

Article

Brenneria goodwinii and Gibbsiella quercinecans as a Threat to Quercus coccifera L.

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Abstract: Acute Oak Decline (AOD) is a complex and rapidly progressing disease affecting several *Quercus* species across Europe. While previously reported in *Quercus ilex* in Italy, this study provides the first evidence of AOD symptoms and associated bacterial infection in *Quercus coccifera* (kermes oak). Symptomatic trees were identified in a Mediterranean forest in southern Italy, and bacterial isolation, qPCR detection, and 16S rRNA sequencing confirmed the presence of *Brenneria goodwinii* and *Gibbsiella quercinecans*. Phylogenetic analyses clustered the isolates closely with known AOD-related strains. Pathogenicity tests on excised *Q. coccifera* branches demonstrated that both bacteria induced wood necrosis and external exudates consistent with natural symptoms, confirming their virulence. These findings expand the known host range of AOD-related bacteria and highlight the potential threat to Mediterranean oak ecosystems. Early detection and monitoring of *Q. coccifera* decline are essential to inform conservation strategies and forest management practices aimed at mitigating AOD spread.

Keywords: kermes oak; biotic stress; Quercus decline; Italy; acute oak decline



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1. Introduction

Kermes oak (*Quercus coccifera* L.) is a shrub-like evergreen oak that generally does not exceed two meters in height [1]. Distinctive of this oak species are the leaves, which are 1.5–4 cm long and coriaceous with spiny margins, and also the acorns, which possess a characteristic cup that exhibits numerous elongated and reflexed scales [2,3]. Regarding its ecology, it mainly forms the garrigue [4], and it can also be associated with taller trees, like pines and other oak species, and with other shrubs [5]. It is a hardy and slow-growing oak, able to grow in harsh conditions, tolerating nutrient-poor and rocky soils, drought, and fire [6]. It is native to the Mediterranean basin and can be found in Portugal, Spain, Greece, Turkey, South France, and the Southernmost areas of Italy [7], where it is commonly found in Sardinia, Sicily, Basilicata, and Apulia [8]. Given its ecological importance and adaptive resilience in Mediterranean environments, investigating the susceptibility of *Q. coccifera* to emerging oak diseases has become increasingly relevant—particularly in light of recent reports of Acute Oak Decline (AOD) in neighboring oak species such as *Q. ilex*. In 2023, bacteria identified on holm oak in Apulia, specifically Salento, were

reported as pathogenic and necrogenic for oaks in many European countries [9–13]. These include *Brenneria goodwinii*, *Gibbsiella quercinecans*, and *Rahnella victoriana*. These bacteria are known to be the biotic cause of a disease called “Acute Oak Decline” (AOD), a plant disease described for the first time in the UK in 2014, on *Q. petraea* (Matt.) and *Q. robur* (L.) [14–16]. This disease is characterized by distinct symptoms such as brownish exudate bleeding from longitudinal bark cracks, rarefaction of the canopy due to the defoliation, and sprouting of epicormic shoots from the trunk and main branches. The disease is referred to as “acute” as it can quickly lead to the tree’s death, usually within a few years of symptoms, and is characterized by sudden regression in the health of oak trees [16–18]. After its first description, the disease has been observed in different European countries, such as *Q. robur* in Switzerland [9,19], *Q. petraea* in Spain [20], *Q. ilex* in Slovakia and Italy [11,13], *Q. cerris* in Poland [10], *Q. suber* Latvia [21], and *Q. suber* in Portugal [12].

This study presents novel insights into the interactions between *Q. coccifera* and bacterial species associated with Acute Oak Decline (AOD), specifically *Brenneria goodwinii*, *Gibbsiella quercinecans*, and *Rahnella victoriana*. The primary aim is to evaluate the pathogenic potential of these bacteria towards *Q. coccifera* and to elucidate their phylogenetic relationships with related taxa.

2. Materials and Methods

2.1. Sampling and Symptom Assessment

The sampled area, designated as “Bosco dei Romani” (39°53′41.514″ N, 18°10′4.393″ E), is located within the Regional Natural Park of the Ugento Coast in Salento, Italy (Figure 1). This area encompasses a dense mixed forest characterized by Mediterranean shrubland, such as *Rosmarinus officinalis* (L.), *Cistus* (L.), and *Rhamnus alaternus* (L.), and oak species, in particular *Q. ilex* and *Q. coccifera*. Within this forest, a group of several kermes oaks exhibiting symptoms of AOD were identified. The symptoms of each specimen were assessed with Finch’s pathogenicity index, which takes into account 31 descriptive parameters (Table S1) of the oak’s phenological and health status [22]. These parameters aim to evaluate each oak’s size, canopy, and trunk conditions. The data collected visually with a measuring tape or with the hypsometer Haglof Vertex 5 (Haglofs, Torsang, Sweden) were immitted in the online tool (<https://jasenfinch.shinyapps.io/decliner/>, accessed on 6 March 2025) to evaluate and indicate the type and severity of the decline through the Phenotypic Decline Index (PDI) and the Decline Acuteness Index (DAI). The PDI indicates the decline severity and ranges between 0 and 1, the maximum severity score. In contrast, the DAI indicates the differences between two types of decline, Chronic Oak Decline (COD) and Acute Oak Decline, and ranges between −1 and 1, where oaks with a more severe COD score are closer to −1 and oaks with a more severe AOD score are closer to 1.

After symptom evaluation, bark samples were collected from the bleeding points. To diagnose symptomatic plants, approximately 1 g of outer bark fragments was collected at the point where the exudates and brown patches were present, using a sterile blade. The wooden pieces were placed in a sterile plastic test tube and properly labelled; for each tree, at least five exudates were sampled.

For bacteria isolations, bark panels (approximately 3 cm × 4 cm), also in correspondence with the brownish patches with exudates, were excised using a sterile scalpel and placed into sterile plastic containers. The wood panels collected consisted of the outer bark, cambium, and xylem, which also exhibited necrotic areas. For isolations, three panels were collected for each tree.

For the pathogenicity test, segments of branches approximately 20–25 cm long and 3 cm in diameter were excised from healthy kermes oak trees that showed no symptoms of AOD, fungi body fruits, or other potential pathogens.

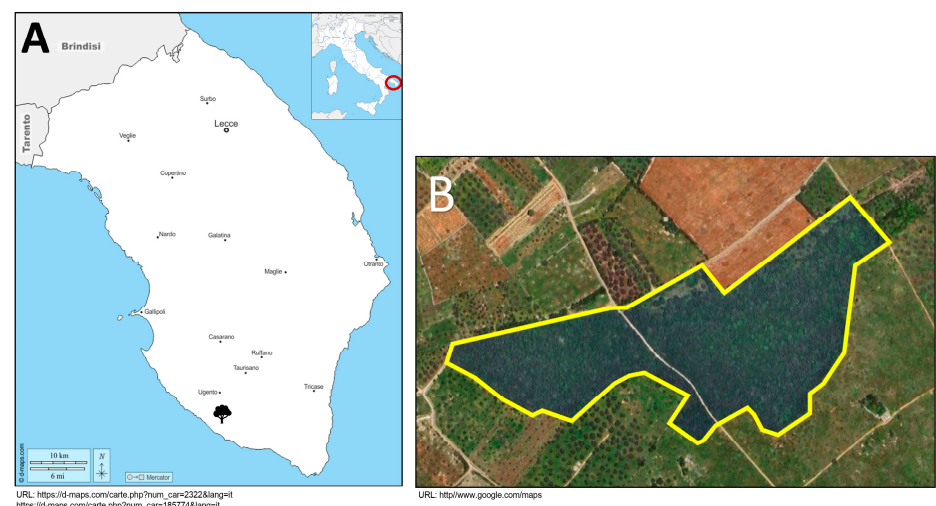


Figure 1. (A) The figure presents the geographical map of Italy, with a focus on the Salento region. The red triangle indicates the geographic location of the sampled area. (B) The figure depicts the aerial view of “Bosco dei Romani”, with the area highlighted in blue.

All samples were maintained at 4 °C and transported to the laboratory.

2.2. Plant Diagnosis

B. goodwinii, *G. quercinecans*, and *R. victoriana* were detected as described by Carluccio et al. [13], based on Crampton et al. [23] with modifications. Bark fragments for each sample were macerated with 1 mL of Sterile Distilled Water (SDW) and gently shaken for 5 min. Then, the suspension was transferred into a sterile 1.5 mL tube and left to settle for a few minutes in order to remove woody debris. After the supernatant was collected, it was centrifugated for 4 min at 9000× g. The resulting pellet was resuspended in 1 mL of SDW, vortexed, and centrifuged again for 4 min at 9000× g; this process was repeated four times in total. Finally, the pellet was resuspended in 50 µL of SDW and stored at −20 °C until use.

To improve specificity and sensitivity, real-time PCR was performed according to Crampton et al. (2020) [23], with species-specific primers validated against a panel of related bacterial species (Table 1). The reaction mixture for *G. quercinecans* consisted of 5 µL of TaqMan™ Environmental Master Mix 2.0 2× (Thermo Fisher, Waltham, MA, USA) and 0.1 µM of each primer and probe, while for the screening of *B. goodwinii* and *R. victoriana*, 0.25 µM of each primer was used. The final volume of the qPCR mixture was 10 µL.

Table 1. Primers and probes used for screening colonies, according to Crampton et al. [23].

Bacteria	Primers and Probes	Sequence (5′–3′)
<i>Brenneria goodwinii</i>	Bg99F Bg179R Bg179P	CTGGCCGAGCCTGGAAAC AGTTCAGGAAGGAGAGTTCGC CCAGAATCTCATATTTCGAACTCCACCATGTT
<i>Gibsiella quercinecans</i>	Gq284F Gq418R Gq342P	GGCTTTGATAGTGGTGGCC CGTTCCGTTATCACCGTGG AACAGTTCAGCGCCATTTTCTTCG
<i>Rahnella victoriana</i>	Rv15F Rv134R Rv57P	CACCCAGACTTACGTGCAT TCAGTGTGATTGGTGAAGGT AGTGATTGGCGATACTGACGTGACC

2.3. Bacterial Isolation and 16S Barcoding

Isolation procedures followed the protocol described by Moradi-Amirabad et al. [24]. Bark sections were sterilized using 70% (v/v) ethanol and rinsed three times with Sterile Distilled Water (SDW). Fragments were then collected from the transition zone between necrotic and healthy tissues as well as from the xylem and outer bark. Each sample was placed in a Petri dish containing 1 mL of SDW and finely chopped using a sterile scalpel. After a 30 min incubation at room temperature, the suspension was streaked onto Nutrient Agar (NA) using a sterile loop. The plates were incubated at 28 °C for five days and monitored daily. DNA was extracted from the resulting colonies [25], and qPCR was performed using the protocol from Crampton et al. [23] to identify colonies of *Brenneria goodwinii*, *Gibbsiella quercinecans*, and *Rahnella victoriana*. Colonies testing positive were subsequently isolated and grown individually on NA.

To confirm the qPCR results, partial 16S rRNA gene sequencing was conducted [26]. PCR reactions were carried out in a final volume of 25 µL, consisting of 12.5 µL of EmeraldAmp® MAX PCR Master Mix 2× (Takara Bio, Kusatsu, Japan), 0.5 µL of each primer (EUB9F: 5′-GAGTTTGATCMTGGCTCAG-3′; EUB1492R: 5′-ACGGYTACCTTGTT-ACGACTT-3′), and 5 µL of DNA template, following the methods described by Petti-
for [25] and Crampton et al. [23]. PCR conditions were as follows: an initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 1 min, 53 °C for 1 min, and 68 °C for 90 s; and a final extension at 68 °C for 5 min.

The resulting amplicons were sequenced via Sanger sequencing (Eurofins, Ebersberg, Germany), aligned with Clustal W [27], and trimmed to equal lengths; positions with gaps or ambiguous bases were excluded before analysis; lastly, they were compared on the NCBI (National Center for Biotechnology Information) database using the Basic Local Alignment Search Tool for nucleotide (BLASTn) [28]. Phylogenetic relationships were reconstructed using partial 16S rRNA sequences in MEGA 11 software [29,30], employing the maximum likelihood method with the Tamura–Nei model and 1000 bootstrap replications to assess clade support [16,29].

2.4. Pathogenicity Tests

Pathogenicity tests were carried out based on Eichenlaub et al. [31]. The branch segments collected were exposed to UV rays for 2 h for superficial sterilization in order to avoid contamination. Subsequently, four inoculation points, representing four replicas for each isolate, were created on each segment by removing a bark disc with a sterile cork borer (1 cm in diameter) along the woody segment length. A one-day-old colony was applied using a sterile loop to each inoculation point. The loop was carefully rubbed on the xylem and on the internal part of the bark disc to ensure a uniform colony distribution. In total, two wood segments were infected for each bacterium plus one branch segment for mock inoculation. Additionally, as a reference, separate twigs were inoculated with isolates of *B. goodwinii* and *G. quercinecans* that were isolated from holm oak and have already been shown to be necrogenic on excised holm oak branches [32].

After repositioning the removed bark discs, the inoculation points and the upper end of each branch segment were labelled and sealed with a plastic film to maintain moisture. Additionally, the lower end of each segment was placed in contact with SDW to prevent the branches from drying out. The prepared segments were then incubated for 1 month at 28 °C with a 12 h photoperiod in a growth chamber.

Following the incubation period, bark was removed from the inoculation sites to examine the underlying tissue. Wood fragments were taken from the boundary between necrotic and healthy areas, specifically from the inner bark and xylem. These samples were surface-sterilized using 70% ethanol, rinsed twice with Sterile Distilled Water (SDW), and

then suspended in 1 mL of SDW and further shredded with a sterile scalpel. After a 30 min incubation at room temperature, the suspension was streaked onto Nutrient Agar (NA) plates using a sterile loop. The plates were incubated at 27 °C for 48 h, after which, the emerging colonies were screened via qPCR as previously described.

3. Results

3.1. Symptomatology and Diagnosis

During the sampling of Bosco dei Romani, an area where symptomatic and AOD-positive holm oaks had previously been identified [13], *Q. coccifera* oaks exhibiting characteristic symptoms of AOD were also observed (Figure 2). These plants exhibited brown exudates on the bark of the trunk and branches, dark patches on the bark due to the wood's impregnation with exudate, and larval galleries corresponding to the exudate. At the canopy level, the foliage appeared sparse. All the kermes oaks sampled were infected with *B. goodwinii* and *G. quercinecans*, but no sample tested positive for *R. victoriana*.

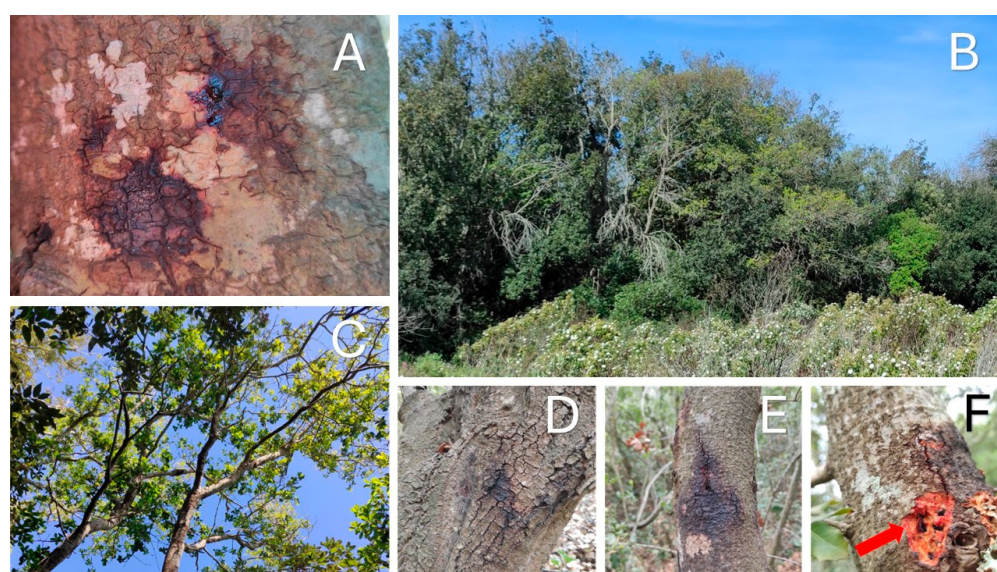


Figure 2. The figure shows the symptoms and conditions observed: (A) brown exudate and patches observed on the bark; (B,C) crown dieback and defoliation; (D,E) dark patches on the bark, given by the necrotic activity of bacteria; (F) red arrow indicates insect galleries under the bark.

Regarding the analysis of symptomatology, the use of the Finch's index revealed that all the plants were affected by AOD, with an average PDI of 0.81 and a DAI of 0.53. These values thus indicate that the oaks examined experience an acute type of decline (DAI close to 1) and similar severity, except for one specimen with a PDI of 0.88, thus showing more severe symptoms (Figure 3).

3.2. Isolation, Sequencing, and Phylogenetic Analysis

After plating, white-colored colonies developed on the NA agar between the third and fifth days of incubation. The identity of the colonies was initially verified by qPCR, and then *B. goodwinii* and *G. quercinecans* colonies were plated individually on NA. No colony tested positive for *R. victoriana*.

Sequencing of the target bacteria by partial 16S rRNA gene revealed that the two isolates were similar to the reference strains, with 99.78% with isolate BY5 (Accession number KY231164) and 99.64% with isolate FRB 185 (Accession number GU562340) for *B. goodwinii* and *G. quercinecans*, respectively. The nucleotide sequences of the isolates were then deposited on GeneBank with the following accession numbers: PQ373172 for

B. goodwinii (isolate BRUG24) and PQ373180 for *G. quercinecans* (isolate BRQS24); based on those sequences, deposited on NCBI, phylogenetic trees were then constructed for each bacterium (Figures 4 and 5). For *B. goodwinii*, the loci OM287486 and MZ612851, respectively, of the strains KKP 3652 and S1T1 of *Serratia fonticola* were used as outliers, while for *G. quercinecans*, the loci NR112555 and EF122435 of the strains DSM 12163 and ICMP 14143 of *Erwinia pyrifoliae* were used as outliers.

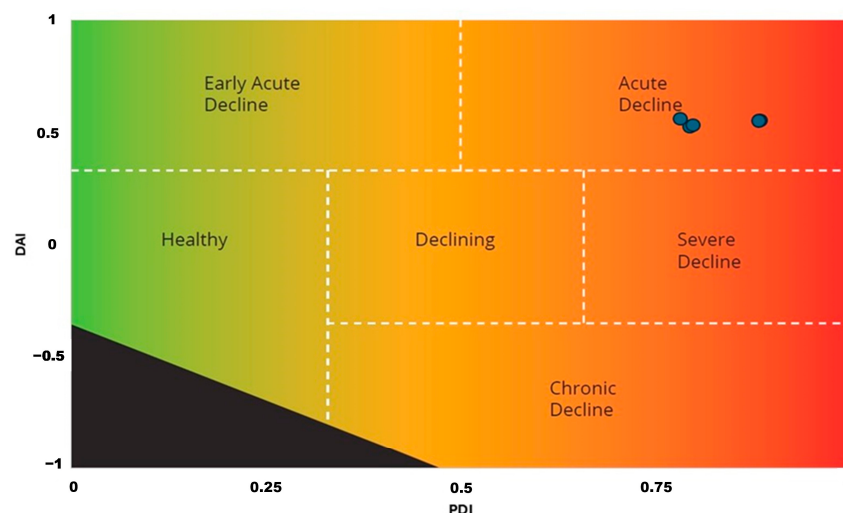


Figure 3. Quantification and classification of decline symptoms on kermes oak: the trees have a positive Decline Acuteness Index (DAI) value, between 0.5 and 1, indicating acute decline, while the Phenotypic Decline Index (PDI) closer to 1 means high severity of symptoms. The blue circles represent samples where AOD symptoms were observed and confirmed. The black area at the bottom left indicates the undefined decline indexes.

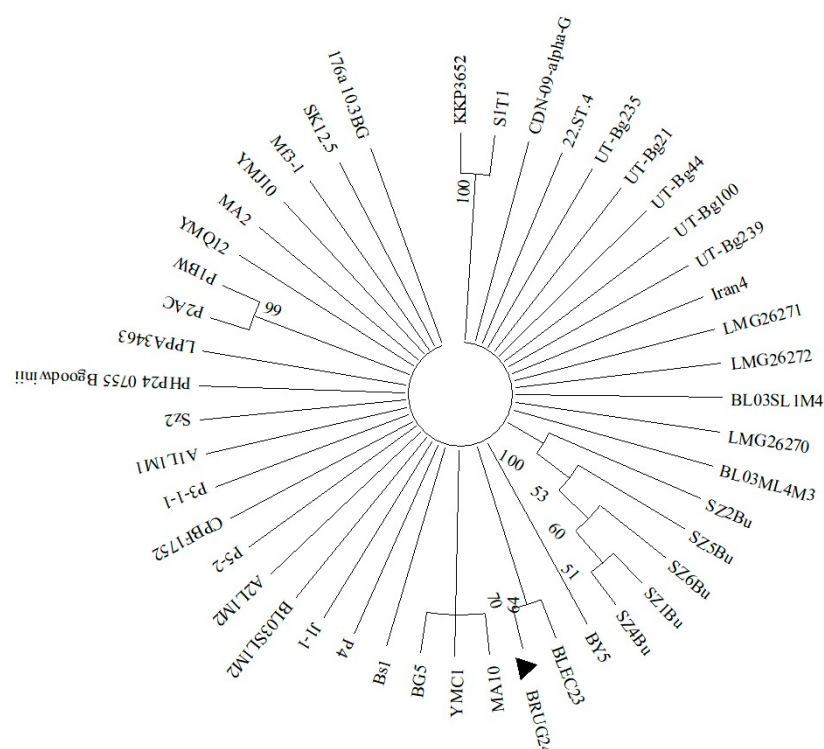
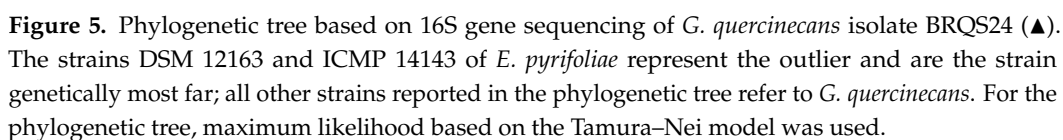
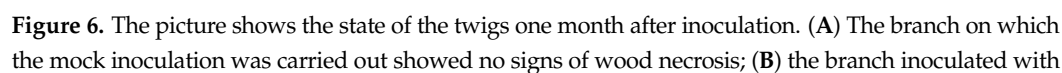


Figure 4. Phylogenetic tree based on 16S gene sequencing of *B. goodwinii* isolate BRUG24 (▲). The strains KKP 3652 and S1T1 of *S. fonticola* represent the outlier and are genetically the farthest strain; all other strains reported in the phylogenetic tree refer to *B. goodwinii*. For the phylogenetic tree, maximum likelihood based on the Tamura–Nei model was used.



The bacteria isolated from kermes oak and identified as *B. goodwinii* and *G. quercineans*, one month after being inoculated on branches excised from healthy oaks, developed necrosis in the xylem and inner bark at all inoculation points as well as exudates external to the bark (Figure 6); the overall results revealed that 100% of the inoculation points showed necrosis from which the respective bacteria could be isolated. The symptoms observed on the branches during the pathogenicity tests entirely resembled those observed in nature. Furthermore, no necrosis was found on the mock-inoculated branch.



B. goodwinii showed extensive wood necrosis, and (C) the bark showed a moist brown patch; (D) the branch inoculated with *G. quercinecans* also showed necrosis of the wood extending beyond the inoculum point; (E) also in this case, the outer bark showed an extensive brown, moist patch with a brownish exudate. As a reference, the bar in the bottom-left-hand corner equals 1 cm.

4. Discussion

In Europe, bacteria associated with AOD have been identified predominantly on plants of the *Quercus* genus, so given that the affected oak species are found in diverse pedoclimatic environments, a comparative analysis of symptom types, severity, and eventual decline recovery across different oak species could provide essential data on the interaction between host and pathogens. Also, since AOD-related bacteria exhibit necrogenic [33,34] activity and bacteria of the same genus are capable of degrading cell walls [35–37], it would be beneficial to investigate the response of different oak species to the infection, as different oaks have different cell wall composition, for example, in terms of chemistry and ultrastructure [38,39]. Thus, understanding these responses and possible differences could provide important insights into the species' diverse resilience and susceptibility to the disease and shed light on specific factors such as lesion dimensions, lesion count, and the bacteria's ability to spread within the plant. These findings support and expand existing hypotheses regarding host range and species specificity of AOD-associated bacteria. Although *Quercus coccifera* is ecologically distinct and highly tolerant to drought and poor soils [6], it appears equally vulnerable to *B. goodwinii* and *G. quercinecans*. This suggests that host susceptibility may not solely depend on environmental adaptation but possibly on specific host–pathogen interactions, as hypothesized in previous studies [33,34]. Furthermore, the confirmation of the necrogenic activity of these bacteria on *Q. coccifera* supports the idea that oak species across various ecological niches can act as viable hosts, which aligns with earlier reports from different European regions on species such as *Q. robur*, *Q. ilex*, *Q. cerris*, and *Q. suber* [9–13,20,21]. Such information can be beneficial for several reasons: (a) guiding reforestation programs by selecting oak species that are less susceptible to AOD; (b) informing plant breeding and screening programs aimed at preserving the biodiversity of oak species and maintaining the integrity of European forest ecosystems [40]; and (c) enhancing our understanding of the abiotic factors involved in the development and spread of AOD (for instance, as there is a correlation between AOD symptoms and drought) [41]. Thus, it would be valuable to compare the susceptibility to AOD between oak species adapted to drier environments, such as *Q. coccifera* [6], and those typical of temperate forests, such as *Q. robur*, which, by contrast, have a lifestyle more dependent on water availability [42].

However, since, outside of Europe, *B. goodwinii* and *G. quercinecans* are reported to infect not only exotic oaks, such as *Q. castaneifolia* and *Q. brantii* [24], but also other species, such as *Elaeagnus angustifolia* [43], *Juglans regia* [44], or *Carpinus betulus* [24], there exists the possibility that these microorganisms may not be strictly oak pathogens and might be a potential vector in Europe. Furthermore, in Europe, such bacteria have been reported on other genera, such as *Tilia* sp. [11], *Malus* sp. ((NCBI) [online], 2021), *Ulmus* sp., and *Acer* sp. ((NCBI) [online], 2019; (NCBI) [online], 2023); no further details about those interactions are available, given the few reports.

Although its role in AOD is not well understood and its role as a proven vector has not been established, *A. biguttatus*, a beetle found throughout Europe, is suspected to be involved in AOD [45]. The literature frequently describes it as an aggressive pest for oaks, especially the species reported in the literature with AOD symptoms, such as *Q. robur*, *Q. pertaea*, *Q. ilex*, and *Q. cerris*, and occasionally other *Fagaceae* [46]. Alongside, there are other European beetles of the same genus, like *A. sulcicollis*, *A. angustulus*, and related buprestid, such as *Coraebus florentinus* or *C. undatus*, whose lifestyles are closely associated

with oak trees that, in the past, have been reported for damaging oaks [46,47], may act as a vector for AOD bacteria, and explain the association between *B. goodwinii*, *G. quercinecans*, and oaks in Europe. Lastly, our findings contribute to the ongoing discussion on the role of insect vectors, particularly xylophagous beetles such as *Agrilus biguttatus*, whose galleries were observed in association with bacterial exudates. While a definitive vector–pathogen relationship remains to be proven, this aligns with previous reports proposing a vector-mediated facilitation of AOD symptoms [45–47].

In addition, the results corroborate previous hypotheses suggesting that AOD pathogens exhibit a broader host range than initially assumed, potentially influenced by bacterial capacity for cell wall degradation [35–37] and the anatomical or chemical features of different oak species [38,39]. The identification of typical symptoms (exudates, necrosis, crown dieback) and the successful re-isolation of the pathogens following inoculation further reinforce the consistency with disease expression patterns described in other *Quercus* hosts [14–18].

While this study offers valuable insights into host–pathogen interactions in AOD across different oak species, certain aspects remain to be further explored. The diversity of environmental conditions among study sites as well as the limited temporal scope may have influenced the interpretation of symptom development and disease progression. Moreover, the complexity of microbial interactions and the potential influence of other biotic or abiotic stressors were beyond the scope of the current analysis.

Future studies could build on these findings by incorporating longer-term monitoring, more detailed analyses of host responses at cellular and molecular levels, and controlled experiments to better isolate species-specific patterns. A deeper investigation into the microbial communities involved may also help clarify the factors contributing to the varying susceptibility and resilience observed among oak species. Such research would complement the current results and contribute to a more comprehensive understanding of AOD dynamics.

5. Conclusions

In this study, symptomatic infection of *Q. coccifera* by *B. goodwinii* and *G. quercinecans* was reported, representing, to our knowledge, the first observation of such a host–pathogen relationship. After detecting kermes oaks with symptoms attributable to AOD, the infection was initially diagnosed by qPCR on symptomatic trees and subsequently confirmed by sequencing the 16S rRNA gene with the construction of phylogenetic trees, where isolates of *B. goodwinii* and *G. quercinecans* clustered with the respective strains. Finally, pathogenicity tests demonstrated that the two bacteria isolated from infected tissue are indeed capable of causing wood necrosis, as inoculation of branch segments of *Q. coccifera* showed that wood necrosis and exudates developed in the presence of the bacteria and the two pathogens were again isolated from necrotic tissue.

This finding opens the way to several lines of research concerning the study of a possible species specificity of AOD-related bacteria and potential vectors, leading to oaks being the main affected plants. It is also important to determine which species are most impacted as well as whether there is a range of symptom intensity or the plant's ability to recover.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f16050789/s1>, Table S1: Phenotypic descriptors collected for each oak used to evaluate the decline type and severity.

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