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

Pasquali M.*, Serchi T., Cocco E., Leclercq, C., Planchon, S., Guignard, C., Renaut J., Hoffmann L.

Department of Environmental Research and Innovation, Luxembourg Institute of Science and Technology, 41, rue du Brill, L-4422 Belvaux, Luxembourg

*corresponding author: matias.pasquali@list.lu

Abstract

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

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

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

Introduction

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

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

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

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

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

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

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

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

Material and methods

Growing conditions and phenotypic measures.

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

The mycelium was incubated in Erlenmeyer flasks containing 100 mL of a medium having as the only nitrogen source glutamic acid or agmatine for 8 days (estimated to be a stage where toxin is already abundant and at the same time when fungal growth is still possible). The chemical composition of the media was the following: 30 g/L sucrose, 2.0 g/L glutamic acid (or 1.15 g/L agmatine) [19], 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7\ H_2O$, 0.5 KCl, 10 mg $FeSO_4 \cdot 7\ H_2O$ in 200 mL of trace elements solution (per 100 mL: 5 g KCl, 5 g $ZnSO_4 \cdot 7\ H_2O$, 0.25 g $CuSO_4 \cdot 5\ H_2O$, 50 mg $MnSO_4 \cdot H_2O$, 50 mg H_3BO_3, 50 mg $NaMoO_4 \cdot 2\ H_2O$). Cultures were incubated in the dark, 150 rpm shaking at 22 °C for 8 days. The experiment was carried out with 4 independent biological replicates for each condition/strain.

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

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

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

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

Carrying out an independent experiment with 5 biological replicates per each strain/condition, a protocol for quantifying agmatine and glutamic acid in the medium at 8 days was developed. The medium was filtered through a 0.2 µm GHP membrane filter (PAL, MI, USA) and diluted 20 times in ultrapure water. Ten µL were then evaporated to dryness under a N_2 flow at room temperature. The sample was re-suspended in 100µL of BSTFA + TCMS, 99:1 / acetonitrile (50/50, v/v). The derivatization was done during 1 hour at 60°C.

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

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

Proteomic analysis

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

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

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

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

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

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

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

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

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

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

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

Results and discussion

Phenotypic observations

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

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

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

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

Proteomic profiles

Three hundred eighty one identified unique protein species with ANOVA or 2-way ANOVA p-value <= 0.05 were selected, corresponding to 189 FGSG numbers [23]. By PCA analysis the medium effect was evident. PC1 accounting for 52.8% was mainly linked to the effect of the medium. PC2 and PC3 accounted for 14.3% and 11 % of the variance respectively, but could not be linked to any observable features. 90% cumulative variance was reached after 9 components (F**ig. 3A**). Treatments could be separated on the PCA plot despite it is obvious that in some cases biological variation in replicates included more variance than strain diversity.

By analysing the protein profile in the agmatine-containing medium, all the three strains could be discriminated. Agmatine modifies the proteome of 453 (the NIV

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

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

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

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

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

Comparing protein profiles to identify key mechanisms shared by strains.

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

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

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

Regulatory changes induced by agmatine

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

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

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

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

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

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

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

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

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

Metabolic and structural changes induced by agmatine

As identified by the FunCat analysis, agmatine partially decreased the activity of primary metabolism including Krebs cycle and TCA as well as respiration. This changes found some confirmation in the available metabolomic and transcriptomic study using agmatine [43] despite cultural conditions and sampling were different. For example the decreased mRNA expression of pyruvate kinase observed by [43] (associated to high level of pyruvic acid in the non-agmatine treated medium) well correlates with our proteomic data, which showed a significant decreased abundance of four different protein species of pyruvate kinase (FGSG_07528) in the agmatine medium, associated to downregulation of the glycolytic cycle as measured by the 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 (glioxylase 2) involved in the detoxification of methylglioxal 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 availbale 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

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

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

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

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

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

Conclusion

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

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

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

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

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

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Figure legends

Fig 1. Pigmentation of the mycelium differs among strains and media.
Mycelium phenotype when grown on PDA for 7 days or before protein extraction after growth for 8 days in liquid medium (see MM) containing as sole nitrogen source glutamic acid or agmatine.

Fig. 2. Agmatine boosts TBs production in a strain specific manner.
Overall trichothecene type B production (expressed in nanograms per mL of liquid medium) by the three strains measured at the end of the 8^{th} incubation day when grown on medium containing agmatine or glutamic acid as unique nitrogen source. The values are the result of 4 biological measures. SD is indicated. Significant differences within the same isolate are indicated with asterisc ($p<0.01$).

Fig. 3. Agmatine medium better separates the strains reducing biological variation among replicates.

(A).PCA performed on all the 381 proteins with single identification and ANOVA <= 0.05. PC1 (related to medium effect) accounts for 52.8% while PC2 for 14.3%; (B) Hierarchical clustering using on both dimensions Euclidean distance and complete linkage performed on all the 381 proteins with single identification (ANOVA <= 0.05).

Fig. 4. Each strain has a unique proteomic profile.

Modified Venn diagram showing the number of shared and unique protein species that are respectively most abundant in agmatine or in glutamic acid medium for each of the three strains used.

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

A. Phosphatase FGSG_07678 protein species identified in this experiment. Numbers refer to the ID number on the 453 proteomic map.
B. Multiple isoforms of FGSG_08737, a precursor of woronin bodies implicated in different mechanisms within the cell. Numbers refer to the ID number on the 453 proteomic map.

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

Hypothetical model of the mechanisms of regulation induced by the agmatine medium. The comparison is done versus glutamic acid medium which is a standard 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.