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1 Impact of an antifungal insect defensin on the proteome of the
2 phytopathogenic fungus *Botrytis cinerea*

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15 **ABSTRACT**

16 ETD151, an analogue of the antifungal insect defensin heliomicin, is an antifungal peptide
17 active against yeasts and filamentous fungi. In order to decipher the mechanisms underlying its
18 molecular action on the phytopathogenic fungus *Botrytis cinerea*, a necrotrophic pathogen
19 responsible for gray mold disease, we investigated the changes in three-day old mycelia upon
20 treatment with different concentrations of ETD151. Optical and fluorescent microscopies were
21 used prior to establishing the peptide/protein profiles through two mass spectrometry
22 approaches: MALDI profiling, to generate molecular mass fingerprints as peptide signatures, and
23 a gel-free bottom-up proteomics approach. Our results show that a concentration of ETD151
24 above the half-maximal inhibitory concentration can alter the integrity of the mycelial structure
25 of *B. cinerea*. Furthermore, reproducible modifications of the peptide/protein composition were
26 demonstrated in the presence of ETD151 within a 1.5-16 kDa mass range. After the robustness
27 of LC-ESI-MS/MS analysis on *B. cinerea* mycelial extracts was confirmed, our analyses
28 highlighted 340 significantly modulated proteins upon treatment with ETD151 within a 4.8-
29 466 kDa mass range. Finally, data mapping on KEGG pathways revealed the molecular impact
30 of ETD151 on at least six pathways. In particular, for the effect on oxidative phosphorylation, we
31 clearly demonstrated that ETD151 does not interact directly with the mitochondrial respiratory
32 chain.

33

34 **Keywords:** Insect defensin, antifungal defensin, *Botrytis cinerea*, proteomics, fungal infection,
35 heliomicin, mechanism of action

36

37 INTRODUCTION

38 Phytopathogenic fungi are a major concern for crops in agriculture. In the last decade,
39 phytopathology experts have alerted the scientific community to several species threatening food
40 security and biodiversity¹. Every year, fungal pathogens destroy more than 125 million tons of
41 the world's top five major crops: rice (rice blast caused by *Magnaporthe oryzae*), wheat (rust
42 caused by *Puccinia graminis*), maize (smut caused by *Ustilago maydis*), potatoes (late blight
43 caused by *Phytophthora infestans*) and soybean (rust caused by *Phakospora pachyrhizi*). These
44 production losses could feed an additional 600 million people per year. Among the
45 phytopathogenic fungi, *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) is a necrotrophic
46 pathogen responsible for gray mold disease causing significant crop losses in more than 200
47 plant species worldwide^{2,3}. Ranked scientifically and economically as the second most important
48 fungal pathogen by the international phytopathological community⁴, *B. cinerea* generates
49 significant costs over a broad host range. Damage occurs in different stages of fruit and
50 vegetable production in open fields or in greenhouses and requires extensive disease control,
51 mainly by fungicide application. Concerns about this fungus have also increased due to the
52 recent discovery of *B. cinerea* fungicide-resistant strains in field populations⁵. The introduction
53 of site-specific fungicides almost 50 years ago revolutionized crop protection and has remained
54 until now the main approach for reducing fungal disease progression. However, plant pathogenic
55 fungi can adapt, leading to a tremendous loss of plant protection effectiveness. Fungicide
56 resistant pathogens have emerged over time and spread globally⁷. Most important classes of
57 antifungals (e. g. benzimidazoles, strobilurins, hydroxyanilides) are concerned by the
58 development of resistance compromising the efficacy of crop protection. As a new major

59 challenge, it is necessary to propose novel fungicides with innovative mechanisms of action or
60 alternative strategies with non-chemical products⁸.

61 Scientists are investigating the field of antimicrobial peptides (AMPs), looking for new
62 molecules with novel targets, reduced toxicity and broad-spectrum activity for a low-rate use.
63 Also known as host defense peptides (HDPs), AMPs are key components of the innate immune
64 system of all organisms, protecting them against attacks by invading pathogens and stressors⁹.
65 Even if a wide range of AMPs have been identified, they can be globally classified according to
66 their amino acid composition, their structure and the organization of their disulfide bonds. One of
67 these classes, the CS α β -defensins (Cysteine-stabilized α -helix β -sheet), groups small, cationic,
68 cysteine-rich peptides with specific 3D organization and conserved disulfide bridge arrays¹⁰⁻¹².
69 Intriguingly, the CS α β -defensins have been found in plants and insects. Plant defensins are well-
70 known and extensively studied for their antifungal activity¹³. On the contrary, only a few insect
71 defensins have been reported to be exclusively antifungal, most of them exhibiting antimicrobial
72 activities (antibacterial and antifungal), with particular efficacy against Gram-positive bacteria¹⁴.
73 The first antifungal insect defensin to be described was drosomycin^{15,16} from the fruit fly
74 *Drosophila melanogaster* (Diptera). It was followed by heliomicin^{17,18} from the tobacco budworm
75 *Heliothis virescens* (Lepidoptera), termicin^{19,20} from *Pseudacanthotermes spiniger* (Isoptera),
76 gallerimycin²¹ from the great wax moth larvae *Galleria mellonella* (Lepidoptera) and ARD1²²
77 from the larvae of *Archaeoprepona demophon* (Lepidoptera).

78 The promising *in vitro* antifungal activity of insect defensins against plant or human
79 pathogens, as well as their non-cytotoxicity, highlights their potential as candidates for crop
80 protection strategies²³ and/or therapeutic applications²⁴. In the 2000s, the French biotechnology
81 company EntoMed SA was the first to develop natural insect AMPs for the treatment of systemic

82 fungal infections affecting immunocompromised patients²⁵. Heliomicin was the working model of
83 a cutting-edge optimization program based on phylogenic exploration and fine-tuned
84 mutagenesis. The results of their work, a 44 amino acid peptide, called ETD151, revealed
85 enhanced antifungal activity against *Candida albicans* and *Aspergillus fumigatus* as well as
86 additional activity against *Cryptococcus neoformans*, as compared to heliomicin²². ETD151 was
87 the principal product developed by EntoMed SA for treating antifungal infections²⁶.

88 However, limited data has been gathered on the antifungal mechanism of action of insect
89 defensins, which include the inhibition of spore germination, the alteration of germ tube
90 elongation and the release of cytoplasmic content^{23,27}. Comparisons based on structural analysis
91 and activity tests on fungal mutants, provided evidence of mechanical commonalities between
92 heliomicin, drosomycin and some antifungal plant defensins such as the radish defensin
93 RsAFP2²⁸⁻³⁰. In contrast to insect defensins, the mechanisms of action of plant defensins have been
94 widely investigated. In most cases, the first step is the interaction with non-protein fungal
95 membrane partners such as sphingolipids or phospholipids³¹. Thereafter, plant defensins can
96 either be internalized or remain outside the fungal cells. Cell death can occur via multiple
97 mechanisms, induction of fungal membrane permeabilization, reactive oxygen species (ROS),
98 apoptosis, or mitochondrial functionality loss³². Effects differ depending on the target organism;
99 the mechanisms of action of the *Medicago* spp. defensin MtDef4 against the ascomycete fungi
100 *Neurospora crassa* and *Fusarium graminearum*³³ are not the same. Upon exposure to antifungal
101 plant defensins, fungal cells will induce mechanisms to defend themselves against these adverse
102 effects. A family of conserved kinases, MAPK, regulate tolerance to plant defensins. In yeast,
103 the MAPK pathways are involved in filamentous growth, cell wall integrity (CWI) and the high
104 osmolarity/glycerol (HOG) response³⁴. All of these elements provide a background for studying

105 the mode of action of ETD151, an optimized peptide analogue of the two lepidopteran insect
106 antifungal peptides heliomicin and ARD1.

107 To decipher the mechanism of action of ETD151 on the fungal phytopathogen *B. cinerea*, a
108 proteomics approach based on matrix-assisted laser desorption/ionization mass spectrometry
109 (MALDI-MS) and liquid chromatography-mass spectrometry coupled to electrospray ionization
110 (ESI-LC-MS) was used. The elucidation of the *B. cinerea* genome was a significant
111 breakthrough for the proteomics research community and has provided for the accurate
112 identification of proteins in this fungus, mainly from mycelial samples³⁵⁻³⁸. In the past ten years,
113 several methodological developments and technological advances in *Botrytis* proteomics have
114 offered effective new tools for achieving a better understanding of *B. cinerea* biology and how
115 its proteins are involved in pathogenic biological processes^{39,40}. Most of this work focused on the
116 secretome or virulence factors produced by the fungus, like cell wall degrading enzymes,
117 considered as key elements in the different steps of the infection cycle of phytopathogenic
118 fungi^{41,42}.

119 Due to the dynamic nature of the proteome, the addition of an antifungal compound should
120 affect processes occurring at the surface and / or within the fungal cell⁴³. In this work we compare
121 the protein profiles of extracts of fresh mycelia treated with ETD151 vs untreated cultures. This
122 proteomics study gives insight into the mechanism of action of this insect defensin on the fungus
123 *B. cinerea*.

124

125 **MATERIALS AND METHODS**

126 **Chemical reagents**

127 A liquid minimum medium based on Tanaka's medium B^{44,45} and a Potato Dextrose Broth
128 medium diluted 1:4 in water (25% PDB) were prepared using reagents from Sigma-Aldrich
129 (St. Louis, MO, USA). Potato Dextrose Agar (PDA) was purchased from Oxoid (Basingstoke,
130 UK). The reagents α -cyano-4-hydroxycinnamic acid (4-HCCA), ammonium bicarbonate (ABC),
131 dithiothreitol (DTT), iodoacetamide (IAA), HPLC-grade dimethyl sulfoxide (DMSO) and LC-
132 MS-grade formic acid (FA) were from Sigma-Aldrich. SYTOX Green Nucleic Acid Stain (5 mM
133 in DMSO) was from Thermo Fisher Scientific (Waltham, MA, USA), *RapiGest* SF surfactant
134 was from Waters (Milford, MA, USA) and sequencing-grade modified trypsin was from
135 Promega (Madison, WI, USA). Acetonitrile (ACN), ethanol absolute anhydrous (EtOH),
136 trifluoroacetic acid (TFA), all LC-MS grade quality or higher, were obtained from Carlo Erba
137 Reagents (Val de Reuil, France). Protein Calibration Standard I (ProtMix) and Peptide
138 Calibration Standard II (PepMix) were purchased from Bruker Daltonics (Germany). For all
139 experiments, MilliQ water was used (Billerica, MA, USA).

140

141 **Biological material**

142 Spores of *Botrytis cinerea* isolated from the field and cryopreserved in 10% DMSO were
143 provided by the La Dargoire Research Center (Bayer CropScience, France). All spores of
144 *B. cinerea* used in this study were derived from the same initial batch. For each experiment, a
145 cryotube was thawed and the stock solution of spores was diluted with sterile MilliQ water
146 resulting in a final concentration of 1.5×10^5 spores per mL (sp/mL) in the presence of 0.1%
147 DMSO. In order to have fresh fungal material, Falcon® culture petri dishes (Corning, NY, USA)

148 containing PDA solid medium were inoculated with 150 μ L of the appropriate spore suspension.
149 Culture dishes were left in the dark for seven days in an incubator (Mettler, Schwabach,
150 Germany) maintained at 20°C and at 70% level of hygrometry. These parameters were defined
151 as the culture conditions for all our experiments. To prepare a fresh spore solution, spores were
152 harvested from one seven-day old culture dish using a Falcon® cell scraper (Corning), filtered on
153 a Falcon® nylon 100 μ m cell strainer (Corning), counted twice with a Malassez chamber and
154 adjusted to a final concentration of 500 sp/mL in minimal medium.

155

156 **Antifungal assay**

157 The bioactive peptide ETD151, provided by Dr. Philippe Bulet, was produced by high cell
158 density fermentation of a modified *Saccharomyces cerevisiae* strain developed by the former
159 company EntoMed S.A. (Illkirch, France) and preserved as a stock solution in saline buffer. ETD
160 151 was desalted by solid phase extraction using Sep-Pak C₁₈ Classic Cartridges (Waters)
161 following the manufacturer's instructions. Solutions from 250 to 0.49 μ M of ETD151 were
162 prepared in sterile MilliQ water. Antifungal assays on *B. cinerea* were performed in sterile 96-
163 well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland). To assess inhibition of
164 mycelium growth, microplate wells were filled with 135 μ L of the fresh suspension at 500 sp/mL
165 prior to incubation in the conditions reported in the Biological material section. Addition of 15
166 μ L of the ETD151 solutions, giving final concentrations of 25 to 0.049 μ M, occurred three days
167 later. The plate was gently shaken and incubated for four additional days. Evolution of the assay
168 was monitored daily by absorbance measurement at 630 nm with an ELx808 microplate reader
169 (BioTek, Winooski, VT, USA). The dose-response curve was generated with Microsoft Excel

170 software and XLfit plug-in (release 5.4). Data were adjusted according to the “Dose-response
171 one site – 205” model to determine the half-maximal inhibitory concentration (IC₅₀).

172

173 **Fluorescence microscopy**

174 A fresh spore solution at 1×10^6 sp/mL was prepared in a 25% PDB medium as reported in the
175 Biological material section and 200 μ L were distributed in several microplate wells. Spores were
176 supplemented with SYTOX Green (final concentration of 30 μ M) prior to shaking and
177 incubation for 24 hours. Young mycelia of *B. cinerea* were treated by addition of ETD151 at
178 final concentrations of 0.78 and 3.12 μ M before a new incubation period of 24 hours.
179 Morphological changes were monitored in transmitted light (TransLight50, 30 ms exposure) with
180 an ImageXpress Micro XL fully automated inverted microscope (Molecular Devices, San Jose,
181 CA, USA) equipped with a Plan Fluor ELWD 40x objective (Nikon). SYTOX Green
182 fluorescence was observed with a Green Fluorescent Protein filter (excitation wavelength,
183 472 nm; emission wavelength, 520 nm; 100 ms exposure).

184

185 **Sample preparation for proteomic studies**

186 In a 96-well plate (TPP product), 135 μ L of the fresh suspension at 500 sp/mL in minimum
187 medium was distributed in six rows of 10 wells each. Mycelia were cultivated by maintaining the
188 plate for three days in the growth conditions defined previously. Subsequently, 15 μ L of sterile
189 MilliQ water was added to the first row as an untreated control. The five other rows were treated
190 with five different concentrations of ETD151, ranging from 31.2 μ M to 1.95 μ M. The
191 experimental plate was maintained for 24 hours in the previously described growth conditions

192 until protein extraction. Each microplate was monitored daily by measuring the absorbance at
193 630 nm with the ELx808 microplate reader (BioTek).

194 **Protein extraction**

195 For each plate, the fungal material (mycelium) from each of the 60 wells was collected by
196 manual pipetting. For each experimental condition (untreated or treated with different
197 concentrations of ETD151), 10 individual wells of a same row were pooled into a single 1.5 mL
198 Eppendorf tube and maintained on ice until use. The fungal material was pelleted by
199 centrifugation for 4 min at 13,000 rpm and 4°C. The fungal pellet was washed twice by up-and-
200 down pipetting with 1 mL of sterile MilliQ water before adding 1 mL of 70% EtOH. The washed
201 mycelia were stored overnight at -80°C. The fungal material was disrupted using a Mill-Mix 20
202 system (Domel, Železniki, Slovenia). The mycelia were ground in 2-mL tubes filled with 0.5 mm
203 zirconium oxide beads for 10 min at 30 Hz. After centrifugation for 4 min at 13,000 rpm and
204 4°C, the pellets were collected and dried under CentriVap vacuum (Labconco, Kansas City, MO,
205 USA). Finally, the proteins were extracted by incubating the dried fungal pellet in the presence
206 of 2 M acetic acid, prepared in 50% ACN, for 4 h at 4°C under gentle shaking. After incubation,
207 the extracts were centrifuged for 4 min at 13,000 rpm and 4°C and the protein supernatants were
208 stored at 4°C until use.

209

210 **Sample preparation for MALDI mass spectrometry analyses**

211 For MALDI MS analyses, a fresh solution of matrix was prepared by adding, up to saturation,
212 a few mg of 4-HCCA in 1 mL of Biotyper solution (Bruker Daltonics) composed of 50% ACN
213 and 2.5% TFA. The samples to be analyzed (0.5 μ L) were spotted and immediately covered with
214 0.5 μ L of the matrix solution on an MTP 96 polished steel MALDI target (Bruker Daltonics).

215 Calibration of the instrument was performed using PepMix complemented with ProtMix. The
216 sample spots were quickly dried under mild vacuum prior to analysis.

217

218 **Peptide mass fingerprint of *B. cinerea* mycelium by MALDI-TOF mass spectrometry**

219 Mass spectra from protein supernatants described in the Protein extraction section were
220 acquired on an AutoFlex III Smartbeam instrument (Bruker Daltonics) using the FlexControl 3.4
221 software (Bruker Daltonics) in positive linear mode. The instrument was set up with the
222 following parameters: 200 Hz laser at a 40% global attenuation offset, 20 kV source voltage 1,
223 18.5 kV source voltage 2, 9 kV lens voltage, 1.82 kV linear detector voltage, 140 ns of pulsed
224 ion extraction delay and 600 Da detector gating. MALDI MS spectra were recorded at the mass
225 range 600 Da to 18 kDa by summing two sets of 1,000 laser shots. Data were previewed using
226 the FlexAnalysis 3.4 software (Bruker Daltonics) and reprocessed in mMass software⁴⁶ (version
227 5.5.0) Average spectra were generated from three selected biological replicates (from a pool of
228 six), based on their fingerprint similarities and their intensities (maximum arbitrary intensity >
229 10,000). The average spectra were cropped from 1,500 to 16,000 m/z , baseline correction was
230 performed with a relative offset of 100 and a precision of 40, along with a smoothing according
231 to the Savitzky-Golay algorithm (window size 5.0 m/z in one cycle). The peaks were
232 automatically picked using a signal-to-noise (S/N) ratio of 2.0, a picking height of 90 and an
233 application of baseline. Finally, the average mass spectra were concomitantly and manually
234 curated to select 100 representative ions.

235

236 **Sample preparation for bottom-up proteomics**

237 Proteins were digested using the modified procedure reported by Masson and colleagues⁴⁷.
238 Briefly, the protein supernatants were dried under vacuum (Labconco), and then suspended with
239 gentle shaking in an ammonium bicarbonate (ABC) buffer (50 mM, pH 7.5) supplemented with
240 0.1% *RapiGest* surfactant. The proteins were reduced with 280 mM dithiothreitol in ABC buffer
241 for 30 min at 56°C in the dark prior to alkylation with 480 mM iodoacetamide in ABC buffer for
242 30 min at room temperature in the dark. Digestions were carried out for 1 h at 37°C by adding
243 0.5 μg of trypsin (0.1 $\mu\text{g}/\mu\text{L}$ in ABC buffer). Following this initial digestion, 0.5 μg of trypsin
244 was added before overnight incubation at 37°C. To stop proteolysis and cleave the *RapiGest*
245 surfactant, samples were acidified with 8 μL of a 10% TFA and 20% ACN solution and
246 incubated for 30 min at 37°C. Finally, samples were centrifuged for 10 min at 13,000 rpm at
247 room temperature. The digests were transferred into injection vials (Agilent Technology, France)
248 containing 5 μL of 2% ACN acidified with a solution of 0.1% TFA.

249

250 **Proteomic analyses by nano-LC-ESI-MS/MS**

251 Nano-LC-ESI-MS/MS instrumentation, software, chromatographic columns and other items
252 were obtained from Thermo Fisher Scientific. Peptides were analyzed using an Ultimate 3000
253 nanoflow HPLC system coupled to a Q-Exactive Orbitrap high-resolution mass spectrometer,
254 controlled by Xcalibur 2.2, Chromeleon Xpress 6.80 and Thermo QExactive Tune 2.5 software.
255 Nano-LC separations were performed according to Pisani and colleagues⁴⁸. Briefly, the digests
256 were loaded at a flow rate of 10 $\mu\text{L}/\text{min}$ for 6 min onto a PepMap 100 C₁₈ precolumn (300 μm x
257 5 mm, 5 μm) and separated at a flow rate of 300 nL/min onto an Acclaim PepMap 100 C₁₈
258 nanoviper column (75 μm x 250 mm, 3 μm) maintained at 35°C. Peptides were eluted using a
259 multi-step linear gradient from 2% to 32% and from 32% to 65% ACN in 0.1% (v/v) formic acid

260 for 94 min and 5 min, respectively. For MS analyses, the mass spectrometer was operating in
261 positive mode and data-dependent acquisition. The instrument acquired a full-range MS scan
262 from 380 to 2000 m/z (70,000 resolution, AGC target $3 \cdot 10^6$, maximum IT 200 ms) and then
263 fragmented the top ten peptide ions in each cycle (17,500 resolution, AGC target $2 \cdot 10^6$,
264 maximum IT 100 ms, intensity threshold $4 \cdot 10^4$, excluding charge-unassigned ions, Normalized
265 Collision Energy 27). Parent ions were then excluded from MS/MS for the next 15 s.

266

267 **Databases searching and annotation**

268 All MS and MS/MS spectral data were analyzed using Proteome Discoverer software
269 (version 2.2.0.388, Thermo Fisher Scientific). Processing and consensus workflows were
270 adapted from manufacturer's basic settings. The acquired MS/MS spectra were matched using
271 the SEQUEST HT[®] search algorithm (University of Washington) against the EnsemblFungi[®]
272 *Botrytis cinerea* B05.10 protein sequence database (http://fungi.ensembl.org/Botrytis_cinerea).
273 The release 42 downloaded on February 16, 2019 contains 13,749 entries. The spectrum
274 identification considered the following parameters: trypsin as proteolytic enzyme with a
275 maximum of two missed cleavages, 6 and 144 amino acids as minimum and maximum peptide
276 lengths, and a tolerance of 10 ppm/0.02 Da for precursor and fragment masses, respectively.
277 Cysteine carbamidomethylation was defined as a static modification. Methionine/tryptophan
278 oxidation, serine/threonine/tyrosine phosphorylation and C-terminal amidation were defined as
279 dynamic modifications. False discovery rates (FDRs), referred to as q-values, and posterior error
280 probabilities (PEPs) for peptide spectral matches (PSMs) were calculated and filtered in the
281 Percolator node using a decoy database^{51,52}. Peptides and identified proteins were respectively
282 validated based on high-confidence (FDR < 1%) and medium-confidence criteria (FDR < 5%).

283 Protein grouping was applied to regroup protein candidates covered by the same set or subset of
284 identified peptides under a representative ‘master’ protein. Annotations were implemented
285 through Proteome Discoverer using Gene Ontology (biological process, molecular function,
286 cellular compartment), protein families from Pfam and biochemical pathway maps from the
287 Kyoto Encyclopedia of Genes and Genomes (KEGG).

288

289 **Protein quantification and functional analysis**

290 Label-free quantification was performed using the Proteome Discoverer software.
291 Chromatographic alignment for LC/MS mapping features, created by the Minora Feature
292 Detector for each individual file, was achieved with a maximum retention time shift of 10 min
293 and a mass tolerance of 10 ppm. Protein abundance was determined on intensity of precursor
294 ions of unique and razor peptides.

295

296 **Activity of complex I/ III and II/III of the respiratory transport chain**

297 *Mitochondrial preparation*

298 Two liters of fresh spores of *B. cinerea* were cultured in glucose yeast mycological peptone
299 medium and incubated in Erlenmeyer flasks with stirring for 72h at 22°C. Mycelia were filtered
300 through a 200 μ m nylon cloth NITEX (Sefar AG, Heiden, Switzerland), before washing with
301 MilliQ ice water, spinning and weighing. The following steps were performed in a cold room
302 (4°C) to preserve functionality of mitochondria. Mycelia were resuspended in a 0.5 M
303 sucrose/1 mM EDTA/0.3% BSA/20 mM MOPS wash buffer adjusted to pH 7.4. After addition
304 of 5 mM cysteine, the mixture was ground with 1 mm glass beads for three cycles of 30 s with
305 1 min pauses to cool down the suspension. Fungal material was filtered on a 100 μ m nylon cloth

306 (Sefar) and beads were rinsed with the washing buffer, prior to centrifugation of the filtrate for
307 10 min at 1,500 g. The supernatant was filtered on a 150 μ m nylon cloth (Sefar) prior to
308 centrifugation for 20 min at 15,000 g. The mitochondrial pellet was resuspended in the wash
309 buffer and fractionated in 2-mL aliquots prior to purification on a 1.8 M/1.2 M sucrose gradient,
310 involving centrifugation for 45 min at 36,000 g followed by slow deceleration. Purified
311 mitochondrial fractions at the sucrose bilayer interface were diluted in the wash buffer and
312 centrifuged for 15 min at 36,000 g. Pellets were combined prior to a new washing step to obtain
313 a single purified fraction of mitochondria.

314 *Measurement of inhibition of complex I/III and II/III by colorimetry*

315 The activity of a molecule as a possible electron transport chain complex inhibitor was
316 measured by a potassium ferricyanide-based colorimetric test³⁹ in the presence of α -ketoglutarate
317 and succinate, substrates of complexes I and II, respectively. Viability of fresh mitochondria was
318 evaluated in 96-well microplates (Greiner Bio One). For complex I, 3 μ L of the purified fraction
319 was diluted in 400 μ L of Mix I solution (Supplementary data S1) before depositing 88 μ L of this
320 mixture into 4 control wells and adding the 0.05 M α -ketoglutarate substrate. After a 3-min
321 incubation at room temperature, the absorbance was measured for 5 min at 405 nm (SpectraMax
322 Plus 384, Molecular Devices). Data was converted to a slope using a linear regression model.
323 Complex II activity was evaluated in the same way using Mix II (Supplementary data S1) and
324 the appropriate substrate. For this assay, the ETD151 peptide diluted in sterile Milli-Q water was
325 compared to two standard inhibitors diluted in DMSO: antimycin A and boscalid. From a
326 dilution range (factor of 3.5) prepared upstream, 2 μ L of the molecule to test (50X) was
327 deposited in a well containing 88 μ L of fresh mitochondria in Mix I or Mix II, for complex I or
328 complex II, respectively. Immediately for complex I or after 7 min of incubation for complex II,

329 10 μL of 0.05 M substrate were added before incubation for 3 min at room temperature. After a
330 brief stirring, the mitochondrial activity was measured as previously described.

331

332 RESULTS AND DISCUSSION

333 Fungal growth inhibition and morphological changes in *Botrytis cinerea*

334 Several antifungal defensins from insects, like heliomicin²³ and drosomycin¹⁵, or from plants,
335 such as RsAFP2²⁴ and VvAMP2⁵⁵, have been reported to be active against *Botrytis cinerea* at a
336 micromolar range. We have previously shown that ETD151 exhibits a broad-spectrum of
337 activities against several yeasts and filamentous fungi²² at the micromolar range. However,
338 ETD151 had not been tested on *B. cinerea*. In this study, the antifungal activity of ETD151 was
339 assessed *in vitro* against three-day-old mycelia of *B. cinerea* prepared from a fresh spore
340 solution. In our experimental conditions, growth inhibition reaches a maximum, 78% as
341 measured by optical density, at 6.25 μM of ETD151 (Figure 1A). Based on this value, the half-
342 maximal inhibitory concentration (IC_{50}) was estimated to be 0.59 μM .

343 The antifungal action of ETD151 was monitored by optical microscopy (Figure 1B). At 0.78
344 and 3.12 μM , ETD151 is able to promote lysis and the release of intracellular content at some
345 points of the growing mycelium, preferentially at the apical end of the hyphae (Fig. 1B, b-c).
346 Such a leakage of cytosolic material suggests a loss of the cell wall integrity. An equivalent
347 observation has already been reported for heliomicin and drosomycin on *B. cinerea* spores, but
348 for these molecules, comparatively weaker effectiveness against the mycelium was recorded²³.
349 Thus, our data confirm the enhanced activity of ETD151 compared to heliomicin, the reference
350 molecule.

351 The permeabilization of the fungal membrane has emerged as a trend in the antifungal mode of
352 action of plant defensins^{32,56}. The results obtained on *B. cinerea* mycelia treated with the insect
353 defensin homologue ETD151 suggest a similar mode of action. Nevertheless, it remains unclear
354 if ETD151 is internalized or if it remains outside the fungal cells. To assess whether ETD151 is

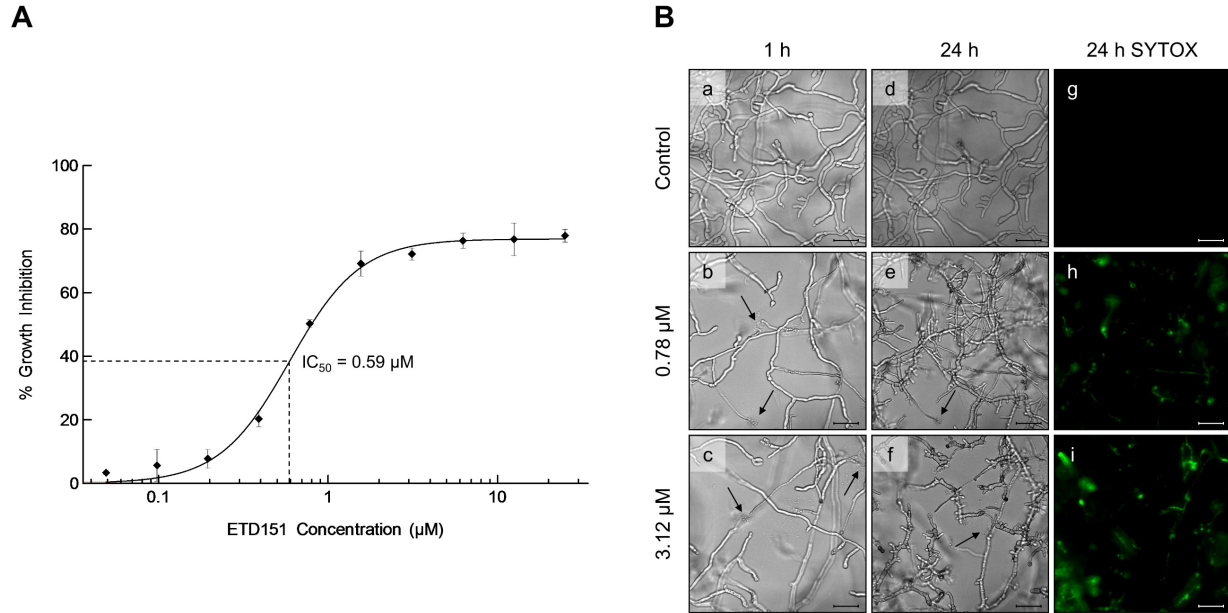
355 able to disturb membrane integrity/permeability, the SYTOX Green nucleic acid stain, a cell-
356 impermeant fluorophore, was used. Compared to the control experiment (Fig. 1B, g), in the
357 presence of ETD151, the dye penetrated locally the hyphae and emitted a green-fluorescent
358 signal revealing a compromised plasma membrane (Fig. 1B, h-i).

359 In addition, ETD151 reduced hyphal elongation with a concomitant disorder in hyphal
360 branching at both tested concentrations (Fig. 1B, e and f, respectively 0.78 and 3.12 μM).
361 Development of the *B. cinerea* mycelium post-treatment highlights the fungistatic aspect of
362 ETD151 activity and may explain the inhibition plateau observed with the fungal growth curve
363 (Fig. 1A). Swelling of hyphae was also noticed with a particularly strong phenotype at 3.12 μM
364 (Fig. 1B, f). These morphological changes defined ETD151 as a morphogenic defensin of
365 *B. cinerea*. This is homologous to observations reported for several plant defensins (RsAFP2,
366 HsAFP1, MsDef1) in filamentous fungi⁵⁷⁻⁵⁹ and confirms commonalities with the antifungal action
367 of the insect defensins. Results obtained after this first series of analyses showed that there are
368 perturbations of the mycelium physiology and morphology, as the optical microscopy pictures
369 and the uptake of the SYTOX Green nucleic acid stain differed between all treatment conditions.

370 Thevissen and colleagues²⁸ demonstrated through an ELISA-based assay with *Pichia pastoris*
371 that heliomicin and RsAFP2 interact with fungal membrane lipids called glucosylceramides. All
372 of our structural and biological data support a common mechanism of action for these three
373 defensins, and lead us to suggest that ETD151 interacts with the same fungal partners.

374 To study the antifungal effects of ETD151 on the intracellular protein content, we used a
375 proteomics study based on a two-step MS workflow, and conducted on *B. cinerea* mycelial
376 extracts. Firstly, a comparison of mass spectrometry (MS) molecular mass fingerprints was
377 conducted by MALDI-MS. This first step was designed to follow the evolution of the peptide

378 protein profiles within the mass range of 600 Da to 18 kDa. Secondly, a bottom-up proteomics
379 analysis by nano-LC-ESI-MS/MS was performed to identify and characterize the proteins
380 changes. The concentration range from 0.19 to 3.12 μ M, which includes the IC₅₀ and covers the
381 entire inhibition profile of ETD151, was adopted for a dose-response approach.



382

383 **Figure 1: Growth inhibition and morphogenic alterations induced by ETD151 in *B. cinerea***

384 (A) Growth inhibition (%) was evaluated on *B. cinerea* three-day-old mycelia produced from
 385 fresh spores incubated in modified Tanaka. The dose-response curve was determined by
 386 incubating mycelia with ETD151 concentrations ranging from 0.049 to 25 μM (final
 387 concentration in \log_{10}) for four days. The dotted lines indicate the IC_{50} . Data are the means of
 388 triplicate measurements. Error bars denote standard deviation. (B) Morphogenic alterations of *B.*
 389 *cinerea* mycelia obtained from a fresh culture of spores were observed microscopically after one
 390 hour (a, b, c) and 24 h (d, e, f) of incubation in Potato Dextrose Broth diluted by $1/4^{\text{th}}$. Mycelia
 391 were incubated in the absence (control experiment, a, d) or the presence of ETD151 at a final
 392 concentration of 0.78 μM (b, e) and 3.12 μM (c, f). SYTOX Green signal was measured by
 393 fluorescence microscopy 24 h post-treatment (h, i) on the same experimental plate wells as the
 394 control experiment (g). Arrows indicate lysis of hyphae. Scale bar = 50 μm .

395 **Protocol development to prepare *B. cinerea* mycelial extracts**

396 Fungal culture, extraction and MALDI-MS analysis protocols have been developed for this
397 study. The extraction protocol is inspired by the work of Schulthess and colleagues⁶⁰, using 35%
398 formic acid for the BioTyping of clinical molds. The objective was to establish the optimal
399 parameters for recording the most representative MALDI peptide signatures/molecular mass
400 fingerprints (MFPs) of *B. cinerea*, with or without different concentrations of ETD151. The
401 following parameters were reviewed: the starting concentration of *B. cinerea* spores, the mycelial
402 culture duration in microplate, the treatment duration with ETD151 and the extraction process to
403 generate mycelial extracts. The development of the protocols was focused on the conditions
404 providing the most reproducible MFPs and with a rich molecular diversity. We settled on a
405 starting concentration of 500 sp/mL prior to three days of growth in microplate and a 24h-
406 treatment of the mycelia with ETD151. Peptides/proteins were extracted by grounding the
407 mycelia with zirconium oxide beads, drying the pellet and incubating it in the presence of 2 M
408 acetic acid solution prepared in 50% ACN (personal communication, Victor Masson and
409 Philippe Bulet). The different parameters for MALDI-MS analysis were optimized for an
410 optimal detection (in terms of sensitivity, resolution, reproducibility and accuracy) of molecular
411 ions between 1,500 – 12,000 mass *m/z* of the *B. cinerea* mycelial extracts.

412

413 **Peptide mass fingerprint of *B. cinerea* mycelium in response to ETD151**

414 Once the experimental methodology had been established, MALDI-MS analyses were
415 performed directly on *B. cinerea* mycelial peptide/protein extracts, and restricted to peptides and
416 small proteins (<20 kDa) as a preliminary approach to assess the antifungal effect of ETD151 at
417 the molecular scale. Three-day-old mycelia were incubated for 24 h with the defensin following

418 the previous concentration range, from 0.19 to 3.12 μM final concentrations. Average spectra
419 (n=3) were generated from biological replicates to first, highlight modifications of the
420 peptide/protein composition in the presence of ETD151 and second, visualize a putative dose-
421 response effect (Figure 2). For each concentration, a manual selection from automatic peak
422 labeling resulted in a representative peak list of 100 ions. Similarities between MALDI MFPs
423 were calculated following the conservation of representative ions (Supplementary data S2).

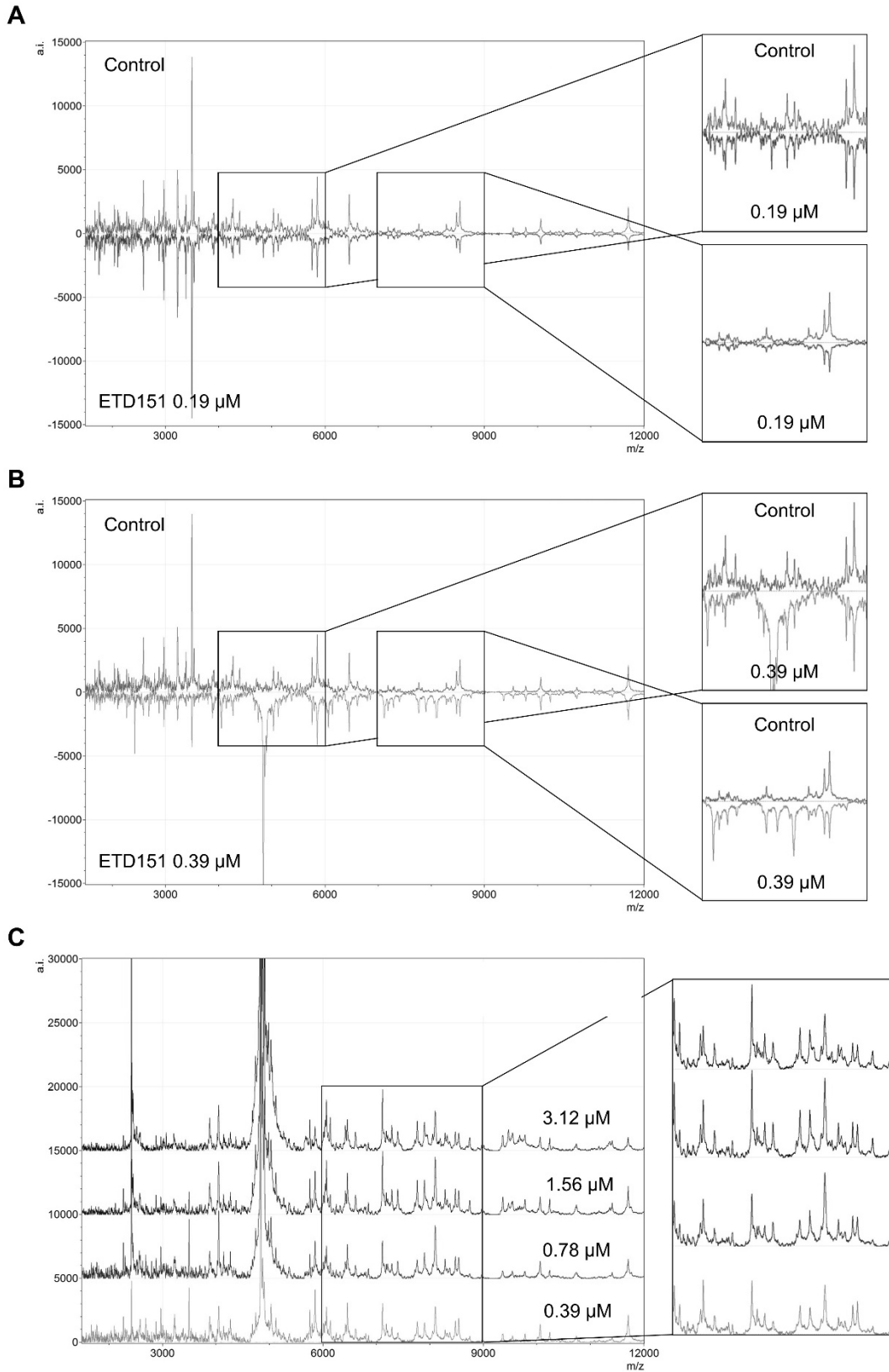
424 In the presence of 0.19 μM of ETD151, no strict molecular change was observed on the MFPs.
425 Compared to the untreated mycelia, MFPs were very similar with 91% identity (Fig. 2A). This
426 observation is consistent with the very reduced growth inhibition effect (less than 10%) observed
427 at this dose (Fig. 1A). From 0.39 μM of ETD151, the antifungal activity of ETD151 has a
428 significant impact on the MFPs recorded. Several modifications were highlighted on the MFPs
429 compared to untreated mycelia, with identity dropping to 68% (Fig. 2B). Differences in the
430 peptide/protein profiles are significant in particular within the 6,000-9,000-mass range
431 (Supplementary data S3). At 0.39 μM , two sets of peptides/proteins within the 9,000-10,000 and
432 14,000-17,000 mass ranges emerged; those were not detectable in the control MFP or at an
433 ETD151 concentration of 0.19 μM .

434 At 0.39 μM or higher doses of ETD151, the MALDI-MS spectra showed similar overall MFPs
435 (Fig. 2C). Indeed, the dose of 0.39 μM shares 92%, 84% and 77% identity with the 0.78, 1.56
436 and 3.12 μM doses, respectively. Several ions within the 9,000-10,000 and 14,000-17,000 mass
437 ranges display intensity variation according to the concentration of the defensin with intensities
438 increasing as the concentration reaches 0.39 μM (Supplementary data S2). These results suggest
439 that MALDI-MS could be a rapid and simple method to assess the impact of an antifungal
440 molecule on its fungal target. The modifications of the MFPs observed between the proteins

441 extracted from the control experiment versus *B. cinerea* mycelia treated with different doses of
442 ETD151 also revealed that ETD151 modified the peptide/protein profiles of *B. cinerea* mycelia,
443 in a dose dependent manner up to a concentration that is more than the IC₅₀ (0.59 μM).

444 Despite the extraction method used, including removal of the culture supernatant and two
445 successive washing steps of the mycelia, the ion observed at *m/z* 4,839 corresponds to ETD151.
446 It is worth mentioning that the intensity of this ion does not decrease with two additional washes
447 (unpublished data). This suggests that in our experimental conditions, ETD151 was interacting
448 with the membrane of the altered mycelium of *B. cinerea*, or was entering the mycelium. Note
449 that the arbitrary intensity (a.i.) of the ETD151 ion increases with the concentration of ETD151
450 used, from 1.9.10³ a.i. at 0.19 μM to 174.103 a.i. at 3.12 μM (Supplementary data S2).

451 As important modifications of the MFPs are observed within the highest molecular mass range
452 detectable in our MALDI-MS conditions, this preliminary approach attests to the antifungal
453 effect of ETD151 on *B. cinerea* and prompted us to analyze the mycelial extract of *B. cinerea*
454 treated with ETD151 at the peptide/protein scale using a bottom-up proteomics approach.



455

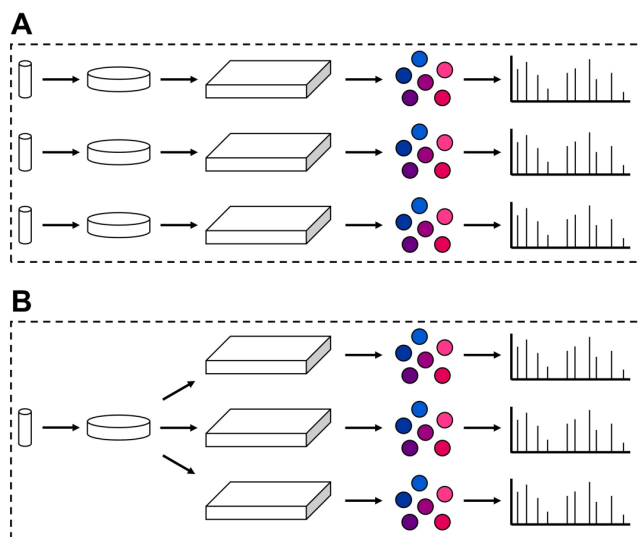
456 **Figure 2: MALDI profiling of extracts of *B. cinerea* mycelia treated with ETD151**

457 The MALDI mass spectra show the average MALDI-molecular mass fingerprint (n=3) from
458 mycelial protein extracts treated or not with the antifungal peptide ETD151. The mass spectra
459 are recorded in the 1,500 – 12,000 mass (m/z) range, for untreated mycelia (Control) *versus*
460 mycelia treated with 0.19 μ M ETD151 (A) and 0.39 μ M ETD151 (B). Zooms show enlarged
461 area at the 4,000-6,000 and 7,000-9,000 mass ranges. The mass spectra recorded when the
462 mycelial cultures were treated with 0.39, 0.78, 1.56 and 3.12 μ M ETD151 are also reported (C).
463 The zoom shows the enlarged mass range (m/z) between 6,000 and 9,000.

464 **Effect of ETD151 on *B. cinerea* mycelium by bottom-up proteomics**

465 Differential proteomics analysis in response to ETD151

466 Our MALDI-MS results showed that the MFPs of the mycelial preparations are altered in
467 response to the different concentrations of ETD151 used to treat *B. cinerea* mycelia. To have a
468 better picture of the molecular response of *B. cinerea* mycelium upon our ETD151 treatment, we
469 used gel- and detergent-free proteomics to monitor protein changes on mycelial extracts. *B.*
470 *cinerea* mycelial peptide/protein acidic extracts prepared for MALDI-MS were dried under
471 vacuum prior to reduction-alkylation-enzymatic digestion. *B. cinerea* protein digests were then
472 analyzed by LC-ESI-MS/MS analysis as a conventional bottom-up proteomics approach. To
473 evaluate the methodology implemented and validate our data analysis strategy, two sets of
474 experiments were performed (Figure 3) allowing to generate two datasets (I and II). First, three
475 individual cultures were harvested, following the same experimental procedure, from the
476 collection of non-treated or treated cultures with different doses of ETD151 (Fig. 3A, dataset I).
477 Second, a triplicate experiment was conducted on three individual plates with three-day-old
478 mycelia (Fig. 3B, dataset II) originating from a unique culture of spores.

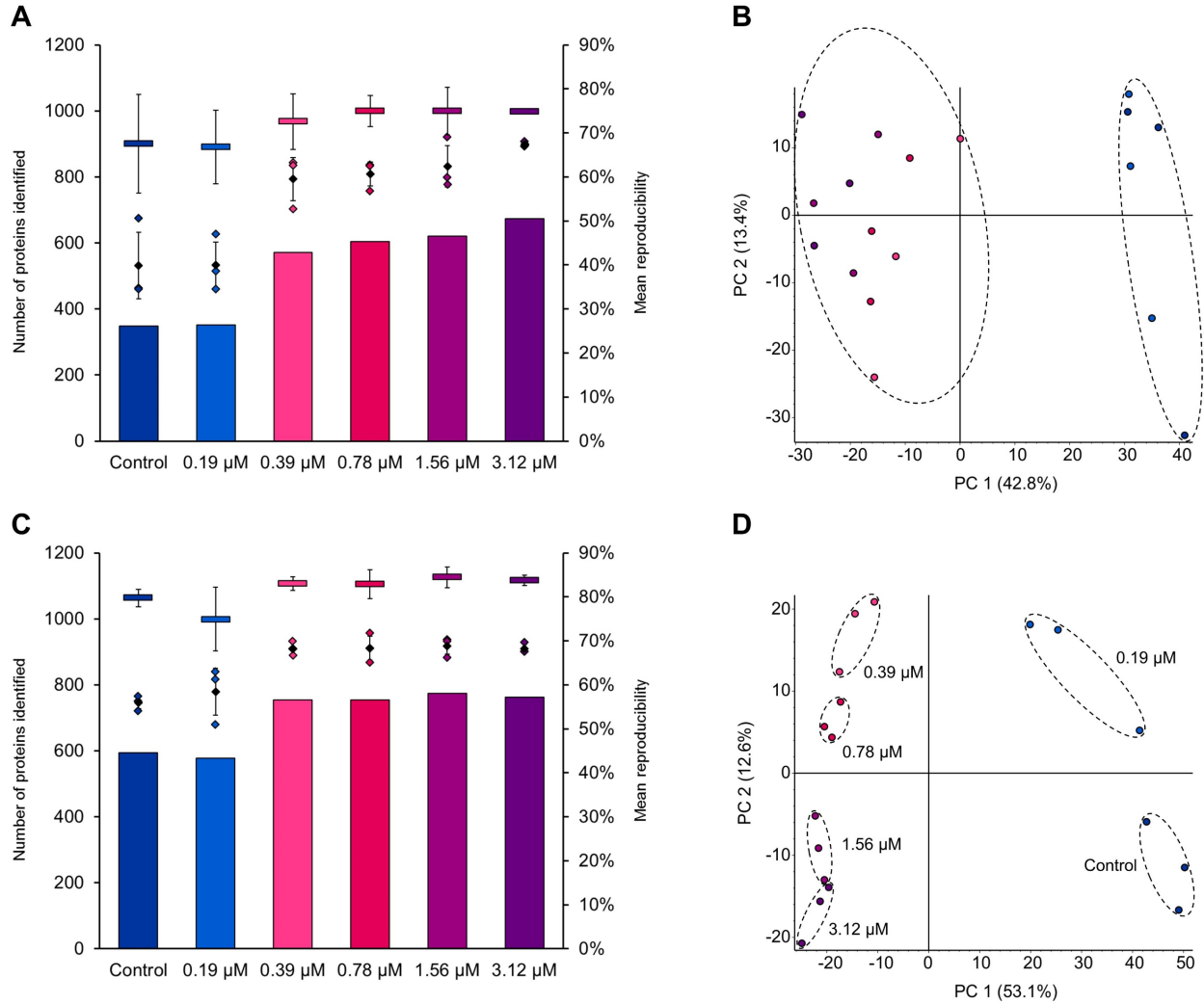


479

480 **Figure 3: Workflow developed for the bottom-up proteomics approach on *B. cinerea***

481 Fresh fungal material was obtained from a calibrated stock solution of *B. cinerea* spores
 482 cultivated for seven days on a PDA medium. A fresh spore suspension was prepared and
 483 distributed in a 96-well plate prior to incubation. Three-day-old mycelia were treated with water
 484 (control experiment, blue) or with different concentrations of ETD151: 0.19 μM (light blue),
 485 0.39 μM (light pink), 0.78 μM (pink), 1.56 μM (light purple) and 3.12 μM (purple). After a 24h-
 486 incubation, mycelia were collected and proteins were extracted. Samples were prepared for
 487 bottom-up proteomics and digests analyzed by LC-ESI-MS/MS prior to data processing. (A) The
 488 dataset I groups three individual cultures, while dataset II (B) is a triplicate experiment
 489 corresponding to three individual plates coming from a unique culture of spores.

490 Dataset I allowed the identification of 1,431 typical proteins with molecular masses ranging
491 from 4.8 to 466 kDa. Among these proteins, 1,430 originate from *B. cinerea* while the last one
492 corresponds to ETD151, detected by LC-ESI-MS/MS with 80% coverage (see raw data). We
493 observed an increase in the number of identified proteins in response to increasing
494 concentrations of ETD151 (Figure 4A, diamond plots). The average number (Fig. 4A, black
495 diamonds) of identified proteins was 532 and 534 for control and 0.19 μM ETD151,
496 respectively. This average number increased to 794 and 899 proteins when the cultures were
497 treated with 0.39 and 3.12 μM ETD151, respectively. The increase in the number of proteins
498 extracted in response to elevated concentrations of ETD151, may reflect a perturbation of the
499 integrity of the cell membrane of the mycelium and/or the stimulation of the expressions of
500 series of genes, and would corroborate microscopic data (see Fig. 1B) that points to an antifungal
501 effect of the defensin on *B. cinerea*. The reproducibility within dataset I was assessed for each
502 concentration of ETD151 as the average ratio between the number of identified proteins in each
503 experiment and the number of recurrent proteins (Fig. 4A, histograms), as identified in all three
504 experiments. The reproducibility within the control experiment and after a treatment with 0.19
505 μM ETD151 was at $68 \pm 11 \%$ and $67 \pm 8 \%$, respectively. For 0.39 μM ETD151, it was 73 ± 6
506 $\%$, and for higher concentrations of ETD151, the reproducibility was 75% with a standard
507 deviation $\leq 6 \%$ (Fig. 4A, square plots). Furthermore, a principal component analysis (PCA) was
508 run on proteins of dataset I and showed a clustering of experiments according to the
509 concentration of ETD151 (Figure 4B). Control and 0.19 μM samples were clearly separated from
510 the other tested concentrations by the first component (42.8%). The first component (PC1, Fig.
511 4B) may reflect the molecular impact of ETD151 on the *B. cinerea* mycelium. Samples from
512 0.39 to 3.12 μM of ETD151 could not be separated according to the second component (13.4%).



513

514 **Figure 4: Protein identification of *B. cinerea* mycelial extracts and assessment of**
 515 **reproducibility**

516 (A) The number of proteins identified in samples of dataset I is shown according to the
 517 concentration of ETD151. Replicates and mean, along with the standard deviation, are
 518 represented by colored and black diamond-shape plots, respectively. Histograms indicate the
 519 number of recurrent proteins between all three replicates, the basis for calculation of
 520 reproducibility. Mean reproducibility is represented by colored rectangles, along with the
 521 standard deviation. (B) The Principal Component Analysis generated by Proteome Discoverer is

522 shown for the 1,431 proteins identified within dataset I. Experiments are visualized according to
523 the concentration of ETD151 and the color code introduced previously. (C) The number of
524 proteins identified in samples of dataset II is reported according to the concentration of ETD151.
525 Values are represented in the same way as dataset I, and data of the Principal Component
526 Analysis generated for the 1,468 proteins identified in dataset II are shown in (D).

527 Dataset II, a triplicate of three individual plates, identified 1,468 master proteins from a 4.8 to
528 466 kDa mass range. The goal of this experimental design was to estimate the impact of the
529 initial culture of spores or of the harvest method on the reproducibility of the data. It should be
530 noted that working simultaneously on three individual microplates significantly complexified the
531 experimental protocol. Treatment of mycelia with ETD151, as well as the manual collection of
532 samples from each microplate, should be completed within a very short period of time. As
533 reported previously for dataset I, the average number of identified proteins increased upon
534 treatment with ETD151 (Figure 4C). As with dataset I, we observed an increase in the number of
535 identified proteins in response to increasing concentrations of ETD151 (Fig. 4C, diamond plots).
536 The average numbers (Fig. 4C, black diamonds) of identified proteins were 745 and 778 for the
537 control and 0.19 μ M ETD151 samples, respectively. This average number increased to 910 and
538 917 proteins when the cultures were treated with 0.39 and 3.12 μ M ETD151 (Fig. 4C, diamond
539 plots). The reproducibility was assessed in the same way as for dataset I (Fig. 4C, histograms). In
540 the control and 0.19 μ M samples, the reproducibility was $80 \pm 2 \%$ and $75 \pm 7 \%$, respectively.
541 For higher concentrations of ETD151, the reproducibility ranged from 83 to 84 % with a
542 standard deviation $\leq 3 \%$. PCA was also run on dataset II (Figure 4D). As observed for dataset I,
543 the first component (53.1 %) separated Control and 0.19 μ M samples from the other
544 concentrations. Unlike the PCA on dataset I, the second component (12.6 %) allowed to separate
545 the different samples according to the concentrations of ETD151.

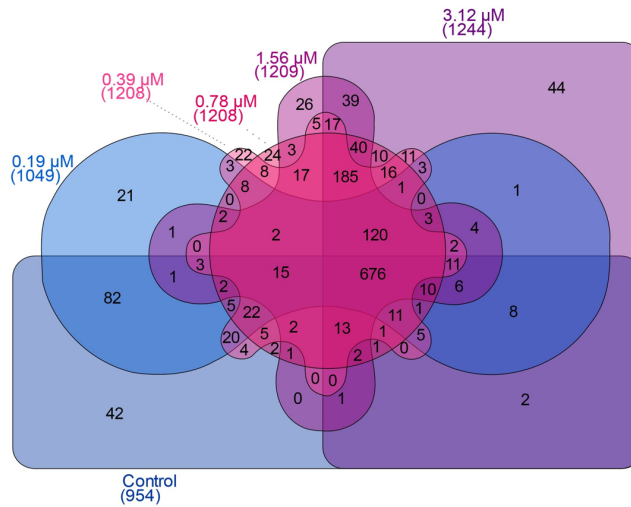
546 This new experimental design improved the robustness of the data as the average
547 reproducibility between dataset I and II increased by 9% while the variability between replicates
548 decreased, as reflected by the reduction in standard deviations. This explains the greater weight
549 in our PCA analyses of the first component, at 43% and 53% in datasets I and II, respectively.

550 Interestingly, in terms of the average number of identified or recurrent proteins, the samples
551 treated with 0.19 μM ETD151 showed values closer to the control experiments (see above),
552 which is supported by the clustering observed for control and 0.19 μM ETD151 replicates on one
553 side within the first component in PCA whereas replicates for higher concentrations aggregated
554 on the other side. Although the results improved in dataset II, samples for 0.19 μM ETD151
555 were the only ones with a reproducibility below 80% and a standard deviation $> 5\%$. This
556 concentration of ETD151 appeared as an inflection point in the molecular effect of the antifungal
557 peptide as the changes induced by ETD151 barely allow to distinguish control from 0.19 μM
558 samples. Finally, datasets I and II were grouped to consolidate data analysis. Indeed, both dataset
559 shared similar properties on the increase in identified and recurring proteins for identical
560 concentrations of ETD151, from 0.39 to 3.12 μM .

561 The protein distributions were represented on a multidimensional Venn diagram based on six-
562 experimental datasets (untreated *vs* treated with five increasing concentrations of ETD151)
563 (Figure 5). The Venn diagram is a simplified tool, free of statistical test, allowing to classify
564 proteins from large datasets and to highlight groups of interest in our study. The diagram we
565 obtained, allowed to represent the logical relations between the 1,592 summed proteins identified
566 in the two datasets I and II. Among these proteins, 42 were specifically found in the non-treated
567 samples and 21 to 26 of them specifically in each concentration of ETD151 (21 in 0.19 μM , 22
568 in 0.39 μM , 24 in 0.78 μM and 26 in 1.56 μM). We found the most abundant number of specific
569 proteins (44) when the mycelial culture was treated at 3.12 μM , the highest concentration of
570 ETD151. Interestingly, as previously discussed, at the inflexion point, 82 proteins were found
571 mutually in the control experiments and in samples treated with 0.19 μM ETD151. Overall, 676
572 proteins were observed to be common to all samples, 120 proteins were found in all treated

573 samples, regardless of the concentration of ETD151, and 185 proteins were specifically found at
574 the concentrations of 0.39 μ M ETD151 and above.

575 Our results demonstrated that ETD151 affected the molecular composition of *B. cinerea*
576 mycelial samples and confirmed, over a wide mass range (4.8 – 466 kDa), the results presented
577 previously by MALDI-MS MFPs up to 18 kDa. This comparative approach allows us to build an
578 overview of the behavior of protein groups following treatment with the peptide and to select by
579 label-free quantification characteristic proteins and metabolic pathways altered upon treatment
580 with ETD151.



581

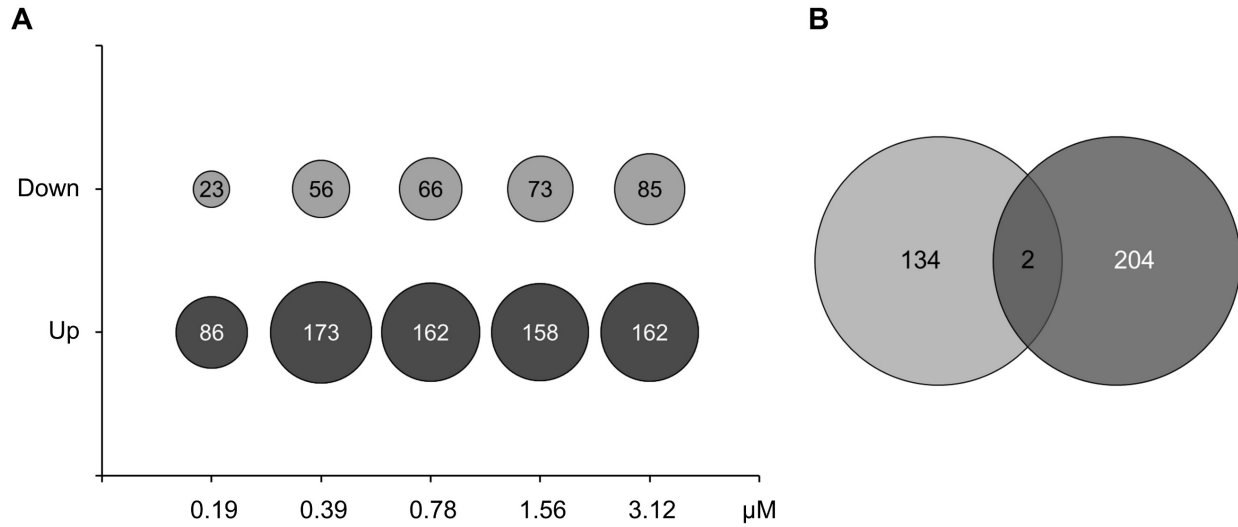
582 **Figure 5: Multidimensional six-set Venn diagram**

583 The Venn diagram generated on www.interactivenn.net displays the 1,592 summed proteins
 584 from datasets I and II according to their presence or absence in mycelia treated with increasing
 585 concentrations of ETD151 : 0.19 μM (light blue), 0.39 μM (light pink), 0.78 μM (pink), 1.56 μM
 586 (light purple) and 3.12 μM (purple).

587 Label-free quantification analysis

588 We identified 1,592 proteins and among them, 1,559 were quantified according to a label-free
589 approach. Abundance ratios and p-values were not calculated for 33 proteins. Significantly
590 dysregulated proteins were selected with volcano plots according to a p-value equal to 0.95 and a
591 \pm 2-fold change (Supplementary data S4). Volcano plots are commonly used to display the
592 results of -omics experiments. This methodology allowed to highlight differentially abundant
593 proteins in the presence of different concentrations of ETD151 (Figure 6A). Increasing
594 concentrations of ETD151 of 0.19, 0.39, 0.78, 1.56 and 3.12 μ M resulted in the downregulation
595 of 23, 56, 66, 73 and 85 proteins, respectively (light grey); and in the upregulation of 86, 173,
596 162, 158 and 162 proteins, respectively (dark grey). Statistical analysis determined that out of the
597 340 proteins identified, 134 were significantly downregulated, and 204 were significantly
598 upregulated (Figure 6B). Two proteins (Bcin02p06900, 40S ribosomal protein S20e, and
599 Bcin12p02600, putative mitochondrial protein), were downregulated in the presence of 0.19 μ M
600 of ETD151 and upregulated at higher concentrations.

601



602

603 **Figure 6: Significantly dysregulated proteins upon treatment of *B. cinerea* mycelium with**

604 **ETD151**

605 (A) Bubble chart summarizes the differentially abundant proteins with a ± 2 -fold change and a P-

606 value ≤ 0.05 as detailed in volcano plots (see Supplementary data S4). (B) The Venn diagram

607 pooling the two sets of proteins modulated upon treatment with ETD151. A total of 340 unique

608 proteins are reported including 134 downregulated (light grey), 204 upregulated (dark grey) and

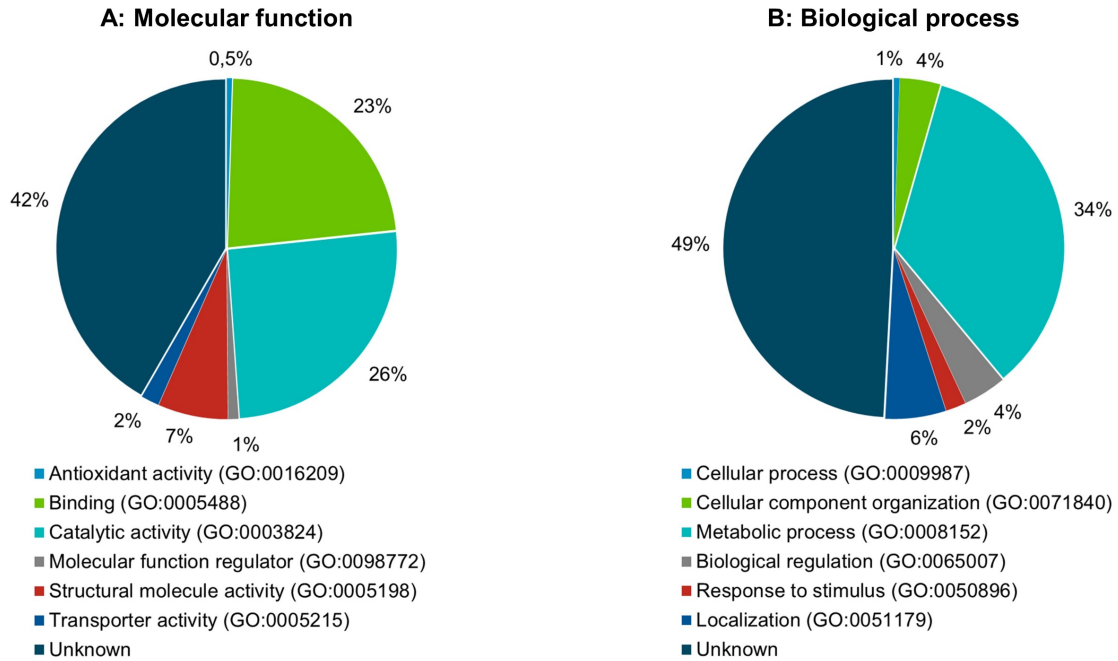
609 two that were both down- and upregulated (intersecting of the two sets).

610

611 Gene ontology and network analysis

612 Proteins were annotated using ProteinCenter (Proteome Discoverer) to investigate molecular
613 functions, biological processes, and cellular components from the Gene Ontology (GO) database
614 (Figure 7). The molecular function-based categorization showed that among the 340 selected
615 proteins, 168 (42%) could not be classified while the others were classified according to the GO
616 terms as follow: 103 (26%) have catalytic activities (GO:0003824), 92 (23%) have binding
617 properties (GO:0005488), 27 (7%) contribute to the structural integrity of a complex or its
618 assembly within or outside a cell (GO:0005198), 7 (2%) act as transporters (e.g. lipids, proteins,
619 oxygen) (GO:0005215), 4 (1%) are regulators of molecular functions (GO:0098772), and finally
620 2 (0.5%) were recognized as having antioxidant activity (GO:0016209) (Fig. 7A). Following the
621 biological process-based categorization, 178 (49%) among the 340 proteins could not be
622 classified. The most affected process is metabolism with 125 proteins (34%) (GO:0008152). The
623 rest of the proteins are involved in biological processes such as (i) localization (21, 6%)
624 (GO:0051179), (ii) regulation (15, 4%) (GO:0065007), (iii) cellular component organization (14,
625 4%) (GO:0071840), (iv) response to a stimulus (7, 2%) (GO:0050896), or (v) cellular process (2,
626 1%) (GO:0009987) (Fig. 7B). Cellular component annotation was also investigated despite a
627 lack of information for *B. cinerea* in this GO categorization. Among the 340 selected proteins,
628 288 (84%) could not be classified (data not shown).

629



630

631

Figure 7: Gene Ontology of dysregulated proteins in *B. cinerea* mycelial extracts

632

Proteins selected (340) according to their differential abundance are categorized according to

633

their (A) molecular function and (B) role in biological processes.

634 **Molecular impact of ETD151 on *B. cinerea* pathways**

635 Significantly modulated proteins upon treatment with ETD151 were searched in *Botrytis*
636 *cinerea* KEGG pathways (117 pathways reported). From the 339 proteins (340 minus ETD151)
637 previously selected, 106 were successfully mapped over 67 different pathways with a varied
638 number of hits, from 1 to 38 matched proteins per map (Supplementary data S5). Pathways with
639 less than five hits were not considered for further investigations, as a low number of matched
640 proteins did not allow us to interpret their relevance in the mechanism of action of ETD151 on
641 *B. cinerea*. Moreover, most of these maps were included in carbohydrate, lipid or amino acid
642 metabolisms. Direct effects on these pathways had been evaluated by measuring the uptake and
643 incorporation of radiolabeled precursors (¹⁴C-glucose, ³H-phenylalaline and ¹⁴C-acetate) by
644 *Botrytis cinerea* spores in the presence of ETD151 (data not shown). ETD151 did not interfere
645 with carbohydrate, protein and lipid biosynthesis confirming that modulated proteins in
646 previously mentioned pathways resulted from global stress in response to an antifungal
647 molecule. The following global pathway maps (metabolic pathways (bfu01100), biosynthesis of
648 secondary metabolites (bfu1110), biosynthesis of antibiotics (bfu01130) and carbon metabolism
649 (bfu01200) were also excluded from the analysis in favor of the study of specific maps. By
650 considering the filters previously described, six pathways were impacted by the treatment,
651 namely ribosome (bfu03010), spliceosome (bfu03040), protein processing in endoplasmic
652 reticulum (bfu04141), endocytosis (bfu04144), MAPK signaling pathway (bfu04011), and
653 oxidative phosphorylation (bfu00190).

654

655 Effect on ribosomes

656 Ribosomes are essentially very complicated nano-factories for producing proteins. In our
657 study, 80 proteins, out of a total of 103 reported in this pathway for *B. cinerea*, were identified in
658 datasets I and II (Supplementary data S6). Upon treatment with ETD151, 25 ribosomal proteins
659 were significantly modulated. Starting at a concentration of 0.39 μ M of ETD151 this included 24
660 up-regulated proteins, as eight in the small subunit 40S: Bcin01p00980/S2e,
661 Bcin02p06900/S20e, Bcin03p05970/S9, Bcin03p06990/S16e, Bcin03p07110/S15Ae,
662 Bcin03p07120/S12e, Bcin07p00830/S13 and Bcin08p00760/S29e, and 16 in the large subunit
663 60S: Bcin01p09660/L35Ae, Bcin03p01900/L39e, Bcin03p06530/L7Ae, Bcin03p07630/L7e,
664 Bcin05p01600/L18Ae, Bcin05p06070/L38e, Bcin07p00520/L27e, Bcin08p03200/L4,
665 Bcin09p03550/L14e, Bcin09p05600/L30e, Bcin10p04290/L17e, Bcin10p04590/L12e,
666 Bcin14p04100/L22e, Bcin08p02490/L32e (starting from 0.19 μ M), Bcin14g04790/L15e
667 (starting from 0.19 μ M) and Bcin08p00590/L15 (at 0.39 and 0.78 μ M). Bcin14p02830, 60S
668 ribosomal protein L3e, is the only downregulated protein, starting from the concentration of
669 ETD151 of 0.39 μ M. To our knowledge, there is no report in the literature concerning antifungal
670 insect or plant defensins directly targeting protein biosynthesis in ribosomes. The modulation of
671 ribosome-related subunits might reflect an attempt to keep protein synthesis at optimal levels as
672 the presence of the antifungal defensin ETD151 disturbs protein biosynthesis and degradation.

673

674 Effect on the spliceosome

675 The spliceosome is a dynamic complex, located primarily within the nucleus of eukaryotic
676 cells, and plays an essential role in the maturation process of mRNA. In our study, 27 proteins,
677 out of a total of 84 reported in this pathway for *B. cinerea*, were identified (Supplementary data

678 S6). Upon treatment with ETD151, 7 proteins were significantly dysregulated.
679 Bcin08p03160/Smd2 and Bcin08p06600/Smd3, small nuclear ribonucleoproteins, and
680 Bcin09p07050/Lsm7, an snRNA-associated Sm-like protein, were upregulated at 0.39 μ M of
681 ETD151 and higher concentrations. In contrast, four proteins were downregulated in the
682 presence of ETD151: Bcin09p06610/PPIL1 peptidyl-prolyl cis-trans isomerase-like from 0.19
683 μ M, Bcin08p00660/Prp1 pre-mRNA-processing factor from 1.56 μ M, Bcin09p06940/SF3b
684 splicing factor and Bcin13p05240/THOC THO complex subunit at 3.12 μ M. There is no clear
685 tendency to interpret the molecular changes induced by ETD151 on this pathway. Moreover, as
686 there is no report in the antifungal defensin literature of a direct effect on the spliceosome, this
687 pathway was not considered for the continuation of the work on the mechanism of action of
688 ETD151.

689

690 Effect on protein processing in the endoplasmic reticulum (ER)

691 The ER is a continuous membrane system found within the cytoplasm of eukaryotic cells
692 allowing for protein synthesis, folding, modification and in some cases transport to the Golgi
693 apparatus through vesicles. In our study, 37 proteins, out of a total of 70 reported in this pathway
694 for *B. cinerea*, were identified (Supplementary data S6). Upon treatment with ETD151, seven
695 proteins were significantly dysregulated. Bcin13p00700/ERGIC53 and Bcin05p05180/OS9 are
696 upregulated starting a concentration of 0.39 μ M. Bcin07p01430/Sec62, Bcin02p05070/Sec24,
697 Bcin01p05490/Bcnp14 and Bcin10p00510 are downregulated starting at 0.39 μ M of ETD151.
698 These proteins play roles in the transport of proteins from the ER. Proteins that are located in the
699 ER and/or Golgi apparatus, are interesting to highlight, as sphingolipids, including
700 glucosylceramides, fungal partners of RsAFP2⁶¹ and possibly ETD151, are synthesized in the ER

701 and Golgi apparatus⁶². Further investigation will be necessary to monitor a direct effect of
702 ETD151 on glucosylceramide biosynthesis and/or export.

703

704 Effect on endocytosis

705 Endocytosis is a cellular process in which substances (e.g. nutrients, plasma membrane
706 proteins, lipids) are brought into the cell. Interestingly, research on antifungal plant defensins
707 demonstrated that certain ones, such as MtDef4, Psd1 and NaD1, can be internalized by fungal
708 cells upon interaction with a membrane partner³². Mechanisms of fungal cell entry are still under
709 investigation, however it is known that MtDef4 and NaD1 internalization are energy dependent
710 and require endocytosis in *Neurospora crassa*³³ and *Candida albicans*⁶³, respectively. In our
711 study, 35 proteins, out of a total of 58 reported in this pathway for *B. cinerea*, were identified
712 (Supplementary data S6). Upon treatment with ETD151, 7 proteins were significantly
713 dysregulated. Three of them were upregulated in the presence of ETD151:
714 Bcin13p05610/CAPZA capping protein (actin filament) muscle Z-line starting at a concentration
715 of 0.19 μ M, Bcin11p01640/VPS26 and Bcin11p01920/VPS35 vacuolar protein sorting-
716 associated protein at a concentration of 0.39 μ M. In contrast, four were downregulated:
717 Bcin01p06150/Arp2/3 actin related protein 2/3 complex subunit, at a concentration of 0.19 μ M
718 only, Bcin03p0497/Epsin membrane protein and Bcin13p04030/ArfGAP stromal membrane-
719 associated protein starting at the concentration of 0.39 μ M, and Bcin13p00990/Hrs hepatocyte
720 growth factor-regulated tyrosine kinase substrate at the concentrations of 0.78 and 1.56 μ M.
721 These results could be valuable for future research on the mechanism of action of ETD151, and
722 specifically the behavior of the antifungal defensin upon its interaction with the fungal
723 membrane.

724

725 Effect on MAPK signaling pathway

726 Fungi, like other eukaryotes, rely on the rapid transduction of signals through the mitogen-
727 activated protein kinase (MAPK) pathway in order to overcome environmental stresses such as
728 the addition of chemical fungicides and antifungal peptides. At lower concentrations, one or
729 more stress response pathways are activated to respond to the cell damage. At higher
730 concentrations, the fungal stress response leads to the inhibition of cell growth or induction of
731 cell death. Several antifungal plant defensins³⁴ (e.g. RsAFP2, Psd1, MsDef1) are known to
732 activate the MAPK pathway, with the cell wall integrity pathway (CWI), inducing changes to
733 cell wall biogenesis, β -glucan synthesis and the organization of the actin cytoskeleton. The
734 Defensin NaD1 activates the high-osmolarity glycerol (HOG) pathway³⁵, leading to an increase in
735 intracellular glycerol which provides protection against osmotic stress. In our study, 23 proteins,
736 out of a total of 56 reported in the HOG pathway (bfu04011) for *B. cinerea*, were identified in
737 datasets I and II (Supplementary data S6). Upon treatment with ETD151, three proteins were
738 dysregulated in the CWI pathway: Bcin08p03780/Rom1,2 (down at 3.12 μ M),
739 Bcin02p06930/Fks2 (up starting from 0.19 μ M) and Bcin16p00490 (up starting from 0.19 μ M);
740 one protein was dysregulated in the HOG pathway: Bcin16p04710 (down starting from 0.39
741 μ M). The protein Bcin16p00490/Cdc28, in both CWI and HOG pathways, was also upregulated
742 starting from 0.19 μ M.

743

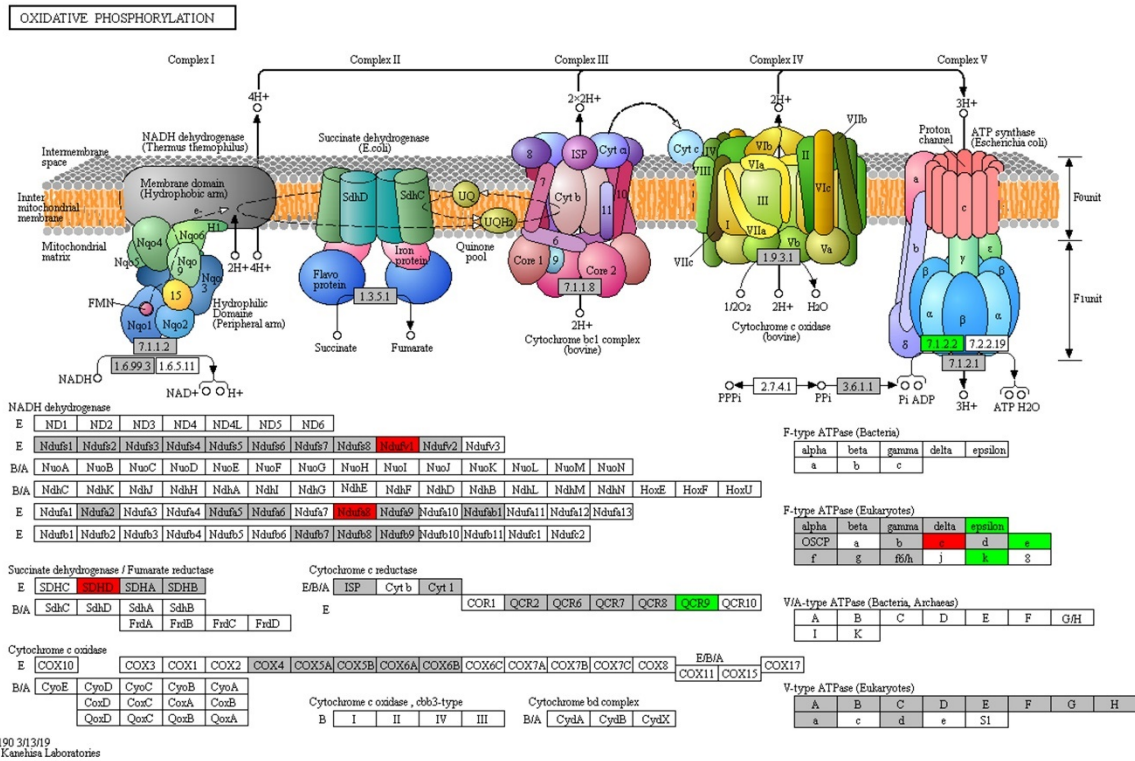
744 Effect on oxidative phosphorylation

745 Oxidative phosphorylation, coupling the cascade of oxidation-reduction of the mitochondrial
746 respiratory chain and the phosphorylation of ADP to ATP, is an essential metabolic pathway for

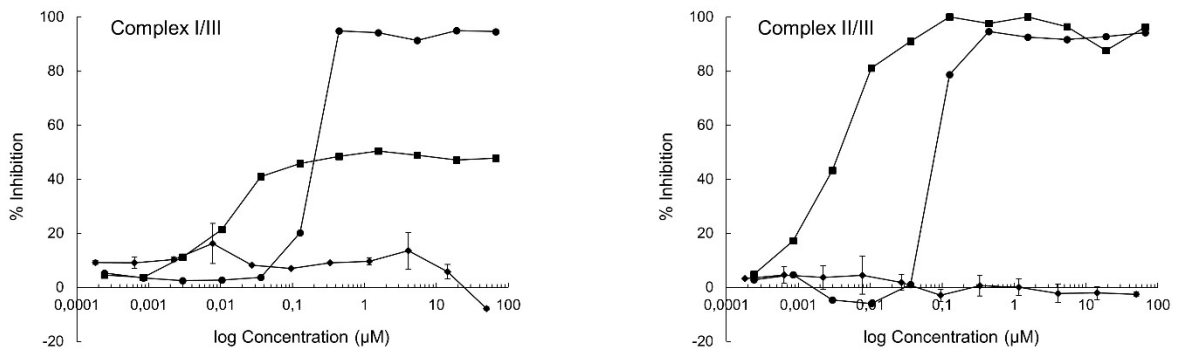
747 the survival of most aerobic eukaryotic organisms. In our study, 60 out of a total of 72 reported
748 proteins in the *B. cinerea* bfu00190 pathway were identified (Figure 8A). Upon treatment with
749 ETD151, two subunits of NADH dehydrogenase (complex I) Bcin08p06780/NDUFV1,
750 Bcin08p01490/NDUFA8 were significantly up-regulated starting from a concentration of
751 0.78 μM of ETD151. Similarly, a subunit of succinate dehydrogenase (complex II)
752 Bcin05p04430 was also up-regulated at 0.19 μM of ETD151. By contrast, a subunit of
753 ubiquinol-cytochrome c reductase (complex III) Bcin03p08370/QCR9 was significantly down-
754 regulated at 0.19 μM of ETD151. Finally, several subunits of F-type H⁺-transporting ATPase
755 (complex V) were significantly modulated upon treatment: Bcin02p02510/epsilon (down at 0.19
756 μM), Bcin10p01500/c (up starting at 0.39 μM), Bcin02p02250/e (down at 3.12 μM) and
757 Bcin12p02610/k (down starting at 1.56 μM). Our proteomics data suggested a mitochondrial
758 dysfunction in the presence of the antifungal defensin ETD151. To go further, the direct activity
759 of ETD151 on complexes I to III of the mitochondrial respiratory chain was assessed and
760 compared to boscalid and antimycin, two molecules providing reference profiles as inhibitors of
761 succinate dehydrogenase and ubiquinol-cytochrome c reductase, respectively (Figure 8B). A
762 colorimetric assay based on the reduction of ferricyanide allowed to measure combined activities
763 of complexes I and III on the one hand, and complexes II and III on the other hand. The activity
764 of boscalid demonstrated a partial inhibition (50%), when testing complexes I and III together,
765 and a total inhibition, when testing complexes II and III together. The activity of antimycin A
766 was characterized by a total inhibition in both associations of complexes. In the presence of
767 ETD151, no inhibition was measured regardless of the tested complex, using up to 50 μM of the
768 antifungal defensin. This result confirmed that ETD151 does not interact directly with complexes
769 of the mitochondrial respiratory chain. Mitochondrial dysfunction highlighted by our proteomics

770 data may result from mitochondrial membrane perturbation, reflecting a secondary effect of
771 ETD151 disturbing cell membrane integrity.

A



B



772

773 **Figure 8: Effect of ETD151 on the *B. cinerea* mitochondrial chain respiration complex**

774

775

776

777

778

(A) Mapping of dysregulated proteins upon treatment with ETD151 on the oxidative phosphorylation pathway (bfu00190) from the KEGG database. For *B. cinerea*, 72 proteins are reported on this map of which 60 were identified in this study (grey). Eight differentially abundant proteins were found, upregulated (red) and downregulated (green). (B) Activity of ETD151 (diamond), boscalid (square) and antimycin A (circle) on complex I/III and complex

779 II/III of *B. cinerea* mitochondria. Results for ETD151 are expressed as the average of a duplicate
780 experiment along with standard deviation.

781 **Conclusions & Perspectives**

782 Through this study, we demonstrated that the proposed approach offers an experimental
783 workflow to evaluate changes in the peptide and protein contents of mycelial extracts of *B.*
784 *cinerea* in response to different concentrations of the antifungal peptide ETD151. As an initial
785 approach, molecular mass fingerprinting by MALDI-MS allowed to highlight molecular changes
786 in *B. cinerea* mycelia treated with ETD151. Even with spectra obtained on a partial mass range
787 (<20 kDa), it could be interesting to compare average fingerprints in the presence of ETD151 to
788 spectra generated with other antifungal defensins. This comparison could direct the study on the
789 mechanism of action of ETD151 toward other peptides. As a second step, bottom-up analyses of
790 mycelial extracts provided robust and reproducible data, necessary to study in-depth molecular
791 changes induced by ETD151 on *B. cinerea*. Thus, we revealed six pathways (ribosome
792 (bfu03010), spliceosome (bfu03040), protein processing in endoplasmic reticulum (bfu04141),
793 endocytosis (bfu04144), MAPK signaling pathway (bfu04011), and oxidative phosphorylation
794 (bfu00190)) impacted by the insect defensin. At least for one of these pathways, we also
795 demonstrated that the observed impact must be indirect. Indeed, we clearly demonstrated that
796 there was no direct effect of ETD151 on the *B. cinerea* mitochondrial respiration chain. As a
797 conclusion, proteomics could offer a way to improve the research strategy into the resolution of
798 antifungal mechanisms of action by targeting preferentially involved proteins or metabolic
799 pathways. Finally, an additional objective for a deeper understanding of the mechanisms of
800 action of ETD151 is to study the interaction of ETD151 with lipid components of the fungal
801 membrane, such as e.g. glucosylceramides.

802

803 **Supporting Information**

804 The following files are available free of charge.

805 S1: Composition of reaction mix for the activity test on respiratory chain complex

806 S2: MALDI-MS fingerprinting of *B. cinerea* mycelial extracts

807 S3: Distribution of MALDI-MS ions in *B. cinerea* mycelium fingerprints

808 S4: Volcano plots depicting significantly dysregulated *B. cinerea* proteins in the presence of

809 ETD151

810 S5: List of KEGG *B. cinerea* pathways with significantly dysregulated proteins

811 S6: Mapping of *B. cinerea* dysregulated proteins on pathways impacted upon treatment with

812 ETD151

813

814 **Author Contributions**

815 Idea and design of the study: PB, CL, TK.

816 Mass spectrometric analysis: TA, PB, SV.

817 Pathway analysis: TA, TK, PB.

818 Statistical analysis: TA, TK, PB, SV.

819 Drafting the manuscript: TA, PB, CL, TK.

820 Review of manuscript for important intellectual content: all authors.

821

822 **Conflicts of Interest:**

823 The authors declare no conflicts of interest.

824

825 **Notes**

826 All relevant data are within the paper and its Supporting Information files.

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838

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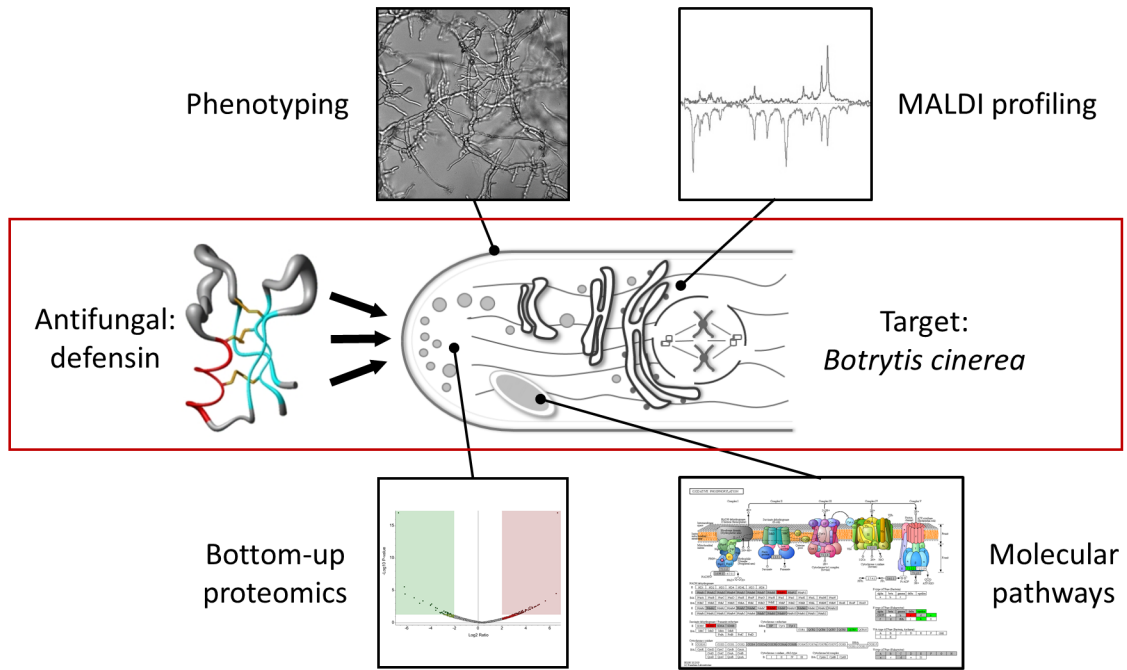
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