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Molecular Characterization of *Fusarium proliferatum* and *F. equiseti* of *Pisum sativum* Seed

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ABSTRACT

Backgraund: The presence or absence of fungi on the seed surface is an important aspect that determines the quality of seeds. Since the data on the presence of *Fusarium* spp. and other seed-borne pathogens on vegetable pea in Serbia are scarce, the aim of this study was to isolate seed-borne pathogens and identify the disease-causing *Fusarium* spp. from the infected pea seed.

Methods: Colony morphology and microscopic characters of isolated *Fusarium* species were recorded from the cultures grown on PDA and CLA, respectively. In order to confirm the morphological identification at the species level, a single-spore culture of the representative isolates was subjected to genomic DNA extraction and gene amplification using the translational elongation factor 1α (TEF-1α) region. The PCR products were sequenced and the sequences were compared against the GenBank nucleotide database by using the BLAST alignment.

Result: Based on morphological and microscopic characteristics, as well as molecular identification by sequencing the TEF gene, the presence of *Fusarium equiseti* and *F. proliferatum* was confirmed on the representative isolates Ps18, Ps19 and Ps1, Ps36 obtained from vegetable pea seeds, respectively. According to our knowledge and research, this is the first report of *F. equiseti* and *F. proliferatum* on vegetable pea seeds in Serbia.

Key words: *F. equiseti*, *F. proliferatum,* Molecular identification, Phylogenetic analysis, *Pisum sativum*.

INTRODUCTION

Grain legumes are valuable worldwide, for their nutritional and health benefits and contribution towards agricultural sustainability (Kahlona *et al*., 2018). Vegetable pea (*Pisum sativum* L., Fabaceae) is a widely grown and popular vegetable crop and is cultivated throughout the world for the consumption of human being and animals. Pea is rich in protein (25%), sugars (8-12%), amino acids, carbohydrates, vitamin A and C, calcium and phosphorus and a small quantity of iron (Kumar *et al*., 2021). The total area in Serbia under vegetable peas is about 13,000 ha, while Vojvodina is the most important region for industrial pea processing with a total production of 6,000 ha (Jovićević, 2011).

Infected seed can provide primary inoculums for infestation of new crops and seed borne pathogens may be dispersed long distances (Dawson and Bateman, 2001). While more than 25 different species of fungi are known to invade stored grains and legumes (Duan *et al*., 2007), *Aspergillus, Penicillium* and *Fusarium* species are responsible for most spoilage and germ damage during storage (Kachapulula *et al*., 2017; Pszczółkowska *et al*., 2019). In addition, they can produce a wide range of secondary metabolites, many of which are toxic to humans or animals, making products unfit for human consumption or lower their market grade (Embaby *et al*., 2013). Moreover, fungal infestation of seed coat decreases seed viability, or may cause seedling abnormalities (Selcuk *et al*., 2008). Seeds for sowing should not contain pathogens, as pathogens are characterized by low germination, high diversity of species and varietal purity (Rathod *et al*., 2012). Therefore, the presence or absence of seed fungi on seed surface is an important aspect of seed quality.

Although morphological observations may not suffice for accurate identification, a great deal of information on a culture is usually obtained at this stage. However, for species that cannot be reliably identified in this way, additional analyses such as PCR assays and DNA sequencing must be conducted. The translation elongation factor 1-(TEF) gene appears to occur consistently as a single copy in *Fusarium*, exhibiting a high level of sequence polymorphism among closely related species even when compared to intron-rich portions of protein-coding genes like calmodulin, b-tubulin and histone H3. Thus, TEF has become the marker of choice as a single-locus identification tools in Fusarium (Geiser *et al*., 2004).

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Data on the presence of *Fusarium* spp. on pea seed in Serbia are scarce. Therefore, the aim of the study was to determine seed-borne fungal pathogens in pea and identify the isolates obtained from *F. proliferatum* and *F. equiseti* in pea seeds by means of molecular and conventional methods using the translation elongation factor gene (1-α TEF) of rDNA, as well as perform molecular characterization of the obtained isolates by comparing them with isolates from all over the world.

MATERIALS AND METHODS

Isolation and morphological identification of *Fusarium* spp.

During routine quality control in 2020, isolates of *Fusarium* spp. originating from pea seeds were collected and used for morphological, pathogenic and molecular characterization.

To avoid contaminants and obtain pure colonies of the pathogen, 400 seeds from each sample were surface-sterilized with a 1% sodium hypochlorite solution (NaOCl) for 2 min, washed with sterile water, dried on sterile filter paper overnight at room temperature, placed onto potato dextrose agar (PDA) and incubated for 7 days at 23±2°C.

After incubation, seeds were examined under a stereoscopic microscope and a compound microscope to detect seed-borne fungi. The fifteen colonies visually identified as *Fusarium* were single-spored by micromanipulation as described by Leslie and Sumerrell (2006) and were transferred to carnation leaf agar (CLA) and PDA for further conventional identification based on macroscopic (colony morphology and pigmentation) and microscopic (the shape, size and type of conidia) characteristics.

Pathogenicity test

The previously obtained isolate of the *Fusarium* species (Ps1, Ps18, Ps19 and Ps36) were tested for their pathogenicity on apparently healthy 20-day-old seedlings of pea (*P. sativum*) and cucumber (*Cucumis sativus*) grown in a glasshouse. The conidial suspension was prepared for each isolate by flooding the 7-day-old culture on PDA plates with sterile distilled water and gently scraping the conidia with a glass rod (Salleh and Sulaiman, 1984). Suspensions were adjusted to 1×10^3 conidia/ml and seedling roots were soaked in 20 ml of this conidial suspension for 20 minutes for root inoculation technique. For the stem inoculation technique, 20 ml of the conidial suspension of each individual isolate was sprayed on the stems. Five plants per isolate were used. Plants inoculated by booth techniques with sterile distilled water were used as negative controls. Inoculated and control seedlings were kept under greenhouse conditions. The presence of symptoms was observed three to four weeks post-inoculation. The inoculated fungi were re-isolated from the artificially infected plants to prove Koch's postulates.

Molecular detection and identification

Isolates morphologically identified as *Fusarium proliferatum* and *F. equiseti* were confirmed by amplifying the partial translation elongation factor-1α (TEF) gene with the specific primers EF1/ EF2 (Geiser *et al*., 2004). Fungal DNA of fifteen isolates was extracted directly from 100 mg of dry mycelium from 7-day-old cultures grown in a potato dextrose broth (PDB) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each 25 µl of reaction mixture contained 12.5 µl of 2X PCR Master Mix (Fermentas, Lithuania), 1.25 µl of each primer (100 pmol/ µl), 1 µl of DNK and 9 µl of RNase-free water. The reaction was performed in a thermal cycler (Eppendorf, Germany) under the following programs: an 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 c × 4 (Vilber Lourmat, Germany).

The amplified products from four selected isolates of fungi were purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced in both directions with an automated sequencer (ABI 3730 XL Automatic Sequencer, Macrogen, South Korea). The sequences generated in this study were deposited in the National Center of Biotechnology Information (NCBI) GenBank database (Table 1). The obtained sequences of the Serbian isolate were analyzed and compared with the previously deposited Fusarium isolates available in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/), using the ClustalW program (Thompson *et al*., 1994) and MEGA7 software (Kumar *et al*., 2016).

Phylogenetic analysis

Phylogenetic tree was created using Maximum Likelihood (ML) implemented in MEGA version 7.0 software (Kumar *et al*., 2016). The edited TEF- sequences generated in this study were compared to the GenBank sequences of 18 known *Fusarium* species (Table 2). Manual comparisons, corrections and alignments of the sequences were performed using Clustalw integrated into MEGA7 software. The reliability of the obtained tree was evaluated using the bootstrap method based on 1000 replicates and bootstrap values <50% were omitted. The best-fitting model of nucleotide substitution was investigated using the Model test implemented in MEGA7 and the Kimura 2-parameter model was chosen.

RESULTS AND DISCUSSION

Morphological characteristics

Seeds play an important role in producing an optimum yield through healthy crop production. Healthy seeds, particularly pathogen-free seeds, are necessary for the maintenance of optimum plant populations and production.

Alternaria spp. was the most frequently isolated fungal species among all isolates obtained in 2020 (70% of fungal isolates) and their average presence per sample was about 10-15%. Followed by species belonging to the genera

Penicillium, Rhizopus and *Cladosporium,* which were present in a slightly smaller percentage.

Fusarium species at were also detected on seed vegetable pea, up to 10% of infected seeds per sample. A total of 15 representative *Fusarium* isolates obtained from diseased pea seeds were examined macroscopically and microscopically. During morphological observation, seven isolates produced abundant aerial white mycelium initially and gradually turned violet with aging pigmentation on PDA (Fig 1a). After transfer to CLA (Fig 1b), these isolates formed microconidia in long chains or cohering in false heads. Slightly curved rather than straight macroconidia were formed, with a curved apical cell, mostly three to five septate, with average dimensions of 31 to 53 × 3.4 to 4.1 µm. Chlamydospores were absent. At the eight isolate noticed abundant, loosely floccose, whitish aerial mycelium with beige pigmentation (Fig 1c). On the CLA (Fig 1d), these isolates formed macroconidia with a tapered and elongated apical cell and prominent foot-shaped basal cell, which were typically four to five septate, with average dimensions of 21 to 60 × 2.8 to 4.6 µm. The isolate formed chlamydospores, but microconidia were not observed. Based on the description given by Leslie and Summerell (2006), cultural and morphological characteristics indicated that the seven isolates belong to *F. proliferatum* and eight isolates were identified as *Fusarium equiseti* (Corda) Sacc, respectively.

Pathogenic fungi of the genus *Fusarium* were identified in the analyzed pea seeds, but the severity of infections was generally low and the presence of this fungus did not significantly affect the quality of the seed. According to our knowledge, this is the first report of *F. proliferatum* and *F. equiseti* presence on pea seeds in Serbia.

Currently, the identification of members of the genus *Fusarium* is based on the characteristic morphology of the colonies and microscopic characters, which include the production of multiseptal sickle conidia called macroconidia (Hue *et al*., 1999). There are reports that mention the difficult recognition of *Fusarium* species when macroconidia are not produced in culture (Guarro and Gene, 1995), because we checked our morphological results using the molecular method based on the TEF gene, whereby the presence of *F. proliferatum* and *F. equisets* on pea seeds was successfully detected and confirmed.

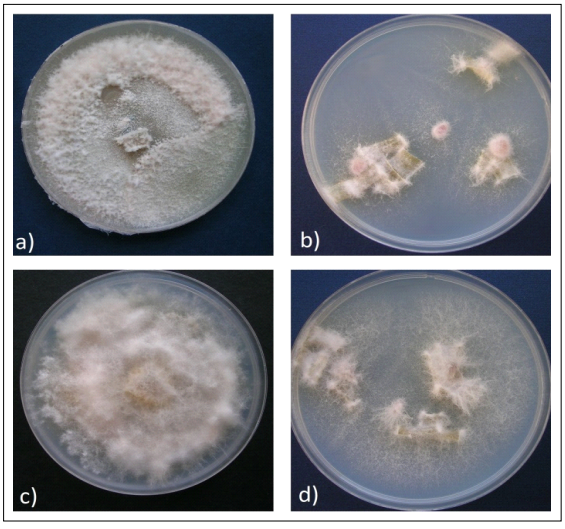

Fig 1: Colony of *Fusarium proloferatum*: (A): PDA (potato dextrose agar), (B): CLA (carnation leaf-piece agar) and *F. equiseti*: (C): PDA, (D): CLA.

Pathogenicity test

Symptoms such as discoloration, necrosis and later brown rot of the stems were observed two weeks after inoculation. The inoculated fungi were consistently isolated from the diseased plants in order to complete Koch's postulates and proved to be the causative agent of the disease. Neither the symptoms were visible nor could the pathogen be isolated from the negative control.

All the symptoms exhibited by inoculated plants are in correlation with earlier descriptions (Chehri *et al*., 2011). According to Agarwal and Sinclair (1997), it is essential to distinguish pathogens from other nonpathogenic fungi before establishing the inoculum thresholds for seeds to avoid a false positive assessment of seed health quality.

Molecular identification and characterisation of *Fusarium* spp.

The identification of four representative isolates (Ps1, Ps18, Ps19 and Ps36) based on morphological features at species level was further confirmed by molecular analyses. Molecular detection utilizing PCR and primers specific for the TEF gene successfully amplified one clear band of approximately 700 bp in all four Serbian isolates, as well as the positive control. The amplified DNA fragment of four isolates were sequenced in both directions using the TEF-1α and deposited in the GenBank. Sequencing the gene for translation elongation factor-1 alpha (EF1a) from the two Serbian isolates Ps18 and Ps19 (MZ351883 and MZ351884, respectively) of *F. equiseti*, revealed that they share 100% nt identity with *Glycine max* isolates Carm34 (MH315929), Carm35 (MH315930) and Carm 36 (MH 315931) from Canada. Isolate Ps1 (MZ351881) of *F. proliferatum* share 100% nt identity with ARSO-4 (KX940970) from USA, while isolate Ps36 (MZ351882) share 100% nt identity with EF10 (MN861748) from Spain.

A maximum likelihood tree (Fig 2), which was reconstructed based on the 1-α TEF sequences of different *Fusarium* species selected from GenBank, shows that Ps1 and Ps36 isolates obtained in this study were grouped with the isolates previously characterized as *F. proliferatum* (Acc. Nos. KF715258, KJ128964 and MK061541) while the Ps18 and Ps19 isolates were grouped with the isolates previously characterized as *F. equiseti* (Acc. Nos. MH315929, MH315930 and MH 315931).

PCR is a highly effective method for identifying pathogenic fungi (Kulik *et al*., 2005). This analysis is particularly useful in diagnosing seed-borne diseases which pose a threat in early stages of plant growth.

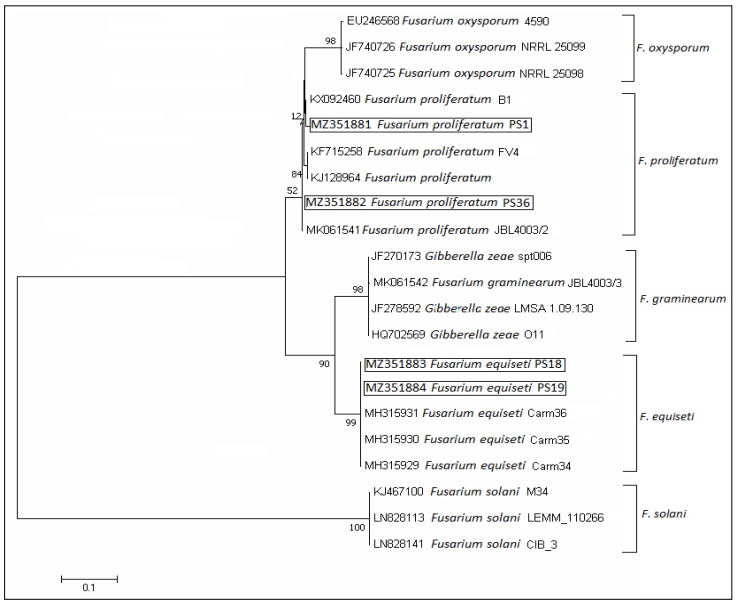

Fig 2: Phylogenetic tree (Maximum likelihood) showing the relationships among *Fusarium proliferatum* (Acc. Nos. MZ351881 and MZ351882) and *F. equiseti* (Acc. Nos. MZ351883 and MZ351884) from *Pisum sativum* compared with isolates of other *Fusarium* species, based on the sequences of elongation factor 1α (EF-1 α) gene. The four Serbian isolates are framed.

O'Donnell (2000) and Aoki *et al.* (2003, 2005) found that the DNA sequences of the ITS region can clearly represent evolutionary links between this complex of species and the TEF-1α gene sequence always offers finer resolution and separates strains of most species of the *Fusarium* complex by species. Therefore, a molecular study using TEF-1α gene was used for accurate identification of *Fusarium* species in this study. Also, molecular detection based on the TEF gene of *Fusarium* species could be a powerful tool in the identification of pathogenic species, giving results in a shorter period of time compared to morphological identification (Pavlović *et al.*, 2016).

Morphological characteristics of pathogenic isolates of *Fusarium* spp., especially conidial width, were important for the selection of isolates for further research. Differences and similarities at the genetic level were confirmed by sequencing the TEF-1α region.

CONCLUSION

The *Fusarium* isolates selected for this investigation were identified as *Fusarium proliferatum* and *F. equiseti* species, based on morphological characteristics and proved by the molecular analysis. Genetic structure and variability of the Serbian isolates remained largely unknown due to the lack of studies of *F. proliferatum* and *F. equiseti* populations in Serbian pea seeds; the study therefore represents the first attempt to characterize pathogens of the genus *Fusarium* associated with pea seeds in Serbia. Furthermore, to the best of our knowledge, this is the first report of *F. proliferatum* and *F. equiseti* presence on pea seeds in Serbia.

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Conflict of interest: None.

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