1 **Elaborated regulation of griseofulvin biosynthesis in *Penicillium griseofulvum* and its role on**
2 **conidiation and virulence**

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14

15 **Abstract:**

16 *Penicillium griseofulvum*, the causal agent of apple blue mold, is able to produce *in vitro* and on
17 apple a broad spectrum of secondary metabolites (SM), including patulin, roquefortine C and
18 griseofulvin. Among them, griseofulvin is known for its antifungal and antiproliferative activity,
19 and has received interest in many sectors, from medicine to agriculture. The biosynthesis of SM is
20 finely regulated by filamentous fungi and can involve global regulators and pathway specific
21 regulators, which are usually encoded by genes present in the same gene cluster as the backbone
22 gene and tailoring enzymes. In the griseofulvin gene cluster, two putative transcription factors were
23 previously identified, encoded by genes *gsfR1* and *gsfR2*, and their role has been investigated in the
24 present work. Analysis of *P. griseofulvum* knockout mutants lacking either gene suggest that *gsfR2*
25 forms part of a different pathway and *gsfR1* exhibits many spectra of action, acting as regulator of
26 griseofulvin and patulin biosynthesis and influencing conidia production and virulence on apple.

27 The analysis of *gsfR1* promoter revealed that the regulation of griseofulvin biosynthesis is also
28 controlled by global regulators in response to many environmental stimuli, such as carbon and
29 nitrogen. The influence of carbon and nitrogen on griseofulvin production was further investigated
30 and verified, revealing a complex network of response and confirming the central role of *gsfR1* in
31 many processes in *P. griseofulvum*.

32

33 **Key words:**

34 Transcription factor, knockout, gene cluster, regulation, patulin, apple blue mold

35 1. Introduction

36 Blue mold of pome fruit is one of the most important postharvest diseases of apple fruit. The major
37 causal agent is *Penicillium expansum*, but many other *Penicillium* spp. can be found together
38 causing blue mold, such as *P. griseofulvum*, *P. aurantiogriseum*, *P. crustosum*, *P. verrucosum*, *P.*
39 *carneum*, *P. commune*, *P. brevicompactum*, *P. solitum* and *P. puberulum* (Moslem et al., 2010,
40 2013; Spadaro et al., 2011; Wu et al., 2019).

41 These species, as other *Penicillium* spp., can produce a wide range of secondary metabolites (SM),
42 which are so called because they are not essential for primary metabolism and growth, but they play
43 an essential role in the interactions, such as competition or communication with other
44 microorganisms, and in many other cellular processes (Brakhage, 2013). Many of these SM have
45 industrial value, such as antibiotics and antitumor compounds, while other metabolites can be toxic
46 for humans and animals and are defined as mycotoxins.

47 In apples, the major concern is related to the presence of patulin, which is a mycotoxin with acute
48 and chronic toxicity, characterized by stability in acid environment and during thermal processes,
49 and therefore can be found in apples, apple juices and other derived products (Reddy et al., 2010).

50 Among causal agents of blue mold, *P. griseofulvum* is able to produce elevate amount of patulin,
51 even higher compared to *P. expansum*, up to 4,500 µg/kg *in vivo*. The European residue limit for
52 patulin is between 10 and 50 µg/kg depending on food commodities (Banani et al., 2016; Moslem et
53 al., 2013; Spadaro et al., 2008).

54 *P. griseofulvum* can also produce other well-known mycotoxins such as roquefortine C and
55 cyclopiazonic acid, and a well characteristic secondary metabolite called griseofulvin (Banani et al.,
56 2016). Griseofulvin was initially recognized as an antibiotic, this antifungal compound is classified
57 as a potential carcinogenic for humans by the International Agency on Research on Cancer (World
58 Health Organization, 2001). Nevertheless, griseofulvin is commonly found in many medical and
59 veterinary products to treat cutaneous dermatophyte infections, and it was recognized in 2017 as an

60 essential medicine by the World Health Organization for its antifungal properties (World Health
61 Organization, 2017). Moreover, its role against cancer cells has been investigated by many
62 researchers (Mauro et al., 2013; Rathinasamy et al., 2010). Because of its antiproliferative activity,
63 griseofulvin and derived compounds have also been investigated for their potential use against
64 fungal pathogens, such as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Magnaporthe grisea*,
65 *Corticium sasaki*, *Puccinia recondita*, *Blumeria graminis* f. sp. *hordei*, *Alternaria solani*, *Fusarium*
66 *solani* and *Colletotrichum gloeosporioides*, showing efficacy both *in vitro* and *in vivo* (Bai et al.,
67 2019; Ribeiro et al., 2018; Stierle and Stierle, 2015; Tang et al., 2015).

68 Due to the importance of griseofulvin in a wide range of applications, ranging from medicine to
69 agriculture, griseofulvin biosynthetic genes were previously identified in *P. aethiopicum* (Chooi et
70 al., 2010). The putative griseofulvin gene cluster consists of 13 genes, including a nonreducing
71 polyketide synthase (PKS), tailoring enzymes and two putative transcription factors. The gene
72 cluster was later determined in *P. griseofulvum* and 10 genes of the cluster were found in the same
73 order, while genes encoding for a reductase (*gsfK*), an hydrolase (*gsfH*) and a transcription factor
74 (*gsfR2*) were not located in the same genomic region (Banani et al., 2016).

75 The putative genes encoding the backbone enzyme (*gsfA*), *O*-methyltransferases (*gsfB*, *gsfC*, *gsfD*)
76 halogenase (*gsfI*), dehydrogenase /reductase (*gsfE*) and cytochrome P450 (*gsfF*) were confirmed by
77 producing deletion mutants (Cacho et al., 2013; Chooi et al., 2010), but the role of the putative
78 transporter as well as the regulatory genes remains to be investigated.

79 Regulatory genes are an interesting target for mutagenesis because silencing or promoting their
80 expression can provide significant enhancement of production of compounds with industrial value.

81 In this work, the regulation of griseofulvin biosynthesis was investigated by characterizing
82 knockout mutants for the putative transcription factors encoded by *gsfR1* and *gsfR2* and comparing
83 them with wild type and deletion mutants for the backbone gene of the cluster, *gsfA*. However, the
84 regulation of SM biosynthesis is incredibly complex and involves not only pathway-specific
85 regulators, which are commonly encoded by genes located inside the gene cluster, but also global

86 regulators expressed in response to various environmental stimuli (Brakhage, 2013). A second
87 approach aimed to study the involvement of global regulators of the cluster was therefore
88 investigated, particularly by evaluating the effect of carbon and nitrogen sources on *P. griseofulvum*
89 growth and on griseofulvin biosynthesis. The findings reported here lead to a better comprehension
90 of griseofulvin biosynthesis and its role in the growth and virulence of *P. griseofulvum*.

91

92 **2. Materials and Methods**

93 *2.1. Fungal strain*

94 *Penicillium griseofulvum* Dierckx strain PG3 was previously isolated from rotten apples in
95 Piedmont (Spadaro et al., 2011) and grown on Potato Dextrose Agar (PDA, Merck KGaA,
96 Darmstadt, Germany) with 50 µg/mL streptomycin (Merck) in the dark at 25 °C. *P. griseofulvum*
97 transformed strains were grown on PDA containing 500 µg/mL of hygromycin B (ThermoFischer
98 Scientific, Waltham, MA USA) in the same conditions. Conidial suspensions were obtained by
99 adding 5 mL of sterile water with 0.01 % (w/v) Tween-20 and gently scraping the surface of fungal
100 cultures grown on Petri plates. The final conidia concentration was measured using a
101 hemocytometer and adjusted to the required concentration, depending on the type of experiment.
102 Conidial suspension of wild-type and transformed strains were maintained in glycerol (40%) at -80
103 °C.

104

105 *2.2 Bioinformatic analysis*

106 The promoter of the genes *gsfR1* and *gsfA* were extracted from the genome of *P. griseofulvum* PG3
107 (Genbank: GCA_001561935.1), and they were scanned for transcription factor (TF) binding using
108 the CIS-BP database (<http://cisbp.cabr.utoronto.ca/TFTools.php>) with default parameters. *P.*
109 *chrysogenum* was used as model species when performing the analysis. Identified *P. chrysogenum*
110 TFs were blasted against PG3 proteome to check for their presence in *P. griseofulvum*, and they
111 were then identified by blasting against NCBI non-redundant protein database.

112

113 2.3 Obtainment of knockout mutants

114 Knockout mutants were obtained as previously described (Ballester et al., 2015), by amplifying 5'
115 (promoter) and 3' (terminator) flanking regions of *gsfA*, *gsfR1* and *gsfR2* genes from the genomic
116 DNA of *P. griseofulvum* PG3 with specific primers pairs (O1/O2 and A3/A4, Tab. S1) containing a
117 tail for USER cloning (Frandsen et al., 2008). Amplified fragments were mixed with pRFHU2
118 vector pre-digested with *PacI* and *NtBbvCI* (New England Biolabs, Ipswich, MA, USA) and treated
119 with USER enzyme (New England Biolabs). Five µL of the USER mixture was directly used to
120 transform chemically competent cells of *Escherichia coli* strain DH5α, with heat shock protocol,
121 then bacterial cells were recovered and plated on Luria Bertani Agar (LB, Miller, Merck)
122 supplemented with 25 µg/mL of kanamycin (ThermoFischer Scientific). Resistant transformants
123 were screened by colony PCR and plasmid DNA from positive colonies containing both promoter
124 and terminator was obtained using NucleoSpin® Plasmid EasyPure kit (Macherey-Nagel, Düren,
125 Germany) following the manufacturer's instructions. The correct integration of promoter and
126 terminator of genes was confirmed by sequencing the amplified fragments with primers pairs
127 RF1/RF6 and RF2/RF5 (Tab. S1). Then, 10 ng of plasmid were introduced by electroporation into
128 electro-competent *A. tumefaciens* AGL1 cells and *A. tumefaciens* mediated transformation of *P.*
129 *griseofulvum* was conducted as described by Buron-Moles et al. (2012). Transformants were
130 maintained on PDA with 500 µg/mL of hygromycin B, according to the results of a sensitivity assay
131 performed on PDA for *P. griseofulvum*.

132

133 2.4 DNA and RNA extraction

134 DNA of *P. griseofulvum* PG3 and knockout mutants was obtained using E.Z.N.A.® Fungal DNA
135 Mini Kit (Omega Bio-tek, Norcross, GA, USA) or using the slightly modified protocol for isolation
136 of high molecular weight DNA from fungal mycelium (Möller et al., 1992). Briefly, a conidial
137 suspension (10^5 conidia/mL) was inoculated in 50 mL of Glucose Yeast Peptone medium (GPY;

138 4% w/v glucose, 0.5% w/v yeast extract and 0.5% w/v peptone) and incubated on a rotary shaker
139 (200 rpm) at 24 °C for 2 days. Fungal mass was collected, dried and frozen in liquid nitrogen. DNA
140 extraction from 0.5 g of frozen mycelium was performed in 50 mL tubes containing 5 mL TES (100
141 mM Tris HCl, pH 8.0, 10 mM EDTA, 2% SDS), 25 µL proteinase K, and 50 µL β-mercaptoethanol.
142 DNA concentration and purity were checked by spectrophotometer (Nanodrop 2000, Thermo
143 Scientific, Wilmington, USA).

144 RNA extraction was performed according to Ballester and collaborators (2015) with some
145 adjustments. First, 5 µL of a conidial suspension (10^6 conidia/mL) were inoculated on PDA with a
146 cellophane membrane and incubated at 24 °C in the dark. One hundred mg of mycelium were
147 collected in 2 mL tube at 5, 7 and 10 dpi and frozen in liquid nitrogen. Two tungsten beads
148 (diameter: 2.7 mm) were added to the mycelium that was crushed using TissueLyser (Qiagen,
149 Hilden, Germany) for 1 min at 20.00 Hz speed. Then, 750 µL of extraction buffer (100 mM Tris-
150 HCl pH 8.0; 100 mM lithium chloride, 10 mM EDTA pH 8.0; 1% SDS; 1% PVP-40; 1% β-
151 mercaptoethanol) and 375 µL of phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) were added,
152 then the mixture was vortexed and incubated at 65 °C for 10 min. After cooling, 375 µL of
153 phenol:chloroform:isoamyl alcohol were added and the extract was vortexed. The homogenate was
154 centrifuged at 13,800 x g for 10 min and the aqueous phase was re-extracted with 750 µL of
155 phenol:chloroform:isoamyl alcohol. Nucleic acids were precipitated by adding 1/10 volume of 3 M
156 sodium acetate pH 5.2 and 2 volumes of cold 100% ethanol and incubating at -20 °C for 60 min.
157 The precipitated RNA was pelleted by centrifugation for 15 min at 13,800 x g. After washing with
158 500 µL of 70% ethanol, pellet was dissolved in 600 µL of TES buffer (10 mM Tris-HCl pH 8; 5
159 mM EDTA pH 8; 0.1% SDS) and precipitated overnight at -20 °C with 200 µL of 12 M lithium
160 chloride. After centrifugation at 13,800 x g for 60 min, the pellet was washed with 500 µL of 70%
161 ethanol, suspended with 250 µL of 3 M sodium acetate, pH 6.0, to remove residual polysaccharides
162 and washed with 70% ethanol. Finally, RNA extract was dissolved in 50 µL of DEPC-water and its
163 concentration was measured spectrophotometrically and verified by gel electrophoresis.

164 DNase treatment was performed using TURBO DNA-free™ Kit (Thermo Fischer Scientific). The
165 samples were diluted 1:2 in a final volume of 50 µL and rigorous DNase treatment followed. First-
166 strand cDNA was conducted with High Capacity cDNA Reverse Transcription Kit (Thermo Fischer
167 Scientific) using 1 µg of total RNA.

168

169 2.5 PCR and qPCR

170 Upstream and downstream fragments of *gsfR1*, *gsfR2* and *gsfA* genes were amplified by PCR with
171 Top-Taq DNA polymerase (Bioron Diagnostics GmbH, Römerberg, Germany) and a T100™
172 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The cycling conditions of the PCR were 5 min at
173 94 °C, followed by 35 cycles of 15 s at 94 °C, 20 s at 58 °C, 90 s at 72 °C and a final extension of 5
174 min at 72 °C. The PCR mixture contained 1X PCR Buffer, 0.2 mM dNTPs, 0.4 µM of each primer,
175 0.5 U of polymerase and 10 ng of genomic DNA of *P. griseofulvum*. Taq DNA Polymerase
176 (Qiagen) was used to perform the other PCRs. To confirm the correct integration of T-DNA in the
177 genome, several PCRs were conducted to amplify the hygromycin cassette and *gsfA*, *gsfR1* and
178 *gsfR2* genes. The cycling conditions of the PCRs were 3 min at 94 °C, followed by 35 cycles of 30 s
179 at 94 °C, 45 s at 58 °C, 90 s at 72 °C and 5 min at 72 °C.

180 To verify the efficiency of the DNase treatment, a PCR was performed using ITS1 and ITS4
181 universal primers. The cycling conditions of the PCR were 3 min at 94 °C, followed by 40 cycles of
182 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and 5 min at 72 °C.

183 RT-qPCRs were performed with StepOne™ and StepOnePlus™ Real-Time PCR System with
184 Power SYBR™ Green PCR Master Mix (ThermoFischer Scientific); cycling conditions were 5 min
185 at 95 °C, followed by 45 cycles of 10 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. In order to
186 determine the number of insertions in the genome of the transformants, the $2^{\Delta\Delta Cq}$ method (Pfaffl,
187 2001) was used using genomic DNA of samples and comparing the amplification of β -tubulin gene
188 or histone H3, with the amplification of the promoter of the corresponding gene target of deletion.

189 To determine the expression of *gsfA* and *gsfR1* genes in Δ *gsfR1* mutants, cDNA was added in the
190 reaction instead of gDNA.

191 All primer sequences used in the PCR and qPCR reactions are listed in Tab. S1.

192

193 2.6 Characterization of mutants in vitro

194 To check growth rate, conidiation and griseofulvin production, deletion and ectopic mutants were
195 compared with wild type *P. griseofulvum* PG3 by inoculating 5 μ l of spore suspension (1×10^6
196 conidia/mL) on PDA and incubating the plates at 25 °C in the dark. Some mutants were also
197 inoculated on Malt Extract Agar (MEA, 2% malt extract, 2% glucose, 0.1 % peptone, 2% agar) and
198 Meat Peptone Agar (MPA, 2.5% meat extract, 0.25% peptone, 2% agar) under the same conditions.
199 Colony diameter (cm), number of asexual spores (conidia/plate) and griseofulvin production
200 (μ g/plate) were measured up to 10 days post inoculation (dpi). At least 5 plates were inoculated for
201 each strain and every assay was performed three times.

202

203 2.7 Characterization of mutants in vivo

204 Apples ‘Golden Delicious’ were purchased from a local supermarket and stored at 4 °C until use.
205 Fruits were surface disinfected with sodium hypochlorite (1% as chlorine), washed with deionized
206 water and allowed to air dry before inoculation, then 21 fruits for each treatment were inoculated by
207 wounding in three points at the equatorial region of the fruits with a sterile needle (3 mm depth, 3-4
208 mm wide) (Spadaro et al., 2013). Ten μ l of conidial suspension (1×10^8 conidia/mL) of each strain
209 were pipetted into each wound, while controls were inoculated with deionized water. Wounded
210 apples were placed in plastic trays, covered with a transparent polyethylene film and stored at room
211 temperature for 3-14 days.

212

213 2.8 Griseofulvin and patulin extraction

214 Griseofulvin and patulin produced *in vitro* were extracted simultaneously from the mycelium and
215 the medium by washing the plates with 3 mL of methanol according to Banani et al. (2016) with
216 some modifications. The plates were scraped and then placed in rotary shaken at 450 rpm for 15
217 min. The liquid was then collected and centrifuged (13000 x g) at room temperature for 5 min.
218 After that, the supernatant was filtered through a 0.45 µm syringe filter and analysed by HPLC.
219 Griseofulvin was extracted from rotten apples as previously described by Banani et al. (2016) with
220 some changes. Briefly, the portion around the inoculation sites from 7 apples was collected and
221 combined. Ten g of each sample were weighed and then placed in a centrifuge tube with 10 drops
222 of pectinase enzyme solution (Sigma Chemical Co., St Louis, USA; 5 U/g juice) and 10 mL of
223 water were added. The mixture was left at 38 °C for 2 hours and then centrifuged at 3,600 x g for 5
224 min. Five mL of clear liquid were placed into a clean tube and griseofulvin was extracted with 5 mL
225 of ethyl acetate (three times). The organic phase was evaporated to dryness using rotary evaporator
226 and the residual dissolved in 500 µL of MeOH:H₂O (1:1, v/v) transferred into a HPLC vial for
227 HPLC-MS/MS analysis.

228

229 2.9 Chemical analyses

230 To assess the production of griseofulvin and patulin on PDA, the analyses were carried out using
231 the method previously described by Banani et al. (2016) with few modifications. A Waters HPLC
232 system equipped with a 600 Consolider pump, a 717 plus Autosampler and a 2996 photodiode array
233 detector were used. The analytical column used was a Kinetex 5 µm Biphenyl 100Å column (150 x
234 4.6 mm; Phenomenex, Torrance, CA, USA) coupled to a SecurityGuard ULTRA Cartridges UHPLC
235 Biphenyl guard column (4.6 mm; Phenomenex). The chromatography conditions were: flow rate of
236 1 mL/min on a linear gradient of 5 to 95 % solvent B in 30 min followed by isocratic 95 % solvent
237 B for another 10 min (solvent A: acid water pH 4.0 with acetic acid; solvent B: acetonitrile); the
238 injection volume was 20 µL. The spectra were acquired between 200 nm and 600 nm wavelength,
239 and patulin and griseofulvin quantifications was performed at 294 nm and 304 nm, respectively.

240 Patulin and griseofulvin were identified in the samples by comparing the retention time and UV-vis
241 spectra with those of chemical standards.

242 Comparison of griseofulvin production on different growth media and *in vivo* was analyzed by
243 liquid chromatography coupled with mass spectrometry. The HPLC-MS/MS system consisted of a
244 binary pump and a vacuum degasser (1260 Agilent Technologies, Santa Clara, CA, USA) connected
245 to a Varian auto-sampler Model 410 Prostar (Hansen Way, CA, USA), equipped with a 20 μ L loop
246 and coupled with a Varian 310-MS TQ Mass Spectrometer. The sample was injected (10 μ L) onto
247 Luna C18(2) (150mm x 2mm i.d. 3 μ m, Phenomenex) and eluted under a flow of 200 μ L/min. The
248 mobile phase was an isocratic mixture of ACN:HCOOH 0.05% (60:40, v/v) for 5 min.

249 The mass spectrometer was equipped with an electrospray ionization (ESI) source operating in
250 positive ion mode. Griseofulvin detection and quantitation were carried out using multiple reaction
251 monitoring (MRM) mode by selecting three transitions: m/z 353 > 285 CE 18 eV (monitoring), m/z
252 353 > 215 CE 16 eV (quantification) and m/z 353 > 165 CE 16 eV (monitoring). The collision gas
253 (Ar) pressure was set at 2 mbar for all experiments.

254 Quantification was performed by external calibration preparing a calibration curve for each
255 mycotoxin. The calibration curves were prepared by dilution of standard solutions of griseofulvin
256 and patulin.

257

258 *2.10 Statistical analysis*

259 All statistical analyses were performed with one-way ANOVA followed by Duncan test using IBM
260 SPSS statistics software version 24 (SPSS Inc., Chicago, IL, USA), $p < 0.05$ was considered
261 significant. The REST 2009 Software (Qiagen) was used for the statistical analysis of RT-qPCR
262 and qPCR results.

263

264 **3. Results**

265

266 3.1 Obtainment of knockout mutants for *gsfR1* and *gsfR2* and role on griseofulvin biosynthesis

267 The role of putative transcription factors encoded by *gsfR1* and *gsfR2* was assessed by obtaining
268 deletion mutants and they were compared with the wild-type strain and a deletion mutant for *gsfA*,
269 the gene encoding the PKS.

270 The deletion of genes was mediated by *A. tumefaciens*, which delivered a T-DNA containing a
271 hygromycin resistance cassette flanked by the promoter and terminator of target genes. Due to the
272 presence of these flanking regions, homologous recombination led to replacement of genes with the
273 antibiotic resistance cassette (Figs. 1-2, S1).

274 The knockout mutants were then assessed by amplifying the hygromycin resistance cassette and
275 target gene by PCR (Fig. 1 B, 2 B, S1 B). As expected, the deleted genes were not amplified in
276 knockout mutants, while hygromycin resistance cassette was only absent in the wild type PG3.
277 Ectopic strains had a functional copy of the target gene and the antibiotic resistance cassette, due to
278 non-target integration into the genome of the T-DNA.

279 To confirm the right insertion of the cassette, PCRs with primer pairs designed on the hygromycin
280 resistance cassette and on 3'UTR or 5'UTR of deleted gene were performed (Fig. 1 C, 2 C, S1 C).
281 These primer pairs can only amplify if the T-DNA has been introduced by homologous
282 recombination at the target sites in the 3'UTR and 5'UTR flanking regions. As expected, the PCR
283 fragments were amplified in the knockout mutants, and failed amplification in the wild type PG3
284 and in the ectopic mutants.

285 The number of integrations in the genome was determined by qPCR by measuring the difference
286 between the quantification cycle (C_q) of target and reference genes in the mutant and in the control
287 wild-type strain (Pfaffl, 2001) (Fig. 1 D, 2 D, S1 D).

288 Three mutants with one single event of integration and two ectopic strains were selected for each
289 knockout event and they were characterized *in vitro* both phenotypically and chemically.

290 Concerning the effect of gene deletion on phenotype, while the deletion of the polyketide synthase
291 did not affect the phenotype (Fig. S2), knockout mutants for *gsfR1* showed the same growth rate as

the wild-type strain (Fig. 3A), but exhibited colonies with a markedly less green color, caused by a marked reduction in conidiation *in vitro* (Fig. 3 B, C). On the other hand, knockout *gsfR2* mutants were similar to the wild type in both growth and sporulation (Fig. 5). The ability of knockout mutants to produce griseofulvin was assessed on PDA. Mutants lacking the *gsfA* gene were no longer able to produce griseofulvin *in vitro* (Fig. S2 B), while knockout mutants for the regulator gene *gsfR1* were found to produce higher quantities of griseofulvin compared to wild-type strain, with an average of about 30%, 50% and 110% higher production compared to wild-type strain at 5, 7 and 10 dpi (Fig. 4 A, Fig. 9). The production of patulin was also investigated and deletion of *gsfR1* showed to influence also the regulation of this mycotoxin, especially at 5 dpi, reaching over 3000 µg/plate in all mutants compared to 2300 µg/plate produced by *P. griseofulvum* PG3, which was significantly different according to Duncan's statistical analysis ($p < 0.05$) (Fig. 4 B). The knockout of *gsfR2* did not affect griseofulvin or patulin production, nor conidiation or growth rate, which were comparable between wild type and knockout and ectopic mutants (Fig. 5, 6).

3.2 *GsfR1* regulates negatively griseofulvin biosynthesis on PDA

The observation that the deletion of *gsfR1* led to a higher griseofulvin production *in vitro* led us to hypothesize that this gene could encode a negative regulator of griseofulvin biosynthesis. To examine this hypothesis, the relative expression of the polyketide synthase gene of three mutants and two ectopic strains was investigated through RT-qPCR. The Δ *gsfR1* deletion mutants showed an increased expression of *gsfA* compared to the wild type, both at 5 and 7 dpi, while ectopic mutants exhibited a similar gene expression to the wild type (Fig. 7).

3.3 Virulence and griseofulvin production on apples

To evaluate the effect of griseofulvin on the pathogenicity of *P. griseofulvum*, the virulence of three knockout mutants was compared with that of the wild-type strain and one ectopic mutant on apples

(Fig. 8). *ΔgsfA* mutants, which were not able to produce griseofulvin, showed a slightly reduced virulence (Fig. 8 A). In this assay, apples infected with E-A ectopic mutant, which contains 4 insertions of the T-DNA, led to a strong reduction of virulence on apples. However, *gsfR2* mutants showed a similar virulence compared to the wild-type strain over a period of 14 days after inoculation (Fig. 8 B). On the contrary, *ΔgsfR1* strains were slightly more virulent (Fig. 8 C) compared to the *P. griseofulvum* parental strain. Nevertheless, the ectopic mutant showed an enhanced virulence as well, although it was less virulent than the mutant in the first stages of the infections. For this reason, we cannot exclude that the enhanced virulence of the mutants was at least partly due to the transformation itself.

To check if there is a relationship between increased virulence and production of griseofulvin *in situ*, the mycotoxin was extracted from apples inoculated with *gsfR1* mutants and quantified. Results showed a significant increased production of griseofulvin from knockout mutants at 10 dpi (Fig. 8 D), while at 14 dpi *gsfR1* mutants produced less griseofulvin compared to wild-type strain.

331

332 3.4 Global regulators in griseofulvin biosynthesis

By using information on the CIS-BP database, the promoters of *gsfR1* and *gsfA* were scanned in order to find putative binding sites for global regulators. Eighty-one and seventy-five transcription factors were predicted to bind the promoters of *gsfR1* and *gsfA*, respectively. Thirteen transcription factors seem to bind only the promoter of *gsfR1*, while 7 are typical of *gsfA* and 68 were predicted to regulate both genes, ensuing a complex scenario (Tab. S2).

338

339 3.5 Carbon and nitrogen have a strong effect on in vitro conidiation and griseofulvin production

Among the different stimuli, the availability of nutritional sources, especially nitrogen and carbon, is a key factor for the biosynthesis of many SMs and for the growth and differentiation of filamentous fungi. Because of this, it is not surprising that we found many putative TFs involved in carbon or nitrogen consumption, which could bind the promoter of *gsfR1* and *gsfA* (Tab. S2).

344 We focused our attention on the effect of carbon and nitrogen on griseofulvin production. To do
345 that, two *gsfR1* deletion mutants and one ectopic strain were grown for ten days on three different
346 media (PDA, MEA and MPA); one knockout mutant for the polyketide synthase gene was also
347 included as control (denoted as KO). These three media are characterized by different C/N ratios:
348 PDA is composed of potato extract and dextrose and has the highest carbon content, while on MEA
349 and MPA, peptone was added, increasing the nitrogen content, which is ten times higher on MPA
350 (thanks to the addition of meat extract) compared to MEA (Núñez et al., 2000). As expected, carbon
351 and nitrogen showed a huge impact both on griseofulvin and conidia production (Fig. 9).
352 Conidiation of the wild-type strain was reduced in both media containing peptone, with a stronger
353 reduction on MEA (Fig. 9A), and a significant reduction of griseofulvin production was observed in
354 MPA (23%) compared to PDA or MEA (Fig. 9B).
355 Conidiation of the *gsfA* knockout mutant, lacking the gene encoding the polyketide synthase, was
356 similar to that observed for the wild-type strain in the three media tested. However, the knockout
357 mutant was not able to produce griseofulvin in any condition.
358 When focusing on *gsfR1* mutants, a more complex scenario was observed. Deletion of *gsfR1* gene
359 led to a significant reduction of conidia on MEA and PDA compared to wild type, ectopic strain and
360 *gsfA* mutants, suggesting that this gene could play a role in the direct or indirect regulation of
361 conidia production. Considering griseofulvin production, a significant increase of griseofulvin
362 production was observed on PDA (Fig. 4 and 9B). Instead, on the other two media, richer in
363 nitrogen, there was a significantly reduced production compared to wild type.

364

365 4. Discussion

366 4.1 Role of transcription factors *GsfR1* and *GsfR2* on griseofulvin biosynthesis

367 The griseofulvin gene cluster previously identified in *P. aethiopicum* includes two genes, *gsfR1* and
368 *gsfR2*, which encode for putative transcription factors. The *gsfR2* gene in *P. griseofulvum* was
369 located in a separated genomic region of the biosynthetic gene cluster, differently from *P.*

370 *aethiopicum*. Previous reports demonstrated the mode in which some TFs regulate the expression of
371 a specific gene cluster even when they are located outside the cluster itself. This is the case of both
372 *fum21* and *zfr1*, that are located inside and outside the gene cluster respectively, and regulate
373 fumonisin biosynthesis in *Fusarium verticilloides* (Brown et al., 2007; Flaherty and Woloshuk,
374 2004). In this work, our aim was to elucidate the role of putative transcription factors in griseofulvin
375 biosynthesis by generating deletion mutants. Deletion mutants for the *gsfA* core biosynthetic gene
376 encoding a polyketide synthase lacking the ability to produce griseofulvin were obtained and used
377 as control (Chooi et al., 2010).

378 Our results show that the *gsfR1* gene encodes a putative transcription factor that not only acts on
379 griseofulvin biosynthesis but also plays an essential role as an important regulator of *P.*
380 *griseofulvum* development and secondary metabolism. GsfR1 seems to regulate griseofulvin
381 biosynthesis as a negative regulator of the cluster when tested *in vitro* on PDA medium and on
382 apples. Indeed, the deletion of this regulatory gene led to an increase of griseofulvin production in
383 these conditions and a higher expression of the *gsfA* gene *in vitro*. Conversely, on media
384 supplemented with peptone, *gsfR1* gene positively regulates the griseofulvin biosynthesis, as all
385 mutant strains were able to produce significantly less amount of the compound compared to wild
386 type. Similar results were obtained studying the *mtfA* gene encoding the TF of aflatoxin in
387 *Aspergillus flavus* and *A. parasiticus*, revealing that the regulatory activity of this class of enzymes
388 is largely dependent on environmental conditions (Zhuang et al., 2016).

389 A differential regulation of patulin production was also revealed in knockout mutants of *gsfR1*,
390 which displayed increased patulin production at 5 dpi *in vitro*. This result suggests that *gsfR1* could
391 be involved in regulation of other SMs produced by *P. griseofulvum*, demonstrating a multiplicity
392 of downstream pathways that are regulated by the same transcription factor.

393 A crosstalk between different SM gene clusters has been largely recognized. For instance, in *A.*
394 *nidulans* the putative transcription factor encoded by *scpR* controls the expression of *inpA* and *inpB*,

two genes involved in the biosynthesis of a NRP-like compound, and the same TF is also involved in regulation of asperfuranone biosynthesis, a polyketide compound (Bergmann et al., 2010). The interconnection of signals and responses is also testified by the complex pattern of TFs that can putatively bind the promoters of *gsfR1* and *gsfA*, triggering the activation or repression of griseofulvin biosynthesis and regulating at the same time other SM gene clusters and many aspects of the biology of *P. griseofulvum*, such as conidiation and virulence. Surprisingly, in the recently released genome of griseofulvin producer *Xylaria flabelliformis* (Mead et al., 2019) the gene *gsfR1* was missing, pointing to a possible regulation of griseofulvin biosynthesis independently of *gsfR1* action.

Considering the second putative transcription factor of the cluster, the deletion of *gsfR2* seems to have no effect on the parameters of *P. griseofulvum* considered, and it is likely that the gene is involved in a different biosynthetic pathway. Indeed, the gene *gsfK* encoding for a putative reductase is located next to *gsfR2*, and its deletion in *P. aethiopicum* did not affect the production of griseofulvin (Cacho et al., 2013). These findings led to the assumption that both *gsfK* and *gsfR2* are probably involved in the biosynthesis of another SM. Moreover, in *X. flabelliformis* (Mead et al., 2019), *gsfJ* and *gsfG* genes were missing, suggesting that these two genes are not necessary for griseofulvin production.

Therefore, considering also that the knockout of *gsfR2* does not impair griseofulvin production, we suggest that the core genes for the biosynthesis of this metabolite are only 7 instead of 13.

414

4.2 Role of global regulators in griseofulvin biosynthesis

Secondary metabolism gene clusters are usually regulated by several global regulatory proteins in response to many environmental stimuli, such as light, carbon and nitrogen levels, pH and redox status, iron concentration and signaling from other organisms (Brakhage, 2013; Hoffmeister and Keller, 2007). Many of these global regulators are well characterized and their putative binding sites in the promoter regions of genes could be detected.

421 One of the transcription factors predicted to regulate *gsfR1* and not *gsfA* is KXG54396.1, a *P.*
 422 *griseofulvum* gene similar to *mtfA*, whose deletion or overexpression in *A. flavus* cause a reduction
 423 of aflatoxins production (Zhuang et al., 2016). Environmental conditions influence the regulatory
 424 activity of the gene in *A. flavus*, where the deletion increases conidiation *in vitro* and reduces it *in*
 425 *vivo* (Zhuang et al., 2016).
 426 Other transcription factors putatively binding *gsfR1* and not *gsfA* included NsdD, which is necessary
 427 for sexual development and represses asexual one (Han et al., 2001), a quinic acid utilization
 428 activator, an homolog of the fluconazole resistance protein and *stuA*, a gene able to regulate cell
 429 pattern formation (Miller et al., 1992), carbon metabolism, effector expression and the synthesis of
 430 penicillin and several mycotoxins (IpCho et al., 2010; Sigl et al., 2011; Yang et al., 2018). Another
 431 transcription factor, KXG45700.1, was similar to LreA, a protein involved in conidiation and
 432 response to light (Igbalajobi et al., 2019), while KXG46600.1 was similar to Res2, necessary for
 433 meiosis (Zhu et al., 1997). KXG47124.1 has 60% identity with the transcription factor Msn2 of *A.*
 434 *parasiticus*, involved in conidiation, hyphal growth and on occasionally stress response in several
 435 fungi (Liu et al., 2013; Song et al., 2018; Tian et al., 2017). KXG48399.1 is homologous to another
 436 transcription factor *flbC* related to asexual development, whose knock-out causes delayed
 437 conidiation in *A. nidulans* (Kwon et al., 2010). The *gsfR1* promoter was predicted to be bound also
 438 by homologs of PAP1, SebA and NapA, transcription factors involved in oxidative stress response
 439 (Asano et al., 2007; Dinamarco et al., 2012; Ikner and Shiozaki, 2005). The presence of the binding
 440 sites for this group of transcription factors suggest a complex regulation of *gsfR1*, that is markedly
 441 influenced by sexual and asexual development and environmental conditions.
 442 The promoter of *gsfA* had only 7 unique binding sites for transcription factor, including SreP, a
 443 repressor of siderophore biosynthesis in presence of high iron concentrations (Haas et al., 1997).
 444 Other transcription factors putatively regulating *gsfA* included two regulators of filamentous
 445 growth, a transcriptional activator of gluconeogenesis, a pathway necessary in *A. nidulans* for
 446 growing with certain carbon sources metabolized via Acetyl-CoA (Hynes et al., 2007), and a

447 homolog of *gal4*, necessary in *Saccharomyces cerevisiae* for growth using galactose or lactose as
448 only carbon sources (Riley et al., 1987). This data seems to suggest an increase in *gsfA* expression,
449 and therefore griseofulvin production, in the presence of difficult carbon sources, but further
450 experiments would be required to check this possibility.

451 Several transcription factors binding the promoters of both *gsfRI* and *gsfA* have not been
452 characterized yet. However, among them, there were homologs of the well-known global regulators
453 AreA and CreA, which are respectively a positive and a negative transcription factor acting in
454 response to nitrogen or carbon (Katz et al., 2008; Wilson and Arst, 1998). Moreover, the binding
455 sites of NirA and the carbon catabolite repressor MIG1 were also found in the promoters of both
456 genes, increasing the possibilities of production of griseofulvin being heavily influenced by carbon
457 and nitrogen availability (Burger et al., 1991; Randhawa et al., 2018). Binding sites for genes
458 regulating the utilization of different carbon and nitrogen sources are also present. These include:
459 *facB*, regulating acetate utilization (Todd et al., 1998), *argR*, regulating arginine metabolism
460 (Dubois et al., 1987), *nirA*, regulating nitrate utilization (Burger et al., 1991), *amdR*, regulating
461 amides, omega amino acids and lactams catabolism (Andrianopoulos and Hynes, 1990), and the
462 quinic acid utilization activator (Wheeler et al., 1996). Other two transcription factors, the cutinase
463 transcription factor 1 beta and SKN7, respectively increase the expression of cutinases and the
464 resistance to ROS respectively, suggesting a role of the griseofulvin cluster in pathogenesis (Cao et
465 al., 2009), as shown in the pathogenicity trials.

466 In addition to those indicated above, other identified transcription factors were related to sexual and
467 asexual development, heat shock, stress resistance, cellobiose response, sulphur catabolism and
468 assimilation, iron homeostasis and adhesion, and hyphal growth (Tab. S2).

469 Both promoters of *gsfRI* and *gsfA* also contain binding sites similar to those recognized by Apa-2
470 (Chang et al., 1993), strengthening the hypothesis that the griseofulvin regulatory system is
471 somewhat similar to the aflatoxins one, regulated by MtfA.

472

473 4.3 Global regulation of griseofulvin biosynthesis is triggered by carbon and nitrogen

474 Nitrogen is a central element for the life of living organisms, and many specific regulatory genes
475 are expressed when nitrogen sources such as ammonium, glutamate or glutamine are scarce. These
476 genes encode transcription factors that repress the utilization of nitrogen sources that are easy to
477 assimilate, in the so-called mechanism “Nitrogen Metabolism Repression” (NMR). All these genes
478 are responsible for the activation of pathways required for the uptake of alternative nitrogen sources
479 (Marzluf, 1997; Tudzynski, 2014). AreA is one of the main enzymes involved in NMR, and
480 putatively regulate griseofulvin gene cluster, acting both on *gsfR1* and *gsfA*. Similarly, the global
481 repressor in response to carbon, CreA, was found to be putatively involved in the regulation of
482 griseofulvin biosynthesis, together with transcription factors that modulate the utilization of
483 different carbon sources.

484 The effect of carbon and nitrogen sources on growth, conidiation and griseofulvin biosynthesis was
485 investigated *in vitro*, inoculating the strains in three different media, characterized by different C/N
486 ratios. Núñez and collaborators (2000) used MEA and MPA to evaluate the effect of carbon and
487 nitrogen on the production of verrucosidin, showing that MEA induce a higher production of this
488 compound and that the type of growth medium had significant influence on mycotoxin production.
489 The wild type showed a similar pattern of griseofulvin production on PDA and MEA, while in
490 MPA a significant reduction was observed. This result is reasonable because carbon is essential for
491 building the polyketide structure.

492 On the other hand, the deletion of *gsfR1* seems to have a differential effect on MEA and MPA. In
493 contrast to what observed on PDA plates, the production of griseofulvin drops drastically in
494 deletants compared to wild type. The high nitrogen concentration of these media likely triggered the
495 NMR, resulting in the activation of SM gene clusters. This could explain the higher production of
496 griseofulvin in the wild type compared to *gsfR1* deletion mutants. This result also indicates that
497 GsfR1 can regulate griseofulvin biosynthesis both in a positive and negative manner, depending on
498 external stimuli.

500 *4.4 Regulation of griseofulvin biosynthesis and conidiation*

501 Fungal development and secondary metabolism are deeply connected and many examples have
502 elucidated that some compounds (especially mycotoxins) can be secreted at the time of conidiation,
503 while other SM such as linoleic-acid in *A. nidulans* or zearalenone in *Fusarium graminearum* can
504 induce sporulation, and some of them are required for formation of sexual and asexual spores and for
505 their survival (e.g. pigments) (Calvo et al., 2002). Knockout mutants for *gsfRI* produce less conidia
506 compared to the parental strain, but this behavior is not surprising since the deletion of regulatory
507 genes is often associated with a reduced conidiation, such as for deletion of *aflR* TF for aflatoxin
508 biosynthesis (Wilkinson et al., 2004). The reduced conidiation at first was thought to be related with
509 the antifungal properties of griseofulvin, which is highly produced on PDA from knockout mutants.
510 From this perspective, the role of *gsfRI* is to avoid an excessive production of griseofulvin, which is
511 known to have an antifungal activity and could have toxic effects on the producer. Nevertheless, a
512 strongly reduced production of conidia was observed in MEA and MPA, where the mutants exhibit
513 a similar and lower griseofulvin production, respectively, compared to production on PDA.
514 Consequently, the reduced conidiation of *gsfRI* mutants cannot be linked with the antifungal
515 activity of griseofulvin, but instead seems to be the result of both the influence of the culture media
516 and the effect of deletion of *gsfRI* itself.

517 Concerning the culture media, MEA and MPA media were supplemented with peptone. The malt
518 extract added in MEA provides carbon and nitrogen content, while in MPA the meat extract
519 contributes to richness in nitrogen. In previous studies on aflatoxin biosynthesis it was shown that
520 simple sugars support fungal growth, sporulation and aflatoxin production, unlike complex sugars
521 and peptone, which strongly repress aflatoxin production (Calvo et al., 2002; Yao et al., 2018).

522 The reduced number of conidia produced by all tested strains in MEA and MPA appears therefore
523 to be related with the nutrient composition of these media. Instead, the reduced conidiation of
524 knockout mutants in all tested media compared to wild type could be explained as a consequence of

525 a direct or indirect positive regulation of conidiation driven by *gsfRI*. Many TFs have a role in co-
526 regulating spore production and mycotoxin formation. MtfA, RtfA, NsdC, RafA and StuA all
527 regulate aflatoxin production, conidiation and sclerotia development in *A. flavus* (Yao et al., 2018).
528 Furthermore, the regulator of patulin biosynthesis was recently proposed to be connected with the
529 development of *P. expansum*, even though the authors suggests that this is only a remote possibility
530 that have to be proved (Snini et al., 2016). Additionally, *gsfRI* is putatively bound by transcription
531 factors involved in asexual and sexual development, that strongly supports this model. Further
532 studies are needed to investigate the role of *gsfRI* in regulation of sporulation or conidiation,
533 together with already known global transcription factors.

534

535 4.5 Role of griseofulvin on pathogenicity of *P. griseofulvum*

536 Considering that SM are not essential for primary metabolism of fungi, along the fact that they
537 confer several advantages to the producer organism, it is reasonable to assume that they could have
538 a role in pathogenicity of fungal pathogens. The relationship between SM production and
539 pathogenicity has been extensively investigated previously, and several examples linking SM and
540 mycotoxins with pathogenesis are well documented (Macheleidt et al., 2016; Scharf et al., 2014).
541 Considering causal agents of blue molds in apples, previous studies have investigated the role of
542 patulin in infection and development of *P. expansum*, but contrasting results emerged. For instance,
543 Ballester et al. (2015) and Li et al. (2015) pointed out that patulin produced by fungal pathogens is
544 not essential for apple infection, as deletion of *patK*, *patL*, and *patN*, genes involved for patulin
545 biosynthesis, did not affect the virulence . Conversely, Sanzani et al. (2012) and Barad et al. (2013)
546 observed a direct correlation with virulence, because mutants which displayed a reduced production
547 of patulin were less virulent on apples. These contrasting results obtained in the same pathosystem
548 can be explained by the use of different strains from different countries and by the great quantity of
549 variable factors involved *in vivo* trials such as temperature, pH, cultivar sensitivity and storage
550 conditions (Barad et al., 2016b). Among these factors, apple variety plays an important role as

551 demonstrated by Snini et al. (2016). Taking into account the previous works reported, patulin could
552 be considered a virulence factor (connected to severity of disease), instead of a pathogenicity one
553 (linked to the ability to cause the disease), helping to establishment of the disease when the
554 pathogen has already colonized the fruit (Barad et al., 2016b). Indeed, the addition of patulin
555 directly on the wound completely restored the wild-type phenotype in less virulent strains (Snini et
556 al., 2016).

557 According to the presented results, griseofulvin may be involved in virulence and not pathogenicity.
558 Indeed, the deletion mutants for the *gsfA* gene are still able to induce the disease, even if they are
559 not able to produce griseofulvin, but the infected apples exhibit reduced lesions. This supports the
560 idea that griseofulvin alone should not be considered a pathogenicity factor but can act to enhance
561 virulence.

562 Δ *gsfR1* mutants were slightly more virulent compared to the wild type, while Δ *gsfR2* strains were
563 comparable to the wild type both in griseofulvin production and in pathogenicity. Considering that
564 Δ *gsfR2* mutants displayed no differences on virulence compared to the wild type, the differences on
565 development of rot diameter of knockout mutants for *gsfA* and *gsfR1* genes could be related to
566 griseofulvin production. The differences seen in the development of rot diameter on apples by
567 Δ *gsfA* and Δ *gsfR1* mutants could suggest a role of griseofulvin production in virulence of *P.*
568 *griseofulvum*. Nevertheless, the ectopic strain showed increased virulence compared to the wild
569 type and therefore further studies are necessary to understand the molecular mechanism/s
570 underlying this increased production.

571 Concerning the griseofulvin produced *in vivo* by Δ *gsfR1* mutants, a higher production was observed
572 at 10 dpi (similarly to what observed on PDA plates) and a lower production at 14 dpi (similarly to
573 what observed on MEA and MPA plates).

574 In establishing the interaction with the host, *P. griseofulvum* would activate a series of well
575 characterized responses, such as biosynthesis of gluconic acid and ammonium depletion, in order to
576 improve the acidification of apples and the biosynthesis of polygalacturonases responsible for cell

577 wall degradation of fruits (Barad et al., 2016a; Prusky et al., 2004). Reduced nitrogen availability,
578 together with high availability of simple sugars, can trigger the activation of SM gene clusters,
579 including griseofulvin, which is also enhanced in presence of ROS and cutinases according to the
580 findings of our work. This hypothesis is supported by the fact that *gsfR1* mutants produced less
581 griseofulvin compared to wild type after 14 days, so it is reasonable to assume that positive
582 regulation of griseofulvin biosynthesis is activated at this point. Moreover, if GsfR1 acts as a
583 negative regulator, it is reasonable to observe an earlier production of griseofulvin in the Δ *gsfR1*
584 knockout mutants, as it was found in infected apples by day 10, when the production of griseofulvin
585 is higher in the knockout mutants. To our knowledge, the current work presents the first attempt to
586 investigate the role of griseofulvin in virulence of *P. griseofulvum* and further investigation is
587 necessary to prove its involvement.

588

589 **5. Conclusions**

590 This work led to a better understanding of the complex regulation of griseofulvin biosynthesis. The
591 role of putative transcription factors was investigated. Based on our results, *gsfR2* is not involved in
592 this pathway and it is probably part of another gene cluster. The putative reductase *gsfK*, *gsfG* and
593 *gsfJ* are putatively not essential for griseofulvin biosynthesis due to their absence in the genome of
594 the producer species *X. flabelliformis*. Therefore, the griseofulvin gene cluster should be considered
595 composed of 7 genes, instead of 13, as previously described.

596 The gene *gsfR1*, on the other hand, is involved in griseofulvin biosynthesis, acting as a negative
597 regulator of the cluster on PDA and on apples. However, in different culture conditions,
598 characterized by nitrogen and complex sugars richness, *gsfR1* could act as a positive regulator of
599 griseofulvin biosynthesis. These findings suggest that *gsfR1* can trigger different responses
600 depending on external stimuli, especially nitrogen and carbon availability. The deletion of *gsfR1* has
601 a huge impact on many aspects of *P. griseofulvum*, ranging from secondary metabolism to virulence
602 and conidiation. It is remarkable to notice that *gsfR1* seems to be involved in the regulation of

603 patulin biosynthesis, which *P. griseofulvum* can produce in high amounts on apples. The regulation
604 of patulin biosynthesis in *P. griseofulvum* was previously suggested to be peculiar because the
605 expression of the specific transcription factor and other biosynthetic genes involved in patulin
606 biosynthesis did not increase with the increased production of the toxin (Banani et al., 2016),
607 differently from what observed in *P. expansum* (Ballester et al., 2015). Therefore, these findings
608 support the idea that *P. griseofulvum* regulate patulin biosynthesis in a distinctive manner,
609 highlighting variability of the regulation of secondary metabolism in different fungal species. The
610 knowledge of the mechanism involved in the regulation of patulin could be useful to conceive
611 strategies to limit its production on apples, by modifying its transcriptional activation, through
612 environmental stimuli.

613 Concerning pathogenicity of *P. griseofulvum*, the deletion mutants for *gsfA* were less virulent than
614 the wild-type strain, while the deletion mutants for *gsfR1* were slightly more virulent *in vivo*
615 suggesting a direct role of griseofulvin on virulence of *P. griseofulvum*. Until now, this is the first
616 study on the role of griseofulvin in pathogenicity, and further investigations are needed to confirm
617 this data and to highlight the mechanisms of action involved.

618 *gsfR1* is also involved in the asexual multiplication of *P. griseofulvum*, as a reduced conidiation was
619 observed compared to the wild type for all *in vitro* conditions tested.

620 In conclusion, *gsfR1* represents a good example of how the regulation of SM biosynthesis and
621 fungal development can be complex and extremely interconnected.

622

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629

630 **Declaration of interest**

631 None

632

633 **Author contributions**

634 Silvia Valente: Conceptualization, Investigation, Formal analysis, Writing - original draft; Agnese
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641

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Figures

Figure 1 – PCR analysis of *gsfR1* mutants. Schematic presentation of the *gsfR1* locus in the wild type and deletion mutants, primers used are presented as arrows (A). Amplification of hygromycin resistance cassette (*HygR*) and *gsfR1* gene (B); confirmation of orientation of inserted T-DNA (C); copies number of T-DNA inserted in *P. griseofulvum* (D) obtained through qPCR on gDNA using primers 5F/6R and histone H3 gene as reference gene. M= GeneRuler 1Kb Plus DNA ladder, WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR1* and E = ectopic strains, Neg. = negative control (PCR mix without DNA).

Figure 2 – PCR analysis of *gsfr2* mutants. Schematic presentation of the *gsfr2* locus in the wild type and deletion mutants, primers used are presented as arrows (**A**). Amplification of hygromycin resistance cassette (*HygR*) and *gsfr2* gene (**B**); confirmation of orientation of inserted T-DNA (**C**); copies number of T-DNA inserted in *P. griseofulvum* (**D**), obtained through qPCR on gDNA using primers 5F/6R and histone H3 gene as reference gene. M= GeneRuler 1Kb Plus DNA ladder, WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfr2* and E = ectopic strains, Neg. = negative control (PCR mix without DNA).

Figure 3 – Effect of *gsfrI* deletion on *P. griseofulvum* growth *in vitro*. Colony diameter (**A**) and conidia production (**B**) from 3 to 7 dpi. Plate view (front **C** and reverse **D**) at 7 dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfrI* and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

944

945 **Figure 4** - Effect of *gsfRI* deletion on griseofulvin and patulin production. Griseofulvin (A) and
 946 patulin (B) production were measured at 5 and 7 dpi. WT = wild type *P. griseofulvum*, Δ = deletion
 947 mutants and E = ectopic strains. Values followed by the same letter are not statistically different by
 948 Duncan's multiple range test ($p < 0.05$).

949

950

951 **Figure 5** – Effect of *gsfr2* deletion on *P. griseofulvum* growth *in vitro*. Colony diameter (**A**) and
 952 conidia production (**B**) from 3 to 7 dpi. Plate view (front **C** and reverse **D**) at 7 dpi on PDA. WT =
 953 wild type *P. griseofulvum*, Δ = deletion mutants for *gsfr2* and E = ectopic strains. Values followed
 954 by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).
 955

Figure 6 - Effect of *gsfR2* deletion on griseofulvin and patulin production. Griseofulvin (**A**) and patulin (**B**) production were measured at 5 and 7 dpi. WT = wild type *P. griseofulvum*, Δ = deletion mutants and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

Figure 7 – Relative gene expression (RGE) of *gsfA* gene, at 5 (**A**) and 7 (**B**) dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR1* and E = ectopic strains. The expression is relative to the expression of the β-tubulin gene.

967

968 **Figure 8** - Effect of gene deletions on virulence and griseofulvin production *in vivo*. Lesion
 969 diameter caused by the wild type strain was compared with rot diameter of knockout ad ectopic
 970 strains for *gsfA* (A) *gsfR2* (B) and *gsfR1* (C). Griseofulvin production ($\mu\text{g/g}$ of apple) was compared
 971 between wild type and $\Delta\textit{gsfR1}$ strains (D). Measurements were taken at 7, 10 and 14 dpi. WT =
 972 wild type *P. griseofulvum*, Δ = deletion mutants and E = ectopic strains. Values followed by the
 973 same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

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976 **Figure 9** – Effect of carbon and nitrogen on conidiation and on griseofulvin production *in vitro*.
 977 Logarithm of conidia per plate (**A**) and griseofulvin produced (**B**) were measured at 10 dpi in three
 978 solid media (PDA, MEA and MPA). Colony view on PDA (**C**), MEA (**D**) and MPA (**E**). WT = wild
 979 type *P. griseofulvum*, Δ7, Δ23 = deletion mutants for *gsfR1*, E3 = ectopic strain and ΔKO = deletion
 980 mutant for *gsfA*. Values followed by the same letter are not statistically different by Duncan's
 981 multiple range test ($p < 0.05$); n.d. = non detectable.

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983

987 **Figure S1** – PCR analysis of *gsfA* mutants. Schematic presentation of the *gsfA* locus in the wild
988 type and deletion mutants, primers used are presented as arrows (A). Amplification of hygromycin
989 resistance cassette (*HygR*) and *gsfA* gene (B); confirmation of orientation of inserted T-DNA (C);
990 copies number of T-DNA inserted in *P. griseofulvum* (D), obtained through qPCR on gDNA using
991 primers 5F/6R and β -tubulin gene as reference gene. M= GelPilot Wide Range Ladder, WT = wild
992 type *P. griseofulvum*, Δ = deletion mutants for *gsfA* and E = ectopic strains, Neg. = negative control
993 (PCR mix without DNA).

Figure S2 – Effect of *gsfA* deletion on *P. griseofulvum*. Colony diameter (**A**) and griseofulvin production (**B**) from 3 to 7 dpi. Plate view (front **C** and reverse **D**) at 7 dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfA* and E = ectopic strains, n.d. = non detectable.

Table S1 – List of primers used in this work.

Primer name	Primer sequence (5'→3')	Description
Construction and analysis of knockout mutants		
O1- <i>gsfR1</i>	GGTCTTAAUGTCGGCATCGGACGA ATTATAG	Insertion of <i>gsfR1</i> promoter in pRFHU2. Tail for USER reaction in red.
O2- <i>gsfR1</i>	GGCATTAAUCATCTTTCTGAGGAG GGAGAGA	
A3- <i>gsfR1</i>	GGACTTAAUGCGCGAAACGGTAT TTCTAT	Insertion of <i>gsfR1</i> terminator in pRFHU2. Tail for USER reaction in red.

A4- <i>gsfR1</i>	GGGTTTAAUGTGTGAGCGTGAATG GTAATG	
O1- <i>gsfR2</i>	GGTCTTAAUTCGGTGTCCACGTTT CTATTC	Insertion of <i>gsfR2</i> promoter in pRFHU2. Tail for USER reaction in red.
O2- <i>gsfR2</i>	GGCATTAAUTTGCTTGCCACGGTT ATCT	
A3- <i>gsfR2</i>	GGACTTAAUGACGAGCCAGTTCTC CTAATG	Insertion of <i>gsfR2</i> terminator in pRFHU2. Tail for USER reaction in red.
A4- <i>gsfR2</i>	GGGTTTAAUCTCCTTGCCCGATGT CTTATC	
O1- <i>gsfA</i>	GGTCTTAAUGCACACCTGAGCAA GATCAA	Insertion of <i>gsfA</i> promoter in pRFHU2. Tail for USER reaction in red.
O2- <i>gsfA</i>	GGCATTAAUTATTGCAGCTGCCGA GAGTA	
A3- <i>gsfA</i>	GGACTTAAUGGTCAATTCGGCGCA CTTAA	Insertion of <i>gsfA</i> terminator in pRFHU2. Tail for USER reaction in red.
A4- <i>gsfA</i>	GGGTTTAAUTCGAGAGGGCAAGA ATGTGT	
RF2	TCTCCTTGCATGCACCATTCCTTG	Confirmation of terminator fusion into pRFHU2 plasmid
RF5	GTTTGCAGGGCCATAGAC	
RF1	AAATTTTGTGCTCACCGCCTGGAC	Confirmation of promoter fusion into pRFHU2 plasmid
RF6	ACGCCAGGGTTTTCCAGTC	
1F- <i>gsfR1</i>	CCCAACATGAGTGAAAGCATAAC	Confirmation of orientation of inserted T-DNA. Upstream region of <i>gsfR1</i>
1F- <i>gsfR2</i>	TCGCACCAGAGCAAACATAC	Confirmation of orientation of inserted T-DNA. Upstream region of <i>gsfR2</i>
1F- <i>gsfA</i>	TGATAGAGCATTCGCGGTCC	Confirmation of orientation of inserted T-DNA. Upstream region of <i>gsfA</i>
HPH1F	ACGAGGTCGCCAACATCTTCTTCT	Confirmation of orientation of inserted T-DNA. Hygromycin cassette.
2R- <i>gsfR1</i>	AGTGCAGTCGGTCAACAATAC	Confirmation of orientation of inserted T-DNA. Downstream region of <i>gsfR1</i>
2R- <i>gsfR2</i>	GCTAGACGATGCGACACTACTA	Confirmation of orientation of inserted T-DNA. Downstream region of <i>gsfR2</i>
2R- <i>gsfA</i>	TTGGGAAACTCGTCGACCTG	Confirmation of orientation of inserted T-DNA. Downstream region of <i>gsfA</i>

HPHPR04	GCACCAAGCAGCAGATGATA	Confirmation of orientation of inserted T-DNA. Hygromycin cassette.
HMBF1	CTGTCGAGAAGTTTCTGATCG	Amplification of hygromycin resistance cassette
HMBR1	CTGATAGAGTTGGTCAAGACC	
R1-F	CTTGCGGGTCTCGAAGTAAA	Amplification of <i>gsfR1</i> gene
R1-R	CCGTCATCCGAATGTCTATCTG	
R2-F	TGCGAATACAACGGCCGATA	Amplification of <i>gsfR2</i> gene
R2-R	CATGAGAACCTCACGGAGAAA	
A-F	GCTTTGGTTTGGTTGTCGAT	Amplification of <i>gsfA</i> gene
A-R	CATCCCCTGGATCTTTCTCA	
Determination of number of integration and qPCR		
5F- <i>gsfR1</i>	GGATCTATCCATGTCGGAACAC	Determine number of integrations of T-DNA in <i>gsfR1</i> mutants and calculate relative expression of <i>gsfR1</i>
6R- <i>gsfR1</i>	CCTCACAGCCTGTTTGGTTA	
5F- <i>gsfR2</i>	GAAGCCGGATGATTAGAGGAG	Determine number of integrations of T-DNA in <i>gsfR2</i> mutants
6R- <i>gsfR2</i>	CATGAGAACCTCACGGAGAAA	
5F- <i>gsfA</i>	TGCTAGAGTGCGTGACAGT	Determine number of integrations of T-DNA in <i>gsfA</i> mutants
6R- <i>gsfA</i>	AGGACGCTCTCAAAGGTGG	
PeHis3_F2	TCTCCGCTTCCAGTCCTCTG	Amplification of histone H3 to determine number of integrations of T-DNA in <i>gsfR1</i> and <i>gsfR2</i> mutants
PeHis3_R2	TTGGTGTCCTCGAAGAGAGAGAC	
Pgr_tub_1F	CGAGTTGACCCAGCAGATGT	Amplification of β -tubuline to determine number of integrations of T-DNA in <i>gsfA</i> mutants and to calculate relative expression of <i>gsfR1</i> and <i>gsfA</i> .
Pgr_tub_2R	GTCTGGACGTTGTTGGGGAT	
<i>gsfA</i> -F	AAAAGACGGACAGTGACAGC	Calculate relative expression of <i>gsfA</i> .
<i>gsfA</i> -R	TATCCGCGATTTCACTCG	

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1003 **Table S2** – Transcription factors putatively binding the promoter of *gsfR1* and *gsfA*. It is reported
1004 the name of homolog and the regulated processes in other organisms with references when
1005 available.

Protein	Homolog	Regulated processes	Reference
Transcription factors binding only the promoter of <i>gsfR1</i>			
KXG45700.1	LreA	Conidiation, response to light	(Igbalajobi et al., 2019)
KXG46329.1	Fluconazole resistance protein	resistance to fluconazole	

KXG46595.1	NsdD	activation of sexual development	(Han et al., 2001)
KXG46600.1	res2	Meiosis	(Zhu et al., 1997)
KXG47124.1	Msn2	Conidiation, hyphal growth and stress response	(Liu et al., 2013; Song et al., 2018; Tian et al., 2017)
KXG47905.1	quinic acid utilization activator	Quinic acid utilization	(Wheeler et al., 1996)
KXG48399.1	FlbC	conidiation	(Kwon et al., 2010)
KXG48752.1	Fluconazole resistance protein 3	Fluconazole resistance	
KXG48995.1	hypothetical protein		
KXG49526.1	Transcription factor PAP1		
KXG53661.1	Transcription factor		
KXG54396.1	MtfA (ID 50%)	regulation of aflatoxin production	(Zhuang et al., 2016)
KXG54691.1	StuA	Regulation of cell pattern formation	(Miller et al., 1992)
Transcription factors binding only the promoter of <i>gsfA</i>			
KXG46092.1	gal4	Using galactose or lactose as only carbon sources	(Riley et al., 1987)
KXG47012.1	Hypothetical protein		
KXG47549.1	SREP	repressor of siderophore biosynthesis in presence of high iron concentrations	(Haas et al., 1997)
KXG47976.1	Zinc finger, C2H2-type/integrase, DNA-binding		
KXG51025.1	transcriptional activator of gluconeogenesis	transcriptional activator of gluconeogenesis	(Hynes et al., 2007)
KXG51359.1	Filamentous growth regulator	Filamentous growth	
KXG52025.1	Achaete-scute transcription factor related		
Transcription factors putatively binding the promoters of both <i>gsfRI</i> and <i>gsfA</i>			
KXG45164.1	Basic-leucine zipper (bZIP)		

	transcription factor		
KXG45306.1	Zinc finger, C2H2		
KXG45454.1	Transcription factor		
KXG45545.1	Hypothetical protein		
KXG46064.1	RlmA	response to cell wall stress, asexual development	(Damveld et al., 2005; Kovács et al., 2013)
KXG46347.1	RosA	represses sexual development	(Vienken et al., 2005)
KXG46776.1	Homeodomain-like		
KXG46828.1	Quinic acid utilization activator	Quinic acid utilization	(Wheeler et al., 1996)
KXG46909.1	Non-histone chromosomal protein 6		
KXG46994.1	Aflatoxin biosynthesis regulatory protein		(Chang et al., 1993)
KXG47056.1	High mobility group, HMG- I/HMG-Y		
KXG47172.1	Winged helix-turn-helix transcription repressor		
KXG47315.1	Protein of unknown function DUF3468		
KXG47412.1	NirA	Nitrate induction	(Burger et al., 1991)
KXG47486.1	TbfI	Coordinated ribosomal protein (RP) gene expression	(Hogues et al., 2008)
KXG47556.1	Protein of unknown function DUF3468		
KXG47599.1	CRZ1	Calcineurin pathway	(Cramer et al., 2008)
KXG47656.1	C6 transcription factor		
KXG47688.1	ARO80	catabolism of aromatic amino acids	(Lee et al., 2013)
KXG47847.1	AmdA	acetate induction	(Lints et al., 1995)
KXG47936.1	Hypothetical protein		
KXG47959.1	Hypothetical protein (RfeB)		
KXG48139.1	PrnA		

KXG48212.1	Homeodomain-like		
KXG48341.1	Activator of stress genes		
KXG48355.1	GATA transcription factor nreB		
KXG48533.1	Putative transcription factor kapC		
KXG48617.1	Protein of unknown function DUF3468		
KXG48682.1	Biofilm and cell wall regulator 1	Biofilm and cell wall	(Nobile and Mitchell, 2005)
KXG48765.1	Homeodomain		
KXG48781.1	Activator of stress genes 1	stress response	
KXG48784.1	Hypothetical protein		
KXG48884.1	AtfA	Spore germination	(Wolfers et al., 2015)
KXG48955.1	Hypothetical protein		
KXG48983.1	TATA-box binding protein		
KXG49138.1	Hypothetical protein		
KXG49157.1	facB	Regulation of genes involved in acetate utilization	(Todd et al., 1998)
KXG49345.1	acu-15	Positive regulator of acetate induction	(Bibbins et al., 2002)
KXG50499.1	carbon catabolite repressor		
KXG50601.1	Hypothetical protein (Sfp1)		
KXG50650.1	ARO80	catabolism of aromatic amino acids	(Lee et al., 2013)
KXG50787.1	transcription factor Prf		
KXG50810.1	HMG box protein		
KXG50911.1	Hypothetical protein		
KXG51030.1	cef1	pre-mRNA splicing and cell cycle control	(Kellner et al., 2014)
KXG51076.1	C6 transcription factor		
KXG51406.1	cellobiose response regulator 2	cellobiose response	

KXG51472.1	Heat shock factor (HSF)- type	Heat response	
KXG51622.1	Fungal transcriptional regulatory protein		
KXG51830.1	Hypothetical protein		
KXG52055.1	CCAAT-binding transcription factor, subunit B		
KXG52059.1	bZIP transcription factor FlbB		
KXG52166.1	C6 transcription factor		
KXG52228.1	Zinc finger, C2H2-like		
KXG52264.1	Basic-leucine zipper (bZIP) transcription factor		
KXG52366.1	Forkhead transcription factor (Sep1)		
KXG52406.1	Hypothetical protein		
KXG52464.1	DNA damage and replication checkpoint protein Rfx1		
KXG52761.1	Respiration factor		
KXG52955.1	Hypothetical protein		
KXG53008.1	stress response transcription factor SrrA/Skn7	response to oxidative stress	(Cao et al., 2009)
KXG53655.1	Acetamidase regulatory protein	catabolism of certain amides, omega amino acids and lactams	(Andrianopoulos and Hynes, 1990)
KXG53834.1	Homeodomain-like		
KXG53935.1	Cutinase transcription factor 1 beta		
KXG54406.1	MCM1	Induces mating, growth rate, pathogenesis and appressorium formation	(Zhou et al., 2011)
KXG54419.1	AreA	nitrogen response	(Katz et al., 2008)
KXG54528.1	Zinc finger, C2H2-like		

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KXG54658.1	Transcription factor		
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