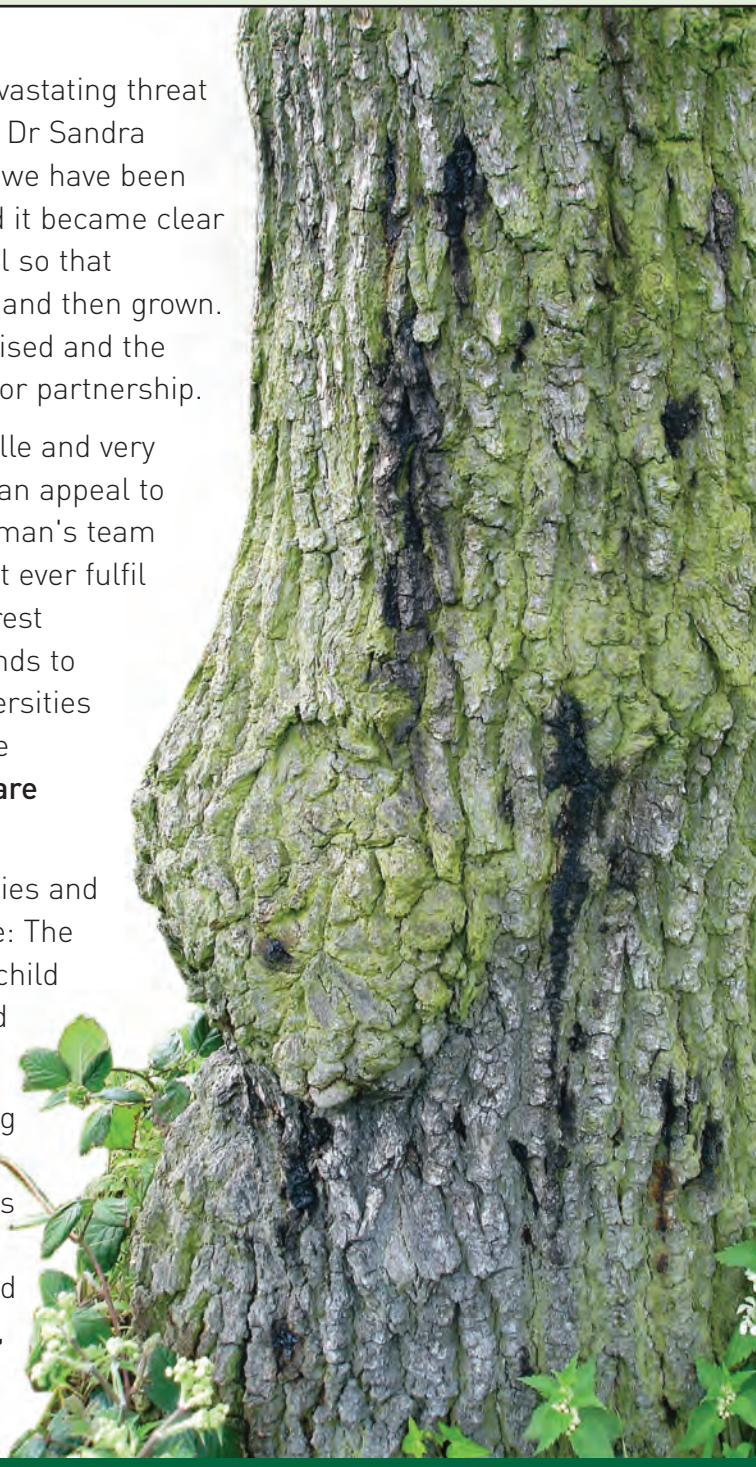
Report on Acute Oak Decline (AOD) research – April 2014 to April 2015



It is now six years since WH realised the potentially devastating threat of Acute Oak Decline (AOD) and first joined forces with Dr Sandra Denman and her team at Forest Research. Since then we have been operating against a backcloth of public sector cuts and it became clear we would need to lead the way in raising private capital so that momentum of research into AOD could be maintained and then grown. Subsequently, significant sums of money have been raised and the result has been a highly successful public-private sector partnership.

Thanks to early intervention by Lord Sainsbury of Turville and very considerable support by The Rufford Foundation, plus an appeal to our wonderful members, we were able to help Dr Denman's team move their research forward. We accept that we cannot ever fulfil the role of DEFRA who are the funding mainstay of Forest Research, but we can raise significant and effective funds to provide the AOD team with "outside" scientists at universities who can bring their talents to the research programme devised by Dr Denman. **These amazing young people are shown at work on the following pages.**

We must also give thanks to the following major charities and foundations who have given so generously to the cause: The J.Paul Getty Jr Trust, The Monument Trust, The Rothschild Foundation, The Duke of Bedford's Woburn Charity and CHK Charities.

We appreciate the immense cooperation of the following universities & colleagues who have worked with us to employ the young scientists who are part of Dr Denman's "outside" team: Bangor University, University of the West of England (U.W.E.), Swansea University, Cranfield University, Harper Adams University, Exeter University, Imperial College, and Rothamsted Research.

Forest Research

AOD Research Update

by Sandra Denman

I am very pleased to be able to report good news to you. The AOD research team has expanded thanks to the help and support we receive from all of you, and the welcome support of Defra and the sustained support from the Forestry Commission. I am particularly grateful to colleagues in the Universities (especially Bangor and University of the West of England) for working with us and facilitating appointments.

I can now announce that Woodland Heritage and the other charities that contribute so much to the AOD Fund, have made provision for the replacement of Dr Glyn Barrett, a Post-Doctoral Research Officer (PDRO), who spent a productive year with me at Alice Holt particularly on the *Armillaria* aspect of the research. In November last year Glyn headed to Oman.

We are excited about welcoming ***Dr Maciej Kaczmarek*** (Mac for short) a plant pathologist who has just completed his PhD at Edinburgh University. Mac will be looking at the populations of the bacteria involved in AOD to establish the degree of diversity within the population which may yield clues about their origins. Mac will also be helping with other aspects of our work over the next two years including finding out whether the *Agrilus* beetle carries the AOD bacteria.

We are sad to bid Rory Vereker farewell. Rory was funded by Defra and the Rufford Foundation. He was the AOD Project Support Officer and helped with databasing, particularly the data from the AOD survey carried out in England and Wales.

Woodland Heritage has also enabled a number of other new appointments. ***Dr Martin Broberg***, a PDRO from Finland, has expertise in microbial genetics and transcriptomics, which helps identify which genes are being expressed (functional aspect) and over the next two years will be helping James McDonald and James Doonan investigate exactly how the *Agrilus* larvae and the bacteria interact by examining the gene products of this interaction.

We have established a new collaboration with ***Dr Mary Gagen***, a dendrochronologist at Swansea University, who will help supervise an M.Phil student to investigate whether there is any evidence of 'predisposition' (weakening) of the trees over previous years making them susceptible to both AOD and Chronic Oak Decline (COD) using tree ring information.

Finally, we are strengthening our ties with colleagues at Alice Holt ***(Dr Elena Vanguelova)*** and Cranfield University ***(Dr Jac Hannam)*** by setting up a PhD in soil science aimed at finding out if there are any soil factors that play a key role in 'predisposing' trees to attack by *Agrilus* and the bacteria. We will link the soil findings with the dendrochronology information so that we can identify key environmental stress factors.

This work, together with other proposed research, will lead us to being able to derive management strategies to prevent trees from becoming susceptible to Decline diseases, and also help trees recover from these diseases. Again, all this has been made possible by the very generous help we receive from you, so my sincere thanks and deepest appreciation for all that you do to help us safeguard the health of our native Oak trees and secure their future in a changing climate.

The good news does not stop here either. I am pleased to share the most recent research results from the AOD team. In fact the Oak team has been so productive that to convey our results to you the Woodland Heritage Journal has had to print this special leaflet to accommodate the results.

Another highlight in 2014 was that Nathan Brown completed his PhD ***(well done Dr Nathan Brown!)***. In this leaflet he gives an overview of the spatial epidemiology of AOD – explaining the distribution patterns of infected trees and the sequence of events that appear to take place.

Katy Reed tells us about her findings with the *Agrilus* beetle and how its life cycle and distribution are affected by temperature and the implications of this in a warming climate. Jozsef Vutz and Mary Sumner describe the Oak tree 'smells' that *Agrilus* find attractive; and James Doonan, James McDonald, Carrie Brady and I review our discoveries on how the bacteria kill the living tissues of the Oak trees. So I wish you enjoyable, interesting reading and hope you are reassured that the research is making excellent progress.

April 2015

Molecular insights into the causes of lesion formation and bark rot characteristic of Acute Oak Decline

by James Doonan, Sandra Denman and James McDonald

Over the past year the strong collaboration between the molecular lab at Bangor University (under the leadership of James McDonald) and Sandra Denman's pathology lab at Alice Holt produced insightful results.

Genomics PhD student James Doonan is addressing the problem of the lesion formation in the Oak trees using molecular approaches, with the aim of providing evidence of lesion forming capability of the bacteria that Sandra's lab isolated consistently from AOD trees, namely *Brenneria goodwinii* and *Gibbsiella quercinecans*. James is unravelling the inherent genetic programming that the bacteria use to damage Oak tree tissue. The basis of the analyses is the whole genome sequences of *G. quercinecans* and *B. goodwinii* that James did last year (see WH Journal 2014). Having sequenced the entire genomes of both bacteria he searched for genes that can cause damage to live Oak bark. This novel work has given us nanoscopic insights into the workings of bacterial pathogenesis.

The essence of life in bacteria is encoded within their DNA *(Fig 1)*; it is here that their idiosyncratic features, whether pathogenic, commensalistic (benefits one without harming the other) or mutualistic (benefits both) are defined. Previously identified bacterial plant and tree pathogens such as *Dickeya dadantii* and *Pseudomonas syringae* have a known repertoire of genes which enable them to produce harmful enzymes. These enzymes are capable of causing pathogenicity in plants, through mechanisms such as maceration of host cell tissues (*D. dadantii*) and intracellular injection of toxins known as 'effectors' (*P. syringae*). *D. dadantii* and *Pectobacterium carotovorum* are recognised phytopathogens which are closely related to *G. quercinecans* and *B. goodwinii*. We have been analysing similarities between these bacteria and have discovered many similar genes in the AOD related species. These particular genes encode destructive plant cell wall degrading enzymes, which previous studies have proven, can lead to disease of crop plants.

Additionally, James has been studying a recently identified mechanism through which bacteria inject effector proteins into neighbouring cells. Genes related to this mechanism have been found in *G. quercinecans* and may also be present in the *B. goodwinii* genome. The research carried out over the past year has shed substantial light on the molecular mechanisms through which *G. quercinecans* and *B. goodwinii* are pathogenic contributors to the tissue rot that characterises Acute Oak Decline syndrome.

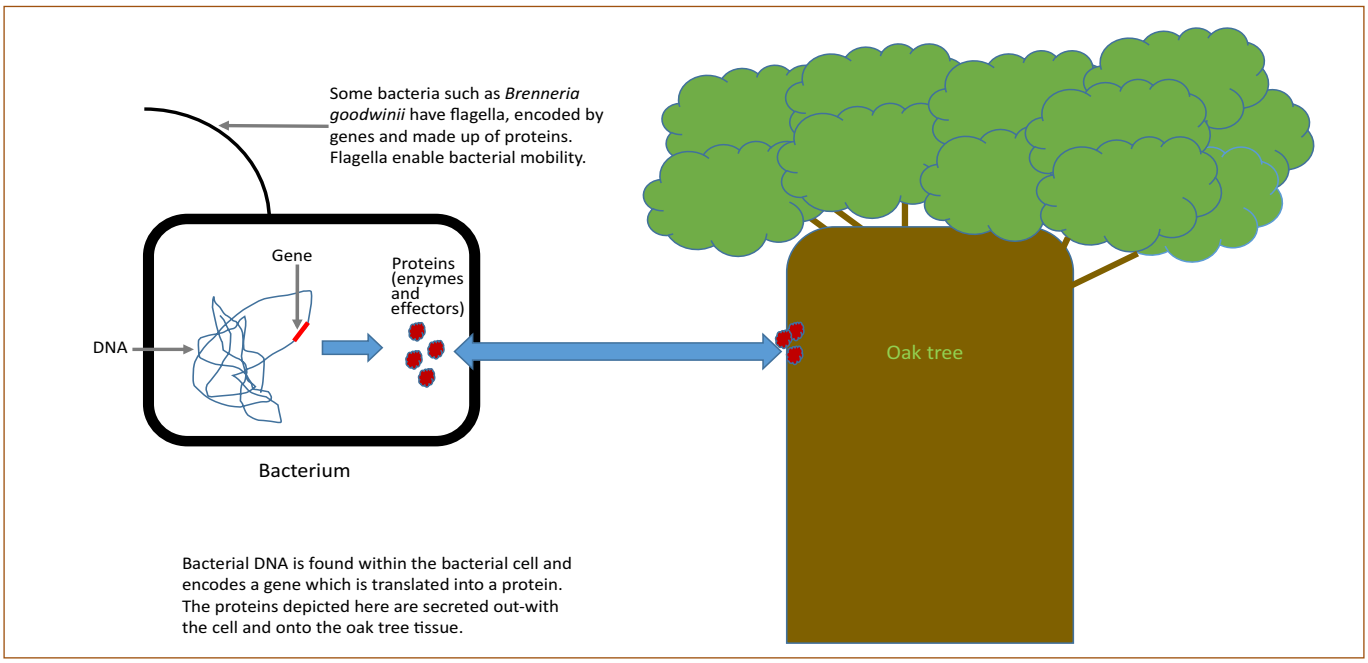


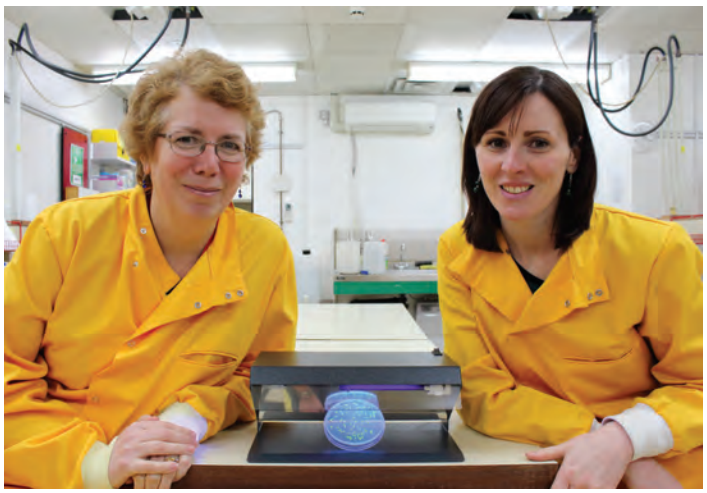
Fig. 1. Schematic diagram illustrating the basic pathway bacteria use to attack tree stems.

Laboratory manipulations of necrogenic bacteria in AOD to visualise pathogenic effects

by Carrie Brady, Dawn Arnold and Sandra Denman

Since the current outbreak of Acute Oak Decline was recognised in Britain, an initial and sizable part of the subsequent research focused on identification and classification of the bacterial species isolated from symptomatic trees, as many of the bacteria found in the symptomatic Oak trees were new to science.

Carrie Brady first started working with Sandra Denman and Susan Kirk in 2008, slightly before official inception of the AOD project, while she was still studying for her PhD degree at the University of Pretoria in South Africa. Luckily for us, and thanks to Woodland Heritage and the Monument Trust, things have worked out such that she has been able to stay with the project where she has already made a major contribution to advancing our knowledge. Now seated in Dawn Arnold's lab at the University of the West of England, in Bristol, they both continue to help us advance the frontiers of knowledge on this difficult problem.



Carrie Brady (right) a talented molecular biologist funded by W.H. and working at the University of West of England (U.W.E.) with her supervisor Dawn Arnold (left).

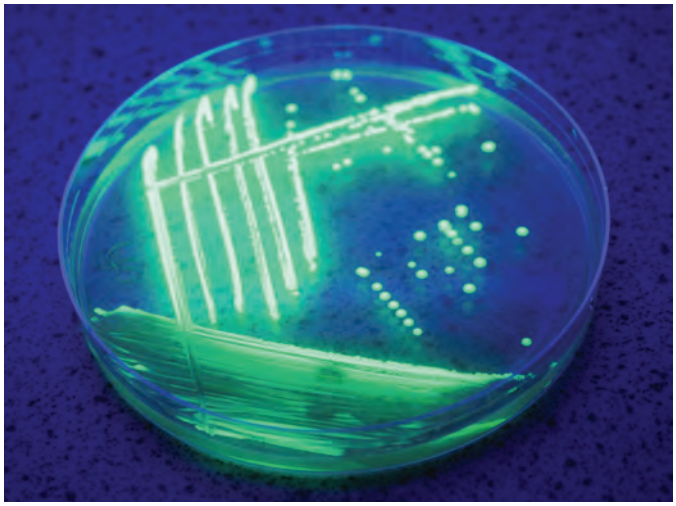
In the past five years, our AOD group has been responsible for the formal description of two novel genera, nine novel species and four novel subspecies for bacteria associated with AOD. The taxonomy of these bacteria has been an important starting point in AOD research, as it has been necessary to be able to differentiate all species associated with AOD for future screening and rapid identification of isolates in the *Enterobacteriaceae*, a bacterial family that includes most known tree bacterial pathogens. Although there are still few bacterial species in the *Pseudomonadaceae* requiring taxonomic clarity, with the taxonomy of the majority of bacterial species in the *Enterobacteriaceae* associated with AOD now resolved, we can start to examine how these species interact with their Oak host and determine how these bacteria cause necrogenesis (pathogenicity).

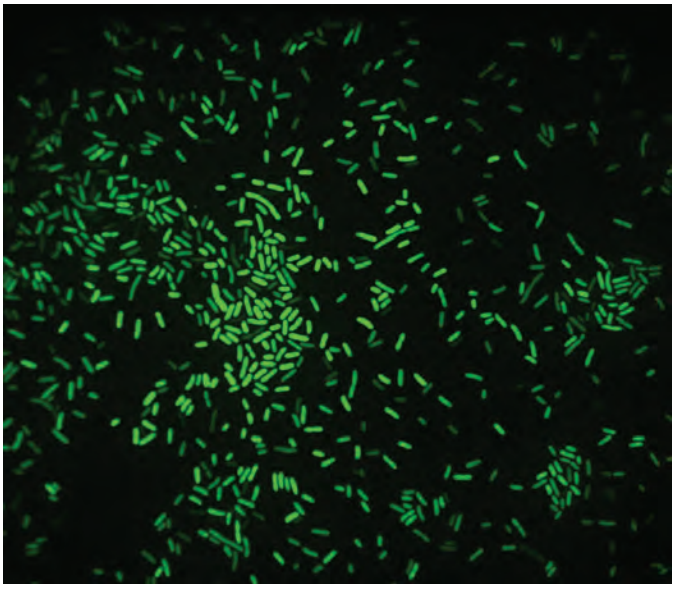
As the two most frequently occurring bacteria isolated from symptomatic Oak (*Gibbsiella quercinecans and Brenneria goodwinii*) are both novel species, and in the case of *G. quercinecans*, a novel genus, we know very little about their origin, pathogenicity, biology or genetics. One way of trying to discover as much as possible about how and where in Oak cells these two bacterial species cause damage, is to manipulate them at the gene level so that the actual operation of the bacteria at various stages in the infection and disease process can be viewed using a microscope.

However, bacteria exist as almost transparent, single cells and as such they are very hard to see. Traditional methods use various stains to colour the bacteria so that they are easy to see, but these usually kill the cells. Furthermore, the plant tissue is often stained as well, making viewing difficult. Modern technology has developed ways of viewing live bacteria at work in their normal way, but this does entail having to make the bacteria visible. Bacteria are able to take up bits of foreign DNA and express them in a process called transformation. An approach to make them more visible is to incorporate proteins that will make the bacteria visible but not affect their functioning. However, some bacterial species are notably fussy and will not allow transformation or will allow transformation to occur, but won't allow expression of the foreign DNA.

To determine whether *G. quercinecans* can be genetically manipulated, we transformed a single strain with an external DNA molecule (called a plasmid) carrying a gene which produces green fluorescent protein (GFP). GFP is produced naturally by the jellyfish *Aequorea victoria* and the gene which encodes the protein is routinely used to report expression of foreign DNA in bacteria. We tested several different plasmids and versions of *gfp* genes before successfully transforming a strain of *G. quercinecans* so it produces large amounts of GFP and thus can be easily visualised with a fluorescence microscope.

The bacterial colonies exhibit a bright green fluorescence when viewed under a UV light *(below)*.





When single cells of the GFP expressing *G. quercinecans* are examined under a confocal microscope, it is clear that each cell produces large quantities of GFP, enough to make the entire bacterial cell fluoresce *(above right)*.

We are currently moving ahead with the next step, which will be to infect young Oak saplings with the transformed *G. quercinecans* strain, monitor them and collect samples over the course of a few weeks. The infected Oak tissue will be very thinly sectioned and viewed under UV light with the confocal microscope to explore the possible pathogen-host relationship. By examining patterns of colonisation of Oak tissue by GFP-expressing bacteria, we hope to gain further insight into how *G. quercinecans* contributes to lesion development and necrosis of Oak.

Future work includes the possible transformation of *B.goodwinii* with either red fluorescent protein (RFP) or yellow fluorescent protein (YFP), and co-infecting Oak tissue to determine whether either of these bacterial species are more dominant in colonising Oak or if they work together to cause symptoms.

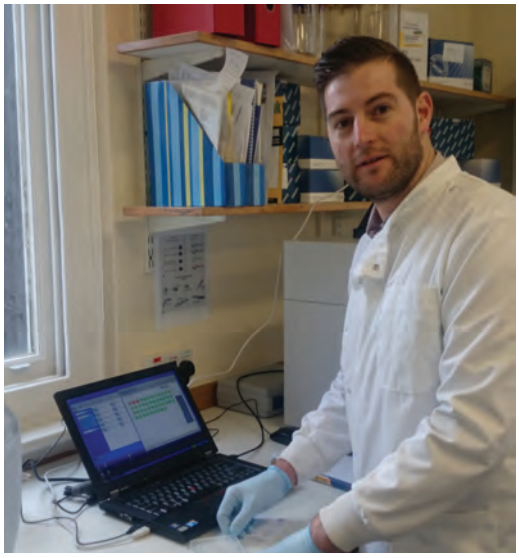
Cages for trapping beetles emerging from Oak logs

*Larval galleries of **Agrilus biguttatus** are present deep in live bark of AOD symptomatic Oak, which if not excavated deep enough go undetected. A key question that requires addressing is whether or not the beetle has a role in AOD, and if so what it is. To be able to investigate this it is critical to have beetles in hand. In the wild they are elusive creatures with a cryptic lifecycle and not easily seen or obtained. To overcome this problem a PhD study (carried out by Katy Reed) was set up to rear beetles in captivity. These photographs show the cages and logs from which we obtain adult beetles.*

Testing the necrotic ability of bacteria associated with Acute Oak Decline

by James McDonald and Sandra Denman

It is clear that microorganisms are a contributing factor to the progression of AOD, but their exact role in this process is currently the focus of investigation. Since Sandra Denman and her team isolated two new bacterial species, ***Brenneria goodwinii*** *and* ***Gibbsiella quercinecans****, from the lesions of Oaks affected by AOD, they have become one of the main foci for AOD research. Through collaboration with Sandra and her team, work at Bangor University has focussed on the hypothesis that* ***G. quercinecans*** *and* ***B. goodwinii*** *represent plant pathogenic microorganisms.*



There are several ways to test this hypothesis, including pathogenicity tests (i.e. inoculating the bacteria onto non-symptomatic tissue and observing the onset of the symptoms), and the detection of potential pathogenicity factors (genes) in *B. goodwinii* and *G. quercinecans* that can elicit the symptoms of AOD.

To address the latter, we have fully sequenced the genome of both *B. goodwinii* and *G. quercinecans* through a PhD studentship between Bangor University and Forest Research, and funded through Woodland heritage (for more details, please refer to article by James Doonan – page 3). This study has revealed that both species possess genes that could potentially cause pathogenicity. This work is important, because it highlights the 'genomic potential' of the microorganisms to cause pathogenicity. However, further experimental characterisation is required to demonstrate that these genes are actually used by these two bacteria when causing tissue necrosis, the primary symptom of AOD.

Testing the ability of *B. goodwinii* and *G. quercinecans* to break down plant cell walls

The ability to produce necrogenic enzymes (that is, enzymes that cause the breakdown of plant tissues) is a key strategy for plant pathogens to attack host tissues. Consequently, the isolation of both *B. goodwinii* and *G. quercinecans* from lesions, and their strong association with necrotic tissue in the inner bark, would suggest that necrogenic enzyme production would be an obvious pathogenic mechanism for these two species on Oak. Oak cell walls are comprised of lignin, cellulose, hemi-cellulose and tannins, all of which represent complex and recalcitrant plant cell wall polysaccharides that must be degraded by plant pathogens.

In order to test the ability of *B. goodwinii* and *G. quercinecans* to degrade plant cell wall polysaccharides, Sandra and I have been conducting comparative laboratory tests on *B. goodwinii* and *G. quercinecans* against a panel of other bacterial species that are known plant pathogens, or harmless colonisers of plants. This enables us to determine if the necrogenic enzyme profile of *B. goodwinii* and *G. quercinecans* more closely resembles other plant pathogens, or harmless plant bacteria.

The bacterial species were grown on agar plates using traditional microbiological culture techniques, but we supplemented the growth medium with several polysaccharides that represent components of Oak tissue to stimulate production of necrogenic enzymes by the bacteria. The ability of certain strains to attack the polysaccharides provided in the growth medium can be visualised by observing zones of clearing around the bacterial colonies (where the enzymes have broken down the polysaccharide – *Fig. 1A*) or by colour changes in the medium around the colony *(Fig. 1B)*. These initial tests provided the first experimental evidence that *B. goodwinii* and *G. quercinecans* possess necrogenic enzymes. *B. goodwinii* was found to have activity against starch, cellulose and gallic acid (a component of tannin), while *G. quercinecans* could degrade a wider range of substrates including starch, cellulose, gallic

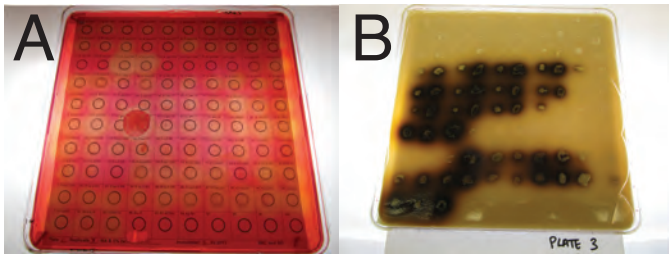


Fig. 1. Enzyme degradation of complex molecules present in Oak tissues.

and tannic acids (a type of tannin), and one strain appeared to degrade xylan (hemi-cellulose).

In order to obtain more sensitive and quantitative data on the enzyme activities of these bacteria, we have also used more sensitive enzyme assays to compare polysaccharide degradation rates of each species over time, enabling comparative analysis of polysaccharide degradation rates of *B. goodwinii* and *G. quercinecans* against a panel of other plant-associated bacteria.

These enzyme activity assays measure the release of a fluorescent molecule attached to the polysaccharide when it is degraded, and this provides a more accurate determination of enzyme activity than the more confirmatory visual determinations shown in *Fig. 1*. This work has provided important insights into the necrogenic capabilities of *B. goodwinii* and *G. quercinecans*.

Analyses of the data is still ongoing, but they suggest that both *B. goodwinii* and *G. quercinecans* possess a range of necrogenic enzymes with activities similar to those observed in other known plant pathogens. *G. quercinecans* also demonstrated strong activities against tannic acid and gallic acids, both components of tannins that are found in Oak bark and considered as a potential host defence against invasion by pathogenic microorganisms; however, tannins clearly do not have this effect on *G. quercinecans*.

The production of necrogenic enzymes by plant pathogenic bacteria represents one of the major mechanisms for pathogenicity in plants, supporting our working hypothesis that *B. goodwinii* and *G. quercinecans* are pathogens of Oak and are involved in the necrosis that occurs in AOD affected trees.

The polysaccharide degradation tests are a good example of how the presence of pathogenicity genes in the genome sequence of *B. goodwinii* and *G. quercinecans* (which suggests the genomic potential to cause necrosis, but does not necessarily confirm actual activity) can be experimentally validated and confirmed using an independent approach.

AOD is a complex Decline syndrome, and as such, an integrated 'systems' approach, where several complementary experimental approaches are used, will be required to provide a more complete picture of the role of microorganisms in AOD.

Sandra Denman's sapling trial - *June 2014*

Eight-year-old stressed Oaks inoculated with bacteria and in some trees day-old larvae inserted into pin-holes using fine bristle paint brushes.

Three months later a panel was cut from an Oak sapling in the trial, revealing astonishing ***Agrilus*** *growth and attack in such a short time.* ***This shows that stressed trees are vulnerable to Agrilus.***

Pathogenicity Tests Carried Out at Alice Holt

by Sandra Denman, Sarah Plummer, Katy Reed, Mary Sumner and Susan Kirk

Methods:

Over the past five years we have been pioneering ways to reproduce AOD under controlled conditions so that we can prove causes of the disease and understand the role each agent plays. AOD is different from primary diseases because it is complex, involving multiple factors, whereas primary diseases usually have only a single causal agent.

Fig. 1. Inoculated logs incubated in growth chambers with controlled temperature and lighting.

Fig. 2. Black fluid (i.e. stem bleed) weeping from an inoculation point. This is one of the four diagnostic symptoms of AOD.

Last year we set up two trials, one on 20 cm diameter logs (60 cm long) which were inoculated and placed in a growth chamber for four months *(Fig. 1)*. The other which is still running used young trees standing outside in our nursery *(see page 7)*. Both experiments tested the same treatments. The AOD bacteria *Brenneria goodwinii* (Bg), *Gibbsiella quercinecans* (Gq), *A. biguttatus* eggs and *A. biguttatus* larvae were applied to the logs singly or in each possible combination through small wounds, controls used water only. Eggs and larvae were tested because we were unsure of their survival, so we wanted to cover all eventualities.

At the end of the experiment lesions were exposed and lesion areas calculated and the bacteria were cultured from the treatments they were applied to. We were excited to observe tiny stem bleeds *(Fig. 2)* just like those in AOD, on many of the inoculation points, and little larval galleries were visible in a lot of the treatments that had *A. biguttatus* eggs – indicating that the eggs hatched and the larvae had been able to survive in the logs for a short while at least.

We did however, have a setback in the trial (☹) because some of the logs became contaminated. This was most probably because they were incubated without coverings i.e. not in plastic bags. Because the growth chamber was small the logs were in very close proximity to each other, so we suspect that micro-insects such as aphids or small flies were the culprits, taking bacteria from one wound to the other.

Many inoculation points escaped contamination however, and the experimental design was robust enough to allow statistical testing yielding results. Next year we will amend the test protocol to prevent this from happening.

Results:

There were significant differences between the treatments ($p<0.001$). ***Combined treatments gave the largest lesion areas*** *(Fig. 3)*: The biggest lesions were obtained with (1) A combination of the bacteria and *Agrilus* eggs (Bg+Gq + *A. biguttatus* eggs); Followed by (2) A combination of the two species of bacteria (Bg+Gq); (3) A combination of the bacteria and *Agrilus* larvae (Bg+Gq + *A. biguttatus* larvae) and finally (4) A combination of Bg + *A. biguttatus* eggs. These results indicated the cumulative effect of the different agents on lesion area and confirm the hypothesis that AOD is a Decline disease rather than a primary disease. The results also showed that:

- *Bg was common to the treatments with the biggest lesions and might be the most important necrogen in AOD.*
- *Similar sized lesions were caused by each bacterial species (Bg or Gq) when applied singly. But the sizes of the lesions were not significantly different to controls which had quite a large wound response. We will attempt to reduce or eliminate the wound in future tests as it clearly affects results.*
- *Gq appears to enlarge lesion areas when combined with Bg but did not cause AOD symptoms in combination with* ***A. biguttatus*** *eggs or larvae.*

We need to repeat the tests to be sure of these findings and also see what the sapling trial yields this spring and summer. However, through the log trial we have demonstrated that we can cause three of the four distinctive signs and symptoms of AOD and have shown (i) the bacteria species Bg and Gq have a role in causing necrosis in AOD and degrading the tissue beneath the stem bleeds, and (ii) that interaction between *A. biguttatus* and these bacteria leads to typical signs and symptoms of AOD.

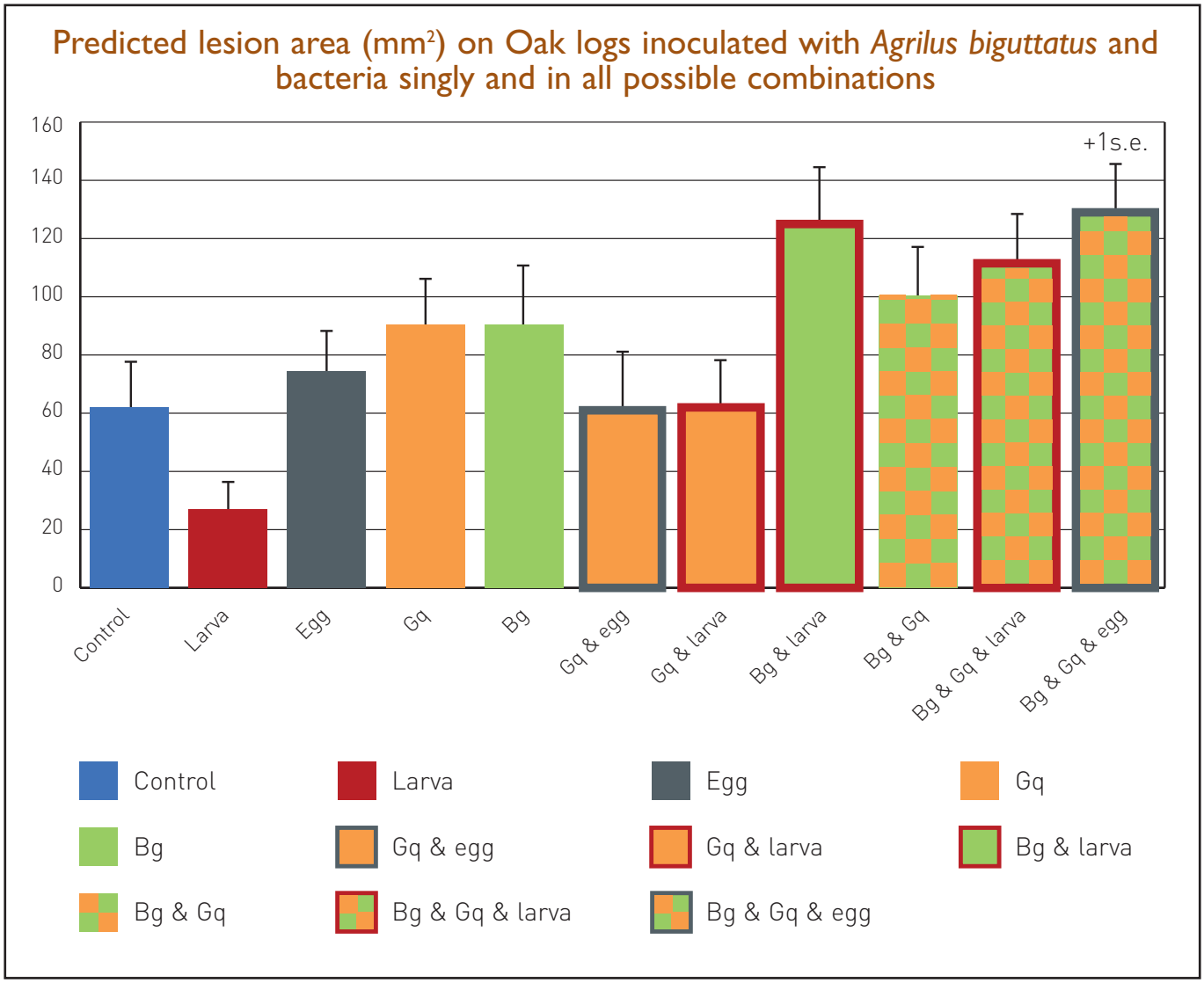


Fig. 3. Results of Log Test Inoculation Trials.

Insights from mapping and monitoring

by Nathan Brown and Sandra Denman

Nathan Brown began work on AOD with Sandra Denman in 2009 as an MSc student. After successful completion of his MSc (obtained with distinction), Nathan spent six months at Forest Research (FR) continuing his work with Sandra (part-funded by Natural England and Woodland Heritage). In 2010 the Forestry Commission (FC) agreed to fund a PhD on the spatial epidemiology of AOD and Nathan was accepted for study at Imperial College under the supervision of Sandra Denman and Prof Mike Jeger. In 2014 Nathan was awarded his PhD. Following this he was successful in being appointed by Rothamsted Research (RRes), to the Population ecology, epidemiology and evolutionary biology group, in the department of Computational and Systems Biology where under the guidance of Dr Frank van den Bosch he works on the Defra funded AOD project with Sandra. Here below Nathan summarises the scope of work he has covered.

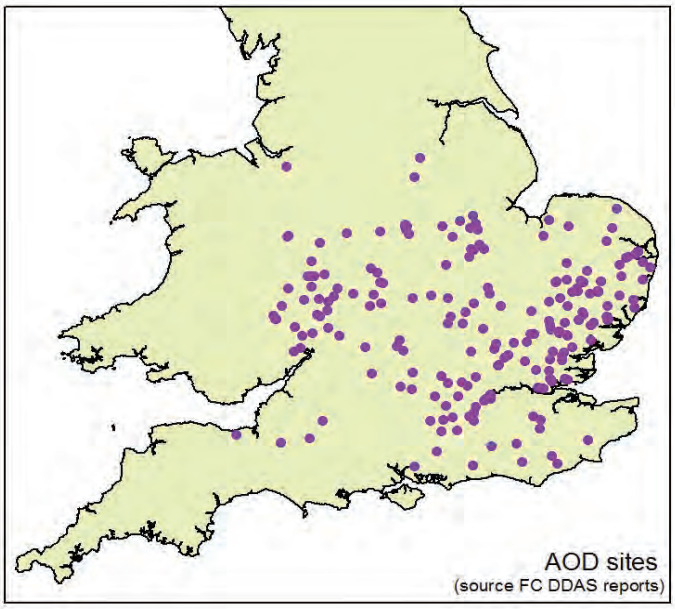
In 2009 reports of declining Oak were arriving at FR with increasing frequency. These reports described distinctive stem symptoms and often mentioned stem "bleeding". Affected Oak wept dark exudate from cracks between the bark plates. Further investigation revealed patches of dead cambial tissue beneath the bleeds. Scientists from FR began looking for the cause, expecting to find something known, however this was not the case. Consistently they found bacteria, of species new to science, and larval galleries of the beetle *Agrilus biguttatus*. These distinctive symptoms and signs were defined as Acute Oak Decline and after much work in the lab the bacteria could be formally named as *Gibbsiella quercinecans* and *Brenneria goodwinii*. With the key elements of the AOD system described, it became important to investigate how they interacted and what impact AOD was having on Oak woodland, so the FC funded a PhD and the establishment of long term monitoring plots.

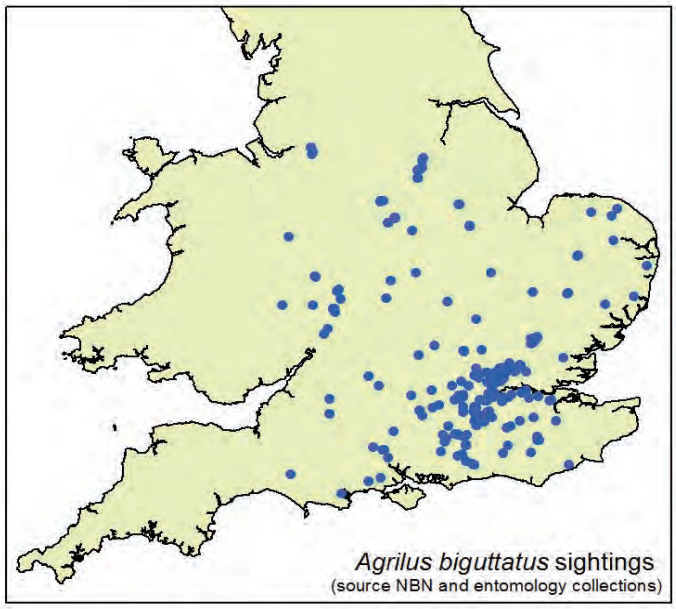
As part of the PhD, eight Oak monitoring plots were established in AOD affected areas across East Anglia and the Midlands. The plots varied in size based on the natural divisions of the landscape, covering the full extent of small woodlands or management blocks in larger forests and parklands. In total 1,200 Oak were individually mapped, and with on-going annual monitoring these plots now form a long term, hugely valuable resource for AOD research. Each monitoring plot was mapped with high accuracy GPS (acquired by funding from Woodland Heritage) so that the location of every tree larger than 15 cm diameter was recorded. All Oak within the plots were assessed visually for crown health and the presence of AOD symptoms. Through repeated visits each summer the changes in AOD symptoms and signs were accurately documented. These records revealed a variety of rates of new infections across the different sites, but also showed some consistent trends:

- *D-shaped exit holes of **A. biguttatus** occur most often on Oak with stem bleeds.*
- *Galleries are found in more than 90% of trees with stem bleeds.*
- *Affected trees occur in localised clusters.*
- *Lightly affected trees can grow callus tissue over infections and galleries, entering remission.*
- *The shortest period from first symptoms to tree death was four years.*

The initial observations from the monitoring sites suggested a link between *A. biguttatus* and the stem bleeds. They both occurred on the same trees, but the nature of the relationship was unclear. The beetle had been little studied, with adult beetles described as elusive; they feed high in the crowns of Oak and are only present for a short period in a single summer. In fact, at the outset of the study the beetles' presence at AOD sites was only inferred from distinctive exit holes shaped like a capital D. Its larvae develop within the main stem of mature Oak feeding on the cambial tissue and phloem over one to two years before adults emerge. Until the exit holes are made there is no sign of *A. biguttatus*, unless the tree is destructively sampled and its galleries are revealed. These are deep in the phloem-sapwood tissues at the interface with the cambium, so presence of the larval phase of the beetle may go undetected.

*Nathan Brown taking a sample panel from an infected Oak to reveal the **Agrilus** beetle activity.*





In order to begin gathering information on the species composition at AOD sites and develop an understanding of the beetle population dynamics across a season, insect traps were trialled at affected woodlands. Initial studies showed purple prism traps to be effective; these traps are used extensively in the USA to monitor another *Agrilus* species, the Emerald Ash Borer (*A. planipennis* – newly arrived in Moscow and unfortunately spreading westwards). The trials at AOD sites showed *A. biguttatus* was the only member of the genus to be found consistently at all AOD sites, although another *Agrilus* species, *A. sulcicollis* was also present across East Anglia. This smaller species also uses the trunks of mature Oak for egg laying and larval feeding, but is thought to prefer smaller and weaker stems.

A final strand of research widened the focus of monitoring efforts, from documenting AOD symptom development within a site to recording the distribution of AOD sites across Great Britain. This was initially achieved using "citizen science" where members of the public, land owners and land managers had reported Decline symptoms to the Forest Research Disease and Diagnostic Advisory Service. By collating all records from 2006 onwards the first snapshots of the national distribution of AOD affected sites could be seen. Increased awareness and reporting has seen this resource grow to contain 450 records and describe a distribution that covers a band across England from East Anglia, through the Midlands, to the Welsh border. This resource is a product of public interest and concern for Oak which has shaped our understanding of the factors that influence the occurrence of AOD and allowed preliminary studies to investigate climate and soils.

An investigation conducted jointly with the pest risk mapping team at FERA suggested that temperature is likely to be the most important limiting factor at the northern edge of the range and has hinted that drought affected Oak maybe more susceptible to AOD. These questions are currently being investigated more fully by scientists at Forest Research, while the accuracy of the AOD distribution is being assessed through a systematic survey of Oak woodland funded by Defra. The survey has involved collaboration between Forest Research and Rothamsted Research. During 2013 and 2014 Forest Research staff visited a random sample of 280 woodlands containing Oak. This process used records held by: the National Forest Inventory; Forestry Commission; Woodland Trust; National Trust; Wildlife Trust; and many private landowners, whom we thank for their cooperation.

Early results match well with those collected through citizen science. Although the northern boundary has expanded into Nottinghamshire and many more sites than expected were found across the south in Kent, Sussex and Surrey, and, most surprisingly of all, in the south west across to Somerset. In order to fully understand the extent of AOD in the south west we ask for your help in reporting all suspected cases to Forest Research.

Knowledge of AOD has come a long way since the outset, however some important questions remain. The differing rates of new infections across the monitoring sites are likely due to varying levels of predisposition at the sites: At each site the soils, climate, the chemical deposition (e.g. atmospheric pollution) and Chronic Oak Decline agents are likely to affect occurrence as are the management histories and the level of between-tree competition. All of these factors require further research.

In addition now that the AOD system is more fully understood it is possible to investigate mitigation and management. Through experimentation and management intervention, options can be developed and, in consultation with land owners, advice can be shaped into best practice.

The two-spotted oak buprestid, *Agrilus biguttatus*: new insights into its lifecycle

by Katy Reed, Daegan Inward and Sandra Denman

Last spring, we wrote in the Woodland Heritage Journal about a pilot study which we conducted on the role of temperature in the development of ***Agrilus biguttatus****, a beetle implicated in Acute Oak Decline.*

Our aim was to collect as much information as possible about the beetle's lifecycle in the laboratory, in order to predict its development time in Oak trees and better understand its distribution and abundance in the UK. Last summer, we collected bark material for *A. biguttatus* emergence by felling a number of infested Oak trees in woodlands across England. Some trees had very high numbers of beetles: over 1,500 *A. biguttatus* emerged from only five trees.

Katy Reed monitoring egg laying.

We measured the lifespan and number of eggs laid by females in the laboratory, and developed a successful technique to culture the larvae on freshly

Sandra checks the feeding and mating chambers.

Sarah Plummer applying one-day old larvae to logs.

cut logs. By dissecting a number of the logs at fixed intervals, we were able to measure the size of the larvae, and to compare their growth rate at five different temperatures. Most of the larvae have now finished feeding, and we are trying to determine what triggers them to begin pupation, the final stage before becoming adults.

We expect that the timing of this transition plays an important role in the overall length of the lifecycle, which in turn influences the abundance of the beetle in the wild. Using our laboratory data, we have started creating statistical models to predict

Logs infested with ***Agrilus****, inspected every two weeks.*

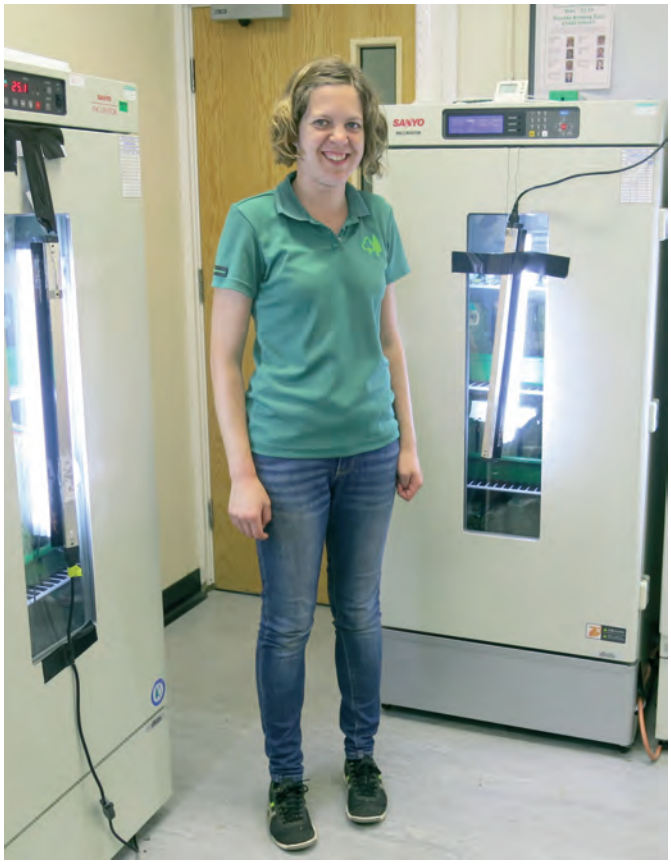
Sandra showing where the bacteria and beetles are applied.

the development time of the eggs and the larvae in Oak trees.

Initial findings suggest that the beetles are unable to survive in northern England or Scotland because temperatures are simply too low for them to develop. This would explain why their distribution is apparently limited to central and southern England.

After we finish collecting data this summer, we will be able to build a lifecycle model that predicts precisely which parts of the UK are suitable for the beetle's temperature requirements, both at present and under future predicted climate change scenarios. Although there is much still to do, as we improve our knowledge of the ecology and lifecycle of *A. biguttatus*, so we continue to advance our understanding of its role in the Acute Oak Decline syndrome.

Dr Daegan Inward – FR Entomologist.



Katy Reed with her logs in the incubators.

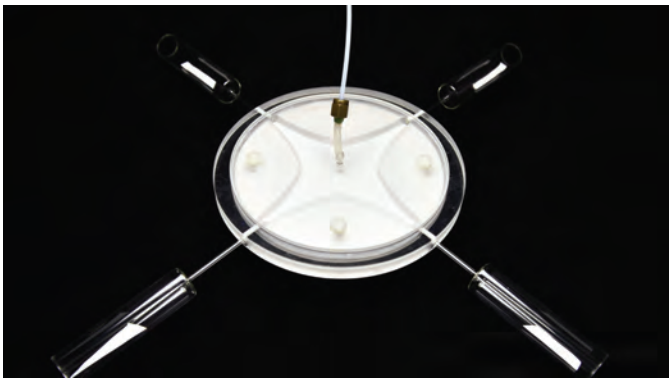
Agrilus biguttatus 'smells AOD Oaks'

by Mary Sumner, Katy Reed, József Vuts, Daegan Inward and Sandra Denman

Mary Sumner joined Forest Research in May 2014 as part of her studies towards an MSc degree in Entomology at Harper Adams University. Her project entailed working over the summer with Katy Reed and Dr Daegan Inward on rearing Agrilus biguttatus beetles (the twin spotted Oak buprestid), as well as carrying out olfactometry studies with Dr József Vuts in the chemical ecology group at Rothamsted Research. This research formed a component of the Defra funded AOD project Th0108 led by Dr Sandra Denman. After obtaining her degree with distinction Mary was awarded an 11 month short term temporary appointment at FR assisting Sandra in her lab and she is taking responsibility for the beetle rearing over this coming summer. Mary gives an overview of her experiences and Jozsef summarises progress with the rest of the chemical ecology research.

Mary Sumner (centre) learning pathology techniques from Susan Kirk and Sandra Denman.

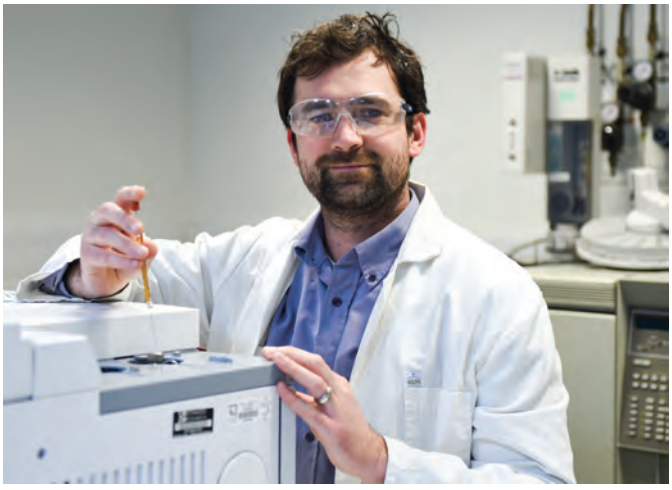
I was very excited to be involved in this project, as it was a perfect chance to work within a very active team on research with real-world significance. Studies the group reported in the WH journal in 2014 showed that *A. biguttatus* are capable of detecting differences between the scents of leaves of AOD-infected and healthy Oak trees, but it was not known whether they were more attracted to leaves of healthy Oak trees or those from symptomatic trees. I used a technique called olfactometry to investigate this. *A. biguttatus* are able to detect volatiles (gases/scents) through their antennae which like most insect antennae trigger set reactions to certain scents in the wild which help them navigate towards positive habitat features. For example it can guide them towards food sources and suitable egg-laying sites but steer them away from threats. Understanding these responses to certain chemicals or blends of chemicals can help explain how pests move within a forest and why they select certain trees; thus allowing us to identify which trees are most at risk.



*This is an 'Olfactometer' used by Mary and Jozsef to find out which volatile blends **A. biguttatus** likes best.*

The research began by placing *A. biguttatus* in a closed arena called an 'Olfactometer' which has four side openings through which scented volatiles are introduced. The olfactometer was connected to an air pump which drew the scents through the openings into the arena. Adult beetles were presented with volatile blends from leaves from healthy trees as well as those from AOD symptomatic trees and controls used clean air only. The beetle's response (movement towards or away from the volatiles) was measured and we found that *A. biguttatus*, especially males, are attracted to the artificially created scent of leaves of AOD trees, suggesting they actively search them out in the wild.

Funding for the initial part of the research on volatiles (up to June 2014) came from the Defra TH0108 project. In October 2014 we were very fortunate to win an award of £50,000 from Defra's



*Dr Jozsef Vuts chemical ecologist working on 'what attracts **A. biguttatus** to Oak trees'.*

Future Proofing Plant Health programme which covered a further 6 months for József Vuts to continue his work on detecting and characterising volatiles from leaves, bark and microbes associated with AOD.

In the Woodland Heritage Journal 2014 the first very exciting findings of this work were reported. This research is a vitally important part of the AOD work as A. biguttatus is pivotal to the problem and there is still so much to be done so we will be doing our best to raise more funding to support József and the Rothamsted Chemical Ecology team.

Another side of the Oak-*A. biguttatus* relationship is finding out how egg-carrying females find trees and sites on those trees in which to lay their eggs. A clue may be found from the discovery that several bacterial species are associated with the necrotic tissues in tree trunks and cause stem bleeding of AOD-symptomatic trees. We suggested that *A. biguttatus* uses volatiles given off by such bacteria to find suitable trees for egg-laying. *We sampled the volatiles emitted by bacterial cultures which were kindly given to us by Sandra Denman's lab,* and tested these compounds on the antennal responses of gravid female *A. biguttatus*, as well as males. The antennae of both sexes showed strong electrical signals for a number of sample constituents.

Also, based on previous observations of *A. biguttatus* colonising trees preferentially attacked by parasitic fungi, such as the honey fungus (*Armillaria* spp.), we sampled the air around non-infested Oak logs and logs from trees infested by the fungus. The antennae of both sexes of *A. biguttatus* reacted strongly to some of the volatiles appearing in samples from *Armillaria*-infested logs, suggesting *A. biguttatus* might also use this cue when looking for a tree to lay eggs. These novel results will enable us to create artifical bacterial and fungal scents, which are identical copies of the original ones, and test if *A. biguttatus* is attracted to them.

Two types of Agrilus beetle

***Agrilus sulcicollis** inside - **A. biguttatus** outside. Note the difference in size.*

Update on *Armillaria* research

by Sandra Denman, Glyn Barrett, Susan Kirk and Martin Coetzee

***Armillaria** spp. occur on Oak in Britain and continental Europe and are frequently implicated in Oak Declines.*

They could play a part in predisposing trees to attack by other organisms and/or contribute to tree mortality. Information about which *Armillaria* species occur on Oak in Britain is lacking. We carried out a small study on *Armillaria* isolates obtained from English Oak with symptoms of poor crowns, dieback and/or stem bleeding from woodlands and gardens at six sites in England. Multilocus sequence analysis (MLSA) was used to determine the identity of the isolates. Four species of *Armillaria* were identified: *A. gallica*, *A. mellea*, *A. ostoyae*, *A. tabescens*: *A. mellea* and *A. tabescens* were not isolated from woodland trees but were found on Oak trees in gardens. The other two species were found on woodland Oak with *A. gallica* being most common. In some cases two species were isolated from the same tree indicating that much is unknown about the ecology and pathogenicity of *Armillaria* on Oak and their impacts on tree health and attraction for other organisms such as *Agrilus biguttatus*. Further research is required to understand the role *Armillaria* spp. play in Oak Declines in Britain

As I write these closing comments I pause to gaze out the window and notice that the Oak trees are flushing – pushing out nourishing new leaves and delicate blossoms from protective bracts, investing precious energy that will sustain growth and lead to acorns forming, ensuring the future of Oak trees. That is what Woodland Heritage is doing too – ensuring the future of Oak trees. Through the AOD Appeal, Woodland Heritage works very hard at raising funds and investing in young scientists to deliver thorough, robust, multidisciplinary science that endeavours to understand the causes of AOD at multiple levels, and develop management strategies to counter the disease. I am sure you will agree that a great deal has been achieved to date (see output table), with some ground-breaking research carried out, but there is still much to do. **Continued research on determining the role *Agrilus* plays in AOD is vital and I appeal for help to achieve this goal.** It is essential to determine how the bacteria are transmitted; where natural sources of inoculum exist; the sequence of events and possible ways of managing beetle populations if deemed necessary. Also crucial are:

- *Determining whether predisposition (tree weakening) is necessary for disease establishment*
- *Ascertaining whether soil factors, climate and management history play a role in predisposition*
- *Establishing predisposition/stress effects on tree function and susceptibility*
- *Linking the factors and their interactive effects, deriving and testing appropriate practical management strategies*

The task is large and requires a well organised, co-ordinated, multidisciplinary team that works closely with landowners, forest managers and land custodians to get to the bottom of the problem. The widespread concern about AOD is emphasised in the array of funding sources we have been so lucky to receive. This has led to the realisation that collaborative funding as well as collaborative research is needed as it brings with it collaborative thought and resources. It is because of this collaborative effort that we have been able to achieve what we have so far.

It is on this note that I thank you all for your fantastic commitment and unwavering support and hope that it will continue into the future so that we truly will ensure the future of Oak in Britain.

Sandra Denman
April 2015

Outputs (April 2009-April 2015)	Number
Publications popular (10) and scientific (13)	23
Radio and television interviews	5
Presentations	44
Posters and conference proceedings	17
Post-Doctoral positions sponsored	4
PhD, MSc or graduate student training	5

"I would like to thank my trustees at Woodland Heritage for their constant support of our charity's AOD Appeal. Dr Sandra Denman and her team have achieved a great deal since 2009 when our English Oaks appeared doomed, but our partnership is both strong and well co-ordinated and will soon be capable of giving Britain's foresters a range of methods to fight off AOD.

To my consultant forester friends who have, since Day One, provided vital information about their declining Oaks and stood alongside me at numerous meetings and conferences, trying to convince the authorities to take AOD seriously, I give my heartfelt thanks: Mike Seville, Gary Battell, Rod Pass, David Taylor and Andrew Falcon must be acknowledged here.

Finally, I must mention Lady Victoria Getty and Patricia Morison – two ladies who have listened, learned and visited Forest Research's AOD team to see for themselves and reported back to their trustees. Without their interest and support our funding would have struggled. Thank you ladies; you have given us all hope."

Peter Goodwin