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Etiology of Bacterial Wetwood of *Quercus robur* L.

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Abstract. Phytopathogenic bacteria in the plant organism form an integral part of the accompanying microflora, as well as pathogens of pathological processes that do not just weaken the plant, but shortly (with acute pathogenesis) lead to degradation and complete dieback. Notably, bacteriosis is described by typical macroscopic signs of the course of the disease, but the exact aetiology of the pathological process can be reliably established only based on bacteriological analysis with the identification of morphological, cultural, and biochemical properties of isolates. The purpose of this study is to experimentally confirm the direct causes of oak degradation caused by bacterial wetwood in the tree stands under study, as well as to investigate the morphological and biochemical properties of the pathogen. This study employed classical microbiological, phytopathological, and biochemical methods that establish the aetiology of the disease, analyse typical symptoms, include microscopy of the affected parts of the oak, isolation, and identification of the pathogen. The properties of bacterial isolates were figured out according to generally accepted methods and using the API 20E test system and the NEFERMtest24 MikroLaTEST®, ErbaLachema a test system. It was experimentally confirmed that by all macroscopic signs (crown openness, exudate discharge from bark cracks, presence of depressed (sunken) necrotic wet wounds in certain areas of cracks, development of a wet pathological core, presence of epicormic sprouts, etc.) the identified disease is a systemic, vascular-parenchymal bacteriosis, known as bacterial wetwood of common oak. The isolated bacterial isolates were identified by morphological, physiological, and biochemical properties as *Lelliottia nimipressuralis* – the causative agent of bacterial wetwood of common oak. This suggests that the aetiology of degradation of common oak in Ukraine is closely related to bacteriosis, and the results of this study allow for early phytosanitary diagnostics of the state of common oak in natural conditions based on typical symptomatic signs

Keywords: common oak, phytopathogenic bacteria, pathogen, pathogenesis, bacteriosis, *Lelliottia nimipressuralis*, sudden dieback

Introduction

In recent decades, trees of the Oak genus (*Quercus*) L., especially *Quercus robur*, are described by reduced competitiveness and resistance, as well as the phenomenon of unregulated accelerated dieback, even in those that grow under optimal conditions [1-3]. Every day, researchers register new reports of an alarming situation within the common oak growing area, associated with the degradation of both individual plants and entire stands of an unknown aetiology [4; 5]. It is known that the life cycle of common oak stands alternates between periods of stabilisation of the sanitary condition and periods of accelerated dieback [2]. Researchers note the cyclicity and established the chronology of this phenomenon [1; 6]. In general, over the past century, there are three recorded waves (periods) of extreme exacerbation of the pathological condition of common oak, which was accompanied by its mass dieback: 1982-1911, 1927-1946, and 1964-1983 [2]. Concerned practitioners and scientists of the forest industry are currently searching for prerequisites for weakening and direct causes of pathological processes in common oak cenoses. The leading role in the mass and dynamic spread of pathological phenomena of common oak is played by synoptic and climatic anomalies (hydrothermal stress) [7], the presence of invasive pests [8-10] and infectious agents [3; 11; 12], etc.

Among the researchers of the causes of oak tree dieback, it is advisable to single out the adherents of infectious aetiology, namely mycotic [13; 14] and bacterial [3; 11; 15]. It is known that common oak is characterised by impaired

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resistance and reduced competitiveness, which leads to the dieback of individual oak trees, as well as entire tree stands. Apart from climatic anomalies and invasive pests, phytopathogenic bacteria play a leading role in the degradation of oak stands. A dangerous feature of phytopathogenic bacteria is the ability to cause diseases that reach the size of an epiphytoties.

Currently, in European countries (Germany, the Netherlands, the United Kingdom, etc.) [16] and in the United States, the phenomenon of "Sudden oak death" [4] is widespread, the aetiology of which is associated with the fungus *Phytophthora ramorum* [14; 17]. The pathogen causes typical symptoms of infection (bleeding wounds, ulcers, and necrosis) on branches and trunks of *Quercus robur*, *Quercus falcata* and *Quercus rubra* [17]. In addition, the scientific literature related to the aetiology of mass dieback of Oak cenoses refers to oak wilt, which is caused by a fungus *Bretziella fagacearum* (Bretz) [13]. There is confirmed information on the mycological analysis of common oak wood with blackened vessels, in which the fungus was isolated *Ophiostoma roboris* [6].

As for the pathogens of bacteriosis, researchers [3; 5; 18] isolated and experimentally confirmed the involvement of phytopathogenic bacteria in the mass dieback of oak trees. One of the first information concerning bacteriosis of common oak belongs to A.L. Shcherbyn-Parfenenko, which specifically refers to bacterial dieback caused by *Plasmopara nigro-quercina* sp. n., *Xanthomonas quercus* sp. n., *Erwinia lignifilla* sp. n. and *Erwinia multivora* sp. n. [15; 19]. Bacterial cancer of common oak trunks and branches is also known to be caused by a polymorphic type of bacteria *Pseudomonas syringae* von Hall. [20], with bacteria *Lonsdalea quercina* subsp. *britannica*, and *Lonsdalea quercina* subsp. *populi* also involved [21]. Similar pathological symptoms (peptic ulcer disease) are caused by *Pseudomonas fluorescens* Migula 1895 and *Pseudomonas* sp. [1]. Brown mucus and other symptoms on common oak are caused by *Micrococcus dendroporthos* Ludw, *Pseudomonas syringae* von Hall, *Erwinia valachica* Georg et Bod, *Erwinia valachica* f. *onaca*, *Erwinia gueieicola* Georg, et Bod. [20].

Currently, there is evidence of a wide spread of bacteria involved in Acute Oak Decay (AOD) in British forests [22], which is generally characterised as a complex syndrome affecting key oak species (namely, *Quercus robur* L. and *Quercus petraea* L. (Matt.) Liebl.), in some cases causing mortality within five years of the development of primary symptoms. The most noticeable symptom is damage to the tree trunk, from which four types of bacteria are isolated: *Brenneria goodwinii*, *Gibbsiella quercinecans*, *Lonsdalea britannica*, and *Rahnella victoriana* [18; 22]. In the northern and mountain forests of Zagros (Iran), symptoms of common oak disease are recorded [23], which are observed in several native species of arboreal plants, including *Quercus castaneifolia* C.A. Mey., *Quercus brantii* Lindl., and *Carpinus betulus* L. There are parallels between the disease in Iran and the AOD reported in the UK, specifically the presence of wet ulcers, which have been associated with a polybacterial complex where *Brenneria goodwinii* is considered a key necrogen [23; 24].

There are also isolated reports of bacterial leaf scorch (BLS) of common oak caused by the bacterium *Xylella fastidiosa*, lives which in xylem vessels (water supply elements) and restricts the flow of water. *Xylella fastidiosa* is transmitted from tree to tree by xylem-feeding insects, such as cicadas and leafhoppers [25].

The Drippy Nut of Oak disease has been spreading in the world (pathogen – *Lonsdalea quercina*) [21], with currently recorded separate data from Spain [26] and Colorado [5]. The symptoms of this disease include loss of colour of acorns and discharge of a viscous brown liquid ("mucus flow"), which slowly scrapes to the soil surface from infected acorns growing on trees. On the territory of Ukraine, the symptoms, features of aetiology (*Erwinia quercina* sp. *nova)* [22], and harmfulness have been known for a long time, thanks to the works of A.F. Goychuk [1]. Apart from the drippy nut of oak, typical manifestations of soft or wet rot of oak acorns (pathogen – *Erwinia carotovora* subsp. *karotovora*) are also recorded [1].

One of the most widespread and dangerous diseases of bacterial origin is bacterial wetwood of common oak [3].

Thus, it becomes clear that pathogens of bacterial diseases – phytopathogenic bacteria, now act not as a concomitant microbiota in the phylosphere of arboreal plant organs but are active and dangerous (sometimes leading) participants in the emergence of pathological phenomena of arboreal plants, specifically common oak. Therefore, they require thorough experimental research and observations.

Since the dieback out of common oak on the territory of Ukraine becomes epiphytotic, and the aetiology of this phenomenon has not yet been established, the purpose of this study lies in experimental confirmation of the immediate causes of this phenomenon, as well as the study of morphological and biochemical properties of the pathogen.

Materials and Methods

The general scheme of pathology studies included reconnaissance and detailed forest pathological surveys of forest stands including common oak with the laying out of 4 experimental plots in the State Enterprise "Fastiv Forestry" of the Kyiv region in the summer and autumn of 2021 according to the SOU 02.02-37-476:2006 "Experimental Plots of Forest Management. Method of Laying Out" (2007) [27]. 7 sample trees were cut down. More than 78 samples (individual tissues and organs) of common oak with visual signs of damage from bacteriosis (trees of the II and III categories of sanitary condition) were selected for microbiological studies.

Bacteriological analysis of the selected samples was performed by homogenisation of plant material, followed by plating in Petri dishes on agarised nutrient media (potato agar, meat-peptone agar, meat-peptone broth, malt extract of agar, etc.) and growing under a thermostat at 28°C for 4-5 days. Colonies were selected for analysis. Glucose-peptone (Eikman's) and Ushinsky media, and medium with asparagine were used to accumulate enterobacteria. King's medium – for identifying Pseudomonas spp based on fluorescein production. Anatomical-morphological and physiological-biochemical characteristics of the selected strains (Gram staining, Voges-Proskauer test, Kovac's oxidase test, dilution of gelatin, growth in 5% NaCl, formation of reducing sugars from sucrose, acid formation from carbohydrates, etc.) were performed respectively to standard protocols and according to the methods of V. Patyka et al. [28] and using the API 20E test system and the NEFERMtest24 MikroLaTEST®, ErbaLachema test system. Omelyansky's mineral medium was used to determine the ability of isolated bacteria to ferment various sources of hydrocarbons (lactose, rhamnose, xylose, trehalose, maltose, raffinose, L-arabinose, sucrose, fructose, galactose, sorbitol, mannitol, glycerol, citrate, salicin) [28]. Milk and gelatin were used to detect proteolytic enzymes in bacteria. Oxidase-negative bacterial isolates were examined to identify them, their properties were investigated and compared with the collection strain *E. nimipressuralis* 8791, and the properties of bacteria given in the Manual of systematic bacteriology [29] and the original works [30]. Pathogenic properties of isolates were detected in laboratory and field conditions by artificial damage to the organs of common oak and indicator plants with a bacterial suspension with a titre of 10^8-10^9 cl×ml^{-1} (according to the turbidity standard). Control – sterile supply water. Subsequently, bacteria were re-isolated from the sites of artificial damage for further comparative studies (Koch's postulates). Latin names of microbiota species are given according to the National Centre for Biotechnology Information [31].

Results and Discussion

Based on the materials obtained during the study, it was found that the mass dieback of common oak covers large areas and all age categories of forests. Identification of the causes of dieback was based on several groups of signs (macroscopic or visual and microscopic). Visual examination of damaged stands revealed macroscopic signs of a bacterial disease – bacterial wetwood, which was later confirmed by laboratory studies [3] based on the D.K. Zabolotnyi Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine. These signs are almost identical to the typical symptoms of bacterial wetwood of arboreal plants described in the literature [15], which indicates persistent symptoms of bacteriosis, regardless of the type of arboreal plant and the region of research. These features include the following morphological, anatomical features and structural modifications of damaged common oak trees:

– local foci of tree death, which cover an average of 6-10 neighbouring trees (Fig. 1a).

Figure 1. Bacterial wetwood on common oak: macroscopic signs (1a – focus of weakened oak trees with an opened crown, water shoots and local ulcers on the trunks; 1b – leakage of bacterial exudate and wet wound development)

– the phenomenon of crown openness, as well as defoliation and dieback of individual apical and lateral shoots, which are atypically curved and coal-black, as if damaged by frost;

– exudate discharge (a dark, usually grey-brown liquid that turns black in the air, with bubbles and a typical smell of butyric acid fermentation) from bark cracks of the affected tree is an important diagnostic sign of wetwood (Fig. 1b). The outflow of bacterial fluid is most often observed in the spring-summer period and does not last long (on average, 10-12 days and significantly depends on the temperature regime), while infected specimens are easily found by formed carboniferous black streaks on the bark of trees, which often go from the primary site of the lesion down the trunk, and then spread out on the soil surface;

– formation of depressed (sunken) necrotic wet wounds in certain areas of the crack. If you remove the top layer of periderm and gradually separate sapwood, you can note that dark brown, sometimes purple-tinged dead areas (ulcers) spread up and down from the primary site of infection by 0.3-0.8 m or more (Fig. 2a), sometimes reaching the basal part of the trunk;

Figure 2. Symptomatic signs of damage by bacterial wetwood of common oak on a cross-section of the trunk: general appearance (2a) and development of a wet pathological core (2b)

– when the bark falls off at the site of wound formation and the source of infection exits, an ulcer with clear edges is formed, the colour of which varies significantly – from dark gray to brown, which is usually associated with the action of saprotrophic mycoflora;

– development of a wet pathological core of rounded or stellated shape (Fig. 2b) with penetration into the shoots and exit along the core rays into cracks and wounds under the pressure of gases that tear the wood fabric. Soft rot with mucus, necrotisation, and maceration of tissues develops in the affected tissues;

– epicormic sprouts on trunks, often underdeveloped, with a light-yellow leaf colour, which indicates the loss of arboreal plants' biological resistance.

All the above-mentioned macroscopic signs of damage from bacteriosis were noted on common oaks of ripening and ripe age, regardless of the growth class and growing conditions. Thus, the detected disease according to all symptomatic signs known from literature sources [15] is a systemic, vascular-parenchymal bacteriosis, known as bacterial wetwood of common oak, which affects all tissues (phloem, cambium, xylem), parts of the plant (branches, trunks, root system) and generative organs (flowers, ovary, fruits, seeds) at all stages of ontogenesis, including self-seeding and seedlings.

It is well-known that based on the analysis of only typical macroscopic symptoms of the disease, it is not advisable to contemplate its aetiology. Therefore, a number of special microbiological experiments (bacteriological analysis) were conducted *in vitro* for the study of morphological, cultural and biochemical properties of isolates (specifically isolates from oak wood samples with typical symptoms of bacterial wetwood) (Table 1).

Table 1. Morphological and biochemical properties of *Lelliottia nimipressuralis*

Test	By Carter	Collection strain (Erwinia nimipressuralis 8791)	Isolated by the authors (2021)
Mobility, peritrich	+	+	+
Gram stain	–	–	–
Yellow pigment	–	–	–
Gelatine dilution	–	–	–
Reaction to milk: coagulation	+	+	+
peptonisation	–	–	–
Litmus serum		+	+
Nitrate reduction	+	+	+
Formation: indole, ammonia	–	–	–
hydrogen sulphide	+	+, –	+, –
Growth on media:			
PA, MPA, MPB, MPA+5% sucrose, Eikman, Ushinsky, with asparagine, Fermi, Liske		+	+
Kohn, Czapek		–	–

Table 1, Continued

Test	By Carter	Collection strain (Erwinia nimipressuralis 8791)	Isolated by the authors (2021)
Absorption of carbohydrates and alcohols:			
arabinose, glucose, maltose, lactose, mannitol	ag	ag	ag
mannose, raffinose, fructose		ag	ag
salicin	+, –	ag	ag
glycerin	+, –	a	a
ramnosa		a*	a
dulcite, inositol		–	–
xylose, sorbitol	ag	ag*	ag*
Assimilation of organic acids:			
ketoglutaric, citric, formic, acetic, malic, succinic, fumaric, lactic		al	al
tartaric, oxalic	–	–	–
Assimilation of amino acids and amides:			
arginine, asparagine, glutamine		al	al
Cysteine, cystine, leucine, tyrosine, tryptophan		–	–
γ- aminobutyric acid		–	–
Enzymatic activity:			
protopectinase, oxidase	–	–	–
catalase, urease		+	+, –
Voges-Proskauer reaction		+	+
Education:			
indole	–	–	–
ammonia		–	–
hydrogen sulphide	+	–*	–

Note: (+) – availability of properties; (–) – lack of properties; (+,–) – variable properties; () – data missing or not researched; (a) – formation of acid; (al) – alkali formation; (g) – gas formation; (r) – reduction; (*) – individual strains have different properties
Source: [3; 32; 33]

It was found that isolates from common oak tissues are straight or ellipsoid gram-negative rods with a size of 0.6-0.8×0.7-1.6 μm, which move using peritrichal flagella. In smears from agar and broth culture, cells are arranged singly, in pairs, and less often – in short chains. They do not form capsules and spores.

On potato agar (PA), colonies are formed rounded, 4-5 mm in diameter, greyish white in colour, less often – with a typical shade of cream colour, translucent to the light, convex in shape, with a weak gloss. The edge of bacterial colonies forms weak waves, occasionally stays smooth, a corrugated strip runs along the periphery, translucent circles and radial rays are clearly visible in the light.

On meat-peptone agar (MPA), bacterial colonies are smaller in size, grey in colour, with a weak gloss, translucent, granular, smooth, slightly convex. The edge of the colonies is slightly radially crossed, ridge-like. In meat-peptone broth (MPB), bacteria grow well, forming a parietal ring, uniform turbidity, swirled sediment, and pellicle.

On Eikman's and Ushinsky's media with asparagine, isolated bacteria also grow well and form a light greyish-white pellicle. Colonies on King's media are characterised by abundant growth and dirty, milky white colour. The lack of growth on a nutrient medium with dulcite is a common property of the strains isolated for this study.

On mineral media with arabinose, galactose, glucose, xylose, lactose, maltose, mannose, mannitol, raffinose, sucrose, sorbitol, salicin, and fructose, all strains form gas and acid. Some amino acids and amides are used as a carbon source. No changes were recorded in the medium with leucine, cystine, tyrosine, cysteine, and tryptophan. Tartaric and oxalic acid strains were not used. During the day, mineral media with sodium salts of succinic, ketoglutaric, citric, lactic, formic, acetic, fumaric, and malic acids were intensively alkalised.

All the bacterial isolates under study had a positive Foges-Proskauer reaction.

The bacteria isolated do not dilute gelatin; coagulate milk quickly, do not form oxidase and protopectinase; form catalase and urease. They do not use Inositol and sorbitol citrate. They do not form indole and hydrogen sulphide (H_2S) but are capable of reducing nitrates. They contain arginine dehydrolase, ornithine decarboxylase, β-galactosidase, but there is no lysine decarboxylase, urease.

Individual properties of bacteria are significantly affected by their habitat, so the current differences between the collection strain (*Erwinia nimipressuralis* 8791) and bacteria by Carter [33] are variability within the species.

Therefore, according to the complex of investigated morphological, cultural, and biochemical characteristics, bacterial isolates from common oak with typical symptomatic signs of bacterial aetiology disease damage are almost identical to the collection strain of the Department of phytopathogenic bacteria at D.K. Zabolotnyi Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine – *Erwinia nimipressuralis* 8791 and correspond to the properties given in the bacterial determinant [29] for *E. nimipressuralis*.

Thus, by their morphological, cultural, and biochemical properties, the isolated bacteria are classified as *Lelliottia nimipressuralis* (Carter 1945) (synonymous names – *Erwinia nimipressuralis* (Carter 1945) Dye 1969 and *Enterobacter nimipressuralis* (Carter 1945), which is a known causative agent of bacterial wetwood of coniferous and deciduous species of arboreal plants [15], including common oak.

Conclusions

One of the main reasons for the mass weakening and dieback of common oak on the territory of Ukraine is the spread of systemic, vascular-parenchymal bacteriosis, which affects all tissues, plant parts, and generative organs at all stages of ontogenesis, including self-seeding and seedlings. According to the identified typical macroscopic symptoms, namely excessive crown openness, as well as defoliation and dieback of 1-2-year-old shoots; cracking of the bark of trunks and the development of depressed necrotic wet wounds, which discharge bacterial exudate during the growing season (especially in the spring) – a gas-filled liquid and mucus of dark (brown or black) colour with a typical smell of butyric acid fermentation; the development of a wet pathological core; the presence of epicormic sprouts, etc., it was found that this bacteriosis is nothing less than bacterial wetwood. At the same time, the prerequisite for active and aggressive development of any disease of infectious aetiology is a decrease in plant immunity due to the development of destructive processes caused or enhanced by adverse abiotic environmental factors. In the case of bacterial dropsy, its mass spread occurs after a dry summer, i.e., it is hydrothermal stress that contributes to the unregulated spread of the causative agent of said bacteriosis and associated organisms (pathogens of root rot, stem pests, and a wide array of microxylotrophs). The bacteriological analysis revealed that the bacterial isolates from common oak with typical symptomatic signs of damage by bacterial wetwood, according to their biochemical, morphological, and cultural properties, are classified as the causative agent of bacterial wetwood of coniferous and deciduous species of arboreal plants, including common oak – *Lelliottia nimipressuralis*. A promising area of future research lies in the development of specific methods and means for protecting arboreal plants, namely for the use of biologics based on Bacillus sp. and other myco- and microorganisms with existing antagonistic properties to phytopathogens.

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Етіологія бактеріальної водянки *Quercus robur* L.

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Анотація. Фітопатогенні бактерії у рослинному організмі виступають невід'ємною компонентою супутньої мікрофлори, а також збудниками патологічних процесів, які не просто ослаблюють рослину, а часто протягом короткого періоду часу (при гострому патогенезі) призводять до явища деградації та повного відмирання. Також варто зауважити, що бактеріозам притаманні типові макроскопічні ознаки перебігу хвороби, проте точну етіологію патологічного процесу можна достовірно встановити лише на основі проведення бактеріологічного аналізу з визначенням морфологічних, культуральних і біохімічних властивостей ізолятів. Метою досліджень є експериментальне підтвердження безпосередніх причин явища деградації дуба, спричиненої бактеріальною водянкою у обстежуваних деревостанах, а також дослідження морфологічних і біохімічних властивостей збудника. В роботі використано класичні мікробіологічні, фітопатологічні та біохімічні методи, за допомогою яких встановлено етіологію захворювання, аналіз типових симптомів, проведена мікроскопія уражених частин дуба, ізоляція та ідентифікація збудника. Властивості ізолятів бактерій визначали за загальноприйнятими методиками та з використанням API 20E тест-системи і тест-системи NEFERMtest24 MikroLaTEST®, ErbaLachema. Експериментально підтверджено, що виявлене нами захворювання за всіма макроскопічними ознаками (ажурність крони, виділення ексудату з тріщин кори, наявність вдавлених (запалих) некротичних мокрих ран на окремих ділянках тріщин, формування мокрого патологічного ядра, присутність водяних пагонів та ін.) є системним, судинно-паренхіматозним бактеріозом, відомим як бактеріальна водянка дуба. Виділені нами ізоляти бактерій, за морфологічними і фізіолого-біохімічними властивостями ідентифіковані як *Lelliottia nimipressuralis* – збудник бактеріальної водянки дуба. Це дає підстави стверджувати, що етіологія деградації дуба звичайного в Україні тісно пов'язана з бактеріозом, а результати наших досліджень дозволяють здійснювати ранню фітосанітарну діагностику стану дуба в природних умовах за типовими симптоматичними ознаками

Ключові слова: дуб звичайний, фітопатогенні бактерії, збудник, патогенез, бактеріоз, *Lelliottia nimipressuralis*, раптове відмирання