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ORIGINAL PAPER

Proteomic analysis of phytopathogenic fungus *Botrytis cinerea* as a potential tool for identifying pathogenicity factors, therapeutic targets and for basic research

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Abstract *Botrytis cinerea* is a phytopathogenic fungus causing disease in a substantial number of economically important crops. In an attempt to identify putative fungal virulence factors, the two-dimensional gel electrophoresis (2-DE) protein profile from two *B. cinerea* strains differing in virulence and toxin production were compared. Protein extracts from fungal mycelium obtained by tissue homogenization were analyzed. The mycelial 2-DE protein profile revealed the existence of qualitative and quantitative differences between the analyzed strains. The lack of genomic data from *B. cinerea* required the use of peptide fragmentation data from MALDI-TOF/TOF and ESI ion trap for protein identification, resulting in the identification of 27 protein spots. A significant number of spots were identified as malate dehydrogenase (MDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The different expression patterns revealed by some of the identified proteins could be ascribed to differences in virulence between strains. Our results indicate that proteomic analysis are becoming an important tool to be used as a starting point for identifying new pathogenicity factors, therapeutic targets and for basic research on this plant pathogen in the postgenomic era.

Keywords *Botrytis cinerea* · Fungal phytopathogen · Fungal proteomics · Virulence factor

Abbreviations

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
MDH	Malate dehydrogenase
PMF	Peptide mass fingerprinting
2-DE	Two-dimensional gel electrophoresis

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Introduction

Botrytis cinerea Pers. Fr. is a phytopathogenic ascomycete that causes significant yield losses in a substantial number of crops (Coley-Smith et al. 1980; Verhoeff et al. 1992; Davidson et al. 2004; Dik and Wubben 2004; Droby and Lichter 2004; Elmer and Michailides 2004). It is considered as a very complex species in which molecular differences among different populations have been widely reported (Buttner et al. 1994; Vallejo et al. 2002). Such differences are expressed as variability in virulence among isolates as

well as phenotypic instability (Vallejo et al. 2003; Beever and Weeds 2004).

In the last years, many research advances regarding the infection process developed by this pathogen have been made. Thus, several virulence factors, such as enzymes involved in penetrating plant tissues, have been shown to be produced by this fungus and research efforts have been made to elucidate which of them are essential for full virulence of the fungus (Staples and Mayer 1995; van Kan et al. 1997; ten Have et al. 1998). It has to be taken into account that *B. cinerea* is a necrotrophic fungus that depends on its own ability to kill plant cells before starting the penetration of the plant tissues. A number of phytotoxins, the best known being botrydial and dihydrobotrydial (Collado et al. 2000), are synthesized and released by this organism and have been shown to be responsible for the typical chlorotic spots displayed by infected leaves (Colmenares et al. 2002). Lately, a positive correlation between phytotoxin production and virulence in different *B. cinerea* isolates has been reported, being those toxins considered to play an important role as virulence factors (Reino et al. 2004).

In spite of these findings, neither the molecular basis of the synthesis and production of different virulence factors nor the mechanisms of phytopathogenicity used by this fungus are yet well understood. Improvements in key proteomics technologies such as protein separation by two-dimensional gel electrophoresis (2-DE) and peptide analysis by mass spectrometry (MS) have allowed the efficient characterization and identification of a large number of proteins from microbial origin (Jungblut and Hecker 2004), but few reports on filamentous fungi are available (Lim et al. 2001; Nandakumar and Marten 2002; Grinyer et al. 2004; Kim et al. 2004; Ebstrup et al. 2005; Medina et al. 2005; Shimizu and Wariishi 2005). A recent report from Fernández-Acero et al. (2006) described the first analysis of *B. cinerea* proteome by 2-DE and MS showing, among others, the relevance of housekeeping enzymes such as MDH. Afterward, a comparative analysis between two *B. cinerea* isolates differing in both virulence and toxin production has been initiated. This proteomic approach has enabled the identification of protein spots that showed qualitative or quantitative differences between mycelial extracts from the two isolates. Due to the absence of protein and DNA databases containing *B. cinerea* sequences, protein identification was achieved by peptide fragmentation fingerprinting (PFF) followed by either database search or de novo peptide sequencing and sequence alignment.

Materials and methods

Botrytis cinerea isolates and culture conditions

Botrytis cinerea strain 1.11 was kindly provided by Dr. I. G. Collado (Department of Organic Chemistry, University of Cádiz, Spain), and strain 2100 (also called *B. cinerea* 1.29) was obtained from the Spanish Type Culture Collection. Conidial stock suspensions were prepared and conserved as previously reported (Vallejo et al. 2002). One mL of conidial suspension (5×10^4 conidia mL^{-1}) was transferred to flasks containing 250 mL of Czapeck–Dox liquid medium and cultures were incubated on an orbital shaker at 180 rpm, 22°C, and 12 h light for 5 days. Mycelia were harvested by filtration, washed with sterile water, lyophilized and stored at –20°C.

Oxalic acid production and pH values

Oxalic acid production was quantified by using a commercial kit (R-Biofarm AG/Roche, Landwehrstr, Germany) following the manufacturer's instructions. Variation of the pH was determined by measuring pH values of the cultures. All assays were performed with two independent cultures of each strain and read for 5 days.

Protein extraction, two-dimensional gel electrophoresis, gel analysis and protein identification

Protein extracts were obtained by potassium phosphate buffer solubilisation and trichloroacetic acid (TCA) precipitation protocol and 2-DE was performed as previously described (Fernández-Acero et al. 2006). Protein concentration was determined in the supernatant using the RC-DC Protein Assay (Bio-Rad, Hercules, CA, USA) with ovalbumin as standard. Samples were frozen in liquid nitrogen and stored at –80°C for later analysis.

Gels were Coomassie stained with CBB G-250 (Merck, Whitehouse Station, NJ, USA), and gel images were captured with a densitometer (GS-800, Bio-Rad), digitalized and analyzed by PDQuest™ software (Bio-Rad) using a guided protein spot detection method (Asirvatham et al. 2002). Spots were named with ordinal numbers. Analytical and biological variability studies were directed at quantifying variations associated with the 2-DE experiments or differences in protein expression levels between independent replicates. For the analytical variance study, aliquots of a pooled extract were used to rehydrate three replicate

IPG strips. Three extracts from a single fungal culture were analyzed to determine the analytical variance. For the biological variance study, three individual cultures from each fungal strain were also harvested and extracted separately. For each spot the following parameters were assessed: isoelectric point (pI), molecular mass (Mr), mean values of protein amount, standard deviation (SD) as a measure of dispersion, and coefficient of variance (CV), a useful tool to compare variability (18). Protein spots were identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF/TOF) or electrospray ion trap mass spectrometry (ESI IT MS/MS), as previously described (Fernández-Acero et al. 2006).

Results

Two-dimensional gel electrophoresis protein profile, gel analysis and analytical and biological variability

For comparative proteome analysis, mycelial protein extracts from *B. cinerea* strains 1.11 (less virulent) and 2100 (more virulent) (Reino et al. 2004; Colmenares et al. 2002; Collado et al. 2000; Durán-Patrón et al. 2004) were used. After 2-DE separation and Coomassie Blue staining of the protein extracts of *B. cinerea* 1.11 and 2100 strains, 380–400 protein spots were resolved and detected by digital image analysis and visual confirmation (Fig. 1). Normalized protein spot volumes were determined for each 2-DE gel, and protein spot quantification was performed using a calibration curve obtained with different quantities of protein standards (Jorge et al. 2005). Normalized spot volumes were linear to protein quantity all over the range. To quantify the variances associated with 2-DE experiments and the differential protein expression between culture batches from the two strains, both analytical and biological variability were calculated. In a previous work (Fernández-Acero et al. 2006), the analytical and biological variances were calculated for *B. cinerea* 2100 (16.1 and 37.5%, respectively). In order to compare between strains, biological variance of *B. cinerea* 1.11 was calculated and the average CV was found to be 51.6%.

This comparative analysis revealed qualitative and quantitative differences in protein expression between the two *B. cinerea* strains. Only those changes consistently present in all the extract replicates and with quantitative differences higher than the corresponding biological variance were considered. Twenty-eight spots were selected on the basis of their higher protein amount and classified according to their relative expression levels. Thus, 6 spots (65, 66, 67, 68, 69 and 70) were only present in strain 1.11 extracts, whereas 15 spots (1, 2, 3, 4, 5, 13, 14, 15, 16, 17, 18, 19, 20, 21 and 22) were only found in strain 2100 extracts. Moreover, seven spots (6, 7, 8, 9, 10, 11 and 12) were significantly overexpressed in strain 2100 ($P < 0.05$) (Figs. 1, 2). These differences may be put down to changes in protein expression or to post-translational or degradation processes.

Protein identification by mass spectrometry

Twenty-eight selected spots were positively identified by MS/MS analysis using MALDI-TOF/TOF and ESI ion-trap MS. The proteins identified are listed in Tables 1 and 2 and numbered on the virtual 2-DE reference map (Fig. 1). Due to the absence of DNA and protein database sequences of *B. cinerea*, database searches with PMF data were unsuccessful. Thus, identification was achieved through PFF by MALDI-TOF/TOF or through homology searching with tentative sequences obtained de novo by ESI ion-trap MS. Positive identifications were achieved by matching well conserved sequence domains within similar proteins from other organisms.

pH values and oxalic acid production

Values of the pH of both *B. cinerea* strains were measured for 5 days. Results showed that pH values in the cultures of the more virulent strain, 2100, were lower than those in the cultures of the less virulent strain, 1.11, showing significant differences ($p > 0.05$) from day 3 onwards (Fig. 3a). At this point, pH values stabilizes at 3.94 (± 0.01) for *B. cinerea* 2100 and 4.53 (± 0.11) for *B. cinerea* 1.11.

Oxalic acid concentration was determined at days 0, 3 and 5. The concentration of oxalic acid in cultures of *B. cinerea* 2100 was 2.7-fold higher than cultures from *B. cinerea* 1.11, at day 3. The concentrations of the acid decreased at day 5 in both cultures, and even at this point, culture of *B. cinerea* 2100 kept presenting 3.4-fold higher oxalic acid concentration than *B. cinerea* 1.11 (Fig. 3b).

Discussion

Two-dimensional gel electrophoresis protein profile, analytical and biological variability

The biological variance obtained for *B. cinerea* 1.11 (51.6%) was higher than that previously reported for *B. cinerea* 2100 (37.5%) that had also been considered

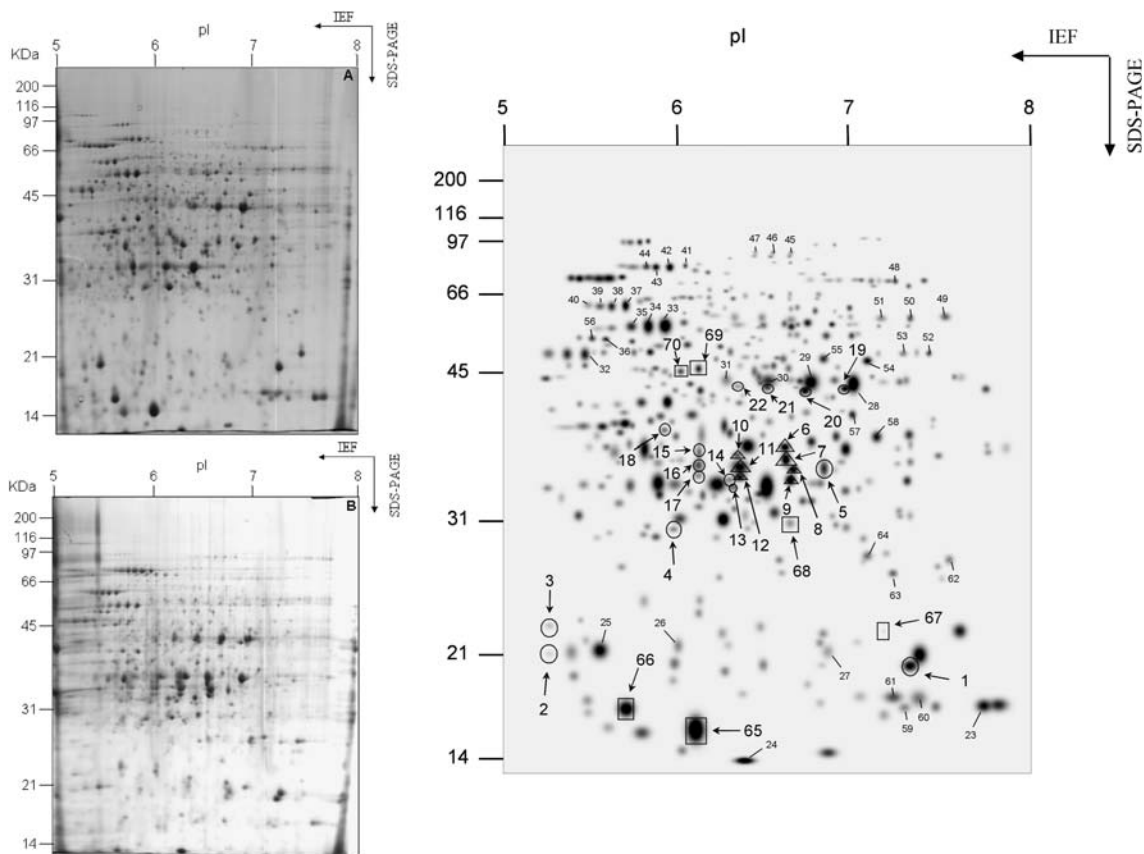

Fig. 1 *Left* Coomassie stained gels from *B. cinerea* 1.11 (**a**) and *B. cinerea* 2100 (**b**). *Right* 2-DE virtual gel showing the spots used for calculating the analytical and biological variances. *Numbers* are indicated in Fig. 2, Tables 1 and 2. *Arrows* point out identified spots. Variable proteins among the two strains: specific proteins from *B. cinerea* 1.11 (*open squares*), specific proteins from *B. cinerea* 2100 (*open circles*), overexpressed proteins in *B. cinerea* 2100 (*open triangles*)

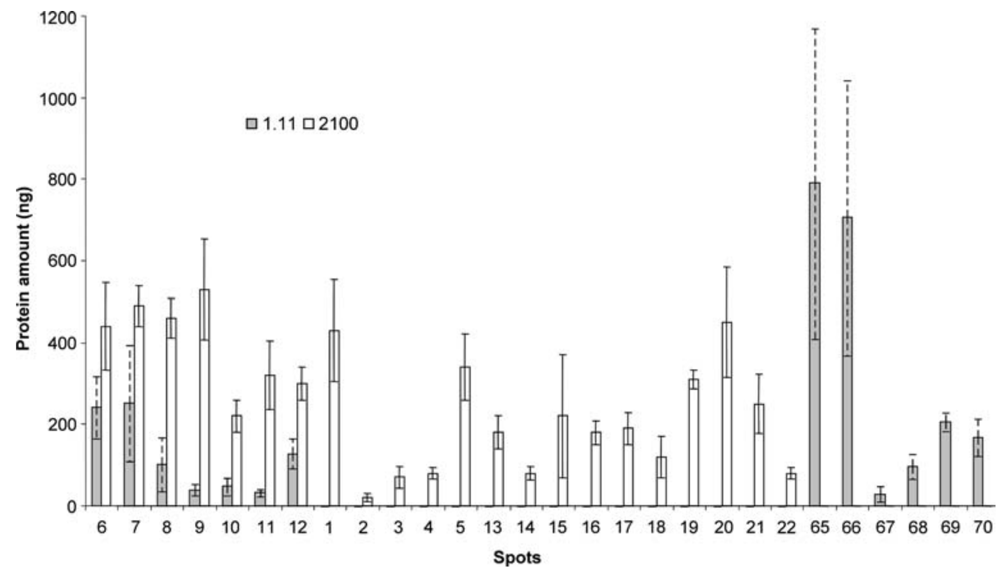

Fig. 2 Protein amount in the 28 spots selected for identification, showing spots specific for each strain or overexpressed in *B. cinerea* 2100

as showing a high biological variance. Indeed, most of the 73 spots analyzed in the present work showed significantly higher biological than analytical variability and were hence classified as variable spots. These high biological variabilities showed by the two *B. cinerea* strains are commonly found in proteomic studies of

Table 1 Proteins identified from *B. cinerea* 1.11

Spot	Protein	Accession	Organism	Sequence	Score[a]	Identified by
67	Malate dehydrogenase, mitochondrial (fragment)	gi/3192929	*Glycine max*	LFGVTTLDVVR	94	MALDI-TOF/TOF
69	Putative mannitol-1-phosphate-5-dehydrogenase	gi/5834650	*Staphylococcus aureus*	AVHFGAGNIGR	81	
	Mannitol-1-phosphate dehydrogenase	gi/22651499	*Aspergillus niger*	GFVAEFLHK	64	
70	Putative mannitol-1-phosphate-5-dehydrogenase	gi/5834650	*Staphylococcus aureus*	AVHFGAGNIGR	94	
	Mannitol-1-phosphate dehydrogenase	gi/22651499	*Aspergillus niger*	GFVAEFLHK	72	
65	Fission yeast SPBC409.12c protein	Q9UUB2	*Schizosaccharomyces pombe*	SNSLYMLVELAK -FVTPAAPNDEMK -FVTPAAPNDEM^{ox}K M^{ox}PLSVYVVSVGEWDR	50	nESI-ION TRAP
66	Large subunit of terminal dioxygenase	P95531	*Pseudomonas putida*	LEDDWVDMSR LEDDWVDM^{ox}SR LEDDWVDM$^{ox/ox}$SR QPTDPDPQSNLK	71	
68	Putative lipoprotein	Q82G17	*Streptomyces avermitilis*	FDLAAAARVQ -LEGWSSGNGR	88	

[a] High and total scores presented in the BLASTP2 results report

Uncertainty in 2–3 first residues in sequences is labelled by hyphens

plants, mice, or human samples, such it has been previous and widely discussed by Fernández-Acero et al. (2006).

Protein identification

Seventeen spots were identified as MDH. All the proteins over expressed in the more virulent strain used in this work, *B. cinerea* 2100 (Fig. 2), corresponded to MDH (spots 6, 7, 8, 9, 10, 11 and 12). Moreover, most of the spots specific for 2100 strain have been identified as MDH species (spots 2, 3, 4, 5, 13, 14, 15, 16 and 17). Fernandez-Acero et al. (2006) had previously described three clusters (A, B, and C) of MDH in the strain 2100. The comparative analysis of the present work reveals that the cluster A and two spots from cluster B are not displayed in the proteome of the strain *B. cinerea* 1.11. Moreover, cluster C is over expressed in *B. cinerea* 2100. Only one spot identified as MDH has been ascribed to *B. cinerea* 1.11 (spot 67). Thus, MDH is produced exclusively, or at higher levels, in *B. cinerea* 2100. The role of MDH as a pathogenicity factor had been previously suggested (Fernández-Acero et al. 2006). This enzyme catalyzes the reversible conversion of oxalacetate and malate. Oxalacetate is an oxalic acid precursor (Kubicek et al. 1988; Lyon et al. 2004) which has been described as a pathogenicity factor in *B. cinerea* (Lyon et al. 2004). The secretion of oxalic acid creates an acidic environment that may generate a more suitable ecological niche for the pathogenic activities of the fungus. Indeed, the influence of pH on the expression and secretion of virulence factors such as cell wall degrading enzymes has been described in *B. cinerea* (Wubben et al. 2000; Manteau et al. 2003). In addition, acidification of the environment generated by oxalic acid would lead to the biosynthesis and secretion of phytotoxins. Botrydial and dihydrobotrydial, two toxins secreted exclusively in acidic media, are over expressed by the more virulent strain used in this study (Durán-Patrón et al. 2004). These toxins are not produced by the less virulent strain 1.11, which is in concordance with its low MDH expression level. Thus, MDH is likely to play a key role in the cascade of events leading to the plant cell death and therefore to be essential for the whole infection process.

In this sense, it is interesting that, although the pH of both strain cultures decreased when growing the fungus, the pH of *B. cinerea* 2100 cultures felt dramatically and showed lower pH values than those of *B. cinerea* 1.11 (Fig. 3a), in a percentage 3.29% on the first day, and 15.38% on the fifth. That is, the difference of pH of both strain cultures increased along the time. These results correlated very well with those obtained when determining oxalic acid production in the cultures. Actually, our results revealed that the concentration of oxalic acid in cultures of *B. cinerea* 2100 was 2.7-fold higher than that in the cultures from

Table 2 Proteins identified from *B. cinerea*. Spots are numbered in Figs. 1 and 2. (*o*) Protein spots specific of *B. cinerea* 2100 extracts, (□) protein spots specific of *B. cinerea* 1.11 extracts, (Δ) protein spots present in both strains and overexpressed in *B. cinerea* 2100

Spot	Expression	Protein	Ref[a]
1	*o*	Hypothetical protein cyclophilin, cytosolic form	1
2	*o*	Nodule-enhanced malate dehydrogenase	1
3	*o*	Nodule-enhanced malate dehydrogenase	1
4	*o*	Malate dehydrogenase	1
5	*o*	Mitochondrial malate dehydrogenase precursor	1
6	Δ	Malate dehydrogenase	1
7	Δ	Malate dehydrogenase	1
8	Δ	Malate dehydrogenase transcriptional regulator metE/metH family	1
9	Δ	Malate dehydrogenase	1
10	Δ	Malate dehydrogenase	1
11	Δ	Malate dehydrogenase transcriptional regulator metE/metH family	1
12	Δ	Malate dehydrogenase	1
13	*o*	Malate dehydrogenase	1
14	*o*	Malate dehydrogenase	1
15	*o*	Malate dehydrogenase	1
16	*o*	Malate dehydrogenase	1
17	*o*	Malate dehydrogenase transcriptional regulator metE/metH family	1
18	*o*	Hypothetical protein	1
19	*o*	Glyceraldehyde-3-phosphate dehydrogenase	1
20	*o*	Glyceraldehyde-3-phosphate dehydrogenase	1
21	*o*	Glyceraldehyde-3-phosphate dehydrogenase	1
22	*o*	Glyceraldehyde-3-phosphate dehydrogenase	1
65	□	Fission yeast SPBC409.12c protein	2
66	□	Large subunit of terminal dioxygenase	2
67	□	Malate dehydrogenase, mitochondrial (fragment)	2
68	□	Putative lipoprotein	2
69	□	Mannitol-1-phosphate dehydrogenase	2
70	□	Mannitol-1-phosphate dehydrogenase	2

[a] References: 1 Fernández-Acero et al. (2006), 2 Table 1

B. cinerea 1.11, at day 3 (Fig. 3b). Although both concentrations decreased afterwards, cultures from *B. cinerea* 2100 presented 3.4-fold higher concentration of oxalic acid than those of *B. cinerea* 1.11, at day 5. That is, the more virulent strain kept showing much higher oxalic acid production than the less virulent one. Taken together, these data (pH values and oxalic acid concentration), support the above suggested hypothesis.

Recent reports show the implication of the *B. cinerea* enzyme cytochrome P450 monooxygenase in abscisic acid (Siewers et al. 2004) and botrydial synthesis (Siewers et al. 2005), representing the first botrydial biosynthetic gene identified. Canonical P450 enzymes use electrons from NADPH to catalyze activation of molecular oxygen of a plethora of substrates (Werck-Reichhart and Feyereisen 2000). NADPH is produced in the MDH reaction and may be used, among others, in the reaction of P450. In prokaryotes, P450 species are soluble proteins, whereas in eukaryotes these enzymes are associated with endoplasmic reticulum or mitochondrial membranes (Werck-Reichhart and Feyereisen 2000), the same localization showed by MDHs. It has been suggested that P450 enzymes interact with multi-enzyme complexes, the nature of these interactions being still controversial (Werck-Reichhart and Feyereisen 2000). Despite that in *B. cinerea* the relation between P450s and MDHs remains to be elucidated, both activities have been reported to increase in response to phenobarbital in rat liver (Kaliman et al. 1991). This suggested relationship between P450 and MDH may indicate a new role as a virulence factor of MDH in *B. cinerea*.

One of the major breakthroughs of proteomic technologies is their capacity to find new targets for diagnostics and vaccine candidates, among others (Jungblut and Hecker 2004). Previous research by Fernandez-Acero et al. (2006) showed the existence of a transcriptional regulator metE/metH family protein comigrating with MDH (spots 8, 11 and 17). This protein family, involved in methionine biosynthesis pathways (Jafri et al. 1995), is considered as potential therapeutic target for fungicide design (Leroux et al. 2002). In this study, these comigrating proteins are down expressed (spots 8 and 11) or absent in the less pathogenic strain 1.11 (spot 17). The variability of these fungicide targets may be involved in the molecular basis of the different fungicide resistance phenotypes described for *B. cinerea* (Leroux et al. 2002).

Several GAPDH protein species were identified when mapping the proteome of *B. cinerea* 2100 strain (spots 19, 20, 21 and 22) (Fernández-Acero et al. 2006). Apart from its well-known role in the glycolytic cycle, GAPDH has been reported to influence many other cellular processes and act as a virulence factor in

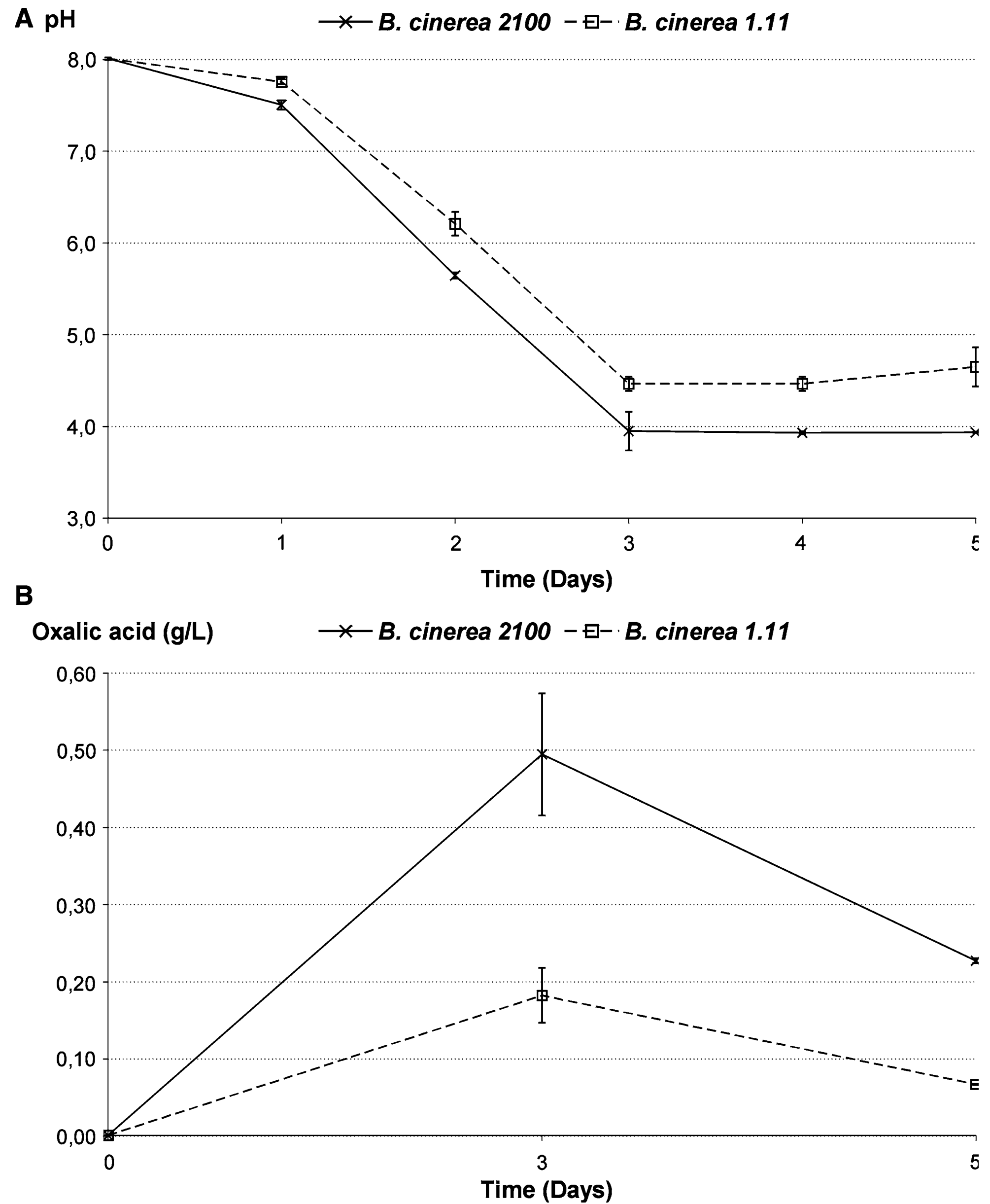

Fig. 3 **a** Evolution of values of pH obtained from day 0 to day 5 in *B. cinerea* 2100 and *B. cinerea* 1.11 cultures. **b** Oxalic acid concentration in *B. cinerea* 2100 and *B. cinerea* 1.11 cultures. Some *errors bars* are too small to be visible

different organisms (Hernández et al. 2004; Alderete et al. 2001; Deveze-Alvarez et al. 2001; Pancholi and Chhatwal 2003). The four spots identified as GAPDH in this study were only present in the more virulent *B. cinerea* strain 2100, supporting the hypothesis that the oxidative metabolism in this strain may be more active than that of the strain 1.11 and suggesting a putative role as virulence factor in *B. cinerea*.

A cytosolic cyclophilin, present in the more pathogenic strain 2100, was absent in the proteome of the less pathogenic strain 1.11. These proteins have been shown to be involved in many different cellular processes, including a role as a virulence determinant in fungal pathogens of human and plant cells (Wang et al. 2001; Viaud et al. 2002). The role of cyclophilin as virulence factor in *B. cinerea* has been previously shown and has been proposed to be involved in the later stages of infection, such as penetration or plant colonisation (Wang et al. 2001; Viaud et al. 2003). For an analysis of the presence of cyclophilin in the proteome of the strain 2100, two facts have been considered: (1) strain 2100 is more virulent, expresses a specific cyclophilin and produces high levels of toxins botrydial and dihydrobotridial; (2) these toxins are encoded by calcineurin-dependent genes (Siewers et al. 2005) which have been suggested to play a role in the first steps of plant infection (Viaud et al. 2003). Taken together, these facts imply that two different cellular

components with distinct roles in two different stages of the infection process are expressed in the more pathogenic strain (cyclophilin and calcineurin-dependent gene expression), whereas none of them are detected in the less pathogenic one, which does not produce toxins**,** and the above described specific cyclophilin form is not displayed in its proteome. Thus, proteomic analysis of the two strains selected for this study, which differ in virulence as well as in toxin production, is very useful for relating different cellular components involved in the infection process.

Six spots detected exclusively in the less virulent *B. cinerea* 1.11 strain (65, 66, 67, 68, 69 and 70) were positively identified using NCBInr database (Table 1). These proteins are involved in various metabolic pathways. Spot 67 was identified as MDH. Spots 69 and 70 were identified as mannitol-1-phosphate dehydrogenase, the first enzyme in the mannitol biosynthesis pathway. Mannitol is the predominant carbohydrate in conidiospores of the filamentous fungus *Aspergillus niger* (Witteveen and Visser 1995), where it is utilized as a reserve carbon source and as an antioxidant, preventing cell damage under stress conditions (Ruijter et al. 2003). Spot 66 was identified as the large subunit of terminal dioxygenase, which plays a key role in the detoxification of toxic aromatic amines in *Pseudomonas putida* (Fukumori and Saint 1997). Hence, this dioxygenase subunit could be involved in the detoxification of toxic compounds in *B. cinerea*. Spots 65 and 68 were identified as two putative proteins from large scale genomic DNA experiments as SPBC409.12c protein from *Schizosaccharomyes pombe* and putative lipoprotein from *Streptomyces avermitilis*, respectively. Unknown functions were inferred from electronic annotation (http://www.expasy.org/uniprot/) and ascribed to DNA binding protein for spots 65 and electron transport for spot 68. Those protein spots, were re-evaluated by using the established Syngenta/Broad Institute *B. cinerea* sequence database (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html) to match the found peptides against predicted proteins in this database. The obtained results were similar to those above described: (1) the same proteins were identified for the spots 67, 69 and 70 using both databases; (2) no relevant protein was ascribed to spot 65 and 66 using the Syngenta database; (3) spot 68 was identified as Glucose oxidase with oxidoreductase activity from *A. niger* using Syngenta database. This FAD binding protein seems to have a role as electron transport as above mentioned.

Botrytis cinerea is a very complex pathogen that utilizes many distinct strategies for killing the host and overcoming host defences through the whole infection process. Results indicate that the differential proteomic analysis of strains differing in virulence contributes to identifying proteins likely involved in the mechanisms of pathogenicity of this fungus, therapeutic targets and for basic research, and thus constitutes an excellent tool to be used as a first and straight step for determining new fungal virulence factors.

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