VCP1 PROTEASE DETECTION IN *Pochonia chlamydosporia* var. *catenulata* STRAIN IMI SD 187

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ABSTRACT: *T*he mode of action of *Pochonia* as a biological control agent is the penetration by means of the hyphae into the target nematode eggs. The egg shell of nematodes has an outer vitelline membrane composed mainly by proteins. Thus, hyphal penetration is the result of a physic pressure and the specific hydrolytic activity of some enzymes such as proteases and chitinases. The most important protease studied in *Pochonia chlamydosporia* var. *chlamydosporia* is VCP1. The aim of this work was to detect and characterize the protease VCP1 in a strain of *P. chlamydosporia* var. *catenulata,* a potential biological control agent for root-knot nematodes. The specific activity of this protease was tested on different culture media supplemented with protein inductors using N-succinyl-Ala-Ala-Phe-p-nitroanilide as substrate. PCR and RFLP analyses with specific primers and probes were also done. Isolates from the *chlamydosporia* variety were used as positive controls. The medium supplemented with chitin was the only one capable of inducing some VCP1 activity but in a low level. The VCP1-encoding gene was not detected in strain IMI SD 187 by RFLP even when the stringency conditions were reduced to the minimum possible. The low level of enzymatic activity detected by biochemical techniques does not support the presence of protease VCP1 in this strain. The low enzymatic activity reached points out to the existence of a VCP1 isoform or another different protease. These hypotheses should be proved widening the range of *catenulata* variety strains and taking the *chlamydosporia* variety (strain 10) as a reference in a comparative study with conclusive results. The results of this enzymatic characterization related to the egg infection process of host nematodes are the first achieved in a *catenulata* variety strain in the world.

(Key words: nematode parasites; RFLP; enzymes; protease)

DETECCCIÓN DE PROTEASA VCP1 EN LA CEPA IMI SD 187 DE *Pochonia chlamydosporia* var. *catenulata*

RESUMEN: El modo de acción de *Pochonia* como agente de control biológico es a través de la penetración de las hifas en los huevos de sus nematodos diana. La cubierta de los huevos de los nematodos posee una capa externa vitelina, compuesta en su mayoría por proteínas. Por tanto, la penetración es el resultado de una presión física y de la actividad hidrolítica específica de algunas enzimas tales como proteasas y quitinasas. La proteasa más importante del género *Pochonia* ha sido estudiada en la variedad *chlamydosporia* y es conocida como VCP1. El objetivo de este trabajo fue la detección y el estudio del comportamiento de la proteasa VCP1 en una cepa promisoria de *Pochonia chlamydosporia* var. *catenulata.* La actividad enzimática de esta proteasa se estudió en diferentes medios suplementados con inductores, empleando como sustrato N-succinil-Ala-Ala-Phe-p-nitroanilida. También se desarrollaron análisis PCR y RFLP con cebadores y sondas específicos. En todos los experimentos, los controles positivos utilizados fueron aislamientos de la variedad *chlamydosporia*. El medio suplementado con quitina fue el único capaz de inducir alguna actividad de la VCP1 pero en un nivel bajo. El gen que codifica para la enzima VCP1 no fue detectado por RFLP en la cepa IMI SD 187, aún cuando las condiciones de la hibridación de la sonda fueron reducidas al mínimo posible. El bajo nivel alcanzado

de la actividad de esta enzima apunta hacia la existencia de una isoforma de la VCP1 u otra proteasa totalmente diferente. Esta hipótesis debe ser comprobada aumentando el rango de cepas de la variedad *catenulata*, teniendo como referencia la cepa 10 de la variedad *chlamydosporia* en un estudio comparativo con resultados conclusivos en este aspecto. Los resultados de esta caracterización enzimática relacionada con el proceso de infección de los huevos del nematodo hospedante son los primeros que se desarrollan en una cepa de la variedad *catenulata*, en el mundo.

(Palabras clave: nematodos parásitos; RFLP; enzimas; proteasas)

INTRODUCTION

Pochonia chlamydosporia is an opportunistic fungal parasite of eggs of sedentary nematodes including *Meloidogyne* spp. (1). Due to differences in the conidial disposition on phialides there is some controversy on whether *Pochonia chlamydosporia* var. *chlamydosporia* and *P. chlamydosporia* var. c*atenulata* are separated species or different varieties. Conidia are disposed as chains in the *catenulata* variety or as false heads in the *chlamydosporia* variety. Gams (2) considered that such differences had a limited importance and proposed to consider *chlamydosporia* and *catenulata* as varieties of the same species, *Verticillium chlamydosporium*. This judgement was sustained by Gams and Zare (3) when they renamed this species in the *Pochonia* genus.

The design of primers based on a nucleotidic sequence of β-tubulin gene intron from *P. chlamydosporia* has allowed the *in situ* detection of the *chlamydosporia* variety in tomato plants infected with root-knot nematodes with high specificity (4). At present, there are specific primers for the detection of the *catenulata* variety in soil samples by conventional PCR, as well as by real time PCR (5,6). Moreover, it has been possible to differentiate both varieties of the fungus and the biotype A of *catenulata* variety by RFLP analyses on the Internal transcribed spacer (ITS) amplified products (1,7).

The mode of action of *Pochonia* as a biological control agent is the penetration of the nematode egg shell by the hyphae (8). The nematode egg shell has an outer vitelline membrane composed mainly by proteins. Thus, fungal penetration is the result of a physic pressure and specific hydrolytic activity of some enzymes such as proteases and chitinases (9). For these reasons, the study of extracellular enzymes and other metabolites excreted by the fungus is an attractive issue for many researchers (10, 11, 12) due to their possible role as virulence factors or as a criterium for strain selection. A serine protease similar to subtilisin was purified and partially characterized from *P. chlamydosporia* var. *chlamydosporia,* and this protein is known as VCP1 (11).

The VCP1 variability among isolates of *P. chlamydosporia* can be due to differences in the subtilisins and they can explain the different niches occupied by this facultative parasite in the soil, its host range as well as the virulence factor (11, 13). Recently, these differences were shown with the sequencing of the VCP1-encoding gene (12). Up to date, all the studies have been carried out with isolates from the *chlamydosporia* variety, using the strain 10 from Rothamsted collection as a reference.

Other studies at DNA or protein level contributing to the intrinsic characterization of both varieties of the fungus, their behaviour during the infection process and the interaction between both, may provide important insights that may lead to a better distancing and taxonomic classification.

The objective of this work was to detect and characterize the protease VCP1 in a strain of *Pochonia chlamydosporia* var. *catenulata* growing in different media supplemented with protein inductors and using RFLP technique.

MATERIALS AND METHODS

Strains and induction media

The strains of *P. chlamydosporia* var. *chlamydosporia* 10, 309, 399, from different geographical areas (Table 1) were kindly donated by the Rothamsted collection, at Nematode Interactions Unit, Rothamsted Research, Harpenden, UK. Strain IMI SD 187 of *P. chlamydosporia* var. *catenulata* and the strain 16 of *P. chlamydosporia* var. *chlamydosporia* were obtained from the Cuban collection, at the National Center for Animal and Plant Health, Havana, Cuba. The strains were maintained on corn meal agar (CMA) and subcultured on the same medium at 28ºC in the dark for two weeks.

TABLE 1. Characteristics of *P. chlamydosporia* strains analyzed./ *Características de las cepas de* **P. chlamydosporia** *analizadas*

Code of Strains	Fungal species and Variety	Source	Origin	Parasitism (>30%)	Colonization (>80%)
IMI SD 187	*P. chlamydosporia* var. *catenulata*	Cuba	*Meloidogyne incognita*	+	+
10	*P. chlamydosporia* var. *chlamydosporia.*	Brasil	*Meloidogyne incognita*	+	+/-
16	*P. chlamydosporia* var. *chlamydosporia.*	Cuba	Soil	Unknown	Unknown
309	*P. chlamydosporia* var. *chlamydosporia.*	Zimbabwe	Soil	+	+
399	*P. chlamydosporia* var. *chlamydosporia.*	China	Soil	+	+/-

Data provided by Rothamsted Research Station, UK

Protein induction in liquid media

For protein induction in a basal liquid medium (14), two compounds "chemically related" to the egg shell structure of *Meloidogyne* eggs were investigated. These compounds are known to induce enzymes with hydrolytic activity (11). The treatments were as follows: i) basal liquid medium (as control); ii) basal liquid medium supplemented with 0,2% gelatine; iii) basal liquid medium supplemented with 1% chitin practical grade (Sigma) (sifted through a 30- mesh- pore screen) (11). For each treatment, 20 mL of medium was dispensed in sterile Corning 50 mL tubes, and inoculated with four 5 mm-diameter disks of fungal mycelium taken from the edge of a colony of a pure culture for each strain. The tubes were incubated at 28ºC, in the dark with continuous agitation in an orbital shaker at 120 rpm. Three replications per fungal strain were assayed.

The supernatants of the cultures were collected 7 days after fungal inoculation and filtered through filter paper Whatman Nº 1, concentrated by freeze-drying, and finally dissolved in 1 mL of sterile distilled water and then, maintained at -20ºC until they were used in the enzymatic analyses. The fungal biomass collected from each replication was dried in an oven at 80ºC for two days to determine the dry weight, except for those coming from the medium supplemented with chitin, using an analytic scale Sartorius, with an accuracy of 0,0001, and the protocol described in the US Pharmacopeia (15).

Quantification of proteins and enzymatic analyses

The concentration of total proteins was determined following the protocol described by Bradford (16), using the BIORAD kit and ELISA plates as support for the assay. The optical densities were recorded at 495 nm in a Multi Scan MRX (Dynex technologies Ltd., Billinghurst, West Sussex, UK). Serum bovine albumin (BSA) at concentrations ranging from 42 to 10 µg mL^{-1}, was used to prepare the standard curve. The VCP1 activity was determined using N-succinyl-Ala-Ala-Phe-p-nitroanilide as substrate according to Morton *et al.* (12). In the assay, 2 µL of the enzymatic extract were mixed by agitation in an eppendorf tube with 100 µL of 2 mM substrate and 98 µL of buffer solution (0,1 M Tris HCl, pH 7,9). The absorbance was immediately read at 410 nm for 3 min, in a spectrophotometer for UV and visible light, CARY 50 using the program Kinetics. An activity unit was defined as the quantity of enzyme that releases 1 µmol of p-nitroanilide . min^{-1} . mL^{-1} (12). Three replications were done for all determination. Data were compared by Duncan's Test of Multiple Ranges using the SAS program (17); the fungal strain was considered as the independent variable and the indicators analysed in the time and in the particular medium.

Detection of VCP1 protease in strain IMI SD 187 by RFLP.

The genomic DNA of strains IMI SD 187 and 399 (used as reference for VCP1 gene) were obtained according to the extraction method described by Morton *et al.* (12).

For the RFLP, the genomic DNA was digested by the restriction enzymes *Bam*HI, *Eco*RI, *Hind*III and *Sac*I (Roche, Lewes, UK), using the buffer solution specified for each restriction enzyme and incubating the reaction mixture in a water bath at 37°C overnight. The digestion products were separated by electrophoresis on 1% agarose gel in 0,5X TAE at 70 volts. The gel was stained with Etidium Bromide (EtBr)

(0,5 mg mL^{-1}) to visualize the digestion products and transferred to a positively charged nylon membrane (Roche, UK) by capillarity overnight. The protocols for gel treatment and transfer were those described by "The DIG system user's for filter hybridisation" (Roche, UK). The gel was checked under UV light and the DNA fixed to the membrane with UV light, in the UV Stratalinker 1800 (Stratagene, UK). The probe used was a fragment of the VCP1 gene from the reference strain amplified by PCR with a couple of specific primers VCP1SF (5'-ATG CAA CTG TCT GTT CTT CTC-3') and VCP1R1 (5'- TAG TCA CAG CTT GTT CTC GCC-3') (12).

The amplified fragment was purified from agarose gel using the "High pure PCR product purification kit" (Roche, UK) and the product was diluted to 50 µL final volume. The labelling was developed using the "PCR DIG samples synthesis kit" (Roche, UK) and it was tested on 1,5% agarose gels. The recommendations followed for both, prehybridisation and hybridisation were described by "DIG high labeling and prevails detection starter kit II" and the user´s manual above referred (Roche, UK). The prehybridisation was carried out for 2 h at 42°C, while the hybridisation was developed overnight at the same temperature. Once hybridisation concluded, washes were as follows: 2 times with 2X SSC-0,1% SDS solution at room temperature for 5 min each time; and 2 times with 0,1X SSC-0,1% SDS solution at 68ºC for 15 min each time. The colour development was carried out according to the specifications described previously for Luminigen as substrate (Roche, UK). The membrane was exposed for 24 h for the final detection in the film.

RESULTS AND DISCUSSION

Protein induction in liquid medium

The levels of VCP1 production are shown in Figs. 1, 2 and 3. In the basal medium (Fig. 1), the highest values were reached by the strain 309 although they were not statistically different from those achieved by the strain 16; they were different from those of the remaining strains. In the medium supplemented with gelatine (Fig. 2), only the strain 399 showed a significant enzyme activity, whereas there were no statistical differences among the other strains. However, in the medium with chitin (Fig. 3), all the strains showed a high enzymatic activity induction, even strain IMI SD 187. In this medium, the strains 399 and 16 reached higher values (P<0,05), although the latter strain did not differ from the strains 10 or IMI SD 187.

Although the induction levels of proteaseVCP1 were higher in the medium with chitin, this medium is not recommended for the characterization and selection of isolates because it offers limited information about the enzymes. These results are similar to those of Segers et al., (11) working with the $chlamydosporia$ variety strains and using the strain 10 as a reference. Therefore, the basic medium was a good alternative to study these enzymes and the potential of the isolates for their production. It has been previously stated that these enzymes are also inducible under conditions of scarce or limited nutrition (9), when the fungus has to face adverse conditions (e.g. lack of a carbon source) and then it synthesizes enzymes that are able to degrade other sources and, therefore, it changes from the saprophytic to its parasitic stage.
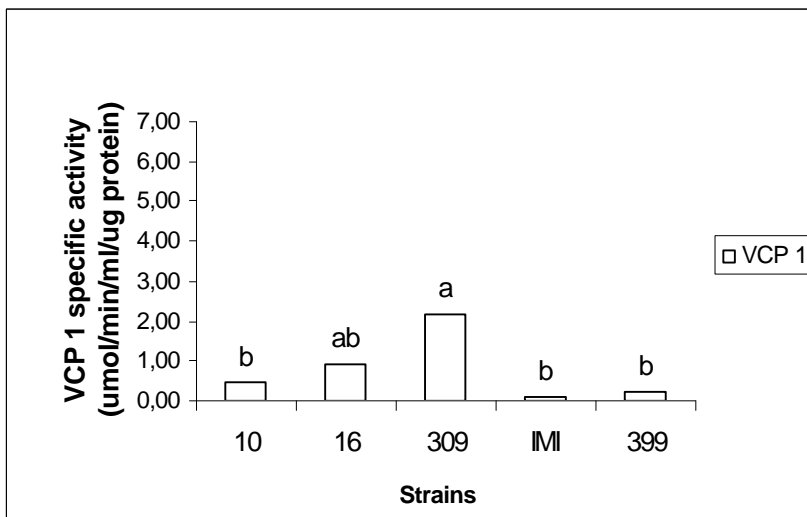
FIGURE 1. VCP1 induction in *P. chlamydosporia* strains in basal liquid media. Means with different letters differ significantly for Duncan's Multiple-Range Test (p≤0,05). SD = 0,80./ *Inducción de VCP1 en cepas de **P. chlamydosporia** en medio basal líquido. Medias con letras diferentes, difieren significativamente para la Prueba de Rangos Múltiples de Duncan (p≤0,05).*
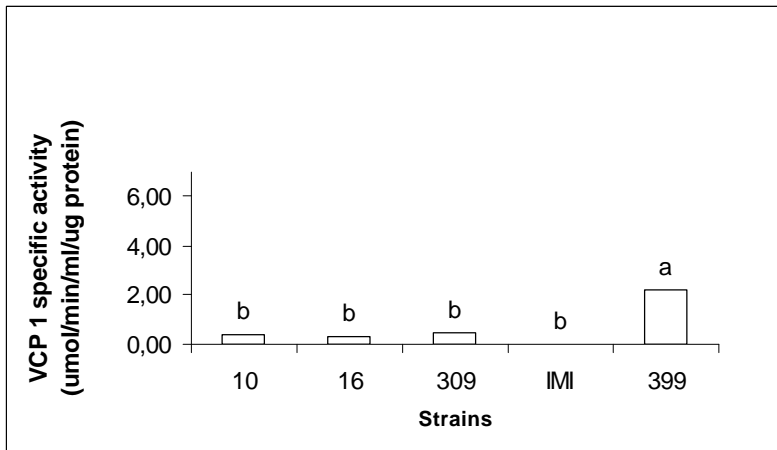
FIGURES 2. VCP1 induction in *P. chlamydosporia* strains in basal liquid media supplemented with gelatine. Means with different letters differ significantly for Duncan's Multiple-Range Test (p ≤ 0,05). SD= 0,66./ *Inducción de VCP1 en cepas de P. chlamydosporia en medio basal líquido suplementado con gelatina. Medias con letras diferentes, difieren significativamente para el Test de Rangos Múltiples de Duncan (p≤0,05).*
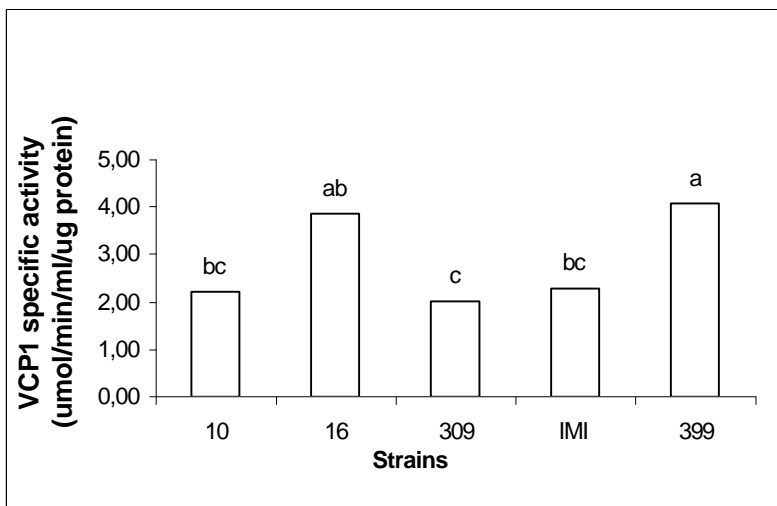
FIGURE 3. VCP1 induction in *P. chlamydosporia* strains in basal liquid media supplemented with chitin. Means with different letters differ significantly for Duncan's Multiple-Range Test (p ≤ 0,05). SD = 0,87./ *Inducción de VCP1 en cepas de P. chlamydosporia en medio basal líquido suplementado con quitina. Medias con letras diferentes, difieren significativamente para el Test de Rangos Múltiples de Duncan (p≤0,05).*

The basal medium is very poor in nutrients and this fact could have a positive effect on the enzymes related to the infection process induction.

According to the structure and chemical composition of the external layer of nematode eggs, *Pochonia* should have a selection of enzymes to degrade each layer, which appears as barriers, to arrive finally to the egg inner content and to infect it (11, 12). This process is complex and depends on molecular recognition, immobilization, penetration, colonization and digestion of the nematode egg.

In addition to the differences of the *catenulata* variety strain with regard to those of the *chlamydosporia* variety from the morphological point of view, variability within the strains of this last variety is also shown to occur because of the different responses resulting from this variety strain studied with different inductors (12, 18).

Detection of VCP1 protease in strain IMI SD 187 by RFLP.

The VCP1-encoding gene was not detected by RFLP in strain IMI SD 187, even when the stringency conditions were reduced to the minimum possible (data are not shown). Such negative results could suggest that the protease VCP1 gene in this strain has some difference from that in the strains of *P. chlamydosporia* var. *chlamydosporia* (11, 12).

At the protein level, Hirsch (personal communication, 2004) had experienced with VCP1 induction in impoverished Czapek medium for a group of strains, including the strain IMI SD 187. The low levels of VCP1 expression, even when studies of repression and induction with glucose and nitrates were carried out for this particular strain, drew this author's attention.

The family of the hydroxyalanine-proteases similar to subtilisin shares similar characteristic such as

molecular mass, maximum activity in alkaline conditions, behaviour in front of inhibitors, but intra-specific differences have been found for the protease VCP1. These are supported by studies in the aminoacidic sequence that may have effects on the substrate used, the kinetics of the enzyme and its surface properties. Such differences could justify the use of different substrates to detect VCP1 activity in isolates from root-knot nematodes and those from cysts nematode (12).

In the present study, it was not possible to detect VCP1 gene in strain IMI SD 187 of *P. chlamydosporia* var. *catenulata* by RFLP/Hybridisation with a specific probe. This strain has also shown a very low protease activity. In the specific case of the strain IMI SD 187 and under these experimental conditions, it is convenient to talk about a VCP1-like protein.

The fact of a negative result at DNA level points towards more variations in the sequence of this protease in strain IMI SD 187. These variations did not allow the hybridisation with the specific probe. Such variations at protein level could produce changes in the recognition sites of the enzyme or steric impediment, resulting in an extremely low activity. The single presence, for example, of a glycine in strains coming from root-knot nematodes, instead of an alanine in those from cyst nematodes, can change the affinity for amino acids in the substrate union site named S3. Such differences among these enzymes caused high effects on their specificity (9, 12).

The low enzymatic activity points out to the existence of a VCP1 isoform or another different protease. But in both cases, a low affinity for the substrate tested was found. Recently, Atkins *et al.* (19), found 71 single nucleotide polymorphisms (SNPs) and 19 amino acid differences for the IMI SD 187 VCP1 sequence in comparison with *P. chlamydosporia* var. *chlamydosporia* isolate 10 (AJ427460), contrasting with the 15 SNPs and 4 amino acid differences seen when six *P. chlamydosporia* var. *chlamydosporia* VCP1 sequences were compared (12). Therefore, at least for this strain, this enzyme should not be considered as the virulence factor.

Segers (11) outlines that it is possible that homologous genes with products that differ in charge (as the isozymes detected in *Pochonia*) differ in their adsorption onto egg nematode shells, with consequences for pathogenicity. These assertions agree with the present results found in *catenulata* variety.

Keeping in mind the role attributed to VCP1 as a virulence factor in nematode egg infection by *Pochonia* (11), the level of the enzyme production could be recommended as a possible biochemical marker related to the strain virulence. This decision would be contradictory with the behaviour observed in IMI SD 187 strain in studies under field conditions. This strain effectively decreases nematode populations once applied to the soil, it is a good parasite, colonizer and survivor, with a high adaptation ability (5, 20, 21).

Lastly and not less important, if they are additionally considered the morphological distinguishing characteristics for the two *Pochonia* varieties (previously pointed out by Gams) and the differences at DNA level detected by the use of specific primers for each varieties (designed by Arora *et al.,* (22); Atkins *et al.,* (7)), the distinctive characteristics found in IMI SD 187 strain and the possible presence of a virulence factor different from VCP1; the distance between the two *Pochonia* varieties will become wider, which could even mean changes in an effective taxonomic classification. The results of this enzymatic characterization related to the egg infection process of host nematodes are the first achieved in a *catenulata* variety strain in the world. These hypotheses should be proved widening the range of *catenulata* variety strains and taking the *chlamydosporia* variety (strain 10) as a reference in a comparative study with more conclusive results in this regard.

ACKNOWLEDGMENTS

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