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ORIGINAL ARTICLE

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POSTHARVEST DECAY OF MANDARIN FRUIT IN SERBIA CAUSED BY *Penicillium expansum*

ABSTRACT: Mandarin fruits are one of the most popular among the *Citrus* genus. They are consumed because of their nutritional and health benefits, as well as pleasant taste and smell. This paper describes the identification and characterization of *Penicillium expansum*, isolated from molded mandarin fruits. The obtained isolates were cultivated on five media [Czapek Yeast Autolysate agar (CYA), MEA (Malt extract agar), Creatine sucrose agar (CREA), Yeast extract sucrose agar (YES), and Oatmeal agar (OA)] and at five different incubation temperatures (5, 15, 25, 30, and 37 °C). Isolates were sequenced for two molecular loci: internal transcribed spacer and beta-tubulin. Based on the results from morphological, physiological, molecular, and phylogenetic analyses, the recovered isolates were identified as *P. expansum*. The isolated species was confirmed as pathogenic to mandarin fruits in a pathogenicity test. To the best of our knowledge, this is the first report of *P. expansum* as a postharvest pathogen of mandarin fruit in Serbia.

KEYWORDS: *Citrus reticulata*, identification, morphological analysis, molecular characterization, pathogenicity

INTRODUCTION

Citrus fruits (oranges, lemons, limes, grapefruit, tangerines, and their varieties) are among the most important crops worldwide. They belong to the Rutaceae plant family and they are cultivated in more than 140 countries across the globe (Liu et al., 2012; Wu et al., 2018). Their nutritional values make them an important part of the healthy human diet.

One of the most popular citrus fruits is mandarin (*Citrus reticulata* Blanco). It encompasses many varieties, hybrids, and mutants that have distinct names, but several terms are used in colloquial speech (mandarins, clementines, tangerines, etc.). Together with citron (*C. medica*) and pummelo (*C. maxima*), mandarins are considered ancestral *Citrus* species (Wu et al., 2018). The tasty

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flavour, alimentary importance, seedlessness in some sorts, the ease of peeling, and the small amount of the residual oils on hands during peeling are some of the reasons for being often the first choice among the consumers of citrus fruits (El-Otmani et al., 2011; Wang et al., 2018). Mandarin fruits are consumed in fresh form, used for mandarin juice production, or applied in the manufacturing of jellies, essential oils, confectionery, and sweets. Mandarin juice is also used for adulteration of other citrus juices or for making fermented beverages (Putnik et al., 2017). Nutritional benefits of mandarin fruit consumption include vitamins C, E, and A, carotenoids (phytoene, β-cryptoxanthin, and violaxanthin), phenolic compounds, sugars, organic acids, potassium, phosphorus, magnesium, amino acids (asparagine, arginine, aspartic acid, proline, and glutamine). These compounds have various antioxidant, anti-inflammatory, anticarcinogenic, and cardioprotective effects on human health (Codoñer-Franch and Valls-Bellés, 2010; Putnik et al., 2017).

Production of mandarin fruit and its varieties in 2018 was 34,393,430 tons with a steadily increase in the last three decades (FAOSTAT, 2020). Mandarins and belonging hybrids are ranked the second of all the citrus fruits produced in the world (Palou et al., 2015). In Serbia, the annual import of mandarin/clementine/tangerine fruits in 2019 was 28,568.4 tons. Mandarin with its varieties was the second of all the citrus fruits, and the fourth of all the fruits imported in Serbia in 2019 (*Statistical Office of the Republic of Serbia*, 2020).

Many fungi can attack citrus fruits (including mandarins) and cause diseases, in the field or after harvest. Pathogens that infect the plants in the orchard often can remain latent and the disease can develop later in the packinghouse. Some of the most important postharvest fungal pathogens from this group are *Lasiodiplodia theobromae*, *Phomopsis citri*, *Alternaria citri*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Phytophthora citrophthora*. Pathogenic fungi from the second group infect the mandarin fruit through injuries or micro-wounds. These are known as wound pathogens and some of the characteristic species that cause postharvest diseases are *Penicillium digitatum*, *P. italicum*, and *Geotrichum candidum* (Barkai-Golan, 2001; Palou et al., 2015). Two listed *Penicillium* species are considered maybe the most important postharvest diseases of citrus fruits, contributing up to 90% of the total loss (Yang et al., 2019). Besides mentioned, other species of *Penicillium* spp. are reported as pathogens of mandarin fruit and its varieties/hybrids: *P. crustosum* (Garcha and Singh, 1976), *P. ulaiense* (Tashiro et al., 2012; Park et al., 2018), and *P. expansum* (Moosa et al., 2019). *P. expansum* was also isolated from mandarin fruit (*Citrus unshiu* (Swingle) Marcov.) in a study by Liu et al. (2009), but the pathogenicity test was not performed.

To the best of our knowledge, the only reported postharvest pathogen of mandarin fruit in Serbia is *C. gloeosporioides* (Živković et al., 2012). Considering that there are no literature data about postharvest diseases caused by *Penicillium* species on stored mandarin fruit in Serbia, the aim of this study was to elucidate the etiology of the *Penicillium*-like decay, to identify the causal agent(s) using the polyphasic approach – combining morphological, physiological, and molecular methods – and to test the pathogenicity of the obtained isolates.

MATERIALS AND METHODS

Sample collection and fungal isolation

Mandarin fruits with blue mold symptoms were collected from the markets in Serbia during 2017–2019. Symptoms included pale, watery spots or the presence of the blue sporulation on the fruit surface. Forty-eight samples have been collected in total and the isolations were carried out immediately upon bringing them to the laboratory. Small pieces on the margin of the diseased and healthy tissue were removed with a sterilized scalpel and surface-sterilized for 3 min in 1% aqueous home bleach solution. After that, pieces were rinsed three times in sterile distilled water, placed and incubated on malt extract agar (MEA) for 7 days at 25 °C. Mycelial fragments were taken from the growing colony margin and transferred to new MEA plates. Ten monosporial isolates were produced and preserved on potato dextrose agar (PDA) slants in the refrigerator at 4 °C.

Morphological and physiological characterization

The morphology of these ten isolates was examined on five media: Czapek Yeast Autolysate agar (CYA), MEA, Creatine sucrose agar (CREA), Yeast extract sucrose agar (YES), and Oatmeal agar (OA). Cultures were three-point inoculated with 1 µl of conidial suspension and incubated for 7 days at 25 °C (Visagie et al., 2014). After the incubation period, colony growth and texture, degree of sporulation, the colour of spores, the presence and colours of soluble pigments and exudates, colony reverse colours, and degree of growth and acid production (on CREA) were recorded.

The isolates were also inoculated on CYA plates and incubated for 7 days in the dark at five different temperatures (5, 15, 25, 30, and 37 °C) to examine the growth and the appearance of the cultures and to detect limiting temperatures for fungal development.

Microscopic observation

Microscopic slides were prepared from 10-day old MEA cultures with 60% lactic acid used as a mounting fluid (Visagie et al., 2014). Conidiophores' branching patterns, shape and texture of phialides, the shape of conidia and ornamentation of the conidial cell wall were recorded for all tested isolates. Observations were performed using an Olympus microscope (model BX51, Olympus Corporation, Japan) and slides were photographed with Olympus camera (model E620, Olympus Corporation, Japan) attached to the microscope. Measurements of conidia (two diameters – length and width) were done in the Quick Photo Camera software program (PROMICRA, s.r.o., Czech Republic).

DNA extraction and PCR

Genomic DNA was extracted from cultures grown on MEA for 7 days at 25 °C. A sterile blade was used to scrape roughly 100 mg of mycelium from the plate surface and to transfer it to a 2 ml centrifuge microtube. Harvested mycelium was frozen with liquid nitrogen and ground using a sterile micropestle. Fungal DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The obtained DNA was preserved at -20 °C until further use.

To confirm morphological identification, partial sequences of the internal transcribed spacer (ITS) region of the rDNA and β-tubulin gene (*BenA*) were amplified in a polymerase chain reaction (PCR), using the fungal specific primers V9G/LS266 and Bt2a/Bt2b, respectively (Glass and Donaldson, 1995; Masclaux et al., 1995; de Hoog and van den Ende, 1998) and following the instructions from Visagie et al. (2014). Amplification conditions were as follows: an initial denaturing step of 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C, and a final denaturing step of 7 min at 72 °C. The compounds and volumes for PCR reactions were prepared as described in our previous work (Stošić et al., 2020).

Sequencing and phylogenetic analyses

The same primers used for PCR amplifications were also used for the sequencing of the obtained PCR products. Purification and sequencing in both directions of the recovered PCR amplicons were done by Macrogen's Europe commercial sequencing service (Macrogen Europe B.V., Amsterdam, the Netherlands). The sequences' quality was examined using FinchTV software (version 1.4.0, www.geospiza.com/finchtv) and the ClustalW algorithm (Thompson et al., 1994) integrated into MEGA7 software (Kumar et al., 2016) used to compute consensus sequences. Nucleotide BLAST search algorithm was applied to compare the similarity of the sequences from this study with the fungal sequences from the NCBI GenBank database. After the similarity search and definitive identification, all generated sequences were deposited in the NCBI GenBank database (Table 1, accession numbers MT556009 and MT556010 (ITS), MT563326, and MT563327 (*BenA*)).

The phylogeny was assessed using MEGA software (version 7.0.26, Kumar et al., 2016). The maximum likelihood (ML) tree was constructed for a combined dataset of ITS and *BenA* sequences (Table 1). The gamma-distributed Tamura-Nei model (G+I) with invariant sites was employed as the best fitting model of nucleotide substitution (5 discrete gamma categories, complete deletion of gaps/missing data treatment, nearest neighbour interchange as ML heuristic method). The reliability of the obtained trees was evaluated with 1,000 bootstrap replications for branch stability. Bootstrap confidence values less than 70% were omitted and the sequences of *Aspergillus niger* (isolate NRRL

326) were designated as an outgroup in all analyses. Phylogenetic trees were edited and prepared for publication in Adobe Illustrator CS6 (Adobe, U.S.A.).

Table 1. Accession numbers of *Penicillium* spp. isolates with collection details used in the phylogenetic analysis; isolates from this study are bolded.

Species	Strain/isolate	Substrate and origin	GenBank accessions	
			ITS	*BenA*
A. niger	NRRL 326 = CBS 554.65	Tannin-gallic acid fermentation, Connecticut, U.S.A.	EF661186	EF661089
P. allii	IBT 3056=CBS 188.88	Food item, U.K.	AJ005484	AY674333
P. crustosum	FRR 1669 = CBS 115503 = IMI 091917	Lemon fruit, Aberdeen, Scotland, UK	AY373907	AY674353
P. crustosum	SFC20140101-M781 = 5501	Unknown	KJ527442	KJ527407
P. crustosum	CV1267 = DTO182I3	*Protea repens* infructescence, Riverlands (Malmesbury), South Africa	JX091401	JX091537
P. crustosum	CV1529 = DTO183C4	*Protea repens* infructescence, Riverlands (Malmesbury), South Africa	JX091402	JX091538
P. crustosum	CV0241 = DTO181D2	*Protea repens* infructescence, Stellenbosch, South Africa	JX091403	JX091536
P. crustosum	CV0251 = DTO181D6	Mite from *Protea repens* infructescence, Stellenbosch, South Africa	JX091404	JX091530
P. crustosum	CNU 6043	Apple fruit, Yesan, Korea	HQ225711	HQ225724
P. digitatum	CBS 112082	Lemon, Italy	KJ834506	KJ834447
P. expansum	CBS 325.48 = ATCC 7861	Apple fruit, U.S.A.	AY373912	AY674400
P. expansum	SFC20140101-M737 = 5537	Unknown	KJ527444	KJ527409
P. expansum	F758	Sugar beet root, Idaho, U.S.A.	MG714838	MG714864
P. expansum	CV2860 = DTO180F6 = CV 407	Soil, South Africa	FJ230989	JX091539
P. expansum	CV2861 = DTO180F7 = CV 432	Soil, South Africa	FJ230990	JX091540
P. expansum	CNU 7003	Apple fruit, Daejoen, Korea	HQ225715	HQ225727
P. expansum	**MP/4**	**Mandarin fruit, Serbia**	**MT556009**	**MT563326**
P. expansum	**MP/5**	**Mandarin fruit, Serbia**	**MT556010**	**MT563327**
P. italicum	CBS 339.48	Citrus fruit, Riverside, CA, U.S.A.	KJ834509	AY674398
P. italicum	SFC20140101-M724 = 5340	Unknown	KJ527447	KJ527412
P. italicum	CNU 6089	Apple fruit, Yesan, Korea	HQ225716	HQ225728
P. polonicum	CBS 222.28 = NRRL 995	Soil, Poland	AF033475	AY674305
P. polonicum	F775	Sugar beet root, Idaho, U.S.A.	MG714841	MG714868
P. solitum	CBS 424.89 = FRR 937	Unknown, Germany	AY373932	AY674354
P. solitum	CNU 4096	Apple fruit, Daegu, Korea	HQ213935	HQ225721
P. viridicatum	CBS 390.48 = DTO 005-C9 = FRR 963	Air, Washington DC, U.S.A.	AY373939	AY674295

Pathogenicity assay

A pathogenicity test was conducted on intact, symptomless mandarin fruits for all obtained isolates. Mandarins were surface-sterilized with 70% ethyl alcohol, allowed to air dry, and then wounded with a sterile needle. Fifty microliters of spore suspension were injected into the wound using an automated micropipette and the corresponding micropipette tip. The conidial suspension was prepared by diluting the spores collected from the 14-day-old MEA cultures in 1 ml of sterile distilled water. Spore concentration was determined and adjusted to a concentration of 1×10^6 spores/ml using a Neubauer hemocytometer. Fruits inoculated with 50 µl of sterile distilled water represented negative control. Three replicates per isolate were used. All tested fruits were placed in plastic boxes and incubated at 25 °C and 95% relative air humidity. Seven days after the inoculation, the developed symptoms were observed and the horizontal and vertical (stem-calyx axis) diameters of the lesions were measured. Reisolations from the inoculated fruits were performed on MEA using the same method as previously described. The symptoms expressed on the artificially wounded fruits, colony appearance and growth, and conidiophore and spore morphology were inspected to check the fulfillment of Koch's postulates.

Data analysis

All experiments were performed in three replicates per isolate. Basic descriptive values (average and standard deviation) were computed for each colony, spore, and lesion diameters. Calculations were done in Microsoft Excel 2007 (Microsoft Corporation, U.S.A.).

RESULTS AND DISCUSSION

Blue mold symptoms were observed on collected mandarin fruits. They included discolored or sometimes brown tissue on the fruit surface, concave, circular, and watery spots. Decay was present in different stages – from small lesions with no evident sporulation to some samples with white mycelia and blue-green sporulation. Ten isolates were recovered from symptomatic fruits (MP/1-MP/10), and two isolates (MP/4 and MP/5) were chosen for sequencing and phylogenetic analysis.

After 7 days of incubation at 25 °C, all isolates had radially sulcate colonies on CYA and plane to moderately radially sulcate on MEA. Sulcation was also present on YES, varying from slight radial to the radial and concentric combined. Cultures manifested different textures on tested media – velvety to loosely floccose on CYA and YES, fasciculate on CREA, plane and weak

fasciculate on MEA and OAT. Reverse, colonies had light brown colour with a brighter margin (CYA), pale yellow with a hint of greenish (MEA), greyish yellow (OAT), purple with a yellow margin (CREA), and cream-yellow with brown center (YES). Strong acid production (change of the medium colour from purple to yellow) followed by base production was noticed on CREA. Intensive conidiogenesis was present on all five media, with blue-green spores on CYA, MEA, and YES, green on CREA, and dull green on OAT. Isolates had white mycelia on all inoculated media, present as the margin of the culture, broad on CYA (6–7 mm width), moderate on MEA, YES, and OAT (2–3 mm) and narrow on CREA (up to 1.5 mm). Clear exudate droplets were noticed on CREA, YES, and OAT, while there was no exudate formation on CYA and MEA.

All tested isolates manifested intensive growth across all five tested media, with YES being the most stimulative medium (58.63 mm). The mycelial development was the least intensive on CREA (37 mm), and the average growth diameters on the remaining three media (CYA, OAT, and MEA) were 41.88, 40.38, and 40.25 mm, respectively (Figure 1). The growth diameters and culture appearance agree with the data from the relevant literature sources (Pitt, 1979; Frisvad and Samson, 2004; Pitt and Hocking, 2009; Visagie, 2012; Vico et al., 2014).

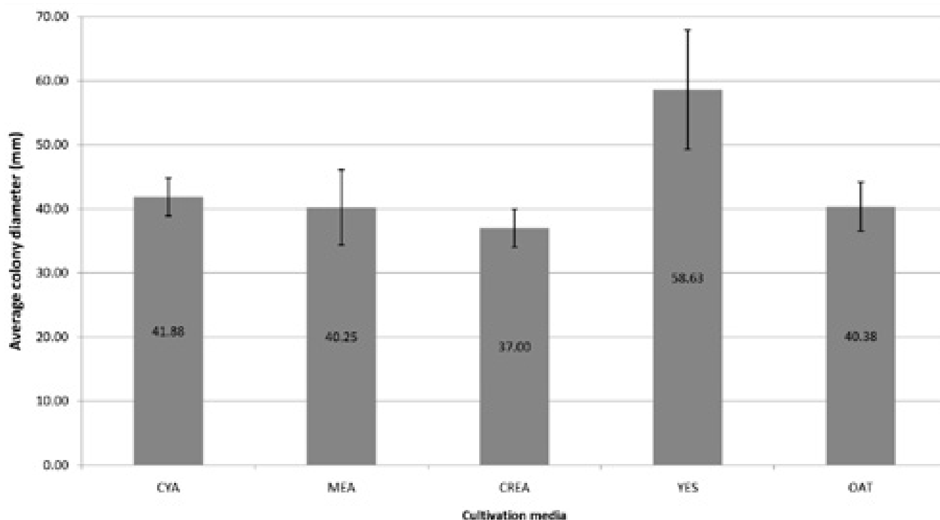

Figure 1. The average colony growth of *P. expansum* isolates on five different media after 7 days of incubation at 25°C. Vertical error bars indicate the standard deviation (SD).

Isolates' cultivation at different temperatures on CYA showed that the optimal (and in the same time maximal) temperature for their development was 25 °C (41.88 mm, Figure 2). Of all tested temperatures, the smallest average

diameter was measured at 5 °C (18.46 mm). Fungal growth was not recorded at 37 °C (Figure 2).

The most restrictive temperature for fungal development was 37 °C and the most optimal was 25°C which is in agreement with previous data (Pitt, 1979; Frisvad and Samson, 2004; Pitt and Hocking, 2009; Visagie, 2012; Vico et al., 2014). Growth was still possible at the lowest tested temperature (5 °C), but our values were closer to those reported by Vico et al. (2014) (18.46 and 14.3–17.6 mm, respectively) than to those noted in Frisvad and Samson (2004), Pitt and Hocking (2009), and Visagie (2012) (up to 5 mm, collectively). The average growth diameter at 30 °C was in concordance with the data in the work by Visagie (2012).

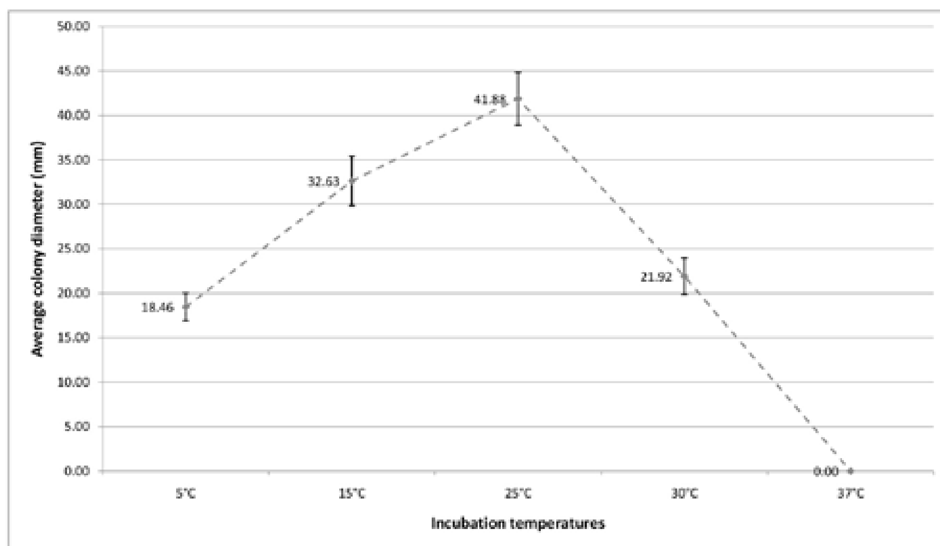

Figure 2. The average colony growth of *P. expansum* isolates on CYA at five different incubation temperatures after 7 days. Vertical error bars indicate the standard deviation (SD).

Microscopic observations revealed that all analyzed isolates formed hyaline, mostly terverticillate conidiophores, with usually smooth stipes and cylindrical phialides. Phialides had a short but distinctive neck. Conidia were subglobose or ellipsoidal, green and with smooth walls, 2.5–3.8–5.25 µm × 2.5–3.52–5.00 µm (minimum-average-maximum values). Ascomata were not formed, as expected (Samson et al., 2010). The listed micromorphological characteristics were in agreement with previous findings (Pitt, 1979; Frisvad and Samson, 2004; Pitt and Hocking, 2009; Samson et al., 2010; Vico et al., 2014).

In the molecular analysis, ITS sequences of the selected Serbian isolates of *P. expansum* from mandarin fruit were 100% identical to each other. BLAST analysis showed 100% similarity with *P. expansum* isolates NRRL 6069,

NRRL 35231, and NRRL 2304 (GenBank Accession numbers DQ339562, DQ339558, and DQ339556, respectively). The sequences of *BenA* of our *P. expansum* isolates shared a 99.75% similarity between each other (1 base pair difference). Nucleotide identity of Serbian *P. expansum* and GenBank *BenA* sequences was in the range of 99.75–100%. The most similar *BenA* sequences from GenBank to ours were the following isolates of *P. expansum*: YC-IK11 sampled from pear (*Pyrus* × *bretschneideri*) in China (Acc. No. MK862430), isolate CMV017H9 from apple in South Africa (MN031410), and isolate DTO 216-G4 from foliar tissue of *Psedotsuga menziesii* in USA (MF990777).

Inferring phylogenetic relationships started with multiple sequence alignments of ITS and *BenA* sequences which were 452 nucleotides (nt) and 387 nt long, respectively. ML phylogenetic tree was constructed using the aligned combined dataset (ITS+*BenA*) which had a total length of 839 nt and it included 26 sequences from 9 taxa (representative isolates of 8 *Penicillium* species and *A. niger* as an outgroup). In a multilocus phylogeny, our isolates of *P. expansum* (MP/4 and MP/5) clustered together with the other *P. expansum* isolates with maximum bootstrap support (100%, Figure 3). Subclades of *P. expansum* and *P. italicum* formed a joint clade with a high confidence level (94%).

Sequences of the ITS region have been used before for resolving *Penicillium* phylogenies (Berbee et al., 1995; Skouboe et al., 1999) and although this region is accepted as the universal genetic barcode for all fungi (Schoch et al., 2012), its resolution is limited at the species level in *Penicillium* (Visagie et al., 2014). With all its disadvantages in mind, *BenA* is proposed as the secondary molecular marker, especially in routine identification procedures (Visagie et al., 2014). We applied ITS and *BenA* sequences in this research and representative isolates were identified as *P. expansum* for both sequenced loci. In a subsequent multilocus analysis, our sequences of the *P. expansum* were clustered in the same clade with other isolates of this species, thereby validating the results of morpho-physiological and molecular characterization.

In a pathogenicity test, isolates of *P. expansum* recovered in this study induced decay on healthy mandarin fruits after 7 days of incubation (Figure 4, A-D). The symptoms on wounded fruits were quite similar to those observed on naturally infected mandarin fruits. The average lesion diameter (± standard deviation, SD) ranged from 30±0 mm to 33.5±2.12 mm. Control fruits remained symptomless. Fungi reisolated from decayed fruits in the pathogenicity test expressed the same morphological characteristics as the original isolates, thus confirming Koch's postulates.

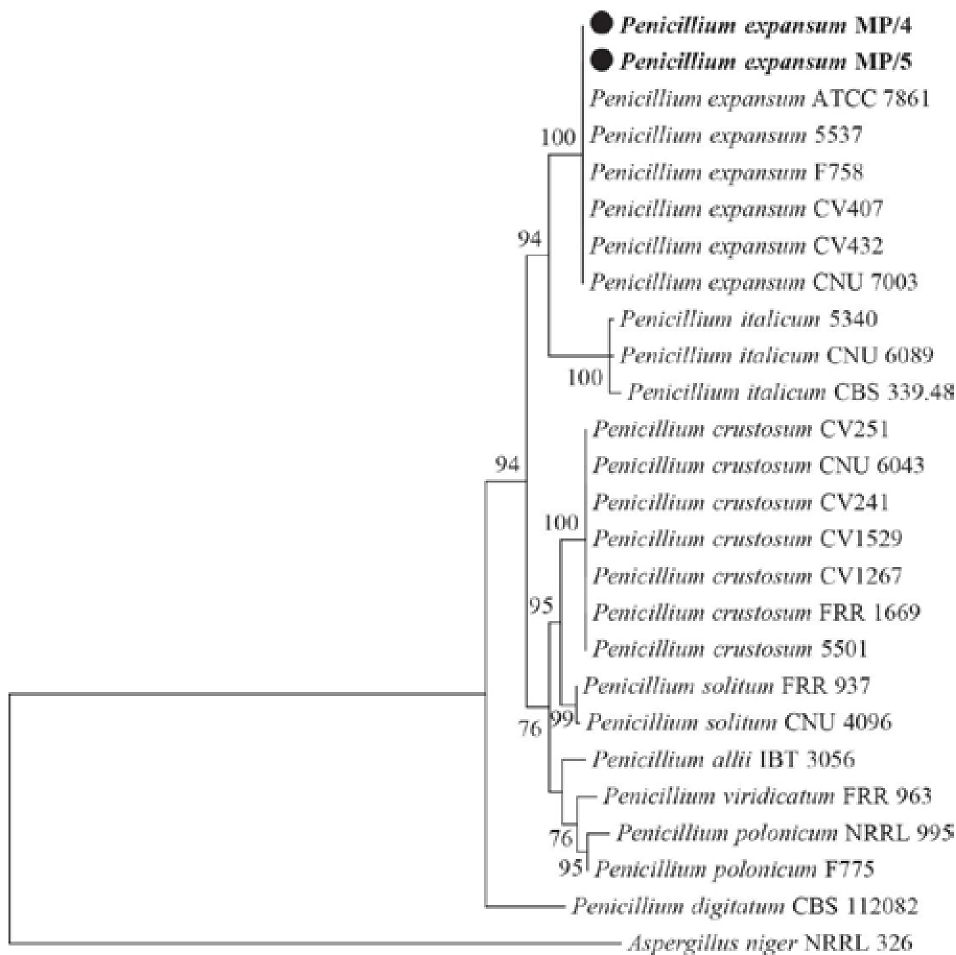

Figure 3. Phylogenetic tree based on combined ITS and *BenA* sequences using the maximum likelihood (ML) method for selected *Penicillium* species. The isolate of *A. niger* NRRL 326 was designated as an outgroup. The bootstrap analysis included 1,000 replicates and only bootstrap values >70% are displayed near the corresponding nodes. The scale represents the number of substitutions per site. Isolates in bold preceded with black dots are from this study.

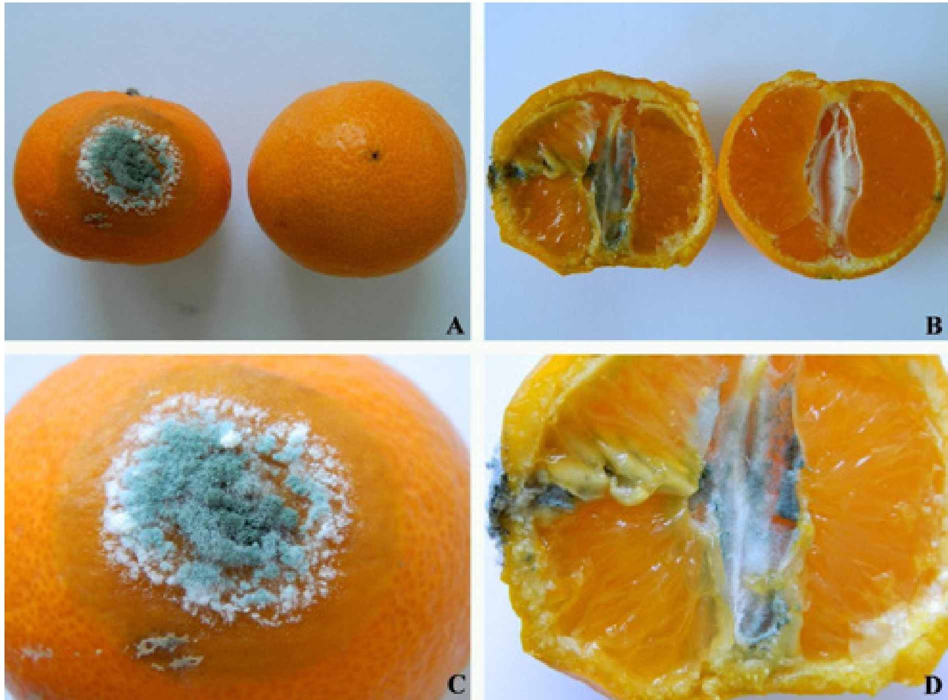

Figure 4. A) Pathogenicity of *P. expansum* in mandarin fruit; B) Cross-sections of the inoculated fruits (left A and B – fruit inoculated with the pathogen suspension, right A and B – control fruit); C) Close view of the lesion. D) Close view of the cross-section of the diseased fruit.

P. digitatum and *P. italicum* are considered among the most important *Penicillium* postharvest pathogens of citrus fruits, including mandarins (Jhalegar et al., 2015; Louw and Korsten, 2015; Saito and Xiao, 2017; Yang et al., 2019; Cheng et al., 2020). Other *Penicillium* species important for the citrus industry as the decay agents are *P. ulaiense*, *P. crustosum*, *P. expansum*, and *P. dierckxii* Biourge 1923 (=*P. fellunatum*), but the data on *P. dierckxii* is not confirmed (Louw and Korsten, 2015).

In this study, *P. expansum* was isolated from molded mandarin fruits. It was identified at the morphological and molecular level and confirmed as the postharvest pathogen in stored fruits of mandarin. Findings from this research report for the first time *P. expansum* as the cause of the mandarin fruit rot in Serbia.

There are only two studies where *P. expansum* was isolated from mandarin fruit. In research by Liu et al. (2009), *P. expansum* originating from decayed mandarin fruit (cv. *Citrus unshiu* Marcov) was used to test the impact of the quorum-sensing molecule, farnesol, on inducing morphogenetic changes in this fungal pathogen. The isolate was identified using morphological and

molecular methods (sequencing of the ITS region). However, the pathogenicity of the acquired isolate was not tested since that was not the aim of the study. Last year, Moosa and co-authors (2019) isolated and confirmed *P. expansum* as the pathogen of the stored mandarin fruit (*Citrus reticulata,* cv. 'Kinnow') in Pakistan. Using morphological methods and ITS sequencing (primers ITS1/ITS4), they identified the isolated fungus as *P. expansum*.

This species was recorded as capable of producing decay in other members of citrus fruits. The pathogenic potential of *P. expansum* was detected in grapefruit, lemon, and orange fruit in the work by Macarasin et al. (2007). The lesion formation occurred, but it was artificially stimulated by the addition of citric, ascorbic, and oxalic acids and enzyme catalase before the inoculation of *P. expansum*. Vilanova et al. (2012) studied compatible (*P. digitatum*) and incompatible (*P. expansum*) orange fruit–pathogen interactions. The results of their study showed that *P. expansum* can be pathogenic in two varieties of orange fruits (*Citrus sinensis*). The rot development caused by *P. expansum* was dependent on the fruit maturity, storage temperature, and the inoculum concentration. Decay was possible in mature and over-matured fruit, higher at the lower storage temperature (4 °C) and with inoculum concentration of 10^6 and 10^7 spores/ml. In the research of Louw and Korsten (2015), *P. expansum* was able to produce decay in two mandarin cultivars (Nules Clementine and Owari Satsuma). The pathogenic isolate was not sampled from citrus fruit, but it originated from a citrus export chain, more specifically from a wall of distributer/repack facility. It is important to point out that this isolate was reintroduced into apples and isolated from the produced lesions.

P. expansum is usually regarded in the literature as the spoilage agent of pomaceous fruits (apples, pears, quinces) (Pitt, 1979; Snowdon, 1990; Frisvad and Samson, 2004; Pianzzola et al., 2004; Amiri and Bompeix, 2005; Pitt and Hocking, 2009; Louw et al., 2014). Worldwide distribution, the occurrence on a very wide variety of living plant tissues, and the ability to cause severe decay in various fruits and vegetables indicate that *P. expansum* is a broad-spectrum pathogen (Pitt, 1979; Pitt and Hocking, 2009; Neri et al., 2010; Samson et al., 2010; Vilanova et al., 2012). The results from our study and other studies with citrus hosts are congruent with the last statement.

Furthermore, beside causing decay on mandarin and other fruits, it is important to note that *P. expansum* is a consistent producer of mycotoxin patulin (Andersen et al., 2004). Consumption of patulin contaminated products can lead to immunological, neurological, and gastrointestinal health disorders (Puel et al., 2010). Therefore, the maximal allowed concentration of patulin in apples and apple products is limited to 0.4 mg/kg of body weight per day by the FAO-WHO Expert Committee (Bennett and Klich, 2003). Besides patulin, *P. expansum* is designated as the possible producer of other secondary metabolites which also can compromise human health: citrinin, chaetoglobosins, communesins, roquefortine C, and expansolides A and B (Andersen et al., 2004).

CONCLUSION

Several *Penicillium* spp. species can cause decay on mandarin fruits. In this research, isolates of *P. expansum* from mandarin fruits were morphologically identified and the results were confirmed using PCR, sequencing, and phylogenetic analysis of the two loci: ITS and *BenA*. The obtained isolates caused rot in the artificially inoculated mandarin fruits in pathogenicity assay. To the best of our knowledge, this is the first report of *P. expansum* as the decay agent of stored mandarin fruits in Serbia, and one of the few in the rest of the world. The findings of this study could provide a basis for future efficient protection measures of the stored mandarin fruits in Serbia.

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REFERENCES

Amiri A, Bompeix G (2005): Diversity and population dynamics of *Penicillium* spp. on apples in pre- and postharvest environments: consequences for decay development. *Plant Pathol.* 54: 74–81.

Andersen B, Smedsgaard J, Frisvad JC (2004): *Penicillium expansum*: consistent production of patulin, chaetoglobosins, and other secondary metabolites in culture and their natural occurrence in fruit products. *J. Agric. Food Chem*. 52: 2421–2428.

Barkai-Golan R (2001): *Postharvest diseases of fruits and vegetables: Development and control*, 1st ed. Elsevier Science B. V., Amsterdam.

Bennett JW, Klich, M (2003): Mycotoxins. *Clin. Microbiol. Rev.* 16: 497–516.

Berbee ML, Yoshimura A, Sugiyama J, Taylor JW (1995): Is *Penicillium* monophyletic? An evaluation of phylogeny in the family Trichocomaceae from 18S, 5.8S and ITS ribosomal DNA sequence data. *Mycologia* 87: 210–222.

Cheng Y, Lin Y, Cao H, Li Z (2020): Citrus postharvest green mold: recent advances in fungal pathogenicity and fruit resistance. *Microorganisms* 8: 449.

Codoñer-Franch P, Valls-Bellés V (2010): Citrus as functional foods. *Curr. Top. Nutraceutical Res*. 8: 173–184.

De Hoog GS, Van den Ende, AHGG (1998): Molecular diagnostics of clinical strains of filamentous Basidiomycetes. *Mycoses* 41: 183–189.

El-Otmani M, Ait-Oubahou A, Zacarías L (2011): *Citrus* spp.: orange, mandarin, tangerine, clementine, grapefruit, pomelo, lemon and lime. In: E. M. Yahia (Ed.), *Postharvest biology and technology of tropical and subtropical fruits*, vol. 2: *Açai to Citrus*. Woodhead Publishing Limited, Cambridge, UK, 437–516.

Food and Agriculture Organization of the United Nations (2020): FAOSTAT Statistical Database, 20.04.2020. Available at: http://www.fao.org/faostat/en/#home.

Frisvad JC, Samson RA (2004): Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*: A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Stud. Mycol.* 49: 1–173.

Garcha HS, Singh V (1976): *Penicillium crustosum*, a new pathogen of *Citrus reticulata* (mandarin) from India. *Plant Dis. Report.* 60: 252–254.

Glass NL, Donaldson GC (1995): Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61: 1323–1330.

Jhalegar MJ, Sharma RR, Singh D (2015): In vitro and in vivo activity of essential oils against major postharvest pathogens of Kinnow (*Citrus nobilis* × *C. deliciosa*) mandarin. *J. Food Sci. Technol.* 52: 2229–2237.

Kumar S, Stecher G, Tamura K (2016): MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33: 1870–1874.

Liu P, Deng B, Long C-A, Min X (2009): Effect of farnesol on morphogenesis in the fungal pathogen *Penicillium expansum*. *Ann. Microbiol.* 59: 33–38.

Liu Y, Heying E, Tanumihardjo SA (2012): History, global distribution, and nutritional importance of citrus fruits. *Compr. Rev. Food Sci. Food Saf.* 11: 530–545.

Louw JP, Korsten L (2014): Pathogenic *Penicillium* spp. on apple and pear. *Plant Dis.* 98: 590–598.

Louw JP, Korsten L (2015): Pathogenicity and host susceptibility of *Penicillium* spp. on citrus. *Plant Dis.* 99: 21–30.

Macarisin D, Cohen L, Eick A, Rafael G, Belausov E, Wisniewski M, Droby S (2007): *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. *Phytopathology* 97: 1491–1500.

Masclaux F, Guého E, de Hoog GS, Christen R (1995): Phylogenetic relationships of human-pathogenic *Cladosporium* (*Xylohypha*) species inferred from partial LS rRNA sequences. *J. Med. Vet. Mycol.* 33: 327–338.

Moosa A, Farzand A, Sahi ST, Gleason ML, Khan SA, Zhang X (2019): First report of postharvest fruit rot of *Citrus reticulata* 'Kinnow' caused by *Penicillium expansum* in Pakistan. *Plant Dis.* 103: 155.

Neri F, Donati I, Veronesi F, Mazzoni D, Mari M (2010): Evaluation of *Penicillium expansum* isolates for aggressiveness, growth and patulin accumulation in usual and less common fruit hosts. *Int. J. Food Microbiol.* 143: 109–117.

Palou L, Valencia-Chamorro S, Pérez-Gago M (2015): Antifungal edible coatings for fresh citrus fruit: a review. *Coatings* 5: 962–986.

Park JH, Hyun J-W, Park MJ, Choi YJ (2018): First report of whisker mold as a postharvest disease caused by *Penicillium ulaiense* on citrus (*Citrus unshiu*) in Korea. *Plant Dis.* 102: 2643.

Pianzzola MJ, Moscatelli M, Vero S (2004): Characterization of *Penicillium* isolates associated with blue mold on apple in Uruguay. *Plant Dis.* 88: 23–28.

Pitt JI (1979): *The genus Penicillium and its teleomorphic states Eupenicillium and Talaromyces*. London: Academic Press.

Pitt JI, Hocking A (2009): *Fungi and food spoilage*, 3rd ed. New York: Springer-Verlag, New York Inc.

Puel O, Galtier P, Oswald IP (2010): Biosynthesis and toxicological effects of patulin. *Toxins* (Basel) 2: 613–631.
Putnik P, Barba FJ, Lorenzo JM, Gabrić D, Shpigelman A, Cravotto G, Bursać Kovačević D (2017): An integrated approach to mandarin processing: food safety and nutritional quality, consumer preference, and nutrient bioaccessibility. *Compr. Rev. Food Sci. Food Saf.* 16: 1345–1358.
Saito S, Xiao CL (2017): Prevalence of postharvest diseases of mandarin fruit in California. *Plant Heal. Prog.* 18: 204–210.
Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B (2010): *Food and indoor fungi*, 1st ed., CBS Laboratory Manual Series. Utrecht: CBS-KNAW Fungal Biodiversity Centre.
Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W (2012): Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl. Acad. Sci. U.S.A.* 109: 6241–6246.
Skouboe P, Frisvad JC, Taylor JW, Lauritsen D, Boysen M, Rossen L (1999): Phylogenetic analysis of nucleotide sequences from the ITS region of terverticillate *Penicillium* species. *Mycol. Res.* 103: 873–881.
Snowdon AL (1990): *Color atlas of post-harvest diseases and disorders of fruits and vegetables*, Vol. 1: General introduction and fruits. Boca Raton: CRC Press.
Statistical Office of the Republic of Serbia (2020): Database of the Statistical Office of the Republic of Serbia, 30.06.2020. Available from: https://data.stat.gov.rs/?caller=SDDB.
Stošić S, Ristić D, Gašić K, Starović M, Grbić MLj, Vukojević J, Živković S (2020): *Talaromyces minioluteus*: new postharvest fungal pathogen in Serbia. *Plant Dis.* 104: 656–667.
Tashiro N, Manabe K, Ide Y (2012): First report of whisker mold, a postharvest disease on citrus caused by *Penicillium ulaiense* (in Japan). *J. Gen. Plant Pathol.* 78: 140–144.
Thompson JD, Higgins DG, Gibson TJ (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.
Vico I, Duduk N, Vasić M, Nikolić M (2014): Identification of *Penicillium expansum* causing postharvest blue mold decay of apple fruit. *Pestic. Fitomed.* 29: 257–266.
Vilanova L, Viñas I, Torres R, Usall J, Jauset AM, Teixidó N (2012): Infection capacities in the orange-pathogen relationship: compatible (*Penicillium digitatum*) and incompatible (*Penicillium expansum*) interactions. *Food Microbiol.* 29: 56–66.
Visagie CM (2012): *The polyphasic taxonomy of Penicillium and Talaromyces spp. isolated from the diverse Fynbos biome*. Doctoral dissertation. Stellenbosch University, Stellenbosch.
Visagie CM, Houbraken J, Frisvad JC, Hong S-B, Klaassen CHW, Perrone G, Seifert KA, Varga J, Yaguchi T, Samson RA (2014): Identification and nomenclature of the genus *Penicillium* *Stud. Mycol.* 78: 343–371.
Wang L, He F, Huang Y, He J, Yang S, Zeng J, Deng C, Jiang X, Fang Y, Wen S, Xu R, Yu H, Yang X, Zhong G, Chen C, Yan X, Zhou C, Zhang H, Xie Z, Larkin RM, Deng X, Xu Q (2018): Genome of wild mandarin and domestication history of mandarin. *Mol. Plant* 11: 1024–1037.
Wu GA, Terol J, Ibanez V, López-García A, Pérez-Román E, Borredá C, Domingo C, Tadeo FR, Carbonell-Caballero J, Alonso R, Curk F, Du D, Ollitrault P, Roose ML, Dopazo J, Gmitter FG, Rokhsar DS, Talon M (2018): Genomics of the origin and evolution of *Citrus*. *Nature* 554: 311–316.

Yang Q, Qian X, Dhanasekaran S, Boateng NAS, Yan X., Zhu H, He F, Zhang H (2019): Study on the infection mechanism of *Penicillium digitatum* on postharvest citrus (*Citrus reticulata* Blanco) based on transcriptomics. *Microorganisms* 7: 672.

Živković S, Trkulja N, Popović T, Oro V, Ivanović Ž (2012): Morphological and molecular identification of *Colletotrichum gloeosporioides* from *Citrus reticulata*. Proceedings, International Symposium: Current Trends in Plant Protection. Institute for Plant Protection and Environment, Belgrade (Serbia), Belgrade, Serbia, 293–298.

ТРУЛЕЖ ПЛОДОВА МАНДАРИНЕ СКЛАДИШТЕНЕ У СРБИЈИ ПРОУЗРОКОВАНА ВРСТОМ *Penicillium expansum*

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РЕЗИМЕ: Мандарина је једна од најтраженијих воћних врста из рода *Citrus*. Конзумира се због својих нутритивних и здравствених добробити, као и због пријатног мириса и укуса. У овом раду описана је идентификација и карактеризација врсте *Penicillium expansum*, изоловане са плесњивих плодова мандарине у Србији. Прикупљени изолати су гајени на пет микробиолошких подлога [Чапекова аутолизатна подлога са додатком квасца (CYA), сладни агар (MEA), креатин сахарозна подлога (CREA), агар са квашчевим екстрактом и сахарозом (YES) и подлога од овсеног брашна (OA)], као и на пет различитих температура (5, 15, 25, 30, 37 °C). Добијени изолати су секвенцирани на два молекуларна маркера (интерни транскрибовани регион и бета-тубулин). Резултати морфолошких, физиолошких, молекуларних и филогенетичких анализа указују да добијени изолати припадају врсти *P. expansum*. Изолована врста је потврђена као патоген плодова мандарине. Према нашим сазнањима, ово је први налаз *P. expansum* као складишног патогена на плодовима мандарине у Србији.

КЉУЧНЕ РЕЧИ: идентификација, морфолошка анализа, молекуларна карактеризација, патогеност, *Citrus reticulata*