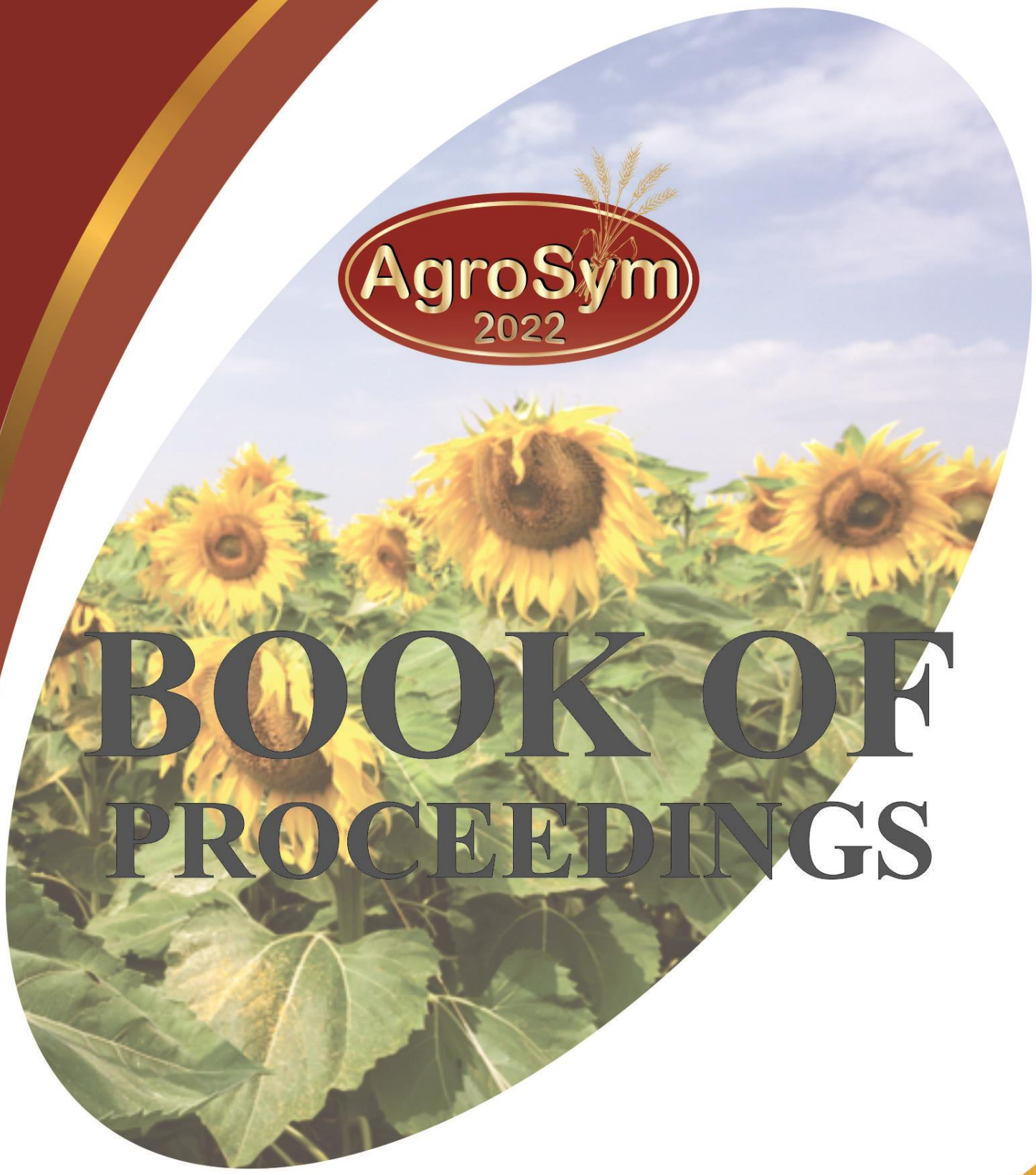

BOOK OF PROCEEDINGS

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PENICILLIUM EXPANSUM AS A POSTHARVEST PATHOGEN OF TOMATO FRUIT IN SERBIA

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Abstract

Tomato (*Solanum lycopersicum*, L.) is one of the most widely cultivated crops with high content of vitamins and antioxidant lycopene, which are very important for human health. During the growing season and postharvest storage tomato is susceptible to various diseases caused by pathogenic fungi. In July 2019, tomato (cv. Balkan) with symptoms of blue mold decay were collected from market in Belgrade, Serbia. Macroscopic morphology of three obtained monosporic isolates were observed after growth on Czapek yeast autolysate agar (CYA), creatine sucrose agar (CREA), and malt extract agar (MEA) for seven days at 25ºC. Also, selected isolates were incubated at 5, 25, and 37°C for one week on CYA to monitor the effect of different temperature incubation conditions. Colony characteristics and micromorphology of the fungi agreed with the literature descriptions of *Penicillium expansum*. The conidiophores of isolates were hyaline, mainly terverticillate; stipes usually smooth-walled; metulae and phialides cylindrical; conidia ellipsoidal (3-3,86-4 × 3-3,13-4 µm), greenish, smooth-walled. Total DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and partial β-tubulin (*BenA*) sequence was amplified with primers Bt2a/Bt2b. *BenA* sequence of representative isolate ParP/1 was deposited in NCBI GenBank (Accession No. ON186699). Phylogenetic analysis clustered our isolate with other isolates of *P. expansum*. Pathogenicity test was conducted on symptomless, detached tomato fruits. All tested isolates caused typical blue mold symptoms on tomato fruits after seven days of incubation. To our knowledge, this is the first report of *P. expansum* causing postharvest fruit decay on tomato in Serbia.

Keywords: *Tomato, Postharvest decay, Penicillium expansum, Identification*

Introduction

Tomato (*Solanum lycopersicum*, L.) is one of the most widely cultivated crops in the world. Tomato fruits are a great source of vitamins C and K, potassium, and antioxidant lycopene which are very important for human health. Water-soluble nutrients and high moisture content in tomato fruits make them perishable and susceptible to a number of postharvest fungal pathogens, such as *Alternaria alternata, Cladosporium cladospirioides, Geotrichum candidum, Botrytis cinerea, Fusarium acuminatum, Rhizopus stolonifer, Talaromyces miniolutes, Penicillium polonicum, P. solitum* and *P. olsonii* (Chatterton *et al.,* 2012; Singh *et al.*, 2017; Petrasch *et al.*, 2019; Ma *et al.,* 2020; Stošić *et al.,* 2020; Slathia *et al.,* 2021, Živković *et al.,* 2021).
Blue mold decay caused by *P. expansum*, is one of the most economically important postharvest plant disease, globally. Cosmopolitan distributions of *P. expansum*, decay-producing capability coupled with a strong virulence are properties that characterize this species as a broad spectrum pathogen (Pitt and Hocking, 2009). Wounds such as punctures created at harvest and during postharvest handling are the primary avenue for infection of fruits by this fungus. Economic

losses caused by the disease are mainly attributed to lower fruit quality and marketability. Also, it is important to note that *P. expansum* is a producer of mycotoxin patulin and other secondary metabolites (citrinin, chaetoglobosins, communesins, roquefortine C, and expansolides A and B) which can compromise human health (Andersen *et al.,* 2004).
In Serbia, postharvest decay caused by *P. expansum* are detected on apple, pear, quince, and medlar fruit, and onion bulbs (Vico *et al.,* 2014; Duduk *et al.,* 2017; Stošić *et al.,* 2021; Žebeljan *et al.,* 2021). To our knowledge, there are no literature data about blue mold fruit decay caused by *P. expansum* on tomato in Serbia. Therefore, the objective of this study was identifying the causal agent of this disease on tomato using morphological, physiological and molecular methods.

Material and methods

Fungal isolation

In July 2019, tomato fruits (cv. Balkan) with symptoms of blue mold decay were collected from market in Belgrade (Serbia). The decayed area of the fruits was pale yellow and tissue was soft and watery. Blue sporulation was abundant on the fruits surface. Small pieces on the margin of diseased and healthy tissue were surface sterilized in 3% NaOCl for 3 min, followed by several rinses with sterile distilled water, and placed on malt extract agar (MEA) for seven days at 25°C. Three monosporial isolates were obtained and characterized using morpho-physiological and molecular methods.

Morphological and physiological characterization

Macromorphology of the isolates were examined on three media: MEA, Czapek Yeast Autolysate agar (CYA), and Creatine sucrose agar (CREA). Cultures were three-point inoculated with 1 µl of conidial suspension and incubated for seven days at 25°C (Visagie *et al.,* 2014). After the incubation, colony growth and texture were noted. Also, the isolates were inoculated on CYA plates and incubated for seven days in the dark at 5, 25, and 37°C to monitor the effect of different temperature incubation conditions. The experiments were performed in three replicates, and basic descriptive values (average and standard deviation) were done in Microsoft Excel 2007 (Microsoft Corporation, U.S.A.). Microscopic slides were prepared from ten day old MEA cultures with 60% lactic acid. Morphological features of conidiophores, phialides and conidia (shape, cell wall ornamentation) were observed using Olympus microscope (BX51, Japan). Measurements of conidia (length and width) were done in Quick Photo Camera software program (Promicra, s.r.o., Czech Republic).

DNA extraction, PCR amplification and phylogenetic analysis

Genomic DNA was isolated from mycelium scraped from the surface of a seven days old MEA culture with DNeasy Plant Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Partial sequence of *BenA* gene was amplified in a polymerase chain reaction (PCR) using Bt2a/Bt2b primer pair (Glass and Donaldson, 1995). Thermal cycle conditions set up as recommended by Visagie *et al.* (2014) - an initial denaturation 5 min at 94 °C; 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72°C; and a final denaturation step of 7 min at 72 °C. Reaction

mixture contained 20 µl of 2×PCR Master Mix (TaqNova-RED, DNA Gdansk, Poland), 4 µl of each primer (Metabion International AG, Germany), 10.4 µl of sterile nuclease-free water (Thermo Fisher Scientific, U.S.A.), and 1.6 µl of fungal DNA. Amplified products were analyzed by 1% agarose gel electrophoresis, stained with Midori Green DNA Stain (Nippon Genetics), and visualized under a UV transilluminator. The products of PCR were purified and sequenced by the Macrogen's Europe commercial sequencing service (Amsterdam, the Netherlands). Clustal W algorhitm (Thomson *et al.,* 1994) implemented in MEGA7 software (Kumar et al, 2016) was employed to assembly sequence contigs. Maximum likelihood (ML) phylogenetic tree was constructed in the same software, using reference and reliable *BenA* sequences from NCBI GenBank database.

Pathogenicity assay

To confirm the pathogenicity of our isolates, symptomless, detached tomato fruits (cv. Balkan) were first surface-sterilized in 70% ethanol, and then inoculated with 50 µl of a spore suspension (1×10^6 conidia/ml) from the cultures grown seven days on MEA. The control fruits were inoculated with 50 µl of sterile distilled water. All inoculations were done in three replicates. The fruits were placed in a sterile plastic container at 25°C, and >95% relative humidity. After seven days reisolation of the pathogenic fungi was performed, and obtained cultures were checked for colony and spore morphology to confirm Koch's postulates.

Results and discussion

Identification and characterization of the causal agent of blue mold decay on tomato is essential to establish the basis of studies on epidemiology and disease control. Morphology and physiology of isolates from tomato fruits (ParP/1, ParP/3 and ParP/4) were uniform. After seven days of incubation at 25°C, all isolates had radially sulcate colonies on CYA (Figure 1B), and plane to moderately radially sulcate on MEA. Cultures manifested different textures on tested media – velvety to loosely floccose on CYA, fasciculate on CREA, plane and weak fasciculate on MEA. Reverse, colonies had light brown colour with brighter margin (CYA), pale yellow with a hint of greenish (MEA), and purple with a yellow margin (CREA). Intensive conidiogenesis was present, with blue-green spores on CYA, and green on MEA and CREA. Isolates had white mycelia on all inoculated media, present as the margin of the culture, broad on CYA (5 mm width), and moderate on CREA (2 mm) and MEA (1.5 mm). Clear exudate droplets were noticed on CREA, while there were no exudate formation on CYA and MEA. All tested isolates manifested intensive growth across all tested media, with CYA being the most stimulative medium (Figure 2A). Isolates cultivation at different temperatures on CYA showed that the optimal temperature for their development was 25°C, and the smallest average diameter was measured at 5°C. Fungal growth was not recorded at 37°C (Figure 2B). Microscopic observations revealed that all isolates formed hyaline, mainly terverticillate conidiophores; stipes usually were smooth-walled; metulae and phialides were cylindrical; and conidia were ellipsoidal (3-3,86-4 × 3-3,13-4 µm), greenish, and smooth-walled. Colony characteristics, micromorphology and physiology of our isolates agreed with the literature descriptions of *P. expansum* (Frisvad and Samson, 2004; Pitt and Hocking, 2009; Visagie *et al.,* 2014; Vico *et al.,* 2014).

All tested isolates caused typical *Penicillium*-like decay on tomato fruits after seven days of incubation. No symptoms were observed on any of the control fruits (Figure 1A). Isolates recovered from inoculated tomato fruits showed the same morphological characteristics as the original isolates, thus completing Koch's postulates.

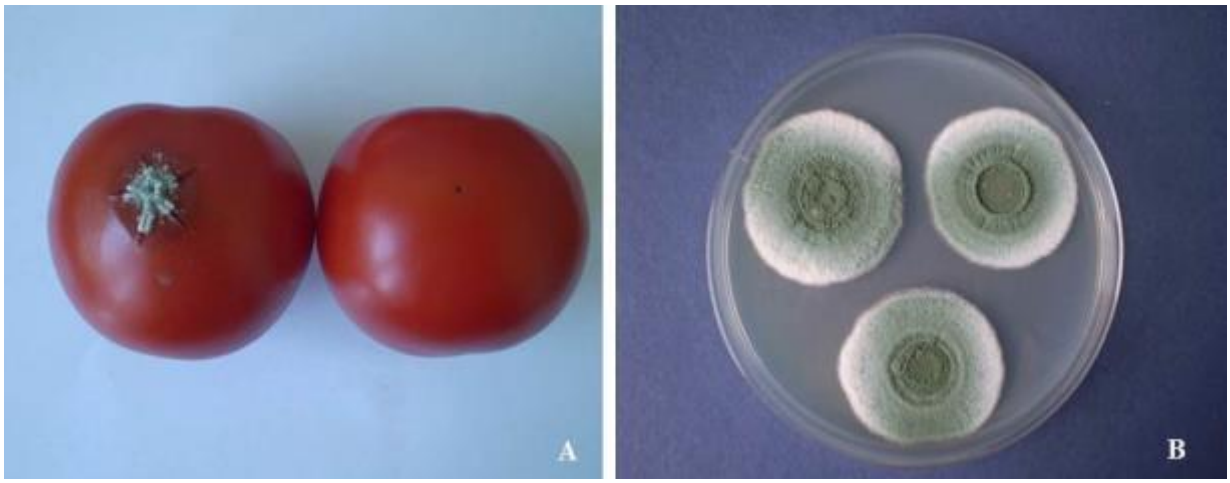

Figure 1. A. Pathogenicity of isolate ParP/1: tomato fruit inoculated with pathogen (left) and control fruit without symptoms (right). B. Colony characteristics of isolate ParP/1 after seven days of incubation at 25°C on CYA.

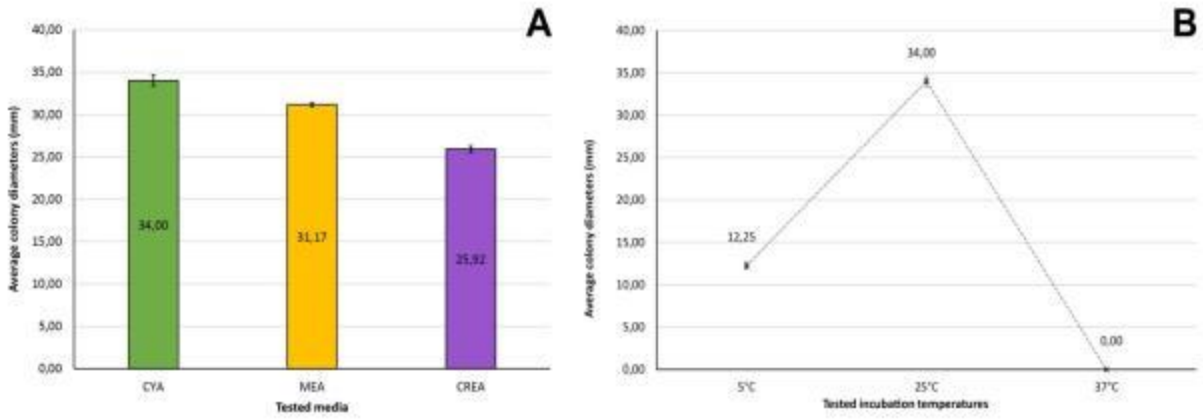

Figure 2. A. The average colony growth of *P. expansum* isolates (mm) on three tested solid media after seven days of incubation at 25°C. B. The average colony growth of *P. expansum* isolates (mm) on CYA after seven days of incubation at three tested temperatures. Vertical error bars represent standard deviation (SD) in both graphs.

Molecular analyses of genomic DNA from tomato isolates confirm the identity of the pathogen. The sequence of representative isolate ParP/1 was submitted to the NCBI GenBank database (Accession No. ON186699). Phylogenetic analysis for selected *BenA* sequences of *Penicillium* revealed that all *P. expansum* isolates (including Serbian) formed a separate clade with a high bootstrap support (98%), (Figure 3). Within that clade, Serbian sequence was separated in one subclade while other *P. expansum* sequences were grouped into the second subcluster. This

separation had relatively high node support (71%). Sequences of the ITS region have been used before for resolving *Penicillium* phylogenies (Skouboe *et al.*, 1999). Because its resolution is limited at species level in *Penicillium*, *BenA* is proposed as the secondary molecular marker, especially in a routine identification procedure (Visagie *et al.*, 2014).

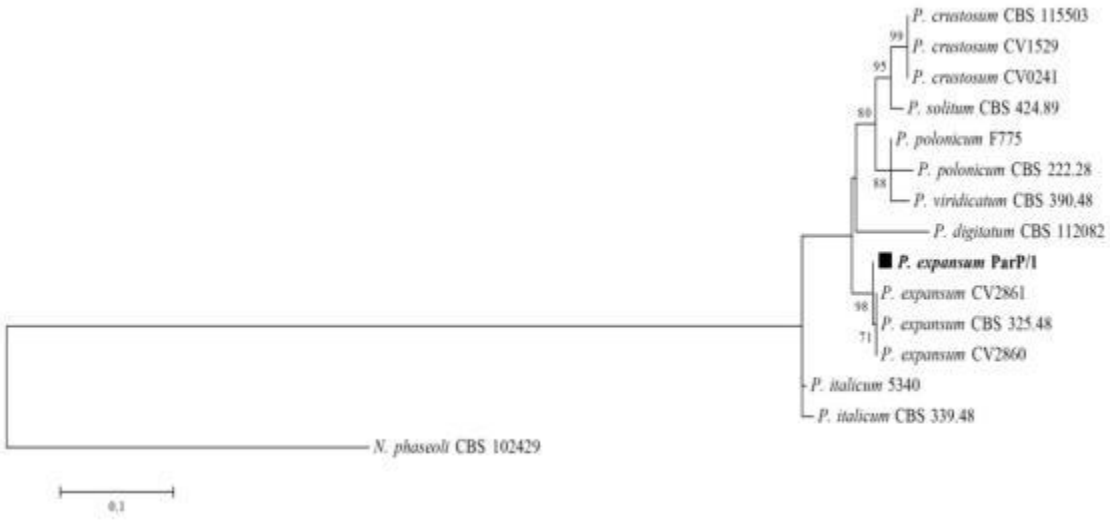

Figure 3. Maximum likelihood (ML) phylogenetic tree based on *BenA* sequences of selected *Penicillium* species. The isolate of *Neocosmospora phaseoli* (CBS 102429) represented an outgroup sequence. Bootstraping was performed in 1,000 replications and bootstrap values <70% are omitted. The scale depicts the number of substitutions per site. Isolate in bold font and with black square is from this study.

Conclusion

Based on morphological and physiological characterization, pathogenicity test, and molecular and phylogenetic analyses, the isolates from this study were identified as *P. expansum*. To our knowledge, this is the first report of *P. expansum* associated with fruit decay on tomato in Serbia. Knowledge of the populations of *Penicillium* species of tomato fruits in Serbia is of great importance due to their ability to reduce quality and shelf life. Also, obtained results provide the base for development of effective disease management strategies.

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