

***Xylella fastidiosa* Pathogen Case Study**

Introduction

The majority of diseases on commercial crops in the UK are caused by fungal pathogens. Historically, bacterial diseases have been a rarity in the British climate, and therefore not of major concern to growers. While there are many plant protection products available for the control of fungal diseases, control options for bacteria are extremely limited. Consequently, the present spread of the bacterium *Xylella fastidiosa* across Europe presents a very significant threat to the UK, both in term of crop production, and plant health in natural environments. The causal organism behind many different plant diseases, this bacterial pathogen has successfully established itself across the American continents and, aided by man, is now spreading to other parts of the world.

In order to develop effective controls for any plant pathogen, it is fundamentally important to have a comprehensive understanding of its biology and behaviour. Based on historical and present-day research, this case study details the aspects of *Xylella fastidiosa* that are important from the plant pathologist's perspective.

Taxonomy

The taxonomic tree for *Xylella fastidiosa* is as follows:

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Xanthomonadales
Family: Xanthomonadaceae
Genus: *Xylella*
Species: *Xylella fastidiosa*

X. fastidiosa is currently the only species belonging to the *Xylella* genus. A Proteobacteria, *X. fastidiosa* is Gram negative, the cells being surrounded by an outer membrane. Fastidious in nature, this organism requires very specific growth media for primary laboratory culturing (Wells *et al.*, 1987). Currently, there are four subspecies of *X. fastidiosa* that are generally accepted, subsp. *fastidiosa*, subsp. *Multiplex*, subsp. *Pauca* and subsp. *Sandyi*, each having evolved separately in a distinct geographical range (Almeida and Nunney, 2015).

A further subspecies, *Morus*, has been proposed, the result of recombination of DNA from subsp. *fastidiosa* and *multiplex* (Nunney *et al.*, 2014b). Additionally, a relative of *X. fastidiosa* thought to be native to Taiwan has been described (Su *et al.*, 2014), but it is possible that this is a separate species (Almeida and Nunney, 2015), and it is not further discussed here. This list of

clades is thought to be incomplete, as it is fully expected that further distinctive varieties will be discovered in future research (Nunney *et al.*, 2014a).

Background

When originally studied in the late 19th and early 20th centuries, *X fastidiosa* was categorised by plant pathologists as a virus (Hewitt, 1939). At this time it was referred to as 'Pierce's Disease (PD), and known primarily as a pathogen of grape vines in the USA. Some details of the pathogen's lifecycle were known at this time, such as the importance on insect vectoring. Due to epidemic levels of PD occurring in 1940's California, extensive research was carried out to improve knowledge of the causal organism (Hewitt *et al.*, 1942). During this time some fundamental discoveries were made. Insect vector species were identified (Winkler, 1949), and it was determined that, within plants, the pathogenic organism was restricted to growing in xylem vessels (Houston, 1947). A particularly crucial observation was that PD had a variety of host plant species, many symptomless but still capable of transmitting the pathogen to insect vectors (Freitag, 1951). However, despite these advances, PD was still thought to be caused by a virus rather than a bacterium.

A major step in re-diagnosing the causal organism came in the early 70's when heat therapy for the treatment of viruses was being investigated. Researchers found that PD could be successfully eliminated with heat regimes known to be ineffective for viral control (Goheen *et al.*, 1973). The resulting theory was that either a Mycoplasma, Mycoplasma-like organism, or Bacterium was responsible for causing PD, but attempts to artificially culture the organism failed (Goheen *et al.*, 1973). The need to create a culture was great, as without it Koch's postulates could not be completed, and the pathogen could not be properly classified.

In the late 1970's a bacterium was finally cultured from PD infected plant material, and through Koch's postulates it was proven to be the causal pathogen of PD (Davis *et al.*, 1978). Further detection and classification work was then carried out using molecular methods. Many further host plants were quickly discovered, and the name of '*Xylella fastidiosa*' was given to the bacterium (Wells *et al.*, 1987). Following large-scale collaboration between various laboratories in Brazil, where *X. fastidiosa* posed a great threat to citrus and coffee industries, the complete genome of *X. fastidiosa* was published in the year 2000 (Simpson *et al.*, 2000). It was the first plant pathogenic bacterium to be sequenced in its entirety.

Life Cycle

X. fastidiosa is unusual among bacterial pathogens, in that it is obligately vectored by insects. The most recent list of confirmed host plants numbers 359 species from 204 genera (Gardi *et al.*, 2015). The list is expected to grow as research continues. Many of these host species, although susceptible to infection, will not go on to develop disease symptoms (Purcel and Saunders,

1999). In fact, *X. fastidiosa* exists as a mostly harmless endophyte in symptomless plants (Chatterjee *et al.*, 2008a), while remaining capable of infecting susceptible feeding insect hosts (Daugherty, 2011). Such plants could therefore be considered 'carriers' of *X. fastidiosa*.

One experiment has shown that a particular strain of *X. fastidiosa* isolated from a grape would readily form a biofilm in uninfected grape xylem fluid, but development of the same strain was significantly inhibited in citrus xylem fluid (Bi *et al.*, 2007). Inside the a host plant, *X. fastidiosa* cells likely gain nutrition from sugars in xylem sap, although there is evidence that the pathogen is capable of producing cell-wall degrading enzymes (Dow and Daniels, 2000), and may also feed on the products of their activity on neighbouring plant cells. These enzymes could also help *X. fastidiosa* spread within a plant, moving between xylem vessels via membrane degradation (Newman *et al.*, 2004).

In symptomatic hosts, bacterial cells multiply to the point where the flow of xylem sap becomes restricted, reducing the transport rate of water to plant tissues, and resulting in drought-stress (McElrone *et al.*, 2003). It is this process that causes the dieback of vegetation, and in some cases entire plants, characteristic of *X. fastidiosa* disease. Such restriction and decline of the host plant is detrimental to the *X. fastidiosa* community, which is totally dependent upon the plant for the supply of nutrients. Clearly there are differences in the way that *X. fastidiosa* populations behave in symptomatic and asymptomatic hosts. Cell-to-cell signalling within bacterial communities has been suggested as an in-built mechanism for *X. fastidiosa* to self-restrict virulence (Chatterjee *et al.*, 2008b). This enables the bacteria to reside merely as an endophyte in most hosts, without causing any disease symptoms. It is unclear why this system breaks down in susceptible species.

A defining characteristic of *X. fastidiosa* is a restriction to xylem vessels, never spreading into other areas of a plant. It has been suggested that the lack of a type III secretion system prevents *X. fastidiosa* from being able to move beyond the xylem (Dow and Daniels, 2000). Common amongst plant pathogenic bacteria, a type III secretion system facilitates the invasion of host cells, and the circumvention of defence strategies (Galán and Collmer, 1999). It has been shown, however, that the system can also be recognised by a host, causing it to initiate defensive responses (Kjemtrup *et al.*, 2000).

Xylem-feeding leafhoppers (Homoptera: Cicadellidae, tribes Cicadellini and Proconiini), are known to be the primary vector of *X. fastidiosa*, with transmission efficiency affected by interspecific relationships between insect species, plant species and the bacterial strain (Redak *et al.*, 2004). Upon penetration of a *X. fastidiosa* infected xylem vessel by the mouthparts of a susceptible leafhopper, bacterial cells are transferred from the plant to the insect (Purcell *et al.*, 1979). The bacterium then establishes a community in the foregut of the insect (Purcell *et al.*, 1979). *X. fastidiosa* will remain in the leafhopper until it moults, at which point the foregut, along with the bacterial colony, is shed (Purcell and Finlay, 1979).

The efficiency of *X. fastidiosa* acquisition by a vector improves with increasing time spent feeding, up to 48 hours (Purcell and Finlay, 1979). As within the pathogen-plant relationship, cell-to-cell communication is thought to have a major role in the colonisation of insect hosts by *X. fastidiosa* (Newman *et al.*, 2004). Such signalling enables *X. fastidiosa* cells to form a biofilm in the foregut of a vector, thereby enabling long-term infectivity (Newman *et al.*, 2004). Experiments using a mutated form of *X. fastidiosa*, have proved that without cell-to-cell signalling the bacteria is quickly removed from within a leafhopper (Newman *et al.*, 2004). After successfully acquiring *X. fastidiosa* from an infected plant, leafhoppers are able to re-transmit the bacterium to an uninfected host within one hour (Purcell and Finlay, 1979).

The lack of a latency period for these insects means that *X. fastidiosa* can spread rapidly from diseased to healthy plants. The efficiency of inoculation from insect to plant increases with increased xylem feeding time of the vector up to 96 hours (Almeida and Purcell, 2003). Once inside a plant, bacterial cells will begin to replicate and form a colony within the xylem. An infected plant is capable of re-infecting an insect host within a week of initial inoculation (Purcell and Finlay, 1979). However, after inoculation a plant does not necessarily remain as a host for its lifetime. Symptomatic plants have been known to recover from disease (Purcell, 1981), and *X. fastidiosa* can also die out in asymptomatic carrier plants (Purcell and Saunders, 1999). It is possible that sub-zero winter temperatures act to restrict development of, or even destroy, *X. fastidiosa* populations within plants (Purcell, 1981).

Distribution

Until relatively recently, the geographical range *X. fastidiosa* was primarily limited to the Americas (Almeida and Nunney, 2015). Based on evidence from molecular analysis, the four generally accepted subspecies of *X. fastidiosa* are thought to have evolved separately in different areas of the continent. Subsp. *multiplex* appears to have originated in North America (Nunney *et al.*, 2012), subsp. *sandyi* in the southern USA (Yuan *et al.*, 2012), subsp. *fastidiosa* in southern Central America (Nunney *et al.*, 2010), and subsp. *pauca* in South America (Nunney *et al.*, 2012). The fifth proposed subsp. *morus* is found in the east of the USA (Almeida and Nunney, 2015). *X. fastidiosa* has an extremely wide range of insect vector hosts, comprising numerous species (Redak *et al.*, 2004) that are present not only across America, but worldwide.

While some of these vector species are polyphagous and therefore significant in terms of disease spread (Redak *et al.*, 2004), there is only one example of a vector becoming invasive in such a way as to introduce *X. fastidiosa* to unaffected areas (Purcell *et al.*, 1999, Stenger *et al.*, 2010). The most likely pathway for *X. fastidiosa* to reach new areas is through the movement of infected host insects and plants by humans (Baker *et al.*, 2015).

X. fastidiosa subsp. *fastidiosa*, although well known for causing PD of grapevines in the USA, is not endemic to this territory. Genetic research has

pointed to Central America as the origin of this subspecies, and it is likely that it became established in the USA after the introduction of an infected plant (Nunney *et al.*, 2010). This finding is supported by the lack of genetic diversity within subsp. *fastidiosa* isolates found in the USA (Yuan *et al.*, 2010), suggesting it was introduced in the relatively recent past.

The scale of distribution of subsp. *fastidiosa* in grape-growing regions of the USA today indicates the efficiency with which this pathogen can establish and spread in new geographic ranges. There is evidence of a separate introduction in the opposite direction, with subsp. *multiplex* proven to be the causal agent of disease to plums in South America (Nunes *et al.*, 2003). As subsp. *multiplex* is native to North America, this epidemic is also likely to have been caused by the inadvertent transportation of diseased plant material to the area. Although the spread of individual *X. fastidiosa* subspecies within the Americas has caused new diseases to arise, the movement of *X. fastidiosa* as a species, away from the American continents, has the potential to cause major disease epidemics in the rest of the world. One such occurrence led to *X. fastidiosa* ending up in Taiwan, evidenced by the development of PD on grapevines in the country (Su *et al.*, 2013).

More recently, the discovery of *X. fastidiosa* subsp. *pauca* causing dieback in olive trees (Saponari *et al.*, 2013), has received much greater international attention. Through molecular analysis the strain of subsp. *pauca* identified has been shown to be distinct from any previously known subsp., and consequently a unique name of *X. fastidiosa* strain CoDiRO has been proposed (Saponari *et al.*, 2013). Known as 'Olive Quick Decline Syndrome', the dieback disease of olive trees caused by *X. fastidiosa* (Elbeaino *et al.*, 2014) is a major threat to the European olive industry. In July 2015 *X. fastidiosa* was reported on the French island of Corsica (EPPO, 2015a), and then on the French mainland in October of the same year (EPPO, 2015b). The type of *X. fastidiosa* found in France is subsp. *multiplex* (EPPO, 2015c), different to the subsp. *pauca* found in Italy, therefore suggesting yet another individual case of *X. fastidiosa* movement out of the Americas. The global distribution of suitable vector species for *X. fastidiosa* (Redak *et al.*, 2004) means that it can readily become established in totally new locations from only a single introduction event.

A possible limitation to the geographical range of *X. fastidiosa* is its lack of long-term cold hardiness. One notable experiment showed a high percentage of recovery among PD infected vines if they overwintered in areas with very low temperatures, while the same plant stock in milder areas remained diseased (Purcell, 1980). The effect of cold winters on *X. fastidiosa* populations can also be seen in the susceptibility of wild grape vine provenances to PD. Species of grape native to areas of the USA with relatively warm winters are more susceptible to PD than those from regions with particularly cold winters, which show some degree of tolerance or resistance (Hewitt, 1958). This indicates that cold winters alone are enough to prevent *X. fastidiosa* from causing disease, such that plants adapted to these environments have not needed to evolve defences to the pathogen in order to survive.

Epidemiology

As previously discussed, *X. fastidiosa* will, in many cases, infect a plant without ever leading to any visible symptoms. However, in those species where disease does develop, it usually causes significant damage and can often lead to plant death. Disease is much more likely to develop if *X. fastidiosa* is vectored in springtime. Infections later in the year can be less severe or even lead to no symptoms whatsoever (Purcell, 1981). Some of the most economically important crops to have been heavily impacted by *X. fastidiosa* are grapes, peaches, citrus, coffee and olive. Although the mechanism behind disease (an obstructive build-up of *X. fastidiosa* in the xylem) is the same for all plant species, the visible symptoms can vary.

In the case of Pierce's disease of grape vines, the initial stage is necrosis of the leaf margin, followed by severe leaf scorch, then loss of the leaf blade but retention of the petiole. As infection develops, dieback of shoots will begin, and may be followed by plant death (Hopkins and Purcell, 2002). Alternatively, vines may recover completely over a winter season (Purcell, 1977).

Phony Peach disease produces markedly different symptoms. Here, the host peach tree will become stunted, with restricted growth of new shoots and a shortening of stem internode length. Any fruit produced will become progressively smaller, becoming an unmarketable crop (Wells *et al.*, 1981). Similarly, dwarfing symptoms are characteristic of Coffee Leaf Scorch Disease, where leaves and fruit remain small. Along with this, scorched areas begin to appear on leaves, followed by early leaf and fruit fall, then dieback of shoots (De Lima *et al.*, 1998).

In the case of Citrus Variegated Chlorosis Disease, initial symptoms present themselves in a way that can be mistaken for a nutrient deficiency. Chlorosis of leaves is the first stage of the disease, explaining its name, further developing into necrotic lesions within the leaves. Fruit produced are undersized and not suitable for sale. Eventually the citrus plant begins to wilt and dieback of shoots and branches occurs (Hopkins and Purcell, 2002; Damsteegt *et al.*, 2006).

Arguably one of the most debilitating diseases caused by *X. fastidiosa* is among those which have been identified most recently. Olive Quick Decline Syndrome begins with leaf scorching and desiccation of branches in the upper part of the tree canopy, extending downwards until the entire crown dies back. In an attempt to halt disease spread within the tree, farmers may prune out affected branches, leading to the production of healthy shoots. However, the new growth quickly succumbs to the disease (Martelli *et al.*, 2016). To the concern of olive grove owners, it is the oldest (and often most prized) trees that are most heavily affected, becoming plagued by opportunistic insects and fungi in later stages of decline (Martelli *et al.*, 2016). *X. fastidiosa* also causes 'Bacterial Leaf Scorch', a disease causing decline and sometimes death in a variety of broadleaf tree species in the USA (Gould and Lashomb, 2005). The EFSA list of susceptible host plant in Europe includes many trees we consider fundamental to UK native forestry (Gardi *et al.*, 2015).

Interestingly, in South and Central America there are as yet no known native plant hosts of *X. fastidiosa* (Almeida and Nunney, 2015). The species that harbour the bacteria in these regions have all been introduced rather than having evolved there. This raises the possibility that *X. fastidiosa* is only pathogenic on plant species with which it did not co-evolve. Such a relationship would certainly explain why any particular *X. fastidiosa* clade is so damaging when introduced to a new geographical region. However, whether in a natural or new range, *X. fastidiosa* displays very particular interactions with plant species. Disease will only develop if the correct isolate interacts with the correct host. An example is Subspecies *pauca*, responsible for both Coffee Leaf Scorch Disease and Citrus Variegated Chlorosis Disease. A bacteria isolate extracted from one plant species will not cause symptoms in the other (Nunney *et al.*, 2012). So although *X. fastidiosa* has a diverse and extensive plant host list, the number that any one isolate will cause disease in is actually very low. Accurate sequencing of *X. fastidiosa* using molecular methods is therefore crucial to understanding, forecasting and controlling disease epidemics.

As seen in Italy, *X. fastidiosa* can rapidly spread from a single location. When disease first broke out in the country a focus zone of 8,000 ha was established, but after just a few months this had to be extended to 23,000 ha (Almeida and Nunney, 2015). Following this a whole series of smaller outbreaks have been identified across the wider region (Almeida and Nunney, 2015). Clearly, the most effective way to prevent *X. fastidiosa* diseases is to prevent the introduction of the organism in the first place. In the event of disease being found in a new location, movement of plants out of the area should be stopped immediately.

A key limitation of *X. fastidiosa* is that it relies on insect vectors to spread between plants. If, therefore, the population of vectors in an area is reduced, spread of the bacteria should be restricted. However, research published on the prevention of PD spread in the USA using insecticides, showed that this technique failed to provide effective control of the disease (Mortensen, 1966). Fortunately, there is another control method that involves targeting vectors. Research is taking place into the use of paratransgenesis to prevent *X. fastidiosa* colonisation of insect vectors, thereby stopping transmission to plant hosts (Bextine *et al.*, 2004). Although still in the research stage, this technique offers a novel and highly targeted way to control *X. fastidiosa* diseases.

The identification, or alternatively breeding and production, of resistant cultivars could provide a way for farmers to continue growing crops that would otherwise be decimated by disease. Grape producers in the southeast of the USA rely heavily on a cultivar resistant to Pierce's disease (Mortensen *et al.*, 1977). Another control solution involves the inoculation of plants with *X. fastidiosa* strains that produce slight or no symptoms, to prevent infection by a highly virulent *X. fastidiosa* strain that would cause severe damage. Research in this field has been carried out with positive results in the prevention of Pierce's disease in grape vine (Hopkins, 1994).

In laboratory experiments, it has been shown that all varieties of *X. fastidiosa* display resistance to penicillin, but are susceptible to a number of other antibiotics (Kuzina *et al.*, 2006). In field studies, stem injection techniques have been shown to provide a level of control in infected trees, capable of reducing or delaying symptoms, but not eliminating *X. fastidiosa* entirely (Gould and Lashomb, 2005; Amanifar *et al.*, 2016). Similar therapeutic effects have been observed with treatments using the growth regulator Paclobutrazol (DeStefano *et al.*, 2007; Hartman *et al.*, 2010).

Conclusion

Many of the diseases caused by *X. fastidiosa* have long been known to crop growers. It is only relatively recently though, and with the use of modern plant pathology techniques, that the complex life cycle of this bacterium has started to be fully understood.

Now that *X. fastidiosa* has been transported out of the Americas and into the rest of the world, research into its properties and behaviour is more valuable than ever. The ability of this pathogen to thrive in a range of environments has allowed it to spread within Europe from its locations of initial introduction, and it will, in all likelihood, continue to do so. The list of host plant species, already numerous, is also expected to increase as more species are tested, and new diseases are detected. There is, as yet, no simple and easy to implement control measure for *X. fastidiosa*. It is essential that further research is carried out with the aim of discovering an economical eradicator treatment for infected plants. Without an internationally co-ordinated approach to prevent further spread of *X. fastidiosa*, global establishment of the bacterium is a real possibility.

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