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Identification, mycotoxin risk and pathogenicity of Fusarium species associated to fig endosepsis in Apulia.

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Identification, mycotoxin risk and pathogenicity of *Fusarium* species associated to fig endosepsis in Apulia.

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Abstract

In a survey carried out on 87 rotted fig fruits samples collected in Apulia region, we have isolated 126 *Fusarium* strains identified as *F. ramigenum* (69 strains), *F. solani* (49 strains), *F. proliferatum* (5 strains) and 3 not identified strains. Investigation on the fertility of the strains belonging to *F. proliferatum* and *F. ramigenum* revealed that only strains of *F. proliferatum* were fertile. The identity of *F. ramigenum* strains was confirmed by sequencing a portion of the translation elongation factor-1α gene. When *Fusarium* species were analyzed for their toxigenicity, 37 out of 69 strains of *F. ramigenum* produced fusaric acid (FA) up to 525 mg/kg;, 30 strains produced beauvericin (BEA) up to 190 mg/kg; 60 strains produced fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) up to 1,575 mg/kg of total FBs; and 2 strains produced fusaproliferin (FUP) up to 345 mg/kg; all 5 strains of *F. proliferatum* produced FA at low levels; 2 strains produced BEA up to 205 mg/kg; 1 strain produced FB_1 and FB_2, 1,100 and 470 mg/kg, respectively; and 1 strain produced FUP, 820 mg/kg; *F. solani* (30 strains) produced FA, 13 strains up to 215 mg/kg. Few fungal extracts showed high toxicity toward brine shrimp larvae and in some cases in relation to BEA and FA content. A pathogenic assay on fig fruits showed that all three species were pathogenic, with higher virulence of *F. ramigenum*. These data report for the first time the production of BEA and FB_1/FB_2 by *F. ramigenum* and show that it is a main agent of fig endosepsis in Apulia and can contribute to the fumonisin contamination of fresh and dried figs.

Keywords: *Gibberella fujikuroi* complex, elongation factor-1α, fumonisins, beauvericin, brine shrimp assay

Introduction

Fig (*Ficus carica* L.) is a typical Mediterranean plant which cultivation is mostly concentrated in the whole Mediterranean Basin. Among the countries that produce figs, Italy keeps an important

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position worldwide with its 17,000 t of fruit production on 2,700 Ha and Apulia is the region with the highest production of dried figs in the whole Italy (ISTAT, 2009). Although different carcinogenic mycotoxins were reported as natural contaminants on fig fruits, such as aflatoxins and ochratoxin A (Karapinar and Gönül, 1989, Iamanaka et al., 2007, Karbancioglu-Güler and Heperkan, 2008), there is only a recent report that refers to contamination of fumonisin B_1 (Karbancioglu-Güler and Heperkan, 2009). Fumonisins are carcinogenic compounds (IARC, 1993), involved in several human and animal diseases such as esophageal cancer in humans, leukoencephalomalacia in horses, and pulmonary edema in swine (Desjardins, 2006). Among members of this class of toxins, especially fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) can both occur in several food commodities such as maize (Rheeder et al., 2002), wheat and barley (Castellá et al., 1999), rice (SCOOP, 2003), asparagus (Logrieco et al., 1998) and sorghum (Jackson and Jablonski, 2004). The fungal species responsible of FB_1 and FB_2 production mainly belong to *Fusarium* genus, although recently, strains of *Aspergillus niger* isolated from different commodities have been reported as also capable of producing FB_2, at a very low extent (Frisvad et al., 2007; Logrieco et al., 2009). Species of *Fusarium* have been recorded frequently as occurring on dried figs at a high incidence (Heperkan, 1989) and they are also considered the main agents of the endosepsis, a fungal disease so called for the internal rot that can affect fig fruits (Michailides et al., 1996). The main species involved in the disease was identified as *F. moniliforme* var. *fici* by Caldis (1927). However, Subbarao and Michailides (1992) excluded the variety name of *fici* because they established that morphological characteristics used to delineate the *F. moniliforme* variety *fici* were not unique to the pathogen and the fungus did not show specificity to figs. Moreover, while the same authors identified also *F. solani* as main agent of endosepsis (Subbarao and Michailides 1993), Marasas (1995) identified *F. proliferatum* as an important member of the *Fusarium* species complex causing the fig endosepsis. Finally, the strains of *F. moniliforme* from fig were re-classified as *F. lactis* and *F. ramigenum*, based on their morphological and phylogenetic traits (Nirenberg and O'Donnell, 1998; O'Donnell et al., 1998) and these two species were considered as

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specific to fig plants. With the exception of *F. solani*, all the above mentioned species involved in the endosepsis belong to *G. fujikuroi* species complex (Leslie and Summerell, 2006). Many species of this complex produce a diverse array of known mycotoxins and secondary metabolites (Desjardins, 2006) that can contaminate animal feeds and human foods. Reports on toxin production by *F. lactis* and *F. ramigenum*, are limited to few or single strains (Moretti et al., 2007; Fotso et al., 2002) and therefore they offer limited information on their toxin profiles. Moreover, data on their fertility, genetic traits and pathogenicity are poor and need deeper investigations and there is a lack of information on the *Fusarium* species that colonize fig fruits.

Our objectives in this study were (i) to identify the main *Fusarium* species occurring on fig fruits in Apulia, one of the most dedicated regions for fig production in Italy, by using all available techniques (morphology, fertility test, and DNA sequencing); (ii) to evaluate the possible production *in vitro* of BEA, FA, FUP, FB_1, and FB_2 by these species; (iii) to test their toxicity on a brine shrimp assay; iv) to evaluate their pathogenicity on fig fruits.

Material and methods

Fungal collection and fungal isolation

We selected 17 localities in Apulia (9 in Bari area, 7 in Lecce area, and 1 locality in Taranto area). In all localities, 5 fields were sampled by selecting 15 fruits with endosepsis-like visible symptoms, with exception of 1 locality, where 7 fields were sampled. Fungal isolations were made by placing the internal part of each fruit on a peptone-PCNB medium (Nash-Snyder medium) semi-selective for *Fusarium* spp. (Nash and Snyder, 1962). Cultures were incubated under fluorescent lights at 25°C with a 12-h–12-h day/night cycle for 4 to 7 days to allow fungal colonies to grow out onto the medium. Each morphologically distinct *Fusarium* colony isolated from each fruit was then transferred to complete medium slants. From these colonies, the cultures were started from single spore isolation of microconidia/macroconidia and a subculture per each strain was frozen for long-

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term storage at -80°C in 18:82 (vol/vol) glycerol/water. One-hundred twenty-six fungal strains, were morphologically identified according to Leslie and Summerell (2006), were deposited in the ITEM culture collection of Institute of Sciences of Food Production, Bari, Italy (ITEM) (web site: http://www.ispa.cnr.it/Collection). *F. ramigenum* ITEM 3570 ex holotype [other collection number 68592 of BBA (Biologische Bundesanstalt fur Land- und Forstwirtschaft culture collection), Berlin, Germany], was used as reference strain for sequence comparison. Due to objective difficulties in morphological identification, all *Fusarium* strains were successively studied to confirm their identity at species level, except those identified as *F. solani* that show well distinguishable traits.

Fertility test

Single spore culture of *F. ramigenum* (69) and *F. proliferatum* (5) were crossed as male parents on carrot agar to standard tester strains of *G. fujikuroi* MPs [MATA-1 (ITEM 3621), MATA-2 (ITEM 3622), MATB-1 (ITEM 3623), MATB-2 (ITEM 3624), MATC-1 (ITEM 3625), MATC-2 (ITEM 3626), MATD-1 (ITEM 3627), MATD-2 (ITEM 3628), MATE-1(ITEM 3629), MATE-2 (ITEM 3630), MATF-1 (ITEM 3631), MATF-2 (ITEM 3632), MATG-1 (ITEM 3633), MATG-2 (ITEM 3634), MATH-2 (ITEM 7616), MATH-2 (ITEM 7617), MATI-1 (ITEM 7642) and MATI-2 (ITEM 7643), kindly provided by J.F. Leslie, Plant Pathology Department, Kansas State University, Manhattan, Kansas], as female parents on CLA (Klittich and Lelslie 1988). All strains were crossed twice to both testers from each MP. Crosses were examined weekly and were considered positive when mature dark blue perithecia were observed with oozing ascospores; they were considered negative after three attempts failed in obtaining fertile perithecia. All strains resulted unfertile in the above mentioned crosses were further crossed each other by testing them as both male and female parents, in order to obtain a fertile cross combination (Leslie and Summerell, 2006).

In vitro toxin production.

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A total of 107 strains, belonging to *F. ramigenum* (69), *F. solani* (30), *F. proliferatum* (5) and unidentified species (3) were cultured on 100 g of autoclaved yellow maize kernels (approximately 45% moisture content) in 500-ml Erlenmeyer flasks and inoculated with 2 ml of an aqueous suspension containing approximately 10^6 conidia/ml. Cultures were incubated at 25°C for 4 weeks in the dark. This procedure was repeated at two additional times to permit evaluation of variation in toxin production by individual strains. Harvested culture material was dried in a forced draft oven at 60°C for 48 h, finely ground, and stored at 4°C until used. Controls were treated in the same way, except that they were not inoculated.

BEA, Fumonisin and FUP analyses.

For BEA and FUP extractions and analyses, the procedure of Moretti et al. (2007) were followed. For fumonisin extraction and analyses, the procedure described by Leslie et al. (2004).

Fusaric acid analyses

For fusaric acid, a 2.5-g sample of each fungal culture was finely ground and added to 25 ml of a 75:25 methanol-water solution containing 1% of KH_2PO_4 adjusted to pH 3. The samples were homogenized in an Ultra-Turrax homogenizer (13,500 rpm for 3 min at room temperature) and then clarified by centrifugation at 4,000 rpm for 5 min at 4°C. The supernatant was then extracted by using 35 ml of chloroform for 3 times and the organic extract was evaporated at 35-40°C under reduced pressure. The residue was re-suspended in 5 ml of chloroform to which the derivative agent diazomethane was added. To eliminate the diazomethane, the derivate complex was dried and then re-suspended in 1 ml. The recovery efficiency of this extraction method was 85%.

The GC-MS analysis was carried out using a Shimadzu 17A gas-chromatography equipped with QP5000 mass spectrometer detector. Chromatography column was a RTX 5% diphenyl (30 m 0.25 mm i.d .x 0.25 um) (Superchrome). Injector temperature was set up at 250°C while the column temperature was kept at 100°C for two min, then raised up to 200°C at 35 C/min speed. The oven

temperature was kept constant for 4 min and again raised of 25°C/min up to 250°C where was kept constant for 3 min. The mass spectrometer source was set up at 280°C with an electron power of 70 eV. The mass range of the detector was fixed from 50 uma to 300 uma. The retention time observed for derivatized fusaric acid was 8.2 min. In Single Ion Monitoring (SIM) mode 193 and 135 *m/z* peaks were selected; these two peaks corresponding to molecular ion of methyl ester of fusaric acid and molecular ion less acetic acid, respectively. The quantification and detection limits were 0.01 mg Kg^{-1} and 0.01 µg Kg^{-1}, respectively. All the analyses were statistically evaluated by Stat View 5.0 Software.

Brine shrimp assays.

Toxicity to brine shrimp (*Artemia salina* L.) larvae was determined as previously described (Logrieco et al., 1996). Briefly, larvae were exposed to fungal culture extracts in 24-well cell culture plates (30 to 40 larvae per well in 500 µl of 3.3% [wt/vol] marine salt in H_2O). The number of dead larvae was recorded after incubation at 27°C for 24 h. The total number of larvae in each cell was counted after killing the surviving larvae by freezing at -20°C for 12 h. Water-soluble extracts, containing fumonisins, in general had little or no toxicity (0 to 3% mortality) (data not shown) towards the brine shrimp larvae, and data are reported only from extracts made in methanol that usually contained fusaproliferin and/or beauvericin. Tests in each experimental run were performed in quadruplicate. Data are based on the averages of three independent experiments (see Table 1).

DNA extraction

The *F. ramigenum* ex holotype ITEM 3570 and all strains (77), except *F. solani* strains, were grown for 48 hours in shake cultures (125 rpm) at 25 °C in 100 ml of Wikerham's medium (4 % D-glucose, 0.5 % peptone, 0.3 % yeast extract, 0.3% malt extract). The resulting mycelium was vacuum-filtered on Whatman N° 4 filter paper, washed with distilled water, frozen, and lyophilised.

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About 25-30 mg of lyophilised mycelium was ground and used to obtain genomic DNA with the Wizard® Magnetic DNA Purification System for Food (Promega, Madison, WI). Extracted DNA was dissolved in 100 µl of sterile water and stored at –20 °C. DNA quality was evaluated by agarose gel electrophoresis and its concentrations was estimated by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) .

DNA sequencing

DNA sequencing was performed only for the 77 strains belonging to *Liseola* section, according to the morphological identification. Sequence analysis of translation elongation factor 1-α (TEF) gene employed PCR conditions and primers described by O' Donnell et al. (2000). PCR products were analyzed by agarose gel electrophoresis to confirm that a ~700-bp fragment was amplified. Fragments were purified by filtration through Shepadex G-50 (Sigma) prior to DNA sequencing. Sequence analysis of both DNA strains was done with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The DNA sequences for the TEF gene were subjected to blast analysis at the FUSARIUM ID database (Geiser et al., 2004). Sequences were aligned in ClustalW (Maddison and Maddison, 2001) by using default settings with Molecular Evolutionary Genetics Analysis (MEGA), Version 4.0 (Tamura et al., 2007). Aligned sequence data were subjected to Neighbor-Joining method analysis. Clade validation was assessed by bootstrap analysis (Felsenstein, 1985), using heuristic searches with 500 replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Previously generated TEF sequences obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) were also included to allow comparative analysis.

Pathogenicity test

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The pathogenicity test was performed by following the procedure reported by Subbarao and Michailides (1993), with some modifications. Briefly, fig fruits of the local variety *Petrelli* were surface-disinfected with a 1% sodium hypochlorite solution for 3 min and air-dried. The fruits were sliced longitudinally through the ostiole by using a sterile knife and placed with the cut surface touching the wire screens in plastic-containers. Forty fruit halves were wounded and inoculated with each strain. Inoculation was performed with a 5 µl conidial suspension (10^6 conidia/ml) following two different ways: on a wound made on the surface of the fruits (external lesions) and on a wound made in the cavity of each half fruit (internal lesions). To each container, 200 ml of distilled water were added in order to increase the relative humidity. The inoculated fruits were incubated at 25 °C for 5 days in the dark. Each inoculated fig half was considered a replication. Data were tested for normality and homogeneity of variance before analysis of variance (SAS, 1990). Means were computed, and strain means were compared with the least significant difference test (Steel and Torrie, 1980). The lesion sizes (length x width of the colony) of all fruits were calculated separately for external and internal lesions because of the difficulty of the internal quantification. Indeed, the inoculation in the cavity of the fruits, made to mimic the natural process of the infection, resulted often in irregular rotting symptoms and lesions difficult to quantify. Moreover, the background inoculum due to *Fusarium* spp. eventually occurring in the fruit cavity, can affect the results. For these reasons, the surface inoculation, which resulted in circular lesions with well defined margins, facilitated the disease quantification and determination of pathogenicity, avoided the background contamination and was therefore more reliable. The strains were divided in different categories according with the size of the lesions that they caused: 0 mm^2, not pathogenic; 1-50 mm^2, weakly pathogenic; 51-150 mm^2, moderately pathogenic; 151-300 mm^2, pathogenic; >300 mm^2, highly pathogenic.

Results

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Fungal incidence, identification and pathogenicity tests

Of the 1,305 fruits examined, fruits from 61 fields were not contaminated by *Fusarium*, while in the remaining 26 fields, the incidence of the contaminated fruits by *Fusarium* strains was 32%. We recovered 126 strains of *Fusarium* that, based on morphology, were identified as *F. solani* (49 strains) while 77 strains were grouped within *Fusarium* section *Liseola* (Nelson et al., 1983) which members belong to *G. fujikuroi* species complex (Nirenberg and O'Donnell, 1998). Further morphological identification showed that 72 strains were *F. ramigenum*, and 5 strains were identified as *F. proliferatum*. The 77 strains belonging to *G. fujikuroi* species complex were tested also for fertility. Of the 77 strains examined, 5 strains belonged to *G. intermedia* (all strains resulted MATD-2), being fertile with MATD-1 standard tester strain of *G. intermedia*, while 72 strains were unfertile in crosses against the standard strains of *G. fujikuroi* species complex (Leslie and Summerell, 2006). The unfertile strains were also crossed each other as both male and female parents, but none of the crosses produced fertile perithecia.

Identification by sequence analysis

Sequences of TEF obtained from the 77 strains belonging to *Liseola* section were subjected to blast analysis at the FUSARIUM ID database (Geiser et al., 2004). Based on BLAST analysis, 69 strains were identified as *F. ramigenum*, 5 strains as *F. proliferatum* and 3 strains (ITEM 2913, ITEM 2921 and ITEM 2922) did not show significant homology with any species sequences deposited at the FUSARIUM ID database. Sequences obtained were compared against the NCBI database sequences and did not show any significant homology. TEF gene sequences were also subjected to phylogenetic analysis. For this analysis, sequences of 13 selected strains were loaded from FUSARIUM ID and NCBI databases to represent 13 different species: *F. ramigenum, F. napiforme, F. lactis, F. brevicatenulatum, F. udum, F. phyllophilum, F. xylarioides, F. subglutinans, F. concentricum, F. fratiflexum, F. proliferatum, F. verticillioides* and *F. solani* were used. The optimal tree of TEF sequences with the sum of branch length of 0.515 is shown in Figure 1.

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Bootstrap analysis showed high values for both the *F. ramigenum* and *F. proliferatum* clusters as well as for the cluster composed by the three not identified strains ITEM 2913, ITEM 2921 and ITEM 2922.

Mycotoxin production and toxicity on brine shrimp larvae

All strains assigned to the *Liseola* section were subjected to mycotoxin analyses, while only 30 out of 49 strains of *F. solani* were analyzed for the mycotoxin production. Results on the mycotoxin production are shown in Table 1. FA was produced by 13 out of 30 strains of *F. solani* ranging from 0.1 to 215 mg/kg, by 37 out of 69 strains of *F. ramigenum* with a range from 0.1 mg/kg to 525 mg/kg, and by all 5 strains of *F. proliferatum* (range, 0.1-4 mg/kg); BEA was produced by both *F. ramigenum* (28 strains out of 69 strains with a range from 2 mg/kg to 190 mg/kg) and *F. proliferatum* (two strains out of 5, producing 10 mg/kg and 205 mg/kg, respectively); FB_1 was produced by 41 strains out of 69 strains of *F. ramigenum* ranging from 0.1 mg/kg to 1,080 mg/kg of FB_1 and 1 strain of *F. proliferatum* (1,100 mg/kg); FB_2 was produced by *F. ramigenum* (44 out of 69 strains and a range of production from 0.1 mg/kg to 655 mg/kg of FB_2) and 1 strain of *F. proliferatum* (470 mg/kg); FUP was produced only by two strains of *F. ramigenum* (155 mg/kg and 345 mg/kg, respectively) and 1 strain of *F. proliferatum* (820 mg/kg). Finally, among the three unidentified strains, two strains produced BEA (from 20 to 40 mg/kg), two strains produced FA (0.1 mg/kg for both strains), and all strains do not produce FB_1, while they produced FB_2 (0.1 mg/kg for two strains and 20 mg/kg for one strain). The toxicity of the fungal cultural extracts (Table 1) showed that within *F. ramigenun*, 32 out of 69 strains were not toxic at all on the brine shrimp larvae assay (0% larvae mortality), while 5 strains out of 69 strains resulted highly toxic at a value of 100%. For the remaining 32 strains, the range of larvae mortality was between 6% and 78%. Among the 5 strains of *F. proliferatum*, only the BEA producing strain ITEM 2802 showed the highest toxicity (100% larvae mortality), while 4 strains were non toxic. The *F. solani* strains were mostly non toxic against the brine shrimp larvae since 20 strains did not show activity, 7

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strains showed a very low range of toxicity (from 6% to 27%), while only three strains proved to be highly toxic (two strain showed 100% of toxicity against the larvae, and 1 strain was toxic at a value of 93%). Finally, among the three unidentified strains, ITEM 2913 was not toxic, while ITEM 2921 and ITEM 2922 showed to cause a limited mortality on brine shrimp larvae (71% and 46% of larvae mortality, respectively).

Pathogenicity test

We arbitrarily selected 19 strains of *F. ramigenum*, 4 strains of *F. solani* and 2 strains of *F. proliferatum* for the virulence test. The data of the pathogenicity test for each strain are summarized in Table 2. Varied degrees of endosepsis were induced by different strains and species of *Fusarium*, but also by the different inoculation way since the internal lesions caused a higher level of virulence. Most of strains of *F. ramigenum* were pathogenic (10 strains) or highly pathogenic (8 strains) in the internal inoculation experiment, with a single strain that resulted moderately pathogenic (Table 2 and Table 3). The evaluation of the external lesions scored most of strains as moderately pathogenic (11 strains) and 7 strains as pathogenic, with a single strain that resulted highly pathogenic. The two strains of *F. proliferatum* were scored pathogenic and highly pathogenic in internal inoculation experiment, while both of them resulted virulent in the external inoculation experiment. Finally, the four *F. solani* strains tested showed to be mostly pathogenic (3 strains) in the internal inoculation experiment (1 strain resulted moderately virulent), while in the external inoculation experiment, only one strain resulted pathogenic, being two strains moderately pathogenic and one strain weakly pathogenic.

Discussion

Our report shows a high contamination of fig fruits by strains classified as *F. ramigenum*. This species has been firstly described by Nirenberg and O'Donnell (1998), based on strains isolated

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from fig in California and un-correctly classified as *F. moniliforme* (Subbarao and Michiailides, 1993). O'Donnell et al. (1998) re-evaluated other strains from Californian figs that were classified by Subbarao and Michiailides (1993) as *F. moniliforme*, and identified them as *F. lactis*. *Fusarium lactis* and *F. ramigenum* are considered two specific *Fusarium* species of fig morphologically and genetically closely related (Leslie and Summerell, 2006; O'Donnell et al., 1998). Of these two species, we identified in our survey only *F. ramigenum*, while *F. lactis* never occurred on fig fruits. Phylogenetic analyses carried out by O'Donnell et al. (1998) revealed 46 species in the *G. fujikuroi* complex, 23 of them as new to science. Among the 46 species, the eleven species identified by biological species concept have been reported identical to the phylogenetic species which indicates that phylogenetic approach can provide the same information as biological approach and that other MPs need to be still identified. We carried out experiments to obtain the sexual stage of *F. ramigenum* by performing the fertility tests described by Leslie and Summerell (2006), but all crosses within the *F. ramigenum* population resulted negative. On the other hand, Munaut and van Hove (2004) reported preliminary data on the sexual stage of *F. ramigenum*, *G. ramigena*, described as a member of *G. fujikuroi* complex. The five *F. proliferatum* strains isolated from fig fruits in our survey were all fertile in crosses with the *G. intermedia* (syn. mating population D of *G. fujikuroi* complex) tester strain MATD-1 ITEM 3628, being all same mating type MATD-2. Based on this data, *F. proliferatum*, which is a heterothallic species, seems to have few possibilities to recombine in fig fields in Apulia, although the size of strains sampled is extremely limited. Among the 5 strains of *F. proliferatum*, only a single strain, ITEM 2829, was able to produce FB_1 and FB_2. *Fusarium proliferatum* is considered, together with *F. verticillioides*, the major fumonisin producing species worldwide, and rarely its strains were reported as non-fumonisin producing strains (Desjardins, 2006). If the lack of fumonisin production can be a prevalent specific trait of the *F. proliferatum* population occurring on fig in Apulia, needs to be deeper investigated within a wider set of strains, in order to understand the existence of possible mechanisms that silence the mycotoxin production in the interaction *F. proliferatum*/fig plant. With respect to fumonisin

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production, several strains of *F. ramigenum* here reported proved to produce FB_1 and FB_2 *in vitro* at high levels, although a wide variability within the population was also recorded. This is in contrast with a previous report of Fotso et al. (2002) that, examining the ex-type culture of *F. ramigenum* ITEM 3569, revealed the lack of fumonisin production by this strain. Therefore, this is the first report of FB_1 and FB_2 production by *F. ramigenum* and shows that this species may contribute to the FB_1 contamination of dried figs, as recently reported for the first time in Turkey (Karbancioglu-Güler and Heperkan, 2009). Indeed, the authors did not provide any information on the identity of *Fusarium* species occurring on dried fig contaminated by FB_1, therefore a wider investigation in the main areas where the fig is cultivated in the Mediterranean Basin is worthwhile in order to more realistically assess the role that *F. ramigenum* could play as causal agent of fig endosepsis and contamination of figs by FB_1. Indeed, little is known of the ecology or biology of this fungus or of its ability to act as a plant pathogen. The pathogenicity test on fig fruits, performed with well characterized strains of *F. ramigenum*, provided useful indications, since showed that several strains of this species were aggressive on fig fruits, being their capability to colonize the fig fruits comparable, if not slightly higher, to *F. proliferatum*, and *F. solani* strains. Besides FB_1 and FB_2, we analyzed other toxins which are tipically produced by members of *G. fujukuroi* species members such as BEA, FA, and FUP. While FUP was produced only by two strains of *F. ramigenum*, we show in this report for the first time that FA is produced by this species, even though its production was highly variable and only two strains (ITEM 2824 and ITEM 2852) produced it at a high level (220 and 525 mg/kg, respectively). On the other hand, BEA resulted commonly produced by *F. ramigenum* (Table 1). This is the first report on BEA production by *F. ramigenum*. Previous reports (Fotso et al., 2002; Moretti et al., 2007) have shown the lack of BEA production by *F. ramigenum* ITEM 3570 (ex-type) and ITEM 3569, a further strain used by Nirenberg and O'Donnell (1998) for firstly describing this species. Levels of production resulted variable in the population investigated, with almost half of strains capable to produce this toxin also at a high value (28 strains out of 69 strains up to 190 mg/kg). These results show that also BEA could represent a contaminant to control

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in the risk management of fig in Italy. Beauvericin can induce apoptosis on mammalian cell lines (Logrieco et al., 2002). Moreover, BEA showed negative effects on heart rate and contraction strength in guinea pig hearts (Lemmens-Gruber et al., 2000). Thus, it may be involved in human and animal pathology, representing a serious risk for health when occurring as a contaminant in dried fig. Finally, the eventual simultaneous production of BEA and FB_1 and FB_2, assessed for some of *F. ramigenum* strains, is very important since the co-occurrence of different toxic metabolites could imply additive and/or synergistic effects on target organisms (Muller and Schwadorf, 1993). Most of strains included in this study showed a level of toxin production that was related also to their ability to be toxic toward brine shrimp larvae. This bioassay has been used to identify *Fusarium* toxins as the major toxic component in an organic extract from cultures of *Fusarium* species (Moretti et 1994; Ritieni et al., 1995). Beauvericin was shown to be toxic in the brine shrimp bioassay and was correlated significantly, with some exceptions, to the larvae mortality recorded in the bioassay shrimp performed (Moretti et al., 2007). In this study, some strains, ITEM 2812, ITEM 2928, and ITEM 2932, were highly toxic in the brine shrimp bioassay although any known toxin was detected in their culture extracts. This shows that new toxic metabolites need to be further characterized within these species and also within this complex as suggested by Moretti et al. (2007). Finally, ITEM 2913, ITEM 2921 and ITEM 2922, could not be assigned to any species by comparing sequences of TEF. Further phylogenetic investigations will be needed to better characterize such strains in order to understand if they belong to species not yet described occurring on fig plants. In conclusions we showed that in Apulia, there is a high risk of fig contamination by *Fusarium* species and that the main species associated with fig endosepsis are *F. ramigenum* and *F. solani*, which include highly virulent strains. High variability in toxin production was observed in *F. ramigenum* population. However, the capability of many strains to produce both FB_1 and FB_2, make it a good candidate as the main responsible for the contamination of FB_1 of dried fig recently reported.

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Table 1. Production of beauvericin, fusaric acid, fumonisin B_1 and fumonisin B_2 by strains of *Fusarium ramigenum* isolated from fig fruits in Apulia and mortality of their cultural extracts toward brine shrimp larvae.

Strain (ITEM[a]) and *Fusarium* species[b]	BEA[c] mg/kg	FA[d] mg/kg	FB_1[e] mg/kg	FB_2[f] mg/kg	Mortality on brine shrimp larvae (%)
F. ramigenum					
2799	nd	1	270	170	11
2800	nd	2	0.2	0.1	0
2801	2	1	200	5	34
2803	190	2	1	0.4	100
2807	2	0.2	1	1	0
2809	nd	0.3	1	0.3	0
2812	nd	0.1	nd	nd	100
2813	nd	0.2	5	2	44
2818	nd	1	nd	nd	56
2824	15	220	2	nd	0
2826	120	1	nd	nd	0
2827	nd	9	0.4	0.2	0
2828	3	nd	470	70	0
2830	15	nd	1,080	490	40
2831	10	0.2	290	25	13
2832	15	2	625	390	0
2833	nd	nd	930	100	0
2834	nd	nd	1	0.5	0
2835	nd	nd	540	80	0
2836	nd	nd	85	0.1	0
2837	nd	nd	960	6	0
2844	nd	nd	nd	nd	0
2845	nd	nd	20	nd	0
2846	nd	nd	15	1	0
2847	nd	15	55	2	0
2848	nd	nd	nd	nd	0
2849	nd	nd	435	655	0
2850	nd	40	40	1.3	0
2851	nd	2	0.3	0.1	0
2852	nd	525	0.5	0.2	100
2853	nd	nd	nd	nd	0
2854	nd	1	nd	nd	0
2901	nd	2	nd	nd	0
2902	nd	1	0.1	0.1	0
2903	105	nd	nd	nd	29
2904	nd	1	nd	nd	14
2905	35	0.2	nd	nd	27
2906	35	1	nd	nd	19
2907	25	0.3	nd	nd	0
2908	nd	0.3	nd	nd	73

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2909	55	0.3	nd	0.1	27
2910	40	1	0.1	0.1	19
2912	nd	0.3	0.1	0.1	36
2914	nd	0.5	nd	0.2	0
2915	nd	1	nd	0.2	0
2918	nd	20	0.1	0.2	12
2919	nd	nd	nd	0.2	0
2920	15	nd	nd	0.4	0
2923	nd	nd	nd	25	12
2928	15	nd	nd	nd	100
2929	nd	nd	nd	nd	75
2930	35	nd	1	0.3	6
2931	55	nd	0.1	nd	78
2932	50	nd	nd	nd	100
2933	40	nd	120	5	68
2934	25	nd	0.1	nd	0
2935	35	nd	0.1	nd	17
2939	nd	nd	nd	nd	25
2940	nd	nd	415	7	10
2941	15	nd	325	15	0
2942	25	nd	nd	3	0
2943	nd	nd	165	0.3	19
2944	nd	10	nd	nd	16
2945	nd	5	nd	0.2	15
2947	45	2	0.1	0.1	28
2948	10	1	nd	nd	8
2960	nd	7	0.1	0.2	12
2963	5	nd	170	3	60
2964	nd	nd	220	3	62
F. proliferatum					
2802	205	4	nd	nd	100
2811	nd	0.1	nd	nd	0
2819	nd	0.4	nd	nd	0
2823	nd	0.5	nd	nd	0
2829	10	1	1,100	470	0
Unidentified strains					
2913	nd	2	0.1	0.1	0
2921	40	0.1	nd	0.1	71
2922	20	nd	nd	20	46

[a] Institute of Science of Food Production Fungal Collection.

[b] Strains were cultured on 100 g of autoclaved yellow maize kernels at 25°C for 4 weeks in the dark.

[c] Beauvericin

[d] Fusaric acid

[e] Fumonisin B_1

[f] Fumonisin B_2

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Table 2. Virulence test on selected strains of *F. ramigenum*, *F. solani* and *F. proliferatum* by evaluation of the external and internal lesions size after fungal inoculation.

Strain ITEM[a]	Lesion size (mm^2)[b] ±SE[c]	
	External	Internal
F. ramigenum		
2824	59±14	178±73
2825	84±14	222±41
2826	147±24	291±50
2827	118±27	383±52
2828	127±17	288±31
2830	205±40	268±34
2831	260±31	291±39
2832	134±16	218±45
2833	271±32	382±40
2834	88±10	352±36
2835	184±22	277±40
2836	202±28	436±59
2837	155±22	300±32
2844	339±23	390±37
2845	151±21	197±58
2846	253±33	429±25
2847	117±16	71±31
2848	116±15	206±33
2849	74±26	335±67
F. solani		
2898	10± 3	251±61
2899	42±10	128±28
2900	82±12	254±37
2911	176±27	246±47
F. proliferatum		
2823	185±58	286±88
2829	189±22	406±39

[a] Institute of Science of Food Production Fungal Collection.

[b] Means represent the average of 40 inoculated fig fruits.

[c] Standard error of the mean.

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Table 3. Number of strains from *F. proliferatum*, *F. ramigenum*, and *F. solani* tested on fig fruits, classified in different lesions-size classes.

Lesion-size class (mm^2)	***F. ramigenum***		***F. solani***		***F. proliferatum***	
	external[a]	Internal[b]	external	internal	external	internal
0	0	0	0	0	0	0
1-50	0	0	2	0	0	0
50-150	10	1	1	1	0	0
150-300	8	9	1	3	2	1
>300	1	9	0	0	0	1

[a] The lesion sizes (length x width of the *Fusarium* colony) were calculated on the external surface of the fig fruit due to the inoculation performed with a 5 µl conidial suspension (10^6 conidia/ml) on the surface of the fruits on a wound made (external lesions).

[b] The lesion sizes (length x width of the *Fusarium* colony) calculated on the internal surface of the fig fruit due to the inoculation performed with a 5 µl conidial suspension (10^6 conidia/ml) on a wound made in the cavity of each half fruit (internal lesions).

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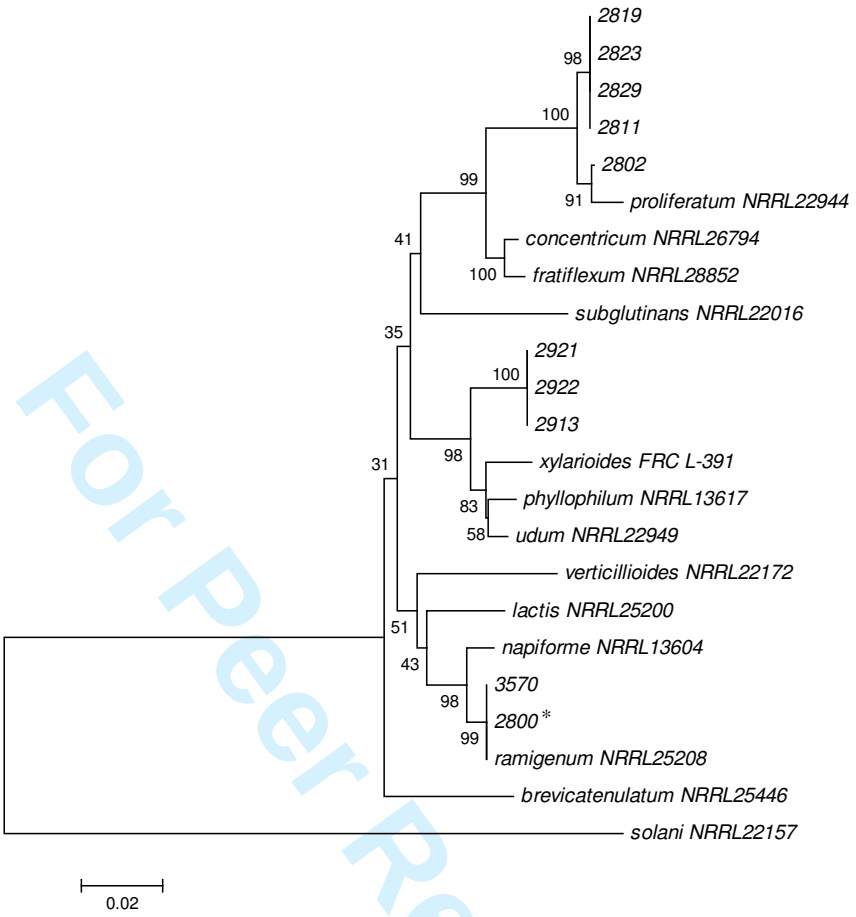

Figure 1 Phylogenetic tree generated by Neighbor-Joiney method (Saitou et al., 1987) on translation elongation factor sequences of 77 strains of *Fusarium* and 13 sequences representative for related species from GenBank accessions. Branch lengths are in the same units of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of base substitutions per site. Numbers at the major branches indicate the percentage occurrence of the cluster to the right of the branch in 500 bootstrapped data sets.

* all other *F. ramigenum* strains sequenced were identical to ITEM 2800 and they share the same values of bootstrap.

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