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Is exploitation competition involved in a multitrophic strategy for the biocontrol of Fusarium Head Blight?

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Manuscripts

Is exploitation competition involved in a multitrophic strategy for the biocontrol of Fusarium Head Blight?

Sabrina Sarrocco[1*], Fabio Valenti[1], Sara Manfredini[1], Pilar Esteban[1], Rodolfo Bernardi[1], Grazia Puntoni[1], Riccardo Baroncelli[2], Miriam Haidukowski[3], Antonio Moretti[3], Giovanni Vannacci[1]

[1] Università degli Studi di Pisa – Dipartimento di Scienze Agrarie, Alimentari e Agro-Ambientali (DISAAA-a), Plant Pathology & Mycology lab - Via del Borghetto 80 – 56124 Pisa, Italy.

[2] Instituto Hispano-Luso de Investigaciones Agrarias (CIALE), University of Salamanca, Calle del Duero, 12; 37185 Villamayor (Salamanca), Spain

[3] ISPA-CNR, Via Amendola, 122/O - 70126 Bari, Italy

Corresponding author: S. Sarrocco; E-mail address: sabrina.sarrocco@unipi.it

18 **ABSTRACT**

19 Sarrocco S., Valenti F., Manfredini S., Esteban P., Bernardi R., Puntoni G., Baroncelli
20 R., Haidukowski M., Moretti A. and Vannacci G. 2018. Is exploitation competition
21 involved in a multitrophic strategy for the biocontrol of Fusarium Head Blight?
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24 *Trichoderma gamsii* T6085 was used in combination with a *Fusarium oxysporum*
25 isolate (7121) in order to evaluate, in a multitrophic approach, their competitive ability
26 against *Fusarium graminearum*, one of the main causal agents of Fusarium Head Blight
27 (FHB) on wheat.
28 The two antagonists and the pathogen were co-inoculated on two different natural
29 substrates, wheat and rice kernels. Both T6085 and 7121, alone and co-inoculated,
30 significantly reduced the substrate colonization and mycotoxin production by the
31 pathogen, The two antagonists did not affect each other. Using a metabolic approach
32 (Biolog) we investigated whether exploitation competition could explain this
33 antagonistic activity. The aim was to define whether the three fungi co-exist or if one
34 isolate nutritionally dominates another one. Results obtained from Biolog suggest that
35 no exploitative competition occurs between the antagonists and the pathogen during the
36 colonization of the natural substrates. Interference competition was then preliminary
37 evaluated to justify the reduction in the pathogen's growth and to better explain
38 mechanisms. A significant reduction of *F. graminearum* growth was observed when the
39 pathogen grew in the cultural filtrates of *T. gamsii* T6085, both alone and co-cultured
40 with *F. oxysporum* 7121, thus suggesting the involvement of secondary metabolites.

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As far as we know this is the first time that an ecological study has been performed to explain how and which kind of competition could be involved in a multitrophic biocontrol of FHB.

Keywords Exploitation competition_ *Fusarium graminearum* _ *Trichoderma gamsii* _ *Fusarium oxysporum* _ FHB

INTRODUCTION

Fusarium Head Blight (FHB) causes significant yield losses worldwide in crops such as wheat, maize, rice and minor cereals (Parry et al. 1995; Xu and Nicholson 2009). Many different *Fusarium* species contribute to the disease, but *F. graminearum* is one of the main causal agents (Parry et al. 1995). Infections of wheat by *F. graminearum*, as well as by other *Fusarium* species, do not only reduce grain size, weight, germination rate, protein content, and baking quality of the flour but also the feed and food safety, due to the contamination of the grains with mycotoxins (Pieters et al. 2002). Strategies for controlling the disease and preventing mycotoxin accumulation, such as fungicides, host resistance or agronomical approaches, do not fully reduce the impact of FHB and new and sustainable strategies are greatly needed.

At the beginning of the 20th century, competitive interaction within coexisting population was suggested as a mechanism to limit plant disease and to reduce pathogen populations (Kinkel et al., 1995). Two competition strategies are generally applied to fungal communities: exploitation competition and interference competition (Hartley 1921; Leben 1965, Fokkema 1971; Wilson and Lindow 1994). Exploitation is the direct competition for resources by rapid scavenging from a common pool, while interference competition (or indirect competition) involves habitat monopolization by antagonistic

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66 combat (Keddy 2001). These definitions have now been adapted to biological control:
67 exploitation competition is used for resources (oxygen, carbon, nitrogen, and other
68 essential resources), while interference competition is used for space via antibiosis,
69 where a biological control agent inhibits the pathogen through the effects of toxic
70 secondary metabolites or other means of combat (Jensen et al. 2017).
71 During the disease cycle, *F. graminearum* uses cultural debris to overwinter between
72 two consecutive cultural cycles, while flowering is the most susceptible wheat growth
73 stage for spike infection (Parry et al. 1995; Champeil et al. 2004). Because this
74 pathogen is generally considered a poor competitor over time compared to other
75 organisms that colonize crop residues (Pereyra and Dill-Macky 2008), competition
76 could thus be a valid strategy to control the production of the primary inoculum (Leplat
77 et al. 2013). If biocontrol agents are inoculated on cultural debris in soil, they gain
78 access to territory or resources previously held by the pathogen (Holmer and Stenlid
79 1993; Boddy 2000; Jensen et al. 2016). Many fungi are able to outcompete with *F.*
80 *graminearum* and are studied for their ability to limit the survival of the pathogen. Of
81 these, *Trichoderma atroviride*, *Trichoderma harzianum* and *Clonostachys rosea*, as well
82 as *Microsphaeropsis*, are good candidates due to their ability to colonize wheat residues
83 or to reduce *F. graminearum* sporulation on cultural debris (Bujold et al. 2001; Naef et
84 al. 2006; Gromadzka et al. 2012; Sarrocco and Vannacci 2018). *Trichoderma gamsii*
85 T6085 has been used both in laboratory and field experiments for the control of FHB
86 causal agents, and has reduced *F. graminearum* and *F. culmorum* growth by acting as
87 an antagonist and a mycoparasite (Matarese et al. 2012; Sarrocco et al. 2013; Baroncelli
88 et al. 2016). In plate tests, on boiled rice, the activity of *T. gamsii* T6085 also resulted in
89 a significant reduction in deoxynivalenol (DON) production by the pathogens after 21

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days, thus showing the potentiality of reducing the risk of accumulation of this mycotoxin (Matarese at al. 2012). However, not much is known about the competitive ability of *T. gamsii* T6085, although *Trichoderma* spp. are among the main competitors that aggressively colonize the crop residues of maize and wheat throughout the decomposition process (Broder and Wagner 1988). Among the competitive species for cultural debris, *Fusarium oxysporum* is an effective colonizer of partially decomposed wheat residues, and has a greater saprophytic ability than common FHB causal agents (Pereyra and Dill-Macky 2004). From a large collection of *F. oxysporum* isolates recovered from wheat soils by straw baits, the isolate *F. oxysporum* 7121 used in the present work is also considered particularly interesting due to its ability to grow in presence of DON (Sarrocco et al. 2012).

In this work *T. gamsii* T6085 was used, for the first time, in combination with *F. oxysporum* 7121 against *F. graminearum*. We used a metabolic approach to investigate whether exploitation competition could explain the antagonistic activity performed by the two beneficial isolates against the pathogen on natural substrates. As far as we know this is the first time that an ecological study has been performed to explain the kind of competition that could be involved in a multitrophic biocontrol of FHB.

MATERIAL AND METHODS

Fungal isolates

Trichoderma gamsii T6085 was isolated in Crimea (Ukraine) from uncultivated soil (Matarese et al. 2012). *Fusarium oxysporum* 7121 belongs to a wider collection of *F. oxysporum* strains isolated from wheat straw in soils close to Pisa (Italy) with a previous history of wheat cultivation. This isolate was selected because it is able to grow in

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114 presence of 50ppm of DON (Sarrocco et al. 2012). Both the beneficial isolates are
115 deposited in the Fungal Collection of the Plant Pathology & Mycology Lab (DISAAA-
116 a, University of Pisa).
117 *Fusarium graminea*rum ITEM 124, isolated from rice, belongs to the fungal collection
118 of the CNR-ISPA (http://www.ispa.cnr.it/Collection Bari, Italy), and its genome was
119 recently sequenced, annotated and released (Zapparata et al. 2017). All fungi were
120 maintained on PDA (Potato Dextrose Agar, BD, Difco, USA) under mineral oil at 4°C
121 for long-term storage and grown on PDA (*T. gamsii* and *F. oxysporum*) or OA (Oat
122 Meal Agar, Difco, BD) (*F. graminearum*) at 24°C, under photoperiod of 12h light / 12h
123 darkness, when actively growing cultures were needed. The pathogen has been regularly
124 passaged through the host plant to maintain its virulence.
125

126 **Competition test on natural substrates**

127 In order to estimate the effects of *T. gamsii* T6085 and *F. oxysporum* 7121 on *F.*
128 *graminearum* growth and trichothecene production, a competition test was performed
129 on two natural substrates, rice and wheat. Twenty grams of rice or wheat kernels and 8
130 mL of distilled water were put in a 100 mL Erlenmeyer flask and autoclaved twice, with
131 24-hour interval, for 30 min at 121°C. Kernels were inoculated with 1 mL of a 2.0 x10^6
132 conidia mL^{-1} water suspension, made from two week-old PDA (*T. gamsii* T6085 and *F.*
133 *oxysporum* 7121) or OA (*F. graminearum*) fungal cultures, grown at 24°C, under
134 photoperiod of 12h light / 12h darkness. The pathogen was inoculated alone (FG) as
135 biotic control and co-inoculated with each antagonist (FG+T; FG+FO) or in the
136 presence of both (FG+T+FO). Uninoculated rice and wheat were used as abiotic

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1 2 3
4 137 controls. Flasks were incubated at 24°C 12h light / 12h darkness for 21 days. The
5 6 138 experiment was independently repeated three times.
7 8 139 At the end of the incubation time, the growth of *T. gamsii* T6085 and *F. oxysporum*
9 10 140 7121, and their effects, alone and in combination, on the growth of *F. graminearum* and
11 12 141 mycotoxin production were evaluated as described below. Values of both fungal growth
13 14 142 and mycotoxin production were submitted to ANOVA (Tukey post hoc test) using
15 16 143 Systat (Systat Software, Inc, Chicago, IL, USA) and assuming $P \leq 0.05$ as the
17 18 144 significance level.
19 20 145 Fungal growth: the growth of *T. gamsii* T6085, *F. oxysporum* 7121 and *F. graminearum*
21 22 146 on natural substrates were expressed as the DNA concentration of the two antagonists
23 24 147 and of the pathogen, and measured by Real-time PCR (absolute and relative). Genomic
25 26 148 DNA from inoculated seeds was extracted according to Doyle and Doyle (1990) with
27 28 149 some modifications. Briefly, 0.5 g of inoculated and control wheat and rice seeds were
29 30 150 used. Seeds were ground in liquid nitrogen and homogenised in a CTAB extraction
31 32 151 buffer [NaCl 1.4 M; EDTA 20 mM; Tris-HCl 100 mM;pH 8.0; CTAB 3% (w/v); 2-
33 34 152 Mercaptoetanol 0.2% (v/v)] in a 6:1 ratio (v/w). The mixture was incubated for 20 min
35 36 153 at 60°C, then extracted twice with chloroform:isoamyl alcohol (24:1). The upper phase
37 38 154 was recovered; 2/3 volumes (v/v) of cold isopropyl alcohol were added, and DNA was
39 40 155 precipitated at -20°C for 1 h. The pellet obtained after centrifugation was washed with
41 42 156 absolute ethanol and dissolved in TE pH 7.8 (Tris-HCl 10 mM ph 7.8, EDTA 1 mM).
43 44 157 Solubilized DNA was treated with RNaseA from bovine pancreas (Sigma) at a final
45 46 158 concentration of 1 μg ml^{-1}, incubated at 37ºC for 1 h and extracted with an equal
47 48 159 volume of chloroform:isoamyl alcohol (24:1). The aqueous layer was recovered and 0.1
49 50 160 volume (v/v) of sodium acetate 3M (ph 6.6) and 2 volumes of ice-cold ethanol were
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161 added. DNA was precipitated at -20°C for 1 h. The pellet obtained after centrifugation
162 was washed with 70% ethanol, dissolved in sterile water and stored at -80°C. As a
163 positive control, the DNA of all isolates was extracted from 100 mg of mycelium
164 collected from a PDA plate, using DNeasy Plant Mini Kit (Qiagen, Germany) following
165 the manufacturer's instructions. All DNA samples were quantized using a Qubit dsDNA
166 HS Assay Kit (Thermo Fisher, USA), which is highly selective for double-stranded
167 DNA (dsDNA) over RNA. DNA content was checked on 0.7% agarose gel
168 electrophoresis.
169 For the absolute Real-Time PCR, the standard curve method was used in order to
170 determine the absolute target quantity in samples according to Standard Curve
171 Experiments (Applied Biosystems StepOne™ and StepOnePlus™Real-Time PCR
172 Systems PN 4376784F, Foster City, CA, USA). Measurements of fungal DNA in
173 wheat and rice seeds were taken by interpolation from a standard curve generated with a
174 standard DNA, which was amplified in the same PCR run. The standard curve was
175 generated from 5-fold serial dilutions (ranging from 10 ng to 0.1 pg per tube) of a
176 known concentration of DNA and analyzed in triplicate in three independent assays.
177 The amount of DNA was expressed as pg μl^{-1}. qRT-PCR reactions (20 µL) were carried
178 out with DNA from fungal pure mycelium or from seed samples, 250 nM primers
179 (FusGRAfw: 5'-TCTGCTCTTCCATCTCGTCGG-3'; FusGRArev: 5'-
180 CGTGGCAGTAGTGACTGAACAAACC-3'; TGAMfw: 5'-AACAACCTCCAAAAGTCCGCC-3';
181 TGAMrev1:5'-CGGAAGAGCCGTTGTAGATACC-3'; FOXYfw:5'-TCGATTTCCCCTACGACTCG-
182 3';
183 FOXYrev: 5'-TCAAGTGGCGGGGTAAGTGC-3') and 1x PowerUp™ SYBR™ Green Master
184 Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's
185 instructions. PCR was run in a StepOne realtime PCR System (Applied Biosystems,

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4 186 Foster City, CA, USA) using the recommended thermal cycling conditions (hold 95°C
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6 187 for 20s; 40 cycles at 95°C for 3s and 60°C for 30s). Primer specificity and the absence
7
8 188 of primer–dimer formation during qRT-PCR analysis were indicated in each sample by
9
10 189 the presence of a single peak in the dissociation (melt) curve at the end of the
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12 190 amplification programme. Amplification efficiency, linearity and working range were
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14 191 determined by linear regression analysis of serial dilutions of DNA.
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16 192 As an endogenous control gene could not be used due to the presence of both the
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18 193 substrate (seeds) and fungal DNA in a variable ratio in each sample, a relative Real-
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20 194 Time PCR was performed to compare each sample with the C_t obtained by amplifying
21
22 195 the DNA extracted from seeds treated only with each isolate, using the same primers
23
24 196 previously described. qRT-PCR reactions (20 µL) were carried out with 10 ng of DNA
25
26 197 extracted from rice or wheat seeds inoculated with *F. graminearum* and *F. oxysporum*
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28 198 and/or *T. gamsii*, 250 nM primers and 1x PowerUp™ SYBR™ Green Master Mix
29
30 199 (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.
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32 200 PCR was run in a StepOne realtime PCR System (Applied Biosystems, Foster City, CA,
33
34 201 USA) using the same recommended thermal cycling conditions described for the
35
36 202 absolute Real-Time PCR. Amplifications of the target were run using three biological
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38 203 replicates, each with three technical replicates, and analyzed on the same plate in
39
40 204 separate tubes. The relative abundance of amplicons was calculated by using the
41
42 205 $2^{-\Delta\Delta Ct}$ method (Livak and Schamittgen 2001).
43
44 206 Mycotoxin production: the effect of *T. gamsii* T6085 and *F. oxysporum* 7121, alone and
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46 207 in combination, on mycotoxin production was evaluated and expressed as amount of
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48 208 trichothecenes DON, 3-acethyl DON (3ac-DON) and 15-acethyl Deoxynivalenol (15ac-
49
50 209 DON) produced by *F. graminearum* on rice and wheat. For the analysis of the
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210 trichothecenes, the sample preparation was performed as follows: 1 g of ground infected
211 kernels were extracted with 10 mL acetonitrile/water (84:16, v/v), on an orbital shaker
212 for 2 h. After filtration through filter paper (Whatman N.4), 6 mL of the extract was
213 passed through a Mycosep® #227 column (Romer Labs Diagnostic GmbH, Tulln,
214 Austria) (Weingaertner et al., 1997). The purified extract (3 mL; equivalent to 0.3 g
215 sample) was dried under an air stream at 50°C. The dried residue was reconstituted in
216 500 µL LC mobile phase, i.e. water:methanol (85:15, v/v). A UPLC-PDA analysis was
217 performed according to Pascale et al. (2014). UPLC (Waters) instrument was used with
218 column Aquity UPLC BEH C18 (2.1 x 100 mm, 1.7 µm). The isocratic flow was set at
219 0.350 mL min^{-1} and the mobile phase was water:methanol (85:15 v/v). The injection
220 volume was 10 µL in a full loop injection system. Toxins were detected in 220nm with
221 PDA spectra. The temperature of the column was set at 50°C. Mycotoxin standards
222 (purity> 99%) were supplied by Sigma-Aldrich (Milan, Italy). Water was of Milli-Q
223 quality (Millipore, Bedford, MA, USA). All solvents (HPLC grade) were purchased
224 from J.T. Baker (Deventer, The Netherlands). Aliquots of the stock solution (100 µg
225 mL^{-1} in acetonitrile) were evaporated to dryness under a stream of nitrogen at 50°C. The
226 residue was dissolved in the solvent and water:methanol (85:15, v/v) was added for
227 dilution. In the standard mix the mycotoxin standard was used in 0.1 and 10 µg mL^{-1}
228 concentration. The detection limits of this method were 0.05 mg kg^{-1} for DON and 0.1
229 mg kg^{-1} for 3ac-DON and 15ac-DON.
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231 **Metabolic analysis**

232 In order to verify whether exploitation competition was the mechanism used to control
233 *F. graminearum* growth on a natural substrate, metabolic requirements of *T. gamsii*

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234 T6085, *F. oxysporum* 7121 and *F. graminearum* were analyzed using the Biolog
235 microarray system. A total of 100 µL of a water spore suspension (10^6 spore mL^{-1}) of
236 each of the three fungal isolates were inoculated in each well of a Biolog multiwell plate
237 (FF - for Filamentous Fungi - MicroPlate[tm], Hayward, CA, USA) containing water and
238 95 different carbon sources
239 (http://www.biolog.com/pdf/milit/00A%20010rB%20FF%20Sell%20Sheet%20Mar07.
240 pdf) and incubated at 24°C 12 h light/ 12 h darkness. Three independent replicates were
241 carried out for each strain. Fungal growth was spectrometrically measured as
242 absorbance (OD) at 595nm (Gardiner et al. 2009) for eleven days, every four hours (12
243 hours during the night). OD values were used to calculate growth curves (GraphPad
244 Software Inc., La Jolla, CA, USA) and the slopes of the linear phase were used to create
245 a heatmap showing a qualitative picture of the different metabolic abilities of each
246 isolate. Hierarchical clustering of species and substrates according to slope values was
247 performed and visualized using the heatmap package (Kolde 2015) in R (R Core Team,
248 2012).
249 For each isolate, slopes of the linear phase of growth curves on each of the 95 substrates
250 were submitted to analysis of variance of regression to compare the slopes with the
251 growth curve in water. The substrates whose slopes were significantly higher from those
252 in water were considered as metabolized ($P_{slope} \leq 0.05$) and used to calculate the total
253 number of carbon sources used by each isolate. For each substrate, the linear growth
254 curves of two isolates (assumed as isolates A and B) were submitted to analysis of
255 variance of regression to compare their slopes. This detected which and how many
256 substrates were used in the same way by the two isolates ($P_{slope} \geq 0.05$).

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257 These data were then used to calculate the Niche Overlapping Index (NOI) between
258 isolate A and isolate B, according to Wilson and Lindow (1994), with a small
259 modification, as shown below:
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$$NOI_{AB} = \frac{\text{no. of carbon sources used in the same way (Pslope} \geq 0.05\text{) by A and B}}{\text{Total no. of carbon sources used by A (Pslope} \leq 0.05 \text{ compared to water)}}$$

265
266 NOI was calculated for each combination of isolates A and B, and expressed as NOI_{AB}
267 and NOI_{BA}.
268 Finally, all OD data were used to create growth curves for each isolate on each
269 substrate. Each substrate was classified as slow (supporting a poor growth), medium
270 (medium growth) and high (fast growth) by a semi-parametric functional clustering
271 method. The functional clustering was performed according to the following two steps
272 implemented in the Functional Clustering Algorithms (Funcy) R package version 0.8.6.
273 (Yassouridis 2017), which was built to implement methods described by Chiou and Pai-
274 Ling (2007):
275 - growth curves were estimated non parametrically using spline basis functions, with
276 coefficients chosen so to include all time points in the estimation;
277 - curve clustering was obtained by applying mixed effect models to the base coefficients
278 as implemented in the funcit function (method option 3 i.e. iter Sub space described by
279 Chiou and Pai-Ling (2007).

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280 The procedure includes information on the entire curve and its behaviour in the
281 classification procedure.
282

283 **Antibiosis and mycoparasitism**

284 In order to preliminary assess which mechanism could be involved in the interference
285 competition between *F. graminearum* and the two beneficial isolates *F. oxysporum*
286 7121 and *T. gamsii* T6085, two antibiosis and one mycoparasitism test were performed.
287 Antibiosis in dual cultures: in the first experiment, PDA discs of 6 mm diameter, cut
288 from the edge of actively growing colonies of each antagonist and of the pathogen, were
289 placed at opposite sides (4.5 cm from each other) on PDA plates. Plates were incubated
290 at 24°C, under photoperiod of 12h light / 12h darkness. The radius of each fungal
291 colony, both facing the colony of the other isolate and in a perpendicular (control)
292 direction (Matarese et al., 2012) were measured three times a day, until contact. Since
293 the combination *T. gamsii* T6085 vs *F. graminearum* ITEM124 was already tested by
294 Matarese et al. (2012), in the present work the experiment was performed by inoculating
295 *F. oxysporum* 7121 against *F. graminearum* and against *T. gamsii* T6085, this last to
296 exclude a possible interaction between the two beneficial fungi. Each combination was
297 set up in triplicate. Growth values were used to create curves further subjected to an
298 analysis of variance of regression to compare the slope and the elevation of curves in
299 the presence/absence of the other isolate, assuming $P<0.05$ as a significant level.
300 Evaluation of the effect of secondary metabolites: antibiotic effect of secondary
301 metabolites produced by the beneficial isolates was evaluated against *F. graminearum*.
302 PDB (Potato Dextrose Broth, BD, Difco, USA) spore suspensions of *F. oxysporum*
303 7121 (FO) and of *T. gamsii* T6085 (T) were inoculated, alone and in combination, in

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304 250 mL Roux bottles containing 100 mL of PDB, at the final concentration of 10^4
305 conidia mL^{-1}. Uninoculated medium was used as controls. Each thesis consisted of three
306 independent replicates. Bottles were incubated on a shaker at 100 rpm, 24°C, under
307 photoperiod of 12h light / 12h darkness for 21 days. At the end of the incubation period,
308 culture broths were collected by filtering through a Miracloth layer and sterilized by
309 filtration (20 µm). Filtrates were used to evaluate *F. graminearum* growth in 96-well
310 microplates (PbI International, Milan, Italy) using a spectrophotometric approach. In
311 details, each well was filled with 150 µL of filtered broth (from *T. gamsii* and *F.*
312 *oxysporum* cultures, alone and in co-culture) inoculated with 5 µL of an aqueous spore
313 suspension (at the final concentration of 10^5 conidia mL-1) of *F. graminearum*
314 prepared from OA cultures grown for 1 week at 24°C under photoperiod of 12h light /
315 12h darkness. Three replicates (for each of the three independent replicated liquid
316 cultures), with 6 wells each, were used. Fresh PDB inoculated with the pathogen was
317 used as a control. Fungal growth was monitored for a period of 120 h by reading the
318 absorbance of the suspensions at 595 nm (OD) every 4h (12h during the night) in a
319 microplate reader 680 (Bio-Rad Laboratories, Hercules, CA, USA). OD values were
320 used to create growth curves. Data were subjected to analysis of variance of regression
321 in order to compare the slope of the curve of the pathogen grown on PDB with those of
322 the pathogen grown in the filtered media used to cultivate the antagonists, assuming
323 $P<0.05$ as a significant level.
324 Mycoparasitism: PDA discs of 6 mm diameter, cut from the edge of actively growing
325 colony of each antagonist and pathogen, were placed at opposite sides (4.5 cm from
326 each other) on a sterile cellophane membrane (10 cm diameter, food grade) laid on
327 water agar (WA) medium (20 g bacteriological agar l21, BD, Difco, USA). Since the

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combination *T. gamsii* T6085 vs *F. graminearum* ITEM124 was already tested by Matarese et al. (2012), in the present work the experiment was performed by inoculating *F. oxysporum* 7121 against *F. graminearum* and against *T. gamsii* T6085, this last to exclude a possible interaction between the two beneficial fungi. Each fungal combination was set up in triplicate. Plates were incubated at 24°C, under photoperiod of 12h light / 12h darkness and, after 21 days, signs of mycoparasitism (coilings and short loops) between tested fungi were microscopically evaluated.

RESULTS

Competition test on natural substrates

A competition test was performed on both wheat and rice kernels, in order to evaluate the growth of *T. gamsii* T6085 and *F. oxysporum* 7121, and their effects, alone and in combination, on the growth and mycotoxin production of *F. graminearum* after 21 days of incubation. Results obtained from the test and submitted to statistical analysis are shown here.

Fungal growth

Data obtained by absolute Real-Time PCR for all three fungi both on wheat and rice, are showed in Figure 1. Since data obtained by relative Real-Time PCR analysis confirmed what obtained by absolute Real-Time PCR, they were not shown. No DNA contamination of *F. graminearum*, *T. gamsii* and *F. oxysporum* was detected in uninoculated wheat and rice kernels.

When inoculated on wheat, the growth of *F. graminearum* was significantly reduced by the presence of *F. oxysporum* 7121. With *T. gamsii* T6085, the pathogen's growth was even more strongly reduced, resulting in a significant difference when compared with

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352 the effect caused by *F. oxysporum* 7121 alone. However, when inoculated together, the
353 two antagonists caused the highest significant growth inhibition of the pathogen (Figure
354 1A). The same trend was observed on rice: growth of *F. graminearum* was significantly
355 reduced by the two antagonists, both alone and co-inoculated, with the highest
356 significant effect obtained when *T. gam*sii T6085 and *F. oxysporum* 7121 were
357 inoculated together (Figure 1B).
358 When inoculated either on wheat or on rice, the growth of *T. gamsii* was significantly
359 reduced by the presence of *F. oxysporum* and *F. graminearum*, both alone and co-
360 inoculated. However, no difference in *T. gamsii* growth was detected among these three
361 latter samples (T+FO, T+FG, T+FG+FO) (Figures 1C and 1D).. In any case, even if
362 statistically significant, the reduction was not so evident as for *F. graminearum*.
363 When inoculated either on wheat or on rice, the growth of *F. oxysporum* was
364 significantly reduced by *T. gamsii*, both alone and co-inoculated with the pathogen,
365 while no significant difference was detected with the pathogen alone (Figures 1E and
366 1F).
367 Mycotoxin production
368 When inoculated on wheat, after 21 days of incubation, *F. graminearum* produced all
369 the three mycotoxins analysed here, although in different amounts. The presence of *F.*
370 *oxysporum* 7121 significantly reduced the amount of 15Ac-DON, while there was non-
371 statistically significant reduction of DON and 3Ac-DON. When in the presence of *T.*
372 *gamsii* T6085, both alone and in combination with *F. oxysporum* 7121, the production
373 of all three mycotoxins was significantly reduced, with no different values compared to
374 those detected in the uninoculated control (Table 1).

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375 When inoculated on rice, after 21 days of incubation, *F. graminearum* produced all the
376 three mycotoxins analysed. When in presence of *F. oxysporum* 7121 there was no
377 statistically significant reduction of DON, 3Ac-DON and 15Ac-DON. When in
378 presence of *T. gamsii* T6085, both alone and in combination with *F. oxysporum* 7121,
379 the production of all three mycotoxins was lower, with a significant reduction for both
380 DON and 3Ac-DON (Table 2).
381

382 **Metabolic analysis**

383 OD data were submitted to statistical analyses in order to define whether compounds
384 included in the Biolog multiwall (FF) MicoPlates could be considered as a potential
385 source of competition. This test helps in analysing whether exploitation competition
386 could explain the effects of both the antagonists on the growth of the pathogen on wheat
387 and rice kernels. Figure 2 shows the results of the hierarchical clustering analysis by a
388 heatmap. Many compounds are either not, or poorly, utilized by all fungi (for example
389 all the compounds in cluster 2, which includes the water control), while some
390 compounds are better utilized by a single fungus (compounds in clusters 3 by *F.*
391 *graminearum*, in cluster 4 by *T. gamsii*, in cluster 5 by *F. oxysporum*). Almost all the
392 compounds included in cluster 1 are better utilized by the two *Fusarium* species (*F.*
393 *graminearum* better than *F. oxysporum*) than by *T. gamsii.* Of note is the very high
394 utilization rate of the compounds, in cluster 1, from D-Gluconic acid and L-Malic acid
395 by *F. graminearum*. This analysis suggests that the two antagonists are more related,
396 from a metabolic point of view, to each other than to the *F. graminearum* isolate, the
397 latter being able to use more substrates with higher slopes.

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398 The compounds rapidly utilized by the various fungi do not overlap, apart from those
399 from cluster 1, which are partially shared between the two *Fusarium* species. The
400 overlap in nutrient is better understood by the Niche Overlapping Index (NOI). When
401 slope values of the linear phase were used to calculate the NOI, according to the
402 formula shown in Materials and Methods, all three isolates seem to occupy different
403 niches, as shown in Table 3 (NOI<0.9).
404 Figure 3 shows the growth curves of the three isolates on 93 substrates (two substrates,
405 i.e. Sebacic acid G2 - and Adenosine H10 gave unclear results and were withdrawn
406 from these analyses) calculated by the semi-parametric "functional clustering" method,
407 considering data collected during the whole experimental period. The substrates could
408 thus be subdivided into three categories regarding their utilization: i) high (green
409 curves), ii) medium (red curves), and iii) slow (black curves) (Figure 3). *F.*
410 *graminearum* mainly showed curves in the "high" group (Figure 3B) whereas the
411 curves referring to *F. oxysporum* 7121 and *T. gamsii* T6085 showed mainly substrates
412 in the "medium" and "slow" groups (Figures 3C and D, respectively).
413 In Table 4, for each category (slow, medium and high) substrates are grouped according
414 to the isolates (alone or in all the possible different combinations) they are metabolized
415 by. Data presented in Table 4 and Figure 3 indicate that *F. graminearum* utilizes the
416 carbon sources tested better than the other two fungi (17 in the slow category, 22
417 medium and 54 high), followed by *F. oxysporum* (31 slow, 62 medium and 0 high) and
418 then by *T. gamsii* (70 slow, 23 medium, 0 high). Within the "slow" category, 12
419 substrates are common to all the three fungi. All, except four substrates, are in the same
420 sub-cluster of cluster 2 along with water, as shown in the heatmap (Figure 2), thus
421 confirming that they are not metabolized by our fungi. There are more substrates in

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422 common between the two antagonists (28) than between the pathogen and each of the
423 two beneficial isolates (11), thus in accordance with Table 4.
424 If the hierarchical clustering (heatmap) (Figure 2) is compared to the functional
425 clustering outputs (Figure 3), a different picture appears, but always in favour of a better
426 exploitation of substrates by *F. graminearum*. A total of 27 substrates coded in the
427 white-blue range for the pathogen in the heatmap are now included in the "high" group.
428 This suggests that the evaluation of the whole curve (richer in information if compared
429 to the linear phase) gives us a better information about the metabolization of substrates.
430

431 **Antibiosis and mycoparasitism**

432 In order to evaluate if interference competition, by direct combat, could be the
433 mechanism used by the two beneficial isolates, alone and in combination, against *F.*
434 *graminearum*, antibiosis and mycoparasitism tests were performed. Results obtained by
435 antibiosis tests and microscopic observations are reported. These results will be
436 discussed together with those obtained in our earlier work (Matarese et al. 2012) (*T.*
437 *gamsii* vs *F. graminearum*)
438 Antibiosis in dual cultures: *F. oxysporum* 7121 did not affect the growth of *F.*
439 *graminearum* and *T. gamsii* T6085 in dual cultures on PDA. As shown in Table 5, there
440 were no significant differences in slope (P=0.24) or elevation (P=0.17) of growth curves
441 of *F. graminearum* in presence/absence of *F. oxysporum* 7121. The same trend was
442 observed for *T. gamsii* T6085 that showed no significant difference in slope (P=0.89) or
443 elevation (P=0.66) of curves when in presence of the other beneficial isolate, compared
444 to control.

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445 Evaluation of the effect of secondary metabolites: In Table 6 the effect of the cultural
446 filtrates of *F. oxysporum* 7121 and *T. gamsii* T6085, alone and co-cultured, on the
447 growth of *F. graminearum* is shown. The growth of the pathogen was significantly
448 reduced when inoculated in the cultural filtrates of *T. gamsii* T6085 alone or co-cultured
449 with *F. oxysporum* 7121 (P_{slope}<0.0001 and P_{slope}=0.001, respectively). Metabolites
450 present into the cultural filtrate of *F. oxysporum* 7121 seem to affect only the elevation
451 of *F. graminearum* growth curve, causing a delay in the beginning of the germination of
452 its spore ($P_{elevation}$=0.03), but without affecting the growth rate (P_{slope}=0.090).
453 Mycoparasitism: we investigated whether *F. oxysporum* 7121 would coil or produce
454 short loops on the hyphae of the pathogen, which are considered to be signs of
455 mycoparasitism. *F. oxysporum* 7121 did not produce coils or short loops around the
456 hyphae of the pathogen, thus excluding a direct mycoparasitic activity. When the zone
457 of interaction between *F. oxysporum* 7121 and *T. gamsii* T6085 were microscopically
458 observed, no signs of mycoparasitism between these two beneficial isolates were
459 detected, thus excluding a detrimental interaction between them.
460
461
462 **DISCUSSION**
463 One of the most important parts of the life cycle of *F. graminearum* takes place outside
464 the plant where the pathogen produces a plethora of enzymes, which allow it to use
465 nutrient sources, such as cultural debris, both for its saprotrophic growth and for
466 increasing the initial inoculum. In this environment there is a strong competition not
467 only against other *Fusarium* species belonging to the FHB species complex but also
468 against other inhabitants with good saprotrophic fitness. To the best of our knowledge,

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469 this is the first study to use two or more beneficial fungi to control growth of *F.*
470 *graminearum* and mycotoxin contamination. The present work evaluates for the first
471 time the role of competition in a multitrophic approach involving a well known
472 beneficial isolate of *T. gamsii* and a *F. oxysporum* strain, the latter selected for its ability
473 to colonize straw debris (Matarese et al. 2012; Sarrocco et al. 2012; 2013).
474 Since *F. graminearum* survival is enhanced by high quantities of available crop residues
475 (Leplat et al. 2013), in the present work we used two very nutrient rich substrates, i.e.
476 wheat and rice kernels, on which *F. graminearum* grows very well and produces a high
477 amount of DON (Matarese et al. 2012). In these conditions, its growth was significantly
478 reduced by both the antagonists, with the co-presence of *T. gamsii* and *F. oxysporum* as
479 the most effective, on both substrates. Under the same condition, *T. gamsii* growth was
480 slightly reduced in the presence of both the pathogen and *F. oxysporum*, either alone or
481 co-inoculated and on both substrates. This led us to hypothesize that the reduced growth
482 of *T. gamsii* was related more to a lower availability of nutrients than to the presence of
483 a given isolate. The behaviour of *F. oxysporum* was slightly different since it was not
484 affected by the presence of the pathogen alone, but showed a growth reduction when co-
485 occurring with *T. gamsii,* either alone or with *F. graminearum*, on both substrates.
486 However, the strong positive effect of *F. oxysporum* against the pathogen, when co-
487 inoculated together with *T. gamsii*, suggests that the use of this mix would broaden its
488 spectrum of applicability, while the possible additive/synergic effect of the two
489 beneficial isolates needs to be better evaluated. Finally, on both substrates, the
490 mycotoxin content was significantly reduced, thus demonstrating a double effect of the
491 two competitive isolates on pathogen growth and mycotoxin production. This was
492 reported also by Naef et al. (Naef et al. 2006), who found a 36% reduction in DON

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493 production, when *F. graminearum* was inoculated in the presence of *Trichoderma*
494 *atroviride* on autoclaved maize leaves. Recently, Tian et al. (2016), demonstrated the
495 occurrence of modified mycotoxin deoxynivalenol-3-glucoside (D3G) – a well
496 known detoxification product of DON in plants - in *F. graminearum* and
497 *Trichoderma* interaction. This provides evidence that *Trichoderma* isolates, able to
498 reduce *F. graminearum*'s growth, also possess a self-protection mechanism, as
499 plants have, to detoxify DON into D3G when competing with this pathogen.
500 The two beneficial fungi seemed to have the capacity to outcompete the pathogen, at
501 least in these semi-*in vivo* conditions. Although artificial media are unrealistic and fail
502 to reflect the complexity of the natural ecosystems, they offer a simpler model that
503 enables those mechanisms to be extensively investigated, that are sometimes difficult to
504 demonstrate in natural conditions (Crowther et al. 2017). Despite the need to consider
505 additional environmental factors such as temperature, water, light and O_2 - key
506 conditions for *F. graminearum* growth and the development of its sexual reproduction
507 structures in nature – the system described here gives us a positive indication that the
508 two antagonists have the potential to compete with the pathogen, thus reducing its
509 development and mycotoxins production.
510 Exploitation competition can play a role in the control of pathogen growth and we used
511 the Biolog Phenotype MicroArray, a high throughput system for the identification of
512 carbon sources and other nutrients used for the growth of various microorganisms that
513 can be used to characterize the fungal metabolism and for comparing growth and
514 defining nutritional utilization patterns (Druzhinina et al. 2006; Mohale et al. 2013).
515 This information can be used to calculate the niche overlap index, in order to study how

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4 516 two or more organisms can compete for nutrients, but excluding at the same time any
5
6 517 direct interaction among the organisms.
7
8 518 We adopted two analyses, based on different assumptions: the “hierarchical clustering”
9
10 519 (according to slope values of the linear phase of curves) performed and visualized using
11
12 520 the heatmap package (Kolde 2015) within R (R Core Team, 2012) and, for the first
13
14 521 time, the semi-parametric “functional clustering” method (Chiou and Pai-Ling 2007)
15
16 522 which takes into account the whole curve. Hierarchical clustering is a simplified method
17
18 523 to compare fungal growth by comparing the slopes in the linear phase of growth curves.
19
20 524 However this adds uncertainty to the evaluation, as the linear phase is not so easy to
21
22 525 define when 279 curves are taken into consideration. Moreover, the use of linear phase
23
24 526 slopes does not take into consideration the lag phase required by a fungus to adapt to a
25
26 527 novel nutrient or the area under the curve which represents the total amount of biomass
27
28 528 produced by that fungus on that substrate. However, linear phase slopes better explain
29
30 529 how the fungus grows and metabolizes single substrates over time compared with the
31
32 530 more classical method that only takes into consideration the absorbance at one point in
33
34 531 calculating the niche overlapping index (Mahale et al. 2013).
35
36 532 In addition to carbon sources, Biolog multiwell (FF) MicroPlates also contain 26
37
38 533 nitrogen sources, including 12 L-amino acids. Figure 2 shows that 17 (including 11 L-
39
40 534 amino acids out of the 12 tested) out of the 26 N containing substrates are in clusters 1
41
42 535 and 3 (and almost all in the “high” group), indicating that these substrates play an
43
44 536 important role in competition. Among the nitrogen substrates in cluster 1 and in the
45
46 537 “high” group, L-Ornithine and the plant stress metabolite Putrescine are both known to
47
48 538 stimulate DON production (Gardiner et. al., 2009). DON could play an important role,
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50 539 which is not yet completely understood, during the interactions between the pathogen
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540 that produces it and one or more beneficial organisms. In the multitrophic interactions
541 that *F. graminearum* faces during its saprotrophic growth, the production of L-
542 Ornithine and Putrescine is therefore significant (Abid et al. 2011).
543 Our results confirm that *F. graminearum* can grow very fast, as reported also by Pereyra
544 and Dill-Macky (2008). We also found no evident exploitation competition (direct
545 competition for nutrients) among the pathogen and the two beneficial organisms,
546 suggesting that interference competition could play a major role in the reduction of
547 growth and mycotoxin production registered in the first experiment. Interference
548 competition can be due to different mechanisms: physically, by direct hyphal contact
549 (Rayner and Todd 1979), or with the involvement of soluble or volatile chemicals (Dix
550 and Webster 1995). Secondary metabolites play a pivotal role in the antagonistic
551 activities of some biocontrol species of *Trichoderma,* resulting in the suppression of
552 fungal pathogens (Vinale et al 2008; Vinale et al 2014). Our early interference
553 competition study showed that *T. gamsii* T6085 significantly reduced the growth of *F.*
554 *graminearum* on PDA and the antagonist produced coilings and short loops around the
555 hyphae of the pathogen on WA (Matarese et al. 2012). In the present work the same
556 experiments were made in order to verify if also *F. oxysporum* 7121 could reduce the
557 growth of the pathogen and parasitize its mycelium. Our results showed neither growth
558 inhibition nor signs of mycoparasitism of *F. graminearum* by *F. oxysporum* 7121. *F.*
559 *oxysporum* 7121 also showed no antagonistic or mycoparasitic activity against *T. gamsii*
560 T6085. To verify if the co-culturing of the two antagonists could modify the effects
561 recorded on PDA, cultural filtrates of *T. gamsii* T6085 and *F. oxysporum* 7121, alone
562 and from co-culture, were tested on spore germination and germlings growth of *F.*
563 *graminearum*. Our results showed that *T. gamsii* T6085 is able to produce, after 21 days

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of incubation in PDB - alone and co-cultured with *F. oxysporum* 7121 - secondary metabolites that can significantly reduce the growth of the pathogen. *F. oxysporum* seems to be less effective in producing active secondary metabolites, since only a delay in the beginning of *F. graminearum* growth was registered.

Further investigations are required in order to move from the lab to the field the use of both these beneficial fungi in a multitrophic approach aimed at controlling FHB on wheat. However, a deeper knowledge of the ecological basis of FHB biocontrol by a multitrophic approach is a pre-requisite to implement such a strategy as a wheat protection practice.

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AUTHOR CONTRIBUTIONS STATEMENTS

SS conceived the study, designed the experimentation, discussed results, wrote the manuscript and insured relations between all co-authors. FV and GP performed Biolog experimentation and processed samples. RBa analyzed Biolog data and discussed results. SM, PE and GP performed substrate colonization experimentation and processed samples. RBe set up the Real-Time PCR protocol, analyzed samples and discussed RT-PCR results. MH and AM performed

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588 mycotoxins analyses and discussed mycotoxins results. GV conceived the study,
589 designed the experimentation, discussed results and reviewed the manuscript. All
590 authors commented on the manuscript.
591

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4 725 **CAPTIONS FOR FIGURES**
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11 728 **Figure 1.** DNA concentration (measured by absolute Real-Time PCR and submitted to
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13 729 log transformation) of *F. graminearum* in wheat (A) and rice (B) kernels; of *T. gamsii*
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15 730 T6085 in wheat (C) and rice (D) kernels; of *F. oxysporum* in wheat (E) and rice (F)
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17 731 kernels. CONT: uninoculated kernels; FG: kernels inoculated with *F. graminearum*;
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19 732 FG+FO: kernels inoculated with *F. graminearum* and *F. oxysporum* 7121; FG+T:
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21 733 kernels inoculated with *F. graminearum* and *T. gamsii* T6085; FG+FO+T: kernels
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23 734 inoculated with *F. graminearum*, *T. gamsii* T6085 and *F. oxysporum* 7121. Different
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25 735 letters, within the same graph, correspond to significantly different values (ANOVA,
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27 736 $P \leq 0.05$).
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32 738 **Figure 2.** Hierarchical clustering of isolates (*F. graminearum*, *F. oxysporum* 7121 and
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34 739 *T. gamsii* T6085) and substrates (included into the Biolog Phenotype MicroArray FF
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36 740 microplates) according to slope values of the linear phase of growth curves was
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38 741 performed and visualized using the heatmap package (Kolde, 2015) within R (R Core
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40 742 Team 2012). Slopes values were calculated for each fungal growth curve for each
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42 743 substrate, using the OD values measured by the Biolog system. Letters S, M and H
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44 744 (slow, medium and high, respectively) within coloured rectangles refer to the groups
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46 745 obtained by the semi-parametric method (Table 4). Some clusters are numbered (from 1
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48 746 to 5) as they are commented on in the text. Underlined substrates contain nitrogen.
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748 **Figure 3.** Growth curves on each of the Biolog substrates: all three fungi together (A);
749 *F. graminearum* (B); *F. oxysporum* 7121 (C); and *T. gamsii* T6085 (D). Substrates are
750 grouped into three categories: slow (S, black), medium (M, red) and high (H, green),
751 according to a semi-parametric "functional clustering" method (Chiou and Pai-Ling,
752 2007).
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Table 1. Effects of *T. gamsii* T6085 and *F. oxysporum* 7121 on mycotoxin production by *F. graminearum* on wheat.

Samples	DON	3Ac-DON	15Ac-DON
CONT	n.d. a	n.d. a	n.d. a
FG	683.90 b	20.23 b	11.70 b
FG+FO	278.10 ab	6.59 ab	6.77 a
FG+T	5.60 a	n.d. a	n.d. a
FG+FO+T	10.10 a	n.d. a	n.d. a

Mycotoxins values are expressed as $\mu g\ g^{-1}$. *F. graminearum* (FG); *F. graminearum* and *F. oxysporum* 7121 (FG+FO); *F. graminearum* and *T. gamsii* T6085 (FG+T), *F. graminearum*, *F. oxysporum* 7121 and *T. gamsii* T6085 (FG+FO+T), no inoculation (CONT). Different letters, within the same column, correspond to significantly different values (ANOVA), $P \leq 0.05$. When mycotoxin contents were under the detection limits (n.d.), the detection limit values (DON= 0.05 mg kg^{-1}; 3Ac-DON and 15Ac-DON= 0.1 mg kg^{-1}) were used to perform the ANOVA.

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Table 2. Effects of *T. gamsii* T6085 and *F. oxysporum* 7121 on mycotoxin production by *F. graminearum* on rice.

Samples	DON	3Ac-DON	15Ac-DON
CONT	n.d. a	n.d. a	n.d. a
FG	2814.70 b	23.20 b	134.35 a
FG+FO	1888.60 b	16.78 ab	58.35 a
FG+T	90.60 a	n.d. a	n.d. a
FG+FO+T	95.20 a	n.d. a	n.d. a

Mycotoxins values are expressed as μg g^{-1}. *F. graminearum* (FG); *F. graminearum* and *F. oxysporum* 7121 (FG+FO); *F. graminearum* and *T. gamsii* T6085 (FG+T), *F. graminearum*, *F. oxysporum* 7121 and *T. gamsii* T6085 (FG+FO+T), no inoculation (CONT). Different letters, within the same column, correspond to significantly different values (ANOVA), $P \leq 0.05$. When mycotoxin contents were under the detection limits (n.d.), the detection limit values (DON= 0.05 mg kg^{-1}; 3Ac-DON and 15Ac-DON= 0.1 mg kg^{-1}) were used to perform ANOVA.

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Table 3. Niche Overlapping Index (NOI) of *F. graminearum*, *T. gamsii* T6085 and *F. oxysporum* 7121, according to the ability to grow on the 95 carbon source included in the Biolog FF plates.

	Isolate B		
Isolate A	*F. graminearum*	*T. gamsii* T6085	*F. oxysporum* 7121
F. graminearum	1.00	0.17	0.13
T. gamsii T6085	0.10	1.00	0.22
F. oxysporum 7121	0.14	0.37	1.00

NOI values are between 1 and 0, and define whether, or not, fungi co-exist. They occupy the same niche ($NOI_{A/B}$ and $NOI_{B/A} > 0.9$); they occupy separate niches ($NOI_{A/B}$ and $NOI_{B/A} < 0.9$); one strain dominates ($NOI_{A/B} > 0.9$ and $NOI_{B/A} < 0.9$ means isolate A nutritionally dominates over isolate B).

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Table 4. Substrates included in the Biolog Phenotype MicroArray FF microplates have been grouped into three categories: slow (S) medium (M) and high (H), according to a "functional clustering" semi-parametric method on the basis of the growth curves shown in Figure 2, and assigned to each fungus or group of fungi according to their ability to metabolize them.

	Substrates metabolized by*						
Category	**FG, T, FO**	**FG, FO**	**FG, T**	**T, FO**	**FG**	**FO**	**T**
Slow (S)	A5, B1, B2, B6, B8, C1, C2, C9, C10, D6, D10, E3	C8	A3, A8, A9, F6	B4, B11, C6, D7, E8, F2, F5, F7, F8, G6, G7, H1, H3, H7, H11, H12		A6, E2,	A4, A11, B10, C3, C4, C7, C11, D3, D4, D5, D8, D9, D11, D12, E1, E4, E5, E6, E11, F1, F3, F4, F9, F10, F11, F12, G1, G3, G4, G5, G9, G11, H2, H4, H5, H6, H8, H9
Medium (M)	A2, A12, B5, B7, B9, B12, C5, D2	D9, D12, E1, G3, H5, H8		A7, A10, B3, C12, D1, E7, E9, E10, E12, G8, G10, G12	B4, B11, C6, D7, F2, F8, G6, H3,	A3, A4, A8, A9, A11, B10, C3, C4, C7, C11, D3, D4, D5, D8, D11, E4, E5, E6, E11, F1, F3, F4, F6, F9, F10, F11, F12, G1, G4, G5, G9, G11, H2, H4, H6, H9	A6, C8, E2,
High (H)					A4, A6, A7, A10, A11, B3, B10, C3, C4, C7, C11, C12, D1, D3, D4, D5, D8, D11, E2, E4, E5, E6, E7, E8, E9, E10, E11, E12, F1, F3, F4, F5, F7, F9, F10, F11, F12, G1, G4, G5, G7, G8, G9, G10, G11, G12, H1, H2, H4, H6, H7, H9, H11, H12		

***F. graminearum* (FG), *T. gamsii* T6086 (T) and *F. oxyporum* 7121 (FO)**

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Table 5. Antagonistic effect, expressed as fungal growth inhibition, of *F. oxysporum* 7121 against *F. graminearum* and *T. gamsii* T6085 on PDA plate.

Dual culture		Slope	R^2	P	P_{slope}	$P_{elevation}$
F. graminearum	**CONT**	0.39 ± 0.01	0.97	< 0.0001		
	vs *F. oxysporum*	0.42 ± 0.02	0.97	< 0.0001	0.24	0.17
***T. gamsii* T6085**	**CONT**	0.62 ± 0.05	0.92	< 0.0001		
	vs *F. oxysporum*	0.60 ± 0.06	0.90	< 0.0001	0.89	0.66

Analysis of variance of regression of growth curves of *F. graminearum* and *T. gamsii* T6085 in dual culture with *F. oxysporum* 7121 on PDA. Slope: fungal growth rate expressed as mm h^{-1}; R^2 and P: significance of regression line; P_{slope}: significance of differences between slopes of growth curves of the fungal isolate along the control radius (CONT) and along the radius facing *F. oxysporum* 7121; $P_{elevation}$: significance of differences between elevations of growth curves of the fungal isolate along the control radius (CONT) and along the radius facing *F. oxysporum* 7121.

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Table 6. Antagonistic effect of cultural filtrates of *F. oxysporum* 7121 and *T. gamsii* T6085 (alone and co-cultured) on *F. graminearum* growth, expressed as OD.

Thesis	Slope	R^2	P	P_{slope}	$P_{elevation}$
PDB	0.009 ± 0.000	0.85	< 0.0001		
PDB *F. oxysporum* 7121	0.009 ± 0.000	0.85	< 0.0001	0.090	0.003
PDB *T. gamsii* T6085	0.003 ± 0.000	0.70	< 0.0001	< 0.0001	
PDB *F. oxysporum* 7121 + *T. gamsii* T6085	0.006 ± 0.000	0.68	< 0.0001	0.001	

Analysis of variance of regression of growth curves of *F. graminearum* in PDB and in cultural filtrates of the two antagonists. Slope: fungal growth rate expressed as OD; R^2 and P: significance of regression line; P_{slope}: significance of differences between slopes of growth curves of *F. graminearum* in PDB (control) and in PDB used to culture the two antagonists (alone and co-cultured); $P_{elevation}$: significance of differences between elevations of the growth curves of *F. graminearum* in PDB (control) and in PDB used to culture the two antagonists (alone and co-cultured).

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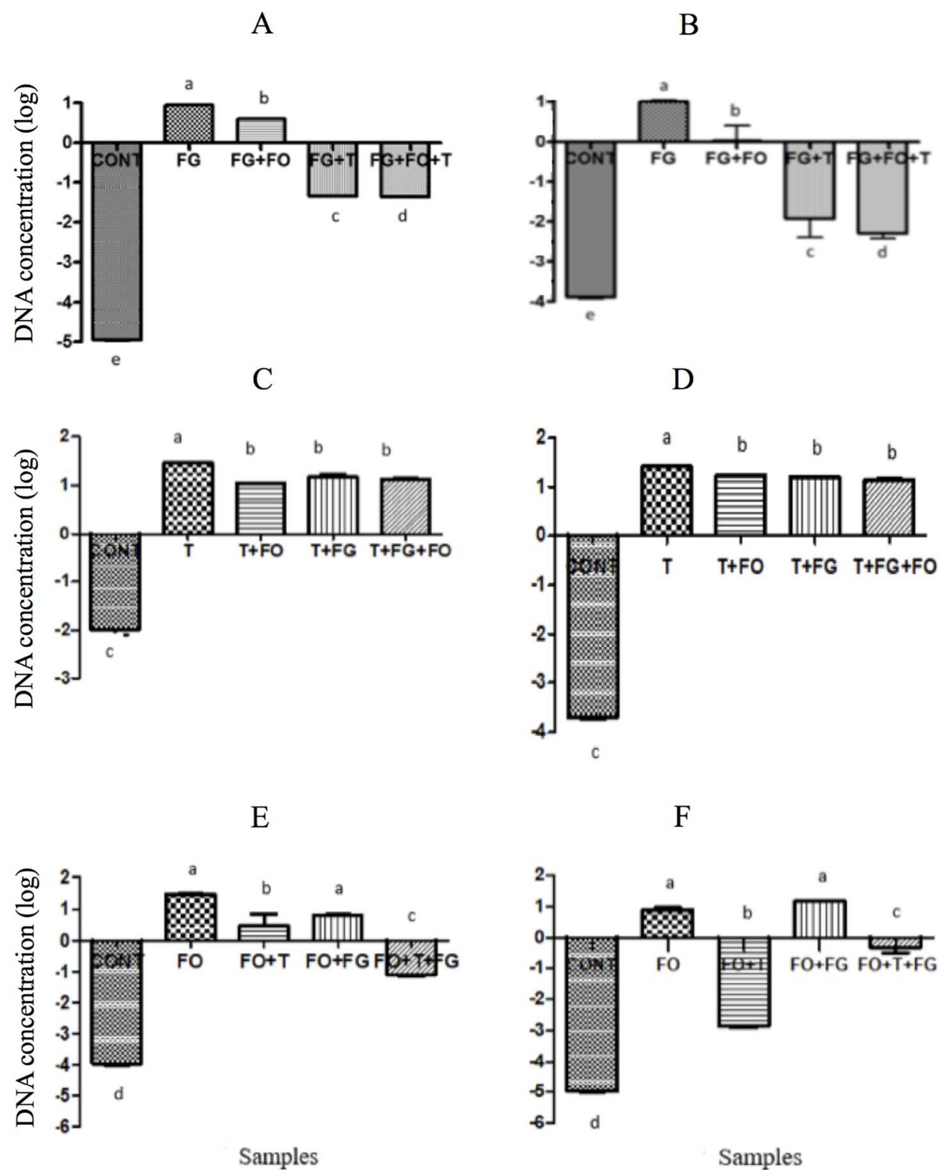

Figure 1. DNA concentration (measured by absolute Real-Time PCR and submitted to log transformation) of F. graminearum in wheat (A) and rice (B) kernels; of T. gamsii T6085 in wheat (C) and rice (D) kernels; of F. oxysporum in wheat (E) and rice (F) kernels. CONT: uninoculated kernels; FG: kernels inoculated with F. graminearum; FG+FO: kernels inoculated with F. graminearum and F. oxysporum 7121; FG+T: kernels inoculated with F. graminearum and T. gamsii T6085; FG+FO+T: kernels inoculated with F. graminearum, T. gamsii T6085 and F. oxysporum 7121. Different letters, within the same graph, correspond to significantly different values (ANOVA, $P<0.05$).

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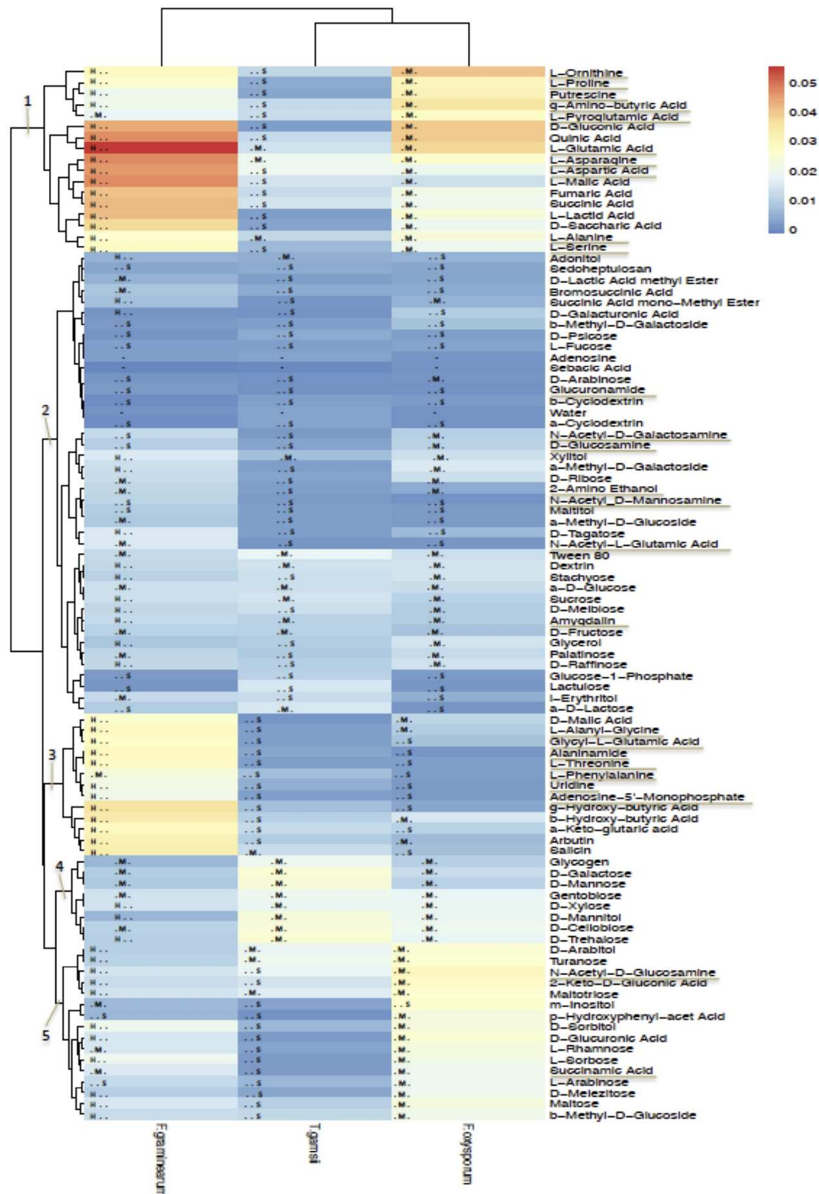

Figure 2. Hierarchical clustering of isolates (F. graminearum, F. oxysporum 7121 and T. gamsii T6085) and substrates (included into the Biolog Phenotype MicroArray FF microplates) according to slope values of the linear phase of growth curves was performed and visualized using the heatmap package (Kolde, 2015) within R (R Core Team 2012). Slopes values were calculated for each fungal growth curve for each substrate, using the OD values measured by the Biolog system. Letters S, M and H (slow, medium and high, respectively) within coloured rectangles refer to the groups obtained by the semi-parametric method (Table 4). Some clusters are numbered (from 1 to 5) as they are commented on in the text. Underlined substrates contain nitrogen.

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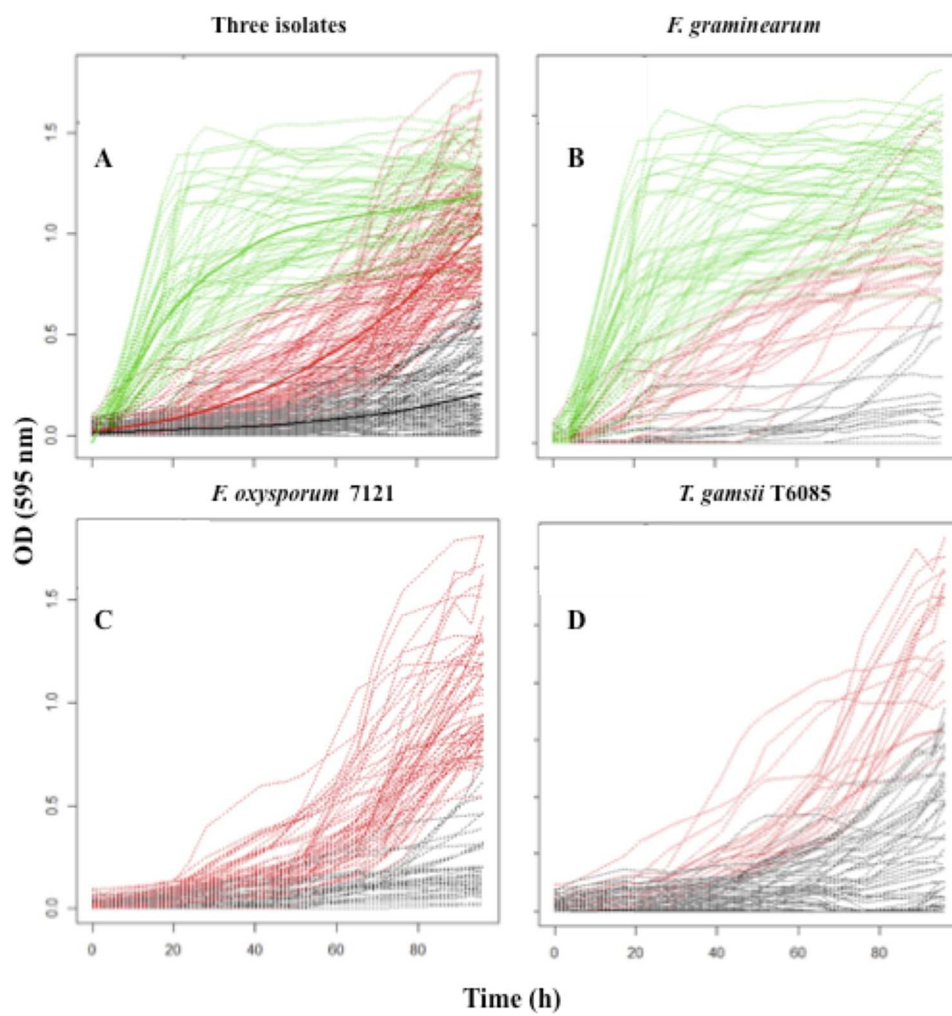

Figure 3. Growth curves on each of the Biolog substrates: all three fungi together (A); F. graminearum (B); F. oxysporum 7121 (C); and T. gamsii T6085 (D). Substrates are grouped into three categories: slow (S, black), medium (M, red) and high (H, green), according to a semi-parametric “functional clustering” method (Chiou and Pai-Ling, 2007).

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