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ORIGINAL SCIENTIFIC ARTICLE

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# FIRST REPORT OF *Fusarium tricinctum* ON NARROW-LEAVED ASH (*Fraxinus angustifolia* Vahl.) IN SERBIA

SUMMARY: Monitoring the health status of narrow-leaved ash tree seedlings (forest office Morović, locality Vinična) in the early spring of 2015, after the catastrophic floods in May 2014, revealed presence of large dark necrotic areas on 1–2 year old sprouts. The isolation of the fungal pathogen was done by standard phytopathological protocols. Three representative isolates (K41, K42 and K78) were preliminary detected and purified by a single-spore technique for further morphological, molecular analyses and pathogenicity testing. Morphological characteristics classified the isolates as *Fusarium tricinctum.* Tested isolates on narrow leaved ash sprouts caused reddish brown elongated necrotic lesions averaged 20.1 mm. Two marker genes, translation elongation factor 1-alpha (*TEF1-α*) and internal transcribed spacer (*ITS1*), were used in this study. Using the Basic Local Alignment Search Tool (BLAST) searching engine, nucleotide sequences were compared to all related sequences. Alignement score resulted in 98.9% identities with *F. tricinctum* for isolate K78, while isolates K41 and K42 showed 94.1% and 94.3% identities with *F. tricinctum* complex respectively. To the best of our knowledge, this is the first report of *F. tricinctum* pathogen infection on flood stressed narrow-leaved ash trees in Serbia.
KEYWORDS: sprout necrosis, plain forests, floods

## INTRODUCTION

Narrow-leaved ash (*Fraxinus angustifolia* Vahl) is one of the most important tree species of lowland floodplain forests in Europe with great ecological and economic importance, due to its rapid development and valuable wood

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(Drvodelić et al., 2016). In Central Europe, the Pannonian Basin and Balkans, narrow-leaved ash occurs mainly in the lowlands, in riparian and floodplain forests along large rivers, where it used to form vast and continuous populations, now with more limited extent. Narrow-leaved ash forests, in the area (southwest part of Srem, Vojvodina, Serbia) of the river Sava lower course, are the most valuable and the best preserved forests of this species in Serbia (Bobinac et al., 2010). They form pure and mixed forests (mainly with common oak), which are conditioned by additional moisture – flood or high levels of underground water (Cvijetićanin et al., 2014).

Over recent years, young plantations, tree seedlings and stands of narrow-leaved ash are endangered by the fungus *Hymenoscyphus fraxineus* (Marković et al., 2016). This fungus is named as a main factor of narrow-leaved ash decline in neighbouring countries (Milotić et al., 2016) and throughout Europe (Kowalski and Holdenrieder, 2009). Furthermore, Kranjec et al. (2017) reported about the possible role of *Pythium* and *Phytopythium* soil borne species in declining of narrow-leaved ash forests in Croatia.

However, forest stands decline is caused by synergy of biotic and abiotic factors. Air pollution, flooding in the vegetation period and the absence of regular winter and spring floods, coupled with consecutive dry periods, are crucial stress factors that exert an adverse impact on narrow-leaved ash forests (Tikvić et al., 2008).

The region of Southwest Srem was exposed to the catastrophic floods by the Sava River during the May 2014. Next year, in the early spring, narrow-leaved ash tree seedlings (aged 4-7 years) were examined in forest office Morović at the locality Viničn a (sect. 11 and 16) in a course of routine monitoring of their health status. Beside the necrotic lesions typical for *H. fraxineus,* large dark necrotic areas with bark lesions were observed on some one and two year old sprouts. The aim of this study was to identify the causal agent of these lesions and to test its pathogenicity.

## MATERIAL AND METHOD

### Isolation of fungi and morphological characterization

Symptomatic branches were collected and ten cuttings of belonging tissue were surface disinfected with 2% sodium hypochlorite solution for 5 min, rinsed three times in sterile distilled water, air dried on sterilized filter paper and plated on potato dextrose agar (PDA) and water agar (WA) amended with streptomycin sulphate. After seven days incubation in the dark at 25 °C, three representative isolates (K41, K42 and K78) were chosen for further analyses and purified by a single-spore technique (Leslie and Summerell, 2006). Colony characteristics of single spore isolates were evaluated on PDA media 14 days after the incubation in the dark at 25 °C. Shape and size of microconidia; size and septation of macroconidia and presence of chlamydospores were observed in two weeks on isolates grown on WA media under the black light. Macroconidia

and microconidia were photographed and measured in a light microscope (DM 1000 LED, equipped with a camera MC190HD and LAS V4.9-imaginig and analysis software, Leica Germany). Per each isolate 100 macroconidia and 50 microconidia were measured and standard deviation was calculated using using software *STATISTICA*, ver. 13.2 (Dell, Inc., USA).

## Molecular identification

DNA from the seven-day-old *F. tricinctum* isolates (K41, K42 and K78) used for inoculation as well was extracted using the cetyltrimethylammonium bromide (CTAB) protocol (Permingeat et al., 1998). To confirm morphological characterization of the isolates, several fragments were amplified and sequenced.

IGS rDNA region for the K41 and K42 isolates was amplified using species specific primer pair tri1 (5' CGT GTC CCT CTG TAC AGC TTT GA 3') and tri2 (5' GTG GTT ACC TCC CGA TAC TCT A 3') Kulik (2008). The PCR was carried out in 25 µl volumes containing 40 ng DNA, 1x TaqBuffer (containing KCl, Thermo-Fisher Scientific), 0.5 µM each of forward and reverse primers, 0.2 mM of each nucleotide, 2 mM of $MgCl_2$ and 1.25 units of TaqDNA Polymerase (Thermo-Fisher Scientific). The amplification protocol was as follows: initial denaturation for 5 min at 94 ºC, followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 65 ºC for 30 s and elongation at 72 ºC for 20 s, followed by a final elongation step at 72 ºC for 5 min.

The internal transcribed spacer (ITS) region amplified and sequenced with primers ITS1 (5'CTT GGT CAT TTA GAG GAA GTA A3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC3') (White et al., 1990). The PCR was carried out in 25 µl volumes containing 30 ng DNA, 1x TaqBuffer (Thermo-Fisher Scientific), 0.2 µM each of forward and reverse primers, 0.2 mM of each nucleotide, 1.5 mM of $MgCl_2$ and 1.20 units of TaqDNA Polymerase (Thermo-Fisher Scientific). Amplification protocol was following: initial denaturation for 5 min at 97 °C, followed by 37 cycles with 94 °C for 30 s, 60 °C for 60 s and 72 °C for 45 s. Final elongation was at 72 °C for 5 min.

Translation elongation factor 1α (TEF1-α) gen for the isolates K41 and K42 were amplified using EF1728F (5'CAT CGA GAA GTT CGA GAA GG3') and EF1986R (5'TAC TTG AAG GAA CCC TTA CC3') primers (Rehner and Buckley, 2005). The PCR was carried out in 25 µl volumes containing 30 ng DNA, 1x TaqBuffer (Thermo-Fisher Scientific), 0.2 µM each of forward and reverse primers, 0.15 mM of each nucleotide, 1.5 mM of $MgCl_2$ and 1.20 units of TaqDNA Polymerase (Thermo-Fisher Scientific). Amplification was done according to the protocol: initial denaturation for 1 min at 97 °C, followed by 36 cycles with 96 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s. Final elongation was at 72 °C for 2 min.

PCR products were separated on a 1.5% agarose gel in 1x TBE buffer, stained with ethidium bromide, and visualized under UV light. The amplification products were purified with QIAEX II Gel Extraction Kit (Qiagen, USA) and sent for sequencing to Macrogen Inc. (Macrogen Europe B.V., the Netherlands).

### Pathogenicity test

Pathogenicity tests were conducted on one-year-old narrow-leaved ash sprouts, disinfected with 70% ethanol. Mycelial plugs taken from actively growing PDA colonies of *F. tricinctum* isolates were applied in shallow wounds (0.5 cm in diameter) made by scalpel. Sterile agar plugs were placed in the wounds of a control sprouts. Inoculation was performed on four sprouts per isolate and control respectively, with two cuts per sprout. Inoculated cuts were separately wrapped with moist sterile cotton and covered by aluminium foil to prevent drying. The inoculated branches were planted in plastic containers filled with sterile ground in the open field to incubate for three weeks (April, 2017).

### Statistical analysis

The obtained data were analysed using Statistica 13.2 (Dell Inc., USA). The results for pathogenicity were tested by analysis of variance followed by a comparison of means by the Bonferroni test ($P < 0.01$).

## RESULTS AND DISCUSSION

Naturally infected sprouts had two types of symptoms. Elongated-diamond shaped lesions brown with discoloration in the bark typical for *C. fraxinea* (Barić et al., 2012) and dark elongated necrotic almost black areas (Figure 1). From these dark necrotic areas *Fusarium* species were isolated.

*Figure 1.* Symptoms of narrow-leaved ash branches resulting from natural infection

Isolates K 41, K42 and K78 on PDA were growing rapidly and formed abundant dense mycelia that were initially white, but with age became red with yellowish fragments. It also exuded red pigments in the agar (Figure 2).

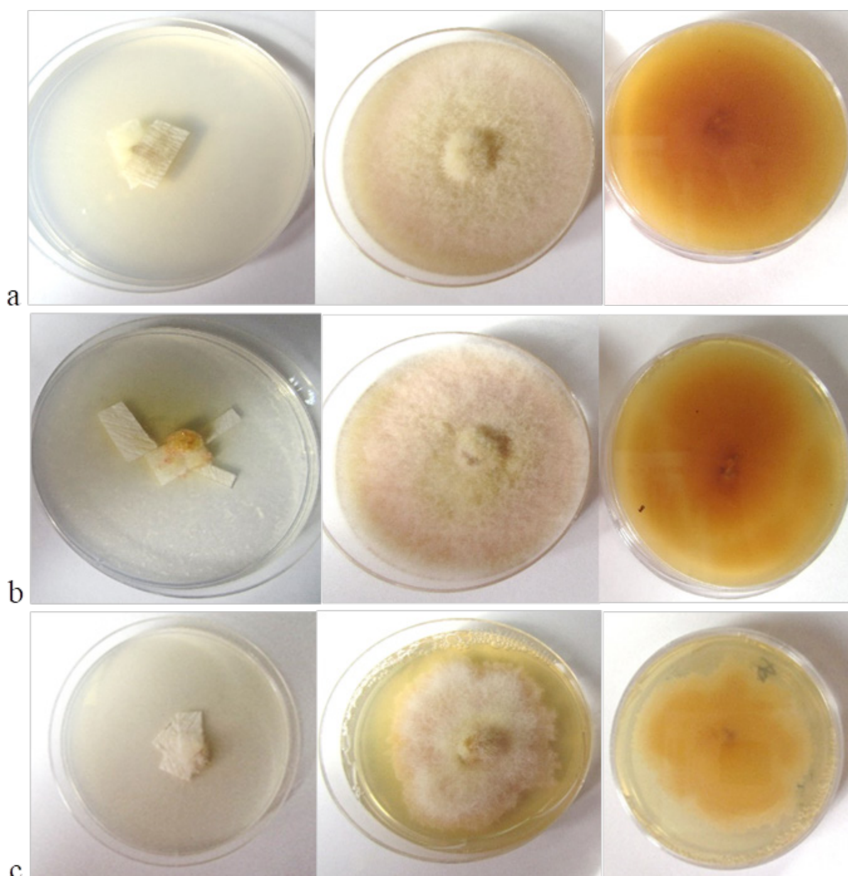



*Figure 2.* Colony morphology of the isolates a) K41, b) K42, c) K78 on WA and PDA media

On WA medium isolate K-78 formed 3–5 septate macroconidia with curved tapering apical cell and an obviously foot-shaped basal cell. Microconidia were oval, usually non-septate, but occasionally with one septa. Microconidia were clustered on monophialides in small heads. Napiform conidia were rare (Figure 3c). Chlamydospores were absent. Isolates K41 and K42 isolates sporulated on WA medium only after two weeks period under black light. Isolate K41 formed orange sporodochia with the clusters of macroconidia. Macroconidia of isolate K41 were the most slender and did not form microconidia. On the other hand, macroconidia of isolate K42 were sickle shaped, elongated, one-septate microconidia (Figure 3 a, b). The dimensions of different types of conidia are given in the Table 1.

*Table 1.* Dimensions of macro and microconidia of tested isolates

| isolate | conidia type | average dimension±SD* (μm) | min-max dimension (μm) |
|---|---|---|---|
| K 41 | macrocinida | 45.81 ± 0.79 x 3.17 ± 0.05 | (23.4–63.7) x (2.2–4.4) |
| K 42 | macrocinida | 52.35 ± 0.89 x 3.36 ± 0.05 | (28.8–69.4) x (2.3–4.9) |
| K 42 | microcinida (1 cell) | 12.5 ± 0.91 x 2.8 ± 0.4 | (11.5–13.7) x (2.3–3,2) |
| K 42 | microcinida (2 cell) | 21.95 ± 4.76 x 3.04 ± 0,29 | (15.0–26.9) x (2.5–3.3) |
| K 78 | macrocinida | 35,24 ± 0,81 x 3,65 ± 0,08 | (21.3–52.6) x (1,8–6.2) |
| K 78 | microcinida (1 cell) | 12.3 ± 2.8 x 2.7 ± 0.7 | (8.5–20.6) x (1.7–5.4) |
| K 78 | microcinida (2 cell) | 16.3 ± 2.7 x 3.0 ± 0.7 | (9.0–21.3) x (2.2–5.4) |

*SD standard deviation

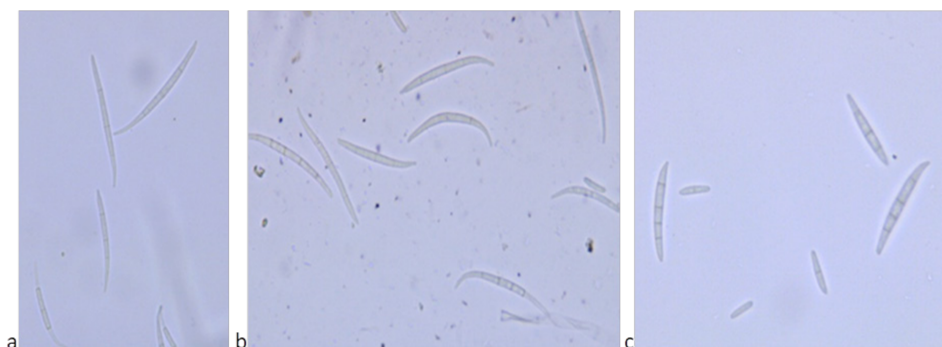



*Figure 3.* Macro and microconidia of *Fusarium* isolates: a) K-41, b) K-42, c) K-78

Observed morphological characteristics indicated that isolates belonged to *F. tricinctum* (Leslie and Summerell, 2006; Lević, 2008) species which was confirmed by molecular tools.

Based on a BLAST search of the FUSARIUM-ID nucleotide database, the IGS sequences for isolates K41 and K42 (submission no. MZ749901 and MZ749902 respectively) matched with sequence FD_01725_EF-1a (*F. tricinctum* complex) 94.1% and 94.3%, respectively. Based on a BLAST search of the NCBI nucleotide database, the ITS sequence of isolate K78 (GenBank MK928426.1) had 100% identity with *F. tricinctum* strain ZMXR6 (MT446111.1). The TEF1-α sequence of the isolate K78 (MN822227.1) had 98.9% identity with *F. tricinctum* isolate SPF003 (MG704914.1). This was the best match of the obtained isolates with those from the GenBank. Although Kulik (2008) reported that primers specific for *F. tricinctum* amplified aplicons of *F. acuminatum* and *F. nurragi*, the specificity of the primers, in this case, cannot be disputed because molecular identification confirmed the morphological findings.

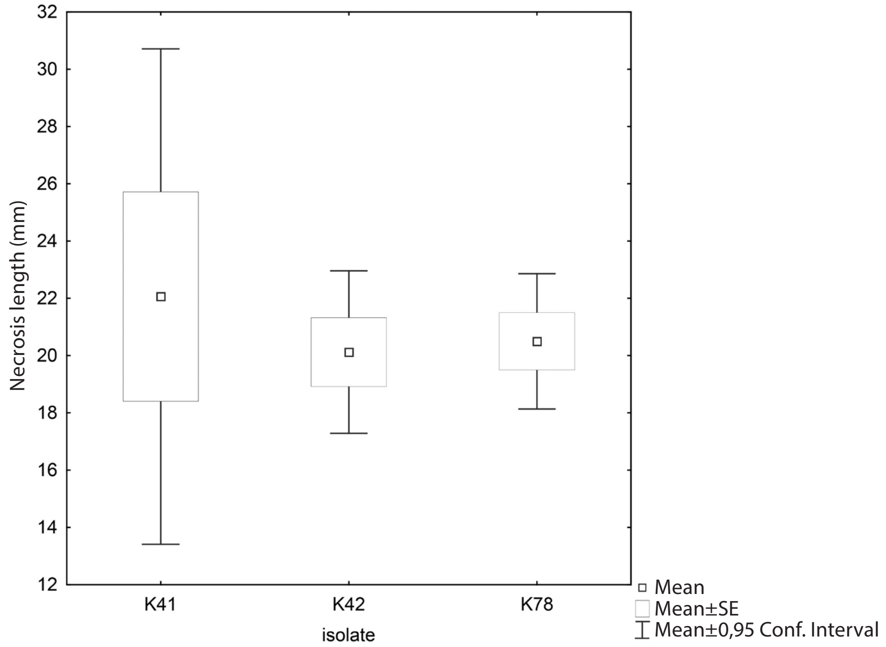
*F. tricinctum* is worldwide distributed species which usually occurs as a saprophyte or a weak parasite in temperate regions (Leslie and Summerel, 2006). However, recent studies confirmed this species as a causal agent of disease in many agricultural plants such as wheat crown rot (Shikur et al., 2018), pink rot of

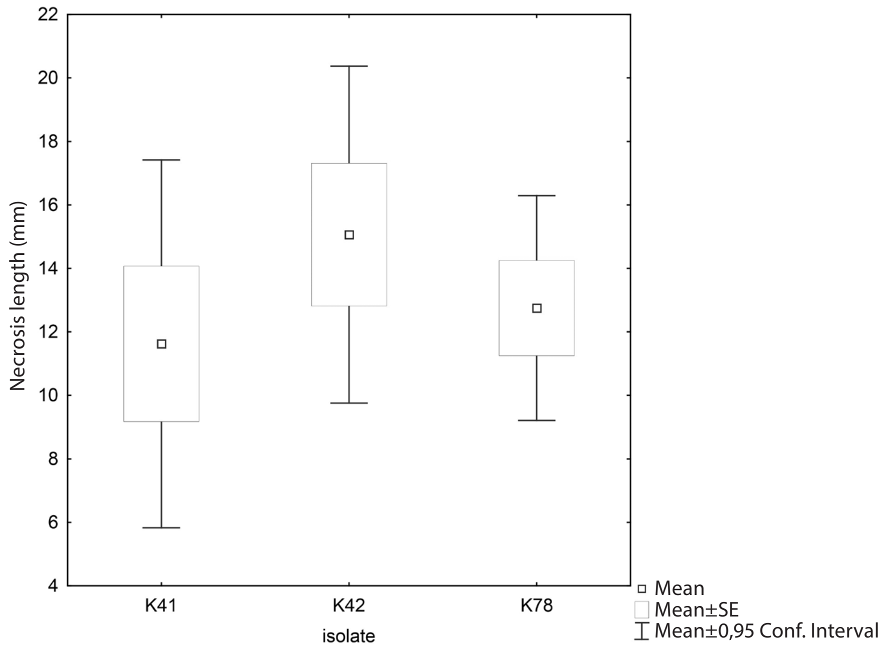
onion bulbs (Carrieri et al., 2013), postharvest fruit rot of pumpkin (Aktaruzzaman et al., 2018), and also causal agents of root rots in soybean (Chitrampalam and Nelson, 2014) and alfalfa (Jiang et al., 2020). Taking in the account woody species *F. tricinctum* proved to be weakly virulent or non-virulent to pine seedlings inhibited root growth, but in the greenhouse tests caused small necrotic wounds, which did not differ significantly in size from those observed in the control seedlings (Davydenko et al., 2018). The pathogenicity test was performed in order to check the capability of the isolates to cause the symptoms in the bark. All tested isolates caused symptoms on narrow-leaved ash sprouts in the form of elongated oval shaped lesions with reddish brown border (Figure 4).

*Figure 4.* Symptoms on sprouts inoculated by *F. tricinctum*: a) K42 isolate, b) K78 isolate

The lesion length and width ranged from 20.1 to 22.1 mm and from 11.6–15.1 mm respectively, but there were no differences in the size of lesions among the isolates. The isolate K-41 showed higher variability in lesion length (Figure 5).

The isolate K-78 caused girdling of an inoculated sprout. Control sprouts inoculated with sterile PDA plug were symptomless. The pathogen was successfully re-isolated from symptomatic sprouts, while no fungi were isolated from control sprouts. Compared to this, the necrosis length on individual one-year-old *F. excelsior* trees of seed origin caused by *Chalara fraxinea* varied from 1.1 to 28.7 cm (on average, 7.2 cm), although about 20% of the trees inoculated with the fungus, necrosis did not exceed 1.5 cm (Bakys et al., 2009a).




$F(4, 40)=0.81608, p=0.52252$

*Figure 5.* Necrosis length and width on narrow-leaved ash sprouts caused by *F. tricinctum* isolates

There are not many data in the literature about fungi from the genus *Fusarium* on *F. angustifolia* and *F. excelsior* – European ash. Although it is known that *Fraxinus* species are currently suffering from ash dieback disease caused by the fungus *Hymenoscyphus fraxineus*, there are co-occurrences of large numbers of other fungi with similar endophytic as well as pathogenity among *Fusarium* species (Ivanova et al., 2020). According to Trapiello et al. (2017), one of the most frequently isolated species from European ash leaves with symptoms such as leaf spots and petiole discoloration were *Fusarium* species. *F. lateritium* did not cause any symptoms on stems of *F. excelsior* seedlings, while *F. solani* induced brown discolouration around the inoculation points on two-year-old plants (Przybył, 2002). *Fusarium* sp. was isolated in minor percent in *F. excelsior* petioles and symptomatic shoots (Davydenko et al., 2013). Kranjec-Orlović et al. (2019) among the other species on mycobionta of narrow- leaved ash seed isolated *Fusarium oxysporum*. *Fusarium lateritium* and *F. solani* were isolated from the root and stem base of *F. angustifolia,* whereas the connection to the symptoms of trees crown defoliation up to 60% were confirmed only for *F. solani* (Kranjec-Orlović et al., 2020).

The appearance of the necrosis of *F. tricinctum* on narrow-leaved ash spouts at the locality Vinična (sect. 11 and 16) could be explained by the retention of the water in the stands for almost two months. Symptoms typical for *Fusarium* necrosis appeared sporadically. Forests in Vinična are in the protected area, however, in this case the embankment breaching cause that the entire management unit was flooded. As it was mentioned, the necrosis on narrow ash leaved sprouts were at 50 cm height which was at the level of flood water. According to obtained data it can be assumed that *F. tricinctum* spores were brought by the flood waters of Sava river. Additionally, the *Fusarium* species have not been isolated from the ash sprouts after that period. It can be concluded that young narrow plants exposed to the water stress became susceptible for the fungus colonization.

This conclusion is in the agreement with Pukacki and Przybyl (2005) who isolated fungi from the genus *Fusarium* (*F. avenaceum, F. lateritium, F. solani and F. sambucinum)* from the necrotic buds and shoots of *F.excelsior,* but these authors assumed that stress in this case freezing injury could have been the primary factor, predisposing damaged organs to fungal colonization. This assumption was confirmed by the findings of Bakys et al. (2009b) who reported that the majority of trees inoculated with *Gibberella avenacea*, remained visually healthy, most probably due to favourable growing conditions. The environmental factors and fungal interactions in addition to genetic resistance should be considered as possible modifiers of the pathogenicity of ash mycobiota in nature (Trapiello et al., 2017). Therefore, the possibility cannot be excluded that pathogenicity of certain otherwise naturally occurring endophytic, saprotrophic or opportunistic fungi could be triggered by environmental factors (Bakys et al., 2009b).

## CONCLUSION

*Fusarium* species are known as cosmopolites and pathogenic to vast number of plants, some of them has been reported on ash trees, but yet there has been no formal report of the disease or its causal fungus on narrow leaved ash in Serbia. To the best of our knowledge, this is the first report of *F. tricinctum* on narrow-leaved ash trees in Serbia. Considering the fact that *Fusarium* species, in this particular case *F. tricinctum,* under stress conditions could cause necrosis in the narrow ash sprouts, their occurrence and pathogenicity should be monitored.

## ACKNOWLEDGEMENTS

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ОРИГИНАЛНИ НАУЧНИ РАД

# ПРВИ НАЛАЗ *Fusarium tricinctum* НА ПОЉСКОМ ЈАСЕНУ (*Fraxinus angustifolia* Vahl.) У СРБИЈИ

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РЕЗИМЕ: Пољски јасен (*Fraxinus angustifolia* Vahl.) је дрвенаста врста распрострањена у низијским шумама у Европи. Регион југозападног Срема где се налазе највредније шуме ове врсте у Србији, био је изложен катастрофалним поплавама у мају 2014. Праћењем здравственог стања садница јасена (шума Моровић, локалитет „Винична") у рано пролеће 2015. године откривено је присуство великих тамних некротичних подручја на избојцима старим 1–2 године. Са оболелих избојака, стандардним фитопатолошким поступком, урађена је изолација патогена. Према морфолошким карактеристикама добијени изолати су детерминисани као *Fusarium tricinctum*. За даље анализе су узета три репрезентативна моноспорна изолата (К41, К42 и К78). BLAST претрагом NCBI нуклеотидне базе утврђено је 98,9% сличности TEF1-α секвенце изолата К78 (MN822227.1) са *Fusarium tricinctum* и 100% поклапања ITS секвенце овог изолата (GenBank MK928426.1) са изолатом *F. tricinctum* ZMXR6 (MT446111.1). IGS секвенце изолата К41 и К42 (MZ749901 и MZ749902) имале су 94,1% и 94,3% сличности са *F. tricinctum* комплексом, на основу BLAST претраге FUSARIUM-ID нуклеотидне базе. Тестови патогености на избојцима јасена старим годину дана показали су црвенкасто смеђе издужене некротичне лезије просечне дужине 20,1 mm. Патоген је реизолован из избојака са симптомима. Према нашим сазнањима, ово је први налаз *F. tricinctum* на стаблима пољског јасена у Србији.

КЉУЧНЕ РЕЧИ: пољски јасен, *Fusarium tricinctum*, некроза избојака