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**Light affects fumonisin production in strains of *Fusarium fujikuroi*, *Fusarium proliferatum*, and *Fusarium verticillioides* isolated from rice**

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## UNIVERSITÀ DEGLI STUDI DI TORINO

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22 **Light affects fumonisin production in strains of *Fusarium fujikuroi*, *Fusarium***  
23 ***proliferatum*, and *Fusarium verticillioides* isolated from rice**

24  
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38 **ABSTRACT**

39

40 Three *Fusarium* species associated to bakanae disease of rice (*Fusarium fujikuroi*, *Fusarium*  
41 *proliferatum*, and *Fusarium verticillioides*) were investigated for their ability to produce fumonisins  
42 (FB<sub>1</sub> and FB<sub>2</sub>) under different light conditions, and for pathogenicity. The conditions that highly  
43 stimulated the fumonisin production compared to darkness were yellow and green light in *F.*  
44 *verticillioides* strains; white and blue light, and light/dark alternation in *F. fujikuroi* and *F.*  
45 *proliferatum* strains. In general, all light conditions influenced positively the fumonisin production  
46 with respect to the dark. Expression of the *FUM1* gene, which is necessary for initiation of  
47 fumonisin production, was in accordance with the fumonisin biosynthetic profile. High and low  
48 fumonisin-producing *F. fujikuroi* strains showed typical symptoms of bakanae disease, abundant  
49 fumonisin-producing *F. verticillioides* strains exhibited chlorosis and stunting of rice plants, while  
50 fumonisin-producing *F. proliferatum* strains were asymptomatic on rice. We report that *F. fujikuroi*  
51 might be an abundant fumonisin producer with levels comparable to that of *F. verticillioides* and *F.*  
52 *proliferatum*, highlighting the need of deeper mycotoxicological analyses on rice isolates of *F.*  
53 *fujikuroi*. Our results showed for the first time the influence of light on fumonisin production in  
54 isolates of *F. fujikuroi*, *F. proliferatum*, and *F. verticillioides* from rice.

55

56 *Keywords:* Bakanae disease; Rice; *Fusarium*; Pathogenicity; FB<sub>1</sub>; FB<sub>2</sub>

## 57 **1. Introduction**

58

59       The fungal genus *Fusarium* is composed of a large number of species that can be pathogenic on  
60 plants. *Fusarium* species are causal agents of various diseases affecting many economically  
61 important cereals, such as rice (*Oryza sativa* L.). *Fusarium fujikuroi* Nirenberg [teleomorph  
62 *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura] is an important rice pathogen, causing the  
63 bakanae disease or disease of foolish seedlings (Carter et al., 2008; Desjardins et al., 1997), and is a  
64 member of the polyphyletic taxon *Gibberella fujikuroi* species complex (GFSC; O'Donnell et al.,  
65 1998a). Beside *F. fujikuroi* other *Fusarium* species, such as *Fusarium proliferatum* and *Fusarium*  
66 *verticillioides*, have been associated with bakanae disease on rice (Desjardins et al., 2000; Nur Ain  
67 Izzati and Salleh, 2010; Wulff et al., 2010).

68       *Fusarium* spp. produce a wide range of biologically active secondary metabolites, among them  
69 there are mycotoxins which are harmful to animals and humans (Desjardins and Proctor, 2007), and  
70 they are considered the most important toxigenic fungi in the Northern temperate areas (Gutleb et  
71 al., 2002). Fumonisins are an important class of *Fusarium* mycotoxins. Among the *Fusarium* spp.  
72 isolated from rice, *F. verticillioides* and *F. proliferatum* are reported as the most abundant  
73 fumonisin producers, whereas *F. fujikuroi*, the causal agent of the bakanae disease, has a lower  
74 capacity of fumonisin production (Stępień et al., 2011; Wulff et al., 2010). The main chemical  
75 structure of fumonisins is a diester of propane-1,2,3-tricarboxylic acid and a pentahydroxyicosane  
76 containing a primary amino acid (Gurung et al., 1999). Due to their structural similarity to the lipid  
77 sphingosine, the mechanism of action of fumonisins might include competition with sphingosine in  
78 the sphingolipid metabolism (De Lucca, 2007; Riley et al., 1996).

79       The fumonisin B series, including FB<sub>1</sub>, FB<sub>2</sub>, FB<sub>3</sub>, and FB<sub>4</sub>, is the most toxic to plants and animals  
80 (Abbas et al., 1998a). FB<sub>1</sub> is generally found in corn, rice, triticale, sorghum, beans, and asparagus.  
81 It can cause equine leucoencephalomalacia and porcine pulmonary oedema (Scott, 2012). FB<sub>1</sub> has  
82 been associated to human oesophageal cancer in humans (Chu and Li, 1994; Sydenham et al.,



109 citrinin under white, red and blue light, whereas *Penicillium verrucosum* stimulated citrinin  
110 production under yellow and green light (Schmidt-Heydt et al., 2011). Wavelengths within the  
111 visible spectrum (from red to blue) increased the fumonisin biosynthesis compared to darkness in  
112 two species of *Fusarium* originating from maize, in particular red and blue light in *F. proliferatum*,  
113 and red and royal blue light in *F. verticillioides* (Fanelli et al., 2012a; 2012b).

114 The aim of our study was to investigate the fumonisin biosynthesis and *FUM1* relative  
115 expression in different light conditions between three *Fusarium* species (*F. fujikuroi*, *F.*  
116 *verticillioides*, and *F. proliferatum*) originated from rice and to compare their pathogenicity.

117

## 118 2. Materials and methods

119

### 120 2.1. Fungal strains

121

122 Fungal strains of *F. fujikuroi* (Augusto2), *F. verticillioides* (19-115), and *F. proliferatum* (19-  
123 113), isolated and characterized from Italian bakanae-associated rice samples (Amatulli et al., 2010)  
124 were used in this study. Three additional bakanae-associated isolates (I1.3 and CSV1 of *F. fujikuroi*,  
125 and 11-471 of *F. proliferatum*) from Northern Italy were included in this study. Reference  
126 *Fusarium* rice strains from other countries obtained from the Fusarium Research Centre  
127 (Pennsylvania State University, USA) were also used: *F. fujikuroi* M-1149 from Taiwan, *F.*  
128 *verticillioides* M-5331 from China, and *F. proliferatum* M-6580 from Thailand (Table 1). The  
129 strains were stored at 4 °C in Spezieller nährstoffarmer agar (0.2 g sucrose, 0.2 g glucose, 1.0 g  
130 KNO<sub>3</sub>, 1.0 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.5 g NaCl, and 12 g agar per litre).

131

### 132 2.2. DNA extraction and *EF-1α* /*FUM1* amplification

133





159 Fungal cultures were subcultured on PDA plates for 10 days at 20 °C. A spore suspension of the  
160 strains was prepared from the plates in sterile distilled water. One hundred  $\mu\text{l}$  of a  $10^6$  spores  $\text{ml}^{-1}$   
161 solution was inoculated in 50 ml of three growing media: (a) potato dextrose broth (PDB, Merck  
162 KGaA), (b) Czapek-Dox broth (Sigma-Aldrich, Buchs, Switzerland), and (c) GYAM (0.24 M  
163 glucose, 0.05% yeast extract, 8.0 mM L-asparagine, 5.0 mM malic acid, 1.7 mM NaCl, 4.4 mM  
164  $\text{K}_2\text{HPO}_4$ , 2.0 mM  $\text{MgSO}_4$ , and 8.8 mM  $\text{CaCl}_2$ ), by shaking at 100 rpm for 10 days in darkness.  
165 Growing media were evaluated for their efficiency for induction of fumonisin production in  
166 different *Fusarium* strains. The best fumonisin-producing medium was selected for the tests of light  
167 conditions and pathogenicity, as described below. The experiment was carried out three times at  
168 constant temperature.

169

## 170 2.5. Light conditions

171

172 *Fusarium* strains were grown in PDB under different light conditions: red (685 nm), yellow  
173 (580 nm), green (535 nm), blue (475 nm), and white. In addition, the growth under dark and  
174 light/dark alternation were examined. To investigate the influence of light and various wavelengths,  
175 the growth chambers were provided with a lighting system containing five RoHs comforted bulbs.  
176 Each bulb contained 36 Light Emitting Diodes (Super Bright LEDs Inc., St. Louis, USA) that  
177 emitted at corresponding wavelengths as indicated above. The bulbs were fixed at the four corners  
178 and in the middle of the upper side of the chambers, and were kept at a 20 cm distance from the  
179 orbital shaker where fungal strains were grown for 10 days at 100 rpm. Only one chamber was not  
180 provided with the lighting system, and it was used as a dark control. The luminous flux of each bulb  
181 was 72 lumen, and the light intensity was  $4.0 \text{ lumen/cm}^2$  or  $76 \text{ mW/cm}^2$  on the fungal culture.  
182 Continuous light or dark was applied in the chambers for each experiment, with the exception of the  
183 growth under light/dark (12 h: 12 h). The experiments were carried out three times at constant  
184 temperature of 20 °C maintained by the control system of the growth chambers.

185

186 *2.6. Fumonisin analysis*

187

188 For fumonisin quantification, the strains grown in PDB were filtered through sterile cheese cloth  
189 (Merck KGaA) to separate the supernatant and the mycelia. The supernatant was used for FB<sub>1</sub> and  
190 FB<sub>2</sub> analyses, whereas the mycelium was used for subsequent RNA extraction and obtainment of  
191 the dry mycelium described as follows. The collected mycelium was weighed and divided into two  
192 equal parts. One part was immediately processed for RNA extraction, and the other part was dried  
193 at 70 °C for 24 h to measure the mycelial dry weight, that was multiplied by two (including also the  
194 mycelium used for RNA extraction) to obtain the total dry mycelium. The total dry mycelium was  
195 used to normalize the final FB<sub>1</sub> and FB<sub>2</sub> values obtained as described below.

196 The recovered supernatant was additionally filtered through regenerated cellulose 0.45 µm filter  
197 and analysed by HPLC coupled with a triple quadrupole mass spectrometer. Liquid chromatography  
198 was performed with Varian Model 212-LC micro pumps (Hansen Way, CA, USA) coupled with a  
199 Varian 126 autosampler Model 410 Prostar. A Synergi 4u Fusion-RP 80A (100 mm x 2.0 mm,  
200 Phenomenex, Castel Maggiore, Italy) analytical column was used coupled with Fusion-RP (4 x 2.0  
201 mm) security guard for LC separation. The chromatographic conditions were: column temperature  
202 at 45 °C; mobile phase consisting of eluent A (buffer solution with 20 mM ammonium formate-  
203 formic acid at pH 3.75) and eluent B (methanol), using a flow rate of 0.3 ml/min. A gradient elution  
204 was applied as follows: 0 min 30% B; 0.1-5 min 30-90% B; 5.1-10 min 90-30% B. Five minutes of  
205 post run were necessary for column conditioning before the subsequent injection. The injection  
206 volume was 10 µl.

207 The triple quadrupole mass spectrometer (Varian 310-MS) was operated in the negative/positive  
208 electrospray ionization mode (ESI/ESI<sup>+</sup>). To select the MS/MS parameters for the analysis of  
209 fumonisins by multiple reaction monitoring (SRM), ESI mass spectra for each analyte were initially  
210 analysed introducing the stock fumonisin standard solution (Sigma-Aldrich) with direct injection

211 into the spectrometer by a Harvard 11 plus infusion pump. The  $m/z$  706 positive ion and  $m/z$  720.4  
212 negative ion were used, respectively, as parent ions for FB<sub>1</sub> and FB<sub>2</sub>. The most intense daughter  
213 ions, resulting from collision-induced dissociation with argon, used to detect and quantify the  
214 fumonisin content were:  $m/z$  336 at 36 eV of collision energy (CE) and 318 at 318 eV for FB<sub>1</sub>;  $m/z$   
215 156.7 at 30 eV CE and 562.4 at 16.5 eV for FB<sub>2</sub>. The limits of detection (LOD; signal-to-noise  
216 ratio: 3) and quantification (LOQ; signal-to-noise ratio: 10) were, respectively, 0.62 µg/l and 1.55  
217 µg/l for FB<sub>1</sub>, 2.09 µg/l and 5.17 µg/l for FB<sub>2</sub>. Each analysis was performed in triplicate.

218

### 219 2.7. RNA extraction, reverse transcription and real-time PCR for *FUM1*

220

221 Total RNA of fungi was extracted from mycelium with Aurum Total RNA fatty and fibrous  
222 tissue kit (Bio-Rad, Richmond, CA, USA). Total RNA was DNase treated using TURBO DNase  
223 (Ambion, Foster City, CA, USA) according to the manufacturer's instructions to remove  
224 contaminating DNA, and then subjected to reverse transcription using the High capacity cDNA  
225 reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA mixture  
226 contained the total RNA (ranging from 0.1 to 1000 ng), 2 µl 10X RT buffer, 0.8 µl 25X dNTP mix,  
227 2 µl 10X RT random primers, 1 µl Multiscribe™ reverse transcriptase, and 1 µl RNase inhibitor in a  
228 total volume of 20 µl. cDNA synthesis conditions were: 10 min at 25 °C, 120 min at 37 °C, and 5  
229 min at 85 °C. The resulting cDNA was used as a template for real-time PCR.

230 Real-time PCR was used for the quantification of the *FUM1* transcript expression under different  
231 light conditions. Primers for *FUM1* gene sequence were designed: Fum1fp1 (5'-  
232 AGGATTGGCTGGATCTTCAC-3') and Fum1fp2 (5'-TAATACGGTTGGAAATGGCA-3') for  
233 *F. fujikuroi* and *F. proliferatum* on the basis of the GenBank accession no. AY577454 (nt position  
234 149-241) giving an amplicon of 93 bp, and Fum1univs (5'- CCAGCTGTTTTTCCCTGCTA-3')  
235 and Fum1univa (5'- CGATTTCCCATCAGCAAGAT-3') for *F. verticillioides* designed from the  
236 sequence AF155773 (nt position 25785-25868) giving an amplicon of 103 bp. PCR conditions were

237 as follows: an initial step at 95 °C for 5 min, and 40 cycles at 95 °C for 15 s (denaturation), 60 °C  
238 for 30 s (annealing), and 72 °C for 30 s (extension). The reactions were carried out in an iCycler  
239 (Bio-Rad) and contained 1 µl cDNA, 10 µl SsoFast™ EvaGreen® Supermix 2X (Bio-Rad), 2.5 mM  
240 each primer in a total volume of 20 µl. PQTUB-F and PQTUB-R primers designed from the  
241 sequence of the β-tubulin gene *tub2* were used as a control for the constitutive expression (Glass  
242 and Donaldson, 1995). To investigate the range of concentrations where the target RNA and C<sub>T</sub>  
243 values were linearly correlated, and to determine the reaction efficiency for both sets of *FUM1*  
244 primers, the reactions were performed by using the cDNA synthesized from serially diluted RNAs.  
245 Baseline range and C<sub>T</sub> values were automatically calculated using the iCycler Optical System  
246 Software v. 3.0. The expression of the *FUM1* gene was normalized to that of *tub2*, by subtracting  
247 the *tub2* C<sub>T</sub> value from the C<sub>T</sub> value of the *FUM1* gene resulting from the ΔC<sub>T</sub>. The expression ratio  
248 was calculated from equation  $2^{-\Delta\Delta C_T}$ , where ΔΔC<sub>T</sub> represents the ΔC<sub>Tsample</sub> – ΔC<sub>Tcontrol</sub> (Livak and  
249 Schmittgen, 2001).

250

## 251 2.8. Pathogenicity tests

252

253 Fungal strains were cultured in PDB for 10 days in darkness in acclimatized chamber at 20 °C.  
254 The strains were filtered through sterile cheese cloth to a final spore concentration of 10<sup>6</sup> ml<sup>-1</sup> in  
255 sterile distilled water. The rice cultivar Galileo susceptible to *F. fujikuroi* was used for  
256 pathogenicity tests. Rice seeds were surface-disinfected in 1% sodium hypochlorite for 2 min and  
257 rinsed in sterile distilled water. A total of 120 seeds were soaked in 100 ml spore suspension by  
258 shaking for 30 min at room temperature. Control seeds were soaked in sterile distilled water. Seeds  
259 were sown in plastic pots (40 x 24 x 12 cm) in triplicate (40 seeds per pot) in a sterile mixture of  
260 peat and sand (60% : 40%). The plants were kept in greenhouse conditions (25 °C day : 17 °C  
261 night) by watering 3 times per day. Disease symptoms were evaluated 30 days after germination.

262 We searched for bakanae symptoms including thin and elongated internodes typical of *F. fujikuroi*,  
263 and for other less specific symptoms, such as chlorosis, necrosis and delayed growth.

264

## 265 2.9. Statistical analysis

266

267 Data from all the experiments were submitted to analysis of variance (ANOVA) by using the  
268 Statistical Package for Social Science (SPSS, IBM, Chicago, IL, USA) version 17.0. The statistical  
269 significance was judged at  $P < 0.05$ . Statistically significant differences among light treatments with  
270 a reference to the dark incubation were determined by the *t* test. Pearson's correlation coefficient  
271 between expression levels of *FUM1* gene and FB<sub>1</sub> content was also calculated.

272

273

## 274 3. Results

275

### 276 3.1. Species identification

277

278 The nine isolates of *Fusarium* spp. originated from rice were identified through analysis of the  
279 *EF-1 $\alpha$*  and *FUM1* sequences (Table 1). Both genes permitted to identify four strains of *F. fujikuroi*,  
280 three strains of *F. proliferatum* and two strains of *F. verticillioides*. The phylogenetic studies of the  
281 *FUM1* gene portion (amplicons from 1030 to 1039 nt) allowed the separation of the three *Fusarium*  
282 species similarly to those performed with the *EF-1 $\alpha$*  portion (amplicons from 649 to 661 nt). Thus,  
283 phylogenetic analyses of the nucleotide (Supplementary Fig. 1) or amino acid (data not shown) of  
284 the *EF-1 $\alpha$*  and *FUM1* fragments grouped the *Fusarium* strains into two clusters; one cluster  
285 contained the strains of *F. fujikuroi* and *F. proliferatum*, and the another one the strains of *F.*  
286 *verticillioides*. The first cluster was further divided into two subclusters allowing the separation of

287 *F. fujikuroi* and *F. proliferatum*. On the basis of the results obtained, *FUM1* gene sequence,  
288 corresponding to the acetyltransferase domain of the PKS protein, could be used to differentiate  
289 species of the *Gibberella fujikuroi* species complex. *F. fujikuroi* and *F. proliferatum* had 91.1%  
290 *FUM1*-nucleotide sequence identity. A lower nucleotide identity was found when *F. verticillioides*  
291 strains were compared to the strains of *F. proliferatum* and *F. fujikuroi* (77.3% and 78.7%,  
292 respectively).

293

### 294 3.2. Selection of growing medium

295 The medium which allowed the highest production of FB<sub>1</sub> (Fig. 1 and Supplementary Table  
296 1) and FB<sub>2</sub> (data not shown) in three *Fusarium* species was PDB. It induced 3.2 to 5.0 times higher  
297 FB<sub>1</sub> production compared to the GYAM medium. Czapek-Dox was not a satisfactory growing  
298 medium for the production of fumonisins since it allowed very low production of FB<sub>1</sub> in only two  
299 *Fusarium* strains. On the basis of these results, lighting and pathogenicity tests were performed by  
300 using PDB as a growing medium.

301

### 302 3.3. Fumonisin production in three *Fusarium* species under dark

303

304 Strains belonging to three *Fusarium* species were compared for fumonisin production after their  
305 growth in dark. The highest FB<sub>1</sub> producing strains were *F. verticillioides* M-5331, *F. fujikuroi*  
306 Augusto2, and *F. fujikuroi* M-1149 (Table 2). The other *Fusarium* strains showed lower FB<sub>1</sub>  
307 production. FB<sub>2</sub> biosynthesis was obtained in both strains of *F. verticillioides*, and in two out of four  
308 strains of *F. fujikuroi* (I1.3 and Augusto2), while none of the *F. proliferatum* strains produced FB<sub>2</sub>.  
309 Interestingly, *F. verticillioides* M-5331 and 19-115, and *F. fujikuroi* I1.3 produced more FB<sub>2</sub> than  
310 FB<sub>1</sub> (16.7, 17.3 and 25.9 times more, respectively).

311 By considering the geographic origin of the *F. verticillioides* strains, the M-5331 strain  
312 originating from China produced 2.3 and 2.2 times more FB<sub>1</sub> and FB<sub>2</sub>, respectively, than the Italian

313 strain 19-115 (Table 2). *F. fujikuroi* Augusto2 originating from Italy was a higher FB<sub>1</sub>/FB<sub>2</sub> producer  
314 compared to the Taiwanese strain of *F. fujikuroi* (M-1149). The Italian strain of *F. proliferatum*  
315 (11-471) showed higher level of FB<sub>1</sub> production compared to the Thai strain (M-6580).

316

#### 317 3.4. Fumonisin production in different light conditions

318

319 All the species of *Fusarium* isolated from rice showed a higher production of fumonisins (Fig. 2  
320 and Table 2) under different light conditions compared to darkness. *F. proliferatum* strains had the  
321 highest FB<sub>1</sub> increase under white and blue light, and under light/dark. Red, green and yellow light  
322 were also stimulating the FB<sub>1</sub> biosynthesis compared to the dark incubation. FB<sub>1</sub> production was  
323 highly stimulated in both strains of *F. verticillioides* under yellow and green light. The other light  
324 conditions also activated the FB<sub>1</sub> production in *F. verticillioides*, but to a lower extent. *F. fujikuroi*  
325 Augusto2 and M-1149 stimulated the FB<sub>1</sub> biosynthesis under white and blue light, and light/dark.  
326 The other two strains of *F. fujikuroi* (I1.3 and CSV1) ceased FB<sub>1</sub> production under different  
327 wavelengths and light conditions, with the exception of blue light and the light/dark alternation  
328 (Fig. 2 and Table 2). Conclusively, the highest production of FB<sub>1</sub> was obtained in *F. fujikuroi* strain  
329 M-1149 (white) and *F. verticillioides* M-5331 (yellow light).

330 FB<sub>2</sub> production was activated by different wavelengths of light; red and blue light in the three  
331 strains of *F. proliferatum* and in *F. fujikuroi* Augusto2 and M-1149. It was also stimulated under  
332 light/dark alternation in *F. proliferatum* and most *F. fujikuroi* strains. Red and yellow light activated  
333 FB<sub>2</sub> biosynthesis in *F. verticillioides* (Table 2 and Supplementary Fig. 2). Red light induced a  
334 higher FB<sub>2</sub> production compared to FB<sub>1</sub> in both strains of *F. verticillioides* (Table 2).

335 We observed that the light-pattern of fumonisin synthesis was generally more similar between *F.*  
336 *fujikuroi* and *F. proliferatum*, compared to *F. verticillioides*. Additionally, a stimulation of  
337 mycelium production was also observed under different light conditions compared to the dark  
338 incubation in all three *Fusarium* species (Supplementary Fig. 3). All light conditions that we

339 applied (light of different wavelengths, white light and the light/dark alternation) had a significant  
340 effect on the mycelium production with respect to dark after 10 days of growth in PDB.

341

### 342 3.5. Expression of the *FUM1* gene in different light conditions

343

344 Ct values and the logarithm of RNA concentrations ranging from 1000 to 0.1 ng were linearly  
345 correlated for both sets of *FUM1* primers. An average squared regression ( $R^2$ ) of 0.998 and a  
346 reaction efficiency of 97.5% were obtained for the *FUM1* primer pair designed for *F. fujikuroi* and  
347 *F. proliferatum*, while similar values of 0.999 ( $R^2$ ) and 97.8% (reaction efficiency) were obtained  
348 for the *FUM1* primer pair for *F. verticillioides*. These results allowed the use of both set of primers  
349 in the subsequent real-time PCR reactions to investigate the light influence.

350 The *FUM1* expression was activated under different light conditions in all three species of  
351 *Fusarium* from rice compared to the dark. It was highly activated under blue, and white light, and  
352 light/dark alternation in *F. proliferatum* and *F. fujikuroi* strains Augusto2 and M-1149, but it was  
353 also activated under green light in *F. proliferatum*, and under red light in *F. fujikuroi* Augusto2 and  
354 M-1149 (Fig. 3). The other two strains of *F. fujikuroi* (I1.3 and CSV1) showed significant *FUM1*  
355 expression only under blue light and light/dark alternation. Wavelengths that highly stimulated  
356 *FUM1* expression in *F. verticillioides* were yellow and green.

357 The highest increase was observed in *F. fujikuroi* M-1149 (486-fold change; white), *F. fujikuroi*  
358 Augusto2 (384-fold change; white light) and *F. proliferatum* 11-471 (267-fold change; blue light)  
359 (Table 2). The *FUM1* expression level was found in correspondence with FB<sub>1</sub> production and the  
360 value for the Pearson's correlation coefficient was 0.78 for all three species. When the Pearson's  
361 correlation coefficient was calculated for each species separately, a higher correlation was found in  
362 *F. verticillioides* (0.97) and *F. fujikuroi* (0.80) compared to *F. proliferatum* (0.57).

363

### 364 3.6. Pathogenicity tests



365

366 Five of the 9 tested strains showed to be pathogenic for rice: *F. fujikuroi* M-1149, I1.3, and  
367 CSV1 showed typical bakanae symptoms with a death incidence of 93%, 91%, and 83%,  
368 respectively, while *F. verticillioides* M-5331 and 19-115 caused chlorotic leaves and delayed  
369 growth with no death incidence (about one month after germination). Comparing the two *F.*  
370 *verticillioides* strains, strain M-5331 caused a higher incidence of leaf chlorosis and plant stunting  
371 than 19-115 (Table 3 and Fig. 4). On the other hand, one strain of *F. fujikuroi* (Augusto2) and all  
372 three strains of *F. proliferatum* showed to be asymptomatic for the plants. Comparing pathogenic *F.*  
373 *fujikuroi* and *F. verticillioides* strains, it can be observed that they influenced the growth of the  
374 plants differently; *F. fujikuroi* with a plant internode elongation, and *F. verticillioides* with stunting.  
375 Both growth ways affected negatively the general plant behaviour, but plant death occurred only  
376 when the plant underwent a rapid elongation.

377

## 378 **Discussion**

379 There are reports of natural contamination of rice with fumonisins from Korea (Chung and Kim,  
380 1995), United States (Abbas et al., 1998b), China (Trucksess, 2000), Brazil (Mallmann et al., 2001),  
381 Argentina (Lerda et al., 2005), Japan (Kushiro et al., 2008), and Thailand (Tansakul et al., 2012). A  
382 huge number of samples of rice plants and rice food products were analysed, and no fumonisin  
383 contamination was found in Italian samples (data not published).

384 For this reason, fumonisins were measured from fungal strains grown *in vitro* in PDB, which was  
385 previously selected. In general, there is limited information on the use of PDB as a growing medium  
386 for mycotoxin production (Spadaro et al., 2010). We know that secondary metabolites, such as  
387 gibberellins and bikaverin in *F. fujikuroi*, and fumonisins in *F. verticillioides*, are repressed by high  
388 amounts of nitrogen (Mihlan et al., 2003; Schönig et al., 2008; Kim and Woloshuk, 2008). In this  
389 work, the PDB medium non-supplemented with additional nitrogen sources showed to have  
390 favourable nitrogen conditions for fumonisin production in comparison with other previously used

391 substrates, including GYAM and Czapek-Dox that contained other nitrogen sources (Proctor et al.,  
392 2008; Amatulli et al., 2012).

393 *F. fujikuroi* Augusto2 was asymptomatic in pathogenicity tests on rice, but it showed a high  
394 fumonisin-producing ability. On the contrary, another high fumonisin-producing strain (M-1149)  
395 and two low fumonisin-producing strains (I1.3, and CSV1) of *F. fujikuroi* were more aggressive on  
396 rice with hyper-elongation of the stems. This divergence may be related to the previous findings  
397 showing that other components besides mycotoxins, such as phytohormones may influence the  
398 pathogenicity of *F. fujikuroi* on rice (Wulff et al., 2010). Taking into consideration two other  
399 species of *Fusarium*, we found a higher level of production of fumonisins in *F. verticillioides* which  
400 showed pathogenicity on rice, while the fumonisin-producing strains of *F. proliferatum* were  
401 asymptomatic. In *F. verticillioides*, the M-5331 strain from China induced a higher incidence of rice  
402 stunting and a more abundant synthesis of fumonisins than the strain 19-115 from Italy. Stunted  
403 growth could be associated with the inability of *F. verticillioides* to produce gibberellin, therefore  
404 other factors might be causing the symptoms observed.

405 In this study, we showed for the first time the influence of light on fumonisin production in  
406 isolates of *F. fujikuroi*, *F. proliferatum*, and *F. verticillioides* from rice. Fumonisin production was  
407 considered to be higher during dark incubation, so most previous studies were performed by  
408 growing fumonisin producing isolates in the dark (Alberts et al., 1990; Desjardins et al., 2000;  
409 Wulff et al., 2010). However, recent findings indicated that light and different wavelengths of light  
410 might have a stimulatory effect on fumonisin production in *F. verticillioides* and *F. proliferatum*  
411 from maize compared to the dark incubation (Fanelli et al., 2012a; 2012b). Our data showed that  
412 dark incubation induced fumonisin synthesis in all tested *Fusarium* species from rice, but the light  
413 conditions were more stimulating for fumonisin production.

414 *F. fujikuroi* is reported to produce little or no fumonisin compared to *F. verticillioides* and *F.*  
415 *proliferatum* (Desjardins et al., 2000; Stępień et al., 2011; Wulff et al., 2010). Here we report for the  
416 first time that two strains of *F. fujikuroi* from rice have the ability to produce fumonisin levels

417 comparable to those of *F. verticillioides* and *F. proliferatum*. FB<sub>1</sub> and FB<sub>2</sub> production was highly  
418 increased under white light in *F. fujikuroi* Augusto2 and M-1149. In these conditions, *F. fujikuroi*  
419 Augusto2 from Italy and M-1149 from Taiwan proved to be the most abundant fumonisin  
420 producers.

421 Many fungal species use specific wavelength receptors and all receptors contain an organic  
422 molecule of low molecular weight, such as flavin, retinal or tetrapyrrols for blue-, green-, or red-  
423 light perception, respectively (Rodriguez-Romero et al., 2010). Red-light sensing of fungi was  
424 involved in sporulation and mycotoxin synthesis. In *Botrytis cinerea*, a red-light reversible  
425 photoreaction was found in the recovery from the blue-light inhibition of sporulation (Tan, 1974).  
426 *P. expansum* produced a high quantity of citrinin under red, blue and white light, whereas *P.*  
427 *verrucosum* did so under yellow and green light (Schmidt-Heydt et al., 2011). Fanelli et al. (2012a;  
428 2012b) found that the visible spectrum from red to blue increased fumonisin production in *F.*  
429 *proliferatum* and *F. verticillioides* originated from maize.

430 As far as we know, little is known about the light wavelength sensing in fumonisin production of  
431 *F. fujikuroi*. We reported here that white light, followed by blue light and light/dark alternation had  
432 a strong stimulatory effect on FB<sub>1</sub> or FB<sub>2</sub> synthesis in two *F. fujikuroi* strains (Augusto2 and M-  
433 1149). Under white light, FB<sub>1</sub> and FB<sub>2</sub> productions were increased, respectively, 75 and 15 times in  
434 *F. fujikuroi* Augusto2, and 326 and 1367 times in *F. fujikuroi* M-1149. Our data indicated that a  
435 better effectiveness of fumonisin production in *F. fujikuroi* was generally obtained with light  
436 regimes compared to darkness. Positive influence of white light on fumonisin-producing profile was  
437 found, and the result is in agreement with increase of production of secondary metabolites in other  
438 organisms such as DON in *Fusarium graminearum*, citrinin in *P. expansum*, and aflatoxins in  
439 *Aspergillus parasiticus* (Bennett et al., 1981; Schmidt-Heydt et al., 2011). The activation of  
440 fumonisin biosynthesis found by light-dark alternation, may be attributed to the night-day cycle of  
441 fungi. Many fungal species possess a circadian clock which is influenced by light and temperature  
442 (Dunlap and Loros, 2006). Other two *F. fujikuroi* strains (I1.3 and CSV1) did not show an

443 activation in fumonisin production with light application, with the exception of light/dark  
444 alternation (6 and 10 times FB<sub>1</sub> increase) and blue light (6 and 11 fold increase). It would be  
445 interesting to study the expression of genes involved in fumonisin production, such as the *FUM*  
446 cluster, the white collar gene, and the velvet-like complex, in low and high fumonisin-producing *F.*  
447 *fujikuroi* strains.

448 Concerning *F. proliferatum*, white and blue light stimulated the FB<sub>1</sub> synthesis, whereas the FB<sub>2</sub>  
449 production was activated under red light and the light/dark alternation compared to darkness. Our  
450 data fit with Fanelli et al (2012a), but we found additional induction of fumonisin biosynthesis  
451 under white light and light/dark alternation. Strains of *F. proliferatum* showed 11 to 32 times more  
452 FB<sub>1</sub> production under blue light, and 19 to 36 times more FB<sub>2</sub> production under red light.

453 Our data showed that different wavelengths, particularly yellow and green light, favour  
454 fumonisin production in *F. verticillioides*. These wavelengths were also found stimulatory in the  
455 previous work of Fanelli et al. (2012b). In our work, both strains of *F. verticillioides* showed 47 to  
456 171 times more synthesis of FB<sub>1</sub> under yellow light, and 17 to 20 fold increase under green light in  
457 comparison to darkness.

458 FB<sub>2</sub> production was higher compared to FB<sub>1</sub> in most strains of the three *Fusarium* species under  
459 light/dark alternation, and in both strains of *F. verticillioides* under red light and dark. *Fusarium*  
460 isolates able to produce more FB<sub>2</sub> than FB<sub>1</sub> have been already reported (Musser and Plattner, 1997),  
461 and it could be possible that some light conditions are more favourable for FB<sub>2</sub> production, by  
462 inducing a higher expression of *FUM2* gene, involved in the switch from FB<sub>1</sub> to FB<sub>2</sub> (Proctor et al.,  
463 2003).

464 In conclusion, *F. fujikuroi* and *F. proliferatum* showed a similar light-regulation profile of  
465 fumonisin biosynthesis that could be associated with their closely related phylogenetic relationship  
466 within *Gibberella fujikuroi* species complex (Amatulli et al., 2010; Hsuan et al., 2011). On the other  
467 hand, *F. proliferatum* and *F. verticillioides* showed different wavelength-regulation pattern for  
468 fumonisin production which can be attributed to variability in phenotypic fumonisin biosynthesis

469 between these two species (Visentin et al., 2009). Indeed, *F. proliferatum* and *F. verticillioides*  
470 exhibited different regulation profiles of fumonisin biosynthesis under different environmental  
471 conditions, such as temperature and water stress (Marín et al., 2010).

472 Previous studies reported that *FUM1* gene expression showed a correlation with fumonisin  
473 production, offering a diagnostic tool for the rapid and sensitive detection of metabolically active  
474 fumonisin-producing *Fusarium* species (López-Errasquín et al., 2007; Jurado et al. 2010; Fanelli et  
475 al., 2012a). In our work we also found a positive and high relationship between mRNA levels of  
476 *FUM1* gene and FB<sub>1</sub> production. Some cases of lower correspondence between *FUM1* transcripts  
477 and FB<sub>1</sub> content could be explained by slightly different time-points of transcription and mycotoxin  
478 production.

479 Our results report fumonisin production and *FUM1* gene expression in members of the  
480 *Gibberella fujikuroi* species complex isolated from rice. Fumonisin production was activated by  
481 specific light conditions in three different *Fusarium* species. The variations have been found  
482 between different *Fusarium* species, but a closer fumonisin-production profile was found between  
483 *F. proliferatum* and *F. fujikuroi*, compared to *F. verticillioides*. Abundant and low fumonisin-  
484 producing strains were found in *F. fujikuroi* under different light conditions. The data obtained in  
485 this study highlight the need for a deeper analysis of fumonisin production in *F. fujikuroi*, the major  
486 causal agent of the bakanae disease, and a re-examination of its mycotoxigenic capacity, which can  
487 be additionally supported with recent information coming from the *F. fujikuroi* genomic and  
488 transcriptomic data (Jeong et al., 2013; Wiemann et al., 2013).

489

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491

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496

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675 **Table 1**  
 676 List of *Fusarium* strains used in this study.  
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Strain	Species	Origin	Host	Accession no. ( <i>EF-1<math>\alpha</math></i> gene)	Reference ( <i>EF-1<math>\alpha</math></i> gene)	Accession no. ( <i>FUM1</i> gene)	Reference ( <i>FUM1</i> gene)
11-471	<i>F. proliferatum</i>	Italy	<i>Oryza sativa</i>	KC121066	This study	KC188784	This study
19-113	<i>F. proliferatum</i>	Italy	<i>Oryza sativa</i>	GQ848533	Amatulli et al., 2010	KC188785	This study
M-6580	<i>F. proliferatum</i>	Thailand	<i>Oryza sativa</i>	JN092336	Amatulli et al., 2010	KC188786	This study
19-115	<i>F. verticillioides</i>	Italy	<i>Oryza sativa</i>	GQ848530	Amatulli et al., 2010	KC188787	This study
M-5331	<i>F. verticillioides</i>	China	<i>Oryza sativa</i>	AY337449	Yergeau et al., 2005	KC188788	This study
I1.3	<i>F. fujikuroi</i>	Italy	<i>Oryza sativa</i>	GQ848523	Amatulli et al., 2010	KC188789	This study
CSV1	<i>F. fujikuroi</i>	Italy	<i>Oryza sativa</i>	KC121067	This study	KC188790	This study
Augusto2	<i>F. fujikuroi</i>	Italy	<i>Oryza sativa</i>	KC121068	This study	KC188791	This study
M-1149	<i>F. fujikuroi</i>	Taiwan	<i>Oryza sativa</i>	HM243234	Amatulli et al., 2010	KC188792	This study

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**Table 2**

Relative expression of FUM1 gene (FC), and FB<sub>1</sub> and FB<sub>2</sub> production (µg per g of mycelial dry weight) of *Fusarium* strains grown under different light conditions.

<i>F. proliferatum</i>												
	11-471			19-113			M-6580					
	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)
Red	1.77±0.21	43±4.0	24±1.9	1.28±0.06	36±3.6	36±2.9	1.78±0.17	29±3.2	19±2.3			
Yellow	2.33±0.13	27±2.2	ND*	2.23±0.22	30±4.1	ND	1.96±0.16	48±3.3	ND			
Green	3.64±0.44	128±9.2	9±1.6	3.13±0.51	56±4.2	ND	2.43±0.31	43±3.0	ND			
Blue	267±21.16	214±20.8	22±0.9	253±22.31	289±25.7	15±1.4	194±21.07	290±32.1	21±2.5			
White	10.73±1.09	412±52.9	ND	5.63±0.72	159±18.1	ND	7.38±0.64	286±30.4	ND			
Light/Dark	4.56±0.54	145±11.9	320±35.5	9.42±0.68	201±24.2	489±50.4	5.92±0.71	249±30.5	276±21.6			
Dark	1±0.15	20±1.8	ND	1±0.14	12±1.3	ND	1±0.10	9±0.7	ND			

  

<i>F. verticillioides</i>						
	19-115			M-5331		
	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)
Red	5.98±0.47	35±4.4	494±43.2	8.93±1.13	278±25.9	1694±139.2
Yellow	9.40±0.53	1029±149.6	435±38.2	185±23.64	8524±822.6	1930±185.3
Green	22.43±1.31	444±28.5	15±3.9	15.84±1.02	843±62.1	82±8.5
Blue	3.59±0.47	151±17.9	222±23.7	6.11±1.09	296±35.3	ND
White	1.22±0.09	27±3.1	ND	1.75±0.18	123±15.5	273±30.4
Light/Dark	3.81±0.49	162±18.4	225±23.3	6.02±1.08	272±30.1	393±42.9
Dark	1±0.11	22±2.9	380±30.1	1±0.20	50±6.2	834±92.9

  

<i>F. fujikuroi</i>												
	I1.3			CSV1			Augusto2			M-1149		
	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)	FUM1	FB <sub>1</sub> (µg / g)	FB <sub>2</sub> (µg / g)
Red	1.05±0.02	ND	ND	1.09±0.03	ND	ND	8.61±1.02	249±28.3	59±4.8	10.86±0.84	107±13.1	42±3.9
Yellow	1.19±0.11	ND	ND	1.22±0.13	ND	ND	1.73±0.13	64±5.5	17±6.8	1.22±0.15	68±6.6	ND
Green	1.09±0.09	ND	ND	1.14±0.11	ND	ND	1.19±0.07	59±3.4	ND	1.33±0.12	54±6.6	ND
Blue	4.32±0.27	39±3.6	ND	5.20±0.98	171±15.6	ND	20.8±1.76	346±40.2	46±3.7	23.47±1.28	404±44.8	58±4.9
White	1.12±0.09	ND	ND	1.25±0.08	ND	ND	383.78±34.29	3650±387.3	534±78.6	485.73±45.06	14002±1633.6	1367±80.0
Light/Dark	3.08±0.46	44±4.6	319±35.7	4.66±0.93	157±20.8	239±18.9	7.33±1.41	359±38.3	ND	2.08±0.16	130±10.9	185±23.1
Dark	1±0.14	7±1.2	181±24.1	1±0.15	16±2.1	ND	1±0.19	49±4.8	36±2.6	1±0.13	43±3.9	ND

\*ND- not detected

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686 **Table 3**  
 687 Pathogenicity tests of *Fusarium* strains on the rice cultivar Galileo.  
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Strain	Germination (%)	Elongated plants with chlorotic leaves (%)	Stunted plants with chlorotic leaves (%)	Death incidence (%)
<i>F. proliferatum</i> 11-471	98	0	0	0
<i>F. proliferatum</i> 19-113	75	0	0	0
<i>F. proliferatum</i> M-6580	75	0	0	0
<i>F. verticillioides</i> 19-115	60	0	21	0
<i>F. verticillioides</i> M-5331	58	0	87	0
<i>F. fujikuroi</i> I1.3	83	91	0	91
<i>F. fujikuroi</i> CSV1	92	83	0	83
<i>F. fujikuroi</i> Augusto2	93	5	0	5
<i>F. fujikuroi</i> M-1149	52	93	0	93

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691 **Supplementary Table 1**

692 FB<sub>1</sub> production (µg per g of mycelial dry weight) in *Fusarium* strains grown in different media under dark.

Isolate	FB <sub>1</sub> production (µg / g)		
	PDB	Gyam	Czapek-Dox
<i>F. proliferatum</i> 11-471	22±4.21	6±0.52	ND <sup>694</sup>
<i>F. proliferatum</i> 19-113	13±3.08	4±0.03	ND
<i>F. proliferatum</i> M-6580	10±2.07	3±0.02	ND <sup>695</sup>
<i>F. verticillioides</i> 19-115	23±3.55	6±1.13	ND
<i>F. verticillioides</i> M-5331	52±7.08	13±1.54	3±0.20 <sup>696</sup>
<i>F. fujikuroi</i> I1.3	8±1.03	2±0.16	ND
<i>F. fujikuroi</i> CSV1	17±3.06	5±0.34	ND
<i>F. fujikuroi</i> Augusto2	50±9.17	10±1.24	2±0.12 <sup>697</sup>
<i>F. fujikuroi</i> M-1149	42±7.32	11±0.95	ND

698 \* ND- not detected

699

700 **Figure captions**

701

702 **Fig. 1.** Production of FB<sub>1</sub> in strains of three *Fusarium* species: *F. proliferatum*, *F. verticillioides*,  
703 and *F. fujikuroi* in three different growing media: PDB, Gyam and Czapek-Dox. Strains were  
704 grown for 10 days by shaking (100 rpm) in darkness at 20 °C. Error bars show standard deviations  
705 for triplicate assays.

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707 **Fig. 2.** Production of FB<sub>1</sub> in strains of three *Fusarium* species: *F. proliferatum*, *F. verticillioides*,  
708 and *F. fujikuroi* under different light conditions. Dark incubation was used as a reference. Strains  
709 were grown in PDB for 10 days by shaking (100 rpm) at 20 °C. Error bars show standard deviations  
710 for triplicate assays. Statistical significance:  $P < 0.05$  for all comparisons.

711

712 **Fig. 3.** Relative expression of *FUM1* gene by real-time RT-PCR in strains of *F. proliferatum*, *F.*  
713 *verticillioides*, and *F. fujikuroi* under different light conditions. Dark incubation was used as a  
714 reference. Strains were grown in PDB for 10 days by shaking (100 rpm) at 20 °C. Error bars show  
715 standard deviations for triplicate assays. Statistical significance:  $P < 0.05$  for all comparisons.

716

717 **Fig. 4.** Symptomatology induced on 1-month old rice plants (cultivar Galileo) artificially inoculated  
718 with *Fusarium* strains. The following *Fusarium* species were used for pathogenicity tests: *F.*  
719 *fujikuroi* (1. I1.3; 2. M-1149; 3. CSV1; 4. Augusto2), *F. proliferatum* (5. 19-113; 6. 11-471; 7. M-  
720 6580), and *F. verticillioides* (8. 19-115; 9. M-5331).

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723 **Supplementary Fig. 1.** Phylogenetic analyses based on the partial nucleotide sequences of *EF-1 $\alpha$*   
724 and *FUM1* genes from *Fusarium* strains: *F. fujikuroi* (I1.3, Augusto2, CSV1 and M-1149), *F.*  
725 *proliferatum* (11-471, 19-113 and M-6580), and *F. verticillioides* (19-115 and M-5331). Reference  
726 isolates of *F. fujikuroi* (HF679028), *F. proliferatum* (JF740718) and *F. verticillioides* (AB674289)  
727 for *EF-1 $\alpha$*  gene, and *F. fujikuroi* (HF679031) and *F. verticillioides* (AF155773) for *FUM1* gene are  
728 shown in bold. Phylogenetic analyses were performed by neighbor-joining method using MEGA 5  
729 (Tamura et al., 2011). Bootstrap analyses were supported with 1,000 replications.

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731 **Supplementary Fig. 2.** Production of FB<sub>2</sub> in strains of three *Fusarium* species: *F. proliferatum*, *F.*  
732 *verticillioides*, and *F. fujikuroi* under different light conditions. Dark incubation was used as a  
733 reference. Strains were grown in PDB for 10 days by shaking (100 rpm) at 20 °C. Error bars show  
734 standard deviations for triplicate assays. Statistical significance:  $P < 0.05$  for all comparisons.

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736 **Supplementary Fig. 3.** Dry mycelium weight (g) of *Fusarium* strains grown at 20 °C under  
737 different light conditions. Strains were grown in PDB for 10 days by shaking (100 rpm). Error bars  
738 show standard deviations for triplicate assays.