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Secretome analysis identifies potential virulence factors of *Diplodia corticola*, a fungal pathogen involved in cork oak (*Quercus suber*) decline

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ABSTRACT

The characterisation of the secretome of phytopathogenic fungi may contribute to elucidate the molecular mechanisms of pathogenesis. This is particularly relevant for *Diplodia corticola*, a fungal plant pathogen belonging to the family *Botryosphaeriaceae*, whose genome remains unsequenced. This phytopathogenic fungus is recognised as one of the most important pathogens of cork oak, being related to the decline of cork oak forests in the Iberian Peninsula.

Unfortunately, secretome analysis of filamentous fungi is limited by the low protein concentration and by the presence of many interfering substances, such as polysaccharides, which affect the separation and analysis by 1D and 2D gel electrophoresis. We compared six protein extraction protocols concerning their suitability for further application with proteomic workflows. The protocols involving protein precipitation were the most efficient, with emphasis on TCA–acetone protocol, allowing us to identify the most abundant proteins on the secretome of this plant pathogen. Approximately 60 % of the spots detected were identified, all corresponding to extracellular proteins. Most proteins identified were carbohydrate degrading enzymes and proteases that may be related to *D. corticola* pathogenicity.

Although the secretome was assessed in a noninfection environment, potential virulence factors such as the putative glucan-β-glucosidase, neuraminidase, and the putative ferulic acid esterase were identified.

The data obtained forms a useful basis for a deeper understanding of the pathogenicity and infection biology of *D. corticola*. Moreover, it will contribute to the development of proteomics studies on other members of the *Botryosphaeriaceae*.

Introduction

Fungi from the family *Botryosphaeriaceae* can infect plants causing diseases and often death (Alves *et al.* 2004; Damm *et al.* 2007; Marincowitz *et al.* 2008; Mehl *et al.* 2011; Úrbez-Torres & Gubler 2009). Their ecological and economic impact is considerable – particularly when they infect profitable trees such as cork oak. The involvement of *Diplodia corticola* (a member of the

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Botryosphaeriaceae) on the decline of cork oak forests is well known (Alves *et al.* 2004; Linaldeddu *et al.* 2009). It causes symptoms like dieback, canker, and vascular necrosis in oak trees, but the mechanism of pathogenesis is unknown.

In the last decade, proteomics of phytopathogenic fungi has been increasingly applied to study plant–pathogen interactions (Gonzalez-Fernandez & Jorrín-Novo 2012). More specifically, secretome characterisation of phytopathogenic fungi may contribute to elucidate the infection mechanism and provide information for the development of disease management strategies.

Remarkably, only Cobos *et al.* (2010) used proteomics to characterise a member of the *Botryosphaeriaceae* family. This gap is due to the low number of sequenced genomes of that family making protein identification difficult. Nevertheless, 2-D gel-based proteomics followed by *de novo* sequencing allows identifying proteins from organisms with unsequenced genomes, such as *D. corticola* (Rogowska-Wrzesinska *et al.* 2013; Tannu & Hemby 2007). Another possible reason for the low number of proteomics studies of the *Botryosphaeriaceae* family is that the analysis of the secretome is constrained by the low concentration of extracellular proteins, the high amount of polysaccharides, and the presence of low-molecular-weight metabolites also secreted by fungi (Chevallet *et al.* 2007; Erjavec *et al.* 2012). These molecules interfere with protein extraction and separation methods, making the choice of an adequate extraction method crucial for proteomics (Lemos *et al.* 2010).

We aimed to optimize an extraction protocol suitable for secretome analysis by 1D and 2D electrophoresis and to identify the major extracellular proteins of *D. corticola*. This is the first report describing the secretome of *D. corticola*, providing a first view on the machinery this organism could exploit in plant infection.

Material and methods

Microorganisms and culture conditions

The strain used in this study was *Diplodia corticola* CBS112548. Cultures were maintained on Potato Dextrose Agar (PDA) medium (Merck, Germany). For secretome extraction, a mycelium plug with 0.5 cm diameter from a 6-d-old PDA plate was inoculated into a 250 mL flask containing 50 mL of Potato Dextrose Broth (PDB), and statically incubated for 12 d at room temperature (±25 °C). All assays were performed in triplicate. Culture supernatants were individually collected by gravitational filtration through filter paper and stored at −20 °C until use. The dry-weight of mycelia was determined to evaluate the fungal biomass. For this, filtered mycelia were dried at 50 °C for 4 d before weighting. The extracellular protein fraction was then concentrated as described below.

Extracellular protein extraction protocols

Protocol 1 (Trichloroacetic acid (TCA)–acetone) was based on a previously described method (Cobos *et al.* 2010). After thawing, the culture supernatant (35 mL) was centrifuged (48 400×*g*, 1 h at 4 °C) to discard precipitated polysaccharides. One volume of ice-cold TCA/acetone [20 %/80 % (w/v)] with 0.14 % (w/v) DTT was added to the supernatant and incubated at −20 °C (1 h). Precipitated proteins were recovered by centrifugation (15 000×*g*, 20 min, 4 °C) and excess TCA was removed from the precipitate by washing with 10 mL of ice-cold acetone (2×) and 10 mL of ice-cold 80 % acetone (v/v). Residual acetone was air-dried and the protein pellet was resuspended in 500 μL of lysis buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris-base) and stored at −20 °C.

Protocol 2 (TCA–phenol) was adapted from a previously described method (Fernández-Acero *et al.* 2009). After thawing, the culture supernatant (35 mL) was centrifuged (48 400×*g*, 1 h at 4 °C) to discard precipitated polysaccharides. Proteins were precipitated by the addition of one volume of ice-cold TCA/acetone [20 %/80 % (w/v), 1 h, −20 °C], and collected by centrifugation at 15 000×*g* (20 min, 4 °C). The precipitate was successively washed with 10 mL of ice-cold TCA/acetone [20 %/80 % (w/v), twice], 10 mL of 20 % TCA (w/v), and twice with 10 mL of ice-cold 80 % acetone (v/v). Residual acetone was air-dried and the protein pellet was resuspended in 5 mL of dense Sodium dodecyl sulfate (SDS) buffer [30 % (w/v) sucrose, 2 % (w/v) SDS, 0.1 M Tris–HCl pH 8.0, 5 % (v/v) 2-mercaptoethanol] adding then 5 mL of phenol equilibrated with 10 mM Tris–HCl, pH 8.0, 1 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma–Aldrich, USA). The resulting solution was vigorously mixed and centrifuged at 15 000×*g* (10 min, 4 °C). The phenol phase was transferred to a tube to which five volumes of cold 0.1 M ammonium acetate in methanol were added and incubated at −20 °C overnight to promote protein precipitation. Afterwards, proteins were recovered by centrifugation and washed twice with 10 mL of cold 0.1 M ammonium acetate in methanol, followed by two washes with 10 mL of ice-cold 80 % acetone (v/v). The air-dried pellet was finally resuspended in 500 μL lysis buffer and stored at −20 °C.

Protocol 3 (ultrafiltration with protein cleaning): polysaccharides were separated as described for method 1 and the resultant supernatant was concentrated by ultrafiltration with Vivaspin concentrator (MWCO 3 kDa, Sartorious), at 4000 rpm (4 °C). Retained proteins were purified with 2-D Clean-Up kit (GE Healthcare, USA; from now on mentioned as protein cleaning), according to the manufacturer's instructions. The proteins were solubilized in 500 μL of lysis buffer and stored at −20 °C.

Protocol 4 (ultrafiltration without protein cleaning): this method is identical to method 3 with the exception of the final cleaning step. Therefore, the proteins were immediately resuspended in 500 μL of lysis buffer and stored at −20 °C after their concentration.

Method 5 (ultrafiltration without polysaccharide precipitation): this method is similar to method 3 with the exception of the initial polysaccharide removal step. After protein cleaning, the resultant pellet was solubilized in 500 μL of lysis buffer and stored at −20 °C.

Protocol 6 (lyophilisation): culture supernatant (35 mL) was concentrated by lyophilisation (Snijders Scientific) for 24 h at −50 °C. Afterwards, proteins were cleaned as previously described, solubilized in 500 μL of lysis buffer and stored at −20 °C.

Protein concentration determination

Protein concentration was determined with the 2-D Quant Kit (GE Healthcare, USA), according to the manufacturer's instructions.