structure is composed of clonal lineages, the occurrence of multiple electrophoretic phenotypes in race 2 would support the conclusions of Leonard and Leath (12) that race 2 has evolved over a longer period of time than race 3. In addition, the similarity between races 1 and 3 and the race 2 isolates within EP-3 and EP-5 might suggest a common precursor, but with the limited number of isolates used in this study, the possibility of random associations because of sampling error cannot be ruled out.

The ability to differentiate races of *C. carbonum* on the basis of isozyme analysis provides a useful tool to follow the evolution of a fungal plant pathogen. Different mechanisms of pathogenicity in this pathogen suggest a complex evolutionary history. Panaccione and co-workers (22) have cloned the *Tox1* locus from race 1, which contains the genes necessary for HC-toxin biosynthesis. No homology to the *Tox1* locus was found in either race 2 or race 3, suggesting an independent origin of this virulence factor. A more recent example of evolution in the pathogen is the identification of a new pathotype of *C. carbonum,* designated race 4 (9). Race 4 has a lesion phenotype distinct from races 1, 2, and 3, and it is virulent on maize genotypes that are resistant to race 3. The recent occurrence of this race provides an opportunity to use isozyme analysis to determine where it originated. The extent of isozyme polymorphisms found in *C. carbonum* also will be useful in genetic analysis. Isozyme polymorphisms can be used as neutral genetic markers to determine the inheritance of virulence (17,28) and also to enhance the use of DNA methodology, such as RFLP analysis (19) and polymerase chain reaction techniques (32–34), by providing an initial screen to determine degree of polymorphism present in a particular isolate.

## LITERATURE CITED


1. Blanco, M. H., Nelson, R. R., Ayers, J. E., Hill, J. P., and Dalmacio, S. 1974. Racial composition of *Helminthosporium maydis* and *H. carbonum* on corn hybrids in normal cytoplasm in Pennsylvania in 1973. Plant Dis. Rep. 58:934-936.
2. Bonde, M. R., Peterson, G. L., Dowler, W. M., and May, B. 1984. Isozyme analysis to differentiate species of *Peronosclerospora* causing downy mildews of maize. Phytopathology 74:1278-1283.
3. Bosland, P. W., and Williams, P. H. 1987. An evaluation of *Fusarium oxysporium* from crucifers based on pathogenicity, isozyme polymorphism, vegetative compatibility, and geographic origin. Can. J. Bot. 65:2067-2073.
4. Burdon, J. J., Luig, N. H., and Marshall, D. R. 1983. Isozyme uniformity and virulence variation in *Puccinia graminis* f. sp. *tritici* and *P. recondita* f. sp. *tritici* in Australia. Aust. J. Bot. 36:403-410.
5. Burdon, J. J., and Roelfs, A. P. 1985. Isozyme and virulence variation in asexually reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. Phytopathology 75:907-913.
6. Burdon, J. J., and Roelfs, A. P. 1985. The effect of sexual and asexual reproduction on the isozyme structure of populations of *Puccinia graminis.* Phytopathology 75:1068-1073.
7. Clayton, J. W., and Tretiak, D. N. 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. J. Fish. Res. Board Can. 29:1169-1172.
8. Dalmacio, S. C., MacKenzie, D. R., and Nelson, R. R. 1976. Heritability of differences in virulence between races of 2 and 3 of *Cochliobolus carbonum.* Philipp. Phytopathol. 15:47-50.
9. Dodd, J. L., and Hooker, A. L. 1990. Previously undescribed pathotype of *Bipolaris zeicola* on corn. Plant Dis. 74:530.
10. Hooker, A. L., Mesterhazy, A., Smith, D. R., and Lim, S. M. 1973. A new Helminthosporium leaf blight of corn in the Northern corn belt. Plant Dis. Rep. 57:195-198.
11. Leonard, K. J. 1978. Polymorphisms for lesion type, fungicide tolerance, and mating capacity in *Cochliobolus carbonum* isolates pathogenic to corn. Can. J. Bot. 56:1809-1815.
12. Leonard, K. J., and Leath, S. 1990. Genetic diversity in field populations of *Cochliobolus carbonum* on corn in North Carolina. Phytopathology 80:1154-1159.
13. Leung, H., and Williams, P. H. 1986. Enzyme polymorphism and genetic differentiation among geographic isolates of the rice blast fungus. Phytopathology 76:778-783.
14. Levy, M., Romano, J., Marchetti, M. A., and Hamer, J. E. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell 3:95-102.
15. Liu, Z., Nickrent, D. L., and Sinclair, J. B. 1990. Genetic relatedness among isolates of *Rhizoctonia solani* anastomosis group-2 based on isozyme analysis. Can. J. Plant Pathol. 12:376-382.
16. Lodge, D. J., and Leonard, K. J. 1990. A cline and other patterns of genetic variation in *Cochliobolus carbonum* isolates pathogenic to corn in North Carolina. Can. J. Bot. 62:995-1005.
17. Melchinger, A. E. 1990. Uses of molecular markers in breeding for oligogenic disease resistance. Plant Breeding 104:119.
18. Micales, J. A., Bonde, M. R., and Peterson, G. L. 1986. The use of isozyme analysis in fungal taxonomy and genetics. Mycotaxon 27:405-449.
19. Michelmore, R. W., and Hulbert, S. H. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Annu. Rev. Phytopathol. 25:383-404.
20. Nei, M., and Li, W. H. 1979. Mathematical models for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76:5269-5273.
21. Nelson, R. R., Blanco, M. H., Dalmacio, S., and Shain-Moore, B. 1973. A new race of *Helminthosporium carbonum* on corn. Plant Dis. Rep. 57:822-823.
22. Panaccione, D. G., Scott-Craig, J. S., Pocard, J.-A., and Walton, J. D. 1990. Characterization of the Tox locus of *Cochliobolus carbonum.* (Abstr.) Phytopathology 80:1009.
23. Pringle, R. B., and Scheffer, R. P. 1967. Isolation of the host-specific toxin and a related substance with nonspecific toxicity from *Helminthosporium carbonum.* Phytopathology 57:1169-1172.
24. Rohlf, J. 1985. Numerical Taxonomy System of Multivariate Statistical Programs. Department of Ecology and Evolution, State University of New York at Stony Brook, Stony Brook.
25. Scheffer, R. P., Nelson, R. R., and Ullstrup, A. J. 1967. Inheritance of toxin production and pathogenicity in *Cochliobolus carbonum* and *Cochliobolus victoriae.* Phytopathology 57:1288-1291.
26. Selander, R. K., Smith, M. H., Yang, S. Y., Johnson, W. E., and Gentry, J. B. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus.* I. Variation in the old-field mouse (*Peromyscus polionotus*). Univ. Tex. Publ. 103:49-90.
27. Soltis, D. E., Haufler, C. H., Darrow, D. G., Gastony, G. J. 1983. Starch gel electrophoresis of ferns: A compilation of grinding buffers, gel and electrode buffers, and staining schedules. Am. Fern J. 73:9-27.
28. Spielman, L. J., Sweigard, J. A., Shattock, R. C., and Fry, W. E. 1990. The genetics of *Phytophthora infestans:* Segregation of allozyme markers in F2 and backcross progeny and the inheritance of virulence against potato resistance genes R2 and R4 in F1 progeny. Exp. Mycol. 14:57-69.
29. Tooley, P. W., Fry, W. E., and Villarreal-Gonzalez, M. J. 1985. Isozyme characterization of sexual and asexual *Phytophthora infestans* populations. J. Hered. 76:431-435.
30. Tuite, J. 1969. Plant Pathological Methods. Burgess Publishing Co., Minneapolis, MN.
31. Ullstrup, A. J. 1941. Inheritance of susceptibility to infection by *Helminthosporium maydis* race 1 in maize. J. Agric. Res. (Washington, D.C.) 63:331-334.
32. Vilgalys, R., and Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J. Bacteriol. 172:4238-4246.
33. Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213-7218.
34. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingley, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.