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MSc in Environmental Engineering

ANALYSIS OF MICROBIAL COMMUNITIES ASSOCIATED WITH THE INFECTION OF WALNUT TREE

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Sopron

2025

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MASTER DIPLOMA PIECE TASK STATEMENT

Name of the author of diploma piece: **ALEKSANDR PNEV**

Title of diploma piece: **ANALYSIS OF THE MICROBIAL COMMUNITIES ASSOCIATED WITH THE INFECTION OF WALNUT TREES**

Tasks set for writing the diploma piece:

1. Participating in collecting samples in walnut plantations to reveal the connection of infections and the walnut husk fly.
2. DNA isolation from infected walnuts, leaves and walnut husk flies.
3. Analysis of sequencing data, discussion of the microbial diversity and the potential spreading diseases transmitted by the walnut husk fly.

Consultant: Dr. György Sipos PhD, Dr. Tamás Rétfalvi PhD

The length of the essay is not limited. Please prepare the diploma piece following the formal requirements for this type of work, submit 1 hardcopy an electronic version to the university repository in pdf format, identical to the hardcopy by the deadline specified in the study regulations for the academic year 2024/2025.

Sopron, February 03, 2025

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INTRODUCTION

Walnut trees (*Juglans regia*) are of significant ecological and economic value, which are cultivated around the world for timber and nuts. However, their productivity and viability are increasingly threatened by climate change and drought due to which the plant's immunity is reduced, allowing pathogen invasion, which can lead to substantial economic losses and ecosystem impacts [1]. These challenges highlight the significance of sustainable and ecologically informed plant health strategies.

Microbial communities are the complex networks of bacteria, fungi, and other microorganisms living in and around plants—play a crucial role in plant health and disease. *Xanthomonas arboricola* pv. is mentioned among the bacterial pathogens that cause damage to walnuts. *X. juglandis* (xaj) (bacterial burn, brown tip necrosis) [2], *Brenneria nigrifluens* and *B. rubrifaciens* (cancer) [3], *Ophiognomonia leptostyla* (anthracnose) [4], *Fusarium, Alternaria* (brown tip necrosis) [5] and *Geosmithia morbida* (thousand canker disease) [6]. Studies of listed communities included the study of morphological, biochemical and physiological characteristics of isolates, as well as pathogenicity tests.

Insect vectors play an important role in the transmission of infections. One of these insects is the walnut husk fly (*Rhagoletis completa*), the larvae of which develops in the pericarp layer of walnuts [1].

The **scientific novelty of the work** consists in the need to better understand the microbial ecosystems associated with walnut tree infections. By comparing the microbial profiles of healthy and infected trees, this research seeks to identify key microbial indicators of disease presence and potential protective roles.

Practical Relevance: the findings of this study aim to support the development of sustainable, biology-based disease monitoring and control strategies in walnut cultivation. By identifying microbial indicators and understanding their roles, the research may contribute to early detection methods and eco-friendly alternatives to chemical treatments, promoting long-term tree health and environmental sustainability

Thus**, the final thesis aims** were to examine the microbiota of the walnut husk fly (*Rhagoletis completa*), to identify pathogenic microorganisms capable of infecting walnut trees, and to assess the potential role of the fly in the spread of these pathogens.

This objectives were realized by the following tasks:

1. To determine the composition of the microbiota characteristic to the different developmental stages of the walnut husk fly using modern next-generation amplicon sequencing methods [7].
2. To compare the composition of the microbiota of the walnuts and leaves collected in different counties in Hungary and at different symptomatic stages of walnut trees [8, 9].
3. Based on the data obtained, formulate hypotheses about the role of the microbiota of the walnut husk fly in the health of Walnut (*Juglans regia*) and the potential spread of diseases.

Points to be presented:

During this thesis, several key themes and research components will be explored to address the central problem.

- Establishing a solid theoretical foundation through a critical review of existing literature on plant-associated microbial communities and disease ecology in walnut trees [10].
- Outlining how different parts of the plant samples were collected, how microbial DNA was extracted and sequenced, and which computational tools were employed to analyze the microbial data [11, 1].
- Highlighting the differences in microbial composition between healthy and infected trees using the analyzing results. Particular attention will be paid to identifying microbial taxa that appear to be consistently associated with disease symptoms or, conversely, with tree health [1].
- Discussing Research Implication, including its relevance for biological control strategies and sustainable disease management in walnut cultivation.

Structure of the paper: the thesis is structured as follows: an initial literature review provides the scientific context, followed by a methodology section detailing sample collection, DNA analysis, bioinformatic techniques. The results are then presented and interpreted in the discussion section, with conclusions summarizing key findings and offering practical recommendations for future research and applications

1 LITERATURE REVIEW

1.1 GENERAL INFORMATION

Walnut *(Juglans regia*) is the most widely cultivated in the world's leading commercial nut producer. It is of considerable economic importance worldwide and has a long history of cultivation [12]. *Juglans regia* is distributed in Central and East Asia, Europe and the United States [13], being the most common Juglans species in Europe, and its natural range extends to Central Asia [14]. Walnut (*Juglans regia*) are susceptible to various diseases, including bacterial blight caused by the bacterium *Xanthomonas arboricola pv. juglandis (Xaj)* [2], anthracnose caused by the fungus *Gnomonia leptostyla [4]*, apical necrosis, and bark cancers [3] caused by various bacteria and fungi.

1.2 PATHOGENS RELATED TO THE BROWN APICAL NECROSIS OF WALNUTS

Brown apical necrosis (BAN) is a relatively recently recognized walnut disease (*Juglans regia*) that causes premature fruit loss and significant crop losses in walnut growing regions, including Hungary [12]. This disease has become economically significant in recent years and often affects *Juglans regia* [6].

In the early stages of fetal development, detailed monitoring of externally and internally affected tissues is required to distinguish apical necrosis from walnut blight. Studies conducted in Spanish and Turkish orchards have contributed to the description of the external and internal symptoms of affected fruits that remain on the tree or fall off.

The initial external symptoms appear after fruit tying, in the form of small dark brown or blackish spots without a watery halo located at the apical end of the nuts, sometimes limited by the stigma and the surrounding area. As the fetus grows, the symptoms become more noticeable, representing brown lesions, often round with smooth edges, ranging in size from 2 to 15 mm. The lesions may enlarge and turn brown or reddish-brown with more uneven edges. The external spread of infection on infected fruits remaining on trees is less extensive than on fallen fruits, in which external necrosis can reach the equatorial zone of nuts. On fallen fruits, old apical infections are sometimes covered with a white surface of fungal mycelium [12].

The infection progresses internally from the e picarp to the mesocarp and can reach the seed. Epicarp and mesocarp lesions are dry and hard, but rot develops when

the seed is affected. After the shell hardens, the lesions remain limited to the epicarp and the mesocarp. Apical necrosis on fallen fruits is more severe and spreads to all internal tissues. Sometimes the degree of external and internal lesions is not interrelated, and a small external necrosis may be associated with a severe infection affecting all internal tissues of the fetus. These symptoms coincide with those described for the brown apical necrosis affecting Italian walnut orchards, which corresponds to a brown or dark brown spot that occurs exclusively on the flower end of the pericarp, and brown or blackish rot of the internal tissues [12, 15].

Brown apical necrosis can be considered as a complex disease. Recent in-depth studies have shown that the bacterium *Xanthomonas arboricola pv. juglandis* (*Xaj*) is the causative agent of bacterial blight of walnuts and apical necrosis of fruits, which often severely affects *Juglans regia*. Studies have shown that this pathogen begins the infection process at the tip of the fetus [6].

In addition to *Xaj*, fungi of the genera *Fusarium spp*. and *Alternaria spp*. also appear to be involved in the development of apical necrosis, causing secondary infections or developing as saprophytes on bacterially infected tissues, thereby exacerbating the symptoms and severity of the disease. Etiological studies of brown apical necrosis in Italian walnut orchards have led to the conclusion that *Fusarium* species may play an important role. Various *Fusarium* species have been isolated alone or in association with other fungi (*Alternaria spp.* or *Colletotrichum spp.*) from the affected walnut fruits, whereas *Xaj* was isolated only sporadically.

However, the role of *Xaj* in the apical necrosis of the Italian walnut has not been definitively established until recently. Preliminary studies in Spanish walnut orchards have shown that *Xaj* is the most frequently isolated microorganism from fruits affected by apical necrosis, alone or in association with *Fusarium* and *Alternaria* species. These results are consistent with field studies conducted over 10 years in the northern regions of Spain [12].

The first report of apical necrosis on walnut varieties from the northern part of Argentine Patagonia was made in 2017. [16]

A study of the distribution of populations of *Xaj* and *Alternaria* sp.-grps. associated with BAN at different phenological stages of walnut in Argentina showed that *Xaj* was detected at 9 of the 11 studied phenological stages, and *Alternaria* at all

stages. During the phenological stages, the fungus (*Alternaria*) was detected more often than the bacterium (*Xaj*). However, in diseased fetuses with typical symptoms of BAN, the bacterium (*Xaj*) was found more often than the fungus (*Alternaria*), which was found only sporadically and always accompanied by *Xaj*. The bacterium appears to have a greater ability to colonize external tissues, reaching higher epiphytic biomass [2].

In addition to *Xaj* and *Fusarium* and *Alternaria* fungi, *Pantoea* agglomerants has been reported as the causative agent of brown apical necrosis of walnuts in China. In this study, an isolate of *Pseudomonas oryzihabitans* was also isolated from a walnut fruit with blight symptoms [11].

1.3 BACTERIAL BLIGHT OF WALNUTS

Bacterial blight of walnuts caused by the bacterium *Xanthomonas arboricola pv. juglandis* (*Xaj*), is one of the most significant walnut diseases. It is widespread in all regions of the world where walnuts are grown and can lead to significant crop losses.

The causative agent, *Xaj*, is an aerobic, G ram-negative, rod-shaped bacterium that does not form spores. The pathogen was originally isolated in California in 1896 by Pierce (1896) and named *Pseudomonas juglandis* [17]. More recent phylogenetic studies conducted by Voterin et al., led to the proposal of the name *Xanthomonas arboricola pv. juglandis* [10, 17]. This proposal was accepted in one of the reviews, although it is not universally accepted [17]. There is significant genetic variability among *Xaj* strains from different geographical areas.

The disease causes serious damage to leaves, shoots, buds, petioles, rachis, male and female catkins, ovaries and kerne. If it develops strongly in humid and mild climates, especially after several rainy summers, it can even lead to tree death. Bacterial blight is considered to be the main reason for the decrease in fruit yield and tree vitality. The symptoms of the disease on *Juglans regia* fruits in Tasmania are similar to those observed in other regions of the world [10].

Bacterial blight disease that usually occurs in conjunction with a complex disease known as Brown Apical Necrosis (BAN) [16, 17]. *Xaj*, together with the fungi *Alternaria, Fusarium, Aspergillus* and *Botryosphaeria*, leads to the development of BAN. Lesions caused by bacterial blight can be infected with fungi

(*Botryosphaeria, Alternaria* or *Fusarium*), which leads to fruit loss. Apical necrosis, a key symptom of BAN, begins with necrosis at the stigma end of the fetus [17]. *Xaj* is most often associated with apical necrosis and can cause infections in young fetuses [2, 11, 12, 16, 17]. Observations in Spain show that *Xaj* is the most frequently isolated microorganism in apical necrosis, sometimes in association with *Fusarium* and *Alternaria* [12]. In Italy, *Xaj* was isolated less frequently than fungi [2].

The development of bacterial blight strongly depends on environmental conditions in spring. The humid and mild climate contributes to the strong development of this disease [17]. The development of the disease in fetuses under ten weeks of age requires at least 12-24 hours of humidification at temperatures from 15 to 25 °C. The bacterium is transmitted through contaminated pollen. It may be in an epiphytic phase on tree buds and fruits. Buds are considered fundamental to *Xaj* epidemiology [12, 17]. The spread of bacteria in the garden occurs mainly with rain [2, 12, 17]. There were significant differences in the temporal development of morbidity in fruits of different varieties ('Franquette' and 'Vina') in different years of cultivation, while the incidence before harvest ranged from 19% to 100%. The final percentage of infected trees positively correlated with the amount of precipitation during the first 4 weeks after budding [17]. S oil characteristics and nutrition may be an additional factor associated with infection with apical necrosis [2, 11, 12, 16, 17].

At the moment, no resistance to *Xaj* has been found in walnuts; differences are observed only in the severity of symptoms in varieties growing under the same conditions. One of the main objectives of breeding is to obtain resistant varieties, and their introduction into production is considered as a preventive measure against *Xanthomonas* infection [17]. The susceptibility of varieties can be assessed using tests on immature fruits [12] [17, 18].

Current control strategies often include the use of antimicrobial copper-containing drugs and fungicides. Due to the development of resistance of *Xaj* strains against copper [17], as well as the complexity of processing mature trees, protection work is difficult. For finding innovative protection methods, programs with reduced use of copper were evaluated [12, 18]. Biological control methods using antagonistic bacteria are also being investigated [18].

At the moment, to improve prevention and control programs, it is recommended to consider the meteorological factor of the spring season and walnut phenophases, trying to move away from using fixed treatment calendars [17]. Many aspects of apical necrosis are still unknown, making it difficult to identify effective disease management strategies [11, 16, 17].

Predisposing factors and epidemiology

Soil characteristics and the content of phenolic compounds in walnut fruits can also predispose trees to infection and increase the severity of apical necrosis. Soils that favor the development of the disease are sandy soils, acidic soils, light textured soils, and clay soils [12].

The walnut husk fly, *Rhagoletis completa*, native to North America, invaded Western Europe in the late 1980s, causing significant damage to its main host - the walnut tree *Juglans regia* . In gardens where *Rhagoletis completa* is present and not controlled, up to 100% of walnut trees can be infected, resulting in nut yield losses of up to 80%. The fly is widespread in Europe. The pest lays eggs in the pericarp, the larvae develop inside, the pupae in the soil, and the adults fly above the ground [14].

Recent studies are investigating the role of insects such as *Rhagoletis completa* as potential vectors of pathogens that damage walnut production, including pathogens of bacterial blight and brown apical necrosis (*Xanthomonas arboricola pv. juglandis*, *Fusarium, Alternaria*) [14]. The purpose of one of the studies was to observe the link between the spread of pathogens and the nut fly.

The buds were the main overwintering site of the bacterium (*Xaj*), which showed higher epiphytic abundance.

Many aspects of apical necrosis are still unknown, and control strategies cannot be clearly defined, making the disease difficult to control. The results of studies on the distribution of *Xaj* and *Alternaria* populations may contribute to a better understanding of BAN disease [2].

1.4 BACTERIAL AND FUNGAL CANCER (CANKER)

Walnut trees are susceptible to a number of diseases, many of which are manifested by the formation of ulcers or necrotic areas on the bark and branches, which is generally described as cancer (canker). Bacterial walnut cancer has

historically been associated primarily with bacteria of the genus *Brenneria.* Symptoms include vertical cancers with fluid leakage on the trunks and branches of the Persian walnut (*Juglans regia*), which appear mainly in summer [19]. The bacterium was isolated from diseased trees in various regions of France. Identification is based on physiological and biochemical characteristics. *B. nigrifluens* cause dark lesions in the inner bark and dark lines in the inner wood after inoculation, although external cancers are not always observed with artificial infection experiments [3].

Deep bark cancer of walnut can be caused by *Brenneria rubrifaciens* [19]. Symptoms include cancerous growths on trunks and branches with dark outflows that appear mainly in summer. Interestingly, the bacterium was identified based on physiological and biochemical characteristics, fatty acid profiles, and ELISA (enzyme-linked immunosorbent assay) [20]. The whole genomes of *Brenneria rubrifaciens* and *Brenneria nigrifluens* were sequenced by paired-end sequencing (2 × 250-bp paired-end sequencing) which was performed on an Illumina MiSeq instrument . It was found that both genomes encode type III secretion systems. The genome of *B. rubrifaciens* also contained two almost identical clusters of genes encoding a type IV secretion system [21].

1.5 INFECTION PROCESS OF THOUSAND CANKERS DISEASE

Thousand Cankers Disease (TCD) is a complex of diseases caused by the fungus *Geosmithia morbida* Kolařik (Ascomycota, Hypocreales) and its vector, the walnut twig beetle *Pityophthorus juglandis* Blackman 1928 (Coleoptera, Scolytinae) [22]. Since the mid-1990s, this disease has caused widespread mortality of many nut species in the United States [5]. TCD is an aggressive emerging disease affecting species of the genera *Juglans* and *Pterocarya*. It was first identified in Colorado in 2007 and has since spread to the western and seven eastern states of the United States and has also been found in Italy [22].

The role of a vector in the spread of TCD is performed by the walnut twig beetle *Pityophthorus juglandis*, which carries the fungus *Geosmithia morbida*. Symptoms of TCD on walnut tree *Juglans regia* include yellowing of foliage, wilting and dying of the youngest twigs. Small brown ulcers appear on the bark. Examination of longitudinal and radial incisions through ulcers reveals gray or brown coloration of

the phloem and bark, as well as the passages of walnut twig beetles [5]. In addition to walnuts, TCD also affects black walnut (*Juglans nigra L.*). Sensitivity to *Geosmithia morbida* has been shown in various species of walnut and hickory. Other *Juglans* and *Pterocarya* species may also be affected [22].

In Europe, TCD was first recorded on black walnut (*Juglans nigra*) in Italy in May 2014. Several other walnut trees with symptoms of the disease and the presence of fungus and beetle were also found near infected black walnut plantations [5].

Studies of *Geosmithia morbida* isolates from Colorado have revealed three different genetic clusters. It was noted that the virulence of *Geosmithia morbida* isolates in the experiments was not related to their genetic groups. The population structure of *Geosmithia morbida* in the USA has also been investigated, as well as genetic differentiation and spatial structure. Along with *Geosmithia morbida*, the pathogen *Fusarium solani* is also often isolated from trees affected by TCD. The extent of *Fusarium solani* contribution to the development of the disease, as well as its interaction with *Geosmithia morbida*, is currently not fully known. Studies have shown that co-inoculation with these two pathogens does not lead to a synergistic increase in symptoms. *Fusarium solani* of phylogenetic species 25 was reported to be associated with the early stages of thousand cankers disease on *Juglans nigra* in Italy [22].

Standard microbiological and molecular methods are used to identify microorganisms associated with TCD [5]. Isolation can be carried out on nutrient media such as potato dextrose agar (PDA). The identification is based on the study of the morphological characteristics of the fungus and beetle. Molecular methods include the amplification and sequencing of certain genetic regions, for example, the ITS (internal transcribed spacer) region of the rRNA gene coding DNA for the fungus *G. morbida* and the barcode region of the mitochondrial cytochrome oxidase I gene for the beetle *P. juglandis*. The obtained sequences are analyzed, including using BLAST, for comparison with known genetic data and confirmation of species affiliation [5].

1.6 WALNUT ANTHRACNOSE

Walnut anthracnose is caused by the ascomycete fungus *Gnomonia leptostyla*. Its anamorphic stage is called *Marssonina juglandis*. [8]. This disease is considered one of the most widespread, occurring in almost all regions where walnut is cultivated

[15]. Anthracnose causes significant economic damage, leading to a decrease in the quantity and quality of the nut harvest, as well as weakening the trees. In some cases, crop losses in terms of quality and quantity can reach 60-80%. The disease can cause deterioration of the core's fullness, darkening, and decreased quality [8].

Disease symptoms and the morphology of infected sites

The pathogen affects all terrestrial parts of the walnut tree, including leaves, young shoots and fruits. On the leaves, the symptoms appear as dark brown, rounded spots that can merge to form extensive necrotic areas [8]. Small black fruiting bodies of the fungus known as acervules develop within these necrotic zones, especially on the underside of the leaves. Acervules contain numerous conidia [4]. Conidia (*Marssonina juglandis*) are colorless, usually crescent-shaped, pointed at one end and rounded at the other, divided by one septum into two approximately equal cells. With severe infection, premature leaf fall (defoliation) may occur [8]. The spots on the petioles are smaller and elongated than on the leaves. The affected young fruits have dark spots and often fall off before ripening. Early infection of fruits can lead to their deformation. Symptoms on young shoots appear as elliptical necrotic lesions [4].

The perfect (teleomorphic) stage of the fungus, *Gnomonia leptostyla*, overwinters on fallen infected leaves. Overwintered plant remains are the main source of inoculum (a portion of the pathogen fungus) [8]. In spring, perithecia form on the fallen leaves. The perithecia have a long neck and contain numerous asci with ascospores. Ascospores are colorless (hyaline), elongated (fusiform), and bicellular (bicellular). Ascospores serve as the primary inoculum in spring [4]. Secondary infections during the growing season are caused by conidia. The development of the disease is favored by wet and rainy conditions. High relative humidity (over 95%) contributes to leaf infection. Temperatures in the range of 10-32 °C do not significantly affect the development of the infection, but it decreases at temperatures below 10 °C [8]. Both life cycles of the fungus (asexual and sexual) are involved in the development of the disease.

Host susceptibility and disease management of walnut anthracnose

Different species of the genus *Juglans* and even different varieties of *Juglans regia* show varying degrees of susceptibility to anthracnose [15]. For example,

Juglans nigra can be considered a resistant species, while *J. regia* is susceptible. Hybrids (*J. nigra x J. regia*) have intermediate resistance [8]. Observations have shown that some wild species may be less susceptible to the pathogen [4].

Control measures include cultural practices and the use of fungicides. An important measure is the collection and destruction of infected fallen leaves to reduce the supply of inoculum. Pruning of affected branches and shoots can also be used. The use of fungicides based on copper is recommended. Adequate nitrogen fertilizers can also reduce the severity of the disease [4, 8].

1.7 CARRIERS OF WALNUT INFECTIONS

In the context of wal nut tree diseases, vectors, especially insect pests, play a critical role in the spread of pathogens. The analysis of microbial communities associated with the infection of walnut trees often includes the study of microorganisms associated with such vectors.

Walnut twig beetle (*Pityophthorus juglandis*)

One of the most studied examples of the interaction of a vector insect and a pathogen that causes a serious disease of walnut trees is the thousand cankers disease. This disease is caused by the fungus *Geosmithia morbida*, and its main vector in the United States is the walnut twig beetle *Pityophthorus juglandis*. Adult beetles penetrate the branches and trunks of walnut trees. By creating passages under the bark for nutrition and reproduction, they transfer the spores of the *G. morbida* fungus and inoculate them into the phloem through the wounds they create [22]. This partnership between beetle and fungus is a key aspect of the spread of TCD. The disease affects various species of the genus *Juglans* and *Pterocarya*, including walnut (*Juglans regia*) and black walnut (*Juglans nigra*).

Walnut husk fly (*Rhagoletis completa*)

In addition to the walnut weevil beetle, other insects associated with walnut trees can carry various microorganisms. For example, the walnut husk fly [7], known as a pest of nut fruits, like other members of the *Tephritidae* family, is associated with diverse bacterial communities in its intestines and digestive system [23]. These bacteria may include genera such as *Klebsiella*, *Citrobacter*, *Enterobacter*,

Providencia, *Erwinia*, *Proteus*, and *Bacillus*. *Klebsiella oxytoca* and Pantoea agglomerans (*Enterobacter agglomerans)* have often been isolated from the digestive tract and esophagus of *Rhagoletis* spp. [24]. Symbiotic bacteria may play a role in the nutrition and development of flies, providing them with essential amino acids or vitamins, or participating in the transmission of bacteria to offspring. Some bacteria present in the gut of flies, such as representatives of the genera *Pseudomonas* and *Serratia*, may be opportunistic pathogens [23]. However, based on the literature, there is no clear evidence that the wal nut husk fly or other nut flies act as vectors for specific, well-described diseases of wal nut trees caused by bacteria such as *Xanthomonas arboricola pv. juglandis* or *Brenneria* species [24].

As for other significant diseases of walnut trees, such as bacterial walnut blight caused by *Xanthomonas arboricola pv. juglandis*, and walnut anthracnose [17] caused by the fungus *Gnomonia leptostyla*, as well as b acterial bark ulcers caused by *Brenneria rubrifaciens* and *Brenneria nigrifluens*, previous studies describe the pathogens, symptoms, taxonomy, identification methods [4] and sources of inoculum (for example, overwintered leaves for *Gnomonia leptostyla* bearing ascospores, or pollen as a source of *Xanthomonas arboricola pv. juglandis* [2]), but do not indicate specific insects as vectors of these diseases.

Mentioning pollen as a source of X. *arboricola pv juglandis* infection may indirectly indicate the role of insect pollinators in the transmission of infected pollen, including the walnut husk fly [4].

2 MATERIALS AND METHODS

2.1 SAMPLING SITES AND SAMPLING

Felsőpáhok (F1) - walnut plantation

Walnut plantations were visited near Felsőpáhok (GPS: 46°47'38.2"N 17°09'15.7"E), Zala County, Hungary. The trees showed various states of infection: uninfected, infected and severe infection. Walnuts had a brown or black discoloration in spots or the whole fruits were necrotized. Most of the leaves also showed small brown spots and some of them had gall mite galls (Fig. 2.1.). The sampling was conducted in August 2024. We have collected healthy walnuts (F1W1) and leaves (F1L1) as control, infected walnuts (F1W3, Fig. 2.2., Fig. 2.3) and small brown-spotted leaves (F1L2) into sterile containers.

Figure 2.1: Walnut with black discoloration and brown-spotted leaves in the plantation of Felsőpáhok.

Figure 2.2: Infected walnut sample (F1W3) collected from the plantation showing brown discoloration.

Hidegség (H1)

To expand the areas of sampling places, walnut plantations were visited near Hidegség (GPS: 47°37'32.5"N 16°44'31.8"E).

Figure 2.3: Infected walnut sample (H1W3) collected from the plantation in Hidegség showing black discoloration.

2.2 CULTIVATION

In a study related to the walnut husk fly (Rhagoletis completa), infected walnuts were collected from trees growing in the orchard near Felspáhok, then the nuts were kept on sterile neutral soil to collect larvae and raise flies (imagos). Pupae (P1) and imagos (I1) of the cultivated flies were collected for DNA extraction.

All samples were kept under 4°C until processing.

2.3 DNA EXTRACTION

Sterile scalpel was used to cut samples from the healthy and the infected part of walnuts collected (Fig. 2.4.).

Figure 2.4: Samples collected from the walnuts for DNA extraction with orange arrows, from left to right: F1W1, F1W3, H1W3.

0.25 g of samples were taken from every sample. They were carefully ground into a fine powder using a sterile and pre-cooled mortar and pestle in the presence of liquid nitrogen. The use of liquid nitrogen helps to freeze tissue instantly, making it brittle and easier to grind, which is critical for effective cell lysis and protecting DNA from degradation. The Qiagen PowerSoil Pro kit (Qiagen) was used to isolate DNA. The procedure was performed according to the manufacturer's protocol.

The extracted DNA was stored at -20 °C until further analysis. Low temperature helps to preserve the integrity of DNA and prevent its degradation.

Subsequent processes including PCR, quality checking and sequencing were performed in the Hungarian Centre for Genomics and Bioinformatics, Szentágothai Research Centre, University of Pécs.

2.4 PCR

For PCR, 16S primers were used: forward (Fw) with the sequence 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse (Rev) with the sequence 5'-TACGGYTACCTTGTTACGACTT-3'. The GoTaq® Long PCR Master Mix produced by Promega Corporation, Madison, USA was used as the master mix. Thermal conditions for amplification included initial denaturation at 98 °C for 3 minutes. This was followed by 30 cycles, each consisting of denaturation at 98 °C for 30 seconds, annealing at 60 °C for 30 seconds, and elongation at 72 °C for 90 seconds. The program ended with the final elongation at 72 °C for 5 minutes.

2.5 SEQUENCING

Sequencing was performed on the Oxford Nanopore MinION platform using Flow Cell R10 Version, after library preparation using Native Barcoding Kit 24 V14 according to the manufacturer's protocol.

Table 2.1: Oxford Nanopore Sequencing Protocol

Step	Description	Reagents / Tools	Conditions / Time
2b. PCR Cleanup (if used)	Remove primers, dNTPs, polymerase	AMPure XP Beads (1×) or spin columns	Elute in 20–30 µL EB
3. End-Repair and A-Tailing	Blunt-ending and A-tailing	NEBNext FFPE Repair Mix + Ultra II ER/dA Module	20°C for 5 min → 65°C for 5 min
4. Barcoding	Ligation of native	Native Barcoding	20°C, 15 min

Step	Description	Reagents / Tools	Conditions / Time
	barcodes	Kit 24 V14, Blunt/TA Ligase	
5. Cleanup after Barcoding	DNA purification	AMPure XP Beads (1×)	Elute in 10 µL EB
6. Pooling	Combine barcoded samples	—	Total volume ≤60 µL
7. Adapter Ligation	Add sequencing adapters	Adapter Mix II, Blunt/TA Ligase	20°C, 15 min
8. Library Cleanup	Remove unligated adapters	AMPure XP Beads (0.4×–0.5×)	Elute in 15 µL EB
9. Flow Cell Preparation	Check pores and flush if needed	MinKNOW, Flow Cell Flush Kit	>800 active pores recommended
10. Library Loading	Mix with SB + LB, load to flow cell	Sequencing Buffer, Loading Beads	Load gently into SpotON port
11. Sequencing	Launch run in MinKNOW	Choose: R10.4.x, NBD114.24 kit	24–72 hours
12. Basecalling & Demultiplexing	Basecall and split by barcode	Guppy or Dorado	GPU mode preferred
13. QC & Visualization	Assess read quality and yield	NanoPlot, pycoQC, MinIONQC	After sequencing

2.6 BIOINFORMATIC EVALUATION

The evaluation was conducted with the help of Simang Champramary. Raw 16S rRNA Nanopore sequencing reads were processed to ensure high-quality input for downstream taxonomic analysis. All analyses were performed in R v 4.4.3 (R Core Team) [54] using the ShortRead [55], dada2 [56], and tidyverse [57] packages.

Initially, raw FASTQ files were concatenated and imported. Sequences were filtered and trimmed using dada2. Reads were trimmed by 100 base pairs at both the 5' and 3' ends, and only sequences with lengths between 1200 and 1800 base pairs were retained. Filtering parameters were chosen to optimize for full-length 16S rRNA gene sequences, accounting for the expected read length generated by nanopore sequencing. A summary of the filtering outcomes was recorded in a CSV file for quality control purposes.

Following quality filtering, taxonomic classification was performed. For each filtered FASTQ file, a random subsample of 100 sequences was selected to standardize the number of reads used for classification across samples. Taxonomic assignment was conducted using the assignTaxonomy() function from the dada2 package with a

custom-formatted SILVA reference database (SILVA nr99 v138.2, trimmed to the genus level) [58]. A minimum bootstrap confidence threshold of 50% was applied to ensure reliable taxonomic calls. Reverse complement matching was allowed to accommodate sequencing orientation variability. Taxonomic assignments for each sample were saved as individual CSV files for further analysis.

3 RESULTS

According to the protocol, the raw readings of 16S rRNA from the Oxford Nanopore MinION platform were processed to ensure high quality. During filtering and pruning using the DADA 2 package, 100 base pairs were cut off at both ends of the reads, and only those with a length of 1,200 to 1,800 base pairs were saved. These parameters were selected to optimize for full-length sequences of the 16S rRNA gene. Based on the processed data, it was shown that the average total number of raw reads per sample was ≈ 12,030 (a total of 84,210 reads for 7 samples), while the average number of filtered sequences per sample was ≈ 8,363 (a total of 58,841 sequences for 7 samples). On average, about 69.6% of the raw reads were saved after filtering and pruning (58,841 out of 84,210). The filtering results were recorded for quality control purposes (Table 3.1).

Table 3.1: Results of filtering and pruning 16S rRNA sequencing data

	Total number of reads	**Number of filtered sequences**
P1	2119	178
I1	13793	9596
F1W1	10751	8750
F1W3	27781	19875
F1L1	13886	10459
F1L2	2263	322
H1W3	13617	9361

- P1 - Pupae Samples
- I1 - Imago Samples
- F1W1 - healthy walnuts samples collected from Felsőpáhok
- F1W3 - Infected walnuts samples collected from Felsőpáhok
- F1L1 - healthy leaves samples collected from Felsőpáhok
- F1L2 - small brown-spotted leaves samples collected from Felsőpáhok
- H1W3 - Infected walnut samples collected from Hidegség

Taxonomic composition of the samples

At the phylum level, the sequencing data showed significant differences between the samples. In samples P1 (pupae), I1 (imago), F1W3 (infected walnuts

from Felsőpáhok) and H1W3 (infected walnuts from Hidegség), the *Pseudomonadota* dominated, accounting for 87.76% in P1, 84% in I1, 99% in H1W3 and with high percentage in F1W3 - 26% .

In contrast, the *Cyanobacteriota* prevailed in samples F1W1 (control walnuts), F1W3 (infected walnuts from Felsőpáhok), F1L1 (control leaves) and F1L2 (infected leaves), reaching 99% in F1W1, 71% in F1W3, 100% in F1L1 and 38.38% in F1L2. The *Bacillota* phylum was present only in sample P1 with a proportion of 2.04%, while *Bacteroidota* was present in P1 (1.02%) and F1L2 (2.02%). *Actinomycetota* was detected in samples I1 (8%), F1W3 (2%) and F1L2 (4.04%). There is also a proportion of Unknown bacteria in P1 (1.02%), I1 (1%), F1W3 (1%) and F1L2 (1.01%).

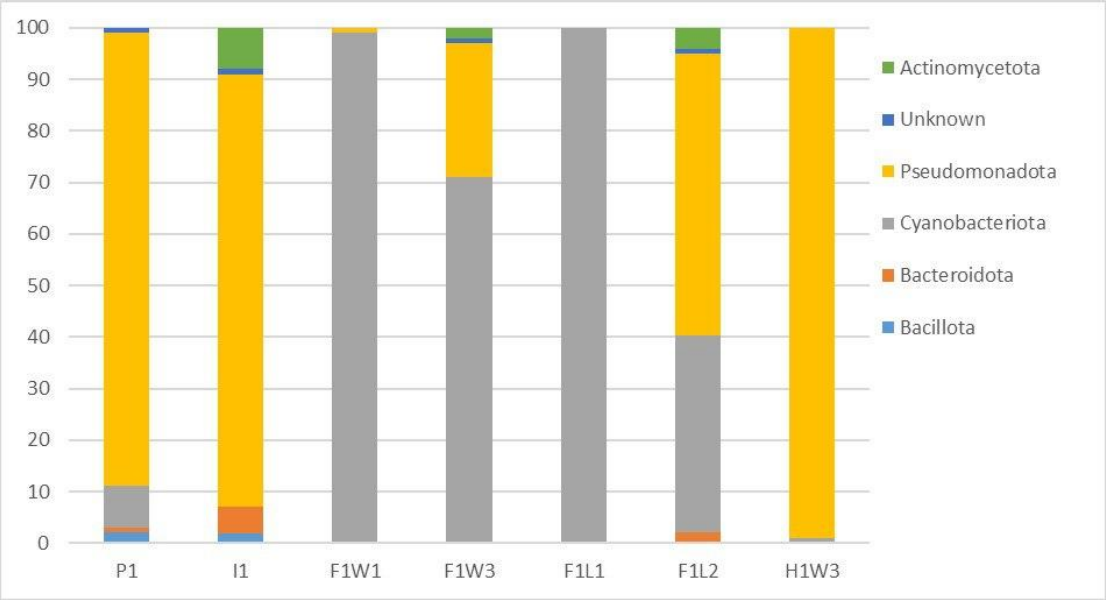

Figure 3.1: Analysis of the phyla composition present in samples.

At the class level, a more detailed distribution of the dominant phyla was observed. Within the phylum of *Pseudomonadota*, the classes *Alphaproteobacteria* and *Gammaproteobacteria* were the main ones. *Alphaproteobacteria* accounted for 5.1% in P1, 40% in I1, 1% in F1W1, 2% in F1W3 and 3.03% in F1L2. *Gammaproteobacteria* were very abundant in P1 (82.65%), I1 (44%), F1W3 (23%) and H1W3 (99%), but were absent in F1W1, F1L1 and F1L2. The *Cyanobacteria* class closely corresponds to the distribution of the *Cyanobacteriota*, dominating F1W1 (99%), F1W3 (71%), F1L1 (100%) and F1L2 (38.38%). *Bacilli* were found only in P1 (2.04%), and *Bacteroidia* - in P1 (1.02%) and F1L2 (2.02%). *Actinobacteria* were represented in I1 (8%), F1W3 (2%) and F1L2 (4.04%).

At the level of order, the data show further specialization. *Cyanobacteriota* is very abundant in F1W1 (99%), F1W3 (71%), F1L1 (100%) and F1L2 (38.38%). Among the *Pseudomonadota*, the order *Pseudomonadales* dominated in P1 (60.2%), was present in I1 (13%), F1W3 (2%), F1L2 (12.12%) and H1W3 (2%). The order *Lysobacterales* was very numerous in H1W3 (97%) and F1W3 (13%), and was also found in F1L2 (4.04%).

Enterobacterales was significantly present in P1 (8.16%), I1 (30%), F1W3 (7%) and F1L2 (33.33%). *Hyphomicrobiales* was abundant in I1 (40%) and P1 (5.1%), also found in F1W1 (1%) and F1W3 (2%). Other orders, such as *Bacillales*, *Burkholderiales*, *Lactobacillales*, *Bacteroidales*, *Micrococcales*, *Sphingobacteriales*, *Mycobacteriales*, *Chitinophagales* and *Propionibacteriales*, were present in smaller quantities or in a limited number of samples.

At the level of the family, the distribution becomes even more detailed. *Pseudomonadaceae* follow the trend of *Pseudomonadales*, being numerous in P1 (60.2%), I1 (13%), F1W3 (2%), F1L2 (12.12%) and H1W3 (2%). *Lysobacteraceae* dominated in H1W3 (97%), were also noticeable in F1W3 (13%) and F1L2 (4.04%). The *Rhizobiaceae* was numerous in I1 (40%) and P1 (4.08%), was present in F1W1 (1%) and F1W3 (2%). *Enterobacteriales* (including *Erwiniaceae*, *Morganellaceae*, *Pectobacteriaceae*, *Yersiniaceae*, which are also mentioned separately and have different fractions) were significant in I1 (20%), P1 (3.06%), F1W3 (3%), F1L2 (25.25%) and H1W3 (including *Morganellaceae* 1.01% and *Yersiniaceae* 1.01%). The category of "Unknown" at the family level is very high in samples with a predominance of *Cyanobacteriota* (F1W1 99%, F1L1 100%) and is also noticeable in F1W3 (75%) and F1L2 (42.42%), as well as in P1 (15.31%), I1 (7%) and H1W3 (1%). Other families present in some samples included *Alcaligenaceae*, *Bacillaceae*, *Comamonadaceae*, *Devosiaceae*, *Beutenbergiaceae*, *Micrococcaceae*, *Sphingobacteriaceae*, *Nocardiaceae*, *Chitinophagaceae, Nocardioidaceae* and HAW-RM37-2.

Finally, at the genus level, the data provides the most specific information. *Pseudomonas* follows the trend of its family, dominating in P1 (59.18%) and present in I1 (13%), F1W3 (2%), F1L2 (12.12%) and H1W3 (2%) (Figure 8). The genus *Stenotrophomonas* was abundant in H1W3 (15%). The genus *Pseudoxanthomonas* was also prominent in H1W3 (50%) and F1W3 (7%) and was also present in F1L2 (2.02%). Among *Enterobacterales*, *Enterobacter* genera were present in P1 (1.02%,

also listed separately with 0% in all samples), I1 (2%), F1L2 (3.03%). *Falsochrobactrum* was abundant in I1 (25%) and was present in F1W3 (2%). *Raoultella* was prominent in I1 (7%) and F1L2 (10.1%). *Daeguia* was abundant in I1 (13%) and present in F1L2 (3.03%). A significant proportion of sequences at the genus level remained Unkown, especially in samples F1W1 (100%), F1L1 (100%), F1W3 (81%) and F1L2 (55.56%), as well as in P1 (20.41%), I1 (21%) and H1W3 (18%). Other genera such as *Brackiella, Brucella, Comamonas, Coralloluteibacterium, Erwinia, Providencia, Schinkia, Falsarthrobacter, Lelliottia, Margalitia, Moellerella, Nissabacter, Paenarthrobacter, Pantoea, Pseudochrobactrum, Sphingobacterium, Orrella, Rhodococcus, Samsonia, Aquincola, Ferruginibacter, Marmoricola and Yokenella*, were found in smaller proportions or in single samples (Figure 3.1).

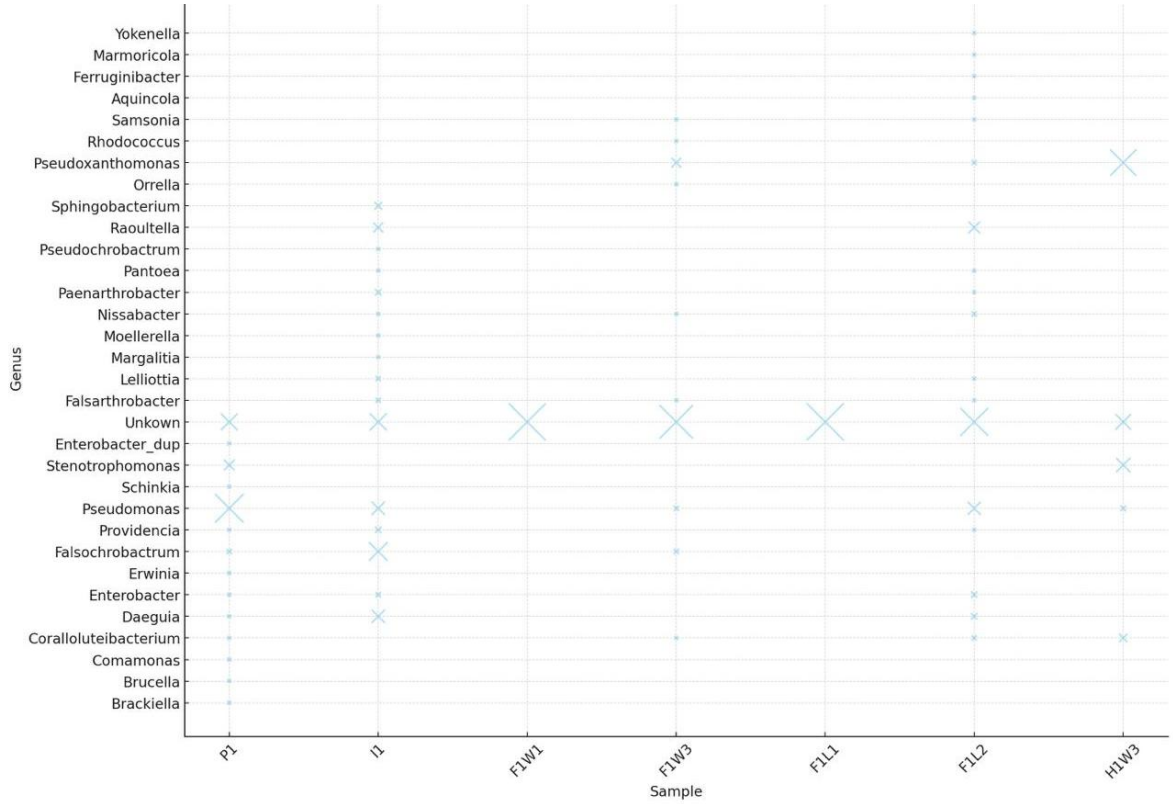

Figure 3.1: Bubble plot showing the relative abundance of dominant bacterial genera across samples.

Samples with taxonomy data include insect samples (P1, I1) and walnut and leaf samples (F1W1, F1W3, F1L1, F1L2, H1W3). Analysis of the relative abundance of taxa showed significant differences between insect samples (P1 - pupae, I1 - adults) and plant samples (walnut and leaves). Insect samples were mainly represented by bacteria of the phyla *Bacillota, Bacteroidota, Cyanobacteriota* and *Pseudomonadota*, with *Pseudomonadota* predominating (up to 87.76% in P1). At genus level, *Pseudomonas* dominates in sample P1 (59.18%), while *Falsochrobactrum* (25%), *Daeguia* (13%), *Pseudomonas* (13%) and *Raoultella* (10.1%) are prominent in sample I1.

In contrast, control plant tissue samples (F1W1 - uninfected walnut, F1L1 - control leaf) consisted almost entirely of sequences classified as *Cyanobacteriota / Chloroplast* (99-100%), likely reflecting the presence of plant chloroplasts classified as bacteria.

Infected or diseased plant tissue samples (F1W3 - infected walnut, F1L2 - leaf with spots, H1W3 - infected walnut) show a significant decrease in the relative abundance of chloroplasts and a dramatic increase in the proportion of bacteria, especially *Pseudomonadota*. The abundance of various genera including *Stenotrophomonas* (up to 15% in F1W3), *Pseudomonas* (up to 12.12% in F1L2), and especially *Pseudoxanthomonas* (up to 50% in H1W3 and 7% in F1W3) increases in these samples. The presence of bacteria such as *Pseudomonas* and *Pseudoxanthomonas* is associated with diseased walnut tissues[1].

Figure 3.1 shows the relative abundance of the most abundant genera in each of the analyzed walnut samples (F1W1 - control, F1W3 - infected Felsőpáhok, H1W3 - infected Hidegség). The control walnut F1W1 is dominated by unclassified sequences (Unkown 100%), presumably chloroplasts. In the infected F1W3 walnut, there is a notable presence of *Stenotrophomonas* (15%) and *Pseudoxanthomonas* (7%). In infected H1W3 walnut, *Pseudoxanthomonas* is the dominant identified bacterial genus (50%), with a significant presence of *Lysobacteraceae* (97% at the family level).

4 DISCUSSIONS OF RESULTS

The aim of this study was to investigate the microbiota of the walnut husk fly (*Rhagoletis completa*) and compare the microbial communities of healthy and infected walnut (*Juglans regia*) tissues to identify potential pathogens and assess the role of the fly in their spread. The sequencing data obtained revealed significant differences in microbial composition between insect and plant tissue samples, as well as between healthy and infected plant parts.

Taxonomic composition of microbial communities.

Analysis of 16S rRNA sequencing data showed a clear separation between insect and plant microbiota. *Pseudomonadota* dominated the walnut husk fly (*Rhagoletis completa*) samples (pupae P1 and adults I1), accounting for up to 87.76% in pupae samples and 84% in imago samples. For the class, *Alphaproteobacteria* and *Gammaproteobacteria* were abundant, and at the order level, *Pseudomonadales*, *Lysobacterales, Enterobacterales* and *Hyphomicrobiales* were dominant. Among the genera, *Pseudomonas* (up to 59.18% in P1), *Falsochrobactrum* (25% in I1), *Daeguia* (13% in I1) and *Raoultella* (10.1% in I1) were visibly present in insect samples.

A different pattern was observed in plant tissue samples. Healthy control samples (nuts F1W1, leaves F1L1) consisted almost entirely of sequences assigned to *Cyanobacteriota* (99%). This presumably reflects the presence of plant chloroplasts, which are classified as bacteria in the database used.

Stenotrophomonas, Pseudomonas, Pseudoxanthomonas

In infected or symptomatic plant tissue samples (F1W3 and H1W3 infected nuts and F1L2 stained leaves), there was a significant decrease in the relative abundance of chloroplast sequences and a significant increase in the proportion of bacteria, especially of the *Pseudomonadota* phylum. This indicates a change in the microbial community associated with the health status of the plant.

The abundance and diversity of bacterial genera increased in infected walnut and leaf tissues. In particular, there was an increase in the relative abundance of genera such as *Stenotrophomonas* (up to 15% in F1W3), *Pseudomonas* (up to 12.12% in F1L2) and especially *Pseudoxanthomonas* (up to 50% in H1W3 and 7% in F1W3). The presence of bacteria such as *Pseudomonas* and *Pseudoxanthomonas* is associated with diseased walnut tissues in research. The control walnut F1W1 was dominated by Unkown sequences with 99%, presumably chloroplasts.

The symptoms observed on infected nuts (brown or black discoloration in spots or fruit necrotization) and on leaves (small brown spots) are similar with Brown Apical Necrosis (BAN) and Bacterial Blight [59]. The bacterium *Xanthomonas arboricola pv. juglandis (Xaj)* and various types of fungi *Fusarium* and *Alternaria* are often associated with it. In Italian gardens, *Xaj* was isolated sporadically, more often finding *Fusarium* and *Alternaria* species, whereas in Spain Xaj was the most frequently isolated microorganism, sometimes in association with Fusarium and *Alternaria*. *Botryosphaeria, Alternaria* and *Fusarium* fungi can also infect lesions caused by bacterial blight [12]. Thousand Canker Disease (TCD) is caused by the fungus Geosmithia morbida in association with the *Pityophthorus juglandis [5.]*. Fusarium solani is also associated with early stages of TCD. The sequencing results in this study revealed significant presence of *Pseudomonadota* including genera *Stenotrophomonas*, *Pseudoxanthomonas* and *Pseudomonas* in infected tree tissues. The presence of these groups was directly related to the presence of disease symptoms in the samples analyzed.

According to the literature, although some *Pseudomonas* strains may be beneficial, others have been associated with pathogenicity on walnut. For example, *Pseudomonas vancouverensis* strain PAN4 showed plant-stimulating effect and biocontrol ability on walnut seedlings[11]. However, *Pseudomonas oryzihabitans* isolate could infect walnut fruits and *Pseudomonas flavescens* isolate caused bacterial cancer of walnut shoots in South Korea [11]. The phytopathology, systemic migration and disease symptoms caused by *Pseudomonas syringae pv. avellanae* were investigated in hazelnut shoots [6].

In (H1W3), which, related to diseased tissues, the relative abundance of *Pseudoxanthomonas* was 50%. This fact indicates a close association of *Pseudoxanthomonas* with diseased walnut tissues. In addition to the association with walnut, the ecological niche of *Pseudoxanthomonas* may include aquatic environments or biofilms, as *Pseudoxanthomonas broegbernensis* has been isolated from biofilters [64]. There is currently no evidence to call *Pseudoxanthomonas* a direct pathogen of walnut, but its high relative abundance in affected samples makes it a potentially important factor in the development of walnut diseases.

Ahmadi and colleagues detail the role of *Stenotrophomonas maltophilia* in the context of Persian oak desiccation in Iran. *S. maltophilia* has been identified as one of the potentially causative bacteria involved in this disease. It is noted that the damage

characteristic of oak desiccation is likely to have a polymicrobial cause rather than being caused by a single organism. [60]. In the case of *Pinus* genera disease, *Stenotrophomonas maltophilia* showed high pathogenicity, but the underlying cause of the disease is related to the interaction between the bacterium and the Pine wood nematode (*Bursaphelenchus xylophilus*). *S. maltophilia* can affect gene expression of the nematode and enhance its virulence [60].

Like *Pseudomonas, Stenotrophomonas* is mainly associated with insects. *Stenotrophomonas* has been identified in the gut of fruit flies (*Bactrocera carambolae*). [61]. According to this information and the data obtained (8.16% in P1 and 15% in H1W3), it can be said that the *Stenotrophomonas* bacteria are included in the life cycle of walnut husk fly and walnut trees, similarly as *Bactrocera carambolae*.

Thus, *Stenotrophomonas*, is a genus with a complex ecological role including both pathogenic and potentially beneficial aspects for plants. Its presence in diseased walnut tissues requires further study to determine its specific role in walnut pathological processes, as discussed in the context of oak desiccation.

The walnut husk fly (*Rhagoletis completa*) is mentioned as a potential vector of infections, with larvae developing in the nuts. One of the objectives of the study was to evaluate the potential role of the *Rhagoletis completa* in the pathogens transmission. The microbiota of the fly contained dominantly members of the phylum *Pseudomonadota* (up to 59.18% in P1 and 13% in I1). *Pseudomonadota* was also found in infected plant tissues (up to 12.12% in F1L2 and 2% in F1W3 and H1W3). This indicates a link between the walnut husk fly (*Rhagoletis completa*) microbiota and the bacteria present in infected walnut tissues.

Although the survey indicates the possibility of *Xaj* transport by pollen and indirectly links insect pollinators including the walnut fly to this, the sequencing results in this study did not show a high abundance of *Xaj* in the samples, but did identify other bacteria associated with disease symptoms [2]. The presence of *Pseudomonas* in both insects and infected plant tissues supports the hypothesis of a potential role of the walnut husk fly as a vector of bacteria associated with walnut diseases, even if these bacteria are different from those most commonly cited in the literature in association with BAN (*Xaj, Fusarium, Alternaria*).

The identification of specific bacterial groups such as *Pseudoxanthomonas, Stenotrophomonas* and *Pseudomonas* associated with disease symptoms in these samples and their presence in microbiota of a potential vector walnut husk fly

(*Rhagoletis completa*) shows scientific novelty and may contribute to the development of new methods for monitoring and controlling walnut diseases.

Enterobacteriales

As previously reported, members of *Enterobacterales* have a dominant presence in the results of sequences. They suggest a role in walnut diseases based on their prevalence in infected samples.

The genus *Raoultella* (10.1% in F1L2) and *Pseudoxanthomonas* (7% in I1) present in insect and leaf samples in high percentage. The genus *Raoultella* was previously detected in the bacterial community of the gut of fruit fly larvae (*Anastrepha fraterculus)*. These larvae were collected from diseased peach and guava fruits. In our study, *Raoultella* was detected as part of the gut microbiome of the larvae, accounting for 1.353% of the total reads. Salgueiro and colleagues' investigated how host-fruit (peach or guava) and geographical origin affect the gut bacterial community of *Anastrepha fraterculus* [62] larvae. There is no information on the pathogenic ability of *Raoultella* to plants. Therefore, it can be inferred that their presence in plant environment is related to ecological functions. But it can be assumed that bacteria of this genus are also closely related to the microbiota of walnut husk fly (*Rhagoletis completa*), due to their detected presence in the body of adults (I1).

Lelliottia

Lelliotta bacteria have been observed to be present in low numbers in I1 and F1L2. *Lelliottia* is a genus of Gram-negative, motile and facultatively anaerobic bacteria belonging to the family *Enterobacteriaceae*. According to available studies, some bacteria of this genus possess plant growth-promoting effects, carried out through solubilization of phosphorus and production of siderophores, which in turn positively affect iron uptake by plants from soil [63]. Presumably they have a positive effect on tree growth when living on roots. Interestingly, they were also detected from the adult walnut husk fly sample with a low abundance.

Pantoea

Brown apical necrosis (BAN) research shows that it is a complex disease involving a variety of microorganisms [12]. These mainly include fungi such as Fusarium and *Alternaria*, and bacteria including *Xanthomonas* and *Pantoea*. The genus *Pantoea* has also been previously reported in association with BAN, in

particular *Pantoea agglomerans* has been reported as the cause of brown apical necrosis of walnut in China [11].

Although *Xanthomonas arboricola pv. juglandis* is indicated as the dominant bacterial pathogen in BAN of walnut [11], the results of microbiome studies indicate that *Pantoea* is one of the major bacterial genera present along with *Xanthomonas* [11]. The sequencing results revealed the presence of *Pantoea* genera in samples I1 and F1L2. The information obtained confirms that the bacteria may play a role in secondary infections or grow as saprophytes on tissues (e.g. leaves) [12] infected with the bacteria, thereby increasing the symptoms and severity of the disease.

Thus, the data indicate that *Pantoea* is an important component of the microbial community associated with walnut brown apical necrosis and, along with other pathogens, contributes to this complex disease.

Erwinia

Brenneria nigrifluens and *Brenneria rubrifaciens*, which were previously synonymous with E*rwinia nigrifluens* causing shallow bark canker and *Erwinia rubrifaciens* causing deep bark canker, are bacterial pathogens of walnut [19]. These diseases weaken tree trunks and branches over time and can lead to progressive wilting, reduced immunity and as a consequence provide an opportunity for parallel infection by other walnut pathogens [21]. It is worth noting that previous studies indicate the possibility of co-infection of walnut by species of the genus *Erwinia*. In addition, they may exist as quiescent endophytes, becoming pathogenic when the tree is exposed to environmental stressors [21]. According to sequencing data, the presence of these bacteria was detected in both larvae (P1) and adults (I1), and the presence of this genus was also detected on leaves of infected trees (F1L2).

Although the genus *Erwinia* is not considered in the sources as a direct causative agent of walnut diseases, related species, that were, assigned to the genus *Brenneria*, recently, play a significant role in the development of bark cancer, a serious disease that weakens trees.

Lysobacterales

The genus *Coralloluteibacterium* belonging to *Lysobacterales* has its presence in samples (P1 - 1.02%) and (F1L2 - 2.02%). It is not stated whether the bacteria of the genus *Coralloluteibacterium* is pathogenic, symbiotic, endophytic or just part of the general microbial flora of the surface or tissues of walnut. Thus, based on available sources, we know that bacteria of the genus *Coralloluteibacterium* are

present on walnut in some cases, but their influence or function in the context of walnut has not been described [64].

Other Genera

Bacteria of the genera *Rhodococcus, Pseudochrobactrum, Margalitia, Moellerella, Samsonia, Aquincola, Ferruginibacter, Marmoricola, Yokenella, Sphingobacterium, Orrella, Brackiella, Brucella, Comamona*s were detected in samples only individually in either animal or plant samples in low numbers, suggesting that they have no association with the interaction between the microbiota of walnut husk fly and walnut trees. Nevertheless, the following genera may have an impact on the trees as they have been found to be present on leaves and fruit *Rhodococcu*s (F1W3 - 1%), *Samsonia* (F1L2 - 1.01%, F1W3 - 1%), *Aquincola* (F1L2 - 1.01%), *Ferruginibacter* (F1L2 - 1.01%), *Marmoricola* (F1L2 - 1.01%), *Yokenella* (F1L2 - 1.01%), *Orrella* (F1W3 - 1%).

CONCLUSION

Sequencing results highlight a significant change in the microbial community in walnut tissues in the presence of disease symptoms, characterized by an increase in the abundance of bacteria, particularly *Pseudomonadota*, and a predominance of genera such as *Pseudoxanthomonas*, *Stenotrophomonas* and *Pseudomonas* in infected samples. Several other possible plant pathogenic bacteria were detected with low relative abundances that can act synergistically to induce disease symptoms. The walnut husk fly's microbiota, based on data obtained from P1 and I1 samples, is also rich in *Pseudomonadota*, including the genus *Pseudomonas*, thus confirming the relationship between the presence of these genera in the walnut husk fly (*Rhagoletis completa*) microbiota and disease initiation based on the assumption of pathogenicity of *Pseudomonata*, and the role of flies as vectors.

The findings are an important step towards the identification of microbial indicators of disease and may serve as a basis for the development of sustainable, environmentally friendly strategies for managing the health of walnut orchards.

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