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ORIGINAL SCIENTIFIC PAPER

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POLYPHASIC IDENTIFICATION OF DECAY AGENTS OF LEMON FRUITS IN SERBIA

SUMMARY: Lemon fruits are an important source of vitamin C, potassium, folate, carotenoids, polyphenols, coumarins and terpenes. These lemon compounds have antioxidant and anti-inflammatory properties which have beneficial effects on human health. This research aimed to elucidate the etiology of blue and green molds detected on lemon fruits in Serbia. Using integrative identification approach, the obtained isolates were characterized from morphological, physiological, molecular, phylogenetic and pathological aspects. Colony growth and morphology were examined on Czapek yeast autolysate agar (CYA), Malt extract agar (MEA) and Creatine sucrose agar (CREA), and on CYA at two additional incubation temperatures (5 and 37 °C). For molecular identification, ITS and partial β-tubulin (*BenA*) genes were sequenced. Phylogenetic relationships were investigated using maximum-likelihood method. A pathogenicity test was carried out and the possible difference in pathogenicity among isolates was assessed with analysis of variance (ANOVA) and subsequent Tukey's test. Four species were identified: *Penicillium expansum*, *Penicillium digitatum*, *Penicillium polonicum* and *Talaromyces rugulosus*. All four species proved to be pathogenic on lemon fruits, producing symptoms similar to those observed on naturally infected fruits. The results of this study are the first records of the beforementioned *Penicillium*/*Talaromyces* species as postharvest pathogens on lemon fruits in Serbia and the first world report of *T. rugulosus* as phytopathogenic on the same host.

KEYWORDS: *Citrus limon*, morphological analysis, molecular characterization, multilocus phylogeny, pathogenicity, *Penicillium*, *Talaromyces*

INTRODUCTION

Lemon (*Citrus limon* (L.) Osbeck) is an evergreen plant from the family Rutaceae with leathery, lanceolate leaves, and yellow, edible berry fruits. The

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inner part of the fruit is divided into segments which are full of juicy pulp. Exocarp of the fruit contains carotenoid pigments and oil vesicles (Klimek-Szczykutowicz et al., 2020). Lemons are probably the result of crossbreeding two other *Citrus* species – bitter orange (*C. aurantium*) and citron (*C. medica*) (Wu et al., 2018). Although the originating habitat of the lemons is not known precisely, it is considered that the first growing areas of this plant were northwest and northeast India (Klimek-Szczykutowicz et al., 2020).

Lemons are popular and widely utilized fruit throughout the world. They are consumed in fresh form or used in processed food such as juices, jams, jellies, molasses etc. (González-Molina et al., 2010). Beside the usage in food production, lemon fruit and its extracts are important compounds in cosmetic and pharmaceutical industry (Klimek-Szczykutowicz et al., 2020).

Lemon fruits are an important source of vitamin C, potassium, folate, carotenoids, flavonoids, phenolic acids coumarins and terpenes. These lemon compounds have antioxidant and anti-inflammatory properties which have beneficial effects on human health (Klimek-Szczykutowicz et al., 2020).

Some of the major fungal pathogens of citrus trees and fruits (including lemons) are: *Alternaria alternata*, *Colletotrichum acutatum, Diaporthe citri*, *Elsinoë fawcettii*, *E. australis*, *Zasmidium citri* (=*Mycosphaerella citri*) (Timmer et al., 2004). Beside mentioned, other fungi are important as decay agents of citrus fruits in the postharvest phase – *Penicillium digitatum*, *P. italicum*, *Geotrichum citri-aurantii*, *Aspergillus niger*, and *A. flavus* (Wang et al., 2022).

World production of this crop rise every year reaching 21,353,502 t in 2020 (FAOSTAT, 2022). Serbian annual import of this crop in 2021 was 30,366 t and it has an increasing trend in the last ten years according to the data of the Republic Statistical Office (2022).

To our knowledge, there are no literature records on postharvest fungal pathogens of lemon fruits in Serbia. Considering the amount of import of lemon fruits to our country and the possibility to introduce new phytopathogenic fungi with them, the aim of this research was to explore the etiology of blue and green mold causal agents on this crop.

MATERIAL AND METHODS

Isolation procedure and fungal cultures

Samples of lemon fruits with *Penicillium*-like symptoms were collected from supermarkets and open markets in Serbia as part of a broader study, during 2015–2021. Isolation of the fungi was achieved following standard phytopathological procedures. Small parts of the fruit tissue (on the line healthy-diseased tissue) were removed with sterilized scalpel, immersed in 1% NaOCl for 3 min and then rinsed three times with sterile distilled water. Rinsed tissue pieces were placed on Malt extract agar (MEA) and incubated for 5 days at 25 °C in the dark. Developed cultures were examined and only clean, uncontaminated

cultures were chosen for further subculturing and monosporial isolate production (Crous et al., 2009). Isolated fungi were deposited in the Fungal collection of the Institute for Plant Protection and Environment (Belgrade, Serbia). The polyphasic approach was employed in identification of the recovered isolates which represents a combined use of morphological, physiological, molecular and phylogenetic methods.

Morphological and physiological studies

Phenotypic appearance of the colonies was studied on Czapek Autolysate agar (CYA), MEA (Malt extract agar) and Creatine sucrose agar (CREA), after incubation for 7 days at 25 °C. Growth of the cultures was tested on two additional incubation temperatures (5 and 37 °C), on CYA. Micromorphological observations were carried out from isolates grown on MEA, using Olympus BX51 microscope equipped with an Olympus camera (model E620). Quick Photo Software program (Promicra, Czech Republic) were used for photographing and measuring fungal reproductive structures. Media composition, inoculation methods, preparation of media and microscopic slides, and evaluation procedures followed instructions described in Visagie et al. (2014).

Molecular identification and phylogenetic analyses

Genomic DNA was extracted with DNeasy Plant Mini Kit (Qiagen, Germany) from 7 day-old cultures incubated at 25 °C on MEA. The extracted DNA was preserved at -20 °C. Two genetic loci were amplified and sequenced – partial ITS and β-tubulin (*BenA*), using primers V9G/LS266 (ITS) and Bt2a/Bt2b or T10/Bt2b (for *BenA*). DNA extraction procedure, PCR conditions and compound volumes were the same as in one of our previous study (Stošić et al., 2020). Bidirectional sequencing of the amplified products was completed in Macrogen Europe commercial sequence service facilities (Amsterdam, the Netherlands). FinchTV software (Geospiza) served for visual inspection of the sequences' quality and assembly of the consensus sequences was achieved using ClustalW algorithm (Thompson et al., 1994) implemented in MEGA7 program (Kumar et al., 2016). The obtained sequences were compared with previously deposited sequences in the NCBI GenBank database with BLASTn algorithm.

Phylogenetic relationships were inferred by constructing maximum likelihood tree with combined ITS and *BenA* sequences (Table 1) in MEGA7 software (Kumar et al., 2016). "Find best model" option in the same computer program was used to analyze and propose the best model of nucleotide substitution. Construction of the tree was based on Kimura 2-parameter model with 5 discrete gamma categories. The robustness of phylogeny was tested by performing 1000 bootstrap replicates and the sequences of *Neocosmospora phaseoli*

(isolate CBS 102429) were used to root the obtained tree. Adobe Illustrator CS6 (Adobe, USA) served for visual editing of the tree.

Table 1. GenBank accession numbers of the sequences of *Penicillium* and *Talaromyces* species used in phylogenetic analysis, isolates in bold are from this research

Species	Strain/isolate	Substrate and origin	GenBank accessions	
			ITS	*BenA*
Neocosmospora phaseoli (=*Fusarium solani*)	CBS 102429	Tree bark, Australia	KM231808	KM232069
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. digitatum	**21-7**	**Lemon fruit, Serbia**	-	**ON988101**
	CBS 112082	Lemon, Italy	KJ834506	KJ834447
P. expansum	**LiP/4**	**Lemon fruit, Serbia**	-	**ON988099**
	CBS 325.48 = ATCC 7861	Apple fruit, U.S.A.	AY373912	AY674400
	F758	Sugar beet root, Idaho, U.S.A.	MG714838	MG714864
P. italicum	CBS 339.48	Citrus fruit, Riverside, CA, U.S.A.	KJ834509	AY674398
	SFC20140101-M724 = 5340	Unknown	KJ527447	KJ527412
P. polonicum	**LiP/4**	**Lemon fruit, Serbia**	-	**ON988100**
	CBS 222.28 = NRRL 995	Soil, Poland	AF033475	AY674305
	F775	Sugar beet root, Idaho, U.S.A.	MG714841	MG714868
P. solitum	CBS 424.89 = FRR 937	Unknown, Germany	AY373932	AY674354
P. viridicatum	CBS 390.48 = DTO 005-C9 = FRR 963	Air, Washington DC, U.S.A.	AY373939	AY674295
T. flavus	CBS 310.38	Unknown, New Zealand	JN899360	JX494302
T. islandicus	CBS 338.48	Unknown, Cape Town, South Africa	KF984885	KF984655
T. minioluteus	CBS 642.68	Unknown	JN899346	KF114799
	CBS 270.35	*Zea mays*, U.S.A.	KM066172	KM066129
T. rugulosus	**LiP/1**	**Lemon fruit, Serbia**	-	**ON988098**
	CBS 371.48T	Rotting potato tubers (*Solanum tuberosum*), U.S.A.	*KF984834*	*KF984575*
	CBS 378.48 = NRRL 1073	Type of *P. tardum* & *P. elongatum*, decaying twigs, France	KF984832	KF984579
T. trachyspermus	CBS 373.48	Unknown, U.S.A.	JN899354	KF114803

Pathogenicity test

Pathogenicity of the isolated *Penicillium*/*Talaromyces* species was tested on healthy, uninjured lemon fruits. Each fruit was surface-sterilized by thorough wiping with paper towel soaked in 70% ethanol and left to air dry. Spore suspensions of all isolates were prepared in 1 ml of sterile distilled water from 14-day-old MEA cultures. The final desired concentration of suspensions (1×10^6 spores/ml) were achieved through serial dilutions. A small wound on fruit rind was made using sterile needle and 50 µl of the pathogen conidial suspension were inserted into the wound. Control fruits were inoculated with the same volume of sterile distilled water. Three replicates per isolate/control were used. Incubation of the inoculated fruits was in covered plastic boxes, at 25 °C and 95% relative air humidity. Disease symptoms were evaluated seven days post-inoculation. Width and height of develepod lesions were recorded and reisolations from those lesions were conducted to verify Koch's postulates.

Statistical analysis

Three replicates for each isolate were used in all assays. Descriptive statistics was calculated (mean value and standard deviation) for each colony, conidia and lesion radiuses. One-way analysis of variance ($p<0.05$) and Tukey's test were used to determine degree of differences in lesion diameters.

RESULTS AND DISCUSSION

Symptoms on the originally collected lemon fruits varied – discoloration or browning of the infected fruit surface, sometimes followed by development of white mycelia and substantial production of blue and green conidia. On some fruit samples tissue was soft and watery. Twelve isolates were obtained from the diseased fruits, and four representative isolates (LiP/1, LiP/2, LiP/4 and 21-7) were selected for in detail analysis.

Using the polyphasic approach, four species were identified: *Penicillium expansum*, *P. digitatum*, *P. polonicum* and *Talaromyces rugulosus*. In morphological assays, all isolates exhibited moderate to intensive growth on CYA, MEA and CREA, except isolate LiP/1 (determined as *T. rugulosus*) which had weak growth on these media. Isolate LiP/2 (identified as *P. expansum*) had the most intensive growth of all species on three tested media. The only isolate that did not form any colonies on CREA was 21-7 (subsequently identified as *P. digitatum*, Figure 1). Radial segmentation was noticed in LiP/4 (identified as *P. polonicum*) and isolates of *P. expansum* on CYA, whereas *P. digitatum* and *T. rugulosus* had compact colonies. Velutinous cultures were observed in all species excluding *P. expansum* where the variation of the textures was recorded – from fasciculate to synnematous. Intensive spore production was present in all species on CYA and MEA, with different conidial colors (Table 2).

Acid production on CREA was present in cultures of *P. expansum* and *P. polonicum* and lacked in the other two species.

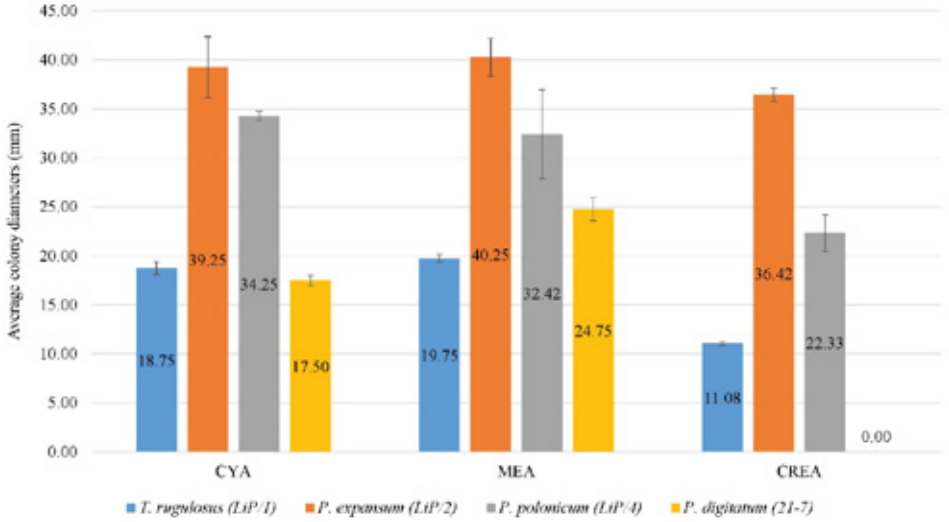

Figure 1. Mean colony diameters of *Penicillium* and *Talaromyces* isolates on three tested media (7 days of incubation, 25 °C). Vertical error bars represent standard deviation of the mean (SD).

In microscope examination, typical features for each of the isolated species were recorded (Table 2). Conidiophores' branching was: terverticillate in *P. expansum* and *P. polonicum*, biverticillate in *T. rugulosus* and irregular in *P. digitatum*.

Table 2. Conidial characteristics of *Penicillium* / *Talaromyces* isolates from this study

Species (Isolate)	**Dimensions, µm** (minimum – **average** – maximum)	**Shape**	**Cell wall ornamentation**	**Color en mass (MEA)**
P. digitatum (21-7)	3.62–**6.52**–7.75 × 3.04–**3.47**–3.98	ellipsoidal to cylindrical	smooth	olive green
P. expansum (LiP/2)	2.75–**4.23**–5.00 × 2.75–**4.06**–5.00	subglobose or ellipsoidal	smooth	green
P. polonicum (LiP/4)	2.75–**3.37**–3.75 × 2.75–**3.35**–3.75	subglobose	smooth	green with a blue shade
T. rugulosus (LiP/1)	2.50–**3.03**–3.75 × 2.50–**2.77**–3.75	ellipsoidal	roughened	dark green

When incubated at 5 °C, only *P. expansum* and *P. polonicum* formed colonies with *P. expansum* having higher values of mycelial growth. The absence of growth was observed at 37 °C for all species. Temperature of 25 °C

was the most optimal for fungal development, as expected (Figure 2). The phenotypic appearance of the isolates, growth on tested media and temperatures and micromorphological traits were in agreement with previous species descriptions (Frisvad and Samson, 2004; Pitt and Hocking, 2009; Samson et al., 2010; Visagie, 2012; Yilmaz et al., 2014).

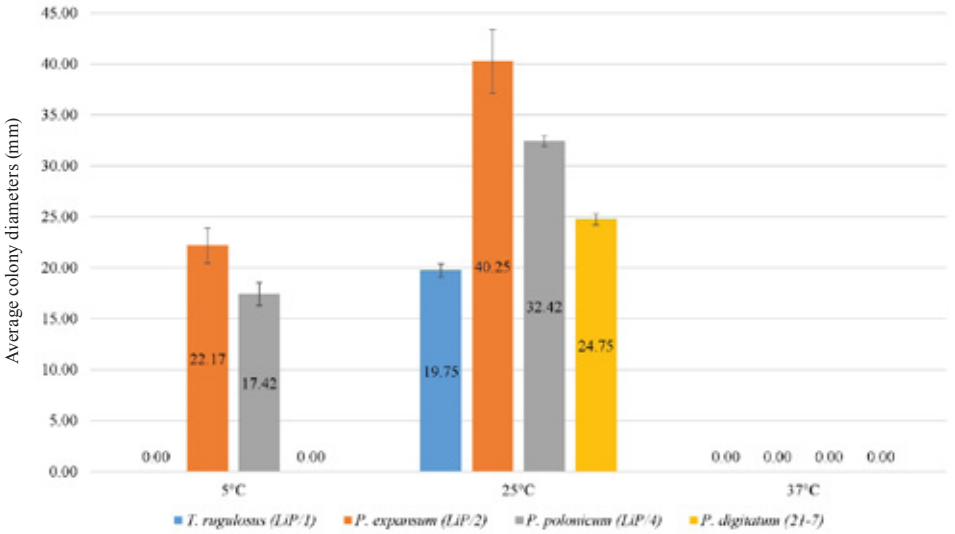

Figure 2. Mean colony diameters of *Penicillium* and *Talaromyces* isolates on three tested incubation temperatures (7 days of incubation, CYA). Vertical error bars represent standard deviation of the mean (SD).

BLAST search of sequences obtained in this study showed that the nucleotide identities with GenBank sequences were in the range 99.62%–100% for *BenA* (all isolated species) and 100% for ITS (3 species). The only exception was the BLAST comparison of Serbian *P. polonicum* ITS sequence which yielded inconclusive results. Variety of species were listed in the search results, proving again that ITS is not powerful enough to discriminate species of *Penicillium* (Visagie et al., 2014). BLAST search of *BenA* sequence of this isolate revealed that it belongs to *P. polonicum*, confirming preliminary identification in morphological and physiological experiments.

Construction of the multilocus phylogenetic tree began with separately aligned sequences of ITS and *BenA* which had lengths of 430 nucleotides (nt) and 298 nt, respectively. A combined, aligned set of ITS and *BenA* (728 nt long) was utilized for final analysis and it involved 24 sequences of representative species of *Penicillium* and *Talaromyces* (Table 1). Multilocus phylogenetic analysis based on the abovementioned molecular markers revealed clustering of the isolates from this study with the other isolates of the corresponding species (Figure 3), confirming the identity of the obtained isolates.

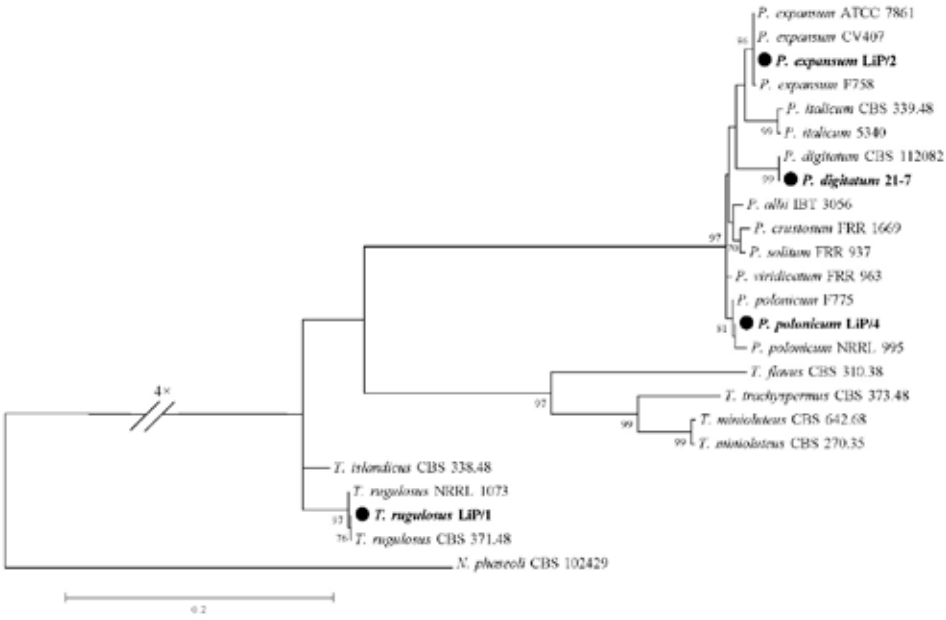

Figure 3. Maximum likelihood phylogenetic tree based on combined ITS and *BenA* sequence alignments of selected *Penicillium* and *Talaromyces* isolates. Tree was rooted with *Neocosmospora phaseoli* (=*Fusarium solani*). Bootstrap values >70% are displayed near to the corresponding nodes and isolates from this study are highlighted in bold text and with a black circle.

In pathogenicity assay, seven days after inoculation recorded symptoms varied among species – from small, spot necroses caused by *T. rugulosus* and *P. polonicum*, or dark brown spots induced by *P. expansum*, to complete cover of the inoculated fruits with heavy conidial mass (*P. digitatum*, Figure 5). Longitudinal cross-sections revealed that all pathogens, despite small outer lesions, induced typical softening of the inner tissues of the fruits (Figure 5). This phenomenon was followed with brightening of the diseased inner fruit tissue, making it more distinct and easy to detect. On cross sections it was also possible to notice that all four species were able to sporulate inside the fruit. Statistically significant differences ($p<0.05$) in virulence have been determined between species – *P. digitatum* was the most virulent, *P. polonicum* and *T. rugulosus* were the least virulent, while *P. expansum* virulence was moderate (Figure 4). Fullfilment of the Koch's postulates was completed by isolating the MEA cultures which resembled to the cultures recovered from the originating hosts.

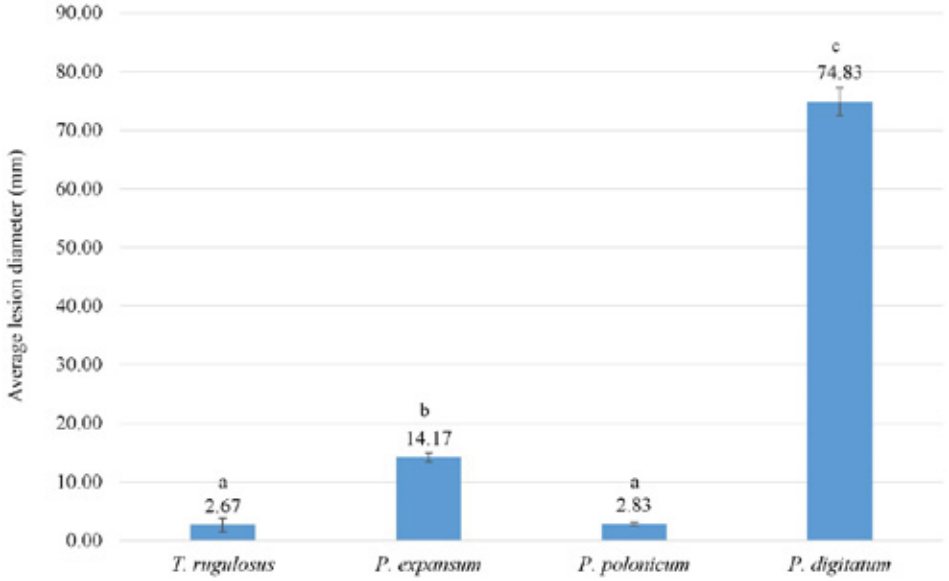

Figure 4. Average lesion diameters of *Penicillium* and *Talaromyces* isolates in pathogenicity test on lemon fruits. Different letters indicate values that are significantly different in Tukey's test ($p<0.05$), and vertical error bars represent standard deviation of the mean (SD).

P. polonicum and *T. rugulosus* caused small outer visible necroses on the lemon fruits in this study (~3 mm). This may seem insignificant at first, so it is plausible to ask about the magnitude of the economic damage that these species could cause. Nevertheless, softening of the tissue and fungal sporulation were noticed on cross-sections of the inoculated fruits. These phenomena surely decrease quality of the infected fruits making them undesirable for market placement. At the same time, produced spores could easily spread to the neighbouring fruit of this, or the other, more susceptible hosts.

P. expansum is not usually associated with citrus fruit decay – that place is reserved for *P. italicum* and *P. digitatum* which are considered the primary *Penicillium* pathogens on these crops (Frisvad and Samson, 2004). However, *P. expansum* is recently isolated and reported as rot agent of lemon fruits in Egypt (El-Dawy et al., 2021) and China (Khokhar et al., 2021). Pathogenic potential of this species on citrus fruits has been recorded earlier in the literature, but the hosts or substrates from which the isolates were obtained were not citrus fruits or in some studies the artificial inoculation was chemically assisted (Louw and Korsten, 2015; Macarisin et al., 2007; Vilanova et al., 2012). This is a very expansive species (as its Latin name suggests), with worldwide distribution, capable to invade and reside on a number of different plant hosts (Neri et al., 2010; Pitt, 1979; Pitt and Hocking, 2009).

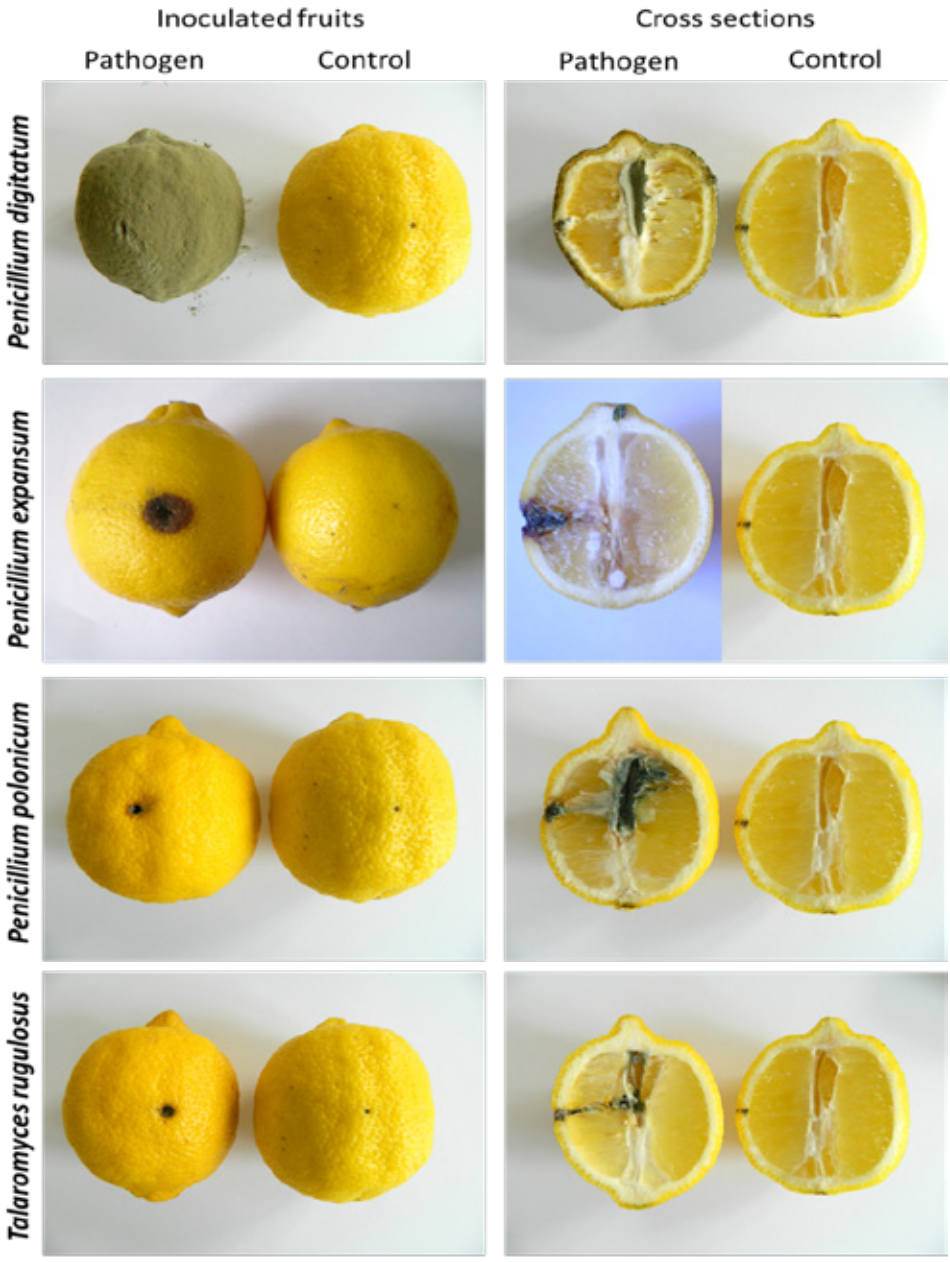

Figure 5. Pathogenicity test of the isolated species on lemon fruits

P. polonicum is also not considered a typical lemon fruit pathogen. Nevertheless, like *P. expansum*, it has been recently detected on this host, together with other citrus fruits such as tangerines and oranges (El-Dawy et al., 2021). In the cited study *P. polonicum* virulence was not tested on lemon but on orange fruits, thus the comparison to our results is not possible. This species has also been isolated from citrus fruits in the studies of Kim et al. (2008) in Korea and Chen et al. (2017) in China but with no further details about the species of citrus fruits.

One of the pathogenic species on lemon fruits determined in this study was *P. digitatum*, which is in agreement with earlier findings (Holmes and Eckert, 1999; Louw and Korsten, 2015; Palou, 2014; Raper and Thom, 1949; Smilanich et al., 2006). This species was the most potent pathogen in our research when compared to the other three identified species. It completely covered the artificially inoculated lemon fruts with characteristically green spores. Sporulation was also present inside the fruit, coupled with evident water loss, which would lead to mummifying of the infected fruit. Mummification could serve as one of the diagnostic characters to differ the *P. digitatum* and *P. italicum* infection on citrus fruits (Smith et al., 1988). This species and *P. italicum* are typical pathogens of lemon and other commercial citrus fruits, but also related genera like *Fortunella*, *Poncirus* and *Citrofortunella*. Alongside fruits, *P. digitatum* is a common resident of soils where citrus plants are grown (Palou, 2014).

Pathogenicity of *T. rugulosus* on lemon fruits has been confirmed in our research, which is the first report on this type of fruit in the world. This species can survive on various plant hosts and can cause decay but in most of the studies procedure of the confirmation of the Koch's postulates is not complete (Amiri and Bompeix, 2005; Dugan and Roberts, 1994; Norin and Rumpunen, 2003; Radenkovs and Juhnevica-Radenkova, 2018). The exceptions from the mentioned studies represent research by Vismer and co-workers (1996) and Strausbaugh (2018) who isolated *T. rugulosus* from apple fruits and sugarbeet roots (respectively) but the fungi were not pathogenic on these hosts. Barkai-Golan (1974) conducted a research in Israel and confirmed this species as capable to cause lesions on fruits of apple, pear, grape and tomato. In one of our previous studies (Stošić et al., 2021) we identified *T. rugulosus* as spoilage agents of stored pear fruits in Serbia. Pitt and Hocking (2009) claim that this species could be pathogenic on plants probably more then the current data suggest.

The results of our study are the first confirmations of the beforementioned *Penicillium*/*Talaromyces* species as postharvest pathogens on lemon fruits in Serbia.

CONCLUSION

Four species of *Penicillium* and *Talaromyces* were identified and confirmed as pathogens of lemon fruits in Serbia: *P. digitatum*, *P. expansum*, *P. polonicum* and *T. rugulosus*, using polyphasic approach. To the best of our knowledge, these are the first records of the mentioned species as pathogens

of lemons in our country, as well the first world report of *T. rugulosus* as decay agent on the same host.

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ОРИГИНАЛНИ НАУЧНИ РАД

ПОЛИФАЗНА ИДЕНТИФИКАЦИЈА ПРОУЗРОКОВАЧА ТРУЛЕЖИ ПЛОДОВА ЛИМУНА У СРБИЈИ

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РЕЗИМЕ: Плодови лимуна су важан извор Це витамина, калијума, фолата, каротеноида, полифенола, кумарина и терпена. Ови састојци лимуна имају антиоксидативно и противупално дејство што су позитивни ефекти на здравље људи. Циљ овог истраживања био је да одгонетне етиологију плавих и зелених плесни на плодовима лимуна у Србији. Коришћењем интегративног приступа у идентификацији, добијени изолати су окарактерисани са морфолошког, физиолошког, молекуларног, филогенетског и патолошког аспекта. Раст и морфологија колонија је испитана на Чапековој аутолизатној подлози са додатком квасца (CYA), агару са сладним екстрактом (MEA), креатин-сахарозном агару (CREA), као и на CYA на две додатне температуре инкубације (5 и 37 °C). Секвенцирани су интерни транс-

крибовани регион (ITS) и ген за бета-тубулин ради молекуларне идентификације изолата. Филогенетски односи испитани су коришћењем метода максималне вероватноће. Урађена је и провера патогености а могуће разлике у патогености одређених изолата поређене су применом једнофакторске анализе варијансе и *Tukey* тестом. Идентификовано је укупно четири врсте: *Penicillium expansum*, *Penicillium digitatum*, *Penicillium polonicum* и *Talaromyces rugulosus*. Све четири врсте потврђене су као патогени плодова лимуна, изазивајући сличне симптоме као на природно инфицираним плодовима. Резултати ове студије су први налази наведених врста *Penicillium* / *Talaromyces* као складишних патогена плодова лимуна у Србији и први налаз *T. rugulosus* као фитопатогена на истом биљном домаћину.

КЉУЧНЕ РЕЧИ: *Citrus limon*, морфолошка анализа, молекуларна карактеризација, мултилокусна филогенија, патогеност, *Penicillium*, *Talaromyces*