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SHORT COMMUNICATION

Brenneria roseae subsp. roseae, a new pathogen of walnut (*Juglans regia* L.) in Europe

Klementina Kalmár[1] · Szabolcs Ádám[2] · Tímea Tóth[2] · Tamás Lakatos[1]

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Abstract
Walnut production in Hungary has grown dynamically in the 2000s, with the area planted almost quadrupling. The production is mainly based on local varieties, using almost exclusively Hungarian nurseries produced graftings as propagating material. In the summer of 2023, numerous symptoms with bark cracking and brown oozing spots of varying size appeared in a mature walnut orchard, in the central part of the country. In order to identify the causative pathogen, samples of necrotic tissue of sapwood were taken from two symptomatic trees. Bacteria of round-shape, light cream colour, convex colonies could be obtained from both samples. Based on the 16S rRNA gene sequence, the two newly isolated bacterial strains (III/9 and IV/11) were 100% identical to each other and showed 99.7% similarity to the type strain of *Brenneria roseae subsp. roseae*. The phylogenetic analysis based on four housekeeping protein coding gene (*atpD*, *gyrB*, *infB* and *rpoB*) sequences of the newly isolated strain III/9 confirmed the taxonomic position. The Hungarian stain III/9 clustered together *Brenneria roseae subsp. roseae*, including strain Br190, which was isolated from walnut in Iran (Allahverdipour et al., 2021). Pathogenicity test on two-years old walnut graftings cv. 'Milotai intenzív' and 'Bonifác' demonstrated that *Brenneria roseae subsp. roseae* strain III/9 can cause tissue necrosis and oozing on woody parts of the walnut trees. Based on these results, *Brenneria roseae subsp. roseae* should be added to the list of pathogens of walnut with a proven European occurrence.

Keywords *Brenneria spp.* · *Brenneria roseae subsp. roseae* · *Juglans regia* · Bacterial diseases of walnut trees

Introduction

Persian walnut (*Juglans regia*) is the most important shell fruit species and is grown in temperate climates throughout the world. According to the FAO, the world's total nut production has reached 3.903 million tonnes in 2022, harvested from about 1.234 million hectares of land (FAOSTAT 2025). In Hungary, walnut production has grown dynamically in the 2000s, reaching 7957 hectares and a total production of 5502 tonnes by 2023 (HCSO 2025). Hungarian walnut production is mainly based on local varieties under extensive or semi-intensive conditions, using almost exclusively Hungarian nurseries produced graftings as propagating material.

✉ Tamás Lakatos
lakatos.tamas@uni-mate.hu

[1] Research Centre for Fruit Growing, Institute of Horticultural Science, Hungarian University of Agriculture and Life Sciences, Elvira Major, Érd 2030, Hungary

[2] Research Institute of Újfehértó, University of Debrecen, Vadastag 2, Újfehértó 4244, Hungary

Walnut production is threatened by several major bacterial pathogens. *Xanthomonas arboricola pv. juglandis* (Vauterin et al. 1995) causes bacterial blight mainly on leaves, the green husk of the nut and fresh shoots. According to EPPO (2025), the pathogen is present in most countries in Europe, including Hungary. Bacteria of the genus *Brenneria* cause bark cankers mainly on woody parts, thicker branches and the trunk. *Brenneria nigrifluens* (Hauben et al. 1998) causes shallow bark canker, and the dark brown areas of dying tissues can be found in the outer bark. In contrast, *Brenneria rubrifaciens* (Hauben et al. 1998) causes necrosis and cracking of deeper bark tissues. Both diseases are characterised by a brownish oozing from the affected tissues. Recently described *Brenneria izbisi* (Gašić et al. 2022) causes longitudinal black lesions and bark cracking on young walnut trees. *Brenneria roseae subsp. roseae* was described from sessile and pedunculate oak trees affected by Acute Oak Decline (Denman et al. 2014; Brady et al. 2014; Bakhshi-Ganje et al. 2020); however, a recent study reported occurrence of this bacterium in walnut trees with shallow bark canker symptoms (Allahverdipour et al. 2021). *Brenneria rubrifaciens*

is known to occur in Europe only in Spain (González et al. 2002). *Brenneria nigrifluens* is more widespread in Europe, including Spain, Italy, France, Serbia and Hungary (López et al. 1994; Saccardi et al. 1998; Ménard et al. 2004; Popovic et al. 2013; Végh et al. 2014, respectively). *Brenneria izbisi* is known only from Serbia (Gašić et al. 2022), while walnut shallow bark canker caused by *Brenneria roseae subsp. roseae* is reported only from Iran.

During the summer of 2023, in the central region of Hungary, numerous symptoms characteristic of bark canker caused by *Brenneria* bacteria, with bark cracking and brown oozing spots of varying sizes, appeared in a mature walnut orchard of 4 ha (Fig. 1). The proportion of symptomatic trees was close to 10%. In order to identify the causative pathogen, samples of necrotic tissue of sapwood were taken from two symptomatic trees.

Material and methods

The sampling area is located near the town of Érd (N 47° 20′ 13″ E 18° 51′ 48″). The orchard is about 20 years old, planted with varieties typical of Hungarian walnut growing (Alsószentiváni 117, Milotai 10). The trees were planted in 8 m inter-row and 10 m between row spacing. The orchard is unirrigated. The trees selected for sampling showed characteristic symptoms (longitudinal bark cracking, brown oozing), and at the time of sampling their canopy development was similar to that of the asymptomatic trees. Samples were taken with a sterile scalpel from lesion margins. The samples were placed in sterile plastic bags and transported to the laboratory in a refrigerated box and prepared in the day of sampling. In the laboratory, samples were macerated in 10 ml of sterile distilled water for 30 min while shaking them using a laboratory orbital shaker (120 rpm), then the solutions were diluted 10x, 100x, 1000x, and all dilutions plated on tryptic-soy agar plates (TSA; 17 g tryptone, 3 g soy peptone, 2.5 g glucose, 5 g NaCl, 2.5 g K_2HPO_4 and 15 g agar–agar in 1000 ml distilled water). After incubation at 25 °C for 48 h, TSA plates were checked, and those were selected on which the bacterial colonies were separated from each other. Among the dominant morphological types of bacteria, those single colonies were selected that showed the typical morphological characteristics of *Brenneria* bacteria (round-shape, white or light cream colour, convex colonies), and pure cultures were obtained.

Fig. 1 Emerging symptoms observed on the trunk of walnut trees. Dark brown bleeding longitudinal cracks (left), smaller wetting crack (top right), and tissue necrosis under the bark (bottom right). Érd, Central Hungary, 2023

For rapid identification of selected isolates, amplification of 16S rRNA gene was performed according to Tailliez et al. (2006). Genomic DNA was purified from freshly harvested cells of an overnight shaken flask culture using a Gentra Puregene DNA extraction kit (Qiagen, Germany) following manufacturer's instructions. The PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels in 1 × TBE buffer and were purified using QIAquick PCR & Gel Clean Up kit (Qiagen, Germany). Sanger sequencing was performed by Microsynth (Balgach, Switzerland). The overlapping partial sequences were aligned by ClustalX and trimmed manually. Sample DNA sequences were compared with the database of the National Centre for Biotechnology Information (NCBI) using the BLAST search tool.

To confirm the taxonomic position of the new bacterial isolates, the sequences of four protein-encoding housekeeping genes, *atpD*, *gyrB*, *infB* and *rpoB*, were determined according to Brady et al. (2008). Evolutionary analysis was performed using maximum likelihood method and Tamura–Nei model (Tamura and Nei 1993) based on the concatenated sequences of the four genes of isolate III/9 and those deposited in the NCBI GenBank database corresponding to reference strains of the genus *Brenneria*. NCBI accession numbers of the sequences used in this study are listed in Supplementary Table 1. The phylogenetic tree was constructed using MegaX (Kumar et al. 2018).

Pathogenicity test was conducted with the new isolate III/9 and *Brenneria nigrifluens* DSM 30175^T, as a positive control. The bark of two-year-old potted walnut graftings cv. 'Milotai intenzív' and 'Bonifác' was surface-sterilized with 70% of ethyl alcohol, and a cross-shaped cut of ca. 2 cm was made in the bark using a sterile scalpel. Ten microliters of a bacterial suspension (ca. 10^6 CFU/ml) was pipetted in the wound, and it was covered with laboratory film. A control was made using sterile distilled water. Five plants of both walnut varieties were treated with each bacterial isolate, using the same number of control plants. The walnut plants were placed in a greenhouse equipped with screen (isolator net) against possible vectors of pathogens. After four weeks, the symptoms were assessed using a simple infection scale (0—no visible symptom, 1—tissue necrosis at the edge of the cuts, 2—spreading necrosis that is no greater than the original cut, 3—a spreading necrosis larger than the original cut). The classification values were used to calculate an infection index, $I = (1 \cdot n_1 + 2 \cdot n_2 + 3 \cdot n_3)/n_{tot}$, where $n_1..n_3$ is the number of trees in the infection category 1..3 and n_{tot} is the total number of infected trees.

Results

The 16S rRNA gene sequences of the newly isolated bacteria strains (III/9 and IV/11) were 100% identical to each other, and they had the highest, 99.7% similarity with that of *Brenneria roseae subsp. roseae* FRB 222^T (accession number KF308291), based on a 1344 bp part sequence of the 16S rRNA gene. The 16S rRNA sequence of strain III/9 was deposited in the GenBank, under accession number PQ896493 (Fig. 2).

The phylogenetic tree based on the concatenated sequences of four housekeeping protein coding genes showed (Fig. 3) that the isolate III/9 clustered with *Brenneria roseae subsp. roseae*, including the Iranian strain BR190, which was isolated from walnut (Allahverdipour et al. 2021), so this result confirmed the taxonomic identity of the newly isolated bacteria.

On control trees, moderate dry tissue browning was observed on the edges of the cut surfaces, while on trees infected with the new isolate III/9 or *B. nigrifluens* DSM 30175^T, tissue necrosis was observed over a larger area, darker in colour and moderate tissue oozing was also observed (Fig. 2). The 'Bonifác' variety proved to be more susceptible, with an infection index of 0.4 for the control and 2.2 for both bacterial treatments. The 'Milotai intenzív' variety had a control value of 0, compared to 1.8 for infection with the *Brenneria nigrifluens* bacterium and 1.6 for the *Brenneria roseae subsp. roseae* bacterium. Bacteria were isolated from one sample each of 'Milotai intenzív' and 'Bonifác' walnut trees infected with *Brenneria roseae subsp. roseae* III/9 using the method described above and identified by 16S rRNS gene sequencing, which confirmed the presence of *B. roseae subsp. roseae* in the tissue showing necrotic symptoms.

Fig. 2 Symptoms in the sapwood of walnut cv. 'Boifác' infected with *Brenneria* bacteria after 28 days of the inoculation. Control (left), *Brenneria nigrifluens* DSM30175^T (middle) and *Brenneria roseae subsp. roseae* III/9 (right)

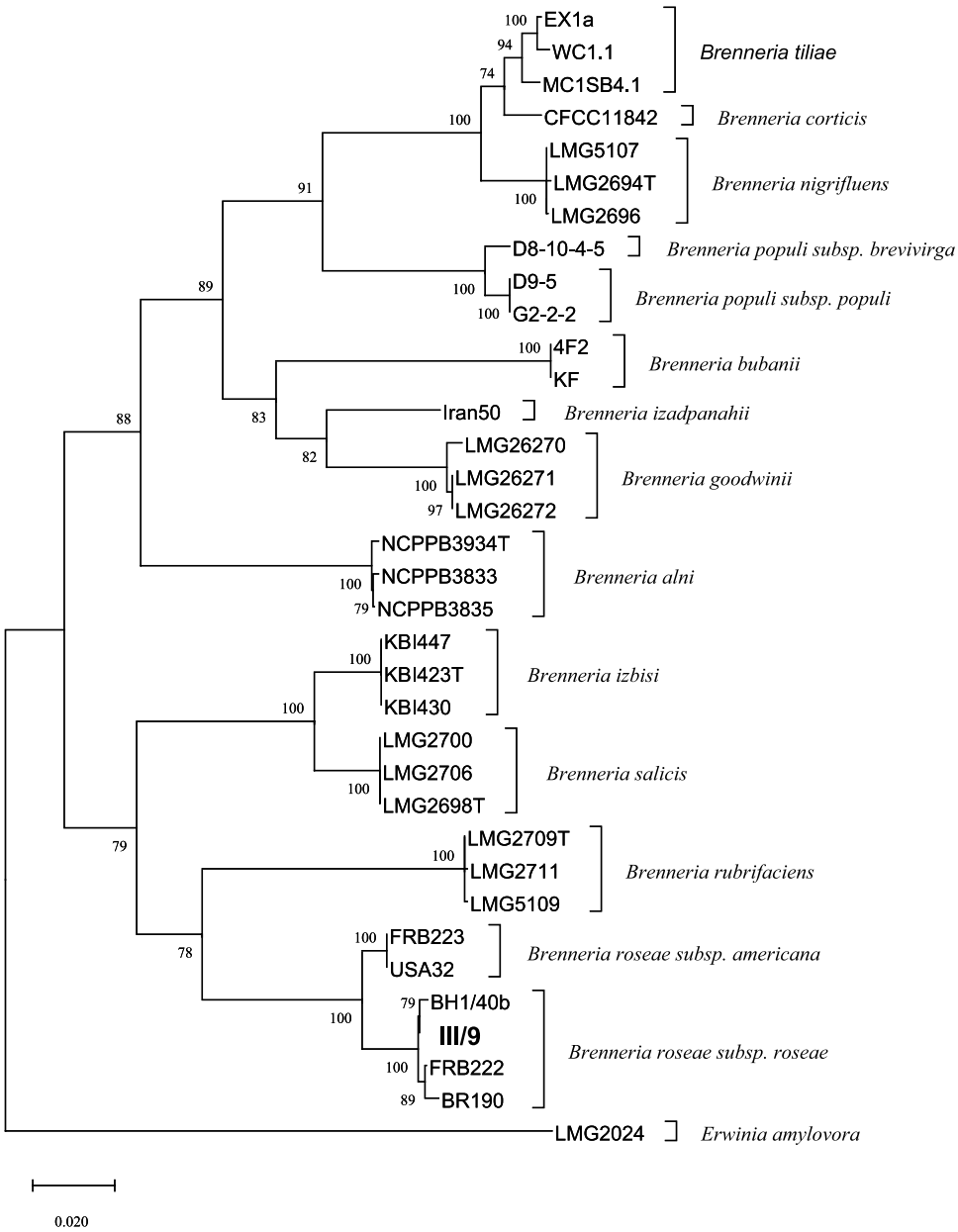

Fig. 3 Phylogenetic tree was inferred by using the maximum likelihood method based on concatenated sequences of *atpD, gyrB, infB* and *rpoB* genes, showing the position of strain III/9 clustered with *B. roseae subsp. roseae*. The evolutionary distances were computed using Tamura–Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 2400 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). Sequence accession numbers are provided in Supplementary Table 1

Discussion

Bacteria of the *Brenneria* genus cause bark canker and tissue necrosis in various deciduous tree species. Among them, *B. nigrifluens* and *B. rubrifaciens*, which cause shallow and deep bark canker diseases of walnut, were known previously in Europe, while *B. izbisi* has been described recently from young walnut trees in Serbia. In 2023, when weeping bark cracks of varying size appeared in a walnut orchard in Central Hungary, one of the three species listed above was suspected as a possible pathogen. However, the bacteria isolated from samples of symptomatic trees belonged to the *B. roseae subsp. rosea* group, which originally described from Acute Oak Decline affected *Quercus petraea* and *Q. robur* trees in Great Britain. *B. roseae subsp. rosea* has been reported from Iran on walnut trees with bark canker disease (Allahverdipour et al. 2021), where it co-occurred with *Gibbsiella quercinecans*. In Hungary, *B. roseae subsp. roseae* was the only pathogenic bacterium detected in newly found symptomatic walnut trees. Pathogenicity tests on walnut graftings confirmed that *B. roseae subsp. roseae* can cause tissue necrosis independently.

Although the present study has demonstrated that *Brenneria roseae subsp. roseae* can cause tissue necrosis on walnut trees, the actual economic significance of the symptoms remains to be explored.

The high prevalence (ca. 10%) of symptomatic trees in the affected orchard suggests that there may be a general

problem behind the appearance of symptoms. A possible explanation could be that Hungary was hit by a very severe drought in the year before the symptoms appeared (Toreti et al. 2022), so that the unirrigated orchards may have been weakened by drought stress. Other *Brenneria* species have been shown to have a higher incidence in stands weakened by abiotic stress factors (Reed et al. 2020; Tkaczyk et al. 2021), but in the present case, this is of course only a speculative explanation and requires further investigation.

We have not received any reports of similar problems in other Hungarian walnut orchards, but one possible reason is that *Brenneria nigrifluens* is already known to be present in Hungarian walnut production (Végh et al. 2014) and the bacterium is less harmful, generally it causes damage in the outer bark only, so Hungarian growers do not pay attention to similar symptoms.

A further difficulty in assessing the current outbreak is that there are very few data available on the distribution and host spectrum of *Brenneria roseae subsp. roseae*. Documented occurrence of this bacterium has so far only been known from Great Britain and Iran. In Great Britain, it was isolated from *Quercus robur* and *Q. petraea* affected by Acute Oak Decline; however, there are no clear data on its pathogenicity in these oak species. In Iran, this bacterium was found in samples of *Q. castaneifolia*, *Q. macranthera* and *Juglans regia*, and it was able to cause tissue necrosis on all three tree species in artificial infection tests (Bakhshi-Ganje et al. 2020; Allahverdipour et al. 2021).

The data presented here are the first to confirm the presence of *Brenneria rosea*e *subsp. roseae* in Central Europe and also confirms that this bacterium is capable of causing bark canker on walnut trees, thus highlighting a potential pathogen threatening European walnut production.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s41348-025-01092-3.

Author contribution K. Kalmár contributed to the pathogen isolation and manuscript compilation and carried out pathogenicity test; Sz. Ádám contributed to the culture of bacterial strains and molecular biological laboratory work; T. Tóth evaluated molecular biological information and assisted in the pathogen identification; T. Lakatos was involved in the conceptualisation and writing and editing the manuscript.

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