The distribution of bacteria *Gibbsiella quercinecans* and *Brenneria goodwinii* in oak (*Quercus robur* L.) stands in Latvia

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**Abstract.** Acute Oak Decline (AOD) is a complex plant disease affecting species of native oaks, for example, *Quercus robur*, in Latvia. Typical AOD symptoms are bark cracks with dark exudate. In other European region countries, like the United Kingdom, AOD is associated with bark insects, for example, *Agrilus biguttatus*. From the results of the forest survey, it can be concluded that in oak forest stands located in Latvia, there is practically no damage by the bark insect *A. biguttatus*, which may indicate the existence of another AOD spreading vector. In 2019, from one oak, which was positive for AOD in the spring, repeated stem bleed samples were taken in the autumn period, the laboratory analysis results of the repeated samplings were negative. These findings suggest that the activity of bacteria *Brenneria goodwinii* and *Gibbsiella quercinecans* are influenced by climatic conditions.

1. Introduction

Pedunculate oak (*Quercus robur* L.) belongs to the beech family (*Fagaceae. Dumort*) [1]. Latvia is close to the northern limit of the pedunculate oak distributional range (figure 1) [2]. Oaks are light-demanding trees that prefer to grow in fertile and moist soil. Fluctuation in ambient temperature can affect oak growth and development. They are most vulnerable to frost in late spring, when the new foliage is developing, for example, if the temperature reaches -3°C, it can kill new foliage [3].

In Latvia, according to the evaluation by P.Saksa in the 1960s [4], oaks, ashes, small-leaved lime and beech dominate stands covering approximately 15,000 ha of the total forest area. With an increase of forest territory, the territory covered by oak, ash, small-leaved lime and beech dominated stands increased, reaching 3,200 ha dominated by oaks and 10,200 ha by ashes (0,8% of the total forest area) in 1983 [5].

According to the information provided by the State Forest Service, in the previous decade’s areas covered with oak-dominated stands have slowly decreased. In 2001, 10051 ha were covered with oak-dominated stands, but in 2005, it was 10028ha. In 2011 only 0.34% (9,734 ha) of the total forest area was covered with oak-dominated stands (unpublished data, State Forest Service, 2011). In the past decade, oak stand areas have not changed significantly, in 2020 it was 9527,85 ha (unpublished data, State Forest Service, 2020).
Pedunculate oak forest covered areas are still reducing not only in Russia but also in many other countries, for that reason protection and conservation are still problems. Every year many researchers publish data about new and rapid outbreaks of oak forest diseases not only in Russia but also in many other countries: in the United Kingdom, USA, Germany, Netherlands, Denmark, Poland, etc. Scientists indicate that qualitative decrease in forest conditions and mortality is influenced by abiotic, biotic and anthropogenic factor complexes. In the scientific literature, it is possible to find many reasons for forest mortality- unfavourable weather conditions: drought, hard frost or insect attacks.

An increase of various disease distribution was observed in temperate climatic zone forests due to climate change. The decline of trees can be a fast process, or it can take several years or decades. The reason can be biotic or abiotic factors or a complex of both. These factors can reduce natural protection mechanisms and make it more predisposed to diseases.

Acute oak decline (AOD) is relatively recently described, and is characterised by the presence of newly identified bacterial species – *Gibbsiella quercinecans* and *Brenneria goodwinii*, as well as probably by other, not yet clearly explored factors. It might be a complex disease, development of which is affected by many factors (not only bacterial influence). The main characteristic of the disease is dark, sticky exudate on the bark of the tree and tree decline. It can cause tree mortality in 3-5 years. 50 years old trees with a diameter larger than 30 cm are more predisposed to AOD.

According to the recent findings *B. goodwinii* and *G. quercinecans*, and some other bacteria species found in necrotic lesions, for example, *Rahniella victoriana*, *R. variigena* and *Lonsdalea britannica*, have phytopathogenic virulence genes. However, *B. goodwinii* and *L. britannica* have higher genome encoded phytopathogenical potential, compared to other mentioned bacteria. *B. goodwinii* have T3SS (type 3 secretion system) associated harpins and effectors, that may cause tissue necrosis. It is suggested that *G. quercinecans* acts as a pathobiont enhancing disease caused by other bacteria.

Recent studies show that *G. quercinecans* can survive in rainwater up to 84 days, but in forest soil up to 28 days, it is enough for spreading from one host to another. *G. quercinecans* can survive outside the host plant and it shows that the bacteria is widespread in the ecosystem. Whereas *B. goodwinii* cannot survive outside the host plant, it is an oak specific endosymbiont and outside the host, it enters a viable, but non culturable state.

In 2017, AOD was confirmed in Switzerland for the first time. Samples from typical AOD lesions were plated on nutrient agar. Morphologically different bacteria colonies were chosen and sequenced (partial genes 16S rRNA and gyrB). The result indicated AOD associated bacteria occurrence:
B. goodwinii, G. quercinecans and R. victoriana. Also pathogenic tests were done to prove that B. goodwinii, G. quercinecans and R. victoriana are pathogenic and can develop typical symptoms in oak trees [15]. Also in the spring of 2021, AOD associated bacteria B. goodwinii and G. quercinecans for the first time were confirmed in Poland. Confirmation was made using molecular methods: real time PCR and sequencing [16].

In 2017, AOD was detected for the first time in Latvia (Kurzeme), and since then researchers are working to determine the distribution of G. quercinecans, B. goodwinii and also to develop solutions to prevent this disease from spreading. This is important because AOD affects not only oak trees in forests, but also in parks, farmsteads and green areas where pedunculate oaks and other oak species are growing.

2. Material and methods

To get more information about AOD associated bacteria B. goodwinii and G. quercinecans distribution in Latvia, and in addition to analyse typical AOD symptoms and factors that can affect disease development, and to develop a management strategy of oak stands, the study “Development of an action plan for the inhibition of Acute Oak Decline at the Forest Research station territory Talsu pauguraine, and possible expansion to other regions in Latvia” was created. This project unites several Latvian organizations that are associated with plant pathology and forestry fields.

An observational study on the symptoms and signs that characterize AOD in Latvia was carried out during the period between the end of 2018 and October of the year 2019.

2.1. Characteristics of the research site

The climate of the territory of Latvia is characterized as a transition zone between the maritime and continental climates. The coldest month is January, but the hottest is July. The average temperature in January 2018 was -1.6°C, but in July +19.8°C. The average annual precipitation was 472.7 mm per year, which was 32% below the defined average annual precipitation (692.3 mm), therefore the year 2018 was the 4th driest year in weather observation history. Also, the average annual temperature was 1.2°C above the defined average, meaning that this year was the third hottest year in weather observation history [17].

The year 2019 also was hot and dry. The average temperature in January was -4.0°C, in June it was +18.6°C, but the average annual precipitation was 629.2 mm (9% below the defined average annual precipitation of 692.3 mm). The average annual temperature was +8.2°C, which is the highest observed average annual temperature since the year 1924 [18].

The most common soil types in Latvia are moraine relief with sandy loam and loam soil [19]. Because of soil diversity, there is a great diversity of forest types and tree species in the forest [20].

2.2. Methodology for selection of oak stands

Oak-dominated stands were chosen from the State Forest Service supported State Register of Forests, which contains information about forest stand inventory data of all Latvia territory (figure 2). The State Forest Service selected the surveyed forest stands based on the following criteria: stand age - at least 50 years; stand composition (forest stand formula Oz) – 4, 5, 6, and 10 oaks; plot area – at least 1 ha. Corresponding data were collected into a database (MS Excel) and divided into three forest districts: Ziemeļkuzemes, Dienvidkurzemes un Zemgales.

A total of 745 sites were selected that met the set criteria. To determine the prevalence of AOD, the State Forest Service surveyed 652 forest stands in 2018 and 436 forest stands in the summer and autumn of 2019.
2.3. Sampling and characterization
Notes on site history, characteristics and management practices were made. The location of each tree was recorded using GPS (Trimble Geo7x – Global Positioning Systems, figure 3). Symptomatic parts of the trees were photographed, documenting the characteristics of the external symptoms. Indications were recorded, i.e. evidence of pests such as D-type exit holes indicating the bark insect (*Agrilus biguttatus* Fabricius) or pathogens. The measurement data were stored in an application created specifically for service use and further processed in the GIS system to create map layers. If a suspicious oak was found in the surveyed forest stand, its coordinates were recorded (in the LKS-92 system), while if several suspicious oaks were found in the stand, the coordinates of at least one tree were recorded.

The employees of the State Forest Service organized surveys according to guidelines in the database. Surveys were made in the period between the end of 2018 and October of the year 2019. Selected forest stands were surveyed following to developed methodology: the stand was surveyed along the longest diagonal, visually inspecting and evaluating growing oaks. In practice, it was found that the optimum placement of the transects was diagonally across the stand in the SE → NW direction. If the stand area was not large, the entire stand survey was made. During the surveys, attention was also paid to stand borders and to individual sunny places, where theoretically the detection risk of AOD can be higher. When inspecting individual oak trees, attention was paid to stem parts exposed to the South. Symptomatic oaks were marked with at least 3 cm wide bright color line.

![Figure 2. Location of oak stands in Latvia.](image)
If the typical stem bleeding was detected, swabs of the lesion fluid were collected (figure 4). Sample were labelled and delivered to the National Phytosanitary Laboratory for further analysis.

2.4. Laboratory methods
Screening tests for *Brenneria goodwinii* and *Gibbsiella quercinecans* were adapted from the Forest Research in the United Kingdom. Swabs were processed according to the methodology developed in Forest Research [21]. Real-time PCR assays were performed according to the Forest Research protocols with some modifications. Real time PCR assays were performed using a Rotor-Gene Q thermocycler (Qiagen). For the *G. quercinecans* assay, primer pair GQ gyrB qPCR F / GQ gyrB qPCR were used [14]. Real-time PCR reactions were carried out with 18 µL master mix (consisting of 0.15 µM forward primer, 0.15 µM reverse primer and 1x PCR master mix (5x HOT FIREpol EvaGreen qPCR Mix Plus
(no ROX) and molecular grade water) and 2 µL of bacterial cell suspension. Real-time PCR conditions were: 95°C for 12 min followed by 40 cycles of (95°C for 15 s and 62°C for 30s). The fluorescence was measured at the end of each cycle. Immediately after PCR melting curve analysis was done. In this process, the amplicon DNA was heated gradually from around 50°C up to around 95°C. Specific melt peaks were identified with reference to the positive amplification control.

For the *B. goodwinii* assay, the primer pair BG-F/BG-R and hydrolysis probe BG-P [22] were used. Real-time PCR reactions were carried out with 19 µl master mix, consisting of 0.6 µM forward primer, 0.6 µM reverse primer, 0.3 µM probe and 1x PCR buffer (Rotor gene probe PCR kit), molecular grade water and 1 µL of bacterial cell suspension. Real-time PCR conditions were: 95°C for 3 min followed by 40 cycles of (95°C for 15 s and 63°C for 40s). The fluorescence measurement was performed at the end of each cycle.

3. Results and discussion

It has already been reported that the AOD in Latvia was first detected in 2017 in the Talsi hillock natural park area [23]. AOD is still a very little studied and identified disease. It is still unclear how oaks become infected with AOD and what are the vectors. The disease is thought to be caused by bacteria but is influenced by a combination of biotic and abiotic factors [14].

To determine the prevalence of AOD in 2019, the State Forest Service surveyed 436 forest stand areas in summer and autumn. In total, 46 exudate and bark panel samples were collected and analysed in the National Phytosanitary Laboratory, of which 7 samples proved to be positive. In 2018, the western part of Latvia was studied more, because this region has a larger number of oak stands. During 2018, State forest Service employees performed a total of 652 surveys of oak stands. At the same time, AOD was laboratory-confirmed in 16 cases.

The laboratory analysis results of the surveyed oak stand in the period 2017-2020 are shown in the figure 5. From these results, it can be concluded that in oak forest stands in Latvia there is practically no damage to bark insects (*Agrilus biguttatus* Fabricius), which may indicate the existence of another AOD spreading vector. On contrary, in The United Kingdom AOD cases are associated not only with typical bacteria *B. goodwinii* and *G. quercinecans*, but also with bark insects, especially with *A. biguttatus*, which is one of the main vectors [7].

In 2019, from one oak, which was positive for AOD in the spring, repeated stem bleed samples were taken in the autumn period. The results of laboratory analysis of the repeatedly sampled stem bleeds were negative. These findings suggest that the activity of bacteria *B. goodwinii* and *G. quercinecans* is influenced by the climatic conditions.

Research on these issues should be continued, because the winter in 2020 was atypical for Latvian climatic conditions, being relatively warm. This probably affected typical AOD symptom development on the oak stems, because no bleeding was observed in the spring. Also, due to changing climatic conditions, practically no active wounds and new wounds were observed in 2020, except for individual trees that were previously infected. On the stem of these four trees, many wounds were observed, but the decline (formation of dry branches) slowed down.

Also, there is a possibility that AOD symptom development was consequence of historical stress. Dendrochronology studies in The United Kingdom have shown that oaks can reduce their radial growth many years before typical ADO symptom development, meaning that historical stress periods can affect AOD development even after several decades [24].
4. Conclusion
This research showed AOD distribution in Latvia and led us to start to consider all possible reasons for AOD spreading in our country. In the future, new scientific research must be carried out, to get more information about AOD spreading vectors, disease development, for example, fungal and abiotic effects on AOD development. Also, it would be useful to observe symptomatic trees for multiple years to see if the disease has progressed. Resampling exudate and necrotic tissue in different seasons and years would give information about bacterial composition differences in samples around the year, this information could give as more information about the bacteria life cycle and can be compared with typical symptom development severity. Also, it is necessary to test samples for another with AOD associated bacteria, for example, Lonsdalea britannica and Rahniella victoriana. All mentioned information can be useful for developing recommendations for AOD containment and management of oak stands.

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Figure 5. Map of distribution of AOD in Latvia.
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