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# USE OF ISOZYME ELECTROPHORESIS TO DIFFERENTIATE AMONG ISOLATES OF *Macrophomina phaseolina*

**Aly, A.A.[1]; M.A. Abdel-Sattar[2] and M.R. Omar[1]**

**[1] Plant Pathology Research Institute, Agric. Res. Center, Giza, Egypt.**

**[2] Dept. of Agric. Bot., Faculty of Agric., Suez Canal Univ., Ismailia, Egypt.**

## ABSTRACT

The banding patterns of malate dehydrogenase (EC1.1.1.37), glycerol dehydrogenase (EC1.1.1.72), peroxidase (EC1.11.1.7), esterase (EC3.1.1.1), and acid phosphatase (EC3.1.3.2) were determined by polyacrylamide gel electrophoresis for 28 isolates of *Macrophomina phaseolina*. A total of 96 phenotypes was detected for all the enzymes. Esterase gave the greatest number of polymorphic banding patterns. Cluster analysis of the isozyme banding patterns by the unweighted pair-group method based on arithmetic means placed the isolates in several groups; however, geographic origin of the isolates or source (host) did not exhibit clear correlation with the isozyme electrophoretic grouping results.

## INTRODUCTION

*Macrophomina phaseolina* (Tassi) Goid., is a deuteromycete soil-borne fungus found throughout the world. It is also one of the most destructive plant pathogens causing charcoal rot, dry-root rot, wilt, leaf blight, stem blight, and damping-off diseases in a wide range of host plants (Dhingra and Sinclare, 1978). *M. phaseolina* is a monotypic species with a wide host range involving more than 500 species in 75 families (Sinclair, 1982 and Wyllie, 1988).

Differentiation among the pathogenic isolates of *M. phaseolina* is important for improving our understanding of the ecology of these isolates and the epidemiology of the diseases caused by this pathogen. The conventional method of differentiation among pathogen isolates is the observation of the differences in virulence when the isolates interact with a set of host genotypes (Aly, 1988; Ahmed *et al.*, 1991; Schilder and Bergstrom, 1990, and Porta-Puglia *et al*., 1996). However, this method is expensive, time consuming, and may be influenced by variability inherent in the experimental system (Aly, 1988 and Bhatti and Kraft, 1992). Furthermore, the differential disease reactions do not provide information about the genetic relationship among the pathogen isolates (Perez-Artes *et al*., 1995). Therefore, another reliable method, either alternative or complementary to that based on the differential interaction between *M. phaseolina* isolates and host genotypes, is required for identification of the pathogenic isolates of *M. phaseolina*. Electrophoresis of isozymes seems to be a suitable method to achieve this goal because the amino acids sequences of polypeptides (components of isozymes) are dependent on nucleotide sequences of their coding genes; therefore an analysis of isozymic variation among isolates of *M. phaseolina*, approximates an analysis of their genetic variation (Markert and Faulhaber, 1965).

Isozymes (Isoenzymes) are defined as genetically determined multiple molecular forms of an enzyme. There are three main causes of formation of multiple molecular forms of enzymes. These are 1) the presence of more than one gene locus coding for the enzyme, 2) the presence of more than one allele at a single gene locus coding for the enzyme, and 3) the post-translation modifications of the formed enzymatic polypeptides resulting in formation of nongenetic or so-called "secondary" isozymes. The term isozymes is usually used to denote multiple molecular forms deriving from different genetic loci, where the term "allozymes" is used to denote multiple molecular forms deriving from different alleles of the same genetic locus. The term "allelic isozymes" is also used by some isozymologists (Manchenko, 1994). Isozymes 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 (Bonde *et al.*, 1993).

Electrophoresis of isozymes has been widely used for studying variation in fungal populations. For example, Sweetingham *et al.* (1986) examined the pectic isozymes of 140 isolates of *Rhizoctonia*-like fungi by electrophoresis. The isolates were placed in 11 distinct zymogram groups (ZGs). Isolates within a ZG had a similar cultural and morphological appearance and were all either multinucleate or binucleate. Neate *et al.* (1988) proposed pectic zymogram patterns as a fast and reliable method of determining AG of *R. solani*. Liu *et al.* (1990) studied the genetic relationship among 14 isolates of *R. solani* (AG-2) by evaluating electrophoretic data derived from 11 enzyme systems. Three closely related groups, designated A, B and C, were differentiated by isozyme analysis. Laroche *et al.* (1992) applied isozyme electrophoresis to study the genetic relationship between AGs 3 and 9 of *R. solani*. The banding patterns of seven enzymes were determined by protein PAGE. A total of 76 phenotypes were detected for all the enzymes. Diaphorase gave the most polymorphic banding patterns, followed by esterase and malate dehydrigenase. On the basis of principal components analysis and cluster analysis, the isolates were subsequently divided into two dissimilar and genetically distant groups (I and II). These groups were in agreement with previous AGs 3 and 9. Group I represented all isolates belonging to Ag-9. Group II represented all isolates belonging to AG-3. The isozymes results indicate that the anastomosis grouping concept is genetically based. Damaj *et al.* (1993) characterized 50 isolates of binucleate *Rhizoctonia* representing 12 Japanese and five North American anastomosis groups (AGs) by isozyme electrophoretic patterns. Of the 23 enzyme systems screened, eight enzymes with a total of 63 phenotypes were applied to study the genetic relationship among the AGs. Hexokinase and malate dehydrogenase displayed the most polymorphic banding patterns. Cluster analysis of isozyme bands generated four distinct groups (I, II, III, and IV). Isozyme groups of binucleate *Rhizoctonia* were consistent with prior groupings determined by hyphal anastomosis and by DNA restriction pattern analysis. This study provides evidence that isozyme phenotypes are good indicators of genetic diversity among anastomosis groups of binucleate

*Rhizoctonia* species. MacNish *et al.* (1993) used pectic isozyme (Zymogram) to demonstrate the existence of five distinct groups within *R. solani* AG-8.

In the present study, polyacrylamide gel electrophoresis (PAGE) of five enzyme systems (malate dehydrogenase, glycerol dehydrogenase, peroxidase, esterase, and acid phosphatase) was employed as biochemical tool to differentiate among 28 isolates of *M. phaseolina*.

## MATERIALS AND METHODS

### Fungal isolates

Isolates of *M. phaeolina* (Table 1) used in the present study were obtained from the fungal collection of Cotton Disease Research Section, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt. These isolates were originally recovered from cotton and other hosts.

**Table 1. Geographic origins and sources of *M. phaseolina* isolates used in electrophoresis.**

| Isolate no. | Geographic origin | Source |
|---|---|---|
| 1 | Giza | Soybean |
| 2 | Giza | Sunflower |
| 3 | Beheira | Cotton |
| 4 | Kafr E-Skeikh | Cotton |
| 5 | Faiyoum | Sesame |
| 6 | Giza | Sesame |
| 7 | Beheira | Cotton |
| 8 | Giza | Cotton |
| 9 | Daqahliya | Cotton |
| 10 | Daqahliya | Cotton |
| 11 | Kafr El-Sheikh | Cotton |
| 12 | Giza | Soybean |
| 13 | Gharbiya | Cotton |
| 14 | Sharqiya | Cotton |
| 15 | Assiute | Cotton |
| 16 | Minya | Cotton |
| 17 | Assiute | Cotton |
| 18 | Giza | Cotton |
| 19 | Giza | Sunflower |
| 20 | Gharbiya | Cotton |
| 21 | Sohag | Cotton |
| 22 | Nobariya | Sesame |
| 23 | Giza | Sunflower |
| 24 | Minufiya | Cotton |
| 25 | Sohag | Cotton |
| 26 | Minufiya | Cotton |
| 27 | Sharqiya | Cotton |
| 28 | Minya | Purslane |

**Extraction of fungal proteins**

Protein extracts from *M. phaseolina* isolates were prepared according to Hussein (1992) in the following way: Fungal isolates were grown for 22 days at 22-30°C on liquid Czapek medium. The mycelium was harvested by filtration through cheesecloth, washed with distilled water several times, and freeze-dried. This frozen mycelium was suspended in phosphate buffer pH 8.3 (1-3 ml/g mycelium), mixed thoroughly with glass beads, and ground in liquid nitrogen to a fine powder. The ground mycelium was centrifuged at 19,000 rpm for 30 minutes at 0°C. The protein content in the supernatant was estimated according to Bradford (1976) by using bovine serum albumin as a standard protein. If protein concentration was low, protein was precipitated from the clarified supernatant by adding ammonium sulphate at 70% of saturation (60g/100 ml) then kept in the refrigerator for 30 hr. Pellets, collected by centrifugation at 11,000 rpm for 30 minutes, were resuspended in phosphate buffer pH 8.3 and subjected to dialysis for 24 hr against the buffer and centrifugation at 11,000 rpm for 30 minutes. Protein was estimated in the obtained supernatant.

**PAGE of native proteins**

Thawed protein-extract supernatant was mixed with an equal volume of a solution containing 20% glycerol (vol/vol) and 0.1% bromophenol blue (vol/vol) in 0.15 M tris-HCl, pH 6.8. Twenty microliters of the resulting suspension (40 to 60 μg of protein) was subjected to electrophoresis in 25 mM Tris buffer containing 192 mM glycine at pH 8.3. Electrophoresis was conducted at room temperature (approximately 20 to 25°C) for 9 hr on a 15% polyacrylamide gel with a 6% stacking gel, at 20 and 10 mA, respectively, until the dye reached the bottom of the separating gel (Laemmli, 1970 and Latrre *et al.*, 1995). Electrophoresis was performed in a vertical slab mold (16.5x14.5x0.1 cm). Gels were stained according to Manchenko (1994) for the detection of malate dehydrogenase (EC1.1.1.37), glycerol dehydrogenase (EC1.1.1.72), peroxidase (EC1.11.1.7), esterase (EC3.1.1.1), and acid phosphatase (EC3.1.3.2.) isozymes.

**Gel analysis**

A gel documentation system (Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6C, Fullerton CA 92631) was used to document the results of PAGE and to cluster the electrophoretic patterns by the unweighted pair-group method based on arithmetic mean (UPGMA).

## RESULTS AND DISCUSSION

Isozyme analysis includes the results obtained from five enzyme systems resolved on polyacrylamide gels. Bands of each enzyme system (Figs. 1, 3, 5, 7, and 9) constituted a number of enzyme banding patterns (zymograms), which varied from one enzyme system to another. Thus, 19, 17, 20, 27, and 13 zymograms were found for malate dehydrogenase, glycerol dehydrogenase, peroxidase, esterase, and acid phosphatase, respectively (Figs. 2, 4, 6, 8, and 10). The geographic origin of the isolates or source (host) did not exhibit clear correlation with the isozyme electrophoretic grouping results.

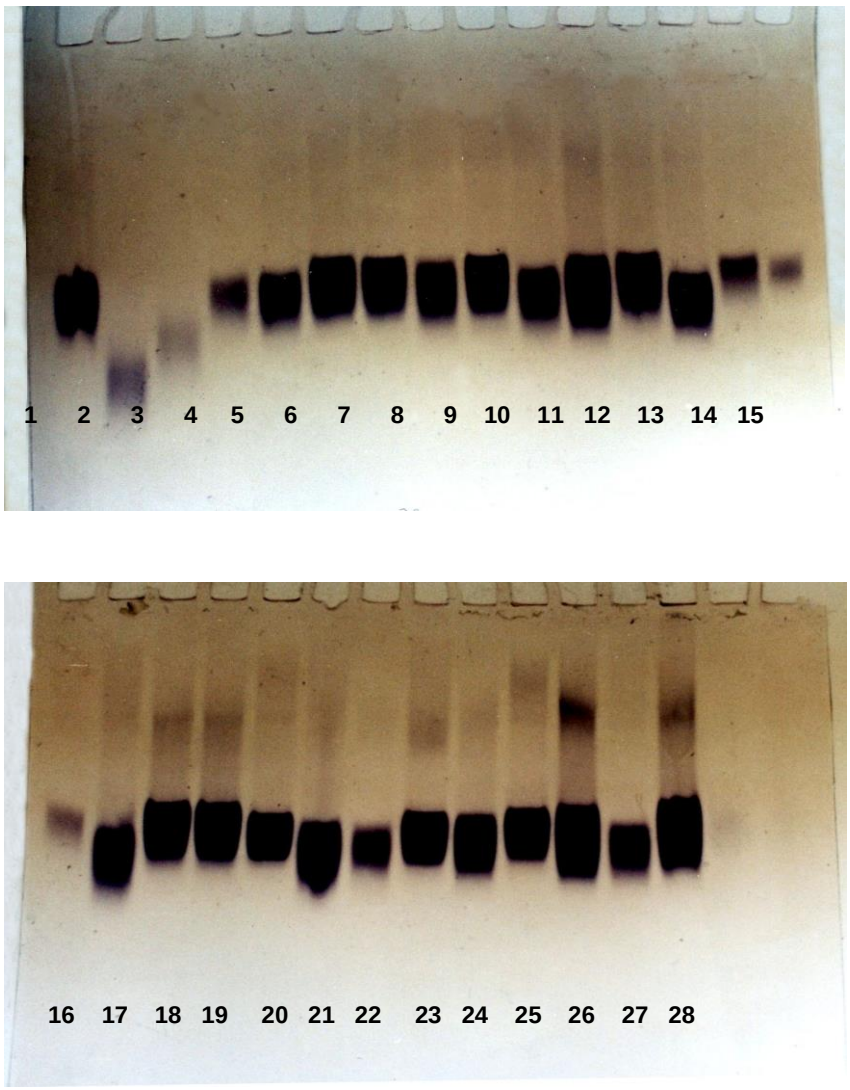


**Fig. 1. Malate dehydrogenase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

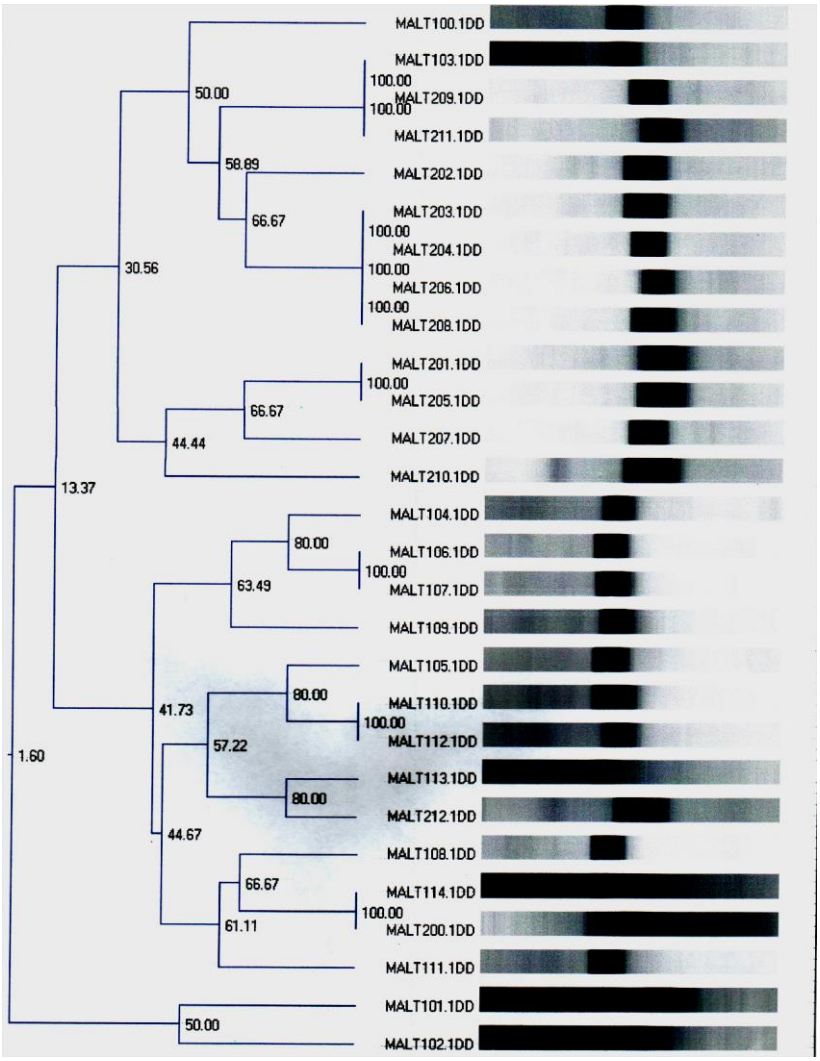


**Fig. 2. Phenogram based on average linkage cluster analysis of electrophoretic malate dehydrogenase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

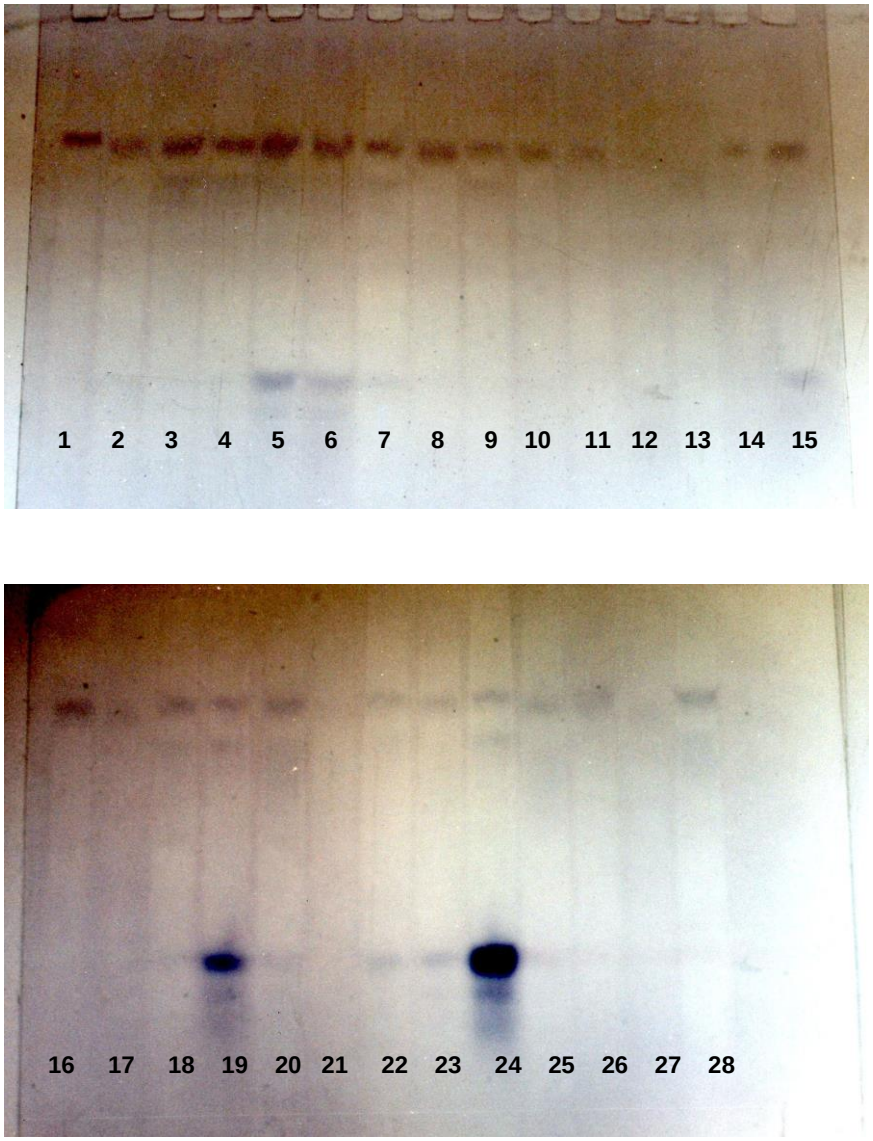


**Fig. 3. Glycerol dehydrogenase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

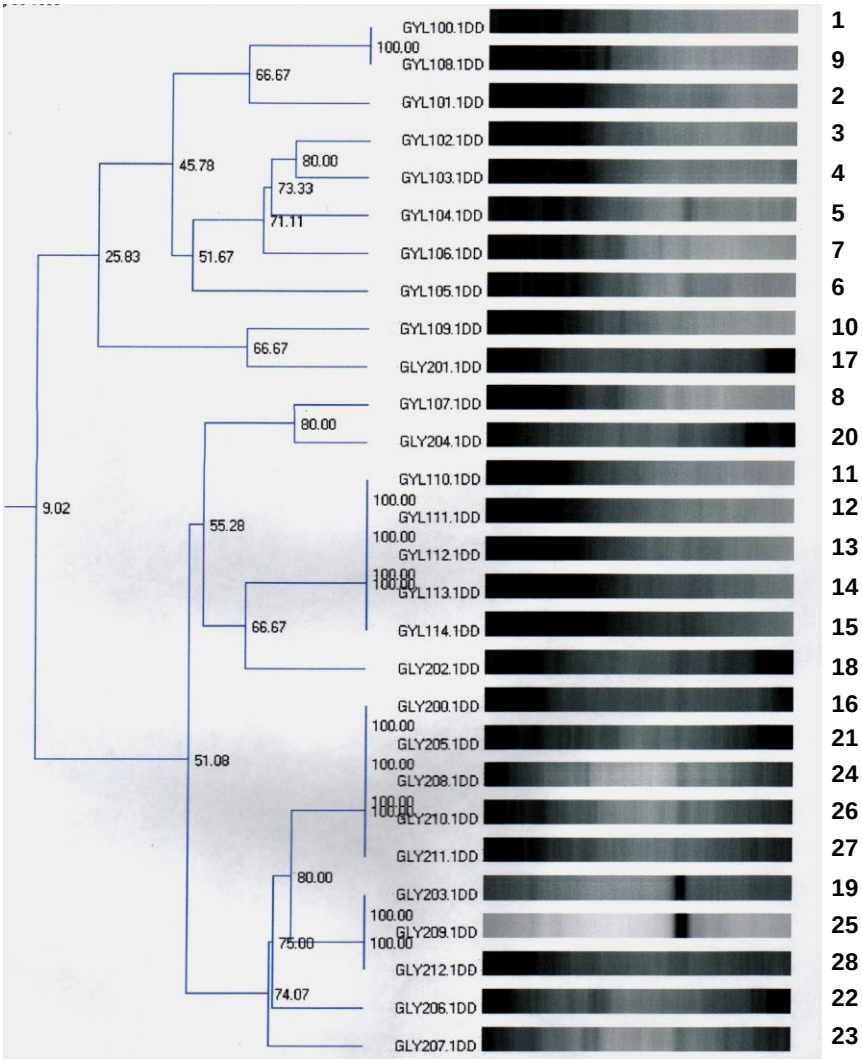


**Fig. 4. Phenogram based on average linkage cluster analysis of electrophoretic glycerol dehydrogenase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

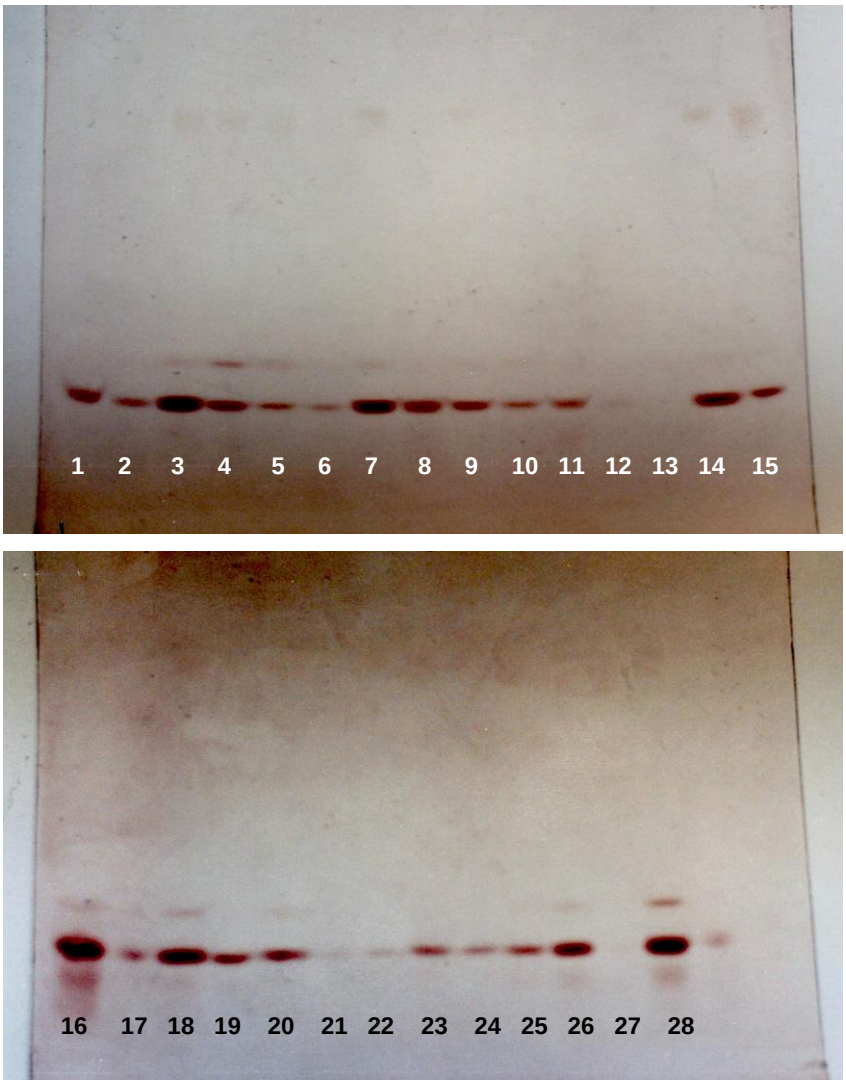


**Fig. 5. Peroxidase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

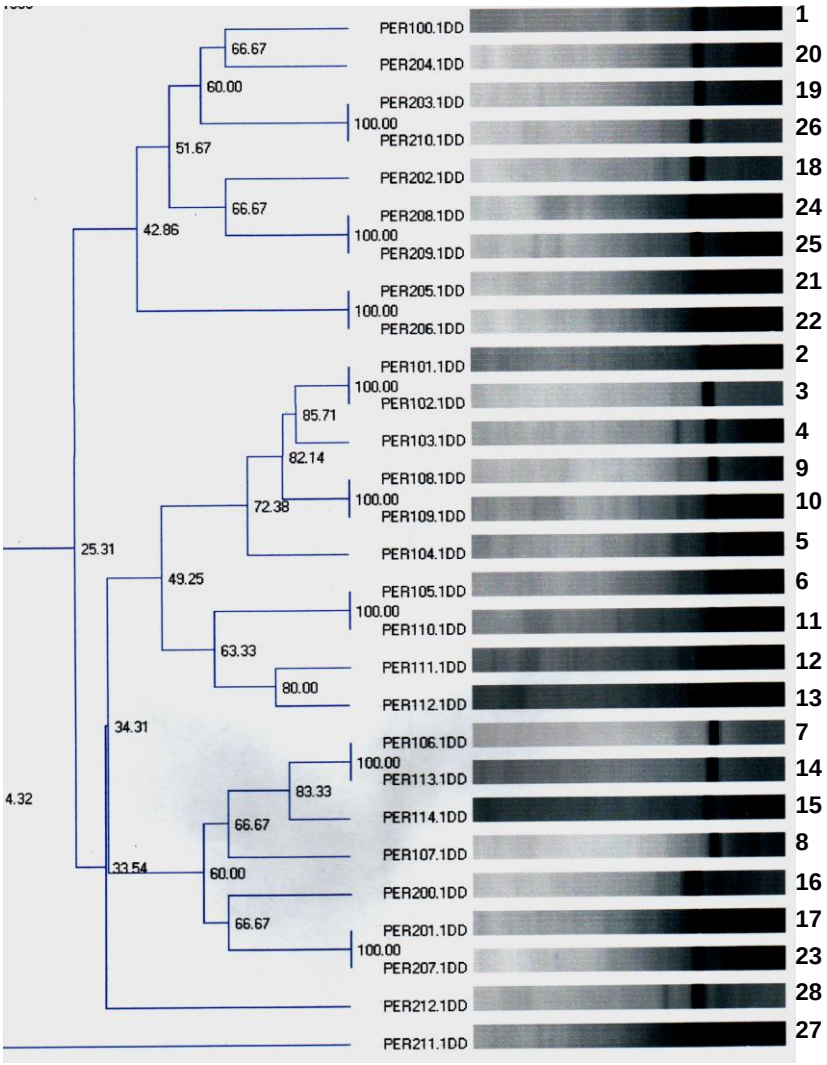


**Fig. 6. Phenogram based on average linkage cluster analysis of electrophoretic peroxidase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

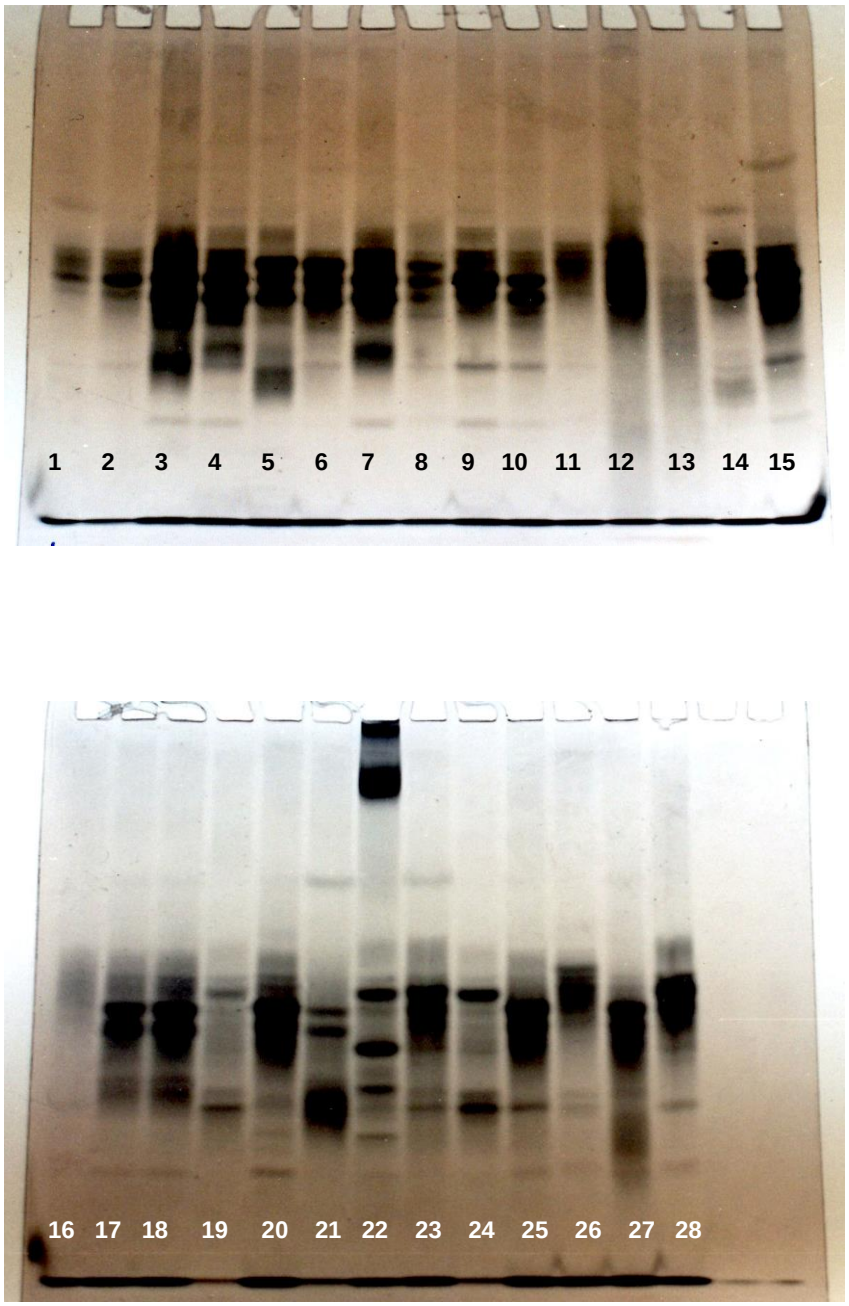


**Fig. 7. Esterase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

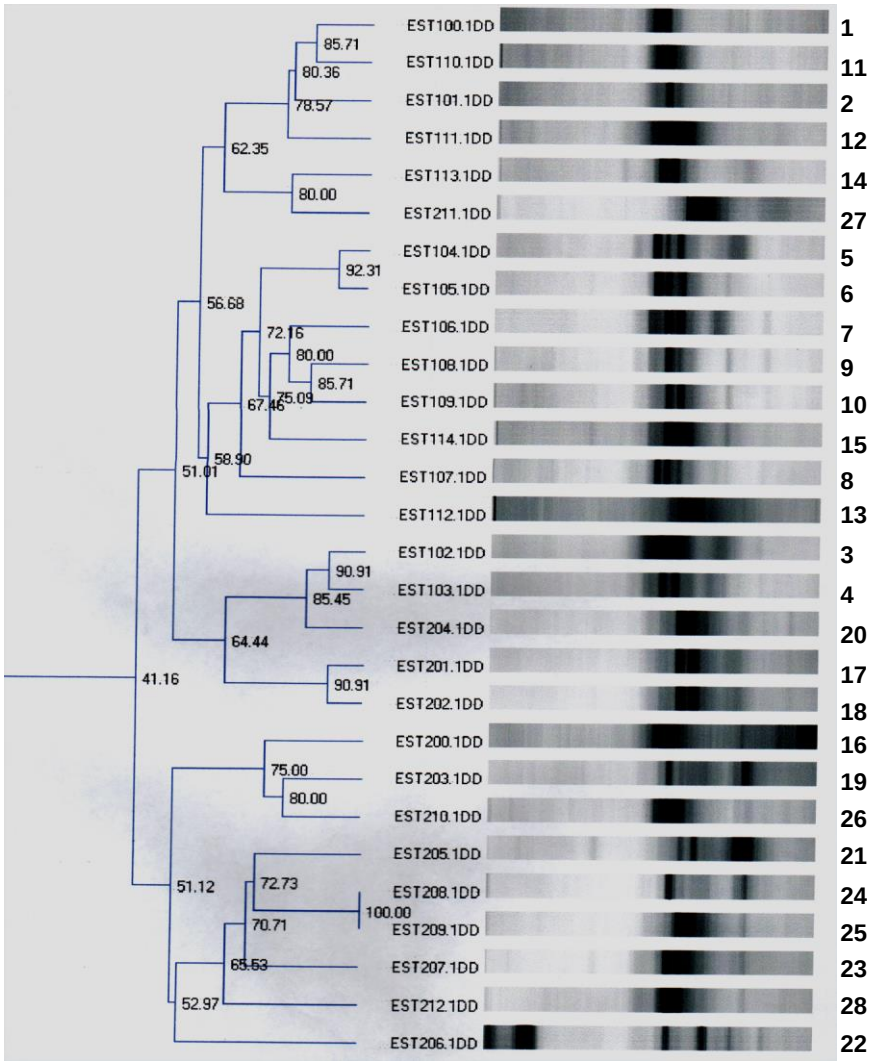


**Fig. 8. Phenogram based on average linkage cluster analysis of electrophoretic esterase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

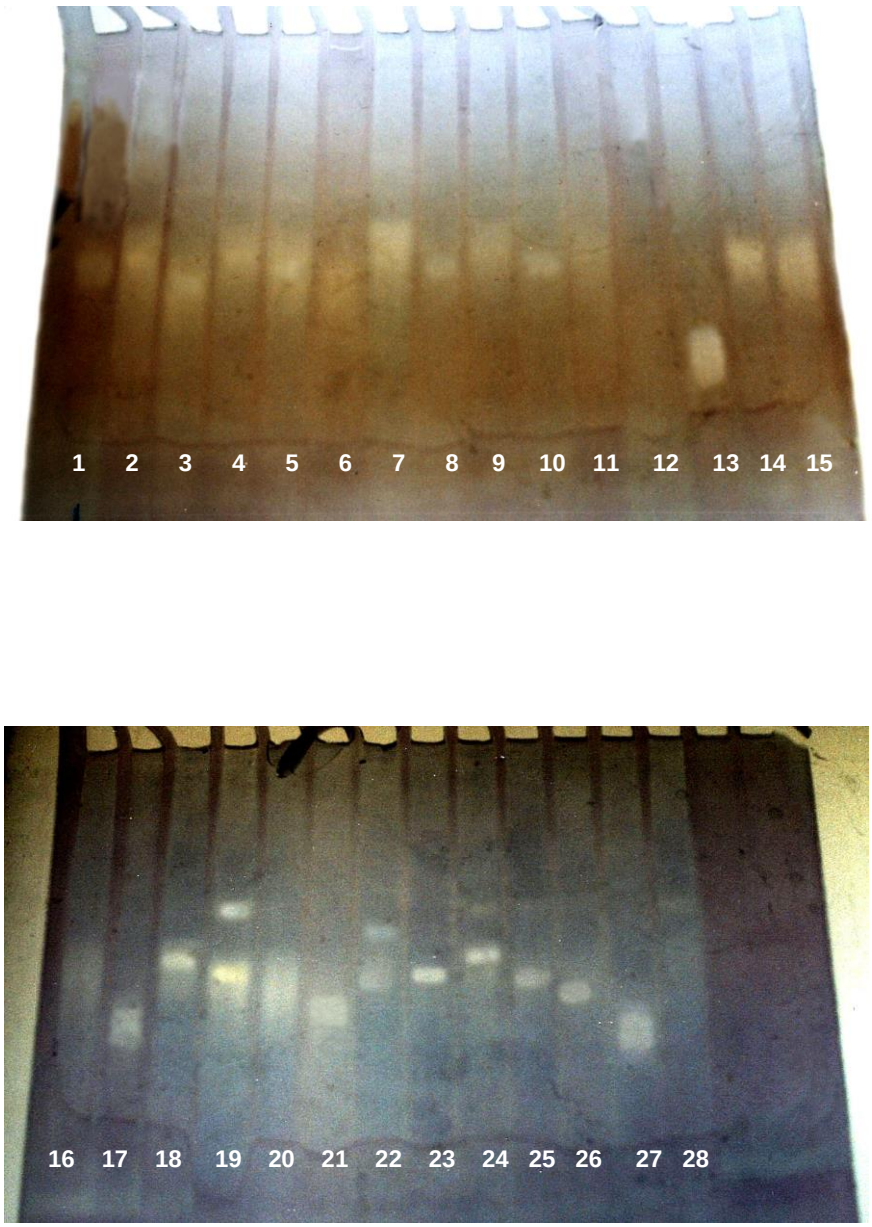


**Fig. 9. Acid phosphatase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

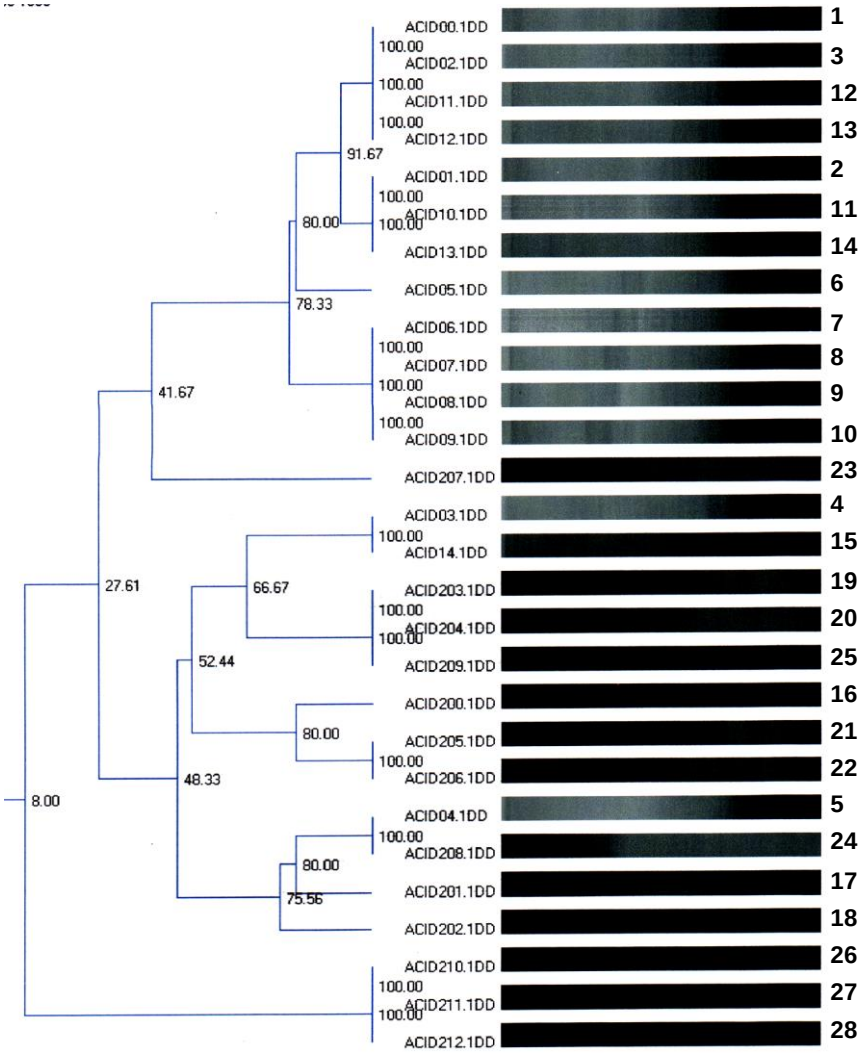


**Fig. 10. Phenogram based on average linkage cluster analysis of electrophoretic acid phosphatase isozyme patterns obtained by PAGE from 28 isolates of *M. phaseolina*.**

Isozyme analysis was chosen to identify genetic diversity among *M. phaseolina* isolates because this technique for isolate characterization offers several advantages over techniques for total protein visualization. Isozyme banding patterns are less complex than total protein patterns and are easier to differentiate and interpret. Also, isozyme analyses provide information about protein function, whereas other electrophoretic techniques only separate proteins on the basis of physical properties (Nygaard *et al*., 1989).

The present study included only 28 isolates of *M. phaseolina*. It is unlikely that this limited number of isolates represents the full range of variation within the fungus. Despite this limitation, a high level of isozymic variation was observed among the isolates. At first, this finding was surprising because a low level of genetic variation is usually observed in populations of fungi that do not reproduce sexually as occurs with *M. phaseolina*; however, in retrospect, it may suggest that parasexualism with fusion of cells from different hyphae may form heterokaryons that contribute to the variation identified. Isozymic variability in the pathogen population may also reflect the lack of resistance among the currently cultivated commercial host cultivars (Almeida *et al.*, 2003).

The high isozymic similarity observed among isolates from different governorates is strong evidence suggesting that such isolates did not evolve independently from each other; therefore, they may be considered as part of the same ancestral population (Almeida *et al.*, 2003). Presumably, the spread of this population from one location to another was by physical means such as seeds, contaminated equipment, or soil infested with microsclerotia.

The isolates of *M. phaseolina* used in the present study were recovered from 5 hosts belonging to 5 different genera. Thus, homogeneity of the isozyme data among isolates from different hosts suggests that selective pressure for host specificity in *M. phaseolina* in the sampled population has been minimal (Tuskan *et al.*, 1990).

The present study demonstrated that the amount of variation in electrophoretic banding patterns of the tested enzyme systems among *M. phaseolina* isolates is adequate for the application of cluster analysis; therefore, the refinement of isozyme analysis employed in the present work by analyzing more isolates of each host in combination with cluster analysis of the resulting zymograms could provide a reliable method for: (1) rapid grouping of isolates; (2) allocation of unknown isolate to a group and its possible identification; (3) storage of large number of isozymic patterns in data banks for reference; (4) information on epidemiological spreading of isolates (Kersters and DeLey, 1975).

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## إستعمال التفريد الكهربى للمشابهات الإنزيمية للتفرقة بين عزلات فطر ماكروفومينا فاسيولينا

**على عبد الهادى على[١] ، محمد أنور عبد الستار[٢] و معوض رجب عمر[١]**

**١- معهد بحوث أمراض النباتات ، مركز البحوث الزراعية ، الجيزة ، مصر.**

**٢- قسم النبات الزراعى ، كلية الزراعة ، جامعة قناة السويس ، الإسماعيلية ، مصر.**

أجريت دراسة مقارنة لأنواع المشابهات الإنزيمية المستخلصة من ٢٨ عزلة لفطر ماكروفومينا فاسيولينا ، وذلك باستعمال تقنية التفريد الكهربى للبروتين الخام. إستعملت خمسة نظم صبغ لإظهار المشابهات الإنزيمية لكل من ماليت ديهيدروجنيز وجليسرول ديهيدروجنيز وبيرأوكسيديز وإستريز والفوسفاتيز الحامضى. أمكن الحصول على ٩٦ نمط مظهرى للإنزيمات الخمسة. أظهر إنزيم الإستريز أكبر عدد من الأنماط المظهرية. إستعمل إسلوب التحليل العنقودى لتصنيف العزلات إلى مجموعات بناءً على ما بينها من تماثل فى الأنماط المظهرية للإنزيمات وتم التعبير عن النتائج فى فينوجرامات. أظهرت الدراسة أن تقسيم العزلات إلى مجموعات لم يرتبط بأصولها الجغرافية أو بالعائل الذى عزلت منه.