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EPICOCCUM NIGRUM PATHOGEN OF SUNFLOWER SEED IN SERBIA

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Abstract: Forty samples of sunflower seed (*Helianthus annuus* L.) were analyzed in period 2017-2018. Based on morphological characterization and pathogenicity test the fungus isolated from the tissues were initially identified as *Epicoccum* spp. The presence of *E. nigrum* was further confirmed by PCR and sequencing. A comparison of the obtained sequence with those available in GenBank confirmed the presence *E. nigrum* in sunflower seed samples.This study represents the first attempt to characterize pathogens of genus *Epicoccum* associated with sunflower seeds in Serbia.

Key words: sunflower, *Epicoccum* spp., sequencing, characterization

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oil seed crops grown throughout the world (Lentz et al., 2001, Balalić et al., 2012). In Serbia, sunflower is also the main oil crop with growing surface varies from 160,000 to 210,000 ha and seed yield ranging from 1.7 to 2.3 t/ha (Miklič et al., 2015). Sunflower seeds contain 38% to 50% high-quality oil, primarily used for human consumption (Castro and Leite, 2018).

Sunflower is affected by a large number of diseases caused by various fungi and other phytopathogenic microorganisms (Godika et al., 1996). The most important seed-borne pathogens represented the genera *Alternaria*, *Aspergillus, Cladosporium, Drechslera, Epicoccum, Fusarium* and *Penicillium* which could cause different levels of losses in its production (Khan 2007; Sharfun-Nahar and Mushtaq 2007; Lević et al., 2012).

Epicoccum nigrum L. (*E. purpurascens*) is an anamorphic Ascomycota with worldwide distribution. It is mainly associated with decay of plant tissues (Mims and Richardson 2005) and sometimes stated as a weak plant pathogen (Bruton et al., 1993; Schulz and Boyle 2005; Arnold, 2007). *E. nigrum* has been detected as the most frequent seed-borne fungi able to infect a wide variety of plant species in different countries (Mathur and Manandhar, 2003; Favaro, 2011). In Serbia, *Epicoccum* spp. is identified on seeds of soybean, sorghum, maize, sunflower, and coriander in low intensity, and the presence of this fungus does not affect significantly the quality of the seed (Lević et al., 2008, 2012; Ristić et al., 2012; Pavlović et al, 2014). In plant pest *E. nigrum* can be used as a biological control (Punja, 1997, Pieckenstain et al., 2001, Larena, 2004, De Cal, et al., 2009).

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Data on the presence of *E. nigrum* on sunflower grown in Serbia are scarce. The emergence of *E. nigrum* was first described by Aćimović (1998), but its presence has not been studied since. The main aim of the study was to identify the isolates obtained from *E. nigrum* in sunflower seeds by means of molecular and conventional methods, applying ITS regions of rDNA, as well as to perform molecular characterization of the obtained isolates by comparing them with isolates from all over the world.

Material and methods

Isolation of the fungal pathogen

During 2017-2018, isolates of *Epicoccum* spp. originating from sunflower seeds were collected and used for morphological, pathogenic and molecular characterization. In order to obtain pure colonies of the pathogen, 400 seeds of forty samples were previously sterilized with 1% NaOCl, washed with sterile water, dried on sterile filter paper, placed onto a potato dextrose agar (PDA; 200 g potato, 20 g dextrose, 17 g agar and 1 liter of distilled H_2O) and incubated for 5 days at 25°C. *Epicoccum*-like colonies were transferred onto fresh PDA and water agar (WA, 17 g agar and 1 liter of distilled H_2O) to obtain monosporial isolates.

Morphology of the pathogen

Pure cultures of four selected 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 of conidia) after 7 days.

Pathogenicity test

To confirm pathogenicity the sunflower plants grown in pots were inoculated with the previously obtained isolate of *E. nigrum*. All plants were inoculated with a suspension of conidia of 7-day-old fungal culture on PDA. Suspensions were adjusted to $1x10^3$ conidia/ml and injected plants 1 cm above soil. Plants injected with sterile water were used as negative control. Ten plants per isolate were used. After inoculation, plants were kept under greenhouse conditions. The presence of symptoms was observed three to four weeks post-inoculation. Reisolation was carried out from artificially infected plants in the same way as described earlier.

Molecular detection and identification

The molecular identity of the *E. nigrum* was confirmed by universal primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′), which hybridizes at the end of 18S rDNA, and ITS4 (5′TCCTCCGCTTATTGATATGC3′), which hybridizes at the beginning of 28S rDNA (White et al., 1990). Fungal DNA was extracted from 100 mg of dry mycelium from 7-days old cultures grown on potato dextrose broth (PDB) by DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Each 25 µl of reaction mixture contained 12.5 µl of 2X

PCR Master Mix (Fermentas, Lithuania), 1.25 µl of each primer (100pmol/ 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: initial denaturation of 2 min at 94°C, followed by 35 cycles of 2 min at 94°C, 30 s at 57°C, and 1 min at 72°C, with a final extension of 10 min at 72°C.

RT-PCR products were separated using electrophoresis on 1.5% agarose gel containing ethidium bromide (0.5 g/mL) and visualized using a UV light whith Bio-print cx4 (VilberLourmat, Germany).

The amplified product from one representative isolate (63Sun) was sequenced directly after purification with a QIAquick PCR Purification Kit (Qiagen). Sequencing in both directions was performed on an automated sequencer (ABI 3730XL Automatic Sequencer Macrogen, Korea). The sequence generated in this study was deposited in the National Centar of Biotechnology Information (NCBI) GenBank database. Obtained sequences of the Serbian isolate 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 MEGA5 software (Tamura et al. 2011).

Results and discussion

The isolates obtained in this study originated during 2017-2018 from sunflower seed were collected and identified on the basis of the morphological, pathogenic and molecular features.

Morphological characterization

Morphological features of all four selected isolates were uniform. Aerial mycelia of all isolates were strong yellow to orange, while the underside of colonies was orange-brown (Figures. 1 and 2). Colonies grow quickly, reaching about 6 cm in diameter in 2 days. The isolates formed single conidia on the slightly pigmented conidiophores. The size of conidia of all isolates ranged from 16 to 24µm. This characteristic is typical of genus *Epicoccu*m, as described by many authors (Aćimović, 1998; Mims and Richardson, 2005; Fávaro et al., 2011; Ristić et al., 2012; Nighat et al., 2016; Chen et al., 2017).

Slika 1. *Epicoccum nigrum*: izgled kolonije na PDA
Figure 1. Epicoccum nigrum: colony appearance on PDA media

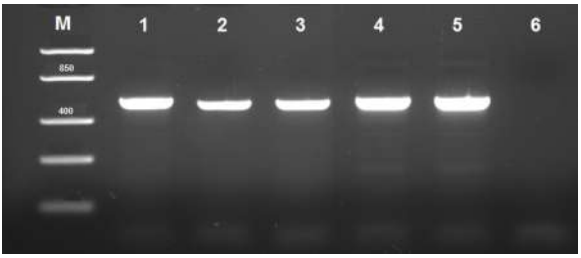
Slika 2. Konidije *Epicoccum nigrum*
Figure 2. Conidia of Epicoccum nigrum

The pathogenicity test

The pathogenicity test showed that all four selected isolates of *E. nigrum* caused development of prominent symptoms on inoculated sunflower seedlings, confirming their pathogenicity and satisfying the Koch's postulates. Five days after inoculation, the first changes were observed in stems of inoculated plants as dark necrotic spots surrounding the point of inoculation. Distinct changes on the roots were observed 10 days post inoculation. In all symptomatic sunflower seedlings, the presence of *E. nigrum* was confirmed by re-isolation and morphological comparison with a respective isolate. Neither the symptoms were visible nor could the pathogen be isolated from negative control, seedlings injected with sterile water. All inoculated plants produced the symptoms which are in correlation with earlier descriptions (Fávaro et al., 2011; Ogórek and Pląskowska, 2011)

Molecular detection and identification

Identification based on morphological features was further confirmed by ITS sequencing. Molecular detection utilizing PCR and primers specific for ITS region successfully amplified one clear band of the predicted size between 500-600 bp in all four Serbian isolates, as well as the positive control. (Figure 3). No amplification was obtained in the negative control (PCR mix with RNase-free water).



Slika 3. Elektroforetska analiza izolata Epicoccum, korišćenjem para prajmeraITS1/ITS4. Kolone: M -FastRulerTM Low Range DNA ladder, ready-to-use (Fermentas Life Sciences GmgH, Lithuania), 1- 61Sun, 2- 63Sun, 3- 73Sun, 4- 25Sun, 5- pozitivna kontrola, JBL539 (KX752419), 6-negativna kontrola (PCR mix sa vodom)
Figure 3. Electrophoretic analysis of four Epicoccum isolates using primer pair ITS1/ITS4. Lines: M -FastRulerTM Low Range DNA ladder, ready-to-use (Fermentas Life Sciences GmgH, Lithuania), 1- 61Sun, 2- 63Sun, 3- 73Sun, 4- 25Sun, 5- positive control, JBL539 (KX752419), 6-negative control (PCR mix with RNase-free water)

The amplified DNA fragment of representative 63Sun isolate was sequenced in both directionsand deposited in the GenBank (Acc.No. MH496036). The ITS sequences of our isolate showed 100% homology with the *E. nigrum* isolate MK051176 from China, the isolate MG813222 from Ireland, the isolate MH258972 from Iran, as well as two isolates (JQ619838, JQ619839) from Serbia.

Wu et al. (2017) and Colavolpe et al., (2018) also distinguished the *Epicoccum* species using universal primers ITS1/ITS4 for sequencing products the size of 550 bp in all the tested isolates.

Conclusion

The *Epicoccum* isolates selected for this investigation were identified as *Epicoccum* nigrum 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 *E. nigrum* populations in Serbian sunflower seeds; the study therefore represents the first attempt to characterize pathogens of genus *Epicoccum* associated with sunflower seeds in Serbia.

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