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

Antonio Moretti^a, Lia Ferracane^b, Stefania Somma^a, Vincenzo Ricci^a, Giuseppina Mulè^a, Antonia Susca^a, Alberto Ritieni^b, and Antonio F. Logrieco^a.

^a *Institute of Sciences of Food Production, Research National Council (ISPA-CNR), Via Amendola 122/O, Bari, 70126, Bari, Italy*

^b *Department of Food Sciences, University of Naples "Federico II", Portici, 80055, Italy*

Corresponding author: Antonio Moretti

e-mail address: antonio.moretti@ispa.cnr.it

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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-1 α 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 B₁ (FB₁) and fumonisin B₂ (FB₂) 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 FB₁ and FB₂, 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 FB₁/FB₂ 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-1 α , 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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3 43 position worldwide with its 17,000 t of fruit production on 2,700 Ha and Apulia is the region with
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5 44 the highest production of dried figs in the whole Italy (ISTAT, 2009). Although different
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8 45 carcinogenic mycotoxins were reported as natural contaminants on fig fruits, such as aflatoxins and
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10 46 ochratoxin A (Karapinar and Gönül, 1989, Iamanaka et al., 2007, Karbancioglu-Güler and
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12
13 47 Heperkan, 2008), there is only a recent report that refers to contamination of fumonisin B₁
14
15 48 (Karbancioglu-Güler and Heperkan, 2009). Fumonisin are carcinogenic compounds (IARC,
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17 49 1993), involved in several human and animal diseases such as esophageal cancer in humans,
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20 50 leukoencephalomalacia in horses, and pulmonary edema in swine (Desjardins, 2006). Among
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22 51 members of this class of toxins, especially fumonisin B₁ (FB₁) and fumonisin B₂ (FB₂) can both
23
24 52 occur in several food commodities such as maize (Rheeder et al., 2002), wheat and barley (Castellá
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26
27 53 et al., 1999), rice (SCOOP, 2003), asparagus (Logrieco et al., 1998) and sorghum (Jackson and
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29 54 Jablonski, 2004). The fungal species responsible of FB₁ and FB₂ production mainly belong to
30
31 55 *Fusarium* genus, although recently, strains of *Aspergillus niger* isolated from different commodities
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33 56 have been reported as also capable of producing FB₂, at a very low extent (Frisvad et al., 2007;
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36 57 Logrieco et al., 2009). Species of *Fusarium* have been recorded frequently as occurring on dried
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38 58 figs at a high incidence (Heperkan, 1989) and they are also considered the main agents of the
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40
41 59 endosepsis, a fungal disease so called for the internal rot that can affect fig fruits (Michailides et al.,
42
43 60 1996). The main species involved in the disease was identified as *F. moniliforme* var. *fici* by Caldis
44
45 61 (1927). However, Subbarao and Michailides (1992) excluded the variety name of *fici* because they
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47
48 62 established that morphological characteristics used to delineate the *F. moniliforme* variety *fici* were
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50 63 not unique to the pathogen and the fungus did not show specificity to figs. Moreover, while the
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53 64 same authors identified also *F. solani* as main agent of endosepsis (Subbarao and Michailides
54
55 65 1993), Marasas (1995) identified *F. proliferatum* as an important member of the *Fusarium* species
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57
58 66 complex causing the fig endosepsis. Finally, the strains of *F. moniliforme* from fig were re-
59
60 67 classified as *F. lactis* and *F. ramigenum*, based on their morphological and phylogenetic traits
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63 68 (Nirenberg and O'Donnell, 1998; O'Donnell et al., 1998) and these two species were considered as

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3 69 specific to fig plants. With the exception of *F. solani*, all the above mentioned species involved in
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5
6 70 the endosepsis belong to *G. fujikuroi* species complex (Leslie and Summerell, 2006). Many species
7
8 71 of this complex produce a diverse array of known mycotoxins and secondary metabolites
9
10 72 (Desjardins, 2006) that can contaminate animal feeds and human foods. Reports on toxin
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13 73 production by *F. lactis* and *F. ramigenum*, are limited to few or single strains (Moretti et al., 2007;
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15 74 Fotso et al., 2002) and therefore they offer limited information on their toxin profiles. Moreover,
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17 75 data on their fertility, genetic traits and pathogenicity are poor and need deeper investigations and
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19
20 76 there is a lack of information on the *Fusarium* species that colonize fig fruits.

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22 77 Our objectives in this study were (i) to identify the main *Fusarium* species occurring on fig fruits in
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24 78 Apulia, one of the most dedicated regions for fig production in Italy, by using all available
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27 79 techniques (morphology, fertility test, and DNA sequencing); (ii) to evaluate the possible
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29 80 production *in vitro* of BEA, FA, FUP, FB₁, and FB₂ by these species; (iii) to test their toxicity on a
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32 81 brine shrimp assay; iv) to evaluate their pathogenicity on fig fruits.

33 34 82 35 36 83 **Material and methods**

37 38 84 39 40 41 85 *Fungal collection and fungal isolation*

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43 86 We selected 17 localities in Apulia (9 in Bari area, 7 in Lecce area, and 1 locality in Taranto area).
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45
46 87 In all localities, 5 fields were sampled by selecting 15 fruits with endosepsis-like visible symptoms,
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48 88 with exception of 1 locality, where 7 fields were sampled. Fungal isolations were made by placing
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51 89 the internal part of each fruit on a peptone-PCNB medium (Nash-Snyder medium) semi-selective
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53 90 for *Fusarium* spp. (Nash and Snyder, 1962). Cultures were incubated under fluorescent lights at
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55 91 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
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58 92 medium. Each morphologically distinct *Fusarium* colony isolated from each fruit was then
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60 93 transferred to complete medium slants. From these colonies, the cultures were started from single
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100 99 spore isolation of microconidia/macroconidia and a subculture per each strain was frozen for long-

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3 95 term storage at -80°C in 18:82 (vol/vol) glycerol/water. One-hundred twenty-six fungal strains,
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5 96 were morphologically identified according to Leslie and Summerell (2006), were deposited in the
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8 97 ITEM culture collection of Institute of Sciences of Food Production, Bari, Italy (ITEM) (web site:
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10 98 <http://www.ispa.cnr.it/Collection>). *F. ramigenum* ITEM 3570 ex holotype [other collection number
11
12 68592 of BBA (Biologische Bundesanstalt für Land- und Forstwirtschaft culture collection), Berlin,
13 99 Germany], was used as reference strain for sequence comparison. Due to objective difficulties in
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15 100 morphological identification, all *Fusarium* strains were successively studied to confirm their
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17 101 identity at species level, except those identified as *F. solani* that show well distinguishable traits.
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25 104 *Fertility test*

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27 105 Single spore culture of *F. ramigenum* (69) and *F. proliferatum* (5) were crossed as male parents on
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29 106 carrot agar to standard tester strains of *G. fujikuroi* MPs [MATA-1 (ITEM 3621), MATA-2 (ITEM
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31 3622), MATB-1 (ITEM 3623), MATB-2 (ITEM 3624), MATC-1 (ITEM 3625), MATC-2 (ITEM
32 107 3626), MATD-1 (ITEM 3627), MATD-2 (ITEM 3628), MATE-1 (ITEM 3629), MATE-2 (ITEM
33
34 108 3630), MATF-1 (ITEM 3631), MATF-2 (ITEM 3632), MATG-1 (ITEM 3633), MATG-2 (ITEM
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36 109 3634), MATH-2 (ITEM 7616), MATH-2 (ITEM 7617), MATI-1 (ITEM 7642) and MATI-2 (ITEM
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38 110 7643), kindly provided by J.F. Leslie, Plant Pathology Department, Kansas State University,
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40
41 111 Manhattan, Kansas], as female parents on CLA (Klittich and Lelslie 1988). All strains were crossed
42
43 112 twice to both testers from each MP. Crosses were examined weekly and were considered positive
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46 113 when mature dark blue perithecia were observed with oozing ascospores; they were considered
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48 114 negative after three attempts failed in obtaining fertile perithecia. All strains resulted unfertile in the
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51 115 above mentioned crosses were further crossed each other by testing them as both male and female
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53 116 parents, in order to obtain a fertile cross combination (Leslie and Summerell, 2006).
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60 119 *In vitro* toxin production.

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3 120 A total of 107 strains, belonging to *F. ramigenum* (69), *F. solani* (30), *F. proliferatum* (5) and
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6 121 unidentified species (3) were cultured on 100 g of autoclaved yellow maize kernels (approximately
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8 122 45% moisture content) in 500-ml Erlenmeyer flasks and inoculated with 2 ml of an aqueous
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10 123 suspension containing approximately 10^6 conidia/ml. Cultures were incubated at 25°C for 4 weeks
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13 124 in the dark. This procedure was repeated at two additional times to permit evaluation of variation in
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15 125 toxin production by individual strains. Harvested culture material was dried in a forced draft oven at
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17 126 60°C for 48 h, finely ground, and stored at 4°C until used. Controls were treated in the same way,
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20 127 except that they were not inoculated.
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22 128 23 24 129 *BEA, Fumonisin and FUP analyses.*

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26
27 130 For BEA and FUP extractions and analyses, the procedure of Moretti et al. (2007) were followed.
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29 131 For fumonisin extraction and analyses, the procedure described by Leslie et al. (2004).
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32 132 33 34 133 *Fusaric acid analyses*

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36 134 For fusaric acid, a 2.5-g sample of each fungal culture was finely ground and added to 25 ml of a
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39 135 75:25 methanol-water solution containing 1% of KH_2PO_4 adjusted to pH 3. The samples were
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41 136 homogenized in an Ultra-Turrax homogenizer (13,500 rpm for 3 min at room temperature) and then
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43
44 137 clarified by centrifugation at 4,000 rpm for 5 min at 4°C. The supernatant was then extracted by
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46 138 using 35 ml of chloroform for 3 times and the organic extract was evaporated at 35-40°C under
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48 139 reduced pressure. The residue was re-suspended in 5 ml of chloroform to which the derivative agent
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51 140 diazomethane was added. To eliminate the diazomethane, the derivate complex was dried and then
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53 141 re-suspended in 1 ml. The recovery efficiency of this extraction method was 85%.
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55 142 The GC-MS analysis was carried out using a Shimadzu 17A gas-chromatography equipped with
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57
58 143 QP5000 mass spectrometer detector. Chromatography column was a RTX 5% diphenyl (30 m 0.25
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60 144 mm i.d .x 0.25 μm) (Superchrome). Injector temperature was set up at 250°C while the column
145 temperature was kept at 100°C for two min, then raised up to 200°C at 35 C/min speed. The oven

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3 146 temperature was kept constant for 4 min and again raised of 25°C/min up to 250°C where was kept
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6 147 constant for 3 min. The mass spectrometer source was set up at 280°C with an electron power of 70
7
8 148 eV. The mass range of the detector was fixed from 50 uma to 300 uma. The retention time observed
9
10 149 for derivatized fusaric acid was 8.2 min. In Single Ion Monitoring (SIM) mode 193 and 135 *m/z*
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13 150 peaks were selected; these two peaks corresponding to molecular ion of methyl ester of fusaric acid
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15 151 and molecular ion less acetic acid, respectively. The quantification and detection limits were 0.01
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17 152 mg Kg⁻¹ and 0.01 µg Kg⁻¹, respectively. All the analyses were statistically evaluated by Stat View
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19
20 153 5.0 Software.

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25 155 *Brine shrimp assays.*

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27 156 Toxicity to brine shrimp (*Artemia salina* L.) larvae was determined as previously described
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29 157 (Logrieco et al., 1996). Briefly, larvae were exposed to fungal culture extracts in 24-well cell
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32 158 culture plates (30 to 40 larvae per well in 500 µl of 3.3% [wt/vol] marine salt in H₂O). The number
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34 159 of dead larvae was recorded after incubation at 27°C for 24 h. The total number of larvae in each
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36 160 cell was counted after killing the surviving larvae by freezing at -20°C for 12 h. Water-soluble
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39 161 extracts, containing fumonisins, in general had little or no toxicity (0 to 3% mortality) (data not
40
41 162 shown) towards the brine shrimp larvae, and data are reported only from extracts made in methanol
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43
44 163 that usually contained fusaproliferin and/or beauvericin. Tests in each experimental run were
45
46 164 performed in quadruplicate. Data are based on the averages of three independent experiments (see
47
48 165 Table 1).

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51 16652
53 167 *DNA extraction*

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55 168 The *F. ramigenum* ex holotype ITEM 3570 and all strains (77), except *F. solani* strains, were grown
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58 169 for 48 hours in shake cultures (125 rpm) at 25 °C in 100 ml of Wikerham's medium (4 % D-
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60 170 glucose, 0.5 % peptone, 0.3 % yeast extract, 0.3% malt extract). The resulting mycelium was
171 vacuum-filtered on Whatman N° 4 filter paper, washed with distilled water, frozen, and lyophilised.

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3 172 About 25-30 mg of lyophilised mycelium was ground and used to obtain genomic DNA with the
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6 173 Wizard® Magnetic DNA Purification System for Food (Promega, Madison, WI). Extracted DNA
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8 174 was dissolved in 100 µl of sterile water and stored at –20 °C. DNA quality was evaluated by
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10
11 175 agarose gel electrophoresis and its concentrations was estimated by using a NanoDrop ND-1000
12
13 176 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) .
14

15 177

17 178 *DNA sequencing*

19
20 179 DNA sequencing was performed only for the 77 strains belonging to *Liseola* section, according to
21
22 180 the morphological identification. Sequence analysis of translation elongation factor 1-α (TEF) gene
23
24
25 181 employed PCR conditions and primers described by O' Donnell et al. (2000). PCR products were
26
27 182 analyzed by agarose gel electrophoresis to confirm that a ~700-bp fragment was amplified.
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29
30 183 Fragments were purified by filtration through Shepadex G-50 (Sigma) prior to DNA sequencing.
31
32 184 Sequence analysis of both DNA strains was done with the Big Dye Terminator Cycle Sequencing
33
34 185 Ready Reaction Kit (Applied Biosystems, Foster City, CA). The DNA sequences for the TEF gene
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36
37 186 were subjected to blast analysis at the FUSARIUM ID database (Geiser et al., 2004). Sequences
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39 187 were aligned in ClustalW (Maddison and Maddison, 2001) by using default settings with Molecular
40
41 188 Evolutionary Genetics Analysis (MEGA), Version 4.0 (Tamura et al., 2007). Aligned sequence data
42
43
44 189 were subjected to Neighbor-Joining method analysis. Clade validation was assessed by bootstrap
45
46 190 analysis (Felsenstein, 1985), using heuristic searches with 500 replicates. The evolutionary
47
48
49 191 distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004)
50
51 192 and are in the units of the number of base substitutions per site. Previously generated TEF
52
53 193 sequences obtained from the National Center for Biotechnology Information (NCBI)
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56 194 (<http://www.ncbi.nlm.nih.gov>) were also included to allow comparative analysis.
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59 60 196 *Pathogenicity test*

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3 197 The pathogenicity test was performed by following the procedure reported by Subbarao and
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6 198 Michailides (1993), with some modifications. Briefly, fig fruits of the local variety *Petrelli* were
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8 199 surface-disinfected with a 1% sodium hypochlorite solution for 3 min and air-dried. The fruits were
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10 200 sliced longitudinally through the ostiole by using a sterile knife and placed with the cut surface
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12 201 touching the wire screens in plastic-containers. Forty fruit halves were wounded and inoculated
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14
15 202 with each strain. Inoculation was performed with a 5 µl conidial suspension (10^6 conidia/ml)
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17 203 following two different ways: on a wound made on the surface of the fruits (external lesions) and on
18
19
20 204 a wound made in the cavity of each half fruit (internal lesions). To each container, 200 ml of
21
22 205 distilled water were added in order to increase the relative humidity. The inoculated fruits were
23
24
25 206 incubated at 25 °C for 5 days in the dark. Each inoculated fig half was considered a replication.
26
27 207 Data were tested for normality and homogeneity of variance before analysis of variance (SAS,
28
29 208 1990). Means were computed, and strain means were compared with the least significant difference
30
31 209 test (Steel and Torrie, 1980). The lesion sizes (length x width of the colony) of all fruits were
32
33
34 210 calculated separately for external and internal lesions because of the difficulty of the internal
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36 211 quantification. Indeed, the inoculation in the cavity of the fruits, made to mimic the natural process
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38
39 212 of the infection, resulted often in irregular rotting symptoms and lesions difficult to quantify.
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41 213 Moreover, the background inoculum due to *Fusarium* spp. eventually occurring in the fruit cavity,
42
43
44 214 can affect the results. For these reasons, the surface inoculation, which resulted in circular lesions
45
46 215 with well defined margins, facilitated the disease quantification and determination of pathogenicity,
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48 216 avoided the background contamination and was therefore more reliable. The strains were divided in
49
50
51 217 different categories according with the size of the lesions that they caused: 0 mm², not pathogenic;
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53 218 1-50 mm², weakly pathogenic; 51-150 mm², moderately pathogenic; 151-300 mm², pathogenic;
54
55 219 >300 mm², highly pathogenic.
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Results

222

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3 223 *Fungal incidence, identification and pathogenicity tests*
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6 224 Of the 1,305 fruits examined, fruits from 61 fields were not contaminated by *Fusarium*, while in the
7
8 225 remaining 26 fields, the incidence of the contaminated fruits by *Fusarium* strains was 32%. We
9
10 226 recovered 126 strains of *Fusarium* that, based on morphology, were identified as *F. solani* (49
11
12 227 strains) while 77 strains were grouped within *Fusarium* section *Liseola* (Nelson et al., 1983) which
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14
15 228 members belong to *G. fujikuroi* species complex (Nirenberg and O'Donnell, 1998). Further
16
17 229 morphological identification showed that 72 strains were *F. ramigenum*, and 5 strains were
18
19
20 230 identified as *F. proliferatum*. The 77 strains belonging to *G. fujikuroi* species complex were tested
21
22 231 also for fertility. Of the 77 strains examined, 5 strains belonged to *G. intermedia* (all strains resulted
23
24 232 MATD-2), being fertile with MATD-1 standard tester strain of *G. intermedia*, while 72 strains were
25
26
27 233 unfertile in crosses against the standard strains of *G. fujikuroi* species complex (Leslie and
28
29 234 Summerell, 2006). The unfertile strains were also crossed each other as both male and female
30
31
32 235 parents, but none of the crosses produced fertile perithecia.
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34 236
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36 237 *Identification by sequence analysis*
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38
39 238 Sequences of TEF obtained from the 77 strains belonging to *Liseola* section were subjected to blast
40
41 239 analysis at the FUSARIUM ID database (Geiser et al., 2004). Based on BLAST analysis, 69 strains
42
43 240 were identified as *F. ramigenum*, 5 strains as *F. proliferatum* and 3 strains (ITEM 2913, ITEM
44
45
46 241 2921 and ITEM 2922) did not show significant homology with any species sequences deposited at
47
48 242 the FUSARIUM ID database. Sequences obtained were compared against the NCBI database
49
50
51 243 sequences and did not show any significant homology. TEF gene sequences were also subjected to
52
53 244 phylogenetic analysis. For this analysis, sequences of 13 selected strains were loaded from
54
55 245 FUSARIUM ID and NCBI databases to represent 13 different species: *F. ramigenum*, *F. napiforme*,
56
57
58 246 *F. lactis*, *F. brevicatenuatum*, *F. udum*, *F. phyllophilum*, *F. xylarioides*, *F. subglutinans*, *F.*
59
60 247 *concentricum*, *F. fratiflexum*, *F. proliferatum*, *F. verticillioides* and *F. solani* were used. The
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optimal tree of TEF sequences with the sum of branch length of 0.515 is shown in Figure 1.

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3 249 Bootstrap analysis showed high values for both the *F. ramigenum* and *F. proliferatum* clusters as
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6 250 well as for the cluster composed by the three not identified strains ITEM 2913, ITEM 2921 and
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8 251 ITEM 2922.

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13 253 *Mycotoxin production and toxicity on brine shrimp larvae*

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15 254 All strains assigned to the *Liseola* section were subjected to mycotoxin analyses, while only 30 out
16
17 255 of 49 strains of *F. solani* were analyzed for the mycotoxin production. Results on the mycotoxin
18
19
20 256 production are shown in Table 1. FA was produced by 13 out of 30 strains of *F. solani* ranging from
21
22 257 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
23
24 258 mg/kg, and by all 5 strains of *F. proliferatum* (range, 0.1-4 mg/kg); BEA was produced by both *F.*
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26
27 259 *ramigenum* (28 strains out of 69 strains with a range from 2 mg/kg to 190 mg/kg) and *F.*
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29 260 *proliferatum* (two strains out of 5, producing 10 mg/kg and 205 mg/kg, respectively); FB₁ was
30
31 261 produced by 41 strains out of 69 strains of *F. ramigenum* ranging from 0.1 mg/kg to 1,080 mg/kg of
32
33
34 262 FB₁ and 1 strain of *F. proliferatum* (1,100 mg/kg); FB₂ was produced by *F. ramigenum* (44 out of
35
36 263 69 strains and a range of production from 0.1 mg/kg to 655 mg/kg of FB₂) and 1 strain of *F.*
37
38
39 264 *proliferatum* (470 mg/kg); FUP was produced only by two strains of *F. ramigenum* (155 mg/kg and
40
41 265 345 mg/kg, respectively) and 1 strain of *F. proliferatum* (820 mg/kg). Finally, among the three
42
43 266 unidentified strains, two strains produced BEA (from 20 to 40 mg/kg), two strains produced FA
44
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46 267 (0.1 mg/kg for both strains), and all strains do not produce FB₁, while they produced FB₂ (0.1
47
48 268 mg/kg for two strains and 20 mg/kg for one strain). The toxicity of the fungal cultural extracts
49
50
51 269 (Table 1) showed that within *F. ramigenun*, 32 out of 69 strains were not toxic at all on the brine
52
53 270 shrimp larvae assay (0% larvae mortality), while 5 strains out of 69 strains resulted highly toxic at a
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55 271 value of 100%. For the remaining 32 strains, the range of larvae mortality was between 6% and
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58 272 78%. Among the 5 strains of *F. proliferatum*, only the BEA producing strain ITEM 2802 showed
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60 273 the highest toxicity (100% larvae mortality), while 4 strains were non toxic. The *F. solani* strains
274 were mostly non toxic against the brine shrimp larvae since 20 strains did not show activity, 7

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275 strains showed a very low range of toxicity (from 6% to 27%), while only three strains proved to be
276 highly toxic (two strain showed 100% of toxicity against the larvae, and 1 strain was toxic at a
277 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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3 301 from fig in California and un-correctly classified as *F. moniliforme* (Subbarao and Michailides,
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6 302 1993). O'Donnell et al. (1998) re-evaluated other strains from Californian figs that were classified
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8 303 by Subbarao and Michailides (1993) as *F. moniliforme*, and identified them as *F. lactis*. *Fusarium*
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10 304 *lactis* and *F. ramigenum* are considered two specific *Fusarium* species of fig morphologically and
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13 305 genetically closely related (Leslie and Summerell, 2006; O'Donnell et al., 1998). Of these two
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15 306 species, we identified in our survey only *F. ramigenum*, while *F. lactis* never occurred on fig fruits.
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17 307 Phylogenetic analyses carried out by O'Donnell et al. (1998) revealed 46 species in the *G. fujikuroi*
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20 308 complex, 23 of them as new to science. Among the 46 species, the eleven species identified by
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22 309 biological species concept have been reported identical to the phylogenetic species which indicates
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25 310 that phylogenetic approach can provide the same information as biological approach and that other
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27 311 MPs need to be still identified. We carried out experiments to obtain the sexual stage of *F.*
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29 312 *ramigenum* by performing the fertility tests described by Leslie and Summerell (2006), but all
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32 313 crosses within the *F. ramigenum* population resulted negative. On the other hand, Munaut and van
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34 314 Hove (2004) reported preliminary data on the sexual stage of *F. ramigenum*, *G. ramigena*,
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36 315 described as a member of *G. fujikuroi* complex. The five *F. proliferatum* strains isolated from fig
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39 316 fruits in our survey were all fertile in crosses with the *G. intermedia* (syn. mating population D of
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41 317 *G. fujikuroi* complex) tester strain MATD-1 ITEM 3628, being all same mating type MATD-2.
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43 318 Based on this data, *F. proliferatum*, which is a heterothallic species, seems to have few possibilities
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46 319 to recombine in fig fields in Apulia, although the size of strains sampled is extremely limited.
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48 320 Among the 5 strains of *F. proliferatum*, only a single strain, ITEM 2829, was able to produce FB₁
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51 321 and FB₂. *Fusarium proliferatum* is considered, together with *F. verticillioides*, the major fumonisin
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53 322 producing species worldwide, and rarely its strains were reported as non-fumonisin producing
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55 323 strains (Desjardins, 2006). If the lack of fumonisin production can be a prevalent specific trait of the
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58 324 *F. proliferatum* population occurring on fig in Apulia, needs to be deeper investigated within a
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60 325 wider set of strains, in order to understand the existence of possible mechanisms that silence the
326 mycotoxin production in the interaction *F. proliferatum*/fig plant. With respect to fumonisin

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327 production, several strains of *F. ramigenum* here reported proved to produce FB₁ and FB₂ *in vitro* at
328 high levels, although a wide variability within the population was also recorded. This is in contrast
329 with a previous report of Fotso et al. (2002) that, examining the ex-type culture of *F. ramigenum*
330 ITEM 3569, revealed the lack of fumonisin production by this strain. Therefore, this is the first
331 report of FB₁ and FB₂ production by *F. ramigenum* and shows that this species may contribute to
332 the FB₁ contamination of dried figs, as recently reported for the first time in Turkey (Karbancioglu-
333 Güler and Heperkan, 2009). Indeed, the authors did not provide any information on the identity of
334 *Fusarium* species occurring on dried fig contaminated by FB₁, therefore a wider investigation in the
335 main areas where the fig is cultivated in the Mediterranean Basin is worthwhile in order to more
336 realistically assess the role that *F. ramigenum* could play as causal agent of fig endosepsis and
337 contamination of figs by FB₁. Indeed, little is known of the ecology or biology of this fungus or of
338 its ability to act as a plant pathogen. The pathogenicity test on fig fruits, performed with well
339 characterized strains of *F. ramigenum*, provided useful indications, since showed that several strains
340 of this species were aggressive on fig fruits, being their capability to colonize the fig fruits
341 comparable, if not slightly higher, to *F. proliferatum*, and *F. solani* strains. Besides FB₁ and FB₂,
342 we analyzed other toxins which are typically produced by members of *G. fujukuroi* species members
343 such as BEA, FA, and FUP. While FUP was produced only by two strains of *F. ramigenum*, we
344 show in this report for the first time that FA is produced by this species, even though its production
345 was highly variable and only two strains (ITEM 2824 and ITEM 2852) produced it at a high level
346 (220 and 525 mg/kg, respectively). On the other hand, BEA resulted commonly produced by *F.*
347 *ramigenum* (Table 1). This is the first report on BEA production by *F. ramigenum*. Previous reports
348 (Fotso et al., 2002; Moretti et al., 2007) have shown the lack of BEA production by *F. ramigenum*
349 ITEM 3570 (ex-type) and ITEM 3569, a further strain used by Nirenberg and O'Donnell (1998) for
350 firstly describing this species. Levels of production resulted variable in the population investigated,
351 with almost half of strains capable to produce this toxin also at a high value (28 strains out of 69
352 strains up to 190 mg/kg). These results show that also BEA could represent a contaminant to control

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3 353 in the risk management of fig in Italy. Beauvericin can induce apoptosis on mammalian cell lines
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6 354 (Logrieco et al., 2002). Moreover, BEA showed negative effects on heart rate and contraction
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8 355 strength in guinea pig hearts (Lemmens-Gruber et al., 2000). Thus, it may be involved in human
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10 356 and animal pathology, representing a serious risk for health when occurring as a contaminant in
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13 357 dried fig. Finally, the eventual simultaneous production of BEA and FB₁ and FB₂, assessed for
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15 358 some of *F. ramigenum* strains, is very important since the co-occurrence of different toxic
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18 359 metabolites could imply additive and/or synergistic effects on target organisms (Muller and
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20 360 Schwadorf, 1993). Most of strains included in this study showed a level of toxin production that
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22 361 was related also to their ability to be toxic toward brine shrimp larvae. This bioassay has been used
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25 362 to identify *Fusarium* toxins as the major toxic component in an organic extract from cultures of
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27 363 *Fusarium* species (Moretti et 1994; Ritieni et al., 1995). Beauvericin was shown to be toxic in the
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29 364 brine shrimp bioassay and was correlated significantly, with some exceptions, to the larvae
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32 365 mortality recorded in the bioassay shrimp performed (Moretti et al., 2007). In this study, some
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34 366 strains, ITEM 2812, ITEM 2928, and ITEM 2932, were highly toxic in the brine shrimp bioassay
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36 367 although any known toxin was detected in their culture extracts. This shows that new toxic
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39 368 metabolites need to be further characterized within these species and also within this complex as
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41 369 suggested by Moretti et al. (2007). Finally, ITEM 2913, ITEM 2921 and ITEM 2922, could not be
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44 370 assigned to any species by comparing sequences of TEF. Further phylogenetic investigations will be
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46 371 needed to better characterize such strains in order to understand if they belong to species not yet
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48 372 described occurring on fig plants. In conclusions we showed that in Apulia, there is a high risk of
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51 373 fig contamination by *Fusarium* species and that the main species associated with fig endosepsis are
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53 374 *F. ramigenum* and *F. solani*, which include highly virulent strains. High variability in toxin
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55 375 production was observed in *F. ramigenum* population. However, the capability of many strains to
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58 376 produce both FB₁ and FB₂, make it a good candidate as the main responsible for the contamination
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60 377 of FB₁ of dried fig recently reported.

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Table 1. Production of beauvericin, fusaric acid, fumonisin B₁ and fumonisin B₂ 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 <i>Fusarium</i> 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 (%)
<i>F. ramigenum</i>					
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

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
<i>F. proliferatum</i>					
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 B₁

^f Fumonisin B₂

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
<i>F. ramigenum</i>		
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
<i>F. solani</i>		
2898	10± 3	251±61
2899	42±10	128±28
2900	82±12	254±37
2911	176±27	246±47
<i>F. proliferatum</i>		
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.

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)	<i>F. ramigenum</i>		<i>F. solani</i>		<i>F. proliferatum</i>	
	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 (10⁶ 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 (10⁶ conidia/ml) on a wound made in the cavity of each half fruit (internal lesions).

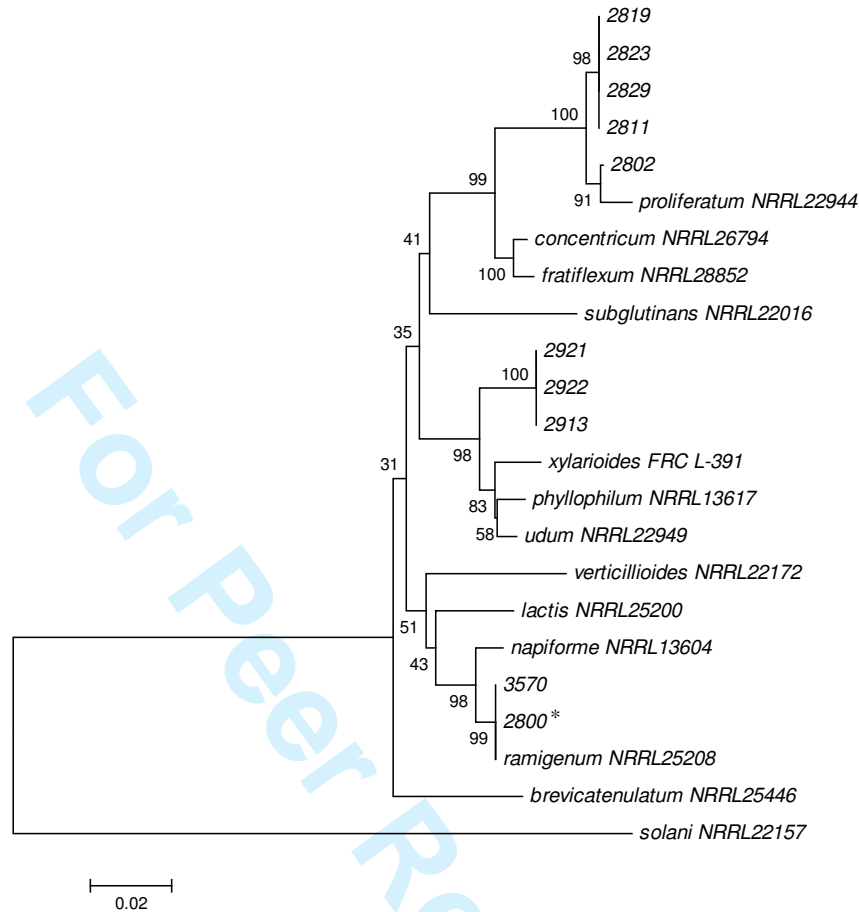


Figure 1 Phylogenetic tree generated by Neighbor-Joining method (Saitou et al., 1987) on translation elongation factor sequences of 77 strains of *Fusarium* and 13 sequences representative for related species from GenBank accessions. Branch lengths are in the same units of the evolutionary distances 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.