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Original scientific paper

Etiology of bacterial diseases of young walnut trees in Serbia

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SUMMARY

In the summer and autumn of 2019-2020, young walnut orchards were monitored for the presence of bacterial diseases. Diseased walnut samples comprising trunks and branches with symptoms of vertical oozing canker (VOC), walnut bacterial blight (WBB) and superficial bark necrosis were collected from eight locations in Serbia. Based on phenotypic features, pathogenicity, and molecular assays using PCR with specific primers, 49 isolates obtained from samples showing VOC and WBB symptoms were identified as *Xanthomonas arboricola* pv. *juglandis*, while further two isolates obtained from bark necrosis were identified as *Brenneria rubrifaciens*. One tested *X. a.* pv. *juglandis* isolate obtained from a VOC sample produced deep cankers in the bark of inoculated trunks of young walnut trees (cultivars Chandler, Franquette and Šejnovo). Therefore, this is the first report of an association between *X. a.* pv. *juglandis* and VOC symptom in Serbia. Considering that *X. a.* pv. *juglandis* significantly endangers walnut production, the presence of this pathogen in walnut transplant imports needs to be assessed by an authorised laboratory. Furthermore, as this is also the first report of *B. rubrifaciens* on walnut trees in Serbia, it is noteworthy that this pathogen is not particularly harmful to young walnut trees.

Keywords: walnut, phytopathogenic bacteria, vertical oozing canker, pathogen identification

INTRODUCTION

Walnut (*Juglans regia* L.) cultivation has a long tradition in Serbia and has taken the form of intensive production in recent years. Newly developed orchards, raised mostly from imported transplants, are now established in many parts of Serbia. Turkey is among the main walnut producers and distributers in the world, and its growers own considerable expertise in walnut propagation, grafting and budding (Akça & Yilmaz, 2017). At present, approximately 2.0 to 2.5 million walnut transplants are produced in Turkey each year (Özaktan et al., 2015), and Serbia is just one of many countries that imports walnut transplants from that country.

Walnut is exposed to attacks by numerous plant pathogens, including bacteria. Bacterial blight caused by *Xanthomonas arboricola* pv. *juglandis* Vauterin et al. 1995 is one of the most important diseases affecting

walnut around the world (Hajri et al., 2010; Lang & Evans, 2010; Moragrega et al., 2011; Özaktan et al., 2015; Giovanardi et al., 2016; Fernandes et al., 2018; Kim et al., 2021). This bacterium causes necrosis on leaves, catkins, twigs and fruits, and thus induces significant economic losses. Besides walnut bacterial blight (WBB), *X. a.* pv. *juglandis* also causes symptoms such as brown apical necrosis (BAN) in immature walnut fruits (Moragrega et al. 2011). Vertical oozing canker (VOC), which is the most severe symptom, has been observed in walnut plantations and nurseries (Hajri et al., 2010). All commercially grown walnut cultivars are considered susceptible to *X. a.* pv. *juglandis* (Lindow et al., 2014), while 'Chandler', 'Hartley' and 'Şebin' are deemed highly susceptible (Özaktan et al., 2015). Stomata or wounds represent the main infection route for *X. a.* pv. *juglandis*, and this bacterium overwinters in diseased buds and twigs (Frutos, 2010). In Serbia, bacterial blight of walnut caused by *X. a.* pv. *juglandis* was first reported by Gavrilović and Arsenijević (1998) and was later characterised by Ivanović et al. (2015). Deep bark canker (DBC) and shallow bark canker (SBC) caused by species of the genus *Brenneria* (*rubrifaciens* and *nigrifluens*, respectively) are other bacterial diseases that are able to affect walnut production (McClean et al., 2008; Frutos, 2010; Biosca & López, 2012; Popović et al., 2013). *Brenneria nigrifluens*, the causal agent of shallow-bark canker on walnut trees in Serbia, was reported for the first time by Popović et al. (2013) in a 30-year-old orchard in the Fruška Gora region.

Although bacterial pathogens on walnut had been studied in Serbia previously, symptoms that are currently present in newly raised orchards (manifesting as severe canker on young walnut trees and leading to trunk deformation and bacterial blight) are not yet well understood and therefore require etiological studies of their causal pathogens. Consequently, the aim of the present study is to determine the role of bacterial pathogens in walnut canker disease, and specifically to identify the causal agent of VOC. For this purpose, data was gathered over a two-year period across different regions in Serbia and was analysed using conventional and molecular methods.

MATERIAL AND METHODS

Bacterial isolation

Diseased walnut samples were collected from young, 1- to 3-years-old commercial orchards in eight locations in Serbia (Kelebija, Zrenjanin, Sombor, Miličinica, Sirig, Rimski Šančevi, Vrbas and Crvenka) during 2019-2020. Symptoms consisted of VOC, bacterial blight and superficial local necrosis, and 3-5 walnut samples (trunks, branches) were taken from each monitored location (Table 1).

Samples were first surface sterilised with 1% sodium hypochlorite solution (NaOCl) for 1 min, and then rinsed under tap water and dried using sterile filter paper. Small fragments (ø 2-3 mm) taken from the margins of healthy and diseased tissues were macerated in sterile distilled water (SDW) and kept for one hour at room temperature. To target the bacteria of the genera *Xanthomonas* and *Brenneria* as the most likely walnut pathogens, the extracts obtained from plant tissues were plated onto two media: (i) Nutrient Agar supplemented with 5% w/v sucrose (NSA), and (ii) Yeast extract-Dextrose-Calcium carbonate agar (YDC) (Hajri et al., 2010). Plates were incubated at 26 °C for 2-4 days.

Isolation of fungal pathogens as potential causal agents of walnut canker was performed by placing small fragments (ø 2-3 mm) taken from the margins of healthy and diseased tissues onto Potato-Dextrose Agar (PDA). Plates were kept at 25 °C for 14 days.

Further study was performed with a total of 51 purified bacterial isolates (Table 1), selected on the basis on their appearance and/or predominance on NSA and YDC plates.

Molecular detection

Polymerase chain reaction (PCR) was used for preliminary detection of plant pathogenic bacteria in the collected walnut samples, as well as for identification of the obtained walnut isolates.

DNA extraction

Total genomic DNA was obtained from the extracts of diseased plant tissues (previously used for isolation) using a modified CTAB procedure as described by Ausubel et al. (2003). Total genomic DNA from pure cultures was extracted using the boiling method (Freschi et al., 2005).

Polymerase chain reaction (PCR)

PCR was performed using the XajF/XajR primer pair specific for detection of *X. a.* pv. *juglandis* (Gironde et al., 2009) and two primer pairs (F1/C3 and BR1/BR3) specific for detection of the genus *Brenneria*. PCR amplification using XajF/XajR was programmed for initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing

at 61 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 10 min. PCRs with the primer pair F1/C3 specific for *B. nigrifluens* and primer pair BR1/BR3 specific for *B. rubrifaciens* involved initial denaturation at 94 °C for 5 min, followed by 39 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 2 min (Loreti et al., 2008; McClean et al., 2008).

Each PCR was performed in a 25 µl volume, using DreamTaq Green master mix (TermoScientific, Lithuania), 1 µl of bacterial DNA as a template and 100 pM of appropriate primer. Amplified PCR products were visualized by gel electrophoresis on 1.5% agarose gel stained with ethidium bromide under UV light.

Biochemical characterization

All isolates were subjected to the following biochemical tests: Gram staining, oxidative and fermentative metabolism of glucose (O/F test), hydrolysis of aesculin, production of H_2S and indole, and oxidase and catalase activity (Schaad et al., 2001).

Pathogenicity

The pathogenicity of 51 walnut isolates was evaluated using immature young walnut fruitlets (cv. Chandler) which were first disinfected with 1% NaOCl for 2 min and rinsed with SDW. Mesocarp was then punctured with sterile needle and wounds were filled with 100 µl of bacterial suspensions of each test isolate adjusted to the final concentration of 10^7 CFU ml^{-1}. SDW was used as negative control treatment.

The inoculated fruits were maintained on wet wool in plastic boxes at ambient temperature under high humidity (≈ 80%) for 14 days. Development of symptoms was rated on a daily basis.

Role of walnut bacterial pathogen in VOC symptom expression

To demonstrate the etiology of VOC as the most dominant symptom in the walnut plantations monitored during the 2019-2020 study period, one isolate of *X. a.* pv. *juglandis* (coded as XOS4, isolated from VOC, Sirig location) and one isolate of *B. rubrifaciens* (coded as BOV2, isolated from superficial bark necrosis, Vrbas location) were used. This test was conducted by artificial inoculation of the central trunk of disease-free one-year-old potted walnut plants of three cultivars–Chandler (originating from the USA), Franquette (France) and Šejnovo (Bulgaria). Inoculation was performed by wounding the bark until splitting it, and then infiltrating the wound with 1 ml of bacterial suspensions prepared in SDW and adjusted to the final concentration of 10^9 CFU ml^{-1}. The inoculated sites were wrapped with wet wool and aluminium foil to keep bacterial suspension inside the wound. The potted plants were kept for several months (May-September) under open field conditions. SDW was used as the negative control treatment.

Bacteria were reisolated from walnut cankers onto YDC and NSA, and confirmed to be the same as the original using the PCR primer set XajR/XajF or BR1/BR3, in line with Koch's postulates.

RESULTS AND DISCUSSION

The present study provides evidence of the presence of a bacterial population structure in walnut trees under Serbian production conditions. A field survey was carried out at eight walnut growing locations during 2019-2020 on three cultivars: Chandler, Lara and Franquette. All transplant material was imported from Turkey, and was mostly grafted on black walnut (*J. nigra* L.). Initial symptoms included vertical cankers on trunks or branches during springtime (Figures 1 and 2). In the summer, brown to black exudate started oozing from the cankers, staining the bark, which is a typical VOC symptom. As a result, all diseased trees exhibited trunk deformities (Figure 3), and dieback (as the final stage of disease development) was noted in some cases. Under Serbian production conditions, VOC symptoms were most prevalent in the cultivar Chandler (25% disease incidence), indicating that this cultivar is the most susceptible. Disease incidence noted for Lara and Franquette was 5-10%. Symptoms of bacterial blight (WBB) were found at several locations, Sombor and Miličinica in particular (Figure 4), and superficial local necrosis of bark was recorded at one location (Vrbas).

Following pathogen isolation, typical *Xanthomonas*-like colonies were formed on samples from all of the eight monitored locations (i.e., from 32 collected walnut samples) on YDC and NSA media after three days (Table 1). The colonies formed on YDC were pale yellow, slimy, circular, mucoid and round, while those formed on NSA were light yellow and round. In addition to the formation of *Xanthomonas*-like colonies, light cream to whitish, circular and smooth colonies (*Brenneria*-like colonies) were formed on the NSA medium of one sample from the Vrbas location (Table 1). No growth of fungal pathogens as potential causative agents of walnut canker was observed on PDA medium.

Figure 1. *Xanthomonas arboricola* pv. *juglandis.*
VOC symptom: canker formation and bark staining

Figure 2. *Xanthomonas arboricola* pv. *juglandis.*
VOC symptom: bark cracking and necrosis of internal tissues

Figure 3. *Xanthomonas arboricola* pv. *juglandis.*
VOC symptom: deep internal necrosis and trunk deformation

Figure 4. *Xanthomonas arboricola* pv. *juglandis.*
WBB symptom on shoots

Table 1. Isolation of causative agents of walnut bacterial diseases

Location	Symptom type	Collected samples	Colony type	Collected isolates	Date of isolation
Kelebija	VOC	5	Yellow	7	Oct 4th 2019
Zrenjanin	VOC	4	Yellow	5	Aug 5th 2019
Sombor	VOC	2	Yellow	2	Aug 15th 2019
	WBB	1	Yellow	2	
Miličinica	VOC	2	Yellow	5	Sep 3rd 2020
	WBB	2	Yellow	3	
Sirig	VOC	4	Yellow	6	Oct 15th 2020
Rimski Šančevi	VOC	4	Yellow	7	Aug 22nd 2020
Vrbas	VOC	3	Yellow	4	Oct 20th 2020
	SN	1	Whitish	2	
Crvenka	VOC	5	Yellow	8	Oct 15th 2020
Total		33	/	51	/

VOC – vertical oozing canker; WBB – walnut bacterial blight; SN – superficial local necrosis

The bacterium *X. a. pv. juglandis* was detected in 32 tested walnut samples, as well as all of the 49 *Xanthomonas*-like isolates by PCR using the specific primer pair XajF/XajR. In all positive samples/isolates, a product of 216 bp size was amplified. One sample (from Vrbas location), including two isolates, yielded positive results when PCR was performed with the BR1/BR3 primer pair specific for *B. rubrifaciens* after the amplification of products 409 bp in size. PCR performed with the F1/C3 primer pair was negative in all cases. The primers used in this study had been proven in many earlier studies as very specific for the tested species (McClean et al., 2008; Gironde et al., 2009; Ivanović et al., 2015).

The 49 selected *Xanthomonas*-like isolates were Gram negative, aerobic (+/-), catalase positive and oxidase negative, and capable of hydrolysing aesculin. Further, these isolates did not produce H_2S or indol. Two *Brenneria*-like isolates were Gram negative, facultative anaerobic (+/+), catalase positive and oxidase negative, hydrolysed aesculin, and did not produce H_2S or indol. Our findings, yielded by fast detection and identification of *X. a. pv. juglandis* and *B. rubrifaciens* isolates using conventional and molecular methods, concurred with those reported by other authors (Biosca et al. 2003; McClean et al., 2008; Gironde et al., 2009; Biosca & Lopez, 2012; Ivanović et al., 2015; Kim et al., 2021).

Inoculation of immature walnut fruits (cv. Chandler) resulted in brown lesions, which were observed on the inoculation sites seven days after inoculation in the case of *X. a.* pv. *juglandis* isolates (Figure 5A). After 14 days, lesions expanded and progressed under the pits, and tissue necrosis occurred (Figure 5B). Similar symptoms were observed in two *B. rubrifaciens* tested isolates (Figure 6A). Negative controls were symptomless (Figures 5C and 6B).

Figure 5. *Xanthomonas arboricola* pv. *juglandis* – pathogenicity on immature walnut fruits. A: symptoms noted 7 days after inoculation; B: fruit cross-section 14 days after inoculation; D: control fruit treated with SDW

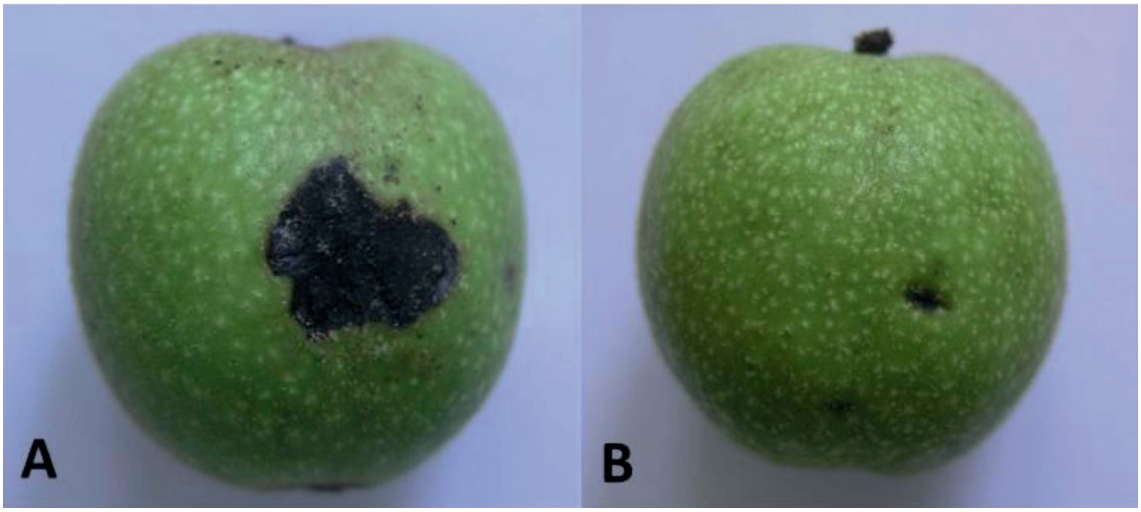

Figure 6. *Brenneria rubrifaciens* – pathogenicity on immature walnut fruits. A: symptoms noted 7 days after inoculation; B: control fruits treated with SDW

After artificial inoculation, the isolate *X. a. pv. juglandis* (XOS4) caused typical VOC symptoms on the trunks of young walnut trees, which were similar to those observed on naturally diseased walnut trees. First, darkening occurred on the inoculation sites, and two months after inoculation necrosis spread along the bark in the form of elongated lesions. Three to four months after inoculation, the affected bark became necrotic, cracked and vertical cankers appeared with reddish to brown exudate. Similar symptoms were observed in all three inoculated walnut cultivars (Chandler, Franquette and Šejnovo). On the other hand, *B. rubrifaciens* isolate (BOV2) did not produce such symptoms on the three tested walnut cultivars, as only local superficial necrosis was evident on the bark wound site. Control plants inoculated with SDW remained healthy. In addition, *X. a. pv. juglandis* was reisolated from the inoculated walnut trees after symptom development and was found to be identical to the original using PCR with the XajR/XajF primer set. Similar VOC symptoms were reported by Hajri et al. (2010) on walnut in France as a part of a field and laboratory examination of VOC on walnut during 2001-2003. The authors concluded that *Xanthomonas* sp. was the only causal agent, as the bacterium *B. nigrifluens* could only cause limited bark necrosis, which supports the findings in the present study.

Our etiological study conducted on canker disease, trunk deformations and bacterial blight affecting young walnut trees confirmed that the bacterium *X. a.* pv. *juglandis* is the main walnut pathogen in Serbia. This pathogen is commonly present throughout walnut-growing regions and severe disease outbreaks have been reported worldwide over the last few decades (Frutos, 2010; Lang & Evans 2010; Buchner et al. 2010; Giovanardi et al. 2016; Temperini et al. 2017; Kim et al., 2021). According to Buchner et al. (2010), a combination of high initial inoculums, young developing shoots and nutlets and warm, moist spring weather is favourable for walnut blight infections. *X. a.* pv. *juglandis* as the cause of bacterial disease symptoms was detected in all Serbian walnut orchards monitored in this study. According to Hajri et al. (2010), VOC caused by *X. a.* pv. *juglandis* is the most severe symptom on walnut, and thus presents a serious threat to walnut production. In addition to symptoms of walnut bacterial blight and brown apical necrosis caused by *X. a. pv. juglandis* reported by other authors (Gavrilović & Arsenijević, 1998; Ivanović et al., 2015), our investigation offers the first evidence of VOC in Serbia. Increase in the production of walnut transplant cultivars and grafts, and more widespread use of agrotechnical measures in newly established walnut orchards have likely contributed to the emergence of VOC. In the first spring after walnut planting, tree top pruning to 30 cm height with the aim of providing more energy to the graft presents one of the main agrotechnical practices. Considering that wounds are the main infection route for *X. a.* pv. *juglandis*, the critical time for pathogen attack on young walnuts is ensured by applying this measure. Moreover, persistence of pathogens in walnut organs should be considered, as Buchner et al. (2012) determined that walnut infections

are strongly related to the presence and magnitude of *X. a.* pv. *juglandis* overwintering under dormant bud scales. Furthermore, walnut transplants grafted on black walnut are susceptible to low temperatures and frost, and these conditions contribute to *X. a. pv. juglandis* infection. In addition, young walnut trees exhibiting VOC symptoms on their trunks tend to break under strong gusts of wind. Consequently, walnut pruning, along with selection of cultivars and grafts suitable for the specifics of Serbian production is recommended.

The bacterium *B. rubrifaciens* was found in only one walnut sample/orchard isolated from a localized bark necrosis spot. *Brenneria* species are usually present in older walnut trees (McClean et al. 2008; McClean & Kluepfel, 2009; Popović et al., 2013). Although we have not established the role of *B. rubrifaciens* in walnut canker etiology, this study provides the first record of this pathogen on walnut trees in Serbia. McClean et al. (2008) reported that *B. rubrifaciens* was not a threat to young walnut trees, and hypothesised that the pathogen may be present in host tissue for many years before plants develop disease symptoms. Both *B. nigrifluens* and *B. rubrifaciens* have been reported as the causal agents of walnut bark disease across Europe. For example, Morone et al. (1998) found *B. nigrifluens* among resident microflora that infect stressed plants in Italy.

CONCLUSION

During the two-year (2019-2020) monitoring of walnut orchards under intensive production, the bacterium *X. a. pv. juglandis* was identified as the causal agent of VOC and bacterial blight symptoms that posed a significant threat to young trees. Since there are no measures that can be effectively applied to control bacterial diseases, the use of pathogen-free transplants, along with an assortment of domestic walnut cultivars, and grafts suitable for Serbian agroecological conditions are recommended. Moreover, rapid detection of causative pathogens by authorised laboratories and phytosanitary authorities is required.

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Etiologija bakterioznih oboljenja mladih zasada oraha u Srbiji

REZIME

Tokom leta i jeseni u periodu 2019-2020, praćeno je prisustvo bakterioznih oboljenja u mladim zasadima oraha. Oboleli uzorci stabala i grana sa simptomima vertikalnog raka (VOC), bakteriozne plamenjače (WBB) i površinske nekroze kore prikupljeni su sa osam lokaliteta u Srbiji. Na osnovu fenotipskih karakteristika, patogenosti i molekularnih testova korišćenjem PCR-a i specifičnih prajmera, 49 izolata sa simptomima VOC i WBB je identifikovano kao *Xanthomonas arboricola* pv. *juglandis* i dva izolata sa simptoma površinske nekroze kore identifikovana su kao *Brenneria rubrifaciens*. Izolat *X. a.* pv. *juglandis* dobijen iz VOC simptoma

na inokulisanim stablima mladih sadnica oraha (sorte Chandler, Frankquette i Šejnovo) je prouzrokovao duboke rak rane na kori. Ova istraživanja daju prve rezultate o povezanosti *X. a.* pv. *juglandis* sa VOC simptomom u Srbiji. S obzirom da *X. a.* pv. *juglandis* značajno ugrožava proizvodnju oraha preporučuje se da se testiranje na prisustvo ovog patogena vrši prilikom uvoza sadnica od strane ovlašćene laboratorije. Dalje, rezultati ove studije predstavljaju i prvi dokaz o prisustvu *B. rubrifaciens* na orahu u Srbiji, ali je utvrđeno da patogen ima manji uticaj na mlada stabla oraha.

Ključne reči: orah, fitopatogene bakterije, vertikalni rak, identifikacija patogena