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Isozyme Characterization and Pathogenicity of *Paecilomyces fumosoroseus* and *P. lilacinus* to *Diabrotica speciosa* (Coleoptera: Chrysomelidae) and *Meloidogyne javanica* (Nematoda: Tylenchidae)

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Ten monosporal fungal isolates of *Paecilomyces fumosoroseus* (Wize) Brown and Smith and 14 isolates of *Paecilomyces lilacinus* (Thom) Samson were characterized biochemically by separating mycelial proteins using isoelectric focusing (IEF). Eleven isozyme systems were used to detect polymorphism among these isolates. According to the cluster analysis of the isozyme data, isolates of *P. fumosoroseus* were separated into two phenetic groups with approximately 40% similarity. The *P. lilacinus* isolates were a homogenous group with high (>85%) internal similarity. Both taxa exhibited approximately 30% similarity. Among the isolates of *P. fumosoroseus* and *P. lilacinus*, most infected <40% of the eggs of the coleopteron, *Diabrotica speciosa* (Germar), and the root-knot nematode, *Meloidogyne javanica* (Treub) Chitwood; only, isolate CG 177 of *P. lilacinus* was highly virulent to both *D. speciosa* and *M. javanica* eggs. The differential virulence observed among the isolates was not correlated with the groups obtained by cluster analysis of isozymes.

KEY WORDS: **biological control; corn rootworm; root-knot nematode; entomopathogenic fungi; nematophagous fungi; soil-borne fungi; isozymes.**

Fourteen species in the fungal genus *Paecilomyces* are known pathogens of various arthropod and nematode hosts found on plants and in the soil throughout the world (Samson, 1974). For each species, many strains appear morphologically similar but differ genetically and pathogenically. A strain of *Paecilomyces fumosoroseus* (Wize) Brown and Smith designated PFR 97 (=CG170), originally isolated from an infected mealybug, infects the whitefly, *Bemisia tabaci* (Germar) (Osborne and Landa, 1992). Because of its high virulence to this agricultural pest, this PFR 97 strain is being developed commercially as a biopesticide in the United States (McCoy and Tigano-Milani, 1992).

Another soil-inhabiting species, *Paecilomyces lilacinus* (Thom) Samson, has been frequently isolated from eggs of root-knot nematodes *Meloidogyne* spp. (Godoy *et al.*, 1982) and cysts of *Heterodera glycines* Ichinohe (Gintis *et al.*, 1983) and *Globodera pallida* (Stone) Behrens (Jatala *et al.*, 1979). Greenhouse studies showed that *P. lilacinus* parasitizes eggs of *Meloidogyne arenaria* (Neal) Chitwood. According to Carneiro and Cayrol (1991), 100% of egg masses were colonized at high inoculum levels (10^6 spores/g soil), but only 50% of the eggs were infected. Experiments conducted in several countries have indicated that *P. lilacinus* controls *Meloidogyne incognita* (Kofoid and White) Chitwood and does so more effectively than a number of commonly used nematicides (Jatala, 1985).

In Brazil, the corn rootworm, *Diabrotica speciosa* (Germar), and the nematode, *Meloidogyne javanica* (Treub) Chitwood, are major soil pests of numerous agricultural crops. Preliminary data suggest that both *P. fumosoroseus* and *P. lilacinus*, common in Brazilian soils (Tigano-Milani *et al.*, 1993), infect the eggs of these important pests (Carneiro, 1992; Tigano-Milani, unpublished data). Research to determine the biological characteristics and identification of strains is basic to the selection of a strain(s) for development of fungi as a biopesticide (McCoy and Boucias, 1989). This study was conducted with *P. fumosoroseus* and *P. lilacinus* isolates to determine their pathogenicity to the eggs of *D. speciosa* and *M. javanica* and to investigate polymorphism among these isolates using isozyme analysis.

MATERIALS AND METHODS

Fungal cultures. Ten strains of *P. fumosoroseus* isolated from soil and a wide range of insects and 14 strains of *P. lilacinus* isolated from nematodes, insects,

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TABLE 1

Host Distribution and Geographical Location for the Different Isolates of *Paecilomyces*

Species/strain[a]	Geographical location[b]	Host substrate	Isozyme cluster
P. fumosoroseus			
CG123	Manaus/AM/Brazil	*Spaethiella* sp.	1
CG170[c]	Apopka/Florida/USA	*Pseudococcus* sp.	1
CG203	Naples/Florida/USA	*Bemisia tabaci*	1
CG204	Tecoman/Mexico	*Bemisia* sp.	1
CG205	Florida/USA	*Bemisia tabaci*	1
CG297	Vaucluse/France	*Pyrrhalta luteola*	2
CG323	Saitana/Japan	*Bombyx mori*	2
CG325	Manila/Philippines	*Nilaparvata lugens*	1
CG326	Brittany/France	*Musca domestica*	2
CG344	Florida/USA	Unknown	1
P. lilacinus			
CG36	Brasília/DF/Brazil	*Deois flavopica*	7
CG301	Campos/RJ/Brazil	*Cerotoma* sp.	4
CG172	Florence/Italy	*Meloidogyne* sp.	4
CG175	Castanhal/PA/Brazil	*Meloidogyne* sp.	7
CG177	Belém/PA/Brazil	*Meloidogyne* sp.	6
CG303	Peru	*Meloidogyne* sp.	7
CG179	SC/Brazil	*Meloidogyne incognita*	7
CG180	Londrina/PR/Brazil	*Meloidogyne javanica*	7
CG313	Brasília/DF/Brazil	*Meloidogyne javanica*	7
CG299	Florida/USA	Soil	4
CG331	MT/Brazil	Soil	3
CG332	MT/Brazil	Soil	3
CG333	Campinas/SP/Brazil	Soil	5
CG178	Paris/France	Soil	4

[a] CG, CENARGEN/EMBRAPA Collection, Brasília, DF, Brazil.

[b] Letter abbreviations refer to Brazilian states: AM, Amazonas; DF, Federal District; RJ, Rio de Janeiro; PA, Pará; PR, Paraná; SC, Santa Catarina; MT, Mato Grosso; SP, São Paulo.

[c] Same as PFR 97 (Osborne and Landa, 1992).

and soil from various geographical locations were selected for study (Table 1). Most isolates were obtained from the culture collections of scientists located at CENARGEN (Brazil), USDA/ARS, and the University of Florida. Initially, a monosporal isolate of each strain was obtained from a colony growing on a potato dextrose agar (PDA) plate. These monosporal cultures were used in all biochemical and pathogenicity studies to assure homogeneity within populations. Conidia needed for bioassays were obtained from cultures grown on PDA at 28°C for 15 days. Conidia were harvested by scraping the surface of the plate lightly after flooding with a sterile distilled water (SDW) containing 0.1% Tween 80 (Sigma, St. Louis, MO). Mycelia needed for biochemical studies were obtained by inoculating 50 ml of Sabouraud dextrose broth in 250-ml flasks with a final conidial suspension of 10^6 conidia/ml. Flasks were incubated on a rotary shaker maintained at 150 rpm and 26°C. After 7 days, the mycelia were separated from the supernatant by vacuum filtration. The mycelial mat was then washed several times in SDW and in a 0.05 *M* Tris–HCL, pH 7.8, buffer to remove residual broth. Mycelial were collected and concentrated by vacuum filtration, weighed, fragmented with liquid nitrogen, and centrifuged at 30,000*g* for 30 min at 4°C. The supernatants were placed in 1.5-ml ampoules and frozen at −80°C.

Electrophoresis. Hydrophilic proteins were separated via isoelectric focusing (IEF) as described by Riba *et al.* (1986a). Zymograms were obtained using 10% polyacrylamide gel containing 6% ampholytes (Pharmacia, Sollentuna, Sweden). Ten microliters of the samples was layered on the gel, and IEF was done in a pH gradient from 3 to 10 at constant power of 8 W. After migration, the gels were incubated in the appropriate staining mixtures. Although 26 enzymes systems were initially tested, we selected 11 that were polymorphic, possessed well-resolved bands, and could be consistently scored for all isolates. All monosporal isolates were tested for acid phosphatase (ACP), alkaline phosphatase (AKP), diaphorase (DIA), galactose-6-phosphate dehydrogenase (GAL-6-PDH), glutathione reductase (GR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isocitrate dehydrogenase (IDH), leucine dehydrogenase (LAP), peroxidase (PER), phosphoglucomutase (PGM), and phosphogluconate dehydroge-

nase (PGD) activity. Staining procedures were adapted from Shaw and Prasad (1970).

Pathogenicity against D. speciosa eggs. The pathogenicity of the *Paecilomyces* spp. strains was determined by using 45 one-day-old eggs of *D. speciosa*. The eggs were obtained from females collected in the field and maintained in an incubator at 26°C, 70% RH, and photophase of 14 h. The eggs were collected from a slightly wet cheesecloth kept inside cages (cylindrical containers of 13.5 × 13.0 cm and screened top) for adult individuals. Cohorts of 15 eggs were immersed in a conidial suspension (10^8 conidia/ml) for approximately 10 s. After the treatment the eggs were transferred to a petri dish containing wet cotton and a layer of filter paper. Eggs receiving only 0.1% Tween served as a control. The treatments were replicated three times. The treated eggs were incubated at 28°C and 70% RH in darkness. After 12 days, the number of unhatched eggs exhibiting fungal infection was determined microscopicaly.

Pathogenicity against M. javanica eggs. The pathogenicity of the *Paecilomyces* isolates was evaluated using 100 egg masses of *M. javanica* per treatment. The egg masses were collected from tomato plants that were grown in greenhouse, axenized with 1% streptomycin sulfate and 1% mercuriothiolic acid for 5 min, and washed using SDW. Twenty-five egg masses were placed on a screen (1-mm openings) with a sterile cotton filter on the base (four replicates per treatment). These screens were placed in a water-filled petri dish, which kept the filter wet and allowed the juveniles to hatch in water. The egg masses were placed proximate to one another, but not in contact, and inoculated with 0.3 ml of conidial suspension (10^8 conidia/ml) of each *Paecilomyces* isolate. The control was treated with the same volume of SDW. The petri dishes were covered and kept at 28°C and 90% RH in the dark. After 15 days, the percentage of egg masses colonized by the fungus (completely covered by fungal conidiophores), and the number of hatched juveniles was determined.

Statistical analysis. A dendrogram was constructed from the isozyme data using NTSYS-pc program (Rohlf, 1993). A similarity matrix was created using the Jaccard coefficient, and cluster analysis was done using the unweighted pair group arithmetic mean method (UPGMA) (Sneath and Lokal, 1973). For ANOVAs and mean comparisons, the percentage of infected eggs were transformed using the arcsin transformation, and the number of *M. javanica* juveniles hatching was transformed to logarithmic scale. Means groups were subsequently obtained using the cluster analysis method described by Scott and Knott (1974).

RESULTS AND DISCUSSION

Eleven of 26 isozymes were selected, based on the number of bands, for the biochemical separation via IEF of 24 fungal strain mycelial proteins. Each enzyme expressed a combination of bands in two or three patterns on the gels, depending on the isolate, indicating considerable molecular homogeneity. The enzyme profiles for the enzyme systems PGD, PGM, and ACP showed no common banding positions for isolates of the two taxa (Fig. 1). As shown in the dendrogram (Fig. 2), isolates of *P. fumosoroseus* were clustered in two phenetic groups with approximately 40% similarity. The strains CG123, CG170, CG203, CG204, CG205, CG325, and CG344 (cluster 1) were identical for the characters analyzed, as were the strains CG297, CG323, and CG326 (cluster 2). These groups did not correlate by geographical origin or host, and the low similarity between them indicates the possibility of subgroups within the species, or even the existence of an aggregate species, as has already been shown for other entomopathogenic fungi (St Leger *et al.*, 1992a,b).

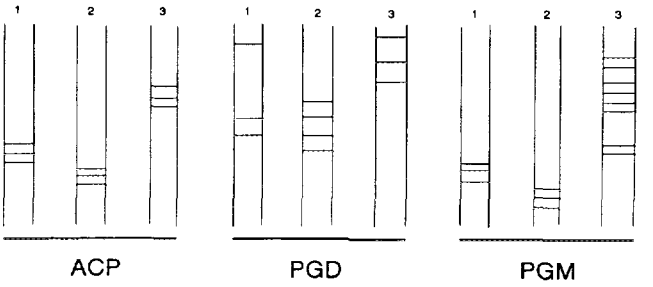

FIG. 1. Electrophoretic polymorphism of acid phosphatase (ACP), phosphogluconate dehydrogenase (PGD), and phosphoglucomutase (PGM) systems in the genus *Paecilomyces*. The first, second, and third lanes (or profile) for each enzyme represent data for CG123, CG170, CG203, CG204, CG205, CG325, and CG344; CG297, CG323, and CG326; and *P. lilacinus* strains (Table 1), respectively.

The 14 isolates of *P. lilacinus,* collected from different geographic locations and from insects, nematodes or soil, clustered together in a homogenous group at more than 85% similarity (Fig. 2). Each cluster represented identical isolates or only one isolate: cluster 3 (CG331 and CG332), cluster 4 (CG172, CG178, CG299, and CG301), cluster 5 (CG333), cluster 6 (CG177), and cluster 7 (CG36, CG175, CG179, CG180, CG303, and CG313).

These results support the taxonomic separation of *P. lilacinus* from *P. fumosoroseus* based on morphological differences and agree with previous isozyme studies within other taxa, such as *Hirsutella* and *Tolypocladium* (Boucias *et al.*, 1982; Riba *et al.*, 1986b). Therefore, 11 isozymes are useful to differentiate between typical *P. fumosoroseus* and *P. lilacinus* strains. In fact, isozyme polymorphism have proved useful for genetic studies for a range of fungi. With other biochemical characteristics, isozymes can also be useful for improving the classical taxonomy of entomopathogenic fungi, e.g., the genus *Beauveria* (Mugnai *et al.*, 1989). But, to

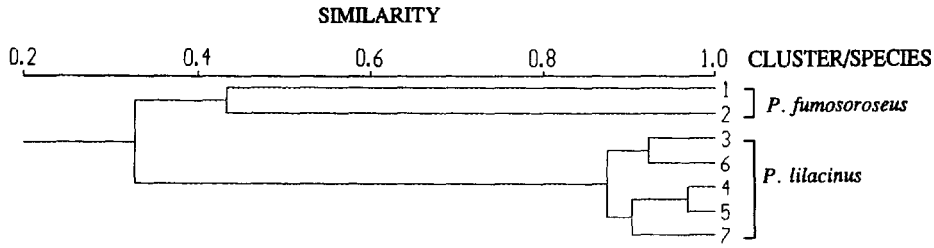

FIG. 2. Dendrogram constructed from isozymes data, indicating the relationships among *Paecilomyces* isolates. A similarity matrix was calculated based on Jaccard coefficient, and the tree was generated from this matrix by the unweighted pair group method, arithmetic mean (UPGMA). For classification of isolates based on cluster analysis, see Table 1.

better elucidate the intra- and interspecific variability within the genus *Paecilomyces* more isolates should be tested.

The results of the pathogenicity tests are represented in Table 2. Most rootworm eggs were resistant to *Paecilomyces,* and infection was usually less than 40%. Two isolates of *P. fumosoroseus* (CG170 and CG204) and two of *P. lilacinus* (CG177 and CG332), however, were virulent, killing more than 75% of the eggs. Infectivity of *M. javanica* egg varied widely among isolates. One

TABLE 2

Pathogenicity of *P. fumosoroseus* (Pfr) and *P. lilacinus* (P) to *D. speciosa* and *M. javanica* Egg Masses

		M. javanica		
Isolates	*D. speciosa* Infected eggs (%)[a,b,c]	Infected egg masses (%)[a,c,d]	Juveniles hatching[a,d,e]	Estimated Control[f]
CG177 (P1)	83.3 *b*	93.0 *a*	90 *a*	96.6
CG332 (P1)	80.0 *b*	57.0 *d*	645 *c*	75.9
CG170 (Pfr)[g]	76.7 *b*	17.0 *g*	1533 *h*	42.8
CG204 (Pfr)	96.0 *a*	6.0 *h*	2650 *l*	1.1
CG344 (Pfr)	10.0 *d*	71.0 *c*	180 *b*	93.3
CG303 (P1)	20.0 *c*	65.0 *d*	283 *b*	89.5
CG333 (P1)	30.0 *c*	84.0 *b*	109 *a*	95.9
CG313 (P1)	0.0*	54.0 *d*	770 *d*	71.3
CG178 (P1)	30.0 *c*	38.0 *e*	773 *d*	71.2
CG123 (Pfr)	40.0 *c*	18.0 *g*	1550 *h*	42.2
CG325 (Pfr)	20.0 *c*	29.0 *f*	1123 *e*	58.1
CG180 (P1)	23.3 *c*	54.0 *d*	748 *d*	72.1
CG299 (P1)	30.0 *c*	50.0 *e*	810 *d*	69.8
CG301 (P1)	30.0 *c*	32.0 *f*	1300 *f*	51.5
CG179 (P1)	20.0 *c*	24.0 *f*	1350 *f*	49.6
CG297 (Pfr)	16.7 *c*	50.0 *e*	1610 *i*	39.9
CG331 (P1)	20.0 *c*	18.0 *g*	1979 *j*	26.5
CG36 (P1)	6.7 *d*	29.0 *f*	1500 *h*	44.0
CG203 (Pfr)	3.3 *d*	15.0 *g*	1433 *g*	46.5
CG175 (P1)	0.0*	47.0 *e*	583 *c*	78.3
CG205 (Pfr)	10.0 *d*	20.0 *g*	2265 *j*	15.5
CG172 (P1)	3.3 *d*	10.0 *h*	2523 *k*	5.9
CG323 (Pfr)	0.0*	25.0 *f*	1345 *f*	49.8
CG326 (Pfr)	13.3 *d*	5.0 *h*	2589 *k*	3.4
Control	0.0*	0.0*	2680 *l*	0.0

[a] Means followed by the same letter were consideed a homogenous set of means ($\alpha = 0.05$) (Method by Scott and Knott, 1974).
[b] Values are means of three replicates with 15 eggs/replicate.
[c] Percentage data transformed to arcsin (square root percent/100) for analysis and retranslated.
[d] Values are means of four replicates with 25 egg masses/replicate.
[e] Means of juveniles hatching were transformed to logarithmic scale for analysis and retranslated.
[f] EC = $(1 - T/C) \times 100$, where T is the mean of juveniles hatching in the treatment and C is the mean of juveniles hatching in the control.
[g] Same as Pfr 97 (Osborne and Landa, 1992).
* Treatment not submitted to Anova.

isolate of *P. fumosoroseus* (CG344) and three isolates of *P. lilacinus* (CG177, CG303 and CG333) caused more than 90% mortality. The ability of *P. fumosoroseus* to infect insect eggs has already been demonstrated in lepidopterons (Fargues and Rodrigues-Rueda, 1980) but not in nematodes such as *Meloidogyne* spp. *P. lilacinus* isolate CG177 was the only pathotype effective against the eggs of both *D. speciosa* and *M. javanica*. The isolate CG177 was even more virulent against *M. javanica* than isolate CG303, which is from Peru and has been used in most field tests against nematodes (Jatala, 1985). In some other experiments, *P. lilacinus* provided variable efficacy, but in these cases some basic requirements to estimate properly the potential of a biological control agent were not satisfied (Kerry, 1990). In fact, the performance obtained by in vitro bioassays does not necessarily represent the same efficacy of the strains in the field. However, as greenhouse and field experiments are time consuming and labor intensive, the tests *in vitro* allow preliminary selection of the isolates. So in terms of crop management, *P. lilacinus* should be further evaluated in soil as a biological agent to control both *D. speciosa* and *M. javanica*, major pests in Brazilian soils.

The two characteristics analyzed in this work, isozymes and pathogenicity, have not shown any correlation. The most highly virulent isolates of *P. fumosoroseus* and *P. lilacinus* (CG170, CG177, CG204, CG303, CG332, CG333, CG344) against rootworm and (or) rootknot nematode eggs (Table 2) were not related to any specific cluster (Fig. 2, Table 1) obtained by isozymes analysis.

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