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Original Article

Morphology, Pathogenicity and Molecular Identification of *Fusarium* spp. Associated with Anise Seeds in Serbia

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Abstract

Anise (*Pimpinella anisum* L.) is an important medicinal spice plant that belongs to the family *Apiaceae*. Anise seeds are rich in essential oils and this is a reason why anise production in Serbia has increased over the last decade. During a routine health inspection on anise seeds collected from three localities in the province of Vojvodina (Mošorin, Veliki Radinci and Ostojićevo) during 2012 and 2013, it was found out that *Fusarium* spp. were a commonly observed fungi. The presence of *Fusarium* fungi on the seed samples ranged from 3.75-13.75%. The aim of this study was to isolate and identify the strains of *Fusarium* species present on anise seed samples as it is necessary that commercially used anise seeds are completely free of *Fusarium*. Based on morphological, microscopic characteristics and a molecular identification by sequencing of TEF gene, the presence of the following species was confirmed on the anise seeds: *F. tricinctum*, *F. proliferatum*, *F. equiseti*, *F. oxysporum*, *F. sporotrichoides*, *F. incarnatum* and *F. verticillioides*. According to our knowledge and research, this is the first report of *F. tricinctum* and *F. sporotrichoides* as pathogens on anise seeds in the world. All seven isolates of *Fusarium* species are pathogenic to the anise seedlings, while the most virulent species were *F. oxysporum*, *F. tricinctum* and F. *incarnatum*.

Keywords: morphological characteristics, *Pimpinella anisum*, PCR detection, seed infection

Introduction

Anise (*Pimpinella anisum* L.) is an annual medicinal plant that belongs to the family *Apiaceae,* widely cultivated for its fruit and essential oils. Anise is an important raw natural material mostly used in medicine, perfumery and cosmetic industries (Simon *et al*., 1984). Due to increasing demand, anise production has increased in Serbia during the last decade (Dražić *et al*., 2007).

The mycoflora of anise seeds has not been studied sufficiently. Bokhari (2007) found out that the dominant species on the anise seeds in Saudi Arabia were from the genus *Aspergillus*. The same results were obtained by Saleem *et al*. (2013), while Saber *et al*. (2009) found *Puccinia pimpinellae* on anise seeds in Egypt. Several fungal species of the 21 genera, have been reported to be associated with anise seeds in Egypt (Ghoneem *et al*., 2012), the predominant species being *Alternaria alternata*, *Drechslera tetramera*, *Cladosporium* sp. and *Stemphiulium* sp., while the species from genus *Fusarium* were present in a low percentage. Bulajić *et al.* (2009) identified *Alternaria alternation* on anise seeds in Serbia. Knowing which seed-borne pathogens are present in or on anise seeds, is of practical importance as this affects the transmission mode of the pathogens. One of the important means of the disease transmission is through seeds. Planting infected seeds may result in a widespread distribution of disease within the crop, and an increased number of initial infection sites from which the disease can spread (Desjardins, 2006). As the fungi are the largest group of pathogens, it is almost impossible to keep the seeds completely pathogen free. In addition, the presence of the fungi in medicinal plants reduces their quality and usefulness (Essono *et al*., 2007). The use of chemical fungicides for plant disease control can be problematic as currently, some may have carcinogenic and teratogenic

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characteristics as well as residual toxicity (Skandamis *et al*., 2001).

Numerous fungi produce mycotoxins. The fungi from genus *Fusarium* contain zearalenone, fumonisin, moniliformin, fusarin and other toxins (Desjardins, 2006; Frisvad *et al*., 2006). The quality of anise is determined mainly on the basis of the essential oil content and its composition (Orav *et al*., 2008). If anise seeds contain mycotoxins, this will be toxic to humans and represents a threat to public health (Jackson and Jablonski, 2004). Fungi of the genus *Fusarium* are already reported to cause seed "rot" disease that affects different stages of the host, seeds, seedlings and the crowns of developing plants (Moya-Elizando, 2013). When seed rot and seedling blight are caused by the same organism, seedlings fail to emerge due to a pathogen attack either before or after germination. There are a number studies on the mode of attack by *Fusarium* on ungerminated seeds. Numerous investigations have been conducted with *Fusarium* species that infect maize seed (Fandohan *et al*., 2003; Duncan *et al*., 2010; Madania *et al*., 2013). In a more specialized study regarding the mode of seed infection by *Fusarium* species, Sauerborn *et al*. (1996) showed the manner of pathogen penetration in the seed.

Fusarium spp. is widespread fungi, which can cause diseases on host plants with a serious economic impact (Wang *et al*., 2011). The morphological characteristics such as: the colony colour, growth rate, size and shape of macro- and microconidia, formation of chlamydospores and conidiogenous cells, have been used as preferred methods for identification of *Fusarium* species (Summerell *et al*., 2003). However, the genus *Fusarium* is complex and morphological differences may be difficult to observe. Therefore, the DNA analysis is needed for accurate identification and characterization of the species. Molecular data in addition to the distinctive morphological characteristics have been used to identify *Fusarium* species by many authors (Geiser *et al*., 2004; Wang *et al*., 2011; Zhu *et al*., 2014).

The presence of *Fusarium subglutinans* has already been detected in anise seed during 2011 in Serbia (Ristić *et al*., 2015). Based on all these studies, the aim of this investigation was to (i) identify other *Fusarium* species on anise seeds, (ii) check incidence of seed infection and (iii) observe pathological effects of the *Fusarium* species *in vivo*.

Materials and Methods

Samples

The samples were collected during 2012 and 2013 from each of the three localities in the Vojvodina Province, Republic of Serbia: Mošorin (45°18' N, 20°09'E, 111 m above sea level), Veliki Radinci (45° 02'N, 19°40'E, 110 m above sea level) and Ostojićevo (45°54'N, 20°09' E, 88 m above sea level). Sampling was conducted on the commercially available, organically produced anise seed N-210 cv. three months after harvesting.

An analysis of the health status was performed by the incubation of anise seeds on the filter paper and on the potato dextrose agar (PDA). Four hundred seeds (4 trials, each with 100 seeds) from each locality were sterilized with NaOCl for 3 minutes and then rinsed with sterile water and transferred to the filter paper on Petri dishes, 15 cm in diameter. Ten seeds from each locality in four repetitions were transferred to the PDA medium following the seed surface sterilization. After the eight-day incubation at 25 °C, parts of the mycelia taken from well-developed colonies were transferred to the PDA in order to be further examined (Mathur and Kongsdal, 2003). According to the appearance of mycelia, seven isolates were selected (A6, A7, A8, A9, A10, A11 and A12) for further investigations. The *Fusarium* spp. present on each seed was recorded as percentage of infected seeds in two investigated years.

Morphological characterization

Pure cultures obtained from a single spore of each isolate were grown on PDA incubated at 25 °C, with a 12-h photoperiod and examined macroscopically (colony morphology and pigmentation) and microscopically (the shape, size and type and manner of conidia formation, production of microconidia, macroconidia, conidiogenous cells, chlamydospores, sclerotia) after 14 days. A hundred micro- and macroconidia were measured in every isolate. The isolates were identified according to the morphological characteristic described by Leslie and Summerell, (2006).

The pathogenicity test

Fifty 30 days-old anise seedlings grown under controlled condition on the filter paper were selected for their healthy and uniform appearance for the pathogenicity test. The roots of the healthy seedlings were injured using scissors and then soaked for 30 min in the spore suspension of the *Fusarium* sp. isolates in order to infect the root cells. *Fusarium* spore concentration of 5×10^6 conidia ml^{-1} was measured by a haemacytometer. The control plants were planted in the sterile soil without any spore suspensions. After inoculation, the plants were potted in the sterilized soil and grown in the glasshouse with day and night temperatures of 27-30 °C and 23-25 °C respectively. Four replications were performed for each isolate. A development of symptoms of root necrosis on the inoculated and control plants was observed after 30 days. The level of root necrosis was calculated according to the scale 0-3 (0 – a healthy seedling, 1 – root tip necrosis, 2 – root and lower part of the stem necrosis, 3 – completely rotted). The intensity of infection (II) was calculated according to the modified Mc-Kinney's formula by Filion *et al*. (2003):

$$II = \sum (nv)/ 4N*100,$$

where:

II= intensity of infection;

n= number of roots in each category;

v= category of infection;

N= total number of evaluated roots.

Fungi from the inoculated plants were re-isolated from the infected plants to prove the Koch's postulates.

Data were subjected to the analysis of variance (ANOVA). The significance was evaluated at $p<0.05$ for all tests. Statistical analyses were performed by using the STATISTICA v.7.

DNA extraction, polymerase chain reaction amplification and sequencing

A genomic DNA extraction of all monoconidial *Fusarium* species was done by obtaining a mycelial mat from the potato dextrose broth in Erlenmeyer flasks inoculated with the 7 day

old pure cultures and making use of DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The molecular identity of the fungus was confirmed by amplifying the partial translation elongation factor-1α (TEF) gene with the specific primers ef1 (ATGGGTAAGGAGGACAAGAC) and ef2 (GGAAGTACCAGTGATCATGTT) (O'Donnell *et al*., 1998).

The TEF region was amplified in a 25 µl reaction mixture containing 12.5 µl 2 X PCR Master mix (K071, Fermentas, Lithuania), 9 µl RNase-free water, 1.25 µl each of both forward and reverse primers (100 pmol μl^{-1}, Metabion International, Germany) and 1 µl template DNA. The PCR conditions were as follows: pre-denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 2 min and final extension at 72 °C for 10 min. Amplified products were analyzed by 1% agarose gel electrophoresis, stained with Midori Green DNA Stain (Nippon Genetics), and visualized under a UV transilluminator. The PCR product was sequenced and deposited in the National Center of Biotechnology Information (NCBI) GenBank database. The sequence was compared with sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) by using the ClustalW program (Thompson *et al*., 1994) and MEGA5 software (Tamura *et al*., 2011).

Results and Discussion

Occurrence

The presence of *Fusarium* species was detected in three localities in both years. The percentage increase of *Fusarium* infection on anise seeds in three localities in 2013 was 200-250% compared to 2012 (Table 1).

Morphological characterization

All seven *Fusarium* isolates were examined macroscopically and microscopically. During morphological observation, aerial mycelia of all isolates were white at the initial stage, while the underside of colonies became pale pink, violet, purple and brown in the later stages on PDA (Figs. 1a and 2a).

Based on their morphological characteristics (shape, form, separation, size, microconidia, macroconidia, phialides, chlamidospores, sclerotia), the isolates were identified as: A6-*F. tricinctum*, A7-*F. proliferatum,* A8-*F. equiseti*, A9-*F. oxysporum*, A10-*F. sporotrichoides*, A11-*F. incarnatum* and A12-*F. verticillioides* (Table 2, Figs. 2 and 3 b, c, d, e).

The pathogenicity test

The results of the pathogenicity test revealed that all seven isolates of *Fusarium* species are pathogenic to the anise seedlings. They showed symptoms like crown rot and root necrosis or root rot. These symptoms were observed within 30 days after inoculation as necrosis and brown discoloration of the root, lower part of the stem necrosis, and a complete rottenness/decomposition. According to the description scale 0-3, the pathogenicity of each *Fusarium* isolate was classified into three groups: virulent (II > 30): *F. oxysporum*, *F. tricinctum* and F. *incarnatum*, moderately virulent (II = 20-30):*F. proliferatum* and *F. sporotrichoides,* and mildly virulent (II < 20):*F. equiseti*and, *F. verticillioides* (Table 3). Inoculated fungi were re-isolated from the diseased plants, but not from negative control plants, thus fulfilling the Koch's postulates.

Molecular identification of Fusarium spp.

To confirm the morphological identification of the seven studied strains (A6, A7, A8, A9, A10, A11 and A12), molecular analyses were performed. Amplification of the TEF gene of the isolates generated a product of expected size (Fig. 3). The amplified and purified DNA fragments of *Fusarium* isolates were sequenced in both directions. Sequences of amplified TEF gene showed that fragments obtained from seven isolates were 440-651 bp in length. The sequences obtained from the isolates were deposited in the GeneBank (NCBI Acc.No. KP126607-13) (Table 4) and showed 100% homology (100% query coverage) with the *F. tricinctum*, *F. proliferatum*, *F. equiseti*, *F. oxysporum*, *F. sporotrichoides*, *F.* cf. *incarnatum* and *F. verticillioides* isolates.

Infected seeds contribute significantly to the disease epidemiology. Identification of seeds health status and the use of *Fusarium* free seeds are the most important steps in disease control. Anise seed infections can result in reduced germination and yield loses (Bottalico, 1998; Perkowski *et al*., 2002). Until this investigation was conducted, there was no information available on the health status of commercial anise seed production in Serbia.

This study represents the first attempt to characterize pathogens of genus *Fusarium* associated with anise seeds in Serbia. The *Fusarium* isolates selected for this investigation from the three localities in Serbia were identified as: *F. tricinctum*, *F. proliferatum*, *F. equiseti*, *F. oxysporum*, *F. sporotrichoides*, *F. incarnatum* and *F. verticillioides* species, based on morphological characteristics and proved by the molecular analysis. Bokhari (2007) also isolated 5 *Fusarium* species: *F.oxysporum*, *F.moniliforme* and *F. subglutinans* in Saudi Arabia on anise seeds. Ghoneem *et al.* (2012) reported the presence of *F. equiseti*, *F. oxysporum*, *F.incarnatum*, *F. solani* and *F.verticillioides* on anise seeds in Egypt based on

Table 1. Percentage of *Fusarium* infected anise seeds during 2012 and 2013 in three localities in Serbia

Locality	% of *Fusarium* infected seeds	
	2012	2013 (% increase infection)
Mošorin	3.75	7.5 (200.0)
Veliki Radinci	4.75	11.75 (247.4)
Ostojićevo	5.50	13.75 (250)

Table 3. The intensity of infection (II) based on the pathogenicity test on anise seedlings

Species	The intensity of infection (II)*
F. tricinctum	34±0.87c
F. proliferatum	24.5±087b
F. equiseti	19.6±0.76a
F. oxysporum	38.5±1.4c
F. sporotrichoides	24.5±3.5b
F. incarnatum	31.8±0.67c
F. verticillioides	18.7±1.76a

*Means±standard deviation followed by the same letter are not significantly different (p<0,05), according to Duncan's range test

Table 2. Morphological characteristics of *Fusarium* spp. isolated from anise seeds

	F. tricinctum	*F. proliferatum*	*F. equiseti*	*F. oxysporum*	*F. sporotrichioides*	*F. incarnatum*	*F. verticillioides*
Microconidia	+	+	-	+	+	-	+
Form	Lc	Lc, Fh		Fh			Lc, Fh
Phialides	Mono	Mono, poly	Mono	Mono	Mono-poly	Mono-poly	Mono
Shape	Np	Ov		Ov-El	Ol,Py,El		Ov
Separation	0	0		0-1	0-1		0-1
Size (µm)	7.0-13.0 × 4.2-7.7	3.0-19.5 × 2.0-5.0		7.5-9.0 × 3.0-5.5	7.0-20.0 × 4.5-7.5		5.2-18.0 × 1.1-5.5
Macroconidia	+	+	+	+	+	+	+
Shape	Sp	Cr		Sp	El	Sp-Sr	Cr
Septate	3-5	3-5	3-5	3-5	3-5	3-5	3-5
Size (µm)	22.0-48.0 × 3.5-4.5	16.5-73.0 × 2.5-5.0	40.5-57.5 x 3.75-5.0	49.0-89.5 × 3.0-10.0	22.0-45.0 × 3.5-5.5	19.0-62.5 × 2.0-4.5	16.3-79.0 × 3.0-5.0
Clamidospore	+	-	+	+	+	+	-
Sclerotia	+	-	+	-	-	-	-

+ = Presence; - = absence; Cr = crecent; Lc = long chains; El = ellipsoidal; Fh = false heads; Np = napiform; Ov = ovoid; Ol = oval; Py = pyriform; PSr = sp

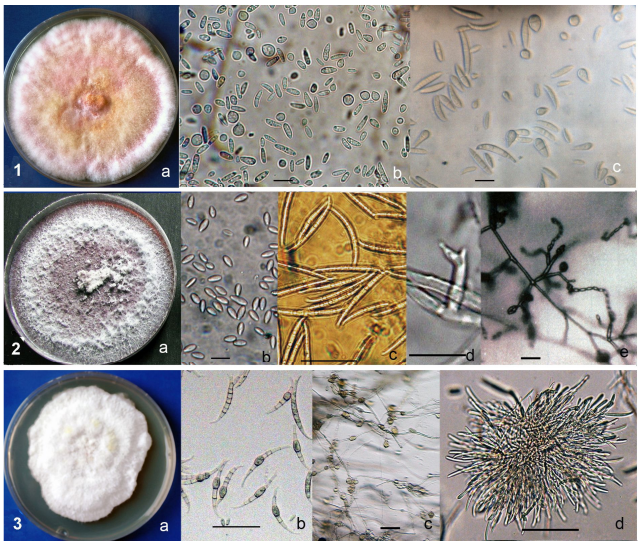

Fig. 1. Morphological characters of *Fusarium* isolate: 1 = *F. tricinctum*: a - colony on PDA, b - microconidia, *bar* - 10 µm, c – micro and macroconidia, *bar* -20 µm, 2 = *F.proliferatum*: a – colony on PDA, b - microconidia, *bar* - 20 µm, c – macroconidia, *bar* - 50 µm, d) polyphialides *in sity*, bar - 20 µm, e) microconidia in chains and false heads on conidiophores *in sity*, bar - 20 µm, 3 = *F. equiseti*: a - colony grown on PDA, b – macroconidia, bar - 50µm, c – chlamydospores *in sity*, bar - 40 µm, d) conidiogenous cells, bar – 50 µm

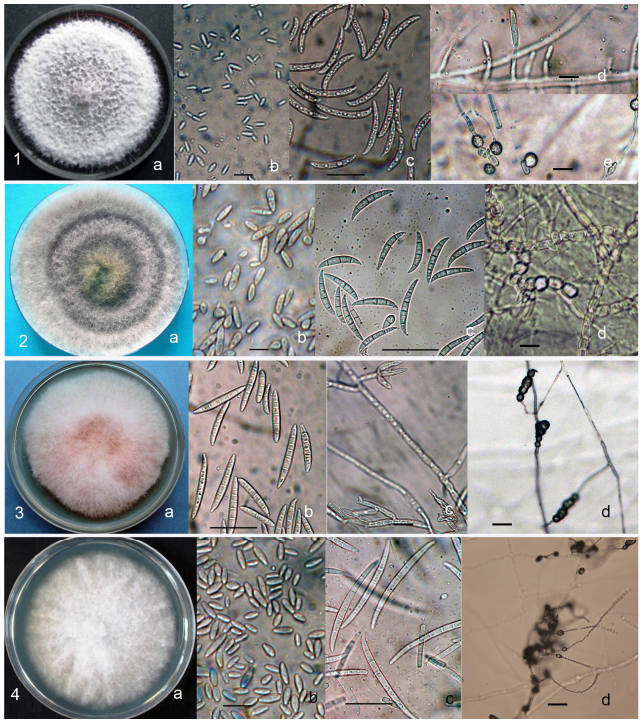

Fig. 2. Morphological characters of *Fusarium* isolate: 1 = *F. oxysporum*: a - colony on PDA, b - microconidia, bar - 10 µm, c - macroconidia, *bar* - 50 µm, d) monophialides, *in sity*, bar - 20 µm, e) chlamydospores, *in sity*, bar - 10 µm, 2 = *F. sporotrichioides*: a - colony on PDA, b - microconidia, bar - 10 µm, c - macroconidia, bar - 50 µm, d) chlamydospores, *in sity,* bar - 10 µm, 3 = *F. incarnatum*: a - colony on PDA, b - macroconidia, bar - 50 µm, c - polyphialides *in sity*, bar - 10 µm, d) chlamydospores *in sity*, bar - 10 µm, 4 = *F. verticilioides*: a - colony on PDA, microconidia, bar - 20 µm, c - macroconidia, bar - 50 µm, d) microconidia in monophyalide chains, *in sity,* bar - 10 µm

morphological and microscopic characteristics. Saleem *et al.* (2013) used the same method to identify *F. merismoides*, *F. oxysporum*and *F. proliferatum* on anise seeds in Egypt. Our results confirmed earlier reports that *F. equiseti*, *F. oxysporum*, *F. incarnatum*, *F. solani, F .proliferatum*and *F. verticillioides* were present on anise seeds in Serbia. According to our knowledge, this is the first report of *F. tricinctum* and *F. sporotrichoides* presence on anise seeds in the world. As several reports mentioned the impossibility of distinguishing among some *Fusarium* species, we checked our morphological results with molecular method based on the TEF gene.

The level of anise seed infection with *Fusarium* species was variable, ranging from 3.75 to 17.75%. The percentage infection of anise seed with *Fusarium* species has been increasing from year to year because the infected seeds are used for sowing. In addition, most *Fusarium* species exists in the soil as chlamydospores which, in favourable conditions, can survive for up to 20 years (Palmero *et al.*, 2014). Chlamydospores present in the soil are the main source of infection. All of the mentioned *Fusarium* species were transmitted by seeds and

Table 4. Identification of *Fusarium* spp. associated with anise seeds based on morphological analysis and TEF gene sequencing

No	Isolate	Locality	Morphological identification	Identification by TEF gene	NCBI accession no.
1	A6	Ostojićevo	*F. tricinctum*	*F. tricinctum*	KP126607
2	A7	V. Radinci	*F. proliferatum*	*F. proliferatum*	KP126608
3	A8	Mošorin	*F. equiseti*	*F. equiseti*	KP126609
4	A9	Ostojićevo	*F. oxysporum*	*F. oxysporum*	KP1266010
5	A10	V. Radinci	*F. sporotrichoides*	*F. sporotrichoides*	KP1266011
6	A11	Ostojićevo	*F. incarnatum*	*F.* cf. *incarnatum*	KP1266012
7	A12	Mošorin	*F. verticillioides*	*F. verticillioides*	KP1266013

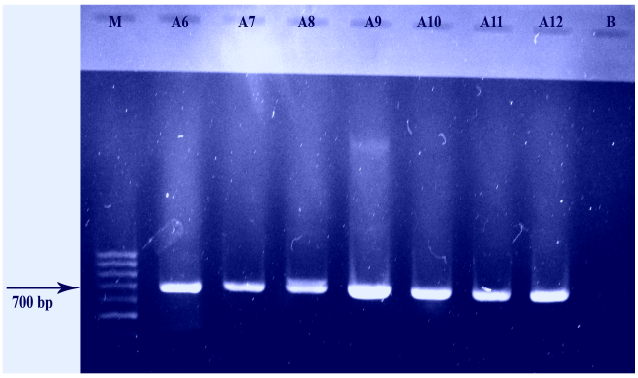

Fig. 3. PCR amplification of the seven *Fusarium* isolates with primer pair ef1/ef2. Lanes: M - Mass-Ruler^{TM}DNA ladder Mix (Fermentas Life Sciences GmgH, Lithuania); 1 - *F. tricinctum*; 2 - *F. proliferatum*; 3 - *F. equiseti*; 4 - *F. oxysporum*; 5 - *F. sporotrichoides*; 6 - *F.* cf. *incarnatum*; 7 - *F. verticillioides*; 8 - negative template control (PCR mix with RNase-free water)

pathogenic to anise seedlings. On the basis of the pathogenicity test, *F. oxysporum*, *F. tricinctum* and *F. incarnatum*, manifested the highest pathogenicity on the anise plants, followed by *F. proliferatum,* and *F. sporotrichoides*. The lowest pathogenicity was expressed by *F. equiseti* and *F. verticillioides*.

Fusarium species have already been recorded all over the world and are proved to be pathogens of many plants (Boughalleb *et al*., 2005; Mehl and Epstein, 2007). The virulence may be attributed either to a single gene or to a set of genes that confer a specific characteristic to the pathogen, such as production of host-specific toxins (Friesen *et al*., 2006; Van der Does and Rep, 2007). *F. oxysporum* has already been shown as very virulent pathogen. Anise seed has been detected as host to some *Fusarium* species (Bokhari, 2007; Ghoneem *et al*., 2012; Saleem *et al*., 2013), but they didn't prove the pathogenicity. Of the 101 most economically important plants, at least 81 are hosts to *Fusarium* pathogens (Nayaka *et al*., 2013). We added anise as the host plant to the two *Fusarium* species: *F. tricinctum* and *F. sporotrichoides*.

In this study, both morphological and molecular approaches (Leslie and Summerell, 2006) successfully detected the same *Fusarium* species, because all of our seven *Fusarium* isolates were precisely identified. Molecular detection based on the TEF gene of *Fusarium* species on anise seeds could be a powerful tool in identification of the pathogenic species, giving results in a shorter period of time compared to the morphological identification.

Morphological identification, followed by sequencing of TEF region fragments amplified by ef1/ef2 primers and BLAST analysis of all our isolates that showed a high level of genetic similarity with DNA sequences of fungal species available in GenBank, confirmed that our isolates were *F. tricinctum*, *F. proliferatum*, *F. equiseti*, *F. oxysporum*, *F. sporotrichoides*, *F.* cf. *incarnatum* and *F. verticillioides*. In conclusion, this study is the first known extensive research of the characterization of the fungal pathogens associated with anise seed production in Serbia.

The translation elongation factor 1-a (TEF) gene, which encodes an essential part of the protein translation machinery, has high phylogenetic utility because it is highly informative at the species level in *Fusarium*. Non-orthologous copies of the gene have not been detected in the genus and universal primers have been designed that barcode successfully amplify this region for all species of the genus (Summerell *et al*., 2003; Geiser *et al*., 2004; Kristensen *et al*., 2005).

The results obtained in this study and a successful application of the molecular identification protocol to identify *Fusarium* species based on the TEF gene sequence (which codes for the factor-1α) represent a starting point for the study of phylogeographic distribution of *Fusarium* sp in Serbia. It is also useful to have a clear genetic characterization and an effective method for species identification. The ability to sequence a large number of isolates by using additional genome parts and being able to compare their relationship with other isolates, all contribute to a precise identification of *Fusarium* population. This can lead to an effective control of these dangerous pathogens.

Generally, a management of root rot disease, caused by *Fusarium* species, is usually based on the crop rotations to reduce the inoculum levels in soil (Davis *et al*., 2006) as well as using healthy seeds or genetically resistant cultivars. In protection of anise seeds of *Fusarium* species we could use beneficial bacterial strains which demonstrate an antagonistic effect to fungi.

Conclusions

Based on morphological, microscopic characteristic and molecular identification by sequencing of TEF gene we proved the presence of *F. tricinctum*, *F. proliferatum*, *F. equiseti*, *F. oxysporum*, *F. sporotrichoides*, *F. incarnatum* and *F. verticillioides* species on anise seeds in three localities in Serbia during 2012 and 2013. Based on our knowledge and research, this is the first report of *F. tricinctum* and *F. sporotrichoides* presence on anise seeds in the world.

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