Targeting *Fusarium graminearum* control via polyamine enzyme inhibitors and polyamine analogues

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1 1 **Targeting *Fusarium graminearum* control via polyamine enzyme**
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24 10 **Abstract**
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26 11 *Fusarium graminearum* has major impact in wheat cultivation as it not only reduces
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28 12 yield and seed quality but also constitutes a risk to public or animal health owing to its
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30 13 ability to contaminate grains with mycotoxins. Under high inoculum conditions,
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32 14 chemical fungicides are the main measure to control *F. graminearum*. However,
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34 15 resistance problems are emerging and control strategies based on new targets are
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36 16 needed. Polyamines are low molecular weight cations which have a key role in growth,
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38 17 development and differentiation. In this work, the possibility of using polyamine
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40 18 metabolism as a target to control *F. graminearum* has been assessed. It was found that
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42 19 the polyamine putrescine induces mycotoxin production, correlating with an over
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44 20 expression of *TRI5* and *TRI6* genes that are involved in deoxynivalenol (DON)
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46 21 biosynthesis and regulation, respectively. In addition, a homolog of the *Saccharomyces*
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48 22 *cerevisiae* *TPO4*, which is involved in the excretion of putrescine, was up-regulated as
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50 23 the concentration of putrescine in the medium increased while *DUR3* and *SAM3*
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52 24 homologues, involved in putrescine uptake, were down-regulated. When 2.5 mM D, L-
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54 25 α -difluoromethylornithine (DFMO), an inhibitor of the enzyme ornithine decarboxylase
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that catalyzes the first and committed step in the polyamine synthesis, was added to the medium, DON production decreased from 3.2 to 0.06 ng/mm² of colony, while the growth was lowered by up to 70 per cent. Interestingly, an over expression of the ornithine decarboxylase gene was observed when the *F. graminearum* culture was amended with DFMO. However, exogenous putrescine could overcome the effects of DFMO on growth and mycotoxin production. Because polyamines are regulated by its biosynthesis, degradation and transport, five polyamine transport inhibitors were also tested against *F. graminearum*. AMXT-1505 was able to completely inhibit *in vitro* both growth and DON production. When wheat spikes were treated with DFMO and subsequently inoculated with *F. graminearum* conidia, DFMO did not display inhibitory effects on the fungus. Nevertheless, AMXT-1505 blocked *F. graminearum* growth in inoculated wheat spikes, reducing DON mycotoxin contamination from 76.87 µg/g present at the positive control to 0.62 µg/g. Therefore, the polyamine transport inhibitor AMXT-1505 may be useful as a target specific fungicide against *F. graminearum*.

Keywords: *Fusarium graminearum*; FHB; deoxynivalenol; wheat; polyamines; DFMO

1. Introduction

Fusarium graminearum is one of the most important causal agents of the fungal disease Fusarium Head Blight (FHB) of wheat and other small-grain cereals that not only reduces yields but also reduces seed quality and contaminates grains with mycotoxins (Parry *et al.*, 1995; Stępień and Chelkowski, 2010). The disease occurs throughout much of the world and often with a devastating impact on wheat crops (Goswami and Kistler, 2004; McMullen *et al.*, 1997). The most common mycotoxins

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3 51 associated with *F. graminearum* are deoxynivalenol (DON), nivalenol (NIV) and
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5 52 zearalenone (ZEA) which pose a health risk to humans and animals through food and
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7 53 feed with accumulated mycotoxins (D'Mello *et al.*, 1999; Desjardins and Proctor,
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9 54 2007). However, DON is known to be the most commonly detected mycotoxin in cereal
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11 55 grains, usually at high concentrations (Canady *et al.*, 2001). At a cellular level, DON
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13 56 can inhibit protein synthesis by binding to the ribosome and activating cellular protein
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15 57 kinases involved in signal transduction related to proliferation, differentiation, and
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17 58 apoptosis (Pestka and Smolinski, 2005). In mammals, DON can cause emetic effects,
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19 59 anorexia and immune dysregulation as well as growth, reproductive and teratogenic
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21 60 effects (Pestka, 2010).
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25 61 To control fungal contamination, several management strategies have been
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27 62 employed, which include FHB-resistant wheat cultivars obtained by breeding processes
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29 63 or through transgenic approaches, chemical fungicides, biological control and cultural
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31 64 practices (Blandino *et al.*, 2012; Yuen and Schoneweis, 2007). However, under high
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33 65 inoculum conditions, chemical fungicides are the main measure to control the impact of
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35 66 the *F. graminearum* infection. Among the fungicides reported to be effective against
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37 67 FHB, triazols are the most used, which are sterol biosynthesis inhibitors (Paul *et al.*,
38
39 68 2010). Nevertheless, since fungi are able to evolve and adapt, fungicide-resistant strains
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41 69 may arise. Indeed, a decrease of triazol fungicide efficiency has been found (Becher *et*
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43 70 *al.*, 2010; Klix *et al.*, 2007; Yin *et al.*, 2009). Additionally, several authors have
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45 71 reported that sub-lethal doses of some of these fungicides may increase mycotoxin
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47 72 production from *F. graminearum* (D'Mello *et al.*, 1998; Müllenborn *et al.*, 2008; Ochiai
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49 73 *et al.*, 2007). Consequently, there is a clear need to design control strategies based on
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51 74 new targets.
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Over the past few years a number of studies have analyzed the role of polyamines (PA) in fungal metabolism, which has been recently reviewed by Valdés-Santiago *et al.* (2012). Polyamines are low molecular weight aliphatic polycations with two or more primary amino groups, ubiquitously present in all living cells. PA are involved in growth, development and differentiation, being its concentration highly regulated. Main PA in fungi are putrescine, spermidine, and spermine, although some fungal species do not produce spermine. Interestingly, it has been observed that the PA biosynthetic pathway is induced in the wheat as an early response to FHB. Moreover, it has been reported that intermediates of the PA pathway promote DON biosynthesis (Gardiner *et al.*, 2010; Gardiner *et al.*, 2009). In this study we have considered PA metabolism as a target to design new control strategies (Figure 1). To this aim, we studied the effect of putrescine and D, L- α -difluoromethylornithine (DFMO), an inhibitor of the enzyme ornithine decarboxylase which catalyzes the first and committed step in the synthesis of PA (Metcalf *et al.*, 1978), on growth, DON production and infection capacity. Additionally, we investigated the possibility of controlling *F. graminearum* with lipophilic polyamine analogues, produced by N-acylation or N-alkylation of the ϵ -amine group of the lysine portion of the Lys-spermine conjugates, which can contribute to the inhibition of polyamine transport. As PA transport has a key role in the regulation of PA content in cell, we also analyzed the gene expression of six proteins putatively involved in PA transport.

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96 **2. Results and discussion**

97 **2.1 Influence of putrescine on *F. graminearum***

98 To examine the effect of exogenous putrescine on *F. graminearum*, the fungus
99 was grown on plates with increasing amounts of putrescine (0, 50, 500, 1000 and 5000

100 μM). As Figure 2A indicates, DON increases proportionally to putrescine concentration,
101 resulting in almost a 23 fold induction when plates were amended with 5000 μM of
102 putrescine. These results are similar to those reported previously by Gardiner *et al.*
103 (2009) who conducted a large-scale nutrient profiling to assess the role that different
104 carbon and nitrogen sources play in the production of DON. These authors found that
105 DON production was strongly induced in liquid culture by some amine compounds
106 which included putrescine. Thus, it was confirmed that putrescine has a key role on
107 mycotoxin production. In contrast to DON production, the growth of *F. graminearum*
108 was not affected by the putrescine concentrations assayed. However, it would appear
109 that the effect of putrescine on growth depends on the fungal species as Rajam and
110 Galston (1985) found that the addition of putrescine to the culture medium resulted in a
111 promotion of growth in *Botrytis cinerea*, *Monilia fructicola* and *Rhizoctonia solani*.

112 Additionally, Figure 2B shows the colonies of the *F. graminearum* strain
113 incubated with increasing amounts of putrescine, which suggests that other metabolites
114 as carotenoids may also be influenced by putrescine due to the variation of colony color
115 among putrescine concentrations. Indeed, Rodríguez-Ortiz *et al.* (2009) showed
116 regulatory connections between nitrogen metabolism and carotenogenesis and
117 secondary metabolism in *Fusarium fujikuroi*.

118 To further elucidate the influence of putrescine on *F. graminearum*, a gene
119 expression analysis was carried out (Figure 3). We studied *TRI5* and *TRI6* genes
120 involved in DON biosynthesis and regulation (Hohn *et al.*, 1993; Proctor *et al.*, 1995),
121 the ornithine decarboxylase (*ODC*) and polyamine oxidase (*PAO*) which are involved in
122 PA biosynthesis and oxidation, respectively, and *TPO1*, *TPO4*, *TPO5*, *UGA4*, *DUR3*
123 and *SAM3*, identified by Valdes *et al.* (2012) by homology through an *in silico* search of
124 well-characterized PA transport proteins of *Saccharomyces cerevisiae*. Figure 3 reveals

that the increment of DON production observed in Figure 2A was correlated with an overexpression of *TRI5* and *TRI6* genes, showing at 5000 μ M of putrescine a log₂ Ratio of 11.5 and 8.2 for *TRI5* and *TRI6*, respectively, which represent a 2896.3 and 294.1-fold change. Consequently, it appears that putrescine can modulate DON biosynthesis affecting the transcriptional regulator encoded by *TRI6* and the trichodiene synthase *TRI5*, which catalyzes the first step in DON biosynthesis. It should be noted that *TRI5* was found to be positively regulated by *TRI6* (Proctor *et al.*, 1995). These results concur with the findings of Gardiner *et al.* (2009) who observed that putrescine induced the fluorescence of a *F. graminearum* strain that contained a GFP gene regulated by the *TRI5* promoter.

By contrast, it was found that the addition of exogenous putrescine to a *F. graminearum* culture had no effect on the *ODC* and *PAO* expression. Because *ODC* catalyzes the first and committed step in the synthesis of PA, it seems that the concentration of putrescine is not self-regulatory at a transcriptional level in *F. graminearum*. Concerning to the gene expression of the putative PA transporters analyzed in this study, we found an up-regulation of *TPO4* and a down-regulation of *DUR3* and *SAM3* when cultures were amended with increasing putrescine concentrations (Figure 3). Transport of PA in fungi has been poorly investigated and the existing studies are mainly focused in *S. cerevisiae* (Igarashi and Kashiwagi, 2010; Valdés-Santiago *et al.*, 2012). In *S. cerevisiae*, TPO1 and TPO4, located mainly in the plasma membrane, are the major PA excretion proteins (Tomitori *et al.*, 2001; Tomitori *et al.*, 1999; Uemura *et al.*, 2005). Nevertheless, TPO5 which is located on Golgi or post-Golgi secretory vesicles and UGA4, located on the vacuolar membrane, can also catalyze the excretion of PA (Tachihara *et al.*, 2005; Uemura *et al.*, 2004). Furthermore, *DUR3* and *SAM3* are the major PA uptake proteins in yeasts (Uemura *et al.*, 2007).

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3 150 Assuming that these proteins may have similar functions in *F. graminearum*, the
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5 151 increase of putrescine in the culture media would lead to an overexpression of *TPO4* to
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7 152 raise the excretion of putrescine, and a downregulation of *DUR3* and *SAM3* to reduce
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9 153 the putrescine uptake.
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14 155 **2.2 Inhibition of putrescine biosynthesis in *F. graminearum* by DFMO**
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16 To reduce PA biosynthesis, we tested the DFMO capacity to inhibit ODC, which
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18 157 catalyze the first step in the synthesis of PA (Figure 1). When 2.5 mM of DFMO was
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20 158 added to the culture media, growth and DON production were greatly reduced as shown
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22 159 in Figure 4. DON production decreased from 3.2 to 0.06 ng/mm² of colony while the
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24 160 growth was lowered by up to 70 per cent. However, when the culture media was
25
26 161 supplemented with 1 mM of putrescine, the DON production and the growth were
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28 162 recovered. Similar behavior was observed when *Penicillium citrinum*, *Aspergillus*
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30 163 *parasiticus* and *Aspergillus nidulans* where treated with PA inhibitors, as mycelial
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32 164 growth and citrinin, aflatoxin and sterigmatocystin production, respectively, were
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34 165 reduced by PA inhibitors (Giridhar *et al.*, 1997; Guzmán-de-Peña *et al.*, 1998; Guzmán-
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36 166 de-Peña and Ruiz-Herrera, 1997). Additionally, PA inhibitors were able to *in vitro*
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38 167 inhibit the growth of other phytopathogenic fungi such as *B. cinerea*, *R. solani*,
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40 168 *Cochliobolus carbonum*, *Colletotrichum truncatum*, *Fusarium oxysporium* and
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42 169 *Phytophthora infestans* (Gamarnik *et al.*, 1994; Rajam and Galston, 1985; Walters,
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44 170 1995).
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49 171 As putrescine increased *TRI5* and *TRI6* expression and DON production (Figure
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51 172 3), it might be expected that DFMO, which binds to ODC decreasing its activity and
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53 173 consequently the putrescine biosynthesis, will reduce *TRI5* and *TRI6* expression.
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55 174 However, *TRI5* and *TRI6* gene expression was up-regulated when DFMO was added to
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the media (Figure 5). Supplementation of 1 mM of exogenous putrescine together with 2.5 mM of DFMO resulted in an increase of *TRI5* and *TRI6* gene expression at similar levels to those found when *F. graminearum* cultures were amended with just 1 mM of putrescine (Figure 3 and 5). Therefore, there must be a DON regulation system downstream of putrescine production. Another interesting finding was the up-regulation of *ODC* when *F. graminearum* cultures were treated with DFMO to inhibit the *ODC* activity, showing a log₂ Ratio of 4.8 which represents a 27.9-fold change. With respect to PA transporters, *F. graminearum* showed an up-regulation of *TPO1*, *TPO4* and *UGA4* as well as a down-regulation of *DUR3* in response to DFMO. Based on these results, it would seem that DFMO induces PA excretion proteins and transport to the vacuolar membrane and represses PA uptake proteins but further analysis needs to be performed.

2.3 Inhibition of PA transport in *F. graminearum*

Although DFMO inhibits largely DON production, the fungi may import PA from the wheat during the infection process, overcoming the inhibition of putrescine biosynthesis. Intracellular PA concentrations are regulated by not only biosynthesis and degradation but also transport. Therefore, PA transport may be a useful target to control *F. graminearum* infection and DON contamination. For some years now, the inhibition of PA transport has been a goal for medical research as elevated levels of PA have been associated with different types of cancer (Nowotarski *et al.*, 2013). In this context, Burn *et al.* (2001; 2009) developed a group of lipophilic polyamine analogues that potently inhibit the cellular PA uptake. To investigate whether the PA transport could be inhibited in *F. graminearum*, five different PA analogs, kindly provided by Aminex Therapeutics (USA), were tested. To this aim, *F. graminearum* was inoculated in plates

200 amended with 1 mM of putrescine as this could be a common PA concentration during
201 plant infection. Results are presented as relative with respect to the control (1 mM
202 putrescine) (Figure 6). The observed effect on growth and DON production largely
203 depends on the Polyamine Transport Inhibitor (PTI) used. While AMXT-1483 reduced
204 growth and increased DON production by as much as 180 %, AMXT-1505 reduced
205 completely both DON production and mycelial growth when 600 μ M of this PTI was
206 added to the medium. AMXT-2030 had little effect on DON production and mycelial
207 growth and AMXT-3016 increased DON production by 270 % but did not affect to the
208 growth. In addition, AMXT-3946 reduced DON production up to 70 % and increased
209 the mycelial growth up to 210 %. Of them all, we should highlight the AMXT-1505
210 PTI, as it was able to efficiently control *F. graminearum*. Additionally, when AMXT-
211 1505 was applied at sub-lethal doses, not only it does not increase DON production as
212 observed for other fungicides but reduces it, minimizing the risk of mycotoxin
213 contamination (D'Mello *et al.*, 1998; Müllenborn *et al.*, 2008; Ochiai *et al.*, 2007).

214 To further investigate the possible mode of action of AMXT-1505, gene
215 expression analysis of genes involved in DON biosynthesis and regulation, PA
216 biosynthesis and oxidation and PA transport was carried out in cultures amended with
217 100, 300 and 500 μ M of this PTI (Figure 7). However, little changes were observed
218 with respect to the control, showing a concentration dependent down-regulation of
219 *TPO1* and to a lesser extent *TPO5*. In the case that AMXT-1505 was really able to
220 inhibit PA transport, it may lead to PA accumulation inside the cell which in turn may
221 cause cytotoxicity. In fact, excessive level of PA may be coupled to the induction of
222 apoptosis (Schipper *et al.*, 2000). Nonetheless, it is difficult to speculate a mode of
223 action for AMXT-1505 and more research is needed.

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2.4 Antifungal activity of DFMO and AMXT-1505 against *F. graminearum* in wheat

To verify whether DFMO and AMXT-1505 are able to control *F. graminearum* growth and mycotoxin production in wheat, spikes of wheat were treated with these compounds to be later point inoculated by placing a droplet of conidia suspension in the middle of the spikes. The conditions upon which inoculated spikes were incubated were highly favorable for the *F. graminearum* growth as the inoculation was performed in spikes harvested at anthesis and incubated at 26 °C and 100 % RH for 12 days. Firstly, it should be pointed out that spikes were free from external contamination as shown by the negative control in the Figure 8. Additionally, it should be noted that *F. graminearum* grew in all the spikes from the positive control, forming a white mycelium that covered almost the entire length of the spike. Interestingly, although DFMO reduced the fungal growth *in vitro* by 70 %, when spikes were pre-treated with DFMO, *F. graminearum* was able to grow even more than in the positive control. Conversely, AMXT-1505 was capable of controlling *F. graminearum* with only light growth in two of the spikes. As for the DON production, a concentration of 76.87 µg/g was found in the positive control while pre-treated spikes with DFMO and AMXT-1505 contained 212 and 0.62 µg/g, respectively. From the results obtained, noteworthy is the need of validating the results obtained *in vitro* as DFMO switched from a 98 % reduction to a 276 % increase of DON production. These findings may be explained considering the possibility that the fungus overcomes the inhibition of putrescine biosynthesis importing the putrescine from the wheat. Bharti and Rajam (1995) found that some DFMO treatments caused an increase of PA levels in wheat that may be responsible for the DON increase observed in Figure 8 since higher putrescine concentrations produce higher amounts of DON as shown in Figure 2.

Although this kind of PTIs have not been tested before as fungicides against *F. graminearum*, some researchers have reported other PA analogues and conjugates used to control phytopathogenic fungi. The putrescine analogue 1-aminooxy-3-aminopropane did not reduce mycelial growth of the phytopathogenic fungus *Sclerotinia sclerotiorum*, but perturbed polyamine metabolism inhibiting the enzyme ornithine decarboxylase, spermidine synthase, and S-adenosyl-methionine decarboxylase in mycelial extracts (Gárriz *et al.*, 2003). In addition, it was found that two spermidine analogues, N,N-dimethyl-N1-(3-aminopropyl)-1,3-diaminopropane trihydrochloride and N,N-dimethyl-N1-(3-aminopropyl)-1,4-diaminobutane trihydrochloride, reduced growth of the fungal plant pathogen *Pyrenophora avenae* (Mackintosh *et al.*, 2001). Walters *et al.* (2001) found PA conjugates, isolated from pollen of *Quercus alba*, which have antifungal activity against *P. avenae* and Mellon and Moreau (2004) showed a combination of two polyamine conjugates that reduced aflatoxin production but had no effect on growth of *Aspergillus flavus*.

The results reported in this study indicate that the use of PA analogs is a promising approach for the control of the FHD and DON contamination. Since there is a need to find new targets to overcome resistance problems, AMXT-1055 may be useful as a target specific fungicide against *F. graminearum*, contributing to limit crop losses and to reduce the risk of contamination by mycotoxins in food and feed.

3. Material and methods

3.1 Strain and growth conditions

The *F. graminearum* strain used in this study was L1-2/3D, kindly provided by Dr. Belén Patiño from the Department of Microbiology III, Faculty of Biology, University Complutense of Madrid (Spain). This strain was isolated from Spanish wheat

and identified by PCR using Fg16F and Fg16R primers (Nicholson *et al.*, 1998). To enable production of macroconidia, the isolate was sub-cultured on plates containing, per liter, 30 g sucrose, 2 g NaNO₃, 1 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.5 g KCl and 100 µL of trace element solution (per 100 mL, 5 g citric acid, 5 g ZnSO₄·7 H₂O, 0.25 g CuSO₄·5 H₂O, 50 mg MnSO₄·H₂O and 50 mg H₃BO₃) under white light (12 h/12 h light/dark) at 26 °C for 6 days. Conidia were collected with a sterile solution of 0.005% (v/v) Tween 80, and adjusted to 10⁵ conidia/mL using a Thoma counting chamber. Plates with the media mentioned above but containing 1 gram of glutamine instead of 2 g NaNO₃, were inoculated centrally with 5 µL of conidia suspensions to study the effect of putrescine (SIGMA), DFMO (Enzo Life Sciencies) and five polyamine transport inhibitors (PTI), detailed in Table 1, kindly provided by Aminex Therapeutics (USA). Putrescine concentrations assayed were 50, 500, 1000 and 5000 µM, the DFMO concentration tested was 2.5 mM, while the PTIs concentrations analyzed ranged from 50 to 1000 µM. Putrescine, DFMO and the PTIs were dissolved in water, sterilized by passing the solution through a 0.45 µm pore size filter and added to the autoclaved culture media. For growth assessment, two perpendicular diameters of the growing colonies were measured after incubation for 3 days under white light (12 h/12 h light/dark) at 26 °C.

3.2. Extraction and detection of DON from culture

To extract DON, one agar plug (7 mm in diameter) was removed from the center of the colonies incubated for 7 days under white light (12 h/12 h light/dark) at 26 °C. The agar plug was shaken for 60 s in 750 µL of water and left for 60 min at room temperature. Then, vials were shaken and the extracts were filtered (Millex-HV 0.45 µm, 25 mm, Millipore Corporation) into another vial. DON extracts were stored at -20

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300 °C until quantification. DON was measured using the RIDASCREEN® DON enzyme
301 immunoassay test from R-BIOPHARM, according to the manufacturer's instructions.

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303 **3.3. Gene expression analysis**

304 Mycelia were collected from 7 days old cultures grown on media amended with
305 putrescine, DFMO or PTIs. The mycelium was frozen in liquid nitrogen and stored at
306 -80 °C before nucleic acid extraction. RNA was extracted from the mycelium ground to
307 a fine powder with a mortar and pestle using liquid nitrogen. Powdered mycelium was
308 added to a pre-heated (65 °C) mixture of 10 mL of extraction buffer (100 mM Tris-
309 HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% (w/v) sodium dodecyl sulfate, 1% (w/v)
310 polyvinyl-pyrrolidone 40, 1% (v/v) β-mercaptoethanol) and 5 mL of Tris-equilibrated
311 phenol. The extract was incubated at 65 °C for 15 min and cooled before adding 5 mL
312 of chloroform:isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at 3900 ×g
313 for 20 min at 4 °C, and the aqueous phase was re-extracted with 10 mL of
314 phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). RNA was precipitated during 3 h at
315 -20 °C by adding 3.3 mL of 12 M LiCl. After centrifugation at 27,200 ×g for 60 min,
316 the pellet was washed with 500 µL of 70 % ethanol. The resulting pellet was re-
317 extracted with 250 µL of 3 M sodium acetate (pH 6.0) to remove residual
318 polysaccharides. Then, RNA was washed again with 500 µL of 70 % ethanol, and,
319 finally, dissolved in 200 µL of water. RNA concentration was measured
320 spectrophotometrically and verified by agarose gel electrophoresis (1.2 %) and
321 ethidium-bromide staining. Total RNA was treated with DNase (TURBO DNase,
322 Ambion) to remove contaminating genomic DNA. Single-strand cDNA was synthesized
323 from 5 µg of total RNA using SuperScript III reverse transcription kit and an oligo(dT)
324 primer, according to the manufacturer's instructions (Invitrogen).

APT5newF and APT5newR were used as gene-specific primer set to amplify the *TRIS* gene (Mudge *et al.*, 2006). The rest of the primer sets were designed with OLIGO Primer Analysis Software V.7 (Table 2). Real-time RT-PCR reactions were performed on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD) monitoring cDNA amplification with SsoAdvanced™ SYBR® Green Supermix (BIO-RAD). The primer pair BETA-6_F and BETA-6_R was designed within the beta tubulin gene for using it as a reference gene. The standard protocol included one cycle at 98 °C for 2 min, followed by 40 cycles at 98 °C for 5 s and 56 °C for 30 s. The corresponding real-time PCR efficiency (E) in the exponential phase was calculated according to the equation: $E = 10[-1/\text{slope}]$. The relative expression of the target genes was calculated based on the E and the Crossing point (Cp) value of the sample versus the control. The Cp value is the cycle at which fluorescence from amplification exceeds the background fluorescence. The relative expression of the target genes was expressed in comparison to the beta tubulin gene (reference gene), according to the following equation (Pfaffl, 2001; Rasmussen, 2001): $\text{ratio} = (E_{\text{target}})^{\Delta C_p \text{ target}(\text{control} - \text{sample})} / (E_{\text{ref}})^{\Delta C_p \text{ ref}(\text{control} - \text{sample})}$. Gene expression measures were derived from biological triplicates.

3.4 Plant growth and inoculation

Wheat plants (*Triticum aestivum*), cultivar Califa sur, were grown in a greenhouse with temperatures that ranged from 14 to 32 °C during the day and 7 to 12°C at night. Spikes of wheat were harvested at or shortly before anthesis. Spikes were surface-sterilized by dipping them into a NaClO solution (0.1% Cl) for 15 min, followed by 2 min in ethanol (70 %). Excess of ethanol was removed by placing spikes in a laminar flow bench. The DFMO and AMXT-1505 PTI treatments were performed by dipping the spikes into the solutions for 2 minutes and placing the spikes in a laminar flow bench until they were

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3 350 dried (about 10 minutes), after which spikes were inoculated. Four different treatments
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5 351 were tested consisting of spikes point inoculated in the middle of the spike with 10 µL
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7 352 of conidial suspension (10^5 conidia/mL) or water, as positive and negative controls, and
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9 353 spikes treated with 2.5 mM DFMO or 700 µM AMXT-1505 inoculated with 10 µL of
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11 354 conidial suspension. Transparent plastic boxes 300 mm x 160 mm x 180 mm deep were
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13 355 filled with a beaker with water to give a RH of 100 %, a data logger (Escort iLog RH)
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15 356 recording temperature and RH at 30 min intervals and a rack where spikes were placed
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17 357 vertically separated from one each other. Boxes were kept in an incubator at 26 °C with
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19 358 12 h/12 h light/dark photoperiod for 12 days. A box with 10 spikes was used for each
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21 359 treatment.
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38 366 **4. References**

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Figure captions

Figure 1: Polyamines biosynthetic pathway.

Figure 2: A) Effect of putrescine on DON production (left y-axis) and relative colony diameter (%) referred to the control (right y-axis) (control = 100 %). B) Effect of putrescine on *F. graminearum* colonies incubated for 7 days at 26 °C (12 h/12 h light/dark).

Figure 3: Relative expression of *TRI5*, *TRI6*, *ODC*, *PAO*, *TPO1*, *TPO4*, *TPO5*, *UGA4*, *DUR3* and *SAM3* in *F. graminearum* grown in the presence of different concentrations of putrescine with respect to expression level in the same medium without putrescine. Error bars indicate standard errors.

Figure 4: A) Effect of DFMO on DON production (left y-axis) and relative colony diameter (%) referred to the control (right y-axis) (control = 100 %). B) Effect of DFMO on *F. graminearum* colonies incubated for 7 days at 26 °C (12 h/12 h light/dark).

Figure 5: Relative expression of *TRI5*, *TRI6*, *ODC*, *PAO*, *TPO1*, *TPO4*, *TPO5*, *UGA4*, *DUR3* and *SAM3* in *F. graminearum* grown in the presence of DFMO and putrescine with respect to expression level in the same medium without DFMO or putrescine. Error bars indicate standard errors.

Figure 6: Effect of PTIs on DON production and colony diameter referred to the control (1000 µM putrescine) in percentage (control = 100 %).

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544 **Figure 7:** Relative expression of *TRI5*, *TRI6*, *ODC*, *PAO*, *TPO1*, *TPO4*, *TPO5*, *UGA4*,
545 *DUR3* and *SAM3* in *F. graminearum* grown in the presence of 1000 µM putrescine
546 different concentrations of AMTX-1505 with respect to expression level in the same
547 medium without AMTX-1505. Error bars indicate standard errors.

548 **Figure 8:** Activity of DFMO and AMXT-1505 against *F. graminearum* in wheat.
549 Spikes of wheat were harvested at or shortly before anthesis, point inoculated by
550 placing a droplet (10 µL) of conidia suspension (10⁵ conidia/mL) and incubated at
551 26°C-100% RH with 12 h/12 h light/dark photoperiod for 12 days.
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553 **Table legends:**

554 **Table 1:** Polyamine transport inhibitors tested against *F. graminearum*.

555 **Table 2:** Primers used in this study.

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Name	Structure
AMXT-1483	<p>Chemical structure of AMXT-1483: A long-chain amide with a branched amine side chain. The structure features a long alkyl chain (14 carbons) attached to an amide group (H-N-C=O). The amide nitrogen is connected to a side chain that includes a secondary amine (N) and a tertiary amine (N) linked by a methylene group. The side chain also contains a methyl group and a hydrogen atom.</p>
AMXT-1505	<p>Chemical structure of AMXT-1505: A long-chain amide with a branched amine side chain. The structure features a long alkyl chain (14 carbons) attached to an amide group (H-N-C=O). The amide nitrogen is connected to a side chain that includes a secondary amine (N) and a tertiary amine (N) linked by a methylene group. The side chain also contains a methyl group and a hydrogen atom.</p>
AMXT-2030	<p>Chemical structure of AMXT-2030: A long-chain amide with a branched amine side chain. The structure features a long alkyl chain (14 carbons) attached to an amide group (H-N-C=O). The amide nitrogen is connected to a side chain that includes a secondary amine (N) and a tertiary amine (N) linked by a methylene group. The side chain also contains a methyl group and a hydrogen atom.</p>
AMXT-3016	<p>Chemical structure of AMXT-3016: A long-chain amide with a branched amine side chain. The structure features a long alkyl chain (14 carbons) attached to an amide group (H-N-C=O). The amide nitrogen is connected to a side chain that includes a secondary amine (N) and a tertiary amine (N) linked by a methylene group. The side chain also contains a methyl group and a hydrogen atom.</p>
AMXT-3946	<p>Chemical structure of AMXT-3946: A long-chain amide with a branched amine side chain. The structure features a long alkyl chain (14 carbons) attached to an amide group (H-N-C=O). The amide nitrogen is connected to a side chain that includes a secondary amine (N) and a tertiary amine (N) linked by a methylene group. The side chain also contains a methyl group and a hydrogen atom.</p>

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Gene number	Gene	Primer name	Primer sequence (5'→3')
FGSG_03537	TRI5	APT5newF	CACTTTGCTCAGCCTCGCC
		APT5newR	CGATTGTTTGGAGGGAAGCC
FGSG_16251	TRI6	TRI6_F	CACCTTCACACGGCCAAGCAA
		TRI6_R	ATGCCGCCTAAAGTCCCGTCC
FGSG_05903	ODC*	ODC_F	ACCGCATCACTGATAGCACTCG
		ODC_R	TGGTGTATGCGCCCATATCCTC
FGSG_10284	PAO*	PAO_F	ACTCTGAGAGCGATCCCGACT
		PAO_R	GCAGACCCTCCCGCATTGTCC
FGSG_04370	TPO1*	TOP1_F	GCTTGCCGCCAACAACGGTA
		TOP1_R	GGTGTATCCAGTCCATCCGAACCA
FGSG_03725	TPO4*	TOP4_F	CCATCTTTGCTGCCGCTCCC
		TOP4_R	CCATGACCCATCGCCAACCAG
FGSG_02439	TPO5*	TOP5_F	GCCACCATCTGTCTCGCCTCC
		TOP5_R	TCCACACGACGCAGATACCGTTG
FGSG_06473	UGA4*	UGA4_F	GCCCTCCATGATCCCTGTTACCC
		UGA4_R	TGACCCCAGGCCCAGTACCAA
FGSG_03111	DUR3*	DUR3_F	AGACTTATCGCCATCTCGCACAC
		DUR3_R	ACGCCCATCCAGACATATAGCC
FGSG_00613	SAM3*	SAM3_F	ATATTCGCTTCCGCCGTGCCT
		SAM3_R	ATCCATGAGCCCACGACACCA
FGSG_09530	BETA TUBULIN	BETA-6_F	AGCTCACCCAGCAGATGTTTCG
		BETA-6_R	GCGCATCTGGTCCTCAACCTCC

*Homologous genes identified by Valdes et al. (2012) by homology with an *in silico* search of well – characterized *Saccharomyces cerevisiae* proteins.

A)

B)

A)

0 mM P / 0 mM DFMO 0 mM P / 2.5 mM DFMO 1 mM P / 2.5 mM DFMO

B)

AMXT-1483

AMXT-1505

AMXT-2030

AMXT-3016

AMXT-3946

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Negative control

Infected

Infected
2.5 mM DFMO

Infected
700 µM AMXT-1505