THESIS OF PH.D. DISSERTATION

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BUDAPEST

2024

Hungarian University of Agriculture and Life Sciences

Biological diversity of species of *Brenneria* genus

DOI: 10.54598/005550

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Budapest
2024

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1. INTRODUCTION AND OBJECTIVES

The popular deciduous ornamental trees in the streets, public spaces, and parks of Hungarian towns and villages include plane, willow, poplar, birch, horse chestnut, elm and mulberry trees. These fast-growing, long-lived, large trees (e.g. plane species) have been planted for centuries in urban parks throughout Europe as ornamental plants and as street trees in urban areas. While some of these trees thrive in urban environments, others face challenges. However, their presence positively impacts their environment, including people, and they are usually referred to as ecosystem services in the literature. Plants in our environment can provide four main groups of ecosystem services: provisioning, regulating, supporting, and cultural services. However, these services provided by urban trees are significantly affected by changes in climatic conditions, leading to potential declines in tree health and resilience to biotic and abiotic factors. Trees along streets are in the worst situation, because they are almost always tightly surrounded by concrete, and the area with little soil can become compacted, allowing less water and less oxygen to reach the roots. Salinisation (increased soil salinity caused by de-icing salting) poses a further risk to ornamental trees planted in public places, and construction work near construction sites increases the likelihood of damage to these trees. They are also less resilient to fluctuations in environmental factors, and the deterioration of their condition exacerbates the challenges they face. Therefore, our changing climate further hinders their survival. Plants are exposed to these various environmental conditions to which they have limited or no ability to adapt, rendering them vulnerable. Weakened conditions and various types of damage contribute to the colonisation of pathogens and increase the disease risk.

The Hungarian Ornamental Horticulturist Association publishes the “List of Trees in Public Areas” biennially, providing a compilation of species and varieties of woody plants deemed suitable for street planting. The list outlines key characteristics, including their tolerance to urban environments, major pests, and susceptibility to lime. One-third of the plant species listed in the “List of Trees in Public Areas” are categorised as poorly tolerant to urban environments and may eventually face a fate similar to the horse chestnut. A few years ago, *Aesculus hippocastanum* was removed from the list, and the genus is now represented solely by *Aesculus* x *carnea* 'Briotii'. The International Union for Conservation of Nature (IUCN) Red List also designates the endangered species, primarily due to the damage inflicted by the horse chestnut leaf miner. The presence of bacterial species causing bark diseases on deciduous trees in public areas and parks, coupled with the absence of sufficient plant protection, exacerbates the situation. Bark cracking, necrosis, canker, and the distinctive dark, blackish, or brownish bacterial oozing from wounds represent typical symptoms associated with bacterial species of

the genera *Brenneria* and *Lonsdalea*, which are widespread pathogens worldwide. The genus *Brenneria* currently comprises 11 species (*B. alni*, *B. bubanii*, *B. corticis*, *B. goodwinii*, *B. izbisi*, *B. nigrifluens*, *B. populi*, *B. roseae*, *B. rubrifaciens*, *B. salicis*, *B. tiliae*), and the genus *Lonsdalea* comprises four species (*L. britannica*, *L. iberica*, *L. populi*, *L. quercina*). In Europe, various species have been reported in countries including Great Britain, Belgium, the Netherlands, France, Italy, Spain, and Serbia. Bacterial species from the *Brenneria* and *Lonsdalea* genera have been isolated from plant species such as *Alnus*, *Ficus*, *Juglans*, *Populus*, *Salix*, *Tilia*, and *Quercus*. In Hungary, *Brenneria* and *Lonsdalea* species have also been identified, with four bacterial species recognised so far: *Brenneria nigrifluens* affecting Persian walnut and London plane, *B. salicis* on willow, *B. bubanii* on Persian walnut and hybrid poplar, and *Lonsdalea populi* on hybrid poplar. All these species induce similar symptoms. The presence of bacterial species, *Brenneria* and *Lonsdalea*, in Hungary increases the challenge of plant protection in public areas. These pathogens mainly target old trees, posing difficulties in treatment. Firstly, there are no permitted and effective plant protection products against these bacteria in public areas. Secondly, treatments can only be carried out in the evening or at night after adequate public notification [FVM Decree 43/2010 (V.23.) on plant protection activities]. On the other hand, it is crucial to prevent the bacteria's spread during pruning by following hygiene practices, such as disinfecting tools. However, this may lead to a slowdown in the work, and necessitate a change in the routine for pruning workers.

In Hungary, bacterial bark diseases affecting Persian walnut and deciduous ornamental trees are among poorly studied areas, and there is little experience and research available in relation to bacterial species belonging to the genus *Brenneria*. Therefore, we set the following goals:

1. Isolation and identification of bacterial species causing bark disease on deciduous ornamental trees and Persian walnut trees in public areas and parks in different parts of Hungary using traditional and molecular methods,
2. Analysis of the phylogenetic relationships of isolates of the genus *Brenneria*, comparison of Hungarian isolates available in the National Centre for Biotechnology Information (NCBI) database.

2. MATERIALS AND METHODS

2.1. Location and date of sampling

Between 2012 and 2019, samples were collected from various ornamental tree species and Persian walnut trees in public spaces, parks, and woodland, across different regions of Hungary. The identification of pathogens responsible for the symptoms took place between 2015 and 2019 at the laboratory of the Department of Plant Pathology, Institute of Plant Protection, Buda Campus, Hungarian University of Agriculture and Life Sciences.

2.2. Materials

2.2.1. *Isolates*

A total of 39 isolates of eight plant species (*Aesculus hippocastanum*, *Betula pendula*, *Juglans regia*, *Morus alba*, *Platanus* x *acerifolia*, *Populus nigra*, *Salix alba*, *Ulmus* sp.) were collected from 13 sites.

2.2.2. *Primers*

To identify the genus level of pathogens, an analysis of the 16S rRNA gene was conducted using the 63F/1389R oligonucleotide primers (Brosius et al., 1978). Based on the literature, the 16S rRNA gene sequence is unsuitable for the differentiation of closely related bacterial species (Hedegaard et al., 1999, Spröer et al., 1999). Housekeeping genes are more reliable for phylogenetic analysis, and their multilocus sequence analysis (MLSA) has proven to be effective in resolving taxonomic questions across various bacterial genera (*Pantoea*, *Tatumella*, *Erwinia*) (Brady et al., 2009, 2010, Moretti et al., 2011). When selecting the four housekeeping genes, the literature related to the description of *Brenneria* species was considered. Generally, the gene sequences analysed included *atpD*, *infB*, *gyrB,* and *rpoB* (Brady et al., 2012, 2014, Denman et al., 2012, Kile et al., 2022).

2.3. Methods

2.3.1. *Observation of symptoms*

Symptoms' characteristics of *Brenneria* and *Lonsdalea* species were primarily observed on ornamental trees within urban environments; especially during the late summer and autumn the warm, humid weather conditions can have a favourable effect on the growth of bacteria. These often lead to oozing from small cracks and wounds. Under favourable conditions, bacterial oozing can darken the trunk of trees for several meters in length.

2.3.2. *Isolation and morphological characterisation*

Following sampling, the bark and exudate samples were refrigerated at four ºC until processing. Small sections of bark were placed in sterile plastic tubes, and sterile distilled water was added. The same procedure was applied to the exudate samples. A volume of 100 µl was spread onto a medium (King B) (King et al., 1953).

IDENTIFICATION OF PATHOGENS BY TRADITIONAL METHODS

2.3.3. *Gram test*

One colony was removed from a 24-hour pure culture of isolates using a sterile toothpick, and placed on a slide, and homogenised with a drop of 3% potassium hydroxide (KOH) solution (Suslow et al., 1982).

2.3.4. *Hypersensitivity test*

The hypersensitive response was tested on tobacco plants. A suspension of the isolates (10^7 CFU) prepared from a 24-hour pure culture, was injected into the leaves and assessed after 24 and 48 hours (Klement, 1963). Each test included a positive control suspension, using either our *Erwinia amylovora* (Ea1) isolate or *B. rubrifaciens* type strain (DSM 4483).

2.3.5. *Biochemical characteristics*

Biochemical tests were performed using API 20E Kit (BioMérieux). According to the manufacturer's instructions, the microtubes were filled with bacterial suspension, incubated at 26 ºC and 24 hours later evaluated.

2.3.6. *Pathogenicity test*

Pathogenicity tests were conducted on one- and two-year-old saplings of *Aesculus hippocastanum, Betula pendula, Juglans regia, Morus alba, Platanus* x *acerifolia, Populus nigra, Populus* x *euramericana, Salix alba* and *Ulmus pumila*, and were inoculated with bacterial suspension (10^7 CFU) of our isolates. Controls were treated with sterile distilled water, and the positive controls were inoculated with *Brenneria salicis* DSM 30166 and *Lonsdalea populi* NY060. Two methods were conducted to prove the pathogenicity of the isolates. In the first method, bacterial suspension was injected into the leaf base of leaves at the shoot tips. Following inoculation, the infected shoots were enclosed in transparent plastic bags for 72 hours, and sterile distilled water was sprayed inside the bags to provide humidity for the infection. In the second method, as outlined in a previous study (Li et al., 2014), the trunks of the saplings were disinfected with 75% ethanol, 1-1.5 cm long wounds were cut with a sterile scalpel and were inoculated with bacterial suspension. After inoculation, the wounds were sealed with Parafilm to prevent drying. In both tests, the symptoms were evaluated three months later.

2.3.7. *Rapid identification of Brenneria nigrifluens on Persian walnut fruits*

Based on the rapid identification method of Moretti and Buonaurio (2010), the fruit was inoculated with bacterial suspension. Following inoculation, the fruits were placed in sealable plastic boxes with layers of paper towels soaked in distilled water. Distilled water was then sprayed on the inner sides before sealing the boxes, to provide humidity for the infection. The fruits were then kept at room temperature (~26 ºC) for seven days, until evaluation.

IDENTIFICATION OF PATHOGENS BY MOLECULAR METHODS

2.3.8. *Analysis of nucleic acid sequences*

To generate phylogenetic trees, gene sequences for 16S rRNA, *atpD*, *gyrB*, *infB* and *rpoB* from the *Brenneria* and *Lonsdalea* genus strains available in the NCBI GenBank. The substitution model and parameters were selected based on the model test available in MEGA7 (Molecular Evolutionary Genetics Analysis) software (Nei and Kumar, 2000, Kumar et al., 2016). The phylogenetic tree was generated using the MEGA7 software (Kumar et al., 2016).

For the multilocus sequence analysis (MLSA), four alignments were concatenated using MEGA11 software (Tamura et al., 2021). The substitution model and parameters selected were based on the model test available in MEGA11. The phylogenetic tree was generated using the MEGA7 version.

The phylogenetic tree was generated using the MEGA7 software, similar to the 16S rRNA gene. In both cases, pairwise comparison was conducted in the CLC Genomics Workbench 8.5 software on alignments created to the phylogenetic trees (Internet1).

3. RESULTS

Between 2012 and 2019, samples were collected from trees with bark disease and bacterial oozing at 13 sites. A total of 39 isolates were characterised and analysed by traditional and molecular methods. Two isolates were from *Juglans regia* host, collected in Zánka and Látrány. One isolate was from the host *Salix alba*, collected in Budapest. Eleven isolates were collected from *Platanus* x *acerifolia* in Zamárdi, Bázakerettye, Siófok and Balatonfüred. Ten isolates were from *Ulmus* host, collected in Budapest and Kecskemét (elm forest). Seven isolates were collected from *Betula pendula*, host in Budapest, Szentendre, Leányfalu and Nyírderzs. One isolate was collected from *Populus nigra*, and two isolates were collected from *Morus alba* hosts in Budapest. Five isolates were collected from *Aesculus hippocastanum* host in Budapest, Ebes and Mátészalka.

3.1. Symptoms caused by the pathogens

Between 2012 and 2019, characteristic symptoms were observed on ornamental trees and Persian walnuts across different locations in Hungary. The trunk of the trees showed shallow vertical cracks in varying sizes, except willow, on which substantial longitudinal bark crack and partial branch dieback were seen. From late summer bacterial oozing was observed from these bark cracks, pruning wounds, and injection points on trees subject to plant protection treatments (horse chestnut). The quantity (smaller volume), consistency, and colour (dark brown) of exudates were comparable to those found on walnuts, mulberries, poplar, birches, planes, and horse chestnuts. The appearance of bacterial ooze differed slightly on elms, with a denser, slimier consistency, a shiny surface, and a higher amount on the bark of the trunk. Symptoms were observed on woody plant species in public areas, parks and gardens. In urban environments, plants are subjected against stress, presumably reducing their resistance and making them more susceptible to various biotic factors. In addition, climate change is another factor that may contribute to a further weakening of tree conditions.

3.2. Results of traditional methods

3.2.1. *Morphological characteristics*

The isolates from Persian walnut, white mulberry, white willow, black poplar, silver birch, London plane, elm, and horse chestnut grew on King's medium B at room temperature (~26 ºC). The colonies of our four isolates were identical: convex, round, white, slightly bluish, had entire margins, and slightly raised from the surface of the medium.

3.2.2. *Gram test*

The 3% KOH solution dissolved the bacteria's cell wall in all isolates, so the species isolated from Persian walnut, white mulberry, white willow, black poplar, silver birch, London plane, elm, and horse chestnut were Gram-negative.

3.2.3. *Hypersensitivity test*

In all cases, strains of *Erwinia amylovora* (Ea1) and *Brenneria rubrifaciens* (DSM 4483) caused necrosis on leaves of tobacco plants (*Nicotiana tabacum* L. cv ˈXanthiˈ) inoculated with their suspensions. Tissue necrosis did not develop on tobacco leaves inoculated with suspensions of 39 isolates from Persian walnut, white mulberry, white willow, black poplar, silver birch, London plane, elm, and horse chestnut.

3.2.4. *Biochemical characteristics*

API 20E biochemical tests were performed with the suspension of all 39 isolates.

The biochemical characteristics of the five isolates collected from horse chestnut were the same in most cases (18 reactions), more or less homogeneous, and compared to the Aes1 isolate, giving different results in two reactions. In addition, the Aes1 isolate has given the same results for all reactions as the London plane isolates. The other isolates from horse chestnut were identical to the Juglans1 strain in 18 characteristics.

The seven isolates from silver birch trees have given different results for tryptophan deaminase, glucose fermentation/oxidation and sorbitol fermentation/oxidation reactions but the same results for all other reactions. The difference in the properties of the isolates suggests that they form a heterogeneous group. Furthermore, the Bet1 isolate was identical to the Juglans2 strain in 18 characteristics. Bet2 and Bet3 isolates were similar to the Juglans2 strain in 17 characteristics. Strain Bet4 has given the same results in 17 reactions to Pop1, Juglans2 and ten strains from London plane.

The Bet5 isolate was identical to Pop1 and Juglans2 isolates in 17 characteristics. For strains Bet6 and Bet7, the isolate Pop1 has given the same results in 18 reactions.

The ten isolates collected from elm trees in Budapest and Kecskemét had the same characteristics and formed a homogeneous group. In addition, the Juglans2 and Pl6 isolates were identical to them in 18 properties.

Compared to each other, Juglans1 and Juglans2 isolates gave different result for almost half of the reactions (9 reactions). Juglans1 was identical with Pl1, Pl2, Pl3, Pl4, Pl5, Pl7, Pl8, Pl9, Pl10 and Pl11 in 18 characteristics, and Juglans2 has given the same results in 18 properties to Aes1, Bet1, ten elms and eleven London plane isolates.

The ten strains from London plane formed a homogeneous group, giving identical results in all biochemical reactions, with one exception, the Pl6 isolate, which differed in two reactions. In addition, Pl6 has given the same results in 18 characteristics to Aes1, Juglans2 and ten isolates from elm trees.

The biochemical reactions of the two isolates collected from mulberry trees were homogeneous and identical in all characteristics. Compared to isolates from other host plants, 14 reactions have given the same results.

3.2.5. *Pathogenicity test*

Pathogenicity tests were performed on 37 of our isolates.

Shoot infection:

Small necrotic lesions at the infection site were observed in all our isolates. In one isolate from willow, ten from elms and five from London planes, wilting, browning, drying and shoot dieback were observed close to the inoculation site. When the shoots were cut in half, black bands and weaker symptoms were visible in the case of our isolates from one walnut (Juglans1), two mulberry trees, one black poplar, seven silver birches, ten London planes and five horse chestnuts. Disease-associated bark cracking and oozing was not observed in the case of our isolates – even after three months.

Trunk wounding:

Trunk wounding was performed on 18 of our isolates. The wounds on the trunk healed in most cases; necrosis and brown bands became visible by cutting the trunk in half. These symptoms were observed in two isolates from mulberry, one from black poplar, seven from birch, five from London plane and three from horse chestnut. All isolates from birch trees showed different symptoms, in 1-1 replicates. Around the wound blackening was observed two months after infection, and when the shoots were cut in half, it was evident that it did not penetrate deeper. No necrosis or dark bands were observed in control plants inoculated with water.

3.2.6. *Rapid identification of Brenneria nigrifluens on Persian walnut fruits*

Following the rapid identification method developed by Moretti and Buonaurio (2010), 29 isolates from one Persian walnut, one white willow, six London planes, ten elms, seven silver birches, one black poplar, two white mulberries and one horse chestnut were used to inoculate green walnut fruits.

In the case of Bet1, Bet2, Bet3, Bet5, Bet6, Bet7, Pl6, Salix1, Ulmus1, Ulmus2, Ulmus3, Ulmus4, Ulmus5, Ulmus6, Ulmus7, Ulmus8, Ulmus9, Ulmus10, Morus1 and Morus2 isolates, cells were necrotic around the inoculation site, but no bacterial oozing was visible and the kernel remained healthy. When evaluating infection with isolates Aes1, Juglans1, Pl7, Pl8, Pl10 and Pl11, necrosis was observed around the inoculation site, and the nut kernel was slightly browned and became sunken and water-soaked. Infection with Bet4 and Pl9 isolates resulted in necrosis around the inoculation site, and blackening and damaging of the kernel were seen. In the case of Pop1 isolate, in addition to necrosis around the inoculation site, bacterial sap oozing was also observed.

3.3. Results of molecular methods

3.3.1. *Identification based on the analysis of the 16S rRNA gene*

A DNA fragment of approximately 1300 base pairs was amplified using the 36F/1389R primer pair. Subsequently, the amplified fragments were sequenced after cloning. During the pairwise comparison, the sequences of 37 of our isolates were compared with the sequences of 22 *Brenneria* strains downloaded from the NCBI GenBank. The following isolates were the closest relationship with *B. populi* Hauben et al. 1998 strain YMAPO5: Juglans1, Juglans2 (94,45-94,52%), Salix1 (97,26%), Pl1-11 (91,19-94,60%), Ulmus1-10 (93,41-93,86%), Bet5 (85,05%), Pop1 (96,60%), Morus1, Morus2 (92,75-92,97%), and Aes1, Aes2, Aes3 (91,71%). In addition, the sequence of *B. nigrifluens* strain OR1 showed 89,40-89,92% identity with Bet1, Bet3, Bet4, and Bet7 isolates and 92,27% identity with Aes4 isolate. Furthermore, the sequence of isolate Bet2 was 89,14% and isolate Aes5 was 90,10%, identical to the sequence of *B. tiliae* strain EX1a.

The phylogenetic tree based on partial 16S rRNA gene sequences shows the relationships of 37 isolates' sequences to sequences of 35 strains of the *Pectobacteriaceae* family (*Brenneria* spp., *Dickeya* spp., *Lonsdalea* spp., *Pectobacterium* spp.). Isolates collected from Persian walnut (Juglans1), ten isolates from London planes and five isolates from horse chestnuts were located near each other on a separate branch. Of the isolates of London plane, Pl1, Pl2, Pl7, Pl10, and Pl11 formed a cluster, isolates Pl3, Pl4, Pl5, Pl9, Juglans1, and Pl8 were grouped in a separate cluster, and isolates collected from horse chestnut (Aes1, Aes2, Aes3, Aes5) were located in a third cluster. Strain Aes4 was located separately from London plane and horse chestnut isolates. Among the *Brenneria* species available in the NCBI GenBank database, the closest related strains to these isolates were *B. corticis* gBX10-1-2 from hybrid poplar, *B. tiliae* (WC1b.1, EX1a) isolated from linden, *B. nigrifluens* (DSM 30175, OR1, and BNK1) isolated from Persian walnut, and our isolates Juglans2 and Pl6. The isolates collected from silver birch trees differed slightly but were grouped into a distinct clade. They were located between two clades on the phylogenetic tree, *B. corticis*, *B.*

tiliae and *B. nigrifluens* species mentioned above, and *B. alni* (from alder) and *B. populi* Li et al. 2015 (from hybrid poplar). Our isolate Pop1, collected from black poplar, and the type strain *B. populi* Hauben et al. 1998 NCPPB 4299, from hybrid poplar, and the strain *B. populi* YMAPO5 from black poplar, were grouped on the same branch. The isolate with the identification code Salix1 (from willow) was located on the same branch with the type strain *B. salicis* LMG 2698, isolated from willow. Our two mulberry isolates were grouped on a separate branch, which were closely related to the *B. izbisi* (KBI 423^{T}, KBI 429) strains collected from Persian walnut, the type strain *B. salicis* (LMG 2698), and the *B. populi* Hauben et al. 1998 (NCPPB 4299, YMAPO5) isolates. The elm isolates were almost identical and were located on the same branch in the same clade. The closest related species were *B. bubanii* (4f2, Kf) - identified in Hungary - and *B. goodwinii* (LMG 26270, LMG 26272, Mf3-1, J1-1, Sz2) species.

3.3.2. *Identification based on the analysis of the housekeeping genes*

In the pairwise comparison, the sequences of 12 of our isolates were compared with the sequences of 15 *Brenneria* strains downloaded from the NCBI GenBank. The sequences of isolates from London plane trees showed an identity ranging from 83,01% to 83,83% with the sequences of *B. populi* subsp. *brevivirga* D8-10-2-5. Similarly, the isolates collected from elm trees were identical to the sequences of *B. populi* subsp. *brevivirga* D8-10-2-5 in the range of 86,90% to 89,05%.

The phylogenetic tree based on the partial sequences of four housekeeping genes from five London plane trees (Pl7, Pl8, Pl9, Pl10, Pl11) and seven elm tree (Ulmus1, Ulmus2, Ulmus4, Ulmus5, Ulmus6, Ulmus7, Ulmus8) shows the relationship to 35 bacterial strains of the family *Pectobacteriaceae* (*Brenneria* spp., *Dickeya* spp., *Lonsdalea* spp., *Pectobacterium* spp.). The isolates collected from elm trees differed slightly from each other but were located on a separate branch, distinct from the *Brenneria* type strains. They were the most closely related to the type strains *B. populi* Li et al. 2015 D9-5 and *B. populi* subsp. *brevivirga* D8-10-2-5, isolated from a canker of *Populus* x *euramericana*. Isolates from London plane trees differed and were grouped in a separate cluster. The Pl7 isolate was located alongside but distinct from the other strains. The closest related species are *Brenneria goodwinii* LMG 26270 and LMG 26262 isolates, isolated from oak trees showing AOD symptoms.

4. CONCLUSIONS, RECOMMENDATIONS

In Hungary, our research contributes to a complete knowledge of the bacterial species causing bark disease and bacterial oozing on Persian walnut and ornamental trees. These are considered necessary because the horse chestnut species studied are already listed as threatened on the IUCN Red List, mainly due to the damage caused by *Cameraria ohridella* and its consequences (Allen and Khela, 2017). There are many unanswered questions about *Brenneria* and *Lonsdalea* species, their distribution, environmental requirements, tolerance, and long-term effects of infection that need to be known to consider which species should be prevented from spreading further, as they further degrade our already declining and valuable urban ornamental trees. We also need to prevent more species from becoming endangered and thus even closer to extinction.

Since 2012, bark cankers and bacterial oozing were observed on Persian walnut trees, ornamental trees (*Aesculus hippocastanum*, *Betula pendula*, *Morus alba*, *Platanus* x *acerifolia*, *Populus nigra*, *Salix alba*, *Ulmus* sp.), and in an elm forest. The symptoms closely resembled those documented in the literature for *Brenneria* and *Lonsdalea* on various plant species. The morphology of bacterial strains isolated from symptomatic trees aligned with the descriptions provided for previously published *Brenneria* species. Additionally, the isolated bacterial strains showed consistency with findings in the literature for *Brenneria* and *Lonsdalea* species based on their Gram characteristics (negative) and hypersensitivity (no tissue necrosis, except for *B. rubrifaciens*) (Surico et al., 1996, Saccardi et al., 1998 , Brady et al., 2012, Hauben and Swings, 2015, Kile et al, 2022).

Two methods were performed to confirm pathogenicity: infecting shoots with 37 isolates and inoculating the trunk with a suspension of 18 isolates. During the evaluation three months later, no bacterial oozing was observed, and no cankers formed, consistent with observations by Kile et al. (2022), who infected linden shoots with *B. tiliae,* reporting only necrotic lesions 11 weeks after inoculation. A Spanish study (one month after infection) also noted no bacterial oozing or canker development but observed callus formation at the infection point, absent in the control (Biosca et al., 2006). Additionally, in a study on the pathogenicity of *B. izbisi*, strains were inoculated into 2-year-old walnut plants. After 14 months, lesions appeared on the bark, revealing brown necrotic bands upon removal, although no exudate oozing or cankers were observed (Gašić et al., 2022). However, in a pathogenicity test with *B. nigrifluens*, Saccardi et al. (1998) observed oozing and blackening five months after inoculation. Considering these findings, members of *Brenneria* might require more time, particularly under optimal environmental conditions, to develop cankers and bacterial oozing. Additional factors, such as

the age of the plants used for inoculation, could also influence the outcomes. These factors likely contribute to the observed symptoms, explaining why the method we used - as published by Li et al. (2014) – did not result in the same. Notably, in their field inoculations Li et al. (2014) observed cankers on poplar trees along with the characteristic white exudates oozing.

The biochemical characteristics of all our isolates aligned consistently with those of the type strains (*Brenneria alni* PVfi20, *B. bubanii* 4f2 ^{T}, *B. corticis* gBx10-1-2^{T}, *B. goodwinii* LMG 26270^{T}, *B. izbisi* KBI 423^{T}, *B. nigrifluens* DSM 30175^{T}, *B. populi* Li et al. D9-5^{T}, *B. salicis* LMG 2698^{T} és *B. tiliae* WC1b.1^{T}) in the following reactions: lysine decarboxylase, indole production, gelatinase, sucrose fermentation/oxidation, and arabinose fermentation/oxidation.

The isolates collected from five horse chestnut trees demonstrated identity with the type strain *B. corticis* (Aes1: 17 reactions; Aes2-5: 15 reactions) and the type strain *B. nigrifluens* (Aes1: 17 reactions; Aes2-5: 15 reactions) in most reactions. Isolates from birch trees showed greater diversity than type strains with similar characteristics, although differences existed among isolates. Bet1 showed similarity with *B. bubanii*, *B. corticis*, and *B. nigrifluens* in 17 reactions, while Bet2 and Bet3 isolates showed similarity with the same species in 16 characteristics. The Bet4 isolate was identical to *B. bubanii*, *B. corticis*, *B. nigrifluens*, *B. salicis*, and *B. tiliae* in 18 reactions. The Bet5 isolate resembled the strain *B. corticis* in 18 reactions. Isolates Bet6 and Bet7 were identical in 17 reactions with *B. corticis*, a *B. nigrifluens*, and *B. tiliae*. Isolate Pop1, obtained from poplar, showed similarity in 17 reactions with *B. tiliae*. The isolates from elm trees were similar in 18 characteristics to *B. goodwinii*. Our Juglans1 isolate showed similarity in 14 reactions with the type strain *B. alni*, and our Salix1 isolate was identical in 14 reactions with the type strains *B. alni*, *B. corticis*, and *B. nigrifluens*. Juglans2 isolate and those from London planes (except for Pl6) were identical to *B. corticis* and *B. nigrifluens* in 17 reactions. The Pl6 isolate aligned with the *B. goodwinii* in 18 characteristics. Isolates collected from mulberry trees showed similarity with *B. corticis* and *B. goodwinii* strains in 14 reactions.

The tests on green walnuts revealed that all isolates induced symptoms. Even though isolates of the type strains, including *B. salicis* DSM 30166, did not infect the fruits, our isolate (Salix1) collected from willow trees induced blackening at the inoculation point. Additionally, our other isolates collected from other host plant species caused various symptoms - such as necrosis and blackening of the walnut kernel.

In molecular studies, certain gene sequences (*atpD*, *gyrB*, *infB*, *rpoB*) in a few isolates could not be amplified even after multiple attempts or multiple non-specific products were generated. Despite our efforts to identify the source of the problem, we were unsuccessful. Brady et al. (2008, 2014) and Kile et al. (2022) have reported that adjusting the primer annealing

temperature, using lower temperatures such as 46 ºC and 50 ºC instead of 55 ºC improved the amplification of housekeeping gene sequences of some strains. However, another study achieved favourable results by increasing the annealing temperature (Erjavec et al., 2019). Considering these findings, it might be possible to amplify the missing gene sequences of our isolates by adjusting the annealing temperature or by modifying the concentration of specific PCR components ($MgCl^2$, primers, and DNA template).

Based on the phylogenetic tree analysis of partial 16S rRNA gene sequences and biochemical characteristics, isolates from Persian walnut, London planes, and horse chestnuts were found to be most closely related to *B. corticis* isolated from hybrid poplar and *B. nigrifluens* isolated from Persian walnut. However, in the phylogenetic tree based on concatenated sequences of four housekeeping genes, the closest related species was *B. goodwinii* (LMG 26270^T and LMG 26262), isolated from oak trees. Nevertheless, the pairwise comparison of four housekeeping gene sequences showed the highest similarity to *B. populi* subsp. *brevivirga* (83,01-83,83%). Additionally, isolates collected from silver birches were positioned on the phylogenetic tree adjacent to *B. corticis* and *B. nigrifluens* species, which are closely related based on their biochemical characteristics as well. They were grouped on the same branch as our isolate Pop1, collected from black poplar, and strains of the bacterial species *B. populi* Hauben et al. 1998 (hybrid poplar). The 16S rRNA sequence of *B. populi* showed similarity to our isolate's sequence by 91,32-96,60%, according to the pairwise comparison. However, in contrast, the biochemical characteristics showed more similarities with *B. tiliae*. The isolate Salix1 (from willow) was positioned on the same branch as *B. salicis* LMG 2698, isolated from willow. Nevertheless, pairwise comparison and biochemical characteristics revealed differences; with *B. populi* Hauben et al. 1998 strain YMAPO5, the two sequences were 97,26% identical to *B. corticis* and *B. nigrifluens*. Our two isolates from mulberry trees were placed on a separate branch, closely related to the two *B. izbisi* (KBI 423^T, KBI 429) isolates collected from Persian walnut, *B. salicis* type strain (LMG 2698), and *B. populi* Hauben et al. 1998 (NCPPB 4299, YMAPO5) isolates identified from poplar. Additionally, pairwise comparison indicated 92,75-92,97% similarity with *B. populi* Hauben et al. 1998 strain YMAPO5 but a higher similarity in biochemical characteristics with other species (*B. corticis*, *B. goodwinii*).

The isolates from elm trees were almost identical and were on a separate branch. The most closely related species to them, based on the 16S rRNA gene sequences, were *B. bubanii* (4f2, Kf) - identified in Hungary - and *B. goodwinii* (LMG 26270, LMG 26272, Mf3-1, J1-1, Sz2) species. Based on the multilocus sequence analysis, *B. populi* Li et al. 2015 and *B. populi* subsp. *brevivirga* were the most closely related species, and the pairwise comparison of the sequences of four housekeeping genes results also showed the highest similarity with *B. populi* subsp.

brevivirga (86,90-89,05%). However, the biochemical characteristics showed the highest similarity with *B. goodwinii* in most reactions.

The phylogenetic tree based on the sequences of the 16S rRNA gene differed from the MLSA phylogenetic tree in several aspects: the type strains were situated differently, as were the strains isolated from London planes and elm trees. However, other studies have concluded that within the order *Enterobacterales*, 16S rRNA gives reliable results for genus-level determination but not for species-level identification (Naum et al., 2008, Maddock et al, 2022). On this basis, the phylogenetic tree of 16S rRNA indicates that our isolates belong to the genus *Brenneria* within the family *Pectobacteriaceae*. In addition, it is assumed, that the Salix1 strains isolated from willow is *Brenneria salicis*. Furthermore, isolate Pop1 collected from black poplar is presumed to belong to the species *B. populi* Hauben et al. 1998, which is also identified in poplar, but its biochemical characteristics are not available to confirm this.

The 16S rRNA and MLSA phylogenetic tree showed similarly that the sequences of isolates from elm and London plane were on separate branches, suggesting that they may be novel *Brenneria* species - but further studies are needed to confirm this. In addition, the strains isolated from Pl7, Pl8, Pl9, Pl10, and Pl11 from London planes differed slightly. Similar experiences were reported by Brady et al. (2012), who observed a genetic variability in *Lonsdalea britannica* isolates collected from oak trees based on 16S rRNA and four housekeeping genes (*atpD*, *gyrB, infB, rpoB*), which they assumed could be due to their different collection sites. In addition, Kile et al. (2022) reached a similar conclusion for *B. tiliae*. These also explain the differences in our isolates (Pl7, Pl8, Pl9, Pl10, Pl11) from the same host plant but with differences in the phylogenetic tree.

Recommendations for further research:

- Performing additional molecular tests: Optimise PCR conditions, including modifications of magnesium, primer, and DNA concentrations in the PCR mixture; consider Gradient PCR. These optimisations will enable the supplementation of phylogenetic trees.
- Conduct fatty acid analysis and DNA-DNA hybridisation with the isolates to gather comprehensive data on their characteristics.
- Perform additional biochemical tests using API 20 NE, API 50 CHB/E, and Biolog GN2 Microplate to supplement the biochemical profiles of the isolates.
- Generate electron microscopic images to determine the morphological characteristics of bacterial cells.
- Assessing additional symptomatic plant species and oak trees, including those affected by acute oak decline.

- Detecting potential vectors involved in the spread of bacterial diseases.
- Conducting research in the rhizosphere microbiome in Hungary based on studies conducted in the United Kingdom. Compare bacterial species occurring in the rhizosphere of healthy and symptomatic plant species.
- Examining the interaction of bacterial species, based on the research of Brady et al. 2022.

5. NEW SCIENTIFIC RESULTS

In Hungary, the identification and characterisation of *Brenneria* species causing bark disease on various ornamental tree species is an under-researched field. In this thesis, a substantial amount of new information has been provided, and the following key points need to be highlighted:

1. We characterised first in Hungary 39 isolates of *Brenneria* – two from Persian walnut, one from white willow, eleven from London plane, ten from elm, seven from silver birch, one from black poplar, two from white mulberry, and five from horse chestnut – using traditional (morphological, Gram, biochemical characteristics, hypersensitivity and pathogenicity tests) and molecular methods (16S rRNA and four housekeeping genes), contributing valuable data to the international research community.
2. We have found that our isolates from *Platanus* x *acerifolia* (Pl7–Pl11) and *Ulmus* sp. (Ulmus1, Ulmus2, Ulmus4–8) could be potential members of two novel species of the genus *Brenneria*. This study also presented, for the first time, sequence data on the four housekeeping genes (*atpD*, *gyrB*, *infB*, *rpoB*) of bacterial species isolated from London plane and elm hosts and analysed them by multilocus sequence analysis.
3. We demonstrated that our isolates from Persian walnut (Juglans1), eleven London planes (Pl1–Pl11), and five horse chestnuts (Aes1–Aes5) could potentially belong to novel species, indicating polyphagy or different subspecies within the same species.
4. Our isolates (Bet1–Bet5, Bet7, Morus1, Morus2) from *Betula pendula* and *Morus alba* trees were found to be distinct from known *Brenneria* species based on their biochemical characteristics and partial 16S rRNA gene sequences and may be novel members of the genus.
5. We identified the pathogen isolated from *Populus nigra* (isolate Pop1), a bacterial species of *B. populi* Hauben et al. 1998. In Hungary, we reported for the first time sequence data on this species's partial 16S rRNA gene sequence and biochemical characteristics.
6. Based on our studies, we have shown that isolates collected from Persian walnut (Juglans2) and London plane (Pl6) belong to the species *Brenneria nigrifluens*, and the London plane is a new host for the pathogen based on the analysis of the partial 16S rRNA gene sequence.
7. We firstly described a *Brenneria* species from the host *Aesculus hippocastanum* and reported the characteristics of the isolates, partial 16S rRNA gene sequence data – which distinguish them from known *Brenneria* species; the closest related species are *B. corticis*, *B. nigrifluens* and *B. tiliae*.

6. PUBLICATIONS CONNECTED TO THE DISSERTATION

Articles published in journals with impact factor

Zlatković, M., **Tenorio-Baigorria, I.**, Lakatos, T., Tóth, T., Koltay, A., Pap, P., Marković, M., Orlović, S. 2020. Bacterial Disease on *Populus* x *euramericana* Caused by *Lonsdalea populi* in Serbia. Forests, 11(10): 1080. e-ISSN: 1999-4907; IF: 2,59 (2020)

Tenorio-Baigorria, I., Botyánszki, G., Gyuris, R., Zsigó, Gy., Palkovics, L., Végh, A. 2022. *Brenneria nigrifluens* Isolated from *Aesculus hippocastanum* L. Bark in Hungary. Forests, 13(2): 227. e-ISSN: 1999-4907; IF: 3,23 (2021)

Articles published in peer-reviewed scientific journals

Tenorio-Baigorria, I., Végh, A. Galambos, N., Dávid, O., Palkovics, L. 2017. Díszfák kéregbetegségét okozó baktériumfajok. Növényvédelem, 78(53): 11. 477-484.

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Tenorio-Baigorria, I., Végh, A., Palkovics, L. 2017. Díszfákat fenyegető, újonnan megjelenő baktériumfajok. XX. Bolyai konferencia, Budapest, p. 33.

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