

Penicillium allii – A new postharvest pathogen of onion bulbs in Serbia

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Abstract: Onion (*Allium cepa* L.), a member of the Amaryllidaceae family, is among the oldest and most widely cultivated crops, valued both in cuisine and traditional medicine. Between 2019 and 2021, onion bulbs showing blue-green mould symptoms were collected from storage facilities in Serbia. The isolated fungi were identified and characterised using a combination of morpho-physiological, molecular, phylogenetic, and pathogenicity approaches. Results from morphological and multilocus genetic analyses confirmed that the isolates belonged to *Penicillium allii*. Pathogenicity assays demonstrated that this species is a pathogen of onion. This is the first record of *P. allii* on onion bulbs in Serbia as a postharvest pathogen. Monitoring and controlling its occurrence in stored onions is essential to prevent potential health risks and reduce economic losses in onion production.

Keywords: *Allium cepa*; blue mould; onion rot; morphological characterisation; molecular analyses

Onion (*Allium cepa* L.), a member of the Amaryllidaceae family, is one of the oldest cultivated crops, with evidence of use dating back over 4 000 years (Khosa et al. 2016; Teshika et al. 2019). The bulb is the most valuable part, widely used in culinary practices and traditional medicine, and cultivated globally as an annual vegetable (Griffiths et al. 2002). Onions are rich in flavonoids, fructo-oligosaccharides, sulfur compounds, vitamins, and minerals, and exhibit antitumor, antiplatelet, antithrombotic, antiasthmatic, and antimicrobial properties (Suleria et al. 2015; Li et al. 2020). However, they are highly susceptible to postharvest fungal pathogens, and some of the most important ones are *Agroathelia rolfsii* (*Sclerotium rolfsii*), *Alternaria porri*, *Aspergillus niger*, *A. alliaceus*, *Botrytis* spp., *Colletotrichum circinans*, *Fusarium oxysporum* f. sp. *cepae*,

Penicillium spp., *Peronospora destructor* (Snowdon 1991; Yurgel et al. 2018). Latest data from 2024 show that onions are cultivated on 3 953 ha in Serbia, with the total yield of 31 964 t (Statistical Office of the Republic of Serbia, 2025). Reports of *Penicillium*-induced postharvest diseases are scarce, apart from Duduk et al. (2017), who detected *Penicillium glabrum*, *P. polonicum* and *P. expansum* on this crop. The goal of our study was to investigate whether some other *Penicillium* spp. are the causal agents of blue mould in stored onion bulbs in Serbia.

MATERIAL AND METHODS

Sampling and pathogen isolation. Mouldy onion bulbs were collected from the storage fa-

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cilities of small local producers in Serbia (around Smederevo, Pančevo, and Kraljevo) without strict control of air humidity and temperature over three years (2019–2021). In total, 48 symptomatic onion bulbs were sampled. Isolations were done as follows: the parts on the margin between healthy and diseased tissue of the onion bulb were incised and removed with a sterilised scalpel, then surface-sterilised in 1% water solution of NaOCl for 2 min, rinsed three times with sterile distilled water and placed in Petri plates containing potato dextrose agar (PDA). Petri dishes were incubated for 7 days in the dark at 25 °C. Single-spore culturing on PDA was employed to establish pure fungal cultures. Finally, 12 isolates were kept at PDA slants in the refrigerator at 4 °C until subsequent analyses.

Morphological and physiological assays. Cultures were inoculated at three points on 90-mm Petri plates, using spore suspensions made in semi-solid agar (Pitt 1979). Macroscopic characters were studied on Czapek Yeast Autolysate agar (CYA), Malt extract agar (MEA) and Creatine sucrose agar (CREA), after 7 days of incubation at 25 °C. Additionally, isolates' growth was tested on CYA at 5 °C and 37 °C. Two perpendicular diameters were measured for each colony (6 per isolate), and the average was calculated. The overall culture appearance was assessed (growth, texture, obverse and reverse colony colours, sporulation density, exudate presence) and microscopic features, as described in Visagie et al. (2014). Quick Photo Camera software (version 3.0) was used to measure conidial length and width ($n = 100$).

DNA extraction, polymerase chain reaction (PCR) and sequencing. Fungal DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Four genetic regions [ITS, β -tubulin (*BenA*), calmodulin (*CaM*), and RNA polymerase II second-largest subunit (*RPB2*)] were amplified and sequenced according to previously described methods (Visagie et al. 2014; Stošić et al. 2020). Sequencing was performed in Macrogen Europe (Amsterdam, the Netherlands). The quality of the recovered sequences was assessed using FinchTV software (version 1.4.0), and consensus sequences were generated in MEGA (version 7) (Kumar et al. 2016) using the ClustalW algorithm (Thompson et al. 1994). Comparison was done with the previously deposited GenBank sequences using BLASTn search, and the final version of our sequences

was then submitted to the GenBank database (Accession numbers ITS: PX136356-PX136358; *BenA*: PX206557-PX206559; *CaM*: PX206560-PX206562; *RPB2*: PX206563-PX206565).

Phylogeny. The maximum likelihood (ML) method was used to analyse the aligned data of ITS, *BenA*, *CaM* and *RPB2* in MEGA (version 7). Sequences from this work were added to the reliable, previously published sequences in GenBank to examine their phylogenetic relationships within the *Penicillium* genus. The dataset for each gene was aligned using the ClustalW algorithm (Thompson et al. 1994) in the MEGA (version 7) software package (Kumar et al. 2016). Node confidence was evaluated across 1 000 bootstrap replicates, and values below 70% were disregarded. The sequences of *Aspergillus niger* (isolate NRRL 326) served as an outgroup. The recovered phylogenetic tree was edited and prepared for publication using Adobe Illustrator CS6.

Pathogenicity test. The rot-causing potential of the isolated species was tested on healthy onion bulbs. Bulbs were surface sterilised with 70% ethanol, air dried and wounded with a sterile needle. Conidial suspensions were made by adding one fungal disk ($\varnothing$ 5 mm) from 7-day-old MEA cultures in Eppendorf tubes containing 1 mL of sterile distilled water. Tubes were briefly vortexed, and conidial concentration was adjusted using a Neubauer hemocytometer. Fifty microliters of conidial suspension (1×10^6 spores/mL) was injected into the wound. Control bulbs were inoculated with the same volume of sterile distilled water. The bulbs were incubated in plastic boxes at 25 °C and 95% relative air humidity. After 7 days, the lesions were measured, and reinoculations were performed to verify the fulfilment of Koch's postulates.

All assays mentioned were carried out in three replicates per isolate, and the average diameter and standard deviation (SD) were calculated for each colony, spore, and lesion diameter.

RESULTS AND DISCUSSION

Typical blue mould symptoms were observed on the collected onion bulbs – soft, discoloured, slightly sunken, and watery outer tissue; the presence of white mycelia and masses of blue spores. Twelve isolates were obtained: 3 from producers located around Smederevo (CLP/1, CLP/2, CLP/8),

4 from Pančevo (CLP/9-CLP/12) and 5 from Kraljevo (CLP/13-CLP/17). The isolates were characterised at the macro- and micromorphological levels. They exhibited moderately fast growth on CYA, fast growth on MEA and weak growth on CREA at 25 °C (36.00 ± 1.14 mm, 36.17 ± 3.23 mm, 28.50 ± 3.49 mm, respectively). Growth dimensions on CREA suggest that the colonies are moderately developed, but the growth was assessed as weak because the mycelia were sparse. The common features of the first two media were also the white mycelia visible at the edges of the cultures and intensive sporulation with spores of a light blue-green colour. Cultures obverse were compact on CYA, velvety, with light radial sulcation (visible in the centre, Figure 1A). On MEA, fungi had compact colonies with granular to corneal texture on the obverse (Figure 1B). Weak growth on CREA was followed by the absence of acid production (no change of medium colour from purple to yellow, Figure 1C). On the microscope, conidiophores were terverticillate, with short, rough-walled stipes. Metulae and phialides were cylindrical, and

spores were (sub)globose, smooth-walled and conidial diameters were $2.50\text{--}(3.50)\text{--}4.00$ $\mu\text{m} \times 2.75\text{--}(3.75)\text{--}4.25$ μm . The isolates showed weak growth at 5 °C, with an average colony diameter of 4.17 mm, and no growth was observed at 37 °C. All observed morpho-physiological traits of the isolates corresponded to previous descriptions of *Penicillium allii* (Frisvad & Samson 2004; Samson et al. 2010).

All tested isolates exhibited similar morphology; therefore, three of them (CLP/2, CLP/8, CLP/9) were chosen for molecular and phylogenetic analyses. Multilocus sequence typing revealed that the isolates belong to *P. allii*, confirming the results of morphological identification. A phylogenetic tree was constructed using the best-fit substitution model (Tamura-Nei with gamma distribution, TN93+G), based on sequences from 4 loci. Isolates of *P. allii* from this study clustered with other *P. allii* isolates from the USA, South Africa, and Serbia, with very high node support (Figure 2).

In the pathogenicity test, the isolates caused typical discolouration and slight indentation of onion

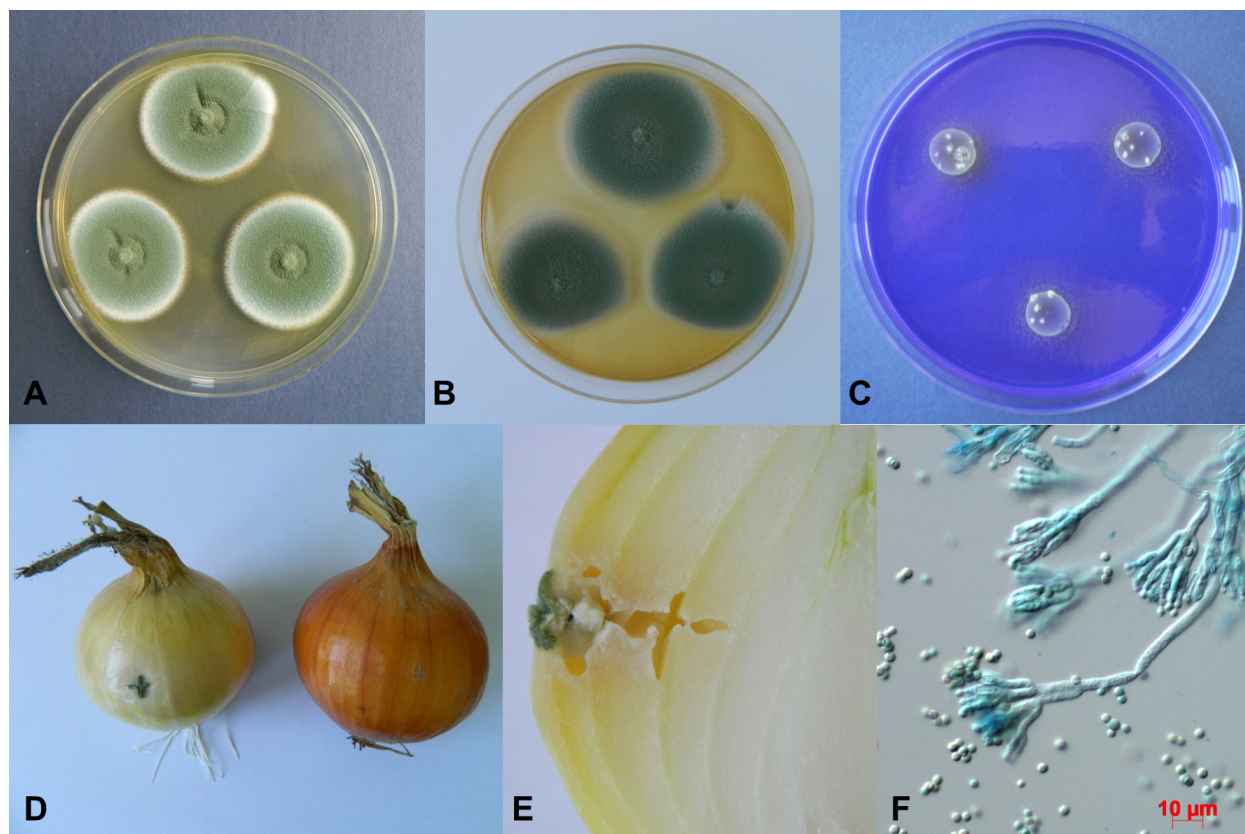


Figure 1. *Penicillium allii* (isolate CLP/2)

(A–C) morphological characteristics after 7 days of incubation at 25 °C on CYA, MEA, CREA, respectively; (D) pathogenicity test on onion bulbs, left – bulb inoculated with the pathogen, right – control; (E) cross-section of the infected bulb; (F) conidiophores and conidia; bar = 10 μm

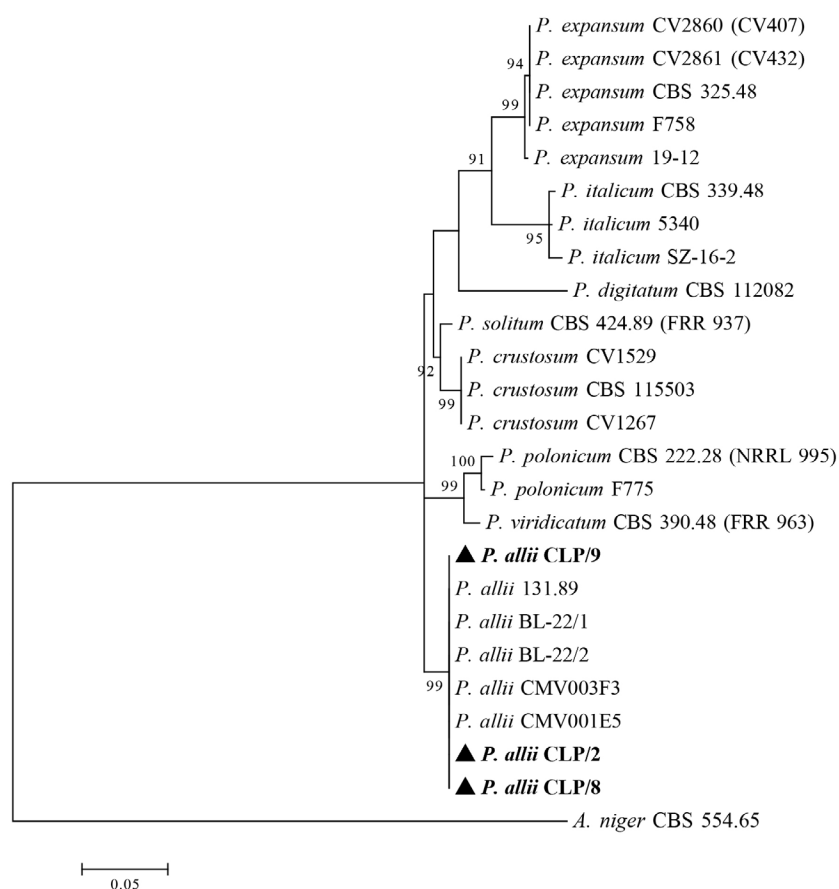


Figure 2. Multilocus consensus phylogenetic tree based on maximum-likelihood analysis of the combined ITS, *BenA*, *CaM* and *RPB2* sequences of *Penicillium* species; bootstrap values higher than 70% are displayed. Isolate of *Aspergillus niger* (CBS 554.65) represented an outgroup sequence; the isolates from this research are denoted in bold font and with a black triangle

bulb tissues, followed by the development of green conidia (Figure 1D). The average lesion diameter across all isolates was 6.00 mm (SD = 1.00 mm). Vertical cross-sections demonstrated the softening of the inner leaves of the bulbs (Figure 1E). The phenotypic appearance of the reisolates obtained from the artificially inoculated bulbs corresponded to that of the originally obtained cultures, thus confirming Koch's postulates. Control bulbs did not show any disease symptoms.

This study represents the first report of *P. allii* as a postharvest pathogen of onion bulbs in Serbia. The isolates were identified using a combination of conventional and molecular methods. In previous research by other authors, this fungus has been confirmed as the pathogen of onion, but the isolates originated from garlic bulbs (Overly et al. 2005; Dugan et al. 2014). *P. allii* was originally described as a new species by isolating it from mouldy garlic bulbs (Vincent & Pitt 1989). It was previously detected on this host in Argentina, Spain and Egypt (Valdez et al. 2006; Moharam et al. 2013; Gálvez & Palmero 2021). Some authors state that this species is the main cause of garlic losses in storage (Dugan et al. 2011).

To the best of our knowledge, our study is the first in the world in which *P. allii* isolates were obtained specifically from onion bulbs and subsequently confirmed as pathogens of that plant host. The other previously detected pathogens of stored onion bulbs in Serbia were *P. expansum*, *P. polonicum* and *P. glabrum* (Duduk et al. 2014, 2017). *P. allii* is a proven producer of mycotoxin roquefortin C, which exhibits neurotoxic properties (Frisvad et al. 2004; Otero et al. 2020). Monitoring and controlling its presence on stored onions is crucial to avoid harmful health effects and significant economic losses in onion cultivation and production.

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