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Evidence and characterisation of *Xanthomonas arboricola* pv. *juglandis* causing bacterial blight of walnut in Montenegro

Jelena Latinović[1], Nedeljko Latinović[1], Bogoljub Kandić[1], Aleksandra Jelušić[2], Tatjana Popović Milovanović[3]

[1]*Biotechnical Faculty, University of Montenegro, Podgorica, Montenegro*
[2]*Institute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia*
[3]*Institute for Plant Protection and Environment, Belgrade, Serbia*

Corresponding author: tanjaizbis@gmail.com

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Abstract: This study represents the first evidence of the bacterial blight caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*) on walnut trees in Podgorica (Montenegro). Disease symptoms appeared on leaves in the form of dark, angular leaf spots surrounded by yellow-green haloes and lesions spread across the whole leaf. Isolated bacteria were preliminarily identified using PCR with pathovar-specific primer pair XajF/XajR and further characterised based on multi-locus sequence analysis with nine housekeeping genes (*fusA*, *gapA, gltA*, *gyrB1*, *lepA*, *rpoD*, *dnaK*, *fyuA,* and *gyrB*2). Montenegrin walnut isolates were homogeneous among themselves and the most closely related to different *X. arboricola* strains originating from *Juglans regia* isolated elsewhere. The pathogenicity of isolates was confirmed on walnut leaves, fruits, and branches. All inoculations resulted in the formation of necrotic lesions that initially developed at the site of bacteria entry, with later developing chlorotic areas on leaves along the leaf veins. This finding of *Xaj* causing leaf blight symptoms on walnuts in Montenegro highlights its expanding distribution across Europe and indicates a potential threat to walnut plantations in Montenegro.

Keywords: *Juglans regia*; detection; identification; pathogenicity; multi-locus sequence analysis

Walnut (*Juglans regia* L.) production has increased considerably in recent years and presents the third fastest-growing product on the nuts market, after peanuts and almonds. China, the United States, Chile, the European Union, and Ukraine are the world's top walnut producers, accounting for 95% of worldwide production. Besides their health benefits as a rich source of nutrients, antioxidants, and omega-3 fatty acids, walnuts also have a significant traditional role in Balkan countries for preparing sweet cakes. During cultivation, walnuts are exposed to attack pathogens due to technology and natural factors, particularly *Xanthomonas arboricola* pv. *juglandis* (*Xaj*), which causes bacterial blight, reduces its yield and damages its quality. This bacterium can cause yield reduc-

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tion of up to 70%, as it attacks all plant parts: leaves, twigs, immature fruits, and stems (Scortichini et al. 2001; Moragrega 2012; Kim et al. 2021). In addition to *Xaj*, two other *X. arboricola* pvs, i.e, *corylina*, and *pruni*, responsible for diseases of nuts and stone fruits that have emerged worldwide, are classified as Regulated Non-Quarantine Pest quarantine organisms in the European Union (RNQP). In Montenegro, symptoms of walnut bacterial blight were previously observed by Mijušković (1990), but until now, the experimental presence of *Xaj* has never been confirmed. Since bacterial blight is present in many European countries (https://gd.eppo.int/taxon/XANTJU/distribution), this work was undertaken to detect, identify and characterise the causal bacterium of bacterial blight-like disease observed lately on walnut trees in Podgorica (Montenegro).

MATERIAL AND METHODS

Isolation. In July 2024, symptoms of bacterial blight, in the form of dark, angular leaf spots surrounded by yellow-green haloes and lesions spread across the entire leaf (Figure 1), were observed on walnut trees in Podgorica (Montenegro). The walnut orchard is located on a limited area of 20 trees and belongs to the experimental and commercial fields of the University of Montenegro, Biotechnical Faculty (locality Lješkopolje-Podgorica). At the time of sampling, the disease incidence was approximately 30%. Diseased leaves were collected for isolation, rinsed under tap water, and then dried on sterile filter paper. Small leaf fragments, taken from the boundary between the healthy and diseased tissues, were macerated in sterile distilled water and plated onto Nutrient Agar supplemented with 5% w/v sucrose (NSA) and Yeast extract-dextrose-calcium carbonate agar (YDC) (Iličić et al. 2021). Plates were incubated at 26 °C for 3 days. Pure cultures were obtained from a predominant bacterial colony.

Molecular identification. The genomic DNA of the obtained isolates was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Popović et al. 2019). Their preliminary identification was performed by amplifying the DNA with pathovar-specific primer pair XajF/XajR (Ivanović et al. 2015; Iličić et al. 2021). Amplifications were performed according to the following program: 5 min of initial denaturation at 94 °C, then 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 10 min of final extension step at 72 °C. PCR products were separated by electrophoresis on 1% agarose gel stained with Midori Green Advance (Nippon Genetics Europe GmbH, Germany) and checked for the presence of a specific band in relation to the 200–10 000 bp DNA ladder (SmartLadder MW-1700-10; Eurogentec, Belgium) and *Xaj* strain isolated in Serbia (coded as XOS4, Iličić et al. 2021) serving as positive control.

Two representative isolates (MNW124 and MNW524) were randomly selected and further characterised using two Multi-locus sequence analysis (MLSA) schemes commonly employed to identify and characterise *Xanthomonas* sp. These schemes are based on nine housekeeping genes: *rpoD*, *dnaK*, *fyuA*, and *gyrB*1 (Young et al. 2008) and *fusA*, *gapA*, *gltA*, *gyrB*1, and *lepA* (Almeida et al. 2010). Cycling programs were as described by Popović et al. (2019).

Figure 1. Bacterial blight on walnut tree, natural infection (Podgorica, Montenegro)

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Table 1. *Xanthomonas* spp. strains from the NCBI database used for phylogenetic analysis

Strain	Host	Country	Year	Acc. No.
Xanthomonas arboricola				
CPBF 237	*Juglans regia*	Portugal (Ponte de Lima)	2015	LR877307
CPBF 554	*J. regia*	Portugal (Carrazeda de Ansiães)	2016	LR962896
CPBF 796	*J. regia*	Portugal (Alcobaça)	2016	LR877306
CPBF 1483	*J. regia*	Portugal (Alcobaça)	2014	LR962646
CPBF 1567	*J. regia*	Portugal (Bombarral)	2015	LR962645
CPBF 1586	*J. regia*	Portugal (Loures)	2014	LR962897
1314c	*Vaccinium corymbosum*	Poland	2013	HG992337
X. arboricola* pv. *juglandis				
CPBF 765	*Carya illinoinensis*	Portugal	–	HG999365
CPBF 1494	*C. illinoinensis*	Portugal	2014	HG999362
IVIA 2499	*J. regia*	Spain	2001	CP076726
IVIA 1317	*J. regia*	Spain	1993	CP076725
3 (= CPBF 427)	*J. regia*	Portugal	2016	LR861807
WHRI 5692A	*J. regia*	United Kingdom	1996	CP168206
X. arboricola* pv. *pruni				
NCPPB 416^{T}	*Prunus salicina*	New Zealand	1953	CP167239
15-088	*P. persica*	South Korea	2015	CP044334
CITA 33	*P. dulcis*	Spain	2009	CP076701
X. arboricola* pv. *corylina				
NCPPB 935^{T}	*Corylus maxima*	USA	1939	CP166095
CFBP 6600	*C. avellana*	France	1977	HG992342
CFBP 1846	*C. avellana*	France	1975	CP076619
X. campestris* pv. *campestris				
ATCC 33913 ^{T*}	*Brassica oleracea* var. *gemmifera*	United Kingdom	1957	AE008922

^{T}pathotype strain; *outgroup strain

PCR products were purified and sequenced by the Macrogen sequencing service (the Netherlands) using the same primers that were used for the amplification. Sequences of all nine genes were manually checked for quality and compared to those available in the NCBI (National Centre for Biotechnology Information) database using the nucleotide BLAST (BLASTn) function. Before constructing the Neighbour-joining (NJ) phylogenetic tree, partial coding sequences of all nine genes from the two representative isolates in this study, along with reference strains of *X. arboricola* pvs. *juglandis*, *pruni* and *corylina*, retrieved from the NCBI database (Table 1), were aligned and trimmed to the same length using the automated ClustalW alignment feature of the BioEdit program (version 7.0.5). Two distinct NJ phylogenetic trees were constructed using MEGA software (version 7) based on concatenated sequences for each MLSA scheme. Trees were rooted with *Xanthomonas campestris* pv. *campestris* pathotype strain ATCC 33913, also from the NCBI. Genetic distances were computed using the Kimura two-parameter nucleotide substitution model (Kimura 1980). Sequences of the two selected isolates from this study were deposited to the NCBI under the following Accession numbers: *dnaK* (PQ468289-90), *fyuA* (PQ468291-92), *gyrB1* (PQ468293-94), *rpoD* (PQ468295-96), *fusA* (PQ468297-98), *gapA* (PQ468299-300), *gltA* (PQ468301-02), *gyrB2* (PQ468303-04), and *lepA* (PQ468305-06).

Pathogenicity. Pathogenicity of isolates was assessed using three methods: *(i)* detached leaves via syringe-infiltration method with a dose of bacterial inoculum 10^8 cfu/mL, *(ii)* branches, and *(iii)* immature walnut fruits by puncturing method, which involved using a sterile toothpick, previously dipped

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in a bacterial culture grown for 3 days at 26 °C on YDC medium. Toothpicks were slightly twisted while being withdrawn to ensure the release of the bacteria. After inoculations, conditions with high relative humidity (80–100%) and a temperature 28 ± 2 °C were provided for symptom development. The appearance of progressive necrotic lesions through tissues as a sign of the presence of pathogenic isolates was evaluated 7 and 14 days after inoculation (DAI). To fulfil Koch's postulates, bacteria were re-isolated from all inoculated organs on YDC medium and confirmed to be the same as the original isolates using the PCR with primer pair XajR/XajF.

RESULTS AND DISCUSSION

From diseased walnut leaves exhibiting blight-like symptoms, the isolations were positive. They yielded bacterial, typical *Xanthomonas*-like colonies formed after three days of incubation on YDC (circular, pale yellow, slimy, mucoid) and NSA (round, light yellow) media. Medium YDC is widely used for bacteria isolation from walnut tissues and is recommended by many diagnostic protocols (Schaad et al. 2001).

PCR performed with pathovar-specific primer pair XajF/XajR amplified a specific band at 216 bp for leaves samples and for the selected isolates, indicating the presence of *Xaj* (Gironde 2009). BLASTn analysis results for the nine sequenced housekeeping genes revealed that Montenegrin walnut isolates share 100% identity with *Xaj* strains based on genes *dnaK, rpoD, gapA, gltA*, and *gyrB2*. The isolates show 100% identity with *X. arboricola* strains, not only with these five but also for *fyuA* and *fusA* genes (Table 2). A slightly lower percent identity with *Xaj* and/or *X. arboricola* was observed using *gyrB1* and *lepA* genes, 99.28% and 98.45%, respectively (Table 2).

Table 2. Percent identity of the two representative isolates from this study with the reference strains deposited in the NCBI database according to the Nucleotide BLAST (BLASTn) function for the nine sequenced genes

MLSA scheme	Housekeeping gene	Reference strain from NCBI	Percent identity (%)
Young et al. (2008)	dnaK	*Xanthomonas arboricola* strains CPBF 1567, CPBF 796, CPBF 1483, CPBF 554, CPBF 237; *X. arboricola* pv. *juglandis* strains 3, ICMP 35, IVIA 1317, IVIA 2499, WHRI 5692A, Xaj 417	100.00
	fyuA	*X. arboricola* strains 1314c, ICMP 9894	100.00
	gyrB1	*X. arboricola* strains CPBF 1567, CPBF 796, ICMP8452, ICMP 4939, CFBP 1483; *X. arboricola* pv. *pruni* strains NCPPB 416, Xcp1, 15-088; *X. arboricola* pv. *juglandis* strains 3, ICMP 35, IVIA 1317, IVIA 2499, WHRI 5692A, Xaj 417	99.28
	rpoD	*X. arboricola* strains CPBF 1567, CPBF237, CPBF 1497; *X. arboricola* pv. *juglandis* strains ICMP 35, WHRI 5692A	100.00
Almeida et al. (2010)	fusA	*X. arboricola* strains CPBF 1567, CPBF 796	100.00
	gapA	*X. arboricola* strains CPBF 1567, CPBF 796, CPBF 237; *X. arboricola* pv. *juglandis* strain WHRI 5692A	100.00
	gltA	*X. arboricola* strains CPBF 1567, CPBF 796, G3, R2, CFBP 1483; *X. arboricola* pv. *juglandis* strains 3, IVIA 1317, IVIA 2499, WHRI 5692A, Xaj 417; *X. arboricola* pv. *corylina* strains A7, NCPPB 935, CFBP 6600, IVIA 3978, CFBP 1159	100.00
	gyrB2	*X. arboricola* strains CPBF 1567, CPBF 796, XA5, CPBF 1483; *X. arboricola* pv. *pruni* strains NCPPB 416, Xcp1, Xp219, 15-088; LMG852; *X. arboricola* pv. *juglandis* strains 3, LMG747, Xaj417, IVIA 1317, WHRI5692A; *X. arboricola* pv. *corylina* strain IVIA 3978	100.00
	lepA	*X.* sp. strains NCPPB 2866, NCPPB 2865; *X. arboricola* strains 1314c, CPBF 1586	98.57; 98.45

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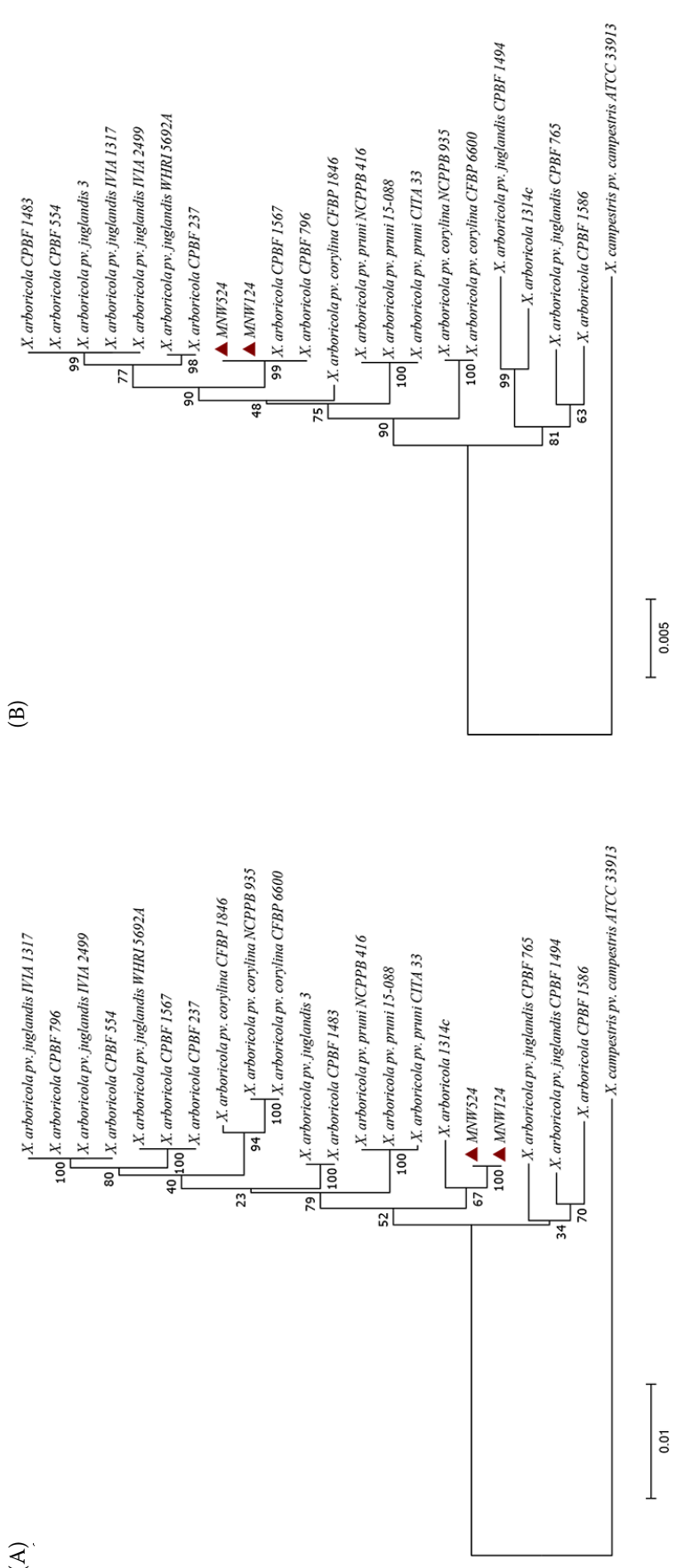

Figure 2. Neighbour-joining phylogenetic trees based on the concatenated sequences
(A) four housekeeping genes (*dnaK*, *fyuA*, *gyrB1* and *rpoD*) according to Young et al. (2008) and (B) five (*fusA*, *gapA*, *gltA*, *gyrB1*, and *lepA*) given by Almeida et al. (2010) for two Montenegrin walnut isolates (MNW124 and MNW524) and *X. arboricola* strains and pathovars retrieved from NCBI; the tree was rooted with *X. campestris* pv. *campestris* strain ATCC33913

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Constructed NJ phylogenetic trees based on the concatenated sequences according to two schemes given by Young et al. (2008) and Almeida et al. (2010) are presented in Figures 2A and 2B, respectively. The two Montenegrin walnut isolates (MNW124 and MNW524) were found to be homogeneous among themselves and most closely related to *X. arboricola* strain 1314c (isolated from *Vaccinium corymbosum* in Poland) (Figure 2A) and *X. arboricola* strains CPBF796 and CPBF1567 (isolated from *Juglans regia* in Portugal) (Figure 2B).

According to Ivanović et al. (2015), 15 Serbian walnut strains were placed into a single cluster of a phylogenetic tree based on *gyrB1* gene sequences. The same authors stated that Serbian strains did not match 100% with any *Xaj gyrB1* sequences in the NCBI database. This finding aligns with the results from our study, suggesting that the isolates from Serbia and Montenegro are likely similar. Marcelletti et al. (2010) typed 55 *X. arboricola* pv. *Juglandis* strains from *Juglans regia* are cultivated in different countries using genes *acnB, gapA, gyrB,* and *rpoD*. The dendrograms revealed two major and two minor phylotypes and a recombination breakpoint was detected within the *rpoD* gene fragment. Kim et al. (2021) used 16S rDNA sequence analysis and MLSA with genes *atpD, dnaK, efp*, and *rpoD* to characterise Korean walnut strains.

Inoculations of all tested organs resulted in developed necrosis, which is an indicative sign of the pathogen presence (Scortichini et al. 2001; Vagelas et al. 2012; Ivanović et al. 2015; Iličić et al. 2021; Kim et al. 2021). On the inoculated leaves, necrosis was developed at the sites where the bacterial suspension was infiltrated, and by 14 DAI, yellow chlorotic areas appeared around the necrotic spots and along the leaf veins (Figure 3). On inoculated branches, dark lesions were formed 5–7 DAI at the point of entry of the toothpick. Chlorosis and necrosis of top foliage occurred along the leaf veins 15 DAI (Figure 3). Inoculated fruits developed necrosis at the inoculation site, which expanded into the surrounding tissue (2–5 mm) by 10–15 DAI, and when cut transversely, necrosis progression toward the shell was evident (Figure 3). The same pathogen was re-isolated from all symptomatic tissues.

The results of identifying the causal agent of the observed symptoms in walnut leaves indicate the presence of the plant pathogenic bacterium *Xaj*. Its presence has been confirmed in many European countries, including Austria, Azerbaijan, Bulgaria, Denmark, France, Georgia, Germany, Greece, Hungary, Italy, Lithuania, Moldova, Netherlands, Poland, Portugal, Romania, Russia, Serbia, Slovenia, Spain, Switzerland, Turkey, Ukraine, United Kingdom. Therefore, discussing the intro-

Figure 3. *Xanthomonas arboricola* pv. *juglandis*. Symptoms on inoculated leaves, fruits, and stem of walnut

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duction and/or tracing of this pathogen in Montenegro can only be speculative. *Xaj* can be transported by asymptomatic plantlets and propagative material from infected nurseries or disseminated over short distances through pollen (Lamichhane 2014). Even though symptoms similar to those caused by *Xaj* infection were also observed on walnut fruits, the negative isolations we obtained may be due to the presence or prevalence of other walnut pathogens causing similar symptoms on the fruits. Further monitoring of the walnut orchard in Podgorica will be part of a future study of this pathogen.

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