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Occurence, Identification and Phylogenetic Analysis of *Fusarium proliferatum* on Bean Seed (*Phaseolus vulgaris* L.) in Serbia

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Summary: During the routine quality control analysis on bean seeds in 2015, *Fusarium* fungal infection was observed on an average of 17% of the bean seed. The objective of this paper was isolation and identification of *Fusarium* sp. based on the pathogen's morphological and molecular characteristics. Morphological identification of *Fusarium* isolates was performed on PDA and CLA. DNA of 14 *Fusarium* sp. isolates was extracted directly from the mycelium (~ 100 mg wet weight), with a Dneasy Plant Mini Kit (Qiagen, Hilden, Germany). Following DNA extraction, the translation elongation factor 1-alpha region was amplified by PCR with the primer pair EF1 and EF2. An amplicon of 700 bp was amplified in all tested isolates. Identification of one isolate was performed by sequencing the translation elongation factor *EF-1a* gene. Completed morphological and molecular characteristics of isolates, as well as the results of sequencing confirmed that *Fusarium proliferatum* was the causal agent of bean seed rot.
Key words: beans, *EF-1a* gene, *Fusarium proliferatum*, *Phaseolus vulgaris*, sequencing

Introduction

Species of *Fusarium* has been described as the most common seed-borne fungi of great economic importance which attacks and can be transmitted by bean seeds. Seed-borne pathogens are responsible for the reduction of seed growing energy and seed germination, and therefore can jeopardize production of this crop (Mahmoud et al., 2013; Jasnić et al., 2005; Pavlović et al., 2012). Lević et al. (2009) indicate that *Fusarium* species periodically cause significant diseases, especially the wilting type of disease, on onion, garlic, tomato, but there is a lack of information about *Fusarium* species associated with bean seed (*Phaseolus vulgaris* L.) in Serbia. The most common seed-borne fungi on dry beans are: *Fusarium* sp., *Alternaria* sp., *Botrytis* sp., *Penicillium* sp., *Rhizopus* sp., *Cladosporium* sp. and *Trichotecium* sp. (Domijan et al., 2005). The objective of this study was isolation and identification of the causal agent of bean seed rot based on the pathogen's morphological and molecular characteristics.

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Materials and Methods

Isolation and morphological characterization

Bean seeds were immersed in NaOCl solution (1% available chlorine) for 5 minutes, and then drained. Seed subsamples (containing 400 seeds) were taken from 18 seed samples. Plating was performed aseptically and 10 seeds were placed onto blotter surface of each plate. Plates were incubated for 7 days at 25 °C in the dark. Observation of each seed was carefully performed under a stereo-microscope and the microscopic examination was done with different magnifications. The fragments of diseased seed were sterilized with 3% NaOCl for 3 minutes, dried and placed on potato dextrose agar (PDA), and then incubated for seven days at 25 °C. A total of 14 isolates of *Fusarium* spp. were obtained from the infected bean seed samples collected from storages and warehouses in Serbia. Fungi isolates were single-spored and sub-cultured, using single spore technique (Leslie and Summerell, 2006), on both PDA and Leaf carnation agar (CLA) for 7-10 days at 25 °C, in alternating cycles of 12 hours light and 12 hours darkness. Morphological identification of *Fusarium* isolates was done on PDA and CLA according to the taxonomy of Nelson et al. (1983) and Leslie & Summerell (2006).

Pathogenicity test

Bean seeds (ten seeds of each sample/two repetition) were artificially infected by immersing seeds in spore suspension (du Toit & Inglis, 2003). Plates were incubated at 25 °C and examined on daily basis for 7-10 days. *Fusarium* isolates were re-isolated and subcultured on PDA. Seed treated with known strain Fu12 were used as the positive control. Non-treated seeds were used as the negative control.

PCR and DNA sequencing

To identify 14 representative strains, amplification of TEF-1α gene, using polymerase chain reaction (PCR) was performed with the primer pair: EF1 and EF2 (O'Donnell et al., 1998), which directs the amplification of the 700-bp DNA fragment. Amplifications were carried out in a Mastercycler ep gradient S thermal cycler (Eppendorf, Germany) under the following programs: - 35 repeated cycles: 94 °C 1 min, 53 °C 1 min, 72 °C 2 min. The PCR mixture with a total volume of 25 µl consisted of 2x Eppendorf Master Mix (Taq DNA polymerase 1.25 U, 30mM Tris-HCl, 50mM KCl, 1.5 mM $MgCl_2$; 0.1% Igepal-CA630; 0.2 mM dNTP); 0.6 µM of each primer, and 1µl of fungal DNA. DNA fragments were amplified on the Eppendorf Mastercycler PCR device using the modified program by Abdel-Satar et al. (2003). The PCR products thus obtained were separated by electrophoresis on a 1% agarose gel over a period of 60 minutes using a constant voltage of 100 V, after which they were observed on a UV-transilluminator. The appearance of fragments with an expected size of 700 bp was considered a positive reaction. After completion of the reaction, PCR products were transferred to a pre-labelled 1.5 ml tubes and sent to sequencing. Purification and sequencing of the amplified fragments was done in Company MACROGEN, Seoul, South Korea (http://dna.macrogen.com). Sequences were analysed in the program FinchTV Version 1.4.0., and filed in the GenBank database under the National Centre for Biotechnology Information (NCBI).

Phylogenetic analysis

Manual corrections of aligned database, phylogenetic and molecular evolutionary analyses were conducted using MEGA 6 software package (Tamura et al., 2013). These gene sequences were assembled and edited using FINCHTV v.1.4.0 (http://www.geospiza.com). Multiple alignments and comparisons with reference strains for each of the genes were performed using CLUSTALW integrated into MEGA 6 software (Tamura et al., 2013). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the analysed taxa (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are presented as units of the number of base substitutions per site.

Results and Discussion

Morphological characterization of Fusarium species

During the routine quality control analysis on bean seeds in 2015, *Fusarium* fungal infection was observed on an average of 17% of the bean seed. Symptoms of *Fusarium* sp. on seeds appeared as white mycelium, usually with purple pigmentation around the infected seeds. Presence of *Fusarium* sp. was confirmed by microscopic examination of conidia. Pigments produced on PDA varied from light pink to dark red. Aerial mycelium was fast growing, with or without concentric rings, with an average growth rate of 8 cm in 7 days. On CLA microconidia were mostly in chain of single-celled microconidia, dimensions of 4-12 µm × 2-3 µm. Macroconidia were hyaline, almost straight, mostly with 3 septate. The size of macroconidia averaged 20-42 µm × 2-3 µm. All the isolates were classified as *F. proliferatum* based on morphological characteristics similar to *F. proliferatum* as reported by Leslie & Summerell (2006). All 14 tested isolates were found to be pathogenic. The symptoms of seed rot developed after 10 days of incubation at 25 °C. Infected seeds were covered with white mycelium usually with red to purple pigmentation, on blotter, under the infected seeds. On non-treated bean seeds symptoms of seed rot were not recorded. After re-isolation on PDA and CLA, fungi were identified based on the colony characters and spore morphology.

F. proliferatum is a common pathogen infecting field and vegetable crops worldwide. To increase the yield of bean, growers need healthy quality seeds with high percentage of germination (Jasnić et al., 2005). According to Neergard (1997) many important diseases are caused by seed-borne fungi. Species of *Fusarium* has been described as the most common seed-borne fungi of great economic importance which attack and can be transmitted by vegetable seeds, but there is a general lack of data about fusarioses transmitted by bean seeds in Serbia.

Sequencing and phylogenetic analysis

Genetic analysis of the translation elongation factor 1-α (TEF) sequence, confirmed that isolates originating from bean belong to *F. proliferatum* species showing 100% homology with *F. proliferatum* strain from GenBank (Accession number KM462983). TEF partial gene sequence of *F. proliferatum* was analysed to conduct a phylogenetic tree. Sequences generated in this study were added to the sequences of different *Fusarium* species selected from a BLAST search in NCBI GenBank for better understanding of their phylogenetic relationship (Geiser et al., 2004). A NJ tree constructed showed that the bean isolates were grouped together

with next reference strains from data base: *F. proliferatum* JX065071, *Gibberella intermedia* JX065073, *Gibberella fujikuroi* var. *intermedia* AM404116, *F. proliferatum* KM462983, *F. proliferatum* AB725613, *F. proliferatum* JX118983 and *F. thapsinum* JX268969 (Fig. 1). Morphological identification of *Fusarium* species is time consuming and molecular methods provide precise identification (Ignjatov et al., 2012). Completed morphological and molecular characteristics of isolates, as well as results of sequencing confirmed that *Fusarium proliferatum* is the causal agent of bean seed rot. Using primers EF1 and EF2, a specific band at 700 bp was obtained by PCR for 14 isolates. This part of the genome sequence is considered to be highly significant information on species level for the entire *Fusarium* genus (Summerell et al., 2003; Geiser et al., 2004) and one isolate (P1) was identified by sequencing the translation elongation factor *EF-1a* gene. For better determination of this pathogen, this study included the use of molecular characters to revealed genetic similarity with different *Fusarium* taxa. This study based on analysis TEF gene sequences confirmed that strain originated from bean had 100% homology to sequences of *F. proliferatum* strain KM462983 obtained from NCBI data base. In the previous studies TEF gene sequences were used to understand phylogenetic relationship among the members of the *Fusarium* group (Stępień et al., 2011; 2012).

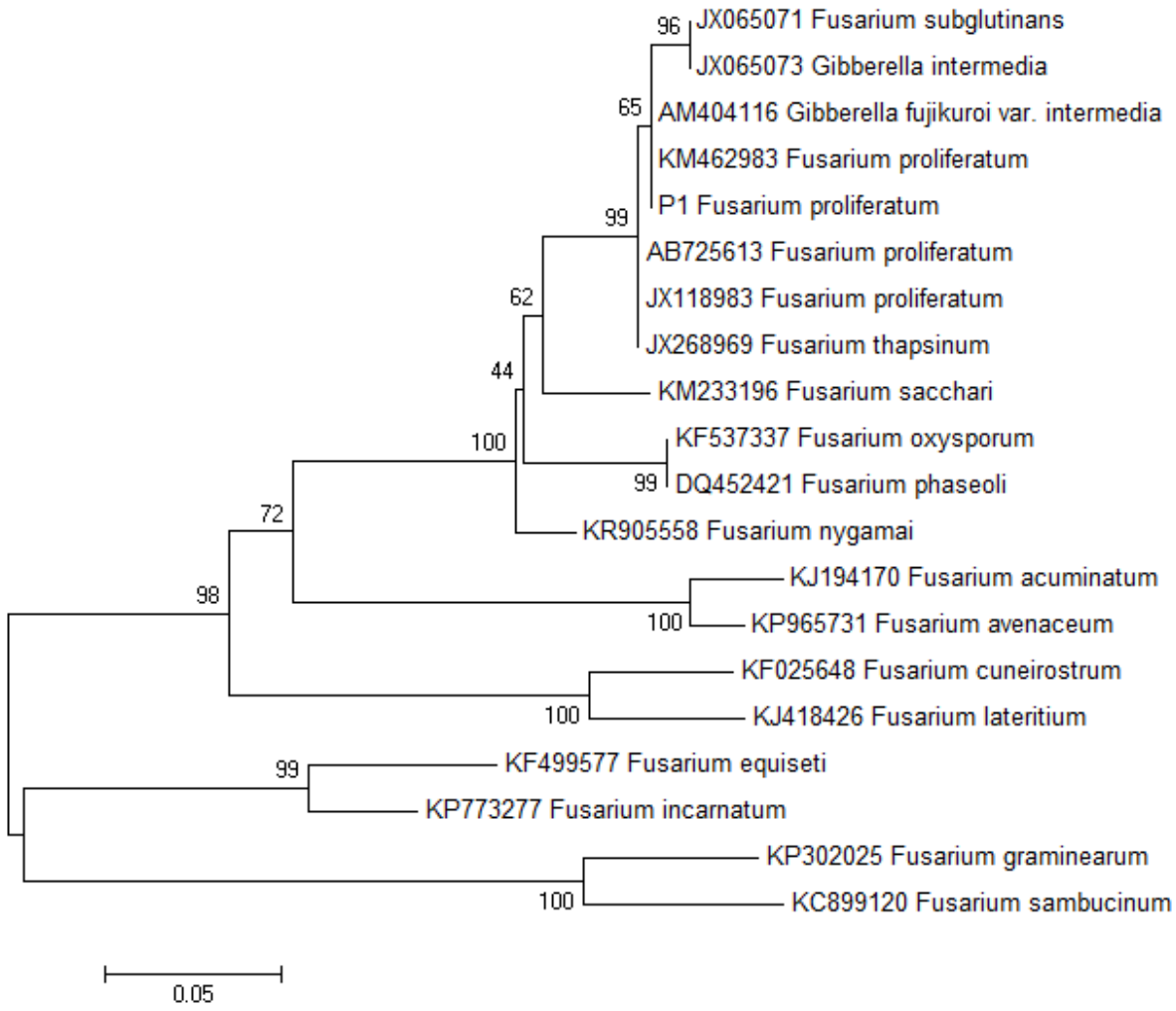

Figure 1. Phylogenetic tree based on Neighbour-Joining (NJ) analysis of TEF gene sequences for *F. proliferatum* isolates from bean and other *Fusarium* reference strains from NCBI data base. Bar – estimated nucleotide substitutions per site is 0.05.

Conclusions

Based on the morphological characteristics of fungal isolates, we confirmed presence of *Fusarium* sp. on bean seed. Completed morphological and molecular characteristics of isolates and sequencing results confirmed that *Fusarium proliferatum* is the causal agent of bean seed rot. Our study clearly revealed genetic differences of our isolates with other *Fusarium* species. The presence of this pathogen could reduce seed germination and decrease bean production in Serbia. These results will improve the base for the effective disease management strategies.

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Pojava, identifikacija i filogenetska analiza *Fusarium proliferatum* prouzrokovača truleži semena pasulja (*Phaseolus vulgaris* L.) u Srbiji

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Sažetak: Tokom rutinske kontrole kvaliteta semena pasulja (*Phaseolus vulgaris* L.) u 2015. godini ustanovljeno je prisustvo *Fusarium* sp. u visokom procentu, u proseku 17%. Cilj ovog rada bio je izolacija i identifikacija prouzrokovača truleži semena pasulja, na osnovu morfoloških i molekularnih karakteristika patogena. Odgajivačke karakteristike 14 odabranih izolata ocenenjene su na PDA i CLA podlogama. Ekstrakcija DNK 14 odabranih izolata obavljena je direktno iz 100 mg sveže micelije, korišćenjem Dneasy Plant Mini Kit (Qiagen, Hilden, Nemačka). Amplifikacija DNK obavljena je pomoću PCR korišćenjem para prajmera EF1 i EF2. U svim proučavanim izolatima formirani su amplikoni veličine 700 bp. Identifikacija jednog odabranog izolata izvršena je sekvenciranjem translacionog faktora *EF-1a* gena. Na osnovu dobijenih rezultata ustanovljeno je da je prouzrokovač truleži semena pasulja *Fusarium proliferatum*.

Ključne reči: EF-1a gen, *Fusarium proliferatum*, pasulj, *Phaseolus vulgaris*, sekvenciranje

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