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# Polyphasic Approach Used for Distinguishing *Fusarium temperatum* from *Fusarium subglutinans*

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## ABSTRACT

**Morphological, biological, and phylogenetic approaches were undertaken for the identification of pathogenic species *F. temperatum* in the Serbian population of *F. subglutinans* collected in the 1999-2010 period from *Zea mays* (3 root, 15 stalk, and 6 seed samples), *Sorghum bicolor* (two seed samples), *Hordeum vulgare* (one seed sample) and *Taraxacum officinale* (one seed sample). Based on interspecies mating compatibility analyses and the maximum parsimony analysis of *EF-1α* sequences, only two strains, originating from *S. bicolor* seed (MRIZP 0418 and MRIZP 0552), were identified as *F. temperatum*, while the remaining 26 single-spore strains were identified as *F. subglutinans* Group 2. *In situ* detached barley leaf assay and artificially stalk and ear inoculation of two maize hybrids demonstrated that both *F. temperatum* and *F. subglutinans* strains were medium and strong pathogens under laboratory and field conditions, respectively. These are the first data on the *F. temperatum* as seed-borne pathogens of sorghum, as well as pathogenicity of *F. temperatum* strains on maize.**

**Keywords:** Maize, Maximum parsimony analysis, Polyphasic approach of identification, Sorghum seed.

## INTRODUCTION

*F,usarium temperatum* Scauflaire and Munaut is a new species morphologically similar and phylogenetically closely related to *F. subglutinans* (Wollen. & Reinking) P. E. Nelson. Toussoun & Marasas, *F. circinatum* Nirenberg & O'Donnell and *F. konzum* Zeller, Summurell, Block & Leslie (Scauflaire *et al*., 2011a). At first, Steenkamp *et al*. (2002) revealed the presence of two major groups (Group 1 and Group 2) representing cryptic species in *F. subglutinans*. In addition, results obtained by Scauflaire *et al*. (2011 a) strongly suggested that *F. subglutinans* Group 1 and *F. temperatum* represent highly similar evolutionary entities, if not the same thing. On the other hand, *F. subglutinans* strictly circumscribes the second group, namely, *F. subglutinans* Group 2 (Scauflaire *et al*., 2012 b).

*F. temperatum* was first detected in Belgium (Scauflaire *et al*., 2011b) and then in Australia, Germany, Italy, Netherlands, Poland, Slovakia, South Africa, Turkey, USA (Susca *et al*., 2013, https://colloque6.inra.fr/efs2013/Media/Fichier/EFS12-Book-of-abstrcts/), Spain (Pintos *et al*., 2013) and China (Wang *et al*., 2014) as a pathogen of maize, and in the France (Pinson-Gadais *et al*., 2013, https://colloque6.inra.fr/efs2013/Media/Fichier/EFS12-Book-of-abstrcts/). This species has not been identified so far in Serbia.

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A total of 325 pure cultures of *Fusarium* strains morphologically identified as *F. subglutinans*, mostly originating from maize, since it is the most important pathogen of maize in Serbia, are stored in the fungus stock cultures (MRIZP) at the Maize Research Institute, Zemun Polje (Lević *et al*., 2012; Lević, 2008). The objective of this study was to detect *F. temperatum* within the Serbian population of *F. subglutinans sensu lato*, originating from different plant species, applying a polyphasic approach involving a morphological, biological, and phylogenetic approach.

## MATERIALS AND METHODS

### Fungal Strains and Culture Conditions

Single-spore cultures were selected out of 28 strains of *F. subglutinans* from the MRIZP Culture Collection, originated from various hosts and plant parts (Table 1), and collected in the 1997-1999 period. *F. subglutinans* cultures were grown in 5-mL vials on Potato Dextrose Agar (PDA) slants, Carnation Leaf Agar (CLA) and Synthetic Nutrient-poor Agar (SNA) and maintained at 4ºC. The first two media were prepared according to Burgess *et al*. (1994), and the third one after Nirenberg (1976). Additional two single-spore cultures of Belgian tester strains of *F. temperatum* designated as MUCL 52438 (*MAT1-2*) and MUCL 52463 (*MAT1-1*) were used (Scauflaire *et al*, 2012a).

### Identification of Strains

### Molecular Identification and Phylogeny

The molecular identification was performed as previously described by Scauflaire *et al*. (2011a). Fungal DNA was extracted and purified using the Invisorb Spin Plant MiniKit (Invitek GmbH, Germany) according to the manufacturer's recommendations. The amplification of the *Elongation Factor 1α* (EF-1α) gene was carried out by using the amplification conditions described by O'Donnell *et al*. (1998). The PCR products were sequenced using both the forward and reverse primers in a 3,100 Genetic Analyser (Applied Biosystems, USA). Sequences were edited with the Version 4.8 of the Sequencher Software (Gene Codes Corporation, USA) and the most related sequence was obtained using the GenBank Blast (NCBI-National Centre for Biotechnology Information). For the phylogenetic analysis of the *EF-1α* region, 28 sequences were obtained from the strains used in this study and eight sequences were retrieved from GenBank (*F. subglutinans* NRRL 22016, AF160289; *F. temperatum* MUCL 52436, HM067684; *F. konzum* MRC 8544, EU220235; *F. anthophilum* NRRL 13602, AF160292; *F. guttiforme* NRRL 22945, AF160297; *F. verticillioides* NRRL 22172, AF160262; *F. proliferatum* NRRL 22944, AF160280; and *F. oxysporum* NRRL 22902, AF160312). The final dataset consisted of 13 sequences after selecting representatives of haplotypes from the sequence comparison. Maximum Parsimony (MP) analyses were conducted using PAUP* v4.0b10 (Swofford, 2000), with the heuristic search option with 1,000 random sequence additions, tree-bisection-reconnection branch swapping and MulTrees effective. The remaining indels present in datasets were treated as fifth character states (NewState). Tree topology was supported by analyzing 1,000 parsimony bootstrap replications. The Consistency Index (CI) and the Retention Index (RI) were calculated to obtain the amount of homoplasy in the dataset.

### Mating Studies

The Mating Population (MP) technique (Klittich and Leslie, 1988) as modified by Kovačević *et al*. (2013) was used for the biological identification of the selected strains to the *F. temperatum* mating





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**Table 1.** Frequency, median and the average number of septa of sporodochial macroconidia of studied *Fusarium* strains.

| Strain code number[a] | Strain origin[b] | Number of septa Frequency (%) | | | | Median[c] | Mean±*SD* |
|---|---|---|---|---|---|---|---|
| | | 3 | 4 | 5 | 6 | | |
| *Fusarium subglutinans* | | | | | | | |
| MRIZP 0043 | *Zm*-sp | 54.7 | 44.0 | 1.3 | | 3 | 3.47 ± 0.53 |
| MRIZP 0555 | *Zm*-sp | 39.3 | 48.7 | 12.0 | | 4 | 3.73 ± 0.66 |
| MRIZP 0556 | *Zm*-sn | 41.3 | 52.0 | 6.7 | | 4 | 3.65 ± 0.60 |
| MRIZP 0559 | *Zm*-s | 48.7 | 39.3 | 12.0 | | 4 | 3.63 ± 0.69 |
| MRIZP 0560 | *Zm*-s | 32.7 | 51.3 | 16.0 | | 4 | 3.83 ± 0.68 |
| MRIZP 0706[d] | *Zm*-r | 96.7 | 3.3 | | | 3 | 3.03 ± 0.18 |
| MRIZP 0707 | *Zm*-sn | 35.3 | 52.0 | 12.7 | | 4 | 3.77 ± 0.66 |
| MRIZP 0712 | *Zm*-sn | 25.3 | 54.7 | 20.0 | | 4 | 3.95 ± 0.67 |
| MRIZP 1025 | *Zm*-r | 28.0 | 55.3 | 15.3 | 1.4 | 4 | 3.90 ± 0.69 |
| MRIZP 1033 | *To*-s | 65.3 | 32.0 | 2.7 | | 3 | 3.38 ± 0.55 |
| MRIZP 2197 | *Hv*-s | 56.0 | 34.0 | 10.0 | | 3 | 3.54 ± 0.68 |
| MRIZP 2406 | *Zm*-s | 33.3 | 57.7 | 8.0 | | 4 | 3.75 ± 0.59 |
| MRIZP 2419 | *Zm*-s | 50.7 | 47.3 | 2.0 | | 3 | 3.51 ± 0.51 |
| MRIZP 2796 | *Zm*-sn | 25.3 | 62.0 | 12.0 | 0.7 | 4 | 3.89 ± 0.60 |
| MRIZP 2797 | *Zm*-sp | 96.7 | 3.3 | | | 3 | 3.03 ± 0.18 |
| MRIZP 2798 | *Zm*-sube | 48.0 | 42.0 | 10.0 | | 4 | 3.62 ± 0.66 |
| MRIZP 2800 | *Zm*-sn | 82.0 | 16.7 | 1.3 | | 3 | 3.19 ± 0.43 |
| MRIZP 2803 | *Zm*-s | 26.0 | 54.0 | 19.3 | 0.7 | 4 | 3.95 ± 0.69 |
| MRIZP 2804 | *Zm*-s | 92.6 | 6.7 | 0.7 | | 3 | 3.08 ± 0.30 |
| MRIZP 2807 | *Zm*-se | 90.6 | 8.7 | 0.7 | | 3 | 3.10 ± 0.32 |
| MRIZP 2808 | *Zm*-sn | 41.3 | 48.0 | 10.3 | | 4 | 3.69 ± 0.65 |
| MRIZP 2828 | *Zm*-sn | 64.7 | 31.3 | 4.0 | | 3 | 3.39 ±0.57 |
| MRIZP 2970 | *Zm*-sp | 73.3 | 25.3 | 1.4 | | 3 | 3.28 ± 0.48 |
| MRIZP 3016 | *Zm*-sp | 70.7 | 27.3 | 2.0 | | 3 | 3.31 ± 0.51 |
| MRIZP 3019 | *Zm*-sp | 44.7 | 50.0 | 5.3 | | 4 | 3.61 ± 0.59 |
| MRIZP 3037 | *Zm*-r | 73.3 | 26.0 | 0.7 | | 3 | 3.27 ± 0.46 |
| *Fusarium temperatum* | | | | | | | |
| MRIZP 0418 | *Sb*-s | 58.6 | 38.7 | 2.7 | | 3 | 3.44 ± 0.55 |
| MRIZP 0552 | *Sb*-s | 24.6 | 66.0 | 8.7 | 0.7 | 4 | 3.85 ± 0.58 |
| MUCL 52438 | *Zm*-s | 36.7 | 31.3 | 24.0 | 4.0 | 4 | 3.95 ± 0.88 |
| MUCL 52463 | *Zm*-sp | 32.0 | 48.7 | 17.3 | 2.3 | 4 | 3.89 ± 0.75 |

[a] MRIZP: Culture Collection of the Maize Research Institute, Zemun Polje, Belgrade, Serbia; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain-Neuve, Belgium.

[b] *Zm - Zea mays*, *Sb*: *Sorghum bicolour*, *To*: *Taraxacum officinale*, *Hv: Hordeum vulgare*, sp: Stalk parenchyma, s*:* Seed, sn: Stalk node, r: Root, sube: Stalk subepidermis, se: Stalk epidermis.

[c] Number in the middle of the set of given septa numbers.

[d] Strains that formed sparse sporodochia after the two-week incubation on CLA and under the light (combined fluorescence and near ultraviolet)/dark regime.

population of the *Fusarium fujikuroi* species complex. After two weeks, cultures of crossed testers and strains were examined at weekly intervals under the stereomicroscope to determine the fertility of progenies. Highly fertile (oozing of ascospores out of numerous perithecia), sparingly fertile (sparse perithecia with formed asci and ascospores), nearly sterile (formed perithecia with immature asci), nearly barren (formed protothecia), and completely barren (without protothecia or sclerotia) were the terms used to describe fertility of progenies (Perkins, 1994).





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### Morphological Studies

The color of colonies, type of conidiophores, conidia formation and number of conidial of the putative 28 *F. subglutinans* strains were studied according to the procedure described by Burgess *et al*. (1994). The number of septa was determined in 150 sporodochial macroconidia two weeks after single-spore cultures were incubated on CLA under a 12 hour light (cold fluorescent light and near ultraviolet light - NUV)/dark regime at 20$^{o}$C. In order to determine the number of septa, a suspension consisting of 3-5 sporodochia, was prepared in 1 mL of sterile water.

### Pathogenicity Tests

### In Vitro Detached Leaf Assay

Artificial inoculation of injured leaves of commercial barley cultivar was used to investigate potential pathogenicity of the 28 strains originating from Serbia and two tester strains of *F. temperatum* (Imathiu *et al*., 2009). A 4 cm long leaf segment (facing up) was injured in the centre of the adaxial surface using a sterile 5-µL micropipette tip. Ten µL of conidial suspension, adjusted to the $1.75\pm0.33\times10^{6}$ conidia $mL^{-1}$, were placed on each injury. A control leaf was injured as the inoculated leaf, but instead of the inoculum, 10 µL of sterile distilled water was used. Eight segments of inoculated or control leaves were placed in each of 14-cm Petri dish and incubated at 20ºC under 12 hours light (combination of cold fluorescent light and near ultraviolet light)/dark regime. Lengths of lesions in a form of necrotic areas on detached barley leaves were measured seven days after inoculation. Necrotic lesions were identified by placing the detached leaves under a lighted magnifying glass (Imathiu *et al*, 2009).

### Pathogenicity Test under Field Conditions

For pathogenicity test in field conditions, 20 out of 28 MRIZP strains were selected due to limited experimental field size. These strains were tested on stalks and ears of two maize hybrids, differing in maturity (medium late and late, FAO 500 and 700 maturity groups, respectively), under experimental field conditions of the Maize Research Institute, Zemun Polje, in 2011. Each inoculated stalk and ear and the control variant encompassed 80 plants per hybrid or 20 plants per replication.

Inoculation of maize stalks and ears was done by applying a modified toothpick method (Chambers, 1988) and spore suspension into the silk channel (Reid *et al*., 1996). The area of pathological change in the tissue of the inoculated internode was visually evaluated on the stalk longitudinal section on the 1-6 scale (1= Necrosis localised at the inoculation spot; 6= Necrosis spreads to other internodes) 6-7 weeks after stalk inoculation (Chambers, 1988). Disease severity rating of each individually inoculated ear was evaluated on the 1-7 scale (1= No symptoms; 7= 76-100% of infected kernels) (Reid *et al*., 1996).

### Statistical Analysis

A disease index score was calculated for each strain using the equation devised by Jenkinson and Parry (1994). The data were transformed to arcsine-transformed values and subjected to the Analysis Of Variance (ANOVA) and the sets were performed using the MSTAT-C for the Randomized Complete split-plot Block design (RCB). Duncan's multiple range test was performed to test the significance ($P< 0.01$) of differences between the strain pathogenicity using the detached leaf method under laboratory conditions and stalk and ear inoculation under field conditions. The interrelations of the strains aggressiveness under laboratory and field conditions were





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determined by the Pearson product–moment correlation test (P< 0.01).

## RESULTS

### Molecular Identification and Phylogeny

Two strains (MRIZP 0418 and MRIZP 0552) were identified as *F. temperatum* based on molecular data, while the 26 remaining presented 99-100% *EF-1α* sequence similarity to *F. subglutinans*. The two *F. temperatum* strains, MRIZP 0418 and MRIZP 0552, were of the same haplotype. Three different haplotypes found within the *F. subglutinans* clade and strains, i.e. MRIZP 1025 (= MRIZP 0043, MRIZP 0556, MRIZP 0555, MRIZP 2970, MRIZP 3016), MRIZP 1033 (= MRIZP 2807, MRIZP 0706, MRIZP 2797, MRIZP 2828, MRIZP 2796, MRIZP 2808, MRIZP 0712, MRIZP 0707, MRIZP 2800, MRIZP 2803, MRIZP 2804, MRIZP 0560, MRIZP 0559, MRIZP 2197, MRIZP 2406, MRIZP 2419, MRIZP 3019, MRIZP 3037) and MRIZP 2798, were selected as representatives for the phylogenetic analysis.

The final alignment of the *EF-1α* sequences generated a total of 632 characters, 94 of which were parsimony uninformative and 66 were parsimony informative. One of the two most parsimonious trees retained is shown (Figure 1). The tree length was 227; the CI and the RI for the trees generated were 0.903 and 0.836, respectively. All strains of *F. subglutinans* from this study formed a strongly monophyletic clade [Bootstrap (B)= 100], while strains of *F. temperatum* were placed in a distinct, well supported clade (B= 100). The overall tree topology was similar to those presented for the *Fusarium fujikuroi* species complex (Geiser *et al*., 2013).

### Mating Studies

Crossing of 28 MRIZP single-spore cultures of *Fusarium* strains identified as *F. subglutinans* to a standard tester *F. temperatum MAT1-2*, designated as MUCL 52438 in this manuscript, did not produce any fertile progeny (data are not shown). Contrary to this, these strains crossed to a standard tester *F. temperatum MAT1-1* (MUCL 52463) produced two fertile progenies, nine sparingly fertile, three nearly sterile, eight nearly barren, and six completely barren (Table 2).

**Table 2.** Fertility and infertility of Serbian *Fusarium* strains morphologically identified as *F. subglutinans* strains crossed to the standard tester of *F. temperatum MAT1-1* designated as MUCL 52463.

| | Progeny[a] | | | |
|---|---|---|---|---|
| HF | SF | NS | NB | CB |
| **MRIZP 0418** | MRIZP 0555 | MRIZP 0556 | MRIZP 0560 | MRIZP 0043 |
| **MRIZP 0552** | MRIZP 2197 | MRIZP 0559 | MRIZP 0706 | MRIZP 0707 |
| | MRIZP 2406 | MRIZP **1025** | MRIZP 0712 | MRIZP 2797 |
| | MRIZP 2419 | | MRIZP **1033** | MRIZP 2800 |
| | MRIZP 2796 | | MRIZP **2798** | MRIZP 2803 |
| | MRIZP 2970 | | MRIZP 2804 | MRIZP 2807 |
| | MRIZP 3016 | | MRIZP 2808 | |
| | MRIZP 3019 | | MRIZP 2828 | |
| | MRIZP 3037 | | | |

[a] HF: Highly Fertile; SF: Sparingly Fertile; NS: Nearly Sterile; NB: Nearly Barren; CB: Completely Barren, terms according to Perkins (1994). MUCL 52463 tester determined by Scauflaire *et al*. (2012a). Bold-marked are halotypes.





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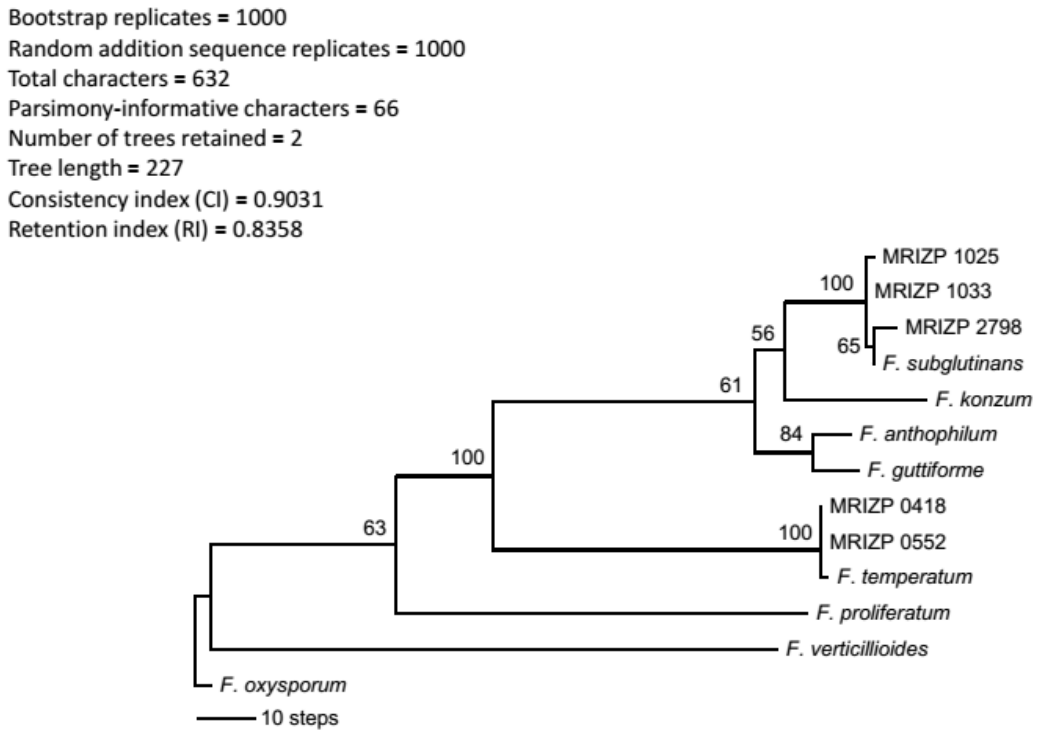


**Figure 1.** Phylogenetic tree based on the maximum parsimony analysis of *EF-1α* sequences of *Fusarium subglutinans*, *F. temperatum* and relatives from GenBank. Percent of 1,000 Bootstrap replications (B) are indicated at branch nodes and branches in boldface= B≥ 70%. The bar represents the substitutions expected per site. *Fusarium oxysporum* was used as an outgroup.

## Morphological Studies

Results obtained on the analysis of macroscopic traits of single-spore cultures of 28 *Fusarium* strains showed that some strains developed cream (MRIZP 2797), pale salmon (MRIZP 2798), cream to violet (MRIZP 2796, MRIZP 712) or violet to cream colonies (MRIZP 2808, MRIZP 2828 and MRIZP 556), while all remaining strains formed violet colonies on PDA at 25°C in the dark. Based on the colony appearance, the strain MRIZP 2798 was the most similar to the reference strains of *F. temperatum* designated as MUCL 52438 and MUCL 52463.

According to routine *in situ* analyses of pure single-spore cultures grown on CLA and native preparations of these cultures, it was determined that 28 strains of similar morphological properties belonged to *F. subglutinans*. All strains formed mono- and polyphialides and microconidia in false heads, while they did not form chlamydospores. Straight or falcate macroconidia with dorsal and ventral surfaces parallel, a constricted apical cell and a distinct foot cell were abundantly formed within orange sporodochia during the two-week growth on CLA and under near ultraviolet light. All strains had 3-5 septa, apart from four MRIZP strains (MRIZP 0552, MRIZP 1025, MRIZP 2796 and MRIZP 2803) and two *F. temperatum* testers, which had some macroconidia with six septa (Table 1). On average,





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macroconidia with four septa prevailed in the majority of strains.

### Strains Pathogenicity

All single-spore strains caused lesions on *in situ* detached barley leaves after inoculation at 20ºC under 12 hours near ultraviolet light - NUV. No disease developed on the control leaves treated with sterile distilled water. The majority of strains had a round to oval and dark necrotic ring around the lesion (e.g. MRIZP 1025 or MUCL 52438), while strains MRIZP 0418, MRIZP 0552, MRIZP 1033, MRIZP 2197, MRIZP 2970, MRIZP 3019, and MUCL 52463 caused elongated lesions (Figure 2).

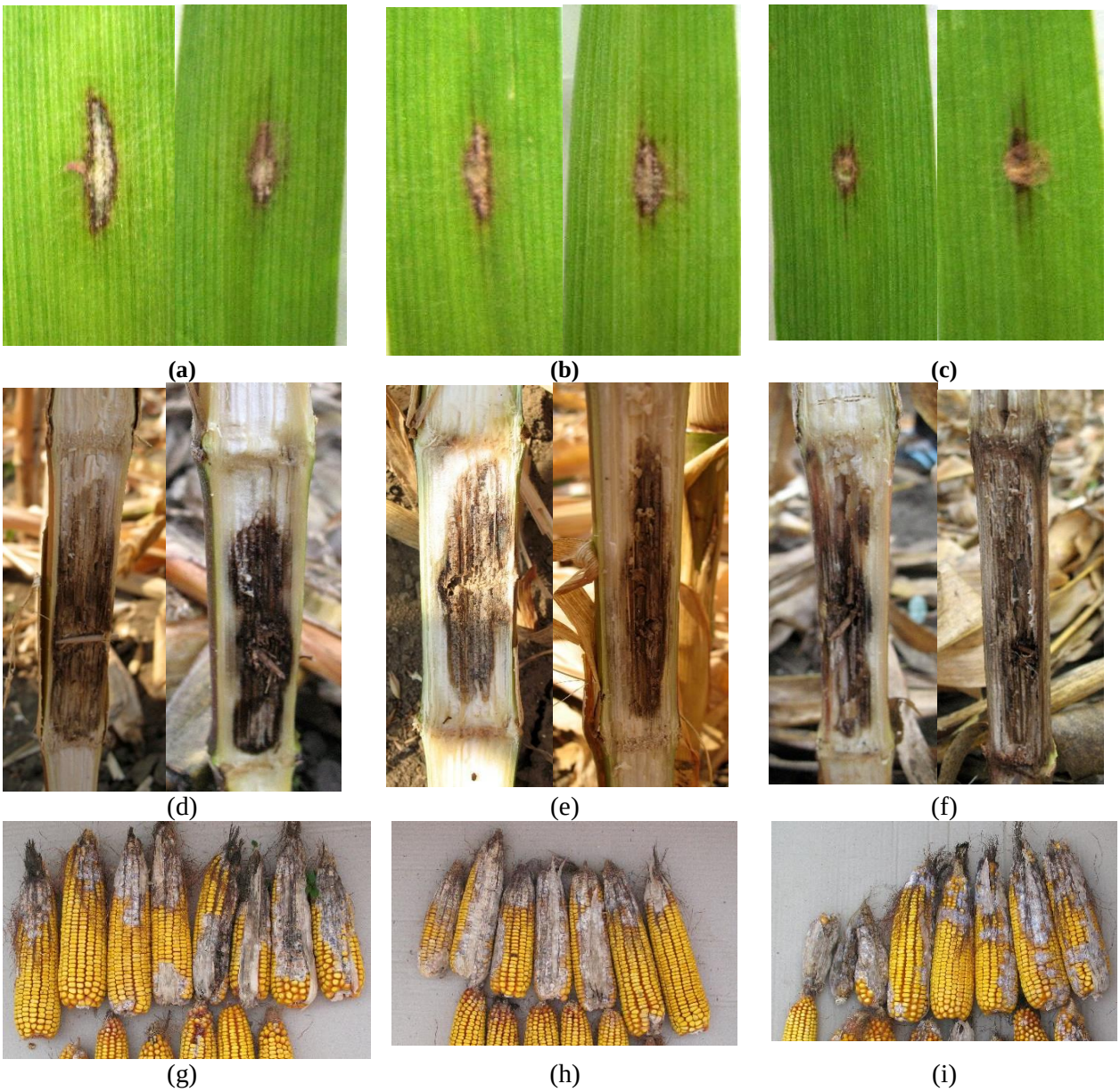


**Figure 2.** Lesion on detached barley leaves developed 7 days after inoculation at 25ºC with strains of *F. temperatum* (A – MRIZP 0418, B – MRIZP 0552) and *F subglutinans* (C – MRIZP 0556); stalk and ear after artificial inoculation with strains of *F. temperatum* (D,G – MRIZP 0418, E,H – MRIZP 0552) and *F subglutinans* (F,I – MRIZP 0556).





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Significant (P< 0.01) differences were obtained in aggressiveness of strains measured by the lesion length. The largest significant lesion length was determined seven days after inoculation for MRIZP 1033 in comparison to the other studied strains (Table 3). There was no significant difference between the average lesion lengths for *F. subglutinans* (5.7±1.2 mm) and *F. temperatum* (6.6±1.1 mm).

**Table 3.** Lesion length (mm) on detached barley leaves under laboratory conditions and the mean disease index for maize stalks and ears artificially inoculated with strains of *F. temperatum* and *F. subglutinans* under field conditions.

| Strain code number[A] | Detached barley leaf assay | Field assay[C] | | | |
|---|---|---|---|---|---|
| | Lesion length[B] (mm) | Maize stalk | | Maize ear | |
| | After 7 d | $H_1$ | $H_2$ | $H_1$ | $H_2$ |
| | | *Fusarium subglutinans* | | | |
| MRIZP 0043 | 5.8$^{cdefghi}$ | ... | ... | ... | ... |
| MRIZP 0555 | 5.2$^{efghi}$ | 37.4$^{abc}$ | 35.1$^{ab}$ | 32.5$^{ab}$ | 38.9$^{abc}$ |
| MRIZP 0556 | 4.0$^{i}$ | 41.0$^{ab}$ | 35.9$^{ab}$ | 31.1$^{ab}$ | 39.1$^{abc}$ |
| MRIZP 0559 | 5.7$^{cdefghi}$ | ... | ... | ... | ... |
| MRIZP 0560 | 7.1$^{abcd}$ | 39.0$^{abc}$ | 31.9$^{ab}$ | 31.6$^{ab}$ | 37.1$^{bcde}$ |
| MRIZP 0706 | 4.6$^{hi}$ | 38.2$^{abc}$ | 32.2$^{ab}$ | 31.6$^{ab}$ | 31.9$^{ef}$ |
| | | nt | nt | n | nt |
| MRIZP 0707 | 5.1$^{fghi}$ | | | t | |
| MRIZP 0712 | 4.4$^{i}$ | nt | nt | nt | nt |
| MRIZP 1025 | 4.8$^{hi}$ | nt | nt | nt | nt |
| MRIZP 1033 | 8.7$^{a}$ | nt | nt | nt | nt |
| MRIZP 2197 | 7.8$^{ab}$ | 37.7$^{abc}$ | 37.6$^{ab}$ | 29.2$^{b}$ | 36.8$^{bcde}$ |
| MRIZP 2406 | 4.7$^{hi}$ | 42.7$^{a}$ | 36.1$^{ab}$ | 34.2$^{ab}$ | 37.0$^{bcde}$ |
| MRIZP 2419 | 4.7$^{hi}$ | 39.7$^{abc}$ | 34.5$^{ab}$ | 32.7$^{ab}$ | 38.2$^{bcd}$ |
| MRIZP 2796 | 4.4$^{i}$ | 36.6$^{abc}$ | 31.7$^{b}$ | 27.6$^{b}$ | 32.1$^{def}$ |
| MRIZP 2797 | 5.3$^{defghi}$ | nt | nt | nt | nt |
| MRIZP 2798 | 6.5$^{bcdefgh}$ | 32.7$^{c}$ | 31.5$^{ab}$ | 30.6$^{ab}$ | 37.7$^{bcde}$ |
| MRIZP 2800 | 5.7$^{cdefghi}$ | 34.1$^{bc}$ | 34.2$^{ab}$ | 30.4$^{ab}$ | 35.8$^{cdef}$ |
| MRIZP 2803 | 5.2$^{efghi}$ | 35.9$^{abc}$ | 37.8$^{ab}$ | 40.4$^{a}$ | 29.9$^{f}$ |
| MRIZP 2804 | 5.6$^{cdefghi}$ | 38.3$^{abc}$ | 32.5$^{ab}$ | 30.1$^{b}$ | 33.3$^{cdef}$ |
| MRIZP 2807 | 6.7$^{bcdefg}$ | nt | nt | nt | nt |
| MRIZP 2808 | 6.8$^{bcdefg}$ | 35.9$^{abc}$ | 31.3$^{b}$ | 34.2$^{ab}$ | 32.9$^{cdef}$ |
| MRIZP 2828 | 4.9$^{hi}$ | 34.1$^{bc}$ | 33.6$^{ab}$ | 35.9$^{ab}$ | 35.7$^{cdef}$ |
| MRIZP 2970 | 7.0$^{bcde}$ | 35.9$^{abc}$ | 35.7$^{ab}$ | 35.5$^{ab}$ | 34.0$^{cdef}$ |
| MRIZP 3016 | 6.3$^{bcdefgh}$ | 38.3$^{abc}$ | 35.5$^{ab}$ | 34.3$^{ab}$ | 32.4$^{def}$ |
| MRIZP 3019 | 6.9$^{bcde}$ | 37.9$^{abc}$ | 32.3$^{ab}$ | 30.1$^{b}$ | 44.3$^{a}$ |
| MRIZP 3037 | 5.0$^{ghi}$ | 42.7$^{a}$ | 35.0$^{ab}$ | 31.9$^{ab}$ | 36.3$^{bcde}$ |
| | 5.7±1.2 | 37.7±2.8 | 34.1±2.1 | 32.4±3.0 | 35.7±3.4 |
| | | *Fusarium temperatum* | | | |
| MRIZP 0418 | 7.3$^{abc}$ | 38.9$^{abc}$ | 34.4$^{ab}$ | 28.5$^{b}$ | 36.9$^{bcde}$ |
| MRIZP 0552 | 7.4$^{abc}$ | 36.7$^{abc}$ | 39.1$^{a}$ | 30.7$^{ab}$ | 41.9$^{ab}$ |
| MUCL 52438 | 4.7$^{hi}$ | nt | nt | nt | nt |
| MUCL 52463 | 6.8$^{bcdef}$ | nt | nt | nt | nt |
| | 6.6±1.1 | 37.8±1.6 | 36.8±3.3 | 29.6±1.6 | 39.4±3.5 |

[A] MRI: Culture Collection of the Maize Research Institute, Zemun Polje, Belgrade, Serbia; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain-Neuve, Belgium. [B] Values are means of four replicates. Values with the same letter are not statistically different based on Duncan's test (P< 0.01). [C] Arcsine-transformed values of the stalk and ear disease index. $H_1$: Medium late hybrid or FAO 500 maturity group, $H_2$: Late maturity hybrid or FAO 700 maturity group, nt: Strains which are included in field test. Values with the same letter are not statistically different based on Duncan's multiple range test (P< 0.01).





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After inoculation under field conditions, all strains successfully infected stalks and ears in both maize hybrids in comparison with the control treatments. Visible discoloration in control treatments of stalks and ears were localized only at the inoculation spot (Figure 2).

All 20 tested strains caused dark necrosis of the maize stalk pith tissue after inoculation of the second internode (Figure 2). Results of the pathogenicity test performed on maize ears showed that strains could cause severe ear rot in both hybrids (Figure 2). Tested strains caused symptoms of whitish, pinkish, or violet ear rot, which spread over a group of kernels or over a greater part of the ear, and in some cases, it spread over the entire ear. The strain MRIZP 2803 was the most aggressive and caused the most intensive disease progress in ears of $H_2$. On the other hand, strains MRIZP 2796 were the least aggressive on $H_1$. No statistically significant correlation was found between aggressiveness under laboratory and field conditions ($P< 0.01$).

## DISCUSSION

The present study shows that *F. temperatum* was hidden within the Serbian population of *F. subglutinans*. This species was identified on the basis of interspecies mating compatibility and by the molecular identification, while it could not be identified by the routine morphological studies, as it is similar to the closely related *F. subglutinans*. Our studies show that one strain of *F. temperatum* (MRIZP 0418) did not form macroconidia with six septa and that three *F. subglutinas* strains formed macroconidia with up to six septa. On the other hand, the average number of four septa was recorded in both species. These results differ from results obtained by other authors, who stated that *F. temperatum* formed macroconidia with 3-6 septa (Scauflaire *et al*., 2011 a), or three to five septa (Wang *et al*., 2014) or usually four septa, while *F. subglutinans* most often formed three septa (Scauflaire *et al*., 2011a). We assume that the difference between our results and results obtained by other authors is due to our use of CLA to cultivate fungi, while other authors used PDA and SNA. According to Scauflaire *et al*. (2011a), morphological differences established between these two species are not sufficient for the routine identification of *F. temperatum*. As indicated by Scauflaire *et al*. (2012a) *F. temperatum* can be distinguished from *F. subglutinans* and from other *Fusarium* species within the *Fusarium fujikuroi* species complex with the AFLP fingerprint profile, differences in the translation elongation factor *1-*$\alpha$ and $\beta$-tubulin DNA sequence and interspecies mating compatibility analysis. Furthermore, mycotoxin analyses demonstrated that *F. temperatum* was a beauvericin and moniliformin producer, and, therefore, this species represents a greater toxicological risk than its sister species *F. subglutinans* (Scauflaire *et al*., 2012b).

Results of mating compatibility analyses of 28 *F. subglutinans* strains with the tester strains of *F. temperatum* showed considerable differences in fertility, from being highly fertile to being completely barren. Two strains (MRIZP 0418 and MRIZP 0552) were reliably identified as *F. temperatum*, while there were some doubts relating to another nine strains, because, although few in number, they had formed perithecia with asci and ascospores (sparingly fertile). Obtained results are in accordance with interpretations given by Perkins (1994) that there are transitions among different strains within the same species, especially when individuals originating from geographically different populations are combined. According to the same author, this observation was significant in providing evidence of a close relationship, even though ascospores were few in number, slow to appear, and imperfect in their maturation. All 28 isolates of *F. subglutinans* and *F. temperatum* are potentially MAT1-1, no MAT1-2.





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Results of one laboratory and two tests of pathogenicity under field conditions successfully demonstrated that both *F. subglutinans* and *F. temperatum* had the capacity to cause diseases in infected plant tissue in both barley and maize. However, it was not possible to distinguish between *F. subglutinans* and *F. temperatum* based on symptoms induced in inoculated plants. These results are in agreement with results gained by both Scauflaire *et al*. (2012b) and Wang *et al*. (2014), who established that based on aggressiveness, *F. temperatum* was similar to *F. subglutinans* under conditions of glass-house and field, respectively. In contrast, data presented by Scauflaire *et al*. (2012b) suggest that this fungus is less virulent to maize in comparison to other *Fusarium* species (*F. culmorum*, *F. verticillioides*, *F. avenaceum*, *F. crookwellense*, and *F. graminearum*).

Results achieved in this study show that *F. temperatum* originated from diseased sorghum was pathogenic to maize. Wang *et al*. (2014) noticed that strains of *F. temperatum* isolated from maize kernels could successfully infect maize and wheat spikes. In this context, it could be concluded that a wide distribution of this species may be possible in other plants and countries (Scauflaire *et al*., 2011b; Susca *et al*., 2013).

In conclusion, the obtained results confirm that *F. temperatum* species is present in sorghum in Serbia. This species expressed pathogenic properties on maize stalks and ears under field conditions. With regard to pathogenic properties and responses of maize genotypes, there were no statistically significant differences between *F. temperatum* and *F. subglutinans*. Molecular tools such as *EF-1α* gene sequencing are the only efficient and rapid method that clearly distinguish *F. temperatum* from its sister species *F. subglutinans*.

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# استفاده ازرویکرد چند فازی برای تشخیص *Fusarium temperatum* از *Fusarium subglutinans*

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## چکیده

در این پژوهش، برای تشخیص گونه های بیماریزای *F. temperatum* درجمعیت های *F. subglutinans* موجود در صربستان که در دوره ۲۰۱۰-۱۹۹۹ از مزارع ذرت *Zea mays* (۳ نمونه ریشه،۱۵ نمونه ساقه، و ۶ نمونه بذر) و سورگم *Sorghum bicolor*(دو نمونه بذر) وجو *Hordeum vulgare* (یک نمونه بذر) و گیاه *Taraxacum officinale* (یک نمونه بذر) تهیه شده بود، از رویکرد های مورفولوژیکی، زیست شناسی، و فیلوژنتیکی استفاده شد. بر مبنای تحلیل سازگاری جفتگیری بین گونه ها و تجزیه حداکثر پارسیمونی ( maximum parsimony) توالی *EF-1α*با منشاء بذر *S. bicolor*( MRIZP 0418 و MRIZP 0552) تنها دو ریسه به عنوان *F. temperatum* شناسایی شد در حالیکه بقیه ۲۶ ریسه های تک-اسپوری (single-spore) به عنوان *F. subglutinans* Group 2 تشخیص داده شد. آزمون در محل(in situ)برگ بریده از جو و ساقه و بلال از دو هیبرید ذرت که به طور مصنوعی آلوده شده بود نشان داد که هر دو ریسه *F. temperatum* و *F. subglutinans* در شرایط آزمایشگاهی و مزرعه به ترتیب بیمارگرهای متوسط و قوی بودند. این نتایج اولین داده ها در مورد *F. temperatum* به عنوان بیمارگر بذر بُرد(*seed borne*) سورگم ونیز در مورد بیمارگربودن ریسه *F. temperatum* روی ذرت می باشد.





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