

Proteomic profiling of *Botrytis cinerea* conidial germination

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Received: 20 May 2014 / Revised: 21 July 2014 / Accepted: 12 August 2014 / Published online: 21 August 2014
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Abstract *Botrytis cinerea* is one of the most relevant plant pathogenic fungi. The first step during its infection process is the germination of the conidia. Here, we report on the first proteome analysis during the germination of *B. cinerea* conidia, where 204 spots showed significant differences in their accumulation between ungerminated and germinated conidia by two-dimensional polyacrylamide gel electrophoresis and qPCR. The identified proteins were grouped by gene ontology revealing that the infective tools are mainly preformed inside the ungerminated conidia allowing a quick fungal development at the early stages of conidial germination. From 118 identified spots, several virulence factors have been identified while proteins, such as mannitol-1-phosphate dehydrogenase, 6,7-dimethyl-8-ribityllumazine synthase or uracil phosphoribosyltransferase, have been disclosed as a new potential virulence

factors in botrytis whose role in pathogenicity needs to be studied to gain new insights about the role of these proteins as therapeutic targets and virulence factors.

Keywords *Botrytis cinerea* · Virulence factors · Pathogenicity factors · Fungal phytopathogens · Fungal proteomics · Conidia germination

Abbreviations

SDW Sterile distilled water
HPI Hour post inoculation
GO Gene ontology
ID Identification

Introduction

Botrytis cinerea is a phytopathogenic fungus capable to infect more than 200 plant species, including fruits, flowers and leaves at any stages of plant development (Elad et al. 2004; Tudzynski and Kokkelink 2009). Since its relevance as a phytopathogen, *B. cinerea* has become a model fungus for molecular phytopathology, representing the second position of the top ten lists of fungal pathogens (Dean et al. 2012) behind *Magnaphorte oryzae*, the causal pathogen of the rice blast disease.

Botrytis cinerea exhibits a wide arsenal of strategies to produce its infection cycle, causing severe damage, during pre- and post-harvest. The initial step of this cycle is the germination of fungal conidia, producing the initial symptoms in crops, acting as an initial trigger for the sequential flow of events that occurs during disease development. The germination of fungal spores involves the transformation of a dormant cell into growing hypha. Germination occurs in response to hydration in the presence of appropriate

Communicated by Erko Stackebrandt.

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Electronic supplementary material The online version of this article (doi:10.1007/s00203-014-1029-4) contains supplementary material, which is available to authorized users.

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condition (Leroch et al. 2013). Conidial germination is a very complex and varied process depending on the fungal specie, in which several morphological and biological changes occur during the first hours. These changes generally include swelling of conidia, adhesion, increasing intracellular osmotic pressure, uptake of water (Taubitz et al. 2007) and nuclear decondensation during the first hours of germination (Osherov and May 2001; Oh et al. 2010). Because its relevant role in the establishment of the fungal disease, the germination process has been widely studied; (Oh et al. 2010; Cooper et al. 2007; Taubitz et al. 2007; Hagag et al. 2012; Leroch et al. 2013). Previous studies have indicated that DNA and RNA synthesis are apparently not essential for early germination in *Neurospora crassa*, *Fusarium solani* or *Aspergillus nidulans* (Hagag et al. 2012). Other studies had highlighted the role of regulatory pathways, including the cAMP/protein kinase A (Fillinger et al. 2002; Lafon et al. 2005, 2006) and the ras/mitogen-activated protein kinase pathway (Hagag et al. 2012). On the other hand, the role of protein accumulation during conidial germination in *N. crassa*, *A. nidulans* and *F. solani* has been investigated using either protein-synthesis inhibitors or temperature-sensitive mutants defective in this process (Cochrane and Cochrane 1970; Hagag et al. 2012).

Currently, proteomics approaches have shown its potency to unravel complex biological questions, being established that is the proteome the relevant level of analysis (Rossignol et al. 2009) allowing the collection of a vast set of biological information without the selection of any gene as previous candidate. The relevance of this approach is based on the concept that proteins are finally controlling the observed phenotype, avoiding the problems of correlation between accumulated proteins and transcriptome. Integrated proteome-transcriptome studies have shown that approaches, based on mRNA by itself, are a poor indicator of protein accumulation levels at large scale (Hack 2004). However, the detection of specific RNAs still remains as a suitable way to check a specific gene expression, being required an analysis of the presence of the transcript by RT-PCR.

Therefore, proteomic analysis has an increasing importance in the search of virulence factors in plant pathogenic fungi (Garrido et al. 2010; Gonzalez-Fernandez and Jorrin-Novo 2012). Recently, an increasing number of *B. cinerea* proteomics approaches has been published, ranging from total proteome analyses to various subproteome studies based on gel or gel-free techniques (Fernández-Acero et al. 2006, 2007, 2009, 2010; Shah et al. 2009a, b; Espino et al. 2010; Cherrad et al. 2012; Li et al. 2012; Gonzalez-Fernandez et al. 2013; Davanture et al. 2014). These advances have been facilitated due to the recent publication of two different genome

databases of *B. cinerea* (http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/MultiHome.html and <https://urgi.versailles.inra.fr/Species/Botrytis>). The optimization of protein extraction and separations protocols combined with matrix-assisted laser desorption/ionization—time-of-flight mass spectrometry (MALDI-TOF/TOF MS) and electrospray ionization coupled to ion trap mass spectrometry (ESI-IT MS/MS)—has led to the identification of several hundreds of *B. cinerea* proteins (Garrido et al. 2010; Gonzalez-Fernandez and Jorrin-Novo 2012). Until now, no previous proteomic studies have been reported concerning the intracellular proteins accumulated during germination process in *B. cinerea*. This process has been studied by transcriptome profiling (Zheng et al. 2011; Leroch et al. 2013) describing the up-regulation of secreted proteins, different germination-specific expression pattern and corresponding secondary metabolic pathways. Only a few proteomic approaches concerning the germination process have been reported (Noir et al. 2009) describing the first annotated proteome map of ungerminated conidiospores of *Blumeria graminis* f.sp hordei, the causal agent of powdery mildew in barley, concluding that spores are equipped with the complete molecular machinery to trigger the germination process on a suitable host surface, since most of the identified proteins in these ungerminated conidiospores are involved in carbohydrate, lipid and protein metabolism. Comparative proteomic approaches to the study of germination process have allowed to highlight the key role of the transcriptional regulator COM1 in reprogramming genes implicated in melanin biosynthesis, carbon and energy metabolism and others cellular processes indispensable for conidia development and appressoria penetration in *Magnaporthe oryzae* (Bhadauria et al. 2007). Recently, a first comprehensive study of the proteome during the early stage of the *Colletotrichum acutatum* conidial germination by comparing the protein profiles of ungerminated and germinated conidia has been published (El-Akhal et al. 2013).

All these reports used gel-based approaches combining a protein separation by two-dimensional polyacrylamide gel electrophoresis (2-DE) and subsequent mass spectrometric analyses of the tryptically digested proteins, usually tandem matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS/MS). In the last years, differential gel electrophoresis (DIGE), a fluorescence-based method (Unlu et al. 1997) is being used to increase sensitivity and resolution of 2-DE for the comparative proteomic analysis and quantification of proteins, which has been successfully applied to analysis and quantification of the proteome from different fungal plant pathogens (Wartenberg et al. 2012; Yao et al. 2012).

In the present study, we have used DIGE in combination with mass spectrometry, to detect and identify statistically significant changes in the fungal proteome during the early phase of conidial germination in *B. cinerea*. Proteins were extracted from germinated and ungerminated conidia, labeled with cyanine-based fluorescent tags, and separated by 2-DE yielding the detection of 674 unique spots. After statistical analysis, 204 of these spots show different accumulation patterns, and a total of 118 of these were identified by MALDI-TOF/TOF MS. Since conidia germination is the initial step of the plant infection process, we assume that most of the identified proteins should be of high relevance for the development of the fungal infection cycle. The characterization of the *B. cinerea* proteome during conidial germination may result in more information on the biochemical mechanisms, infection strategies and life cycle of this important pathogenic fungus but also in new potential targets for antifungal drug screening.

Materials and methods

Strain, culture media and growth condition

The strain of choice for this study was *B. cinerea* B05.10. Isolates were grown on malt extract agar medium (MA), containing 20 % (w/v) malt extract and 20 % (w/v) agar, under alternating 12-h light/dark cycles at 22 °C. Conidia of *B. cinerea* were harvested from sporulating 15-day-old MA cultures by adding NaCl 0.9 % (w/v), subsequent mixing with a sterile loop, and two-fold filtration (30- μ m filters, Nytal; Maissa, Barcelona, Spain). Conidia were pelleted by centrifugation at 5,000 $\times g$ (5 min) and resuspended in SDW at a final concentration of 1×10^8 conidia mL⁻¹. A 1-mL aliquot of the conidial suspension was centrifuged again; the pellet resuspended in 1 mL of pure acetone, supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and finally stored at -80 °C until protein extraction. For conidial germination, 500-mL flasks contained 250 mL of liquid Vogel medium [15 % (w/v) sucrose, 3 % (w/v) C₆H₅Na₃O₇ $\times$ 2H₂O, 3 % (w/v) K₂HPO₄, 0.2 % (w/v) MgSO₄ $\times$ 7H₂O, 0.1 % (w/v) CaCl₂ $\times$ 2H₂O, 2 % (w/v) NH₄NO₃, and pH 6.0] supplemented with 0.5 % (w/v) of deproteinized tomato cell walls, which was subsequently sterilized and filtrated (45 μ m filters, Nytal; Maissa, Barcelona, Spain). The cultures were inoculated with 1×10^8 conidia and incubated in an orbital shaker at 180 rpm at 22 °C. To determine the optimal time to collect the germinated conidia, different samples of conidia were grown as described above for 24 h being periodically visualized (each hour) by light microscopy. Samples collected at 0 and 5 hpi were finally selected. Four independent biological replicates were used *per* assay.

Isolation of total RNA, cDNA synthesis and quantitative real-time PCR

For RNA isolation, one milliliter of 1×10^8 conidia mL⁻¹ suspension from ungerminated (T0) and germinated (T5) conidia samples was separately homogenized with 450-mg glass beads (Sigma-Aldrich, St. Louis, USA) and two 1/4 Ceramic Sphere (Q-Biogene, Valencia, USA) using Fast-Prep (Fast-Prep®-24, MP Biomedicals) 1×60 s at 6.0 rpm and Trizol (Invitrogen, Gibco BRL) for RNA isolation following Invitrogen recommended protocol (http://tools.lifetechnologies.com/content/sfs/manuals/trizol_reagent.pdf). Three independent biological replicates were used *per* assay.

Samples of RNA were purified using RQ1 RNase-Free Dnase (Promega, USA) according to standard protocols. The RNA purity and integrity was determined in a NanoDrop spectrophotometer (NanoDrop Technologies). Gel electrophoresis was also performed to verify intact RNA. For quantitative RT-PCR analyses, 1 μ g of RNA of each sample was reverse-transcribed into cDNA in a 20- μ L total volume reaction containing 4 μ L 5 $\times$ iScript reverse transcription supermix for RT-qPCR (Bio-Rad, Munich, Germany) and 12.5 μ L nuclease-free water. The cDNA was synthesized in a three-step cycle, 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. A control reaction was performed without reverse transcriptase for all the isolates to verify the absence of genomic DNA contamination.

A total of 14 genes were selected according to identified candidate proteins to study their gene expression profiles by RT-qPCR (Table S2—Supporting information). Tests were carried out in triplicate and in three separate experiments. RT-qPCR was carried out using the CFX 96 Touch™ Real-Time PCR Detection System (BIORAD), and the amplifications were done using the SYBR Green PCR Master Mix (Applied Biosystems) following manufacturer's instructions. For normalization, actin and β -tubulin genes were selected as the most stable candidates. The thermal cycling conditions were composed of an initial denaturalization step at 95 °C for 3 min, followed by 45 cycles at 95 °C for 10 s, 58 °C for 20 s. Melting curve analysis and gel electrophoresis were performed to confirm specificity of the product. Relative transcriptomic expressions of the selected genes were statistically analyzed using methods from Gil-Salas et al. (2007). This method calculates the Mean Normalized Expression using the average of Cq values and slope-intercept point from each regression analysis, including the calculation of variance and errors. The sequences of primers used in this study are listed in Table S2 in the supplemental material.

Protein extraction

The method used for cell lysis was carried out as described by Maddi et al. (2009) with minor modifications: Aliquots

(1 mL) of germinated or ungerminated conidia suspended in acetone and were ruptured in 2 mL ice-cold tubes containing 450 mg glass beads (Sigma-Aldrich, St. Louis, USA) and two 1/4 Ceramic Spheres (Q-Biogene, Valencia, USA) using the FastPrep Instrument (Q-Biogene). Samples were homogenized for 12 cycles at 6.0 speed for 20 s and cooling intervals on ice (1 min) after each cycle. Protein extraction, precipitation and further purification were performed according to Fernandez-Acero et al. (2009). The resulting lysate was transferred to 15 mL tubes containing 10 mL of 100 % ice-cold acetone and after vortexing (1 min) centrifuged at $5,000\times g$ for 5 min. The pellet was sequentially washed twice with 10 % w/v TCA/acetone, once with 20 % (w/v) TCA and finally twice with 80 % (v/v) acetone. The pellet was air-dried and purified by a phenol-based extraction procedure according to Fernández-Acero et al. (2009) Proteins were quantified using RC/DC assay (Bio-Rad, München, Germany) prior to 2-DE separation.

Labeling of proteins with CyDye

For each experimental group, consisted of four biological replicates, 50 µg of precipitated and air-dried proteins from the different replicas was solubilized separately in labeling buffer (7 M urea, 2 M thiourea, 30 mM Tris-HCl, 4 % CHAPS, pH 8.5) to a concentration of 5 µg/µL. The pH was adjusted to 8.5 using NaOH 100 mM, and the protein concentration was measured using the Bio-Rad Protein Assay. For labeling of the proteins, 400 pmol of CyDye in 1 µL was mixed with 10 µL of sample containing 50 µg of protein and incubated on ice for 30 min in the dark. The labeling reaction was terminated by adding 1 µL of 10 mM lysine and subsequent incubation on ice for 10 min. Each sample was covalently labeled with a fluorophore, either Cy3 or Cy5. In order to avoid biasing, 50 % of the samples from each experimental group were labeled with Cy3 and the other 50 % with Cy5. A mixture of equal amount of protein from every sample in the experiment was labeled with Cy2 and used as internal standard.

2D-DIGE gel imaging and image analysis

For analytical 2D-DIGE analysis, 50 µg each of Cy3, Cy5 and Cy2 labeled samples (150 µg of protein) was combined and mixed with an equal volume of $2\times$ IEF buffer (7 M urea, 2 M thiourea, 4 % w/v CHAPS, 0.002 % w/v Bromophenol Blue, 1 % w/v DTT, 1 % v/v ampholytes) and incubated for 10 min on ice. Each mixture contained one internal standard sample and two individual samples each belonging to a different experimental group. For the first dimensional separation by isoelectric focusing (IEF), sample mixtures were filled up to 100 µL with IEF buffer and

loaded onto 17 cm IPG strips (3–10 nonlinear pH gradient, Bio-Rad). IEF was performed in a Bio-Rad Protean IEF cell. Rehydrating conditions and the voltage ramp protocol were according to the manufacturer's recommendations. After IEF, the strips were equilibrated for two intervals of 15 min in equilibration buffer (0.1 M Tris-HCl pH 8.8, 6 M urea, 30 % v/v glycerol, 2 % w/v SDS) supplemented with 2 % DTT (w/v) in the first equilibration step and with 2.5 % iodoacetamide (w/v) in the second step. The second dimension was run on an Ettan Dalt-six system (GE Healthcare/Amersham Biosciences) vertical unit using self-made 12 % Tris-Glycine Laemmli-SDS-PAGE gels. Electrophoresis was carried out as follows: first for 1 h at 80 V, 10 mA/gel and 0.5 W/gel and subsequently overnight at 150 V, 25 mA/gel and 1.5 W/gel. Protein fluorescent gel imaging of the Cy3-, Cy2- and Cy5-labeled samples was visualized using a Typhoon FLA9000 laser scanner (GE Healthcare/Amersham Biosciences) according to the manufacturer's recommendations. Digital images were edited and studied using the Delta 2D image analysis software 4.3 version (Decodon GmbH, Greifswald, Germany). Four replicates gel were done. Before to statistical analysis, spot detection was done automatically and occasionally corrected manually. A normalization in the total of pixels for each fluorescent signal against total volume of all valid spots was performed using the Delta2D software for the overlays of four replicate gels each. Spots with an average gray value below 0.015 were deleted. Significant changes in spot patterns of the different groups were determined using Student's *t* test (confidence interval ≥ 95 %) based on the relative spot volume. Spots with more than 1.5-fold change were counted as significantly accumulated.

In-gel tryptic digest, sequence data and mass spectrometry-based protein identification

After 2D-DIGE imaging and analysis, gels were post-stained with a Silver stain protocol and scanned on a day-light scanner linked to the Proteineer spII spotting robot (Bruker Daltonic, Bremen, Germany). Differential spots were localized on the gel by comparing the silver-stained spot pattern with the 2D-DIGE protein pattern of the same gel, and the selected spots were punched robotically. The excised spots were tryptically digested and spotted on AnchorChipTM targets by a Proteineer dp robot (Bruker Daltonics). MALDI MS and MS/MS analyses were performed on an UltraflexIII MALDI-TOF/TOF MS (Bruker Daltonics) as described in the Supporting Information material. The resulting PMFs were submitted to database searches as described below. MS/MS spectra were collected on selected precursors in order to confirm the PMF-based identifications or to characterize further nonidentified peptide fragments. Both MS and MS/MS data were used to

search against the December 2012 release of the *B. cinerea* protein database (<http://www.broad.mit.edu/>) and the NCBI nonredundant database (<http://www.ncbi.nlm.nih.gov>) using MASCOT (<http://www.matrixscience.com>) (see Supporting Information material for details).

Results and discussion

Sample extraction and 2-DE protein profiles

Botrytis cinerea strain B05.10 was used to determine the optimal sampling time during conidial germination. Several aliquots of germinating conidia were visualized each hour by light microscopy during the 24-h period of post inoculation (24 hpi), and the length of germination tubes was determined (Figure S1—Supporting information). A harvesting time after 5 h post inoculation (5hpi) was selected when more than 95 % of the conidia showed germination tubes with an approximate size similar to the diameter of the original conidia, which correlates with the previous investigations of conidial surfaces (Leroch et al. 2013), where *B. cinerea* 05.10 germinating conidia were not observed until 4–5 hpi.

The cell lysis procedure and the subsequent extraction protocol have been carried out with minor modifications according to El-Akhal et al. (2013). Using the optimized protocol for the extraction of ungerminated and germinated conidia, we analyzed by comparative proteomic studies the changes in the early phase of conidial germination to identify differentially accumulated proteins.

Protein extracts were obtained from four independent cultures from each conidial stage, ungerminated (T0) and 5 hpi germinated conidia (T5). Protein samples from each of these experimental groups were labeled with a fluorophore (CyDye). After 2-DE, protein fluorescent profiles of the Cy3-, Cy2- and Cy5-labeled samples were obtained after laser scanning. A total of 4 gels were run, and 12 images were obtained. The distribution of the spots according to their molecular weight (Mw) was mostly ranging between 200 to 14 kDa and from 3 to 10 of pI (Fig. 1). Using the Delta2D software, DIGE-stained proteins were analyzed. Only those spots with a *t* test >0.95 were included in the analysis.

A total number of 674 detected spots were included into the analysis, among those 470 did not show statistical significance in terms of regulation and were excluded from our further studies. The remaining 204 spots were classified into two different categories: (1) spots more abundant (>1.5-fold) in ungerminated conidia T0 (110 spots) and (2) spots more abundant (>1.5-fold) in germinated T5 (94 spots). From the total number of these relevant spots, 118 were identified by MALDI-TOF/TOF MS; 56 protein spots (27.45 %) derived from T0 and 62 proteins (30.39 %) from T5 (Fig. 2).

Protein identification of differentially accumulated proteins

After DIGE analysis, relevant spots in terms of regulation were excised from 2-DE gels, processed by automated in-gel tryptic digestion and identified by MALDI-TOF/TOF MS analysis. Both MS and MS/MS data were used to search against the August 2012 release of the *B. cinerea* protein database (<http://www.broad.mit.edu/>) (Staats and Van Kan 2012) using MASCOT (<http://www.matrixscience.com>) as a search engine (see Supporting Information material for details). A total of 118 protein spots yielded identifications with 149 positive hits, which corresponded to 105 unique proteins.

The identified proteins are listed in Table 1, 111 of 118 identified spots were present in both stages (Table 1; Spots 1–14, 18–41, 43–53, 55–109, 112–118); only two spots were identified specifically in T0 (Table 1; Spots 110 and 111) and five spots only in T5 (Spots 15, 16, 17, 42 and 54) (Fig. 2). Co-migration of multiple proteins was detected in 21 of the identified spots (18 %) (spots more abundant 15, 16, 32, 35, 36, 39, 44, 54 in T5 and spots more abundant 63, 66, 70, 72, 79, 80, 81, 82, 83, 92, 94, 96, 102, 106, 107, 111 in T0) (Table 1). Furthermore, 17 proteins from the total identified proteins (16.1 %) were detected in more than one spot, i.e., spots 2, 3, 4 and 5 (Table 1) are all spots corresponded to a translation elongation factor eEF-3 up-regulated in T5, and spots 56 and 100 (Table 1) are corresponded to an ascorbate peroxidase with up-regulation in T0 for spot 100 and up-regulation in T5 for spot 56. These results suggest varying posttranslational modifications of the same gene product (i.e., proteolysis, glycosylation or phosphorylation) or the presence of isoforms of these proteins (Fernandez-Acero et al. 2006). Proteins annotated as “hypothetical protein” (Table 1) were annotated by BLAST searches against the NCBI nonredundant database (<http://www.ncbi.nlm.nih.gov>). For a complete list of the identified proteins and peptides as well as BLAST annotations, see supplementary Table S1.

Gene ontology classification

The identified proteins from ungerminated and germinated conidia of *B. cinerea* were categorized according to their molecular functions and biological processes based on gene ontology classification, using GoAnna from AgBase Mississippi State University (McCarthy et al. 2006). Most of the identified proteins were assigned to the generic categories biological processes (Fig. 3) molecular function (Fig. 4), especially when the annotations of gene products were not enough defined, as it currently happens with the present version of the *B. cinerea* database.

The classification of T0-identified proteins by biological processes yielded twenty different groups (Fig. 3a).

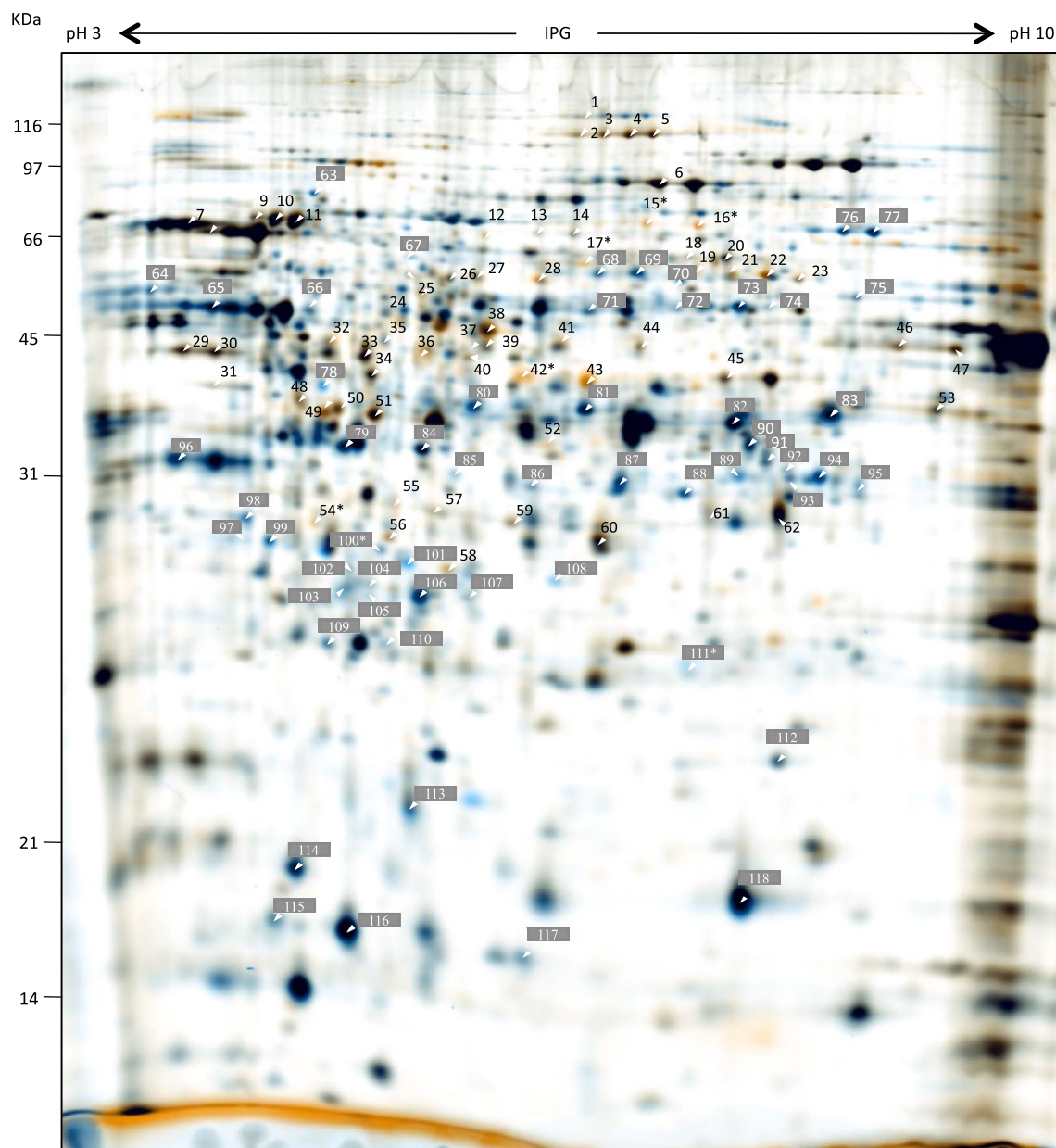


Fig. 1 2D-DIGE gel image of samples from germinated (T0) and ungerminated (T5) conidia of *B. cinerea* B.05.10. Labeled spots indicate the differential accumulated spots successfully identified by MS analysis and correspond to the proteins listed in Table 1. Shaded

labeled spots (63–118) represent overaccumulated proteins in T0, and unshaded labeled spots (1–62) represent overaccumulated proteins in T5. Spots marked with an asterisk are exclusive spots

More than 60 % of the total proteins from T0 are classified into four categories: (1) biological processes (41.85 %) (2) response to stress (13.78 %), (3) catabolic processes (5.01 %) and (4) small-molecule metabolic processes (4.76 %). The classification of T5 proteins by biological processes yielded twenty-two different groups (Fig. 3b), and more than 65 % of the total proteins from T5 were classified into six categories: (1) biological processes (36.53 %) (2) small-molecule metabolic processes (8.18 %), (3) biosynthetic processes (6.59 %), (4) cellular

nitrogen compound metabolic processes (5.39 %), (5) cellular amino acid metabolic processes (4.59 %) and (6) anatomical structure development (4.59 %). Response to stress the GO term is defined as any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a disturbance in an organism, as it happens during conidial germination. This category is reduced from 13.78 % in T0 to 2 % in T5, showing its direct relationship with this process. The next main

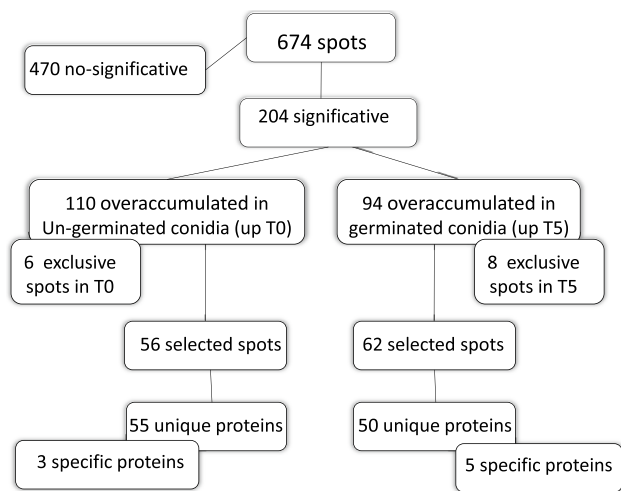


Fig. 2 Flow chart of DIGE analysis and protein identification: among of the 654 analyzed spots, only 240 showed statistically significant differences in terms of accumulation between the two compared stages: germinated (T5) and ungerminated conidia (T0). A total number of 110 are overaccumulated in ungerminated conidia (up T0); six spots are exclusively present at this stage. Within this group, a total of 56 spots were selected and robotically picked, and 55 unique proteins have been identified by MALDI-TOF/TOF MS/MS. On the other hand, 94 spots are overaccumulated in germinated conidia (up T5); eight exclusive spots were detected at this stage. Sixty-two spots were picked, and 50 unique proteins were identified by MALDI-TOF/TOF MS/MS

category is catabolic processes; the percentage of identified proteins belonging to this category is reduced from 5.01 % in T0 to 2.59 % in T5, which may correlate with the internal degradation of the endogenous energy reserves of ungerminated conidia. Besides the presence of a number of categories that appeared exclusively in T0 such as “lipid metabolic processes” or “carbohydrate metabolic processes,” all relate to energy metabolism. It may show that the germination machinery seems to be preformed inside the conidia, as it has been observed in *Blumeria graminis* and *C. acutatum* (Noir et al. 2009; El-Akhal et al. 2013). On the contrary, small-molecule metabolic processes, related to the chemical reactions and pathways involving small molecules, any low molecular weight, monomeric, nonencoded molecule, appear more abundant in T5, which may be related to a more active metabolism in the growing mycelia. Other categories specifically presented in germinated conidia support the same hypothesis, i.e., cellular nitrogen compounds, metabolic processes or cellular amino acid metabolic processes.

The classification by molecular function shows in T0, ten different groups (Fig. 4a); 94 % of the proteins belong to either (1) molecular function (44.96 %), (2) oxidoreductase activity (30.23 %), (3) ion binding (10.08 %), (4) mRNA binding (4.65 %) or (5) peptidase activity (4.65 %). Other categories include lipid binding, RNA binding,

transcription factor binding, nucleotidyltransferase activity and isomerase activity, whereas the classification in T5 yielded sixteen different groups (Fig. 4b); major categories, 77 %, belong to either (1) molecular function (28.81 %), (2) oxidoreductase activity (13.56 %), (3) ligase activity (9.32 %), (4) enzyme binding (8.47 %), (5) ion binding (8.47 %), (6) translation factor activity, nucleic acid binding (4.24 %) or kinase activity (4.24 %). Other categories include RNA binding, transferase activity (methyl, aryl or alkyl), lyase activity and enzyme regulator activity. The fact that the overaccumulation of oxidoreductase activity in T0 samples, related to the transformation of reactive oxygen species (ROS) species produced by plants as a defense mechanism, fits with the overaccumulation of response to stress proteins obtained in the biological process comparison. The presence of specific categories in T5, such as, kinase activity, methyl transferase activities, ligase activities, or translation factor activity, nucleic acid binding (Fig. 4) may be related to a higher metabolism rate in T5.

Comparison of gene and protein accumulation pattern for ungerminated and germinated conidia

In order to compare the results obtained about accumulated proteins and the expression level of genes, we used quantitative real-time PCR (RT-qPCR) as a control of our 2D-DIGE results. This comparison may illustrate how good is the correlation between specific genes and its proteins to check the relevance in a specific biological function.

To that end, 14 proteins were selected from the whole list of identified proteins as follows: (1) proteins only present in ungerminated (T0) conidia: 6,7-dimethyl-8-ribitylumazine synthase (spots 110), acetoin(diacetyl) reductase and imidazoleglycerol-phosphate dehydratase (spot 111), (2) proteins only present in germinated (T5) conidia: ATP-binding cassette sub-family F member 3 (spot 15), asparagine synthetase (spot 17), acetolactate synthase small subunit, mitochondrial precursor (spot 54) and S-adenosylmethionine synthetase (spot 42), (3) known virulence factors identified in our approach such as peptidyl-prolyl cis–trans isomerase (spot 118), Superoxide dismutase (spot 117) and GTP-binding proteins (spot 53 and spot 62) and (4) some proteins related with DNA synthesis, protein synthesis and oxidative respiration such as CTP synthase (spot 12), translation elongation factor eEF-3 (spot 5) and peroxiredoxin-5, mitochondrial precursor (spot 114), respectively, to correlate the expression level of based metabolism key proteins. (Table S2—Supporting information).

Standard curves were constructed from all the selected and housekeeping genes by serial dilution of DNA samples. Data were normalized using both housekeeping genes, in independent statistical analysis, and Mean Normalized

Table 1 List of identified spots from *B. cinerea* ungerminated and germinated conidia

Spot	Accession	pI	MW (KDa)	Cov (%)	Mascot		Protein identification	Ratio T5/T0
					MS	MS/MS		
1	BC1G_05327	6.11	132.6	10.9	84.1		Botrytis cinerea pyruvate carboxylase (1210 aa)	1.567
2	BC1G_15638	6.23	105.4	12.9	72.5	38.38	Botrytis cinerea translation elongation factor eEF-3 (947 aa)	2.307
3	BC1G_15638	6.23	105.4	25.7	190		Botrytis cinerea translation elongation factor eEF-3 (947 aa)	2.622
4	BC1G_15638	6.23	105.4	23.8	153		Botrytis cinerea translation elongation factor eEF-3 (947 aa)	2.714
5	BC1G_15638	6.23	105.4	26.3	206	39.54	Botrytis cinerea translation elongation factor eEF-3 (947 aa)	2.752
6	BC1G_12307	6.04	86.2	58	331	153.18	Botrytis cinerea 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (768 aa)	1.588
7	BC1G_06164	4.85	59.8	60	244	194.4	Botrytis cinerea heat shock 70 kDa protein 2 (551 aa)	1.534
8	BC1G_10846	5.12	45.9	36.3	145	133.7	Botrytis cinerea heat shock protein SSB (425 aa)	1.643
9	BC1G_11661	5.73	73.4	27.2	97	233.16	Botrytis cinerea heat shock protein. mitochondrial precursor (680aa)	1.674
10	BC1G_11661	5.73	73.4	54.1	322	254.07	Botrytis cinerea heat shock protein. mitochondrial precursor (680 aa)	1.862
11	BC1G_11661	5.73	73.4	50.4	289		Botrytis cinerea heat shock protein. mitochondrial precursor (680 aa)	1.958
12	BC1G_11620	5.74	61.3	38.7	158	25.9	Botrytis cinerea CTP synthase (552 aa)	2.866
13	BC1G_06223	5.93	69.8	26.4	134		Botrytis cinerea lysyl-tRNA synthetase (608 aa)	1.681
14	BC1G_06223	5.93	69.8	37.4	107	44.81	Botrytis cinerea lysyl-tRNA synthetase (608 aa)	1.619
15*	BC1G_05116	5.64	27.5	34	83.2	104	Botrytis cinerea ATP-binding cassette sub-family F member 3 (254 aa)	3.306
	BC1G_05115	8.72	32.5	4.5		33.46	Botrytis cinerea conserved hypothetical protein (293 aa)	
16*	BC1G_05116	5.64	27.5	48.2	125	121.49	Botrytis cinerea ATP-binding cassette sub-family F member 3 (254 aa)	3.795
	BC1G_05115	8.72	32.5	50.7	106		Botrytis cinerea ATP-binding cassette sub-family F member 3 (293 aa)	
17*	BC1G_04186	6	65.4	47.5	249	46.97	Botrytis cinerea asparagine synthetase (582 aa)	2.761
18	BC1G_15666	6.28	61.8	3.7		79.11	Botrytis cinerea phosphoribosylaminoimidazole carboxylase (575 aa)	1.604
19	BC1G_12242	6.46	64	39.1	153	129.01	Botrytis cinerea sulfate adenylyltransferase (574 aa)	1.82
20	BC1G_05305	6.9	56.6	51.8	222	56.2	Botrytis cinerea pyruvate kinase (518 aa)	1.643
21	BC1G_12242	6.46	64	44.3	139	364.85	Botrytis cinerea sulfate adenylyltransferase (574 aa)	1.572
22	BC1G_12242	6.46	64	29.1	84.5	167.21	Botrytis cinerea sulfate adenylyltransferase (574 aa)	2.333
23	BC1G_07479	9.06	65.6	28.1	116	49.67	Botrytis cinerea prolyl-tRNA synthetase (589 aa)	2.145
24	BC1G_03578	5.7	59.8	37.8	204		Botrytis cinerea GMP synthase (544 aa)	1.57
25	BC1G_04775	9.09	60.4	6.1		79.31	Botrytis cinerea hypothetical protein similar to mitochondrial import inner membrane translocase subunit precursor (542 aa)	1.951
26	BC1G_09186	6.56	65	21.2	88.5	217.99	Botrytis cinerea dihydroxy-acid dehydratase (610 aa)	1.696
27	BC1G_13131	5.85	58.2	4.7		70.01	Botrytis cinerea inosine 5-monophosphate dehydrogenase (550 aa)	2.075
28	BC1G_13131	5.85	58.2	22.8	87.5	80.32	Botrytis cinerea inosine 5-monophosphate dehydrogenase (550 aa)	2.847
29	BC1G_00466	4.99	44.8	22.1	46.7	139.49	Botrytis cinerea eukaryotic initiation factor 4A (399 aa)	1.823
30	BC1G_00466	4.99	44.8	37.4	102	271.7	Botrytis cinerea eukaryotic initiation factor 4A (399 aa)	1.848
31	BC1G_15906	5.85	47.9	35.5	132	32.68	Botrytis cinerea ATP-specific succinyl-CoA synthetase beta subunit (446 aa)	1.826
32	BC1G_06095	5.79	51	22.3	70.3	163.51	Botrytis cinerea D-3-phosphoglycerate dehydrogenase (472 aa)	1.754
	BC1G_00939	5.28	46.9	34.2	121	111.15	Botrytis cinerea elongation factor 1-gamma 1 (416 aa)	
33	BC1G_07167	5.65	43.6	39	128	135.62	Botrytis cinerea adenylosuccinate synthetase (401 aa)	1.852
34	BC1G_03241	5.55	48.08	38.1	193	106.16	Botrytis cinerea adenosylhomocysteinase A (450 aa)	1.768
35	BC1G_06095	5.79	51	37.4	96.8	130.03	Botrytis cinerea D-3-phosphoglycerate dehydrogenase (472 aa)	1.839
	BC1G_14197	6.31	46	35.8	68.1	26.12	Botrytis cinerea argininosuccinate lyase (412 aa)	
36	BC1G_01188	5.79	38.2	25.7	21.1	463.91	Botrytis cinerea hypothetical protein similar to glycine-rich protein (359 aa)	1.898
	BC1G_07167	5.65	43.6	45.8	202	192.67	Botrytis cinerea adenylosuccinate synthetase (401 aa)	
37	BC1G_13490	5.9	49	65.6	262	190.48	Botrytis cinerea NADP-specific glutamate dehydrogenase (451 aa)	1.538
38	BC1G_13490	5.9	49	72.4	436	171.33	Botrytis cinerea NADP-specific glutamate dehydrogenase (451 aa)	3.497
39	BC1G_12973	9.19	58.4	48.6	135	108.97	Botrytis cinerea fumarate hydratase (540 aa)	1.853
	BC1G_15049	5.9	49.3	4.5		142.94	Botrytis cinerea saccharopine reductase (449 aa)	
40	BC1G_11707	5.66	43.9	57.5	145	185.47	Botrytis cinerea translation elongation factor EF-Tu (399 aa)	1.911

Table 1 continued

Spot	Accession	pI	MW (KDa)	Cov (%)	Mascot		Protein identification	Ratio T5/T0
					MS	MS/MS		
41	BC1G_15049	5.9	49.3	56	192	149.17	Botrytis cinerea saccharopine reductase (449 aa)	1.732
42*	BC1G_04602	5.9	43.1	48.6	162	154.43	Botrytis cinerea S-adenosylmethionine synthetase (396 aa)	4.121
43	BC1G_04602	5.9	43.1	68.9	239	171.02	Botrytis cinerea S-adenosylmethionine synthetase (396 aa)	5.628
44	BC1G_15049	5.9	49.3	28.8	109	128.51	Botrytis cinerea saccharopine reductase (449 aa)	2.043
	BC1G_06312	6.13	51.2	38.7	164	180.79	Botrytis cinerea WD repeat-containing protein 12 (479 aa)	
45	BC1G_02443	8.67	33	26.6	55.4	116.6	Botrytis cinerea citrate synthase. mitochondrial precursor (302 aa)	1.56
46	BC1G_06851	7.83	52.7	22.2	42.2	172.55	Botrytis cinerea serine hydroxymethyltransferase (478 aa)	1.878
47	BC1G_09492	9.76	50	36.1	135		Botrytis cinerea elongation factor 1-alpha (461 aa)	1.644
48	BC1G_11454	5.18	36.1	70.3	274	64.7	Botrytis cinerea activator of heat shock protein 90 (324 aa)	2.168
49	BC1G_01260	5.4	38.2	61.7	187	148.33	Botrytis cinerea 3-isopropylmalate dehydrogenase (359 aa)	1.974
50	BC1G_12011	5.54	40.6	25.5	61.5	180	Botrytis cinerea glutamine synthetase (369 aa)	2.492
51	BC1G_04443	9.3	44.1	35.7	103	463.57	Botrytis cinerea ketol-acid reductoisomerase. mitochondrial precursor (399 aa)	1.969
52	BC1G_07905	5.87	42.1	61.4	218	221.72	Botrytis cinerea sterol 24-C-methyltransferase (Delta(24)-sterol C-methyltransferase) (379 aa)	1.798
53	BC1G_04778	9.23	41	30.1	90.7		Botrytis cinerea GTP-binding protein (370 aa)	1.744
54*	BC1G_04555	6.41	35.4	67.2	157	187.13	Botrytis cinerea acetolactate synthase small subunit. mitochondrial precursor (324 aa)	2.312
	BC1G_07168	5.48	28.6	60.8	89.8		Botrytis cinerea urease accessory protein ureG (269 aa)	
	BC1G_05306	5.22	73.6	29.4	56.4		Botrytis cinerea hypothetical protein (664 aa)	
55	BC1G_00841	5.39	27.6	43.2	158	58.46	Botrytis cinerea pyridoxine biosynthesis protein PDX1 (258 aa)	1.858
56	BC1G_08301	9.2	40.7	48.9	177	214.87	Botrytis cinerea ascorbate peroxidase (373 aa)	1.594
57	BC1G_00062	5.92	28.3	22.5	56.9		Botrytis cinerea eukaryotic translation initiation factor 4E-2 (259 aa)	1.653
58	BC1G_07808	10.2	42	20.5	22.8	76.79	Botrytis cinerea hypothetical protein similar to nucleolar protein 13 (382 aa)	2.65
59	BC1G_00062	5.92	28.3	36	57		Botrytis cinerea eukaryotic translation initiation factor 4E-2 (259 aa)	1.646
60	BC1G_07409	8.85	35.6	76.2	274	182.18	Botrytis cinerea malate dehydrogenase. mitochondrial precursor (342 aa)	1.615
61	BC1G_10054	6.63	34.9	32.3	74.8	288.08	Botrytis cinerea guanine nucleotide-binding protein beta subunit (317 aa)	1.509
62	BC1G_10054	6.33	34.9	47.5	98.9	564.95	Botrytis cinerea guanine nucleotide-binding protein beta subunit (317 aa)	1.661
63	BC1G_03729	5.35	78.7	18.2	89.3	24.6	Botrytis cinerea hypothetical protein similar to A Chain A (714 aa)	0.488
	BC1G_07200	9.34	64.5	18.1	56.8		Botrytis cinerea SNW domain-containing protein 1 (576 aa)	
64	BC1G_05299	4.83	54.1	28.7	130	184.71	Botrytis cinerea beta-Ala-His dipeptidase (489 aa)	0.574
65	BC1G_12487	5.32	55.1	20.6	66.6	121.92	Botrytis cinerea predicted protein (487 aa)	0.586
66	BC1G_07152	5.36	48.5	32.5	83.1	33.65	Botrytis cinerea hypothetical protein (447 aa)	0.604
	gil347836367	5.24	48.1	22	82.4		Hypothetical protein [Botryotinia fuckeliana]	
67	BC1G_06097	5.65	51.9	15.5	71.7	37.85	Botrytis cinerea hypothetical protein similar to GTP cyclohydrolase (471 aa)	0.659
68	BC1G_06374	6.46	53.7	29.8	136	223.2	Botrytis cinerea hypothetical protein similar to DyP(477 aa)	0.557
69	BC1G_06374	6.46	53.7	50.6	271	264.17	Botrytis cinerea hypothetical protein similar to DyP(477 aa)	0.593
70	BC1G_09386	6.25	63.8	40.7	167	114.3	Botrytis cinerea peroxisomal catalase (564 aa)	0.623
	BC1G_09861	6.22	62.2	28.2	73.3		Botrytis cinerea glutamate decarboxylase 2 (558 aa)	
71	BC1G_15108	5.63	38.1	43.9	126	64	Botrytis cinerea glucose-6-phosphate isomerase (343 aa)	0.655
72	BC1G_01968	6.25	57.4	29.1	126	67.58	Botrytis cinerea peroxisomal catalase (510 aa)	0.591
	BC1G_10557	6.6	58.6	16.2	66.7	108.18	Botrytis cinerea UDP-glucose pyrophosphorylase (526 aa)	
73	BC1G_01968	6.25	57.4	18.9	57	122.21	Botrytis cinerea peroxisomal catalase (510 aa)	0.468
74	BC1G_01968	6.25	57.4	9.2	37.5	98.1	Botrytis cinerea peroxisomal catalase (510 aa)	0.637
75	BC1G_01297	6.57	54.4	18.4	75.4	36.55	Botrytis cinerea 26S proteasome nonATPase regulatory subunit 12 (475 aa)	0.66

Table 1 continued

Spot	Accession	pI	MW (KDa)	Cov (%)	Mascot		Protein identification	Ratio T5/T0
					MS	MS/MS		
76	BC1G_02144	6.67	74.3	31.8	143	131.13	Botrytis cinerea hypothetical protein (667 aa)	0.532
77	BC1G_02144	6.67	74.3	45.8	306		Botrytis cinerea hypothetical protein to alcohol oxidase A (667 aa)	0.452
78	BC1G_11550	5.85	11.5	45.8	56.1	199.12	Botrytis cinerea mannitol-1-phosphate 5-dehydrogenase (108 aa)	0.515
79	BC1G_15343	5.46	37.5	24.6	110	470.16	Botrytis cinerea L-threonine 3-dehydrogenase (347 aa)	0.552
	BC1G_04836	5.32	39.2	6.1		140.83	Botrytis cinerea fructose-bisphosphate aldolase (361 aa)	
80	BC1G_12319	8.78	26.9	49.4	94.1	120.83	Botrytis cinerea NAD-dependent formate dehydrogenase (246 aa)	0.592
	BC1G_13607	5.78	40.2	22.6	51.5	113.75	Botrytis cinerea peptidyl-prolyl cis-trans isomerase (372 aa)	
81	BC1G_12319	8.78	26.9	77.6	217	273.4	Botrytis cinerea NAD-dependent formate dehydrogenase (246 aa)	0.648
	BC1G_09640	5.86	38.8	28	54.2	72.57	Botrytis cinerea bifunctional aspartokinase/homoserine dehydrogenase 2 (365 aa)	
82	BC1G_02930	6.81	43.5	90	293	105.96	Botrytis cinerea predicted protein (400 aa)	0.598
	BC1G_10113	6.38	32.1	43	89.8	533.45	Botrytis cinerea hypothetical protein similar to allergen (301 aa)	
	BC1G_05297	6.19	46.4	51.6	129	121.19	Botrytis cinerea phosphatidylserine decarboxylase proenzyme (408 aa)	
83	BC1G_02930	6.81	43.5	72.9	243	111.38	Botrytis cinerea predicted protein (400 aa)	0.547
	BC1G_00258	9.88	4.9	69.8	55.2		Botrytis cinerea predicted protein (44 aa)	
84	BC1G_00094	5.55	37.5	42.3	97.1	220.46	Botrytis cinerea hypothetical protein similar to toxD (351 aa)	0.65
85	BC1G_06844	8.81	37.9	32.6	107	79.7	Botrytis cinerea sorbose reductase SOU1 (354 aa)	0.418
86	BC1G_06844	8.81	37.9	27.2	81.5	190.09	Botrytis cinerea sorbose reductase SOU1 (354 aa)	0.518
87	BC1G_04788	6.13	35.5	49.2	141	245.65	Botrytis cinerea alcohol dehydrogenase (324 aa)	0.532
88	BC1G_11083	6.29	35.9	62.6	219	192.15	Botrytis cinerea alcohol dehydrogenase (341 aa)	0.534
89	BC1G_06394	6.81	37.4	54.1	157	163.45	Botrytis cinerea alcohol dehydrogenase 1 (352 aa)	0.445
90	BC1G_10113	6.38	32.1	52.3	82.8	544.31	Botrytis cinerea hypothetical protein similar to allergen (301 aa)	0.59
91	BC1G_10113	6.38	32.1	51.7	113	113.33	Botrytis cinerea hypothetical protein similar to allergen (301 aa)	0.521
92	BC1G_10113	6.38	32.1	60.3	140	123.43	Botrytis cinerea hypothetical protein similar to allergen (301 aa)	0.559
	BC1G_11466	8.59	57	34.8	115	50.02	Botrytis cinerea quinone oxidoreductase (518 aa)	
93	BC1G_01745	5.67	39.9	70.1	194	225.34	Botrytis cinerea NADP-dependent leukotriene B4 12-hydroxydehydrogenase (369 aa)	0.546
94	BC1G_06394	6.81	37.4	74.6	245	162.85	Botrytis cinerea alcohol dehydrogenase 1 (352 aa)	0.376
	BC1G_03072	6.87	37.1	30	129	27.71	Botrytis cinerea alcohol dehydrogenase (351 aa)	
95	BC1G_14078	6.93	35.7	57	218	285.87	Botrytis cinerea dihydroflavonol-4-reductase (331 aa)	0.529
96	gil347442007	4.97	35.4	49.8	185		Botrytis cinerea transaldolase [Botryotinia fuckeliana]	0.631
	BC1G_00555	4.23	11	13		78.1	Botrytis cinerea transaldolase (101 aa)	
97	BC1G_02778	6.12	31.6	20.4	46.1	109.31	Botrytis cinerea hypothetical protein (285 aa)	0.45
98	BC1G_02415	5.25	33.8	23.8	58.2	83.07	Botrytis cinerea catechol 1,2-dioxygenase 2 (304 aa)	0.507
99	BC1G_02778	6.12	31.6	22.2	87.7	188.21	Botrytis cinerea hypothetical protein (285 aa)	0.397
100	BC1G_08301	9.2	40.7	33.3	54.8	271.48	Botrytis cinerea ascorbate peroxidase (373 aa)	0.625
101	BC1G_02778	6.12	31.6	43	104	224.86	Botrytis cinerea hypothetical protein (285 aa)	0.368
	BC1G_00298	9.22	35.5	38.8	98	41.73	Botrytis cinerea sorbitol utilization protein SOU2 (328 aa)	
102	BC1G_05298	5.39	29.4	19.4	31.3	132.83	Botrytis cinerea hypothetical protein (264 aa)	0.492
	BC1G_06967	5.54	72.6	11.1	64		Botrytis cinerea plastin-3 (651 aa)	
103	BC1G_15849	5.76	28.9	35.8	74.3		Botrytis cinerea Acetoin(diacetyl) reductase (275 aa)	0.617
104	BC1G_09569	5.71	24.8	41.5	102	199.95	Botrytis cinerea uracil phosphoribosyltransferase (235 aa)	0.538
106	BC1G_04734	5.84	18.6	10.2		107.38	Botrytis cinerea S-formylglutathione hydrolase (167 aa)	0.576
	BC1G_12981	5.68	27.7	42.9	173	131.93	Botrytis cinerea proteasome subunit alpha type-6 (255 aa)	
107	BC1G_15849	5.76	28.9	57.7	155	279.15	Botrytis cinerea Acetoin(diacetyl) reductase (275 aa)	0.628
	BC1G_14906	5.67	30.4	40.9	94.1		Botrytis cinerea hypothetical protein (277 aa)	
108	BC1G_02778	6.12	31.6	7		91.44	Botrytis cinerea hypothetical protein (285 aa)	0.286
109	BC1G_03377	5.59	27.7	78.2	137	192.96	Botrytis cinerea hypothetical protein similar to cyanamide hydratase (249 aa)	0.611
110*	BC1G_00088	6.24	21.7	36.1	88.2		Botrytis cinerea 6,7-dimethyl-8-ribityllumazine synthase (209 aa)	0.644

Table 1 continued

Spot	Accession	pI	MW (KDa)	Cov (%)	Mascot		Protein identification	Ratio T5/T0
					MS	MS/MS		
111*	BC1G_15849	5.76	28.9	44.2	88.6	68.94	Botrytis cinerea Acetoin(diacetyl) reductase (275 aa)	0.349
	BC1G_00669	6.1	23.8	55.8	73.8	44.39	Botrytis cinerea imidazoleglycerol-phosphate dehydratase (225 aa)	
112	BC1G_01910	9.5	25.1	9.1		69.88	Botrytis cinerea manganese superoxide dismutase (231 aa)	0.64
113	BC1G_14488	6.44	21.8	42.9	193	166.29	Botrytis cinerea hypothetical protein similar to scytalone dehydratase (192 aa)	0.565
114	BC1G_05133	5.22	16.3	22.4	36.7	328.59	Botrytis cinerea peroxiredoxin-5, mitochondrial precursor (157 aa)	0.572
115	BC1G_16031	5.68	16.7	63.1	140	115.97	Botrytis cinerea predicted protein (150 aa)	0.515
116	BC1G_16031	5.68	16.7	76.5	139	144.82	Botrytis cinerea predicted protein (150 aa)	0.511
117	BC1G_00558	5.85	15.9	78.6	140	125.5	Botrytis cinerea superoxide dismutase Cu–Zn (155 aa)	0.649
118	BC1G_01740	6.38	19.6	54.1	142	193.75	Botrytis cinerea peptidyl–prolyl cis–trans isomerase (182 aa)	0.638

Spot: Number of the corresponding spot indicated on the 2D-DIGE images Figs. 1, 2. Spots that appear exclusively in germinated (T5) or ungerminated (T0) are marked with an asterisk. Accession: Accession number refers to predicted proteins from the *B. cinerea* B.05.10 genome or the NCBI nonredundant protein database. Protein identification: description of the corresponding protein accession. pI: Theoretical isoelectric point, Mw: Theoretical molecular weight. Cov: Percentage of the complete protein sequence covered by matching peptides from the PMF and or additional MALDI-TOF/TOF. MASCOT: MASCOT MS Scores for PMF data. MASCOT MS/MS scores for fragmentation data. Ratio T5/T0: ratio for the normalized spot volumes of the fluorescent signals in (T5) and (T0). For statistically significant differentially accumulated proteins, 1.5-fold accumulation is required, so the value greater than 1.5 indicates protein more abundant in T5 while values below 0.65 indicate proteins more abundant in T0. For further information and an extended version of this table, see supplementary material

Expression was calculated according to Gil-Salas et al. (2007). In the analysis, actin and β -Tubulin genes were selected as the most stable candidate gene for normalization (Mamarabadi et al. 2008; Dekkers et al. 2012). The resulting curves demonstrated good efficiencies and lineal regressions for all the genes (Table S2—Supplemental material). Analyzing the ratio T5/T0 shown in all the cases, we found that the results were totally coincident with those shown by 2D-DIGE analysis, and these results were confirmed using both housekeeping genes for normalization (Table S2). All these data confirm the differential expression levels of proteins identified by 2D-DIGE in this study.

Proteome mining

Inside the observed GO categorization of the total 118 identified spots, a set of proteins has been found, which was overaccumulated in T5 samples and apparently involved in an active metabolism (1), such as citrate synthase (spots 45), adenylosuccinate synthetase (spot 33 and 36) and 3-isopropylmalate dehydrogenase (spot 49), which are responsible for energy production within the tricarboxylic acid cycle. Following proteins were classified as (2) amino acid biosynthesis proteins, i.e., asparagine synthetase (spot 17), *S*-adenylmethionine synthetase (spot 42 and 43), acetolactate synthase (spot 54) or (3) ribosomal proteins, for example, phosphoribosylaminoimidazole carboxylase (spots 18), nuclear protein 13 (spot 58) as well as (4) proteins with transmembrane transport activity, e.g., ATP-binding cassette sub-family F member 3, (spots 15

and 16) and finally (5) proteins implicated in nucleic acid synthesis, for example, CTP synthase (spot 12). The fact that most of these proteins are exclusively present in T5 (spots 15, 16, 17, 42 and 54) may be due to an increased metabolic activity during the early stages or conidia germination. The role of mannitol as a first substrate for endogenous respiration in the earlier stage of conidia germination is well known (Horikoshi et al. 1965; d'Enfert et al. 1999; Ruijter et al. 2003). Mannitol dehydrogenase converts mannitol into fructose, providing energy for the germination. The two-fold increase of mannitol-1-phosphate dehydrogenase (MPD) (spot 78) in T0 (Table 1) is consistent with previous results of Dulerio et al. (2010). The authors reveal the existence of new mannitol pathway in which mannitol catabolism can occur through phosphorylation of mannitol via MPD. This is another evidence for the key role of this enzyme during *B. cinerea* conidia germination.

Another important accumulated protein in T0 in relation to energy metabolism is the 6,7-dimethyl-8-ribityllumazine synthase (spot 110), a precursor for riboflavin (vitamin B2). This pathway has been reported as essential for virulence in early germination steps for *Candida albicans* (Becker et al. 2010). In our study, we have found 6,7-dimethyl-8-ribityllumazine synthase (spot 110) to be exclusively expressed in T0. This finding is consistent with our RT-PCR data (Fig. 5), which may suggest a relevant role of this protein during conidial germination.

The uracil phosphoribosyltransferase (spot 110) has been found specifically in ungerminated conidia. This enzyme synthesizes Uridine 5'-monophosphate, a common precursor

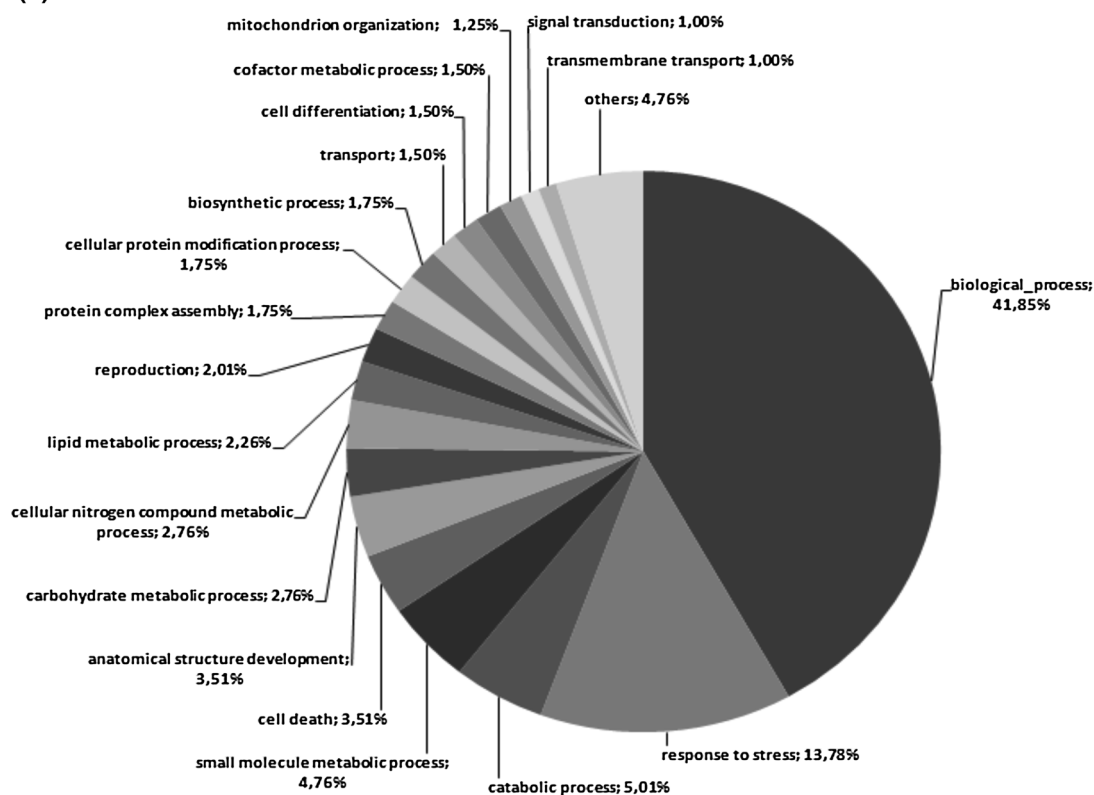
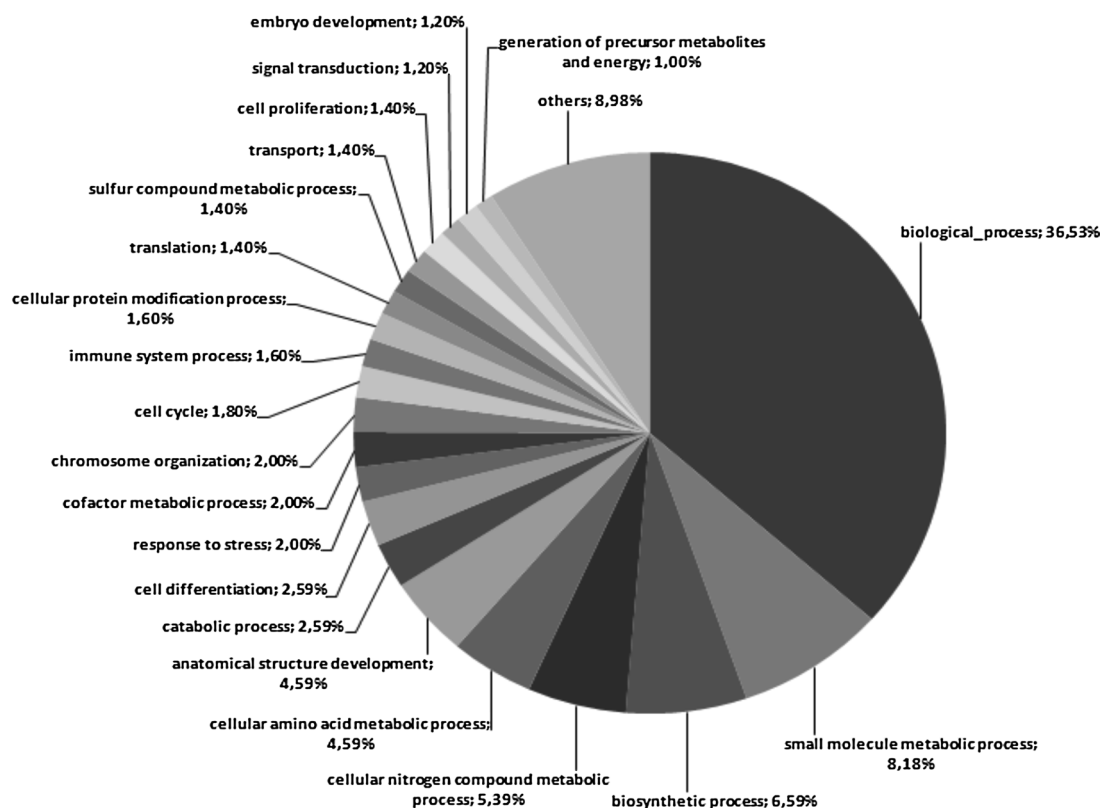
(a) Biological Processes T0**(b) Biological Processes T5**

Fig. 3 Gene ontology classification by biological process. Gene ontology categorization of identified proteins from the proteome of **a** nongerminated conidia and **b** germinated conidia of *B. cinerea* according to their involvement in biological processes

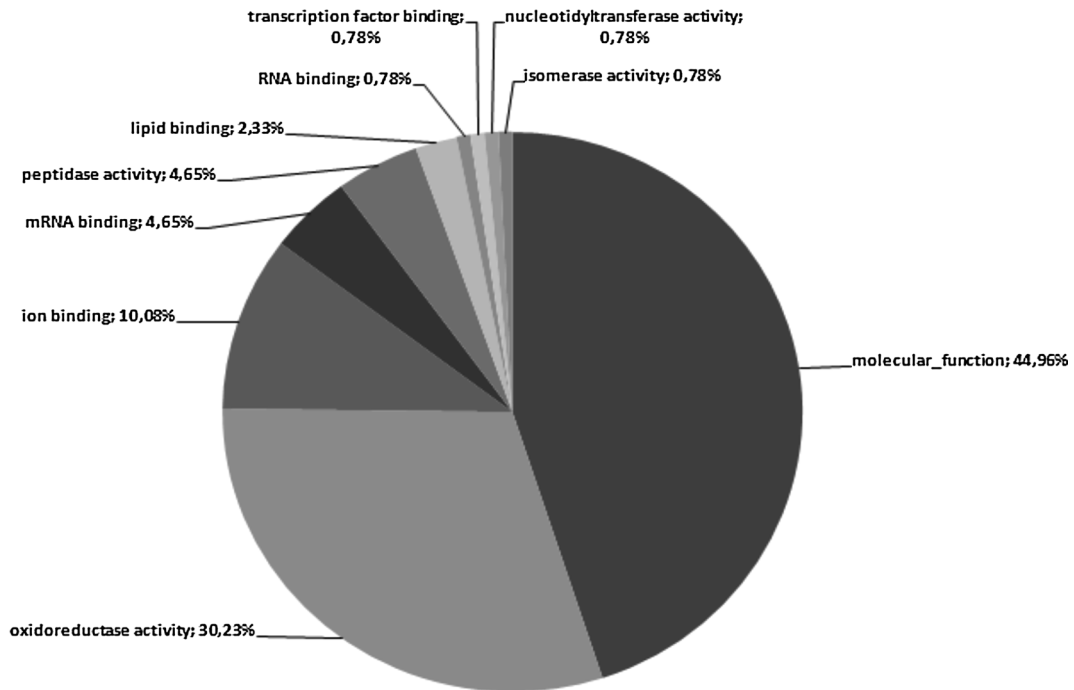
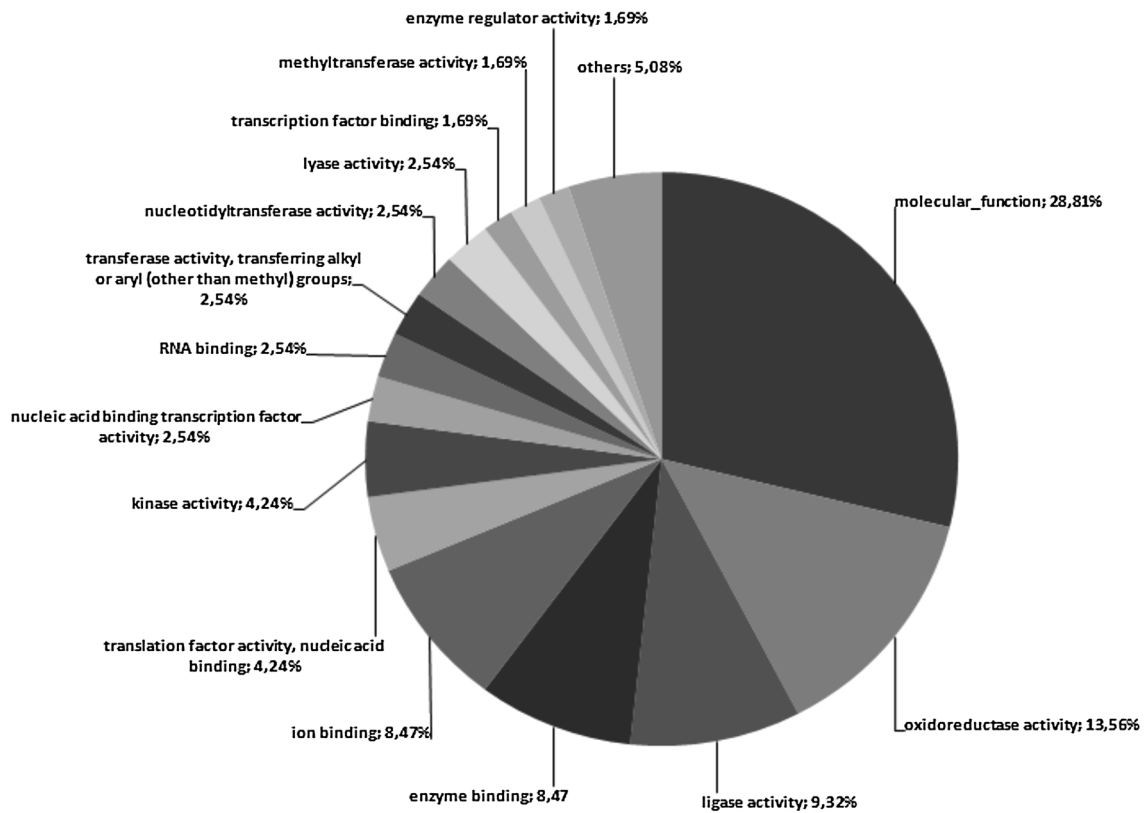
(a) Molecular function T0**(b)** Molecular function T5

Fig. 4 Gene ontology classification by molecular function. Gene ontology categorization of identified proteins from the proteome of **a** nongerminated conidia and **b** germinated conidia of *B. cinerea* according to their molecular function

of all pyrimidine nucleotides. A role of this enzyme has been suggested during the latent state and/or virulence in *M. tuberculosis* (Villela et al. 2013). In *Candida albicans*, it has been related to primary resistance to flucytosine, an antifungal reagent (Hope et al. 2004), which may indicate a role of the pyrimidine “de novo” synthesis as a therapeutic target for fungicide design and the development of fungal virulence.

The correlation of early stages of conidial germination with the accumulation of proteins related to translation has been reported in previous studies for different filamentous fungi as *N. crassa*, *A. nidulans* and *F. solani* (Osherov and May 2001). These studies are consistent with our results since we have identified a set of proteins related to protein translation, which were overaccumulated in germinated conidia, such as (1) ribosomal proteins, e.g., phosphoribosylaminoimidazole carboxylase (spots 18), nucleolar protein 13 (spot 58) and (2) a considerable amount of elongation and translational initiation factors like elongation factors eEF-3 (spots 2, 3, 4, 5) and 1-gamma 1 (spot 32), the translation elongation factor EF-Tu (spot 40) and elongation factor 1-alpha (spot 47), the translational initiation factor 4E-2 (spot 57 and 59), as well as (3) heat shock proteins (spot 7, 8, 9, 10, 11 and 48) (see Table 1), which are functionally linked to translation elongation factors and ribosomal proteins to maintain the integrity of the folding confirmation of nascent polypeptides (Cooper et al. 2007). All these data may suggest an increment of protein translation in early stage of *B. cinerea* conidia germination.

Proteasomes are implicated in stress response by removing proteins that are damaged, denatured or misfolded (Goldberg 2003). They are involved in the adaptation of metabolism by degrading transcriptional regulators (Hershko and Ciechanover 1998). Recent studies have demonstrated the relation between ubiquitin-mediated proteolysis, conidial germination and pathogenicity in *M. oryzae* (Oh et al. 2012). In our study, the 26S proteasome nonATPase regulatory subunit 12 (spot 75) and proteasome A-type (spot 106) were identified in the conidial proteomes of both stages, but up-regulated in ungerminated conidia. The overaccumulation of these proteins in T0 may suggest the necessity of these proteins from the outset of germination, working as a mechanism of degradation of transcription regulators factors (Hershko and Ciechanover 1998), and playing an important role in the flux of substrates through metabolic pathways, cell signaling, the selective elimination of abnormal proteins and within the cell cycle (Coux et al. 1996).

Reactive oxygen species play a major role in pathogen–host interactions (Temme and Tudzynski 2009). It has been described in *B. cinerea* that this pathogen shows not only resistance against oxidative burst, but even stimulates it on host plants. Catalase and superoxidase also protect conidia by opposing oxidative stress that occurs

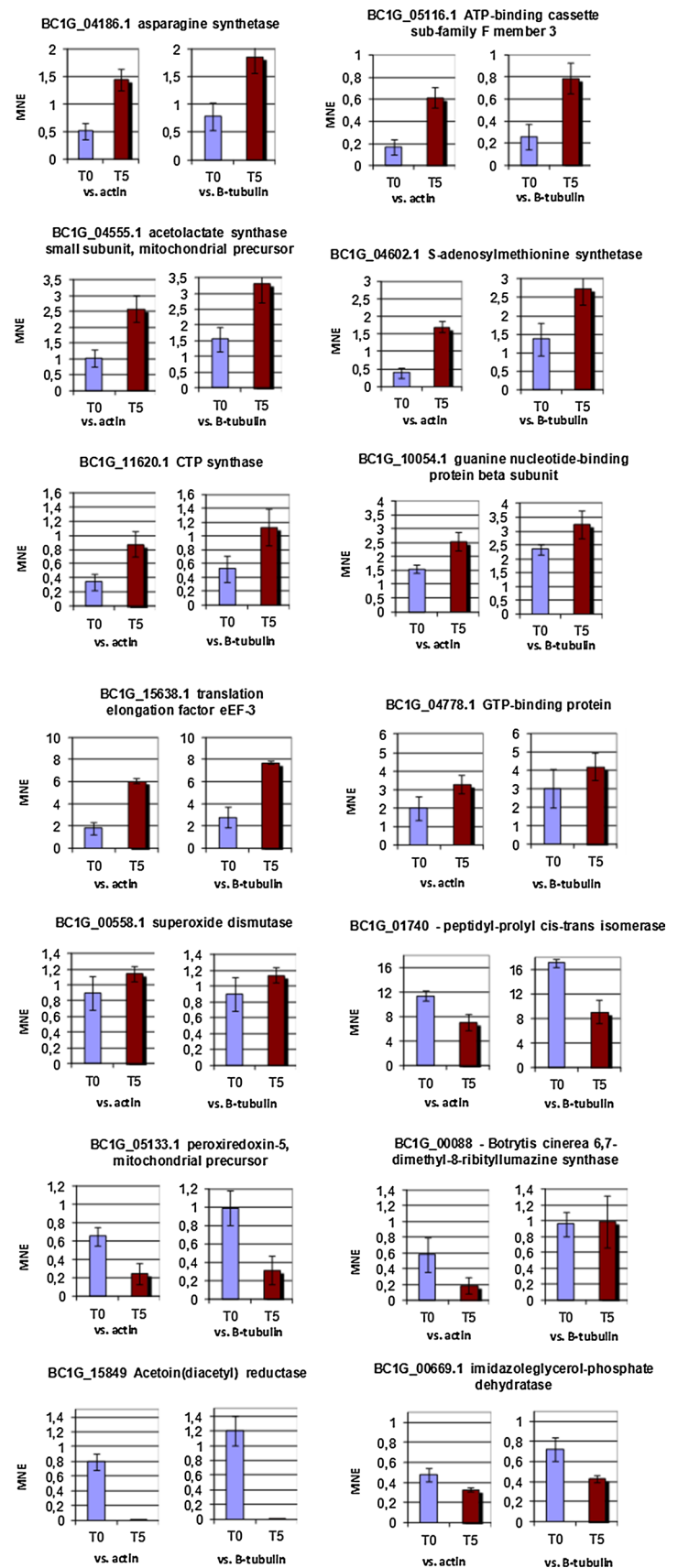
during air transport or after rewetting of dried spores. A peroxiredoxin-5 mitochondrial precursor (spot 114), two peroximal catalases (spot 70 and spots 72, 73 and 74) and two superoxide dismutases (spots 112 and 117) were identified in dormant cells. These data suggest that stress resistance of conidia may be developed strongly during very early stages of germination (van Leeuwen et al. 2013). In addition, it is known that *B. cinerea* induce oxidative shock to plant cells during early stages of infection process, accumulating hydrogen peroxide in germ tube (Choquer et al. 2007). This fact could also explain how peroxiredoxin and catalases enzymes are down-regulated during the germination process, in order of maintenance of high level of hydrogen peroxide in germ tube during germination.

Concerning the identification of proteins related to the pathogenicity process, we have detected experimentally validated virulence factors for *B. cinerea* listed in the Pathogen—Host Interaction Database (<http://www.phi-base.org/>), and most of them overaccumulated in T0, e.g., peptidyl-prolyl cis-trans isomerases (spot 80 and 118) (Viaud et al. 2003) and superoxide dismutase Cu-Zn (spot 117) (Fernandez-Acero et al. 2006; Rui and Hahn 2007). This data support the hypothesis that the ungerminated conidia accumulate a significant amount of these factors for a rapid fungal development and host invasion.

Conidial pigment biosynthesis scytalone dehydratase Arp1 gene (spot 113) was found overaccumulated in dormant conidia of *B. cinerea*. This protein is related to the melanin biosynthesis, which is the conidial pigment, and known to contribute to virulence (Tsai et al. 1997), including filamentous fungi as *B. cinerea* (Schumacher and Tudzynski 2012). Other relevant proteins found in our study are related to GTP-binding proteins (spot 53, 61 and 62) and were upregulated in T5. These proteins have been demonstrated to be essential for cell growth, asexual and sexual development, virulence and secondary metabolite production in plant pathogenic filamentous species including *B. cinerea* (Gronover et al. 2001).

As far as we know, this is the first proteomic approach to investigate the specific protein profile of the *B. cinerea* conidia. Using 2D-DIGE, 204 spots were detected showing significant accumulated differences between ungerminated (110) and germinated conidia (94). Identified proteins were classified by gene ontology into its involvement in specific biological processes and by its molecular functions, revealing that most of the infective tools are preformed inside the ungerminated conidia allowing a quick fungal development and host infection at the early stages of conidial germination. From the 118 identified spots, several virulence factors have been identified, such as cyclophilin. However, the functional roles of most of the identified spots need further investigation. Nevertheless, mannitol-1-phosphate

Fig. 5 Gene expression profiles by RT-qPCR. Study of differentially accumulated proteins by RT-qPCR. Figure shows the expression profiles for the fourteen selected genes normalized using Actin and β -Tubulin expression level results. T0 and T5 indicate the time of sampling, ungerminated conidia (T0) and 5 h post inoculation (T5)



dehydrogenase, 6,7-dimethyl-8-ribityllumazine synthase, uracil phosphoribosyltransferase, the proteasome machinery or the scytalone dehydratase Arp1 gene are potential targets for further molecular and genetic analysis. This research brings new clues to the scientific community about the role of new proteins as therapeutic targets and virulence factors.

Acknowledgments We thank the Prof. Dr. Tudzynski for donated the strain *B. cinerea* B05.10 used in this study. This research has been financed by the Spanish Government DGICYT—AGL2009-13359-CO2/AGR and AGL2012-39798-CO2-02 (www.micinn.es/portal/site/MICINN/), by the Andalusian Government (Junta de Andalucía, PO7-FQM-002689; www.juntadeandalucia.es/innovacionciencia/empresas), and by the CeiA3 International Campus of excellence in Agrifood (18INACO177.002AA; <http://www.uco.es/cei-A3/>). Victoria E. González-Rodríguez was supported by the grant FPU of the Ministerio de Educación, Government of Spain (AP2009-1309). Eva Liñeiro was supported by a FPI grant from the University of Cadiz (2010-152).

Conflict of interest The authors have declared no conflict of interest.

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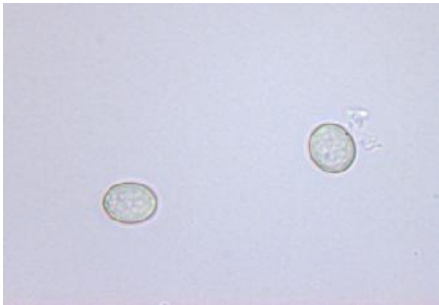
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SUPPLEMENTARY MAT

Table S1.- Detailed identification table (Excell file)

Figure S1.- Germination process of *B. cinerea* conidia during the first five hours post inoculation. A harvesting time of five hours post inoculation (5hpi) was selected due to more than 95% of the conidia showed germination tubes with an approximately size similar to the diameter of the original conidia.

2 hpi	3 hpi	4 hpi	5 hpi
			

MS/MS supporting information

Parameters for MS and MS/MS data collection with Bruker UltraflexIII.

PMF collection:

For thin layer preparations a relative laser intensity of 44% has been found to be generally optimal for PMF's on our UltraflexIII. This intensity was used for the first round MS data collection. 1200 shots were collected for each PMF. Calibration using peptide standards was performed every four sample spots. In addition, tryptic self-digestion peptide masses were used (when present) as an additional internal calibrant.

MS/MS via MALDI-TOF/TOF LIFT collection:

Up to 5 precursors were selected automatically based on their suitability for fragmentation (signal strength and relative isolation) and on the following policy:

Where a PMF identification was achieved a maximum of 2 matched precursors were fragmented to confirm the identification. Otherwise unidentified precursors ranked by quality were fragmented in order to identify other sample components. The parameters for the LIFT MS/MS acquisition (outlined in Suckau et al. 2003) were as follows:

Parent ion mode:

Following re-crystallization an initial relative laser intensity of 58% was used as the starting point for collecting parent ion spectra to verify isolation prior to fragmentation. This intensity was adjusted automatically based on initial signal quality. 300 satisfactory spectra (shots) were collected on the parent mass before switching to MS/MS mode.

MS/MS mode :

The following parameters were used for LID (laser induced dissociation): Detector Boost 130%, Laser Power Boost 40%, 800 satisfactory spectra were collected before proceeding to the next precursor. Spectra were processed in FlexAnalysis (Bruker). Peak files were generated from smoothed calibrated spectra as follows: PMF – max 200 peaks sig/noise > 4
MS/MS – 200 peaks sig/noise > 3

Database searching

Mass tolerances:

MS- PMF searches - 50 ppm (when post calibration was successful – 200 ppm when postcalibration failed)

MS/MS searches – 0.4 Daltons for both parent masses and fragment masses

Fixed Modification : Carbamidomethyl (C)

Variable Modification : Oxidation (M)

MASCOT score thresholds for 95% certainty for the Botrytis Protein database:

PMF – 55

MS/MS – 25

In cases where multiple MS/MS analyses were performed on a sample (Both MALDI and ESI-MS/MS) the coverage represents the total extent of the sequence observed in all measurements combined.

Table S2. qPCR Data. Reference of genes studied in this work and the corresponding RT-qPCR data for the standard curves. Table shows the name and reference of the genes from Broad Institute Database; Sequences of primers (5' → 3'); Efficiency, slope and R² for the lineal regression corresponding to normalize the data using actin and β -tubulin housekeeping genes; Relative expression of the genes between 5 and 0 hpi.

BC1G_ Database Reference ^a	Primer Forward ^b	Primer Reverse ^b	Efficiency (%)	Slope	R ²	T5/T0 Actin	T5/T0 β -Tubulin
BC1G_04186.1 asparagine synthetase	GGTTACCTATACTTCCACGC	GGCTCTCAAGCAGTCAGATA	103,85	-3,233	0,992	2,796	2,357
BC1G_05116.1 ATP-binding cassette sub-family F member 3	CGGTCTCTCTGATCGTGTTA	GTTCCATGGTTGTGTCGTTT	105,07	-3,206	0,998	3,659	3,073
BC1G_04555.1 acetolactate synthase small subunit, mitochondrial precursor	GAGGAATTGTTGGCGCATCA	CCTTCAACTCTGCCAATCGC	104,76	-3,213	0,995	2,52	2,145
BC1G_04602.1 S-adenosylmethionine synthetase	GGACATCGGATACGATGACT	ACCTTGAGCGATATCTGGGG	102	-3,275	0,995	4,329	1,03
BC1G_11620.1 CTP synthase	ATCTGGGTTGAAGCTGAGCA	CCAGGAACGAGAATACCAT C	103,76	-3,235	0,998	2,544	2,145
BC1G_10054.1 guanine nucleotide-binding protein beta subunit	CGCATCCTGGGACAAGACTC	TCTGTTATCGGCAGAGAAGG	104,26	-3,224	0,999	1,638	1,393
BC1G_15638 Traslation elongation factor eEF-3	CCAGCATTCAACTGGCAATC	CCTCAACTTGCTCGTTGTTG	102,21	-3,27	0,993	3,32	2,79
BC1G_04778.1 GTP-binding protein	GGGTGTTGAATCCGCTTTAC	CCACTGACGGACTTCATCAG	104,16	-3,226	0,997	1,653	1,396
BC1G_00558.1 superoxide dismutase Cu-Zn	TGACTCCAAGATCTCCGGCA	TCAGCATTGGCATCGTTTCC	104,57	-3,217	0,998	1,28	1,275
BC1G_01740 - peptidyl-prolyl cis-trans isomerase	CGAGAACTTCCGTGCTCTCT	GGTGAAGTCACCTCCTTGGA	101,86	-3,278	0,992	0,627	0,534
BC1G_05133.1 peroxiredoxin-5, mitochondrial precursor	GGTCAACATTGCTGCCGAGA	AACCAGGAACGTGAGAGTC A	103,27	-3,246	0,997	0,377	0,321
BC1G_00088 Botrytis cinerea 6,7-dimethyl-8-ribityllumazine synthase	TTCGATGCCATCATCGCCAT	AATTGGATGCGCATGAGACC	108	-3,144	0,993	0,329	1,029
BC1G_15849 Botrytis cinerea Acetoin(diacetyl) reductase	TTCGGTCGAATCGATGTCGT	CATCAGGTTGGCTCCACTT	105,35	-3,2	0,997	0,0064	0,0054
BC1G_00669.1 imidazoglycerol-phosphate dehydratase	CCAGCATTCAACTGGCAATC	GATACTTGGAAGCATGTCC	106,57	-3,174	0,991	0,701	0,594

Figure S2.- 2-DE DIGE of representative gel. Images for T0 (Cy3) (ungerminated) and T5 (Cy5) (5hpi) of *B. cinerea* conidia.

