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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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ABSTRACT

Three Fusarium species associated to bakanae disease of rice (Fusarium fujikuroi, Fusarium proliferatum, and Fusarium verticillioides) were investigated for their ability to produce fumonisins (FB₁ and FB₂) under different light conditions, and for pathogenicity. The conditions that highly stimulated the fumonisin production compared to darkness were yellow and green light in F. verticillioides strains; white and blue light, and light/dark alternation in F. fujikuroi and F. proliferatum strains. In general, all light conditions influenced positively the fumonisin production with respect to the dark. Expression of the FUM1 gene, which is necessary for initiation of fumonisin production, was in accordance with the fumonisin biosynthetic profile. High and low fumonisin-producing F. fujikuroi strains showed typical symptoms of bakanae disease, abundant fumonisin-producing F. verticillioides strains exhibited chlorosis and stunting of rice plants, while fumonisin-producing F. proliferatum strains were asymptomatic on rice. We report that F. fujikuroi might be an abundant fumonisin producer with levels comparable to that of F. verticillioides and F. proliferatum, highlighting the need of deeper mycotoxicological analyses on rice isolates of F. fujikuroi. Our results showed for the first time the influence of light on fumonisin production in isolates of F. fujikuroi, F. proliferatum, and F. verticillioides from rice.

Keywords: Bakanae disease; Rice; Fusarium; Pathogenicity; FB₁; FB₂
1. Introduction

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

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

The fumonisin B series, including FB\(_1\), FB\(_2\), FB\(_3\), and FB\(_4\), is the most toxic to plants and animals (Abbas et al., 1998a). FB\(_1\) is generally found in corn, rice, triticale, sorghum, beans, and asparagus. It can cause equine leucoencephalomalacia and porcine pulmonary oedema (Scott, 2012). FB\(_1\) has been associated to human oesophageal cancer in humans (Chu and Li, 1994; Sydenham et al.,...
1990). FB$_2$ is found often in lower concentrations than FB$_1$. Although FB$_1$ is the primarily studied fumonisin, FB$_2$ is considered 3 to 4 times more cytotoxic than FB$_1$ (Dombrink-Kurtzman et al., 1994).

Fumonisin biosynthetic (FUM) gene clusters were reported in *F. verticillioides*, *F. proliferatum*, and *Fusarium oxysporum* (Proctor et al., 2003; Proctor et al., 2008; Waalwijk et al., 2004). The gene *FUM1* encodes a polyketide synthase (PKS) necessary for the production of fumonisins, which catalyses the initial steps in fumonisin biosynthesis (Bojja et al., 2004). Interestingly, the flanking regions of the fumonisin cluster are not significantly similar between *Fusarium* spp., suggesting an independent species acquisition of the cluster (Proctor et al., 2008). It is not yet clear how the sequence divergence within the cluster affects the fumonisin biosynthesis, but it was evidenced that a single-point mutation can cause the occurrence of a nonproduction phenotype (Proctor et al., 2006). Beside the FUM cluster, other genes are involved in fumonisin biosynthesis. *FfVel1* and *FfLae1*, components of a velvet-like complex, and *FvVE1* co-regulate the biosynthesis of the fumonisins in *F. fujikuroi* and *F. verticillioides*, respectively (Myung et al., 2009; Wiemann et al., 2010). *WcoA*, a component of a white collar photoreceptor family, *Ffg1* and the cAMP-mediated signalling pathway affect other secondary metabolite pathways in *F. fujikuroi* (Estrada and Avalos, 2008; Studt et al., 2013).

Fungi are microorganisms exposed to environmental stimuli on a circadian rhythm, such as temperature, light, and humidity. During the evolution, they developed complex genetic mechanisms to respond to those environmental variables. Light affects fungal growth, reproduction and pigment biosynthesis, depending on the species (Rodriguez-Romero et al., 2010). Recently, it has been found that light also affects the secondary metabolism of fungi, particularly the mycotoxin production. Regarding the light-sensing function in fungi, many fungal species use a number of various wavelength-specific receptors. Wavelengths from both sides of the spectrum (blue and red) had the strongest inhibitory effect on ochratoxin A production compared to the dark control in *Aspergillus niger* (Schmidt-Heydt et al., 2011). *Penicillium expansum* increased the production of
citrinin under white, red and blue light, whereas *Penicillium verrucosum* stimulated citrinin production under yellow and green light (Schmidt-Heydt et al., 2011). Wavelengths within the visible spectrum (from red to blue) increased the fumonisin biosynthesis compared to darkness in two species of *Fusarium* originating from maize, in particular red and blue light in *F. proliferatum*, and red and royal blue light in *F. verticillioides* (Fanelli et al., 2012a; 2012b).

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

2. Materials and methods

2.1. Fungal strains

Fungal strains of *F. fujikuroi* (Augusto2), *F. verticillioides* (19-115), and *F. proliferatum* (19-113), isolated and characterized from Italian bakanae-associated rice samples (Amatulli et al., 2010) were used in this study. Three additional bakanae-associated isolates (I1.3 and CSV1 of *F. fujikuroi*, and 11-471 of *F. proliferatum*) from Northern Italy were included in this study. Reference *Fusarium* rice strains from other countries obtained from the Fusarium Research Centre (Pennsylvania State University, USA) were also used: *F. fujikuroi* M-1149 from Taiwan, *F. verticillioides* M-5331 from China, and *F. proliferatum* M-6580 from Thailand (Table 1). The strains were stored at 4 °C in Spezieller nährstoffarmer agar (0.2 g sucrose, 0.2 g glucose, 1.0 g KNO₃, 1.0 g KH₂PO₄, 0.5 g MgSO₄ · 7H₂O, 0.5 g NaCl, and 12 g agar per litre).

2.2. DNA extraction and EF-1α/*FUM1* amplification
Total DNA of nine fungal strains was extracted with the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) from mycelium grown on potato dextrose agar (PDA, Merck KGaA, Darmstadt, Germany) plates according to the manufacturer’s instructions. Twenty ng of fungal DNA were used for the PCR reaction performed with a mixture containing 10X PCR buffer (Qiagen, Düsseldorf, Germany), 0.5 μM of each primer, 0.5 mM of dNTPs (Qiagen), 2 U Taq DNA polymerase (Qiagen) and the final volume adjusted to 40 μl with sterile distilled water. Three Fusarium isolates (I1.3, CSV1, and 11-471) not characterized by Amatulli et al. (2010) were amplified in the portion of the EF-1α gene by using specific primers (O’Donnell et al., 1998b). Fum1F1 and Fum1R2 primers were used for the amplification of a part of the FUM1 gene in the nine strains (Stępień et al., 2011). The PCR program was initiated with a denaturation step of 94°C for 5 min, followed by 35 cycles at 94 °C for 45 sec, 55 °C for 45 sec, and 72 °C for 2 min, and a final extension step of 7 min at 72 °C.

2.3. Sequence analysis

EF-1α and FUM1 amplicon sequences were deposited in GenBank (Table 1) and comparison with sequences available was done by using the BLAST program (www.ncbi.nlm.nih.gov). Multiple sequence alignment of nucleotide (nt) and amino acid (aa) sequences and identification of open reading frames were done by using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, Maryland, USA) with the Clustal W algorithm (Thompson et al., 1994). Phylogenetic analyses were performed using MEGA 5 (Tamura et al., 2011). Neighbor-joining (NJ) trees were constructed with 1,000 bootstrap replications.

2.4. Selection of growing medium
Fungal cultures were subcultured on PDA plates for 10 days at 20 °C. A spore suspension of the strains was prepared from the plates in sterile distilled water. One hundred μl of a 10⁶ spores ml⁻¹ solution was inoculated in 50 ml of three growing media: (a) potato dextrose broth (PDB, Merck KGaA), (b) Czapek-Dox broth (Sigma-Aldrich, Buchs, Switzerland), and (c) GYAM (0.24 M glucose, 0.05% yeast extract, 8.0 mM L-asparagine, 5.0 mM malic acid, 1.7 mM NaCl, 4.4 mM K₂HPO₄, 2.0 mM MgSO₄, and 8.8 mM CaCl₂), by shaking at 100 rpm for 10 days in darkness. Growing media were evaluated for their efficiency for induction of fumonisin production in different Fusarium strains. The best fumonisin-producing medium was selected for the tests of light conditions and pathogenicity, as described below. The experiment was carried out three times at constant temperature.

2.5. Light conditions

Fusarium strains were grown in PDB under different light conditions: red (685 nm), yellow (580 nm), green (535 nm), blue (475 nm), and white. In addition, the growth under dark and light/dark alternation were examined. To investigate the influence of light and various wavelengths, the growth chambers were provided with a lighting system containing five RoHs comforted bulbs. Each bulb contained 36 Light Emitting Diodes (Super Bright LEDs Inc., St. Louis, USA) that emitted at corresponding wavelengths as indicated above. The bulbs were fixed at the four corners and in the middle of the upper side of the chambers, and were kept at a 20 cm distance from the orbital shaker where fungal strains were grown for 10 days at 100 rpm. Only one chamber was not provided with the lighting system, and it was used as a dark control. The luminous flux of each bulb was 72 lumen, and the light intensity was 4.0 lumen/cm² or 76 mW/cm² on the fungal culture. Continuous light or dark was applied in the chambers for each experiment, with the exception of the growth under light/dark (12 h: 12 h). The experiments were carried out three times at constant temperature of 20 °C maintained by the control system of the growth chambers.
2.6. Fumonisin analysis

For fumonisin quantification, the strains grown in PDB were filtered through sterile cheese cloth (Merck KGaA) to separate the supernatant and the mycelia. The supernatant was used for FB₁ and FB₂ analyses, whereas the mycelium was used for subsequent RNA extraction and obtainment of the dry mycelium described as follows. The collected mycelium was weighed and divided into two equal parts. One part was immediately processed for RNA extraction, and the other part was dried at 70 °C for 24 h to measure the mycelial dry weight, that was multiplied by two (including also the mycelium used for RNA extraction) to obtain the total dry mycelium. The total dry mycelium was used to normalize the final FB₁ and FB₂ values obtained as described below.

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

The triple quadrupole mass spectrometer (Varian 310-MS) was operated in the negative/positive electrospray ionization mode (ESI/ESI⁺). To select the MS/MS parameters for the analysis of fumonisins by multiple reaction monitoring (SRM), ESI mass spectra for each analyte were initially analysed introducing the stock fumonisin standard solution (Sigma-Aldrich) with direct injection
into the spectrometer by a Harvard 11 plus infusion pump. The \( m/z \) 706 positive ion and \( m/z \) 720.4 negative ion were used, respectively, as parent ions for FB\(_1\) and FB\(_2\). The most intense daughter ions, resulting from collision-induced dissociation with argon, used to detect and quantify the fumonisin content were: \( m/z \) 336 at 36 eV of collision energy (CE) and 318 at 318 eV for FB\(_1\); \( m/z \) 156.7 at 30 eV CE and 562.4 at 16.5 eV for FB\(_2\). The limits of detection (LOD; signal-to-noise ratio: 3) and quantification (LOQ; signal-to-noise ratio: 10) were, respectively, 0.62 µg/l and 1.55 µg/l for FB\(_1\), 2.09 µg/l and 5.17 µg/l for FB\(_2\). Each analysis was performed in triplicate.

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

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

Real-time PCR was used for the quantification of the FUM1 transcript expression under different light conditions. Primers for FUM1 gene sequence were designed: Fum1fp1 (5’-AGGATTGGCTGGATCTTCAC-3’) and Fum1fp2 (5’-TAATACGGTTGGAAATGGCA-3’) for F. fujikuroi and F. proliferatum on the basis of the GenBank accession no. AY577454 (nt position 149-241) giving an amplicon of 93 bp, and Fum1univs (5’- CCAGCTGTTTTTCCCTGCTA-3’) and Fum1univa (5’- CGATTTCATCAATCGAAGAT-3’) for F. verticillioides designed from the sequence AF155773 (nt position 25785-25868) giving an amplicon of 103 bp. PCR conditions were...
as follows: an initial step at 95 °C for 5 min, and 40 cycles at 95 °C for 15 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 30 s (extension). The reactions were carried out in an iCycler (Bio-Rad) and contained 1 μl cDNA, 10 μl SsoFast™ EvaGreen® Supermix 2X (Bio-Rad), 2.5 mM each primer in a total volume of 20 μl. PQTUB-F and PQTUB-R primers designed from the sequence of the β-tubulin gene tub2 were used as a control for the constitutive expression (Glass and Donaldson, 1995). To investigate the range of concentrations where the target RNA and C\text{\textsubscript{T}} values were linearly correlated, and to determine the reaction efficiency for both sets of \textit{FUM1} primers, the reactions were performed by using the cDNA synthesized from serially diluted RNAs. Baseline range and C\text{\textsubscript{T}} values were automatically calculated using the iCycler Optical System Software v. 3.0. The expression of the \textit{FUM1} gene was normalized to that of \textit{tub2}, by subtracting the \textit{tub2} C\text{\textsubscript{T}} value from the C\text{\textsubscript{T}} value of the \textit{FUM1} gene resulting from the ΔC\text{\textsubscript{T}}. The expression ratio was calculated from equation 2^{-\Delta\Delta\text{CT}}, where ΔΔC\text{\textsubscript{T}} represents the ΔC\text{\textsubscript{T}}\text{\textsubscript{sample}} − ΔC\text{\textsubscript{T}}\text{\textsubscript{control}} (Livak and Schmittgen, 2001).

2.8. Pathogenicity tests

Fungal strains were cultured in PDB for 10 days in darkness in acclimatized chamber at 20 °C. The strains were filtered through sterile cheese cloth to a final spore concentration of 10^6 ml\textsuperscript{-1} in sterile distilled water. The rice cultivar Galileo susceptible to \textit{F. fujikuroi} was used for pathogenicity tests. Rice seeds were surface-disinfected in 1% sodium hypochlorite for 2 min and rinsed in sterile distilled water. A total of 120 seeds were soaked in 100 ml spore suspension by shaking for 30 min at room temperature. Control seeds were soaked in sterile distilled water. Seeds were sown in plastic pots (40 x 24 x 12 cm) in triplicate (40 seeds per pot) in a sterile mixture of peat and sand (60% : 40%). The plants were kept in greenhouse conditions (25 °C day : 17 °C night) by watering 3 times per day. Disease symptoms were evaluated 30 days after germination.
We searched for bakanae symptoms including thin and elongated internodes typical of *F. fujikuroi*, and for other less specific symptoms, such as chlorosis, necrosis and delayed growth.

2.9. Statistical analysis

Data from all the experiments were submitted to analysis of variance (ANOVA) by using the Statistical Package for Social Science (SPSS, IBM, Chicago, IL, USA) version 17.0. The statistical significance was judged at P < 0.05. Statistically significant differences among light treatments with a reference to the dark incubation were determined by the *t* test. Pearson’s correlation coefficient between expression levels of *FUM1* gene and FB1 content was also calculated.

3. Results

3.1. Species identification

The nine isolates of *Fusarium* spp. originated from rice were identified through analysis of the *EF-1α* and *FUM1* sequences (Table 1). Both genes permitted to identify four strains of *F. fujikuroi*, three strains of *F. proliferatum* and two strains of *F. verticillioides*. The phylogenetic studies of the *FUM1* gene portion (amplicons from 1030 to 1039 nt) allowed the separation of the three *Fusarium* species similarly to those performed with the *EF-1α* portion (amplicons from 649 to 661 nt). Thus, phylogenetic analyses of the nucleotide (Supplementary Fig. 1) or amino acid (data not shown) of the *EF-1α* and *FUM1* fragments grouped the *Fusarium* strains into two clusters; one cluster contained the strains of *F. fujikuroi* and *F. proliferatum*, and the another one the strains of *F. verticillioides*. The first cluster was further divided into two subclusters allowing the separation of
F. fujikuroi and F. proliferatum. On the basis of the results obtained, FUM1 gene sequence, corresponding to the acetyltransferase domain of the PKS protein, could be used to differentiate species of the Gibberella fujikuroi species complex. F. fujikuroi and F. proliferatum had 91.1% FUM1-nucleotide sequence identity. A lower nucleotide identity was found when F. verticillioides strains were compared to the strains of F. proliferatum and F. fujikuroi (77.3% and 78.7%, respectively).

3.2. Selection of growing medium

The medium which allowed the highest production of FB₁ (Fig. 1 and Supplementary Table 1) and FB₂ (data not shown) in three Fusarium species was PDB. It induced 3.2 to 5.0 times higher FB₁ production compared to the GYAM medium. Czapek-Dox was not a satisfactory growing medium for the production of fumonisins since it allowed very low production of FB₁ in only two Fusarium strains. On the basis of these results, lighting and pathogenicity tests were performed by using PDB as a growing medium.

3.3. Fumonisin production in three Fusarium species under dark

Strains belonging to three Fusarium species were compared for fumonisin production after their growth in dark. The highest FB₁ producing strains were F. verticillioides M-5331, F. fujikuroi Augusto2, and F. fujikuroi M-1149 (Table 2). The other Fusarium strains showed lower FB₁ production. FB₂ biosynthesis was obtained in both strains of F. verticillioides, and in two out of four strains of F. fujikuroi (I1.3 and Augusto2), while none of the F. proliferatum strains produced FB₂. Interestingly, F. verticillioides M-5331 and 19-115, and F. fujikuroi I1.3 produced more FB₂ than FB₁ (16.7, 17.3 and 25.9 times more, respectively).

By considering the geographic origin of the F. verticillioides strains, the M-5331 strain originating from China produced 2.3 and 2.2 times more FB₁ and FB₂, respectively, than the Italian
strain 19-115 (Table 2). *F. fujikuroi* Augusto2 originating from Italy was a higher FB$_1$/FB$_2$ producer compared to the Taiwanese strain of *F. fujikuroi* (M-1149). The Italian strain of *F. proliferatum* (11-471) showed higher level of FB$_1$ production compared to the Thai strain (M-6580).

3.4. Fumonisin production in different light conditions

All the species of *Fusarium* isolated from rice showed a higher production of fumonisins (Fig. 2 and Table 2) under different light conditions compared to darkness. *F. proliferatum* strains had the highest FB$_1$ increase under white and blue light, and under light/dark. Red, green and yellow light were also stimulating the FB$_1$ biosynthesis compared to the dark incubation. FB$_1$ production was highly stimulated in both strains of *F. verticillioides* under yellow and green light. The other light conditions also activated the FB$_1$ production in *F. verticillioides*, but to a lower extent. *F. fujikuroi* Augusto2 and M-1149 stimulated the FB$_1$ biosynthesis under white and blue light, and light/dark. The other two strains of *F. fujikuroi* (I1.3 and CSV1) ceased FB$_1$ production under different wavelengths and light conditions, with the exception of blue light and the light/dark alternation (Fig. 2 and Table 2). Conclusively, the highest production of FB$_1$ was obtained in *F. fujikuroi* strain M-1149 (white) and *F. verticillioides* M-5331 (yellow light).

FB$_2$ production was activated by different wavelengths of light; red and blue light in the three strains of *F. proliferatum* and in *F. fujikuroi* Augusto2 and M-1149. It was also stimulated under light/dark alternation in *F. proliferatum* and most *F. fujikuroi* strains. Red and yellow light activated FB$_2$ biosynthesis in *F. verticillioides* (Table 2 and Supplementary Fig. 2). Red light induced a higher FB$_2$ production compared to FB$_1$ in both strains of *F. verticillioides* (Table 2).

We observed that the light-pattern of fumonisin synthesis was generally more similar between *F. fujikuroi* and *F. proliferatum*, compared to *F. verticillioides*. Additionally, a stimulation of mycelium production was also observed under different light conditions compared to the dark incubation in all three *Fusarium* species (Supplementary Fig. 3). All light conditions that we
applied (light of different wavelengths, white light and the light/dark alternation) had a significant effect on the mycelium production with respect to dark after 10 days of growth in PDB.

3.5. Expression of the FUM1 gene in different light conditions

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

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

The highest increase was observed in F. fujikuroi M-1149 (486-fold change; white), F. fujikuroi Augusto2 (384-fold change; white light) and F. proliferatum 11-471 (267-fold change; blue light) (Table 2). The FUM1 expression level was found in correspondence with FB1 production and the value for the Pearson’s correlation coefficient was 0.78 for all three species. When the Pearson’s correlation coefficient was calculated for each species separately, a higher correlation was found in F. verticillioides (0.97) and F. fujikuroi (0.80) compared to F. proliferatum (0.57).

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

**Discussion**

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

For this reason, fumonisins were measured from fungal strains grown *in vitro* in PDB, which was previously selected. In general, there is limited information on the use of PDB as a growing medium for mycotoxin production (Spadaro et al., 2010). We know that secondary metabolites, such as gibberellins and bikaverin in *F. fujikuroi*, and fumonisins in *F. verticillioides*, are repressed by high amounts of nitrogen (Mihlan et al., 2003; Schönig et al., 2008; Kim and Woloshuk, 2008). In this work, the PDB medium non-supplemented with additional nitrogen sources showed to have favourable nitrogen conditions for fumonisin production in comparison with other previously used
substrates, including GYAM and Czapek-Dox that contained other nitrogen sources (Proctor et al., 2008; Amatulli et al., 2012).

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

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

F. fujikuroi is reported to produce little or no fumonisin compared to F. verticillioides and F. proliferatum (Desjardins et al., 2000; Stępień et al., 2011; Wulff et al., 2010). Here we report for the first time that two strains of F. fujikuroi from rice have the ability to produce fumonisin levels
comparable to those of *F. verticillioides* and *F. proliferatum*. FB₁ and FB₂ production was highly increased under white light in *F. fujikuroi* Augusto2 and M-1149. In these conditions, *F. fujikuroi* Augusto2 from Italy and M-1149 from Taiwan proved to be the most abundant fumonisin producers.

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

As far as we know, little is known about the light wavelength sensing in fumonisin production of *F. fujikuroi*. We reported here that white light, followed by blue light and light/dark alternation had a strong stimulatory effect on FB₁ or FB₂ synthesis in two *F. fujikuroi* strains (Augusto2 and M-1149). Under white light, FB₁ and FB₂ productions were increased, respectively, 75 and 15 times in *F. fujikuroi* Augusto2, and 326 and 1367 times in *F. fujikuroi* M-1149. Our data indicated that a better effectiveness of fumonisin production in *F. fujikuroi* was generally obtained with light regimes compared to darkness. Positive influence of white light on fumonisin-producing profile was found, and the result is in agreement with increase of production of secondary metabolites in other organisms such as DON in *Fusarium graminearum*, citrinin in *P. expansum*, and aflatoxins in *Aspergillus parasiticus* (Bennett et al., 1981; Schmidt-Heydt et al., 2011). The activation of fumonisin biosynthesis found by light-dark alternation, may be attributed to the night-day cycle of fungi. Many fungal species possess a circadian clock which is influenced by light and temperature (Dunlap and Loros, 2006). Other two *F. fujikuroi* strains (I1.3 and CSV1) did not show an
activation in fumonisin production with light application, with the exception of light/dark alternation (6 and 10 times FB$_1$ increase) and blue light (6 and 11 fold increase). It would be interesting to study the expression of genes involved in fumonisin production, such as the FUM cluster, the white collar gene, and the velvet-like complex, in low and high fumonisin-producing F. fujikuroi strains.

Concerning F. proliferatum, white and blue light stimulated the FB$_1$ synthesis, whereas the FB$_2$ production was activated under red light and the light/dark alternation compared to darkness. Our data fit with Fanelli et al (2012a), but we found additional induction of fumonisin biosynthesis under white light and light/dark alternation. Strains of F. proliferatum showed 11 to 32 times more FB$_1$ production under blue light, and 19 to 36 times more FB$_2$ production under red light.

Our data showed that different wavelengths, particularly yellow and green light, favour fumonisin production in F. verticillioides. These wavelengths were also found stimulatory in the previous work of Fanelli et al. (2012b). In our work, both strains of F. verticillioides showed 47 to 171 times more synthesis of FB$_1$ under yellow light, and 17 to 20 fold increase under green light in comparison to darkness.

FB$_2$ production was higher compared to FB$_1$ in most strains of the three Fusarium species under light/dark alternation, and in both strains of F. verticillioides under red light and dark. Fusarium isolates able to produce more FB$_2$ than FB$_1$ have been already reported (Musser and Plattner, 1997), and it could be possible that some light conditions are more favourable for FB$_2$ production, by inducing a higher expression of FUM2 gene, involved in the switch from FB$_1$ to FB$_2$ (Proctor et al., 2003).

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

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

Previous studies reported that *FUM1* gene expression showed a correlation with fumonisin production, offering a diagnostic tool for the rapid and sensitive detection of metabolically active fumonisin-producing *Fusarium* species (López-Errasquín et al., 2007; Jurado et al. 2010; Fanelli et al., 2012a). In our work we also found a positive and high relationship between mRNA levels of *FUM1* gene and FB₁ production. Some cases of lower correspondence between *FUM1* transcripts and FB₁ content could be explained by slightly different time-points of transcription and mycotoxin production.

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

**Acknowledgments**

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**References**


pathogens and fumonisin producers *Fusarium verticillioides* and *Fusarium proliferatum*. FEMS Microbiology Ecology 73, 303–311.


### Table 1

List of *Fusarium* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Origin</th>
<th>Host</th>
<th>Accession no. (<em>EF-1α</em> gene)</th>
<th>Reference</th>
<th>Accession no. (<em>FUM1</em> gene)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-471</td>
<td><em>F. proliferatum</em></td>
<td>Italy</td>
<td><em>Oryza sativa</em></td>
<td>KC121066</td>
<td>This study</td>
<td>KC188784</td>
<td>This study</td>
</tr>
<tr>
<td>19-113</td>
<td><em>F. proliferatum</em></td>
<td>Italy</td>
<td><em>Oryza sativa</em></td>
<td>GQ848533</td>
<td>Amatulli et al., 2010</td>
<td>KC188785</td>
<td>This study</td>
</tr>
<tr>
<td>M-6580</td>
<td><em>F. proliferatum</em></td>
<td>Thailand</td>
<td><em>Oryza sativa</em></td>
<td>JN092336</td>
<td>Amatulli et al., 2010</td>
<td>KC188786</td>
<td>This study</td>
</tr>
<tr>
<td>19-115</td>
<td><em>F. verticillioides</em></td>
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<td><em>Oryza sativa</em></td>
<td>GQ848530</td>
<td>Amatulli et al., 2010</td>
<td>KC188787</td>
<td>This study</td>
</tr>
<tr>
<td>M-5331</td>
<td><em>F. verticillioides</em></td>
<td>China</td>
<td><em>Oryza sativa</em></td>
<td>AY337449</td>
<td>Yergeau et al., 2005</td>
<td>KC188788</td>
<td>This study</td>
</tr>
<tr>
<td>11.3</td>
<td><em>F. fujikuroi</em></td>
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<td><em>Oryza sativa</em></td>
<td>GQ848523</td>
<td>Amatulli et al., 2010</td>
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<td><em>Oryza sativa</em></td>
<td>KC121067</td>
<td>This study</td>
<td>KC188790</td>
<td>This study</td>
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<td>Augusto2</td>
<td><em>F. fujikuroi</em></td>
<td>Italy</td>
<td><em>Oryza sativa</em></td>
<td>KC121068</td>
<td>This study</td>
<td>KC188791</td>
<td>This study</td>
</tr>
<tr>
<td>M-1149</td>
<td><em>F. fujikuroi</em></td>
<td>Taiwan</td>
<td><em>Oryza sativa</em></td>
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Table 2
Relative expression of FUM1 gene (FC), and FB1 and FB2 production (µg per g of mycelial dry weight) of Fusarium strains grown under different light conditions.

### F. proliferatum

<table>
<thead>
<tr>
<th></th>
<th>11-471</th>
<th>19-113</th>
<th>M-6580</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FUM1 (µg / g)</td>
<td>FB1 (µg / g)</td>
<td>FB2 (µg / g)</td>
</tr>
<tr>
<td>Red</td>
<td>1.77±0.21</td>
<td>43±4.0</td>
<td>24±1.9</td>
</tr>
<tr>
<td>Yellow</td>
<td>2.33±0.13</td>
<td>27±2.2</td>
<td>ND</td>
</tr>
<tr>
<td>Green</td>
<td>3.64±0.44</td>
<td>128±9.2</td>
<td>9±1.6</td>
</tr>
<tr>
<td>Blue</td>
<td>267±21.6</td>
<td>214±20.8</td>
<td>22±0.9</td>
</tr>
<tr>
<td>White</td>
<td>10.73±1.09</td>
<td>412±52.9</td>
<td>ND</td>
</tr>
<tr>
<td>Light/Dark</td>
<td>4.56±0.54</td>
<td>145±11.9</td>
<td>320±35.5</td>
</tr>
<tr>
<td>Dark</td>
<td>1±0.15</td>
<td>20±1.8</td>
<td>ND</td>
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### F. verticillioides

<table>
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<th>19-115</th>
<th>M-5331</th>
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<tbody>
<tr>
<td></td>
<td>FUM1 (µg / g)</td>
<td>FB1 (µg / g)</td>
</tr>
<tr>
<td>Red</td>
<td>5.98±0.47</td>
<td>35±4.4</td>
</tr>
<tr>
<td>Yellow</td>
<td>9.40±0.53</td>
<td>102±9.6</td>
</tr>
<tr>
<td>Green</td>
<td>22.43±1.31</td>
<td>44±28.5</td>
</tr>
<tr>
<td>Blue</td>
<td>3.59±0.47</td>
<td>151±17.9</td>
</tr>
<tr>
<td>White</td>
<td>1.22±0.09</td>
<td>27±3.1</td>
</tr>
<tr>
<td>Light/Dark</td>
<td>3.81±0.49</td>
<td>162±18.4</td>
</tr>
<tr>
<td>Dark</td>
<td>1±0.11</td>
<td>22±2.9</td>
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### F. fujikuroi

<table>
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<th>CSV1</th>
<th>Augusto2</th>
<th>M-1149</th>
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<tbody>
<tr>
<td></td>
<td>FUM1 (µg / g)</td>
<td>FB1 (µg / g)</td>
<td>FB2 (µg / g)</td>
<td>FUM1 (µg / g)</td>
</tr>
<tr>
<td>Red</td>
<td>1.05±0.02</td>
<td>ND</td>
<td>ND</td>
<td>1.09±0.03</td>
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<tr>
<td>Yellow</td>
<td>1.19±0.11</td>
<td>ND</td>
<td>ND</td>
<td>1.22±0.13</td>
</tr>
<tr>
<td>Green</td>
<td>1.09±0.09</td>
<td>ND</td>
<td>ND</td>
<td>1.14±0.11</td>
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<tr>
<td>Blue</td>
<td>4.32±0.27</td>
<td>39±3.6</td>
<td>ND</td>
<td>5.20±0.98</td>
</tr>
<tr>
<td>White</td>
<td>1.12±0.09</td>
<td>ND</td>
<td>ND</td>
<td>1.25±0.08</td>
</tr>
<tr>
<td>Light/Dark</td>
<td>3.08±0.46</td>
<td>44±4.6</td>
<td>319±35.7</td>
<td>4.66±0.93</td>
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<tr>
<td>Dark</td>
<td>1±0.14</td>
<td>7±1.2</td>
<td>18±24.1</td>
<td>1±0.15</td>
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*ND- not detected
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<tr>
<th>Strain</th>
<th>Germination (%)</th>
<th>Elongated plants with chlorotic leaves (%)</th>
<th>Stunted plants with chlorotic leaves (%)</th>
<th>Death incidence (%)</th>
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<td>98</td>
<td>0</td>
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<td><em>F. proliferatum</em> 19-113</td>
<td>75</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td><em>F. verticillioides</em> M-5331</td>
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<td>0</td>
<td>87</td>
<td>0</td>
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<tr>
<td><em>F. fujikuroi</em> I1.3</td>
<td>83</td>
<td>91</td>
<td>0</td>
<td>91</td>
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<tr>
<td><em>F. fujikuroi</em> CSV1</td>
<td>92</td>
<td>83</td>
<td>0</td>
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<tr>
<td><em>F. fujikuroi</em> Augusto2</td>
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<td>5</td>
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<td><em>F. fujikuroi</em> M-1149</td>
<td>52</td>
<td>93</td>
<td>0</td>
<td>93</td>
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</tbody>
</table>
**Supplementary Table 1**

FB$_1$ production (µg per g of mycelial dry weight) in *Fusarium* strains grown in different media under dark.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>FB$_1$ production (µg / g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDB</td>
</tr>
<tr>
<td><em>F. proliferatum</em> 11-471</td>
<td>22±4.21</td>
</tr>
<tr>
<td><em>F. proliferatum</em> 19-113</td>
<td>13±3.08</td>
</tr>
<tr>
<td><em>F. proliferatum</em> M-6580</td>
<td>10±2.07</td>
</tr>
<tr>
<td><em>F. verticillioides</em> 19-115</td>
<td>23±3.55</td>
</tr>
<tr>
<td><em>F. verticillioides</em> M-5331</td>
<td>52±7.08</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> I1.3</td>
<td>8±1.03</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> CSV1</td>
<td>17±3.06</td>
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<tr>
<td><em>F. fujikuroi</em> Augusto2</td>
<td>50±9.17</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> M-1149</td>
<td>42±7.32</td>
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</tbody>
</table>

* ND- not detected
Figure captions

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

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

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

**Fig. 4.** Symptomatology induced on 1-month old rice plants (cultivar Galileo) artificially inoculated with *Fusarium* strains. The following *Fusarium* species were used for pathogenicity tests: *F. fujikuroi* (1. I1.3; 2. M-1149; 3. CSV1; 4. Augusto2), *F. proliferatum* (5. 19-113; 6. 11-471; 7. M-6580), and *F. verticillioides* (8. 19-115; 9. M-5331).
**Supplementary Fig. 1.** Phylogenetic analyses based on the partial nucleotide sequences of EF-1α and FUM1 genes from *Fusarium* strains: *F. fujikuroi* (II.3, Augusto2, CSV1 and M-1149), *F. proliferatum* (11-471, 19-113 and M-6580), and *F. verticillioides* (19-115 and M-5331). Reference isolates of *F. fujikuroi* (HF679028), *F. proliferatum* (JF740718) and *F. verticillioides* (AB674289) for EF-1α gene, and *F. fujikuroi* (HF679031) and *F. verticillioides* (AF155773) for FUM1 gene are shown in bold. Phylogenetic analyses were performed by neighbor-joining method using MEGA 5 (Tamura et al., 2011). Bootstrap analyses were supported with 1,000 replications.

**Supplementary Fig. 2.** Production of FB2 in strains of three *Fusarium* species: *F. proliferatum*, *F. verticillioides*, and *F. fujikuroi* under different light conditions. Dark incubation was used as a reference. Strains were grown in PDB for 10 days by shaking (100 rpm) at 20 °C. Error bars show standard deviations for triplicate assays. Statistical significance: P<0.05 for all comparisons.

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