

1 **A *Fusarium graminearum* strains-comparative proteomic approach identifies**
2 **regulatory changes triggered by agmatine.**

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14 **Abstract**

15 Plant pathogens face different environmental clues depending on the stage of the
16 infection cycle they are in. *Fusarium graminearum* infects small grain cereals
17 producing trichothecenes type B (TB) that act as virulence factor in the interaction
18 with the plant and have important food safety implications. This study addresses at the
19 proteomic level the effect of an environmental stimulus (such as the presence of a
20 polyamine like agmatine) possibly encountered by the fungus when it is already
21 within the plant. Because biological diversity affects the proteome significantly, a
22 multistrain (n=3) comparative approach was used to identify consistent effects caused
23 on the fungus by the nitrogen source (agmatine or glutamic acid). Proteomics analyses
24 were performed by the use of 2D-DIGE. Results showed that agmatine augmented TB
25 production but not equally in all strains. The polyamine reshaped drastically the

26 proteome of the fungus activating specific pathways linked to the translational control
27 within the cell. Chromatin restructuring, ribosomal regulations, protein and mRNA
28 processing enzymes were modulated by the agmatine stimulus as well as metabolic,
29 structural and virulence-related proteins, suggesting the need to reshape specifically
30 the fungal cell for TB production, a key step for the pathogen spread within the spike.

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35 **Keywords:** deoxynivalenol; 15-acetylated deoxynivalenol; nivalenol; 3-acetylated
36 deoxynivalenol; polyamine regulation; 2D-DIGE; strain diversity; glutamic acid.

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39 **Introduction**

40 Infection of cereals by *Fusarium* species can have an important impact on human and
41 animal health due to contamination of crops by trichothecenes [1], sesquiterpenes
42 compounds which have powerful protein synthesis inhibiting activity. *Fusarium*
43 *graminearum* s.s. [2] is a worldwide spread species belonging to the *Fusarium*
44 *graminearum* species complex (FGSC), and it represents the major cause of
45 trichothecene B (TB) accumulation in wheat and other small grain cereals. It has been
46 ranked as the 4th most important fungal pathogen in plants [3].

47 TB are synthesized by a gene cluster (*tri*) that, depending on its structure, can mainly
48 lead to production of deoxynivalenol (DON) and 15-acetylated DON (15ADON) or
49 DON and 3-acetylated DON (3ADON) or nivalenol (NIV) [4]. Within *F.*
50 *graminearum* a genetic chemotype can be defined by the genetic diversity within the

51 *tri* cluster [5]. As TBs differ for their toxicity, the major toxin produced by the fungus
52 has important implications for food safety and it becomes therefore important to
53 understand how fungal diversity affects the quality and the quantity of toxin
54 accumulation in the plant. Because the toxin acts also as a virulence factor in wheat
55 [6], understanding mechanisms of toxin regulation and pathogen adaptation to the
56 environment that triggers toxin production is important for both food safety and plant
57 protection purposes.

58 Polyamines play a diverse set of roles in every living organisms including
59 physiological responses to pathogens in plants [7]. In fungi they are involved in
60 metabolic and regulatory functions [8] as well as stress coping functions [9].
61 Moreover they are known to induce DON production in *F. graminearum* [10].
62 Inhibitors of polyamine import and synthesis have been proposed for limiting DON
63 production in *F. graminearum* [11].

64 A relatively large set of proteomic studies on *Fusarium graminearum* have been
65 carried out [12] but all focused on single strain analysis or on comparing mutants
66 obtained from the same isolate [13]. Because the effect of plant polyamines such as
67 agmatine on the proteome of this fungus has never been investigated before, here we
68 introduce a comparative experimental design that takes into account strain diversity,
69 including genetic chemotype diversity, to: 1) differentiate the core of proteins that are
70 induced by the selected *in vitro* conditions; and 2) describe the proteome profiles
71 which fluctuate strain-dependently.

72 Therefore this work shall contribute also to understand how fungal diversity plays a
73 role in modulating toxin synthesis when triggered by a plant derived compound [14].

74 A whole-cell 2D-DIGE proteomic study on three strains of *F. graminearum* s.s.
75 belonging to three different genetic chemotypes (15ADON, 3ADON, NIV) was

76 performed. Production of toxin was induced by addition to the fungal culture medium
77 of agmatine or glutamic acid as the sole nitrogen source. The comparison of the
78 proteomes obtained in the two media lead to the identification of a set of shared
79 regulatory processes triggered in the fungal cell by agmatine.

80

81 **Material and methods**

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84 *Growing conditions and phenotypic measures.*

85 Fungal material was grown in liquid cultures as described in [15]. In particular three
86 *F. graminearum* strains with diverse geographic origin were selected (453 [16],
87 NRLL28336 [17], Ph1 [18], **table 1**)

88 The mycelium was incubated in Erlenmeyer flasks containing 100 mL of a medium
89 having as the only nitrogen source glutamic acid or agmatine for 8 days (estimated to
90 be a stage where toxin is already abundant and at the same time when fungal growth
91 is still possible). The chemical composition of the media was the following: 30 g/L
92 sucrose, 2.0 g/L glutamic acid (or 1.15 g/L agmatine) [19], 1 g/L KH_2PO_4 , 0.5 g/L
93 $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.5 KCl, 10 mg $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ in 200 mL of trace elements solution
94 (per 100 mL: 5 g KCl, 5 g $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.25 g $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 50 mg $\text{MnSO}_4 \cdot$
95 H_2O , 50 mg H_3BO_3 , 50 mg $\text{NaMoO}_4 \cdot 2 \text{H}_2\text{O}$). Cultures were incubated in the dark,
96 150 rpm shaking at 22 °C for 8 days. The experiment was carried out with 4
97 independent biological replicates for each condition/strain.

98 For toxin analysis, the medium was filtered through a 0.2 µm GHP membrane filter
99 (PAL, MI USA) and diluted in methanol (medium/methanol, 9/1, V/V) in order to be
100 in the appropriate solvent ratio for chromatographic analysis. Toxin separation and

101 detection were achieved by LC coupled to tandem mass spectrometry (LC-MS/MS,
102 Dionex Ultimate 3000; Applied Biosystems API 3200) in multiple reaction
103 monitoring (MRM) in negative mode for DON, NIV and acetylated forms of toxins.
104 For separation, an Alltima HP RP-C18 column (Grace Davison, IL) was used
105 (150x2.1 mm; 3 μ m) with a mobile phase consisting of methanol and water with 2.5
106 mM of ammonium acetate in a linear gradient. All mycotoxins were quantified by
107 external calibration based on pure standards (Biopure, Tulln, Austria). The
108 differentiation of 3ADON and 15ADON was obtained by calculating the ratio of two
109 different selected fragment ions (397->337 and 397-> 307).

110 Ef-1alpha sequence of strain 453 was obtained following the protocol and the
111 procedure described in [20].

112 The pH value was checked daily in the flasks using colorimetric strips.

113 Carrying out an independent experiment with 5 biological replicates per each
114 strain/condition, a protocol for quantifying agmatine and glutamic acid in the medium
115 at 8 days was developed. The medium was filtered through a 0.2 μ m GHP membrane
116 filter (PAL, MI, USA) and diluted 20 times in ultrapure water. Ten μ L were then
117 evaporated to dryness under a N₂ flow at room temperature. The sample was re-
118 suspended in 100 μ L of BSTFA + TCMS, 99:1 / acetonitrile (50/50, v/v). The
119 derivatization was done during 1 hour at 60°C.

120 For separation and detection of analytes an Agilent 7890B gas chromatograph
121 coupled to a 5977A MSD detector (Agilent, Waldbronn, Germany) was used.
122 Instruments were controlled by the Mass Hunter software. A volume of 1 μ L of
123 derivatized sample was injected at 250°C in splitless mode. An HP 5MS column (30m
124 x 0.25mm, 0.25 μ m; Agilent) was operated at a constant helium flow of 1.2mL/min.
125 The initial oven temperature was set at 60°C. The oven was heated at 280°C

126 (10°C/min) and then at 325°C (40°C/min). This temperature was kept for 5 min. The
127 MSD interface was kept at 280°C. The source was kept at 230°C and the quadrupole
128 at 150°C. The detector was used in SIM mode. Effects of medium or strain was
129 measured by Kruskal-Wallis one-way ANOVA on Ranks with Dunn's method as
130 implemented in SigmaPlot (v 12.5). The same filtrates were also used to obtain
131 UV/Vis spectra using a Nanodrop 1000 (Thermo Scientific, USA) spectrophotometer.

132

133 *Proteomic analysis*

134 Full protein extraction was carried out as described in [21]. Briefly, mycelia were
135 ground with liquid nitrogen and extracted with ice-cold acetone containing 20% w/v
136 trichloroacetic acid (TCA) and 1% w/v dithiothreitol. Proteins were let to precipitate
137 overnight at -20 °C and then washed three times with ice-cold acetone.
138 Resolubilization of the precipitated proteins was carried out in lysis buffer (7 M urea,
139 2 M thiourea, 4% w/v CHAPS, 30 mM Tris, pH 8.5) containing protease inhibitor mix
140 (Roche) for 1 hour in a rotary shaker at room temperature. The protein extracts were
141 quantified using the Bradford method. 30 (thirty) µg of proteins for each sample (or
142 internal standard) were labelled with 240 pmol of fluorochromes (CyDyes™, GE
143 Healthcare) following the manufacturer's instructions.

144 Due to the presence of diverse pigmentation levels in the different strains, for the
145 following labelling step, the samples were divided in 3 groups, each one representing
146 one strain (4 biological replicates for each growing condition for each group giving a
147 total of 8 samples for each group and 24 samples for the whole experiment). One
148 internal standard was produced for each group. The four biological replicates were
149 labelled using the dye swap technique: 2 replicates of the same growing condition
150 were labelled with the Cy3 label and the other 2 replicates were labelled with the Cy5

151 label. The four gels belonging to the experimental groups 453 were obtained and
152 reported before in the proteomic map previously published [21]. A total of 12 gels
153 were compared, each gel containing two biological replicates of the strains used and
154 the respective internal standard, resulting in total protein load per gel of 90 μ g. IPG
155 buffer (Bio-Rad) and DeStreak reagent (GE Healthcare) were added to the mixed
156 samples and internal standard prior the loading on the strip. Strips were passively
157 rehydrated and proteins were loaded on 24 cm NL pH 3–10 IPG-strips (Bio-Rad) and
158 isoelectric focusing (at 22 C° till approximately 100000 Vh) was carried out with
159 IPG-phor system 3 (GE Healthcare). Strips were then kept in equilibration solution
160 with 1% w/v DTT for 15 min and then 2.5% w/v iodoacetamide for 15 min. The
161 second dimension was carried out with 12.5% polyacrylamide pre-cast gels
162 (Gelcompany) following manufacturer's instructions. Images were acquired using a
163 Typhoon9400 (GE Healthcare) and analyzed by DeCyder v.7.0 software (GE
164 Healthcare). After confirming lack of preferential labelling, exclusion filters and
165 manual detection of spots were applied to each gel in order to obtain the most
166 representative gel image. Gels were exported to the biological variation analysis
167 (BVA) module. Twenty spots were manually landmarked to allow the software to
168 perform inter-gel matching. Extensive manual spot matching was then done to ensure
169 correct matching of spots [22]. The EDA module allowed linking, standardizing and
170 comparing the different groups for the subsequent statistical analysis.

171 Spots considered to be consistent and reproducible (at least present in 75% of
172 biological replicates and with 1-way ANOVA p-value ≤ 0.05) were subjected to
173 statistical analysis. Within the same *F. graminearum* strain, mycelia grown in the
174 presence of agmatine were pairwise compared to those grown in the presence of
175 glutamic acid: spots resulting in a difference of at least $\pm 30\%$ and with a p-value (T-

176 Test) ≤ 0.05 were considered as spots of interest and selected for subsequent picking
177 and protein identification. In order to compare the effects of the strain and medium
178 and their interaction, 2-way ANOVA multivariate analysis was performed. Moreover
179 also spots resulting in a p-value ≤ 0.05 in at least one among 2-way ANOVA
180 *Fusarium* strain, 2-way ANOVA medium or 2-way ANOVA interaction were added
181 to the list of the spots of interest and selected for the subsequent picking and protein
182 identification.

183 Spots were picked from the gel mainly from the 453 map [21] and few random
184 verifications were carried out on the two master gels of the other experimental groups.
185 All picked spots were then digested by trypsin for 6 h at 37 °C using an Ettan Dalt
186 Spot Handling Workstation (GE Healthcare) before acquisition of peptide mass
187 spectra with a 4800 MALDI-TOF-TOF analyzer (ABSciex).

188 One MS spectrum accumulating 1500 laser shots in total was acquired and the highest
189 8 precursors, having a signal-to-noise ratio of more than 30, were automatically
190 selected for subsequent MS/MS analysis.

191 The MIPS *Fusarium graminearum* database v 3.2 was used for Mascot analysis using
192 a combined approach of protein mass fingerprint and MS/MS. Complete NCBI
193 proteins database check was also performed on all unknown proteins. All searches
194 were carried out using a mass window of 150 ppm for protein mass fingerprint and
195 0.75 Da for the MS/MS analysis of selected precursors. Up to two trypsin missed
196 cleavages were allowed. The search parameters allowed for carbamidomethylation of
197 cysteine (fixed modification), oxidation of methionine as well as oxidation of
198 tryptophan, tryptophan to kynurenine and double oxidation of tryptophan to N-
199 formylkynurenine (as variable modifications). Only identifications with a Mascot p-
200 value ≤ 0.05 were considered, manually checked and validated. Significance

201 threshold for the combined MOWSE score was ≥ 54 , while for fragmented peptides
202 the significance threshold was ≥ 30 . The overall list of selected protein species
203 identified which respected the above cited criteria of significance can be found in
204 [23]. Here with the purpose of comparing the two media effects, a sublist based on T-
205 test < 0.05 (glutamic acid vs agmatine medium) and abundance ratio $> \pm 1.3$
206 comparing the two media was generated. Further analysis of the protein lists
207 according to the biological processes (FunCat and GO) was carried out using MIPS
208 FunCat as implemented on the FungiFun webpage [24]. Significant
209 overrepresentation of categories was calculated using the Bejamini-Hochberg
210 procedure for correcting p-values. Protein species associated to the same gene name
211 were considered only for specific discussions within the manuscript. When no
212 specifications on the behaviour of different protein species associated to the same
213 FGSG number are given it has to be assumed that only a single protein species was
214 identified or that all isoforms behave identically.

215 Analysis of protein relationships and involvement in known biological processes was
216 done using String v.10 [25].

217 Unless otherwise specified, all chemical and reagents were purchased from Sigma-
218 Aldrich (Schnelldorf, DE).

219

220 **Results and discussion**

221 *Phenotypic observations*

222 The three strains were selected to account for morphological and genetic diversity
223 within *F. graminearum* s.s. They had diverse genetic and chemical chemotypes, as
224 well as diverse features on PDA plates (**Table 1, Fig. 1**). Growth of the three fungal
225 species occurred similarly in the two nitrogen sources, despite the coloration of the

226 mycelium differed among agmatine grown and glutamic acid grown cultures
227 confirming historical and recent reports showing the effect of nitrogen source on
228 pigmentation [26,27]. The colour is the result of the combination of different ratio of
229 compounds such aurofusarin, rubrofusarin, nor-rubrofusarin [28] and activation of
230 some but not all the genes in the pathway [29]. Type of pigments produced and their
231 intensity changed between strains and media (**Fig.1**). Small variations on colour
232 intensity were also visible among biological replicates but the effect was minor when
233 compared to the effect of the media or the strain (**Fig.1**). While 453 and Ph1 showed
234 an increased yellowing/browning pigmentation in glutamic acid with more pale-
235 orange/whitish mycelium in agmatine medium, NRRL28336 showed on average pale
236 mycelium in both media again with slight increase in the orange component in the
237 agmatine medium. Our results are consistent with other morphological reports
238 showing the effect of agmatine on mycelia colour [11]. The effect is not exclusively
239 linked to pH as the two media were both acidic, agmatine being the most acidic one at
240 8 days (due to the effect of fungal acidification of the media which dropped the pH
241 from 4 to 2) compared to glutamic acid that increased the pH from 2 to 4 in 8 days.
242 We also tested if pigments could be detected in the medium by UV/Vis
243 spectrophotometric comparison of the curves but no significantly different profiles
244 (UV/Vis) linked to a strain or a compound could be detected. (data not shown)
245 suggesting that changes induced by the nitrogen source acts on pigments that are
246 mainly in the mycelium compartments or that the simple spectrophotometric analysis
247 is not sufficiently sensitive.

248 No difference among strains was observed in the pH of the media suggesting that the
249 mechanism of acidification is not influenced by strain diversity while on the contrary
250 the toxin production was strain dependent. Indeed trichothecene type B production

251 differed significantly among strains and between the two media with the exception of
252 strain 453 which did not drastically shift its production of nivalenol and acetylated
253 form, maintaining in both cases a relatively low level of production (**Fig. 2**). This
254 confirms results obtained earlier in our laboratory that shows that some strains do not
255 respond to the agmatine stimulus for what concerns TB production [19]. On the
256 contrary Ph1 strain showed a 4-fold increase in toxin production while NRRL28336 a
257 1.5 fold increase confirming responses described in [10] which were verified on a
258 DON producing isolate. By measuring the two nitrogen sources in the media at 8 days
259 we could show that biological variability is the major cause of variability in the use of
260 nitrogen source, therefore no significant differences in the amount of nitrogen
261 consumption were observed among the strains and comparing the two media. This
262 suggests that the effects observed at the proteomic level cannot be simply explained
263 by a diversity in nitrogen concentration and availability (**supplementary figure 1**) .
264

265 *Proteomic profiles*

266 Three hundred eighty one identified unique protein species with ANOVA or 2-way
267 ANOVA p-value ≤ 0.05 were selected, corresponding to 189 FGSG numbers [23].
268 By PCA analysis the medium effect was evident. PC1 accounting for 52.8% was
269 mainly linked to the effect of the medium. PC2 and PC3 accounted for 14.3% and 11
270 % of the variance respectively, but could not be linked to any observable features.
271 90% cumulative variance was reached after 9 components (**Fig. 3A**). Treatments
272 could be separated on the PCA plot despite it is obvious that in some cases biological
273 variation in replicates included more variance than strain diversity.
274 By analysing the protein profile in the agmatine-containing medium, all the three
275 strains could be discriminated. Agmatine modifies the proteome of 453 (the NIV

276 strain) towards NRRL28336 (the 3ADON strain) separating the latest from Ph1 (**Fig.**
277 **3B**). Whether this data is linked to the toxin production level, Ph1 strain being by far
278 the first (and most induced) TB producer of the lot, can be hypothesized. This would
279 indicate that the proteomic profile reflects at least partially the ability and the cellular
280 reshaping needed to produce such a high amount of toxins. Agmatine contributes to
281 increase uniformity of the proteomic profiles among biological replicates as it can be
282 observed from the Euclidean distance tree. This finding suggests that a medium
283 containing agmatine would increase the possibility to identify diversity among strains
284 at the proteomic level diminishing intra-replicates variability (**Fig. 3B**).

285 Differentially regulated protein species (differing for at least 30% abundance) were
286 analysed by 2-way ANOVA: 320 protein species corresponding to 165 genes varied
287 due to the effect of the medium . These numbers included also isoforms that account
288 for half of the diversity observed.

289 Strain-dependent protein species shifts were 107, corresponding to 65 genes while
290 259 protein species (corresponding to 133 genes) fluctuated significantly due to the
291 interaction of strain and medium [23].

292 Strain diversity influenced the shift in abundance of protein species as suggested by
293 the number of significantly-regulated shared and unique spots identified for each
294 strain (**Fig. 4**).

295 In order to have reliable data on the core of the effects of the medium (agmatine vs
296 glutamic acid) on the fungal proteome we decided to focus only on concordant pattern
297 of abundance shared by all the strains..

298

299 *Comparing protein profiles to identify key mechanisms shared by strains.*

300 By selecting specifically only those spots that are consistently more abundant or less
301 abundant in all the three strains due to the medium effect, it is possible to identify
302 shared mechanisms which are common to the 3 strains when they are cultured in the
303 two different media. Therefore considering all the 3 strains as replicates of the same
304 experiment (12 replicates treated in agmatine and 12 treated in glutamic acid) a total
305 of 115 protein species were more abundant in agmatine (T- test \leq 0.05 and
306 abundance ratio \geq 1.3) and 133 in glutamic acid which corresponded respectively to
307 80 and 55 FGSG numbers (**supplementary table 1A**). Protein isoforms were detected
308 for 24 FGSG numbers when the strains were grown in glutamic acid and for 18 FGSG
309 numbers when grown in agmatine (**supplementary table 1B**). Eight FGSG numbers
310 coded for more than one isoform showing opposite behaviour for at least one of the
311 isoforms between the two media (**supplementary table 1C**). FunCat analysis showed
312 that three categories were overrepresented in agmatine more abundant proteins
313 (adjusted $p < 0.05$) (16.01, protein binding; 12.04, translation; 01.05.02.04, sugar,
314 glucoside, polyol and carboxylate anabolism), while 17 functional categories were
315 found to be significantly overrepresented in the set of proteins obtained from the
316 strains grown in glutamic acid medium (including 01.01.03.02.01, biosynthesis of
317 glutamate; 01.05.02.07, sugar, glucoside, polyol and carboxylate catabolism;
318 01.05.02.04, sugar, glucoside, polyol and carboxylate anabolism; 2.1, tricarboxylic-
319 acid pathway (citrate cycle, Krebs cycle, TCA cycle); 2.01, glycolysis and
320 gluconeogenesis; 02.13.03, aerobic respiration, 16.21.08, Fe/S binding; 2.11, electron
321 transport and membrane-associated energy conservation; 01.01.06.05.02, degradation
322 of methionine; 01.05.06.07 C-2 compound and organic acid catabolism)
323 (**supplementary table 2**).

324 If a very stringent approach of strain comparison is selected (by considering only
325 those proteins that shared significant values of abundance in all the three strains) a
326 total of 34 protein species (20 genes) were more abundant in glutamic acid (ratio
327 $>\pm 1.3$) and 36 protein species (27 genes) more abundant in agmatine medium (**Fig. 4**
328 **and supplementary table 3**).

329 We opted for a combined approach that guaranteed robustness of the data (having 12
330 biological replicates) and biological significance of the data trying to avoid too many
331 false negatives. Explicitly, when significant differences were identified considering
332 the 12 replicates for each medium we included in our analysis only those results that
333 showed identical trend for the 3 strains even if significance for each strain considered
334 independently was not achieved. Results are therefore discussed taking into account
335 only those proteins that are also listed in the **supplementary table 1A**. For the full set
336 of data which included also strain significant effects of the medium we refer to the
337 complete dataset [23].

338

339 ***Regulatory changes induced by agmatine***

340 Strikingly, more than half of the protein species which augmented in the agmatine
341 medium belonged to protein binding and translation categories suggesting a strong
342 regulatory shift that reshaped drastically the cell. Whether this reshaping process
343 mimics at least partially the *in planta* fungal specialization required to produce DON
344 and derivatives to spread in the spike [30] is our hypothesis, despite we are aware that
345 the resulting proteome is due to the direct effect of nitrogen source and to the
346 processing of the medium by the fungus.

347 The nature of the medium influences how light stimulates or decreases the growth rate
348 in fungi [31]. Here there is evidence to suggest that the type of nitrogen source

349 induced a differential regulation of circadian cycle controlled proteins despite no
350 difference in light condition was applied to the cultures. Indeed nine out of ten
351 isoforms of a proteins similar to ccg7 glyceraldehyde-3-phosphate dehydrogenase
352 (FGSG_16627), a clock controlled gene in *Neurospora* [32] as well as BLi3
353 homologous FGSG_17247 protein, which is activated by light but possibly co-
354 regulated by other mechanisms [33], had increased abundance in the agmatine
355 medium. At the same time different molecular species, all identified as glutamine
356 synthetase (FGSG_10264), a light responsive protein, were all less abundant in the
357 agmatine medium. Overall this differential abundance of light controlled proteins
358 confirms in *F. graminearum* the known overlapping regulation of inducible light
359 genes by nitrogen sources [34] and suggests further levels of regulation occurring on
360 light controlled proteins that are independent on nitrogen availability (**supplementary**
361 **figure 1**).

362 Recently, ribosomal regulation of stress related genes has been postulated by Barna
363 [35]. The process is putatively regulated by RPL40 that, in yeast, specifically controls
364 translation of 7% of total mRNA including specifically stress-response mRNAs [36].
365 As agmatine increased the abundance of FGSG_01956 protein (homologue of RPL40)
366 it is tempting to speculate that also in *F. graminearum* specific ribosomal mechanisms
367 of regulation are occurring. Together with FGSG_01956 also FGSG_02523
368 (interacting protein with role in microtubule stabilization) and FGSG_07292
369 (probable 40S protein S12), involved in translation and constituent of the ribosomes,
370 increased their abundance in agmatine supplemented medium, suggesting their
371 common participation in the ribosomal activity induced by agmatine. Similarly other
372 ribosome associated proteins involved in transcription activation such as RAP1

373 (FGSG_10905), co-expressed and interacting in yeast with FGSG_01008 (EFb1),
374 were more abundant when the strains were grown in the agmatine medium.

375 The increased abundance of two ubiquitin proteins (FGSG_01956 and FGSG_02029)
376 in the agmatine medium indicates a more consistent ubiquitin mediated protein
377 turnover. Other evidence for the protein turnover occurring in the agmatine medium is
378 the increased abundance of three isoforms of a cell signalling homologue of
379 cyclophilin B (FGSG_00777) known to accelerate protein folding [37] as well as the
380 increased amount of a proteasome constituent protein corresponding to gene
381 FGSG_01200. Also increased abundance of FGSG_07938 (related to RPN2 protein) a
382 proteasome regulatory protein suggests that agmatine medium induced protein
383 reshaping via proteasome processing.

384 Chromatin regulation seems to be implicated in the agmatine effect on the cells.
385 Indeed agmatine medium increased the abundance of FGSG_16147 protein,
386 homologue of TAF14, involved in negative regulation of chromatin silencing [38].
387 Indeed the role of histone deacetylation in the regulation induced by agmatine is
388 supported by the increased abundance of the NAD-dependent histone deacetylase
389 (FGSG_13552).

390 Another transcriptional regulator with increased abundance in the agmatine medium
391 in different isoforms was FGSG_03028, the homologue of UMrrm75 of *Ustilago*
392 *maidis* [39] whose increased amount at the mRNA level in filamentous growth and
393 low pH suggests a direct link with the *in vitro* conditions (confirmed by the lower pH
394 measured in the agmatine medium compared to the glutamic acid medium after 8
395 days).

396

397 Also mRNA turnover is probably actively regulated by agmatine, increasing the
398 abundance of some specific RNA binding and processing proteins. Protein coded by
399 FSGS_09864 gene (mRNA splicing factor) as well as 3 isoforms of the FGSG_11064
400 (glycin rich RNA binding protein implicated in positive regulation of translation and
401 reported to be upregulated at the gene transcriptional level in both Fusarium Head
402 blight (FHB) and crown rot (CR) [40]) as well as a probable LSM2 - Sm-like (Lsm)
403 protein (FGSG_00360) involved in pre-mRNA splicing and a probable BRT1 protein
404 (FGSG_00609) involved in regulation of translational reinitiation [41] were all more
405 abundant in the agmatine medium suggesting the activation of RNA processing.
406 Agmatine seems also to induce RNase T1 (FGSG_11190) that can be secreted [42] as
407 well as act an internal RNA processing protein. RNA turnover seems therefore
408 significantly affected by agmatine.

409

410

411 ***Metabolic and structural changes induced by agmatine***

412 As identified by the FunCat analysis, agmatine partially decreased the activity of
413 primary metabolism including Krebs cycle and TCA as well as respiration. This
414 changes found some confirmation in the available metabolomic and transcriptomic
415 study using agmatine [43] despite cultural conditions and sampling were different.
416 For example the decreased mRNA expression of pyruvate kinase observed by [43]
417 (associated to high level of pyruvic acid in the non-agmatine treated medium) well
418 correlates with our proteomic data, which showed a significant decreased abundance
419 of four different protein species of pyruvate kinase (FGSG_07528) in the agmatine
420 medium, associated to downregulation of the glycolytic cycle as measured by the
421 metabolomic study done by Suzuki et al. [43].

422 At the same time, probably to generate precursors for DON synthesis, fatty acid and
423 steroid synthesis such as acetoacetyl-coA thiolase (FGSG_09321) in 3 isoforms as
424 well as members of the farnesyl pyrophosphate pathway such as FGSG_09722
425 (probable isopentenyl-diphosphate delta-isomerase), precursor for DON synthesis,
426 were more abundant in the agmatine medium. Different isoforms of malate
427 dehydrogenases (FGSG_02461 and FGSG_02504), possibly involved in the
428 production of NADPH needed for oxidative stress balancing within the cell, were
429 more abundant in agmatine. The abundance of malate dehydrogenase is also in
430 accordance with the findings of Suzuki et al. [43] that, after supplementation of
431 agmatine to the medium, found high level of oxalate that are possibly due to
432 decreased abundance of oxalate decarboxylase observed at the protein level in the
433 agmatine medium (FGSG_06612). Moreover agmatine seems to control glutamate
434 dehydrogenase (FGSG_07174) diminishing its abundance as was already noted in
435 other eukaryotes [44].

436 Oxidative stress response related proteins differed significantly in the glutamic acid
437 and agmatine medium: SOD (Mn type) (FGSG_04454) was less abundant in the
438 agmatine medium. Catalases isoforms shifted strain-specifically without any
439 consistently significant effect due to the medium, while glutathione metabolism was
440 triggered by agmatine as suggested by the higher abundance of FGSG_13072
441 (glyoxylase 2) involved in the detoxification of methylglyoxal and other reactive
442 aldehydes as well as glutaredoxin (FGSG_01317) and URE2 (FGSG_02000) which
443 acts as glutathione peroxidases [45].

444 Inventories of secondary metabolites clusters have been generated in *F. graminearum*
445 [46,47]. By measuring the toxin we could only indirectly monitor the *tri* cluster
446 because no differentially abundant proteins of the cluster could be detected in our

447 experiment. This was probably due to the sensitivity of our proteomic technique. A
448 general regulator of secondary metabolite activation is glutamine synthetase
449 (FGSG_10264) which can control secondary metabolites production in the closely
450 related species *F. fujikuroi* [48]. Here different molecular species, all identified as
451 glutamine synthetase (multiple isoforms), showed all increased abundance in the
452 glutamic acid medium suggesting an occurring modulation of the secondary
453 metabolite production [48] in agreement with the increased abundance of some
454 proteins belonging to secondary metabolite clusters. Indeed two key enzymes in the
455 aurofusarin cluster were significantly more abundant in the glutamic acid medium.
456 These results are consistent with the different degrees of pigmentation observed in our
457 experiment and previously reported colouration of mutants
458 (http://www.rasmusfrandsen.dk/fusarium_mutants.htm). Interestingly FGSG_02325,
459 also belonging to the aurofusarin cluster, was more abundant upon agmatine
460 exposure. This suggests that the cluster is not uniformly regulated at the protein level
461 while those three genes were uniformly regulated by high nitrogen at the
462 transcriptional level [49]. Homologues of FGSG_02325 have been found in different
463 scaffolds in other fungal species [47], having diverse evolutionary, and possibly
464 regulatory, origins. Further studies are therefore welcome to further elucidate the
465 complex post transcriptional level of regulation of the aurofusarin cluster that can be
466 indeed modulated by the available nitrogen source [50].

467 A member of the butenolide cluster FGSG_08077 [51] was less abundant in agmatine
468 and was shown to be regulated by tri6 in *F. sporothrichiodes* and under opposite
469 regulation with DON in the wild type and the *mgvkinase1* knockout mutant [52].
470 Assuming that the increased DON production is the result of increased activity of
471 proteins involved in its synthesis, we can confirm that in our study we observed that

472 agmatine is modulating differentially secondary metabolite clusters favouring DON
473 and for example repressing butenolide.

474 This specific modulation can be linked to specific need of the pathogen to adapt its
475 development during different ecological stages. Interestingly rubrofusarin has
476 previously been described to have antifungal properties [53] that can be important
477 during growth as saprophyte or before head colonization but not essential when, after
478 infection, the pathogen needs to invade the plant tissue, requiring a timely and
479 significant amount of DON production. Similarly butenolide was suggested to play an
480 ecological role to protect the source of food of the infecting fungus against bacteria
481 and other organisms [51].

482 Ectophosphatases (like FGSG_07678) do show a wide array of glycosylation and
483 other modifications and are thought to be involved in host-microorganism interaction
484 and establishment of the infection [56]. Interestingly all the protein species identified
485 as FSGS_07678 were less abundant in the agmatine medium (**Fig. 5A**). Similarly,
486 secreted and structural fungispumin like FGSG_08122, similar to phiA protein from
487 *Aspergillus* [57], potentially playing a role in coping with the host environment was
488 less abundant in the agmatine medium. Interestingly this 2 FGSG numbers showed
489 opposite behaviour at the mRNA level being upregulated in the agmatine medium (at
490 4 days) [58]. A RNA binding protein (FGSG_08421) member of a putative network
491 of likely-virulence factors [59] involved in RNA stability was also less abundant in
492 the agmatine medium as well as two isoforms of CAP20 gene homologue
493 (FGSG_05177) which is a pathogenicity gene in *Colletotrichum* [60] and was found
494 more abundantly on a proteomic study carried out on plant derived material [61]. A
495 pathogenicity as well as stress related gene (FGSG_08737), Hex 1, a precursor of
496 woronin body [62] showed extensive PTM regulation comparing the two media.

497 Further studies on the functional protein modifications occurring on the main
498 constituent of the woronin body may help elucidating the effect of post transcriptional
499 modifications determined by agmatine on proteins with double role in the cell. (**Fig.**
500 **5B**).

501 Structural as well as secondary metabolites modulation caused by agmatine would
502 favour the hypothesis that agmatine determines a restructuring of the cell towards a
503 specialized configuration [63] which includes cell wall reshaping, diminishing
504 activities that are specific for initial step of infection or for environmental competition
505 which are not essential for the interaction with the host at a stage where the “toxin
506 weapon” need to be released to further invade the host. The identification of bulbous
507 structures [63] that were also observed in different abundance in all the three isolates
508 grown in agmatine in our study (**supplementary figure 2**) is probably the *in vitro*
509 phenotypic manifestation of this process. Whether agmatine (or polyamines) is the
510 triggering factor for structural changes necessary for the in planta interaction [64]
511 remains to be investigated. The concentrations used in these study are within the
512 range of reported agmatine concentration that can be found in wheat apical parts
513 [65,66] supporting the physiological value of the study.

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518 **Conclusion**

519 Strain diversity using proteomics has been seldomly explored [67,68]. Here we
520 proposed to strengthen proteomic data by using multiple strains to study the effect of
521 the nitrogen source on the proteome of the fungus. We successfully identified shared

522 mechanisms of regulation induced by agmatine (**Fig. 6**) in all the strains which differ
523 for geographic origin, genetic chemotype, morphology and date of isolation. Our
524 work allowed to identify novel candidates for functional analysis that would put in
525 relation the regulatory phenomena induced by agmatine with the ability of the fungus
526 to adapt to the stage of toxin production *in planta*. With our study we showed the
527 usefulness of exploring strain proteomic diversity within the species not only to
528 characterize the level of diversity among strains with different phenotypic
529 manifestations [69] but also to facilitate the process of inferring general biological
530 mechanisms by identifying shared biological processes among the strains.

531 Our study suggests also that strain variability can be the cause of some discordant
532 results among laboratories using different strains, as 8% of the protein species showed
533 opposite abundance ratio in the 2 media among strains [23]. As the cost of *omics*
534 experiments is dropping, experimental designs should possibly include strain diversity
535 1) as a procedure to validate proteomic findings and; 2) as an exploratory tool to
536 understand the level of diversity within a species.

537 We are aware that by sampling a single time-point at a late cultural stage we cannot
538 exclusively attribute the effects observed on the proteome to the agmatine
539 supplementation. The proteome profile is the result of the nitrogen supplementation
540 and the transformations of the metabolites that are changing the medium for the 8
541 days of culture. Nonetheless it is evident that the quality of nitrogen source (and not
542 the amount), being the only factor changing in our experiment, is the original cause of
543 the shifts. The lack of notable agreement with a microarray data performed at 4 days
544 growth stage [58], (**supplementary table 4**) confirms previous findings in
545 filamentous fungi that suggested that at least 60% of differences in the protein profiles
546 are not linked to mRNA abundance [70].

547 The drastic change of the proteomic fungal profile as well as the phenotype suggests
548 that agmatine is affecting deeply cellular processes in the fungal cell. It is tempting to
549 hypothesise an ecological role of agmatine (a polyamine) that potentially determines
550 the specialization of structures necessary for massive toxin production that ultimately
551 lead to complete reshaping of the fungal cell. Gardiner et al [14] showed that there is
552 no direct correlation between the amount of polyamines in wheat cultivars and the
553 toxin accumulation, but was unable to measure agmatine in the plant. Indeed toxin
554 accumulation *in planta* depends on multiple factors including how the plant responds
555 to infection and copes with toxin. The fact that, at least *in vitro*, the fungus changes
556 drastically its status, indicates that targeting agmatine sensors can be a way to tackle a
557 crucial step in the infection process of the fungus [11].

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770

771 **Figure legends**

772

773 **Fig 1.** Pigmentation of the mycelium differs among strains and media.

774 Mycelium phenotype when grown on PDA for 7 days or before protein extraction
775 after growth for 8 days in liquid medium (see MM) containing as sole nitrogen source
776 glutamic acid or agmatine.

777

778 **Fig. 2.** Agmatine boosts TBs production in a strain specific manner.

779 Overall trichothecene type B production (expressed in nanograms per mL of liquid
780 medium) by the three strains measured at the end of the 8th incubation day when
781 grown on medium containing agmatine or glutamic acid as unique nitrogen source.

782 The values are the result of 4 biological measures. SD is indicated. Significant

783 differences within the same isolate are indicated with asterisc (p<0.01).

784

785 **Fig. 3.** Agmatine medium better separates the strains reducing biological variation
786 among replicates.
787 (A).PCA performed on all the 381 proteins with single identification and ANOVA <=
788 0.05. PC1 (related to medium effect) accounts for 52.8% while PC2 for 14.3%; (B)
789 Hierarchical clustering using on both dimensions Euclidean distance and complete
790 linkage performed on all the 381 proteins with single identification (ANOVA <=
791 0.05).

792

793 **Fig. 4.** Each strain has a unique proteomic profile.
794 Modified Venn diagram showing the number of shared and unique protein species
795 that are respectively most abundant in agmatine or in glutamic acid medium for each
796 of the three strains used.

797

798 **Fig. 5.** Localization of protein species on the gel.

799 A. Phosphatase FGSG_07678 protein species identified in this experiment.

800 Numbers refer to the ID number on the 453 proteomic map.

801 B. Multiple isoforms of FGSG_08737, a precursor of woronin bodies implicated
802 in different mechanisms within the cell. Numbers refer to the ID number on
803 the 453 proteomic map.

804

805 **Fig. 6.** The effects of agmatine on the fungal cell.

806 Hypothetical model of the mechanisms of regulation induced by the agmatine
807 medium. The comparison is done versus glutamic acid medium which is a standard
808 nitrogen source used to induce toxins *in vitro*. In red the increased abundance and in

809 blue the decreased abundance caused by agmatine of proteins identified in this work
810 and associated to cell functions and activities.