Genetic, pathogenic and toxigenic variability by *F. proliferatum* isolated from maize kernels

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Genetic (mating population and vegetative compatibility), pathogenic and toxigenic variability of *F. proliferatum* isolated from maize kernels originating from different localities in Serbia were studied. Investigated isolates of *F. proliferatum* were fertile and most of them (8/13) belonged to the mating type MATD-2. Based on the complementary test isolates, *F. proliferatum* were grouped into 11 vegetative compatibility groups (VCGs) and two isolates were self-incompatible. All tested field isolates of *F. proliferatum* and nit mutants were pathogenic and most expressed medium pathogenicity. Field isolates of *F. proliferatum* showed a higher potential for biosynthesis of FB₁ (up to 1,246.00 µg g⁻¹) in relation to their nit1 (up to 53.41 µg g⁻¹) and NitM mutants (up to 56.17 µg g⁻¹). Based on genetic, pathogenic and toxigenic properties of 13 isolates of *F. proliferatum*, relatively high variability of this fungus originated from maize grain was found.

**Key words:** *Fusarium proliferatum*, mating type, vegetative compatibility, pathogenicity, fumonisin B1.

INTRODUCTION

The fungus *Fusarium proliferatum* (Matsushima) Nirenberg occurs worldwide on a variety of plant hosts and along with *F. graminearum* Schw., *F. verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* Sheld.) and *F. subglutinans* (Wollen. and Rein.) Nelson, Toussoun and Marasas, it is one of the most important fungus associated with maize causing kernel rot, an important maize disease in temperate climates (Nelson et al., 1983). Maize samples were found to be infected by *F. proliferatum* from 1% in Meksiko (Gallardo-Reyes et al., 2006) to 70.0% in India (Hossain et al., 2002). In Croatia, the most frequent fusaria found in maize were *F. verticillioides*, *F. subglutinans* and *F. proliferatum*. *F. proliferatum* was found in almost all the analysed maize samples and *F. verticillioides* in 8 out of 15 samples, which is in agreement with the high frequency of FB₁ positive samples. Depending on the sample, the incidence of *F. proliferatum* varied from 2 to 40% (Domijan et al., 2005). In Serbia also, after *F. verticillioides*, *F. subglutinans* and *F. graminearum*, *F. proliferatum* is the most important pathogen of maize (Lević et al., 1997).

*F. proliferatum* belongs to *Liseola* section of the *Fusarium* genus (Nelson et al., 1983) and its teleomorph, *Gibberella intermedia* (Kuhlman) Samuels, Nirenberg and Seifert, belongs to the *G. fujikuroi* (Sawada) S. Ito complex, composed of at least nine reproductively isolated biological species (mating populations [MPs]), denoted by letters A to I (Leslie et al., 2004). Although *F. proliferatum* is one of the most common fusaria on maize worldwide (Reyes et al., 2006), it is still poorly known and characterized. 

Abbreviations: MATD-1, Mating population D, mating type 1; MATD-2, mating population D, mating type 2; VCGs, vegetative compatibility groups; FBs, fumonisins; FB₁, fumonisin B₁; PDA, potato dextrose agar; CLA, carnation leaf agar; ELISA, enzyme-linked immunosorbent assay.
proliferatum has a teleomorph, G. fujikuroi, the perithecia are rarely found in nature. The perithecia can readily be produced in the laboratory by pairing with female fertile tester strains that belong to the "D" mating group of G. fujikuroi (Leslie, 1995). Genetic variations within each mating population have been investigated using several mycological characters such as a distribution of a mating type (Leslie, 1991), frequency of hermaphrodite (Leslie and Klein, 1996), and vegetative compatibility (Leslie, 1993).

F. proliferatum is a toxigenic species, producing a broad range of toxins, such as fumonisin B1, moniliformin, beauvericin, fusaproliferin, fusarin and others (Logrieco et al., 1995; Miller et al., 1995; Ritieni et al., 1995; Fandohan et al., 2003, Lević et al., 2004). Maize grains infected by F. proliferatum are primarily contaminated with fumonisins (FBs) that may have cancer-promoting activities in humans. Oesophageal cancer (Marasas et al., 1981), leukoencephalomalacia in horses, as well as pulmonary edema in swine are fatal diseases of humans and animals caused by FBs-contaminated food and feed (Thiel et al., 1991). The elevated fumonisin level in maize has become an area of concern since ingestion of fumonisin-contaminated maize associated with livestock loss and human health risks have occurred (Wang et al., 2008). The relatively high intake of raw materials with the diet of livestock can lead to nutrient losses and have adverse effects on animal health and on productivity (Biagi, 2009).

Knowledge about the genetic variation of important fitness traits (like fertility and aggressiveness) of Fusarium spp. in maize is important for resistance, estimating the evolutionary risk of the pathogen, and for planning the agricultural management practices (Leslie, 1991). In order to determine the variation of F. proliferatum the following was studied: (i) the fertility of isolates of F. proliferatum and assigning their classification into a specific mating types; (ii) distribution of isolates over different VCGs; (iii) pathogenicity to maize seed under laboratory conditions; and (iv) the taxo-

logical profile of the isolates.

MATERIALS AND METHODS

Fungal isolates

Isolates of F. proliferatum were obtained from maize kernels from different localities in Serbia. One hundred maize kernels from each sample (ten ears), were collected during harvest in 2006 and 2007 and after disinfection in 1% sodium hypochlorite (NaOCl) for 5 min were directly placed on Petri dishes (five kernels per plate) containing potato dextrose agar (PDA) and incubated in the dark at 25°C for one week. The identification of the Fusarium species was made according to the taxonomic system developed by Burgess et al. (1994) and Leslie and Summerell (2006). Single conidium cultures on PDA and on carnation leaf agar (CLA), according to

Burgess et al. (1994) and Leslie and Summerell (2006), were used for further morphological observations, vegetative compatibility and pathogenicity studies. For examining mating populations, vegetative compatibility groups, pathogenicity and production of fumonisin B1, 13 field isolates of F. proliferatum were selected. The pathogenicity test and analysis of fumonisin included nit mutants, nit1 and NitM of field isolates.

Crosses

Sexual crosses were made on carrot agar as previously described by Klittich and Leslie (1988). Briefly, testers (3628 MATD-1 and 3627 MATD-2) or the male strains were grown on sloping CLA in glass test tubes for seven days at 25°C under alternating 12 h of combined light (fluorescent and near ultraviolet light) and dark, while the field isolates or the female strain were grown on Petri dishes on a carrot medium at 20°C in the dark. After crossing, 1 ml of the tester suspension was carefully displaced through the culture of field isolates. The plates were incubated for 4-6 weeks under the same growing conditions, used for the growth of testers. The presence of perithecia and mature perithecia with ascospore mass extruded from the ostioles were examined weekly. In all of the successful crosses with the female strain standard tester the unknown field isolates were classified as the male strain. Tests for female fertility of the unknown isolates were made by reversing the roles of the two strains in the cross. A cross was scored as infertile if mature perithecia were not formed after two attempts. The effective population size \( N_{e} \) was calculated by using the equations of Leslie and Klein (1996).

Vegetative compatibility tests

Nitrate non-utilizing mutants (nit mutants) were generated on minimal agar with chlorate according to the method of Correll et al. (1987). The physiological genotypes of nit mutants recovered from the representative isolates were interpreted on the basis of their growth on media containing different nitrogen sources. Complement-
transformed into arcsin using Microsoft Office Excel 2007 according to the equation $\text{ASIN} \left( \sqrt{\frac{X}{100}} \right) \times \frac{180}{\pi}$, where $X = \text{average value of germination expressed as a percentage}$, and $\pi \approx 3.14159$. Seeds used in these studies were obtained from a seed lot of maize hybrids whose germination amounted to 97%.

### Potential for fumonisin B<sub>1</sub> production

Thirteen isolates each of field isolates, nit1 and Ntt1 mutants, or a total of 39 isolates of *F. proliferatum* were used for the analysis of fumonisin B<sub>1</sub> (FB<sub>1</sub>) by ELISA (Enzyme linked immunosorbent assay). Investigated isolates of *F. proliferatum* were grown on 50 g maize kernels in 250 ml Erlenmeyer flasks. Maize kernels, with previously increased humidity to about 40%, were autoclaved. Kernels were inoculated with three fragments (5 x 5 mm) of the *F. proliferatum* colony, previously developed on PDA. Cultures were incubated at 27°C for three weeks. Harvested cultures were dried in a fan oven at 50°C for 72 h, finely ground, and stored at 4°C. Control kernels were treated the same way, except that they were not inoculated.

Furthermore, 5 g of ground sample were mixed with 25 ml of methanol and 1 g NaCl. This sample was mixed in a blender for 3 min and then was strained through a Whatman filter paper No. 1, and the filtrate was collected. The dilution of the filtrate was carried out based on the concentration of toxin in the sample and the ELISA procedure was performed by following the manufacturer’s recommendations (Tecna S. r. I., Trieste, Italy). Absorbance was determined using the spectrophotometer Elisa reader BioTek EL x 800™ (Absorbance Microplate Reader) at 450 nm.

### RESULTS AND DISCUSSION

According to the Republic Hydrometeorological Service of Serbia in 2006 and 2007, the moderate warm (daily temperatures of around 25°C) and mostly dry weather with infrequent precipitations during the reproductive period of maize were recorded. These climatic conditions were convenient for developing *Fusarium* species on maize. The optimal conditions for *F. verticillioides* and *F. proliferatum* maize ear rot tend to be hot and dry (Doohan et al., 2003). According to Miller (2001), the incidence of *Fusarium* kernel rot (*F. verticillioides* and *F. proliferatum*) is higher in warmer climates under dry conditions. In such environments, insect damage is well recognised as a collateral factor.

### Crosses

Both MATD-1 and MATD-2 individuals were identified among 13 isolates of *F. proliferatum* collected from maize kernels from different locations. Five, that is eight isolates out of the isolates selected for the pathogenicity test, were MATD-1 and MATD-2, respectively (ratio 1: 2.46), according to the ratio found in the whole population tested (ratio 1:2.60) (Table 1). This ratio reduced the effective population number $N_{e_{in}}$ to 94.69%. Moreover, among 13 fertile isolates, two isolates (1574 and 1609) were hermaphrodites and 11 isolates were female sterile, giving an $N_{e_{in}}$ of 2.67% of the count (total population). One out of the two hermaphrodites belonged to the mating type MATD-1 and the other to MATD-2.

Also, the report of Stankovic et al. (2007) showed that MATD-2 was predominant over the MATD-1 in this
population (ratio 2.6:1) with the similar estimated ratio in population of *F. proliferatum* isolated from onion in Serbia. However, this ratio reduced the effective population number and could be a consequence of the lack of female fertility that was detected among the analysed isolates. These results suggest that the mating type MATD-2 dominates on maize kernels in Serbia. On the other hand in Iran, isolates from maize mainly belonged to MATD-1, while the isolates from rice roots belonged to MATD-2 (Alizadeh et al., 2010). According to these authors, isolates originating from the roots of sugarcane were equally represented with MATD-1 and MATD-2, while all isolates from the roots of onion belonged to MATD-1.

The lowest $N_{e(0)}$ values were recorded in isolates belonging to the mating population D, suggesting that sexual reproduction was relatively infrequent, but that there was potential for sexual reproduction within the Serbian population D of *G. fujikuroi* and thus an expansion of the genetic diversity by recombination. Sexual recombination probably occurs often enough to ensure that most populations usually will appear to be randomly mating. The effective population size is affected much more severely by a decrease in the number of female-fertile strains than it is by any known inequalities in the relative numbers of strains carrying the different mating-type idiomorphs (Leslie and Klein, 1996).

<table>
<thead>
<tr>
<th>Collection number</th>
<th>Seed germination$^a$</th>
<th>Fumonisin B$_1$ (μg g$^{-1}$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Field isolates</td>
<td>NitM</td>
</tr>
<tr>
<td>1275</td>
<td>47.1$^{a}$</td>
<td>59.5$^{bc}$</td>
</tr>
<tr>
<td>1598</td>
<td>65.3$^d$</td>
<td>62.3$^{bc}$</td>
</tr>
<tr>
<td>1574</td>
<td>70.3$^a$</td>
<td>68.6$^{bc}$</td>
</tr>
<tr>
<td>1345</td>
<td>84.2$^b$</td>
<td>67.7$^c$</td>
</tr>
<tr>
<td>1555</td>
<td>70.3$^a$</td>
<td>62.3$^{bc}$</td>
</tr>
<tr>
<td>1584</td>
<td>70.3$^a$</td>
<td>57.5$^{ab}$</td>
</tr>
<tr>
<td>1616</td>
<td>74.4$^d$</td>
<td>60.2$^c$</td>
</tr>
<tr>
<td>1609</td>
<td>84.2$^b$</td>
<td>59.5$^{bc}$</td>
</tr>
<tr>
<td>1374</td>
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</tr>
<tr>
<td>1322</td>
<td>78.3$^c$</td>
<td>53.7$^c$</td>
</tr>
<tr>
<td>1653</td>
<td>84.2$^b$</td>
<td>60.9$^{ab}$</td>
</tr>
<tr>
<td>Average</td>
<td>72.2</td>
<td>61.7</td>
</tr>
</tbody>
</table>

$^a$Data transformed into arcsin. Mean values followed by the same letters are not significantly different (P > 0.05) according to Duncan’s multiple range test. $^b$Isolates grown on autoclaved maize kernels in the dark at 25°C for four weeks.

### Vegetative compatibility

Thirteen isolates of *F. proliferatum* were examined for their ability to sector spontaneously on the toxic chlorate medium. Moreover, 69% of over 155 colonies examined formed chlorate-resistant mutant sectors. After the growth of isolates on media that contained one of three nitrogen sources, the nit mutants were divided into three phenotypic classes. The nit mutants occurred in seven loci: a structural gene for nitrate reductase (*nit1*), a regulatory gene specific for the nitrate reduction pathway (*nit3*), and five genes (*nit2*, *nit4*, *nit5*, *nit6* and *nit7*) controlling the production of a molybdenum-containing cofactor that is necessary for nitrate reductase activity (NitM). Mutations of *nit1* were recovered most frequently (11.1 to 90%, depending on the strain) followed by NitM (10 to 50%) and *nit3* mutations (0 to 13.3%). Based on the inability of pairings between *nit1* and NitM, mutants of isolates 1345 and 1598 on the minimum medium revealed that these isolates were self-incompatible. These isolates belonged to the mating types MATD-1 (isolate 1345) and MATD-2 (isolate 1598). Among the 13 isolates, 11 VCGs were identified on the basis of positive complementation reactions of *nit1* and NitM mutants. The distribution of *F. proliferatum* isolates obtained from maize from various locations into different VCGs is reported for the first time in Serbia. We identified 11
VCGs among 13 isolates with a genetic diversity number of VCGs (number of VCGs/number of isolates) of 0.86. Alizadehl et al. (2010) identified 10 VCGs of 20 F. proliferatum isolates collected from maize in different regions of Iran. Nine isolates belonged to VCG 1, two isolates in each to VCG 3 and VCG 4, while seven isolates belonged to different VCGs (VCG 2, VCG 8, VCG 9, VCG 10, VCG 11, VCG 12 and VCG13). Some isolates of the fungus, which were isolated from the roots of rice and sugar cane, were classified into VCG 1 and VCG 2, while all isolated originated from onion were classified into VCG 1. The same authors concluded that natural populations of F. proliferatum in Iran were probably genetically divergent and included isolates representing a potential risk for disease development. Elmer (1991) and Elmer et al. (1999) collected 110 and 77 isolates of F. proliferatum from asparagus originating from different locations in the USA and seven regions in Australia, respectively, and they grouped them into 20 VCGs. Lim et al. (2001) also paired the six isolates from the mating population D originating from sorghum in Korea and received three different VCGs.

**Pathogenicity**

The study of F. proliferatum pathogenicity, both for field isolates and nit mutants, revealed that all isolates affected seed germination (Table 2), but effects of some of field isolates were stronger than effects of their nit mutants. On the average, field isolates had a lower effect on seed germination (72.2%) compared to nit1 (61.7%) and NitM mutants (58.9%). Germination of inoculated seeds, with a spore suspension of field isolates, nit1 and NitM mutants ranged from 47.1 to 84.3%, 53.7 to 68.6% and from 51.9 to 64.5%, respectively. Only two out of 13 field isolates (1275 and 1374) significantly affected seed germination (47.1 and 50.7%) compared to the other field isolates or their nit mutants. The greatest effect on seed germination was expressed by nit1 mutants isolates 1322 and 1584 (53.7 and 57.5%, respectively) and by NitM isolates 1653 (51.9%) and 1584 and 1248 (57.5%).

All 13 field isolates of F. proliferatum exhibited lower pathogenicity under laboratory conditions compared to nit mutants (13 nit1 and 13 NitM). The results achieved by Pascale et al. (2002) showed F. proliferatum to be highly pathogenic to maize under field conditions. On the basis of the effect on seed germination of maize field isolates, they can be classified into six groups, while 11 VCGs among 13 isolates were identified by the complementation test. Discrepancies between the results of pathogenicity and the complementation test are likely a result of the methodology applied in studying pathogenicity. Elmer (1991) also found no correlation between VCG and virulence of isolates of F. proliferatum.

**Toxigenic potential for fumonisin B1**

The analysis of FB1 showed that field isolates of F. proliferatum, except the isolate 1322, had showed a higher production of this mycotoxin than their isolates of nit mutants. Field isolates biosynthesised FB1 at a concentration ranging from 2.37 to 1.246 µg g⁻¹ with an average of 206.64 µg g⁻¹, while their nit1 and NitM mutants produced 2.77 to 53.41 (average 27.27 µg g⁻¹) and 3.75 to 56.17 µg g⁻¹ (average 28.08 µg g⁻¹), respectively. On the average, the field isolates biosynthesised 7.6 times more FB1 than their nit mutants. Typically, isolates belonging to the mating type MATD-1 produced higher concentrations of the mycotoxin FB1 than isolates of the mating type MATD-2. In relation to this, there were no differences between field isolates and their nit mutants. Only one field isolate (1345) produced 1,246.00 µg g⁻¹ FB1, while the remaining field isolates, nit1 and NitM mutants, produced FB1 to 219.50 µg g⁻¹, 53.41 µg g⁻¹ and 56.17 µg g⁻¹, respectively. Ross et al. (1990) identified isolates of F. proliferatum that biosynthesised FB1 in the range from 1.670 to 2.790 µg g⁻¹, while Nelson et al. (1992) found that strains of F. proliferatum (19 of 31.61%) produced this mycotoxin in amounts ranging from 155 to 2.936 µg g⁻¹, similar to our results for field isolates. On the average, isolates of the mating type MATD-1 produced a higher concentration of FB1 in relation to the isolates of the mating type MATD-2. Similar results were also obtained from Leslie et al. (2004).

However, contrary to the results of pathogenicity tests, field isolates produced higher concentrations of FB1 (in average 206.64 µg g⁻¹) in relation to nit1 (27.27 µg g⁻¹) and NitM mutants (28.01 µg g⁻¹). A positive correlation between production of FB1 and the effect on seed germination was found only for nit1 mutants (r = 0.41 significant at P0.10). Desjardins and Hohn (1997) working on the preliminary genetic analysis of G. fujikuroi mating type A (F. verticillioides) indicated a possible link between the ability of a fumonisin production and virulence expressed in kernels of maize.

**Conclusion**

F. proliferatum showed relatively high variability based on comparison of results of mating types, vegetative compatibility groups, pathogenicity and the production of FB1. This can be explained by isolations of the pathogen from different locations in Serbia, as well as high genetic diversity within F. proliferatum species and its teleomorph G. intermedia. Genetic diversity of a fungal population is an important factor when developing a biocontrol strategy against that fungus. This is also necessary in order to develop management strategies in the prevention of...
mycotoxin contamination, as well as the selection of maize genotypes resistant to Fusarium kernel rot.

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REFERENCES


