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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 (FB_1 and FB_2) 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; FB_1; FB_2

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 KNO_3, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 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-1*α 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-1*α 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 10^6 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 K_2HPO_4, 2.0 mM $MgSO_4$, and 8.8 mM $CaCl_2$), 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/cm^2 or 76 mW/cm^2 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 FB_1 and FB_2 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 FB_1 and FB_2 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'- CGATTTCCCATCAGCAAGAT-'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_T values were linearly correlated, and to determine the reaction efficiency for both sets of *FUM1* primers, the reactions were performed by using the cDNA synthesized from serially diluted RNAs. Baseline range and C_T values were automatically calculated using the iCycler Optical System Software v. 3.0. The expression of the *FUM1* gene was normalized to that of *tub2*, by subtracting the *tub2* C_T value from the C_T value of the *FUM1* gene resulting from the ΔC_T. The expression ratio was calculated from equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ represents the $\Delta C_{Tsample} - \Delta C_{Tcontrol}$ (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^{-1} in sterile distilled water. The rice cultivar Galileo susceptible to *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 FB_1 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-1*α 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-1*α 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-1*α 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 FB_1 (Fig. 1 and Supplementary Table 1) and FB_2 (data not shown) in three *Fusarium* species was PDB. It induced 3.2 to 5.0 times higher FB_1 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 FB_1 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 FB_1 producing strains were *F. verticillioides* M-5331, *F. fujikuroi* Augusto2, and *F. fujikuroi* M-1149 (Table 2). The other *Fusarium* strains showed lower FB_1 production. FB_2 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 FB_2. Interestingly, *F. verticillioides* M-5331 and 19-115, and *F. fujikuroi* I1.3 produced more FB_2 than FB_1 (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 FB_1 and FB_2, 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 FB_1 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*. FB_1 and FB_2 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 tetrapyrrols 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 FB_1 or FB_2 synthesis in two *F. fujikuroi* strains (Augusto2 and M-1149). Under white light, FB_1 and FB_2 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 FB_1 production. Some cases of lower correspondence between *FUM1* transcripts and FB_1 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).

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Table 1

List of *Fusarium* strains used in this study.

Strain	Species	Origin	Host	Accession no. (*EF-1*α gene)	Reference (*EF-1*α gene)	Accession no. (*FUM1* gene)	Reference (*FUM1* gene)
11-471	*F. proliferatum*	Italy	*Oryza sativa*	KC121066	This study	KC188784	This study
19-113	*F. proliferatum*	Italy	*Oryza sativa*	GQ848533	Amatulli et al., 2010	KC188785	This study
M-6580	*F. proliferatum*	Thailand	*Oryza sativa*	JN092336	Amatulli et al., 2010	KC188786	This study
19-115	*F. verticillioides*	Italy	*Oryza sativa*	GQ848530	Amatulli et al., 2010	KC188787	This study
M-5331	*F. verticillioides*	China	*Oryza sativa*	AY337449	Yergeau et al., 2005	KC188788	This study
I1.3	*F. fujikuroi*	Italy	*Oryza sativa*	GQ848523	Amatulli et al., 2010	KC188789	This study
CSV1	*F. fujikuroi*	Italy	*Oryza sativa*	KC121067	This study	KC188790	This study
Augusto2	*F. fujikuroi*	Italy	*Oryza sativa*	KC121068	This study	KC188791	This study
M-1149	*F. fujikuroi*	Taiwan	*Oryza sativa*	HM243234	Amatulli et al., 2010	KC188792	This study

Table 2

Relative expression of FUM1 gene (FC), and FB_1 and FB_2 production (µg per g of mycelial dry weight) of *Fusarium* strains grown under different light conditions.

F. proliferatum

	11-471			19-113			M-6580		
	FUM1	**FB1 (µg / g)**	**FB_2 (µg / g)**	**FUM1**	**FB_1 (µg / g)**	**FB_2 (µg / g)**	**FUM1**	**FB_1 (µg / g)**	**FB_2 (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

F. verticillioides

	19-115			M-5331		
	FUM1	**FB_1 (µg / g)**	**FB_2 (µg / g)**	**FUM1**	**FB_1 (µg / g)**	**FB_2 (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

F. fujikuroi

	I1.3			CSV1			Augusto2			M-1149		
	FUM1	**FB_1 (µg / g)**	**FB_2 (µg / g)**	**FUM1**	**FB_1 (µg / g)**	**FB_2 (µg / g)**	**FUM1**	**FB_1 (µg / g)**	**FB_2 (µg / g)**	**FUM1**	**FB_1 (µg / g)**	**FB_2 (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

Table 3

Pathogenicity tests of *Fusarium* strains on the rice cultivar Galileo.

Strain	Germination (%)	Elongated plants with chlorotic leaves (%)	Stunted plants with chlorotic leaves (%)	Death incidence (%)
F. proliferatum 11-471	98	0	0	0
F. proliferatum 19-113	75	0	0	0
F. proliferatum M-6580	75	0	0	0
F. verticillioides 19-115	60	0	21	0
F. verticillioides M-5331	58	0	87	0
F. fujikuroi I1.3	83	91	0	91
F. fujikuroi CSV1	92	83	0	83
F. fujikuroi Augusto2	93	5	0	5
F. fujikuroi M-1149	52	93	0	93

Supplementary Table 1

FB_1 production (µg per g of mycelial dry weight) in *Fusarium* strains grown in different media under dark.

	FB_1 production (µg / g)		
Isolate	PDB	Gyam	Czapek-Dox
F. proliferatum 11-471	22±4.21	6±0.52	ND*
F. proliferatum 19-113	13±3.08	4±0.03	ND
F. proliferatum M-6580	10±2.07	3±0.02	ND
F. verticillioides 19-115	23±3.55	6±1.13	ND
F. verticillioides M-5331	52±7.08	13±1.54	3±0.20
F. fujikuroi I1.3	8±1.03	2±0.16	ND
F. fujikuroi CSV1	17±3.06	5±0.34	ND
F. fujikuroi Augusto2	50±9.17	10±1.24	2±0.12
F. fujikuroi M-1149	42±7.32	11±0.95	ND

* ND- not detected

Figure captions

Fig. 1. Production of FB_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_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-1*α and *FUM1* genes from *Fusarium* strains: *F. fujikuroi* (I1.3, 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-1*α 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 FB_2 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.