Distinction Between *Penicillium canescens* and *Penicillium janczewskii* by Means of Polygalacturonase and Esterase Isozyme Analysis

R. Nicoletti*, R. Caiazzo, A. Carella and E. Lahoz

Council for Research and Experimentation in Agriculture, Research Unit of Scafati, Scafati, Italy

Abstract: Classification of many *Penicillium* species has been recently revised through the application of several biochemical and molecular methods. The distinction between the species *Penicillium canescens* and *Penicillium janczewskii* is problematic due to the existence of isolates with intermediate morphological features forming an interface between the two taxa. Electrophoretic analysis of polygalacturonase and esterase isozymes carried out on 20 isolates belonging to these species yielded two well-defined zymograms that supported their identity as separated species. The method also allowed ascription to *P. janczewskii* of some intermediate isolates that could not be unequivocally classified according to their morphological features. The use of zymography as a practical tool for the classification of isolates belonging to these closely related species is discussed.

Keywords: *Penicillium* taxonomy, section *Divaricatum*, zymograms.

INTRODUCTION

Classification of *Penicillium* species is sometimes controversial and revision of species status of a number of taxa is ongoing with the introduction of new biomolecular methods [1, 2]. Within the section *Divaricatum* (subgenus *Furcatum*), the separation between the species *Penicillium canescens* Sopp and *Penicillium janczewskii* Zalessky is not consolidated. In fact, despite a general agreement on their consideration as different species, isolates with intermediate morphological features are reported whose ascription is uncertain, and one or more species, such as *Penicillium kapuscinskii* Zalessky and *Penicillium albidum* Sopp, have been proposed for their placement [3-6]. Moreover, some biomolecular evidences are rather indicative of their similarity [7]. Pitt [6] has expressed a point of view that intermediate species are unjustified, and that a pragmatic solution should be adopted consisting in ascribing problematic isolates to whichever of the two species is closer according to an overall examination of morphological and cultural features. However, it is quite obvious that a generalized adoption of such principle may generate even greater uncertainty in view of the possibility that some mistakenly identified strains may be used as references. Therefore, the existence of the interfacing isolates should be considered more carefully by searching a method allowing a more objective separation of the two taxa, or eventually demonstrating the opportunity to establish one or more intermediate species.

A group of 18 isolates referable to either *P. canescens* or *P. janczewskii* was recovered from cropped soils with suppressive properties toward the plant pathogen *Rhizoctonia solani* [8]. At least 4 isolates showing intermediate features could not be definitely ascribed to one species or the other according to morphological observations. A chemotaxonomic approach based on HPLC profile of extrolites was followed to obtain further data for a more appropriate classification, but results were unsatisfactory since profiles of all isolates were quite similar [9]. Other biochemical methods have been used for a more accurate discrimination of closely related fungal species. In particular, isozyme gel-electrophoresis has proved to be very useful in this respect [10-12]. Several practical applications of this method to *Penicillium* taxonomy have been carried out so far, concerning the distinction of species in the subgenus *Penicillium* [13-15], the characterization of *P. chrysogenum* and some related species [16], the distinction of variants of *P. citrinum* [17], the characterization of the species *P. nodositanum* [18] and of interspecific hybrids between *P. chrysogenum* and *P. roqueforti* [19]. Thus, zymography is now considered a promising criterion for polyphasic taxonomy in *Penicillium* [1]. Previous experiences in our laboratory concern the identification of a new infraspecific group of *Rhizoctonia solani* AG-2-1 by means of polygalacturonase isozymes analysis [20], and the use of esterase isozymes for the characterization of isolates of *Pyrenochaeta lycopersici* [21]. We have therefore undertaken a study on polygalacturonase and esterase polymorphisms to evaluate if zymography supports the current status of *P. canescens* and *P. janczewskii* as separated species, and may eventually represent an objective tool for a correct species ascription of intermediate isolates.

MATERIALS AND METHODOLOGY

Penicillium Isolates

Isolates of *P. canescens* and *P. janczewskii* employed in the present study (Table **1**) were recovered from soil samples collected in two Italian regions (Apulia and Umbria) as previously reported [8]. Isolate PL4G of the closely related species *Penicillium melinii*, used as an outgroup reference, was recovered in the same conditions. Three additional isolates were obtained from other institutions: CBS221.28 and A248 (*P. janczewskii*), and CBS340.48 (*Penicillium janthinellum*, outgroup reference). All isolates are included in the myco-

*Address correspondence to this author at the Council for Research and Experimentation in Agriculture, Research Unit of Scafati, Scafati, Italy; Tel: +39.0818563631; Fax: +39.0818506206;
E-mail: rosario.nicoletti@entecra.it

1874-4370/08

logical collection of the Tobacco Experiment Institute which is part of the National Collection of Microorganisms of Agricultural and Agroindustrial Relevance sponsored by the Italian Ministry of Agricultural and Forest Policy. Isolates were cultured on Czapek-yeast extract agar (CYA), malt extract agar (MEA, Oxoid), yeast extract-sucrose agar (YES) and 25% glycerol-nitrate agar (G25N) according to Samson *et al.* [22] and Pitt [6], and incubated in darkness at 25°C for 1 week. Microscopic observation of morphological characters was carried out at 1000x magnification through a Olympus BX-51 microscope. Biometric data were recorded by means of DP-Soft 3.2 software (Olympus).

Table 1. *Penicillium* Isolates Employed in the Present Study

Isolate	Species	Origin
PK3A	*P. canescens*	Umbria, Città di Castello
PK3B	*P. canescens*	Umbria, Città di Castello
PK5A	*P. canescens*	Umbria, Città di Castello
PL5B	*P. canescens*	Apulia, Presicce
PL5D	*P. canescens*	Apulia, Presicce
PL7C	*P. canescens*	Apulia, Nardò
PL8G	*P. canescens*	Apulia, Nardò
PL8H	*P. canescens*	Apulia, Nardò
PL8I	*P. canescens*	Apulia, Nardò
PL8L	*P. canescens*	Apulia, Nardò
PL8M	*P. canescens*	Apulia, Nardò
PL9A	*P. canescens*	Apulia, Nardò
A248	*P. janczewksii*	Sardinia
CBS221.28	*P. janczewksii*	Poland
PL3A	*P. janczewksii*	Apulia, Presicce
PL4E	*P. janczewksii*	Apulia, Presicce
PL8C	*P. janczewksii*	Apulia, Nardò
PL10D	*P. janczewksii*	Apulia, Copertino
PL20A	*P. janczewksii*	Apulia, Mancaversa
PL20B	*P. janczewksii*	Apulia, Mancaversa
PL4G	*P. melinii*	Apulia, Presicce
CBS340.48	*P. janthinellum*	Nicaragua

Polygalacturonase Isozyme (EC 3.2.1.15) Analysis

All the isolates collected were cultured in 50 mL Erlenmayer flasks containing 10 mL of the liquid medium used by Cruickshank and Pitt [13]. After 9-day incubation at 24°C, the cultures were filtered through 0.45 µm cellulose acetate filters (Millipore). Electrophoresis was performed at 4°C in discontinuous vertical pectin-polyacrilamide gel. The stacking gel (4% acrylamide) contained 5 mL Tris-HCl (48 mL HCl 1N, 5.98 Tris, 0.46 mL TEMED, distilled water up to 100 mL total volume, pH 6.7), 10 mL acrylamide solution (10 g acrylamide, 2.5 g NN′-methylenbisacrylamide, distilled water up to 100 mL total volume), 5 mL riboflavin solution (4%), 20 mL distilled water. The resolving gel (13% acrylamide) contained 10 mL Tris-HCl (48 mL HCl 1N, 36.6 g Tris, 0.23 mL TEMED, distilled water up to 100 mL, pH 8.9), 26.6 mL acrylamide solution (28 g acrylamide, 0.74 g NN′-methylenbisacrylamide, distilled water up to 100 mL), 0.6 mL ammonium persulfate solution (10%), 16 mL citrus pectin solution (Acros, 0.5%), 23.4 mL distilled water. Electrode buffer (pH 8.3) was composed by 6 g Tris, 28.8 g glycine, distilled water up to 100 mL total volume. Gels (thickness 2 mm, width 160 mm, length 180 mm) were run at 50 mA for about 3 h until the bromophenol blue tracer reached the opposite edge, then incubated for 1 h at room temperature in 0.1 M DL-malic acid and stained overnight at 4°C in 0.01% ruthenium red. After washing three times during 1 hr with distilled water, a photographic record was taken.

Esterase Isozyme (EC 3.1.1.1) Analysis

All the isolates were cultured in 300 mL Erlenmeyer flasks containing 150 mL Czapek-Dox broth (Oxoid). After a 10-day incubation at 24°C, the cultures were filtered through filter paper (Whatman nr. 1) under reduced pressure. The mycelium was harvested, washed three times with sterile distilled water, lyophilized and ground under liquid nitrogen. Protein extraction was performed as described by Somè and Tivoli [12], with the following modifications: 1.5 mL extraction buffer (0.1 mM Tris, 10 mM KCl, 0.1 mM $MgCl_2$, 10 mM EDTA, 140 mM mercaptoethanol, 0.1 mM ascorbic acid and 0.2 g mL^{-1} polyvinilpyrrolidone) was used for 150 mg lyophilized mycelium; the mixture was then centrifuged at 11000 g for 20 min at 4°C. The supernatant was analysed immediately or stored at -20°C. Electrophoresis was performed in discontinuous vertical polyacrilamide gel in the same conditions reported for polygalacturonases. The stacking gel was made as reported for polygalacturonases, while the composition of the resolving gel (9% acrylamide) was: 10 mL Tris-HCl (48 mL HCl 1N, 36.6 g Tris, 0.23 mL TEMED, distilled water up to 100 mL, pH 8.9), 18.4 mL acrylamide solution (28 g acrylamide, 0.74 g NN′-methylenbisacrylamide, distilled water up to 100 mL), 0.6 mL ammonium persulfate solution (10%), 47.6 mL distilled water. Gels were stained according to a published procedure [12]. For both isozyme systems, the amount of proteins in the culture filtrates and the mycelial extracts was determined by the Quick Start™ Bradford protein assay (Bio-Rad), in order to perform electrophoresis with samples with approximately the same protein content. A digital image of the gels was acquired by means of the Gel-Doc system (Bio Rad). The electrophoretic profiles were compared by measuring the relative mobility (R_f) of the enzyme bands, in terms of the ratio between their migration distance and the distance reached by the bromophenol blue tracer.

RESULTS

Taxonomic differences between *P. canescens* and *P. janczewskii* are fundamentally based on culture pigmentation and texture of conidiophores and conidia. Exudates, soluble pigments and reverse side of cultures are darker in *P. canescens*, which also presents longer and conspicuously roughened conidiophores producing smooth to finely roughened conidia, while *P. janczewskii* has smooth stipes and rough or spinose conidia [6]. Based on such features, 12 isolates of our sample (PK3A, PK3B, PK5A, PL5B, PL5D, PL7C, PL8G, PL8H, PL8I, PL8L, PL8M and PL9A) could be as-

cribed to *P. canescens*, while 2 isolates only (PL8C and PL20A) were clearly referable to *P. janczewskii*. The other 4 isolates PL3A, PL4E, PL10D and PL20B presented somewhat intermediate features. In fact PL10D and PL20B had rough stipes and finely roughened conidia, but their cultural appearance was rather respondent to *P. janczewskii*, while PL3A and PL4E showed conspicuously roughened conidia although their cultures had a darker reverse more typical of *P. canescens*.

Electrophoretic analysis of polygalacturonase isozymes of *P. canescens* and *P. janczewskii* isolates showed two distinct phenotypes with no intraspecific variation; outgroup isolates of *P. melinii* and *P. janthinellum* each presented a distinct pattern (Fig. **1**). Zymograms consisted in a couple of bands with a clearly different R_f, that is 0.15 and 0.19 in *P. canescens,* 0.21 and 0.24 in *P. janczewskii*. All the morphologically intermediate isolates (PL3A, PL4E, PL10D and PL20B) exhibited a pattern corresponding to the latter species.

Results of analysis of esterase isozymes were similar. In fact, two different zymograms were obtained for the species under comparison, and outgroup isolates showed peculiar patterns (Fig. **2**). Zymograms presented 4 or 5 bands, one of which (R_f 0.13) was common to all isolates. *P. canescens* phenotype was made of 4 bands at R_f 0.13, 0.17, 0.20 and 0.25, while the one of *P. janczewskii* presented one additional band (R_f 0.13, 0.16, 0.19, 0.22 and 0.27). Again the intermediate isolates originated a zymogram corresponding to the phenotype of *P. janczewskii*.

DISCUSSION

Taxonomic applications of isozyme electrophoresis rely on the correspondence of a zymogram with a definite genotype, and banding patterns (or electromorphs) are interpreted in terms of the presumed alleles of the genetic loci coding for the isozymes. Multiple bands may represent the expression of different loci coding for isoforms of the same enzyme within a defined genome, or rather of mixed genomes [23]. The latter condition depending on heterokaryosis possibly occurs in *Penicillium* species that are known to be multinucleate [3], but in our case the absence of infraspecific variation demonstrated by the observation of the same electromorphs in isolates coming from distant geographic locations may be more indicative of the first hypothesis. Whatever the situation in the case under investigation, it is evident that the limited phenotypic differences observed reflect a consolidated different genetic base. Actually, as observed by Cruickshank and Pitt [14], a zymogram similarity between two taxa does not necessarily indicate that they are identical, while differences in isozyme patterns can be used to discriminate closely related species. The validity of zymography is even more consistent if we consider that other biomolecular methods which measure genetic variation more directly may provide contradictory results. For example, RAPD analysis applied to *Penicillium* species has confirmed taxonomic differences in some cases [24, 25], but otherwise this technique has provided controversial results [26], or it has been unsuccessful because infraspecific differences were emphasized [7].

Several isozyme systems are generally analysed in applications of zymography to fungal taxonomy [10, 16]. Although the use of multiple isozyme systems allow to perform an analysis based on a broader genetic base, and to consider phylogenetic relationships between different taxa, this approach is relatively more complex since a statistical analysis is required for the interpretation of results. Moreover no variation is often displayed by a number of isozyme systems as analysis is carried out on closely related species, which eventually makes part of the work useless. Thus, besides simplifying the methodology, considering the banding patterns as additional phenotypic characters in an analysis restricted to one or two isozyme systems increases the interest of this technique for a routine application. A meaningful example is represented by the use of zymography for infraspecific taxonomy of *R. solani* where, although very accurate studies have been also carried out by using multiple isozyme systems [27, 28], the use of pectic isozymes developed by Australian researchers [29] has provided remarkable results by itself, with the establishment of the concept of zymogram-group (ZG) that has determined an ensuing widespread use of zymography in terms of data brought to the attention of the scientific community.

Zymography based on polygalacturonase and esterase profiles can be therefore regarded as a consistent and reliable

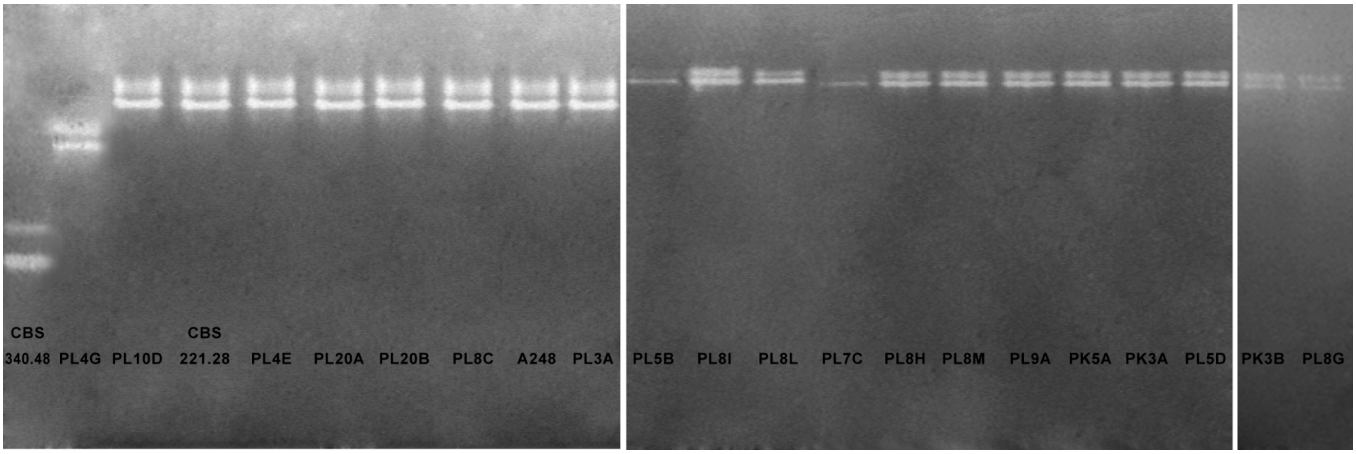

Fig. (1). Polygalacturonase zymograms. From left to right isolates CBS340.48 (*P. janthinellum*), PL4G (*P. melinii*), PL10D, CBS221.28, PL4E, PL20A, PL20B, PL8C, A248, PL3A (*P. janczewskii*), PL5B, PL8I, PL8L, PL7C, PL8H, PL8M, PL9A, PK5A, PK3A, PL5D, PK3B, PL8G (*P. canescens*).

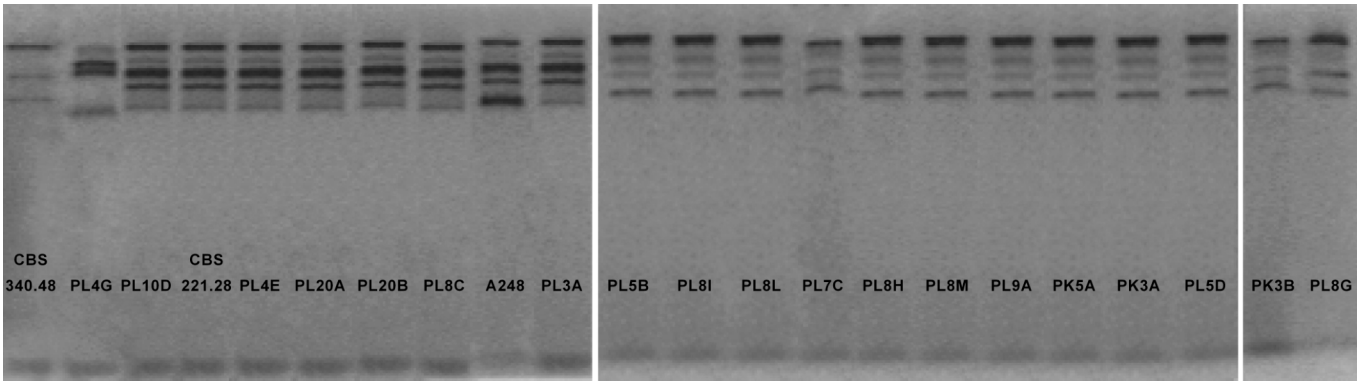

Fig. (2). Esterase zymograms. From left to right isolates CBS340.48 (*P. janthinellum*), PL4G (*P. melinii*), PL10D, CBS221.28, PL4E, PL20A, PL20B, PL8C, A248, PL3A (*P. janczewskii*), PL5B, PL8I, PL8L, PL7C, PL8H, PL8M, PL9A, PK5A, PK3A, PL5D, PK3B, PL8G (*P. canescens*).

method which supports the status of *P. canescens* and *P. janczewskii* as separated taxonomic entities. The finding of just two different phenotypes for both isozyme systems in isolates of the two species, that in some cases had been recovered from the same location, is also indicative that intermediate strains do not eventually derive from interbreeding, although such an hypothesis should be rejected considering that a teleomorph has never been reported in both species. Therefore, unlike morphological features which present a continuous variation and are rather ambiguous in the case under investigation, isozyme patterns are well-defined and result to be discrete phenotypic characters that allowed species ascription of our intermediate strains. Otherwise their classification based on specific morphological characters would have been misleading. In fact, in case we had considered stipe and conidial texture as the prevalent discriminating criterion, isolates PL10D and PL20B would have been incorrectly classified in *P. canescens*, contrasting to an appropriate species ascription of isolates PL3A and PL4E, while the contrary would have resulted, that is a mistaken classification of PL3A and PL4E, if we had opted for the morphology and pigmentation of cultures.

The sample examined was composed by isolates recovered in two distant geographic areas in Italy. Although only two isolates obtained from other institutions were available, the fact that they originated zymograms conforming to their reported ascription to *P. janczewskii* supports the validity of this method. The examination of more isolates showing intermediate morphological features, including those formerly placed in separated taxa, would represent a further qualifying test for its definitive consideration as a tool for a correct ascription of isolates belonging to these closely related species.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Robert A. Samson (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands) and Dr. Anna Benedetti (C.R.A., Plant Nutrition Experiment Institute, Rome, Italy) for providing some strains used in this study. Contribution of Dr. Samson for a correct morphological identification of some isolates is also acknowledged.

REFERENCES

[1] Frisvad JC, Samson RA. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium.* A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Stud Mycol 2004; 49: 1-173.

[2] Peterson SW, Bayer EM, Wicklow DT. *Penicillium thiersii, Penicillium angulare* and *Penicillium decaturense*, new species isolated from wood-decay fungi in North America and their phylogenetic placement from multilocus DNA sequence analysis. Mycologia 2004; 96: 1280-93.

[3] Raper KB, Thom C. A manual of the *Penicillia*. London: Bailliere, Tindall & Cox; 1949.

[4] Fassatiová O, Kubatová A. Evaluation of the diagnostic features of some species of *Penicillium* section *Divaricatum*. In: Samson RA, Pitt JI, Eds. Modern concepts in *Penicillium* and *Aspergillus* classification. New York, Plenum Press 1990; 149-57.

[5] Frisvad JC, Filtenborg O. Revision of *Penicillium* subgenus *Furcatum* based on secondary metabolites and conventional characters. In: Samson RA, Pitt JI, Eds. Modern concepts in *Penicillium* and *Aspergillus* classification. New York, Plenum Press 1990; 159-72.

[6] Pitt JI. A laboratory guide to common *Penicillium* species. 3rd ed. North Ryde: CSIRO Division of Food Processing; 2000.

[7] Sequerra J, Marmeisse R, Valla G, Normand P, Capellano A, Moiroud A. Taxonomic position and intraspecific variability of the nodule forming *Penicillium nodositanum* inferred from RFLP analysis of the ribosomal intergenic spacer and random amplified polymorphic DNA. Mycol Res 1997; 101: 465-72.

[8] Nicoletti R, Raimo F, Carella A. Searching *Penicillium* isolates antagonistic to *Rhizoctonia solani* in suppressive soils in Salento. J Plant Pathol 2003; 85: 295.

[9] Carella A, Nicoletti R. HPLC profile of secondary metabolites in *Penicillium canescens* and *Penicillium janczewskii.* J Plant Pathol 2005; 87: 290.

[10] Bonde MR, Peterson GL, Dowler WM, May B. Isozyme analysis to differentiate species of *Peronosclerospora* causing downy mildew of maize. Phytopathology 1984; 74: 1278-82.

[11] Cruickshank RH. Pectic zymograms as criteria of taxonomy in *Rhizoctonia*. Mycol Res 1990; 94: 938-46.

[12] Somé A, Tivoli B. *Fusarium* spp from potato: polymorphism of esterase phenotypes on polyacrylamide gel electrophoresis. Agronomie 1993; 13: 637-49.

[13] Cruickshank RH, Pitt JI. Identification of species in *Penicillium* subgenus *Penicillium* by enzyme electrophoresis. Mycologia 1987; 79: 614-20.

[14] Cruickshank RH, Pitt JI. The zymogram technique: isoenzyme patterns as an aid in *Penicillium* classification. Microbiol Sci 1987; 4: 14-7.

[15] Paterson RRM, Bridge PD, Crosswaite MJ, Hawksworth DL. A reappraisal of terverticillate *Penicillia* using biochemical, physiological and morphological features. III. An evaluation of pectinase and amylase isoenzymes for species characterization. J Gen Microbiol 1989; 135: 2979-91.

[16] Banke S, Frisvad JC, Rosendahl S. Taxonomy of *Penicillium chrysogenum* and related xerophilic species, based on isozyme analysis. Mycol Res 1997; 101: 617-24.

[17] Zu-tong Q, Zeng-mei S. A taxonomic investigation of *Penicillium citrinum* variants by biochemical and electrophoretic techniques. Mycosystema 1989; 2: 151-8.

[18] Faure-Raynaud M, Daniere C, Moiroud A, Capellano A. Intraspecific variability of isozymes in *Penicillium nodositanum* Valla, a fungus inducing 'myconodules' on the root-system of *Alnus* sp. Plant Soil 1991; 137: 285-91.
[19] Anné J, Peberdy JF. Characterization of interspecific hybrids between *Penicillium chrysogenum* and *P. roqueforti* by isoenzyme analysis. Trans Br Mycol Soc 1981; 77: 401-8.
[20] Nicoletti R, Lahoz E, Kanematsu S, Naito S, Contillo R. Characterization of *Rhizoctonia solani* from tobacco fields related to anastomosis groups 2-1 and BI (AG 2-1 and AG BI). J Phytopathol 1999; 197: 71-7.
[21] Infantino A, Aragona M, Brunetti A, Lahoz E, Oliva A, Porta-Puglia A. Molecular and physiological characterization of Italian isolates of *Pyrenochaeta lycopersici*. Mycol Res 2003; 107: 707-16.
[22] Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O. Introduction to food- and airborne fungi. 6th ed. Utrecht: Centraalbureau voor Schimmelcultures; 2000.
[23] Micales JA, Bonde MR, Peterson GL. The use of isozyme analysis in fungal taxonomy and genetics. Mycotaxon 1986; 27: 405-49.
[24] Boysen M, Skouboe P, Frisvad JC, Rossen L. Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. Microbiology 1996; 142: 541-9.
[25] Dupont J, Magnin S, Marti A, Brousse M. Molecular tools for identification of *Penicillium* starter cultures used in the food industry. Int J Food Microbiol 1999; 49: 109-18.
[26] Pereira JF, Vieira de Queiroz M, Gomes EA, Muro-Abad JI, Fernandes de Araujo E. Molecular characterization and evaluation of pectinase and cellulase production of *Penicillium* spp. Biotechnol Lett 2002; 24: 831-8.
[27] Laroche JP, Jabaji-Hare SH, Charest PM. Differentiation of two anastomosis groups of *Rhizoctonia solani* by isozyme analysis. Phytopathology 1992; 82: 1387-93.
[28] Liu ZL, Sinclair JB. Genetic diversity of *Rhizoctonia solani* anastomosis group 2. Phytopathology 1992; 82: 778-87.
[29] Sweetingham MW, Cruickshank RH, Wong DH. Pectic zymograms and taxonomy and pathogenicity of the Ceratobasidiaceae. Trans Br Mycol Soc 1986; 86: 305-11.

Received: February 6, 2008 Revised: October 10, 2008 Accepted: October 13, 2008