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Mycoparasitic actions against fungi and oomycetes by a strain (CCFEE 5003) of the fungus *Lecanicillium muscarium* isolated in Continental Antarctica

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Abstract - A strain (CCFEE 5003) of *Lecanicillium muscarium,* isolated in Continental Antarctica, *showed* mycoparasitism in agar co-cultures, at 5 and 25 °C, against *Mucor mucedo, Botrytis cinerea*, *Pythium aphanidermatum* and *Phytophthora palmivora*. Different sequential steps were observed in the process leading to parasitism and resulting in a complete host disruption. Parasitism against fungi was characterised by diffused penetration into the host mycelium; with oomycetes, penetration was less evident and the contact between the two organisms was more intimate. Production of glucanolytic and chitinolytic enzymes appeared related to the mycoparasitic process.

Key words: chitinolytic enzymes, Continental Antarctica, mycoparasitism, *Lecanicillium muscarium*.

INTRODUCTION

Many eukaryotic microorganisms cause crop diseases and food spoilage with consistent economic losses due to the diseases themselves and to the necessary control measures. Among fungi, the genus *Botrytis* is known for its pathogenicity while species of *Mucor* are often involved in food spoilage (Fenice *et al.,* 1998). Oomycetes such as *Pythium* and the closely related genus *Phytophthora* are infamous for the severe damages to important cultivated plants (i.e. the genera *Solanum* and *Lycopersicon*). For example, worldwide losses in potato production caused by *Phytophthora* species have been estimated to cost about $3 billion per year (Duncan, 1999).

In recent years, due to undesirable side effects on the environment and human health, the use of chemical pesticides, fungicides and food preservatives has been widely criticised and, as a consequence, microorganisms and/or their products have been studied to develop safer alternatives to chemicals (Chet *et al.,* 1993; Lorito *et al.,* 1994; Fenice *et al.,* 1998, 1999). Most studies concerned *Trichoderma harzianum* a mycoparasite that produces several cell-wall degrading enzymes and which is used as pest biocontrol agent (Chet, 1987; Harman and Kubicek, 1998). Exogenous cell-wall degrading enzymes (i.e. chitinases and glucanases) have been shown to exert aggressive role in mycoparasitism disarranging the dynamic of the host hyphal growth and leading to lysis (Gooday, 1995, 1999).

Lecanicillium muscarium, ex *Verticillium lecanii* (Gams and Zare, 2001; Zare and Gams, 2001), is well known as an entomopathogenic fungus (Hall, 1981; Schreiter *et al.,* 1994; Askary *et al.,* 1999) but it also exerts mycoparasitic activity against fungi involved in food spoilage (Fenice *et al,.* 1998) or plant diseases (Langen *et al.,* 1992; Askary *et al.,* 1996; Verhaar *et al,.* 1996; Benhamou and Brodeur, 2000). In addition, the potential of *L. muscarium* as a biocontrol agent against some fungi causing plant diseases has been proved (Verhaar *et al.,* 1996). Apparently, for this organism that is generally known as an entomopathogenic fungus (Cuthbertson *et al.,* 2005), no parasitic activity against oomycetes has been previously reported.

Lecanicillium muscarium CCFEE 5003, isolated in Continental Antarctica, has been shown to exert mycoparasitism against several moulds via the production of chitinolytic enzymes, also at low temperature (Fenice *et al.,* 1998). The availability of a psychrophilic biocontrol agent could be of value in various situations including biocontrol of pathogenic fungi in cold environments where other biocontrol microorganisms very often fail (Malathrakis and Kritsotaki, 1992).

In this study, we report on the interactions that take place when oomycetes, such as *Pythium aphanidermatum* and *Phytophthora palmivora*, and fungi, such as *Botrytis cinerea* and *Mucor mucedo*, are grown in presence of strain CCFEE 5003 of *L. muscarium.* Some preliminary aspects of the production of cell-wall lytic enzymes by this strain were also investigated.

MATERIALS AND METHODS

Chemicals. Chitin (from crab shells), *N*-acetyl-D-glucosamine, 4-methylumbelliferone (4MU); 4-methylumbellif-

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eryl–β–D–*N*-acetylglucosaminide; 4-methylumbelliferyl–β-D-*N,N'*-diacetylchitobioside; 4-methylumbelliferyl–β–D–*N,N',N''* -triacetylchitotrioside and 4-methylumbelliferyl–β–D–*N,N', N'',N'''*-tetraacetylchitotetraoside were from Sigma Chemicals (St. Louis, MO, USA); potato dextrose agar (PDA) and yeast nitrogen base (YNB) were from Difco Laboratories (Detroit, MI, USA). All other chemicals were of analytical grade.

Colloidal chitin was prepared as already reported (Hankin and Anagnostakis, 1975). After swelling, chitin was resuspended in water and autoclaved (121 °C, 20 min). Glycol chitin was prepared as reported by Molano *et al.* (1979).

Microorganisms and culture conditions. *Lecanicillium muscarium* Petch strain CCFEE 5003, ex *Verticillium lecanii* Zimm strain A3 (Zucconi *et al.,* 1996) and *Botrytis cinerea* Pers. ex Nocca & Balb. were from Dipartimento di Agrobiologia e Agrochimica, University of Tuscia, Viterbo, Italy; *Mucor mucedo* Mich.*, Pythium aphanidermatum* (Edson) Fitzp. and *Phytophthora palmivora* Butler were from the Department of Molecular and Cell Biology, University of Aberdeen, Foresterhill, Aberdeen, Scotland, United Kingdom. During the study, cultures were maintained on PDA slants at 4-6 °C and subcultured every month.

The basal culture medium (BM) was as follows (w/v): glucose 1%, YNB 1%, pH 5.5; the liquid medium for detection of chitinolytic enzymes activity (CM) was: colloidal chitin 1%, YNB 1%, pH 5.5; the liquid medium for detection of glucanase activity (GM) was: laminarin 1%, YNB 1%, pH 5.5. The solid medium for co-cultures was PDA 4%. All media were autoclaved (121 °C, 20 min). The media for chitinase and glucanase production, 50 ml in 250 ml Erlenmeyer flasks (or 20 ml in 100 ml flasks), were inoculated with mycelium from 7-d-old slants of *L. muscarium*.

The chitinolytic activity was also detected using the media obtained as follows: live or killed (autoclaved at 125 °C for 12 min) mycelia obtained from liquid cultures (72 h) in BM of *P. aphanidermatum* or *M. mucedo* were suspended in 50 ml of sterile distilled water. Before merging, mycelia of the above microorganisms were centrifuged (10000 *x g,* 10 min at room temperature), washed extensively in sterile distilled water and re-centrifuged to eliminate any other nutrient.

Media were inoculated using the mycelium obtained by *L. muscarium* 7 d PDA slants.

Shaken cultures were carried out at 28 °C and 180 rpm. Samples were taken every 8, 12 or 24 h and, after centrifugation as above, supernatants were used for the enzyme assays. Experiments were done in triplicate.

Detection of mycoparasitism. To investigate its possible inhibition effects, 1 ml of cultural broth (showing ca. 300 and 200 U l^{-1} of chitinolytic and glucanolytic activity, respectively) of *L. muscarium*, grown on colloidal chitin for 72 h, was filter-sterilised (Millipore filters 0.2 μm) and added to each test organism growing on Petri dishes filled with 15 ml of PDA and incubated at 5 and 25 °C. Controls were done using sterile broth in place of the above enzyme solution.

Antagonism was observed on Petri dishes filled with 15 ml of PDA and inoculated with *L. muscarium* and each test organism placed at opposite sides (co-cultures) and incubated at 5 and 25 °C, as already reported (Fenice *et al.,* 1998). Fungal interactions were observed in the contact zones of the two colonies both under light and scanning electron microscope (SEM). For SEM, samples from dual cultures were aseptically cut with a sharp lancet, fixed and coated as already reported (Onofri *et al.,* 1980) and examined with a "JEOL JSM 35cF" (Jeol, Japan) microscope.

Enzyme assay and activity gels. The molecular weight (MW) of chitinolytic enzymes using one-dimensional activity polyacrylamide gel electrophoresis (PAGE) was determined following the procedure of Trudel and Asselin (1989); markers (range 15-150 KDa) were from Biorad (Ca, USA). MWs and isoelectric points (Pi) using two-dimensional PAGE were determined as reported by Albrecht *et al.* (1994).

β-1,3-Glucanase activity was determined measuring the amount of glucose liberated from laminarin by the enzyme activity following the method of Miller (1959). Overall chitinolytic activity was determined as already reported (Fenice *et al.,* 1998); exo-chitinase, endo-chitinase and chitobiosidase were detected by the release of 4-methylumbelliferone from 4MU substrates by the method of McCreath and Gooday (1992). *N*-Acetyl-D-glucosamine, 4-methylumbelliferone and glucose were used as substrates for each standard curve, respectively. Under the assay conditions, one unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of product per millilitre per minute.

Statistical analysis. One way analysis of variance (ANOVA) and Tukey test were performed using the software SigmaStat (Jandel Corp., San Rafael, CA, USA).

RESULTS AND DISCUSSION

Mycoparasitism of *Lecanicillium muscarium* CCFEE5003 in co-cultures

Experiments were carried out at 5 and 25 °C. At 5 °C microorganisms showed slower growth but the results were similar to those observed at 25 °C (Fig. 1A, 1B; Fig. 2A, 2B).

All co-cultures showed some common features. The tested organisms grew well until they reached a distance of 3-10 mm from the colonies of *L. muscarium* thus entering an "inhibition zone" (Fenice *et al.,* 1998). At this stage, all host organisms stopped their growth while mycelial modifications, never observed in axenic cultures of the same organisms, such as branching (Fig. 1A, 1B), damage, presence of uncommon structures (Fig. 2A, 2B) and sometime protoplast formation became evident. This suggests that *L. muscarium* likely released into the medium some diffusible and inhibiting factors (i.e. lytic enzymes and/or antibiotics). This is typical of mycoparasitic fungi such as *Trichoderma* spp. (Bruce *et al.,* 1995; Inbar *et al.,* 1996; Calistru *et al.,* 1997) or *Verticillium* spp. (Morris *et al.,* 1995; Benhamou and Brodeur, 2000). Similar effects on the same test organisms were observed when filtered (0.4 μm) broth from shaken cultures of *L. muscarium* grown on chitin and containing chitinolytic and glucanolytic activities was added to the Petri dishes in place of the growing fungus (Fig. 1C).

After some time, however, *L. muscarium* began to get in contact with the host and, shortly thereafter, to overwhelm it. Samples of the contact zones from agar co-cultures were observed under light microscopy and SEM.

Always, the contact between the Antarctic strain and the host appeared very firm (Fig. 3A, 3B). Coiling was observed but coils were rather random and not regularly developed around host hyphae as reported for other mycoparasites (Benhamou and Chet, 1993; Inbar *et al.,* 1996).

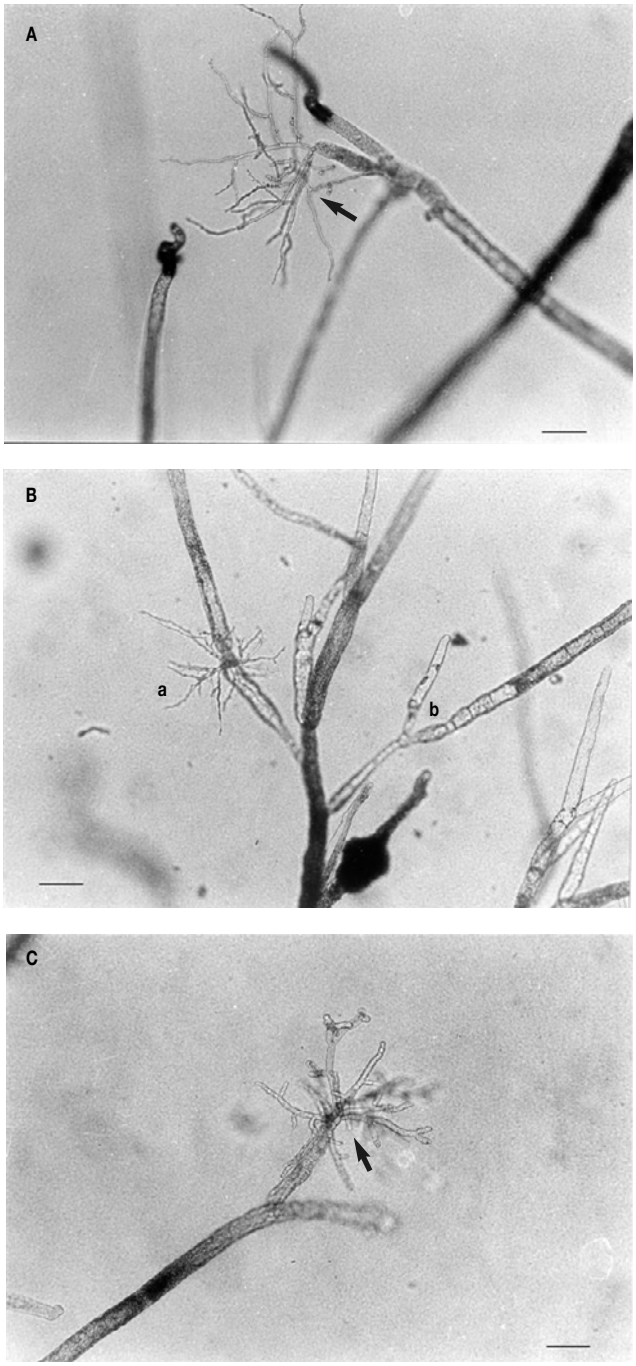

FIG. 1 – Effect of *Lecanicillium muscarium* CCFEE 5003 grown in co-cultures with *Mucor mucedo* (A, B) and effect of the addition of *L. muscarium* culture filtrate on *M. mucedo* cultures (C): observation under light microscope; bar = 50 μm. A) Co-cultures at 5 °C: branched mycelium of *M. mucedo* (arrow); B) co-cultures at 25 °C: branching (a) and vacuolated (b) mycelium of *M. mucedo*; C) branched mycelium of *M. mucedo* (arrow).

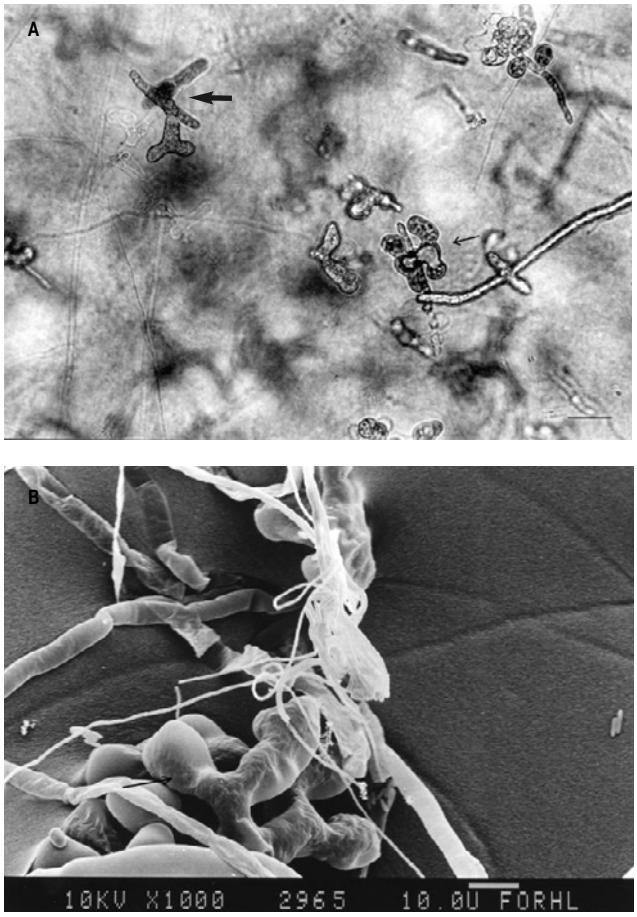

FIG. 2 – Co-cultures at 5 (A) and 25 °C (B) of *Lecanicillium muscarium* CCFEE 5003 and *Pythium aphanidermatum*. A) Uncommon structures of *P. aphanidermatum* (arrows): observation under light microscope; bar = 50 μm; B) same structures observed at SEM (arrow).

The kind of contact exerted by *L. muscarium* type appeared to depend upon the host organism. In the case of fungi, the parasite appeared to follow a typical sequence of events that included attachment to the host, mechanical pressure and production of cell-wall degrading enzymes and diffused penetration into the host cell (Askary *et al.*, 1996; Fenice *et al.,* 1998) (Fig. 3A, 3B). In contrast, no evident penetration but, apparently, firm adhesion and possibly mechanical pressure occurred with the oomycetes appearing the mycelium of *L. muscarium* sometime almost fused with that of the host (Fig. 3B). In all cases, at the late stages of the interaction, *L. muscarium* caused complete destruction of the host that appeared strongly deflated, depressed and invaded by the parasite (Fig. 4A, B). Abundant sporulation of *L. muscarium* was always observed (Fig. 3 and 4).

Bruce *et al.* (1995) described the mycoparasitism as an antagonistic interaction between two fungi during which the aggressor makes intimate contact with the target organism before releasing lytic enzymes to facilitate the degradation of the host cell wall. Others (Benhamu and Chet, 1993; Inbar *et al.,* 1996; Calistru *et al.,* 1997) relate the mycoparasitism to typical actions such as coiling around and penetration into the host; production of lytic enzymes was also mentioned. Sometime, mycoparasitism is simply defined as the attack of a fungus against other fungi (Paul, 1999). Most authors, however, agree that a firm contact between the two organisms must occur (Benhamu and Chet, 1993; Inbar *et al.,* 1996; Calistru *et al.,* 1997).

Sometime, mycoparasites such as *Trichoderma* sp. establish inhibitory interactions with other organisms producing extracellular metabolites and/or volatile compounds with no physical contact between the two organisms (Calistru *et al.,* 1997).

Lecanicillium muscarium CCFEE 5003 always established

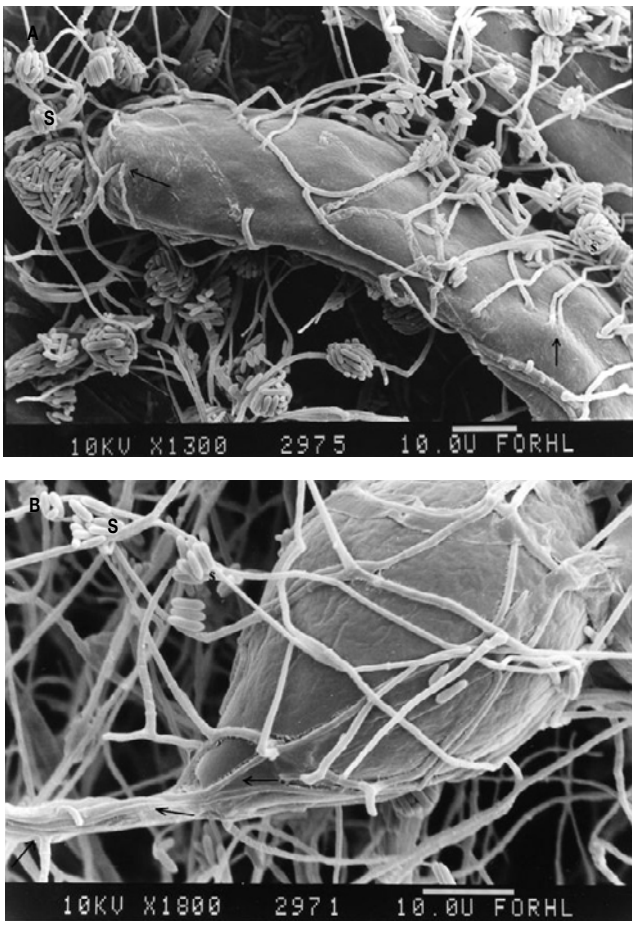

FIG. 3 – Co-cultures at 25 °C of *Lecanicillium muscarium* CCFEE 5003 and *Mucor mucedo* (A) and *Phytophthora palmivora* (B): SEM observations. A) Intermediate stage of the mycoparasitism: *L. muscarium* (small hyphae) took contact with *M. mucedo* (big hyphae), penetrated in it (arrows) and diffusely sporulated (s); B) Intermediate stage of the mycoparasitism: *L. muscarium* (small hyphae) took contact with *P. palmivora* (big hyphae) appearing almost fused with the host (arrows) and diffusely sporulated (s).

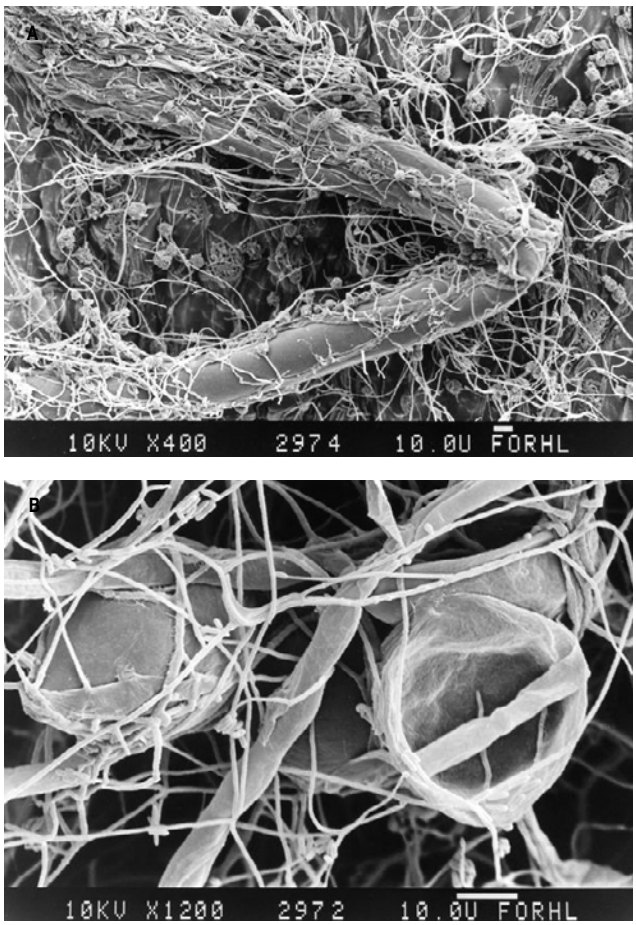

FIG. 4 – Co-cultures of *Lecanicillium muscarium* CCFEE 5003and *Mucor mucedo* (A) and *Phytophthora palmivora* (B) at 25 °C: SEM observations. Late stage of mycoparasitism: all host structures are invaded by *L. muscarium* mycelium and are completely deflated and collapsed.

firm contact with the host after a preliminary phase during which inhibitory compounds were probably released. Always, when the organisms came in contact with *L. muscarium* CCFEE 5003 they were disrupted. The parasitic action appeared particularly effective being exerted also against *P. aphanidermatum* that is itself a mycoparasite (Paul, 1999).

The results of this study show that the Antarctic fungus *L. muscarium* CCFEE 5003 could be considered a promising mycoparasitic organism to be used with pathogenic fungi and, above all, oomycetes. Since the parasitic action occurs at low temperature also, particular applications, such as the use in cold environments, could be supposed. However, the actual possibilities of this organism must be clearly demonstrated: glasshouse and field trials have to be studied to validate its possible role as a biocontrol agent. Moreover, ecological tests must be done prior to release the Antarctic strain in other environments.

Production of lytic enzymes

In order to understand which lytic enzymes were released during *L. muscarium* mycoparasitism, the fungus was shaken

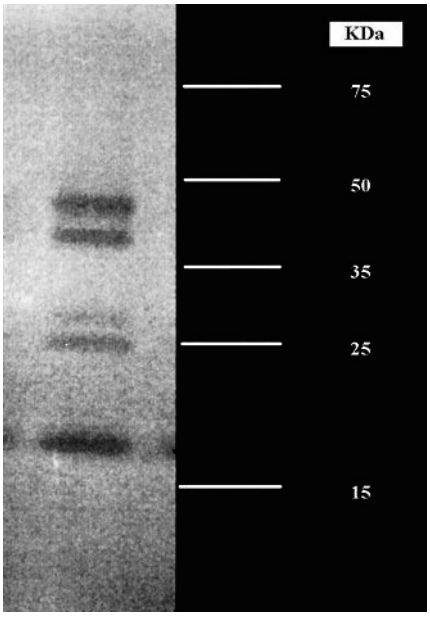

FIG. 5 – One-dimension activity PAGE for chitinolytic enzymes obtained loading cultural broth of *Lecanicillium muscarium* grown on colloidal chitin. Molecular weight markers were "Perfect Protein Markers" from Biorad (15-150 KDa), only the marks in the range 15-75 KDa are shown.

TABLE 1 – Chitinolytic and glucanolytic activities of *Lecanicillium muscarium* CCFEE 5003 grown in shaken cultures on several chitin and glucan sources after 72 h of incubation at 28 °C

Substrate	Enzyme activity (U l^{-1})					
	CHI	β-1,3-Glu	4MU-1	4MU-2	4MU-3	4MU-4
Chitin	312 ± 34^{a}	83 ± 15^{b}	76 ± 5^{a}	74 ± 2^{a}	46 ± 2^{a}	52 ± 4^{a}
Laminarin	134 ± 20^{b}	52 ± 3^{a}	34 ± 4^{b}	32 ± 3^{b}	18 ± 1^{b}	23 ± 2^{b}
P. live	202 ± 19^{bc}	61 ± 6^{a}	46 ± 3^{c}	53 ± 4^{c}	32 ± 1^{c}	29 ± 3^{bd}
P. killed	211 ± 30^{c}	60 ± 8^{a}	41 ± 2^{ce}	50 ± 4^{cd}	33 ± 2^{c}	27 ± 2^{b}
M. live	257 ± 28^{ac}	75 ± 8^{a}	39 ± 4^{c}	57 ± 2^{c}	32 ± 4^{c}	37 ± 3^{cd}
M. killed	263 ± 23^{ac}	72 ± 7^{a}	36 ± 2de	44 ± 1^{d}	36 ± 3^{c}	38 ± 2^{c}

Legend. CHI: Overall chitinolytic activity on colloidal chitin; β-1,3-Glu: Glucanolytic activity on laminarin; 4MU-1, 4MU-2, 4MU-3, 4MU-4: chitinolytic activity detected by the releasing of 4-methylumbelliferon from 4-methylumbelliferyl–β–D–*N*-acetylglucosaminide, 4-methylumbelliferyl–β-D-*N,N′*-diacetylchitobioside, 4-methylumbelliferyl–β–D–*N,N′,N″*-triacetylchitotrioside and 4-methylumbelliferyl–β–D–*N,N′,N″,N‴*-tetraacetylchitotetraoside, respectively; Chitin: colloidal chitin; *P.* live: live mycelium of *Pythium aphanidermatum*; *P.* killed: killed (autoclaved) mycelium of *P. aphanidermatum*; *M.* live: live mycelium of *Mucor mucedo*; *M.* killed: killed (autoclaved) mycelium of *M. mucedo*. Values are mean of three replicates ± SD. Column means followed by the same superscript letter were not significantly different (P<0.05) as determined by the Tukey test.

cultured on colloidal chitin, glucan (laminarin) or mycelium (live or killed in autoclave) of *P. palmivora, P. aphanidermatum* and *M. mucedo*. In all cases, both chitinolytic and glucanolytic enzymes were released into the cultural broth and the assays with the 4MU substrates showed that *L. muscarium* CCFEE 5003 produced a pattern of chitinolytic enzymes with exo, endo and chitobiosidase activities (Table 1).

One-dimensional activity gels showed, that *L. muscarium* released at least five chitinolytic enzymes (Fig. 5) with MWs ranging from ca. 20 to 75 kDa while two-dimensional activity gels showed that isoelectric points of the various enzymes ranged from ca. pH 4.5 to pH 5.0 (data not shown). Previously (Fenice *et al.,* 1998), we purified from *L. muscarium* a chitinolytic enzyme having apparent MW of 45 kDa, isoelectric point of 4.9 and strong inhibiting effects against several moulds. Thus, the 45 kDa enzyme is a part of a complex chitinolytic system. Mycoparasitic fungi, such as *Trichoderma* species, generally show a pool of chitinolytic enzymes having a complete competence in chitin hydrolysis with diversified endo and exo activities (Chet, 1987). On the contrary, fungi that use chitin just for nutrition often present few chitinolytic enzymes with limited competence on chitin hydrolysis (Di Giambattista *et al.,* 2001). In this context, the mycoparasitic role of *L. muscarium* appears also from its ability to produce a wide pattern of cell wall degrading enzymes, chitinases in particular.

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REFERENCES

Albrecht C., Laurent P., Lapeyre F. (1994). Eucalyptus root and shoot chitinases, induced following root colonisation by pathogenic versus ectomycorrhizal fungi, compared on one- and two-dimensional activity gels. Plant Sci., 100:157-164.

Askary H., Benhamou N., Brodeur J. (1996). Ultrastructural and cytochemical investigations of the antagonistic effect f *Verticillium lecanii* on cucumber powdery mildew. Phytopathology, 87: 359-368.

Askary H., Benhamou N., Brodeur J. (1999). Ultrastructural and cytochemical characterization of aphid invasion by the hyphomycete *Verticillium lecanii*. J. Invertebr. Pathol., 74: 1-13.

Benhamou N., Brodeur I. (2000). Evidence for antibiosis and induced host defence reactions in the interaction between *Verticillium lecanii* and *Penicillium digitatum* the causal agent of green mould. Phytopathology, 90: 932-943.

Benhamou N., Chet I. (1993). Hyphal interaction between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold cytochemistry of the mycoparasitic process. Phytopathology, 83: 1062-1071.

Bruce A., Scrinivasan U., Staines H.J., Highley T.L. (1995). Chitinase and laminarinase production in liquid culture by *Trichoderma* spp. and their role in biocontrol of wood decay fungi. Int. Biodeterior. Biodegrad., 4: 337-353.

Calistru C., McLean M., Berjak P. (1997). *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. Mycopathologia, 139: 115-121.

Chet I. (1987). *Trichoderma*-application, mode of action, and potential as biocontrol agent in soilborne plant pathogenic fungi. In: Chet I., Ed., Innovative Approaches to Plant Disease Control. J. Wiley & Sons, New York, pp. 137-160.

Chet I., Barak Z., Oppenheim A. (1993). Genetic engineering of micro organisms for improved biocontrol activity. In: Chet I., Ed., Biotechnological Prospects of Plant Disease Control. Wiley-Liss, New York, pp. 211-235.

Cuthbertson A.G.S., Walters K.F.A., Northing P. (2005). The susceptibility of immature stages of *Bemisia tabaci* to the entomopathogenic fungus *Lecanicillium muscarium* on tomato and verbena foliage. Mycopathologia, 159: 23-29.

Di Giambattista R., Federici F., Petruccioli M., Fenice M. (2001). The chitinolytic activity from *Penicillium janthinellum* P9: partial purification and characterisation and potential applications. J. Appl. Microbiol., 91: 498-505.

Duncan J. (1999). *Phytophthora*, an abiding threat to our crops. Microbiol. Today, 26: 114-116.

Fenice M., Selbmann L., Di Giambattista R., Federici F. (1998). Chitinolytic activity at low temperature of an Antarctic strain (A3) of *Verticillium* cfr. *lecanii*. Res. Microbiol., 149: 289-300.

Fenice M., Di Giambattista R., Leuba GL., Federici F. (1999). Inactivation of *Mucor plumbeus* by the combined action of chitinase and high hydrostatic pressure. Int. J. Food Microbiol., 52: 109-113.

Gams W., Zare R. (2001). A revision of *Verticillium* sect. *Prostrata*. III. Generic classification. Nova Hedwigia, 72: 329-337.

Gooday G.W. (1995). The dynamics of hyphal growth. Mycol. Res., 99: 385-394.

Gooday G.W. (1999). Aggressive and defensive roles for chitinases. In: Jolles P., Muzzarelli R.A.A., Eds, Chitin and Chitinases. Birkhauser Verlag Basel, CH, pp. 153-165.

Hall R.A. (1981). The fungus *Verticillium lecanii* as a microbial insecticide against aphids and scales. In: Burges H.D., Ed., Microbial Control of Pests and Plant Diseases. Academic Press, New York, pp. 483-498.

Hankin L., Anagnostakis S. (1975). The use of solid media for detection of enzyme production by fungi. Mycologia, 67: 597-607.

Harman G.E., Kubicek C.P., Eds (1998). *Trichoderma* and *Gliocladium.* Vol. 2. Enzymes, Biological Control and Commercial Applications. Taylor & Francis, London.

Inbar J., Menendez A., Chet I. (1996). Hyphal interaction between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. Soil Biol. Biochem*.,* 28: 757-763.

Langen G., Beißmann B., Reisener H.J., Kogel K. (1992). A β-1,3-D-endo-mannanase from culture filtrates of the hyperparasites *Verticillium lecanii* and *Aphanocladium album* that specifically lyses the germ pore plug from uredospores of *Puccinia graminis* f.sp. *tritici*. Can. J. Bot., 70: 853-860.

Lorito M., Peterbauer C., Hayes C.K., Harman G.E. (1994). Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. Microbiology, 140: 623-669.

Malathrakis N.E., Kritsotaki O. (1992). Effect of substrate, temperature and time of application on the effectiveness of three antagonistic fungi against *Botrytis cinerea*. In: Verhoeffer K., Malathrakis N.E., Williamson B., Eds, Recent Advances in *Botrytis* Research. Pudoc Scientific Publisher, Wageningen, p. 187-191.

McCreath K.J, Gooday G.W. (1992). A rapid and sensitive microassay for determination of chitinolytic activity. J. Microbiol. Meth., 14: 229-237.

Miller G.L. (1959). The use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem*.,* 31: 426.

Molano J., Polachek I., Duran A., Cabib E. (1979). An endochitinase from wheat germ: activity on nascent and preformed chitin. J. Biol. Chem., 254: 4901-4907.

Morris R.A.C., Ewing D.F., Whipps J.M., Coley-Smith J.R. (1995). Antifungal hydroxymethyl-phenols from the mycoparasite *Verticillium biguttatum*. Phytochemistry, 39: 1043-1048.

Onofri S., Castagnola M., Rossi Espagnet S. (1980). L'impiego della microscopia elettronica a scansione in micologia. Micologia Italiana, 1: 29-32.

Paul B. (1999). *Pythium periplocum*, an aggressive mycoparasite of *Botrytis cinerea* the grey mould disease of grape-wine. FEMS Microbiol. Lett*.,* 181: 277-280.

Schreiter G.T., But M., Beckett A., Verstegaard S., Moritz G. (1994). Invasion and development of in the western flower thrips, *Frankliniella occidentalis*. Mycol. Res., 98: 1025-1034.

Trudel J., Asselin A. (1989). Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal*.* Biochem., 178: 362-366.

Verhaar M.A., Hijwegen T., Zadoks J.C. (1996). Glasshouse experiments on biocontrol of cucumber powdery mildew (*Sphaerotheca fuliginea*) by the mycoparasites *Verticillium lecanii* and *Sporothrix rugulosa*. Biol. Control., 6: 353-360.

Zare R., Gams W. (2001). A revision of *Verticillium* sect. *Prostrata*. IV. The genera *Lecanicillium* and *Simplicillium* gen. nov. Nova Hedwigia*,* 73: 1-50.

Zucconi L., Pagano S., Fenice M., Selbmann L., Tosi S., Onofri S. (1996). Growth temperature preferences of fungal strains from Victoria Land, Antarctica. Polar Biol., 16: 53-61.