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Isolation studies reveal a shift in the cultivable microbiome of oak affected with Acute Oak Decline

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ABSTRACT

Acute Oak Decline is a syndrome within the Oak Decline complex in Britain. Profuse stem bleeding and larval galleries of the native buprestid, *Agrilus biguttatus* characterize the disease. A systematic study comparing healthy with diseased trees was undertaken. This work reports the result of isolations from healthy trees, diseased and non-symptomatic tissue within AOD affected trees, at five sites in England. Bacteria and fungi were identified using the DNA gyrase B gene, or ITS 1 sequencing. A significantly higher proportion of diseased tissues (82%) yielded more bacteria than either healthy (18%) or non-symptomatic tissue in diseased trees (33%). Overall bacterial community compositions varied at each site, but significant similarities were evident in diseased tissues at all sites. *Enterobacteriaceae* dominated in diseased trees whereas *Pseudomonadaceae* dominated healthy trees. Significant associations between diseased tissues and certain bacterial species occurred, implying that the cause of tissue necrosis was not due to random microbiota. *Brenneria goodwinii* and *Gibbsiella quercinecans* were key species consistently isolated from diseased tissue; *Rahnella victoriana* and an un-named *Pseudomonas* taxon were also frequently isolated from both healthy and diseased trees. Most fungi isolated were from the outer bark and had no significant association with tree health status. It was concluded that there was a shift in the cultivatable bacterial microbiome of diseased trees, with *Enterobacteriaceae* strongly represented in symptomatic but not healthy tissues. No single species dominated the isolations from diseased tissues and the tissue degradation in AOD is therefore likely to have a polymicrobial cause.

Introduction

Acute Oak Decline (AOD) has recently been described as a distinctive disease on native oak species, *Quercus robur* L. (pedunculate oak) and *Quercus petraea* (Matt.) Liebl. (sessile oak) within the broader Oak Decline syndrome in Britain [11,14]. Affected trees are characterized by symptoms of bark cracking, dark fluid seeping from the cracks, macerated, decaying tissues underlying the weeping patches, and the presence of larval galleries of the native buprestid *Agrilus biguttatus* (Coleoptera: Buprestidae) (commonly called the two spotted oak borer, or TSOB). AOD occurs throughout southern and midland England extending no further north than the Manchester area, and west along the Welsh borders including its recent discovery in Newport, Wales [11; http://www.forestry.gov.uk/fr/infd-7ul9xr]. Reports from landowners suggest that many thousands of trees are affected, and on monitored sites more than 1.3% of symptomatic trees die every year, but a good proportion (40%) of affected trees appear to go into remission with lesions callusing over, but it is unknown whether this process leads to full recovery [11,14]. Although the cause of death is likely attributable to multiple factors that could include any combination of biotic and abiotic stressors, e.g. soil moisture, root health, insect attack and host primary and secondary metabolic status, the cause of tissue degradation and stem weeping characteristic of AOD is yet to be fully elucidated.

Declining oak trees with symptoms similar to those that characterize AOD have also been reported in continental Europe [4,10,14,16,18–20] where the disease was mostly attributed to TSOB attack, but some reports implicated a bacterial component [1,20]. In the UK, the inner bark lesions show expansion, suggesting a microbial as opposed to insect or abiotic origin [14]. If either insect or abiotic damage was suspected as the primary cause of

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the lesions, they would not have expanding margins, but would heal quickly as wounds caused by injury or pest attack tend to heal soon after being inflicted [10,14,15]. Therefore it appears that the lesions that characterize AOD in the UK could have a causal microbial component.

Initial laboratory diagnoses of AOD cases reported to Forest Research (FR) through citizen science channels, revealed a high incidence of bacteria through isolation and culture, but a more varied and lower level of fungal species was associated with lesions, and many of the fungi isolated were generalist saprophytes. To facilitate rapid, cost effective diagnosis for a large volume of samples submitted, rapid diagnostics were needed [22]. However, many of the isolated bacteria appeared to be previously unknown or un-named species. Thus as a first step to identifying biotic causes of the AOD lesions, a concentrated effort on formally identifying and describing a number of the commonly occurring bacterial taxa was made. Thirteen novel species and two new genera have been created to accommodate frequently detected species [2,4–7,13]. Amongst these are *Brenneria goodwinii*, *Gibbsiella quercinecans*, *Lonsdalea quercina* ssp. *britannica*, *Rahnella victoriana* and *R. variigena*. Nothing is known about the origin, biology or pathogenicity of these species.

To gain insights, and select putative causal agents for the AOD pathosystem, a systematic study examining statistical associations of microbial species with healthy and diseased trees was required. A number of approaches were taken to obtain an overview of the composition of the microbiome, these included metabarcoding (454 pyrosequencing [25]), metagenomics and metatranscriptomics (unpublished results). However, conventional isolation methods were applied in this study, as the main purpose of the current study was to obtain cultures to further study the biology, phenotype and genomic aspects so that appropriate selections for Koch's postulates testing could be made.

The hypothesis behind this study states that there are similarities in the composition of the cultivatable microbiomes of the diseased tissues in AOD symptomatic trees vs healthy trees.

The aims of the study were to: isolate bacteria and fungi from the stems of healthy and diseased native oak trees in the UK, determine similarities and differences in the composition of bacterial and fungal communities at the site level, as well as at the tree level (healthy vs diseased) and at the tissue type levels.

Materials and methods

Field sites

Five sites were selected based on representative spatial distribution of symptomatic trees, and willingness of the landowners/managers to donate trees for research since destructive sampling was used. The number of diseased *Q. robur* trees sampled per site varied because some owners were only willing to allow sampling of one healthy and one diseased tree. The sites chosen were: Attingham (OS Eastings 356033, Northings 310372), Bisham Wood (OS Eastings 485362, Northings 184100), Great Monks Wood (OS Eastings 582100, Northings 225300); Stratfield Brake (OS Eastings 449400, Northings 211900) and Runswood OS Eastings 563207, Northings 310858) [25].

Sample demographics

Samples from both healthy and diseased trees from five representative sites in England were collected. One healthy tree from each of the five sites ($n = 5$ trees) was sampled, and ten symptomatic trees, one tree each from Bisham Woods and Stratfield Brake ($n = 2$ trees); two trees from Great Monks Wood ($n = 2$ trees), and three trees each from Attingham and Runswood ($n = 6$ trees), were sampled. In total five healthy trees and ten symptomatic trees were sampled ($n = 15$ trees).

Tissue condition within healthy trees was considered healthy, but in the diseased trees, tissue condition was either non-symptomatic or symptomatic. The symptomatic tissues consisted of lesions – particularly the dead-live-junction (dlj) area at the advancing margins of the lesions, and galleries of TSOB larvae; whereas the non-symptomatic tissue group consisted of apparently healthy tissue adjacent to, but some distance from the lesions in the diseased trees.

Four tissue types were sampled: Outer bark, inner bark, sapwood and heartwood. Where possible the number of tissue pieces per tissue type per tree was kept constant (10 pieces per tissue type), but this sometimes varied if, for example, the necrotic area was large. A total of 4262 pieces of tissue were processed for isolations.

In the study there were 109 site vs. tissue condition vs. tissue type vs. unique tree combinations (hereafter called sampling combinations), of which 21 produced no bacterial growth, and 42 produced no fungal growth.

Sample taking and preparation for isolation

Sampling was carried out as described by [1,25]. Once the samples had been manually separated into outer bark, inner bark, sapwood and heartwood tissues, they were surface sterilized by submerging in 70% ethanol for 1 min, dipping in 1% sodium hypochlorite (NaOCl) for 1 min, transferring to 70% ethanol for 1 min and rinsing in distilled water for 1 min. Tissue pieces were air dried for 30 min and small chips of tissue (3–5 mm × 3–5 mm) were cut from the lesion margin and plated onto peptone yeast glucose agar (PYGA) bacterial culture medium (yeast extract 5 g, proteose peptone 5 g, glucose 10 g, agar technical no. 3, (5 g, tap water 1000 ml) for bacteria isolation, a non-selective fungal culture medium, malt agar (MA + S) made according to the manufacturer's instructions and amended with streptomycin sulphate (0.05 g L^{-1}), and a selective MAT (malt-antibiotic-thiabendazole) culture medium (malt extract, 10 g L^{-1}; agar, 15 g L^{-1}; penicillin-G, 0.05 g L^{-1}; streptomycin sulphate 0.05 g L^{-1}; polymyxin 0.025 g L^{-1}; thiabendazole lactate (23%) 1 ml L^{-1}) which was autoclaved for 15 min at 121 p.s.i., cooled to 50 °C then dispensed in 20 ml aliquots into disposable 9 cm diameter Petri plates, and Synthetic Mucor Agar (SMA, a selective medium for *Phytophthora* species [8]). In general, ten pieces of tissue per tree part, per medium were plated but in the inner bark and sapwood areas more tissue pieces were often processed, as the symptoms were most prevalent in these tissues, while in the heartwood areas where there were usually no symptoms, fewer pieces were analyzed. Samples from Attingham and Runswood had 1240 pieces plated in total (310 pieces per medium type), Bisham 555 pieces (156 pieces on PYGA and 133 pieces each on the other culture media), Great Monks 904 pieces (226 per medium type), and Stratfield Brake (323 pieces, with 174 of those on PYGA). Isolations were incubated under aerobic conditions at room temperature for two to three weeks, but examined every 2–3 days, and when present, bacteria on PYGA or fungi on the other culture media were sub-cultured by transferring to nutrient agar (NA) for bacteria, or MA + S for fungi. Bacterial subcultures were later streaked onto nutrient agar and processed to obtain single colony cultures [21]. All single colony bacterial strains were KOH tested to infer Gram reaction grouping as this was the quickest method [12]. Fungal cultures were incubated under black light at 25 °C until colonies had developed, after which they were processed for DNA extraction and sequencing.