

Full Length Research Paper

Comparison of multi-locus enzyme and protein gel electrophoresis in the discrimination of five *Fusarium* species isolated from Egyptian cottons

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Electrophoretic studies of multilocus-enzymes (MLEE) and whole-cell protein (SDS-PAGE) were carried out in order to evaluate the parity between different methods for the characterization of five *Fusarium* species recovered from cotton-growing areas in Egypt by numerical taxonomy methods. The obtained data revealed that SDS-PAGE and esterase isozymes are more efficient in grouping isolates in their respective species while peroxidase and malate dehydrogenase isozyme has much limited resolution in organizing all isolates in their respective species-specific clusters. A low correlations was detected between geographical origin of isolates and genetic diversity. Results indicate that the estimated inter-specific variation may be more pronounced with protein markers than with isozymes when the two approaches are applied to the same populations. The level of genetic variability detected within and between *Fusarium* spp. accessions with protein and esterase isozymes analysis suggests that it is a reliable, efficient, and effective marker technology for determining genetic relationships in *Fusarium* genus.

Key words: Cotton, *Fusarium*, Isozymes, polyacrylamide gel electrophoresis.

INTRODUCTION

Numerous biochemical and molecular markers are available for the clarification and delineation of fungal taxa. Isozymes studies have proven useful for the identification of fungal cultures at species (Bonde et al., 1991; Petrunak and Christ 1992) or subspecies level (Julian and Lucas 1990; Damaj et al., 1993).

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Abbreviations: MLEE, multilocus-enzymes electrophoresis; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; UPGMA, unweighted pair group method using arithmetic.

Electrophoretic analysis of whole cell proteins by one-dimensional protein patterns provides a rough measure of the number and physicochemical properties of gene products. One-dimensional polyacrylamide gel electrophoresis of proteins has been used extensively for identification and classification at the strain and species level (Snider, 1973). However, as seen with other methods based on the analysis of gene products, effects such as the influence of the composition of growth media on the banding pattern and superposition of proteins cause problems in the interpretation of data. Highly standardized conditions and computerized comparison of the electropherograms are necessary to obtain reproducibility of results obtained from different laboratories. Isozymes are also useful tools for

differentiating fungal taxa at the specific and intra-specific level. Bosland and Williams (1987) were able to define isozyme electrophoretic types, which could be used to differentiate *Fusarium* species and four *F. oxysporum* formae specialis. The role of *Fusarium* spp. as a pathogen of cotton seedlings, and other crops is well known. Although Johnson and Doyle (1986) reported that *Fusarium* spp. were not important pathogens in cotton seedling disease complex, they are often the most frequently isolated fungi. Isolates of *Fusarium* spp. have previously been shown to be pathogenic on seedling cotton (Colyer, 1988; Aly et al., 1996; Zhang et al., 1996). The objectives of the present study were to differentiate the five closely related *Fusarium* species (*F. oxysporum*, *F. moniliforme*, *F. solani*, *F. avenaceum* and *F. chlamydosporum*) through two main approaches: isozyme analysis and protein gel electrophoresis.

MATERIALS AND METHODS

Fungal cultures

Forty-six isolates of *Fusarium* spp. from diverse geographical origins were used. Mycelium production was carried out by culturing the fungi in 100 ml of potato dextrose broth in 500-ml Erlenmeyer flasks and incubating them at 28°C for 6 days. Mycelial mats were then filtered under vacuum on a Büchner funnel, rinsed twice with distilled water and blotted dry. Mycelium to be used for isozyme and protein analyses was freeze-dried and stored at -20°C.

Preparation of protein extracts

Soluble proteins were extracted by grinding 100 mg freeze-dried mycelium with pestle and mortar in liquid nitrogen and 5 ml buffer solution (0.1M Tris-HCl, pH 6.8). The mixture was centrifuged for 20 min at 17,000 rpm and the supernatant collected. The protein content in supernatant was estimated according to the method of Bradford (1976) with bovine serum albumin as standard protein. Protein content was adjusted to 2 mg/ml per sample.

Standard conditions for SDS polyacrylamide gel electrophoresis (protein fingerprinting)

SDS-PAGE was performed by the method described by Laemmli (1970), and modified according to Hames (1995). Proteins were analyzed on 1.5-mm thick and 15-cm long gels run in a dual vertical slab unit (Hoefer Scientific Instruments, San Francisco, CA, USA, Model SE 600 Series Hoefer® Pharmacia Biotech). From each sample, 25 µl of extract was loaded on a polyacrylamide gel. The separation gel (10%) and stacking gel (3.5%) were prepared from an acrylamide monomer solution (Roth, Karlsruhe, Germany). Electrophoresis was carried out at a constant current of 35 mA through the stacking gel, and at 90 mA through the separation gel at room temperature. After electrophoresis, the gels were stained by silver nitrate (Rabilloud et al., 1988).

Sample preparation for isozymes electrophoresis

One-hundred milligrams of freeze-dried mycelium were homogenized by grinding in liquid nitrogen with a mortar and pestle, and suspended in 1.5 ml of extraction buffer (10 mM Tris-HCl, 1

mM EDTA, 0.1 mM ascorbic acid, pH 7). Extracts were chilled in an ice bath for 1-3 h and centrifuged at 18,000 rpm for 30 min at 4 °C. The supernatant was collected, divided into 100-µl aliquots, stored at -80 °C, and used within 6 months.

Multilocus-enzymes electrophoresis

Isozymes electrophoresis was performed in vertical polyacrylamide gels with a discontinuous buffer system as described by Iglesias et al. (1974). The following enzyme systems were screened: esterase (EST, 3.1.1.1), malate dehydrogenase (MDH, 1.1.1.37), and peroxidase (POX, EC 1.11.1.7). The staining protocols used are described by Gall et al. (1995).

Computing numerical data

Dendrograms for the different MLEE systems and SDS-PAGE were generated by using the one-dimensional software analysis (Advanced American Biotechnology and Imaging, Fullerton CA 92831, USA). For cluster analysis of protein and isozymes banding patterns, the unweighted pair group method using average linkages (UPGMA) was used (Vauterin and Vauterin, 1992).

RESULTS

SDS-PAGE analysis

Cluster analysis of the protein markers data placed the *Fusarium* species into main groups (Figure 1), according to their previous species assignment. The genetic similarity between *Fusarium* spp. isolates ranged from 21 to 54% for inter-specific and 62 to 97% for intra-specific comparisons. The application of UPGMA clustering produced two large clusters within the population with a branched-off at genetic similarity of 21.46%, each consisting of several subclusters (phenons).

The first main cluster included all *F. oxysporum*, all *F. moniliforme* and *F. chlamydosporum* isolates. Within this cluster, Fa1 and Fa2 have a genetic similarity of GS=95.98%. Isolates Fo24 and Fo25 also showed very high genetic similarity of 99.22%. The second cluster consists of *F. solani* isolates at the genetic similarity of 31.37%. Here, isolates Fs3 and Fs4 showed very high genetic similarity of 97.26%. Clustering analysis indicates that the 46 *Fusarium* spp. isolates can be assigned to six protein types. There was low correlation between clustering in the protein dendrogram and geographic origin. These results suggested that protein profiles data clearly separated *Fusarium* spp. isolates.

Isozymes analysis

The activity and resolution of three enzyme-staining procedures for *Fusarium* spp. were tested. Poor resolution was observed for the enzymes peroxidase and malate dehydrogenase (data not shown), while esterase

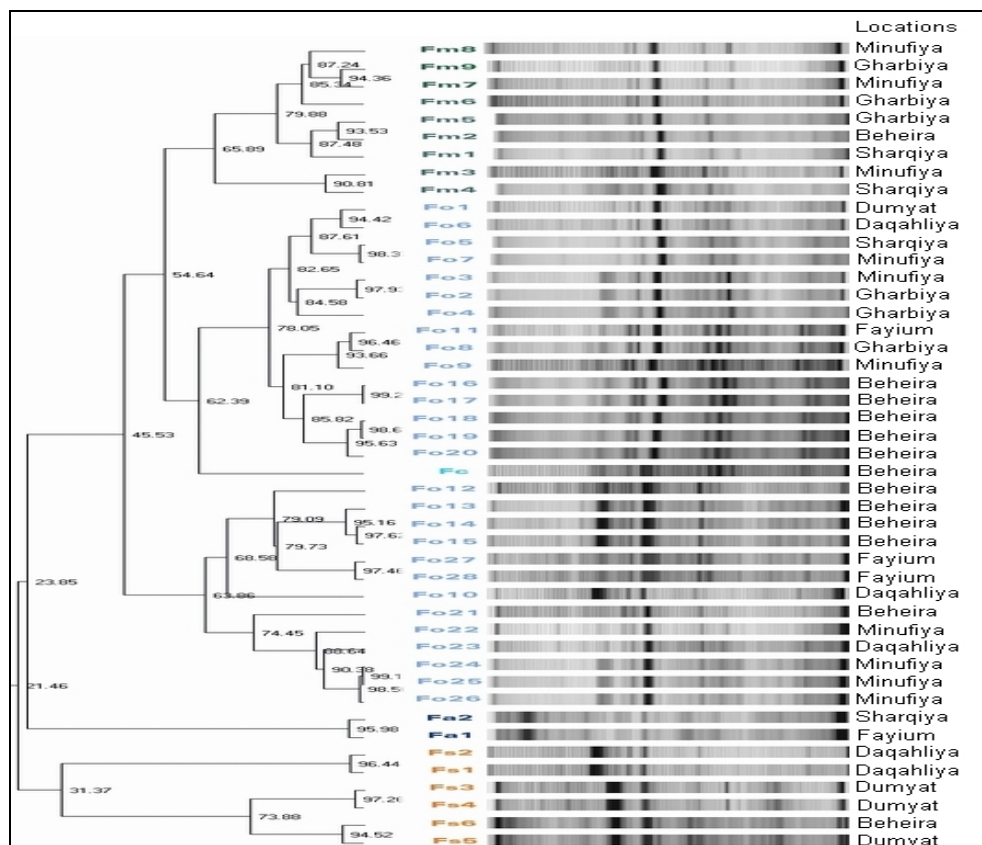


Figure 1. Dendrogram derived from the UPGMA linkage of correlation coefficients between SDS-PAGE profiles from five *Fusarium* spp. isolates. The levels of linkage representing the Pearson product-moment correlation coefficient (r) are expressed as percentages for convenience. The banding profiles against each branch represent normalized and background-subtracted digitized gel strips processed in ABB program.

enzyme showed strong activity and acceptable resolution.

Esterase banding patterns obtained by native PAGE were concatenated and analyzed as a composite by AAB software. The normalization setting used were as follows: a resolution of 500 points, a smoothing factor of 3, and background subtraction by the rolling-disk method with intensity setting of 12, and the patterns were clustered by unweighted pair group method of arithmetic average.

The obtained dendrogram (Figure 2) depicts that all isolates belong to two distinct groups (similarity are above 20%). The genetic similarity between *Fusarium* spp. isolates ranged from 29 to 54% for inter-specific and 60 to 97% for intra-specific comparisons.

The first main cluster included all *F. oxysporum* isolates, *F. moniliforme* and *F. chlamydosporum* isolates, constituted one cluster branched from the first main cluster at level of 61.01 genetic similarity. Here, Fa1 and Fa2 have a genetic similarity of 75.59%. Isolates Fo12 and Fo13 also showed very high genetic similarity of 99.46%. Both were collected from Beheira governorate and could be identical. The second main cluster consist of *F. solani* isolates at the genetic similarity of 73.93%. Isolates Fs1 and Fs2 showed very high genetic similarity

of 97.77%. Within the cluster of *F. oxysporum*, low correlation between the geographic origin of the isolates and their esterase patterns were observed. At the 65% genetic similarity, the 28 *F. oxysporum* isolates could be divided into four subgroups. Some of the *F. solani* isolates that had been collected from the same locations also showed a tendency to cluster. Only one isolate, Fm8, could not be classified by esterase analysis.

DISCUSSION

Biochemical and molecular markers are being increasingly used to characterize fungal plant pathogen populations. They are versatile and highly informative tools for fungal pathogen identification and diagnosis (Majer et al., 1996) and for populations genetics studies (McDonald and McDermott, 1993; McDonald et al., 1999). They can be used to evaluate levels of genetic diversity and phenotypic relationships within and between species, and to identify particular races and pathotypes (Brown, 1996). The electrophoretic separation of protein, including isozymes, is a useful tool for differentiating fungal taxa. Bosland and Williams (1987) have used

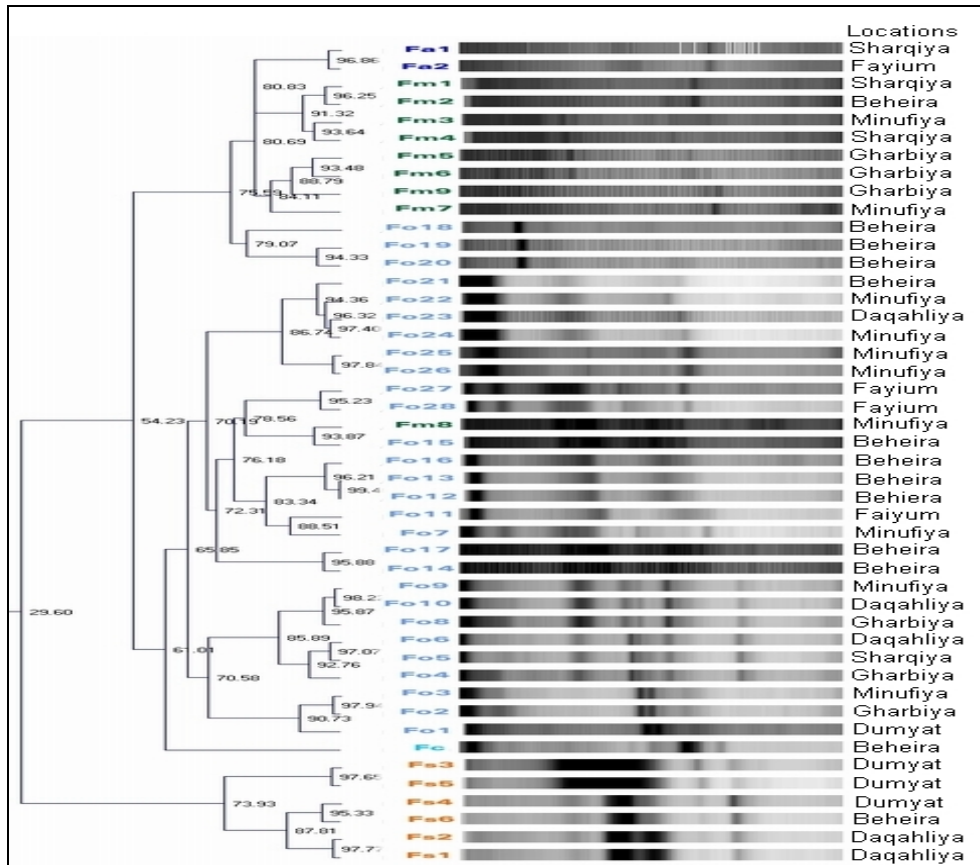


Figure 2. Dendrogram derived from the UPGMA linkage of correlation coefficients between esterase isozyme profiles from five *Fusarium* spp. isolates. The levels of linkage representing the Pearson product-moment correlation coefficient (r) are expressed as percentages for convenience. The banding profiles against each branch represent normalized and background-subtracted digitized gel strips processed in ABB program.

isozymes to differentiate *Fusarium* species and four *F. oxysporum* formae specialis, as well as isolates obtained from different crucifers species. On the contrary, no clear-cut results were found for isozymes study of *F. oxysporum* f. sp. *lycopersici* recovered from different sites and different pathogenic races or vegetative compatibility groups (Elias and Schneider, 1992). SDS-PAGE is used because the method alleviates the need for culturing, and samples are analyzed in a more direct manner. This method is relatively easy and many samples can be analyzed at the same time. It is also cheaper than AFLP fingerprinting. Moreover, the results obtained by SDS-PAGE of whole-cell proteins can discriminate at much the same level as DNA fingerprinting (Priest and Austin, 1993) in some cases.

Cluster analysis of the protein markers data placed the *Fusarium* species into main groups according to their previous species assignment. The genetic similarity between *Fusarium* spp. isolates, similarities ranged from 21 to 54% for inter-specific and 62 to 97% for intra-specific comparisons. The application of UPGMA clustering produced two large clusters within the population. These results suggested that protein profiles

data can clearly separate *Fusarium* spp. isolates with a few exceptions. A low correlation between protein dendrogram and geographic origin of tested isolates was found.

These results agree with those obtained by Mandeel et al. (1994) who compared SDS-PAGE patterns from eight isolates belonging to three *Fusarium* species. Protein profiles were distinct, and each isolate showed a uniquely characteristic profile. The data obtained from protein profiles support the potential use of this experimental approach to help distinguish between different *Fusarium* isolates. On the contrary, Belisario et al. (1998) found no differences when comparing total mycelium protein profiles (SDS-PAGE) of different species and formae species of *F. oxysporum*, *F. solani* and *F. culmorum*.

Isozyme analysis is frequently used for taxonomic purposes, especially when a taxon is morphologically diverse. In most cases, fungal species are easily differentiated by electrophoresis. The technique is commonly used to make recommendations on the separation or combination of species (Otrosina et al., 1992). Zambino and Harrington (1989) have also separated subspecies, varieties, and intersterility groups.

Simple band-counting procedures can be used to distinguish taxa, although cladistic and phylogenetic information can be derived from the allelic frequencies and ratios derived from a genetic interpretation of the data. Isozyme analysis is most successful in distinguishing species and subspecies when the amount of intra-specific genetic variation is limited within a population. Esterase isozyme fingerprinting have been frequently used because of the relatively large differentiation between *Fusarium* spp. in contrast to other enzyme systems (Baayen, 1997).

In the present study, UPGMA cluster analysis of esterase isozyme produced two large clusters within the population, each consisting of several subclusters. Low correlation was observed between clustering in the esterase dendrogram and sampling date of tested isolates. These results suggested that esterase patterns data clearly separated *Fusarium* spp. isolates with a few exceptions. These results, similar to those obtained by Ye and Wu (1985) who showed that the esterase patterns are distinct for 24 *F. graminearum* isolates. The patterns of 7 isolates of *Gibberella zeae* collected from different geographic localities appeared little different from one another and there were some identical bands of esterase isozymes in their patterns. However, no relationship was observed between isozyme patterns and geographic origin, phenotypic distance or virulence of isolates (Etebarian et al., 1996; Yli-Mattila and Hyvonen, 1996; Bosland and Williams, 1987). Also no clear-cut result was detected by malate dehydrogenase and peroxidase isozymes in discriminating between *Fusarium* spp. Isolates (Ye and Wu, 1985). In summary, the genomic diversity within the genus *Fusarium* was determined by using esterase isozyme and SDS-PAGE and each technique could prove useful for the rapid classification of *Fusarium* spp. isolates.

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