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ORIGINAL ARTICLE

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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Fusarium graminearum* Schwabe AS A CAUSAL AGENT OF *Hyssopus officinalis* L. SEED ROT

ABSTRACT: Symptoms of seed rot of *Hyssopus officinalis* L. were noticed during seed health testing in 2018. According to morphological and cultural characteristics, isolates belong to *Fusarium* spp. and *Alternaria* spp.. Based on morphological and pathogenic properties, as well as sequence analysis, isolate designated as 4003/3 was identified as *Fusarium graminearum* deposited in NCBI gene bank under Acc. Number MK061542. To our knowledge *F. graminearum* as the causal agent of *Hyssopus officinalis* L. seed rot in Serbia was noticed for the first time. This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant number: 451-03-9/2021-14/200032.
KEYWORDS: Hyssop, *Fusarium graminearum*, seed rot

INTRODUCTION

Hyssop (*Hyssopus officinalis* L.) is a perennial polymorphous plant species of the Lamiaceae family. It is grown in Southern Europe with essential oil accumulated in the flowers and leaves and often used as condiment and spices in food industries (Fathiazad and Hamedeyazdan, 2011; Ogunwande et al., 2011; Zawiślak, 2013).

Fusarium graminearum Schwabe belongs to a group of important soil- and seed-borne pathogens infecting numerous crop plants and occurring worldwide

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in various climatic zones producing diverse families of secondary metabolites such as zearalenone, deoxynivalenol, and trichothecene (Leslie and Summerell, 2006; Nesic et al., 2014).

During routine quality control of hyssop seeds in 2018, fungal infection followed by seed rot was noticed and confirmed by microscopic examination. Multilocus sequence typing scheme analysis has been applied to members of the *Fusarium* genus and DNA sequence-based identification of some unknown isolates can be achieved using the translation-elongation factor *1-α* TEF gene region. Thus the TEF gene has become the marker of choice as a single-locus identification tool in *Fusarium* (Geiser et al., 2004).

The aim of this research was the identification of the causal agent of hyssop seed rot, based on morphological properties of isolates and molecular features using partial sequences of the translation elongation factor gene (*1-α* TEF).

MATERIAL AND METHODS

Pathogen isolation

Symptoms of infected hyssop seeds appear to be soft, covered with white, light pink, or reddish fungal mycelium, followed with violet pigmentation under the seeds (Figure 1a). In order to perform isolation of the pathogen, infected hyssop seeds were transferred onto a Potato Dextrose Agar (PDA) medium with streptomycin sulfate amended (300 mg/l) (w/v). Plates were incubated for seven days at 25 °C under ultraviolet light ("black light") with a 12^h photoperiod (Mathur and Kongsdall, 2003).

Pathogenicity test

Pathogenicity test was performed *in vitro* using a modified agar slant method in the test tube with PDA amended (Porter et al., 2015). At the bottom of the tube, there was placed a piece of mycelium of each isolate grown on PDA and dried hyssop seed was carefully placed 2 cm above. As a positive control, there was used an identified isolate of *Fusarium graminearum* from the collection of the Institute of Field and Vegetable Crops (Laboratory for Seed Testing). Hyssop seed placed on agar without mycelia was used as a negative control. For each isolate, set of five tubes in four repetitions were inoculated. All test tubes were placed in aseptic sealed plastic boxes and incubated for two weeks at 25 °C. All isolates were re-isolated and sub-cultured on Potato Dextrose Agar (PDA) and Carnation Leaf Agar (CLA) using a hyphal tip transfer technique (Leslie and Summerell, 2006), fulfilling Koch's postulates. Identification of *Fusarium* species and morphological characterization was performed according to Gerlach and Nirenberg (1982) and Leslie and Summerell (2006).

DNA extraction and molecular species identification

Fusarium isolates were grown on PDA plates for 7 days, and mycelia were harvested and ground in liquid nitrogen. DNA was extracted using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany), according to the manufacturer's instructions. The translation elongation factor *1-α* TEF gene region was amplified by PCR with the primers EF1 (ATGGGTAAGGAGGACAAGAC) and EF2 (GGAAGTACCAGTGATCATGTT) (O'Donnell et al., 1998; Geiser et al., 2004). The polymerase chain reaction (PCR) was done in S-thermal cycler (Eppendorf, Germany) with a total volume of 25 µl consisting of 12.5 µl of 2x Eppendorf Master Mix (Fermentas, Lithuania); 1.25 µM of each primer (100 pmol/µl), 1µl of fungal DNA, and 9 µl of RNase-free water. The reaction was performed in a thermal cycler (Eppendorf, Germany) under the following programs: initial denaturation of 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 53 °C, and 2 min at 72 °C, with a final extension of 10 min at 72 °C. PCR products were separated using electrophoresis on 1.5% agarose gel containing ethidium bromide (0.5 g/mL) and visualized using a UV light with Bio-print cx4 (VilberLourmat, Germany).

The amplified product from isolate JBL4003/3 was purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced in both directions with an automated sequencer (Macrogen, Korea). The obtained sequence was compared with the previously reported isolates available in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/), using the ClustalW program (Thompson et al., 1994) and MEGA6 software (Tamura et al., 2013).

The phylogenetic tree was generated using Maximum likelihood implemented in MEGA 6 software (Tamura et al., 2013). The edited *1-α* TEF sequence was compared with other available *Fusarium* species sequences in the GenBank. The reliability of the obtained tree was evaluated using the bootstrap analysis based on 1000 replicates, and bootstrap values <50% were omitted. Kimura 2-parameter model was chosen as the best-fitting model of nucleotide substitution.

RESULTS AND DISCUSSION

Twelve isolates distinguished based on colonies appearances and morphological characteristics were chosen for further investigation (JBL4003/1-4003/12). The presence of *Fusarium* spp. and *Alternaria* spp. (JBL4003/5) was confirmed with microscopic observation. Pigmentation of the most *Fusarium* spp. isolates was variably changing from whitish to pinkish, dark purple to vinaceous with a dash of brown, with macro or microconidia formed which indicated presence of *Fusarium* spp. Isolate JBL4003/3 was distinguished based on cultural and morphological characteristics, and according to the description given by Gerlach and Nirenberg (1982), it belongs to *F. graminearum*. Regarding the daily mycelial growth rate, isolate JBL4003/3 was fast growing causing the

hyssop seed rot after the fifth day. No fungi recovered from the negative control. All isolates were re-isolated from symptomatic tissue thus fulfilling Koch's postulates, and stored by freezing at -70 °C. A previous study showed that hyssop seed is the host of different Fusarium species (Ignjatov et al., 2020). Since 2018, an increase in cases of fusariosis has been observed in Serbia, present on various seeds of many different species (hyssop, garlic, onion, "evening stock" etc.) (Ignjatov et al., 2017, 2020). *Fusarium graminearum* is a major pathogen worldwide and the main causal agent of fusarium head blight, a disease complex of wheat and other small grains. In addition to causing considerable crop yield decrease, it is of particular concern because of the ability of Fusarium species to produce mycotoxins in the grain that are harmful to human and animal consumers (Petru et al., 2016).

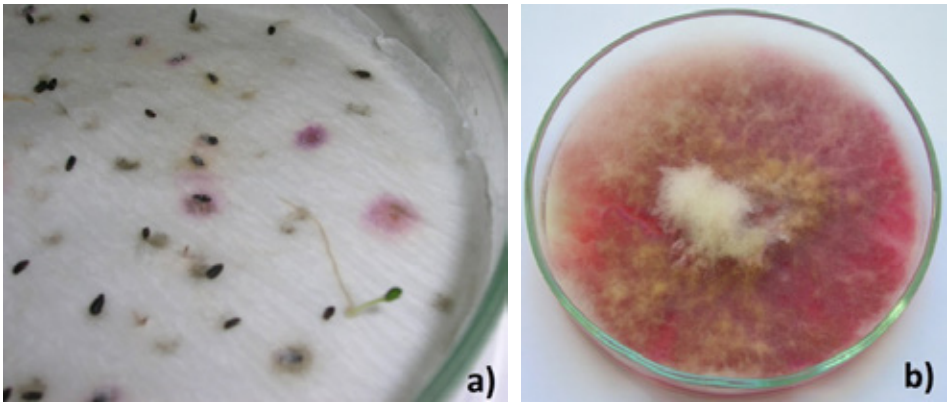

Figure 1. Fusarium graminearum Schwabe on hyssop seed (*Hyssopus officinalis* L.): a) Symptoms of the primary infection of *Hyssopus officinalis* (*Fusarium* spp., *Alternaria* spp.; b) Colony of *F. graminearum* grown on PDA – isolate JBL4003/3

Polymerase chain reaction (PCR) with primers designated as EF1 and EF2 was created as choice of a single locus identification tool in *Fusarium* genus (Geiser et al., 2004). The amplified and purified DNA fragment of representative JBL4003/3 isolate was sequenced in both directions and deposited in the GeneBank under Accession Number MK061542.1. Genetic analysis of the translation elongation factor *1-α* TEF sequence confirmed that Serbian isolate originating from hyssop belongs to *F. graminearum* Schwabe species showing 100% homology with strains from GenBank (Acc. Nos. KM052642 and JX118875). A Maximum likelihood tree (Figure 2), reconstructed based on the *1-α* TEF sequences of different *Fusarium* species selected from GenBank show that JBL4003/3 isolate used in this study was grouped with the isolates previously characterized as *F. graminearum* Schwabe (Acc. Nos. JF270173, JF278592 and HQ702569). Certain groups are well covered in *FUSARIUM*-ID (NCBI) database, particularly *Gibberella fujikuroi* species complex. *Fusarium graminearum* is a common pathogen infecting multiple crops from various climatic zones.

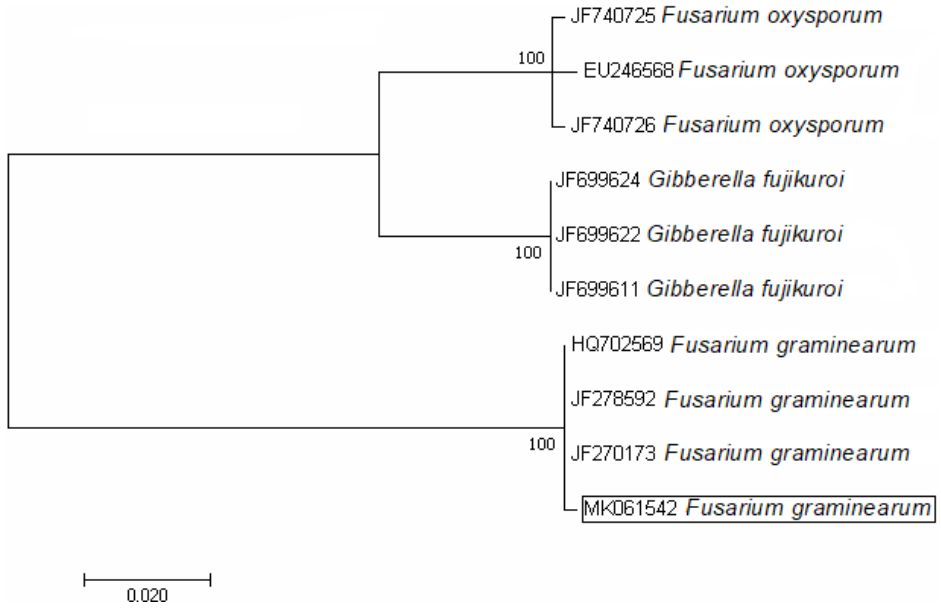

Figure 2. Phylogenetic tree: Phylogram was generated with MEGA6 using bootstrap analysis with 1000 replicates while the bootstrap values (>50%) are shown next to relevant branches. *F. graminearum* Schwabe species showing 100% homology with *F. graminearum* strains from GenBank JF270173, JF278592 and HQ702569

Molecular detection based on the TEF gene of *Fusarium* species could be a powerful tool in identification of the pathogenic species, giving results in a shorter period of time compared to the morphological identification (Pavlović et al., 2016). Generally, a management of 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.

CONCLUSION

Based on morphological and pathogenic properties, as well as sequence analysis, to our knowledge, this is the first case of *F. graminearum* as the causal agent of *Hyssopus officinalis* L. seed rot in Serbia.

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МОРФОЛОШКА И МОЛЕКУЛАРНА КАРАКТЕРИЗАЦИЈА *Fusarium graminearum* Schwabe КАО ПРОУЗРОКОВАЧА ТРУЛЕЖИ СЕМЕНА *Hyssopus officinalis* L.

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РЕЗИМЕ: Симптоми трулежи семена *Hyssopus officinalis* L. примећени су током испитивања здравственог стања семена 2018. године. Према морфолошким и одгајивачким карактеристикама изолати припадају врстама *Fusarium* spp. и *Alternatia* spp.. Идентификација *Fusarium* spp. потврђена је применом ланчане реакције полимеразе са паром прајмера EF1 и EF2, при чему је амплификација и секвенционирање гена TEF-1α извршена за изолат JBL4003/3 (MK061542.1), чиме је потврђено да је изоп нови домаћин врсте *Fusarium graminearum* Schwabe.

КЉУЧНЕ РЕЧИ: изоп, *Fusarium graminearum*, трулеж семена