BIOLOGICA NYSSANA
16 (1) June 2025: 129-135
DOI: 10.46793/BiolNyss.16.1.9V

Original Article

Fusarium oxysporum, the cause of *Fusarium* wilt on cucumber (*Cucumis sativus* L.) in Serbia

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Received: *November 13, 2024*
Revised: *February 28, 2025*
Accepted: *March 06, 2025*

Abstract:

Cucumber (*Cucumis sativus* L.) is a vegetable crop with a global production of approximately 87 million tons in 2021, according to FAO data. It is also an important vegetable species cultivated in both greenhouses and open fields in Serbia. One of the most devastating diseases affecting cucumbers is *Fusarium* wilt, caused by *Fusarium oxysporum*. Infected older plants may exhibit yellowing starting from the oldest leaves, stunted growth, or wilting. Affected plants can develop lesions on the lower stem. The fungus infects the vascular system, and symptoms may remain unnoticed until the plant begins to bear fruit. This paper presents the results of a preliminary study on *Fusarium* wilt in cucumbers in Serbia. Samples were collected in 2022 from the Trebotin locality, Kruševac. A total of 15 samples from *Mirabelle F1* hybrid cucumber plants were analyzed. Our research has shown that, based on morphological, molecular, and pathogenic characteristics, cucumber wilting in Serbia is caused by *F. oxysporum*. The morphological characteristics of the isolates (appearance, size, and septation of macro- and microconidia) were as follows: microconidia dimensions ranged from 3.8–15.5 µm×2.8–4.9 µm, macroconidia from 14.6–37.9 µm×2.4–5.6 µm, with the number of septa ranging from 1 to 4. PCR analysis using *F. oxysporum*-specific primers resulted in the amplification of the expected 315 bp fragment in all tested isolates. The pathogenicity test showed that symptoms of *Fusarium* wilt initially appeared as yellowing of the oldest leaves. The disease progressed, eventually leading to plant wilting and death. The characterization and confirmation of *F. oxysporum* on cucumber in Serbia is a significant finding, emphasizing the need for an effective disease management program to reduce yield losses.

Key words:
cucumber, *Fusarium oxysporum*

Apstrakt:

***Fusarium oxysporum* – prouzrokovač fuzarioznog uvenuća krastavca (*Cucumis sativus* L.) u Srbiji**

Krastavac (*Cucumis sativus* L.) je povrtarska kultura čija je globalna proizvodnja, prema podacima FAO-a za 2021. godinu, iznosila oko 87 miliona tona. Takođe, predstavlja značajnu povrtarsku vrstu koja se u Srbiji gaji u plastenicima i na otvorenom. Jedna od najrazornijih bolesti krastavca je fuzariozno uvenuće, koje izaziva *Fusarium oxysporum*. Infekcija kod starijih biljaka može izazvati žutilo, koje počinje od najstarijih listova, usporavanje rasta ili uvenuće. Pogođene biljke mogu razviti lezije na donjem delu stabla. Gljiva napada i sprovodne sudove, zbog čega zaražene biljke ne pokazuju simptome sve dok ne počnu da plodonose. U ovom radu predstavljamo rezultate preliminarnih istraživanja fuzarioznog uvenuća krastavca u Srbiji. Uzorci su prikupljeni 2022. godine na lokalitetu Trebotin, Kruševac. Analizirano je ukupno 15 uzoraka hibridnih biljaka krastavca *Mirabelle F1*. Naše istraživanje je pokazalo da, na osnovu morfoloških, molekularnih i patogenih karakteristika, uvenuće krastavca u Srbiji izaziva gljiva *F. oxysporum*. Morfološke karakteristike izolata (izgled, veličina i septacija makro- i mikrokonidija) bile su: dimenzije mikrokonidija 3,8–15,5×2,8–4,9 µm, makrokonidija 14,6–37,9×2,4–5,6 µm, dok je broj septi varirao od 1 do 4. PCR analiza sa *F. oxysporum*-specifičnim prajmerima rezultirala je amplifikacijom očekivanog fragmenta od 315 bp u svim testiranim izolatima. Test patogenosti pokazao je da su se simptomi fuzarioznog uvenuća najpre ispoljili kao žutilo starih listova. Bolest je napredovala, a zaražene biljke su venule i na kraju uginule. Karakterizacija i potvrda *F. oxysporum* na krastavcu u Srbiji predstavlja značajan nalaz za razvoj efikasnijih strategija upravljanja ovom bolešću kako bi se smanjili gubici prinosa.

Ključne reči:
krastavac, *Fusarium oxysporum*

Introduction

Cucumber (*Cucumis sativus* L.) is a herbaceous plant belonging to the Cucurbitaceae family. It is widely used as a vegetable in human diets and is considered an annual plant. Native to South Asia, cucumber is now cultivated across most continents (Sharma & Shukla, 2001; Akrami, 2015; Hazirah et al., 2020). In Serbia, cucumber is an important crop grown both in open fields and greenhouses. Due to the limited availability of agricultural land and high market demand, continuous cucumber cultivation is widely practiced.

A large number of pathogenic organisms parasitize cucumber plants, causing significant economic damage. The most important pathogens affecting cucumber include bacteria, viruses, and fungi. The major bacterial pathogens are *Pseudomonas syringae* (angular leaf spot), *Xanthomonas campestris* (bacterial leaf spot), *Erwinia tracheiphila* (bacterial wilt) (Kaiser & Ernst, 2017). Among viral pathogens, Cucumber green mottle mosaic virus (CGMMV), Squash mosaic virus (SqMV), and Watermelon mosaic virus (WMV) are the most significant. Phytoplasma aster yellows also causes severe economic losses in cucumber production (Kaiser & Ernst, 2017).

However, one of the main constraints in cucumber production, leading to yield reduction and quality deterioration, are phytopathogenic fungi. The most important fungal diseases affecting cucumbers include *Pseudoperonospora cubensis* (downy mildew), *Erysiphe cichoracearum* (powdery mildew), *Botrytis cinerea* (gray mold), *Phomopsis sclerotioides* (root rot), *Sclerotinia sclerotiorum* (white mold), *Didymella bryoniae* (gummy stem blight and black rot), *Colletotrichum orbiculare* (anthracnose), and *Fusarium oxysporum* (*Fusarium* wilt). *Fusarium oxysporum* is a soil-borne fungal pathogen that causes *Fusarium* wilt in cucumbers, leading to significant yield losses worldwide. This disease affects the plant's vascular system, disrupting the transport of water and nutrients, ultimately resulting in wilting and plant death (Sharma & Shukla, 2001; Al-Tuwaijri, 2015). *Fusarium oxysporum* is considered one of the most problematic and destructive pathogens in cucumber production (Ye et al., 2004). It is transmitted through soil and water (Sharma & Shukla, 2001; Shen et al., 2008; Al-Tuwaijri, 2015). Cucumber wilt is widespread across many countries, with yield losses ranging from 40% to 70%. Characteristic symptoms include plant weakening before and after the first visible signs of infection, stunted growth, chlorosis, necrosis, and eventual wilting of older leaves and entire plants. Symptom development is also accompanied by vascular tissue discoloration and plant death (Vakalounakis & Fragkiadakis, 1999; Akrami, 2015; Wang et al., 2015; Al-Tuwaijri, 2015; Hazirah et al., 2020; Maymon et al., 2020; Aparicio et al., 2023).

Given the increasing importance of cucumber cultivation in Serbia, this research aimed to identify the causal agent of *Fusarium* wilt in cucumbers to improve understanding of its impact (plant death, yield reduction, and quality deterioration) and to develop more effective disease management strategies.

Materials and Methods

The samples of cucumber plants were collected in the village of Trebotin (near Kruševac) on the Terzić family's estate during the summer vegetation period of 2022. During sample collection, cucumber plants (*Mirabelle F1* hybrid) exhibiting symptoms of wilting, chlorosis of the oldest leaves, and vascular necrosis were taken (**Fig. 1**). A total of 15 samples were collected.

Stem and root segments were washed under running water for 10 minutes and then cut into 1 cm long fragments. These fragments were surface disinfected by immersion in a 5% sodium hypochlorite (NaOCl) solution for five minutes and then rinsed three times in sterile distilled water, each time for five minutes.

Potato dextrose agar (PDA) enriched with 300 µl/L gentamicin sulfate was used for pathogen isolation (Dhingra & Sinclair, 1995). On sterile media in Petri dishes, cucumber plant fragments were aseptically placed, with five fragments per Petri dish. The inoculated Petri dishes were incubated in a thermostat at 24±2 °C in the dark until fungal colonies developed around the fragments. The mycelium completely covered the Petri dish within 7 days.

A total of 15 isolates were obtained, and five were selected for further research: FOS1, FOK2, FOK3, FOM6, and FOT1.

Morphological features

Morphological characteristics of selected isolates of *Fusarium* spp. were studied on a PDA substrate according to the method of Burgess et al. (1994). Dimensions of microscopic structures were calculated based on 30 measurements for macroconidia, microconidia and chlamydospores morphology (shape, color), size (length and width) was based on the descriptions indicated by Nelson et al. (1983). Individual germinating conidia were selected, transferred directly to PDA plates according to the procedures described by Burgess

Fig. 1. Symptoms of *Fusarium* wilt on cucumber in the field

et al. (1994) and stored on PDA in tubes at 4 °C. Colony morphology (color, shape and growth rate) was determined after 7 days of incubation on PDA at 25 °C in darkness. Images were captured by an Olympus SC100 color camera mounted on a CX31 microscope (Olympus, Japan).

Pathogenic characteristics

In order to determine the pathogenic characteristics of selected isolates obtained from infected cucumber plants, a pathogenicity test was performed by inoculating injured cucumber plants (*Mirabelle F1* hybrid) (Vasić, 2007). Four-week-old cucumber seedlings were used. Before inoculation, all plants were wounded at the base of the stem with a sterile scalpel. In this way, the injured plants were inoculated by placing small fragments of the colony of studied isolates in the wounds. Inoculation was performed with isolates of the obtained fungi, 7 days old, grown on PDA medium. Plants inoculated in this way were stored at a temperature of 20-25 °C. The experiment was set up in two replicates with 5 plants per isolate. Cucumber plants that were injured in the same way served as controls, after which they were inoculated with small fragments of the substrate without mycelia. The appearance of symptoms was monitored three, five and ten days after inoculation.

Molecular detection and identification

Colonies of tested isolates were grown on PDA in the dark, at a temperature of 25 °C for 7 days. DNA extraction was performed according to Day & Shattock (1997). In the first step, the mycelium was scraped from the surface of the substrate with a sterile spatula and placed in a 2.0 ml tube with liquid nitrogen. After evaporation of liquid nitrogen, 800 µl of 2% CTAB buffer was poured into the tube and incubated for 1 h at 65 °C. During the incubation, every 15 min the contents of the tube were vigorously shaken.

After incubation, 800 µl of chloroform was added into the microtube and vortexed, then centrifuged for 10 min at 11,000 rpm in a centrifuge at 4 °C (Eppendorf 5804 R, Germany). The supernatant (about 700 µl) was transferred by pipetting into a new 1.5 ml tube, and 0.6 volume (about 420 µl) of isopropanol was added. The mixture was centrifuged for 15 min at 11,000 rpm at 4 °C. After centrifugation, the supernatant was carefully decanted and the tubes were washed with 1 ml of ice-cold 70% ethanol and dried for 10-15 min at room temperature. After drying, the DNA pellet was resuspended in 100 µl of TE buffer.

For the detection of five tested *Fusarium* spp. isolates a PCR method with a *F. oxysporum* specific primers primer pairs Fc-1 (5'-CATACCACTT-GTTGCCTC-3') and Fc-2 (5'-ATTAACGC-GAGTCCCACC3-') was performed (Zhang et al., 2010). PCR analysis was performed in a T-personal thermocycler (Biometra, Germany) in 20 µl reaction volume containing 1 µl of DNA, 2 µl of 10× reaction buffer, 2 µl 2.5 mM dNTPs, 1.2 µl 25 mM of $MgCl_2$, 0.2 µl 10 mM of Fc-1 and Fc-2 primer, and 0.2 µl 5U of Taq DNA polymerase (ThermoScientific, USA) and 13.2 µl nuclease-free water. A negative control contained all PCR reaction compounds except DNA template. PCR cycling conditions are as follows: initial denaturation at 94 °C for 5 min, followed by 40 cycles with denaturation for 30 s at 94 °C, annealing for 1 min at 60 °C and extension for 60 s at 72 °C, with final extension step at 72 °C for 10 min.

PCR products were analyzed in 1.5% agarose gel that was stained with ethidium bromide. PCR fragments were visualized in the Gel Doc EZ System (Biorad, USA) using a UV tray.

Results and discussion

During the vegetation period of 2022, serious symptoms of wilting were observed in cucumber plantations in the village of Trebotin (near Kruševac). Initial symptoms in the form of yellowing were observed on older leaves, and lesions in the lower part of the tree were also observed in infected plants. On the cross-section of the stems of infected plants, necrosis of the vascular tissues was observed, which spread to the roots. Infections in cucumber plants caused stunting and most often wilting.

Morphological characteristics

Morphological characteristics of selected isolates of *Fusarium* spp. were studied on PDA media with 7-day-old fungal cultures. Colony growth rate of five studied isolates of *Fusarium* spp. on the PDA substrate did not differ; on average, for all five isolates, the increase was 5.5 cm after three days. The mycelium completely covers the Petri dish

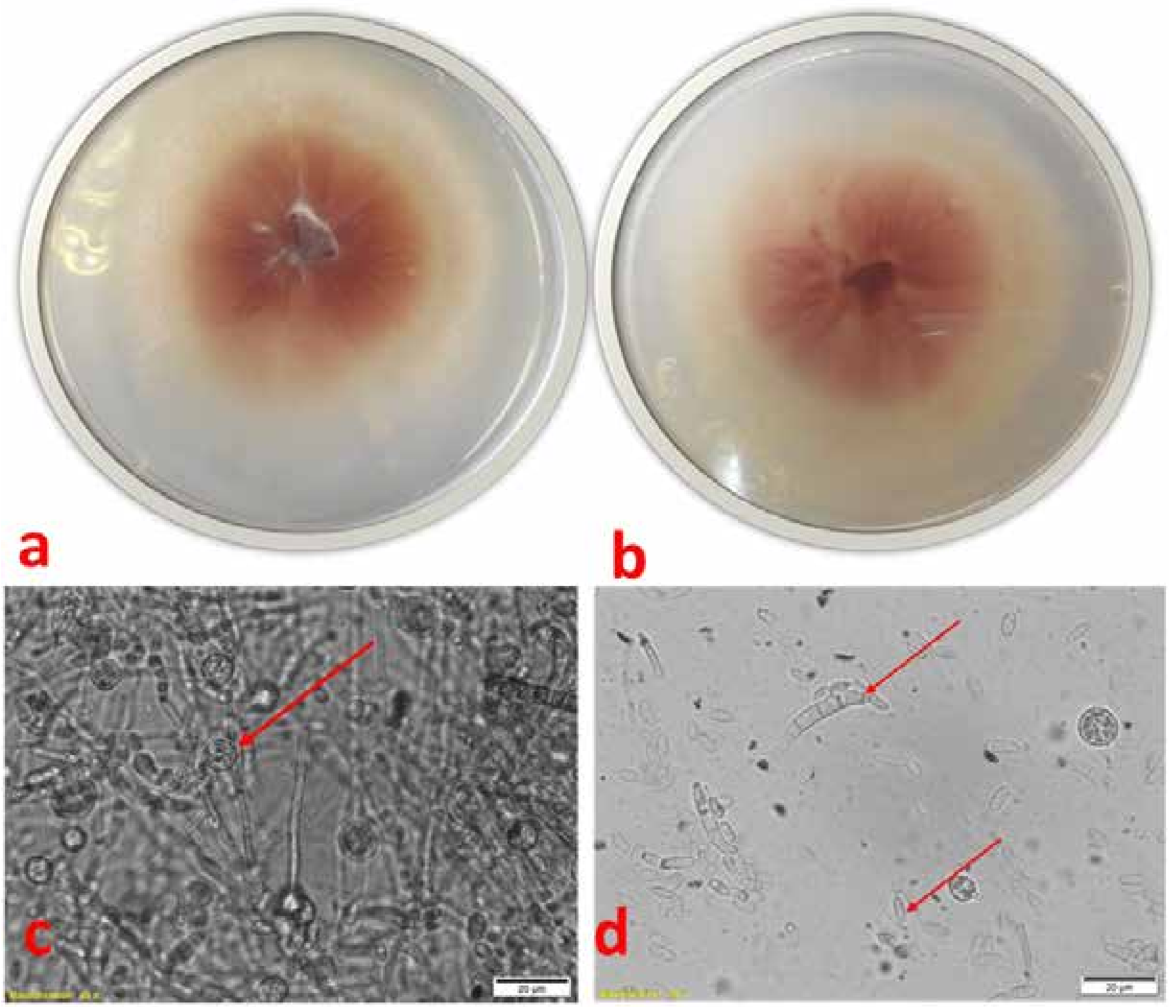

Fig. 2. Appearance of 7-day-old cultures on PDA medium, *F. oxysporum*, isolate FOK2, face (**a**), reverse (**b**), chlamydospores (**c**), microconidia and macroconidia (**d**). Scale bar 20 µm.

within 5–7 days. The color of the colonies varied from creamy whitish to white to light salmon. Some isolates produced salmon-colored pigments in the colonies after 15 to 20 days of incubation at 25 °C on PDA medium. All studied isolates formed macroconidia, microconidia and chlamydospores on the PDA medium (**Fig. 2**). Macroconidia (2.4–5.6 x 14.6–37.9 µm) are tapered and curved to almost straight with a slight hook, thin-walled with usually three septa (1–4) and are produced from monophialides. The microconidia (2.8–4.9 x 3.8–15.5 µm) are oval or elliptical , usually non-septate and form in false heads from short monophialides. Chlamydospores (8-14 µm), both smooth and rough, thick-walled, are produced intercalary or terminal in the hyphae and form abundantly in most isolates, usually in pairs or singular (**Fig. 2**).

Morphological determination of phytopathogenic fungi, in this case *Fusarium* spp., plays a very important role in the early stage of disease detection. This approach is considered more cost-effective but requires trained personnel. Therefore, the morphological identification of phytopathogenic fungi is the primary approach and the most challenging step in the identification process, especially for species of the *Fusarium* genus (Hazirah et al., 2020).

These studies showed that, based on morphological characteristics, the fungi isolated from infected cucumber plants belong to the genus *Fusarium*, more precisely to the species *F. oxysporum*, and are in accordance with the findings published by Nelson et al. (1983), Burgess et al. (1994), and Leslie & Summerell (2006). Vakalounakis & Fragkiadakis (1999), Wang et al. (2015), and Hazirah et al. (2020) determined that the pigmentation of *F. oxysporum* on PDA ranged from white to light salmon. The microconidia had conical apical cells and basal cell-shaped feet, and they were oval and kidney-shaped without septa, which was confirmed in our research.

Pathogenic characteristics

On the fifth day after inoculation, all tested isolates of *Fusarium* spp. exhibited typical symptoms, including wilting of the oldest leaves. Lesions were also observed on the infected plants in the lower part of the stem. With the passage of time, the symptoms on the infected plants were more intense and brown necrosis was observed on the vascular tissue. No changes were observed in the control plants. In their research, Vakalounakis and Fragkiadakis (1999), Akrami (2015), Wang et al. (2015), Al-Tuwaijri (2015), Hazirah et al. (2020), Maymon et al. (2020), and Aparicio et al. (2023) reported that various *F. oxysporum* formae on artificially inoculated cucumber plants caused symptoms such as chlorosis of the oldest leaves, wilting, and necrosis of vascular tissue, which is consistent with our findings. This confirms that our isolates belong to the species *F. oxysporum*.

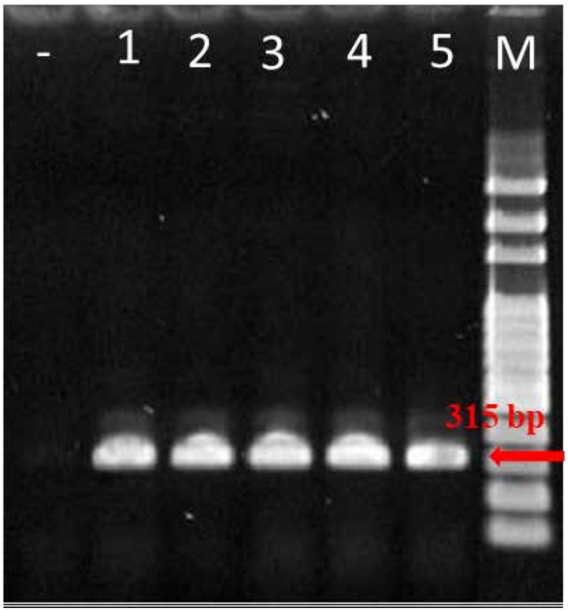

Fig. 3. Electrophoretic analysis of PCR with Fc-1/Fc-2 primer pair. Lines: - negative control, **1** - FOS1 isolate, **2** - FOK2 isolate, **3** - FOK3 isolate, **4** - FOM6 isolate, **5** - FOT1 isolate, **M** - marker 100 bp ladder (Solis BioDyne, Estonia)

Molecular detection and identification of fungi

In PCR analysis with Fc-1/Fc-2 primer pair a fragment of 315 bp was obtained for all five tested isolates from cucumber (**Fig. 3**). No amplification occurred in the negative control.

Fc-1/Fc-2 primer designed by Zhang et al. (2010) proved to be reliable for the detection of *F. oxysporum* isolates from cucumber in Serbia. This primer pair was successfully applied for specific detection of *F. oxysporum* in different hosts. The specificity of the primers was confirmed by analyzing *F. oxysporum* isolates and 33 isolates of other fungal species infecting cucumbers. The primers successfully detected *F. oxysporum* isolates from cucumber, water melon and melon (Zhang et al., 2010). Also, using this primer pair *F. oxysporum* can be reliably detected from DNA extracted from rhizosphere soil (Zhang et al., 2012).

Conclusion

Based on morphological, pathogenic, and molecular characteristics, it has been determined that the fungus isolated from cucumber belongs to the species *Fusarium oxysporum*, which is becoming an increasing problem in commercial production and may threaten cucumber cultivation in Serbia.

In our country, these are preliminary results that highlight the significance of this issue, which is why further research will continue. The confirmed presence of *F. oxysporum* in cucumbers emphasizes the need for greater attention in selecting seed material. This includes the use of cucumber hybrids resistant to *Fusarium*, avoiding continuous planting of cucumbers on the same field for multiple growing seasons, applying beneficial microorganisms such as *Trichoderma* spp. to suppress fungal growth, ensuring proper irrigation, preventing plant injuries, and using disinfection agents.

Acknowledgements. This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, contracts 451-03-137/2025-03/200383 and 451-03-136/2025-03/200215.

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