
МАТИЦА СРПСКА
1826

МАТИЦА СРПСКА
ОДЕЉЕЊЕ ЗА ПРИРОДНЕ НАУКЕ
ЗБОРНИК
МАТИЦЕ СРПСКЕ ЗА ПРИРОДНЕ НАУКЕ

MATICA SRPSKA
DEPARTMENT OF NATURAL SCIENCES
MATICA SRPSKA J. NAT. SCI.

Покренут 1951 / First published in 1951.

Until volume 10, the journal was published under the title *Научни зборник Матице српске: Серија природних наука* (Scientific Proceedings of Matica Srpska: Natural Sciences Series) (1951–1955). Volume 11 was released under the title *Зборник Матице српске: Серија природних наука* (Matica Srpska Proceedings: Natural Sciences Series) (1956), volumes 12–65 under the title *Зборник за природне науке* (Proceedings for Natural Sciences) (1957–1983), and from volume 66 the journal was published under the title *Зборник Матице српске за природне науке* (Matica Srpska Proceedings for Natural Sciences) (1984–). From volume 84 (1993) the journal was published in English under the title *Matica Srpska Proceedings for Natural Sciences* (1993–2012), and since volume 125 under the title *Matica Srpska Journal for Natural Sciences* (2013–)

143

Articales are available in full-text at the web site of Matica Srpska, Serbian Citation Index, CABI, EBSCO, Web of Science Master Journal List, AGRIS FAO (abstracts)

YU ISSN 0352-4906 UDK 5/6 (05)

MATICA SRPSKA JOURNAL FOR NATURAL SCIENCES

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NOVI SAD
2022

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UDC 582.28:75.052(497.11)"16
https://doi.org/10.2298/ZMSPN2243007L
ORIGINAL SCIENTIFIC PAPER

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FROM ON-SITE TO IN-LAB: MICROSCOPIC OBSERVATION OF FUNGAL PROLIFERATION ON 17th CENTURY MURAL PAINTINGS

ABSTRACT: The fungal community of biodeteriorated 17th century mural paintings within the nave and altar portion of the old Church of the Holy Ascension (Veliki Krčimir, Nišava District, Serbia) has been studied via an array of microscopic analyses in order to detect actively growing fungi and assess their potential damage to the painted layer and mortar. In situ microscopy, performed with portable microscopes, together with optical and scanning electron microscopy, has revealed impairments of the painted layer in the form of cracks and biopitting, along with surface salt deposits and hidden, symptomless fungal growth. Various structures, such as fully developed fruiting bodies and melanized mycelia, clusters of microcolonial fungi and lichen soredia, as well as a conidial apparatus and numerous conidia in mass have been observed, all attesting to the presence of actively growing fungal community on the surface of the painted layer and in the interspaces between the painted layer and mortar. Based on the observed reproductive structures, the main agents of biodeterioration have been identified as fungi of *Chaetomium* and *Cladosporium* genera. The documented deterioration symptoms are most likely due to hyphal penetration and formation of fruiting bodies and other fungal structures.

KEYWORDS: biodeterioration, *Chaetomium*, *Cladosporium*, conservation, fungi, *in situ* microscopy, mural paintings, SEM, optical microscopy

INTRODUCTION

Fungal-induced deterioration of mural paintings, as one of the oldest and most important landmarks of humankind, has received much worldwide attention

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in the last several decades with considerable number of published papers (Garg et al., 1995; Pepe et al., 2010; Stupar et al., 2014; to name a few). The porous nature and mineral composition of mortar, as well as the abundance of nutrients in form of adhesives admixed with mineral pigments in the painted layer, provide a variety of ecological niches for fungi. Many of them are known to induce structural and esthetic impairments, such as discoloration, exfoliation, cracking, paint blisters, etc., as a result of growth and metabolism in this unique habitat. Fungi, as ubiquitous organisms, are therefore usually referred to as the main biological agents of deterioration of mural paintings and other cultural heritage objects (Sterflinger, 2010).

To provide conservators and restorers with indications and suggestions necessary for the formulation of an efficient and long-term restorative plan-of-action it is mandatory to gain better understanding of fungal capabilities to colonize and subsequently induce deterioration of these valuable works of art. Use of multimicroscopical approach as the indispensable first step in a survey of fungal-induced decay of mural paintings is emphasized by several research groups (Rios and Ascaso, 2005; Rosado et al., 2015). In view of this, the main aim of the study was to analyze fungal community established on the surface of biodeteriorated 17th century mural paintings in the nave and altar portion of the old Church of the Holy Ascension (Veliki Krčimir, Serbia) via various microscopic techniques (*in situ*, optical and SEM) to detect and identify actively growing fungi and assess their potential damage to the painted layer and mortar.

STUDY SITE

Research was carried out on mural paintings decorating the nave and the altar area of the 17th century Church of the Holy Ascension located in the village of Veliki Krčimir (Gornje Zaplanje, Gadžin Han, Serbia). These preserved fragments of mural paintings with scenes from the Old and New Testaments, dating back to 1629, are a very valuable expression of Serbian fresco painting of the late 16th and early 17th century (Deljanin, 1995; Vučković, 2008). This oldest temple of the Eparchy of Niš is categorized as a cultural monument of great importance and is managed by the Institute for Protection of Cultural Monuments of Niš (SK305, *Official Gazette of RS* No. 28/83).

MATERIAL AND METHODS

In situ microscopy

Direct observation of surface impairments and fungal growth on eight sampling points of the nave and altar mural paintings was performed using two portable digital microscopes: Dino-Lite Edge AM7915MZTL (Figure 1) coupled to ASUS X54Ou notebook and Nikon ShuttlePix P-400R. Image processing

and measurements were achieved using DinoCapture 2.0 v1.5.39.A software and Nikon ShuttlePix Editor v3.4.0.2 software, respectively.

Figure 1. In situ microscopy using Dino-Lite Edge digital microscopes in the altar of the old Church of the Holy Ascension

Optical microscopy

For optical microscopy, samples were collected from all sampling points via adhesive tape method (Urzi and de Leo, 2001). Sample strips were stained with Lactophenol Cotton Blue and put on slides to be analyzed with Zeiss AxioImager M.1 microscope, using AxioVision Release 4.6 software.

Scanning electron microscopy

Samples for scanning electron microscope (SEM) were taken from the surface of the painted layer by means of adhesive carbon tape on aluminium cylinders. Analysis was performed at the SEM laboratory of University of Belgrade, Faculty of Mining and Geology, using JEOL JSM–6610LV microscope with a W filament gun. Sampled fragments of painted layer and mortar were gold coated using Leica EM SCD005 sputter coater (d=15 nm, ρ=19.2 g cm^{-3}). Secondary electron and backscattered electron images were obtained at 20 kV acceleration voltage in high-vacuum mode (15–30 μPa), with magnifications from 150x to 30,000x.

RESULTS AND DISCUSSION

In situ microscopy of mural paintings, performed via two portable microscopes, revealed abundance of surface salt efflorescence in form of white crystal tufts scattered on the deteriorated painted layer (Figure 2A,B). Observed salt is presumably calcium sulfate ($CaSO_4$) as its presence was previously documented on mural paintings of the investigated church (Unković et al., 2016). It is considered one of the most damaging salts for mural paintings that tends to crystallize and re-crystallize on exterior surfaces due to changes in physical parameters, mainly temperature and humidity. Throughout the crystallization process salts accumulated in pores induce additional pressure causing cracking and exfoliation of mural surface, as well as the loss of fragments (Pérez-Alonso et al., 2004; Sterflinger and Piñar, 2013; Ettenauer et al., 2014). Furthermore, visible strucutral alterations, in form of biopitting, loss of mural fragments and cracks filled with organic and inorganic deposits (Figure 2C,D) were evident. Biopitting phenomenon is known to be mainly induced by the mechanical and chemical activity of microcolonial fungi – MCF (Sterflinger and Piñar, 2013). Presence of these fungi was observed in the adhesive tape samples in form of melanized cell clusters (Figure 3C).

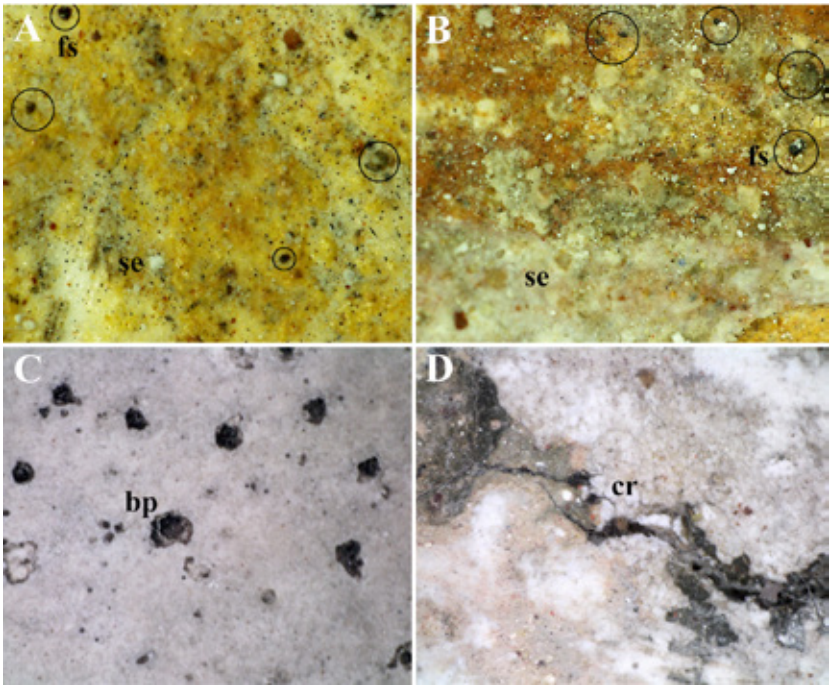

Figure 2. In situ micrographs of deteriorated mural paiting surfaces: A, B. white salt deposits with black fruiting bodies; C. symptoms of biopitting; D. surface crakcs fiilled with organic and inorganic deposits; fs – fungal structures, se – salt efflorescence, bp – biopitting; cr – cracks

Optical microscopy of adhesive tape samples showed presence of a microbial community dominated by melanized micromycetes. In all of the analyzed samples, a significant part of the fungal structures documented between painted layer and mortar support were melanized hyphae, chlamydospores, and conidial chains of microfungi and dark brown clusters of rock inhabiting microcolonial fungi. Four dominant features were detected: perithecia, chaetae, and ascospores of *Chaetomium globosum* (Figure 3A), fragmented melanized mycelia (Figure 3B), numerous clusters of dark microcolonial fungi (Figure 3C) and abundant lichen vegetative propagules – soredia (Figure 3D).

Abundant presence of *Chaetomium* structures, prominent cellulolytic ascomycetes, was expected as previous conservation interventions on mural paintings included cellulose compresses for salt extraction. Such nutrient input, in combination with existing organic matter in the painted layer, plaster, and surface dust deposits, permits proliferation of melanized fungi and formation of dark discolorations on the mural paintings surfaces. In conservation practice, removal of strong dark-pigmented stains that conceal mural motifs is problematic due to a strong melanization and thick cell walls of melanized fungi which makes them highly resistant to physical stress and biocide treatment (Ciferri, 1999; Sterflinger, 2010).

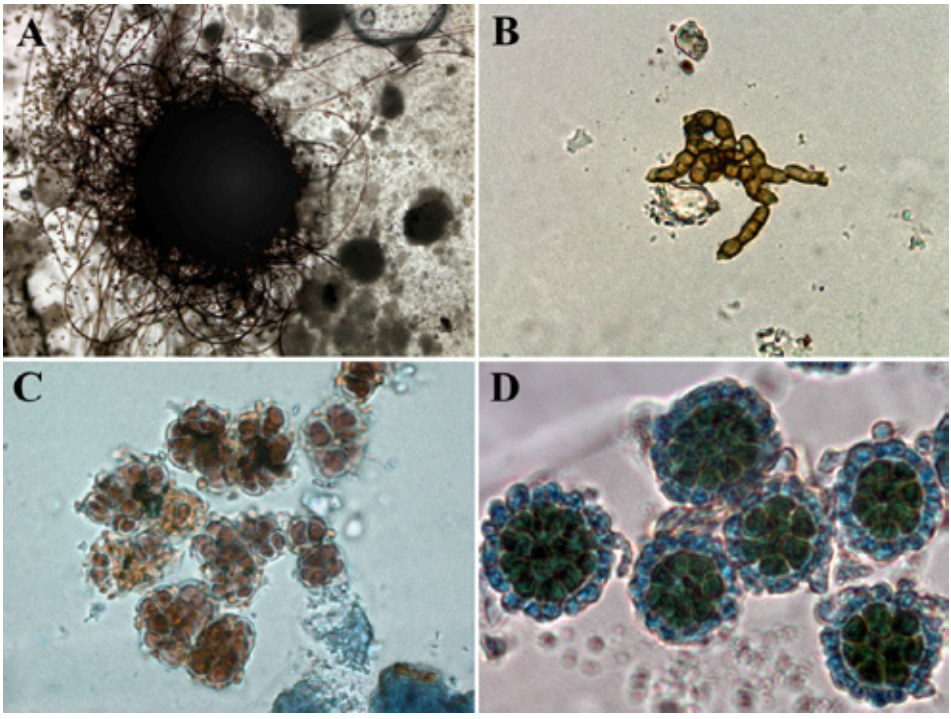

Figure 3. Optical micrographs of adhesive tape samples: A. perithecium of *Chaetomium globosum*; B. fragmented melanized mycelia; C. clusters of microcolonial fungi; D. lichen soredia

SEM analysis of studied mural fragments has indicated the main agent of deterioration. Interspace between painted layer and mortar support was heavily permeated with hyphal network with numerous conidiophores and conidial mass of *Cladosporium cladosporioides* (Figure 4). In addition to various fungal structures, deteriorated material was documented. Abudant presence of *Cladosporium* structures is in accordance to previous investigations which suggests that species of this genus are the one of the most common inhabitants of mural paintings since they possess the ability to not only withstand, but also thrive in a very wide value range of several environmental factors (Ciferri, 1999). Nowadays, *Cladosporium* species are recognized as the the main agents in the process of biodeterioration of mural paintings (Ciferri, 1999; Unković et al., 2016).

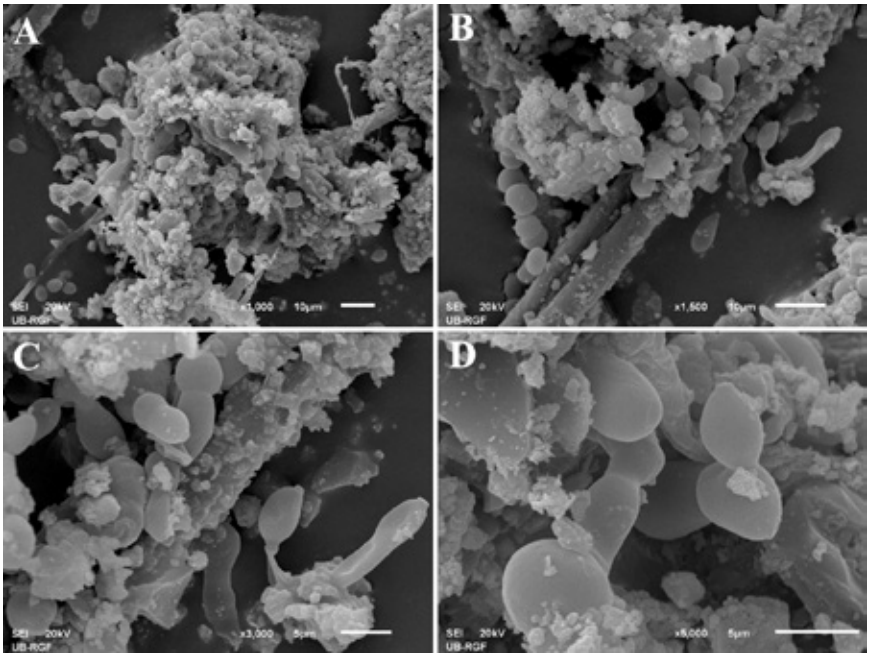

Figure 4. Scanning electron micrographs of *Cladosporium cladosporioides* on carbon tapes: A, B. deteriorated mural fragment permeated with mycelia and conidial chains; C, D. details of various *C. cladosporioides* structures

CONCLUSION

Conducted microscopic analysis of mural paintings decorating 17th century Church of the Holy Ascension (Veliki Krčimir, Serbia) has revealed the main agents of biodeterioration, i.e. fungi from *Chaetomium* and *Cladosporium* genera, and microcolonial fungi. Documented deterioration sypmtoms, such as biopitting, cracks and loss of mural fragments, are most likely formed due to hyphal penetration and formation of fruiting bodies and other structures by

these fungi in the interspace between the painted layer and mortar support. This research emphasizes the importance of application of microscopic methods for the identification of actively growing fungi, as the causative agents of deterioration process. This information is necessary for formulation of conservation plan, adequate biocide selection and application, and eventual stain removal.

ACKNOWLEDGEMENTS

This research was supported by the Science Fund of the Republic of Serbia, PROMIS, #GRANT No. 6066210, PROTECTA and the bilateral project between Serbia and Slovenia (Novel biocides for cultural heritage of Southeast Europe – biocontrol and biomimetic systems for preservation of old masterpieces; No. 337-00-21/2020-09/4).

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ОРИГИНАЛНИ НАУЧНИ РАД

ОД ИСТРАЖИВАНОГ ЛОКАЛИТЕТА ДО ЛАБОРАТОРИЈЕ: МИКРОСКОПСКА АНАЛИЗА ПОРАСТА ГЉИВА НА ЗИДНИМ СЛИКАМА ИЗ XVII ВЕКА

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РЕЗИМЕ: Применом различитих микроскопских техника анализирана је заједница гљива на зидним сликама наоса и олтара старе Цркве Светог Вазнесења Господњег (Велики Крчимир, Нишавски округ, Србија) у циљу детекције активно растућих микрогљива и процене потенцијално штетног утицаја на бојени слој и малтер. Директна микроскопија, спроведена на терену применом преносних дигиталних микроскопа, заједно са оптичком и скенинг електронском микроскопијом указала је на присуство оштећења у форми пукотина и симптома феномена biopitting, као и површинских депозита соли и скривеног раста гљива. Забележене су различите фунгалне структуре, као што су потпуно формирана плодоносна тела, меланизована мицелија, кластери микроколонијалних гљива, соредије лишајева, конидијални апарати и конидије у маси, што указује на присуство активно растуће заједнице гљива на површини бојеног слоја и на месту контакта бојеног слоја и малтерне подлоге. На основу детектованих репродуктивних структура као главни узрочници процеса биодетериорације су идентификоване гљиве родова *Chaetomium* и *Cladosporium*, и закључено је да су присутни симптоми оштећења зидних слика пре свега љуспање и губитак фрагмената последица продирања тургесцентних хифа у супстрат и формирања плодоносних тела и других фунгалних структура.

КЉУЧНЕ РЕЧИ: биодетериорација, *Chaetomium*, *Cladosporium*, гљиве, директна микроскопија, зидне слике, конзервација, оптичка микроскопија, СЕМ

Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. № 143, 15—25, 2022

UDC 582.28
https://doi.org/10.2298/ZMSPN2243015S
ORIGINAL SCIENTIFIC PAPER

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HEMOLYTIC POTENTIAL OF BIOAEROSOL-DERIVED *Aspergillus*, *Penicillium* AND *Talaromyces* MOULD ISOLATES

ABSTRACT: *Aspergillus*, *Penicillium* and *Talaromyces* species are frequently cited as contaminants of various substrata and are often associated with indoor environments. The main purpose of this study was to assess the potential pathogenicity of aerosol-derived fungi from *Aspergillus*, *Penicillium* and *Talaromyces* genera, isolated in the rooms for conservation of cultural heritage artefacts, via estimating hemolytic activity. Hemolysis was detected in 20.58% of tested isolates at 37 ºC (11.76% partial and 8.82% complete) and 64.71% at 25 ºC (38.24% partial and 26.47% complete). The majority of isolates that caused α hemolysis led to the significant oxidation of hemoglobin iron with methemoglobin content in blood agar medium, higher than 80%. *Aspergillus melleus* was the only tested fungi that caused formation of ferry hemoglobin after the incubation at 25 °C. Obtained *I* values (index of activity for hemolytic exoenzymes) for α hemolysis were in range of from 0.13 to 0.60 for 37 °C, while for the temperature of 25 °C values were in range of from 0.08 to 0.50. The same values for β hemolysis were in range of from 0.03 to 0.08 (37 °C), i.e. 0.06 to 0.49 (25 °C). Monitoring of pathogenic airborne fungi in indoor environments and estimation of their virulence is essential for the adequate assessment of human health risks.

KEYWORDS: fungi, blood agar, hemolysis, pathogens, virulence

INTRODUCTION

Aspergillus, *Penicillium* and *Talaromyces* propagules (mostly conidia, rarely ascospores) are very light, with dry cell walls and therefore able to easily

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† We are sad to report that the author Jelena Vukojević is deceased.

detach from conidiogenous apparata and cleistotecia, respectively (Samson et al., 2010; Viegas et al., 2015). This makes them highly dispersible through the air where they are an important component of bioaerosol. Indoor environments, when overloaded with spores and other fungal propagules, could be regarded as serious health risk for anyone who spends a lot of time within (Unković et al., 2018). Hence, nowadays, indoor air fungi are considered one of the major culprits for sick building syndrome (SBS) which includes such symptoms as headache, dizziness, distraction, running nose, dry or sore throat, fatigue, and skin irritation (WHO, 1990). The role of mould fungi from genera *Aspergillus* and *Penicillium* in SBS is linked with their ability to produce toxic metabolites and cause severe allergic reactions (Flanning et al., 2001).

Furthermore, hemolityc activity is regarded as virulence factor for many pathogenic microorganisms (Aktas and Yigit, 2014). In that sense, Vesper and Vesper (2004) hypothesized that fungal hemolysins, enzymes responsible for hemolityc activity, have a crucial role in developement of some SBS symptomes. The process of hemolysis is defined as the rupturing of erythrocytes and the release of their content into surrounding environment. Two types of hemolysis are described: (I) complete (β) hemolysis, which represents total erythrocyte destruction by extracellular metabolites of microorganisms, and (II) partial (α) hemolysis as a result of the oxidation of hemoglobin (Fe^{2+}) to methemoglobin (Fe^{3+}) and ferry hemoglobin (Fe^{4+}); while absence of hemolysis is self-contradictory called γ hemolysis (Buxton, 2005). Upon erythrocyte lysis, free hemoglobin and/or heme are released from the cell and pathogens employ various mechanisms to utilize iron contained in these molecules (Bullard et al., 2012; Subramanian et al., 2014). The ability of pathogens to acquire iron has been documented to be of the upmost importance for their survival within the mammalian host cells, and further, ability to cause infection (Aktas and Yigit, 2014). Therefore, the presence of potential pathogens, especially in indoor environments, where different human activities may occur, must not be neglected. Bearing all this in mind, the main purpose of this study was to evaluate the potential virulence of selected airborne *Aspergillu*s, *Penicillium* and *Talaromyces* species by testing their hemolytic activity and ability to grow at human body temperature.

MATERIAL AND METHODS

Tested fungi

A total of 34 mould fungi belonging to *Aspergillus*, *Penicillium* and *Talaromyces* genera were selected from the culture collection of the University of Belgrade – Faculty of Biology (BEOFB) for screening of the hemolytic activity. All tested fungi were bioaerosol isolates (airborne fungi) obtained from rooms designated for conservation and safeguarding of cultural heritage artifacts in

Belgrade (eg. ateliers for the conservation of stone artifacts; ateliers for the conservation of paintings; ateliers for the conservation of textile). Prior to the experiment, isolates were identified based on macro- and micromorphological characteristics of 7 days old colonies as described previously (Samson et al., 2010). Additionally, morphological identification was confirmed by *ITS* and *β-tubulin* gene sequencing (Savković et al., 2019). All isolates were maintained in cryovials filled with 1.5 ml glycerol (30%) and stored at -80 °C.

Screening of hemolytic activity

The hemolytic activity of the isolates was evaluated by a qualitative method. The selected isolates were inoculated on blood agar (BA) prepared with 5% defibrinated sheep blood (Torlak, Serbia) according to protocol proposed by Buxton (2005). Two incubation temperatures were chosen: human body temperature (37±1 °C) and room temperature (25±1 °C) as a control according to a modified protocol by Pakshir et al. (2016). Inoculated plates were incubated in thermostat (UE 500, Memmert) during 7 days. After the incubation period, the formation of distinctive halo around the colony was observed and three types of hemolysis according to Buxton (2005) were distinguished: α (partial) – the appearance of green or brown halo around the colony; *β* (complete) – the appearance of transparent halo around the colony; γ (absence) – positive growth without forming a halo around the colony. Degrees of α hemolysis were assessed determining the methemoglobin and ferry hemoglobin content using the percent color charts according to Paton et al. (2016) and Kanias and Acker (2010), respectively.

Then diameters of colonies and of the distinctive halos were measured and index of activity for hemolytic exoenzymes (*I*) was calculated according to the formula:

$$I = 1 - D_c D_h^{-1}$$

where D_c and D_h are the diameters of the colony and halo of hemolysis, respectively.

RESULTS

After 7 days of incubation at 37 °C and 25 °C, growth of tested fungi were documented, and both *α* and *β* hemolysis were detected (Figure 1). The growth at human body temperature has been observed for 10 isolates (five *Aspergillus*, three *Penicillium* and two *Talaromyces* species) out of 34 tested, and the oxidation rate of hemoglobin in BA was significant at 37 °C, confirmed by the presence of green ferry hemoglobin (Fe^{4+}) (Figure 2). Partial (*α*) hemolysis, following the incubation at 37 °C, was documented for four isolates (all of them caused the oxidation of hemoglobin to ferry state) while three isolates displayed complete (*β*) hemolysis.

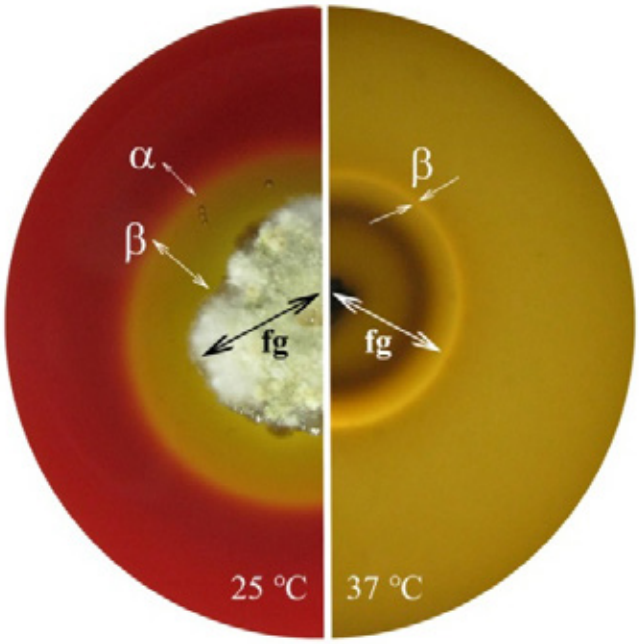

Figure 1. Hemolytic activity of *Aspergillus protuberus* (left) and *Penicillium chrysogenum* (right) on blood agar medium: *α* – partial hemolysis; *β* – complete hemolysis; fg – fungal colony growth.

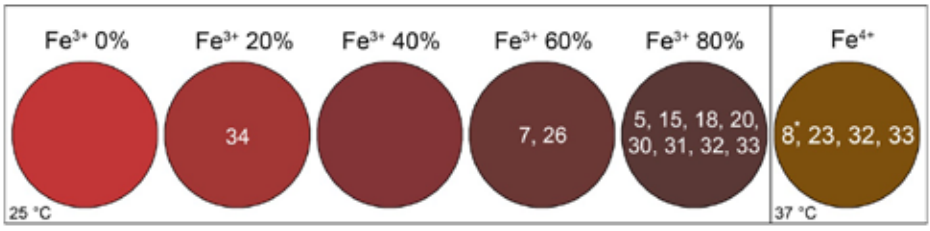

Figure 2. Percent methemoglobin (Fe^{3+}) and ferry hemoglobin state (Fe^{4+}) on inoculated BA medium after 7 days of incubation, modified according to Paton et al. (17) and Kanias and Acker (18). Numbers in circles correspond to isolates' number given in Table 1. Asterisk indicates state on both tested temperatures.

Only five *Aspergilli* isolates (*A. domesticus*, *A. penicillioides*, *A. proliferans*, *A. pseudoglaucus* and *A. versicolor*) were not able to grow on BA at 25 °C (Table 1). Total of 22 isolates (approx. 64.71%) displayed hemolytic activity when being incubated at 25 °C. Among them, the ability of *β* hemolysis was documented for 9 isolates while *α* hemolysis was detected for 13 isolates (Figure 3). The majority of isolates that caused *α* hemolysis (69.20%), led to the significant oxidation of hemoglobin iron with methemoglobin content in BA higher than 80%.

Table 1. Hemolytic activity of tested bioaerosol-derived fungal isolates at 37 °C and 25 °C

No	Isolate	Hemolysis at 37 °C		Hemolysis at 25 °C		No	Isolate	Hemolysis at 37 °C		Hemolysis at 25 °C	
		Type*	Index	Type*	Index			Type*	Index	Type*	Index
1	*Aspergillus amstelodami* BEOFB3160m	-	-	γ	-	**18**	*Penicillium brevicompactum* BEOFB1102m	-	-	α	**0.08**
2	*Aspergillus calidoustus* BEOFB3220m	β	**0.06**	β	**0.19**	**19**	*Penicillium canescens* BEOFB11180m	-	-	β	**0.27**
3	*Aspergillus creber* BEOFB3250m	-	-	γ	-	**20**	*Penicillium carneum* BEOFB11150m	-	-	α	**0.28**
4	*Aspergillus domesticus* BEOFB3270m	-	-	-	-	**21**	*Penicillium chrysogenum* BEOFB11120m	β	**0.08**	β	**0.36**
5	*Aspergillus europaeus* BEOFB382m	-	-	α	**0.30**	**22**	*Penicillium citreonigrum* BEOFB11190m	-	-	γ	-
6	*Aspergillus flavus* BEOFB315m	γ	-	γ	-	**23**	*Penicillium citrinum* BEOFB11110m	α	**0.49**	β	**0.15**
7	*Aspergillus jensenii* BEOFB3200m	-	-	α	**0.11**	**24**	*Penicillium decumbens* BEOFB11160m	γ	-	β	**0.06**
8	*Aspergillus melleus* BEOFB3180m	α	**0.57**	α	**0.26**	**25**	*Penicillium digitatum* BEOFB1112m	-	-	γ	-
9	*Aspergillus niger* BEOFB345m	β	**0.03**	β	**0.14**	**26**	*Penicillium expansum* BEOFB11130m	-	-	α	**0.09**
10	*Aspergillus penicillioides* BEOFB3190m	-	-	-	-	**27**	*Penicillium glabrum* BEOFB11100m	-	-	α	**0.24**
11	*Aspergillus proliferans* BEOFB3280m	-	-	-	-	**28**	*Penicillium sanguifluum* BEOFB11170m	-	-	β	**0.13**
12	*Aspergillus protuberus* BEOFB3240m	-	-	β	**0.46**	**29**	*Penicilliumsolitum* BEOFB1190m	-	-	γ	-
13	*Aspergillus pseudoglaucus* BEOFB3170m	-	-	-	-	**30**	*Penicillium ulaiense* BEOFB11140m	-	-	α	**0.50**
14	*Aspergillus ruber* BEOFB3150m	-	-	γ	-	**31**	*Penicillium viridicatum* BEOFB11200m	-	-	α	**0.50**
15	*Aspergillus sydowii* BEOFB3142m	γ	-	α	**0.17**	**32**	*Talaromyces amestolkiae* BEOFB2610m	α	**0.60**	α	**0.35**
16	*Aspergillus tabacinus* BEOFB3260m	-	-	β	**0.49**	**33**	*Talaromycessayulitensis* BEOFB2600m	α	**0.13**	α	**0.26**
17	*Aspergillus versicolor* BEOFB3133m	-	-	-	-	**34**	*Talaromyces verruculosus* BEOFB2620m	-	-	α	**0.38**

* (*α*) – partial hemolysis (*β*) – complete hemolysis, (*γ*) – growth on BA without visible clearance zone, (-) – no growth

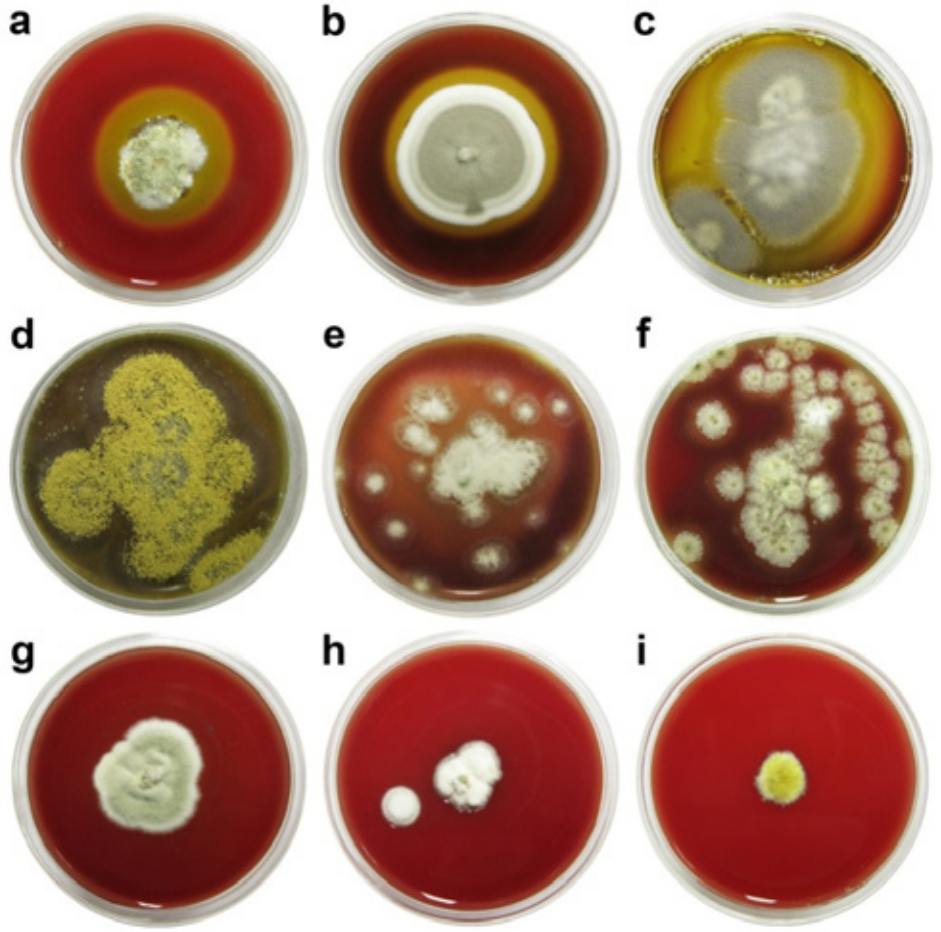

Figure 3. Hemolytic activity of tested airborne isolates on blood agar medium at 25 °C, 7 days: a) *Aspergillus protuberus*; b) *Penicillium canescens*; c) *P. chrysogenum*; d) *A. melleus*; e) *P. glabrum*; f) *Talaromyces amestolkiae*; g) *A. creber*; h) *P. citreonigrum*; i) *A. ruber* (a-c. complete (*β*) hemolysis; d-f. partial (*α*) hemolysis; g-i. γ-hemolysis).

Aspergillus melleus was the only one that caused formation of ferry hemoglobin after the incubation at 25 °C. Methemoglobin content in BA cultures of *A. jensenii* and *P. expansum* isolates was 60%, while *T. verruculosus* ability to cause *α* hemolysis was the lowest with 20% of methemoglobin (Figure 3).

A total of 7 isolates displayed hemolytic activity both on 37 and 25 °C: *A. calisoustus*, *A. mellus*, *A. niger*, *P. chrysogenum*, *P. citrinum*, *T. amestolkiae* and *T. sayulitensis*. Documented *I* values for *α* hemolysis were in range of from 0.13 to 0.60 for 37 °C, while for the temperature of 25 °C values were in range of from 0.08 to 0.50. On the other hand, obtained values for *β* hemolysis were in range of from 0.03 to 0.08 (37 °C), i.e. 0.06 to 0.49 (25 °C) (Table 1).

DISCUSSION

Potential virulence of selected *Aspergillus*, *Penicillium* and *Talaromyces* airborne isolates was estimated through hemolytic potential and the ability to grow at human body temperature. The colony growth on BA is usually used for defining hemolytic activity of bacterial species and absence of this activity was regarded as a lack of virulence for tested bacterial strains (Hof, 1984). However, BA is seldom used for the testing of fungal hemolytic potential (Juntachai et al., 2014), and in most cases virulence of clinical *Candida* isolates was estimated through the ability to grow on BA and to form halo around the colony (Rossoni et al., 2013; Pawar et al., 2014). Also, Aktas and Yigit (2014) used this method to estimate hemolytic activity of dermatophytes isolated from clinical specimens.

Most tested *Aspergillus* and *Penicillium* species are frequently cited as human pathogens, allergens and mycotoxins producers (Samson et al., 2010), but reports regarding their hemolytic potential are rather scarce. However, first records regarding hemolytic activity in *Aspergilli* dated from 1939 by Henrici, who proved this activity for *A. fumigatus* and *A. flavus* using experimental animals (Henrici, 1939). However, there are only limited reports regarding the growth of airborne filamentous fungi on BA (Bogomolova and Kirtsideli, 2009; Anaya et al., 2016; Borrego et al., 2017). Anaya et al. (2016) highlighted the hemolytic potential of fungi isolated from indoor air in variety of rooms of National Archive of the Republic of Cuba. These authors demonstrated hemolytic activity of *A. flavus*, *A. niger* and *P. chrysogenum*, which corresponds with results presented here. On the contrary, in the research of Bogomolova and Kirtsideli (2009), *A. niger* and *P. chrysogenum* isolated from the indoor air of metro stations in St. Petersburg, did not show hemolytic activity. These discrepancies could be explained through the different physiology of tested isolates and different experimental conditions.

Some fungal pathogens are thermophiles and their optimal temperature for growth and proliferation is higher. According to Krijgsheldi et al. (2013) the temperature optimum for *A. niger* ranges from 35 to 37 °C. Hedayati et al. (2007) reported 37 °C as optimal growth temperature for *A. flavus*, which corresponds with the human body temperature. Growth on this temperature is a prerequisite for hyphal proliferation on epithelium and endothelium, including blood vessel cells (Borrego et al., 2017). Hemolytic activity along with ability to grow on human body temperature are therefore considered to be significant virulence factors for human pathogens and hence are of a great importance in medical mycology researches (Schaller et al., 2005; Bogomolova et al., 2007). However, it should be emphasized that this type of testing can only roughly indicate the degree of virulence of the fungi and that a study in experimental animals should be applied for a more adequate assessment (Bogomolova and Kirtsideli, 2009).

Many of the hemolytic agents previously isolated from fungi are peptides, proteins (Greenhill et al., 2010) and specific enzymes involved in hemolytic activity classified as hemolysins (Lineras et al., 2007). Hemolysins have been

defined as exotoxins, capable of lysing erythrocytes as well as nucleated cells, and these compounds are able to interact with specific ligands on the surface of various target cells (Nayak et al., 2013). During the lysis of red blood cells the iron is released, which is an important growth factor for microbes especially during infection (Subramanian et al., 2014). For some fungal isolates which display positive hemolytic activity in this study, the hemolysins have been discovered and characterized. For *P. chrysogenun* and *A. niger* isolates, which both exhibited complete (β) hemolysis in this research, Donohue et al. (2005; 2006) isolated and described hemolysins named *chrysolysin* and *nigerlysin*, respectively. *Aspergillus niger* is considered as a potential strain for acid protease production and hemoglobin degradation with the aim of implementation in biotechnological processes (Li et al., 2019). Other authors identified *aspergilloglutamic peptidase* as one of the compounds responsible for hemolytic action (Shi et al., 2015). Based on a number of characterization and mechanistic studies, several fungal hemolysins have been proposed as fungal virulence factors (Rementeria et al., 2005). Since, all the tested isolates originated from indoor air of cultural heritage conservation premises, where the presence of conservators for a long period of time is often required, hemolytic activity of 47.05% tested isolates as potential health risk factor must not be neglected. The potential role of hemolysins as potential causative agents of SBS has been discussed by Vesper and Vesper (2004). These authors concluded that fungal hemolysins may cause SBS symptoms, and also could be useful as biomarkers of exposure to airborne fungi in indoor environments, since they can be measured in bodily fluids and compared with environmental samples.

CONCLUSION

This study reflects the preliminary assessment of the virulence of indoor air fungal isolates. Given that some species isolated from frequently occupied premises have been confirmed human pathogens (*A. calidoustus*, *A. niger*, *A. flavus*) and along with hemolytic activity and ability to grow on human body temperature *in vitro*, their potential effect on human health should not be neglected. The presence of these species must be monitored in those environments along with estimation of their virulence. However, it is important to emphasize that potential hemolytic activity must be observed in the context of fungal species propagules number and immunity status of exposed individuals.

ACKNOWLEDGEMENTS

This research was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia [Contract No.451-03-9/2021-14/ 200178].

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ХЕМОЛИТИЧКА АКТИВНОСТ ИЗОЛАТА *Aspergillus*, *Penicillium* И *Talaromyces* ИЗ БИОАЕРОСОЛА

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РЕЗИМЕ: Врсте родова *Aspergillus*, *Penicillium* и *Talaromyces* често се наводе као контаминанти различитих супстрата и често се изолују из ваздуха затворених простора. Основни циљ истраживања била је процена потенцијалне патогености гљива родова *Aspergillus*, *Penicillium* и *Talaromyces*, изолованих из аеросола просторија специјализованих за конзервацију објеката културне баштине, на основу способности да врше хемолизу. Хемолитичка активност на температури од 37 ºC је забележена код укупно 20,58% тестираних изолата (11,76% делимичне (α) хемолизе, и 8,82% потпуне (β) хемолизе) и код 64,11% изолата на температури од 25 ºC (38,24% делимичне и 26,47% потпуне). Код већине микромицета код којих је забележена *α* хемолиза дошло је до значајне оксидације хемоглобинског гвожђа до метхемоглобина у медијуму крвног агара (већем од 80%). Изолат *Aspergillus melleus* је једини показао способност формирања ферилхемоглобина након инкубације на 25 °C. Документоване вредности индекса детериорације (*I*) за *α* хемолизу су биле у опсегу од 0,13 до 0,60 (37 °C), док су на температури од 25 °C вредности биле у опсегу од 0,08 до 0,50. Исте вредности индекса за *β* хемолизу износиле су од 0,03 до 0,08 (37 °C), тј. од 0,06 до 0,49 (25 °C). Мониторинг присуства потенцијалних аерогених патогених гљива у затвореним срединама и одређивање њихове вируленције је неопходно за адекватну процену ризика за људско здравље.

КЉУЧНЕ РЕЧИ: вируленција, гљиве, крвни агар, патогени, хемолиза

Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. № 143, 27—38, 2022

UDC 633.11:[615.918:632.25
https://doi.org/10.2298/ZMSPN2243027K
ORIGINAL SCIENTIFIC PAPER

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CONTAMINATION OF DURUM WHEAT LINES KERNELS WITH *Fusarium* SPECIES AND DEOXYNIVALENOL

ABSTRACT: *Fusarium* infection and deoxynivalenol (DON) contamination in seven durum wheat lines kernel (six domestic durum lines ZP 16, ZP 34, ZP 41, ZP 74, ZP 120, ZP DSP 66, and one international durum line Cimmyt 7817) during two harvest seasons (2015–2016) has been studied. The four *Fusarium* species, *F. graminearum*, *F. proliferatum*, *F. sporotrichioides*, and *F. verticillioides*, were identified in 2015. A different structure of the *Fusarium* population, which in addition to *F. graminearum*, *F. sporotrichioides* and *F. verticillioides*, also comprised *F. poae*, *F. semitectum*, and *F. subglutinans*, was identified in 2016. *F. graminearum* was the predominant species in the durum wheat lines kernels and the potential producer of DON. The other *Fusarium* spp. were isolated sporadically and with a low incidence in the kernels. The incidence of *F. graminearum* and DON levels were significantly affected by the wheat genotypes and studied years and these parameters were negatively correlated. The incidence of *F. graminearum* was significantly higher in 2015 (75.86%) than in 2016 (63.43%), while the level of DON was significantly higher in 2016 (3.636 mg kg^{-1}) compared to 2015 (1.126 mg kg^{-1}). Statistically, there was a significantly higher incidence of *F. graminearum* in ZP DSP 66 (73.00%) and ZP 120 (72.75%) durum wheat lines than in the other durum genotypes. DON level was the highest in durum wheat line ZP 120 (3.854 mg kg^{-1}). Considering all treatments tested, the mean DON level was 2.381 mg kg^{-1}, while the mean incidence of *F. graminearum* was 69.64%. Tested durum wheat lines showed susceptibility to *F. graminearum*, resulting in high DON levels in kernels. The results obtained suggest the importance of using the lines with improved resistance to Fusarium head blight in the breeding programs for new durum wheat cultivars.

KEYWORDS: *Fusarium* spp., deoxynivalenol, durum wheat lines

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INTRODUCTION

In Europe, durum wheat (*Triticum turgidum* var. *durum*) is grown on 3 million hectares, mainly in the Mediterranean countries (Italy, Greece, Spain, and France), with an average yield of around 5 t/ha to 6 t/ha. Other European countries have low productivity and quality of durum wheat crops, and they intend to improve breeding processes by focusing on increasing yield and quality features (Gorczyca et al., 2018). In Serbia, an average yield of 2.2 t/ha was reported, which is 30% of the total average yield of bread wheat (*Triticum aestivum* L.) (Đurić et al., 2019).

Fungal pathogens from the genus *Fusarium* cause Fusarium head blight (FHB), one of the most severe diseases of wheat worldwide. Severeal *Fusarium* spp. participate in pathogenesis FHB of which *F. graminearum* is the most predominant. *F. graminearum* is a member of the *F. graminearum* species complex (FGSC) also called *F. graminearum sensu lato*, which includes at least 16 phylogenetic distinct species (van der Lee et al., 2015). It is a monophyletic species complex in which species are localized in different geographical regions, for example, *F. asiaticum* is responsible for FHB in some parts of Asia. However, the term *F. graminearum sensu stricto* is retained for the species most commonly associated with FHB worldwide (Tóth et al., 2005). In Serbia, besides *F. graminearum* strains, one strain of *F. vorosii* was isolated as a member of FGSC and showed pathogenicity on wheat spikes (Obradović et al., 2022). In general, *F. graminearum, F. culmorum, F. avenaceum, F. sporotrichioides, F. langsethiae,* and *F. poae* are the most common FHB pathogens (Pancaldi et al., 2010; Karlsson et al., 2021). *F. graminearum* and *F. poae* appear to cause FHB in humid, warm and dry environments, while *F. culmorum, F. avenaceum F. sporotrichioides,* and *F. langsethiae* are mostly found in cool and wet agroecological conditions. The distribution of FHB is affected by both the genetic diversity of the pathogens and environmental factors (Nielsen et al., 2011; Zhang et al., 2011).

F. graminearum overwinters as a saprobe on the crop residues. The primary inoculum consists of ascospores and macroconidia. Ascospores (sexual spores) develop from perithecia during the flowering wheat stage. Macroconidia (asexual spores) develop from mycelia on the plant infected with *F. graminearum* and can be water splashed onto the adjacent plants and their developing spikes. In general, the most susceptible wheat stages for *Fusarium* infection are from anthesis to soft dough phenophase. Durum wheat is extremely susceptible to FHB and it is very difficult to make resistant cultivars by breeding because of the lack of resistance sources. On the contrary, in bread wheat, there is a range of resistance sources from "exotic" and "native" wheat germplasms and large genetic variation for FHB resistance (Haile et al., 2019).

Direct economic losses of FHB disease are reduced yields and quality of wheat kernels while indirect ones are the consequence of mycotoxin contamination. *F. graminearum* produces trichothecene mycotoxins (the most important are type A and type B) and estrogenic mycotoxin zearalenone. Trichothecenes are sesquiterpenoids that inhibit eukaryotic protein synthesis and cause

numerous human and animal mycotoxicoses. They also exhibit phytotoxicity virulence on sensitive hosts, causing necrosis and proliferation of the pathogen (Starkey et al., 2007; Valverde-Bogantes et al., 2020). The most important B-trichothecenes include deoxynivalenol (DON) and its derivatives [3-acetyl and 15-acetyl deoxynivalenol (3-ADON and 15-ADON)], nivalenol (NIV) and NIV derivative (4-acetyl-nivalenol). *F. graminearum* strains are also capable of producing type A trichothecenes named NX-2. It is a separate population of *F. graminearum*, named NA3 (Valverde-Bogantes et al., 2020), while NA1 and NA2 populations produce 15-ADON and 3-ADON, respectively (Foroud et al., 2019).

In favourable climatic conditions (precipitation and temperature), particular during wheat anthesis, *Fusarium* species can develop and cause FHB. The infection of kernels primarily depends on wheater conditions and the wheat genotype. There are preventive measures to limit FHB development such as agricultural, chemical, and biological control and choice of resistant cultivars. Durum wheat is more susceptible to FHB and DON accumulation than common wheat. Thus, resistance to FHB also includes resistance to DON (Scala et al., 2016). The best timing for fungicide application during the flowering wheat stage has a crucial role in reducing FHB and DON contamination in conducive climatic conditions (Balducci et al., 2022).

Fusarium infection and deoxynivalenol contamination of wheat are the two main constraints for wheat safety worldwide. *Fusarium* species as causers of FHB represent the most dangerous wheat pathogens. Consequently, there is a high risk of DON accumulation in wheat production. Since there is almost no data about natural *Fusarium* and deoxynivalenol occurrence in durum wheat kernels in Serbia, the main object of this study was to determine the spectrum and incidence of *Fusarium* spp. and DON levels on kernels in domestic and international durum wheat lines during two harvest seasons (2015–2016). Data for weather conditions in the years of the study were also analyzed because they have a significant role in *Fusarium* infection and DON production.

MATERIALS AND METHODS

Samples of wheat kernels from six domestic lines (ZP 16, ZP 34, ZP 41, ZP 74, ZP 120, ZP DSP 66) and one international line (Cimmyt 7817) of durum wheat were collected during the harvesting period in 2015 and 2016. The sample size of each line was about 1 kg. Prior to mycological analyses, samples were kept at 4 °C. For determining the moisture content, kernels were first milled in an analytic mill (IKA A11, Germany) and then examined on a moisture analyzer (OHAUS MB35, USA).

In the mycological examination, sub-samples of 200 kernels were disinfected in 1% sodium hypochlorite solution (NaOCl) for about 3–5 min and rinsed twice in sterile water. After drying on sterile filter paper, kernels were plated on Ø90 mm Petri plates (10 kernels per Petri plate) that contained potato dextrose agar with 1.8% salt (18 g NaCl in a 1-litre agar medium) and incubated

for seven days or longer at room temperature. Each sample was done in three replicates. The species of fungi were identified using mycological keys by Leslie and Summerell (2006) and Watanabe et al. (2002). Kernel infection by single fungal species was calculated as the ratio of the number of kernels from which this species was isolated to the total number of analysed kernels, expressed as a percentage of incidence. The base index (BI) was calculated using the formula: BI (%) = Yi / Yo × 100, where Yo is the lowest incidence of *F. graminearum*, i.e. the lowest level of DON, and Yi is the incidence of *F. graminearum*, i.e. level of DON.

The mycotoxicological examinations were performed using the Enzyme-Linked ImmunoSorbent Assay (ELISA). Before analyses, durum wheat kernel samples were dried for 72 h at 60 °C and then milled in an analytic mill (IKA A11, Germany). By an assay procedure of the ELISA test kit *Celer* DON (Tecna, Italy), the quantitative detection of DON in samples was determined on the ELISA reader (Biotek EL x 800TM, USA) at a wavelength of 450 nm. Each sample was assayed in three replicates. The limit of detection for DON in cereals (durum wheat) was 0.12 mg kg^{-1}.

Statistical analysis of variance (ANOVA) (IBM SPSS Statistic 20) was used for testing variables. Tukey's test was used to compare means at a significance level of 5%. Correlation analyses were performed by Pearson's test.

Data for weather conditions (total monthly rainfall and mean monthly temperature) in the years of study were obtained by the Republic Hydrometeorological Service of Serbia for the Belgrade-Surčin area.

RESULTS

Data for weather conditions during vegetation in 2015 and 2016 are shown in Figure 1. The year 2016 was wetter with total rainfall of 481.2 mm in the period from January to July related to 2015 (362.3 mm). Total rainfall and mean temperatures were favourable for *Fusarium* infection during flowering wheat stages in May, but these factors were higher in May 2015 (90.7 mm and 18.5 °C) than in May 2016 (63.4 mm and 17 °C). However, during the maturity stage, in June 2016 total rainfall was very high (156 mm), five and a half times higher than in June 2015 (28.3 mm). In July, the harvest period was also wetter in 2016 (34.7 mm) than in July 2015 (6.5 mm). In both years, the mean temperatures in June and July were above 20 °C.

The mean moisture content of tested durum wheat kernel samples at harvest was 10.44% in 2015 and 14.43% in 2016. By mycological examinations, four *Fusarium* species, *F. graminearum*, *F. semitectum*, *F. sporotrichioides*, and *F. verticillioides* were isolated in 2015. The six *Fusarium* species, *F. graminearum*, *F. poae*, *F. semitectum*, *F. sporotrichioides*, *F. subglutinans*, and *F. verticillioides* were identified in 2016. In both years, the incidence of *F. graminearum* was the highest. Other *Fusarium* spp. were isolated sporadically with low incidence in the kernels. Among other fungi, species from the genera

Alternaria, *Aspergillus*, *Chaetomium*, *Epicoccum*, *Nigrospora*, and *Penicillium* have also been identified, the most frequent being *Alternaria* spp. (data not presented).

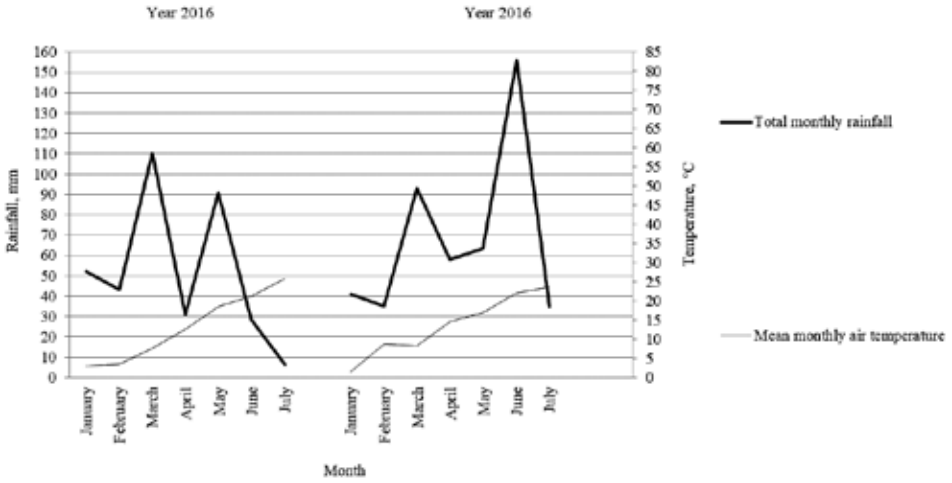

Figure 1. Total monthly rainfall (mm) and mean monthly temperature (°C) during the period January–July in 2015 and 2016 (Belgrade-Surčin area).

The significance of the year and durum wheat line effect on the incidence of *F. graminearum* and DON levels are shown in Table 1 along with the base index values. There was a significant ($P \leq 0.01$) effect of year and durum wheat lines on the incidence of *F. graminearum* and levels of DON. The incidence of *F. graminearum* was significantly higher in 2015 (75.86%) than in 2016 (63.43%) being an increase of 16.39% expressed by the base index. There were no significant differences between durum wheat lines ZP 120 (72.75%) and ZP DSP 66 (73%) and they had a significantly higher incidence of *F. graminearum* than the other studied lines. Line ZP 34 had a 13.18% and 12.79% lower incidence of *F. graminearum* compared to ZP DSP 66 and ZP 120, respectively. On average, in all treatments tested, the incidence of *F. graminearum* was 69.64%. The level of DON was significantly higher in 2016 (3.636 mg kg^{-1}) compared to 2015 (1.126 mg kg^{-1}). The ZP 120 line had the highest (3.854 mg kg^{-1}) and ZP 41 line had the lowest DON level (1.658 mg kg^{-1}) being an increase of 132.45% expressed by the base index. Among the ZP 34, ZP 41, ZP 74, and ZP DSP 66 lines were no significant differences in DON level. On average, in all treatments tested, the mean DON level was 2.381 mg kg^{-1} and was above the maximum limit of 1.750 mg kg^{-1} for unprocessed durum wheat prescribed by the European Regulation 1881/2006/EC. A statistically significant ($P \leq 0.05$) negative correlation was determined between the incidence of *F. graminearum* and DON level ($r = -0.31$).

Table 1. Effect of year and wheat durum line on the incidence of *F. graminearum* and DON level

Factor	Incidence of *F. graminearum* (%)	Index (%)	DON (mg kg^{-1})	Index (%)
Year effect (Y)				
2015	75.86^{a}	83.61	1.126^{b}	100
2016	63.43^{b}	100	3.636^{a}	322.91
F-test	**	-	**	-
Durum wheat lines effects (DWL)				
ZP 16	69.25^{b}	107.36	2.484^{b}	149.82
ZP 34	64.50^{b}	100	1.784^{d}	107.60
ZP 41	72^{b}	111.63	1.658^{d}	100
ZP 74	67.75^{b}	105.04	2.255^{d}	136
ZP 120	72.75^{a}	112.79	3.854^{a}	232.45
ZP DSP 66	73.00^{a}	113.18	2.141^{d}	129.13
Cimmyt 7817	68.25^{b}	105.81	2.491^{c}	150.24
F-test	*	-	**	-
Interactions (F test)				
Y×DWL	**	-	**	-
Means	69.64	-	2.381	-

Means followed by the same letter within a column are not significantly different by the Tukey's test at the $P \leq 0.05$ level.
ns, not significant; *significant at the 0.05 level of probability; **significant at the 0.01 level of probability.

DISCUSSION

This study represents the first report on the occurrence and contamination of durum wheat with *Fusarium* spp. and DON in Serbia during the two harvesting periods (2015–2016). FHB populations of four (*F. graminearum*, *F. semitectum*, *F. sporotrichioides*, and *F. verticillioides*) and six *Fusarium* spp. (*F. graminearum*, *F. poae*, *F. semitectum*, *F. sporotrichioides*, *F. subglutinans*, and *F. verticillioides*) were identified in 2015 and 2016, respectively, with *F. graminearum* as the most prevalent species. This is in agreement with the results of a survey of the main species of the FHB complex on durum wheat kernels in Italy and Poland, in which *F. graminearum* was predominant (Shah et al., 2005; Pancaldi et al., 2010; Covarelli et al., 2015; Gorczyca et al., 2018). In Serbia, according to the previous examination of mycobiota on the bread wheat kernels, *F. graminearum* was also the most isolated while *F. arthrosporioides, F. avenaceum, F. equiseti, F. oxysporum*, *F. poae*, *F. proliferatum*, *F. semitectum*, *F. sporotrichioides*, *F. subglutinans, F. tricinctum* and *F. verticillioides* were sporadically isolated (Stanković et al., 2007; Lević et al., 2008; Krnjaja et al., 2008; 2011a,b; 2014, 2015). Furthermore, Lazzaro et al. (2015) have isolated *F. graminearum* and *F. poae* as the main FHB bread wheat pathogens, where

F. graminearum was predominant in organic wheat and *F. poae* in conventional one. However, the dominant incidence of these species changed depending on the vegetation season, which was influenced by differences in weather conditions during the flowering and anthesis stages in wheat (Lazzaro et al., 2015). Other fungi identified in tested durum wheat line kernels were species from *Alternaria*, *Aspergillus*, *Chaetomium*, *Epicoccum*, *Nigrospora*, and *Penicillium* genera. Depending on the applied agro-technical measures, cultivars, agro-ecological conditions of localities where wheat is grown and the examined years, Stanković et al. (2007), Krnjaja et al. (2008, 2011a,b, 2014, 2015), and Beccari et al. (2018) with *Fusarium* species, *Acremoniella* spp., *Acremonium* spp., *Alternaria* spp., *Arthrinium* spp., *Aspergillus* spp., *Bipolaris* spp., *Cladosporium* spp., *Chaetomium* spp., *Dreschlera* spp., *Epicoccum* spp., *Mucor* spp., *Nigrospora* spp., *Penicillium* spp., *Phoma* spp, *Ramichloridium* spp., *Rhizopus* spp., *Stemphylium* spp., and *Trichoderma* spp. were also isolated from bread wheat kernels.

The *Fusarium* spectrum species associated with FHB on wheat kernels are determined by weather conditions, especially during the wheat anthesis stage (Francesconi et al., 2019). Gorczyca et al. (2018) have reported that low water content during some tested growing seasons affects the fewer *Fusarium* spectrum species. We have also isolated fewer *Fusarium* spp. in a drier season in 2015 than in 2016. However, the incidence of *F. graminearum* was significantly higher in 2015 than in 2016. The reason for that could be favourable weather conditions such as the total rainfall and mean air temperatures, which were higher during the flowering wheat stage in May 2015 (90.7 mm and 18.5 °C) than in May 2016 (63.4 mm and 17 °C). In addition, Beccari et al. (2018) have emphasized that the incidence of *F. graminearum* decreased compared to *F. avenaceum* incidence due to unfavourable climatic conditions during the flowering stage for FHB infection. Balmas et al. (2015) have isolated more *F. culmorum* strains than *F. graminearum* strains in the durum wheat kernel samples from Sardinia indicating the importance of the influence of the growing region on the spectrum of *Fusarium* spp. Depending on localities in Italy, Infantino et al. (2012) have reported a low infestation (of below 2%) of bread and durum organic wheat seed with a few *Fusarium* spp. of FHB complex, *F. avenaceum*, *F. graminearum*, *F. poae*, and *F. verticillioides* of which *F. poae* had the most incidence. Considering co-infection by *Fusarium* spp. on wheat, Pancaldi et al. (2010) have also noticed that *F. poae* is becoming more common in the FHB species complex in different European countries.

All tested lines showed susceptibility to *F. graminearum*, with ZP 120 and ZP DSP 66 having the highest incidence. It was in agreement with the results of Haidukowski et al. (2005), Covarelli et al. (2015), and Gorczyca et al. (2018), reporting susceptibility of different durum wheat cultivars to *Fusarium* spp. of FHB complex. Research results by Bentivenga et al. (2021), from 35 Italian durum wheat cultivars showed that only three cultivars were evaluated as moderately susceptible to FHB, while almost all of the other cultivars were susceptible or very susceptible. FHB resistance is a complex genetic trait controlled by multiple genes and depends on environmental conditions, which leads to the

conclusion that there are almost no completely resistant durum wheat cultivars to FHB. Besides, there are not enough breeding programs for durum wheat, probably due to growing in smaller acreage (Haile et al., 2019).

In this study, the year and durum wheat genotype effects were significant on DON levels. DON level was about three times higher in 2016 (3.636 mg kg^{-1}) than in 2015 (1.126 mg kg^{-1}), which is an increase of 222.91% expressed by the base index (Table 1). This result can be hypothetically explained by abundant rainfall during June 2016 and no positive correlation between *F. graminearum* incidence and DON levels. The average DON level in all treatments (2.381 mg kg^{-1}) was above the allowed limit (1.750 mg kg^{-1}) for unprocessed durum wheat determined by the European Commission (1886/2006/EC). Line ZP 120 had the highest DON level (3.854 mg kg^{-1}). Similarly, Gorczyca et al. (2018) reported that DON synthesis in durum wheat kernels is affected by both the year and cultivars. Scala et al. (2016) have also found higher DON levels in the growing season with more rainfalls. These authors pointed out that DON levels were significantly affected by year and location but not by the cultivars, with low DON levels in all treatments. Shah et al. (2005) and Covarelli et al. (2015) have established that durum wheat cultivars were more susceptible to FHB and DON than bread wheat cultivars. Similarly, Krnjaja et al. (2015) found that DON levels in bread wheat kernels were significantly affected by the cultivars. Balducci et al. (2022) have established the strongest positive correlation between FHB symptoms and DON levels in durum wheat during the wetter growing season. However, in our study, the relationship between *F. graminearum* incidence and DON level was not positively correlated.

CONCLUSION

Fusarium infection and DON contamination were high primarily due to favourable weather conditions in both studied growing seasons and the susceptibility of durum wheat lines to FHB pathogens. Among *Fusarium* species, *F. graminearum* was the most frequently isolated in the kernel of all tested durum wheat lines. This species was also a predominant FHB pathogen. There was a higher incidence of *F. graminearum* in wheat kernels in 2015 than in 2016. On the contrary, DON levels were higher in 2016 than in 2015. The relationship between the incidence of *F. graminearum* and DON level was negatively correlated. All tested durum wheat lines were susceptible to these contaminants. Obtained results indicate the importance of improving and increasing the number of durum wheat breeding programmes. Besides chemical control, the most important and effective agricultural measure in FHB management is sowing less susceptible or resistant wheat cultivars. Moreover, integral strategies can enhance FHB control in durum wheat, including crop rotation, crop residue management, resistant cultivar, fungicide treatments and forecasting.

ACKNOWLEDGEMENT

This research was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia, No. 451-03-68/2022-14/200022 and 451-03-68/2022-14/200040.

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ОРИГИНАЛНИ НАУЧНИ РАД

КОНТАМИНАЦИЈА ЗРНА ЛИНИЈА ДУРУМ ПШЕНИЦЕ СА *Fusarium* ВРСТАМА И ДЕОКСИНИВАЛЕНОЛОМ

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РЕЗИМЕ: Проучавана је *Fusarium* инфекција и деоксиниваленол (ДОН) контаминација зрна седам линија дурум пшенице (шест домаћих ZP 16, ZP 34, ZP 41, ZP 74, ZP 120, ZP DSP 66 и једна међународна дурум линија Cimmyt 7817) у току две жетвене сезоне (2015–2016). Идентификоване су четири *Fusarium* врсте, *F. graminearum, F. proliferatum, F. sporotrichioides,* и *F. verticillioides* у 2015. години. Другачија структура *Fusarium* популације била је у 2016. години, јер је поред *F. graminearum*, *F. sporotrichioides,* и *F. verticillioides*, обухватала и врсте *F. poae*, *F. semitectum* и *F. subglutinans*. *F. graminearum* је била најучесталија врста на зрну дурум линија пшенице и потенцијални продуцент ДОН-а. Друге *Fusarium* spp. изоловане су спорадично и у ниској учесталости на зрну испитиваних линија. Учесталост *F. graminearum* и садржај ДОН-а били су под значајним утицајем генотипова дурум пшенице и проучаваних година и ови параметри били су у негативној корелацији. Учесталост *F. graminearum* била је већа (75,86%) у 2015. него у 2016. години (63,43%), док је садржај ДОН-а био виши (3,636 mg kg^{-1}) у 2016. у поређењу са 2015. годином (1,126 mg kg^{-1}). Утврђена је статистички значајно већа учесталост *F. graminearum* на зрну линија ZP DSP 66 (73,00%) и ZP 120 (72,75%) у односу на друге линије дурум пшенице. Највећи садржај ДОН-а детектован је у зрну линије ZP 120 (3,854 mg kg^{-1}). Разматрајући све испитиване третмане, просечан садржај ДОН-а био је 2,381 mg kg^{-1}, док је просечна учесталост

F. graminearum била 69,64%. Испитиване линије дурум пшенице биле су осетљиве према *F. graminearum* а резултат тога био је и висок садржај ДОН-а у зрну. Добијени резултати указују на значај коришћења линија са добром отпорношћу према фузариози класа у селекционим програмима за стварање нових сорти дурум пшенице.

КЉУЧНЕ РЕЧИ: *Fusarium* spp., деоксиниваленол, дурум линије пшенице

Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. № 143, 39—52, 2022

UDC 582.288:635.25
https://doi.org/10.2298/ZMSPN2243039M
ORIGINAL SCIENTIFIC PAPER

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SEEDBORNE FUNGI ON STORED ONION SEEDS

SUMMARY: Seed as a highly-valuable resource is preserved in collections for many years. Although the seed is kept under optimal conditions, monitoring of germination and the presence of fungi during seed preservation is of great importance. Therefore the aim of this paper is to examine the seed health status and germination of 43 onion accessions kept in the timespan for 15 years in the Institute of Field and Vegetable Crops collection. Germination of seed samples varied from 7–93%. The presence of fungi in the collection was determined on 33 tested samples. Fungi from the genera *Acremonium, Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum*, *Fusarium* and *Penicillium* were developed. The following *Fusarium* species identified on the seeds were *F. proliferatum*, *F. graminearum*, *F. sporotrichioides*, *F. solani*, *F. pseudograminearum* and *F. equiseti*. Based on factor analysis, *Fusarium* and *Penicillium* affected germination, while the occurrence of *Alternaria* species on onion seed is connected to the year of harvest.

KEYWORDS: onion, seed, fungi, germination, collection

INTRODUCTION

Onion (*Allium cepa* L.) is an important vegetable crop grown worldwide as well as in Serbia. In the agroecological conditions of Serbia, onion is grown at the relatively constant production areas, due to favourable natural conditions, tradition and daily use. The production area under this vegetable in Serbia in 2020 was 4,080 ha (Statistical office of Republic of Serbia).

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Onion could be produced from seeds and onion sets. Production from onion sets is typical for Southeast Europe, including Serbia (Gvozdanović-Varga, 2011), therefore the main focus was on fungi attacking onion sets and bulbs. However, special attention should be paid to storage of commercial seed because one of the major constraints in onion cultivation is the limited availability of vigorous seeds. On the other hand, breeders maintain onion genetic collections, also known as breeding or working collections. They contain seeds of accessions of various biological developmental stage, different geographic origin and genetic diversity. These collections should provide enough material for breeding and *ex situ* conservation. It is presumed that the conditions for seed preservation in breeders' collections are optimal.

The quality of onion seeds depends on many factors, such as environmental conditions during the growth of the mother plant and seed development, location of seeds on the plant, time and technology of harvesting and post harvest conditions (Dorna et al., 2013). The main reasons for the low quality of onion seeds besides the long flowering period and storage under suboptimal conditions are fungal infestations (Brocklehurst, 1985). However, it is known that breeders collect seeds from healthy vigorous plants. The quality of fully developed seed and its longevity are largely conditioned by abiotic factors in the storage (humidity, temperature, oxygen), but genetic and pre-storage factors should not be neglected, as well (Kalman et al., 2020; Solberg et al., 2020). Standardization of packaging and keeping conditions could contribute to the satisfying quality of onion planting (Rao et al., 2006).

Multiplication of the higher category of onion seed is expensive, labour demand and time-consuming due to a three or two-year period of production (Ozer and Koycu, 2004). Therefore, that process should be planned accurately, especially if we take into consideration that there is no need for sowing all the accessions from the seed collection every year or every second year. It is very important to monitor the current status of seed viability in the collected samples. The aim of this paper is to examine the seed health status and germination of accessions kept for 1–15 years in onion seed collection in the Institute of Field and Vegetable Crops.

MATERIAL AND METHOD

The presence of seed-borne fungi was analysed in 43 onion seed accessions within the collection of the Institute of Field and Vegetable Crops (Table 1). This survey included 13 varieties, two lines, one experimental hybrid and 11 landraces of *Allium cepa*. After the harvest, the seed samples were kept in the paper bags packed in the closed metal containers in the climate chamber at 5–6 °C, during the period 1–15 years.

Table 1. List of tested seed accessions

Accession / collection number	Year of production	Locality / Country of origin	Biological status of accession
Kupusinski jabučar (KJ)	2000; 2002; 2003; 2004; 2006; 2012; 2013	Serbia	variety
Holandski žuti (HY)	2004; 2005; 2006; 2013	Serbia	variety
Alek (A)	2004; 2005; 2006; 2007; 2010	Serbia	variety
Srebrenjak (S)	2001	Serbia	variety
Ljaskovski 58 (K 121)	2002	Bulgaria	variety
Junski srebrenjak (JS)	2005	Serbia	variety
Makoi bronz (MB)	2005	Hungary	variety
Favorit (K 107)	2006	Hungary	variety
Holland yellow (K 183)	2006, 2009	Crna bara, Serbia	variety
Makoi Feher (K 45)	2007	Hungary	variety
Ema (K 185)	2008	Hungary	variety
Istrian yellow (K 64 Ž)	2012	Croatia	variety
Istrian red (K 64 P)	2012	Croatia	variety
Ptujski red (K 211)	2014	Slovenia	variety
Kupusinski pogačar (KP)	2005, 2009	Serbia	breeding line
Nº 8301 (K 37)	2007	Bulgaria	breeding line
Ptujski x 185 (PT)	2010	Serbia	experimental hybrid
K 51	2007	Turopolje, Croatia	landrace
K 56	2007	Kistanje – Knin, Croatia	landrace
K 58	2007	Golubić – Knin, Croatia	landrace
K 65	2007	Istria, Croatia	landrace
K 76	2008	Breza – Sarajevo, Bosnia and Herzegovina	landrace
K 154	2008	Vrnjačka banja, Serbia	landrace
K 134	2009	Kardeljevo – Komin, Croatia	landrace
K 184	2009	Temerin, Serbia	landrace
K 191	2013	Trebinjski pogačar, Croatia	landrace
K 208	2014	Kupusina, Serbia	landrace
K 210	2014	Valpovo, Croatia	landrace

In order to analyse seed mycoflora, a standard phytopathological method was used. Onion seeds were sterilized in a 1% solution of sodium hypochlorite (NaOCl) for five minutes. Thereafter, seeds were rinsed twice with sterile water and incubated on a PDA medium at 25 °C in the dark. For each sample, ten seeds in three replication were analysed, 30 seeds in total. After seven days of incubation, fungi developed on the seed were identified morphologically by microscopic observations. Fungi from the genus *Fusarium* were identified to the species level according to the key (Leslie and Summerell, 2006). Germination

on PDA medium was recorded. Seed infection and germination rate were calculated according to the following formulas:

$$\text{Seed infection rate (\%)} = \frac{\text{Number of seeds infected by fungi}}{\text{Total number of seeds}} \times 100;$$

$$\text{Seed germination rate (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100.$$

Statistical analysis of data was done using software STATISTICA, ver. 13.2 (Dell, Inc., USA). Data obtained for germination were compared by Analysis of Variance (ANOVA) followed by the Bonferroni test ($p<0.01$). Factor Analysis was performed to reduce the number of variables and to detect relationships between variables. Rotated orthogonal components (varimax normalized method of rotation) with eigenvalues >1 were extracted (Kaiser, 1960) and the relative scores were determined. The occurrence of fungi was shown by graphs in Excel.

RESULTS AND DISCUSSION

Various fungal species have been determined in onion seeds produced in different climatic regions (Koycu and Ozer, 2007). The presence of fungi in the IFVCNS collection was determined on 34, out of 43 tested onion seed samples. Fungi from the genera *Acremonium, Alternaria, Aspergillus, Cladosporium, Epicoccum, Fusarium* and *Penicillium* were developed on the examined seeds. Species from the genus *Fusarium, Botrytis aclada* and *Aspergillus niger* are known as onion seed-borne pathogens (Chilvers and Du Toit, 2006; Southwood et al., 2015). The presence of numerous saprotrophic fungi from the genera: *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum*, *Penicillium* and *Rhizopus* were also detected (Tylkowska and Dorna, 2001).

Fungi of the genus *Fusarium* were identified in eight samples with an infection rate of 3.3–23.3% (Figure 1). The highest number of *Fusarium* species, as well as the highest per cent of the seed infection, was found in sample A (2010). The following *Fusarium* species were identified on this seeds sample *F. proliferatum, F. graminearum*, *F. sporotrichoides, F. solani, F. pseudograminearum* and *F. equiseti.* The most frequent species was *Fusarium proliferatum* which appeared on seven seed samples (Table 2). Haapalainen et al. (2016) reported that *F. oxysporum* was frequently found in onions but only 15% of the isolates caused growth stunting in onion seedlings, while all *F. proliferatum* isolates tested were pathogenic to onion. Seed sample HY (2006) was infected with two *Fusarium* species (*F. proliferatum* and *F. oxysporum*), while on sample K37 (2007) *Fusarium compactum* was identified.

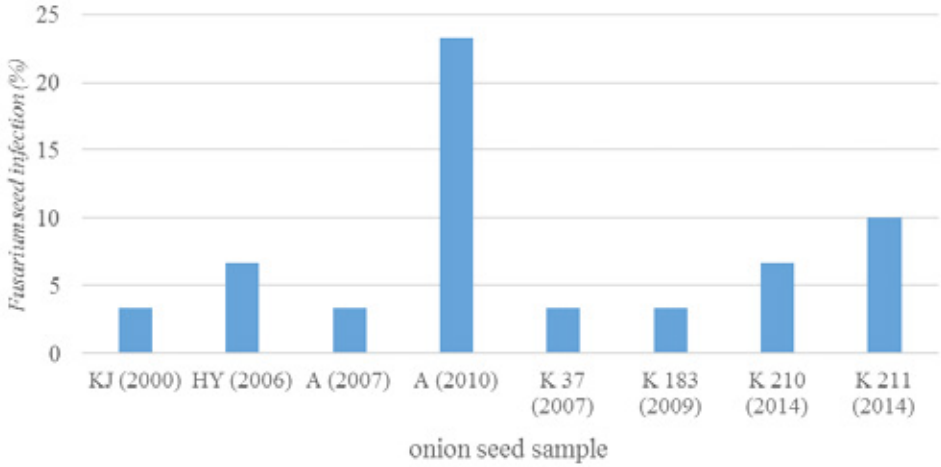

Figure 1. Fusarium onion seed infection

In Serbia, *Fusarium* species periodically cause significant diseases of onion, especially death and rot of seedlings (Klokočar-Šmit et al., 1988; Lević et al., 2009). Following species were reported on onion *F. oxysporum*, *F. solani*, *F. proliferatum*, *F. acuminatum*, *F. cepae*, *F. equiseti*, and *F. verticillioides* (Klokočar-Šmit et al., 1990; Lević, 2008). *Fusarium proliferatum* was reported as a predominant fungal species isolated from the roots and bulbs of onion plants in Serbia (Stanković et al., 2007). Recent findings of *Fusarium* sp. FIESC3, (such as members of FIESC a complex of morphologically similar species *F. equiseti / F. semitectum / F. incarnatum*) as a causal agent of pre-emergence dumping off, decay and rot of onion seed in Serbia was reported by Ignjatov et al. (2017). Apart from *F. proliferatum, F. equiseti*, *F. tricinctum F. sporotrichioides* and *F. poae* were first described in *Allium* sp. in Germany (Boehnke et al., 2015). *F. graminearum* was recorded on onion sets in Turkey (Koycu and Ozer, 1997). According to our knowledge, it is the first report of *F. sporotrichoides*, *F. pseudograminearum* and *F. compactum* on onion seeds in Serbia. The pathogenicity of these species should be tested.

Apart from the fungi of the genus *Fusarium* and other species, *Aspergillus niger* (the causal agent of black mould) can also be phytopathogenic, and its importance is reflected in the potential transmission of infection from seed to onion set (Koycu and Ozer, 1997). Species from the genus *Aspergillus* were isolated from ten onion samples, while *A. niger* was isolated from eight of them. The seed infection by *A. niger* was rated from 3–20% (Figure 2). The causal agent of black mould could be a predominant seed transmitted fungus on onion seeds and the problem is that visual symptoms are not observed because of latent infection (Saranya et al., 2017).

Table 2. Fusarium species isolated from onion seed

Variety Genotype /year	*Fusarium* species on onion seed samples (number of isolates)	seed infection (%)
KJ (2000)	*F. proliferatum* (1)	3.3
HY (2006)	*F. proliferatum* (2) *F. oxysporum* (1)	10.0
K 37 (2007)	*F. compactum* (1)	3.3
K 183 (2009)	*F. proliferatum* (1)	3.3
A (2007)	*F. proliferatum* (1)	3.3
A (2010)	*F. proliferatum* (1), *F. graminearum* (2) *F. sporotrichioides* (1), *F. solani* (1) *F. pseudograminearum* (1), *F. equiseti* (1)	23.3
K 210 (2014)	*F. proliferatum* (2)	6.7
K 211 (2014)	*F. proliferatum* (3)	10.0

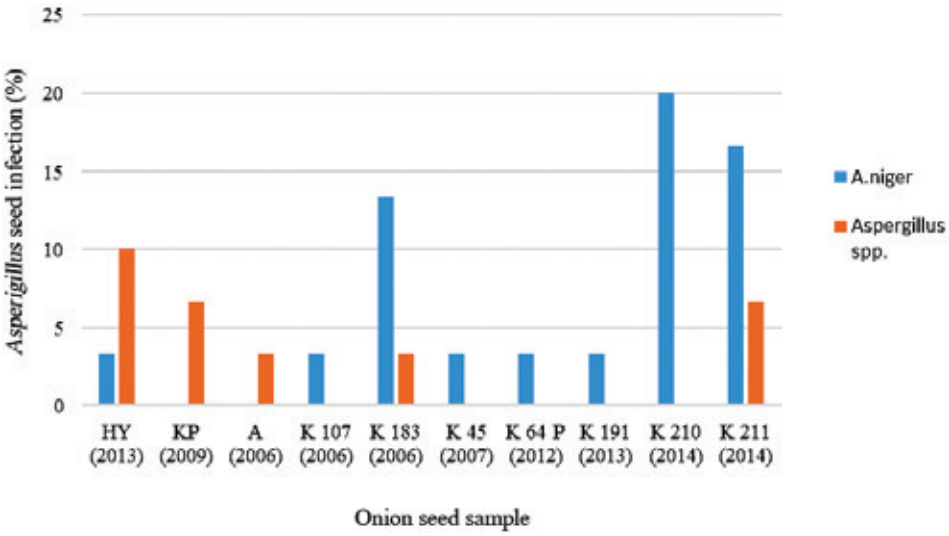

Figure 2. Aspergillus onion seed infection

Alternaria sp. were the most frequent and found on 15 onion samples. Three samples had infection 33%, three were between 20–27% and other samples were from 3–13% (Figure 3). Almost all samples which had *Alternaria* infection above 20% were grown in 2013 and 2014. The pathogenicity of *A. alternata* species on onion is described by Bihon et al. (2015), while *A. porri* is a well-known pathogen of onion and could be seed-borne (Kim et al., 2022). Fortunately, *A. porri* was not detected on the tested seed.

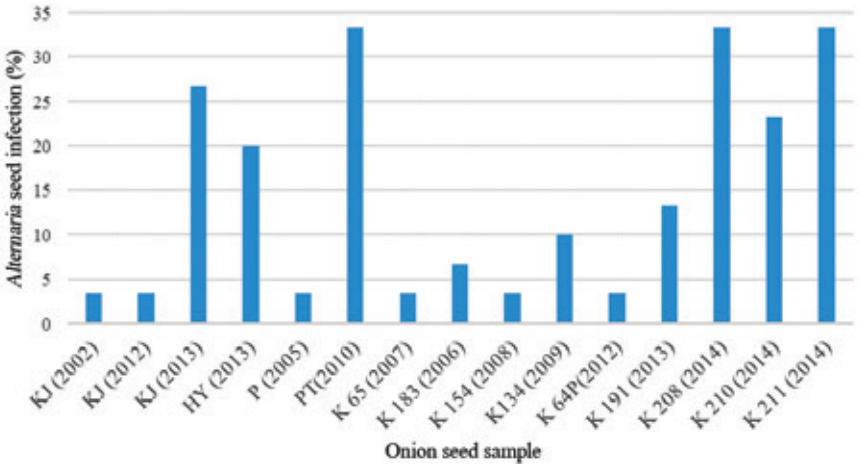

Figure 3. Alternaria onion seed infection

Penicillium infected 14 samples at a rate from 3 to 13.3% (Figure 4). A similar *Penicillium* seed-borne infection rate on onion seed in storage was reported by Adongo et al. (2015), although these authors did not mention the pathogenicity of these isolates.

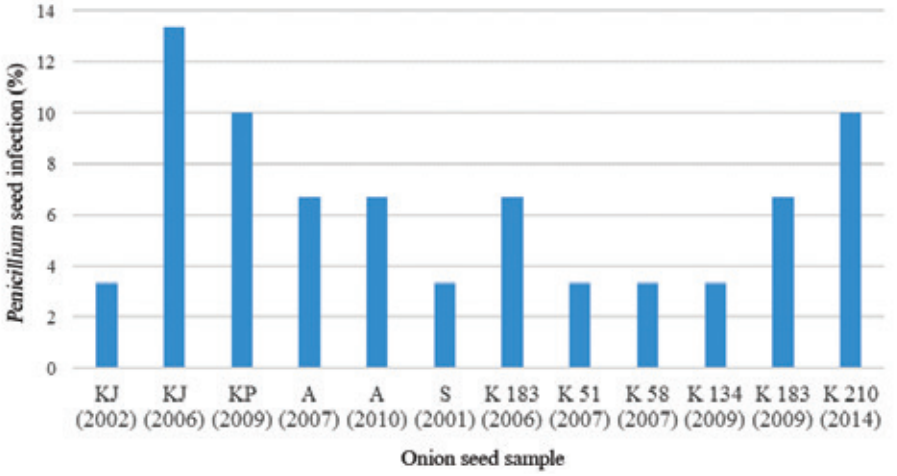

Figure 4. Penicillium onion seed infection

Seeds of K 191 (2013) and K208 (2014) had 16.6% infection with *Acremonium* sp. and other seven samples (K 64P (2012); K 64Ž (2012); K 154 (2008); K 37 (2007); KP (2009); HŽ (2004) and KJ (2006)) had an infection of 3%. *Epiccocum* sp. was found in three seed samples KJ (2006) 13% and A (2010) and K (208) 3%. *Cladosporium* sp. was isolated from three samples P (2005),

JS (2005) and K(208) with infection rate 3, 6.7 and 10% respectively. *Trichoderma* sp. was developed only on sample P (2005). These fungi were determined as saprophytic on onion seeds. Although these species are noted as saprophytic, their role in seed-borne disease of onion has not been revealed (Özer and Köycü, 2004).

Germination of tested genotypes varied from 7–93% (Table 3). Samples classification into homogenous groups was done according to the Bonferroni test. The highest germination had two genotypes stored for six and seven years (A (2007) and K 185 (2008)). Besides the high importance of environmental conditions, storage of the tested accessions (paper bags packed in the closed metal containers which were kept at constant temperature) strongly contributed to the seed viability preservation. It is in the accordance with Rao et al. (2006), who reported that the use of hermetically sealed containers, desiccants and low temperatures improves storability as several physiological and biochemical processes and products are being regulated during dry storage. Various authors indicated a different period in which onion seed retained high germinability in the storage. Doijode (1995) reported that onion seed could retain high germinability up to seven years when stored with silica gel in a moisture impervious container at 5–8 °C. Seeds with an initial germination percentage above 90%, kept at 10 °C, keep their germination potential after 12 months (Dorna et al., 2013).

The lowest germination had samples S (2001) and A (2010) (7 and 13% respectively). These genotypes are significantly different from those which achieved germination above 70% (Table 3). However, the low germination in these genotypes was caused by different factors. Genotype S (2001) had germination below 10% probably due to seed age, while sample A (2010) had 23% of *Fusarium* seed infection (Table 2).

Table 3. Germination of tested seed accessions

Germination (%)	Seed accessions
93	A (2007); K185 (2008)^{a*}
90	K 184 (2009)^{ab}
83	K76 (2008)^{abc}
73–80	K56 (2007); K134 (2009)^{abcd}
67–70	K107 (2006);K65 (2007); KJ (2012)^{abcde}
37–63	K210 (2014); K183 (2009); K64Ž (2012); KJ (2000); HY (2013); A (2006) KJ (2004) KJ (2013) K191 (2013) KJ (2003) MB (2005) K154 (2008) KP (2005) KJ (2006) K58 (2007); K37 (2007); K51 (2007); K45 (2007); KP (2009); K64P (2012)^{abcdef}
33	HY (2004); K 211 (2014); KJ (2002);JS (2005); HY (2005)^{bcdef}
27–30	K 121 (2002); K208 (2014); K183 (2006);A (2004)^{cdef}
23	A (2005), PT (2010); HY (2006)^{def}
13	A (2010)^{ef}
7	S (2001)^{f}

* values with different letters in the columns differ significantly at a significance level of $p<0.05$

Besides the other factors (such as environmental conditions during and after harvest) that were involved in germination, a genotype also influenced this trait. Ilić et al. (2006) reported that seed germination depends on the variety. On the other hand, the reduction in the germination of onion seed is attributed to its chemical composition and fragile seed coat which favours higher lipid peroxidation and fungal incidence (Amalfitano et al., 2019).

Table 4. Total seed infection of tested onion accessions

Total seed fungal infection (%)	Genotype/Variety
0	KJ (2003); KJ (2004); HY (2005); A (2004); A (2005); MB (2005); K65 (2007); K56 (2007); K76 (2008); K185 (2008);
1–5	KJ (2000); KJ (2012); HY (2004); A (2006); S (2001); K121 (2002); K107 (2006); K45 (2007); K51 (2007); K58 (2007); K64Ž (2012)
6–10	JS (2005); K37 (2007); KJ (2002); HY (2006); K154 (2008); K-184 (2009); A (2007); K183 (2009); K64P (2012)
11–30	KJ (2006); KJ (2013); KP (2005); KP (2009); K183 (2006); K134 (2009)
33–59	A (2010); K191 (2013); PT (2010); HY (2013);
60–67	K210 (2014); K211 (2014); K208 (2014);

Further explanation of the obtained results was done through the factor analysis. The Eigenvalues and variance explained by factors were indicated in the Table 5. Only the first five factors were presented because Eigenvalue was over one, and the total cumulative variance of those factors accounted 81.69%. The values in the Table 6, indicated the contribution of each variable to the factors. Only those factor loadings greater than 0.5 were considered important, these values are highlighted in bold. Factor 1 was strongly associated with year, *Alternaria, Aspergillus* and healthy seeds. *Acremonium* and *Cladosporium* were strongly associated with Factor 2.

Table 5. Eigenvalues and variance of the first five factors

Factor	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative %
1	3.357	30.516	3.357	30.516
2	1.796	16.329	5.152	46.845
3	1.521	13.827	6.673	60.672
4	1.269	11.539	7.943	72.211
5	1.043	9.482	8.986	81.693

Table 6. Factor analysis of evaluated parameters

Variable	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
year	**0.827**	0.209	0.040	-0.210	0.172
Alternaria sp.	**0.827**	0.326	-0.130	0.156	0.008
Acremonium sp.	0.227	**0.829**	0.075	-0.071	0.176
Penicillium sp.	0.145	-0.220	**0.872**	-0.032	0.017
Epicocum sp.	-0.078	0.315	**0.844**	0.078	0.032
Aspergillus sp.	**0.797**	-0.308	0.042	0.163	-0.020
Cladosporium sp.	0.086	**0.790**	-0.031	0.116	-0.319
Trichoderma sp.	-0.021	0.055	-0.035	-0.039	**-0.966**
Fusarium sp.	0.289	-0.266	0.320	**0.581**	0.030
healthy seeds	**-0.849**	-0.286	-0.286	-0.264	0.178
germination	-0.018	-0.161	0.095	**-0.923**	-0.016

Based on Factor analysis (Figure 5), the main species which affected germination were *Fusarium* and *Penicillium*. This was in accordance with the results of other authors (Palmero et al., 2012; Kintega et al., 2020) who reported that *Fusarium* species are seed-borne and pathogenic species. Among the saprophytic species, *Aspergillus* spp. and *Penicillium s*pp. could strongly influence the viability of the seeds during storage (Maude, 1996; Fontana et al., 2018).

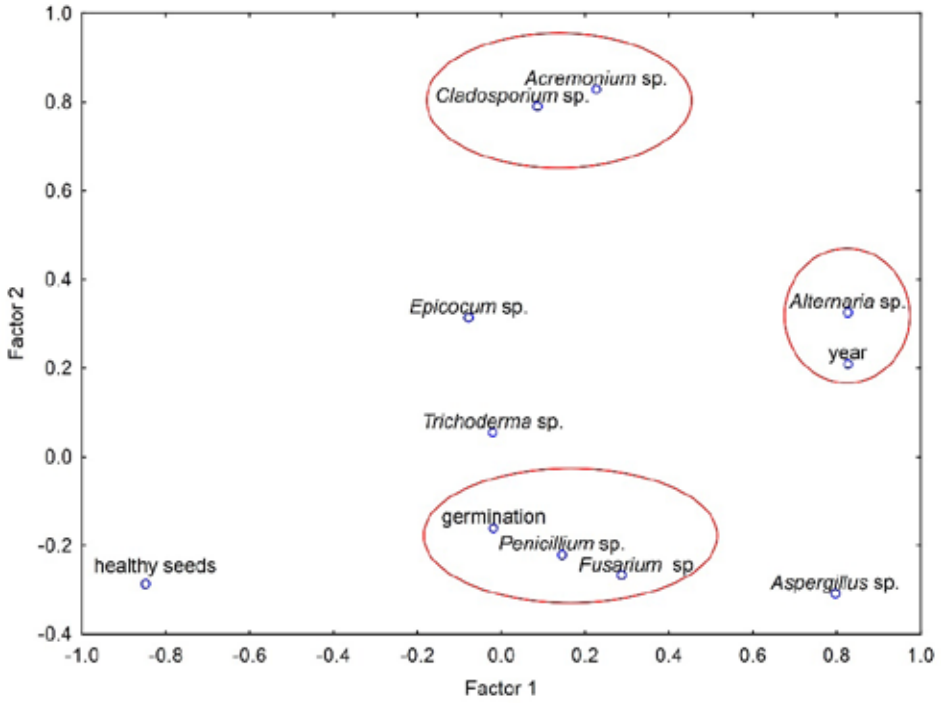

Figure 5. Biplot of evaluated traits in onion seed

Aspergillus niger was reported to reduce seed germination, seedling emergence and vigour (El-Nagerabi and Ahmed, 2001). However, samples K (210) and K (183) with *A. niger* infection 13.3 and 20% respectively, had high germination percent (67–70%) (Table 3). Additionally, the occurrence of *A. niger* was analyzed together with other *Aspergillus* species. Therefore *Aspergillus* species were not connected with germination (Figure 5).

The occurrence of *Alternaria* species on onion seed was connected to the year of harvest. The highest percentage of fungi from the genus *Alternaria* was observed in 2013 and 2014 (Figure 3). Additionally, the samples with the highest total seed fungal infection (33–67%) were harvested in 2010, 2013 and 2014 (Table 3). Meteorological conditions in 2013 and 2014 in Vojvodina, in the period of flower stems formation and beginning of flowering (May and June) were characterised by a doubled amount of precipitations compared to multiyear average and frequent showers. Similar weather conditions in July were followed by high temperatures during the period of seed filling. These conditions such as high humidity, and high temperatures favoured the development of *Alternaria* sp. However, in 2010 when there was the highest occurrence of *Fusarium* species on sample A (2010) the amount of precipitation in May and June was doubled and by 60% more than the multi-year monthly average respectively. In August and July of 2010, the weather was warm with regular rainfall (http://www.hidmet.gov.rs/ciril/meteorologija/agro.php). In Vojvodina, the early onion seed harvest is at the beginning of August (Gvozdanović Varga, 2011). These conditions during the vegetation period probably favoured *Fusarium* seed infection.

Acremonium and *Cladosporium* species grouped closely showing a clear distinction from other traits. Such grouping of these species indicated that they did not have any influence on the germination and health status of the seeds. Healthy seed is segregated from other traits.

CONCLUSION

Seed storage in the breeder's collections is a very important part of seed preservation for a long time. The main prerequisites for quality preservation are optimal storage conditions as well as high-quality seed. Additionally, the year of harvest is very important due to the presence of latent fungal infection on onion seed. Therefore the introduction of samples into the onion seed collection should be under the accurate analysis of their health status and germination.

ACKNOWLEDGEMENTS

"This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant number: 451-03-68/2022-14/ 200032".

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ПОЈАВА ГЉИВА НА УСКЛАДИШТЕНОМ СЕМЕНУ ЦРНОГ ЛУКА

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РЕЗИМЕ: Семе је веома важан ресурс који се чува у колекцији у вишегодишњем периоду. Иако се семе складишти у оптималним условима, веома је важно пратити клијавост и његово здравствено стање. Циљ овог рада је да се испита здравствено стање и клијавост четрдесет три узорка семена црног лука, који су чувани у колекцији Института за ратарство и повртарство 1–15 година. Клијавост црног лука варирала је у опсегу 7–93%. На тридесет три узорка је идентификовано присуство гљива из седам родова: *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum*, *Fusarium* и *Penicillium*. У оквиру рода *Fusarium* утврђено је присуство врста *F. proliferatum*, *F. graminearum*, *F. sporotrichioides*, *F. solani*, *F. pseudograminearum* и *F. equiseti*. На основу факторске анализе врсте које су утицале на клијавост су *Fusarium* и *Penicillium*, док је појава врста из рода *Alternaria* повезана са годином убирања семена.

КЉУЧНЕ РЕЧИ: црни лук, семе, гљиве, клијавост, колекција, *Fusarium*

Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. № 143, 53—63, 2022

UDC 638(497.11)
https://doi.org/10.2298/ZMSPN2243053S
ORIGINAL SCIENTIFIC PAPER

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DIFFERENTIAL DIAGNOSIS OF BEES NOSEMOSIS – PROCEDURE AND SIGNIFICANCE

ABSTRACT: For the living world, in the existing ecosystem, for the reproduction, production and spread of plant species, bees are one of the most important parts of nature. In the pollination of certain field and fruit-vegetable plant species, bees participate with almost 100%. Any factor that contributes to reducing the presence of bees in nature, directly affects the fertility of the plant world, and indirectly affects the rest of the entire living population. Nosemosis is a microorganism that has a great impact on the health of bees. In the previous period, *Nosema sp.* is classified as a single-celled parasite, a protozoan, but today it is classified as a fungus (*Microsporidia*). There are about 30 different species of *Nosema* in nature. For the bee population, especially with regard to the European honey bee (*Apis millifera*), two species of *Nosema* are very important, *Nosema apis* and *Nosema ceranae*. The correct confirmation of the type *Nosema* provides a better understanding of the outcomes and consequences for the apiary in which the clinical picture of nosemosis has occurred. The phenotypic diagnostic method, despite the present morphological differences, does not provide the possibility of reliable confirmation of the *Nosema species*. For these reasons, in order to make a differential diagnosis, it is necessary to determine which type of *Nosema* is present by molecular methods. In our work, by molecular method (PCR), we analyzed bees sampled from two administrative areas. The examination showed that *Nosema ceranae* was found in the two examined areas, while the presence of *Nosema apis* was not confirmed. These results may indicate that *Nosema ceranae* is predominant in the study area and has completely replaced *Nosema apis*.

KEYWORDS: differential diagnosis, bees, nosemosis

INTRODUCTION

Beekeeping is a special branch of animal husbandry. The characteristic of beekeeping is the fact that production is not directly related to land ownership, but beekeepers, producers, can engage in their production even in circumstances when they do not own land. When producers move their bee societies,

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i.e. hives, in order to provide optimal conditions for bee grazing, depending on the season and the vegetative phase of the most important bee nutrition, then we are talking about migratory beekeeping. Stationed beekeeping is based on beekeeping at one location. Both methods of beekeeping require, in addition to the necessary equipment that allows access to areas with desired vegetation, knowledge of legal regulations prescribing the necessary measures of health supervision and control to prevent unwanted transmission of infectious diseases from one area to another (Mutinelli, 2011).

As a segment of agriculture, beekeeping is today a very important link in the chain of obtaining healthy food. Bees are the main pollinators of cultivated plants, which determines the quality and quantity of the obtained crop of oil seeds, fodder plants, fruits and vegetables (Aizen and Harder, 2009; Bradbear, 2009). The immediate benefit of bee products such as honey, pollen, royal jelly and other products makes up to 5% of the profit, while, nowadays, their importance can be fully seen from the aspect of the most important pollinator in the ecosystem. In absent of bees, as natural pollinators, the Earth would be "sterile" for plant vegetation.

The presence of certain infectious bacterial, viral and parasitic diseases leads to health and production problems. CCD (Colony Collapse Disorder) or HBDS (Honey Bee Depopulation Syndrome) syndrome, which in some areas decimates bee colonies and causes great damage, can be caused by the presence of *Nosema spp.* (Adl et al., 2005). Nosemosis is a disease of bee communities that causes broad weakening and increased mortality (Botias et al. 2013).

The subject of our research was the analysis of collected bee samples from two districts on the territory of Vojvodina. The aim of the research was to determine, using modern molecular methods, whether Nosema apis and/or Nosema ceranae are presented in the area of Vojvodina, which can lead to the disappearance of bee communities (CCD).

MATERIAL AND METHODS

Microscopic examination is used to detect nosemosis. In order to determine the existence of the spores *Nosema sp.* it is necessary to collect a sufficient number of bees from the hive (50–100 bees) which are later examined in several grouped samples of 5–10 bees. With the help of laboratory equipment, the abdominal parts should be separated from the thoracic part of the bees and macerated. In that way, the outer cuticular part of the bee's abdomen will be destroyed and the digestive tract and its contents will be released. In prepared macerate it should added 2–3 ml of water after which one drop is taken and placed on the slides, covered with a cover glass and viewed at a magnification of x 400 on a light microscope. Spores are seen under microscopic magnification in the form of rice grains, 5–7 μm long and 3–4 μm wide (*Nosema ceranae* is slightly smaller than *Nosema apis*) (Fries et al., 2013).

PCR method (Polymerase chain reaction)

In order to successfully determine *Nosema apis* and *Nosema ceranae* in the diagnosis of nosemosis and get precisely to know which species is present in bee colonies, the multiplex PCR method was developed. This method provides DNA extraction of the nosema and, with the help of designed primers for selective detection of specific parts of the genome, enables accurate identification of parasites. If the causative agent of nosemosis is found in the processed samples, the PCR method will isolate a part of the genome that is specific only to this type of parasite, and in that way a highly specific result will be obtained, which undoubtedly confirms the type of microorganism.

To isolate the nosema DNA, 10–20 abdominal bees are taken, which have been determined by microscopic methods to be positive for the spores of this parasite. They are macerated with 10 ml of sterile water. The obtained macerate was filtered and centrifuged at 800 g / 6 min (Khezri et al., 2018; Higes et al., 2010). The sediment was incubated in the buffer after removing the supernatant (0.5M sodium chloride, 0.5M sodium bicarbonate, pH 6.0 adjusted with orthophosphoric acid) for 15 min / 37 °C. DNA extraction is performed using a commercial kit (High Pure PCR Template Preparation Kit) (Bokaie et al., 2014).

The obtained DNA of the nosema at this stage of the study can not give us an answer, which type of nosema it is, whether *N. ceranae* or *N. apis* are present in the tested samples. In order to get an answer of the type of nosema, specific primers will be used, which enable the isolation of a specific part of the genome of the nosema that corresponds only to *N. ceranae* or *N. apis*. In the process of obtaining the PCR product, a ready-made commercial Master mix (Roche Diagnostic) kit and a protocol for amplification (increasing the amount of the desired part of the genome) are used. The amplification parameters are: initial activation step 2 min. at 94 °C, followed by 10 cycles of 15 seconds at 94 °C, 30 seconds at 61.8 °C, and 45 seconds at 72 °C, then 20 cycles, 15 seconds at 94 °C, 30 seconds at 61.8 °C and 50 seconds at 72 °C, plus a 5 second elongation cycle for each successful cycle and finally a 7 minute elongation at 72 °C (OIE Terrestrial Manual 2008).

For the final determination of *N. ceranae* or *N. apis* it is necessary to determine the size of the obtained PCR product and thus determine the correct diagnosis. Measurement of the PCR product size was determined by electrophoresis in 2% Agarose gel (Tris acetate-ethylene diamine tetra-acetic acid) stained with ethidium bromide in standard TAE buffer. Color and UV illuminator are used to visualize the obtained product (OIE Terrstrial Manual 2008). Table 1 contains the characteristics of the used primers, with the help of which a specific part of the nosema genome is extracted from the examined samples and on the basis of which the precise diagnosis is determined, i.e. the type of nosema is determined.

Table 1. Selective primers for the detection of *N. ceranae* and *N. apis*

Primers*	Nosem specific sequences	PCR product size (bp)	Specificity
218MITOC FOR	5'-CGGCGACGATGTGATATGAA-ATATTAA-3'	218	*N. ceranae*
218MITOC REV	5'-CCCGGTCATTCTCAAACAAAA-AACCG-3'		
321APIS FOR	5'-GGGGGCATGTCTTTGACGTACTATGTA-3'	321	*N. apis*
321APIS REV	5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3'		

*OIE Terrstrial Manual 2008.

The QIAamp DNA Mini Kit (Qiagen) was used for DNA isolation and the HotSTARTag Master Mix Kit (Qiagen) to obtain the PCR product. For these reasons, the amplification procedure was adjusted. In addition, we did not apply the protocol for multiplex PCR analysis in the assay, so we did not process the samples so that the extracted DNA of the nosema was joined simultaneously with both primers, for *N. ceranae* and *N. apis*. In our analysis, we specifically examined the obtained DNA with a single primer, i.e. with a primer for *N. ceranae*, and especially for *N. apis*. In this way we obtained PCR products that were separated, i.e. especially products for *N. ceranae* and especially for *N. apis*. In the photographs presented in the study, the sizes of base pairs (bp) of PCR products are obtained separately, from *N. ceranae* and *N. apis*, so it will be presented that way. Table 2 contains the data of the amplification procedure according to which the test was performed.

Table 2. Modified PCR amplification protocol (Qiagen kit)

Stages	1	2	3	4	5	6
T °C	95 °C	94 °C	55 °C	72 °C	72 °C	4 °C
Time	15 min	15 sec	30 sec	50 sec	10 min	∞
No. of cycles			40			

RESULTS AND DISCUSSION

Microsporidia, *Nosema ceranae*, migrated from *Apis ceranae*, a South and Southeast Asian honey bee, to *Apis mellifera* (European honey bee) in 1994 (Klee et al., 2007). This transfer of pathogens from one species of bee to another marked the beginning of problems in beekeeping. Studies show that *N. ceranae*, has a much more pathogenic effect on *Apis mellifera* than *N. apis*. The reason for this phenomenon is the short period of cohabitation of these two organisms. Adaptive physiological mechanisms between *N. apis* and *Apis mellifera*, due to the longer period of time, allowed the pathogenic effects, that affect on bee health, to be reduced and parasitism not to be expressed (Utuk et al., 2016; Paxton et al., 2007). *N. ceranae* leads to energy stress, decreased available energy levels, increased food consumption, increased appetite, and

decreased colony numbers (Paxton et al., 2007; Martín-Hernández et al., 2011, Mayack and Naug, 2009). In addition to its impact on energy stress, *N. ceranae* causes immunosuppression and causes secondary infections in bees (Antúnez et al., 2009).

The clinical finding in bees that have suffered from the acute form of nosemosis, especially in early spring, is the presence of brown feces, which is found in large quantities on the honeycomb and frames in the hive. The finding of an increased number of dead bees can in some cases be associated with the lethal effects of some insecticides. For these reasons, it is necessary to determine whether it is nosemosis.

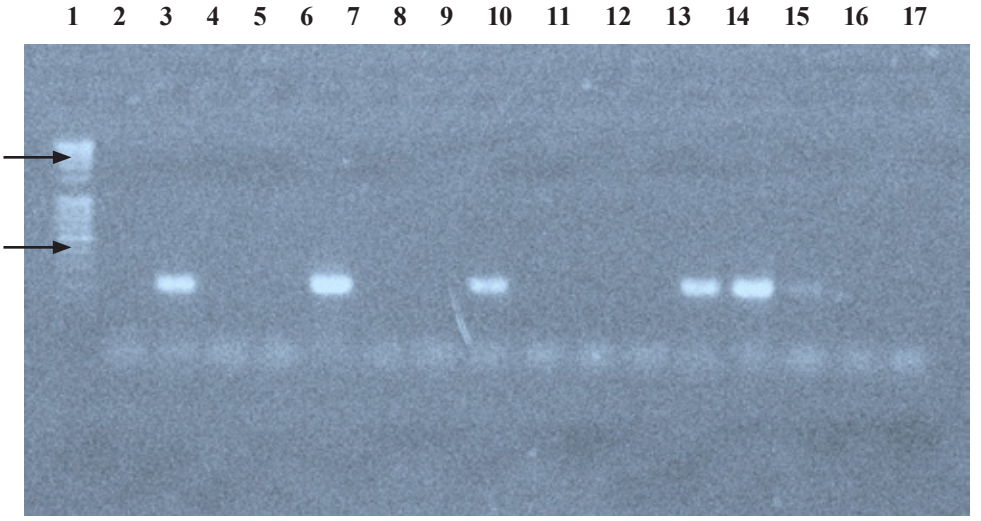
During one year, we examined a total of 494 bee samples for the presence of parasites. The tests aimed at determining whether the samples contained parasites and which species were involved. The analyzes were performed by the method of microscopy by making a native preparation and a clinical examination of honeycombs and bee colonies. Out of the total number of samples examined, the presence of *Nosema sp.* was determined in 114 samples (23.07%).

Microscopic examinations of bee samples in Iran (Khezri et al., 2018), in ten different areas, showed that, taken from a random sample of material, the infestation of bee colonies was 0–50% (on average 25%). This finding corresponds to the results of our research. Similar studies conducted in Saudi Arabia (Mohammad et al., 2017) showed that microscopic examination revealed the presence of *Nosema spp.* in 20.59% of the analyzed samples. Microscopic examination of bee colonies in Turkey in 2016 showed that 16 of 95 bee colonies (16.8%) were infected with *Nosema spp.* (Utuk et al., 2016). Our tests, which were determined by microscopic examination, found that out of the total number of processed samples, 23.07% were positive on *Nosema spp.* and that is fit into the findings of other authors. Microscopic examination is a quick and easy way to diagnose nosemosis but does not provide the possibility of differential diagnosis (Szalanski et al., 2022).

The differential diagnosis of nosemosis provides the insight into the presence of *N. ceranae*, which as a "new" parasite, by transferring to the European honey bee, has created a major health problem for beekeepers. Whether it is important to differentially diagnose the causative agent in clinically manifested nosemosis remains a question and a topic for further research. It is very unclear, however important to determine, which types of *Nosema* are present, when the causative of nosema agents are in bee intestines, but there are no clinical symptoms (subclinical nosemosis). This question can be posted for a reason, due to the already mentioned reduced physiological adaptation of the European bee to *N. ceranae*. Problems related to the diagnosis of nosemosis, as well as the monitoring of clinical symptoms of the disease are also related to determining the degree of infection of the bees and/or society. Determining the number of spores and types *Nosema spp.* in bees, depending on the season, it allows giving a prognosis of the outcome of the infection. However, due to the fact that the burden of bee colonies with *N. apis* or *N. ceranae* does not give a clear picture of the assessment of the outcome of the disease, differential diagnosis will be the "golden key" to giving a prognosis of the disease.

In our research, which referred to molecular tests of bee samples for the presence of the cause of nosemosis, we analyzed two groups of beehives, one from the South Bačka district, and the other from the Srem district. The attached photos show gels that show the results of molecular diagnostics.

Photo 1. Gel electrophoresis. Example for *Nosema ceranae* (218Mitoc F, R)

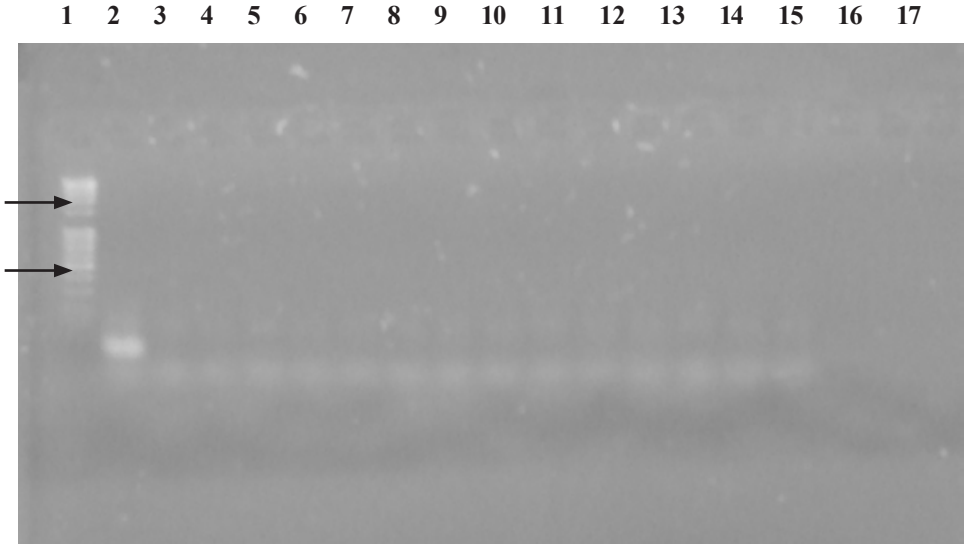


Column: **1.** marker, the upper arrow shows the path100bp, the lower arrow shows the path 200bp, **2.** negative control, **3.** positive control, **4.** sample 1/18, **5.** sample 2/11, **6.** sample 2/15, **7.** sample 4/33, **8.** sample 6/7, **9.** sample 6/12, **10.** sample I/3, **11.** sample 81/4, **12.** sample 185/2, **13.** sample 22/4, **14.** sample 3/30, **15.** sample 5/1, **16**. sample 5/1k, **17.** sample 3/30k.

Photograph 1 shows bands of processed bee samples that were tested for the presence of nosemosis and that were obtained according to the PCR protocol with primers that isolate the 16SrRNA gene corresponding to *N. ceranae*. The photo shows that 5 PCR products obtained from isolated DNA have a weight of 218bp (the lower arrow in the marker column shows where it is 200bp) and that according to the data in Table 2 the weight of base pairs (bp) found in *N. ceranae*. Columns that do not have bands in a position corresponding to the weight of bp for nosema, are samples in which the presence of nosema spores in a very small number (1–2 spores in the field of view) was determined by microscopic findings. A possible explanation for the absence of the presence of PCR products in bands where samples with a small amount of spores were found lies in the fact that during filtration in the phase of preparation of samples for PCR analysis, this small amount of nosema DNA was retained. On the other hand, you should know that after the entry of *Nosema spp.* in the digestive tract, their development and maturation in enterocytes, microsporidia are secreted into the intestinal lumen. During the passage through the digestive tract, spores can reinfect the bee so that empty spores of the nosema can be

found in the feces after excretion (Higes et al., 2007; Higes et al., 2009). Microscopic examination, without staining, makes it difficult to distinguish live from empty spores, and therefore, later molecular examinations can yield an unexpected finding.

Photo 2. Gel electrophoresis. Primer for *Nosema apis* (321Apis F, R)



Column: **1.** marker, the upper arrow shows the path 100bp, the lower arrow shows the path 200bp, **2.** negative control, **3.** positive control, **4.** sample 1/18, **5.** sample 2/11, **6.** sample 2/15, **7.** sample 4/33, **8.** sample 6/7, **9.** sample 6/12, **10.** sample I/3, **11.** sample 81/4, **12.** sample 185/2, **13.** sample 22/4, **14.** sample 3/30, **15.** sample 5/1, **16**. sample 5/1k, **17.** sample 3/30k.

In photo 2, it can be seen that at the place where the lower arrow denoting 200bp is, there are no bands that would testify that the DNA isolate contains a part of the genome that corresponds to *N. apis*. This means that the *N. apis* gene was not detected with the specific primer 321APIS, and could not be found as a PCR product.

The first data on the presence of *N. ceranae* in Europe were reported in the paper (Higes et al., 2006) from 2006. Microscopic examination showed that *N. ceranae* is slightly smaller than *N. apis* with 23–30 polar filaments. Molecular methods found that *N. ceranae* was present in 11 out of the 12 samples examined. Molecular tests involving Spain, France, Switzerland and Germany found that 30.3% of the 290 samples analyzed were negative for the presence of *N. apis* and *N. ceranae*, in 53.8% were positive for *N. ceranae*, while only 9.3% were positive only for *N. apis* and mixed infection with both pathogens was found in 6.6% (Martın-Hernandez et al., 2007). In Turkey, molecular methods found that out of the 84 samples examined, three found *N. ceranae* and four found *N. apis* (Whitaker et al., 2011). It can be observed that the number of positive

samples did not exceed 10%. Molecular tests of honey samples in Bulgaria revealed the presence of only *N. ceranae*. Out of a total of 20 examined honey samples, the presence of nosemosis was confirmed by molecular methods in 30% (Salkova et al., 2022).

CONCLUSION

Microscopic examination revealed the presence of Nosema spp. in 23.07% of the samples. The finding of nosema with this method was higher in relation to molecular tests. That can be explained by the procedure of purification of samples for molecular tests, as well as due to the fact that positive microscopy findings of nosemosis included different degrees of infection of bee colonies. It can be assumed that, when microscopic examination revealed the presence of nosema in small numbers (amount), during the processing of samples for molecular tests, a very small amount of DNA remained in samples or DNA was completely degraded.

The complete prevalence of *Nosema cearane* in the examined samples may mean that in the examined area this species completely suppressed (replaced) *Nosema apis*. This finding may indicate that *N. ceranae* has some other characteristics that are not related only to the physiological adaptation mechanisms of *A. melliferi*, i.e. that the possibility of replacing one species of nosema to another is correlated with some other factors that have not been analyzed.

Differential diagnosis of the cause of bee nosemosis is of great importance from the aspect of knowing the type of nosemosis present in bee colonies, but the question is whether it is important in circumstances when clinical symptoms occur and when societies enter in the clinical picture of nosemosis. This dilemma is primarily related to therapy. If clinical signs of nosemosis occur, then it can be assumed that it does not matter whether *N. apis* or *N. ceranae* is treated.

On the other hand, it is more important to establish a differential diagnosis in the phase when the clinical picture has not appeared yet. It is known that N. ceranae leads to energy stress and consequently reduction of bee protective mechanisms. That leads to depletion of bee colony and accelerated decay. In such a situation, therapy could be preventive too. The answer to the question is, yes, it would be better to find out which Nosema is presented in bees colony before the clinical symptoms occur.

ACKNOWLEDGEMENT

This study was funded by Ministry of Education, Science and Technological development of Republic of Serbia by the Contract of implementation and financing of scientific research work of NIV-NS in 2021, Contract No: 451-03-9/2021-14/200031 from 05/02/2020.

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ОРИГИНАЛНИ НАУЧНИ РАД

ДИФЕРЕНЦИЈАЛНО ДИЈАГНОСТИКОВАЊЕ НОЗЕМОЗЕ ПЧЕЛА – ПОСТУПАК И ЗНАЧАЈ

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САЖЕТАК: У постојећем екосистему, за живи свет, за размножавање, производњу и ширење биљних врста, пчеле представљају један од најважнијих делова природе. У опрашивању појединих ратарских и воћарско-повртарских биљних врста пчеле учествују са скоро 100%. Било који фактор који доприноси смањењу присуства броја пчела у природи, директно утиче на фертилност биљног света, а индиректно и на остатак целокупне живе популације. Ноземоза је микроорганизам који има велики утицај на здравствено стање пчела. У претходном периоду *Nosema* sp. је класификована као једноћелијски паразит (протозоа), али је данас сврстана у гљивице (*Microsporidia*). У природи постоји око 30 различитих врста Нозема. За популацију пчела, поготово кад је у питању европска медоносна пчела (*Apis millifera*) две врсте Ноземе веома су важне: *Nosema apis* и *Nosema ceranae*.

Тачна потврда о којој се врсти *Nosema sp.* ради омогућава боље разумевање исхода и последица за пчелињак у ком је дошло до клиничке слике носемосе. Фенотипски дијагностички метод, и поред присутних морофолошких разлика, не даје могућност сигурног потрвђивања врсте Nosema. Из тих разлога да би се дошло до диференцијалне дијагнозе потребно је молекуларним методама утврдити која врста Nosema је присутна. У овом раду, молекуларном методом (ПЦР), анализирали смо пчеле узорковане из два административна подручја. Испитивање је показало да се на два испитивана подручија налази *Nosema ceranae*, док присуство *Nosema apis* није потврђено. Овакви резултати могу указати на чињеницу да је на испитиваном подручију *Nosema ceranae* предоминантна и да је у потпуности заменила *Nosema apis*.

КЉУЧНЕ РЕЧИ: дифернцијална дијагностика, Ноземоза, пчеле, *Nosema apis*, *Nosema ceranae*

Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. № 143, 65—72, 2022

UDC 582.991.131:579.8
https://doi.org/10.2298/ZMSPN2243065M
ORIGINAL SCIENTIFIC PAPER

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SCREENING OF *Bacillus* spp. AS POTENTIAL BIOCONTROL AGENTS AGAINST SUNFLOWER PATHOGENS

ABSTRACT: *Bacillus* spp. are well known to protect plants from seed or soil-borne pathogens by the synthesis of various metabolites with antimicrobial activity, such as hydrolytic enzymes and antibiotics. This study aimed to select the most effective *Bacillus* spp. from a group of ten antagonistic strains by antifungal activity assay. *Bacillus* strains were primarily isolated from the soil and identified as *B. safensis*, *B. pumilus* and *B. subtilis* by 16S rDNA sequencing. The four analyzed fungi: *Macrophomina phaseolina*, *Alternaria alternata*, *Cladosporium cladosporoides*, and *Sclerotinia sclerotiorum*, were obtained from sunflower seeds and identified using PCR analysis and primers specific for ITS region. The antifungal activity of bacterial strains was examined in a dual plate assay. *Bacillus* spp. demonstrated the highest antagonism against *S. sclerotiorum*, followed by *C. cladosporoides*, *M. phaseolina*, and *A. alternata*, with an average percentage of growth inhibition (PGI) of 77%, 70%, 64% and 59%, respectively. Overall, *Bacillus* spp. included in this study demonstrated a rather strong biocontrol potential, although the effect of particular strain varied depending on the tested fungi. The highest antagonistic effect toward *M. phaseolina* and *A. alternata* was exhibited by *B. safensis* B2 and *B. pumilus* B3. *B. pumilus* B11 and *B. subtilis* B32 were the most efficient against *C. cladosporoides*, whereas *B. pumilus* B3 and *B. subtilis* B7 had the highest antifungal activity against *S. sclerotiorum*. Findings point to the fact that the most effective *Bacillus* spp. could be used as potential biocontrol agents for improving plant health and productivity.

KEYWORDS: *Alternaria*, antifungal activity, *Bacillus*, biocontrol, *Cladosporium*, *Macrophomina*, *Sclerotinia,* sunflower

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INTRODUCTION

Sunflower is one of the most important industrial crops in the world, cultivated in more than 70 countries, with a total production of 50 million tons and average yield of 1.8 tons per hectare (FAOSTAT, 2020). In Serbia, sunflower is grown on 230,000 hectares, with total production of about 700,000 tons and average yield of 3.1 t/ha (Statistical Yearbook of RS, 2021). Plant diseases are the main limiting factors in sunflower production (Tančić Živanov et al., 2021). So far, more than 30 different economically important sunflower pathogens have been identified worldwide (Škorić, 2016). Phytopathogenic fungi are major pathogens that infect sunflower and cause even 80% diseases responsible for high yield reductions, which, depending on environmental conditions, may range from 30% to 50% (Jurković and Ćosić, 2004).

Due to environment and health issues related to the chemical pesticides, efficient management strategy should be based on preventive measures. It is important to identify pathogen, limit its spread and conditions favorable for a disease. Disease control implies cultural, physical, biological and chemical measures such as site selection, sanitation, soil, rotation and water management, using of resistant varieties and healthy seeds, as well as promotion of plant growth and health through proper fertilization and biological control (Poleatewich, 2018). Biological control is defined as the use of beneficial organisms or biological control agents to mitigate the negative effects of plant pathogens (Lazarovits et al., 2014). The most common approach to biological control involves isolation and identification of biocontrol agents, evaluation of biocontrol potential of strains in laboratory, greenhouse and field, bioprocess engineering of selected strains, and development of biopesticides (Poleatewich, 2018).

Bacillus species are among the most investigated biocontrol agents and plant growth-promoting bacteria (PGPB). They are dominant soil, rhizosphere, and endophytic bacteria, able to produce bioactive metabolites and extremely tolerant endospores. *Bacillus*-based biopesticides are developed worldwide and usually contain beneficial strains of *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, *B. velezensis*, and *B. thuringiensis* (Mazzola and Freilich, 2017). Inhibition of pathogen growth by *Bacillus* entails the mechanisms such as competition for nutrients and space, production of antibiotics, hydrolytic enzymes, siderophores, and inducing systemic resistance (ISR). Moreover, *Bacillus* spp. facilitate the uptake of nutrients from the environment via nitrogen fixation and phosphate solubilization, or provide the plant with certain compounds such as plant hormones (Aloo et al., 2019).

The objective of this study was to select most effective antagonistic strains of *Bacillus* spp. against four phytopathogenic fungi affecting sunflower.

MATERIAL AND METHODS

Phytopathogenic fungi

The phytopathogenic fungi were obtained from culture collection of Laboratory for Seed Testing (Institute of Field and Vegetable Crops Novi Sad, IFVCNS). The fungi were originally isolated from sunflower seeds using filter paper method (Mathur and Kongsdal, 2003). Seeds with symptoms were transferred on potato dextrose agar (PDA). After morphological identification, confirmation of pathogenicity was conducted on seedlings using Knop's agar test tube method (Tuite, 1969). Molecular identification was done using PCR analysis of ITS region and ITS1/ITS4 primers (Takamatsu and Kano, 2001). The four analyzed fungi were identified as *Macrophomina phaseolina*, *Alternaria alternata*, *Cladosporium cladosporoides*, and *Sclerotinia sclerotiorum*. Sequences of analyzed fungi are available in the National Center for Biotechnology Information (NCBI) GenBank Database (Table 1).

Table 1. Phytopathogenic fungi used in the study

Isolate code	Isolation source	Species	NCBI
14Sun	Sunflower seeds	*Macrophomina phaseolina*	MH496040
45Sun	Sunflower seeds	*Sclerotinia sclerotiorum*	MH496034
54Sun	Sunflower seeds	*Cladosporium cladosporoides*	MH496035
82Sun	Sunflower seeds	*Alternaria alternata*	MH496037

Antagonistic *Bacillus* spp. strains

Antagonistic strains of *Bacillus* spp. were obtained from culture collection of Laboratory for Microbiology (IFVCNS). In brief, bacterial strains were isolated from soil samples collected in several locations in Serbia, using serial dilution and streak plate methods on nutrient agar (NA) (Bjelić et al., 2018). Morphologically different colonies were recultivated on the same medium to obtain pure cultures. After microscopic observation, strains were selected for molecular identification using 16S rDNA sequencing and universal primers 27F and 1429R (Weisburg et al., 1991). Ten antagonistic strains were identified as *B. safensis* (B2)*, B. pumilus* (B3, B11, B21, B22, B23) and *B. subtilis* (B5, B7, B13, B32). Sequences of the examined *Bacillus* spp. were deposited in the NCBI GenBank Database (Table 2).

Table 2. Antagonistic *Bacillus* strains used in the study

Isolate code	Isolation source	Species	NCBI
B2	Non-agricultural soil	*Bacillus safensis*	KU953932
B3	Rhizosphere (wheat)	*Bacillus pumilus*	KU953923
B5	Rhizosphere (sunflower)	*Bacillus subtilis*	KU953925
B7	Rhizosphere (maize)	*Bacillus subtilis*	KU953927
B11	Non-agricultural soil	*Bacillus pumilus*	KU953931
B13	Rhizosphere (maize)	*Bacillus subtilis*	KX444639
B21	Agricultural soil	*Bacillus pumilus*	KX444647
B22	Rhizosphere (maize)	*Bacillus pumilus*	KX444648
B23	Rhizosphere (wheat)	*Bacillus pumilus*	KX444649
B32	Non-agricultural soil	*Bacillus subtilis*	KX766373

Antifungal activity assay

Antifungal activity of ten antagonistic *Bacillus* spp. strains against four phytopathogenic fungi was examined in a dual plate assay, which implies the simultaneous cultivation of bacterial and fungal culture on PDA. Prior to confrontation, *Bacillus* spp. were cultivated overnight in nutrient broth (NB), while the fungi were grown on PDA for 7–10 days, depending on the fungal species. *Bacillus* strains were streaked by bacteriological loop on PDA along the edge of the Petri dish (R = 85 mm), while fungal discs (6 mm in diameter) were placed on the opposite side. After incubation for 7 days at 25 °C, the fungal growth (in mm) in the control (C) and treated dishes (R1) was measured, and the percent of growth inhibition (PGI) was calculated according to the following formula: PGI (%) = [(C-R1)/C] × 100 (Dimkić et al., 2015).

Statistical analysis

Data was subjected to analysis of variance (ANOVA) using software STATISTICA 12.6 (Statsoft, Tulsa, Oklahoma, USA). Means were separated using Tukey's HSD (honest significant difference) test at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Bacillus spp. are able to control fungal growth and prevent fungal disease, while simultaneously enhancing plant growth and yield (Radhakrishnan et al., 2017). This study confirmed that examined bacterial strains possess very strong potential for the biocontrol of phytopathogenic fungi (Table 3). Antifungal activity assay showed that *Bacillus* spp. exhibited the highest antagonistic effect against *S. sclerotiorum*, with an average PGI of 77%. Moreover, PGI obtained via confrontation of *Bacillus* spp. with *C. cladosporoides*, *M. phaseolina*, and

A. alternata, was 70%, 64% and 59%, respectively. The results also showed different effect of particular strain depending on the tested fungal species. The highest inhibition of *M. phaseolina* and *A. alternata* was recorded by *B. safensis* B2 and *B. pumilus* B3. Strains *B. pumilus* B11 and *B. subtilis* B32 had the highest antagonistic effect on the growth of *C. cladosporoides*, whereas *B. pumilus* B3 and *B. subtilis* B7 showed the largest decrease in growth of *S. sclerotiorum*. Similarly, Dimkić et al. (2015) reported different sensitivity of analyzed fungi in antifungal activity assay using *Bacillus* strains.

Table 3. Antifungal activity of *Bacillus* strains

Bacillus strains	Fungal isolates			
	Macrophomina phaseolina	*Alternaria alternata*	*Cladosporium cladosporoides*	*Sclerotinia sclerotiorum*
	PGI (%) – Percent of growth inhibition			
B. safensis B2	70.83^{a}	65.00^{a}	69.17^{cd}	77.50^{a}
B. pumilus B3	70.83^{a}	65.00^{a}	71.67^{bc}	80.83^{a}
B. subtilis B5	65.00^{a}	60.00^{a}	68.33^{cd}	76.67^{a}
B. subtilis B7	65.00^{a}	61.67^{a}	65.83^{de}	80.83^{a}
B. pumilus B11	64.17^{a}	62.50^{a}	76.67^{a}	76.67^{a}
B. subtilis B13	67.50^{a}	63.33^{a}	74.17^{ab}	79.17^{a}
B. pumilus B21	68.33^{a}	60.83^{a}	73.33^{ab}	78.33^{a}
B. pumilus B22	70.00^{a}	55.00^{ab}	66.67^{d}	78.33^{a}
B. pumilus B23	35.83^{b}	33.33^{b}	62.50^{e}	65.83^{b}
B. subtilis B32	65.00^{a}	63.33^{a}	75.00^{ab}	75.83^{a}
Average	64.25	59.00	70.33	77.00

Mean values (n = 3) of fungal growth inhibition are shown. Values followed by the same letter within columns are not significantly different ($P < 0.05$), according to Tukey's HSD test.

In vitro assay is a good method to examine the antagonistic effect of a large number of strains and provides insight into potential candidates for biological control that need further testing. However, most strains that are effective *in vitro* are not able to inhibit pathogens under environmental conditions, most often because they cannot survive under specific conditions and colonize plant. As spore-forming bacteria, *Bacillus* spp. easily survive and adapt in all habitats, including soil and plant rhizosphere or phyllosphere (Wu et al., 2015). Biocontrol action of *Bacillus* spp. is mainly based on their ability to synthesize antifungal peptides such as surfactin, iturin, fengycin, pumilacidin, mixirin, and/or hydrolytic enzymes such as chitinases, glucanases, cellulases, etc. Due to these antifungal metabolites, bacilli cause changes in the fungal cell wall, cell membrane and intracellular structures. *Baciillus* spp. involved in this study produced various lytic enzymes, while four strains (B5, B7, B13, B32) also produced lipopeptide surfactin (Bjelić et al., 2018). In addition, these antagonistic strains demonstrated broad antifungal and anitbacterial activity against species of *Fusarium*, *Alternaria*, *Diaporthe*, *Xanthomonas*, and other plant

pathogens (Bjelić et al., 2017; Bjelić et al., 2018; Spremo et al., 2018; Miljaković et al., 2022). Successful biological control depends primarily on the interactions between plants, antagonists and pathogens, as well as the environment. Therefore, it is necessary to evaluate the activity of the most effective antagonists *in planta*, after inoculation of pathogens directly into plant tissue and monitoring the development of infection on plants.

CONCLUSION

Findings point to the fact that the most effective *Bacillus* spp. could be used as potential biocontrol agents for improving plant health and productivity. Furthermore, different sensitivity of the analyzed fungi to the action of individual *Bacillus* spp. indicates the possibility of selecting the strain appropriate for each disease and its causative agent. Further evaluation of effective *Bacillus* strains in greenhouse and field experiments is needed to determine their effectiveness in disease suppression and growth promotion of sunflower and/or other field and vegetable crops.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant number: 451-03-68/2022-14/200032.

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ИСПИТИВАЊЕ *Bacillus* spp. КАО ПОТЕНЦИЈАЛНИХ АГЕНАСА БИОКОНТРОЛЕ ПАТОГЕНА СУНЦОКРЕТА

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РЕЗИМЕ: *Bacillus* spp. сузбијају фитопатогене синтезом различитих метаболита са антимикробним деловањем као што су литички ензими и антибиотици. Циљ ових истраживања била је селекција најефективнијих *Bacillus* spp. из групе антагонистичких сојева испитивањем њихове антифунгалне активности. *Bacillus* сојеви су првобитно изоловани из земљишта и идентификовани као *B. safensis*, *B. pumilus* и *B. subtilis* секвенцирањем 16S rDNA. Анализиране гљиве, *Macrophomina phaseolina*, *Alternaria alternata*, *Cladosporium cladosporoides* и *Sclerotinia sclerotiorum*, изоловане су са семена сунцокрета и идентификоване PCR анализом уз примену прајмера специфичних за ITS регион. Антифунгална активност бактерија према фитопатогеним гљивама испитана је методом двојне култивације. *Bacillus* spp. испољили су највећи антагонизам према *S. sclerotiorum*, затим *C. cladosporoides*, *M. phaseolina* и *A. alternata*, са просечним процентом инхибиције раста 77%, 70%, 64% и 59%. Испитивани сојеви *Bacillus* spp. показали су веома јак биоконтролни потенцијал, иако је ефекат одређеног соја варирао у зависности од испитиване гљиве. Највећи антагонистички ефекат према *M. phaseolina* и *A. alternata* имали су сојеви *B. safensis* B2 и *B. pumilus* B3. Најефективнији сојеви против *C. cladosporoides* били су *B. pumilus* B11 и *B. subtilis* B32, док је највећа антифунгална активност према *S. sclerotiorum* утврђен применом сојева *B. pumilus* B3 и *B. subtilis* B7. Резултати ових истраживања показују да се најефективнији сојеви *Bacillus* spp. могу користити као потенцијални агенси биоконтроле за побољшање здравља и продуктивности биљака.

КЉУЧНЕ РЕЧИ: *Alternaria*, антифунгална активност, *Bacillus,* биоконтрола, *Cladosporium*, *Macrophomina*, *Sclerotinia*, сунцокрет

Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. № 143, 73—87, 2022

UDC 338.439:634.334(497.11)
https://doi.org/10.2298/ZMSPN2243073S
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.

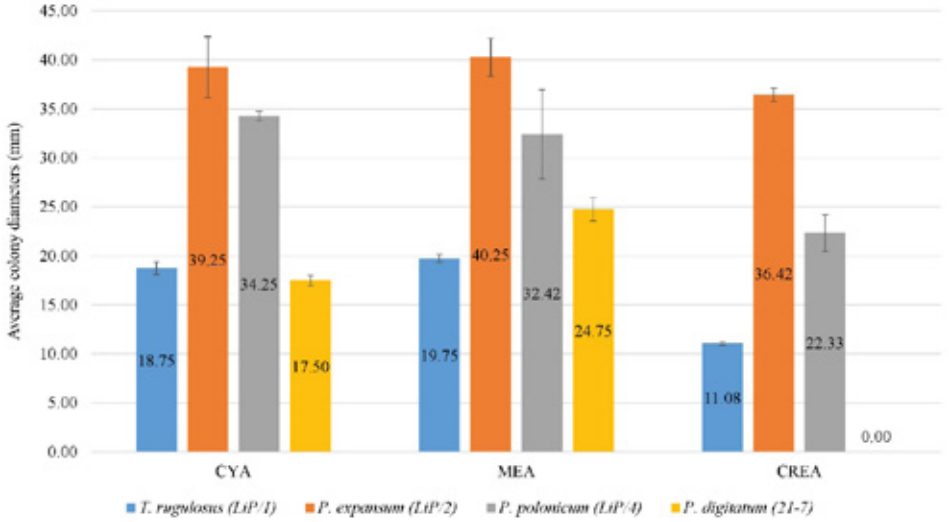

Figure 1. Mean colony diameters of *Penicillium* and *Talaromyces* isolates on three tested media (7 days of incubation, 25 °C). Vertical error bars represent standard deviation of the mean (SD).

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).

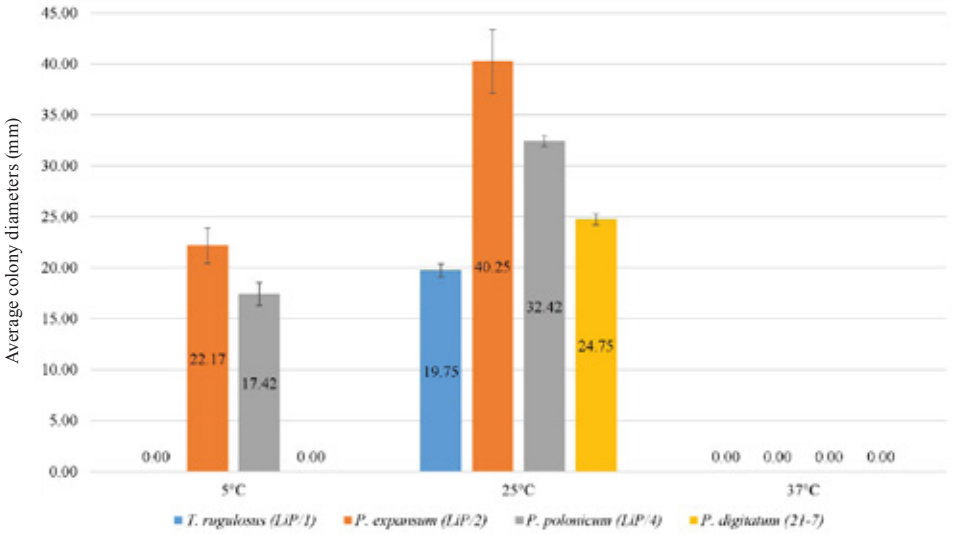

Figure 2. Mean colony diameters of *Penicillium* and *Talaromyces* isolates on three tested incubation temperatures (7 days of incubation, CYA). Vertical error bars represent standard deviation of the mean (SD).

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.

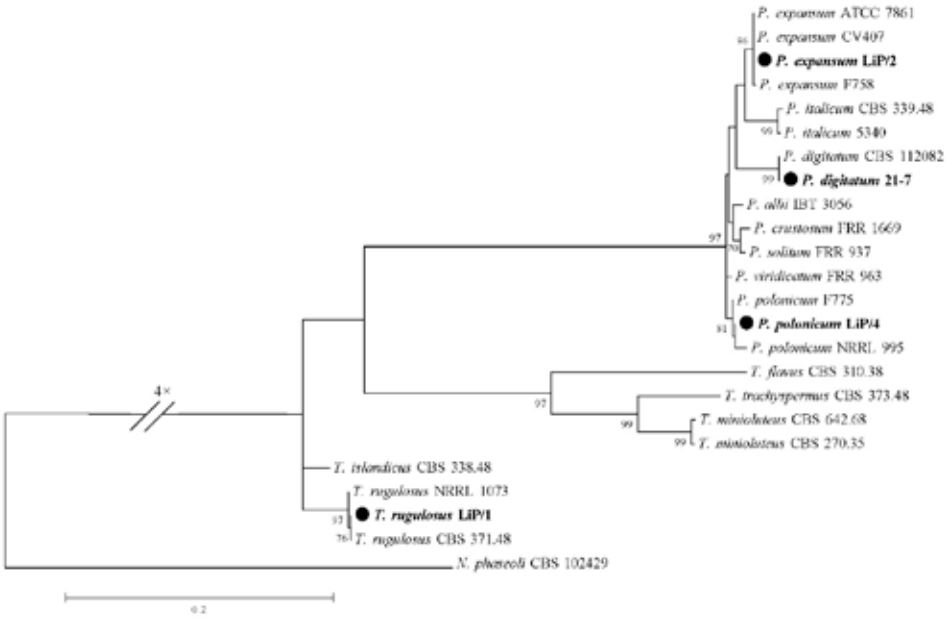

Figure 3. Maximum likelihood phylogenetic tree based on combined ITS and *BenA* sequence alignments of selected *Penicillium* and *Talaromyces* isolates. Tree was rooted with *Neocosmospora phaseoli* (=*Fusarium solani*). Bootstrap values >70% are displayed near to the corresponding nodes and isolates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

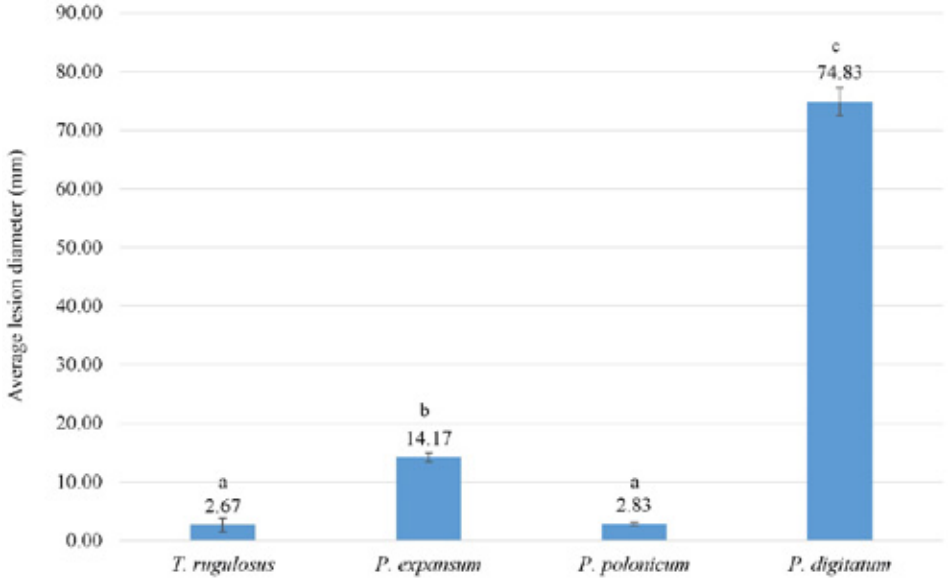

Figure 4. Average lesion diameters of *Penicillium* and *Talaromyces* isolates in pathogenicity test on lemon fruits. Different letters indicate values that are significantly different in Tukey's test ($p<0.05$), and vertical error bars represent standard deviation of the mean (SD).

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).

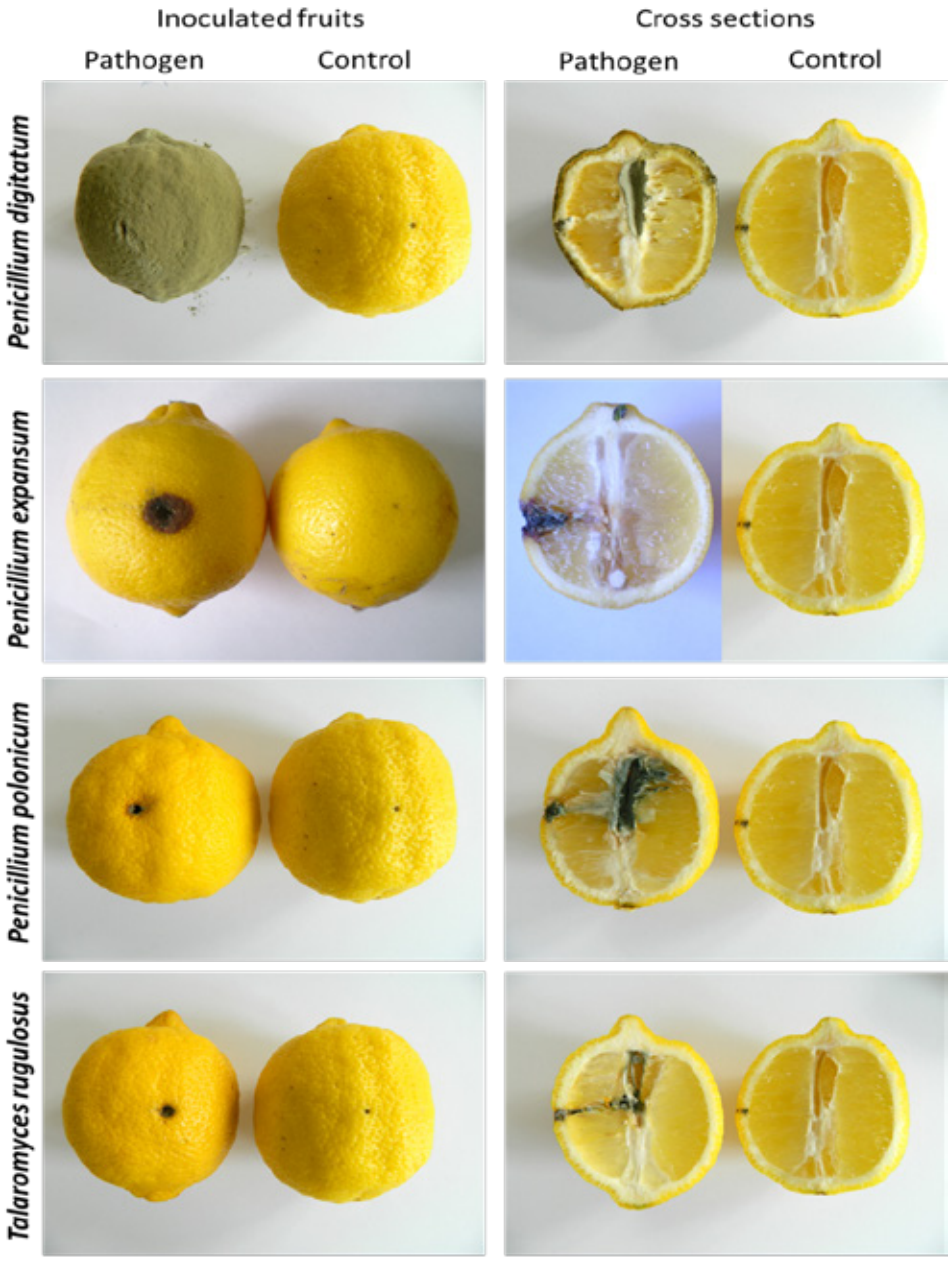

Figure 5. Pathogenicity test of the isolated species on lemon fruits

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.

ACKNOWLEDGEMENT

This research was financially supported by Ministry of Education, Science and Technological Development of the Republic of Serbia, contract number 451-03-68/2022-14/200010.

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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*

Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. № 143, 89—99, 2022

UDC 630*0:63
https://doi.org/10.2298/ZMSPN2243089G
ORIGINAL SCIENTIFIC PAPER

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DEGRADATION OF PRETREATED AGROFORESTRY RESIDUES BY SELECTED MICROMYCETES

ABSTRACT: Nowadays, there are huge amounts of lignocellulosic materials left in agroforestry practice, which can be transformed into useful products. Biomass exploitation could be aiming not only at replacing conventional energy sources but also at preserving biodiversity and natural ecosystems. Five micromycetes were studied with goal to determine their potential to produce active cellulases as well as the ability to decompose pretreated wheat straw and oak sawdust after seven days of solid-state fermentation. Wheat straw was better lignocellulosic substrate than oak sawdust for the production of cellulases in all five micromycetes. Thus, *Penicillium solitum* BEOFB 1190m has shown to be the best producer of highly active forms of xylanases (7532.36 ± 89.37 U/L). The most active endo- and exocellulases (2299.70 ± 72.17 U/L and 195.66 ± 4.64 U/L, respectively) were produced by *Trichoderma harzianum* BEOFB 1230m, while the maximal value of β-glucosidase activity (215.69 ± 3.13 U/L) was detected after *Fusarium graminearum* BEOFB 820m cultivation. *T. harzianum* also showed high efficiency in wheat straw cellulose and hemicellulose depolymerization (23.90% and 33.00%, respectively), which resulted in the highest dry matter loss (36.25%). The results of the study showed great potential of tested micromycetes to synthesize cellulolytic enzymes and consequently transform abundant, low-cost plant residues such as wheat straw into useful products including biofuel.

KEYWORDS: agroforestry residues, cellulolytic enzymes, depolymerization, micromycetes, plant residue

INTRODUCTION

The high standard of living in modern society depends on the availability of natural energy resources but its long-term overexploitation generated its extensive deficit. Therefore, there is a growing need for the reduction of enormous volume of waste, energy consumption and water usage and unless we manage

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and control resources in a sustainable manner its deficit will grow constantly (Lampert, 2019). As a consequence of increased environmental awareness, biotechnological applications came in front of both chemical and physical methods in numerous industrial processes (Viikari et al., 2009). According to Deng et al. (2015) and Terrone et al. (2018) biomass degradation is of great significance because it allows the reuse of lignocellulosic waste generated in agriculture and forestry, food industry as well as municipal waste, and the rational usage of the degradation by-products. Particularly, International Renewable Energy Agency (IRENA) estimated that biomass will remain major renewable energy source beyond 2030 in the European Union (IRENA 2018). Despite the abundance of lignocellulosic biomass and its great potential as a resource for the production of food, feed, biofuels, chemicals and many other industry related products, this material is still improperly deposited and presents serious ballast in nature (Deng et al., 2015; García-Torreiro et al., 2016; Ćilerdžić et al., 2017). Although chemical, physical and physico-chemical methods for biomass degradation are widely used, they are characterized by numerous drawbacks in commercial applications which make them unsatisfactory for preserving the natural environment (Ćilerdžić et al., 2017; Kumar et al., 2019). Thus, the development of high performance biological methods for biomass delignification, hydrolysis and other industrial applications has become a research priority (Chulalaksananukul, 2014).

Wheat is the world's most widely grown crop and according to its abundance with an annual yield of 170 million tons takes the first place in Europe. It presents the cheapest and one of the main lignocellulosic substrates bio converted to ethanol in Europe and North America (Talebnia et al., 2010; Ghaffar et al., 2015; Ćilerdžić et al., 2017). An attractive alternative is also the use of other residues from agriculture and forestry which are widely distributed in numerous regions of the world but still remained unexploited (Ghaffar et al., 2015). However, the development of new methods in seeding technology and increased costs of transportation have negative consequences on the utilization rate of these materials (Xu et al., 2020). Talebnia et al. (2010) and Kucharska et al. (2018) reported that wheat straw cell wall has a complex organizational structure composed of lignin, cellulose and hemicellulose as three main components and their relative abundance depends on species, soil, and climate conditions. Likewise, as these components form an interlinked hetero-matrix, which greatly affects (in)accessibility of its individual components, the transformation of lignocellulose remains a challenge (Dashtban et al., 2009). Nowadays, there is no report for a simple enzymatic scenario for lignocellulose depolymerization but according to Horn et al. (2012) numerous studies showed that fungi are extremely efficient in the degradation of these materials. Likewise, Varnai et al. (2011) reported that enzymes such as xylanases have an important role in enzyme cocktails for lignocellulose mineralization since hemicellulose structure can increase the recalcitrance of cellulose. According to Sajith et al. (2016) and Xue et al. (2020), commonly used commercial enzyme cocktails are produced by *Trichoderma* spp., *Aspergillus* spp., *Penicillium* spp. but since species of the genus *Trichoderma* synthesize larger amounts of cellulases as reported in the

study by Horn et al. (2012) and Pandey et al. (2015), they are the most exploited in lignocellulosic biorafineries. The production process of cellulases is quite expensive and therefore it is necessary to search for organisms that have a potential for high yield of cellulases as well as the use of available and cheap substrates (da Silva et al., 2012). Thus, Gao et al. (2008) and Gautam et al. (2011) reported that the carbon source and the productivity of fungal enzymes are tightly associated with the cost of cellulases production.

Based on everything mentioned, five micromycetes were studied with goal to determine their potential to produce active cellulases as well as the ability to decompose pretreated wheat straw and oak sawdust after seven days of solid-state fermentation.

MATERIALS AND METHODS

Organisms and cultivation conditions

The cultures of micromycetes are maintained on Malt agar medium (NEOGEN Culture Media) at 4 °C, in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB) (Table 1).

Table 1. Studied species

Species	Code	Origin
Aspergillus terreus	BEOFB 352m	Resistivity of various materials OP2 (PQMD, 82j)
Chaetomium globosum	BEOFB 740m	Serbia, Belgrade, National Bank
Fusarium graminearum	BEOFB 820m	Serbia, Belgrade The central Institute for Conzervation, air
Penicillium solitum	BEOFB 1190m	Serbia, Belgrade The central Institute for Conzervation, air
Trichoderma harzianum	BEOFB 1230m	Serbia, Belgrade, Roman stelae

The inoculum preparation was performed by inoculating 100.0 mL of the synthetic medium (glucose, 10.0 g L^{-1}; NH_4NO_3, 2.0 g L^{-1}; K_2HPO_4, 1.0 g L^{-1}; $NaH_2PO_4 \cdot H_2O$, 0.4 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g L^{-1}; yeast extract, 2.0 g L^{-1}; pH 6.5) with 25 mycelial disks (Ø 0.5 cm) of seven-days-old culture. The incubation was performed on Stuart incubator S1600C rotary shaker (22 ± 2 °C, 160 rpm) during seven days. The obtained biomass was washed three times using sterile distilled water (dH_2O) and then homogenized with 100.0 mL of dH_2O in a laboratory blender (Waring, USA). Solid-state fermentation by selected species was carried out in 100 mL flasks containing 2.0 g of agroforestry residues (wheat straw and oak sawdust) previously pretreated with *Pleurotus pulmonarius* HAI 509 and 10.0 mL of the modified synthetic medium (without glucose) at 25 °C in the dark. Thus prepared flasks were incubated with 3.0 mL of homogenized inoculum and the samples were harvested after seven days of cultivation (Stajić et al., 2010).

Assays of enzyme activity and total protein production

The cellulolytic enzymes were extracted by sample stirring with 50.0 mL of dH_2O at 4 °C for 10 min. The obtained supernatant after extract centrifugation in Hettich Zentrifugen, Universal 32 R (4 °C, 3000 rpm, 15 min) was used for measurement of the activity of exo-, endocellulases, β-glucosidases, and xylanases by a spectrophotometer (BioQuest CECIL CE2501, UK). The activities of exo- and endocellulases were determined according to the methods of Bernfeld (1955) using microcrystalline cellulose (1%) and medium viscosity carboxymethyl cellulose (1%), respectively, as the substrates, with glucose as a standard. The activity of β-glucosidases was estimated using 4-nitrophenyl β-D-glucopyranoside and *p*-nitrophenol as a substrate and standard, respectively, while the activity of xylanases were determined using birchwood xylan (1%) as a substrate and xylose as a standard. One unit of exo-, endocellulase and xylanase activity was defined as the amount of enzyme required to produce 1.0 μmoL of glucose or xylose per min at 39 °C (Grujić et al., 2015).

According to the method of Silva et al. (2005) the total protein content (mg/mL) was determined by measurement of Bradford's reagent color change at $\lambda = 595$ nm induced by sample. The bovine serum albumin was used as the standard and further for calculation of the specific enzyme activity (U/mg).

Determination of hemicellulose, cellulose and lignin contents

The loss of substrate dry matter (%) was determined by the formula (M*i* – M*f*)/M*i* x 100, where M*i* represents the initial lignocellulosic mass and M*f* is the mass after fermentation by the studied species.

The determination of hemicellulose, cellulose and lignin contents was performed using the modified methods of Kirk and Obst (1988) and Van Soest et al. (1991). A 1.0 g of dried ground sample was treated with neutral detergent/Na_2SO_3 mixture under refluxing conditions to remove soluble sugars, proteins, lipids, and vitamins and the obtained biomass presented neutral detergent fibers (NDF). Acidic detergent fibers (ADF) were obtained by treatment of these samples with acidic detergent. The difference between obtained fibers represented the hemicellulose content. The lignin content (LC) was defined after ADF incubation with 72% H_2SO_4 at 30 °C and its hydrolysis at 120 °C, and expressed as the percentage of quantity present in the initial sample. The difference between ADF and LC was determined as the cellulose content.

Statistical analyses

All the experiments were done in three replicates and the results were expressed as mean ± standard error. One-way analysis of variance (ANOVA) and Tukey`s test were performed using STATISTICA, version 6.0 (StatSoft, Inc., Tulsa, USA) to test any significant differences among means. Statistical significance was declared at $p<0.05$.

RESULTS AND DISCUSSION

The obtained results showed that wheat straw was much better inducer of cellulolytic enzymes than oak sawdust in all selected micromycetes which demonstrated a significant interspecific diversity in enzymes production after seven days of solid-state fermentation of both substrates (Figures 1, 2).

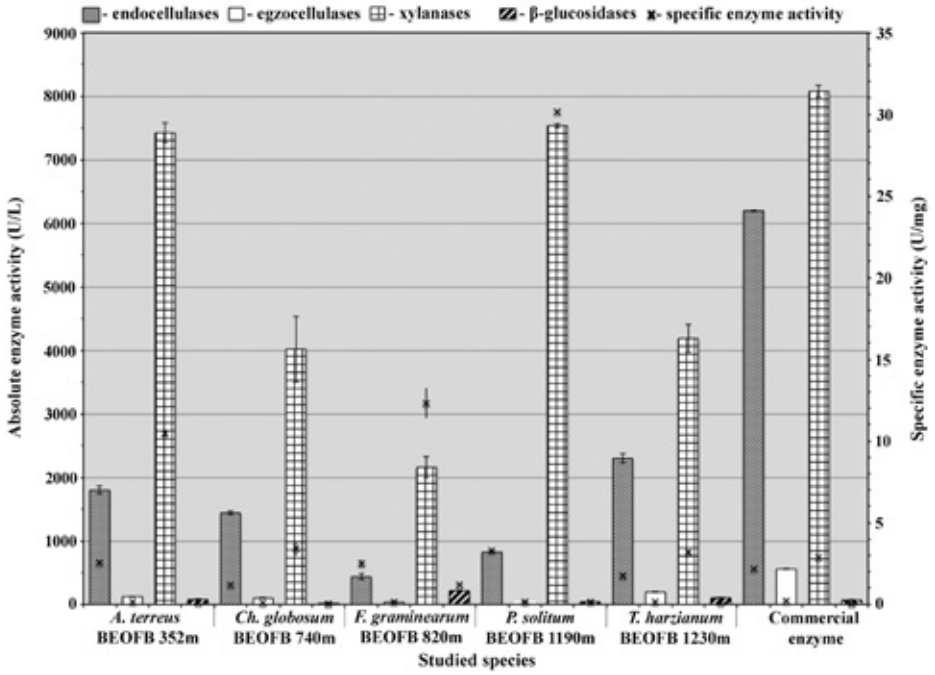

Figure 1. The activity of cellulolytic enzymes after wheat straw fermentation by selected micromycetes

Wheat straw caused the highest xylanase activity of 7532.36 ± 89.37 U/L in *Penicillium solitum* and 7421.35 ± 152.72 U/L in *Aspergillus terreus*, while the lowest activity was noted in *Fusarium graminearum* (2155.91 ± 166.66 U/L) (Fig. 1). Moreover, *T. harzianum* was the best producer of highly active forms of endo- and exocellulases on wheat straw (2299.70 ± 72.17 U/L and 195.66 ± 4.64 U/L, respectively). On the other hand, this residue was weaker stimulator of exocellulase and β-glucosidase synthesis (Figure 1). Although the lowest values of xylanase and exocellulase activities was noted after wheat straw fermentation by *Fusarium graminearum* (Figure 1), this species produced the most active isoforms of β-glucosidases (215.69 ± 3.13 U/L).

Contrary to fungal cellulases, as expected, commercial enzyme cocktail Cellulase from *Trichoderma reesei* (Sigma-Aldrich) was more efficient in endo-, exocellulase and xylanase activities and even weaker than obtained β-glucosidase activities by micromycetes (Figures 1, 2).

Different profile of enzymes activity was observed on oak sawdust (Figure 2).

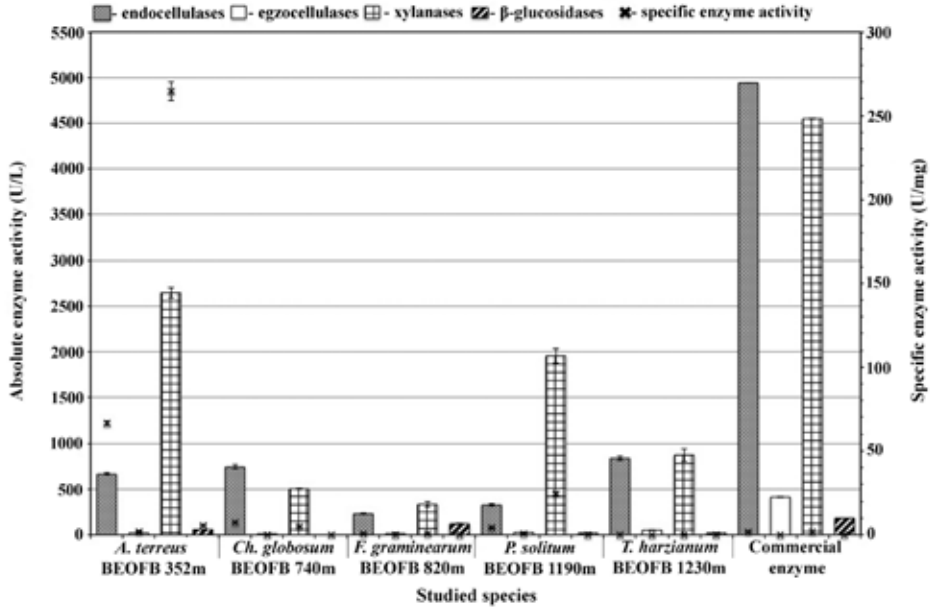

Figure 2. The activity of cellulolytic enzymes after oak sawdust fermentation by selected micromycetes

Namely, the maximal value of xylanases (2645.62 ± 55.07 U/L) was noted after oak sawdust fermentation by *A. terreus*. The highest activity of endo- and exocellulases (835.39 ± 22.66 U/L and 50.03 ± 0.20 U/L, respectively) was detected after *T. harzianum* cultivation, while *F. graminearum* secreted the highest active β-glucosidases isoforms on this substrate (119.81 ± 1.94 U/L) (Figure 2).

The fungal species as well as the plant residues used in this study affected the protein production which consequently affect the specific enzyme activities. Thus, the highest protein content after fermentation of both substrates was detected in *T. harzianum* (3.10 mg/mL after oak sawdust and 1.31mg/mL after wheat straw) while the lowest protein contents after wheat straw and oak sawdust fermentation were noted in *F. graminearum* (0.18 mg/mL) and in *A. terreus* (0.01 mg/mL), respectively. The highest values of xylanase and endocellulase specific activities were detected after *P. solitum* cultivation on wheat straw (30.13 ± 0.12 U/mg and 3.29 ± 0.02 U/mg, respectively), while specific activities of exocellulase and β-glucosidase were noted with *F. graminearum* after fermentation of the same substrate (1.23 ± 0.02 U/mg and 0.18 ± 0.01 U/mg).

Contrary to results obtained by Kurbanmuratovich and Ismailovna (2018) and Vázquez et al. (2019) all tested species in our study produced significantly more active isoforms of cellulases on both substrates. Namely, *T. harzianum* strain in the study of Kurbanmuratovich and Ismailovna (2018) produced enzymes with

activity of only 0.84 U/mL after six days of fermentation of filter paper, while Vázquez et al. (2019) reported extremely low endo- and exocellulase activities (0.002 U/mL and 0.06 U/mL, respectively) after wheat straw fermentation by the same species. On the other hand, species profiled in this study has shown lower cellulolytic enzymes activities compared to some previous reports. Thus, many-fold higher activities of all four enzymes were detected only after seven days of wheat straw fermentation using *Aspergillus niger* strain. In the study by Narra et al. (2012) and Pensupa et al. (2013) it is showed that rice straw as a carbon source stimulated the synthesis of highly active exocellulase isoforms in *A. terreus* strain (10.96 U/g), while Iqbal et al. (2010) demonstrated high activity of endocellulases (278 U/mL) produced by *T. harzianum* cultivated on chemically treated wheat straw. However, despite the fact that chemical treatment modifies the surface of fibers by removing a certain rate of lignin and hemicellulose and thus promotes the hydrolysis of cellulose, the advantage of using fungal enzymatic cocktails is the absence of polluting stages during industrial processes that have harmful effect on the environment (Pandey, 2008; Gupta and Verma, 2015).

Generally, the dry matter loss was affected by both substrate type and the tested species. Thus, higher loss of dry matter was observed for pretreated wheat straw where the level of reduction ranged from 16.26% with *F. graminearum* to 36.25% with *T. harzianum*, while the highest mass reduction of 20% was noted for oak sawdust. The same effect was noted in the case of cellulose and hemicellulose loss (Table 2).

Table 2. The extent of agro-forestry residues depolymerization by micromycetes

Agro-foresty residue	Studied samples	Sample weight (g)	Fibres composition of samples (mg)			Dry matter loss (%)	Extent of polymers degradation (%)		
			Lignin	Cellulose	Hemicellulose		Lignin	Cellulose	Hemicellulose
Wheat straw	Control*	0.80	46.40	124.00	88.00	/	/	/	/
	T. harzianum	0.51	43.10	94.40	59.00	36.25^{b}	7.10^{b}	23.90^{c}	33.00^{c}
	F.graminearum	0.67	46.40	135.10	67.20	16.26^{a}	0.10^{a}	0.00^{a}	23.60^{b}
	A. terreus	0.53	43.10	75.20	66.30	33.75^{b}	7.00^{b}	39.30^{d}	24.70^{b}
	P. solitum	0.65	40.40	138.90	73.00	18.75^{a}	12.90^{c}	0.00^{a}	17.00^{a}
	Ch. globosum	0.54	33.50	107.50	53.50	32.50^{b}	27.80^{d}	13.30^{b}	39.30^{d}
Oak sawdust	Control*	1.00	106.50	222.60	72.70	/	/	/	/
	T. harzianum	0.82	90.00	195.80	57.20	18.00^{b}	15.50^{b}	12.10^{b}	0.00^{a}
	F.graminearum	0.84	92.20	219.10	32.10	16.00^{a}	13.40^{a}	1.60^{a}	43.80^{c}
	A. terreus	0.84	86.40	215.60	62.90	16.00^{a}	18.90^{b}	3.10^{a}	0.00^{a}
	P. solitum	0.84	82.00	220.10	37.70	16.00^{a}	23.00^{c}	1.10^{a}	34.20^{b}
	Ch. globosum	0.80	95.20	184.00	40.00	20.00^{b}	10.60^{a}	17.30^{c}	30.10^{b}

The most effective mineralizators of wheat straw cellulose were *A. terreus* and *T. harzianum* (39.3% and 23.9%, respectively) where depolymerization degree was in positive correlation with the level of enzymes activity, while *Ch. globosum* and *T. harzianum* were the best degraders of hemicellulose (39.3% and 33.0%, respectively). In the case of oak sawdust, the maximum of holocellulose loss was obtained by *Ch. globosum* and *F. graminearum* (17.3% cellulose

and 43.8% hemicellulose, respectively). Although a significant percentage of lignin was removed during pretreatment of wheat straw and oak sawdust with white-rot fungi, micromycetes showed the ability to mineralize lignin as well (Table 2).

According to Varnaitė and Raudonienė (2008), *Ch. globosum* was highly effective mineralizer of rye straw hemicellulose as 87% was degraded after 30 days and 94% after 60 days. Some previous reports showed high ability of *Trichoderma* species to degrade wheat straw cellulose. Namely, 83% of cellulose loss was detected after *T. viride* cultivation on this residue (Nawaz et al., 2018). Comparing the capacity of *Aspergillus fumigatus* and *Trichoderma* sp. to mineralize manure reported in the study by Zulkifli et al. (2018), *A. terreus* and *T. harzianum* in our study had similar capacity to degrade wheat straw hemicellulose and even better in cellulose depolymerization. Singh et al. (2008) showed the ability of *Trichoderma citrinoviride* to delignify sugar cane straw but the observed lignin loss of 8% was significantly lower than that obtained in this study after *Ch. globosum* and *P. solitum* cultivation on both substrates.

CONCLUSIONS

The results of the study showed that tested micromycetes have a great potential of cellulolytic enzymes production and therefore can be used as a potential source of these enzymes for biomass conversion. However, further research is needed to exploit their full potential such as employing manipulation of enzymes at a gene level, protein engineering etc. The use of abundant, low-cost agroforestry residues as substrates also showed to be a promising alternative for the transformation into numerous useful products.

ACKNOWLEDGEMENT

This study was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (451-03-68/2022-14/200178).

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ПОТЕНЦИЈАЛ ОДАБРАНИХ МИКРОМИЦЕТА ДА РАЗГРАДЕ ПРЕТРЕТИРАНЕ ОСТАТКЕ ИЗ ПОЉОПРИВРЕДЕ И ШУМАРСТВА

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РЕЗИМЕ: У агрошумарству су данас присутне огромне количине лигноцелулозног отпада који има велики потенцијал као сировина за производњу хране, хранива, биогорива итд. Експлоатација биомасе могла би за циљ да има не само да замени конвенционалне изворе енергије већ и да има важну улогу у очувању биодиверзитета и природних екосистема. Како би се утврдио њихов потенцијал за синтезу целулаза као и способност да врше деполимеризацију претретиране пшеничне сламе и пиљевине храста након 7 дана чврсте ферментације одабрано је пет врста микромицета. Пшенична слама је била погоднији супстрат од пиљевине храста за синтезу тестираних ензима код свих одабраних врста микромицета. Тако се *Penicillium solitum* BEOFB 1190m показао најбољим продуцентом ксиланаза чија је активност на пшеничној слами износила чак 7532,36 ± 89,37 U/L. Најактивније ендо- и егзоцелулазе (2299,70 ± 72,17 U/L односно 195,66 ± 4,64 U/L) продуковала је *Trichoderma harzianum* BEOFB 1230m, док је *Fusarium graminearum* BEOFB 820m синтетисао изоформе највише активности β-глукозидаза (63 ± 3 U/L). *T. harzianum* је, такође, показао високу ефикасност у деполимеризацији целулозе и хемицелулозе (23,90% односно 33,00%) током деградације пшеничне сламе што је резултирало највећим губитком суве масе (36,25%). Резултати ове студије су показали висок потенцијал одабраних микромицета да синтетишу целулолитичке ензиме и тиме врше конверзију јефтиног и лако доступног биљног отпада попут пшеничне сламе до сировина као што је биогориво.

КЉУЧНЕ РЕЧИ: деполимеризација, лигноцелулозни отпад, микромицете, остаци из пољопривреде и шумарства, целулолитички ензими

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1.1. Matica Srpska Journal for Natural Sciences (short title: Matica Srpska J. Nat. Sci.) publishes manuscripts and review articles as well as brief communications from all scientific fields as referred to in the title of the journal. Review articles are published only when solicited by the editorial board of the journal. Manuscripts that have already been published in extenso or in parts or have been submitted for publication to other journal will not be accepted. The journal is issued twice a year.

1.2. The manuscripts should be written in correct English language regarding the grammar and style. The manuscripts should be submitted electronically as a separate file to vnikolic@maticasrpska.org.rs and enclosed with the author's written consent for the publishing of the manuscript.

1.3. Upon the reception of the manuscript, the author shall be assigned with a manuscript code, which has to be referred to in any further correspondence. The authors will be notified about the manuscript reception within seven days and about the reviewers' opinion within two months from submission. All submitted manuscripts are reviewed and proofread.

2. Planning and preparing of the manuscript

2.1. Type the manuscripts electronically on A4 (21 × 29.5 cm) format with 2.5 cm margins, first line indent, and 1.5 line spacing. When writing the text, the authors should use Times New Roman size 12 font and when writing the abstract, key words, summary, and footnotes use font size 10.

2.2. First name, middle initial and last name should be given for all authors of the manuscript and their institutional affiliations, institution name, and mailing address. In complex organizations, a full hierarchy should be mentioned (e.g. University of Novi Sad, Faculty of Sciences – Department of Biology and Ecology). The institution of employment of each author should be stated below the author's name. The position and academic degrees should not be cited. If there is more than one author, indicate separately institutional affiliation for each of the authors. Put the name and mailing address (postal or e-mail address) of the author responsible for correspondence at the bottom of the first page. If there is more than one author, write the address of only one author, usually the first one.

2.3. Structure the text of the original articles into Abstract, Key Words, Introduction, Material or Methods, or Material and Methods, Results or Results and Discussion, Discussion, Conclusion, References, Summary and Key Words in Serbian language, and Acknowledgement (if there is one). Original articles should not be longer than 10 pages, including the references, tables, legends, and figures.

2.4. Titles should be informative and not longer than 10 words. It is in the best interest of the authors and the journal to use words in titles suitable for indexing and electronic searching of the article.

2.5. The authors should submit the title of the article with last name and the initials of the first author.

(if the article has more than one author, et al. should be used for other authors) and running title of not more than five words.

2.6. List up to 10 key words using words and phrases that describe the content of the article in the best way and that allow indexing and electronic searching of the paper. List the key words alphabetically and divided by commas.

2.7. The Abstract in English language and Summary in Serbian language should be a short and informative presentation of the article. Depending on the length of the article, the Abstract may have from 100 to 250 words. Summary written in Serbian language can be 1/10 length of the article and should contain the title of the article, first, middle initial, and last names of the authors, authors' institutional affiliation and address, and key words.

2.8. Write the information about financial support, advices, and other forms of assistance, if necessary, at the end of the article under the Acknowledgement. Financial support acknowledgement should contain the name and the number of the project, i.e. the name of the program from which the article originated, and the name of the institution that provided the financial support. In case of other forms of assistance the author should submit the first name, middle initial, last name, institutional affiliation, and the address of the person providing the assistance or the full name and the address of the assisting institution.

3. Structure the Review articles in Abstract, Key Words, Text of the manuscript, Conclusion, and References; submit Summary and Key Words in Serbian language.

4. Write brief communication according to the instructions for original articles but not be longer than five pages.

5. References

5.1. List the References alphabetically. Examples:

(a) Articles from journals: Last name CD, Last name CD (2009): Title of the article. Title of the journal (abbreviated form) 135: 122–129.

(b) Chapters in the book: Last name ED, Last name AS, Last name IP (2011): Title of the pertinent part from the book. In: Last name CA, last name IF (eds.), Title of the book, Vol.4, Publisher, City

(c) Books: Last name VG, Last name CS (2009): Title of the cited book. Publisher, City

(d) Dissertations: Last name VA (2009): Title of the thesis. Doctoral dissertation, University, City

(e) Unpublished articles: designation "in press" should be used only for papers accepted for publishing. Unpublished articles should be cited in the same way as published articles except that instead of journal volume and page numbers should write "in press" information.

(f) Articles reported at scientific meetings and published in extenso or in a summary form: Last name FR (2011): Proceedings, Name of the meeting, Meeting organizers, Venue, Country, 24–29.

(g) World Wide Web Sites and other electronic sources: Author's last name, Author's initial. (Date of publication or revision). Title, In: source in Italics, Date of access, Available from: <Available URL>. Use n.d. (no date) where no publication date is available. Where no author is available, transfer the organization behind the website or the title to the author space.

5.2. References in the text should include author's last name and the year of publishing. When there are two authors both should be cited, but in case of three or more authors, cite the first author only and follow with et al.

5.3. If two or more articles of the same author or authors published in the same year are cited, designate the publishing years with letters a, b, c, etc., both in text and reference list.

5.4. The names of the periodicals should be abbreviated according the instructions in the Bibliographic Guide for Authors and Editors (BIOSIS, Chemical Abstracts Service, and Engineering Index, Inc.).

5.5. Do not translate references to the language of the article. Write the names of cited national periodicals in their original, shortened form. For example, for the reference in Serbian language, put (Sr) at the end of the reference.

6. Units, names, abbreviations, and formulas

6.1. SI units of measurement (Système international d'unités) should be used but when necessary use other officially accepted units.

6.2. Write the names of living organisms using Italics font style.

6.3. Abbreviated form of a term should be put into parenthesis after the full name of the term first time it appears in the text.

6.4. Chemical formulas and complex equations should be drawn and prepared for photographic reproduction.

7. Figures

7.1. Authors may use black-and-white photographs and good quality drawings.

7.2. A caption with the explanation should be put below each figure.

8. Tables

8.1. Type tables on separate sheet of papers and enclosed them at the end of the manuscript.

8.2. Number the tables using Arabic numerals.

8.3. Above each table, write a capture with table explanation.

8.4. On the left margin, indicate the place of the tables in the text.

9. Electronic copy of the article

9.1. After the acceptance of the article, send a CD with final version of the manuscript and a printed copy to facilitate technical processing of the text. Articles should be written in Microsoft Word format and sent to the Editorial office of the Matica Srpska Journal for Natural Sciences, 1 Matica Srpska Street, 21000 Novi Sad (Uredništvo Zbornika Matice srpske za prirodne nauke, Matice srpske 1, 21000 Novi Sad).

9.2. Before printing, the manuscripts shall be sent to the authors for the approval of final version. Corrections of the text prepared for printing should be restricted to misspelling and printing errors as much as possible. For major changes of the text, a fee will be charged. Corrected manuscript should be returned to the Editorial office as soon as possible.

Зборник Матице српске за природне науке издаје Матица српска
Излази двапут годишње

Уредништво и администрација:
Нови Сад, Улица Матице српске 1
Телефон: (021) 6615798

Matica Srpska Journal for Natural Sciences
Published twice a year
Editorial and publishing office:
1 Matica Srpska Street, 21000 Novi Sad, Serbia
Phone: +381 21/6615798

E-mail: ljdrazic@maticasrpska.org.rs
zmspn@maticasrpska.org.rs
Website:
https://www.maticasrpska.org.rs/category/katalog-izdanja/naucni-casopisi/zbornik-matice-srpske-za-prirodne-nauke/

The editors of the *Matica Srpska Journal for Natural Sciences*
completed the selection for Issue 143 (2/2022) on Novembre 9, 2022

For Publishers: Prof. Dragan Stanić, PhD
Editorial Staff Secretary: Ljiljana Dražić, Msr
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Proof Reader: Vladimir M. Nikolić
Technical design: Vukica Tucakov

Published in December 2022
Computer set: Vladimir Vatić, GRAFIT, Petrovaradin
Printed by: SAJNOS, Novi Sad

Публиковање овог Зборника помогло је Министарство просвете,
науке и технолошког развоја Републике Србије

Publication of this volume was supported by the Ministry of Education,
Science and Technological Development of the Republic of Serbia

CIP – Каталогизација у публикацији
Библиотека Матице српске, Нови Сад

5/6(082)

ЗБОРНИК Матице српске за природне науке = Matica Srpska Journal for Natural Sciences / главни и одговорни уредник Ивана Максимовић. – 1984, св. 66– . – Нови Сад : Матица српска, Одељење за природне науке, 1984– . – 24 cm

Два пута годишње. – Наставак публикације: Зборник за природне науке. – Текст на енг. језику, резимеи на енг. и на срп. језику.

ISSN 0352-4906

COBISS.SR-ID 5845250