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# The effect of agmatine on trichothecene type B and zearalenone production in *Fusarium graminearum*, *F. culmorum* and *F. poae*

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Agmatine and other putrescines are known strong inducers of deoxynivalenol (DON) production in *Fusarium graminearum*. Other important cereal species produce DON and/or other trichothecene type B toxins (3 acetylated DON, 15 Acetylated DON, Fusarenon-X, Nivalenol) such as *F. culmorum* and *F. poae*. In order to verify if the mechanism of regulation of trichothecenes type B induction by agmatine is shared by different species of *Fusarium*, we tested the hypothesis on 19 strains belonging to 3 *Fusarium* species (*F. graminearum*, *F. culmorum*, *F. poae*) with diverse genetic chemotypes (3ADON, 15ADON, NIV) by measuring trichothecene B toxins such as DON, NIV, Fusarenon-X, 3ADON and 15ADON. Moreover we also tested whether other toxins like zearalenone were also boosted by agmatine. The trichothecene type B boosting effect was observed in the majority of strains used (13 over 19) in all the three species. Representative strains from all the three genetic chemotypes were able to boost toxin production after agmatine treatment. We identified strains not responding to the agmatine stimulus which may contribute to deciphering the regulatory mechanisms that link toxin production to agmatine (and more in general polyamines).

**The effect of agmatine on trichothecene type B and zearalenone production in *Fusarium graminearum*, *F. culmorum* and *F. poae*.**

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**Abstract:** Agmatine and other putrescines are known strong inducers of deoxynivalenol (DON) production in *Fusarium graminearum*. Other important cereal species produce DON and/or other trichothecene type B toxins (3 acetylated DON, 15 Acetylated DON, Fusarenon-X, Nivalenol) such as *F. culmorum* and *F. poae*. In order to verify if the mechanism of regulation of trichothecenes type B induction by agmatine is shared by different species of *Fusarium*, we tested the hypothesis on 19 strains belonging to 3 *Fusarium* species (*F. graminearum*, *F. culmorum*, *F. poae*) with diverse genetic chemotypes (3ADON, 15ADON, NIV) by measuring trichothecene B toxins such as DON, NIV, Fusarenon-X, 3ADON and 15ADON. Moreover we also tested whether other toxins like zearalenone were also boosted by agmatine. The trichothecene type B boosting effect was observed in the majority of strains used (13 over 19) in all the three species.

Representative strains from all the three genetic chemotypes were able to boost toxin production after agmatine treatment. We identified strains not responding to the agmatine stimulus which may contribute to deciphering the regulatory mechanisms that link toxin production to agmatine (and more in general polyamines).

**Keywords:** *Fusarenon-x*; deoxynivalenol; Nivalenol; 3-acetylated deoxynivalenol; 15-acetylated deoxynivalenol, zearalenone; toxin induction.

## 1. Introduction

Mycotoxin regulation mechanisms leading to accumulation in the plant and consequently in grains used for human and animal consumption are still partially unknown. Nutrients and specific molecules are supposed to play a key inducing role in activating toxin pathways in plants. *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein.) Petch], *Fusarium culmorum* (WG Smith) Sacc. and *Fusarium poae* (Peck) Wollenw. are the main species associated with trichothecene type B (TB) production in Fusarium Head Blight (FHB) on wheat in different agricultural areas in the world. Most important Fusarium TB are deoxynivalenol (DON), whose amount is under control in food and feed in many countries, 3-acetylated DON (3ADON), 15-acetylated DON (15ADON), Nivalenol (NIV) and Fusarenol X (FUSX). *F. graminearum* and *F. culmorum* can also produce Zearalenone (ZEA) that is also a legislatively regulated toxin in food and feed due to its estrogenic effect on humans and animals.

Each *Fusarium* strain is able to produce some of these toxins depending on the set of genes present in the genome (Proctor et al., 2009) and the triggering factors leading to toxin production. Because toxigenic risk in food depends also on the type of fungal population colonizing the plant and to its toxigenic potential, lot of efforts have been devoted to studying the epidemiology of different chemotypes worldwide (Pasquali and Migheli, 2014), using genetic means to discriminate populations that are more or less toxigenic (von der Ohe et al., 2011). Chemical determination of chemotypes is still an important approach to confirm genetic analysis based on gene polymorphisms (Desjardins, 2008); therefore liquid media able to stimulate toxin production have been widely employed for determining toxin potential of isolates.

Mechanisms of toxin induction in *F. graminearum* include sugar types (Jiao et al., 2008), pH effects (Gardiner et al., 2009; Merhej et al., 2010), inorganic compounds (Tsuyuki et al., 2011; Pinson-Gadais et al., 2008), oxidative stress (Ponts et al., 2007), fungicides (Magan et al 2008), light (Kim et al., 2014), water activity levels (Llorens et al., 2004; Schmidt-Heydt et al., 2011) and have been linked also to chemotype diversity (Ponts et al., 2009). *F. graminearum* boosts toxin production when grown in medium with agmatine and other putrescines to levels that are comparable to high contaminations observed *in planta* (Gardiner et al., 2009b). Hypotheses on the role of polyamines *in planta* as a cue for production of trichothecene mycotoxins by *F. graminearum* during FHB disease have been formulated (Gardiner et al., 2010) and the use of inhibitors of polyamine transport in the fungal cells have been proposed as a potential novel approach to limit toxin contamination in grains (Crespo-Sempere et al., 2015). Studying the effect of agmatine on other species can elucidate if this mechanism is effective also in other *Fusarium* species that are often found to coexist in agricultural settings (Giraud et al., 2010), directly contributing to toxin accumulation (Beyer et al., 2014). Because the colonization of the

plant by the fungus and the resulting production of toxins are the outcome of the interaction of the environment, the fungus and the plant, here we focused on how fungal diversity is affected by a putative plant derived inducer of toxin production. We therefore tested the level of toxin induction in relation to fungal diversity in 3 *Fusarium* species when confronted with 2 standardized media which are mild and strong inducers of toxin synthesis.

The aims of the work were therefore to evaluate the toxigenic potential *in vitro* of a set of isolates and; to analyse the effect of agmatine as toxin inducing compounds across species and chemotypes.

## Material and methods

### 2.1 Isolates and growth conditions

Isolates used in this study are listed in table 1. They have different geographic origin and belong to different genetic chemotypes as determined in our laboratory (Pasquali et al., 2011). Strains are conserved in the mycological collection of the Luxembourg Institute of Science and Technology. All isolates were grown on V8 (V8 juice 20%, CaCO<sub>3</sub> 2 g, 18 g agar, H<sub>2</sub>O to 1 L) plates for 4 days at 20 °C in the dark. The growing edge of the mycelium was divided into 2 mm squares and one square each was used to inoculate 10 ml media in sterile glass tubes. The assay was carried out with three completely independent biological replicates.

In order to analyse toxin induction, 2 media were used and compared. The first medium (J), from Jiao et al., 2008 (containing 1 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g KCl, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 g L-glutamic acid, 10 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 10 g sucrose in a liter of solution) was compared to a medium (G) from

Gardiner et al., (2009b), containing (30 g sucrose, 1.15 g Agmatine, 1 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g KCl, 10 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  in a liter solution). The two media have approximately the same C/N ratio which is known to play a role in toxin regulation (Hestbjerg et al., 2002) but differ for the source of nitrogen and the amount of carbon source. Tubes were shaken at 180 rpm in the dark for 10 d. Fungal biomass was then filtered and weighted with a precision balance after drying using a freeze drier for 48 h, and the remaining media kept for further toxin extraction.

## 2.2 Chemical analysis

The medium was filtered through a 0.2  $\mu\text{m}$  GHP membrane filter (PAL, MI, USA) and diluted in methanol (extract/methanol, 9/1, V/V) in order to be in the appropriate solvent ratio for chromatographic analysis. The quantification of all mycotoxins except 3-ADON and 15-ADON was performed by LC coupled to tandem mass spectrometry (LC-MS/MS, Dionex Ultimate 3000, AB/Sciex API 3200, Foster City, CA, USA) in multiple reaction monitoring (MRM) in positive/negative switching mode. The LC analytical column was an Agilent Zorbax Eclipse Plus  $\text{C}_{18}$  (2.1X 150mm, 3.5 $\mu\text{m}$ ) with a mobile phase consisting of methanol and water containing 2.5 mM of ammonium acetate in a linear gradient. The specific analysis of 3-ADON and 15-ADON was achieved using a second LC-MS/MS method on an Agilent 1260 LC coupled to an AB/Sciex 4500 QTrap mass spectrometer. The column was an Agilent Poroshell 120 EC- $\text{C}_{18}$  (2.1 x 150 mm, 2.7 $\mu\text{m}$ ) and the eluents were the same as for the first method. All mycotoxins were quantified by external calibration based on pure standards (Biopure, Tulln, AT and Sigma-Aldrich, Schnellendorf, DE). The detection and quantification limits were 1.5 ng/ml of liquid culture for all toxins. The analyses were done in two technical replicates and average value was considered for each biological replicate.

### 2.3 Statistical analysis

All data were analysed using PASW version 19 and SigmaPlot version 12.5. Overall TB production was calculated summing up all trichothecenes type B measured (DON, 3ADON, 15ADON, NIV, FUS-X). Mass-corrected toxin concentrations were calculated and used for determining the effect of nitrogen source on toxin boosting. Mann Whitney Rank Sum test was used to verify the effect of the medium on masses and the effect of nitrogen source in the medium on mass corrected summed trichothecene type B values. Kruskal-Wallis One Way Analysis of Variance on Ranks was used to verify the effect of the medium on the average ratio of TB production in the two media for each strain classified either as species or as chemotype. Significant differences are considered when  $p < 0.05$ .

## 3. Results and Discussion

Growth (dry mass) and toxin production using a multimethod assay which included DON, 3ADON, 15ADON, NIV, FUS-X, ZEA, T2, HT2 were measured on a set of 19 strains (table 1) when grown in the two media. Mycelium growth (estimated as dry mass) was influenced by the medium increasing, as expected, in the high containing saccharose medium ( $p < 0.001$ ). Strains' mass in glutamic acid as nitrogen source containing medium ranged from 3.4 mg to 19.6 mg, while in agmatine/saccharose rich medium masses ranged from 8.9 to 36.1 mg. Growth results are comparable with results obtained by Jiao et al., (2008) for the medium with glutamic acid. We observed that FG and FC had similar range of growth in each medium while FP differed significantly in growth compared to the other two species ( $p > 0.05$ ). No effect of chemotype

( $p=0.328$ ) could be identified. In all the three species there was nonetheless a significant effect of growth caused by the medium being the agmatine/high saccharose medium a booster of mass growth ( $p>0.001$  for all the three comparisons, figure 1). The overall increase in dry mass caused by the agmatine medium can be attributed to the higher amount of saccharose in the medium, carbon being the main constituent of fungal biomass (Newell et al., 1982). To evaluate toxin production we therefore normalized the toxin produced per ml by the dry mass.

The two sources of nitrogen had an overall significant effect on trichothecenes type B mass corrected production across the three species ( $p= 0.003$ ). Agmatine medium induced higher TB production in 13 over 19 strains. Six isolates produced an average of TB toxins in ng/ml corrected by their mass in mg above 150 ng/ml/mg (*F. culmorum* 233, 13-01; *F. graminearum* 734, 37099 and MUCL42825 and *F. poae* 80). All these isolates increased their production in the agmatine containing medium with a boosting factor ranging between 192X of 13-01 to 15X of isolate 80. Four isolates (01-02, 557, MUCL15500, UMW00706) had an opposite behaviour when grown in the agmatine containing medium (figure 2).

We could also confirm that strains with a determined genetic chemotype can also produce a minor amount of the other acetylated and non-acetylated trichothecenes (De Kuppler et al., 2011). Nonetheless there was a good correspondence between the genetic chemotype and the major produced toxin type (Supplementary table 1).

By analyzing average ratio of toxin production in the isolates according to their species or their chemotype we could not detect a clear effect of any of the two categories. Infact the response of toxin production to the two media was not significantly affected by the species ( $p=0.552$ ) nor by the chemotype ( $P = 0.578$ ). Previous hypothesis suggesting differences at the chemotype level on toxin regulation (Ponts et al., 2009), may have been biased by the limited number of strains used.

ZEA production, in the only consistent producer of the lot (*F. culmorum* 01-02), doubled in the agmatine medium suggesting that the mechanism boosting TB production affects potentially also ZEA production. This would imply that agmatine regulation of ZEA and of the trichothecene cluster is common, despite the gene expression data from available microarray studies do not show consistent concordant patterns (Sieber et al., 2014). A larger set of isolates producing ZEA is nonetheless needed to verify this hypothesis.

In our analysis we included also two food and feed monitored trichothecene type A (TA) toxins but no isolates of *F. poae* produced any detectable amount of T2 and HT-2. As the biosynthesis of TAs follows a similar pathway (Kimura et al., 2007) we can expect similar effects due to agmatine on TA that nonetheless we could not confirm with the isolates used in our study.

#### 4. Conclusions

The effect of 2 different media containing agmatine or glutamic acid on toxin induction and growth of a total of nineteen strains belonging to *F. graminearum* ( $n=7$ ), *F. culmorum* ( $n=8$ ), *F. poae* ( $n=4$ ) species was evaluated. With our species comparative study we confirmed that on average the mechanism of triggering toxin production by agmatine is confirmed in the 3 species but that the mechanism is also significantly influenced by strain diversity. The identification of agmatine non-responding isolates may help deciphering the pathways leading to specific agmatine regulation in the fungus. Despite these data were obtained by culturing the fungus *in vitro*, they can contribute to explain the partial diversity of toxin contamination observed *in planta*: indeed, on average, different cultivars accumulate different levels of DON (Ji et al., 2015). Hypotheses on the effect of the plant oxidative status (Waśkiewicz et al., 2014) and of polyamine concentration on toxin synthesis in planta have been formulated (Gardiner et al., 2010). Here we showed that also strain diversity can account for a factor 1000X in the level of

toxin accumulation. Exploring the diversity of interactions between strains and toxin triggering compounds such as polyamines (Valdes Santiago et al., 2012) is fundamental to identify general inhibitors of TB accumulation in the plant.

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275 **Figure 1. Dry mycelium mass measured in the two media in the three species.**

276 Mass in mg of dried mycelium grown 10 days in the two media (Gardiner medium  
277 containing agmatine; Jiao medium containing glutamic acid). Asterisks indicate significant  
278 differences using Mann-Whitney rank test ( $p < 0.05$ ).

279 **Figure 2. Toxins ratio comparing the two media.**

280 Log 10 transformed average ratio of TB production in agmatine vs glutamic acid medium.

281 Values are the sum of all trichothecene type B detected (DON, 3ADON, 15ADON, NIV,

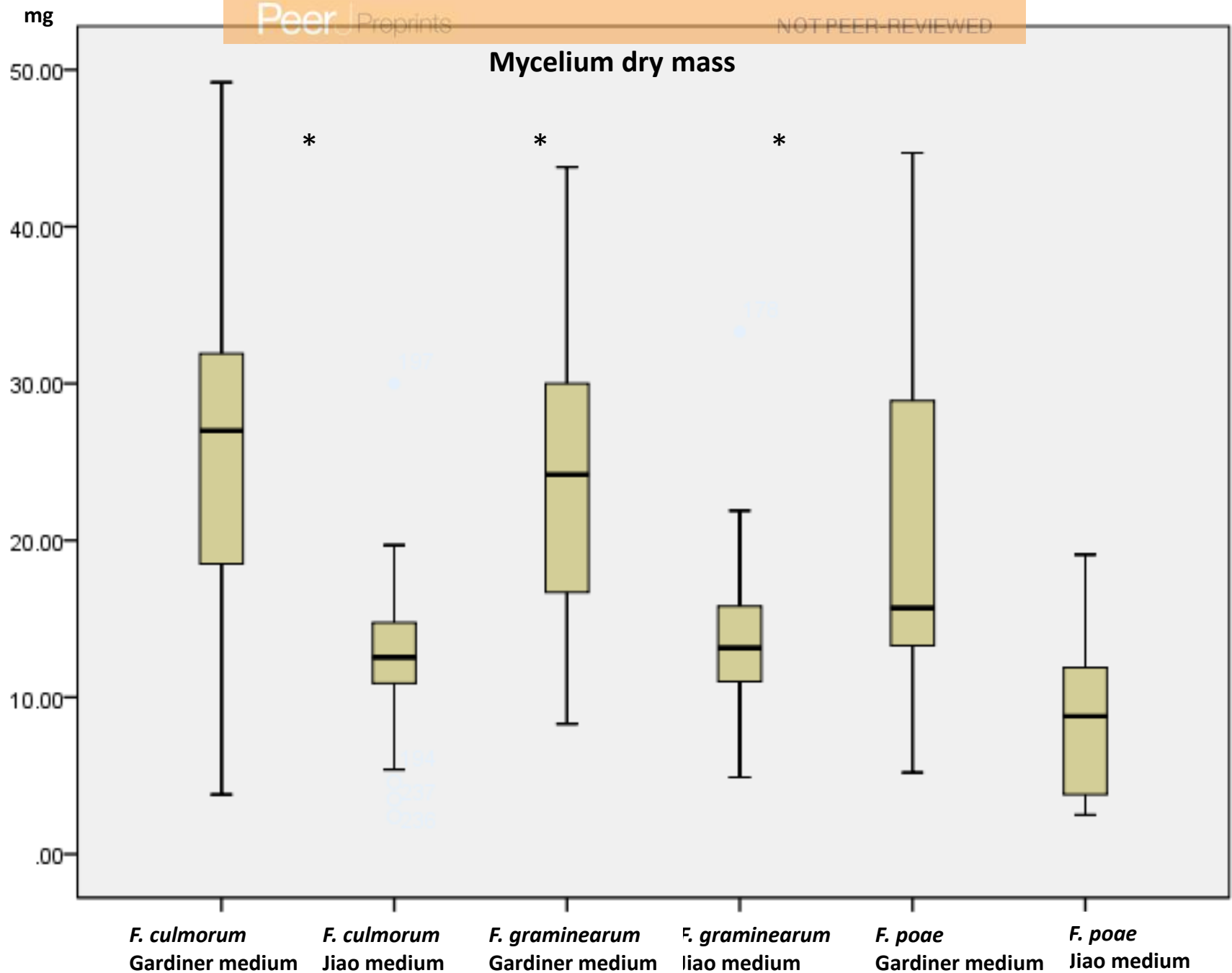
282 FUSX). In red, isolates that have higher amount of TB in the glutamic acid medium while  
 283 in blue those that have higher amount in the agmatine medium. .

284

# **Figure 1**(on next page)

Dry mycelium mass measured in the two media in the three species.

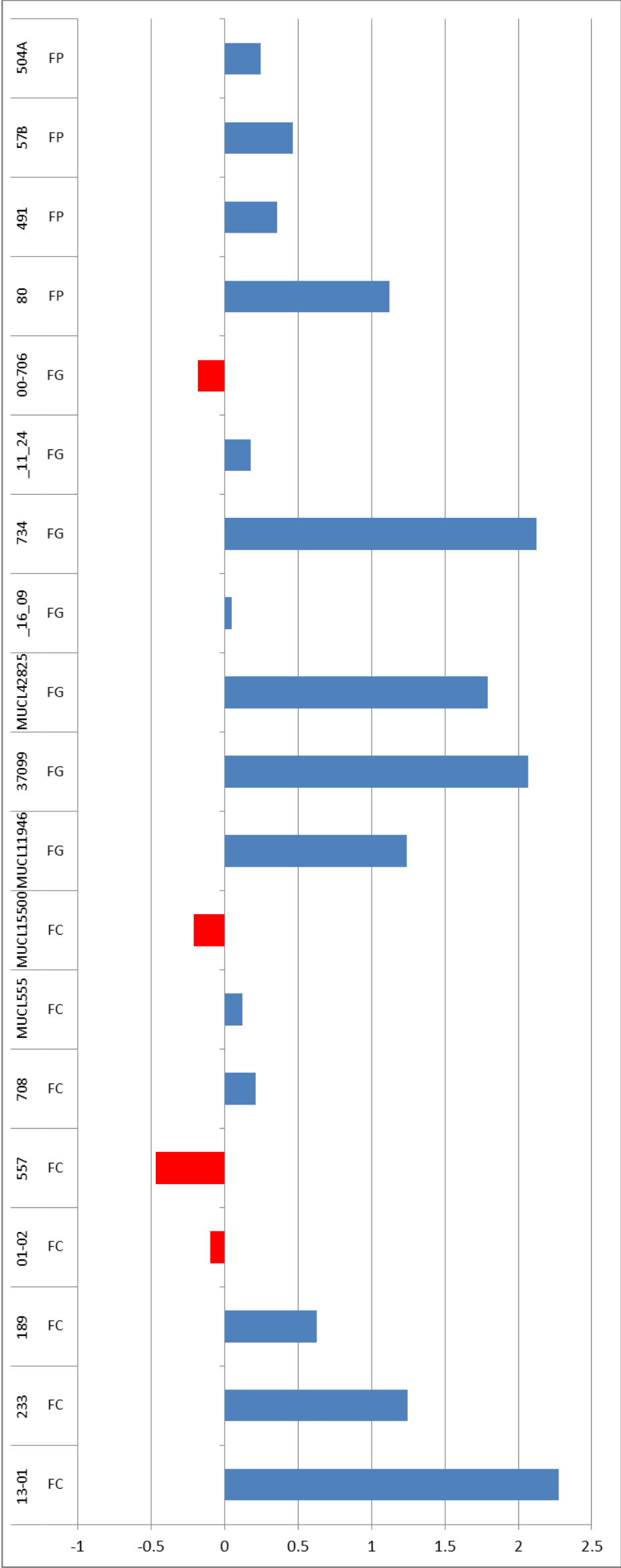
Mass in mg of dried mycelium grown 10 days in the two media (Gardiner medium containing agmatine; Jiao medium containing glutamic acid). Asterisks indicate significant differences using Mann-Whitney rank test ( $p < 0.05$ ).



## Figure 2(on next page)

Toxins ratio comparing the two media.

Log 10 transformed average ratio of TB production in agmatine vs glutamic acid medium. Values are the sum of all trichothecene type B detected (DON, 3ADON, 15ADON, NIV, FUSX). In red, isolates that have higher amount of TB in the glutamic acid medium while in blue those that have higher amount in the agmatine medium.



# **Table 1**(on next page)

## Table 1

Strain identification code, species, genetic chemotype, year of isolation, geographical origin, strain collection where the strain is deposited.

**Table 1.** Strain identification code, species, genetic chemotype, year of isolation, geographical origin, strain collection where the strain is deposited.

Strain identification code	Species	Genetic chemotype	Year of isolation	Geographical origin	Strain collection
13-01	<i>F. culmorum</i>	3ADON	2008	(Hoscheid) Luxembourg	LIST
233	<i>F. culmorum</i>	NIV	2007	(Reisdorf) Luxembourg	LIST
189	<i>F. culmorum</i>	3ADON	2007	(Reisdorf) Luxembourg	LIST
01-02	<i>F. culmorum</i>	NIV	2008	(Kehlen) Luxembourg	LIST
557	<i>F. culmorum</i>	3ADON	2007	(Reuler) Luxembourg	LIST
708	<i>F. culmorum</i>	NIV	2007	(Christnach) Luxembourg	LIST
MUCL555	<i>F. culmorum</i>	NIV	1952	Unknown	MUCL
MUCL15500	<i>F. culmorum</i>	NIV	1946	Netherlands	MUCL
MUCL 11946	<i>F. graminearum</i>	3ADON	1969	Belgium	MUCL
NRRL37099	<i>F. graminearum</i>	3ADON	1994	(Manitoba) Canada	NRRL
MUCL 42825	<i>F. graminearum</i>	NIV	2000	Belgium	MUCL
16-09	<i>F. graminearum</i>	NIV	2008	(Troisvierges) Luxembourg	LIST
734	<i>F. graminearum</i>	15ADON	2007	(Christnach) Luxembourg	LIST
11-24	<i>F. graminearum</i>	15ADON	2008	(Echternach) Luxembourg	LIST
UMW00-706	<i>F. graminearum</i>	15ADON	>2000	USA	Courtesy of L. Gale
80	<i>F. poae</i>	-	2007	(Nothum) Luxembourg	LIST
491	<i>F. poae</i>	-	2007	(Nothum) Luxembourg	LIST
57B	<i>F. poae</i>	-	2007	(Kayl) Luxembourg	LIST
504A	<i>F. poae</i>	-	2007	(Kayl) Luxembourg	LIST

\* LIST: fungal database of the Luxembourg Institute of Science and Technology (LUX); MUCL: *Mycoteque* de l'Université Catholique de Louvain (BEL); NRRL: Agricultural Research Service culture collection (USA).