Isoenzyme Analysis of Isolates of the Entomogenous Fungi *Tolypocladium cylindrosporum* and *Tolypocladium extinguens* (Deuteromycotina; Hyphomycetes)

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Isoenzyme analysis of 11 isolates of *Tolypocladium cylindrosporum* and *T. extinguens* revealed considerable variability between strains of the same species and confirmed great differences between the two species. An exception was the striking similarities in isoenzyme patterns observed for the California isolates of *T. cylindrosporum* isolated from *Aedes sierrensis* larvae from 1972 to 1979. This considerable homogeneity suggests a rather stable genotypic equilibrium in the fungal population which was maintained with little alteration even after repeated subculturing in the laboratory.

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KEY WORDS: *Tolypocladium cylindrosporum*, *Tolypocladium extinguens*; entomopathogenic fungi; strain characterization; isoenzyme analysis.

INTRODUCTION

This paper is an extension of a previous one (Soares et al., 1985) in which virulence, conidiation, and spore size of 11 isolates of *Tolypocladium cylindrosporum* and two isolates of *T. extinguens* were compared. *T. cylindrosporum* infectious for several mosquito species has been isolated from *Aedes sierrensis* (see Soares et al., 1979; Soares, 1982) and *Aedes australis* (see Weiser and Pillai, 1981).

This species has also been isolated from the lovebug, *Plecia nearctica* (Diptera; Bibionidae) in Florida (Kish et al., 1974). However, in this latter case it could not be established whether *T. cylindrosporum* was acting as a true pathogen or as a saprophyte (Kish et al., 1977).

*T. extinguens* has been isolated from the larvae of the New Zealand glow worm *Arachnocampa luminosa* (Diptera: Mycophiliidae). A pathogenic role has not been confirmed. *T. extinguens* is morphologically distinct from other species of *Tolypocladium* and has been described as a new species (Samson and Soares, 1984).

In this present study these same isolates were subjected to enzyme analysis in order to examine biochemical variability within the group and to determine affinities and differences between isolates. Also of interest was to establish whether or not correlations could be made between strain virulence and enzyme patterns. Production of certain enzymes was also examined using standard culture methods.

MATERIALS AND METHODS

Culture conditions and enzyme preparation. Fungal isolates, their sources, and localities are listed in Table 1. Mycelium was produced in liquid shake culture in Adamek medium (Adamek, 1965). Cultures were incubated 3–4 days at 25°C. Mycelium was filtered from the culture medium and washed four times in distilled water and buffer (Tris–EDTA buffer, 0.05 M, pH 8.2). The mycelium was dried with paper toweling then ground in liquid nitrogen.

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TABLE 1
A LIST OF Tolypocladium cylindrosporum AND Tolypocladium extinguenus ISOLATES STUDIED

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>CBS No.</th>
<th>Collector</th>
<th>Locality</th>
<th>Isolate from</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. cylindrosporum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>614.80</td>
<td>D. E. Pinnock</td>
<td>Novato, CA</td>
<td>Aedes sierrensis</td>
<td>1971</td>
</tr>
<tr>
<td>2</td>
<td>612.80</td>
<td>G. G. Soares</td>
<td>Novato, CA</td>
<td>Aedes sierrensis</td>
<td>1976</td>
</tr>
<tr>
<td>3</td>
<td>613.80</td>
<td>G. G. Soares</td>
<td>Novato, CA</td>
<td>Aedes sierrensis</td>
<td>1978</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>G. G. Soares</td>
<td>Novato, CA</td>
<td>Aedes sierrensis</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>J. S. Pillai</td>
<td>New Zealand</td>
<td>Aedes australis</td>
<td>unk.</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>J. S. Pillai</td>
<td>New Zealand</td>
<td>Aedes australis</td>
<td>unk.</td>
</tr>
<tr>
<td>7</td>
<td>276.82</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>717.76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>720.70</td>
<td>W. Veenbaas-Rijks</td>
<td>Netherlands</td>
<td>Soil</td>
<td>1968</td>
</tr>
<tr>
<td>10</td>
<td>719.70</td>
<td>O. Fassatiova</td>
<td>Czechoslovakia</td>
<td>Soil</td>
<td>1967</td>
</tr>
<tr>
<td>11</td>
<td>989.73</td>
<td>L. Kish</td>
<td>Florida</td>
<td>Plecia nearctica</td>
<td>1976</td>
</tr>
<tr>
<td>12</td>
<td>718.70</td>
<td>P. M. Latter</td>
<td>Great Britain</td>
<td>Peat</td>
<td>1965</td>
</tr>
<tr>
<td>T. extinguenus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>345.77</td>
<td></td>
<td>New Zealand</td>
<td>Arachnocampa luminosa</td>
<td>1977</td>
</tr>
<tr>
<td>2</td>
<td>969.72</td>
<td>J. Grinsberg</td>
<td>Chile</td>
<td>Meadow soil</td>
<td>1972</td>
</tr>
</tbody>
</table>

* In INRA, La Miniere culture collection.
  * In Centraalbureau voor Schimmelcultures, Baarn, Netherlands, culture collection.

powder was then mixed with several milliliters of distilled water and centrifuged at 10,000 rpm for 1 ½ hr. The supernatant was frozen in several small plastic tubes.

Electrophoresis. Isoelectric focusing was carried out on a flat-bed apparatus. Enzymes were separated on 115 x 230 x 1 mm 1.5% polyacrylamide gel slabs. Gels were prefocused for 30 min at 10 mA.

Twenty samples were placed on the slab, with each well receiving 12 μl of a given sample. Enzyme were separated at 10 mA for 1 ½ hr at 5°C. After migration, gel slabs were removed from the flat-bed apparatus and incubated in the appropriate staining solution. Depending on the enzymatic system, different pH intervals were chosen for carrier ampholytes (Pharmalyte).

Five systems were analyzed. Total proteins were detected with Coomassie brilliant blue R and destained as described by Davis (1964). Acid phosphatases (AP), esterases (Est), alcohol dehydrogenases (ADH) (isopropanol), and glutamate oxaloacetic transaminases (GOT) were identified by color reactions obtained directly or indirectly by incubating gels in different reagent solutions at 30°C for various periods of time and in the darkness. The esterases were located with naphthyl acetate reagents (Tracey et al., 1975); acid phosphatases were examined according to the methods described by Ayala et al. (1972). Glutamate oxaloacetic transaminase substrates were aspartic acid and α-cetoglutaric acid. Depending on the systems, the substrate coenzymes were phosphate-N-pyridoxal or NAD.

Analysis of data. Correspondence analysis (Cailliez and Pages, 1976) was applied to the data obtained from the isolates. One part of the spectrum was selected for each system, and all bands were located in it. Within this area all the bands were considered and for each sample, presence (1) or absence (0) was noted. Each strain was analyzed three times for each system.

Enzymatic activity. The enzymatic activities were evaluated on Petri dishes by measuring the color of the zone under the colony using special media (lipases using Lipase Reagent Agar Difco) or the diameter of the zone. To examine protease activity we used plates containing 2.3% Difco Media for the detection of amylolytic activity were prepared according to Hanking
and Anagnostakis (1975) and contained 2.5% Difco nutrient agar and 0.2% soluble starch (pH = 6). After 5 days the plates were flooded with iodine, with nonstaining areas indicating amylolytic activity.

RESULTS

Protein and Enzyme Patterns

The relative amount of mycelium produced by the isolates varied from 4 to 7 g wet weight per flask except for TC 8, in which case vegetative growth was very weak. The majority of systems tested produced multiple electromorphs indicating extensive molecular heterogeneity.

The protein patterns of buffer soluble proteins or enzymes extracted from mycelium of each isolate examined were reproducible in different electrophoretic runs. Such profiles also were identical to those of a different culture of the same isolate grown at a different time under identical conditions. Among the total protein patterns, 23 bands were observed. Nine types of protein patterns were distinguished, some of them included only one strain (type 2, 3, 4, 5, 6, 8, 9, 10) and one contained several strains (type 1). All bands, occurring in one or more isolates but not common to all strains could be considered variant sites of activity (Hubby and Lewontin, 1966).

The acid phosphatase patterns (Fig. 1) for the California strains were very similar. The other 9 strains produced unique acid phosphatase zymograms from which any isolate could be distinguished from the other isolates using a combination of isoenzymes comparisons, except for strains 7 and 10.

In alcohol dehydrogenase patterns 7 types could be separated from only 10 bands which had slightly different isoelectric points. Large differences were observed in the glutamate oxaloacetic transaminase system. Six types were distinguished with 12 bands. The T. extinguens strains were very different from the other isolates, possessing enzymes with isoelectric points greater than 7. Also esterase zymograms were very polymorphic with 19 bands well distributed throughout the gel. Five types have been observed.

Groups of Strains and Correspondence Analysis

Table 2 shows the grouping of strains according to similarities in isoenzyme patterns. However, the grouping of strains was dependent on the enzyme system. In order to evaluate the overall similarities between the various isolates a correspondence analysis was applied (Fig. 2). It is evident from the results of this analysis that the T. extinguens strains are very different from all isolates of T. cylindrosporum. Within T. cylindrosporum, isolates 7, 9, and 10 are quite different from isolates 1, 2, 3, 4, 5, 8, 11, and 13. Of this latter group isolates 1, 4, and 5 were found to be virtually identical and very similar to isolates 2, 3, and 11.

Enzymatic Activities of the Isolates

According to their enzymatic activity, 9 types could be distinguished (Table 3). Some strains did not produce lipases (7, 8, 9, 10, 11, 14) or proteases (7) or amylases (11, 13). All the California strains plus TC 13 had similar activities.
TABLE 2

GROUPS OF STRAINS HAVING SIMILAR ISOENZYME PATTERNS FOR FIVE SYSTEMS: TOTAL PROTEINS, ACID PHOSPHATASE (AP), GLUTAMATE OXALOACETIC TRANSAMINASES (GOT), ∂-ESTERASES, ALCOHOL DEHYDROGENASES (ADH)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Proteins</th>
<th>AP</th>
<th>GOT</th>
<th>Esterases</th>
<th>ADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1,2,3,4,5</td>
<td>1.2,4,5,11</td>
<td>1.2,3,4,5,11,13</td>
<td>1.2,3,4,5,8,11,13</td>
<td>1.3,4,5,13</td>
</tr>
<tr>
<td>II</td>
<td>7</td>
<td>3</td>
<td>7</td>
<td></td>
<td>2,11,13</td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>7,10</td>
<td>8</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
<td>9</td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>V</td>
<td>10</td>
<td>13</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>VI</td>
<td>TE 2</td>
<td>TE 2</td>
<td>TE 2</td>
<td></td>
<td>TE 2</td>
</tr>
<tr>
<td>VII</td>
<td>TE 1</td>
<td>TE 1</td>
<td>TE 1</td>
<td></td>
<td>TE 1</td>
</tr>
<tr>
<td>VIII</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>11</td>
<td>8 (no bands)</td>
<td></td>
<td></td>
<td>8 TE 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(no bands)</td>
</tr>
</tbody>
</table>

DISCUSSION

Electrophoretic analysis of mycelial preparations demonstrated extensive variability within the two species of *Tolypocladium* examined. Similar results were obtained with *Hirsutella thompsonii* (Boucias et al., 1982) and *Fomes annosus* (Huttermann et al., 1979). Generally, in the literature the isoenzyme spectrum of fungi is not as variable (Franke, 1973; Reddy, 1973; Gill and Zentmyer, 1978). Because the groups of strains are dependent on the system (Table 2) it is necessary to study genetic relationships between the isolates.

Morphological differences between *T. cylindrosporum* and *T. extinguens* are very slight (Samson and Soares, 1984). However, using biochemical criteria we confirmed large differences between the two species.

Within *T. cylindrosporum* three isolates (7, 9, and 10) were considerably different from the others with respect to isoenzyme patterns while no morphological differences were noted. This indicates a differentiation at the subcellular level not expressed in morphological characters. These strains were also found to differ with respect to infectivity to mosquito larvae (Soares et al., 1985).

On the other hand, strains 1, 2, 3, 4, 5, 11, and 13 were virtually identical or very similar and quite different from the others. With respect to virulence and enzymic activities we confirmed these similarities. For all the biochemical and biological criteria T.c 8 is similar to the other strains except for sporulation, which is very poor. From the standpoint of epidemiology, it is very interesting to consider the high degree of similarity between the California isolates 1, 2, 3, and 4. These were isolated from the
same host, *A. sierrensis*, from the same area but from 1972 to 1979. This shows a good genotypic equilibrium of the fungal population, and of the isolates even after repeated subculturing in the laboratory. De Conti et al. (1980) described similar homogeneity among geographic isolates of *M. anisopliae* in Brazil.

Finally, though both characters are variable, it was not possible to correlate virulence with enzymatic activity or electrophoretic patterns. However, these characterizations may serve as a means of identifying strains and as an aid to defining relationships between isolates.

**ACKNOWLEDGMENTS**

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