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Use of Electrophoretic Patterns of Proteins and Isozymes to Characterize *Trichoderma* Isolates from Cotton Roots

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ABSTRACT

Proteins of 15 isolates of *T. longbrachiatum* and *T. harzianum* were compared by polyacrylamid gel electrophoresis (PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with silver nitrate. Protein banding patterns of the isolates were subjected to cluster analysis by the unweighted pair-group method based on arithmetic mean (UPGMA). Apart from *T. harzianum* isolates T6 in PAGE and T27 in SDS-PAGE, which occupied separate positions in the phenograms, it was possible to delineate the isolates of each species on the basis of the results of PAGE and SDS-PAGE. Peroxidase (EC 1.11.1.7) and esterase (EC 3.1.1.1) isozymes from mycelium and conidia of *Trichoderma* isolates were separated by PAGE, and the obtained banding patterns were visualized by using specific staining systems. Cluster analysis indicated that delineation of the isolates of each species was not possible on the basis of the electrophoretic patterns of peroxidase. On the contrary, patterns of esterase isozymes provided a reliable method for grouping the isolates of each species with the exception of T9 and T14 of *T. longbrachiatum*, which occupied separate positions in the phenogram.

INTRODUCTION

Biological control of root pathogens consists basically of encouraging the growth and activity of *Trichoderma* spp. (Stoppacher *et al*., 2006).

Tichoderma species are capable of producing a variety of antibiotics (Dennis and Webster, 1971) and hydrolytic enzymes (Geremia *et al*., 1993), which play key role in the interaction with pathogenic fungi and are necessary for successful antagonism.

a. Protein electrophoresis:

Protein electrophoresis has been widely used as a biochemical tool in fungal taxonomy. This is because amino acid sequences of polypeptides (components of proteins) are dependent on nucleotide sequences of their coding genes; therefore, an analysis of protein variation among fungal isolates by electrophoresis approximates an analysis of their genetic variation (Markert and Faulhaber, 1965). However, a problem with proteins as markers is the vast number, which can be generated from an organism. Faced with so much data, only sophisticated analysis can help to draw meaningful conclusions. The ready availability of computers has made numerical taxonomy more accessible and same later studies on fungi have proven useful (Manicom *et al.*, 1990).

Aly *et al*. (1997) used PAGE and SDS-PAGE to characterize *F. oxysporum, F. moniliforme,* and *F. solani* isolated from cotton seedlings infected with damping-off. Delineation of isolates of each species was not possible on the basis of electrophoretic banding patterns of native proteins. On the contrary, banding patterns of SDS-dissociated proteins proved a reliable method for grouping isolates for each species. Moreover, phylogenetic relationships between the resulting clusters matched that based on morphological taxonomy.

Aly *et al*. (2000) reported that electrophoretic banding patterns of dissociated proteins provided a reliable method for grouping the four Egyptian races of *F. oxysporum* f.sp. *ciceris*.

Aly *et al*. (2001) compared protein patterns of five isolates of *F. oxysporum* f.sp. *vasinfectum* (FOV) and a nonpathogenic isolate of *F. oxysporum* by PAGE and SDS-PAGE. Both PAGE and SDS-PAGE could not be used to distinguish the highly pathogenic isolates of FOV.

Salama *et al*. (2002) used SDS-PAGE to study the phylogenetic relationships among six *Trichoderma* spp. isolated from the Egyptian soil. Cluster analysis divided the six aggregate species into two groups. The first one included only *T. koningii* aggregate species, whereas the second group was subdivided into two subgroups, the first one included *T. harzianum*, *T. hamatum, T. reesei,* and *T. pseudokoningii* and the second one included only *T. viride*.

b. Isozyme electrophoresis:

Enzymatic properties of isozymes are similar, if not identical; however, isozymes slightly differ in their 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 by differentiated by electrophoresis (Bonde *et al.*, 1993).

Vagujfalvi and Szecsi (1994) collected *Fusarium* spp. from 30 localities and different parts (roots, stalks, and ears) of maize. They used polyacrylamide slab gel electrophoresis of protein extracts of *F. moniliforme, F. proliferatum,* and *F. subglutinans* to obtain esterase (EST) zymograms. The sites of EST activity were recorded, and the R_f values were calculated. Isolates of *F. moniliforme* were divided into two zymogram types (type I and type II) based on EST isozyme patterns. EST zymogram type II was very similar to patterns of *F. proliferatum*. Their data suggested that these isolates are actually *F. proliferatum*.

The present study was initiated to determine whether *Trichoderma* isolates from cotton roots (Table 1) can be characterized by their electrophoretic protein banding patterns separated by PAGE and SDS-PAGE.

Table 1. Geographic origins of *Trichoderma* spp. Isolated from cotton seedlings.

Isolate code	Identification	Geographic origin
T3	*T. harzianum*	Daqahliya, Simbellawain
T4	*T. longibrachiatum*	Assiut, Assiut
T5	*T. longibrachiatum*	Assiut, Assiut
T6	*T. harzianum*	Daqahliya, Simbellawain
T9	*T. longibrachiatum*	Unknown
T10	*T. harzianum*	Daqahliya, Simbellawain
T14	*T. longibrachiatum*	Giza, Giza
T18	*T. longibrachiatum*	Minufiya, Minouf
T23	*T. harzianum*	Gharbiya, El Mahalla El Kobra
T27	*T. harzianum*	Gharbiya, El Mahalla El Kobra
T29	*T. harzianum*	Daqahliya, Simbellawain
T31	*T. harzianum*	Daqahliya, Simbellawain
T38	*T. longibrachiatum*	Minufiya, Shibeen El Kom
T39	*T. longibrachiatum*	Minufiya, Shibeen El Kom
T42	*T. longibrachiatum*	Giza, Giza

Isozymes of peroxidase and esterase separated by PAGE were also tested for ability to differentiate between the isolates.

MATERIALS AND METHODS

1. Extraction of proteins from *Trichoderma* isolates

Proteins were prepared according to Guseva and Gromova (1982), Rataj-Guranowska *et al.* (1984), and Hussein (1992). The protein content in supernatant was estimated according to Bradford (1976) by using bovine serum albumin as a standard protein. If protein concentration was low, protein would be precipitated from the clarified supernatant by adding ammonium sulphate at 70 % of saturation (60 g/100 ml) then kept in the refrigerator for 30 hrs. Pellets, collected by centrifugation at 11,000 rpm for 30 min., were resuspended in phosphate buffer pH 8.3 and subjected to dialysis for 24 hrs. against the buffer and centrifugation at 11,000 rpm for 30 min. Protein was estimated in the obtained supernatant.

2. Electrophoresis of native protein (PAGE)

Electrophoresis was conducted at 10oC for 4 hrs. in a 7.5% polyacrylamide gel with a 3.5 stacking gel, at 15 and 30mA, respectively, until the dye band reached the bottom of the separating gel (Davis, 1964). Electrophoresis was performed in a vertical slab mold (16x18x0.15cm). Gels were stained with silver nitrate for the detection of protein bands (Sammons *et al*., 1981).

3. Electrophoresis of dissociated protein (SDS-PAGE)

Electrophoresis (Laemmli, 1970) and staining of gels were carried out as previously mentioned in PAGE.

4. Electrophoresis of isozymes

Electrophoresis of isozymes was carried out as previously mentioned in PAGE; however, gels were stained according to Manchenko (1994) for the detection of isozymes of esterase (EC.3.1.1.1) and peroxidase (EC.1.11.1.7).

5. Gel analysis

A gel documentation system (Advanced American Biotechnology 1166 E.Valencia Dr. Unit 6 C, Fullerton CA 92631) was used to document the results of electrophoresis and to cluster the electrophoretic patterns of proteins and isozymes by the UPGMA.

RESULTS

1. Electrophoresis of native protein (PAGE)

Fig. 2 showed the phenogram constructed based on similarity levels (SLs) generated from cluster analysis of electrophoretic banding patterns of native (undissociated) proteins shown in Fig. 1.

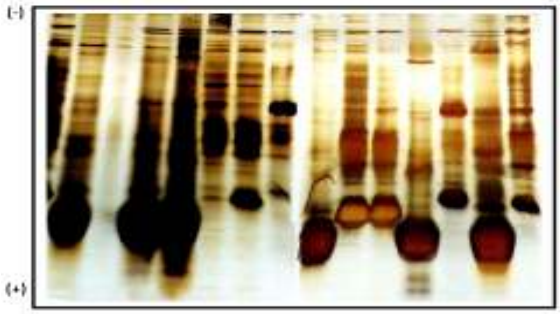

Fig. 1. Protein bands obtained by PAGE from 15 isolates of *Trichoderma* spp. Isolates in lanes from left to right were T18, T39, T38, T42, T4, T5, T14, T9, T27, T23, T29, T10, T3, T31, and T6.

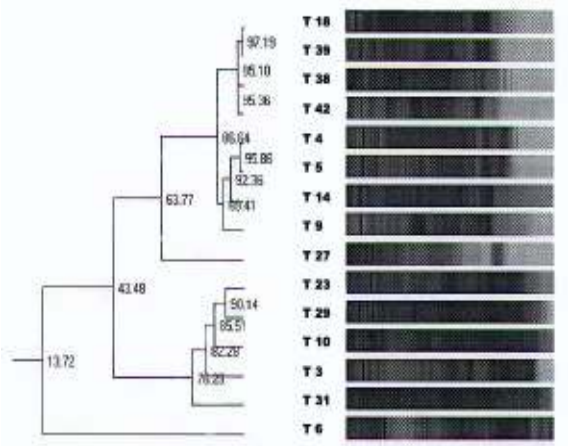

Fig. 2. Phenogram of electrophoretic protein bands obtained by PAGE from 15 isolates of *Trichoderma* spp.

The greater the SL, the more closely the isolates were in their protein composition. In this phenogram, all the isolates of *T. longibrachiatum* were clustered within the SL 86.64%, which illustrated a remarkable overall SL among the tested isolates. On the other hand, only five isolates of *T. harzianum* were placed in a single cluster within the SL 76.29%. A noteworthy peculiarity in the phenogram was the behaviour of the isolates no_s. T27 and T6 of *T. harzianum*, which were remotely related to the other isolates of *T. harzianum*.

2. Electrophoresis of dissociated protein (SDS-PAGE)

The dendrogram shown in Fig. 4 was constructed based on SLs generated from cluster analysis of electrophoretic banding patterns of dissociated proteins shown in Fig. 3. The isolates in the dendrogram formed two large taxonomic groups within the SL 31.41%. The first group (SL = 48.75%) included all the isolates of *T. longibrachiatum*, while the second group included only 6 isolates (85.71%) of *T. harzianum* within the SL 41.30%. Surprisingly, isolate no. T27 of *T. harzianum* formed a separate cluster remotely related to the latter group.

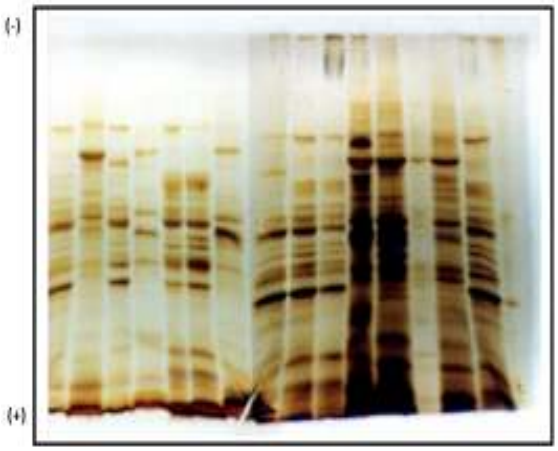

Fig. 3. Protein bands obtained by SDS-PAGE from 15 isolates of *Trichoderma* spp. and a protein marker (M). Isolates in lanes from left to right were T27, T6, T10, T3, T31, T29, T23, T9, T5, T4, T14, T42, T38, T39, T18, and a protein marker.

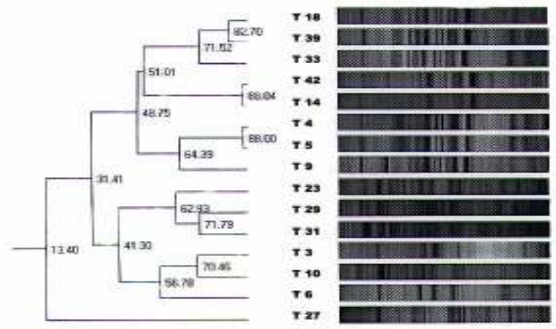

Fig. 4. Phenogram of electrophoretic protein bands obtained by SDS-PAGE from 15 isolates of *Trichoderma* spp.

3. Electrophoresis of isozymes

1. Esterase isozymes

The dendrogram shown in Fig. 6 was constructed based on SLs generated from cluster analysis of electrophoretic esterase isozyme patterns shown in Fig. 5.

The isolates in the dendrogram formed two large taxonomic groups within the SL 51.34%. The first group (SL = 77.25%) included all the isolates of *T. harzianum*, while the second group included only six isolates (75%) of *T. longibrachiatum* within the SL 77.56%. Surprisingly, isolates no$_s$. T9 and T14 of *T. longibrachiatum* formed a separate cluster at SL 47.46%, this cluster was remotely related to the latter group.

Fig. 5. Esterase isozyme bands obtained by PAGE from 15 isolates of *Trichoderma* spp. Isolates in lanes from left to right were T14, T9, T18, T5, T4, T42, T39, T38, T23, T6, T10, T3, T31, T29, and T27.

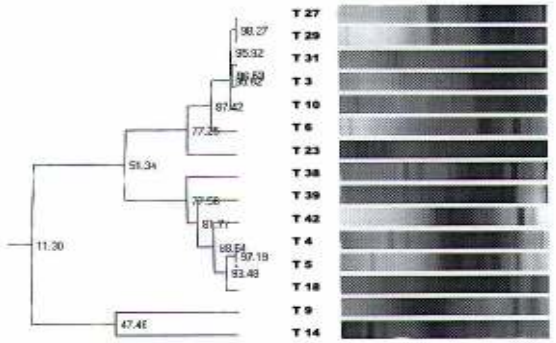

Fig. 6. Phenogram of electrophoretic esterase isozyme bands obtained by PAGE from 15 isolates of *Trichoderma* spp.

2. Peroxidase isozymes

The dendrogram shown in Fig. 8 was constructed based on SLs generated from cluster analysis of electrophoretic peroxidase isozyme patterns shown in Fig. 7. In this phenogram, the delineation of isolates of *T. harzianum* or *T. longibrachiatum* was not possible on the basis of the results of cluster analysis of peroxidase isozyme patterns.

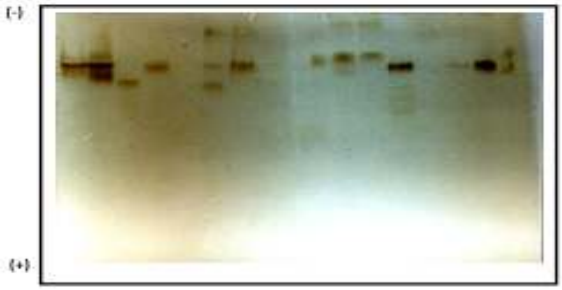

Fig. 7. Peroxidase isozyme bands obtained by PAGE from 15 isolates of *Trichoderma* spp. Isolates in lanes from left to right were T14, T31, T23, T39, T42, T38, T10, T5, T3, T6, T29, T27, T9, T4, and T18.

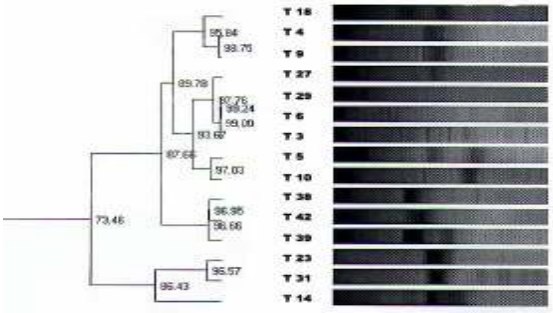

Fig. 8. Phenogram of electrophoretic peroxidase isozyme bands obtained by PAGE from 15 isolates of *Trichoderma* spp.

DISCUSSION

Apart from *T. harzianum* isolates T6 in PAGE and T27 in SDS-PAGE, which occupied separate positions in the phenograms, it was possible to delineate the isolates of each species on the basis of the results of PAGE and SDS-PAGE. However, I am aware that the number of isolates that belonged to each species and were available for study was too limited to arrive unequivocal conclusions regarding the suitability of protein electrophoresis to distinguish isolates of *T. longibrachiatum* from those of *T. harzianum*. Despite this limitation, certain conclusions could be drawn. The amount of variation in electrophoretic banding patterns is adequate for the application of cluster analysis, therefore, the refinement of PAGE and SDS-PAGE employed in the present work by analyzing more isolates of each species, in combination with cluster analysis of the resulting protein profiles, 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 numbers of patterns in data banks for reference; (4) information on epidemiological spreading of isolates (Kerster and De Ley, 1975).

In the present study, in an agreement with Hall (1967), we stress that successful use of PAGE and SDS-PAGE as biochemical aids in *Trichoderma* taxonomy requires that growth, extraction, and electrophoretic procedures must be carried out under standardized rigorously controlled conditions.

Electrophoresis of isozymes is widely used in fungal taxonomic studies because catalytic activity of enzymes can be measured in cell-free systems with precision and ease (Hall, 1967).

Previous studies indicated that isozyme analysis was a useful supplement to morphological analysis in *Trichoderma* taxonomy, particularly when data were analyzed by phylogentic methods (Zamir and Chet, 1985; Stasz *et al*., 1989 and Leuchtmann *et al*., 1996).

In the present study, delineation of the isolates of each species was not possible on the basis of the electrophoretic patterns of peroxidase isozymes. On the contrary, patterns of esterase isozymes provided a reliable method for grouping the isolates of each species with the exception of T9 and T14 of *T. longibrachiatum*, which occupied separate positions in the phenogram.

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استعمال أنماط البروتينات والمشابهات الإنزيمية الناتجة من التفريد الكهربى لتوصيف عزلات التريكوديرما المعزولة من جذور القطن

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أجريت دراسة مقارنة لأنواع البروتينات المستخلصة من عزلات التريكودرما ، وذلك باستعمال تقنية الفصل الكهربى للبروتين الخام أو بعد تفكيكه باستعمال مادة صوديوم دوديسيل سلفيت. استعملت نترات الفضة لإظهار أنماط البروتين المتحصل عليها. استعمل أسلوب التحليل العنقودى لتصنيف العزلات إلى مجموعات بناءً على ما بينها من تماثل فى أنماط البروتين ، وتم التعبير عن النتائج فى فينوجرامات. أمكن باستعمال أنماط البروتين -الخام أو المفكك- التفرقة بين عزلات كل من تريكودرما لونجيبراكياتم وتريكودرما هارزيانم. الجديـر بالذكر أن العزلتيـن T27, T6 مثلتا إستثناءً لما سبق ، إذ أنهما إحتلتا مواقع منعزلة فى الفينوجرامين المتحصل عليهما.إستعملت تقنية التفريد الكهربى لفصل مشابهات إنزيمى البيرأوكسيديز والإستيريز من ميسليوم وكونيديات عزلات التريكودرما ، ثم إستعملت نظم صبغ متخصصة لإظهار أنماط المشابهات الإنزيمية المتحصل عليها . لم يمكن التفرقة بين عزلات تريكودرما لونجيبراكياتم وعزلات تريكودرما هارزيانم باستعمال مشابهات البيرأوكسيديز ، فى حين أمكن ذلك باستعمال مشابهات الإستيريز ، باستثناء العزلتين T14, T9 اللتان إحتلتا موقعين منعزلين فى فينوجرام الإستيريز.