Population diversity and epidemiology of *Bremia lactucae* the cause of lettuce downy mildew

Alicia Ann Farmer

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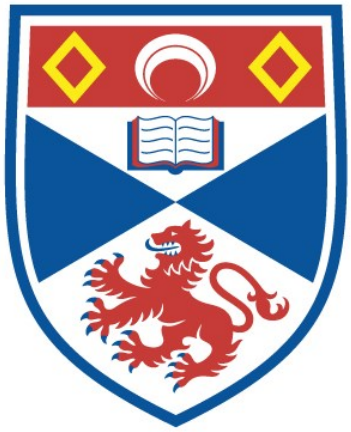

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Population diversity and epidemiology of *Bremia lactucae* the cause of Lettuce Downy Mildew.

Abstract

Bremia lactucae is an obligate biotroph that causes Lettuce Downy Mildew (LDM), a foliar disease of lettuce which negatively affects crop value though reduced yield and quality. Emergence of new strains of *B. lactucae* that can overcome host resistance or result in reduced sensitivity to fungicide active ingredients is a consistent risk to LDM management and crop production.

From the 254 UK samples of *B. lactucae* collected and genotyped successfully using ten SSR loci, 135 multilocus genotypes were identified. Evidence was found of widespread incidence of heterokaryosis and overwintering clonal lineages. At least one UK isolate was able to overcome each differential line in IBEB differential set-C, of which cv Dandie containing *Dm3* was least overcome by isolates tested. Of 15 *B. lactucae* samples tested with the fungicide active ingredients axoystrobin, mandipropamid and dimethomorph, no fungicide insensitivity was observed. No conclusive evidence of association between genotype to fungicide insensitivity nor virulence was obtained. When trials were inoculated with isolates of various pathogen genotypes, cultivar choice was found to greatly influence the population diversity of *B. lactucae*, with specific MLLs associated with some of the trial cultivars.

The study of aerial dispersal of sporangia using the LAMP assay and aerial samplers found it was predominantly local to infected plants (<5m). DNA of *B. lactucae* was detected up to 100m from an inoculum source. In commercial environments detection using the LAMP assay was as early as two days before LDM was reported in field, with symptomatic plants observed ~50-80m away from samplers.

The UK population of *B. lactucae*, based on samples collected, is diverse in genotype and phenotype. LDM management should account for the genetic flexibility of *B. lactucae* heterokaryons, which may contribute to overwintering soil-borne inoculum, and aerial dispersion of sporangia, which transmits the pathogen and can contribute to gene flow.

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Contents

Chapter 1 Introduction

1.1 Lettuce

1.1.1 *Lactuca* spp. taxonomy, classification, and distribution

The genus *Lactuca* L. is currently classified within the subtribe Lactucinae, tribe Cichorieae (also called Lactuceae), subfamily Cichorioideae of the Asteraceae family (previously Compositae) which is the largest family of flowering plants (Kilian et al., 2009; Wei et al., 2017).

The taxonomic position and boundaries of *Lactuca* lineage (or *Lactuca* alliance sensu, Kilian et al., 2017) have been a topic of extensive deliberation, encompassing complications arising from reliance on morphological characteristics and geographical locations (both of which are diverse, Lebeda et al., 2004), and molecular based phylogenetics (Koopman et al., 1998, 2001) for identifying where and what constitutes a *Lactuca* species as reviewed by Koopman et al. (1998); Lebeda et al. (2019); Wang et al. (2013); Wei et al. (2017).

To summarise, only recent comprehensive molecular phylogenetic studies (Kilian et al., 2017; Wang et al., 2013; Wei et al., 2017), using nuclear recombinant DNA (rDNA) internal transcribed spacer (ITS) (Kilian et al., 2017; Wang et al., 2013), neutral chloroplast markers (Kilian et al., 2017; Wang et al., 2013) and chloroplast genes (Wei et al., 2017) on a geographically wide and species diverse combination of accessions has led to a general consensus than most generic groupings do not reflect molecular phylogenetics. For example, the study by Wei et al. (2017) suggested there are two distinct clades within *Lactuca,* the crop clade and *Pterocypsela* clade. However, with biogeographical and divergence dating analysis (Kilian et al., 2017) the resolved lineages were referred as not "practical taxonomical entities", suggesting multiple independent migrations occurred in different geographical locations leading to the complex taxonomic tree.

Prior *Lactuca* groupings suggest there are currently 98 formally described wild *Lactuca* spp. (Lebeda et al., 2004); although estimates suggest that more than 100 *Lactuca* species may exist but roughly 40 are part of the *Lactuca* lineage (Kilian et al., 2017). *Lactuca* species predominately occur naturally in the northern hemisphere in temperate and warm regions (Lebeda et al., 2004) and a majority of wild *Lactuca* spp. are native to Africa and Asia, (Petrželová & Lebeda, 2004).

According to Lebeda et al. (2004) most *Lactuca* spp. are characteristically xerophytes, adapted for dry climatic regions, though there is diversity in the ecological habitats with *Lactuca* spp evident in wasteland environments to rocky slopes, sandy shingles, and woodland habitats.

Sixteen species of *Lactuca* have been documented to be native to Europe (Petrželová & Lebeda, 2004) with four of these; *L. serriola, L. sativa, L. virosa* and *L. saligna* reported in the UK (Carter & Oswald, 1998; Oswald, 2000). Several species in the *Lactuca* genus have been considered to have the common (vernacular) name of "lettuce" but in general this refers to the horticultural crop *Lactuca sativa* and will be referred to in this way hereafter (Frietema, 1994; Lebeda et al., 2002).

1.1.2 Lettuce (*Lactuca sativa*) as a crop

Lettuce is thought to have been domesticated in Egypt, with the presumed first record dated at 4500 BC, (Lindqvist, 1960; Whitaker, 1969). The first evidence of lettuce cultivation in Egypt was as early as 2680 BC, in which the crop was predominately used for producing seed oil (Noumedem et al., 2017). The Egyptian cultivated lettuce was introduced to Greece and Rome from where it spread around Europe, with many varieties being developed in this region between the late 16th and the early 18th century (Noumedem et al., 2017).

One of the attractive qualities of *L. sativa* is its genetic plasticity, which is attributed to its capacity for self-fertilisation. Through selective breeding, this diploid plant can achieve considerable variation in morphology, flavour, and texture (Kim et al., 2016; Whitaker, 1969). Lettuce cultivars are often roughly grouped into morphological forms. According to classification criteria (Křístková et al., 2008), there are seven main cultivar types of *L. sativa*: Butterhead, Crisphead, Cos, Cutting, Stalk (also called asparagus lettuce or celtuce), Latin and Oilseed lettuce. Most lettuce cultivar types are produced for the fresh market, with the exceptions of stalk and oilseed.

The popularity of lettuce types differs between global regions, with the Stalk (or stem) type (*L. sativa* var. *angustana*) being more commonly grown in China and Crisphead lettuce being the most popular type in the US (Kim et al., 2016). FAO statistics (FAOSTAT, 2021), which group chicory with lettuce, indicate that the largest producer of lettuce (and chicory) is China, followed by the US, with 15.5 million tonnes and 3.7 million tonnes produced respectively in 2018. In 2017, 99,000 tonnes of lettuce, worth £184 million, were produced in the UK, utilising 4% of UK land

designated for field crops and representing 16% of the total value of all the UK's field produced vegetable crops (DEFRA, 2023). UK spending on prepared chilled leafy greens and vegetables was in excess of £1.1 million in 2021, which was more than twice that recorded in 2008 (Statista, 2023).

1.1.3 Lettuce breeding

The breeding history of lettuce is complicated due to geographical spread, its capacity to be inter-crossed with wild species, the demand for different morphological types and specific targeting of regional pests and pathogens of lettuce. Cultivated lettuce is considered to have a primary gene pool based on *L. sativa* derived cultivars and land races (Lebeda et al., 2001). In recent years, desirable traits have been introgressed into *L. sativa* from other wild *Lactuca* species, predominately *L. serriola,* as it is fully cross-compatible and interfertile with *L. sativa* (Lebeda et al., 2001, 2009). Other *Lactuca* species are more challenging to cross directly with *L. sativa* (Lebeda et al., 2001; van Soest & Boukema, 1997). However, the observed high interfertility of *L. serriola* with other wild *Lactuca* species, such as *L. aculeata*, and *L. dregeana*, has allowed indirect introgression of traits (Lebeda et al., 2009; Zohary, 1991). For example, there are multiple documentations of introgression of wild varieties to commercial cultivar lines to improve crop resistance to disease (Crute, 1992a, 1992b; Giesbers et al., 2018; Lebeda et al., 2002, 2014; Mikel, 2007); a specific example would be the cultivar Titan developed by Sluis & Groot from introgressing *L. saligna* (accession originally from Israel) into another commercial cultivar providing *Dm6* resistance to *B. lactucae* (Lebeda et al., 2002).

According to Lebeda et al. (2014) the categorisation of *Lactuca* species into *L. sativa* gene pools still requires clarification, this could be in part due to the difficulties in determining the *Lactuca* lineage, as Lettuce is generally believed to be polyphyletic in origin (Lebeda et al., 2019). The *Lactuca* lineage alliance (Kilian et al., 2017) or *Lactuca* subsect *Lactuca,* (sensu Koopman et al. (1998, 2001)) are generally very closely related (conspecific) *Lactuca* species which could be capable donors of useful genetics (Lebeda et al., 2014). Though there are discrepancies in the groupings of donor wild-type species, generally the primary gene-pool reported for cultivated lettuce includes *L. sativa, L. serriola,* and secondary including *L. saligna* and *L. virosa* (Kilian et al., 2017;

Koopman et al., 1998, 2001; Lebeda et al., 2004). Though comparatively studied in less depth than European species, there are Asian and African *Lactuca* species that are reportedly grouped within primary, secondary, and tertiary gene-pools for *L. sativa*, such as *L. dregeana, L. altaica, L. georgica,* all of which could provide genetic material in lettuce breeding (Kilian et al., 2017; Koopman et al., 1998, 2001; Lebeda et al., 2004)

Availability of data on UK lettuce variety pedigrees is limited, however the genealogy and genetic composition of US cultivars from 1970-2010 has been documented (Mikel, 2007, 2013). Despite the genetic diversity available from many wild type accessions and species, limited parental material was often used to select for particular morphologies. The cultivar 'Parris Island Cos' was reported to contribute 25.9% of genes in most US romaine cultivars (Mikel, 2013). As a result of low genetic diversity within each morphotype, cultivars of the same morphotype can be similarly susceptible or resistant to the same pathogen through limited resistance diversity. For example, many modern crisphead (iceberg) lettuce cultivars have a recessive allele *cor* which confers resistance to corky root, a disease caused by a bacterium *Sphingomonas suberifaciens* (Lebeda et al., 2014; Mou et al., 2007). Similarly, modern varieties of crisphead lettuce were conferred with resistance to the *Tombusviridae* virus family that causes lettuce dieback, whereas all romaine cultivars were not (Grube et al., 2005).

Contemporary breeding includes genomic techniques, such as marker assisted selection, that can screen genotype at seedling stage (Collard & Mackill, 2008).Therefore, traits controlled by major resistance (*R*) genes or large effect quantitative trait loci (QTLs), can be identified by early generational testing (Hayes & Simko, 2016). Cultivar traits can be curated with the utilization of CRISPR Cas-9 to precisely modify the genome, a technique known as genome editing. (Rönspies et al., 2021). Genomic regions that favour resistance have been identified, for example, chromosome 2 in lettuce contains *Dm3*/ *RGC2* the largest resistance gene cluster, which includes several *B. lactucae* resistance genes (*Dm),* along with Root aphid (*Ra),* and a *Tombusviridae* resistance locus *(Tvr1)* (Grube et al., 2005; Meyers et al., 1998). There are gene characteristics that can and have been used to identify these important genome regions for breeding, for example leucine-rich repeats (LRRs) are common in major *R* genes (reviewed in Baker et al. (1997)). In

addition to genomics, contemporary breeding can utilise optic sensors for high throughput phenotyping. For example, young lettuce plants were subjected to temperature and salinity stress and were evaluated through the usage of hyperspectral imaging and chlorophyll fluorescence imaging (Simko et al., 2016). In addition to "speed breeding", in which the breeding cycle is reduced through careful management of the growing environment including but not limited to photoperiod, light intensity, CO_2 concentration and temperature (He et al., 2024; Watson et al., 2018).

1.1.4 Lettuce cultivation

Lettuce production is usually grouped into two types; protected and field grown. Protected refers to sheltered or indoor growing, including but not limited to, greenhouses and hydroponic systems. Field production can be achieved by direct sowing of lettuce seeds into the soil, though it is common to sow indoors and transplant seedlings to fields (Wallace et al., 2012). Intensification of lettuce production through practices of monoculture, successive plantings, use of fertilisers and chemical control have contributed to increasing yields for this agricultural crop (Barrière et al., 2014; Michelmore & Wong, 2008).

Lettuce production can generate a potentially large income for a small area of land in the UK. However, as noted in the guide to standards (AHDB, 2022; Assurance, 2015, 2016; Red Tractor Assurance, 2015, 2016) many inputs are required for its cultivation. A US-based study (Tourte et al., 2017) estimated total operating costs to produce and harvest iceberg lettuce on a conventional farm in the central coast region to be $9,947 per acre. However, the study noted that the profit margins can be narrow, dependant on a fluctuation in the amount and costs of input required in a particular growing season.

Lettuce crops tend to have a short growing period, taking 43-65 days from seed to harvest for most varieties, and around 30 days for baby leaf salad (Simko, 2019; Wallace et al., 2012). This allows for several successional plantings in a growing season, thus maximising production on a relatively a small area of land. Lettuce cultivation requires careful management to reduce the impact of pests and diseases whilst maintaining quality.

A key element of lettuce cultivation is to ensure that the crop complies with crop marketing and agricultural regulations. In the UK there are strict guidelines (DEFRA, 2011; MAFF, 1999) on marketing crops, which include the amount of fungicide residue, nutrient levels and overall crop appearance, these traits can determine the marketability of the crop. An example of UK marketing regulations regarding lettuce is the maximum nitrogen level allowed in the marketable lettuce heads. Excessive nitrogen contributes to environmental damage, such as eutrophication, reduces crop yield, and contributes to consumer health problems. For these reasons the UK has set a maximum of 4000 mg nitrate kg^{-1} in lettuce harvested between April and September, and 5000 mg nitrate kg^{-1} in lettuce harvested between October and March (Aydinsakir et al., 2019; Red Tractor Assurance, 2016). A preliminary WRAP study into crop wastage has estimated lettuce crop losses of around 24% of lettuce head weight was left in field due to crop trimming, for 2015 lettuce crop wastage (including trimming) was estimated at £7 million in the UK (Sheane et al., 2017).

1.1.5 Crop protection and common diseases

In general, intensification of lettuce production, such as monoculture and successional planting, has created an environment that is conducive to pests and diseases.

Cultural control measures such as removal of infected crop material, or treatment of crop residues before rotovation into the soil or removal from the field, can reduce the impact and spread of many pathogens (Barrière et al., 2014; Red Tractor Assurance, 2016). Crop rotation can be used for pathogen management and in lettuce production this typically includes Solanaceaeous and Cucurbitaceae crops (Barrière et al., 2014). Prophylactic calendar-based agrochemical application is often used on lettuce to protect the crop, even in the absence of pathogens, to ensure and preserve the marketability of the lettuce crop (Barrière et al., 2014).

Key Lettuce diseases in the UK are Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lactucae* (AHDB, 2019a; Barrière et al., 2014; Taylor et al., 2018), Ring Spot (*Microdochium panattonianum*) (AHDB, 2019a), Grey mould (*Botrytis cinerea)* (AHDB, 2019a), Lettuce Downy mildew (LDM) (*Bremia lactucae)* and *Sclerotinia* (*Sclerotinia minor* and *Sclerotinia sclerotiorum)* (AHDB, 2019a; Barrière et al., 2014; Subbarao et al., 2018). Lettuce crop wastage is generally not quantified based

on individual factors i.e. pests and diseases but is instead often measured in the difference between lettuce planted and heads sold, therefore the overall impact of pests and diseases is not known (Sheane et al., 2017). From the Waste and Resources Action Programme (WRAP) interviews on crop wastage with thirteen UK lettuce growers, four said that pests and diseases were reasons for ploughing whole areas, and ten said that pest and disease were reasons to leave individual lettuce heads in field (Sheane et al., 2017). Reportedly, diseases caused by fungi or oomycetes lead to huge crop losses (AHDB, 2019a), for example Fusarium Wilt is reported to cause losses up to 60% in the UK and Ireland (Taylor et al., 2018).

1.1.6 Lettuce downy mildew (LDM)

Lettuce downy mildew (LDM) is a disease of lettuce, which results in foliar damage that decreases the quality and thus the marketability of lettuce crop. Reduced marketability is due to lettuce being grown predominately for fresh markets requiring high crop quality standards. LDM reduces the marketability of lettuce by foliar damage, visible lesions, and discolouration. Low levels of LDM can increase post-harvest decay in storage or transit (Subbarao et al., 2018), and can incur significant crop trimming to remove infected leaves, reducing crop weight and thus economic value (Hutchinson et al., 1975; Subbarao et al., 2018). High levels of LDM can make a lettuce head completely unmarketable; in pointed lettuce, if the pathogen gets into the lettuce heart the whole head is discarded (G's Fresh, 2020; Subbarao et al., 2018).

In 1966-1969, 2% of California head lettuce weight was reportedly lost to trimming (Hutchinson et al., 1975). According to conversations with commercial lettuce growers in the UK, lettuce heads are usually trimmed by 1-2 leaves, but with LDM symptoms this can increase to 3-4 leaves, any further trimming is considered an economic loss, and the plant will not be harvested (G's Fresh, 2020).

In addition to aesthetic damage, LDM can also render lettuce unmarketable by increasing the risk of secondary pathogen infection. Simko et al. (2015) reported that LDM promoted the colonisation of romaine lettuce by the human pathogens *Escherichia coli* and *Salmonella enterica* effectively rendering the lettuce crop unsafe for consumption.

LDM affects both in-field and greenhouse produced lettuce, and *B. lactucae* can infect lettuce at any growth stage, meaning it is a potential threat throughout the entirety of production. LDM related plant death tends to occur due to early infections of young plants, or indirectly through secondary infections, often through lesions turning necrotic in adult plants (Spring et al., 2018).

Economic damage caused by LDM is not generally quantified, or details are not made publicly available, however losses are estimated to be in the region of £15 million per annum in the UK (AHDB, 2019b). Due to various direct and indirect (stress related) damage caused by LDM it is difficult to define and measure the full impact. In addition, severe LDM epidemics tend to occur at a local or regional level initiated by the emergence of a new virulence phenotype of *B. lactucae* able to overcome host resistance genes used widely in the area (Lebeda & Zinkernagel, 2003b, 2003a), resulting in the economic effect being regionally concentrated (Spring et al., 2018; Subbarao et al., 2018).

1.2 *Bremia lactucae*, the cause of LDM

1.2.1 Oomycetes and the taxonomy of *B. lactucae*

The oomycete pathogen *Bremia lactucae* causes LDM. The oomycetes are eukaryotic stramenopiles that include many destructive plant pathogens (Beakes et al., 2012; Beakes & Thines, 2017; Fry & Grünwald, 2010). The similarities in filamentous structures observed both in fungi and oomycetes led to oomycetes being previously considered fungi (Cavalier-Smith & Chao, 2006); fungal terminology is therefore not uncommon in the literature (i.e. hyphae, fungicides). Molecular based phylogenetic analysis confirmed that oomycetes are more closely related to land plants and algae than fungi (Thines et al., 2010; Thines & Choi, 2016).

The cell wall of oomycetes are predominately composed of cellulose and glucans, (Fawke et al., 2015; Fry & Grünwald, 2010; Thines, 2014). Oomycetes tend to be coenocytic, a multinucleate condition arising from a lack of septa (cell walls) in hyphae (Fry & Grünwald, 2010). These vegetative mycelia typically have diploid nuclei (Fawke et al., 2015; Fry & Grünwald, 2010). Most oomycetes produce wall-less, motile infectious propagules called zoospores which move chemotactically towards host plants over short distances and can have an important epidemiological role in disease spread (Fry & Grünwald, 2010).

One oomycete genus that has lost zoospores is *Bremia,* which resides taxonomically within the *Peronosporaceae* family (Thines, 2014; Thines & Choi, 2016)**.** The *Peronosporaceae* are obligate biotrophs and their survival is intrinsically linked and dependent on obtaining nutrition from a living host (Thines, 2014; Thines & Choi, 2016). *Bremia* species have therefore evolved a range of mechanisms to infect and replicate within a susceptible host, including the ability to penetrate the leaf cuticle and epidermis of suitable hosts (Lebeda et al., 2008; Norwood & Crute, 1983), creating an entry point for the pathogen to colonise leaf tissues without utilising the stomata (Michelmore & Wong, 2008).

The genus *Bremia* was initially distinguished from other *Peronosporaceae* based on sporangial morphology. The genus was presumed to have only two species which were host family specialists; *B. lactucae* which parasitised the Asteraceae (Compositae) and *B. graminaria* which affected Poaceae (Lebeda et al., 2002). However, phylogenetic analysis has since established that

within *Bremia* there are several taxonomically distinct clades, and currently 15 classified species, the majority of which are host-specific and target the Asteraceae family (Choi et al., 2011; Schoch et al., 2020; Thines & Choi, 2016).

1.2.2 *B. lactucae* geographical distribution and hosts

LDM has been reported in several *Lactuca* species, the most common hosts being *L. sativa* and *L. serriola* (Lebeda et al., 2002; Thines et al., 2010). There have been occasional reports of *B. lactucae* infecting non-*Lactuca* species including strawflower (*Helichrysum bracteatum* (Venten.) Andr.) (Koike & Ochoa, 2007). However, reports of infections by *B. lactucae* must be viewed with caution, as it was previously thought to be a monotypic species that affected a broad range of hosts (Choi et al., 2011; Thines et al., 2010).

B. lactucae has been observed wherever cultivated lettuce has been grown, most frequently in temperate climatic regions (Lebeda et al., 2002). To the author's knowledge, in-depth studies of *B. lactucae* distribution in wild *Lactuca* populations have not occurred, with the exception of the Czech Republic (Mieslerova et al., 2013; Petrželová et al., 2013; Petrželová & Lebeda, 2004) and the Netherlands (Hooftman et al., 2007) where surveys looked at wild *Lactuca* populations. Between 44.4% and 63.3% of *L. serriola* populations observed (n>65 populations) in the Czech Republic were reported to have *B. lactucae* infections from a survey during May-September of 2007-2011 (Mieslerova et al., 2013). There are records of the presence of *B. lactucae* in wild *L. serriola* populations in other European countries (Lebeda et al., 2001). In general, most *B. lactucae* isolate monitoring and collection is focused to sampling of commercial environments, mostly with lettuce crops (Nordskog et al., 2014; Souza et al., 2022; van Hese et al., 2016). These studies (Lebeda & Zinkernagel, 2003b; Nordskog et al., 2014; Souza et al., 2022; van Hese et al., 2016) tended to focus on the virulence profile of the *B. lactucae* samples and as such, the overall frequency, distribution, and prevalence of *B. lactucae* is likely under-reported due to this bias towards commercial environments.

1.2.3 *B. lactucae* lifecycle

B. lactucae can reproduce through a polycyclic asexual cycle, and an infrequent sexual cycle as depicted in Figure 1-2. The asexual cycle begins when an aerial spore of *B. lactucae* lands on a

suitable host and germinates directly to form a germ tube (Sargent et al., 1973). The germ tube emerges to form a hooked appressorium, a structure that directly penetrates the plant cell (Michelmore & Wong, 2008; Sargent et al., 1973). Sargent et al. (1973) noted that the appressorium shape, coupled with the shape and profile of the leaf cuticle and epidermal walls during penetration, suggested that mechanical force was minute, or not applied at all. They concluded that with the absence of evidence supporting mechanical force as the main mode of entry, this suggested that penetration utilises enzymatical digestion (Sargent et al., 1973). However, there has since been evidence (Bronkhorst et al., 2021, 2022) of another oomycete *Phytophthora infestans* having an appressorium reinforced with an actin-based 'mechanostat', which is when the actin cytoskeleton restructures to have tip sharpness allowing the hyphae to slice host walls at a diagonal with a 'naifu' cutting action. It is possible that *B. lactucae* could also share this mechanism, though there is currently no direct evidence to support this.

Once inside the host, *B. lactucae* forms a primary vesicle which acts like a "secondary spore" to hold the nutrients from the initial sporangia; then a secondary vesicle from which the pyriform haustoria (the feeding body) and vegetative hyphae emerge approximately 13 hours after sporangia germination (Sargent et al., 1973). The coenocytic (non-septate) vegetive hyphae then grows in the substomatal cavities in the leaf, penetrating surrounding plant cell walls and forming more haustoria (Verhoeff, 1960). At each haustoria-plant cell membrane interface an extra-haustorial membrane is formed to uptake nutrients and to release effectors to inhibit host immune responses.

Pathogen structures called sporangiophores emerge from the infected leaves usually on the abaxial side and produce many multinucleate sporangia (alternatively referred to as conidia) in a process called sporulation (Figure 1-1) (Fry & Grünwald, 2010), which then are dislodged and aerially dispersed to continue the asexual cycle. The duration of the asexual life-cycle can vary from 5 days to one month depending on environmental conditions, predominately temperature and relative humidity (Scherm & van Bruggen, 1994; Verhoeff, 1960).

Figure 1-1, Sporangiophores protruding from foliar lesions on lettuce leaves: B focus on a single sporangiophore, A and C sporulating lesion.

The sexual reproductive cycle of *B. lactucae* results in the production of thick-walled oospores that are tolerant of a range of environmental stressors. The formation of oospores, as documented by Sargent et al. (1977) begins when vegetative hyphae form into the sexual structures the antheridium and the oogonium. A germ tube from the antheridium to the oogonium joins these two structures and allows the antheridium to donate a nucleus to the oogonium. The oogonium has several nuclei, one of which undergoes nuclear fusion with the antheridial nucleus. This fused nucleus is retained and the rest of the oogonial nuclei degrade. The oogonium eventually matures into an oospore, which resides within the plant tissues.

Little is known about the epidemiological importance of *B. lactucae* oospores; they can be stimulated to germinate by the presence of germinating lettuce seed and have been reported to still be capable of germinating up to 12 months after formation when stored *in vitro* in lettuce cotyledons at 24^{o}C (Morgan, 1978, 1983). Oospores are reported to be produced less frequently

than aerial sporangia, (van Hese et al., 2016). It is presumed that the asexual cycle is the preferential reproductive method, and that sexual reproduction occurs when asexual reproduction is suppressed (Michelmore & Wong, 2008). However, oospores were documented frequently in commercial leaf samples in Brazil (Souza et al., 2022), which may suggest that population may vary in preferential reproduction.

Generally, *B. lactucae* is heterothallic, which means that the sexual structures, the antheridium and oogonium, are only compatible when formed by *B. lactucae* isolates of two different mating types (*B1* and *B2*) (Michelmore & Ingram, 1980). It is presumed that each mating type can produce antheridia and oogonia, as observed in *Phytophthora* species (Michelmore & Ingram, 1982). In addition, homothallic, self-compatible, and self-fertile *B. lactucae* isolates have been documented (Michelmore & Ingram, 1982; Michelmore & Wong, 2008). The frequency of homothallism in *B. lactucae* isolates is not extensively studied, however it does present the possibility for some *B. lactucae* isolates to produce overwintering spores without requiring the presence of both mating types.

Genetic variability in *B. lactucae* populations can also be created through somatic variation. Certain *B. lactucae* isolates have been reported to form heterokaryotic mycelia with multiple genetically diverse nuclei (Fletcher et al., 2019). Further research into European *B. lactucae* races, looking at allele balance, suggested that nine of 21 European isolates studied were likely to be heterokaryotic as they were inconsistent with diploidy (Fletcher et al., 2022). A reservoir of diverse genes in a heterokaryon form can increase the probability of rapid adaptation when exposed to selection pressures, such as fungicide insensitivity when exposed to agrochemicals. A polyploid state is usually more stable, and therefore was presumed to be the general means for increasing variation within *B. lactucae* populations (Michelmore & Wong, 2008), but considering these more recent findings, it could be inferred that heterokaryosis seems to play a larger role than expected (Fletcher et al., 2019, 2022).

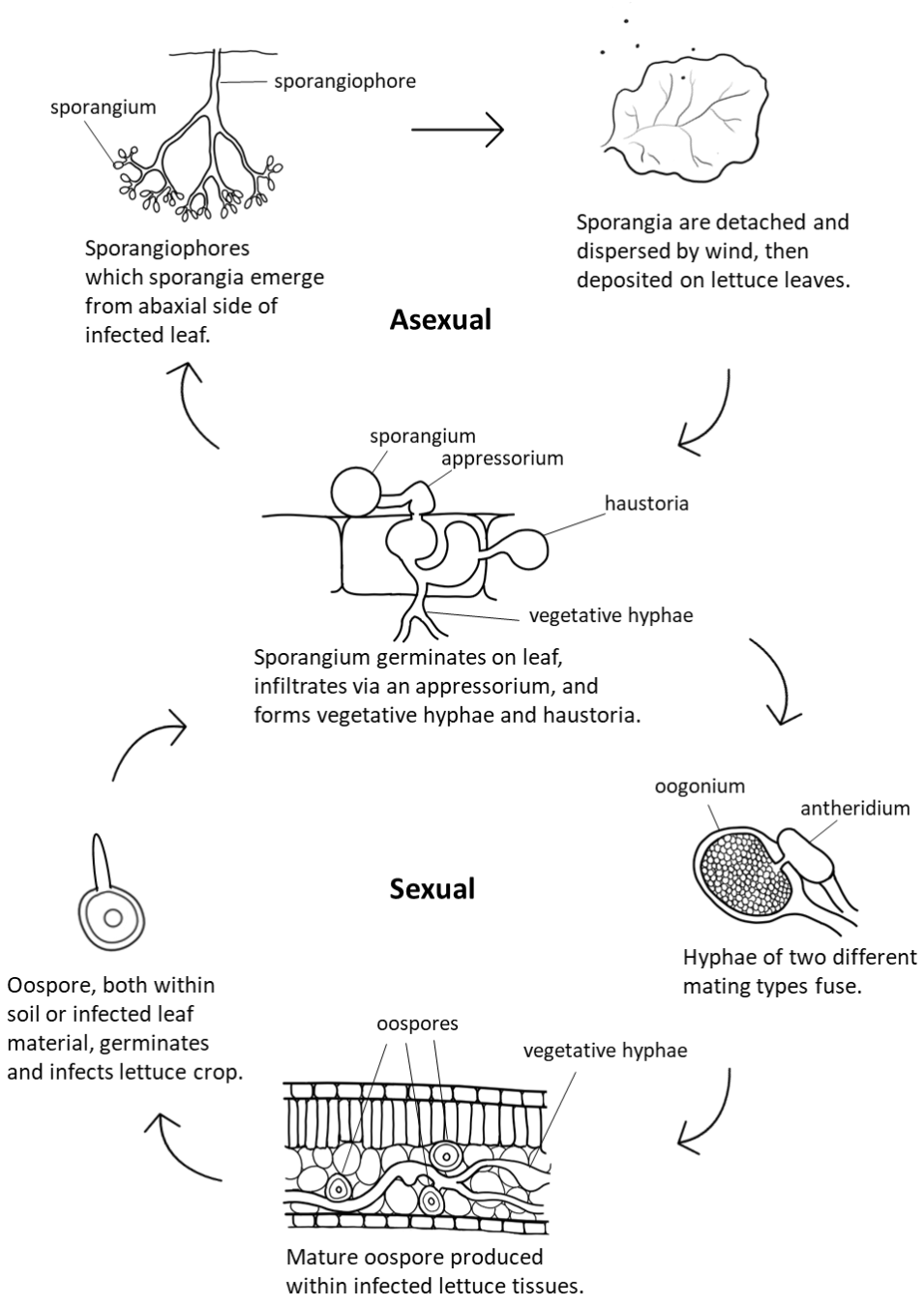


Figure 1-2 Lifecycle of B. lactucae. Diagram depicting the lifecycle of B. lactucae, adapted from infographics from Padgett-Johnson and Laemmlen (n.d.) and Sargent, Ingram, and Tommerup (1977).

1.2.4 Environmental parameters: role in LDM epidemiology

According to the disease triangle proposed by Stevens (1960), disease can only occur if all the following conditions are met, 1) there is a suitable host, 2) the environmental conditions are conducive to disease and 3) the causal agent of the disease is present. The environmental parameters that effect *B. lactucae* have been studied extensively, although the majority of these studies have focused on effects on specific stages of the asexual cycle as summarised in Table 1-1. Most studies of the effect of environmental parameters on *B. lactucae* have reported that cool damp and low light conditions are conducive to most life stages of *B. lactucae*. Therefore, temperature, humidity, light and leaf wetness duration (LWD) are all key environmental factors that affect *B. lactucae* and subsequently the development of LDM on lettuce crops.

It has been reported that optimal conditions in one environmental factor can mitigate the effect of a sub-optimal conditions in another factor, such as temperatures conducive to sporulation (5-10°C) reduced suppression of sporulation by light by <20% compared to sporulation in the dark (Nordskog et al., 2007). Conversely sub-optimal conditions in certain environmental factors can be inhibiting regardless of other variables, for example at wind speeds above 1.8 km/h sporulation was completely inhibited (Su et al., 2004) regardless of optimal relative humidity (RH) and temperature. Therefore "optimum" thresholds for *B. lactucae* can vary for each factor.

In general, *B. lactucae* can survive at temperatures ranging from 5 – 30°C depending on life stage and the duration of a sub-optimal temperature; though most studies found 15°C to be the optimal temperature for *B. lactucae* (Carisse & Philion, 2002; Nordskog et al., 2007; Powlesland, 1954). Temperature can greatly change the rate of sporangial germination with the optimal temperature for germination between 10-15°C (Subbarao et al., 2018; Verhoeff, 1960). Temperature-RH and temperature-time experiments by Wu et al. (2000) found that regardless of the RH, the higher the temperature, and the more prolonged the exposure to high temperature, both resulted in a reduced number of observed germinating sporangia. Constant temperatures have more of an effect on latency period (time between successful infection of host and symptoms expression and/or sporulation) than fluctuating temperatures; with a constant temperature of 20-22°C the latency period of *B. lactucae* is between 4-7 days, and at 6°C, 24-34 days (Scherm & van Bruggen,

1993, 1994). Little is known about the effect of temperature on oospores and reproductive structures in the sexual cycle.

Sporulation of *B. lactucae* was reported to be suppressed when exposed to high intensity light of 400-450 nm wavelengths, or UV light (Nordskog et al., 2007). Solar radiation or UV B exposure of >1.5 W m^{-2} led to degradation of aerial spore infectivity and contributed towards increasing sporangia mortality (Wu et al., 2000). In addition, infection is thought to be unsuppressed under lower light conditions, with overcast days being generally conducive to disease development. Interestingly, sporulation has been linked to photoperiod (Nordskog et al., 2007) and sporangia release is reported to be instigated by low light levels in early mornings suggesting that *B. lactucae* does need light cues to infect plants (Nordskog et al., 2007; Su et al., 2000).

RH and LWD are key environmental factors, with high RH (≥90%) being optimal for sporulation and sporangia germination (Fletcher, 1976; Powlesland, 1954; Su et al., 2000). Sporangial release was reportedly instigated by a small drop in RH, however this RH value is still above 90% (Su et al., 2000). LWD at night was reported to be conducive to infection by *B. lactucae*, and LWD in the early morning was reportedly conducive to sporangial release (Scherm & van Bruggen, 1993).

An additional environmental factor affecting the incidence of LDM is wind, as the asexual spores of *B. lactucae* are aerially dispersed. Carisse & Philion (2002) reported that wind speed was positively correlated to aerial spore concentration of *B. lactucae.* However, the methods of data collection did not verify whether sporangia were still viable. Interestingly, sporulation, the precursor step to sporangial release, was reportedly inhibited by wind speeds above 1.8km/h (Su et al., 2004).

Table 1-1 ***Summary of the effect of environmental conditions on each life stage of B. lactucae.***

Life stage	Environmental conditions	Reference
Sporangial dispersal and survival	Spores can survive >12h at 23°C, 2-5h at 31°C at 33% and 78% RH. Release of spores often corresponds to morning light and reduction in RH. Sporangia can travel <3 km with wind. Sporangium viability dependent on temperature, RH, and solar radiation. UV of 0.5 MJ/m² at short period can lead to spore mortality.	(Wu, Subbarao, and van Bruggen 2000) (Subbarao et al. 2018) (Carisse and Philion 2002) (Wu, van Bruggen, et al. 2001)
Sporangial germination	Can occur 5-20°C at a leaf wetness duration of ≥4h. Not observed at 30°C. Optimum between 10-15°C Relatively lower temperature and water or high humidity conducive for infection.	(Subbarao et al. 2018) (Scherm and van Bruggen 1995b, 1993)
Sporulation	High light intensity (400-450nm) is suppressive. Triggered by RH drop but still in the range of >/=90%RH. Temperature optimal is between 5-15°C, can occur from 5-25°C. Prevented by wind speeds >1.8km/h under high RH.	(Nordskog et al. 2007) (Su et al. 2004) (Scherm and van Bruggen 1994)
Latency period (between infection and symptoms/sporulation)	Determined by temperature, 4-5 days from infection to symptoms at constant 20-22°C. Fluctuating temperatures had reduced latency period.	(Wu et al., 2005) (Scherm and van Bruggen 1994)

Life stage	Environmental conditions	Reference
Overwintering and survival out of crop period	Oospores reported Czech Republic, Netherlands, New York, and Sweden. Not predominate in all populations, e.g. California. Germination can occur at 17°C. Germination tube can be used to directly infect lettuce plants. Alternative hosts such as *L. serriola* thought to be a potential inoculum source when cultivated lettuce is not available.	(Yuen and Lorbeer 1987) (Gustafsson, Liljeroth, and Gustafsson 1985) (Blok 1981) (Ilott, Durgan, and Michelmore 1987) (Petrželová and Lebeda 2004) (Morgan 1978, 1983) (Lebeda et al. 2008)

Oospores are generally considered the likely primary source of inoculum (Fall et al., 2016), however, most studies into environmental parameters of *B. lactucae* have focused on the asexual cycle as this is where most *B. lactucae* infections are presumed to originate from and is an area of interest as the information can be used to target fungicide sprays (Fall et al., 2016; Scherm et al., 1995). The effects of environmental factors on oospores have not been well characterised, probably due to practical difficulties in conducting such studies (Fletcher, 1976; van Hese et al., 2016).

1.2.5 Genetics of *B. lactucae*

A review by Michelmore & Wong, (2008) looked at how contemporary and classical genomics had contributed to the knowledge of *B. lactucae* genomics. The genome was estimated to have several chromosomes (minimum of 7-8) pairs ranging from 3-8Mb in size (Michelmore & Sansome, 1982; Michelmore & Wong, 2008), and the total genome was estimated to be ca. 50Mb. Michelmore & Wong, (2008) suspected this figure to be an underestimation, which was confirmed by Fletcher et al. (2019) who used flow cytometry on 39 isolates of *B. lactucae,* giving a haploid genome estimate of ~152 Mb (+/−3 Mb). Further work by Fletcher et al. (2021) using assembly-free linkage analysis pipeline (AFLAP)-based genetic mapping has led to the genome being grouped into 19 linkage groups; synteny analysis with *P. sojae* suggested that linkage groups 9, 11 and 12 are likely to be one chromosomal group, similarly for linkage groups 1 and 13, putting the estimation of the chromosome number for *B. lactucae* between 7-15 (Fletcher et al., 2021; Michelmore & Wong, 2008).

Exploration of *B. lactucae* genome complexity has shown that approximately 65% of the nuclear DNA consists of repeated sequences and 35% of low copy sequences (Michelmore & Wong, 2008). Heterozygosity in *B. lactucae* isolates ranged from 0.77-1.29%, which is generally higher that found in most oomycetes (<1%) (Fletcher et al., 2019).

Somatic variation has been observed across geographically diverse *B. lactucae* isolates. Hulbert & Michelmore (1988) used restriction fragment length polymorphism (RFLP) analysis of 25 isolates collected from a range of countries on different continents and found that there were differences. Most samples collected from Europe were diploid with high heterozygosity and many genotype

variations, which was attributed to the frequent occurrence of the sexual cycle in this continent (Gustafsson et al., 1985; Michelmore & Wong, 2008). Whereas isolates from Japan, Australia, and Wisconsin (US) were reported to have genetic indicators of polyploidy or heterokaryons through most tested loci having more than two alleles.

Fletcher et al., (2019) when using distinct allele frequency profiles found genomic signatures that suggested heterokaryosis. This conclusion was reached as both diploid and seemingly 'polyploid' isolates had a similar total DNA content from flow cytometry analysis, whereas with polyploids a larger genome size comparative to the diploid would be expected, i.e. with a triploid isolate you would expect 150% size compared to a diploid isolate. Therefore, the data did not support polyploidy in *B. lactucae* (Fletcher et al., 2019), whereas heterokaryosis would explain the allelic variation between isolates without increasing the total DNA content. Heterokaryons were also reported to behave differently to homokaryons phenotypically, and as heterokaryosis was not associated with geographical or temporal sampling it suggests it is a general trait of *B. lactucae* (Fletcher et al., 2019).

To summarise, *B. lactucae* uses sexual reproduction and heterokaryosis which are beneficial in providing allelic variation; an advantageous trait that is conducive to adapting when exposed to selection pressure.

1.3 LDM management

Management of LDM is often heavily reliant on the utilisation of resistant cultivars and the routine and prophylactic use of chemical controls. Cultural control and knowledge of favourable environmental conditions have allowed alternative methods of management that can supplement resistant cultivars and chemical controls. Oospores and wild hosts are thought to be primary sources of inoculum of *B. lactucae*, but disease management relies on the presumption that infection originates from aerially distributed sporangia. Where these sporangia initially come from, especially after non-growing seasons has not been fully defined or quantified in the literature.

1.3.1 Host resistance

Disease resistant cultivars are one of the most widely utilised methods for LDM management, as these cultivars can reduce fungicide usage and can also help meet the organic market requirements. *B. lactucae* is an obligate biotroph, therefore, to successfully colonise its host plant it must avoid detection as triggering a plant immunity response can also result in a hypersensitive response resulting in cell-death (Jones & Dangl, 2006). Due to needing living host material to colonise successfully, *B. lactucae* has a very complex and specialized interaction with lettuce and its "immune" system, these interactions are usually dependent on gene-for-gene (race specific) mechanisms (Parra et al., 2016).

Gene-for-gene resistance is effectively a monogenic trait, which has been used for around a century in lettuce variety development to improve resistance to LDM (Crute, 1992b, 1992a; Ilott et al., 1989). Currently, the repertoire of resistance for *B. lactucae* is a reported 28 resistance genes (*R*-genes or if *B. lactucae* specific *Dm*) and 23 resistance factors (R) that are being used in lettuce variety development (Parra et al., 2016)

Most *Dm* genes are reportedly located in five distinct clusters within the lettuce genome (Jeuken & Lindhout, 2002). Research into the inheritance of major *R* genes in *L. sativa* and *L. serriola* determined inheritance to be Mendelian (Crute & Johnson, 1976). As lettuce is diploid it is relatively easier to breed in desirable *R* genes than crops with higher ploidy. However, breeding

a new cultivar still takes time to develop and the cost of development can lead to an increase in the price of seed.

Lettuce breeding has not been well documented however historically and contemporarily, introgression using wild *Lactuca* spp. have been used to introduce resistance genes. According to Crute (1992b), breeding specifically for resistance for downy mildew in protected lettuce began with a report from MacPherson (1932) who looked at two French cultivars, Gotte a Graine Blanche de Loos and Rosde Printaniere, that were observed to have complete resistance to LDM. European lettuce cultivars in the 1950's utilised genetic material that originated in old German and French cultivars; whereas in the Netherlands in the late 1960s a resistant variety from a breeding program resulted from an interspecific hybridisation between an established cultivar (Hilde) and an accession of *L. serriola* (Crute, 1992b; Lebeda et al., 2014). Despite limited documentation, what we do know from literature on resistance in cultivars is that several *R* genes for *B. lactucae* have been used extensively in European cultivars, notably, *Dm2, Dm3, Dm6, Dm7, Dm11,* and *Dm16* (Crute, 1992a, 1992b). In more recent years the *Dm* profile used in lettuce accessions has changed to include more varied *Dm* sources (Lebeda et al., 2014).

Aside from gene-for-gene resistance, other plant defence responses can be effective against oomycetes including non-host resistance, whereby the entire population of a plant species has immunity to all isolates of a pathogenic organism that affects other plants. The only reported natural host of *B. lactucae* that appears to utilise non-host resistance is *L. saligna*, in which germinating sporangia are "arrested" before normal hyphae develop (Jeuken & Lindhout, 2002; Lebeda et al., 2002). Additionally, host-specific resistance genes have been found in *L. saligna* accessions which are usually present in host plants (Giesbers et al., 2018; Lebeda et al., 2002, 2008; Parra et al., 2016; Zhang et al., 2009). Development in understanding of the basic incompatibility of *L. saligna* and the introgression of *L. saligna* into cultivar lines is ongoing.

1.3.2 Chemical control

Chemical control is a common means of managing LDM, as it allows for more susceptible cultivars to be grown that fulfil the other desired requirements of a lettuce crop such as reliable uniformity, in addition to providing assurance of disease control in the event of new virulent, but

not fungicide insensitive, *B. lactucae* races. These chemicals are often applied in spray programs which can be modified according to pathogen incidence in field and/or by environmental cues that indicate an increased risk: such as high humidity and overcast days (Barrière et al., 2014; Scherm et al., 1995). Chemical control can be contact based, targeting *B. lactucae* at its early stages, usually on the surface of the host tissues, or systemic, in which the mode of action targets later-stage processes of the pathogen within the plant tissues (Gisi, 2002). Chemical control can provide additional benefits by targeting more pathogens than *B. lactucae*, reducing crop damage through prevention of multiple plant diseases.

Several active ingredients are registered for LDM management in the UK, such as metalaxyl-M and mandipropamid, a more extensive list can be observed in Table 1-2 (AHDB, 2019a; Raid, 2012). Historically there were more options for active ingredients (Powlesland & Brown, 1954), but these have been overcome by insensitive strains and/or restricted by legislation (AHDB, 2019a). Some of the UK regulations affecting chemical control in lettuce production include restrictions on the field rates, the frequency of use and combinations of active ingredients. This is so that fungicide spray programs can be established to (1) prevent environmental damage, and (2) to prevent a sub-population of the pathogen developing resistance, through the use of several chemical classes which target multiple sites in sequence or mixtures (Gisi & Sierotzki, 2008). Of these active ingredients permitted for UK use, there are a few systemic types: Metalaxyl-M, dimethomorph, propamocarb, fosetyl-AL and azoxystrobin. Systemic fungicides, specifically phenylamides like metalaxyl, target a single specific site in the pathogen (Gisi, 2002; Gisi & Sierotzki, 2008). Fungicide with single specific target site modes of action can be advantageous as this reduces the chance of phytotoxicity, however this makes the mode of action vulnerable to even the smallest of mutations to the target site. Gisi & Sierotzki (2008) described single-site systemics as "high intrinsic risk" as only slight mutations are required to develop insensitivity and the usage of the systemic exerts selection pressure. There are many documentations of *B. lactucae* sub-populations overcoming single-site systemic fungicides from consistent usage (Brown et al., 2004; Cobelli et al., 1998; Crute et al., 1987).

Table 1-2 Fungicides suitable for *B. lactucae Field application. Information updated and checked on the 13/03/2023. Updates can be accessed via: Pesticides Register - Search Results.*

Active(s) effective actives	**Product** **MAPP No**	**Extent of Authorisation**	**Product Expiry Date**	**Marketing Company**	**Crop(s) and notes**
Azoxystrobin	Amistar 18039	GB and NI	30/06/2027	Syngenta UK Limited	lettuce (outdoor), lettuce (protected)
Mancozeb and metalaxyl-M	Fubol Gold WG 14605	GB and NI	30/04/2024	Syngenta UK Limited	Off-label approval for *B. lactucae* control
Fluopicolide and propamocarb hydrochloride	Infinito 16335	GB and NI	30/11/2025	Bayer CropScience Limited	Off-label approval for *B. lactucae* control
Dimethomorph and mancozeb	Invader 15223	GB Only	30/07/2026	BASF plc	Off-label approval for *B. lactucae* control
Mancozeb	Karamate Dry Flo Neotec 14632	GB Only	30/04/2026	Indofil Industries (Netherlands) B.V.	Off-label approval for *B. lactucae* control
Oxathiapiprolin	Orondis Plus 19305	GB and NI	19/07/2025	Syngenta UK Limited	lettuce (outdoor)
Dimethomorph	Paraat 15445	GB and NI	31/01/2026	BASF plc	Off-label approval for *B. lactucae* control
Fosetyl-aluminium and propamocarb hydrochloride	Previcur Energy 15367	GB and NI	31/10/2025	Bayer CropScience Limited	lettuce (outdoor), lettuce (protected, grown in organic media)
Mandipropamid	Revus 17443	GB and NI	31/01/2026	Syngenta UK Limited	lettuce (outdoor), lettuce (protected)

Contact fungicides are also available for management of *B. lactucae*, including: mandipropamid, a carboxylic acid amide (CAA) fungicide which is absorbed through the waxy cuticle, and disrupts pathogen cell synthesis and spore germination; mancozeb, a dithiocarbonate contact fungicide, which has three modes of action that target the inhibition of glucose oxidation, fatty acid degradation and nucleic acid synthesis (Barrière et al., 2014; Cohen et al., 2008; Gisi, 2002; Gisi & Sierotzki, 2008).

1.3.3 Disease forecasting and other management tools

Integrated Pest Management (IPM) is the utilisation of multiple different approaches to manage pests and pathogens, often with a focus on both prevention and intervention. An example of an IPM approach is the utilisation of the in-depth studies into the environmental conditions optimal for *B. lactucae* survival and persistence, to develop disease forecasting models to be used in decision support sensors (DSS). This relies on the disease triangle theory, aforementioned in section 1.2.4, of which conditions conducive to disease result in the DSS sending alerts suggesting fungicide application based on perceived risk (Scherm et al., 1995; Wu, Subbarao, et al., 2001). An example of environmental parameters beneficial for *B. lactucae* is prolonged LWD during the night, which has been used as an input for likely disease occurrence in forecasting systems (Kushalappa, 2001; Scherm et al., 1995).

There is limited information on the sexual cycle of *B. lactucae*, thus the extent of the epidemiological impact of oospores and environmental parameters affecting the stages of the sexual cycle are relatively unknown. Consequently, most forecasting is calibrated by research on the asexual cycle of *B. lactucae* which may affect accuracy.

Some forecasts have included the detection of LDM symptoms or sporangia of *B. lactucae* as additional variables to improve accuracy of disease risk. Reliance on weather cues for forecasting disease risk does not account for pathogen presence in the field, a key point mentioned by both Kushalappa (2001) and Dhar et al. (2020). Diagnostic assays for *B. lactucae* can also be used in the absence of weather cues and forecasting systems; for example, spore trapping in lettuce fields for the detection of *B. lactucae* (Kunjeti et al., 2016; Kushalappa, 2001). Unlike forecasting, diagnostics take time from sample collection to result, which can delay management decisions.

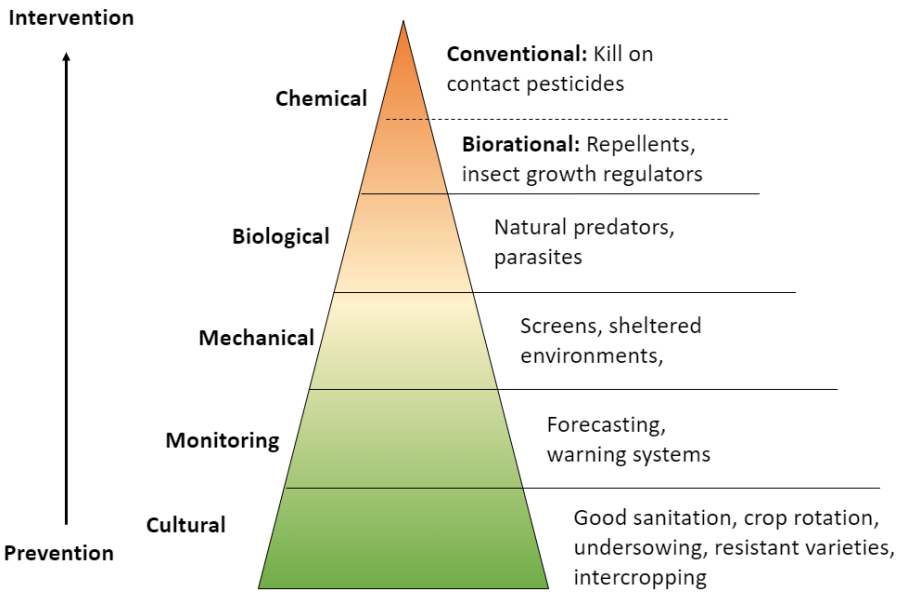


Figure 1-3 IPM Triangle Image adapted from both (Pennsylvania State University and Agriculture n.d.) and (IBMA, IOBC, and PAN Europe 2019).

In informal talks**,** industry partners have mentioned how they have switched to drip-irrigation in some instances to reduce the occurrence of favourable humidity conditions for *B. lactucae* in the field. LDM prevention guidelines recommend drip-irrigation (Matheron, 2015; Subbarao et al., 2018; Turini et al., 2017). Primary research has only compared drip vs furrow irrigation and no significant differences were found (Subbarao et al., 1997). This was, expected due to previous similar results between the two irrigation types (Scherm & van Bruggen, 1995a). There are more than two types of irrigation but results for LDM are not available; for spinach, drip irrigation has been found to reduce downy mildew compared withoverhead irrigation (Montazar et al., 2019) and,this likely holds true for LDM.

Another cultural practise industry partners mentioned using was situating the lettuce growing fields against the prevailing wind during the growing season, this is thought to reduce the likelihood of *B. lactucae* infections as *B. lactucae* has aerially dispersed sporangia. Other cultural practises industry partners mentioned were long-term crop rotations spanning four years and mixing blocks of lettuce varieties in field. Many of these management approaches mentioned by

our industry partners have been recommended in the review of lettuce cultivation with less fungicide by Barrière et al. (2014). Further research in crop management is required for optimal overall practice, as management practises for different diseases can be conflicting. For example, Industry partners noted that protective mesh equipment for preventing aphid predation of lettuce increased the local RH, resulting in increases in the observed LDM.

The vulnerability of infectious spores to light (Nordskog et al., 2007; Wu et al., 2000) has led to the development and testing of UV light as a preventative measure (Paul et al., 2012). Novel techniques of detecting disease prior to main symptoms have been developed (Bauriegel et al., 2014) but further logistical developments are required to implement these on a wide-scale under field conditions. This method of disease detection utilised the natural fluorescence of chlorophyll as an indicator of disease, as chlorophyll degradation from disease damage would reduce the light emitted (Bauriegel et al., 2014). This technique has been considered beneficial for screening for disease resistance in lettuce cultivars, which is a more feasible scale than in field.

Alternatives to fungicides have been explored, and although some provide control of LDM, their efficacy is generally lower than fungicides and they are generally recommended to be used with other management techniques. For example, the plant hormone BABA (Cohen et al., 2010, 2011), and an essential tree oil as a biopesticide in Timorex Gold (Reuveni & Cohen, 2020) both reduced LDM. Similar to fungicides, alternative methods of control can often induce the plant immune system which in itself can become a source of stress and can reduce crop weight (Cohen et al., 2010; Thomas & Puthur, 2017); therefore, research into the optimum thresholds for good crop quality and disease management is needed.

1.3.4 Management challenges

Protection from LDM by use of chemical control and resistant cultivars is consistently under the threat from the high evolutionary potential of *B. lactucae* to develop fungicide insensitivity and avirulence genes. This is particularly the case when *B. lactucae* is subjected to strong selection pressure, such as the use of cultivar monoculture, successional planting, and reliance on a select list of active ingredients and resistance genes. There are many reports of numerous races emerging and *B. lactucae* population shifts occurring in Europe, Australia, and America (Brown

et al., 2004; Cobelli et al., 1998; Crute et al., 1987; Lebeda & Zinkernagel, 2003b; Schettini et al., 1991; Trimboli & Nieuwenhuis, 2011).

B. lactucae has a few mechanisms for creating and maintaining genetic diversity, which contributes to its evolutionary potential. The life-cycle of *B. lactucae* can include genetic recombination with *B1* and *B2* mating types, in addition to the formation of heterokaryons (Fletcher et al., 2019). An additional mechanism which can be overlooked is the gene flow in the population, which can be high with the aerial distribution in combination with human activity as observed with the case of Metalaxyl resistance in the UK (Crute et al., 1987).

Management challenges are in part compounded by previous management choices. For example, in the last 50 years, different European countries utilised different resistance breeding strategies and *Dm* gene deployment, therefore the *B. lactucae* populations in all these regions were subjected to different resistance gene repertoires in lettuce crops (Lebeda et al., 2014). The deployment of resistant genes matters, as the evolutionary flexibility of *B. lactucae* means the potential of these populations to genetically diverge on local scales to adapt to the environment they have been subjected to is highly likely. In other words, microevolution, and coevolution in response to the specific resistance genes deployed would likely occur. For example, studies of German populations of *B. lactucae* by Lebeda & Zinkernagel (2003b, 2003a) noted an increase in new virulent isolates of *B. lactucae* that targeted the *Dm16* and R18 resistance common in lettuce crops in this area. In response to the deployment of new race-specific resistance genes, the populations of *B. lactucae* shifted, with the virulence factor frequencies changing to counteract the resistance genes utilised, even on regional levels (Lebeda & Zinkernagel, 2003b). There is evidence that these virulence factors then tend to remain fixed in the pathogen population, with many pathogen populations containing 'unnecessary' virulence factors to cultivar *R* genes that are either present at low frequencies or not at all (Lebeda & Zinkernagel, 2003b). Therefore, cycling resistance genes in cultivars would not necessarily be an effective strategy in managing *R* gene deployment.

Microevolution and coevolution events with *B. lactucae* populations are not isolated to resistance genes, regional adaptation to fungicides have also been observed. An example is metalaxyl, a popular active ingredient which was used from around the 1970s to control oomycetes including

B. lactucae. By the 1980s resistant strains of *P. infestans* were observed in Europe (Matson et al., 2015), and *B. lactucae* followed this trend. Notably, a study by Crute et al. (1987) recorded metalaxyl insensitivity in Preston, Lancashire, in November 1983, where isolates from several outbreak sites were found to be insensitive. Prior to 5 years of continuous metalaxyl use, isolates collected from this region were sensitive to this active ingredient. Most isolates collected from around a 20 km radius from the initial Preston outbreaks were also reported to have metalaxyl insensitivity. Additional reports of metalaxyl insensitive isolates followed from sites further away which was attributed to movement of plants (Crute et al., 1987).

The Preston metalaxyl insensitivity outbreak case study provides an excellent example of the ability of *B. lactucae* to travel. Wu, van Bruggen, et al. (2001) did a spatial analysis of LDM incidence and reported that the range of dissemination of *B. lactucae* could be between 80-3,000m. This study looked at incidence of *B. lactucae* isolates from presumably commercial environments and did not mention monitoring wild hosts or allotments. Inclusion of more than commercial environments is needed to understand the full extent of the dispersal distances of sporangia of *B. lactucae.* The limited information on dissemination of *B. lactucae* provisions an additional challenge with respect to *B. lactucae* management.

In addition, fungicides are subject to regulations by governmental bodies, and legislation can be implemented preventing or restricting their use. For example, the active ingredient Fenamidone used for the control of LDM, was removed from the market in 2019 by UK governmental regulations (HSE, 2023). In the UK, active ingredients are only approved for use for scheduled blocks of time and lobbying for re-authorisation is consistently needed to prevent withdrawal. Regulation can add a level of complexity in management planning given uncertainties around product availability independent of efficacy.

1.4 Observing and monitoring pathogen population genetics

Early detection of plant pathogens can be useful in informing IPM approaches in lettuce production; genetic monitoring of the pathogen population can also be a useful input to IPM. Monitoring genetic variation in the pathogen population can infer which reproductive method the population frequently uses (Gustafsson et al., 1985; Michelmore & Wong, 2008; Souza et al., 2022) and the potential of the population to overcome control methods (Lebeda & Zinkernagel, 2003b). For example, a population with high genetic variability, would theoretically have a greater chance of adapting to control methods when subjected to a selection pressure and mixed mating systems and major gene resistance can increase the risk (McDonald & Linde, 2002). The development of avirulence or fungicide insensitivity in response to agriculturally imposed stressors at a local or regional level has been recorded in *B. lactucae* multiple times (Brown et al., 2004; Crute et al., 1987; Lebeda & Zinkernagel, 2003b). Therefore, monitoring virulence and fungicide insensitivity in the population would be a highly useful tool for deciding what methods of LDM management to utilise or which remain effective for that region. Jeuken & Lindhout (2002) stated an added danger of aerially dispersed sporangia which could lead to high gene flow between *B. lactucae* populations, which may have been a contributing factor to the spread of Metalaxyl insensitivity in the Preston case study (Crute et al., 1987).

Currently information about populations of *B. lactucae* populations focusses on discovering "Race", grouping isolates by virulence, a standardised practice developed by the International Bremia Evaluation Board (IBEB, 2023). In which, a differential set of lettuce cultivars with known *Dm* genes and *R* factors, or contemporary accessions, are grown and then inoculated with the isolate of interest. The pathogen virulence profile assigns a sextet scoring to the isolate tested on the differentials. If said isolate virulence profile is consistently a threat (prevalent and of economic importance) it is assigned a race name. The official IBEB standardised nomenclature, starts with "Bl:" followed by a chronological number and a suffix that distinguishes continent, for example "US" for isolates from United States and "EU" for European ones. Virulence profiles have been utilised in monitoring and can provide understanding in population studies (Crute, 1992b; Lebeda & Zinkernagel, 2003b, 2003a)

The differential test does have drawbacks, requiring change and modification to the cultivar line up to accommodate emerging virulence factors in the contemporary pathogen population. From 2010 to 2021 differential sets changed from Set B to Set D, with changes in the cultivar composition: Table 1-3. The biotrophic nature of *B. lactucae* makes testing phenotypic attributes challenging, as growing, or maintaining strains of *B. lactucae* is required, especially as there is evidence of heterokaryons being unstable depending on the cultivar used for maintenance, which could alter the virulence profile (Fletcher et al., 2019; Schettini et al., 1991).

Table 1-3 IBEB differential sets B-D

Set B - 2010	**Set C - 2016**	**Set D - 2021**
Lednicky	Dandie	Dandie
UC Dm2	R4T57D	R4T57D
Dandie	UC Dm14	UC Dm14
R4T57D	NunDM15	NunDM15
Valmaine	CGDm16	CGDm16
Sabine	Colorado	Colorado
LSE 57/15	FrRsal-1	FrRsal-1
UC Dm10	Argelès	Argelès
Captain	RYZ2164	RYZ2164
Hilde ll	RYZ910457	RYZ910457
Pennlake	Bedford	Bedford
UC Dm14	Balesta	Balesta
NunDm15	Bartoli	Bartoli
CGDm16	Design	Design
NunDm17	Kibrille	Kibrille
Colorado		Fenston
Ninja		Bataille
Discovery		RYZ20007
Argelès		

Set B - 2010	Set C - 2016	Set D - 2021
RYZ2164		
RYZ910457		
Bedford		
Balesta		
Bellissimo		

The IBEB virulence test examines a single isolate and not its relationship with the pathogen population as a whole, for example synergistic effects or compatible mating types are not included. Additionally, the IBEB testing standards have marginal recording differences between the EU and US counterparts, meaning the race given to an isolate may depend on where it was scored. The binary scoring system (+/- infection) is based on visual results relative to the universally susceptible cultivar, Green Towers. Different interpretations are possible despite reference to a visual scale. Furthermore, some *Dm* genes are dependent on environmental conditions such as temperature for expression and this means that the virulence results may vary on execution under different conditions (Judelson & Michelmore, 1992; Michelmore & Wong, 2008).

A more uniform and robust method of distinguishing isolates in addition to the phenotypic attributes would aid in monitoring the pathogen population. A genomic variability assay could in theory monitor variation. Linking genetic variation to phenotypic traits would comparatively reduce the amount of plant (host) material to work with than traditionally screening phenotype (i.e. the differential assay for virulence); overall making screening changes more manageable in the population. However, this approach requires a substantial amount of data to build up a profile of the population. The survival limits of *B. lactucae* in the absence of a host (as oospores) is not known. Hypothetically, oospores could be a reservoir of different genetic information to the contemporary population. Therefore, the risk of dormant oospores contributing to the pathogen population diversity in a non-chronological manner is also unknown. There is some indication from the literature, for example oospores could still germinate *in situ* after 12 months

(Morgan, 1978, 1983), however, that study does not account for every environmental parameter combination at every life-stage.

As suggested by Cooke & Lees (2004) a standardised test for monitoring pathogen populations must be capable of discriminating isolates both within their respective populations, and outside of their populations. This requires a careful choice of assay, as the level of differentiation would need to be sufficient to distinguish individuals in the same population without creating an exhaustive list of genotypic profiles that no evolutionary links or information can be drawn from. Race determination by the IBEB differential assay is based on an ever-changing list of differentials, therefore comparisons of earlier denominated races cannot be compared with present results unless a live sample still exists.

In addition to the test itself, some of the complexity and diversity expected from *B. lactucae* populations would be in part due to recombination as a result of sexual selection (Michelmore & Ingram, 1981), or from somatic hybridisation (polyploid and heterokaryotic forms) (Hulbert et al., 1988). If no resolved lineages or groupings of isolates occur, then the assay would create a constantly growing list of different multilocus genotypes (MLGs) which would be uninformative. That is why geographical and temporal knowledge of an isolate is equally important to note. Therefore, to understand the population diversity, monitoring should ideally include both a distinguishing assay and also associated key sample information, such as geographical location and temporal collection.

A system looking at genotypic variability coupled with controlled experimental testing of phenotypic pathogen traits has been successfully conducted on another aerial oomycete, *Phytophthora infestans* the causal pathogen of potato late blight (Cooke & Lees, 2004; Lees et al., 2006). *P. infestans*, much like *B. lactucae*, has high evolutionary potential and as such has been classified as "high risk" for developing insensitivity to active ingredients used (McDonald & Linde, 2002). Extensive collaboration therefore goes into monitoring the pathogen population and has provided utility in noting more virulent and/or fungicide insensitive lineages(Abuley et al., 2023; Cooke et al., 2012a; Lees et al., 2006; Lynott et al., 2023). This system monitors the population through measuring allelic frequencies using simple sequence repeat (SSR) markers.

Simple sequence repeats (SSR), also known as microsatellite or short tandem repeats (STR) DNA, are a monotonous repetition of a 1-6 base long nucleotide motif that naturally occur interspersed across eukaryotic genomes. To summarise, as per the reviews by Vieira et al. (2016) and Agarwal et al. (2008) and by Cooke & Lees (2004): SSRs are abundant, codominant and predictable.

SSR regions are generally non-coding, as such they tend to not correspond directly to genes and are theoretically not influenced by selection pressure. Thus, changes in SSR regions are due to occurrences such as slippage during DNA replication resulting in an alteration of tandem repeated units, therefore repeat length. Though point mutations can occur, this tends to be a less frequent occurrence in comparison to slippage, thus SSR loci are generally hypervariable, but with predictable mutation rates as observed in a study by Temnykh et al. (2001) on SSR conducted in rice.

Different alleles for an SSR locus occur by mutations evading correction systems, as *B. lactucae* is diploid for most of its life-cycle (Michelmore & Wong, 2008) this means two different alleles can occur at a single locus, or even more if heterokaryotic. As SSR markers are codominant, present in great abundance in eukaryotic genomes, and have a fairly standard mutation rate, these markers could potentially identify allelic differences in the various forms of *B. lactucae,* be it as heterokaryons, diploid or polyploid. Therefore, an SSR marker could capture the allelic diversity at a locus for a single isolate. However, a review by (Dufresne et al., (2014) mentioned that scoring allele frequencies in polyploids is still challenging due to nulls (no alleles detected) and identical alleles, i.e. two copies of the same allele in a triploid. Therefore, a quantitative aspect to measuring allele frequencies in an SSR based assay is preferential in order to account for triple or quadruple allele copies.

Fortunately, allele quantification is possible with SSR assays. SSRs can be easily amplified using PCR using primers designed to flank the SSR region, one of which is labelled with a fluorescent tag, with the amplicon products analysed using capillary electrophoresis (protocol as described in supplementary information in Cooke et al., 2012). The method allows for high throughput of samples even with small quantities of DNA and the scale of the fluorescent peaks gives an indication of allele ratio and therefore frequency ratio within an isolate. Therefore, this system is

relatively attractive, and has been adopted not only for *P. infestans* but also with other oomycete pathogens such as *Plasmopara viticola* (Gobbin et al., 2003).

To the author's knowledge, no publicly available and/or published SSR marker assay sets exist for *B. lactucae*. Although other markers are available, they have limitations. For example, internal transcribed spacer (ITS) markers do not have the resolution necessary for within population analysis (Choi et al., 2017) and many RFLP and AFLP markers are complicated to carry out for a large number of samples (Sicard et al., 2003). *S*everal SSR markers need to be used in combination in order to capture at least two allelic differences and to reflect the population diversity in *B. lactucae*. As the *B. lactucae* genome of isolate SF5 has recently been made available, the information needed to screen for suitable candidate SSR makers is available(Fletcher et al., 2019) and an assay to monitor the genetic diversity in *B. lactucae* could be developed.

1.5 Literature conclusion, thesis aims and outline

An improved understanding the *B. lactucae* population could provide many benefits for LDM management, particularly in relation to the sustainability of management strategies including fungicide active ingredients and resistance gene deployment.

One of the aims of this study was to investigate the genetic diversity of the *B. lactucae* population, by developing a multiplex SSR marker assay that could distinguish differences between isolates in the UK pathogen population.

The first step for the SSR assay development, was to screen for candidate regions, design suitable primers to amplify these SSR regions and to test whether these markers could distinguish between pathogen isolates. This was carried out using the recently available SF5 genome (Fletcher et al., 2019) and with SSR markers identified by collaborators in the Michelmore laboratory at UC Davis (Chapter 2).

The multiplex SSR assay was then utilised to document and track the genetic diversity of the population of *B. lactucae* over the three years of this PhD using samples collected across the UK. In conjunction, the *B. lactucae* isolates were assessed for phenotypic traits, specifically fungicide insensitivity and virulence to accessions within the IBEB set C differential set (Chapter 3).

To further understand the effect of cultivar selection on population diversity the SSR assay was used to monitor genotype ratios in a mark and recapture trial. Isolates with different virulence profiles and genotypes were used to inoculate various cultivars following which, isolates were recaptured and genotyped (Chapter 4).

As IPM practices utilise multiple means to manage a disease another aim of this PhD project was to validate a diagnostic assay to measure its utility in early pathogen detection in a commercial field environment (Chapter 5). A LAMP diagnostic assay for *B. lactucae* was tested under field conditions and compared with existing qPCR diagnostics. The long-term aim of this diagnostic assay is to help inform fungicide spray programs so that sprays are utilised, when necessary, i.e. according to the presence of *B. lactucae*. Reducing unnecessary sprays would reduce the evolutionary pressures on *B. lactucae* populations, and the economic costs of production. All the findings are summarised and discussed in Chapter 6, along with suggestions for future research.

Chapter 2 Development of a Simple Sequence Repeat marker assay for genotyping samples of *B. lactucae*

2.1 Background to SSR assay development

To analyse population genetic diversity simple sequence repeat (SSR) markers were utilised. SSR markers (also known as microsatellites or short tandem repeats) are repeats of a short DNA motif that are subject to stepwise mutations that change the length at a more rapid rate than other DNA mutations (Vieira et al., 2016). The mutation rate of microsatellites, which encompasses SSRs, is estimated between 10^3 and 10^6 per cell generation (Gemayel et al., 2012). SSR markers are multiallelic, meaning many allele combinations can occur at a single locus within a population, unlike biallelic single nucleotide polymorphisms (SNPs) which are limited to two alleles (in a diploid organism). In addition to this, SSR markers are codominant molecular markers that are widely distributed throughout the genome of eukaryotes (Li et al., 2002; Vieira et al., 2016). Many studies have reported that genomic distribution of SSRs is non-random, with a lower incidence in gene-coding regions of the genome (Vieira et al., 2016).

The abundance of SSR loci in a genome provides many potential candidate markers for genetic analysis. The co-dominant inheritance and multiallelic properties of SSR loci provides a high resolution that is particularly valuable in analysis of organisms with mixed reproductive systems where clonal or low genetic recombination occurs. Despite being neutral as non-coding segments of the genome, SSR loci can be correlated with pathogenic traits (Zhan et al., 2005). SSRs markers are well studied, therefore the mutational mechanisms are understood, and genetic analysis tools are available (Kamvar et al., 2014, 2015).

SSR amplification involves standard PCR techniques followed by capillary electrophoresis to estimate allelic length, and this makes SSR genotyping a relatively straightforward and accessible technique. SSR markers may be multiplexed with the PCR primers labelled with differing fluorescent dyes to discriminate amongst loci with a range of overlapping amplicon sizes (Guichoux et al., 2011). PCR based assays with primers targeting the flanking regions of an SSR locus means that the amplification is specific to the organism being studied. This is especially valuable in the case of biotrophic pathogens like *B. lactucae* in which the lettuce host DNA will be

present in samples (Grünwald et al., 2017). The consistent high rate of mutation, codominant and multiallelic nature therefore makes SSR markers ideal for identification of genetic variability within and between populations. In addition, the availability of data analysis methods (Kamvar et al., 2014, 2015) that cater to populations of mixed ploidy as well as heterokaryons (Fletcher et al., 2019) make the method appropriate for the study of *B. lactucae*.

SSRs have been utilised to investigate the genetic diversity and population structure of oomycete plant pathogens, an example is *Phytophthora infestans* the causal agent of Late Blight disease (Lees et al., 2006). The analysis of SSR data provided evidence of gene flow and differentiation between pathogen populations in different geographical regions. SSRs can serve as a good proxy representation of the selection and rates of mutations in a population when subjected to environmental stressors (Ellegren, 2004). It is important to note that the number of markers required for pathogen population genetic analysis varies according to the objective of the study and the biology and genetic diversity of the pathogen under investigation. For example, it was reported that populations of *Plasmopara* could be analysed effectively using four to five markers (Gobbin et al., 2003, 2005), whereas 21 markers were used for studies on *Venturia inaequalis* (Guérin et al., 2004) and 12 for *P. infestans* (Cooke & Lees, 2004; Li et al., 2013). Fifteen loci were examined in an investigation of the diversity of *P. ramorum* in forests in Oregon but only five proved polymorphic (Carleson et al., 2020). Despite the value of understanding the genetic diversity of *B. lactucae* populations there are no published or standardised methods using SSR markers. Unpublished polymorphic SSR markers for *B. lactucae* have however been identified by Charlotte Acharya in the group of Richard Michelmore at UC Davis California, USA and were kindly shared for the work during this PhD (Acharya et al., 2019).

In establishing SSR assays the key steps are first to identify candidate markers from the literature, collaboration or from screening the genome. Next to design PCR primers to amplify candidate loci, and test markers on a panel of reference samples of *B. lactucae* to ensure amplification is efficient and to demonstrate polymorphism amongst samples of the pathogen to be studied. From this a panel of the best markers are selected and developed into an assay.

SSR assays exists in two formats: singleplex where only one SSR marker is amplified or multiplex where multiple primer pairs are used amplify many SSR markers. Singleplex PCR is used to test amplification of each SSR locus but running multiple separate PCR assays would be logistically challenging, costly in reagents and time, and require an aliquot of sample DNA for each reaction. The latter point would be a challenge when limited amounts of sample DNA is available, and though there is a variety of whole genome amplification (WGA) that could increase sample DNA (Wang et al., 2022), including isothermal kits such as GenomiPhi V2 (Cytiva, Cruaud et al., 2018), additional protocol steps to include WGA would still make the overall genotyping more costly in reagents and time. Multiplex assays reduce genotyping to a single reaction but are complex to develop, as unpredictable interactions may occur between the many primers resulting in interference of target DNA amplification. In addition, standardising PCR conditions to give equal amplification of each locus poses a challenge. The benefits of multiplexing have led to the development of PCR kits, such as Type-it Microsatellite PCR KIT (QIAGEN) that allow for more rapid optimisation of multiplex assays (QIAGEN, 2009).

Establishment of multiplex SSR assays is a potentially lengthy and expensive process as one needs to test the utility of candidate markers and whether they are polymorphic for the organism and population tested. One approach would require relatively expensive fluorescently labelled primers for each candidate locus. Polymorphism is a key component required in genetic variation analysis but testing many markers is costly considering many may not be used in the finalised assay if they are monomorphic or difficult to amplify. However, cost-effective approaches to testing markers have been implemented to improve the development process. One such approach is universal tags, in which the fluorescent dye is shared across multiple primer pairs. Blacket et al. (2012) for example, designed universal tags that work through a 3-primer system, where the unlabelled forward primers of each pair have short (15-18 base pair) tag sequences added to the 5′ end which corresponds to complementary sequences on universal oligonucleotides tagged with different fluorophores. As the PCR progresses the universal tag becomes incorporated in the PCR product. A skewed ratio of the forward to the reverse primers results in a depletion of the forward primer and its replacement by the labelled tag as the forward primer resulting in a labelled product. The advantage of this 3-primer tag system is that only one tag is required per

fluorophore and multiple candidate SSR markers can be tested at relatively little cost compared to using individually purchased fluorescently labelled oligonucleotides.

The process to develop the SSR assay for *B. lactucae* was to first test markers provided by Michelmore's group from UC Davis then adding Hutton markers developed from the SF5 genome (Fletcher et al., 2019). Next, testing the tagged primer system to screen loci for those most polymorphic, informative, and easy to score, before ultimately developing a single 10-plex multiplex SSR assay with individually labelled fluorescent primers. The objective of this work is to screen a range of candidate SSR markers with the goal of developing sufficient polymorphic SSR markers that work as a cost- and time-efficient multiplex assay for genetic discrimination of samples of *B. lactucae*.

The aim of the SSR marker assay is to provide a toolkit to enable pathologists to monitor the genetic diversity of populations of *B. lactucae.* The selected SSR markers would need to distinguish genetic differences both within and between *B. lactucae* populations.

2.2 Methodology

2.2.1 UCD SSR marker selection

Data on candidate SSR loci, amplification, PCR primers and preliminary findings on US isolates of *B. lactucae* was kindly shared by Acharya et al. (2019). From this data, ten SSR markers were selected for this study according to the following criteria:

1. More than two alleles discriminated, and no null alleles reported. This resulted in high polymorphism information content (PIC) values across test samples with a low null allele value.
2. Unique locations in the genome, demonstrated from data on the SF5 genome scaffolds.
3. Diversity in the type of motif (tri or dinucleotide) and a preference for those with a greater number of tandem repeats.

To improve downstream analysis, the ten candidate primers were modified with the PIGTail sequence suggested by Brownstein et al. (1996), in which a short sequence is added to the 5′ end of the reverse primer to reduce stutter and aid allele calling. The primers were also modified to accommodate universal tags at the 5′ end (Blacket et al., 2012)(Table 2-1, Table 2-2). The full marker names adopted by UC Davis and their loci of origin are presented (Table 2-3).

Table 2-1 Modifications used on primers according to method of Blacket et al (2012) and Brownstein et al (1996)

Modification	Name	Sequence	Corresponding dye	Reference
Universal Tag	Tail A	*GCCTCCCTCGCGCCA*	FAM	(Roche Applied (Science, 2006)
	Tail B	*GCCTTGCCAGCCCGC*	VIC	
	Tail C	*CAGGACCAGGCTACCGTG*	NED	(Blacket et al., 2012)
	Tail D	*CGGAGAGCCGAGAGGTG*	PET	
PIGTail (5'-3')		*GTTTCTT*		(Brownstein et al., 1996)

Table 2-2 Candidate SSR primers from Acharya et al. (2019) and the modifications used in this study.

Marker	Unit	length	Dye	Universal Tag Modification		Sequence (5'-->3') with mods all added to the 5' end *1	Primer length
Marker 1	AAT	21	FAM	*GCCTCCCTCGCGCCA*	F	GCCTCCCTCGCGCCACGAGAGGAGTCATCGTGAAT	35
					R	*GTTTCTT*TTTCACACTCGTTGAAAGCG	28
Marker 2	CTG	21	FAM	*GCCTCCCTCGCGCCA*	F	GCCTCCCTCGCGCCAGTCTGAGTTCCGCTGACTTA	35
					R	*GTTTCTT*ACTACGGATGCAACGGATTA	28
Marker 3	AT	18	NED	*CAGGACCAGGCTACCGTG*	F	GCCTTGCCAGCCCGCAGCTAAAGTAAAGGTAGTTCAAAAGA	41
					R	*GTTTCTT*GGGTGATTGTTTGGTTGGTC	28
Marker 4	TA	16	VIC	*GCCTTGCCAGCCCGC*	F	GCCTTGCCAGCCCGCCAGGCAACACGTGTTTACTT	35
					R	*GTTTCTT*GCATCTACGTTCGCACATA	27
Marker 5	CA	12	NED	*CAGGACCAGGCTACCGTG*	F	CAGGACCAGGCTACCGTGTTTGTCCCCTCTTTAACGCT	38
					R	*GTTTCTT*AGGTGGAAAAAGATGCCCAG	28
Marker 6	- *2	-	NED	*CAGGACCAGGCTACCGTG*	F	CAGGACCAGGCTACCGTGCCACTTGCTGGTCATCTTTG	38
					R	*GTTTCTT*CTTTTTCCGCTATGTGTCCC	28
Marker 7	AT	20	NED	*CAGGACCAGGCTACCGTG*	F	CAGGACCAGGCTACCGTGATAATGGGAGTAGCAGGCAG	38
					R	*GTTTCTT*GATGTCACGTGATGGAGAGT	28
Marker 8	TGTA	16	PET	*CGGAGAGCCGAGAGGTG*	F	CGGAGAGCCGAGAGGTGTCCTATCTCACCCAACAAGC	38
					R	*GTTTCTT*CGTGGATACTTTCAAGCGTG	28
Marker 9	GAA	21	PET	*CGGAGAGCCGAGAGGTG*	F	CGGAGAGCCGAGAGGTG GAGTCTACCGCACCTTATGT	38
					R	*GTTTCTT*ATCCATGGCTGGCTGTAAAT	28
Marker 10	ATA	18	PET	*CGGAGAGCCGAGAGGTG*	F	CGGAGAGCCGAGAGGTG GATCAGAAACTAGCGCTTGC	38
					R	*GTTTCTT*AATCACTAGCGATGGCCAC	27

*1 Italics are PIGtail modifications from (Brownstein et al., 1996)

*2 No unit value nor length was provided for marker

Table 2-3 Candidate UCD SSR loci and corresponding primers with Hutton shortened codenames.

Hutton name	UCD Name	Loci	UCD Forward Primer Name	Forward Primer	UCD Reverse Primer Name	Reverse Primer
Marker 1	471c	R18_HRSCAF471b4179358	471c-194FAM	CGAGAGGAGTCATCGTGAAT	471c-328R	TTTCACACTCGTTGAAAGCG
Marker 2	995b	R113_HRSCAF995b1972571	955b-150FAM	GTCTGAGTTCCGCTGACTTA	955b-345R	ACTACGGATGCAACGGATTA
Marker 3	1106s90	R116_HRSCAF1106b352052	1106s90_197NED	AGCTAAAGTAAAGGTAGTTC AAAAGA	1106s90_413R	GGGTGATTGTTTGGTTGGTC
Marker 4	79s975	R3_HRSCAF79b3387454	79s975_193VIC	CAGGCAACACGTGTTTACTT	79s975_459R	GCATCTACGTTCGCACATA
Marker 5	1122s48	R117_HRSCAF1122b211057	1122s48_175NED	TTTGTCCCCTCTTTAACGCT	1122s48_315R	AGGTGGAAAAAGATGCCCAG
Marker 6	1089NED	R115_HRSCAF1089NED140004	1089NED-31NED	CCACTTGCTGGTCATCTTTG	1089NED-249R	CTTTTTCCGCTATGTGTCCC
Marker 7	297s860	R9_HRSCAF297b3110279	297s860_40NED	ATAATGGGAGTAGCAGGCAG	297s860_300R	GATGTCACGTGATGGAGAGT
Marker 8	162s689	R6_HRSCAF162b2668544	162s689_200PET	TCCTATCTCACCCAACAAGC	162s689_309R	CGTGGATACTTTCAAGCGTG
Marker 9	89a	R4_HRSCAF89b9549053	89a-99NED	GAGTCTACCGCACCTTATGT	89a-296R	ATCCATGGCTGGCTGTAAAT
Marker 10	360b	R12_HRSCAF360b848357	360b-144PET	GATCAGAAACTAGCGCTTGC	360b-406R	AATCACTAGCGATGGCCAC

2.2.2 Testing UCD SSR markers in singleplex reactions

The ten candidate markers were tested (both with and without the universal tags) against *B. lactucae* DNA samples in a single PCR per marker to determine whether the anticipated locus amplified efficiently. Each was then tested further to determine whether the SSR locus showed length polymorphisms amongst a selection of isolates of *B. lactucae.* Universal tags with fluorescent labels were synthesised by Applied Biosystems (Thermofisher, Hertfordshire, UK), the other primers were obtained from Eurofins Genomics (Wolverhampton, UK).

2.2.2.1 Protocol without universal tags

Each primer pair, without the universal tags, was tested on DNA from *B. lactucae* sample 2020_Bl2A. Each 25 µl reaction comprised: 13.38 µl of HPLC grade water, 5 µl of 5X Green GoTaq Reaction Buffer (Promega), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each of forward and reverse primers, 0.75 U of Go Taq G2 Flexi DNA polymerase (Promega), and 1 µl of template DNA. Each sample was run in duplicate with HPLC purified water as a negative DNA control. The PCR program started with 95°C for 5 mins, followed by 35 cycles of 94°C, 57°C and 72°C each for 30 seconds, finished by 5 mins at 72°C. The PCR products were run on a 2% agarose gel stained with SYBR SAFE (Invitrogen, Thermofisher) and visualised with UV light using an Azure c200 gel imaging system (Azure biosystems).

2.2.2.2 Protocol with universal tags and tagged primers

The amplification and allele size variation of each marker was tested in singleplex against four *B. lactucae* DNA samples: two from the UK, 2020_Bl2A, 2020_Bl4E, and two reference isolates obtained from Naktuinbouw (NAK). Each 12.5 µl reaction comprised: 6.25 µl Type-it Multiplex PCR Mix (Qiagen), forward primer to a final concentration of 0.1 µM, reverse primer to 0.2 µM, and universal tag to 0.1 µM, 1 µl of DNA template, and HPLC grade water to make up to the final volume. Each sample was run in duplicate along with HPLC water as a negative DNA control template. The PCR program used was 95°C for 15 mins, followed by 40 cycles of 30 seconds at 94°C, 1.5 minutes at 59°C and 1 minute of 72°C.

Prior to running on the capillary sequencer, a master mix of 6 µl of GeneScan™ 500 LIZ™ dye Size Standard (Applied Biosystems) and 1 ml of HiDi Formamide was prepared. After mixing, 10.2 µl of this HiDi/Liz mix was dispensed into each well of a 96-well PCR plate (Abgene AB0600). The PCR product of each reaction was diluted with HPLC grade water to a 1:20 ratio

and an aliquot of 0.6 µl was added to the master mix. The plate was sealed and passed to the Hutton on-site sequencing facility for capillary electrophoresis on an ABI 3730 automated capillary sequencer (Applied Biosystems) according to the manufacturer's instructions. The resultant electropherograms were processed using the GeneMapper (v5.0) software (Applied Biosystems, ThermoFisher, UK).

2.2.3 Testing UCD SSR markers with universal tags in multiplex reactions

Primers for multiple markers were mixed into a single reaction (i.e. a pool) for multiplex amplification. The pools were planned on the basis of the range of anticipated allele sizes with coloured fluorescence tags to discriminate the alleles in the output of the ABI3730. Differences were observed in the alleles detected when loci from the same DNA samples were amplified in either singleplex or multiplex reactions. Adjusting the primer concentrations did not resolve these inconsistencies so further tests were conducted to examine the potential influence of annealing temperature and competition for PCR reagents amongst loci. The aim of these experiments was to obtain a clear allele detection for downstream analysis.

2.2.3.1 Testing the effect of temperature on improving downstream analysis of candidate UCD SSR markers with universal tags

To assess the role of annealing temperature on the generation of discrepancies, a temperature gradient PCR was tested. The temperature gradient PCR was conducted on the two NAK reference isolates over a range of annealing temperatures between 55-62°C. The multiplex PCR followed the same protocol as the universal tags and tagged primers (section 2.2.2.2), except the reaction included all primers and the annealing temperature which ranged from 55-62°C (at eight one degree intervals).

Alleles were called manually in GeneMapper (v5.0), and assigned an arbitrary category of 1, 2, or 3. Categories 1 and 2 were peaks of the predicted product size and identical to that in the singleplex reactions with 1 a peak with a height above 500 relative fluorescent units (RFU) and a category 2 below 500 RFU. Category 3 were low quality peaks in the right size range that could not be called accurately. Each multiplex was compared to that from the singleplex by calculating the percentage of allelic peaks that corresponded between the two reactions.

2.2.3.2 *Testing the effect of split- pooling PCR reaction to improve downstream analysis with universal tags*

To improve allele detection and mitigate against the presumed primer dimers and issues with competition for PCR reagents amongst loci, three single multiplex reactions (of 8 primer pairs for 8 loci) were split to only include one SSR marker per colour channel (4 primers pairs for 4 loci). Post amplification the PCR products were pooled to create a consolidated sample before running on the ABI3730 capillary sequencer. This method will be referred to as "split-pooling". Each individual PCR was independently genotyped. A total of three multiplex split-pools were examined in this study along with two annealing temperatures determined as the most optimal in a previous test. Three SSR multiplexes, comprised of two splits to include a total of eight SSR markers, were each tested on two *B. lactucae* DNA samples, NAK reference 1 and 2.

The three multiplexes were determined by what markers had the fewest predicted dimers as per Multiple Primer Analyzer Thermofisher (https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html) using the standard settings 0.5 μM for primer conditions and sensitivity for dimer detection, along with the default for salt concentration, as the salt level of Type-it kit is not publicly available. The multiplexes tested are shown in Table 2-4.

Table 2-4 Three multiplex tested in PCR split-pool experiments, with the predicted number of dimers each. Dimer prediction used Multiple Primer Analyser by Thermofisher using the standard settings. M is short for marker

Mplx 4		**Mplx 5**		**Mplx 6**	
Split_4.1	**Split_4.2**	**Split_5.1**	**Split_5.2**	**Split_6.1**	**Split_6.2**
M2	M1	M2	M1	M2	M1
M8	M9	M8	M9	M10	M9
M5	M7	M5	M7	M7	M5
M4	M6	M6	M4	M4	M6
1 dimer overall		2 dimers overall		4 dimers overall	

Each split reaction followed the same protocol as the universal tags and tagged primers (section 2.2.2.2), with exception to the PCR programs. There were two PCR programs and machines used to test two different annealing temperatures simultaneously (Table 2-5).

Table 2-5 Two PCR programs used.

PCR program 1			**PCR program 2**		
Temp. (°C)	**Time**		**Temp. (°C)**	**Time**	
95°	15 mins		95°	15mins	
94°	30 sec		94°	30 sec	
61°	1.5 mins	x40	**62°**	1.5 mins	x40
72°	1 min	cycles	72°	1 min	cycles
72°	5 mins		72°	5 mins	

Preparation for genotyping followed the same as in singleplex (section 2.2.2.2; using a 1:20 dilution) with the inclusion of pooling the subsequent splits of each multiplex in duplicate. The output, which are electropherograms, was observed using GeneMapper (v5.0) and data analysis was carried out as described earlier (section 2.2.2.2).

The results were compared by examining the percentage of allelic peaks that corresponded to the expected results from singleplex reactions for both NAK reference races. The split reactions results, and pooled results were calculated separately and compared to the prior temperature gradient results of a complex multiplex set of the NAK reference races at annealing temperatures 61°C and 62°C.

2.2.4 Additional markers developed at Hutton from the *B. lactucae* genome

The tested UCD markers did not provide sufficient polymorphic loci for a multiplex assay on the restricted UK DNA pool tested. The number of candidate markers was therefore extended through a screen of the SF5 genome (Fletcher et al., 2019) or new candidate SSR markers. This bioinformatic screen was conducted by Linda Milne (Information and Computational Sciences (ICS) Group at the James Hutton Institute) using the tandem repeat search tool Phobos (v3.3.12- Mayer, 2006-2010).

2.2.4.1 Selection of Hutton markers and primer design

The SSR marker regions were selected based on the similar parameters as when selecting candidates from UCD. These were, (1) from different assembly scaffolds, to cover different regions of the genome, (2) covering a range of different SSR motif types (di and tri nucleotides), (3) those with 100% perfect repeat sequences.

The online software tool Primer3, version 4.1.0 (https://primer3.ut.ee/) was used to design primers targeting the flanking region of each SSR marker. Standard settings were maintained with a melting temperature defined at 58-61°C. Multiple primer options were considered for candidate markers, with product size being chosen to ensure compatibility with the existing UCD candidate markers in multiplex. Several primer combinations were configured and then tested for dimers with Multiple Primer Analyzer Thermofisher (https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html), the output was used to narrow down the candidate primers to test and retain.

As previously, the primers were modified to the 3-primer system with universal tags (Blacket et al., 2012). Each reverse primer was modified to have a GTTT sequence on the 5'end (Brownstein et al., 1996). These HPSF purified oligonucleotides were supplied by Eurofins Genomics (Wolverhampton, UK). Resultant primers and information are shown in Table 2-6.

2.2.4.2 Testing the Hutton markers in Singleplex

To determine the product sizes for building a multiplex, and to determine which markers were polymorphic, singleplex runs were carried out on the Hutton screened candidate markers. The eleven Hutton candidate SSR markers were tested for efficient amplification of the predicted product from *B. lactucae* DNA. The 16 primers were tested in singleplex PCR reactions with the universal tags on five different *B. lactucae* DNA samples, utilising the above protocol (section 2.2.2.2) with a revised annealing temperature of 61°C. To initially check amplification, the PCR products for one DNA sample were run using the above gel-electrophoresis protocol (section 2.2.2.1) before capillary electrophoresis of all samples.

Table 2-6 The resultant primers from Hutton screen for SSR markers. Numbers in primer name denotes what marker is targeted, up to two primer pairs per marker were considered.

Primer pair name	Oligo	unit	unit length	Uni Tag	Sequence (5'-->3') with mods all 5' end *1	Length	GC%	Product size
1001a	1001aF	AAC	41	PET	**CGGAGAGCCGAGAGGTG**AAGGAGTCCGGCGCAAAATA	20.00	50.00	165.00
	1001aR				*GTT*TCCAAGCGTTCGTCTTTGC	20.00	50.00	
1002c	1002cF	ACG	37	VIC	**GCCTTGCCAGCCCGC**GGCATTCCACATCTGAACCG	20.00	55.00	333.00
	1002cR				*GTTT*GCCCTGTCGTCGTTGAGAAA	20.00	55.00	
1003d	1003dF	ACG	18	FAM	**GCCTCCCTCGCGCCA**GTGTTGATCATCTTGCGGCC	20.00	55.00	306.00
	1003dR				*GTTT*GACTCGTTCCTGCTTCGACA	20.00	55.00	
1003e	1003eF	ACG	18	FAM	**GCCTCCCTCGCGCCA**GTGTTGATCATCTTGCGGCC	20.00	55.00	315.00
	1003eR				*GTTT*CGAAACTCCGACTCGTTCCT	20.00	55.00	
1004c	1004cF	AC	16	NED	**CAGGACCAGGCTACCGTG**TGCATTGGGTGAGTTCGCTA	20.00	50.00	278.00
	1004cR				*GTT*TCCCTGCGCCATATTCCATC	20.00	55.00	
1004e	1004eF	AC	16	FAM	**GCCTCCCTCGCGCCA**TACCGCTCCCTTCATGACAC	20.00	55.00	165.00
	1004eR				*GTTT*GGCCACGCTCTACTTAGACA	20.00	55.00	
1005c	1005cF	AGC	22	VIC	**GCCTTGCCAGCCCGC**CACGTTGGTTAATGCCTGCC	20.00	55.00	171.00
	1005cR				*GTTT*AGCCACCACCCTTTCCATTT	20.00	50.00	
1006c	1006cF	AAT	19	FAM	**GCCTCCCTCGCGCCA**TCCATTCATGCGCGTCACTA	20.00	50.00	364.00
	1006cR				*GTT*TGTGCTTTTCATACGCTGTGC	21.00	47.62	
1006d	1006dF	AAT	19	FAM	**GCCTCCCTCGCGCCA**TGCAGATATCGAGACAGCTTGG	22.00	50.00	237.00
	1006dR				*GTT*TGGGCTCTACATGGGATTTCC	21.00	52.38	
1007d	1007dF	AG	15	VIC	**GCCTTGCCAGCCCGC**GGGAGCGAGAGAGAAAGAGAC	21.00	57.14	250.00
	1007dR				*GTTT*AACTACTACGGCCACAGACAA	21.00	47.62	

Primer pair name	Oligo	unit	unit length	Uni Tag	Sequence (5'-->3') with mods all 5' end*1	Length	GC%	Product size
1008b	1008bF	ACT	22	NED	**CAGGACCAGGCTACCGTG**ACGCAGACAGATCACACGAA	20.00	50.00	355.00
	1008bR				*GTTT*CGGGAAAAGCTTGCACTTCA	20.00	50.00	
1009a	1009aF	AG	19	PET	**CGGAGAGCCGAGAGGTG** TTATTCCGCAGCCTAATCAGC	21.00	47.62	245.00
	1009aR				*GTT*TGCGAGTAGTGCCTTACGTT	20.00	50.00	
1010c	1010cF	AC	8	FAM	**GCCTCCCTCGCGCCA**ACGGTTAAAGCTCTCTCGAAATG	23.00	43.48	175.00
	1010cR				*GTTT*GGACACCCGTTACATCTATCCT	22.00	50.00	
1010e	1010eF	AC	8	PET	**CGGAGAGCCGAGAGGTG**ACGGTTAAAGCTCTCTCGAAATG	23.00	43.48	315.00
	1010eR				*GTTT*CTTCTTGCACTTCATTCGGTACT	23.00	43.48	
1011a	1011aF	AGC	24	NED	**CAGGACCAGGCTACCGTG**CATTTCCTCGTTGCTGGGTG	20.00	55.00	216.00
	1011aR				*GTTT*AACTCGAAACGGGCCAAGTC	20.00	55.00	
1011d	1011dF	AGC	24	NED	**CAGGACCAGGCTACCGTG**AGCACGGCCTGAAGATGTC	19.00	57.89	187.00
	1011dR				*GTTT*GCAACTCGAAACGGGCCAA	19.00	57.89	

*1 Italics are PIGtail modifications from (Brownstein et al., 1996), and bold is the universal tag modification (Blacket et al., 2012).

2.2.4.3 Testing the multiplex SSR assay on pathogen DNA stored on FTA cards

Testing to date was based on a few reference samples (<5), resulting in some markers appearing monomorphic. As per AHDB aerial oomycete project (CP 184) Hutton has a collection of DNA of *B. lactucae* stored in FTA cards.

Previous tests were done on DNA extracted from sporangial suspensions. Before utilising the FTA collection, tests comparing the marker results of *B. lactucae* strains that had both live samples and DNA pressed onto a FTA card were conducted.

A 5 reaction SSR assay was developed including all markers of interest to determine which were most suited for incorporation into the final assay (i.e. polymorphic for UK samples) and to determine whether the genotyping protocol was effective with FTA samples.

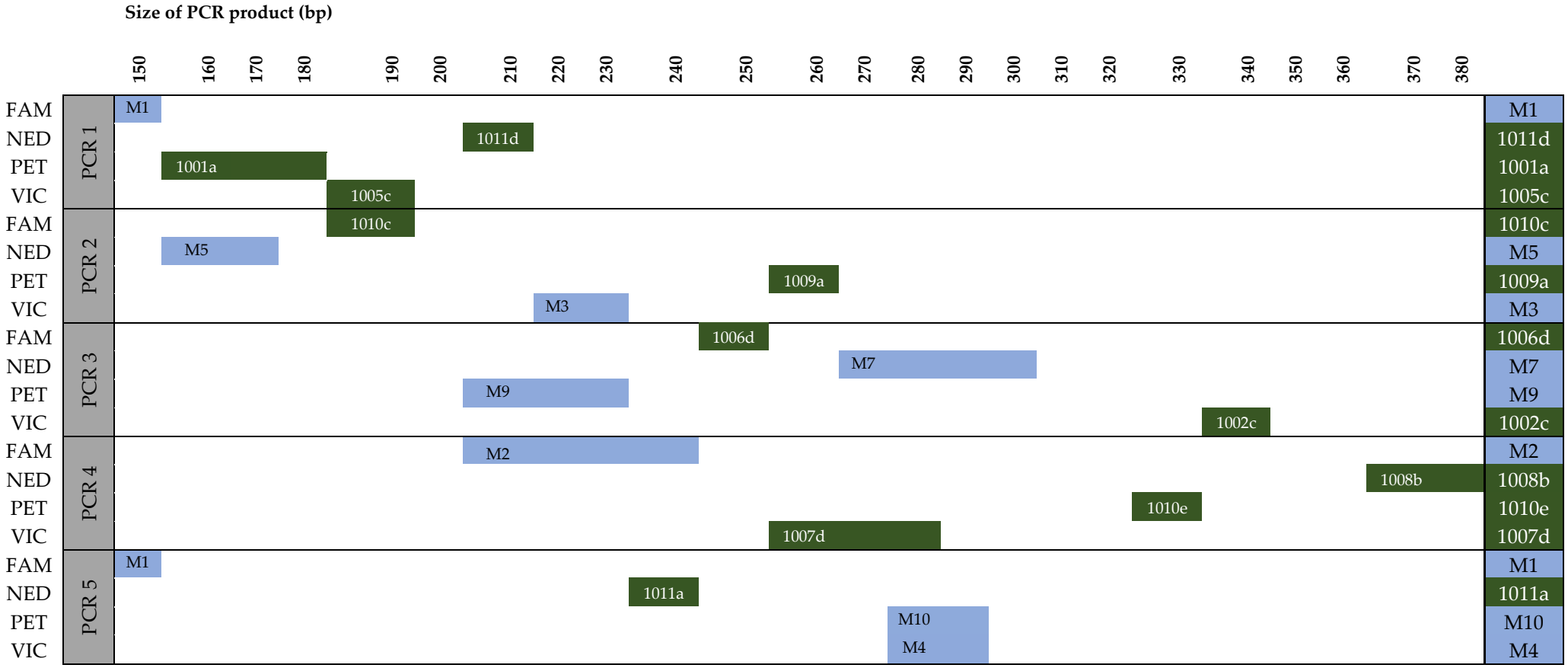
The markers were checked in multiple combinations to determine those that had fewest dimers, no overlap of alleles and to minimise spectral overlap; the resulting combination is shown in Table 2-8. Each reaction consisted of one marker per colour channel, reactions are described in Table 2-7**.** Pooling the 5 reactions was not possible due to product size overlap.

Nine of 2021 UK *B. lactucae* isolates from four different outbreaks were genotyped using the 5 reaction SSR assay (Table 2-7), using the PCR protocol with universal tags with the amended annealing temperature of 61°C. This was repeated with FTA card samples of the same isolates.

Table 2-7 The 5 reaction SSR assay. M short for marker, for the UC Davis screened markers. Hutton primer pair names used for clarification.

	Each Multiplex				**Primer mix**				
	PCR 1	**PCR 2**	**PCR 3**	**PCR 4**	**PCR 5**	**Working Conc.**		**Final Conc (μM)**	**x1 react for 12.5 μl**
F primers	M1	1010c	1006d	M2	M1	10pmol/μl	10μM	0.1	0.125
	1011d	M5	M7	1008b	1011a			0.1	0.125
	1001a	1009a	M9	1010e	M10			0.1	0.125
	1005c	M3	1002c	1007d	M4			0.1	0.125
R primers	M1	1010c	1006d	M2	M1			0.2	0.25
	1011d	M5	M7	1008b	1011a			0.2	0.25
	1001a	1009a	M9	1010e	M10			0.2	0.25
	1005c	M3	1002c	1007d	M4			0.2	0.25
Universal tags	A_FAM	A_FAM	A_FAM	A_FAM	A_FAM			0.1	0.125
	B_VIC	B_VIC	B_VIC	B_VIC	B_VIC			0.1	0.125
	C_NED	C_NED	C_NED	C_NED	C_NED			0.1	0.125
	D_PET	D_PET	D_PET	D_PET	D_PET			0.1	0.125
					2x Qiagen Type-IT Multiplex PCR Mix				6.25
					Water (HPLC)				3.25
					DNA	FTA 2mm disc or ~4ng/ μl			1
					TOTAL volume				12.5

Table 2-8 The 5 reaction SSR assay reaction, split by reaction with expected products for each targeted marker on their respective tagged colour channel. M is the abbreviation for marker, UCD markers are in blue and Hutton in green (primer pair names used for clarification).



2.2.5 Progression from the universal tags to testing unique fluorescently labelled primers

The markers that were reproducible and polymorphic were retained. The exception being Marker 7 which despite poor amplification, was the most polymorphic in samples tested and of great distinguishing value and thus retained. The temperature gradient test demonstrated that low Tm increased the risk of non-specific amplification of DNA and the Type-it kit requires a minimal Tm of 60°C (QIAGEN, 2009). To increase the Tm base pairs were added to the 5' end of the UC Davis primers had in accordance with the SF5 genome sequence so that primers would likely still anneal to *B. lactucae* DNA. Marker 10 was less efficient in amplification and the PCR product size overlapped with many other primers preventing inclusion in one reaction, as such a new primer pair was designed using Primer3 (version 4.1.0; https://primer3.ut.ee/). To minimise overlap, markers with similar PCR product sizes were spaced further apart by changing of the fluorescent label or modifying the primers to amplify an adjusted range. Labelled primer sequences are presented (Table 2-12).

2.2.5.1 Singleplex and multiplex tests of labelled primers

As in previous testing, markers were tested in singleplex to ensure they amplified the predicted loci efficiently from *B. lactucae* DNA samples and that the PCR products were not overlapping. The PCR program followed the recommended primer concentration and program of the Type-it kit (Table 2-9 and Table 2-10). Both reference races from NAK were tested in singleplex in addition to two multiplex variants: Mplx 1 and 2 (Table 2-13)**.** Spectral overlap issues with the initial labelled primers for Marker 7 and 4 led to redesign of Marker 7 with one forward primer with three reverse primer options, 7.1, 7.2 and 7.3 (Table 2-11).

Table 2-9 PCR program for Labelled primers

Type-it Kit Recommended Programme			
DNA solution	95°	5mins	
	95°	30sec	x28 cycles
	57°	1.5mins	
	72°	1min	
	60°	30mins	Final extension
FTA	95°	5mins	
	95°	30sec	x33 cycles
	57°	1.5mins	
	72°	1min	
	60°	30mins	Final extension

Table 2-10 Singleplex PCR mix

Reagent	**Working Conc**	**(µM)**	**Final Conc (µM)**	**x1 react for 12.5µl**
F primers	10pmol/µl	10	0.2	0.25
R primers	10pmol/µl	10	0.2	0.25
2x Qiagen Type-IT Multiplex PCR Mix				6.25
Water (HPLC)				4.75
DNA	4ng/µl			1.00
TOTAL Volume				12.5

Table 2-11 Marker 7 primer redesign

Primer type	**Primer name**	**Primer Seq (5'-->3') w/ Modifications**
Forward	UCD7_F	GACTCGGCGTGGTCACTTAT
Reverse	UCD7.1_R	GTTTGACTGATGTCACGTGATGGA
	UCD7.2_R	GTTTCCCCACTTAAACAACAGTCAC
	UCD7.3_R	GTTTCACTCTCCCCACTTAAACAACA

Table 2-12 Labelled primers, name, motif, modifications, targeted marker and sequence. Initial iteration of Marker 7 primers used.

Marker name	Primer Name	SSR motif	Dye	Primer Seq (5'-->3') with all modifications *1	Company	Purification
1001	1001aF_FAM	AAC	FAM	AAGGAGTCCGGCGCAAAATA	Eurofins	HPLC and salt-free
	1001aR			*GT*TTCCAAGCGTTCGTCTTTGC	Eurofins	HPLC and salt-free
1008	1008bF_FAM	ACT	FAM	ACGCAGACAGATCACACGAA	Eurofins	HPLC and salt-free
	1008bR			*GTTT*CGGGAAAAGCTTGCACTTCA	Eurofins	HPLC and salt-free
1011	1011aF_PET	AGC	PET	CATTTCCTCGTTGCTGGGTG	Thermofisher	desalted
	1011aR			*GTTT*AACTCGAAACGGGCCAAGTC	Eurofins	HPLC and salt-free
Marker 1	UCD_m1F	AAT	NED	**CA**CGAGAGGAGTCATCGTGAAT	Thermofisher	desalted
	UCD_m1R			*GTTTCTT***CCTTA**TTTCACACTCGTTGAAAGCG	Eurofins	HPLC and salt-free
Marker 2	UCD_m2F	CTG	VIC	**C**GTCTGAGTTCCGCTGACTTA	Thermofisher	desalted
	UCD_m2R			*GTTTCTT***TG**ACTACGGATGCAACGGATTA	Eurofins	HPLC and salt-free
Marker 4	UCD_m4F	TA	FAM	**CA**CAGGCAACACGTGTTTACTT	Thermofisher	desalted
	UCD_m4R			*GTTTCTT***GTAA**GCATCTACGTTCGCACATA	Eurofins	HPLC and salt-free
Marker 5	UCD_m5F	CA	PET	**CGA**TTTGTCCCCTCTTTAACGCT	Thermofisher	desalted
	UCD_m5R			*GTTTCTT*AGGTGGAAAAAGATGCCCAG	Eurofins	HPLC and salt-free
Marker 7	UCD_m7F	AT	PET	**CTT**ATAATGGGAGTAGCAGGCAG	Thermofisher	desalted
	UCD_m7R			*GTTTCTT***CT**GATGTCACGTGATGGAGAGT	Eurofins	HPLC and salt-free
Marker 9	UCD_m9F	GAA	FAM	**CAA**GAGTCTACCGCACCTTATGT	Thermofisher	desalted
	UCD_m9R			*GTTTCTT***TC**ATCCATGGCTGGCTGTAAAT	Eurofins	HPLC and salt-free
Marker 10	UCD_m10F	ATA	FAM	CCAGACCAAGAGCAAAAGCA	Eurofins	HPLC and salt-free
	UCD_m10R			*GTTTCTT*CCGATCAAGGCACGAGAATC	Eurofins	HPLC and salt-free

*1 Italics are PIGtail modifications from (Brownstein et al., 1996), bold and underlined is the 5'modification to increase Tm.

Table 2-13 Multiplex primer concentration variations used with the labelled primers throughout assay development. Marker 7 had reiterations of primers, the final version is used.

Marker, primers/ reagents		Primer final concentrations (µM) and other reagents final volume (µl) per multiplex (Mplx)												
		Mplx 1	**Mplx 2**	**Mplx 3**	**Mplx 4**	**Mplx 5**	**Mplx 6**	**Mplx 7**	**Mplx 8**	**Mplx 9**	**Mplx 10**	**Mplx 11**	**Mplx 12**	**Mplx 13**
1001	Forward: 1001aF_FAM	0.2	0.1	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02
	Reverse: 1001aR	0.2	0.1	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02
1008	Forward: 1008bF_FAM	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.09
	Reverse: 1008bR	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.09
1011	Forward: 1011aF_PET	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.25	0.25	0.2
	Reverse: 1011aR	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.25	0.25	0.2
Maker 1	Forward: UCD_m1F	0.2	0.1	0.05	0.05	0.03	0.03	0.03	0.05	0.05	0.02	0.02	0.02	0.02
	Reverse: UCD_m1R	0.2	0.1	0.05	0.05	0.03	0.03	0.03	0.05	0.05	0.02	0.02	0.02	0.02
Marker 2	Forward: UCD_m2F	0.2	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.22	0.22
	Reverse: UCD_m2R	0.2	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.22	0.22
Marker 4	Forward: UCD_m4F	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.09
	Reverse: UCD_m4R	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.09
Marker 5	Forward: UCD_m5F	0.2	0.2	0.2	0.1	0.1	0.05	0.05	0.05	0.03	0.02	0.02	0.02	0.02
	Reverse: UCD_m5R	0.2	0.2	0.2	0.1	0.1	0.05	0.05	0.05	0.03	0.02	0.02	0.02	0.02
Marker 7	Forward: UCD_7F	0.2	0.4	0.4	0.4	0.4	0.6	0.2	0.4	0.4	0.4	0.4	0.4	0.38
	Reverse: UCD_7.1R	0.2	0.4	0.4	0.4	0.4	0.6	0.2	0.4	0.4	0.4	0.4	0.4	0.38
Marker 9	Forward: UCD_m9F	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.08	0.06	0.06	0.06
	Reverse: UCD_m9R	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.08	0.06	0.06	0.06
Marker 10	Forward: UCD_m10F	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.15	0.15	0.14
	Reverse: UCD_m10R	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.15	0.15	0.14
2x Qiagen Type-IT Multiplex PCR Mix		6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25
Water (HPLC)		0.25	0	0	0.75	0.95	0.825	1.825	0.725	0.775	1.05	1.35	1.8	2.05
DNA ~4ng/µl or FTA (2mm disc)		1	1	1	1	1	1	1	1	1	1	1	1	1
Final volume		12.5												

2.3 Analysis of data in GeneMapper

An objective system for categorising the alleles at each locus was required to describe the resultant peaks and confidence in whether they were PCR artifacts or reproducible alleles was developed and utilised. With each genotyping run the analysis became more conservative as repeated and consistent amplification of allelic peaks increased confidence determining what was an allele and what was an artifact.

Marker panels were set-up on the basis of predicted length, then calibrated according to the singleplex runs of each marker. These panels were then applied to all data input into GeneMapper, and the size-standards were checked. After this manual check of the panel allele calling was carried out accounting for sample DNA repeats, and the negative control sample. If a peak was present across all sample repeats but not the negative control in the expected PCR product range, it was considered an allele.

In cases where differences in peak shape/pattern, more than two peaks, or peaks outside the expected range were observed these were noted and retained. This was to accommodate the potential of the samples being heterokaryotic or polyploid. Peak heights were also noted (as relative fluorescent units), to check the peak height ratios between alleles at a locus which may inform on the level of ploidy. To illustrate this concept, two examples are presented: Marker 1 and Marker 5. Triple peaks were common at Marker 1; 142, 145 and 148. Peaks 142 and 145 were both found as homozygous alleles (Figure 2-1 B and E) in some samples, and with a range of height ratios as seen in Figure 2-1 (A, C, D and F). All these peaks have a characteristic and distinct shape and were therefore considered real alleles. Distinct peak patterns and heights were also important with Marker 5; peak 155 would be called if it was higher or equal to the stutter peak just left of 157 (Figure 2-2 A, B and D). However, if the peak was lower as shown in (Figure 2-2, C) it was not called.

The change of primer multiplexing method did not remove 1-2 bp shifts occasionally seen with certain markers, if one allele shifted an indel mutation at the sequence immediately flanking the SSR marker was presumed. Such mutations affected a single rather than multiple alleles at a locus and these instances of 1-2 bp shifts were also logged, but not always retained in final analysis.

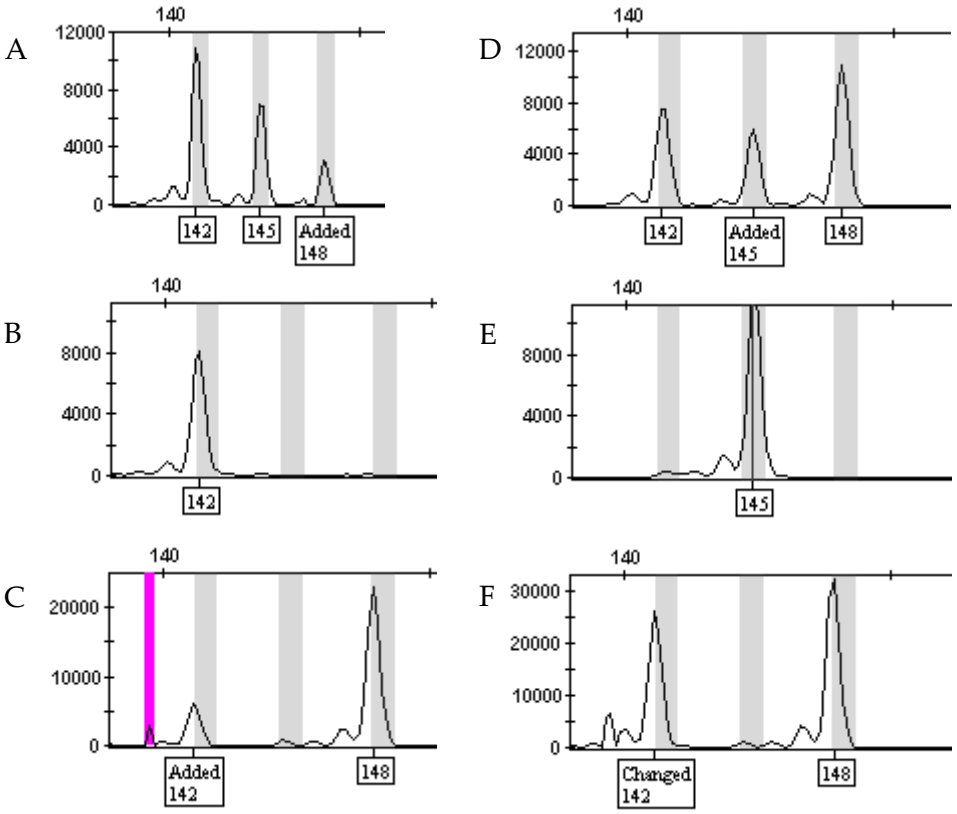


Figure 2-1 Examples of Marker 1 peaks indicating the alleles detected and the range of peak height ratios observed. Numbers are the bp of peaks (x-axis), grey bars are the expected allele bp sizes used by gene mapper to call an allele, y-axis is relative fluorescent units (RFU). 'Added' refers to manually calling an allele, 'changed' refers to manual adjustment of allele already called.

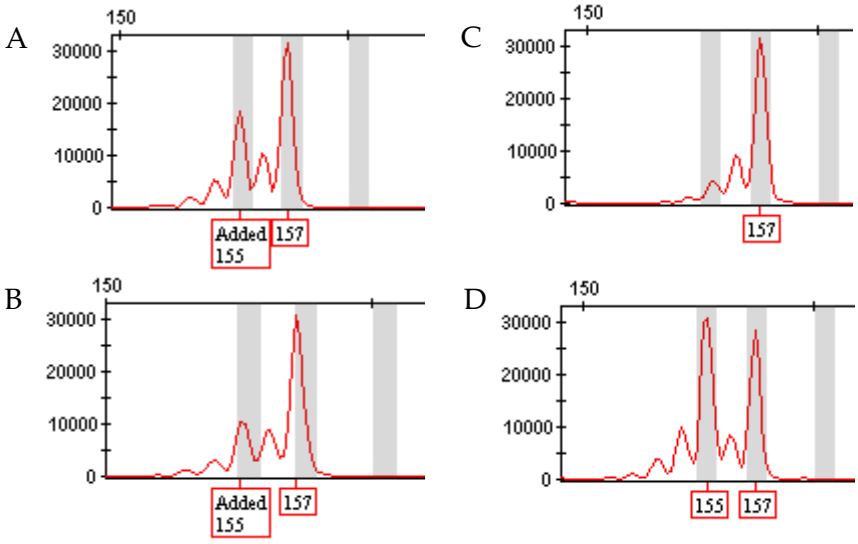


Figure 2-2 Examples of Marker 5 peaks indicating the alleles detected and the range of peak height ratios observed. Panels A, B and D show cases in which allele 155 was called but Panel C where it was absent. No isolate was found with allele 155 in a homozygote form. Numbers are the bp of peaks (x-axis), grey bars are the expected allele bp sizes used by gene mapper to call an allele, y-axis is relative fluorescent units (RFU). Added refers to manually calling an allele.

2.4 Results

2.4.1 UCD SSR Primers amplification in singleplex

Candidate UCD primers amplified predicted SSR loci from *B. lactucae* DNA when tested in singleplex both without universal tags and tested with gel electrophoresis (Figure 2-3), and when tested with capillary electrophoresis with universal tags and tagged primers. Note, the gel electrophoresis was performed to verify the presence of amplified products, with an emphasis on confirming successful amplification rather than assessing exact sizes. Product sizes were however within the expected range of 100-500 bp.

Each tested marker detected at least one allele. Heterozygosity was observed in some markers (such as Marker 1, 7, 5 and 9, Table 2-14) and tri-allelic results were also noted for some DNA samples tested (Figure 2-4 B, Table 2-14). Variation in peak height was observed, with some primers amplifying more efficiently such as Marker 7 and Marker 10 (Table 2-14).

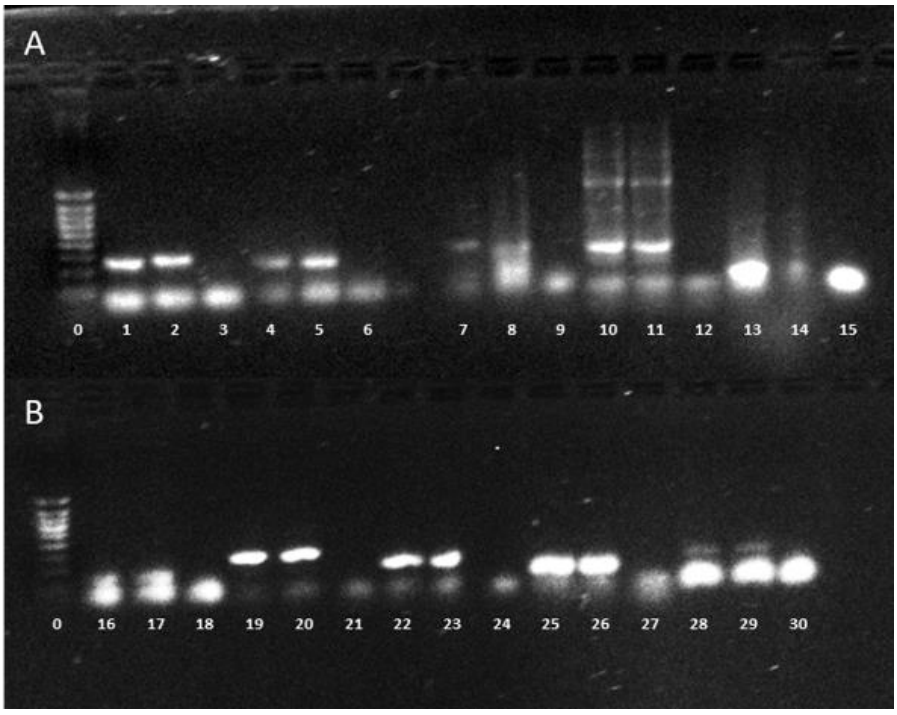


Figure 2-3 Gel Electrophoresis of PCR products from the simplex amplification of each locus. Each test was run in duplicate with its own HPLC grade water control. DNA of the B. lactucae isolate 2020_Bl2 was amplified. 0 refers to the Hyperladder iv 100bp (Bioline, sizes from 100-1000bp). Row A Marker 2 (lanes 1-3), Marker 6 (4-6), Marker 10 (7-9), Marker 7 (10-12), Marker 8 (13-15). Row B primers Marker 5 (16-18), Marker 4 (19-21), Marker 9 (22-24), Marker 1 (25-27), Marker 3 (28-30).

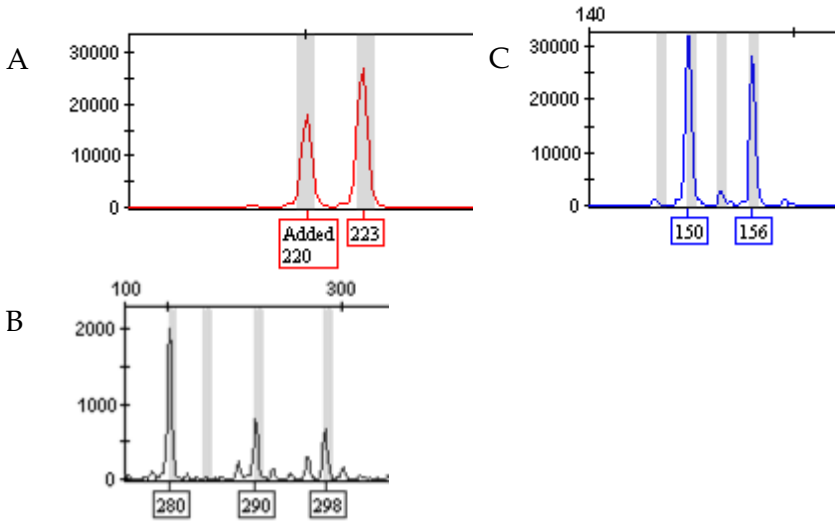


Figure 2-4 Visualisation of amplified loci analysed using fluorescent labelling and high-resolution capillary electrophoresis. A and C are examples of alleles at tri-nucleotide SSR with intervals of 3 base pairs between alleles. B shows a tri-allelic result from a di-nucleotide locus. 'Added' refers to manually calling an allele.

Table 2-14 Genotypic profiles of B. lactucae samples tested in 2021 with singleplex markers. Each row shows a different B. lactucae sample, which was tested in duplicate for each marker. Each set of 2-4 columns is an SSR marker (locus), below each marker the numbers in the column header represents the allele number, the different colours and corresponding number (bp) at each marker represent a different allele.

		Marker 1				Marker 2		Marker 3		Marker 4		Marker 5	
DNA	rep.	1	2	3	4	1	2	1	2	1	2	1	2
2020_Bl2A	1	150	156			242	278	233	233	284	286	170	170
2020_Bl2A	2	150	156			242	278	233	233	284	286	170	170
2020_Bl4E	1	150	156			242	278	233	233	284	286	170	170
2020_Bl4E	2	147	150	153	156	242	278	233	233	284	286	170	170
Reference_1	1	150	150			242	284	233	233	284	284	168	170
Reference_1	2	150	150			284	284	233	233	284	284	168	170
Reference_2	1	150	156			284	284	219	233	284	284	170	170
Reference_2	2	150	156			284*1	284*1	219	233	284	284	170	170
HPLC	1	150	156										

		Marker 6		Marker 7			Marker 8		Marker 9		Marker 10	
DNA	rep.	1	2	1	2	3	1	2	1	2	1	2
2020_Bl2A	1	235	244	288	300		130	130	223	223	287	287
2020_Bl2A	2	235	244	288	300		124	130	223	223	287	287
2020_Bl4E	1	235	244	286	298	300	124	130	223	223	287	287
2020_Bl4E	2	235	244	286	298	300	124	130	223	223	287	287
Reference_1	1	235	238	278*1	288*1		124	130	220	223	284*1	287*1
Reference_1	2	235	238	278	288*1		124	130	220	223	284	287*1
Reference_2	1	235	238	282	288		124	130	220	223	284*1	284*1
Reference_2	2	235	238	282	288		122	130	220	223	284*1	284*1
HPLC	1											

*1 <500 RFU

2.4.2 The effect of annealing temperature in the downstream analysis of UCD candidate markers

Variations in allelic detection were observed at each marker between different annealing temperatures. For example, Marker 1 when amplified using an annealing temperature of 56°C yielded 16.7% of alleles observed in singleplex when counting peaks above 500 RFU, whereas at an annealing temperature 61°C this rose to 50.0% (Table 2-15).

Relatively higher annealing temperature demonstrated an enhancement of allelic resolution in all markers. More alleles were detected using an annealing temperature of 61°C than all other temperatures in the gradient (correspondence to singleplex total of 25.8% at >500 RFU and 66.7% with all peaks).

The initial primer amplification check in singleplex utilised an annealing temperature of 57°C which was not the highest temperature tested in this experiment, thus, direct comparisons of results may be subjected to discrepancies. However, singleplex generally generated peaks, whereas in the cases of multiplexing there were fewer peaks observed.

Some primers failed to reliably amplify *B. lactucae* DNA, specifically those targeting Marker 2 and Marker 7, of which Marker 2 had no peaks corresponding to singleplex. A considerable proportion of the primers failed to generate allelic peak heights above 500 RFU such as Markers 5 and 10. In both cases, it indicates persistent challenges with other aspects of optimising PCR, such as primer dimerization, or competition for PCR reagents amongst loci.

Table 2-15 Comparison of the frequency of peaks at individual markers with expected product range (alleles) amplified from two DNA samples (NAK reference samples 1 and 2) when tested in multiplex at a range of different temperatures 55-62°C. Correct alleles were if the alleles corresponded to the singleplex experimental results of each marker for that isolate which determined the peak (not shown in table).

Temp. (°C)	Type of peak	Marker 1	Marker 2	Marker 3	Marker 4	Marker 5	Marker 6	Marker 7	Marker 8	Marker 9	Marker 10	Total
55	>500 RFU	0.0	0.0	33.3	0.0	0.0	0.0	0.0	50.0	0.0	0.0	7.6
	all	16.7	0.0	66.7	16.7	0.0	12.5	0.0	66.7	37.5	0.0	21.2
56	>500 RFU	16.7	0.0	33.3	0.0	0.0	0.0	0.0	50.0	0.0	0.0	9.1
	all	33.3	0.0	66.7	0.0	0.0	12.5	0.0	83.3	62.5	0.0	25.8
57	>500 RFU	16.7	0.0	16.7	0.0	0.0	0.0	0.0	50.0	0.0	0.0	7.6
	all	33.3	0.0	66.7	33.3	0.0	25.0	0.0	83.3	75.0	0.0	31.8
58	>500 RFU	0.0	0.0	0.0	0.0	0.0	0.0	0.0	66.7	0.0	0.0	6.1
	all	33.3	0.0	33.3	33.3	0.0	25.0	0.0	66.7	100.0	0.0	30.3
59	>500 RFU	33.3	0.0	33.3	16.7	0.0	25.0	0.0	66.7	37.5	0.0	21.2
	all	66.7	0.0	66.7	66.7	37.5	25.0	0.0	66.7	100.0	33.3	47.0
60	>500 RFU	16.7	0.0	16.7	0.0	0.0	0.0	0.0	33.3	0.0	0.0	6.1
	all	66.7	0.0	83.3	66.7	50.0	50.0	0.0	66.7	100.0	0.0	50.0
61	>500 RFU	50.0	0.0	33.3	16.7	0.0	37.5	0.0	50.0	50.0	16.7	25.8
	all	100.0	0.0	66.7	66.7	87.5	87.5	12.5	66.7	100.0	50.0	66.7
62	>500 RFU	50.0	0.0	33.3	16.7	0.0	0.0	0.0	50.0	12.5	0.0	18.2
	all	83.3	0.0	50.0	66.7	75.0	62.5	0.0	66.7	87.5	33.3	54.5

2.4.3 The effect of split-pooling on allele detection from products of the multiplex reaction

A higher frequency of peaks corresponding to singleplex results were observed at an annealing temperature of 61°C than at 62°C, regardless of DNA sample and multiplex tested, (Table 2-16). Pooled multiplex number 4 had the fewest peaks corresponding to singleplex results out of all multiplexes tested, with 4.2% of peaks corresponding to singleplex (using annealing temperature of 62°C). For multiplex 5 and 6 though there were some discrepancies between reference samples, neither multiplex had a corresponding peak frequency to singleplex below 70% at the annealing temperature of 61°C. The genotyping of the split PCR products matched the pooled products confirming that the pooling did not negatively impact the SSR assay.

Table 2-16 Comparison of the frequency of detected peaks in multiplex that corresponded to singleplex product range (alleles) amplified from two NAK reference DNA samples in pooled multiplexes at two different temperatures 61°C and 62°C. Peak size specifications were determined from singleplex experiments (not shown in table). RFU threshold was 500RFU.

	Reference 1						Reference 2					
	Temp 61			Temp 62			Temp 61			Temp 62		
Total Peaks	Mplx 4	Mplx 5	Mplx 6	Mplx 4	Mplx 5	Mplx 6	Mplx 4	Mplx 5	Mplx 6	Mplx 4	Mplx 5	Mplx 6
>500 RFU	15	17	26	1	13	13	16	23	20	2	17	15
all	19	17	26	4	19	17	19	24	21	3	17	18
% freq. (>500RFU)	62.5	70.8	100.0	4.2	54.2	50.0	61.5	88.5	76.9	7.7	65.4	57.7
% freq. (all)	79.2	70.8	100.0	16.7	79.2	65.4	73.1	92.3	80.8	11.5	65.4	69.2

When comparing each marker across different multiplex configurations (multiplex in a single reaction, split reaction, split reaction then pooled), the same reference DNA when run in a single reaction exhibited a lower percentage frequency of allelic detection (Table 2-17). With certain markers this was more pronounced, for example Marker 1 in a single reaction multiplex the frequency of peaks corresponding to singleplex were 50%, whereas each split-pool experiment had an >80% of peaks corresponding to singleplex (Table 2-17).

Markers that did not amplify well in a single multiplex reaction did so in the split-pool reaction, such as Marker 2, 5, and 7. The results suggests that splitting the multiplex provided a better primer performance allowing for clearer downstream analysis. Notably when the annealing temperature was 61°C for both single reaction and split-pooling approach it resulted in higher percentages of allelic peaks corresponding to singleplex results than experiments run at annealing temperature of 62°C (Table 2-17).

Table 2-17 Comparison of the frequency of peaks with correct product range per individual marker amplified from two reference DNA samples in different multiplex combinations. Temperature gradient (Temp. grad.) experiment results were utilised, and only category 1 peaks (>500 RFU peaks).

Markers	Temp. grad. 61°C	Temp. grad. 62°C	Temp. grad. 61°C and 62°C	Only pooled	Temp. 61 pooled and splits	Temp. 62 pooled and splits
Marker 1	50.0	50.0	50.0	83.3	97.2	86.1
Marker 2	0.0	0.0	0.0	75.0	100.0	70.8
Marker 3	33.3	33.3	33.3	55.6	66.7	50.0
Marker 4	12.5	12.5	12.5	47.9	52.1	16.7
Marker 5	0.0	0.0	0.0	80.6	100.0	80.6
Marker 6*[1]	37.5	0.0	18.8	-	-	-
Marker 7	0.0	0.0	0.0	16.7	41.7	2.1
Marker 8	75.0	75.0	75.0	87.5	100.0	87.5
Marker 9	50.0	37.5	43.8	100.0	66.7	62.5
Marker 10	16.7	0.0	8.3	50.0	83.3	0.0

*1 Indel warning so this marker was removed in later analysis

2.4.4 Hutton designed SSR Primer amplification

This screen of the SF5 genome resulted in 40 SSR candidates of varying motif lengths and from a range of genomic locations across the published genome. Of which 11 candidate markers were chosen. All designed primers amplified the predicted SSR loci from *B. lactucae* DNA and did not amplify in negative control reactions, demonstrating successful primer functionality (Figure 2-5). Some markers, such as 1002(c) and 1007(d) amplified multiple bands suggesting heterozygosity (Figure 2-5).

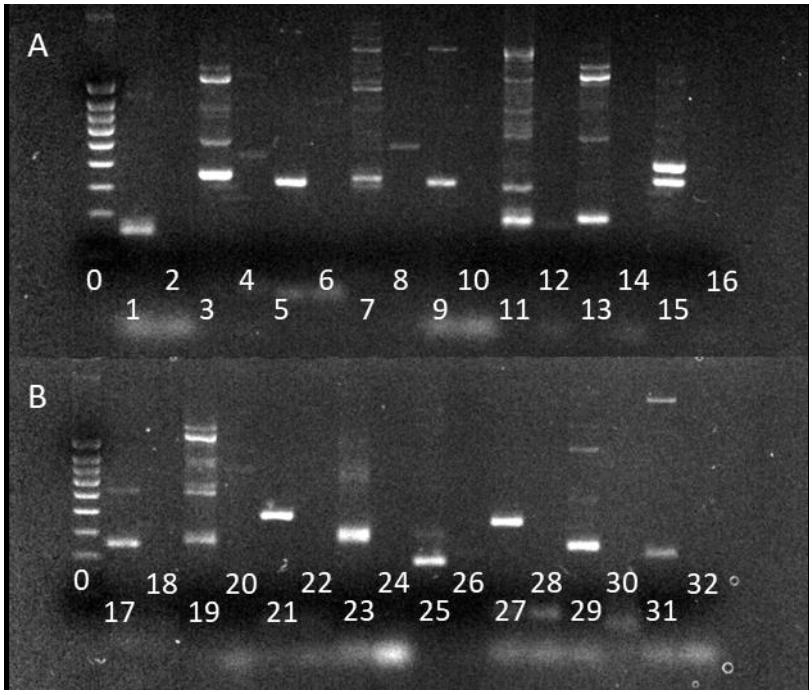


Figure 2-5, Gel Electrophoresis of PCR products from testing the amplification of each primer pair in singleplex. DNA of the B. lactucae isolate 2020_Bl2A was amplified. 0 refers to the Hyperladder iv 100bp (Bioline, 100-1000bp). DNA template of 2020_Bl2A (odd numbers) and a negative template of HPLC grade water (even numbers). Row A: 1001a (1-2), 1002c (2-3), 1003d (5-6), 1003e (7-8), 1004c (9-10), 1004e (11-12), 1005c (13-14), and 1006c (15-16). Row B:1006d (17-18), 1007d (19-20), 1008b (21-22), 1009a (23-24), 1010c (25-26), 1010e (27-28), 1011a (29-30), and 1011d (31-32).

Single genotyping indicated 7 markers (1001, 1003(d), 1004 (c & e), 1006(c), 1007, 1008, 1011) were polymorphic with peaks that represented alleles varying in size according to the dinucleotide and trinucleotide repeats (Table 2-18). The large size difference in peaks detected at markers 1007(d) and 1003(d & e) were presumed to not be from stepwise mutations. With the reference isolates tested four loci were monomorphic, 1010(c & e), 1006(d), 1005(c) and 1002(c). Markers 1004 (c & e) and 1006 (c and d) did not have matching results between primer pairs used, whereas 1010 (c & e) and 1011 (a & d) did match in number of alleles and/or distance between alleles.

Although monomorphic some markers were not yet excluded as the range of candidate isolate samples was limited, and these markers could still be polymorphic when tested against a more diverse panel of *B. lactucae* samples. However, markers which either generated inconsistent results from the two different primer pairs or those generating alleles unlike those expected of SSR loci were removed from the potential list.

Table 2-18 Genotypes for 5 B. lactucae samples (three UK and two NAK reference races) amplified in singleplex assays. Each row shows a different B. lactucae sample, which was tested in duplicate for each marker. Each set of 2-4 columns is an SSR marker (locus), below each marker the numbers in the column header represents the allele number, the different colours and corresponding number (bp) at each marker represent a different allele. Red labels were out of expected range peaks and presumed to indicate indels.

DNA Template		1001(a)		1002(c)		1003(d)		1003(e)		1004(c)		1004(e)		1005(c)		1006(c)		
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	3
2020_Bl2A	R1	160	166	345	345	311	311	320	320	297	297	182	182	192	192	315	378	
	R2	160	166	345	345	311	311	320	350	299	299	182	182	192	192	315	378	
2020_Bl4E	R1	160	166	345	345	311	311			297	297	182	182	192	192	315	378	
	R2	160	166	345	345	311	311			297	297	182	182	192	192	315	378	
2020_Bl4G	R1	160	166	345	345	311	311	320	320	299	299	182	184	192	192	315	378	
	R2	160	166	345	345	311	311	320	320	299	299	182	184	192	192	315	378	
Reference 1	R1	166	166	345	345	311	311	320	320	299	299	182	184	192	192	315	378	
	R2	166	166	345	345	311	311	320	320	297	299	182	184	192	192	315	378	
Reference 2	R1	166	184	345	345	311	356	320	410	297	297	182	182	192	192	381	429	
	R2	166	184			311	356	320	410	297	297	182	182	192	192	315	378	381

DNA Template		1006(d)		1007(d)		1008(b)		1009(a)		1010(c)		1010(e)		1011(a)		1011(d)	
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
2020_Bl2A	R1	252	252	266	288	377	377	264	264	194	194	337	337	239	239	210	210
	R2	252	252	266	288	377	377	264	264	194	194	337	337	239	239	210	210
2020_Bl4E	R1	252	252	266	266	377	377	264	264	194	194	337	337	239	239	210	210
	R2	252	252	266	266	377	377	264	264	194	194	337	337	239	239	210	210
2020_Bl4G	R1	252	252	266	266	377	377	264	264	194	194	337	337	236	239	207	210
	R2	252	252	266	266	377	377	264	264	194	194	337	337	236	239	207	210
Reference 1	R1	252	252	266	288	380	380	264	264	194	194	337	337	236	236	207	207
	R2	252	252	266	288	380	380	264	264	194	194	337	337	236	236	207	207
Reference 2	R1	252	252	266	266	380	380	264	264	194	194	337	337	236	236	207	207
	R2	252	252	266	266	380	380	264	264	194	194	337	337	236	236	207	207

2.4.5 Multiplex testing with integration of the UCD and Hutton markers, and DNA from FTA cards

Of the candidate markers tested in multiplex, Marker 3, 1005, 1006(d), 1007, and 1010 proved monomorphic with the DNA pool tested and the others were polymorphic (Table 2-19). Marker 1009 generated atypical peaks that were too difficult for meaningful and reliable interpretation. Marker 1002 had many peaks, as only one (354) was observed in singleplex, the other peaks were attributed to PCR artifacts resulting from primer dimerization of concurrent primers tested. After removal of the problematic loci the list of candidate markers to take forward was reduced to 10.

There was a noticeable difference between the FTA and the DNA solution results. With the multiplex using DNA from FTA cards as a template generally yielding stronger and more consistent amplification, than DNA extracted from sporangial solutions (Table 2-19). For example, Marker 10 with FTA all samples had alleles detected except for one repeat of 2021_Bl3C and 2021_Bl8A, whereas with DNA solutions only one repeat of 2021_Bl8A amplified.

Differences in Marker 1 results from two PCR reactions (PCR1 and PCR5) highlighted that the primer combination in multiplex can alter results. Specifically, the alleles 158 and 159 (Marker 1) that only appeared in seven repeats in PCR reaction 5 (PCR5) when using DNA extracted from sporangial solution.

Table 2-19 Comparisons of genotyping using DNA from sporangial suspensions or from FTA cards and the 5-reaction SSR assay. Each row is a different B. lactucae sample, tested in duplicate for each marker. Each set of 2-4 columns is an SSR marker (locus), below each marker the numbers in the column header represent the allele number, the different colours and corresponding number (bp) at each marker represent a different allele. Page 1 and 2 of the table show UC Davis screened markers on DNA solution from sporangial suspension, and FTA cards; the following two pages are Hutton screened markers.

DNA type	DNA template		Marker 1 PCR 1				Marker 1 PCR 5				Marker 2		Marker 3		Marker 4		Marker 5		Marker 7			Marker 9		Marker 10	
			1	2	3	4	1	2	3	4	1	2	1	2	1	2	1	2	1	2	3	1	2	1	2
DNA sol.	2021_Bl2B	R1	150	150			150	150			266	282	233	233	284	284	170	170				220	223		
		R2	150	150			150	150					233	233			170	170	293	293		220	223		
	2021_Bl2D	R1					150	150									170	170							
		R2					158	158																	
	2021_Bl3A	R1	150	150			150	156	159				233	233			170	170							
		R2	150	150			159	159							294*1	294*1			286	288		223	223		
	2021_Bl3B	R1	150	150			150	150			266	284	233	233			170	170	284	286		220	223		
		R2	150	150			150	150			266	284	233	233	274*1	274*1	170	170	286	288		220	223		
	2021_Bl3C	R1	153	153			159	159														223	223		
		R2	150	150																					
	2021_Bl4C	R1					159	159							266*1	266*1									
		R2									278	278			291*1	291*1									
	2021_Bl8A	R1					150	150			278	278	233	233			170	170							
		R2					150	150							284	294*1			293	293		220	223	284	287
	2021_Bl8B	R1					159	159					235	235								223	223		
		R2					159	159																	
	2021_Bl8C	R1	150	150																					
		R2	150	150			150	150																	
	Neg. C	R1					159	159			278	278	237	237											

DNA type	DNA template		Marker 1 PCR 1				Marker 1 PCR 5				Marker 2		Marker 3		Marker 4		Marker 5		Marker 7			Marker 9		Marker 10	
			1	2	3	4	1	2	3	4	1	2	1	2	1	2	1	2	1	2	3	1	2	1	2
FTA	2021_Bl2B	R1	147	147			150	150					233	233	284	286	170	170	293	293		220	223	284	284
		R2	150	150			150	150					233	233	284	286	170	170	293	293		220	223	284	284
	2021_Bl2D	R1	150	150			150	150					233	233	284	286	170	170	293	293		220	223	284	284
		R2	150	150			150	150					233	233	284	286	170	170	293	293		220	223	284	284
	2021_Bl3A	R1	147	150			150	150					233	233	284	284	170	170	286	286		220	223	284	287
		R2	150	150			150	150					233	233	284	284	170	170	286	286		220	223	284	287
	2021_Bl3B	R1	150	150			150	150					233	233	284	286	170	170	286	286		223	223	284	287
		R2	150	150			150	150					233	233	284	286	170	170	293	293		220	223	284	287
	2021_Bl3C	R1	150	150									233	233			170	170	286	286		220	223		
		R2	150	150			150	150					233	233	284	284	170	170	286	286		220	223	284	287
	2021_Bl4C	R1	147	150	153	156	147	150	153	156			233	233	284	284	168	170	286	298		223	223	287	287
		R2	147	150	153	156	147	150	153	156			233	233	284	286	168	170	286	298		223	223	287	287
	2021_Bl8A	R1	147	150			150	150					233	233	284	286	170	170						284	287
		R2	147	150			150	150					233	233	284	286	170	170	286	298		220	223	284	287
	2021_Bl8B	R1	150	150			150	150					233	233	284	286	170	170	293	293		220	223	284	287
		R2	147	150			150	150					233	233	284	286	170	170	293	296	298	220	223	284	287
	2021_Bl8C	R1	150	150			150	150					233	233	284	286	170	170	293	296		220	223	284	287
		R2	150	150			150	150					233	233			170	170	293	296		220	223		
	Neg. C	R1	150	153			150	153	156																

DNA type	DNA template		1001		1002				1005		1006		1007			1008		1009		1010(c)		1010(e)		1011(a)			1011(d)		
			1	2	1	2	3	4	1	2	1	2	1	2	3	1	2	1	2	1	2	1	2	1	2	3	1	2	3
DNA sol.	2021_Bl2B	R1	166	166	180*2	218*2	275*2		192	192			240	248	266	380	380	264	264	194	194	338	338	239	239					
		R2	166	166	218*2	275*2			192	192	252	252	266	266		380	380	264	264			338	338							
	2021_Bl2D	R1			197*2	197*2												264	264					208	208		114	114		
		R2			197*2	218*2	275*2											264	264					208	208					
	2021_Bl3A	R1									246	246	266	266				264	266	194	194									
		R2	166	166	180*2	218*2	238*2	275*2	192	192														208	208		201	201		
	2021_Bl3B	R1	166	166	218*2	275*2			192	192	252	252	266	266		380	380	264	264			338	338	229	229					
		R2	166	177	197*2	218*2	275*2		192	192	252	252	266	266		377	380	264	264	194	194	338	338	208	208					
	2021_Bl3C	R1									252	252												208	208					
		R2			197*2	197*2																								
	2021_Bl4C	R1																						207	207					
		R2									252	252																		
	2021_Bl8A	R1											266	266		380	380	264	264	194	194	338	338							
		R2			218*2	275*2	322*2				252	252												236	239					
	2021_Bl8B	R1			238*2	238*2																								
		R2			238*2	238*2																		208	208					
	2021_Bl8C	R1							276	276														208	208					
		R2							291	291			282	282										236	236					
	Neg. C.	R1																												

DNA type	DNA template		1001		1002				1005		1006		1007			1008		1009		1010(c)		1010(e)		1011(a)			1011(d)		
			1	2	1	2	3	4	1	2	1	2	1	2	3	1	2	1	2	1	2	1	2	1	2	3	1	2	3
FTA	2021_Bl2B	R1			180*2	218*2	267*2	275*2					266	266		380	380	264	266	194	194	338	338	236	239				
		R2	166	166	180*2	218*2	267*2	275*2	192	192			266	266		380	380	264	266	194	194	338	338	236	239		207	207	
	2021_Bl2D	R1	166	166	180*2	218*2	275*2		192	192								264	266	194	194			236	239		207	207	
		R2	166	166	180*2	218*2	275*2		192	192								264	266	194	194			236	239		207	210	
	2021_Bl3A	R1	166	166	218*2	275*2	345		192	192	252	252	266	266		377	380	264	266	194	194	338	338	229	236	239	201	207	
		R2	166	166	218*2	275*2	345		192	192	252	252	266	266		377	380	264	266	194	194	338	338	229	236	239	201	207	
	2021_Bl3B	R1	166	166	218*2	275*2	345		192	192	252	252	266	266		380	380	264	266	194	194	338	338	236	239		201	207	210
		R2	166	166	218*2	275*2			192	192	252	252	266	266		380	380	264	266	194	194	338	338	229	236	239	201	207	
	2021_Bl3C	R1	166	166	218*2	275*2	345		192	192	252	252						264	266	194	194						201	207	
		R2	166	166	218*2	275*2	345		192	192	252	252	266	266		377	380	264	266	194	194	338	338	229	236	239	201	207	
	2021_Bl4C	R1	160	166	218*2	275*2	345		192	192	252	252	266	266				264	266	194	194			236	239		207	210	
		R2	160	166	218*2	275*2	345		192	192	252	252	266	266		377	377	264	266	194	194	338	338	236	239		207	207	
	2021_Bl8A	R1	166	166					192	192								264	266	194	194			236	239		207	207	
		R2	166	166	180*2	218*2	238*2	275*2	192	192			266	266		380	380	264	266	194	194	338	338	236	239		207	207	
	2021_Bl8B	R1	166	166	180*2	218*2	275*2		192	192			266	266		380	380	264	264	194	194	338	338	236	239		207	207	
		R2	166	166	180*2	218*2	275*2		192	192			266	266		380	380	264	264	194	194	338	338	236	239		207	210	
	2021_Bl8C	R1	166	166	180*2	218*2	275*2	307*2	192	192			266	266		380	380	264	264	194	194	338	338	236	239		207	207	
		R2	166	166	180*2	197*2	218*2	307*2	192	192			266	266		380	380			194	194	338	338				207	207	
	Neg. C.	R1																											

**1 low RFU and not clear,*

**2 not in fully expected range.*

2.4.6 Amplification observed with labelled primers in singleplex and multiplex testing

Most markers when amplified using labelled primers generated consistent peaks when run in singleplex and multiplex, generally producing peaks above 500 RFU (Table 2-20). This is in comparison to the universal tag system (3-primer system), where in singleplex some lower quality amplification was observed in some markers (Marker 10 only 2 DNA template reps >500 RFU out of 8); and in multiplex some markers did not amplify at all (Marker 2).

Singleplex testing of the labelled primers showed that most still amplified the predicted loci from *B. lactucae* DNA. With the NAK reference 1, labelled primers for Marker 7 exhibited difficulty facilitating amplification, with no replicates amplified in singleplex).

Issues with spectral overlap were observed with the some of the labelled primers. This is due to strong amplification that caused the fluorescent signal at one wavelength to cross into that of the adjacent channel and cause peaks. A lowering of the primer concentration of problematic markers reduced the fluorescence signal and thus the spectral overlap (data not shown).

Testing of the redesigned primers for Marker 7 resulted in an improved marker. All new primers yielded stronger and clearer amplification than the initial labelled primers (Table 2-20, Table 2-21). The primer pair using UCD7.1_R as the reverse generated the best results, yielding PCR products for all multiplex replicates even from lower quality DNA samples such as NAK Reference 1. Primers UCD7.2_R and UCD7.3_R generated PCR products of a similar size to Marker 10 and 1008(b) respectively, whereas UCD7.1_R had no overlap and were therefore selected for the final assay.

Table 2-20 Comparison of universal tag system to labelled primers using the two NAK reference races in singleplex and multiplex. NAK reference 1 = ref 1; reference 2 = ref 2, Neg. C= negative control. Each set of 2-4 columns is an SSR marker (locus), below each marker the numbers in the column header represent the allele number, the different colours and corresponding number (bp) at each marker represent a different allele.

Multiplex	DNA type	DNA Template		Marker 1			Marker 2		Marker 4		Marker 5		Marker 7			Marker 9		Marker 10		1001(a)		1008(b)		1011(a)	
				1	2	3	1	2	1	2	1	2	1	2	3	1	2	1	2	1	2	1	2	1	2
Universal tag Singleplex	DNA solution	Ref. 1	R1	150	150		242	284	284	284	168	170	278	288		220	223	284*1	287*1	166	166	380	380	236	239
			R2	150	150		284	284	284	284	168	170	278	288		220	223	284	287*1	166	166	380	380	236	236
		Ref. 2	R1	150	156		284	284	284	294	170	170	282	288		220	223	284*1	284*1	166	184	380	380	236	236
			R2	150	156		284*1	284*1	284	294	170	170	282	288		220	223	284*1	284*1	166	184	380	380	236	236
		Neg. C	R1	150	156		278	278																	
			R2																						
Universal tag. Multiplex *3	DNA solution	Ref. 1	R1	150	150				284*1	284*1	168*1	170*1				220*1	223*1			-	-	-	-	-	-
			R2	150	150				284*1	284*1	168*1	170*1		288		220	223	284*1	284*1	-	-	-	-	-	-
		Ref. 2	R1	150	156				284	294	170*1	170*1				220	223	284	284	-	-	-	-	-	-
			R2	150	156				284*1	294	170*1	170*1				220*1	223*1	284*1	284*1	-	-	-	-	-	-
		Neg. C	R1																	-	-	-	-	-	-
			R2																	-	-	-	-	-	-
Labelled primer Singleplex	DNA solution	Ref. 1	R1	142	148		272	272	275	275	157	159				206	209	327	327	146	164	362	362	220	220
			R2	142	148		272	272	275	275	157	157				206	209	327	327	146	164	362	362	220	220
			R3	142	148		272	272	275	275	157	157				206	209	327	327	146	164	362	362	220	220
		Ref. 2	R1	142	142		272	272	275	277	155	157	266	276	284	206	209	327	330	146	146	362	362	220	223
			R2	142	142		272	272	275	277	155	157	266	276	284	206	209	327	330	146	146	362	362	220	223
			R3	142	142		272	272	275	277	155	157	266	276	284	206	209	327	330	146	146	362	362	220	223
		Neg. C	R1																						
			R2																						

Multiplex	DNA type	DNA Template		Marker 1			Marker 2		Marker 4		Marker 5		Marker 7			Marker 9		Marker 10		1001(a)		1008(b)		1011(a)	
				1	2	3	1	2	1	2	1	2	1	2	3	1	2	1	2	1	2	1	2	1	2
Labelled primer Multiplex	DNA solution	Ref. 1	R1	142	148		272	272	275	275	157	157	276*1	276*1		206	209	327	327	146	164	362	362	220	220
			R2	142	148		272	272	275	275	157	157				206	209	327	327	146	164	362	362	220	220
			R3	142	148		272	272	275	275	157	157				206	209	327	327	146	164	362	362	220	220
		Ref. 2	R1	142	142		272	272	275	275	155	157	266	276	284	206	209	327	330	146	146	362	362	220	223
			R2	142	142		272	272	275	275	155	157	266	276	284	206	209	327	330	146	146	362	362	220	220
			R3	142	142		272	272	275	275	155	157	266	276	284	206	209	327	330	146	146	362	362	220	220
		Neg. C	R1																						
			R2																						
Labelled primer Mplx 2	DNA solution	Ref. 1	R1	*142*1	145*1	148*1	272	272	275	275	155*1	157				206	209	327	327	146*1	164	362	362	220*1	220*1
			R2	142	148		272	272	275	275	157	157				206	209	327	327	146	164	362	362	220	220
		Ref. 2	R1	142	142		272	272	275	275	155	157	266*1	276*1	284*1	206	209	327	330	146	146	362	362	220	220
			R2	142	142		272	272	275	275	155	157	266*1	276*1	284*1	206	209	327	330	146	146	362	362	220	220
	FTA	Ref. 1	R1	142	148		272	272	275	275	157	157	276	276		206	209	327	327	146	164	362	362	220	220
			R2	142	148		272	272	275	275	157	157	271	276		206	209	327	327	146	164	362	362	220	220
		Ref. 2	R1	142	142		272	272	275	277	155	157	266	284		206	209	327	330	146	146	362	362	220	223
			R2	142	142		272	272	275	275	155	157	266	284		206	209	327	330	146	146	362	362	220	220
		Neg. C	R1																						
			R2																						

*1 below 500 RFU

*2 low DNA sample, pattern sometimes obvious but not always clear

*3 results from 61°C temperature gradient test, which only had UCD marker results

Table 2-21 Results of singleplex and multiplex testing of three redesigned labelled primer combinations of Marker 7. The different colours and corresponding number (bp) at each marker represent a different allele.

Reverse primer used	DNA Template		Singleplex				Multiplex			
			Allele 1	Allele 2	Allele 3	Allele 4	Allele 1	Allele 2	Allele 3	Allele 4
UCD_7.1R	Reference 1	R1					284*1	291*1		
		R2					284*1	291*1		
	Reference 2	R1	281	291	299		281	291	299	
		R2	281	291	299		281	291	299	
	2021_Bl1B	R1	299*1	299*1			299*1	299*1		
		R2	299*1	299*1			299*1	299*1		
UCD_7.2R	Reference 1	R1	318*1	318*1						
		R2					318*1	318*1		
	Reference 2	R1	308	318	326		308	318*1	326*1	
		R2	308	318	326		308	318*1	326*1	
	2021_Bl1B	R1	326*1	326*1			326*1	326*1		
		R2					326*1	326*1		
UCD_7.3R	Reference 1	R1	325*1	325*1						
		R2					325*1	325*1		
	Reference 2	R1	315	325	333		315	325	333*1	
		R2	315	325	333		315*1	325*1	333*1	
	2021_Bl1B	R1	333*1	333*1			315*1	333*1		
		R2	333*1	333*1			333*1	333*1		

*1 below 500 RFU

2.5 Finalised Assay

The final SSR assay for *B. lactucae* comprised a mix of UCD and Hutton markers that were tested initially using a cost-effective universal tagged primer system as a first screen followed by using individually labelled primers to develop this single reaction 10-plex multiplex SSR assay. The lengthy and complex process of optimising the assay running conditions has however resulted in an appropriate choice of markers and product sizes for the multiplex assay for subsequent studies.

Nonetheless adjustments in the primer concentration were carried out during the analysis of the many field and population samples (Table 2-13) in the last 18 months of the study. No further changes to the choice of markers or primer sequence were required. The finalised marker line-up (Table 2-22) and the best primer concentrations for multiplex are presented (Table 2-23).

Table 2-22 Finalised labelled primers for SSR multiplex assay.

Marker name	Primer Name	length	SSR motif	Dye	Primer Seq (5'-->3') with all modifications	Company	Purification
1001a	1001aF_FAM	20	AAC	FAM	AAGGAGTCCGGCGCAAAATA	Eurofins	HPLC and salt-free
	1001aR	22			GTTTCCAAGCGTTCGTCTTTGC	Eurofins	HPLC and salt-free
1008b	1008bF_FAM	20	ACT	FAM	ACGCAGACAGATCACACGAA	Eurofins	HPLC and salt-free
	1008bR	24			GTTTCGGGAAAAGCTTGCACTTCA	Eurofins	HPLC and salt-free
1011a	1011aF_PET	20	AGC	PET	CATTTCCTCGTTGCTGGGTG	Thermofisher	desalted
	1011aR	24			GTTTAACTCGAAACGGGCCAAGTC	Eurofins	HPLC and salt-free
Marker 1	UCD_m1F	22	AAT	NED	CACGAGAGGAGTCATCGTGAAT	Thermofisher	desalted
	UCD_m1R	32			GTTTCTTCCTTATTTCACACTCGTTGAAAGCG	Eurofins	HPLC and salt-free
Marker 2	UCD_m2F	21	CTG	VIC	CGTCTGAGTTCCGCTGACTTA	Thermofisher	desalted
	UCD_m2R	29			GTTTCTTTGACTACGGATGCAACGGATTA	Eurofins	HPLC and salt-free
Marker 4	UCD_m4F	22	TA	FAM	CACAGGCAACACGTGTTTACTT	Thermofisher	desalted
	UCD_m4R	30			GTTTCTTGTAAGCATCTACGTTCGCACATA	Eurofins	HPLC and salt-free
Marker 5	UCD_m5F	23	CA	PET	CGATTTGTCCCCTCTTTAACGCT	Thermofisher	desalted
	UCD_m5R	27			GTTTCTTAGGTGGAAAAAGATGCCCAG	Eurofins	HPLC and salt-free
Marker 7	UCD_7F	20	AT	PET	GACTCGGCGTGGTCACTTAT	Thermofisher	desalted
	UCD_7.1R	24			GTTTGACTGATGTCACGTGATGGA	Eurofins	HPLC and salt-free
Marker 9	UCD_m9F	23	GAA	FAM	CAAGAGTCTACCGCACCTTATGT	Thermofisher	desalted
	UCD_m9R	29			GTTTCTTTCATCCATGGCTGGCTGTAAAT	Eurofins	HPLC and salt-free
Marker 10	UCD_m10F	20	ATA	FAM	CCAGACCAAGAGCAAAAGCA	Eurofins	HPLC and salt-free
	UCD_m10R	27			GTTTCTTCCGATCAAGGCACGAGAATC	Eurofins	HPLC and salt-free

Table 2-23 The final SSR multiplex concentration mix used. Labelled primers were used.

Targeted markers and their primers/ reagents		**Working Conc**	**(µM)**	**Final Conc (µM)**	**x1 react for 12.5 µl**
1001	1001aF_FAM	5pmol/µl	5	0.02	0.05
	1001aR	5pmol/µl	5	0.02	0.05
1008	1008bF_FAM	10pmol/µl	10	0.09	0.1125
	1008bR	10pmol/µl	10	0.09	0.1125
1011	1011aF_PET	10pmol/µl	10	0.2	0.25
	1011aR	10pmol/µl	10	0.2	0.25
Marker 1	UCD_m1F	5pmol/µl	5	0.02	0.05
	UCD_m1R	5pmol/µl	5	0.02	0.05
Marker 2	UCD_m2F	10pmol/µl	10	0.22	0.275
	UCD_m2R	10pmol/µl	10	0.22	0.275
Marker 10	UCD_m10F	10pmol/µl	10	0.14	0.175
	UCD_m10R	10pmol/µl	10	0.14	0.175
Marker 9	UCD_m9F	10pmol/µl	10	0.06	0.075
	UCD_m9R	10pmol/µl	10	0.06	0.075
Marker 5	UCD_m5F	10pmol/µl	10	0.02	0.025
	UCD_m5R	10pmol/µl	10	0.02	0.025
Marker 7	UCD_7F	10pmol/µl	10	0.38	0.475
	UCD_7.1R	10pmol/µl	10	0.38	0.475
Marker 4	UCD_m4F	10pmol/µl	10	0.09	0.1125
	UCD_m4R	10pmol/µl	10	0.09	0.1125
2x Qiagen Type-IT Multiplex PCR Mix					6.25
Water (HPLC)					2.05
DNA ~4ng/µl or FTA (2mm disc)					1
Final volume					12.5

2.6 Discussion

In the development of the SSR assay several key aspects were explored. First, the universal tagged primer system (Blacket et al., 2012) was suited to a first cost-effective screen to identify the polymorphic and informative loci. The universal tags had already been used in multiplex tests for population diversity studies in multiple different organisms which suggested it was effective (Miller et al., 2020; Nipitwattanaphon et al., 2020; Vega-Polo et al., 2020). However, when testing this method on *B. lactucae,* issues were identified that prevented its use for downstream processing of samples. It was challenging to achieve consistent results for multiplex analysis due to the amplification of secondary peaks and unpredictable variation in amplification efficiency between markers using the tagged primer system. The cause of this inconsistency was unclear, but it may have been due to competition for PCR reagents amongst loci combined with the genetic complexity of *B. lactucae.* The pathogen has been shown to have a complex genetic system with variability in the levels of ploidy and heterokaryosis resulting in changing doses of different alleles due to sorting of genetically distinct nuclei in even a single sample (Fletcher et al., 2019). The best resolution obtained with the universal tags was the 5 reaction SSR assay which though more reliable downstream analysis was obtained it was not appropriate for the analysis of samples for which the supply of DNA was limited.

Once the comprehensive list of polymorphic candidate markers was identified the move to shift to individually labelled fluorescent primers improved the clarity and reproducibility of the analysis for genotyping. This shift also proved effective for using in a multiplex assay. This was clearest with Marker 2 and 10 in Table 2-20, where only one replicate amplified effectively with the universal tags system, but with labelled primers all replicates generated alleles for Marker 2, and 13 of 24 replicates amplified alleles at Marker 7.

Some differences between replicate runs of the same sample with different sources of DNA (sporangial or FTA card) were still observed even with labelled primers. In general, clearer downstream analysis was observed when using DNA from FTA than DNA from sporangial suspensions. There are few possible explanations, 1) low or degraded DNA led to poor readings, 2) and difference in sample state in pressed lesions on FTA card compared to the sporangial suspension. Strains of *B. lactucae* have been reported to form heterokaryons

(Fletcher et al., 2019), the heterokaryotic state is thought to be highly transient and the relative frequency of heterokaryosis is not well defined for the UK population. In the literature there are reports of heterokaryons undergoing nuclear sorting or selection of specific genes and alleles over each other with the fungi *Neurospora tetrasperma* (Meunier et al., 2022; Samils et al., 2014).

Therefore, the discrepancies observed between the same isolate genotype when using DNA from sporangial suspensions to DNA from FTA cards may be attributed to nuclear sorting. An effect of nuclear sorting may be more pronounced in a lesion pressed onto an FTA card than in a DNA extraction from a sporangial suspension generated from washing spores from across one or multiple lesions. A single disk cut from a fixed point accommodates location with the plant tissue (FTAs) compared to a sporangial suspension and the spatial differences may explain some of the variation.

Overall, the finalised SSR assay with labelled primers was able to genetically discriminate and characterise samples of *B. lactucae* and was especially effective with lesion DNA stored on FTA cards. It was thus considered appropriate to use to analyse *B. lactucae* populations at a range of scales in the UK.

Chapter 3 The genotypic and phenotypic diversity of the UK population of *B. lactucae*

3.1 Introduction

The lettuce downy mildew pathogen *B. lactucae* is able to generate genetic diversity via a range of mechanisms which, in combination with aerial sporangia that can disperse long distances between crops, provides ample opportunity for gene-flow between sub-populations (Jeuken & Lindhout, 2002). In the longer-term, gene flow is predicted to make LDM disease management more challenging due to the introgression of advantageous traits across the population. Exerting a selection pressure on a genetically diverse population will promote Darwinian selection and adaptation within the population. Management of LDM is generally based on a combination of host resistance, chemical and cultural control. The range of *R* genes (Parra et al., 2016) and fungicide active ingredients are however, limited. Furthermore, introgression of *R* genes into desirable cultivars takes time, and so does the development and regulatory approval of active ingredients (HSE, 2023). A lack of effective host resistance and ineffective chemical control measures can result in poor LDM management and potentially crop failure. Monitoring and thus understanding both the genetic and phenotypic diversity of the pathogen population will inform the stewardship of the *R* gene and fungicide active ingredient resources available (Tör et al., 2023).

There is evidence of populations of *B. lactucae* adapting to management techniques. For example, the changes in the IBEB differential set and the number of pathogen races discriminated over time(IBEB, 2023) suggests the wider population is changing. In addition to many documented reports of population shifts in both virulence (Lebeda & Zinkernagel, 2003b; Trimboli & Nieuwenhuis, 2011; van Hese et al., 2016) and fungicide insensitivity (Brown et al., 2004; Cobelli et al., 1998; Crute et al., 1987; Schettini et al., 1991), some outbreaks have reportedly been exacerbated by human activity such as the distribution of infected plants from specialist plant propagators to growers (Crute, Norwood, and Gordon 1987).

Previous research has indicated that the European population of *B. lactucae* undergoes a sexual reproductive stage which generates new allelic combinations and maintains genetic diversity (Gustafsson et al., 1985; Michelmore & Wong, 2008). Sexual reproduction is typically heterothallic, requiring the presence of two mating types, *B1* and *B2* (Michelmore & Ingram, 1980). Sexual reproduction is problematic for disease management due to both the genetic recombination which can produce novel virulence phenotypes (Michelmore & Ingram, 1981), but also the resultant thick-walled oospores that can survive in soil overwinter and produce primary inoculum in the next season (Morgan, 1978, 1983). Though there are some detailed studies on sexual reproductions on cultivated lettuce (Crute et al., 1987; Gustafsson et al., 1985; Souza et al., 2022) , and *L. serriola* (Petrželová & Lebeda, 2004), there are not many studies on the impact or role of sexual recombination in contemporary European population with most of the samples collected before 2010. Exceptions to the requirement of two mating types in sexual reproduction in the form of secondary homothallism have been documented (Michelmore & Ingram, 1982; Michelmore & Wong, 2008). Contrary to sexual recombination which promotes genetic diversity by inclusion of two different strains, inbreeding driven by homothallism has been demonstrated to reduce heterozygosity by half in every generation (Goodwin, 1997). Regardless of heterothallic or homothallic strains, sexual reproduction results in oospores.

B. lactucae has other means of generating and maintaining genetic diversity. Previous observations have reported somatic fusion leading to the formation of either polyploids or heterokaryons (Hulbert & Michelmore, 1988), and trisomy has been associated with homothallism in sexual reproduction (Michelmore & Sansome, 1982; Michelmore & Wong, 2008). The impact of somatic fusion as a significant source of stable phenotypic variation is not known, but recent studies have shown heterokaryons have somatic stability and superior fitness on non-selective hosts compared to homokaryotic derivatives (Fletcher et al 2019). Hence, it can be said that the genetics underlying *B. lactucae* is complex.

As an asexual polycyclic pathogen *B. lactucae* produces abundant aerially dispersed sporangia that disperse locally but also have predicted maximum dissemination modelled up to 3000 m (Wu et al., 2001). Sporulation at each infection cycle results in multinucleate sporangia, which

have been speculated to be genetically distinct (Fletcher et al 2019). Such sporangia could thus be a key contributor to gene flow in the UK population.

Little is known about the genetics of the UK population of *B. lactucae*. As an island the UK is geographically isolated from mainland Europe, and such, physical separation and lack of gene flow is predicted to lead to genetic divergence from the population of mainland Europe. There is evidence that countries in mainland Europe differ in the composition of mating types in *B. lactucae* populations: mating type *B1* was prevalent in samples from a preliminary study collected from Germany (Lebeda, 1997 as cited in Lebeda & Zinkernagel, 2003b), whereas *B2* was prevalent in samples collected from Czech Republic (Petrželová & Lebeda, 2004). Other studies found sexual reproduction was frequent in Swedish populations which was attributed to both mating types being present (Gustafsson et al., 1985; Souza et al., 2022). Other *B. lactucae* populations on isolated landmasses such as Japan and Australia were reported to be multiallelic and speculated to rely on somatic fusion as a source of genetic variation (Michelmore & Ingram, 1980). There is reportedly an inverse correlation between sexual and asexual reproductive phase, though the reason underpinning this is not well known (Michelmore & Ingram, 1980). Therefore, the reproductive preferences of *B. lactucae* populations may differ between populations, based on whether homothallic strains are present, the frequency of asexual reproduction and somatic fusion, and the composition of mating types within a population.

Monitoring disease and surveillance of other oomycete plant pathogens has documented the emergence and spread of specific aggressive lineages (Cooke et al., 2012) and new virulent races (Taylor et al., 2018). In the case of *B. lactucae*, regional differences in the efficacy of host resistance have been observed (Lebeda & Zinkernagel, 2003b) and such information aids management through informing an appropriate choice of cultivar. Additionally, monitoring will identify any regions of high genotypic or phenotypic variability and therefore regions at a potentially higher risk of management failures. Importantly monitoring can provide records of the population diversity to refer to in future studies.

3.1.1 Aims

This study aimed to understand the genotypic and phenotypic diversity of *B. lactucae* in UK lettuce crops to inform disease management. Isolates were collected via a postal survey and profiled using the previously developed SSR markers to understand their genetic diversity. Live samples were also phenotypically tested for virulence and fungicide insensitivity and this data was evaluated in relation their genetic diversity. The phenotypic and genotypic diversity is examined in geographical and temporal context for patterns that explain the dynamics of the population. The knowledge gained on the evolving contemporary population of *B. lactucae* in the UK will help evaluate the effectiveness of host resistance and fungicides to inform the management practices for industry stakeholders.

3.2 Materials and methods

3.2.1 *Bremia lactucae* isolate collection, maintenance, and storage

3.2.1.1 Isolate collection

Samples of *B. lactucae* were obtained from lettuce plants that were naturally infected with *B. lactucae*. Most samples were obtained from industry stakeholders, who were contacted directly or indirectly through publications and newsletters.

Sample packs were sent to interested parties, including a sample form, instructions, and equipment for sampling along with pre-paid return envelopes. The sample form asked for information on the outbreak the sample was collected from, included the outbreak size, the cultivar, the date the sample was taken and the postcode district. An optimal sample comprised four foliar lesions per outbreak, and an additional four lesions pressed onto a FTA® classic card (Whatman®, Merck/Sigma Aldrich) which stabilises and preserves host and *B. lactucae* DNA. FTA cards provided insurance in cases where the leaf material containing *B. lactucae* did not survive postage or the transfer to susceptible lettuce seedlings at Hutton was not successful. Establishing live cultures maintained on lettuce seedlings was critical for bulking inoculum to test for phenotype.

3.2.1.2 Isolate bulking and maintenance

A sporangial suspension from each sample was obtained by cutting isolated sporulating lesions with flame sterilised tools, placing them in a 15 ml centrifuge tube with deionised water and gently shaking to remove sporangia. Plant material was then removed, and the sporangial suspension prepared for isolate maintenance or for storage.

Lesion samples that had very sparse sporulation were placed in dark and high humidity conditions (in a covered Perspex box with damp paper towels), to promote sporulation and prevent sunlight damage to sporangia. Lesion samples that could not be recovered were pressed onto an FTA card to preserve the pathogen DNA.

Since *B. lactucae* is an obligate biotroph, requiring living host tissues for nutrients, bulking up and maintaining biological material required inoculation and serial passaging on susceptible lettuce seedlings. Seedlings of cultivar Green Towers were sown on damp filter paper inside a Perspex box that was then wrapped in plastic to maintain high humidity (preventing the

filter paper from drying out). The boxes were placed in a north-facing greenhouse (approximate day time temperature of 15°C) and when the seedlings were 7 days old or 2 cm long, they were sprayed with a sporangial suspension of the isolate of interest. Post inoculation the box of seedlings was further wrapped in a white opaque plastic bag to reduce direct light and prevent damage to the sporangia. Only one culture was opened at any time, and each handled separately with sterilised equipment and fresh gloves as a precaution to prevent cross-contamination. After 7-14 days depending on the level of sporulation, sporangia were washed off seedlings to make sporangial suspensions.

3.2.1.3 Isolate storage and DNA extraction of sporangial suspensions

The method of isolate storage depended on planned use. Sporangial suspensions were centrifuged at 4000 rpm for 5 minutes, to remove excess water and approximately 1ml containing the sporangial pellet was retained. The tube containing the pellet and 1ml of water was directly stored, or resuspended then stored, at either -20°C or -80°C. Alternatively, sporulating seedlings were bagged and stored at -20°C.

Aside from FTAs, *B. lactucae* DNA was also sourced from maintained isolates. The 1ml sporangial suspensions would be centrifuged at 4000 rpm for 5 minutes or more until a pellet was visible, excess water was removed. A modified Raeder and Broda (1985) was used, as described in (Lees et al., 2019) in addition to not freeze drying the sporangia. DNA concentration (ng/µl) was quantified using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Loughborough, UK).

3.2.2 Isolate characterization

Isolates were genotyped using the SSR assay developed in Chapter 2. As primer concentrations were adjusted to optimise the peak heights between runs not all strains were typed with the same multiplex primer mix, however all the same primers were used. The details are included in the sample information, as required.

3.2.3 Assessing *B. lactucae* virulence profiles

The standardised virulence test from IBEB was used (IBEB, 2023). Sporangial suspensions of *B. lactucae* isolates were sprayed on 7-day old seedlings of cultivars with differing resistance genes. Post inoculation, seedlings were kept in Perspex boxes at 90% RH and 15°C. After 10

days seedlings were assessed for symptoms following the IBEB index and given a sextet score for their virulence profile.

3.2.4 Fungicide insensitivity testing

The AHDB project CP 184 included fungicide insensitivity testing of 15 samples of *B. lactucae* from 10 outbreaks in 2019 (Pettitt et al., 2020) and 10 samples from 7 outbreaks from 2020-2021 (Pettitt et al., 2023). Isolates were tested in duplicate against a range of doses of three fungicides; Cleancrop, Paraat and Revus, each of which contained different active ingredients; azoxystrobin, dimethomorph, and mandipropamid, respectively. Full details on the protocol and results are available in CP 184 year 1 (Pettitt et al., 2020), and final report (Pettitt et al., 2023). This thesis used the data from this project but did not design nor carry out the experiments. The fungicide sensitivity results were compared against sample genotype based on SSR cluster analysis.

3.2.5 Data Analysis

GeneMapper (v5.0) was used to manually call the alleles from peaks generated at each SSR locus. Some minor adjustments of the allele names were required in a few cases where the expected repeat length did not match that predicted; for example, a 1bp difference where 2bp steps were anticipated. This was required to fit the SSR stepwise mutation model used to calculate Bruvo's distances. Data was exported from GeneMapper and processed with Microsoft Excel pivot tables to group the alleles at all loci for each sample. These data were then imported into R as Genclone objects. Samples with unclear SSR alleles were repeated but retained in the dataset to minimise any selection bias. Limited allelic variation and the presence of null alleles in duplicates of the same sample were observed in some cases. To account for such minor genotyping errors the data was filtered in R, first to remove samples with more than 2 loci missing, followed by a multilocus lineage (MLL) filter function in *poppr* that grouped samples within specific Bruvo's distance thresholds into a single lineage. The following R packages and their dependencies were used to examine the structure and diversity of the population: *poppr* (v2.9.4; Kamvar et al., 2014, 2015), *magrittr* (v2.0.3; Bache & Wickham, 2022), *ggplot2* (v3.4.2; Wickham, 2016), *reshape2* (v1.4.4; Wickham, 2020), *adgenet* (v 2.1.10; Jombart & Ahmed, 2011), *ade4* (v1.7-22; Thioulouse et al., 2018), and *ape* (v5.7-1; Paradis & Schliep, 2019).

AMOVA (Analysis of Molecular Variance) and linkage disequilibrium testing was carried out (in *poppr)* on both clone and non-clone corrected populations to check for clonality in the population and subsequent impact on MLG diversity and allelic linked heritability. *K* cluster analysis was carried out to assess the genetic relatedness of samples without the influence of location or year. Subsets of each *K* cluster were used to produce a UGMA phylogenetic tree based on Bruvo's distance matrix on ten SSR loci. Clusters were chosen based on minimum spanning network output and from the testing multiple *K* values. The genotypic relatedness of samples was also examined with minimum spanning networks.

The virulence data from each tested sample were grouped by year, county, year-county, and year-postcode district, and the proportions of differentials overcome were calculated. Virulence proportions were then grouped by SSR cluster group to assess for any associations between *K* cluster group, county, or year. Additionally, Mapchart.net was used to visualise the sample collection by county (MapChart, 2024).

3.3 Results

3.3.1 Overview of genetic diversity of UK samples of *B. lactucae*

From 2019 to 2022, 298 *B. lactucae* samples were received in Dundee. This comprised 148 leaf samples and 150 FTAs from a total of 46 disease outbreaks from 12 UK counties (Figure 3-1). Not all live samples were recoverable, and for some FTAs insufficient DNA could be recovered leading to a discrepancy between the number of samples received and those processed successfully. Additional FTA-based DNA samples from continental Europe were obtained from industry contacts to observe allelic variation outside of the UK.

Sample numbers varied from year to year depending partly on whether the weather was conducive to LDM infection and transmission. Fewest samples were obtained in 2020 due to Covid-19 which restricted travel to collect samples and caused postal delays which negatively affected the viability of live samples. Nineteen of the sampled disease outbreaks had lettuce cultivars identified, three of the outbreaks referred to the lettuce morphotype instead of cultivar, e.g. iceberg, and two outbreaks referred to cultivar mixes. The majority of samples came from commercial crops; however, a few came from allotments.

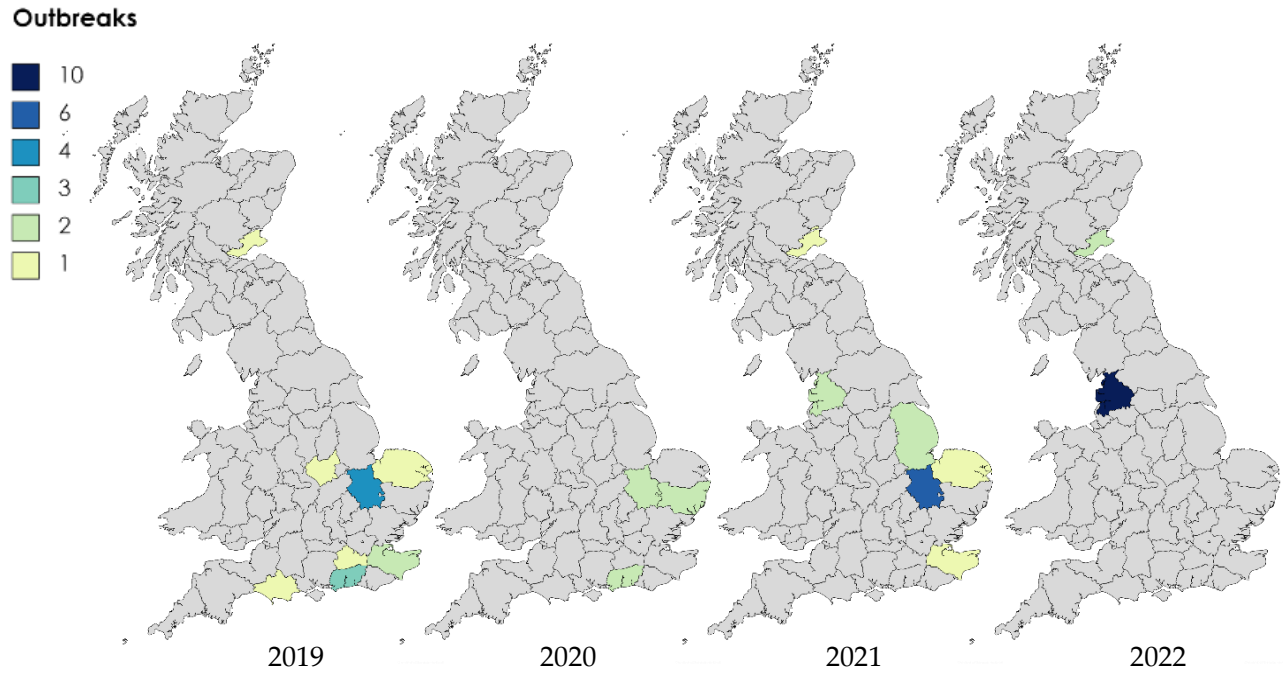


Figure 3-1 Origin of UK B. lactucae *samples collected from the years 2019-2022*

The multiplex SSR assay described in Chapter 2 was successfully applied to UK samples in this work with consistent amplification of allelic peaks across all ten loci that were labelled with different fluorescent dyes (Figure 3-2). In most cases identical genetic profiles were generated from samples from the same outbreak or in technical replicates of the same sample. Despite this, some differences were observed and re-testing of samples with unclear or ambiguous SSR alleles resulted in a total of 292 genotyped samples. Data from samples that were re-genotyped were retained to help detect any evidence of allele sorting and to prevent any inadvertent bias in selection of which samples to remove. Nonetheless data was filtered to remove 57 samples missing data at two or more loci. In total 168 DNA samples generated profiles of sufficient quality with repeats of these samples bringing the total number of profiles to 254.

Due to the many minor differences in SSR alleles and some missing data the MLL approach was applied to collapse the 158 MLGs from 254 UK genotype reads into lineages (Kamvar et al 2015). The average neighbour algorithm was selected and a Bruvo's distance threshold of 0.003906 was determined. The missing data cut-off was set to 0.1 which also removed the data from Marker 2. This process collapsed the 158 MLGs into 135 MLLs that were used for further analysis (Table 3-1).

The number of MLLs observed each year varied with 2021 having the highest (MLL=54) and 2020 having the lowest (MLL=17). However, after correction for sample size the number of MLLs expected (eMLL) and Simpson's diversity value was similar across the years (Table 1-1). No clear trend for change in overall genetic diversity across the years 2019-2022 was noted with eMLL ranging from 17.00 to 20.10, and Simpson's diversity (lambda corrected) ranging from 0.94 to 0.97. The samples collected from the year 2022, had the lowest expected heterozygosity (Hexp = 0.36) observed out of the four sampling years. Notably of 254 MLLs, 168 were found to be tri-allelic or tetra allelic at one or more markers. In addition to samples collected from mainland Europe not showing many novel alleles not found in the UK (data not shown). However, permission has not currently been granted to release these FTA card results for the continental European isolates.

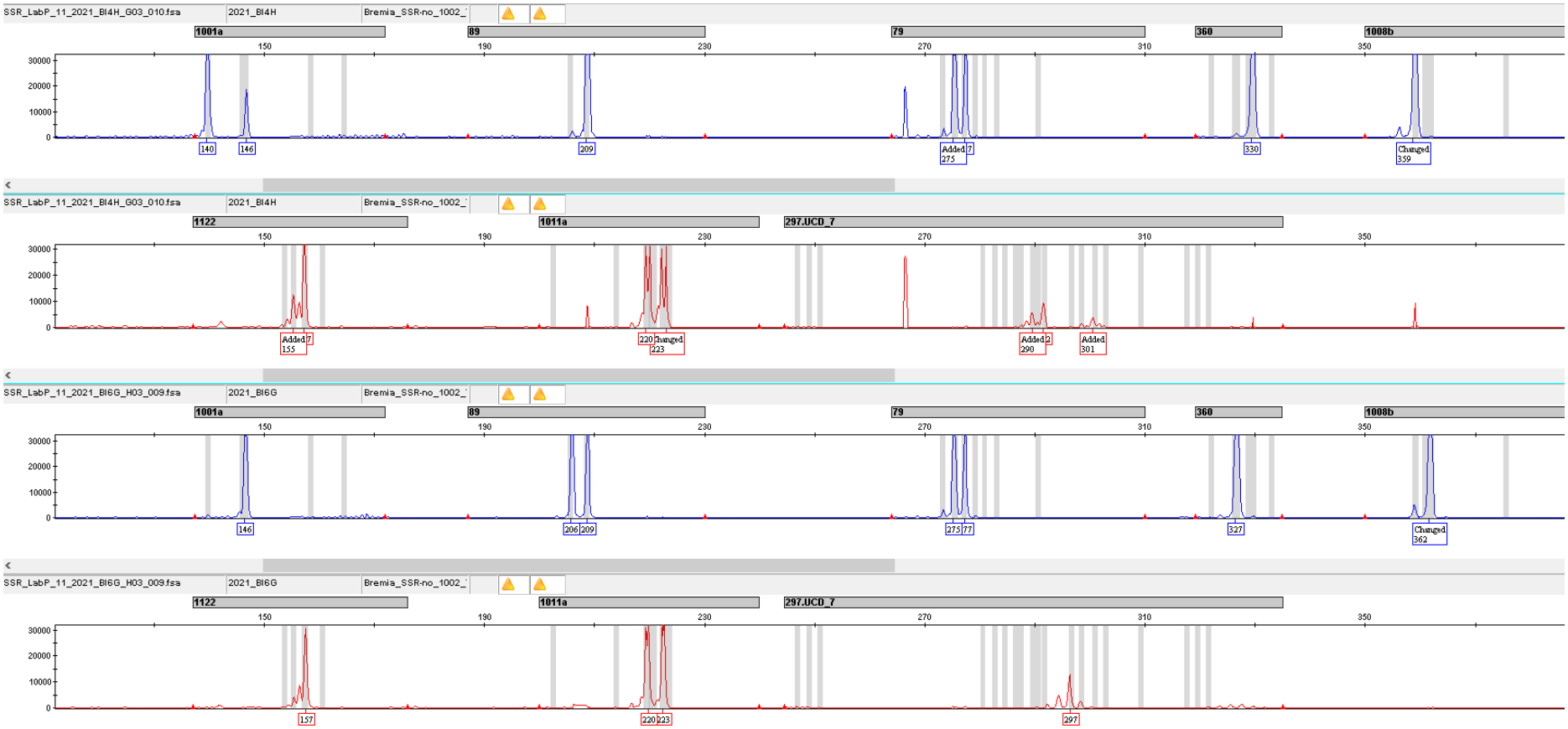


Figure 3-2 Screenshot of the genotype of two isolates from different outbreaks, 2021_Bl4H and 2021_Bl6G, showing representative MLGs of 8 of the 10 SSR loci amplified with the FAM (blue) and PET (red) dyes. Marker names are Marker 9 = 89, Marker 7 = 297.UCD_7, Marker 10 = 360, Marker 5 = 1122. "Added" means allele was manually called, and "changed means allele name was changed manually.

Table 3-1 The genetic diversity of UK B. lactucae samples collected from 2019-2022 based on ten SSR loci with data analysed using poppr. Names of indices of genetic variation and their descriptions are in the table footnotes.

Year	N	MLL	eMLL[a]	SE[b]	H[c]	G[d]	lambda corrected[e]	E.5[f]	Hexp[g]
2019	59	33	17.20	1.82	3.05	12.30	0.94	0.560	0.509
2020	26	17	17.00	0.00	2.63	10.90	0.95	0.770	0.459
2021	94	54	20.10	1.87	3.67	26.60	0.97	0.671	0.469
2022	75	43	18.90	1.84	3.36	15.30	0.95	0.517	0.357
Total	254	135	22.10	1.79	4.47	49.40	0.98	0.559	0.479

[a] eMLL is the expected MLLs at the lowest common sample size (n=26), ≥ 10 based on rarefaction

[b] SE is standard error for rarefaction analysis used to create eMLLs

[c] H is Shannon-Wiener Index of MLL diversity (Shannon, 2001) the higher the number the higher the diversity, includes genotypic diversity and evenness in calculations, is sensitive to number of different MLGs and evenness.

[d] G is Stoddart and Taylor's Index of MLL diversity (Stoddart & Taylor, 1988), the higher the number the higher the diversity, includes genotypic diversity and evenness in calculations

[e] lambda corrected is Simpson's Index (Simpson, 1949) of diversity, corrected to population size, values from 0-1, with 0 being no diversity, and 1 all genotypes are different.

[f] E.5 is Evenness (Grünwald et al., 2003), values from 0-1, with 0 being complete unevenness.

[g] Hexp is Nei's unbiased gene diversity (Nei, 1978), i.e. expected heterozygosity, values from 0-1, with 0 not heterozygous/ no diversity.

3.3.2 Marker discriminatory power

A range in discriminatory power was observed across the ten SSR markers, (Table 3-2). Highly polymorphic loci like Marker 7 with 15 detected alleles, had a greater value in discriminating isolates compared to markers with fewer alleles, such as 1001a with only two alleles. Among the markers tested on UK samples, the markers 1001a, 1008b and Marker 9, resolved three or fewer alleles.

Indexes for diversity and heterozygosity for each locus produced nearly identical scores. Markers tested ranged in Simpsons diversity scores, from high diversity of 1-D= 0.87 to low 1-D =0.22, which was mirrored in expected heterozygosity (Hexp=0.22-0.87). Evenness for all loci was above 0.5, which means the population trended towards more even distribution of alleles. Populations with a dominant clonal lineage would have skewed scores in diversity and evenness as the dominance of one genotype would trend towards zero at 1-D, Hexp, and

Evenness for most markers. Exceptions to this pattern would be shown if several distinct clonal lineages were present or the unlikely scenario where clonal lineages were present having all potential alleles for every marker.

Table 3-2 Allelic diversity statistics for sample UK population

locus	allele[a]	1-D[b]	Hexp[c]	Evenness
Marker 1	4.0	0.37	0.38	0.58
Marker 2	5.0	0.55	0.55	0.78
Marker 4	5.0	0.50	0.50	0.81
Marker 5	4.0	0.33	0.33	0.64
Marker 7	15.0	0.87	0.87	0.78
Marker 9	3.0	0.45	0.45	0.88
Marker 10	4.0	0.43	0.43	0.82
1001a	2.0	0.22	0.22	0.61
1008b	3.0	0.50	0.50	0.92
1011a	4.0	0.56	0.56	0.83
mean	4.9	0.48	0.48	0.77

[a] allele = number of alleles per locus

[b] 1-D = Simpson's index

[c] Hexp = Nei 1978

As a further test of the suitability of the SSR markers for assessing population diversity a genotype accumulation curve of the UK sample population is presented (Figure 3-3). This graphical representation of how many of the MLGs of *B. lactucae* were discriminated by an increasing number of the markers was generated using the genotype curve function of *poppr*. It is analogous to a rarefaction analysis and shows the curve approaching 100% of the MLGs with the addition of the tenth marker and demonstrates that additional markers would not discriminate further genotypes in the population.

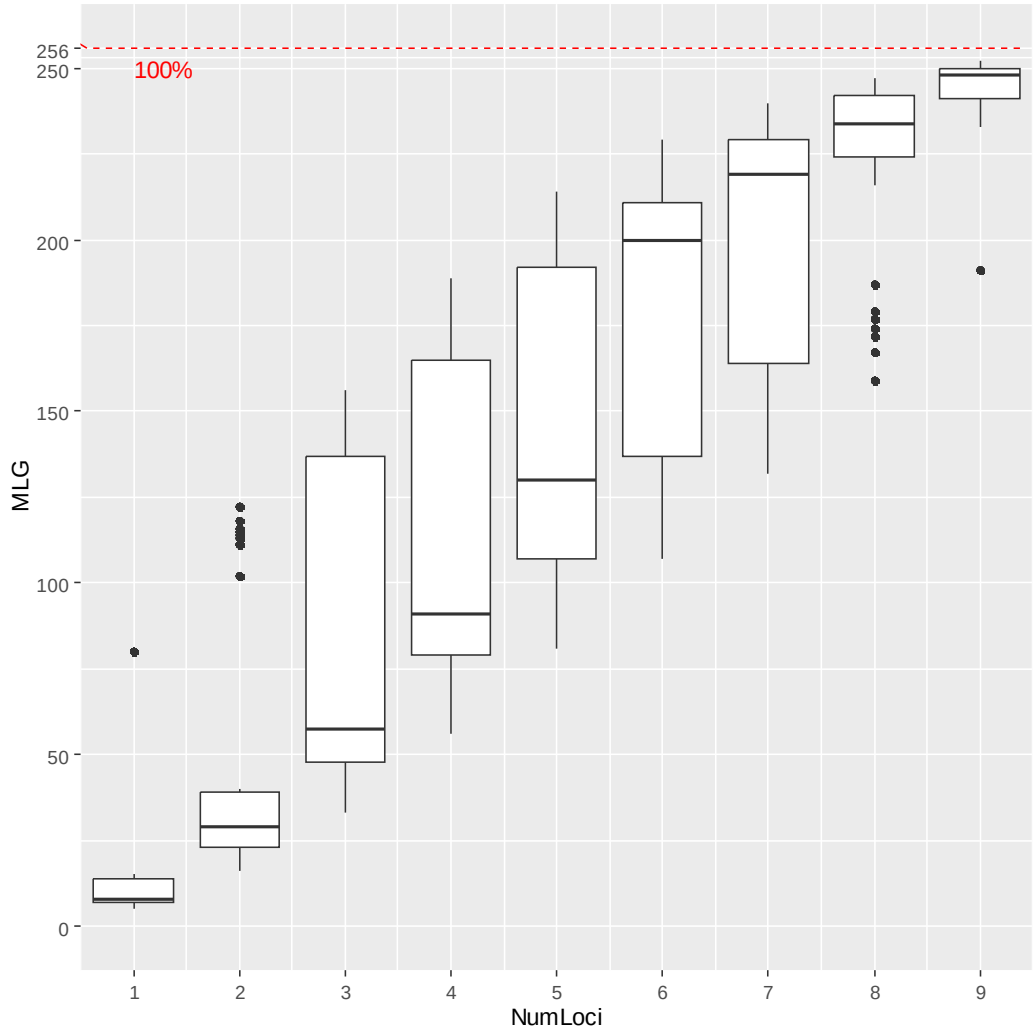


Figure 3-3 Genotype accumulation curve of ten SSR loci on 254 DNA samples of B. lactucae collected from the UK between the years 2019-2022.

3.3.3 Population structure determined by *K*-means clustering

In order to understand population structure within the dataset, unbiased analysis of genetic similarity amongst MLLs was conducted using *K*-means clustering that was then studied in relation to the sample location and year of sampling. *K*-means clustering uses genetic distances between samples to partition data into distinct clusters based on similarity and identifies clusters that minimize within-cluster variance based on many permutations (Grünwald et al., 2017; Jombart et al., 2010). Systematic tests on *K* cluster numbers to find the optimal cluster number to represent data based on BIC (Bayesian Information Criteria) value were carried out.

A preliminary analysis with *K*=15 cluster groups and all *B. lactucae* samples is shown to illustrate the pattern of SSR profiles by county (Figure 3-4). In some countries such as Suffolk and Dorset, only a single genotype was sampled. Many cases can be observed where genetically identical sample types were abundant across many outbreaks within a single county and also shared between counties. One example is the purple cluster in Lancashire that was also sampled in Fife. Similarly, the olive-green coloured group was sampled across Norfolk, Suffolk, Cambridgeshire, Kent, and West Sussex. In subsequent analysis we simplified the cluster groups down to *K*=7 and showed (Figure 3-5) only representative samples rather than the whole dataset.

The *K* clustering analysis (*K*=7) showed the majority of groupings corresponded to year-postcode-district and led to the placement of most samples from the same outbreak in the same cluster group. For example, all samples from the first outbreak of 2021 (2021_Bl1) grouped together in group 6. This indicates that samples from the same location are more likely to be genetically identical or nearly identical and represent a localised clonal population from a single source of primary inoculum (Figure 3-4). This analysis also demonstrated the SSR assay was robust and reproducible in confirming identical profiles in technical replicates (as predicted) but also in *B. lactucae* samples from the same sampled disease outbreak.

Bruvo's genetic distance is a measure specifically optimised for organisms with a range of ploidies (Bruvo et al., 2004). To visualise genotypic relatedness within and between *K* cluster groups, representative samples from each cluster were selected, and their Bruvo's distances utilised in an UPGMA algorithm to construct a phylogenetic tree, as illustrated in Figure 3-5

The phylogenetic tree showed a complex mix of degrees of relatedness with some groups of genetically identical or near identical samples and others with greater Bruvo's distances of up to 0.25 (in the case of an outlier sample Bl6H) from other samples in the same *K* cluster group. The majority of cluster groups were polyphyletic but with distances from 0.00-0.05, suggesting minimal genetic differences. As *K*-means clusters do not perfectly correspond to dendrogram clusters it indicates a level of panmixia (sexual reproduction) and gene flow from other sources.

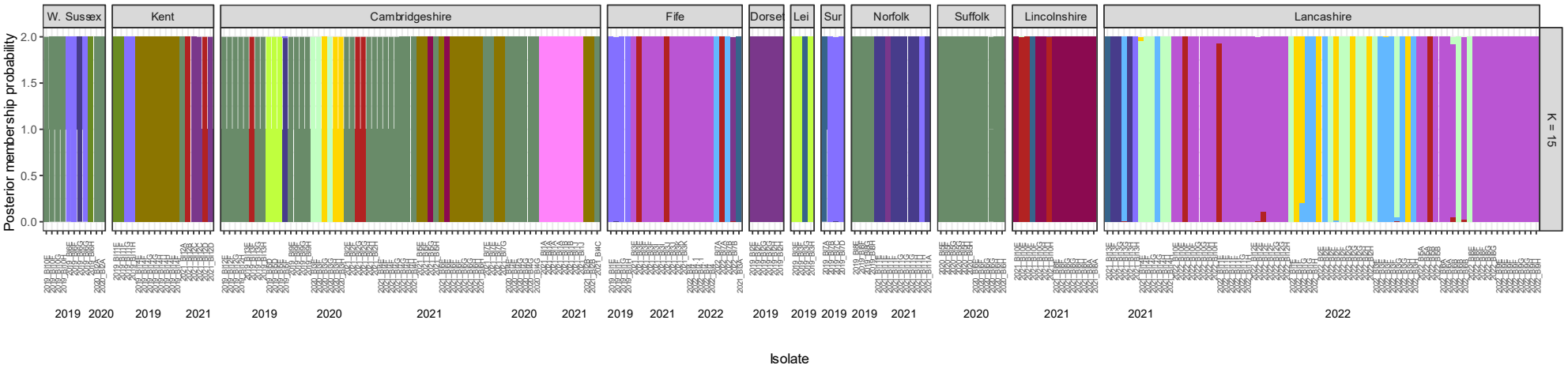


Figure 3-4 The SSR genotypic profiles of all 254 genotyped B. lactucae samples from the UK, plotted by the county they were sampled from, and coloured by K cluster group (K=15). Isolate names are not intended to be legible, outbreaks within a county are offset to upper and lower position to illustrate the common association between outbreak and K cluster. Sampling year labels are presented in the centre of each sample batch from the same year within a county. Lei = Leicestershire, and Sur = Surrey.

In several *K* cluster groups (2, 4, 6, and 7) the clades on the Bruvo's distance tree did not correspond to the *K* clustering. *K* cluster group 2, for example, comprised samples from 2021 and 2022 in Fife, 2019 Surrey, and some 2022 Lancashire samples that were in three different clades of the tree. There were also distinct *K* cluster sub-groups that were monophyletic; for example, samples 2022_Bl5A, 2022_Bl4, 2022_Bl8F, 2022_Bl10F and 2021_Bl13E (from group 2 and 7), formed a monophyletic group with a Bruvo's distance below 0.05, suggesting a genetic similarity between these cluster groups. The biggest difference was with the outlier sampler 2019_Bl6H from West Sussex with a Bruvo's distance of 0.25 to all other samples. Samples from the same outbreak, 2019_Bl6E and 2019_Bl6E 6G, were also in different cluster groups to 2019_Bl6H thus confirming that more than one pathogen genotype may be found within the same outbreak. This was also the case for samples 2021 Bl10F 2 and 2021 Bl10H from the same Lincolnshire outbreak but in distinct parts of the Bruvo's distance tree and in *K* clusters 5 and 4, respectively. In contrast, the three samples from the 2021_Bl1 outbreak in Cambridgeshire all grouped into *K* cluster 3 and formed a single clade indicative of a genetic clonal lineage. Group 5, a *K* cluster group of interest, formed a monophyletic group despite being comprised of samples from multiple counties and years, with samples from outbreaks in Norfolk 2019, West Sussex 2020, and Suffolk 2020. Group 5 additionally contained samples from Cambridgeshire in 2019, 2020 and 2021, indicating a clonal lineage that survived overwinter. Group 1 was similar to group 5; in that it was comprised of samples from different years and locations forming a near monophyletic group. In conclusion some outbreaks comprised a single clone while others were genetically diverse.

A more detailed look at the *B. lactucae* sample SSR data from cluster group 5 shows there is a dominant genotype pattern, (Table 3-3). Noticeably the MLLs include some triploid loci and the samples with only two alleles generally had two of the alleles also present in the triploid. As evident in Figure 3-5, the SSR alleles in *K* means in cluster group 1 were clearly distinct from those in group 5 but the diversity within the group was slightly higher due to variation at three of the ten markers (Marker 4, Marker 7 and 1011a; Table 3-4). Interestingly in Marker 7, the occurrence of two small alleles produced three and four allele peaks. From the SSR data of cluster group 1 it is clear why 2019_Bl6H did not group with the rest of the group and formed an outlier in the phylogenetic tree (Figure 3-5). It likely grouped in *K* cluster group 1 due to shared unique 161 allele in Marker 5 which no other group has.

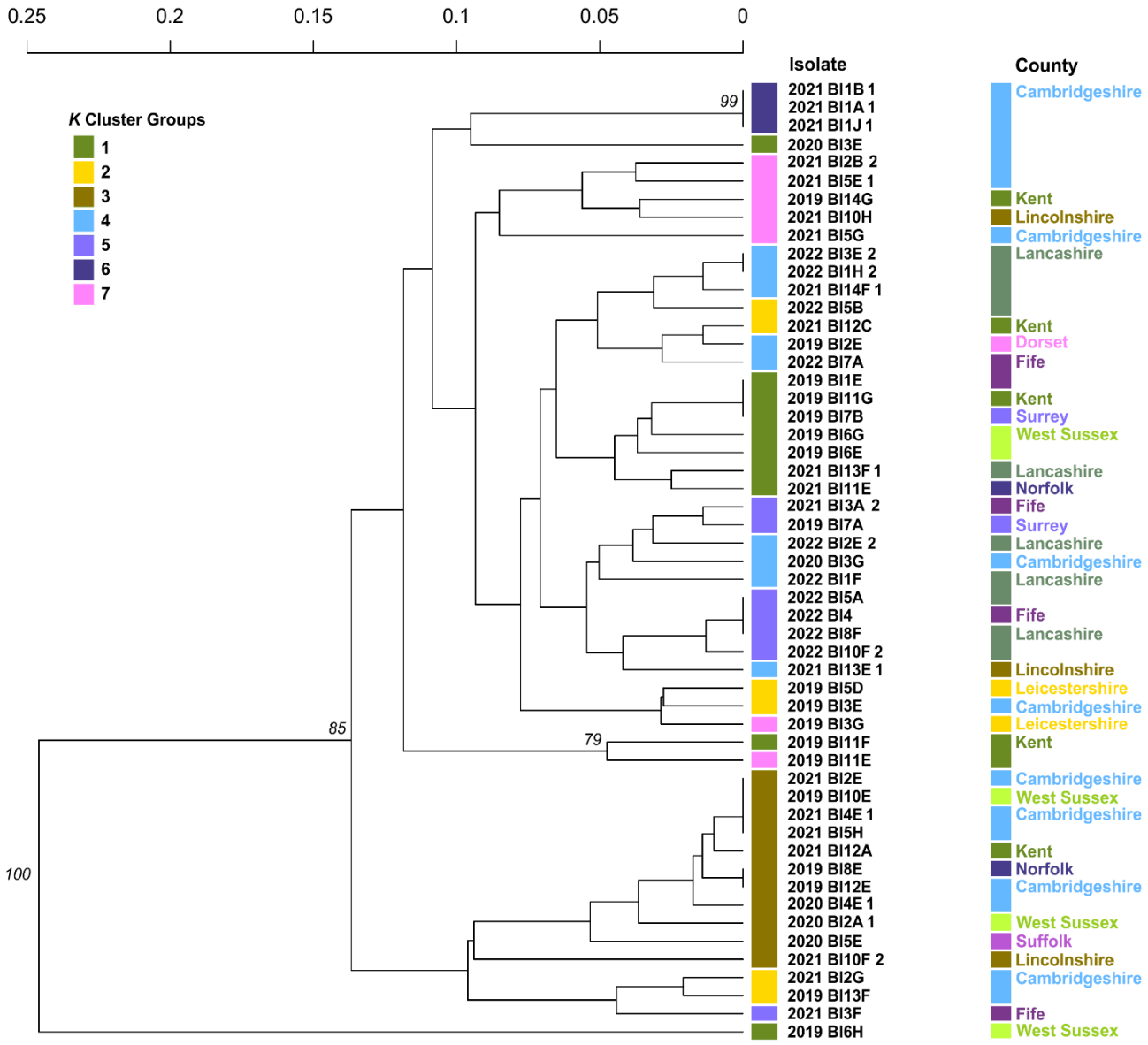


Figure 3-5 Phylogenetic tree constructed using UPGMA algorithm on Bruvo's distances between genotypes from representative samples of each K cluster group. MLL filters were applied to account for minor genotypic differences. Node labels show the percentages bootstrap support amongst 999 trees tested (cutoff at 50%). Colours next to sample names are the K cluster groups, and colours to the right are associated with county (names included). The scale bar above the tree shows the Bruvo's distance of nodes in the tree.

Table 3-3 Genotype profiles of representative samples of K cluster group 5. Below each marker the numbers in the column header represents the allele number. The different colours and corresponding number (bp) at each marker represent a different allele.

		Marker 4			Marker 9		Marker 10		Marker 1			Marker 2		Marker 5		1001a		1008b		1011a		Marker 7			
Sample	**County[c]**	**1**	**2**	**3**	**1**	**2**	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**3**	**4**
2019_Bl12E	CB	275	277		209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223	290	292	303	
2019_Bl8E	Norf.	275	277		209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223	290	292	303	
2019_Bl10E	W. Ssx	275	277		209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223	290	292	301	
2020_Bl5E	Suff.	275	277	291	209	209	330	330	142	145	148	266	266	155	157	140	146	359	376	220	223	290	292	299	
2021_Bl2E	CB	275	277		209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223	290	292	301	
2021_Bl4E_1	CB	275	277		209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223	290	292	301	
2021_Bl5H	CB	275	277		209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223	290	292	301	
2021_Bl12A	Kent	275	277		209	209	330	330	142	145	148	266	266	157	157	140	146	359	359	220	223	292	301		
2020_Bl4E_1[a]	CB	275	277		209	209	330	330	142	148		266	266	155	157	140	146	359	359	220	223	290	292	299	301
2020_Bl2A_1[b]	W. Ssx	275	277		209	209	330	330	142	148		266	266	157	157	140	146	359	359	223	223	292	303		

[a] FTA_Mplx6_2020_Bl4E_R1

[b] FTA_Mplx6_2020_Bl2A_R1

[c] Abbreviations: CB = Cambridgeshire, Norf. = Norfolk, W. Ssx = West Sussex, Suff. = Suffolk,

Table 3-4 Genotype profiles of representative samples of K cluster group 1. Below each marker the numbers in the column header represents the allele number. The different colours and corresponding number (bp) at each marker represent a different allele.

Sample	County[a]	Marker 4		Marker 9		Marker 10		Marker 1		Marker 2		Marker 5			1001a		1008b		1011a			Marker 7			
		1	2	1	2	1	2	1	2	1	2	1	2	3	1	2	1	2	1	2	3	1	2	3	4
2019_Bl6E	W. Ssx	275	275	206	209	327	330	142	142	272	272	155	157	161	146	146	362	362	202	220	223	249	281	299	
2019_Bl1E	Fife	275	275	206	209	327	330	142	142	272	272	155	157		146	146	362	362	220	223		249	251	281	299
2019_Bl6G	W. Ssx	275	277	206	209	327	330	142	142	272	272	155	157		146	146	362	362	220	220		281	299		
2019_Bl11G	Kent	275	277	206	209	327	330	142	142	272	272	155	157		146	146	362	362	220	223		249	251	281	299
2019_Bl7B	Surr.	275	277	206	209	327	330	142	142	272	272	155	157		146	146	362	362	220	223		249	251	281	301
2021_Bl11E	Norf.	275	277	206	209	327	330	142	142	272	272	155	157		146	146	362	362	220	223		281	281		
2021_B13F_1	Lanc.	275	277	206	209	327	330	142	142	272	272	155	157		146	146	362	362	220	223		281	281		
2019_Bl6H	W. Ssx	291	291	206	209	333	333			269	272	161	161		140	146	362	376	220	223		299	299		

[a] Abbreviations: Norf. = Norfolk, W. Ssx = West Sussex, Surr. = Surrey, Lanc. = Lancashire

3.3.4 Analysis of structure and reproductive mode

The above *K* cluster group analysis presented evidence of some clonal lineages spanning multiple years and across counties which suggested inoculum could overwinter. However, there was also a more diverse element of the population suggestive of a sexual or panmictic phase and this mixed reproduction within the UK population required further analysis. Specific tests for the population structure, by year, county, and post-district, and for linkage disequilibrium between markers, using index of association, were conducted.

Analysis of Molecular Variance (AMOVA) is an analysis that partitions genetic variation amongst different population structural levels. AMOVA analysis of a dataset is usually compared with a clone corrected version of the dataset. When partitioning genetic variance of ten SSR loci by year, country, and post-district, using *poppr*, AMOVA analysis showed that the highest percentage of variation observed was attributed to 'within post-district' (>50%) regardless of clone correction. Clone correction increased the percentage of genetic variation for tested loci 'within post-district' (from 50.5% to 63.2%, Table 3-5). Correcting for clones by removing duplicate data points did not change the levels at which structure was observed but further emphasised the structures shown in the non-clone corrected data.

Differentiation between years, and counties 'between years' was minimal compared to strata levels at county and postal district (below <15%,Table 3-5), again suggesting lineages or sub-populations within the UK samples collected. Though the majority of genetic variation was found to occur 'within post-districts', the next largest proportion (>30%) was attributed to samples 'between post-district within county' (Table 3-5). This suggests genetic differentiation at more local and regional levels is common, and that geolocation is more important to genetic differentiation than year.

To objectively measure evidence of mating within populations, linkage disequilibrium analysis was carried out. Linkage disequilibrium assumes panmixia, in which loci are not linked, which in the context of population genetics is presumed from widespread random mating of freely recombining populations. The closer the r_D value to 0 the closer to panmixia, and compared to Ia, r_D accounts for the known number of loci and is less bias. Significant results deviating from the assumption of panmixia would indicate that reproduction is not random and therefore sub-populations or clonal lineages are present.

Table 3-5 AMOVA results on SSR data from samples of the UK population of B. lactucae

Dataset treatment	Populations	Df	Sum Sq	Mean Sq	Sigma	%	Phi	*p*-value[b]
MLL filtered[a]	Between Year	3	3.7	1.2	0.007	6.2	0.06	0.114
	Between County Within Year	15	6.7	0.4	0.013	11.9	0.13	0.418
	Between post-districts Within County	8	3.0	0.4	0.033	31.4	0.38	0.001
	Within post-districts	227	12.2	0.1	0.054	50.5	0.50	0.001
	Total	253	25.5	0.1	0.106	100.0		
Clone-corrected	Between Year	3	1.4	0.5	0.004	4.2	0.04	0.127
and MLL filtered[a]	Between County Within Year	14	3.4	0.2	-0.005	-4.7	-0.05	0.832
	Between post-districts Within County	7	1.6	0.2	0.039	37.3	0.37	0.001
	Within post-districts	110	7.4	0.1	0.067	63.2	0.37	0.001
	Total	134	13.8	0.1	0.106	100.0		

a - MLL filtered with Bruvo's distance using the average distance as it was the highest threshold value, with a threshold of 0.00390625, and a missing data cut-off of 0.1 which removed marker 2

b - Out of 999 permutations

Table 3-6, Linkage disquillibrium analysis of SSR data from B. lactucae *samples from selected counties and post-districts of interest.*

Dataset	N	Ia		r_D	
UK samples	254	1.07	a	0.13	a
UK samples clone corrected	135	0.59	a	0.07	a
Lancashire	78	0.75	a	0.10	a
Lancashire clone corrected	57	0.41	a	0.06	a
PR4	53	0.65	a	0.09	a
PR4 clone corrected	41	0.47	b	0.06	a
Cambridgeshire	68	2.24	a	0.28	a
Cambridgeshire clone corrected	39	1.62	a	0.20	a
CB7	38	1.87	a	0.23	a
CB7 clone corrected	21	0.73	a	0.09	a
Fife	24	0.53	a	0.09	a
Fife clone corrected	20	0.50	b	0.09	a
KY15	11	0.88	b	0.17	a
KY15 clone corrected	8	0.92	b	0.16	a

I_A = Index of association

r_D = rBarD

a, $p<0.001$

b, $p<0.01$

Linkage disequilibrium was tested on the overall UK population using *poppr*, and with the most abundantly sampled regions and post-districts, all suggested asexual reproduction, as all observed values (r_D) differed significantly ($p<0.05$) from expected values if panmixia was met (Table 3-6). Samples collected from Cambridgeshire had a higher proportion of clones in the population (0.43) than samples collected from Lancashire (0.27) and Fife (0.17), suggesting that clonal reproduction predominates in this region. The amount of linkage disequilibrium indicates that there is no evidence of panmixia, as such sexual recombination is likely non-random or not widespread.

Overall, the evidence from the molecular markers suggests that the UK population comprises a mix of asexually reproducing clones (that are also able to overwinter) in addition to limited occurrence of sexual recombination (see later discussion).

3.3.5 Virulence

The IBEB differential assay for the virulence profile was carried out on 52 *B. lactucae* samples from 26 outbreaks from 11 counties across the UK. The IBEB differential set consists of lettuce cultivars and clones each containing different known host resistance (R) genes. 35 isolates were tested on differential set C before set D was released, 12 isolates were tested on set D. All samples could overcome the universally susceptible differential cv Green Towers, indicating that the assay was robust.

In general, the virulence profiles of UK samples did not match any designated IBEB race. Exceptions to this are isolates 2019_Bl2A and 2019_Bl2B from Dorset, which had the same virulence profile to Bl: 35EU. Isolates from the same outbreak tended to have similar but not always identical virulence profiles.

Of the tested *B. lactucae* samples ≥90% of samples tested could overcome the differential lines cv. R4T57 D *(Dm4)* and cv. Colorado (*Dm18*) (Figure 3-6). Of set C the differential lines that had the lowest percentage of samples with LDM symptoms were cv. Dandie (*Dm3)* 13% of samples tested, cv. Balesta (unknown) 23% of samples and cv. Bartoli (unknown) 31% of samples.

Interestingly, only samples that were collected in 2021 and 2022 had samples that could overcome cv. Dandie, cv. Balesta and cv. Bartoli, most of which were collected from Cambridgeshire and Lancashire. Only one differential line, cv. Bataille (set D) was overcome from a single sample collected from Fife in 2022 though fewer *B. lactucae* samples were tested with this set. A general overview of host resistance can be viewed in Table 3-7, where proportion of samples that successfully infected differential lines is grouped by year, county, and post district.

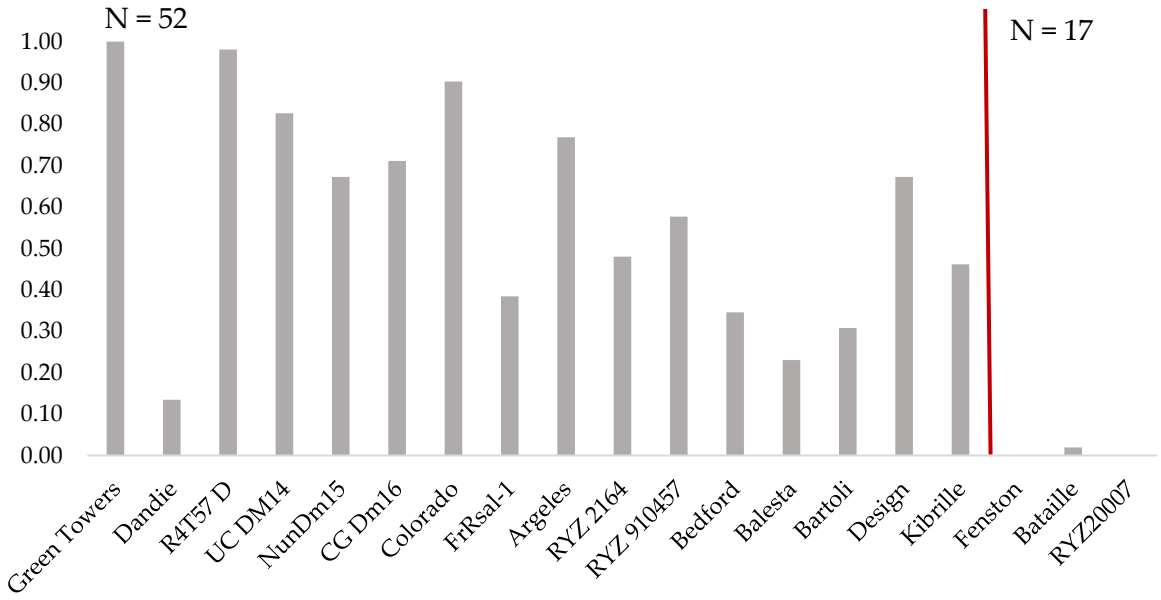


Figure 3-6 Proportion of B. lactucae samples that could overcome each differential line. Right of the red line are the 3 new differentials added to set-C to make set-D, which were only tested against 17 B. lactucae samples.

3.3.6 Association between SSR genotype and virulence

The relationship between genetic fingerprint (by *K* cluster group analysis on ten loci) and virulence profile was examined for cases in which both data sets were available (n=16). For the cases where direct comparison between DNA sample and virulence test could be made, samples from the same outbreak were used.

Some similarity in virulence profile of samples linked with *K* cluster group 5 was observed. A sample from Norfolk 2019 (2019_Bl8A) and one from West Sussex 2019 (2019_Bl10A), for example, had identical virulence profiles (Table 3-8). This identical virulence profile was similar to other samples within the cluster, such as 2020 West Sussex sample (2020_Bl2A) and two 2020 Cambridgeshire samples (2020_Bl4E, 2020_Bl4G) which differed by 1-2 differential lines to the virulence profile of 2019_Bl8A and 2019_Bl10A.

Table 3-7, Proportion of differential lines overcome by B. lactucae samples listed according to year, UK county and post-district. n = number of samples tested. Colour scale ranges Proportion scale ranges from 1 in which all samples can overcome the differential to 0 where no samples could overcome the differential. Colour scale was added to add visualisation of this with red = 1, and green = 0. Each differential line contains a different set of resistance genes or factors (DM or R genes). cv Green Towers is the positive control.Table continues onto the next page..

				Green Towers	Dandie	R4T57 D	UC DM14	NunDm15	CG Dm16	Colorado	FrRsal-1	Argeles	RYZ 2164	RYZ 910457	Bedford	Balesta	Bartoli	Design	Kibrille	Fenston	Bataille	RYZ20007
			R gene	NA	Dm3	Dm4	Dm14	Dm15	Dm16	Dm18	Rsal-1	R38	Dm24/38	R52	R53	R54	R55	R56	Dm11, R57	R65	R59	Dm11, R58
Year	**County**	**Location**	N	S0	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18
2019	**total**	**total**	**15**	**1.00**	**0.00**	**1.00**	**0.67**	**0.80**	**0.93**	**1.00**	**0.47**	**0.80**	**0.40**	**0.93**	**0.53**	**0.00**	**0.00**	**0.87**	**0.60**			
	Cambridgeshire	CB7	4	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50	1.00	0.75	0.00	0.00	0.75	0.75			
	Dorset	DT2	2	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	1.00	1.00			
	Fife	KY15	2	1.00	0.00	1.00	0.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00	1.00			
	Kent	ME19	2	1.00	0.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.50	0.00			
	Leicestershire	LE10	1	1.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	1.00			
	Norfolk	Unknown	1	1.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00			
	Surrey	KT11	2	1.00	0.00	1.00	1.00	1.00	1.00	1.00	0.50	1.00	0.00	0.50	0.50	0.00	0.00	1.00	0.50			
	West Sussex	PO18	1	1.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00			
2020	**total**	**total**	**3**	**1.00**	**0.00**	**1.00**	**0.00**	**0.00**	**1.00**	**1.00**	**0.00**	**1.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.67**	**0.00**			
	Cambridgeshire	CB24	2	1.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00			
	West Sussex	PO22	1	1.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00			

				Green Towers	Dandie	R4T57 D	UC DM14	NunDm15	CG Dm16	Colorado	FrRsal-1	Argeles	RYZ 2164	RYZ 910457	Bedford	Balesta	Bartoli	Design	Kibrille	Fenston	Bataille	RYZ20007
			R gene	NA	Dm3	Dm4	Dm14	Dm15	Dm16	Dm18	Rsal-1	R38	Dm24/38	R52	R53	R54	R55	R56	Dm11, R57	R65	R59	Dm11, R58
Year	**County**	**Location**	N	S0	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18
2021	**total**	**total**	**22**	**1.00**	**0.14**	**1.00**	**0.95**	**0.64**	**0.82**	**0.86**	**0.23**	**0.91**	**0.64**	**0.41**	**0.05**	**0.55**	**0.41**	**0.45**	**0.50**	**0.00**	**0.00**	**0.00**
	Cambridgeshire	CB24	1	1.00	1.00	1.00	1.00	0.00	1.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00			
	Cambridgeshire	CB7	4	1.00	0.25	1.00	1.00	0.25	1.00	1.00	0.00	1.00	0.00	0.00	0.25	1.00	0.00	0.00	0.50			
	Cambridgeshire	CB75	3	1.00	0.33	1.00	1.00	0.00	1.00	1.00	0.00	1.00	0.67	0.00	0.00	1.00	1.00	0.67	0.67			
	Dundee	DD2	1	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	1.00	1.00	0.00			
	Fife	KY14	4	1.00	0.00	1.00	1.00	1.00	1.00	0.50	0.75	0.50	0.50	0.50	0.00	0.00	0.00	0.00	0.25			
	Lancashire	PR4	5	1.00	0.00	1.00	0.80	1.00	0.20	1.00	0.20	1.00	1.00	1.00	0.00	0.20	1.00	0.80	0.20	0.00	0.00	0.00
	Lincolnshire	PE12	4	1.00	0.00	1.00	1.00	0.75	1.00	0.75	0.00	1.00	1.00	0.25	0.00	1.00	0.00	0.75	1.00			
2022	**total**	**total**	**12**	**1.00**	**0.33**	**0.92**	**1.00**	**0.75**	**0.17**	**0.83**	**0.67**	**0.42**	**0.42**	**0.67**	**0.75**	**0.00**	**0.58**	**0.83**	**0.33**	**0.00**	**0.08**	**0.00**
	Fife	KY15	2	1.00	0.00	1.00	1.00	0.50	0.50	0.50	0.50	0.00	0.50	0.50	1.00	0.00	0.00	0.50	0.00	0.00	0.50	0.00
	Fife	KY16?	1	1.00	0.00	1.00	1.00	1.00	0.00	1.00	1.00	0.00	0.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00
	Lancashire	Unknown	2	1.00	0.00	0.50	1.00	1.00	0.00	1.00	1.00	0.00	0.50	0.50	1.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00
	Lancashire	PR4	7	1.00	0.57	1.00	1.00	0.71	0.14	0.86	0.57	0.71	0.43	0.71	0.57	0.00	1.00	1.00	0.57	0.00	0.00	0.00
Grand Total			**52**	**1.00**	**0.13**	**0.98**	**0.83**	**0.67**	**0.71**	**0.90**	**0.38**	**0.77**	**0.48**	**0.58**	**0.35**	**0.23**	**0.31**	0.67	**0.46**	**0.00**	**0.02**	**0.00**

Aside from group 5, in general samples from different outbreaks tended to not have the same virulence profile, in fact a lack of correlation was evident in analysis of the wider dataset. Of which several scenarios were observed, as demonstrated in Table 3-9 and Table 3-10.For example, samples from the same outbreak could be identical or almost identical in genotype and have a virulence profile that only differed at three cultivar lines, as seen with the 2021_Bl1 outbreak. Samples from the same outbreak with different genotypic profile but similar virulence profile were also observed with 2020_Bl4E, and 2020_Bl4G. Samples from outbreaks nearby had similar but not identical genotype and virulence, 2022_Bl4 and 2022_Bl4.1, which though post code district were not the same, these were from fields in approximately the same location. There were also samples from the same outbreak that had mixed genotypes, such is the case with 2021_Bl2 outbreak, of which three samples had similar genotypes (2021_Bl2E, F and H) and one that differed (2021_Bl2G). These samples from the 2021_Bl2 outbreak the virulence profiles of samples tested differed at four differential lines.

3.3.7 Fungicide Insensitivity and association with genotype

There were 15 samples from 10 outbreaks in 2019, and 10 samples from 7 outbreaks from 2020-2021 tested with three fungicides at four different spray rates. The highest fungicide spray rate, which was field rate, gave good control, from 81-100% when compared to untreated (Figure 3-7). As there was no evidence of fungicide insensitivity in samples tested, as such no association no association between genotype cluster and fungicide insensitivity was observed (Figure 3-7).

Table 3-8, Virulence profiles of samples of B. lactucae collected from outbreaks that had samples in K cluster group 5 tested using IBEB Differential Set C. + indicates that the differential line was overcome. Outbreaks are separated by dotted lines.

		Green Towers	Dandie	R4T57 D	UC DM14	NunDm15	CG Dm16	Colorado	FrRsal-1	Argeles	RYZ 2164	RYZ 910457	Bedford	Balesta	Bartoli	Design	Kibrille
	R genes		Dm3	Dm4	Dm14	Dm15	Dm16	Dm18	Rsal-1	R38	Dm24/38	R52	R53	R54	R55	R56	Dm11,R57
	Grid Position		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
County	Sextet Value		1	2	4	8	16	32	1	2	4	8	16	32	1	2	4
Norfolk	2019_Bl8A	+	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-
Cambridgeshire	2019_Bl9A	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
West Sussex	2019_Bl10A	+	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-
Cambridgeshire	2019_Bl12A	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	+
Cambridgeshire	2019_Bl12B	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	+
West Sussex	2020_Bl2A	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-
Cambridgeshire	2020_Bl4E	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-
Cambridgeshire	2020_Bl4G	+	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-
Cambridgeshire	2021_Bl2B	+	+	+	+	-	+	+	-	+	+	-	-	+	+	+	+
Cambridgeshire	2021_Bl2D	+	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+
Cambridgeshire	2021_Bl2H	+	-	+	+	-	+	+	-	+	-	-	-	+	+	-	-

Table 3-9, Examples of UK genotypes of B. lactucae for which the virulence was also tested. Below each marker the numbers in the column header represents the allele number. The different colours and corresponding number (bp) at each marker represent a different allele.

Sample name	Marker 4		Marker 9		Marker 10		Marker 1			Marker 2		1122		1001a		1008b		1011a			Marker 7			
	1	2	1	2	1	2	1	2	3	1	2	1	2	1	2	1	2	1	2	3	1	2	3	4
2022_BL4_2	275	275	206	209	327	330				272	272	157	157	146	146	359	362	214	220	223	283	290	292	
2022_Bl4_3	275	275	206	209	327	330	142	142		266	272	157	157	146	146	359	362	214	220	223	290	292		
2022_BL4.1_2	275	275	206	209	327	330	142	142		272	272	157	157	146	146	359	362	214	220	223	290	292		
2021_Bl2E	275	277	209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223		290	292	301	
2021_Bl2F	275	277	209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223		290	292	301	
2021_Bl2H	275	277	209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223		290	292	301	
2021_Bl2G	275	277	206	209	330	330	142	142		266	266	157	157	140	146	359	362	214	220		290	299		
2021_1A_1	275	275	209	209	330	330	142	145		272	272	157	157	146	146	359	359	220	223		299	299		
2021_1B_1	275	275	209	209	330	330	142	145		272	272	157	157	146	146	359	359	220	223		299	299		
2021_1J_1	275	275	209	209	330	330	142	145		272	272	157	157	146	146	359	359	220	223		299	299		
2020_Bl4G_2	275	277	209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223		275	277	288	
2020_Bl4E_2	275	277	209	209	330	330	142	148		266	266	155	157	140	146	359	362	220	223		275	277	284	286

Table 3-10, Virulence profiles for virulence-genotype associations, comprised of samples of B. lactucae collected from UK outbreaks tested using the IBEB Differential Set C, that also had a corresponding genotype. + indicates that the differential line was overcome. Outbreaks are separated by dotted lines.

	Green Towers	Dandie	R4T57 D	UC DM14	NunDm15	CG Dm16	Colorado	FrRsal-1	Argeles	RYZ 2164	RYZ 910457	Bedford	Balesta	Bartoli	Design	Kibrille
		Dm3	Dm4	Dm14	Dm15	Dm16	Dm18	Rsal-1	R38	Dm24/38	R52	R53	R54	R55	R56	Dm11, R57
2022_Bl4	+	-	+	+	+	+	-	+	-	+	+	+	-	-	+	-
2022_Bl4.1	+	-	+	+	+	-	+	+	-	-	+	+	-	-	+	-
2021_Bl2B	+	+	+	+	-	+	+	-	+	+	-	-	+	+	+	+
2021_Bl2D	+	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+
2021_Bl2H	+	-	+	+	-	+	+	-	+	-	-	-	+	+	-	-
2021_Bl1A	+	-	+	+	+	+	+	-	+	-	-	-	+	-	-	+
2021_Bl1B	+	-	+	+	-	+	+	-	+	-	-	-	+	-	-	-
2021_Bl1J	+	+	+	+	-	+	+	-	+	-	-	-	+	-	-	-
2020_Bl4E	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-
2020_Bl4G	+	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-

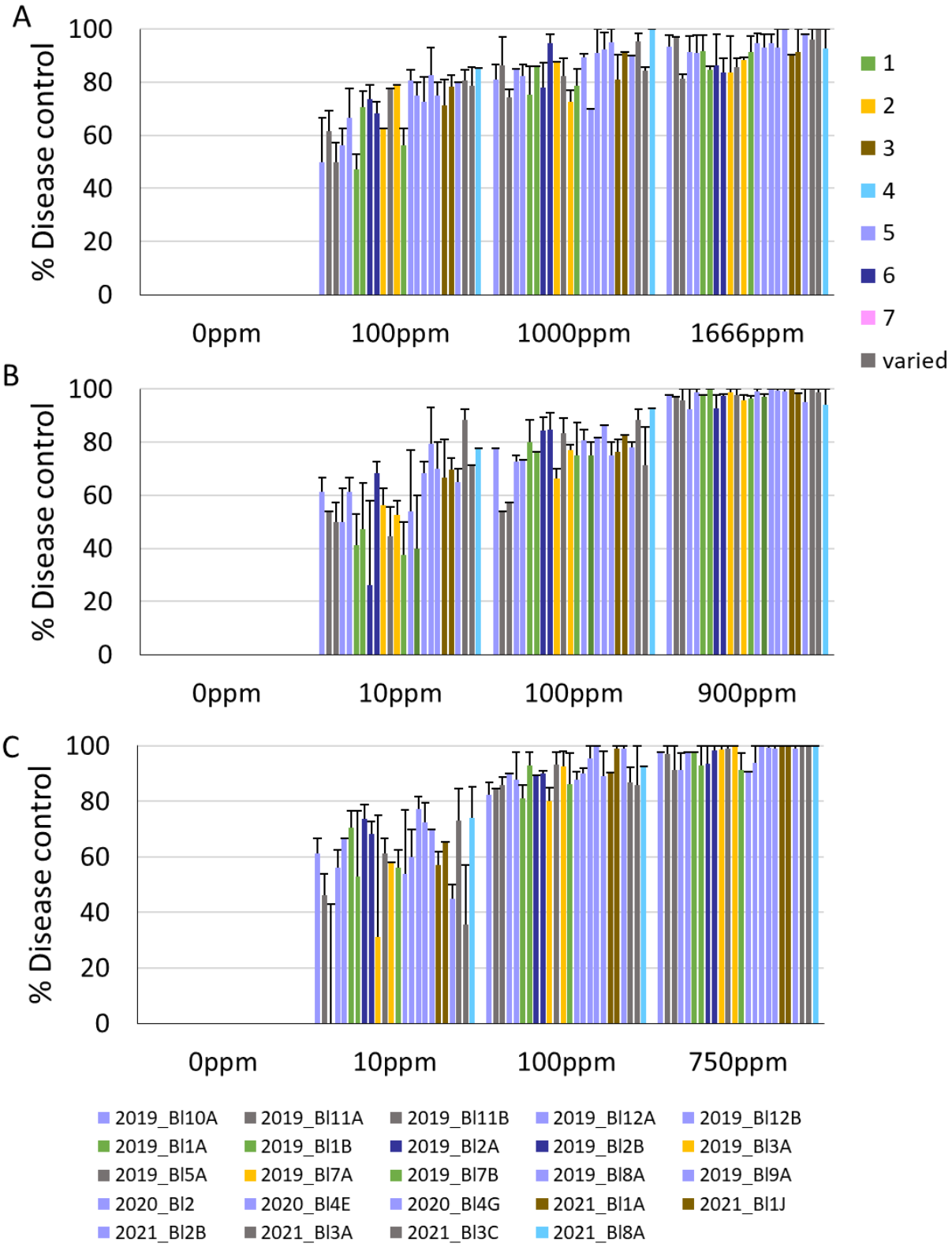


Figure 3-7 Average percentage of disease control of UK B. lactucae *samples from active ingredients A, azoxystrobin, B, dimethomorph, C, mandipropamid, applied at a range of concentrations compared to an untreated control and coloured by genotype cluster group (K=7). Graphs were adapted from CP 184 project data from Pettitt et al. (2020, 2023). Each isolate was tested in duplicate. Error bars represent the difference between replicates; however, the negative range was not displayed to enhance visibility of data. Samples and K cluster are listed in legend below. Note for each product the field rate is the highest fungicide spray used.*

3.4 Discussion

This study is the first usage of SSR markers to survey *B. lactucae* genetic diversity in the UK. The sample UK population collected between 2019 and 2022, showed high genotypic and allelic diversity using ten SSR loci. Each of the ten SSR markers had different discriminatory powers on the UK isolates tested (Table 3-2). Three of the ten markers (1001a, 1008b, and Marker 9) that had relatively lower discriminatory power could, in the future, be replaced with more informative markers. However, the ten markers combined produced a GAC curve that trended towards 100% with UK samples tested (Figure 3-3). Almost all replicate tests of isolates and samples from the same outbreak had identical genotypes which gave confidence to the SSR assay developed. Therefore, with 135 MLLs from 254 samples, the UK population may be considered genetically diverse (Table 3-1).

After adjustment for population size, consistency was observed in the population statistics characterising genetic diversity of UK samples, including Simpson's diversity index, expected MLL, evenness and Nei's unbiased gene diversity, across the years 2019 to 2022 (Table 3-1). Overall, there was a consistency across the sampled years. *K*-means clustering grouped samples mostly by outbreak, though some clusters had wide geographical spread, indicating localised genetic uniformity and a degree of regional gene flow. This was expected given that many outbreaks comprising a single clone were observed, whereas others were genetically diverse. The *K*-means clusters obtained did not perfectly correspond to the Bruvo's distance based dendrogram clusters and suggests an element of sexual reproduction and gene flow from other sources (Figure 3-5). However, the population was dominated by clones, and this was evident through the reduction in sample number from 254 to 135 after clone correction. The presence of clones did not skew allelic diversity metrics (Table 3-2) nor the MLL diversity (Table 3-1), and thus supports the absence of any single dominate clonal lineage in the population. Partitioning the genetic variance by AMOVA (Table 3-5) emphasised these trends of a diverse population; with clear structuring at "within post-district" and "between post-districts within county", which is indicative of local discrete populations spreading between outbreaks at these levels. The absence of an effect by year ("between year"), and "between county within year" indicates similarity in annual genetic diversity which would support clones emerging annually and some long-range spread between counties.

Samples collected from the UK exhibited genotypes with some tri-allelic or tetra-allelic markers. From the genotypes of representative samples from cluster group 5 in Table 3-3, notably there are triploid alleles at Marker 1 and Marker 7. With further examination, most of group 5 samples, if not triploid at these markers, were usually diploid with one or two of the three common alleles, suggesting they were variants of the tri-allelic strain that lost alleles. Multiple alleles at a SSR locus are indicative of somatic fusion, which with *B. lactucae*, heterokaryons can be presumed. The presence of heterokaryons in UK samples was anticipated as samples from the European population had evidence of heterokaryosis (Fletcher et al., 2019), and isolates obtained from Japan, Australia, and Wisconsin (US) also had genetic indicators of polyploidy and heterokaryosis (Gustafsson et al., 1985; Michelmore & Wong, 2008). However, the extent of heterokaryosis within a *B. lactucae* population has not been well documented. Of the 254 MLLs obtained from UK samples 168 had tri-allelic or tetra-allelic markers, providing evidence for widespread incidence of heterokaryosis within the UK population.

This study has also indicated an involvement of heterokaryosis with overwintering. The dominant *K*-cluster group emerging annually with high incidence of clonality, was group 5, which had tri-allelic markers. Trisomy has been linked with self-fertility via secondary homothallism (Michelmore & Sansome, 1982; Michelmore & Wong, 2008). Considering, group 5, and evidence for secondary homothallism reported in *B. lactucae* from an isolate collected from the UK, specifically from isolate IM25 from a NIAB trial ground in Cambridge (Michelmore & Ingram, 1981), homothallism and/or heterokaryosis may be a reproductive strategy of within the UK population. Therefore, there is evidence to support that secondary homothallism and the production of oospores, via heterokaryons, is making a contribution to the overwintering clonal lineages observed in the UK samples collected. Heterokaryons as a common means of propagation would be problematic for LDM management as heterokaryons have been found to improve fitness (Fletcher et al., 2019), allowing the pathogen to adapt to more diverse cultivars and to contain more diverse virulence profiles than non-heterokaryotic strains. Therefore, if trisomy and/or heterokaryons are prevalent and contribute to the source of overwintering oospores, *B. lactucae* management should consider the inclusion of soil treatment (fungicide or biological), or cultural controls similar to other soil-based pathogens (Barrière et al., 2014; Volynchikova & Kim, 2022).

The analysis of the ten SSR loci of UK samples consistently indicated that the population comprises a mix of asexually reproducing clones (some that can overwinter) in addition to the occurrence of sexual recombination. The allelic and expected MLL diversity, the expected heterozygosity and allelic evenness obtained from ten SSR loci from UK samples tested did not correspond to the expected results of a population with highly dominant clonal lineages (Table 3-1, Table 3-2). Structural analysis with AMOVA indicated discrete localised populations with occasional gene flow in and across county regions (Table 3-5). Linkage disequilibrium was not observed at any population strata tested, which indicated a population primarily comprised of asexual clones with MLG variants mostly resulting from allele loss and mutation. However, the presence of multiple novel *K* cluster groups does suggest the creation of new genotypes, thereby a population with mixed modes of reproduction (Table 3-6). This suggests genetic differentiation at more local and regional levels is common, and location is more important to genetic differentiation than year. Reportedly there is an inverse correlation between the occurrence of sexual and asexual reproduction, with the implication that there is suppression from utilising both modes (Michelmore & Ingram, 1980). Though the reason underpinning whether sexual or asexual reproduction occurs is not known, the suppression of reproductive types could explain why analysis indicates a preferentially asexual reproduction in UK representative samples. There is evidence that the European population tends to be diploid with high heterozygosity, and within some populations a frequent occurrence of sexual reproduction (Gustafsson et al., 1985; Michelmore & Wong, 2008). Heterozygosity was observed at moderate levels in UK samples collected, and samples with bi- and tri- allelic markers were observed. However, analysis of samples collected suggests that sexual recombination is not as prevalent as asexual reproduction.

While clonality was evident within the UK population, characterisation and tracking of lineages was more difficult than in other oomycete pathogens such as *P. infestans* (Cooke et al., 2012; Li et al., 2013; Lynott et al., 2023). Notably, most of the samples to represent the UK population were from commercial environments, and few from allotments, gardens or from *L. serriola* which is a wild host to *B. lactucae* present in the UK (Oswald, 2000; Thines et al., 2010). Having a larger sample pool from multiple environments would give more comprehensive information on clonality, heterokaryons, and recombination in the population. Samples that did come from allotments did contain virulence not observed in commercial environments in the same county, for example 2021_Bl4C, and 2021_Bl9A (data not shown) as such they could be a reserve of diverse virulence profiles as cultivars grown on commercial scale are likely different to what is grown by amateur growers. Considering allotments and weeds could be a source of non-commercial inoculum, and that inoculum can be aerially dispersed, public awareness of this disease or monitoring including non-commercial environments would be beneficial (Lebeda & Zinkernagel, 2003b, 2003a). Each differential line in set C was susceptible to at least one of the tested UK samples of *B. lactucae*. This observation raises concerns, given prior reports of *B. lactucae* populations retaining resistance-breaking capabilities, (Lebeda & Zinkernagel, 2003b, 2003a), leading to increasingly complex virulence patterns that ultimately increases the challenge of managing LDM with host resistance cultivars. Despite all differential lines being overcome by at least one UK sample, the proportion of UK samples that could overcome the differential Dm genes ranged from 0.13-0.98. This demonstrates there are still genes, like *Dm3*, that are widely effective against the majority of the UK pathogen population. In Europe, *Dm2*, *Dm3*, *Dm6*, *Dm7*, *Dm11*, and *Dm16* have been used frequently in cultivars. Interestingly from samples collected historically from 1983-1989 (excluding 1986) virulence that could match *Dm3* was present in the pathogen population (Crute, 1992b). Crute (1992) mentions that virulence matching to *Dm2* and *Dm3*, which are some of the earliest host resistances utilised in modern breeding programs, declined in frequency in 1984-1990 with the implication that the reduced use of this host resistance in cultivars meant retention of it was less important to the pathogen population. Virulence that could overcome *Dm7* was also reported as historically frequent in the UK pathogen population Crute (1992b) but IBEB differential set C and D did not test for this resistance gene.

Most of the UK samples did not show identical matched IBEB designated races, which hold significance for lettuce breeding companies in advertising *B. lactucae* resistance within their developed cultivars. Therefore, lettuce varieties with reported resistance to races Bl:16-37 EU can still be infected by *B. lactucae.* It is apparently a common finding, as many European samples exhibit discrepancies in virulence to the limited number of designated races, revealing a considerable degree of variation in virulence profile. According to an IBEB press release (Plantum, 2021), there is a discernible trend towards localised outbreaks being observed. This phenomenon is suggested to be the result of the integration of resistance genes. Given that the UK samples collected had virulence profiles that appeared to have greater similarity within than between outbreaks, it is plausible this phenomenon is true for the UK.

Evidence collected did not give conclusive support for a direct association between virulence and genotypic profiles. Virulence and genotype were more similar per outbreak or local region, than between outbreaks. In most cases, the variance between genotype and virulence in an outbreak was a few alleles or by a few differential lines. However, outbreaks not comprised of single clones were also observed. The associations between the genotype and virulence are more complex than the SSR assay can discriminate. This was not unexpected as SSR markers are theoretically neutral and there are many mechanisms that can alter virulence in oomycetes. Additionally, most resistance genes for *B. lactucae* are clustered together (Parra et al 2016) and the location of the ten SSR loci may not have been close to any *R* genes and therefore not associated with them. No fungicide insensitivity was observed in any samples tested, therefore no correlation between genotype and fungicide insensitivity could be derived. Further analysis of virulence and fungicide insensitivity with more samples could prove more informative considering there was an association reported by Crute (1992b).

Notably, there were virtually no novel alleles found in a range of samples genotyped from mainland Europe despite geographical isolation of the UK from mainland Europe (data not shown). Considering the widespread source of samples from different countries and their similarity in alleles but different allele combination, it seemed unlikely that the ten SSR loci could only effectively discriminate UK samples. It is plausible that the mainland European population is similar through gene flow with the UK.

Importantly, the data shows that the UK population of *B. lactucae* is variable in virulence and genotype, and evidence of prevalent heterokaryosis. As such, the challenge of managing *B. lactucae* is that likely most growers are contending with a selection of multiple diverse nuclei in the same isolate rather than individual races (Fletcher et al., 2019), which can result in multiple virulence profiles. Therefore, careful selection of *R* genes in cultivars for LDM management is needed to mitigate the risk of novel virulence to host resistance emerging. Especially, as there is evidence that virulence can persist in a *B. lactucae* population (Lebeda & Zinkernagel, 2003b).

3.5 Conclusion

Through genotyping 292 samples from more than 40 outbreaks over 3 years with ten SSR markers it was shown that the UK population of *B. lactucae* was highly diverse. Localised outbreaks were usually genetically distinct with a complex mix of virulence. Evidence was presented to show both clonal lineages that overwinter, with evidence of asexual reproduction but also gene-flow suggesting sexual reproduction. Heterokaryotic traits were prevalent in the UK samples collected suggesting heterokaryosis plays a key role in the population structure. As such this study suggests that the *B. lactucae* population in the UK is complex and utilises mixed reproduction methods. Virulence profiles shows that most host resistance can be overcome in set C of the IBEB differential assay. In addition to this, virulence profiles show fluctuating frequencies of *Dm* resistance through the years. Therefore, management of LDM should take into account that the population is comprised of genetically diverse isolates (with potentially multiple nuclei) and varying virulence profiles, with the potential of overwintering.

Chapter 4 Investigating selection in *B. lactucae*

4.1 Background

As previously established, *B. lactucae* can adapt when selection pressure is applied. Several studies have linked selection pressure from *R* genes and fungicides to change in *B. lactucae* populations. Fungicide-insensitive strains, for example to phenylamides (Brown et al., 2004; Cobelli et al., 1998; Crute et al., 1987; Schettini et al., 1991) or novel or more complex virulence profiles may emerge as a result of such selection pressures (Lebeda & Zinkernagel, 2003b; Trimboli & Nieuwenhuis, 2011; van Hese et al., 2016). Strains with **c**omplex virulence profiles or fungicide insensitivity have been noted to persist in the environment (Lebeda & Zinkernagel, 2003) and their presence in the local population is problematic for managing LDM.

Management practices and choice of cultivar and fungicides have been inferred to affect race diversity within the population (Lebeda & Zinkernagel, 2003b). In general *B. lactucae* population studies have focused on phenotype, particularly virulence profile (race), rather than specific genotype (Nordskog et al., 2014; Souza et al., 2022; van Hese et al., 2016). Fletcher et al. (2019) investigated the virulence of variant isolates arising from heterokaryons and examined the genetics of *B. lactucae* heterokaryotic strains. However, tracking of pathogen population change in a disease outbreak under field conditions has not been attempted. The dynamics of multilocus genotypes (MLGs) within a *B. lactucae* population have not been well studied, especially in response to disease management choices such as cultivar resistance.

Cultivar choice is important to agricultural production, with multiple commercial considerations, such as flavour, morphology and size, in addition to disease resistance. Most lettuce cultivars are marketed as having resistance to a range of IBEB designated *B. lactucae* races, without reference to the specific host resistance genes. Economically problematic races are designated by use of a standardised IBEB virulence test (IBEB, 2023). However, the IBEB virulence test has been adapted multiple times due to emergence of more complex virulence than the original assay could capture, and many combinations of virulence occurring in the field differ from IBEB race designation.

Cultivar choice can shape the race structure of *B. lactucae* populations due to gene-for-gene interactions between isolates and *R* genes, (Michelmore et al., 1984; Parra et al., 2016). Heterokaryotic *B. lactucae* isolates have been documented to lose virulence to specific *R* genes when infecting specific cultivars or to 'choose' one group of diploid nuclei over others due to differences in virulence to the host (Fletcher et al., 2019). The population genetic study described in Chapter 3 was unable to determine an effect of cultivar choice on the contemporary *B. lactucae* population in the UK, due to insufficient reporting of cultivars and the wide diversity of cultivars reported. By monitoring allelic diversity and variation in MLGs during artificially created epidemics with a range of pathogen genotypes on genetically diverse cultivars under field conditions this work aimed to gain a further understanding of the effect of cultivar choice on isolate selection.

Allele frequency distribution in a population can be used to infer evolutionary processes (Grünwald et al., 2017), therefore the allelic variation observed using SSR markers can be a proxy for the scale of mutations in the population when subjected to environmental stressors (Ellegren, 2004), which may include the limited availability of a suitable host. With the availability of data analysis techniques appropriate for polyploid organisms, which can also be applied to heterokaryons, the ability to genotype isolates and observe how selection can drive changes in population genetics in an outbreak is feasible (Kamvar et al., 2014).

These experiments focused on the impact of cultivar choice on population genetic diversity, specifically the ratio of MLGs and allelic diversity by cultivar over the course of an epidemic. Evidence for sexual or only clonal reproduction in the *B. lactucae* population was also assessed, to ascertain whether variance between samples could be attributed to genetic crosses between isolates.

Additionally, isolates were resampled from several lettuce cultivars after running the IBEB virulence assay and allelic differences in their genotypes scrutinised to examine if cultivars with differing *R* genes altered the allelic diversity of an isolate, and thus to seek preliminary evidence of a cultivar selection effect.

4.1.1 Aims

This study aimed to examine the effect of cultivar choice on selection for individual isolates of *B. lactucae* over time. Specific isolates of known genotype were released into a trial containing a range of commercial lettuce cultivars of differing resistance background and disease progress was monitored. At several assessment time points the pathogen population was resampled and genotyped using the multiplex SSR assay. Isolate selection according to host cultivar was represented by changes in the ratio of each distinct isolate on each cultivar over time, and the genotypic variation within each distinct isolate on each cultivar over time. In addition, an analysis of the genetic diversity was undertaken to determine whether the pathogen was reproducing via sexual or purely clonal means and if this was shaped by cultivar.

4.2 Methodology

4.2.1 Mark and recapture experiment

To analyse the effect of cultivar on pathogen population genetics under field conditions, a mark and recapture experiment was designed. Genetically distinct or 'marked' isolates, characterised using SSRs, were released in a field trial comprised of 3-4 different susceptible cultivars, and recaptured by sampling throughout the epidemic. Isolate ratios were identified, using the SSR multiplex assay on the samples collected from the trials. In addition, LDM symptoms were scored throughout the experiment. Two iterations of this trial were carried out, each with different isolate mixes and commercial cultivars.

4.2.1.1 Trial 1 2021

The available literature, and industry partners were consulted on relevant LDM susceptible cultivars to include. Four lettuce cultivars (cv Excalibur, Stallion, Actina, and Coventry) were planted in a randomised block design. Each plot comprised one cultivar planted in 2 rows of 12 plants (n=24) surrounded by guard plants and was infected with four genetically distinct UK *B. lactucae* isolates (Figure 1-1B). Lettuce plugs were obtained from Kettle Produce and kept in the glasshouse until transplanted into the field on the 18th of August 2021. Plots were covered with netting to prevent grazing by herbivores. The *B. lactucae* isolates were introduced via infector plants, which were placed in the centre of each plot on the 9th of September 2021.

Preparation of infector plants:

Infector plants were ~4-week-old plants of lettuce cv Green Towers inoculated on the 19th of August 2021 with an equal mix of inoculum of four UK *B. lactucae* isolates (2020_Bl2A, 2020_Bl4E, 2020_Bl4G, and 2021_Bl1B) each adjusted to 20,000 sporangia/ml. Strains 2020_Bl4E and 2020_Bl4G are grouped and referred to as 2020_Bl4 as both are variants of a presumed heterokaryotic strain and were difficult to distinguish genetically. However, they were both included in the analysis to understand changes in the population. After inoculation, infector plants were placed in a growth cabinet overnight in the dark at high RH (>90%). Infector plants were then left in a cool north-facing greenhouse, until angular discolouration was observed. When these symptoms were observed, a mini polytunnel was placed over the infector plants, and the plants were misted regularly to promote sporulation (Figure 1-1 A).

Figure 4-1 Images of Trial; top, apparatus to promote sporulation with infector plants, middle, trial layout including spore traps and netting, bottom right recapturing a single lesion.

When at least one sporulating lesion on each plant was observed and the weather conditions were conducive to infection, the plants were relocated to the field trial and one plant was placed in the centre of each plot.

Disease Scoring and lesion sampling

Field assessments were carried out every 3-4 days, from the 14th of September 2021 until the 14th of October 2021 giving a total of 10 scores. Disease assessments included percentage of leaf coverage that was 1) sporulating and 2) angular damage and discolouration by *B. lactucae* for each plant (Figure 4-2). Assessments were conducted exclusively on the visible foliage of each plant as accurate analysis of all leaves would require destroying the plant. To standardise scores a visual metric was developed and used for each assessment. Any other damage via rots or pests was also noted for each plant. An optimal sample comprised ten single lesions from across the plants in each plot. Each lesion was cut out using scissors that were wiped with 70% ethanol between plants. Each lesion was placed in a single labelled plastic zip-lock bag and returned to the laboratory for processing. Early in the epidemic when few lesions were evident care was taken to ensure that not all the lesions were sampled as it could delay or halt the epidemic. Three full sampling events were carried out, with a single sporulating lesion from each plant collected (example of a single lesion Figure 1-1 C). Single lesions were pressed onto FTA cards, air dried and single 3 mm disks were genotyped using the SSR assay developed in Chapter 2.

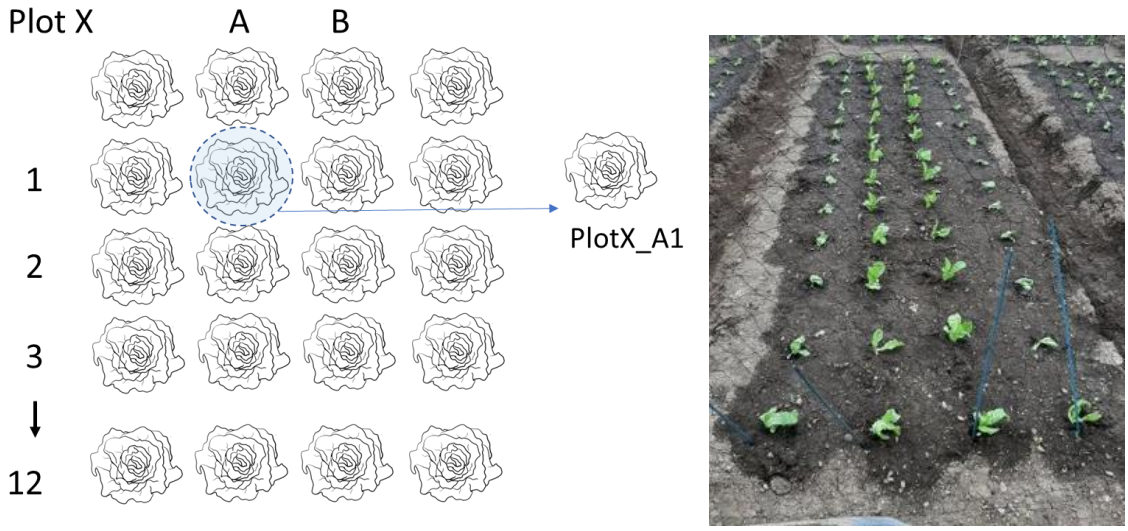


Figure 4-2 Labelling for each plant position within plot that was scored and a picture of a plot. Each plot had a total of 24 plants scored.

4.2.1.2 Trial 2 2022

In Trial 2, three lettuce cultivars (cv Stallion, Actina, and Coventry) were planted in a randomised block design. Cultivar Excalibur was not available, so the trial design was optimised for three cultivars by adding an additional block making five blocks with one plot of each cultivar per block giving a total of 15 plots. The trial was conducted according to the same methods as Trial 1, however, Trial 2 was inoculated with three genetically distinct UK *B. lactucae* isolates originating from the 2021 season at a concentration 5,700 sporangia/ml on the 18th of August 2022.

Lettuce plugs were transplanted in-field on the 28th of July 2022. Infector plants were placed into the centre of each plot on the 26th of August 2022. Disease assessments (percentage of plant foliage visibly damaged by angular discolouration and sporulation) were carried out every 3-4 days for each starting on 26th of August 2022 and ending on 17th of October 2022, using the same metric as Trial 1 Collection and processing of samples was the same as described for Trial 1.

4.2.2 Data Analysis

GeneMapper (v5.0) was used to process the electropherograms and adjust the allele peak calls. For example, alleles were renamed or adjusted to the 2bp or 3bp interval sizes required of the stepwise mutation model in calculating the Bruvo's genetic distance matrix which accommodates comparison of individuals of a different ploidy (Bruvo et al., 2004). Genotype assignment for the expected *B. lactucae* strains was carried out for each lesion to determine the ratio of inoculated strains in the epidemic population. To determine if undesignated samples were genotyping errors, the result of poor DNA, mutations or a new strain, the MLG assignment was also carried out in R. Data was filtered in R, first to remove samples with more than 2 loci missing, followed by a multilocus lineage (MLL) filter that grouped samples within specific Bruvo's distance thresholds into a single lineage. The following R packages and their dependencies were used to examine the structure and diversity of the population: *poppr* (v2.9.4; Kamvar et al., 2014, 2015), *magrittr* (v2.0.3; Bache & Wickham, 2022), *ggplot2* (v3.4.2; Wickham, 2016), *reshape2* (v1.4.4; Wickham, 2020), *adgenet* (v 2.1.10; Jombart & Ahmed, 2011), *ade4* (v1.7-22; Thioulouse et al., 2018), and *ape* (v5.7-1; Paradis & Schliep, 2019). Minimum spanning networks (MSNs) were generated in *poppr* to plot the abundance and genetic relatedness of the sample genotypes using semi-euclidean distances. Friedman's chi

squared and Nemenyi post hoc analysis were used to test the statistical significance of the differences in sporulation per cultivar, per block, and per block and cultivar, these were carried out using R package *PMCMRplus* (v1.9.8, Pohlert, 2014).

4.2.3 Preliminary examination of differential selection

The IBEB differential assay to determine virulence profile was carried out using seven live UK *B. lactucae* isolates. The protocol was the same as described in Chapter 3**.** After virulence scoring, single sporulating lesions on seedlings for every isolate x differential cultivar combination were pressed onto separate FTA cards. The isolates were genotyped using the developed SSR protocol (Chapter 2) and genotypic profiles were compared. As this was a preliminary test, sample numbers were insufficient to carry-out LD nor AMOVAs, therefore only population diversity (*poppr*), and allelic diversity (*poppr*), was carried out on the genotypic data. Data was filtered in R, to remove samples with more than 2 loci missing, and to remove loci that had more than 5% missing data, followed by a MLL filter that grouped samples within specific Bruvo's distance thresholds into a single lineage.

4.3 Results

4.3.1 Trial 1

4.3.1.1 Disease progression

Disease progress scored as angular foliar discolouration from chlorosis and necrosis attributed to LDM followed similar trends to angular sporulation attributed to LDM, in that they both increased over time by approximately the same amount (Figure 4-4). By 14 days after inoculation (DAI) angular sporulating lesions were present on multiple plants per block, with a mean percentage foliar discolouration of 0.23% and sporulation of 0.21%.

During the trial, patches of diffuse sporulation without initial angular chlorosis were observed (Figure 4-3). Sporulation was considered a more reliable indicator of LDM as discolouration could result from other stressors and area of sporulation was therefore used to monitor disease progression.

Despite the increase in LDM symptoms observed throughout the trial, the increases in symptoms were not equal between blocks. The mean percentage of leaf area showing LDM symptoms on living plants between blocks began to diverge for each cultivar at 26 DAI, with the final scoring (35 DAI) generally having the most pronounced difference between block repeats for each cultivar (Figure 4-4). For all cultivars block 1 had the lowest mean values for percentage of leaf area sporulation, which was most evident in the final scoring with cv Stallion, where the mean percentage of leaf area sporulation on living plants for block 1 was 0.54% and all other blocks were ≥8.40% (Figure 4-4).

Due to block repeats exhibiting differences in observed sporulation, the effect of cultivar on percentage leaf area sporulation per block, and the difference in mean percentage foliar sporulation per block were analysed. Overall, blocks did not differ in mean percentage of foliar sporulation (Friedman's chi squared = 7.35, df = 3, p-value = 0.06), but when data was grouped by block and cultivar there was a statistically significant difference (Friedman's chi squared = 40.46, df = 11, p-value <0.01). Nemenyi pairwise analysis showed statistically significant differences (p<0.05) between the mean percentage of leaf area sporulation on cv Stallion block 1 to the sporulation recorded on the following: Coventry block 2 and block 4, Excalibur block 1 and 3, and Stallion block 2 and 3.

Figure 4-3 Lettuce foliage affected by LDM; A, diffuse sporulation, B, clear angular sporulating lesions with faint angular chlorosis, C, angular necrotic lesions; D and E lesions on abaxial and adaxial surfaces of cv Actina leaf with sparse sporulation and highly confined lesions.

Differences in LDM symptoms per cultivar were evident in the final assessment of disease at 35 DAI, in which cv Coventry had a mean percentage of foliar sporulation value of 13.73%, Excalibur a value of 10.36% and Stallion 7.56% (Figure 4-4). This difference between the mean percentage of leaf area sporulation per cultivar was statistically significant (Friedman's chi-squared = 6.75, df = 2, *p*-value <0.05). The lowest *p*-value but highest level of statistical significance obtained from Nemenyi pair-wise analysis on the mean percentage of leaf area sporulation between cultivars was between sporulation observed on Stallion and the other two cultivars (both $p = 0.11$). Despite careful assessment, no disease was observed on cv Actina until the final sampling date (40 DAI) after all assessments occurred and therefore there was no data to be plotted or subject to statistical analysis.

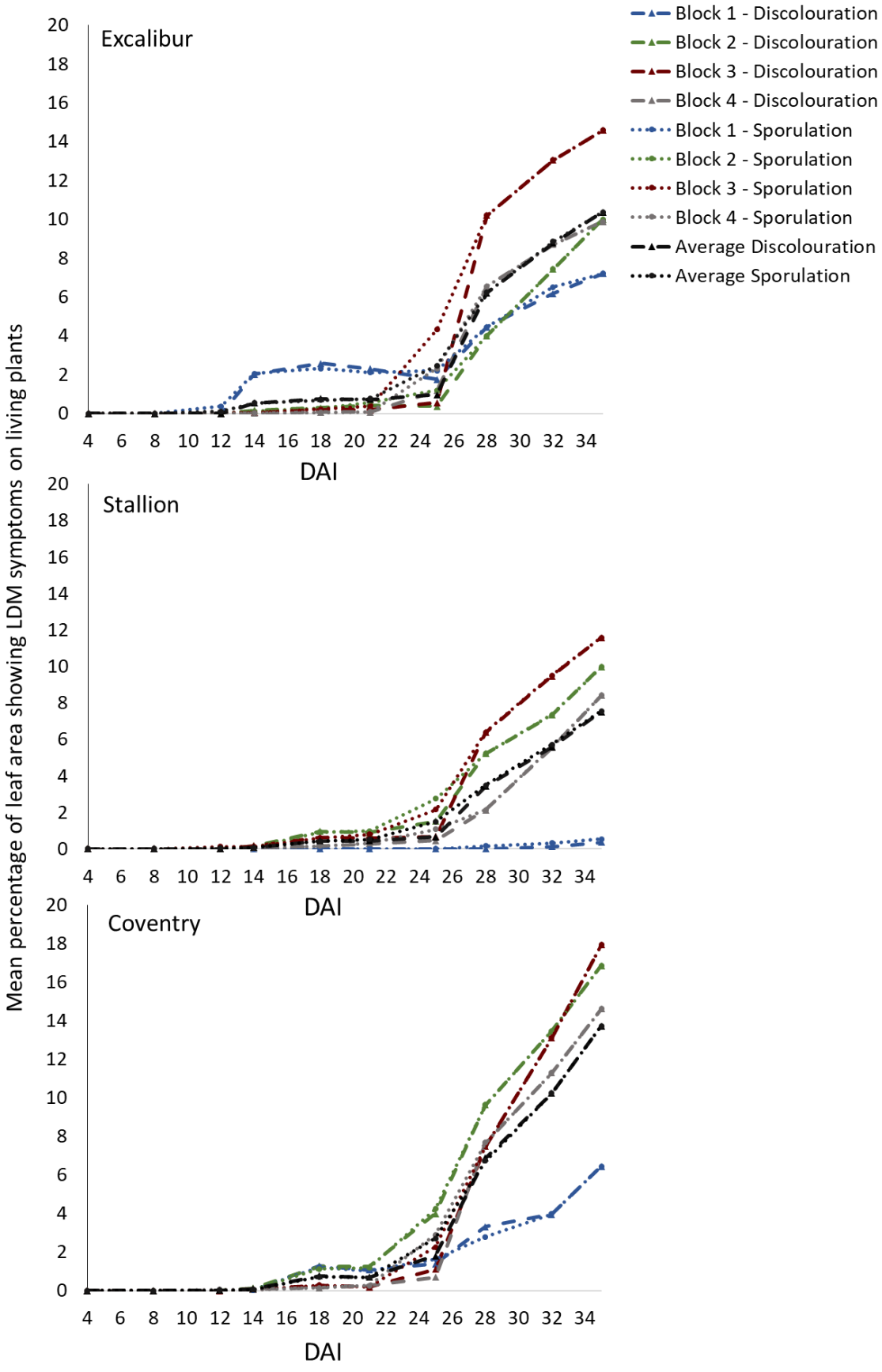


Figure 4-4 LDM progression curves for Trial 1 (Aug to Oct 2021), measured as mean percentage of leaf area of living plants affected by LDM angular discolouration and sporulation. DAI = is days after inoculation.

Table 4-1 Reference SSR genotypes for the B. lactucae *strains used in Trial 1, run in duplicate. Below each marker the numbers in the column header represents the allele number. The different colours and corresponding number (bp) at each marker represent a different allele.*

		Marker 4		Marker 9		Marker 10		Marker 1			Marker 2		Marker 5		1001a		1008b		1011a		Marker 7			
		1	2	1	2	1	2	1	2	3	1	2	1	2	1	2	1	2	1	2	1	2	3	4
2020_Bl2A	R1	275	277	209	209	330	330	142	148		266	266	157	157	140	146	359	359	223	223	292	303		
	R2	275	277	209	209	330	330	142	148		266	266	157	157	140	146	359	359	223	223	281	292	303	
2020_Bl4E	R1	275	277	209	209	330	330	142	148		266	266	155	157	140	146	359	359	220	223	290	292	299	301
	R2	275	277	209	209	330	330	142	148		266	266	155	157	140	146	359	362	220	223	290	292	299	301
2020_Bl4G	R1	275	277	209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223	290	292	301	
	R2	275	277	209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223	290	292	303	
2021_Bl1B	R1	275	275	209	209	330	330	142	145		272	272	157	157	146	146	359	359	220	223	299	299		
	R2	275	275	209	209	330	330	142	145		272	272	157	157	146	146	359	359	220	223	299	299		

4.3.1.2 Population structure in sampled lesions

Despite some minor variation, all four of the reference isolates used to inoculate the trial could be discriminated using the ten SSR loci (Table 4-1). Despite this, when examining the lesions resampled from the trial the isolates 2020_Bl4E and 2020_Bl4G, which were obtained from the same outbreak and had similar MLGs, could not be consistently discriminated at the Marker 1 and Marker 7, and were therefore grouped together as 2020_Bl4.

Isolate 2021_Bl1B was found to have caused disease on all cultivars (Figure 4-5) and was the most frequently occurring isolate (with a total incidence of 459 out of 859 genotyped samples). Isolate 2020_Bl2A had the lowest frequency of all isolates (with a total incidence of 22). Some isolates were constrained to certain cultivars. For example, isolates 2020_Bl2A, and 2020_Bl4 were only collected from cv Excalibur and Stallion. The only designated isolate collected from cv Actina and Coventry was 2021_Bl1B, the rest of the samples collected from these cultivars were undesignated to any released isolate. The cultivar Actina had only one sample collection as LDM symptoms were only observed on the final sampling day resulting in a total of 28 samples.

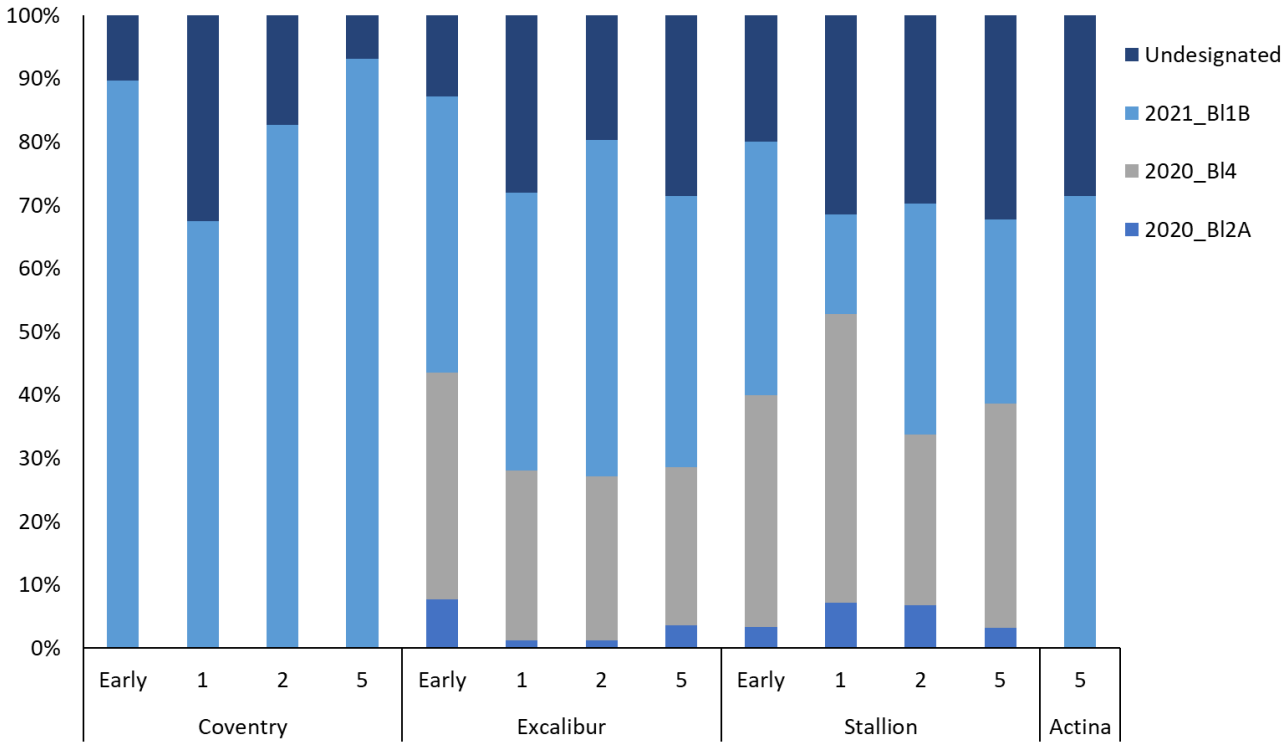


Figure 4-5 Ratio of each isolate's genotype recovered from 853 genotyped LDM lesions sampled on four sampling dates from four cultivars in Trial 1. Lesion numbers: n = 278, 286, 261, and 28 from cultivars Coventry, Excalibur, Stallion and Actina, respectively.

To further test whether cultivar affected isolate selection, all isolates, including those referred to as undesignated were examined in more detail to establish whether they were genetically similar to the initial reference isolates or constituted novel strains. MLGs were therefore combined into MLLs using a Bruvo's distance threshold. From 843 samples obtained over four sample dates, from four cultivars in Trial 1, 95 multilocus lineages (MLL: Bruvo's distance, average threshold 0.008) were identified (Table 4-2). When accounting for population size the number of these MLLs differed per cultivar (Actina eMLL: 3.00, Coventry eMLL: 4.02; Excalibur eMLL: 9.73; and Stallion eMLL: 12.97; Table 1-4). There was minor variation in the number of expected MLL (eMLL) per cultivar between sample collections (time-point), with samples from Coventry ranging between 1-1.00-6.03 eMLLs, samples from Excalibur ranging from 5.4-12.05 eMLLs, and samples from Stallion ranging from 8.79-14.91 eMLLs. The only sample collection from Actina yielded an eMLL score of 3.00.

Simpson's Index of diversity values, reflecting MLL diversity corrected to sample size, also exhibited variability between sample dates per cultivar. Specifically, samples collected from cv Coventry had Simpson's Index of diversity values ranging from 0.00 to 0.46, samples collected from Excalibur ranged from 0.62 to 0.80, and samples collected from Stallion ranging from 0.77 to 0.92 (lambda corrected: Table 4-2). Notably, the maximum Simpson's Index of diversity value from samples collected from cv Coventry was lower than the minimum values obtained from samples collected from cv Stallion and Excalibur, indicating a disparity in MLL diversity between cultivar sub-populations. The only sample collection for Actina had a Simpson's Index of diversity score of 0.14, and an evenness score of 0.41.

The evenness of MLLs observed varied between cultivar and sample collection, with samples from cv Coventry not exceeding an evenness score of 0.41 (E.5), samples from cv Excalibur ranging from 0.41-0.72 (E.5), and samples from Stallion ranging from 0.53-0.67 (E.5: Table 4-2). The highest evenness score was observed with samples collected from Excalibur in the early sample (E.5 = 0.72) and the lowest in samples collected from Coventry (E.5 = 0.35, E.5: Table 4-2). The expected heterozygosity also differed between cultivar and sample collection. Samples collected from Coventry had the lowest score (0.1 in early sample) whereas samples from Stallion had the highest (0.38, Hexp: Table 4-2). These data highlight that samples

collected from cv Coventry had a much lower genetic diversity, evenness of MLLs, and less expected heterozygosity than samples collected from cv Excalibur and cv Stallion.

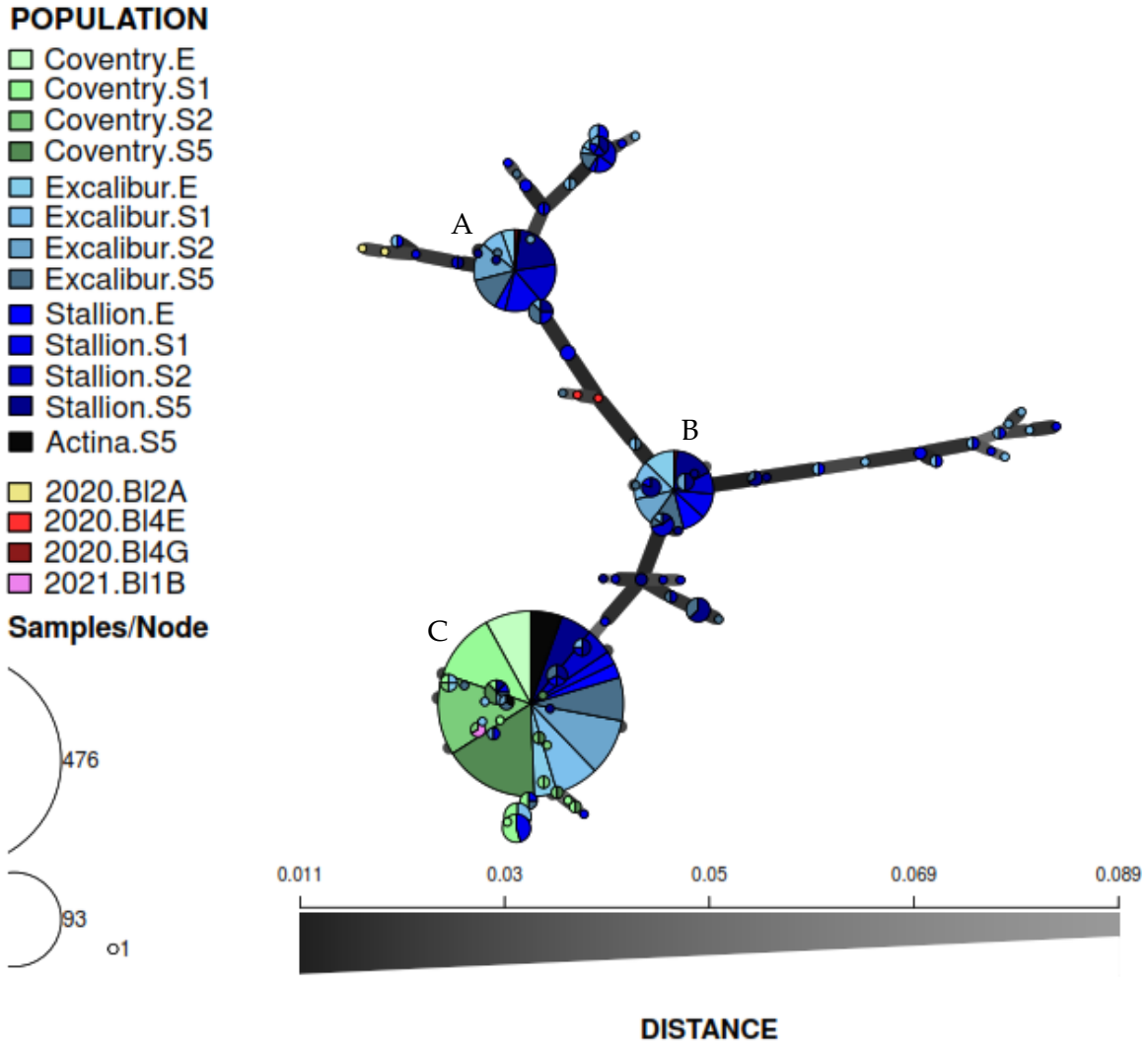


Figure 4-6 Minimum spanning network based on Bruvo's distances calculated SSR genotype data from Trial 1 samples compared to the reference isolates. Nodes are multilocus lineage groupings, and edges represent the Bruvo's distance between MLLs. A, B and C are three main clusters around the three largest nodes. The largest nodes comprised 93 samples (A), 87 samples (B) and 476 samples (C). 2020_Bl4 comprised 2020_Bl4E and 2020_Bl4G combined.

The MSN provides a graphical representation of the Bruvo's distance amongst the samples with each node indicating an MLL with its size proportional to the number of representatives (Figure 4-6). Edges connecting nodes are plotted with the thickness and darkness increasing as the genetic distances decrease, i.e. grey and thinner edges show greater genetic distance. The nodes are coloured according to the lettuce cultivar and sampling date and single nodes indicate the reference genotypes of the isolates as genotyped prior to inoculation. Of the three largest clusters in the MSN (Figure 4-6) cluster C comprised 476 sample genotype reads that were genetically most similar to isolate 2021_Bl1B and cluster B with 87 reads and cluster A

with 93 reads were most similar to the combined isolate 2020_B14. Each cluster contained a main node and minor genetically similar nodes. The genotypic profile of the single lesion samples from the Trial 1 epidemic thus broadly matched the profile of the distinct isolates used to inoculate the trial (Figure 4-6).

The MLL variations up to two nodes apart (with each a Bruvo's distance < 0.03) to the three main isolate nodes were counted, resulting in a total of 729 of 1024 sample genotypes that closely matched a released isolate. This dominant clustering of samples near an isolate MLL indicates that the undesignated samples from Figure 1-5 were closely related to the isolates released and the MLL variation observed stemmed from minor allelic variation of the released isolates. Isolate 2020_Bl2A did not appear to have a larger sample node clustered near its reference isolate genotype read, with instead nodes comprised of 1-2 samples each and being 2 nodes/edges away from the main node A of 2020_Bl4. Consistent with the SSR data in Table 1-1, the reference genotype of isolate 2020_Bl2A was genetically closer to reference isolate 2020_Bl4 and 2020_Bl4 MLL variants than to the 2021_Bl1B in the MSN. As the 2020_Bl4 isolates showed variation in markers that had three alleles that were consistent with being triploid the loss of the third allele may have created the genetic similarity to 2020_Bl2A. Note there were 14 alleles in common between these isolates and only 7 alleles that discriminated them. Consistent with the data shown in Figure 1-5 the genotypic profiles of samples from cv Coventry were genetically similar to the 2021_Bl1B samples in node C than any of the other distinct isolates released, suggesting that the undesignated samples in manual assignment were variants of 2021_Bl1B. Isolate 2021_Bl1B exhibited the lowest level of heterozygosity amongst the isolates tested, with only two loci (Marker 1 and 1011a) showing two or more alleles (Table 4-1). In the MSN generally, samples matching isolate 2021_Bl1B had fewest genetic variants. The main node of cluster C was surrounded by smaller nodes of MLL variants that were mostly only one or two mutational steps (each <0.03 Bruvo's distance) apart from the main node and comprised of ≤8 samples. The furthest MLL variant of 2021_Bl1B was 5 steps/nodes distant from the main cluster. In contrast the isolate 2020_Bl4 had more heterozygous loci and triploid allelic signatures in reference tests than isolate 2021_Bl1B (Table 4-1).

Table 4-2 Trial 1 population genetic diversity of B. lactucae samples based on 10 SSR loci with data analysed by poppr. Names of indices of genetic variation and their descriptions are in the table footnotes. Dataset shows MLL.N = number of samples. E = early sample, and S1, S2 and S5 correspond to lesion sampling at time points 1, 2 and 5.

Cultivar	Time point	N	MLL	eMLL[a]	SE[b]	H[c]	G[d]	lambda corrected[e]	E.5[f]	Hexp[g]
Actina	S5	28	3.00	3.00	0.00	0.31	1.16	0.14	0.44	0.13
Coventry	Total	276	15.00	4.02	1.38	0.67	1.30	0.23	0.31	0.13
	E	38	1.00	1.00	0.00	0.00	1.00	0.00	NaN	0.10
	S1	77	11.00	6.03	1.31	1.12	1.84	0.46	0.41	0.15
	S2	74	6.00	2.89	1.05	0.36	1.15	0.13	0.35	0.12
	S5	87	7.00	3.30	1.10	0.46	1.21	0.18	0.36	0.12
Excalibur	Total	285	50.00	9.73	2.04	2.18	3.73	0.74	0.35	0.35
	E	39	6.00	5.42	0.64	1.35	3.06	0.69	0.72	0.35
	S1	82	26.00	11.65	1.91	2.24	4.43	0.78	0.41	0.35
	S2	80	13.00	6.71	1.42	1.44	2.59	0.62	0.49	0.33
	S5	84	26.00	12.05	1.89	2.31	4.84	0.80	0.42	0.36
Stallion	Total	254	59.00	12.97	2.19	2.80	7.34	0.87	0.41	0.38
	E	29	9.00	8.79	0.41	1.68	3.91	0.77	0.67	0.37
	S1	69	27.00	14.91	1.83	2.78	10.37	0.92	0.62	0.39
	S2	63	17.00	10.35	1.56	2.11	5.19	0.82	0.58	0.36
	S5	93	30.00	12.98	1.96	2.59	7.45	0.88	0.53	0.38
Total		843	95.00	9.42	2.07	2.14	2.97	0.66	0.26	0.32

[a] eMLL is the expected MLLs at the lowest common sample size

[b] SE is standard error for rarefaction analysis used to create eMLLs

[c] H is Shannon-Wiener Index of MLL diversity (Shannon, 2001) the higher the number the higher the diversity, includes genotypic diversity and evenness in calculations, is sensitive to number of different MLLs and evenness.

[d] G is Stoddart and Taylor's Index of MLL diversity (Stoddart & Taylor, 1988), the higher the number the higher the diversity, includes genotypic diversity and evenness in calculations

[e] lambda corrected is Simpson's Index (Simpson, 1949) of diversity, corrected to population size, values from 0-1, with 0 being no diversity, and 1 all genotypes are different.

[f] E.5 is Evenness (Grünwald et al., 2003), values from 0-1, with 0 being complete unevenness.

[g] Hexp is Nei's unbiased gene diversity (Nei, 1978), i.e. expected heterozygosity, values from 0-1, with 0 not heterozygous/ no diversity.

In the MSN, the isolate 2020_Bl4 had two main clusters (A and B). The largest nodes of each of these were four steps apart (of <0.03 Bruvo's distance) and had more variant MLL branches with smaller nodes of 1-3 samples in the MSN going up to 8 steps from the main node of the cluster (Figure 4-6). The distance of the first MLLs from 2020_Bl4 clusters tended to closer to a Bruvo's distance of 0.011 than 0.03, whereas for 2021_Bl1B the first MLL variants tended to be closer to 0.03 Bruvo's distance away from the main cluster.

Analysis of the total genetic variation of tested loci was partitioned to experimental factors using AMOVA. The highest percentage of variation observed for samples collected was always 'Within cultivar' (>70%) rather than 'Sample collection time points', regardless of clone correction (Table 4-3). The percentage of genetic variation for tested SSR loci 'Within cultivar' increased with clone correction (from 76.61% to 87.57%) which suggests the population is likely clonal and asexually reproducing. According to the AMOVA results the population is less likely to be sexually reproducing and genetic material is not being shared between the isolates released. This suggests therefore that the genetic variance found in tested loci was from existing variance in the isolates themselves.

Linkage disequilibrium is an analysis where the null hypothesis assumes panmixia and that loci are completely unlinked, whereas for clonal populations a significant result rejecting the null hypothesis is expected as there is more likely to be linkage amongst loci therefore no linkage disequilibrium. No linkage disequilibrium was observed in the loci within the samples collected from the same cultivar, both with and without clone correction of the genotypes of each isolate group collected from the same cultivar, (Table 4-4). Only for clone corrected samples collected from cv Actina, which had three MLLs, did the observation p= 0.002 for r_D = 0.63 (Table 4-4) fall in the right tail of expected distribution if random mating occurred. The linkage disequilibrium analysis shows that for the isolates collected from same cultivar have high linkage which indicates populations are clonal and therefore asexually reproducing.

Table 4-3 Results of Analysis of Molecular Variance (AMOVA) showing the partitioning of genetic variation among and within sample collections and cultivars during Trial 1. Dataset shows MLL and clone correction Note clone correction was with nine loci omitting LM7.

Dataset treatment	Populations	Df	Sum Sq	Mean Sq	Sigma	%	Phi	*p*-value[b]
MLL filtered[a]	Between Sample collections (time-points)	3	0.19	0.06	-0.00353	-10.21	-0.102	0.942
	Between cultivars within sample collections (time-points)	9	6.93	0.77	0.012	33.60	0.305	0.001
	Within cultivars	830	22.00	0.03	0.027	76.61	0.234	0.001
	Total	842	29.11	0.03	0.035	100.00		
Clone-corrected and MLL filtered	Between Sample collections (time-points)	3	0.17	0.06	-0.00290	-6.21	-0.062	0.686
	Between cultivars within sample collections (time-points)	9	1.10	0.12	0.009	18.64	0.175	0.001
	Within cultivars	123	5.04	0.04	0.041	87.57	0.124	0.001
	Total	135	6.30	0.05	0.047	100.00		

a – MLL filtered with Bruvos distance using the average distance as it was the highest threshold value, with a threshold of 0.007877103.

b – Out of 999 permutations

In addition to an algorithm applied for MLL groupings, data filters were applied to remove samples with low DNA concentrations from the analysis to mitigate genotyping errors. Therefore, the genetic variation observed between MLLs is likely to be derived from genuine biological variation at these loci in the population rather than from genotyping errors. Both the linkage analysis and AMOVA analysis on samples collected from each cultivar showed that clonal lineages were present. Samples presumed to be derived from isolate 2020_Bl4 had more variation in alleles per marker than samples presumed to be derived from 2020_Bl2A and 2021_Bl1B. Such samples from 2020_Bl4 had more MLL variants with more mutational steps than from other isolates in the study. The longest chain of linked MLLs was comprised of nine variants from the main node of 2020_Bl4 (cluster B), compared to a five node branch of sample variants from the main node of 2021_Bl1B (cluster C). This was greater than that observed from 2020_Bl2A and 2021_Bl1B (Figure 4-6). In combination all the data strongly suggests that the genetic variation observed in samples collected is from the isolates themselves rather than sexual reproduction between the released isolates.

Table 4-4 Summary of multilocus LD analysis results for each of the Cultivar populations from Trial 1 both with and without clone correction

Dataset	**Cultivar**	**No. of samples**	**I_A**	**r_D**
MLL filtered	Actina	28	2.70 [a]	0.68 [a]
	Coventry	278	2.23 [a]	0.43 [a]
	Excalibur	285	2.98 [a]	0.52 [a]
	Stallion	254	2.62 [a]	0.42 [a]
	Total	846	3.02 [a]	0.50 [a]
Clone-corrected and MLL filtered	Actina	3	2.35 [b]	0.63 [b]
	Coventry	15	1.45 [a]	0.25 [a]
	Excalibur	50	1.04 [a]	0.14 [a]
	Stallion	59	1.19 [a]	0.15 [a]
	Total	136	1.34 [a]	0.17 [a]

I_A = Index of association

r_D = rBarD

a = p <0.01

b = p <0.05

4.3.2 Trial 2

4.3.2.1 Disease progression

Widespread chlorosis and sporulating lesions were observed after 18 days on the cultivars Stallion and Actina, with the mean percentage of leaf area sporulation 2.6% and 2.5% respectively (Figure 4-7). All of the plots of cultivar Coventry developed disease symptoms later than the other cultivars, with the first incidence of disease 25 DAI (for 3/5 plots), whereas for cultivars Excalibur and Stallion sporulation was first observed 4 DAI. Due to the later onset of disease on cv. Coventry the trial was monitored for longer (52 days) than Trial 1 (35 days), in order to obtain three full sets of lesion samples from cv Coventry.

The longer assessment period resulted in a decline in the observed LDM symptoms which was due to an expanding area of late season lettuce rot which obscured symptoms and ultimately resulted in plant death. In the final assessment 297 plants out of 360 remained of which 83 were scored as showing rotting symptoms. Of all cultivars, rot affected Stallion the most, of a starting total of 120 plants, 35 died, and 62 plants had observed rot by the final assessment. This explains the marked decline in recorded LDM from around day 32 onwards and also the slight declines in blocks of cultivar Coventry (Figure 4-7).

With the exception of the data for Stallion (as described above), the LDM symptoms scored by angular discolouration and sporulation generally increased throughout the duration of the trial. The data for two types of disease score were very similar (usually within 5%) with the main differences being between replicate blocks in the trial (Figure 4-7). Of the three cultivars, Coventry had the largest difference in mean percentage of leaf area sporulation between blocks, which was evident at 45 DAI, where mean percentage leaf area sporulation was 0.1% in block 1 and 19.0% in block 4.

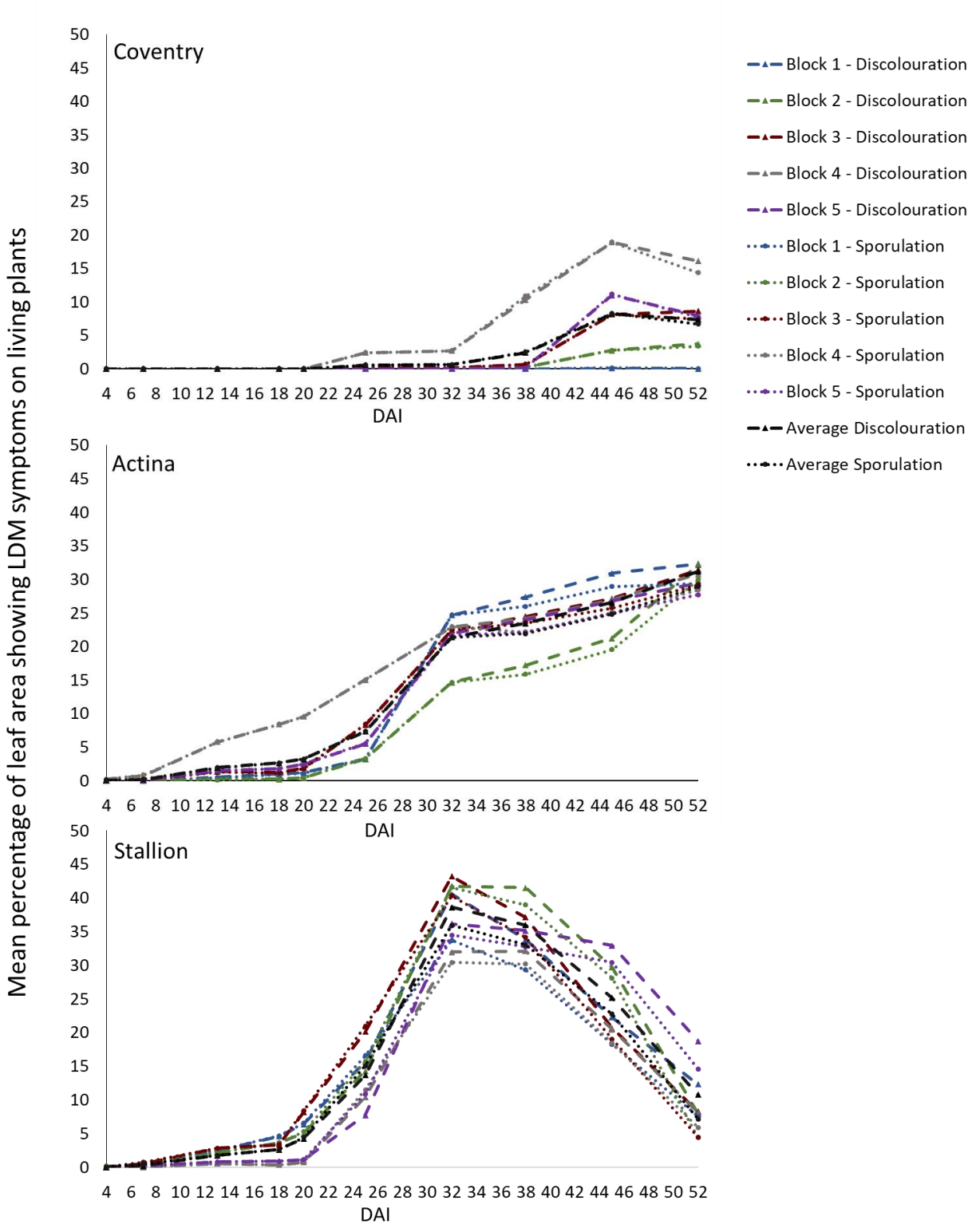


Figure 4-7 LDM progression curves for Trial 2 (Jul to Oct 2022), measured as mean of leaf area of living plants affected by angular discolouration and sporulation. DAI is days after inoculation. High incidence of rot obscuring LDM symptoms led to cv Stallion having a drop in LDM symptoms from DAI 32 onwards.

There were statistically significant differences in levels of foliar sporulation between blocks with Nemenyi pair-wise analysis identifying differences between block 4 and both block 2 and 5 (Friedman chi-squared = 18.64, df = 4, *p*-value <0.01). To further identify the source of this difference, the mean percentage of leaf area sporulation values were grouped per cultivar per block which also showed statistically significant differences (Friedman chi-squared= 94.54, df = 14, *p*-value < 0.01) and pairwise analysis identified multiple combinations of cultivar and block that were significantly different in observed sporulation (data not shown). Noticeably, sporulation on cv Coventry in blocks 1 and 2 were both significantly different (p <0.05) to sporulation observed on cv Stallion in blocks 1, 2, 3, and 5.

4.3.2.2 Population structure in sampled lesions

Marker 2 was difficult to score for samples obtained from Trial 2; it did not amplify efficiently and was subsequently removed from the analysis. The data on the genotypes amplified from the lesions sampled from each cultivar Trial 2 showed the epidemic was dominated by two main genotypes (Figure 1-8). One matched the released isolate, 2021_Bl8A and the other a common pattern that did not match any released isolates but was subsequently named the T3_283 strain. The isolate 2021_Bl8A was observed to be highly cultivar specific, only being isolated from cv Coventry. Isolates 2021_Bl3A and 2021_Bl4C were present in later samples (sample collections 2 and 3) collected from cultivars Actina and Stallion. During manual MLL assignment one single incidence of the isolate T3_283 was detected out of 267 Coventry samples from S2. Similarly to Trial 1, the proportion of undesignated samples varied between time points and cultivars with the highest proportion of undesignated samples being 38% in samples collected from cv Actina in full sample collection 1, and the lowest proportion being 5% in samples collected from cv Stallion in sample collection 2.

The 916 sample genotypes obtained from ten SSR loci were analysed using *poppr* in R which identified 52 multilocus lineages (MLL: Bruvo's distance, farthest threshold 0.011;Table 4-5). Of the three cultivars, samples from Coventry had the lowest expected MLL (14.00 eMLL), followed by samples from Actina (19.20 eMLL), and then samples from Stallion (23.60 eMLL). In addition, samples collected from cv Coventry had the lowest observed Simpson diversity value (lambda corrected = 0.32) when accounting for population size, (Samples from Actina = 0.36, samples from Stallion = 0.47, Lambda corrected: Table 4-5).

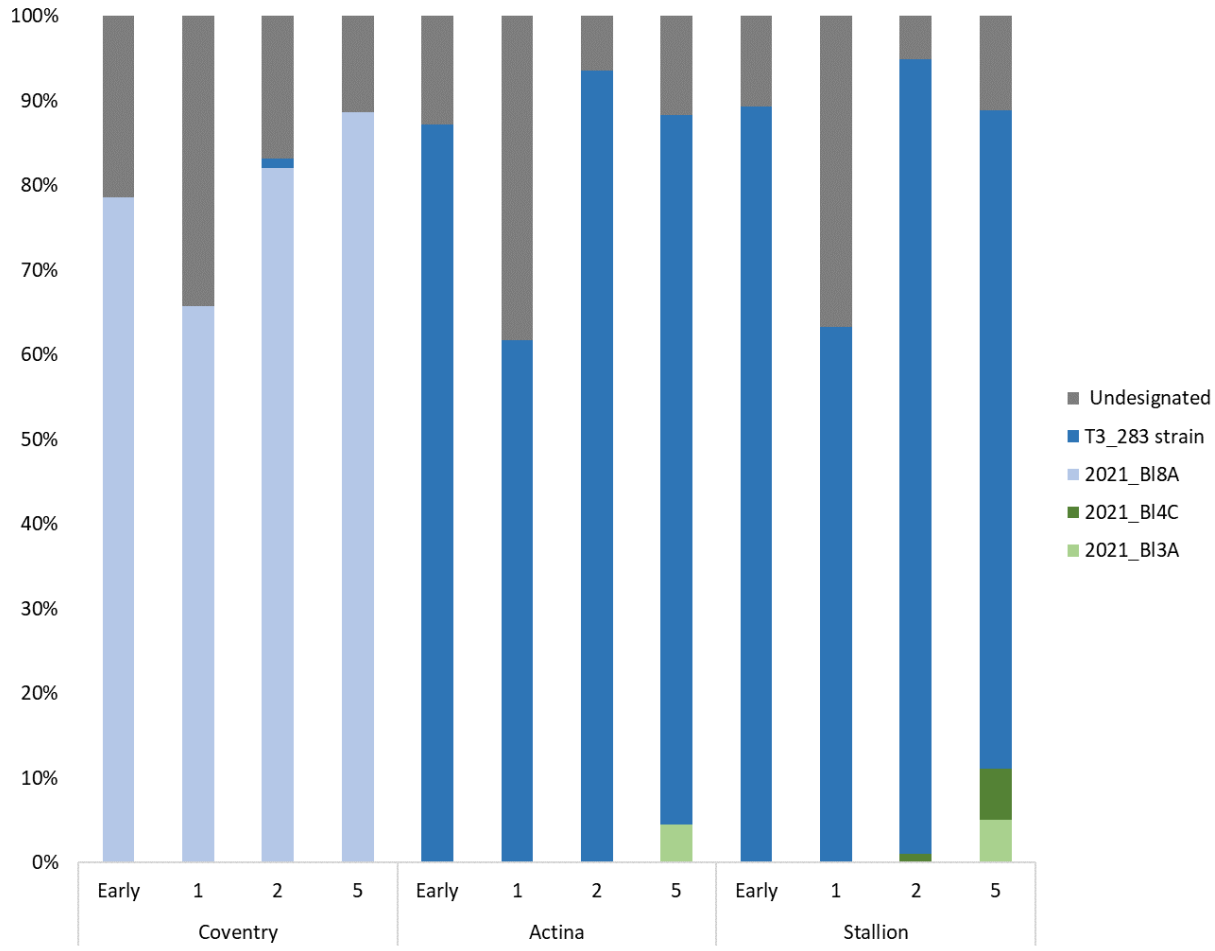


Figure 4-8 Ratio of each isolate's genotype recovered from 947 genotyped LDM lesions sampled on four sampling dates from four cultivars in Trial 2. Lesion numbers: n = 264, 360, and 323 from cultivars Coventry, Actina and Stallion, respectively.

An objective MSN analysis of designated and undesignated sample genotypes using Bruvo's distances identified two large clusters centred around large nodes comprised of many samples; cluster A which had a main node of 232 samples, and B with a main node of 617 samples (Figure 4-9). Cluster A corresponded to isolate 2021_Bl8A and Cluster B corresponded to the non-released T3_283 strain. Genotypes matching isolates 2021_Bl3A and 2021_Bl4C were uncommon and formed smaller clusters with the main nodes comprised of 2 (cluster C) and 9 (cluster D) samples respectively (Figure 4-9).

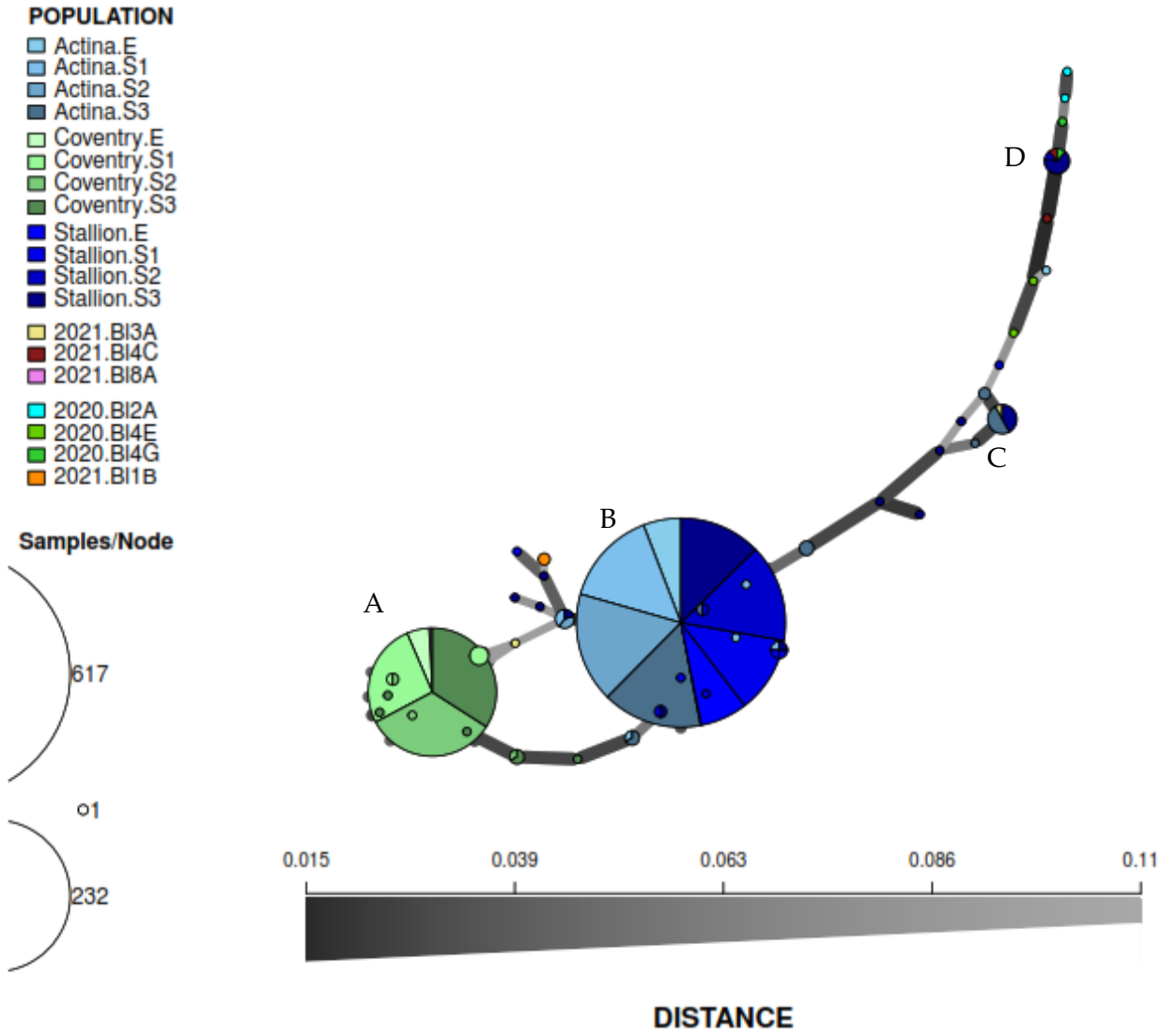


Figure 4-9 Minimum spanning network of Trial 2 samples against the reference races using Bruvo's distances with average threshold. Reference isolates from Trial 1 and 2 were included. Nodes are multilocus lineage (MLL) groupings, and edge colour and shade in proportion to the Bruvo's distance between MLLs. A, B, C and D are four main clusters around the four largest nodes. The largest nodes were comprised of 232 samples (Cluster A), 617 samples (B), 12 samples (C) and 9 samples (D).

The MLL nodes up to two mutational steps away (with each node a Bruvo's distance below 0.03 apart) from each cluster's main node were counted, resulting in a total of 902 of 947 genotype reads closely corresponding to an isolate genotype. This indicates that undesignated strains in Figure 4-8 were mostly closely related to the isolates released and or isolate T3_283. A single sample from cultivar Coventry was noted within the cluster B dominated by the non-released genotype T3_283 strain that was mainly collected from Stallion and Actina and these may represent errors in labelling or processing or perhaps a rare cross over of genotypes from one cultivar to another due to mutation (see discussion).

The T3_283 strain was checked against the reference isolates from Trial 1 to see if it was a variant of any released isolate. Interestingly, the closest reference isolates to the T3_283 strain were 2021_Bl1B (from Trial 1) and 2021_Bl3A. However, both isolate reference reads were two or more nodes removed with Bruvo's distances 0.03 and above. When checking for common alleles (at all markers except Marker 2), T3_283 had 7 alleles in common with 2021_Bl1B and 10 with 2021_Bl3A and differed by 6 alleles to both 2021_Bl1B and 2021_Bl3A. However, at Marker 7, T3_283 had a novel allele suggesting that was a different lineage or had mutated.

There were many smaller nodes of similar MLLs around the isolates 2021_Bl4C and one replicate of 2020_Bl3A, all from the final sample of cv Actina and Stallion. This suggests that these low numbers of these two isolates in manual assignment was in part attributed to the number of variant strains of these isolates, which would have been classified as undesignated in the manual assignment. Interestingly, isolate 2021_Bl4C appeared to be closely related to 2020_Bl4 isolates from Trial 1. The 2020_Bl4 samples came from Cambridgeshire (CB24), which was also where isolate 2021_Bl4C originated, and were both part of the *K* cluster group 5 (Chapter 3). Therefore, similarities observed between these samples are likely due to them being from the same lineage.

Analysis of the partitioning of total genetic variation for Trial 2 samples was tested by AMOVA with sample populations grouped by factors such as sample collection date and the cultivar they were isolated from. The highest percentage of genetic variation observed in AMOVA was 'Between cultivars within sample collection time-points' at 124.16% when data was not clone corrected (Table 4-7), which likely indicates clonal reproduction, as a proliferation of genetically similar *B. lactucae* samples within cultivars would result in greater genetic differentiation between cultivar sub-populations of *B. lactucae*. An increase in the percentage of genetic variation portioned in the 'Within cultivars' sub-populations of *B. lactucae* with clone correction (from 16.53% to 73.08%) also suggests the population is likely clonal and asexually reproducing. The AMOVA results indicates that the Trial 2 population of *B. lactucae* is clonally reproducing which in turn implies that the genetic variance found in tested loci was not from genetic recombination of loci but from other sources (see discussion).

Table 4-5 Trial 2 population genetic diversity of B. lactucae samples based on 10 SSR loci with data analysed by poppr. Names of indices of genetic variation and their descriptions are in the table footnotes. Dataset shows MLL. N= the number of samples. E = early sample, and S1, S2 and S3 correspond to lesion sampling 1, 2 and 3.

Cultivar	Time point	N	MLL	eMLL[a]	SE[b]	H[c]	G[d]	lambda corrected[e]	E.5[f]	Hexp[g]
Actina	Total	351	23	19.20	1.61	1.01	1.56	0.36	0.32	0.18
	E	37	4	2.05	0.80	0.37	1.18	0.16	0.40	0.12
	S1	97	13	4.18	1.17	1.36	2.44	0.60	0.50	0.17
	S2	107	5	1.70	0.74	0.29	1.12	0.11	0.36	0.10
	S3	110	14	3.52	1.25	1.02	1.59	0.37	0.33	0.21
Coventry	Total	246	14	14.00	0.00	0.84	1.46	0.32	0.35	0.33
	SE	13	2	2.00	0.00	0.43	1.35	0.28	0.66	0.29
	S1	67	6	3.45	0.88	0.99	1.91	0.49	0.54	0.25
	S2	81	8	2.47	0.98	0.59	1.30	0.23	0.37	0.34
	S3	85	8	2.58	1.02	0.62	1.31	0.24	0.36	0.34
Stallion	Total	319	28	23.60	1.80	1.25	1.87	0.47	0.35	0.18
	E	47	5	2.46	0.88	0.54	1.30	0.24	0.43	0.12
	S1	76	9	3.95	1.06	1.26	2.42	0.60	0.57	0.17
	S2	97	8	2.17	0.94	0.48	1.21	0.18	0.34	0.19
	S3	99	19	4.99	1.34	1.65	2.79	0.65	0.43	0.23
Total		916	52	4.48	1.28	1.66	2.73	0.64	0.41	0.26

[a] eMLL is the expected MLLs at the lowest common sample size.

[b] SE is standard error for rarefaction analysis used to create eMLLs.

[c] H is Shannon-Wiener Index of MLL diversity (Shannon, 2001) the higher the number the higher the diversity, includes genotypic diversity and evenness in calculations, is sensitive to number of different MLLs and evenness.

[d] G is Stoddart and Taylor's Index of MLL diversity (Stoddart & Taylor, 1988), the higher the number the higher the diversity, includes genotypic diversity and evenness in calculations

[e] lambda corrected is Simpson's Index (Simpson, 1949) of diversity, corrected to population size, values from 0-1, with 0 being no diversity, and 1 all genotypes are different.

[f] E.5 is Evenness (Grünwald et al., 2003), values from 0-1, with 0 being complete unevenness.

[g] Hexp is Nei's unbiased gene diversity (Nei, 1978), i.e. expected heterozygosity, values from 0-1, with 0 not heterozygous/ no diversity.

No linkage disequilibrium was observed between the loci on isolates collected from the same cultivar when sampled from cv Actina or Stallion, both with (Actina: $r_D = 0.11$, $p < 0.01$; Stallion: $r_D = 0.11$, $p < 0.01$) and without clone correction (Actina: $r_D = 0.35$, $p < 0.01$; Stallion: $r_D = 0.45$, $p < 0.01$; Table 4-6). The clone corrected data from samples collected from Coventry showed observations falling into the tail of the resampled distribution with indications of linkage disequilibrium ($r_D = 0.07$, $p < 0.05$). All other samples showed that there was significant linkage between loci which implies that the sub-populations of *B. lactucae*, which were collected from the same cultivar, are not sexually reproducing.

Table 4-6 Summary of multilocus linkage disequilibrium analysis results for each of the Cultivar populations from Trial 2 both with and without clone correction

Dataset	**Cultivar**	**No. of samples**	**I_A**		**r_D**	
MLL filtered	Actina	351	1.94	a	0.35	a
	Coventry	246	0.46	a	0.16	a
	Stallion	319	3.16	a	0.45	a
	Total	916	1.77	a	0.32	a
Clone-corrected and MLL filtered	Actina	17	0.86	a	0.11	a
	Coventry	11	0.17	b	0.07	c
	Stallion	22	0.93	a	0.11	a
	Total	83	1.10	a	0.13	a

I_A = Index of association

r_D = rBarD

a = $p<0.01$

b = $p>0.05$

c = $p<0.05$

Table 4-7 Results of Analysis of Molecular Variance (AMOVA) showing the partitioning of genetic variation among and within sample collections and cultivars during Trial 2. Dataset shows MLLs with and without clone correction.

Dataset treatment	Populations	Df	Sum Sq	Mean Sq	Sigma	%	Phi	*p*-value[b]
MLL filtered[a]	Between Sample collections (time-points)	3	0.57	0.19	-0.01462	-40.69	-0.407	0.906
	Between cultivars within sample collections (time-points)	8	26.82	3.35	0.045	124.16	0.883	0.001
	Within cultivars	904	5.37	0.01	0.006	16.53	0.835	0.001
	Total	915	32.76	0.04	0.036	100.00		
Clone-corrected and MLL filtered	Between Sample collections (time-points)	3	0.24	0.08	-0.00371	-7.10	-0.071	0.669
	Between cultivars within sample collections (time-points)	8	0.97	0.12	0.018	34.02	0.318	0.001
	Within cultivars	48	1.83	0.04	0.038	73.08	0.269	0.001
	Total	59	3.04	0.05	0.052	100.00		

[a] - MLL filtered with Bruvo's distance using the average distance as it was the highest threshold value, with a threshold of 0.007877103.

[b] - Out of 999 permutations

Table 4-8 Reference SSR genotypes for the B. lactucae *strains used in Trial 2 along with dominant resampled genotype termed T3_283. Below each marker the numbers in the column header represents the allele number. The different colours and corresponding number (bp) at each marker represent a different allele.*

	Marker 4		**Marker 9**		**Marker 10**		**Marker 1**			**Marker 2**		**Marker 5**		**1001a**		**1008b**		**1011a**			**Marker 7**		
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**3**
T3_283	275	277	206	209	330	330	142	142				157	157	146	146	362	362	220	220		283	283	
2021_Bl3A	275	277	206	209	327	330	142	142		266	266	157	157	146	146	359	362	215	220	223	290	292	
2021_Bl4C	275	277	209	209	330	330	142	145	148	266	266	155	157	140	146	359	359	220	223		290	292	301
2021_Bl8A	275	277	206	209	327	330	142	142		266	266	157	157	146	146	359	362	220	223		297	299	301

4.3.3 Selection of alleles of isolates of *B. lactucae* on lettuce differentials

The preliminary experiment to explore the effect of passaging isolates of *B. lactucae* through differential cultivars (and the *R* genes they contain) indicated genetic differences in the SSR profile in many cases. Seven isolates were tested before and after virulence testing with the number of tested samples ranging from 6 to 9 as it was dependent on the number of differentials overcome. A total of 51 lesions from differentials and despite the limitations of only a single replicate lesion per isolate differential combination the findings were of note.

Marker 2 was removed from analysis by filtering in R to remove loci that had more than 5% missing results, showing inconsistent amplification of Marker 2. From the 51 lesions, 27 MLGs were identified, which were combined into 22 MLLs using a Bruvo's distance threshold (MLL: Bruvo's distance, furthest threshold 0.016). No consistent pattern of allele loss was observed; mean allele number was generally lower in the reference isolate than in the isolates resampled from the differentials (Table 4-9). The isolate with the largest difference was 2022_Bl7A with 1.56 alleles in the reference compared with a mean of 2.33 alleles in the six resampled lesions.

Nei's unbiased gene diversity (Nei, 1978), which indicates expected heterozygosity, was between 0.24-0.39 for both reference isolates and samples collected from differentials. For some isolates, the reference isolate genotypes had a higher Nei's diversity value than those collected from differentials, such as 2022_Bl4.1 (Ref: Hexp = 0.39; Diff: Hexp = 0.31) and 2022_Bl5A (Ref: Hexp = 0.39; Diff: Hexp = 0.28). Conversely, reference isolate genotypes of 2022_Bl5B (Ref: Hexp = 0.27) and 2022_Bl7A (Ref: Hexp = 0.24), had a lower Nei's diversity value, than those resampled from the differentials (2022_Bl5B Diff: Hexp = 0.32; 2022_Bl7A Diff: Hexp = 0.36). Allele evenness was largely consistent for differentials and reference genotypes, with values between 0.77-1.00

Of all the isolates tested in this experiment, 2022_Bl7A was the most variable with only three of all ten markers having identical alleles across all six genotyped samples which unsurprisingly lead to multiple MLLs in the differential sub-group, (Figure 4-9).

New alleles not previously observed in the reference isolates were recorded. For example, within data from isolate 2022_Bl4, there was one genotype from a sample collected from a differential that had four alleles at Marker 7 whereas, the reference genotype only had two or three alleles at the same marker (data not shown). For isolate 2022_Bl6A, samples collected

from the differentials cv Green Towers (Set C- S0; the universal susceptible) and cv Bartoli (Set C – S13) had an additional allele in Marker 4 (data not shown). Isolate 2022_Bl5B had the most new alleles in samples collected from differentials; all six differential samples were heterozygous at Marker 10 and 1008b unlike the reference isolate that was homozygous at these markers (data not shown).

Table 4-9 Allelic diversity analysis results between the reference (Ref) B. lactucae *isolates and the samples resampled from differentials (Diff) based on nine SSR loci with data analysed by poppr. Names of indices of allelic variation and their descriptions are in the table footnotes.*

Isolate		Sample Number	Mean allele number per locus	Lambda corrected[a]	Hexp[b]	Evenness[c]
2022_Bl4	Ref	3	1.78	0.31	0.36	0.98
	Diff	9	1.89	0.31	0.32	0.96
	Total	12	1.89	0.31	0.32	0.96
2022_Bl4.1	Ref	2	1.67	0.30	0.39	1.00
	Diff	9	1.67	0.29	0.31	0.99
	Total	11	1.67	0.29	0.31	0.99
2022_Bl5A	Ref	2	1.67	0.30	0.39	1.00
	Diff	9	1.67	0.26	0.28	0.91
	Total	11	1.67	0.27	0.29	0.94
2022_Bl5B	Ref	2	1.44	0.18	0.27	0.98
	Diff	6	1.67	0.30	0.32	1.00
	Total	8	1.78	0.30	0.31	0.95
2022_Bl6A	Ref	2	1.67	0.27	0.35	0.92
	Diff	6	1.78	0.32	0.35	0.93
	Total	8	1.78	0.31	0.33	0.91
2022_Bl6B	Ref	3	1.67	0.28	0.34	0.89
	Diff	6	1.67	0.29	0.31	0.97
	Total	9	1.78	0.30	0.31	0.88
2022_Bl7A	Ref	2	1.56	0.19	0.24	0.98
	Diff	6	2.33	0.33	0.36	0.81
	Total	8	2.56	0.32	0.34	0.77
All	Ref	16	2.33	0.31	0.32	0.77
	Diff	51	2.33	0.31	0.31	0.86
	Total	67	2.78	0.31	0.31	0.77

[a] Lambda corrected = Simpson's Index (Simpson, 1949) of diversity, corrected to population size, values from 0-1, with 0 being no diversity, and 1 all genotypes are different

[b] Hexp = Nei's unbiased gene diversity (Nei, 1978), i.e. expected heterozygosity, values from 0-1, with 0 not heterozygous/ no diversity.

[c] Evenness (Grünwald et al., 2003), values from 0-1, with 0 being complete unevenness.

In most cases where an allele was not previously recorded in reference isolates, the peak height was the same or lower than the alleles present in the reference sample genotype (data not shown). An example would be the sample of 2022_Bl7A collected from (Set C- S6; Colorado), which had two novel alleles (283 and 297) at Marker 7 with peak heights of 1209 and 563 relative fluorescent units (RFU) respectively. Comparatively the two commonly occurring alleles, 290 and 292, had peak heights of 1714 and 1960 RFU.

Not all variation was generated from novel alleles. Variation observed in sample genotypes was initially present in reference genotypes for the isolates 2022_Bl4.1, and 2022_Bl5A, where the allelic variation in genotype reads was in general from losing an allele and becoming homozygous at some markers (data not shown). This is reflected by the decrease in the Nei diversity value from genotype reads of the isolate reference (2022_Bl4.1 Ref: Hexp = 0.39; 2022_Bl5A Ref: Hexp = 0.39), to the samples collected from the differentials (2022_Bl4.1 Diff: Hexp = 0.31; 2022_Bl5A Diff: Hexp = 0.28). The loss of alleles in differential collected samples for isolates 2022_Bl4.1 and 2022_Bl5A, did not counteract the impact of novel alleles, with the Nei's diversity value genotypes of samples collected from differentials (Hexp = 0.32) only marginally higher than from references (Hexp = 31;Table 4-9).

There were more MLLs observed in the samples collected from differentials (15 MLLs out of 26 MLL; Figure 4-10) than the reference genotype reads. MLL's recurred across isolates released: MLL 19 was assigned to reference reads of isolate 2022_Bl4, 2022_Bl4.1, 2022_Bl5A, and was assigned to at least one sample collected from differentials (excluding 2022_Bl7A). Every read for all isolates except 2022_Bl7A samples had seven shared alleles, with an additional eight alleles in most reads. Notably the origin of isolates tested were from two counties, Lancashire, and Fife, both of which have had associations in genotype with 2019 samples in *K* – cluster group 1 (Chapter 3).

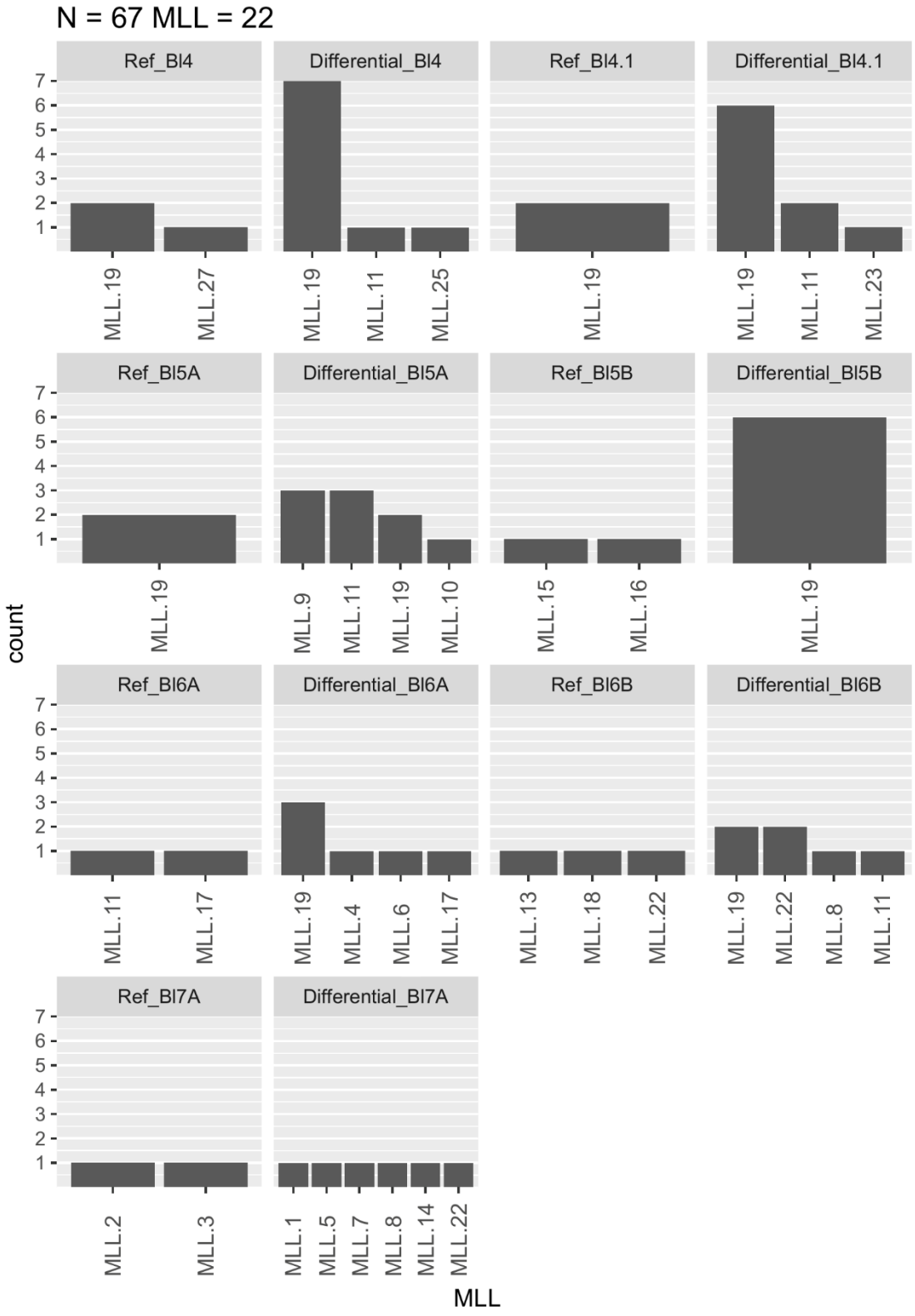


Figure 4-10 Plot of the named MLLs and their frequency observed in seven reference isolates (the upper seven plots and then the same isolates resampled after infecting differential lines of lettuce (lower seven plots). Individual plot headings show isolate names with the prefix Ref for reference isolate names and Diff (Differential) for the same isolates sampled after infecting differential lettuce seedlings.

4.4 Discussion

The results obtained from the two field trials showed a clear effect of cultivar on the frequency of different isolates sampled from the trial *B. lactucae* population. In both trials genotyping proved that a distinct pathogen population developed on lettuce cultivar Coventry than on the other cultivars in the trials. These case-studies demonstrate the gene-for-gene effect (Michelmore et al., 1984), where isolates are either able or unable to infect a cultivar depending on the efficacy of the host resistance to *B. lactucae* present and show the potential importance of cultivar choice as a management tool by growers to alter the pathogen population present in field.

Another key observation was that in these trials the extensive sampling and genotyping of many hundreds of lesions did not yield any strong evidence of sexual recombination amongst the isolates within the epidemics. The number of MLLs in the population were in general consistent throughout the trials. This was of interest as there were multiple different isolates present on the same cultivar, which would have presented the opportunity for recombination or competition, both of which would modify the MLL number. Genetic variation in isolates before and after exposure to cultivars did not appear to be as a result of crosses between isolates, as the population was predominantly clonal. In support of this, AMOVAs and linkage disequilibrium analyses showed that asexual reproduction was predominant in both field trials. Sexual reproduction in populations of *B. lactucae* has been documented in European fields (Gustafsson et al., 1985; van Hese et al., 2016), and in general, European populations were considered to utilise sexual reproduction rather than heterokaryosis and polyploidy which appeared to be common in isolates from Australia, and Japan (Gustafsson et al., 1985; Michelmore & Wong, 2008). More recent research found heterokaryosis to be common in European isolates (Fletcher et al., 2019). Once an isolate is established on a host it undergoes repeated asexual cycles producing aerial sporangia. Therefore, the prevalence of clonal reproduction in the Hutton trials was in part expected. The lack of evidence for sexual reproduction obtained from this study could be due to incompatibility between isolates, as generally two mating types are required for sexual reproduction (Michelmore & Ingram, 1980). Inability to obtain standard *B1* and *B2* strains prevented compatibility type assignment to isolates used.

The many distinct MLGs and MLLs detected in the trials and the fact that reproduction was asexual suggests that genetic variation either came from novel mutations or represented genetic variation that was pre-existing within the isolates. The latter scenario can emerge from heterokaryosis where multiple genetically distinct diploid nuclei are present at variable ratios in the same isolate. Consistent with this, the increase in genotype variation was particularly clear in the isolates with more than two alleles at a marker, which may be presumed to be heterokaryotic. Using whole genome sequencing Fletcher et al, (2019) reported that some heterokaryotic strains were stable in heterokaryosis, but others separated into homokaryotic derivatives when under selection pressure. Unstable heterokaryosis may therefore have contributed to the allelic variation observed in the isolates recaptured after exposure to the cultivars in these trials. It cannot be ruled out that some lesions pressed onto FTA cards were mixed, particularly as lesions became more difficult to distinguish, resulting in some variation. However, this is unlikely to have had a significant effect overall, as variations of released isolates were observed earlier in the trial when lesions were sparse and distinct.

Heterokaryons have been reported to generally grow faster than homokaryotic derivatives (Fletcher et al., 2019). Isolate 2020_Bl4 is presumed to be heterokaryotic, and it therefore seems counter intuitive that MLLs in this isolate were minimal in Trial 1 in comparison to 2021_Bl1B. However, a key aspect noted by Fletcher et al. (2019) is that homokaryons do perform better than heterokaryons if one of the nuclear products of the heterokaryon is recognised by a host resistance and the homokaryon products are not. This could explain why isolates 2021_Bl1B and T3_283 performed well (i.e. dominated) in Trial 1 and Trial 2 respectively, despite not having strong indications of trisomy/tri-alleles in the genotype data. Conversely, for isolate 2020_Bl4 which was tri-allelic, the MSN figure shows (Figure 4-6) it had higher numbers of MLL variants, none of which matched the reference isolates of 2020_Bl4 in the MSN.

In this study isolate selection by cultivar was shown as plots of the changes in the frequency of each distinct isolate on each cultivar over time (Figure 4-5 and Figure 4-8). There was an expectation that the frequency of isolates on each cultivar would change over the course of the epidemic reflecting that some isolates would be better at infecting the cultivars used in the trials. This is what was observed in a similar 'mark and recapture' type of trial with *P. infestans* (Cooke et al., 2012). However, no significant shift in isolate frequency was observed in either

trial across multiple sample time points. The only differences in isolate frequency was between different cultivars, indicating strong selection on isolates by cultivar. In particular a greater diversity of isolates was recovered on Excalibur and Stallion in Trial 1 and on Actina and Stallion in Trial 2 than on Coventry that appeared to be infected by only a single genotype in both trials.

An explanation for the difference in frequency of these isolates may be due to differing aggressiveness. Aggressiveness refers to quantitative traits that affect the variation of pathogenicity on a susceptible host, such as lesion size, sporulation rate or infection efficiency (Pariaud et al., 2009). If an isolate was less efficient in infection or had a lower sporulation rate, then the first transfer from infector plant to the field could skew the population proportions, in this case the population ratio, as observed with the Cooke et al. (2012) trial. Aggressiveness of each isolate could only be reflected in the frequency of isolates obtained, as it is difficult to quantify aggressiveness from latency and sporulation observed as neither could be directly linked to an isolate.

In each trial there was one cultivar that exhibited symptoms of LDM comparatively later than the other cultivars, cv Actina in Trial 1 and cv Coventry in Trial 2. The late development of symptoms in these two cultivars were attributed to different reasons. Notably in Trial 1, cv Actina presented LDM symptoms in the form of relatively small lesions with necrotic dots and more sparse sporulation than observed in other cultivars, whereas cv Coventry had clear angular discolouration and sporulation typical of LDM. The IBEB scoring sheet for virulence often considered necrotic speckles, even with sparse sporulation, as an indicator of partial resistance (IBEB, 2023). Partial resistance with hypersensitive necrosis has been reported with some *Dm* genes (Crute & Norwood, 1978), and the effectiveness of *R* genes has been reported to decrease with low temperatures (Judelson & Michelmore, 1992). The *R* genes present in cv Actina and cv Coventry are not publicly available therefore efficacy of *R* genes in these cultivars be it partial, or temperature based is unknown. The isolate that was collected from cv Actina at the end of Trial 1 was prevalent on samples collected from the other cultivars, and sporulation was sparse with necrotic dots This suggested that a combination of partial resistance and/or the high pressure of disease and inoculum load ultimately rendered cv Actina susceptible. By contrast, in the case of Trial 2, the isolate that affected cv Coventry,

2021_Bl8A was highly cultivar specific, and only collected from this cultivar. There were susceptible plants present (infector plants) that could have provided a reservoir for 2021_Bl8A, however it is likely that the isolate 2021_Bl8A was less 'aggressive' than the other prevalent isolate T3_283, in that its latent period may have been longer or its infection efficiency was lower (Pariaud et al., 2009). Latency period has been reported to change depending on temperature and RH (Scherm & van Bruggen, 1994), which did change during the trial. Considering cv Coventry in Trial 2 was predominately affected by 2021_Bl8A and had less foliar sporulation than the other two cultivars which had T3_283 present (Figure 4-7 and Figure 4-8) and no acute necrosis was observed, it supports the idea that it was not partial resistance that led to delayed symptoms but rather that the isolate 2021_Bl8A was less aggressive than the T3_283 isolate.

Selection was further examined with the comparison of the SSR genotypes of seven reference isolates of *B. lactucae* to that of DNA of same isolates resampled from FTA cards from lesions that had infected seedlings of cultivars in the IBEB differential assay. The hypothesis being tested here was that the selection pressure from the differential resistance genes may be reflected in the change in of MLL. In this analysis, the resampled samples exhibited more allelic diversity than the initial reference genotype reads. If an isolate had heterokaryotic signatures and cultivar selection led to certain alleles being preferentially selected, the allelic diversity would be higher in the reference sample for that isolate. This was not observed in the data collected. In general, the allelic variability observed was thought to have come from the isolates themselves rather than *de novo* mutation. That is, nuclear sorting allowing for one genetically distinct diploid nucleus to be preferentially selected over another, as seen by Fletcher et al. (2019). However, some novel alleles were observed in the recollected samples that were not previously recorded in the references. A plausible explanation for the presence of the new alleles can be from replication slippage (Dufresne et al., 2014), which can occur naturally in the pathogen or may occur during PCR. Changes in peak height ratios were also observed in this experiment and it is possible that some rarer alleles were undetectable in the reference isolate, but that infection of the differential cultivar imposed strong selection that increased the frequency of the nuclei containing these rare alleles to detectable levels. It was not possible to relate specific genetic changes to individual differential lettuce cultivars in this study so the results for the 6 to 9 resample isolates were pooled in each case (Figure 4-10 and

Table 4-9). Further research into cultivar selection through a standardisation of collecting FTAs to genotype after the virulence test would be beneficial in part to measure whether the isolate was polyploid or heterokaryotic. To be sure that cv Green Towers is filtering out complex virulence or preferentially selecting nuclei, an FTA sample prior to bulking inoculum on cv Green Towers and before sporangial suspension use in the differential assay would need to be included.

Another limitation is the lack of knowledge on the *R* genes within the cultivars utilised, which was not publicly made available or is unknown. Knowing the resistance genes in the cultivars used would help relate back to the sextet virulence profile of the isolates released allowing the determination of whether cv Actina host resistance broke down in Trial 1, and to give more information into which *R* genes prevented Coventry from being infected by 2020_Bl4 and 2020_Bl2A in Trials 1 and 2 respectively.

An interesting avenue for future studies would be to continue the trial in the same location, to see if previously used isolates re-emerge and influence the population ratio. Oospores have been found at lettuce outbreak sites and some studies have shown that they can lead to infection (Blok, 1981; Morgan, 1983; Yuen & Lorbeer, 1987). Trisomy in *B. lactucae* has been linked to self-fertility and the production of oospores (Michelmore & Sansome, 1982; Michelmore & Wong, 2008). Evidence of the prevalence of overwintering clonal heterokaryotic (tri and tetra-allelic) strains in the UK population was unknown until analysis of the UK population (Chapter 3) in this work. The Hutton trials did not take into consideration the impact of overwintering oospores in the population structure.

4.5 Conclusion

This study aimed to look at the effect of selection from cultivars on a *B. lactucae* population over an outbreak by examining two artificially induced field epidemics using SSRs. The SSR assay was able to capture differences in MLLs. The results suggested that cultivar does affect the population genetics as evidenced by the different proportion of MLLs in samples collected from each cultivar. Different levels of sporulation between cultivars in light of different predominating MLLs suggests differing aggression or virulence between isolates. Cultivar choice therefore does influence the local pathogen population.

Further data analysis suggested genetic variation in samples collected likely came from within the isolates themselves, and was not the result sexual reproduction, supporting the idea of preferential asexual reproduction as suggested by van Hese et al., (2016) and the benefits of the heterokaryotic state (Fletcher et al., 2019). Data from a preliminary test into cultivar selection on the genetic variants within an isolate found that genotypic variation was exposed after selection by a range of differential lines.

Empirical evidence into cultivar selection on a single heterokaryotic isolate and further homokaryotic derivatives, would be beneficial in furthering the knowledge of cultivar selection in the presence of heterokaryotic isolates.

Chapter 5 Validation and application of a real-time LAMP assay for the detection of *B. lactucae*

5.1 Background information

5.1.1 Benefits of diagnostic assays:

Early pathogen detection plays a pivotal role in curtailing plant disease epidemics and their subsequent impacts. Rapid detection enables prompt interventions to mitigate further propagation and spread (Buja et al., 2021; Crandall et al., 2018). In the context of managing LDM, early detection is particularly important as the aesthetic and foliar damage caused can reduce marketability of the lettuce crop. The causal pathogen *B. lactucae,* has been reported to complete its asexual cycle in as few as four days (Verhoeff, 1960), producing aerially dispersed sporangia which can facilitate rapid field-wide transmission. Consequently, effective disease prevention is of paramount importance in lettuce production, often involving prophylactic use of chemical control. Use of chemical control in the absence of inoculum of *B. lactucae* increases production costs in an industry challenged by increasing agrochemical and seed costs (DEFRA, 2022). Prophylactic application of fungicides, especially if one active ingredient is used repeatedly, also exerts selective pressure on the pathogen population, increasing the risk of fungicide insensitive pathogen strains emerging (Brown et al., 2004; Cobelli et al., 1998; Crute et al., 1987).

For sustainable cropping of lettuce, stewardship of pesticides is required. One approach is to alter the timing of fungicide application in line with the presence of the pathogen, ultimately reducing environmental impact and production costs without compromising crop quality. Thus, the development and validation of an assay to detect inoculum under field conditions can aid in optimising fungicide applications, benefitting the environment and improving cost efficiency, whilst concurrently mitigating selective pressure for fungicide resistance.

5.1.2 *B. lactucae* diagnostic assays

Early detection of *B. lactucae* has previously focused on the asexual aerial sporangia, which are presumed to be the main source of crop infection (Michelmore & Wong, 2008; van Hese et al., 2016). Previous studies have monitored the aerial space for sporangia using samplers, from which sporangia can be counted or from which DNA can be extracted and subsequently tested

for the presence of pathogens using diagnostic assays (Carisse & Philion, 2002; Fall et al., 2015; Kunjeti et al., 2016). Research has also focussed on altering spray programs, with fewer applications made when the weather is not conducive to disease development (Hovius et al., 2007; Scherm et al., 1995; Wu, Subbarao, et al., 2001). There have been efforts to utilise aerial sampling for the prediction of disease, by correlating the number of sporangia detected to weather parameters (Dhar et al., 2020; Kunjeti et al., 2016). For example, to measure the weather conditions suitable for sporangial release in the Canadian lettuce growing regions (Carisse & Philion, 2002; Fall et al., 2015), or to obtain more accurate localised disease forecast predictions and decision making with regard to fungicide spray programs (Dhar et al., 2020; Fall et al., 2016).

Diagnostic assays for the quantification of aerial sporangia of *B. lactucae* exist. In the past, time consuming manual methods such as counting sporangia captured on silicon grease coated impaction tapes were used (Carisse & Philion, 2002; Fall et al., 2015), while more recently qPCR has been used (Dhar et al., 2020; Kunjeti et al., 2016). Although diagnostic assays are useful, they also need to be economically viable, robust in a field environment and provide a quick turn-around to be a useful warning service to the lettuce growing industry.

Another key requirement for the diagnostic assay is the effective collection of samples. As the main presumed source of inoculum of *B. lactucae* in commercial environments is aerial sporangia, a means to sample the air space near to the crops is required. In general, most aerial samplers rely on impaction, whereby the sporangia adhere to the sticky surface of a collection device (e.g. Rotorod, tape or filter disc) on impact. Some alternative methods, such as the multi-vial sampler use a reverse vortex airflow to deposit aerial material within a vial. With impaction traps that utilise adherence, surface overloading can occur, where the entirety of the surface is blocked by material collected (Jackson & Bayliss, 2011; West & Kimber, 2015). Overloading is less commonly a problem with multi-vial samplers, although they rely on airflow and the material is unable to be moved once deposited, so contaminants such as insects and soil can be an issue.

In order to improve detection of *B. lactucae* in the field, a loop-mediated isothermal amplification (LAMP) assay that amplified DNA of *B. lactucae* was developed prior to this

PhD study in an associated project funded by the AHDB (project CP184) and was planned to be used with aerial sampling in commercial environments.

5.1.3 LAMP assays and their use as early warning tools

The LAMP assay, which amplifies DNA at a consistent temperature (isothermic), was devised by Notomi et al. (2000). The basic premise of a LAMP assays is 4-6 primers are used to amplify 6-8 regions of the target DNA using a polymerase that has high strand displacement and extension activity at temperatures where DNA does not denature, allowing for annealing and extension at a consistent temperature. Typically, the amplified products of LAMP are designed to form a dumbbell structure that accelerates amplification. More information on the LAMP assay, including how it works, is provided in the reviews of Parida et al. (2008), Becherer et al. (2020) and the original publication of Notomi et al. (2000). The LAMP assay is an alternative to conventional and quantitative PCR, providing a high tolerance for crude samples (Notomi et al., 2015; Wong et al., 2018). The isothermal nature of the LAMP reaction means that thermocyclers are not required (Wong et al., 2018). LAMP assays can have a reduced run time compared with qPCR assays and can be made suitable for in-field detection devices or adapted for less sophisticated heating apparatus (e.g. water-baths)(Notomi et al., 2000; Wong et al., 2018). LAMP assays can be modified in design to suit several purposes (Becherer et al., 2020). For example, DNA amplification by the LAMP assay can be tracked in real-time with the inclusion of DNA targeting fluorophores (intercalating dyes), such as SYBR green, and a set of known DNA standards to estimate the level of DNA in a sample (Cai et al., 2008; Lucchi et al., 2010). This type of LAMP assay has previously been referred to as RtF-LAMP (Cai et al., 2008), or RealAMP (Lucchi et al., 2010), with RT-LAMP referring to reverse-transcription (Fukuta et al., 2003) a PCR modification that includes the reverse transcription of RNA into DNA. Despite the real-time LAMP assay not being able to quantify inoculum present in a sample absolutely, assays can be adapted using fluorescent signals to make them suitable as point of care/in-field tests (Lucchi et al., 2010). However, this is dependent on whether the samples taken require further processing that can also be adapted for in-field testing e.g. DNA extractions or further sample purification. LAMP assays are inherently less sensitive than PCR assays and have a relatively higher false positive rate (Wong et al., 2018). Therefore, they are not a complete replacement for qPCR based assays. The lower sensitivity of LAMP assays compared with qPCR means that there is potential for false negative results

at low levels of DNA of *B. lactucae*. Thus, it is important to determine the detection limit of the LAMP assay and to check whether the pathogen can be detected reliably in commercial field samples taken at the onset of epidemics.

Early samples of aerial sporangia in a commercial environment, i.e., adjacent to lettuce crops, can be exposed to pesticides and environmental variables that may introduce contaminants, as observed by Kunjeti et al. (2016). Therefore, a diagnostic that is robust and reliable with crude samples and provides a quick turnaround for monitoring purposes would be required for in-field detection. The LAMP assay previously developed at the James Hutton Institute is a real-time assay which uses an DNA-intercalating dye to visualise the amplified DNA, and the protocol includes a serial dilution of DNA of *B. lactucae* to provide an approximate quantification. Prior validation tests examined assay specificity but not the effects of collection method or the specificity and sensitivity with samples collected from field environments. This work aims to bridge this laboratory-to-field gap by a comparison of the conventional qPCR assay with the LAMP assay.

5.1.4 Validation of diagnostic assays

To validate the LAMP diagnostic assay as an early warning tool for use in a field environment, knowledge of how to interpret assay results, set the criteria for positives, and then test the assay with the relevant field samples is required. Conventional PCR and LAMP assays usually result in a binary outcome, i.e., positive, or negative. Real-time diagnostic assays produce experimental outputs that are continuous for each sample and are more complex to interpret with regard to recording a positive result (Burns & Valdivia, 2008). Although LAMP is an isothermal process without thermal cycles, the terminology of "cycles" can be used, with cycles being a unit of time depending on the amplification program and machinery used.

One of the key measured outputs for both diagnostic assays is the cycle threshold (C_T), at which the fluorescent signal detected for a sample exceeds the background noise. After the C_T cycle the relative fluorescent signal is detected at the end of each subsequent cycle and the value plotted against cycle number (or time) to form a curve, with sigmoidal curves expected for positive samples. The longer that real-time assays are run, the presumed higher chance of non-specific amplification or degradation of the probe-based fluorophore through cross-contamination, which could lead to a false positive result. Therefore, limitations on an

acceptable amplification curve, relative fluorescent threshold, and C_T cut-offs (arbitrary cycle where any amplification after is not considered positive) are implemented.

More than one threshold of detection measure can be used to determine C_T cut-offs. The limit of detection in DNA/RNA diagnostics according to Burns and Valdivia (2008) is the minimum amount of DNA/RNA that can, with good probability, be distinguished from a 'blank'/negative value. A good probability for limit of detection was interpreted by Nutz et al. (2011) to be a 95-100% incidence of positive when the sample should be positive. These authors suggested using the Youden index with receiver operating character (ROC) curve analysis to determine C_T cut-off, by comparing "positive" and "negative" assignments for samples using different C_T cut-offs with the "true" positive sample status. The "true" positive is denoted by a gold standard test or through spiking the sample with analyte (e.g. DNA). Essentially the highest value of the Youden index is the optimal cut-off, with the Youden index (J) calculated by sensitivity (SE) and specificity (Sp) in the equation:

$$J = \mathrm{SE} + \mathrm{Sp} - 1$$

With sensitivity and specificity defined in the expanded equation:

$$J = \left(\frac{true\ positives}{true\ positives + false\ negatives}\right) + \left(\frac{true\ negatives}{true\ negatives + false\ \ positives}\right) - 1$$

The limit of detection/sensitivity value is comprised of several factors, including the initial sampling, the sample handling, DNA/RNA extraction protocols and whether the sample contains inhibitors or competitors (Burns and Valdivia, 2008). This sentiment was echoed by Forootan et al. (2017) who advocated for a higher number of sample replicates, and Nutz et al. (2011) who suggested using melt-curves to determine true positives and negatives in samples as use of spiked samples or a dilution series as controls would not account for the variables encountered in the field.

The Hutton designed real-time LAMP assay is a six primer assay, targeting mitochondrial DNA, specifically between 11914bp – 12118bp of NCBI GenBank accession: MH271689.1. Prior validation of the Hutton real-time LAMP assay for *B. lactucae* examined specificity with eighteen *B. lactucae* strains from seven outbreaks and other plant pathogens (three *Peronospora spp.*, 26 *Phytophthora spp.*, one *Pythium ultimum* and 2 *Alternaria spp.*). However, the sensitivity with field samples had not been tested, nor had a C_T cut-off value for positive detection been

provided. In addition, the Hutton developed LAMP assay had not been tested on samples collected from the field environment. Therefore, further validation of the LAMP assay with regard to the effect of collection method with Rotorod samplers on the limit of detection of the assay was required in order to produce a test suitable for in-field use.

5.1.5 Aims and objectives

This work aimed to validate a previously developed LAMP detection assay for use in commercially grown crops and field trials and to compare its utility with a conventional qPCR assay previously developed by Kunjeti et al. (2016).

The study first (Part A) looks at assessing criteria for determining a positive result using the real-time LAMP assay, along with evaluating the sensitivity of the real-time LAMP assay for the detection of *B. lactucae* when simulating a collection method.

In part B, the real-time LAMP assay is evaluated for the detection of *B. lactucae* in commercial field and field trials using aerial samplers. Additionally, the study uses the real-time LAMP assay to investigate gaps in the knowledge of lettuce downy mildew, by studying the effect of distance from a disease outbreak on detection sensitivity and the effect of timing of sampling (AM/PM periods) on the incidence of detection of the pathogen relating to sporangial release. Unless specified as 'conventional', any reference to a LAMP assay hereafter refers to the real-time LAMP assay developed.

Part A. Evaluating sensitivity of the LAMP assay

5.2 Materials and methods

5.2.1 DNA extraction from sporangial suspensions

For the standards (positive controls) in each diagnostic assay, sporangial suspensions were obtained from isolates maintained on lettuce cotyledons and genomic DNA of *B. lactucae* was extracted using a method modified from Raeder and Broda (1985), as described in (Lees et al., 2019) in addition to replacing freeze dried material with the 1 ml sporangial solutions from 3.2.1.3. DNA concentration (ng/µl) was quantified using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Loughborough, UK).

5.2.2 LAMP assay protocol

Each LAMP assay was carried out in a final reaction volume of 25 µl, which included 15 µl Isothermal Master Mix ISO-001 or ISO-004 with ds-DNA binding fluorescent dye (Optigene), 5 µl primer mix containing loop primers (F-loop and B-loop) at 10 µM, external primers (F3 and B3) at 5 µM and internal primers (FIP and BIP) at 20 µM final concentrations, and 5 µl of template DNA or a negative control of HPLC grade water (Table 5-1).

Table 5-1 LAMP primer names and sequences

Primer Name	Sequence 5′ ---> 3′
F3	TTCATAGTACCGGCGATT
B3	ACAGGCAGTTGCTTATAGAC
FIP(F1c+F2)	AAGCAGGTGATGTTTCGGCATACACACTAATAGCCGGTCTT
BIP(B1c+B2)	GCAGTTAATGAACCTCCACCGAAGAGAAGCTTATCCAGGTGA
LoopF	TTCAATCACGGATGGTCAA
LoopB	GCTGCTCTTTCCAATAAACGAG

The real-time LAMP assay was carried out in a StepOnePlus™ (Thermofisher), with a program of 20 seconds at 65°C, followed by 60 cycles at 65°C for 30 seconds, during which the product was visualised every 15 seconds using the FAM detection channel. A C_T of 60 (equivalent of 30 minutes) was assigned to samples where no *B. lactucae* was detected or when

the amplification curve was abnormal. All other samples were considered positive unless non-specific amplification was detected in melt-curve analysis. A melt-curve was included at the end of every program to check for non-specific amplification, with a maximum temperature of 95°C and data was collected after each 0.3°C increase in temperature. To allow for approximate quantification of DNA of *B. lactucae* and for comparison between real-time LAMP assay plates, standards comprising DNA of *B. lactucae* were included in each plate. For these, DNA was extracted from known sporangial sample concentrations and unless otherwise stated DNA of *B. lactucae* was serially diluted to give concentrations of 10 ng/µl, 1 ng/µl, 100 pg/µl, 1 pg/µl, and 0.1 pg/µl.

5.2.3 qPCR assay protocol

Quantitative (real-time) PCR (qPCR) was performed according to the method of Kunjeti et al. (2016). Each qPCR assay was carried out in a final reaction volume of 25 µl containing 7.5 µl of HPLC grade water, 12.5 µl of TaqMan™ Universal PCR Master Mix, no AmpErase™ UNG (Thermofisher), 3 µl of primer mix containing a final concentration of 5 µM each of probe, forward and reverse primers, and 2 µl of the template DNA. The sample template and DNA standards were as described for the LAMP assay. The qPCR was run with a 10-minute incubation period at 95°C, and 55 cycles of 10 seconds at 95°C and 30 seconds at 56°C. C_T values were plotted against the log of the initial concentration of DNA to produce a standard curve. A C_T of 55 (equivalent to ~37 minutes excluding incubation time**)** was assigned to samples where no *B. lactucae* was detected or abnormal amplification curves were observed (different from the genomic standards), all other samples were considered positive.

5.2.4 Sensitivity of detection of DNA of *B. lactucae* from aerial sampling rods to which sporangia were added artificially

Aerial samplers can be used to monitor airspace for aerial sporangia of *B. lactucae.* A common type of these is impaction samplers which use sampling rods. To test the limit of sporangial detection of the LAMP assay using samples obtained from aerial samplers, and to further check the sensitivity of the real-time LAMP assay, a modified protocol from Lees et al. (2019) was used to simulate the collection and processing of Rotorod aerial sampler collected

samples. Four replicates each consisting of two Perspex air sampling rods smeared with Vaseline® in a 2 ml screw-cap tube with 100 µl of a sporangial suspension of sporangia of *B. lactucae* added were prepared. Sporangial suspensions, collected from seedlings, were counted using a Haemocytometer. Two sporangial dilution series were tested; 1) 1000, 100, 10 and 1 sporangia, and 2) 1000, 100, 85, 70, 55, 40, 25, 10 sporangia per reaction These values were chosen according to the results of preliminary experiments estimating sensitivity of the assay to be between 1000-100 sporangia added (data not shown). Samples were then frozen at -20°C to replicate storage conditions prior to DNA extraction of field samples. DNA was extracted using the MasterPure™ Yeast DNA Purification Kit (Lucigen/Cambio, Cambridge UK) with minor modifications (Appendix i). Extracted DNA was tested in duplicate for the presence of *B. lactucae* DNA using both the real-time LAMP and qPCR assay. Genomic DNA of *B. lactucae* was used as a positive control in a standard dilution, equivalent to 1000, 100, 10, 1, 0.1 and 0.01 sporangia per reaction. The amount of template DNA for each reaction differed, 2µl of template was used in the qPCR assay and 5 µl was used in the LAMP assay, therefore the estimated sporangial equivalents per reaction for each diagnostic assay differed (Table 5-2). For comparison between the two assays, samples were referred to by the number of sporangia added to sample.

Table 5-2 Estimated number of sporangia of B. lactucae *to detect in each reaction compared with initial number added to sample before DNA extraction. Sporangia were counted using a haemocytometer. Each sample contained 100 µl of a sporangial suspension, DNA extractions were to a volume of 35 µl.*

Number of sporangia added to sample	**1000**	**100**	**85**	**70**	**55**	**40**	**25**	**10**	**1**
LAMP assay	142.86	14.29	12.14	10.00	7.86	5.71	3.57	1.43	0.14
qPCR assay	57.14	5.71	4.86	4.00	3.14	2.29	1.43	0.57	0.06

5.2.5 Melt-curve analysis

Melt-curve analyses on the LAMP assay products derived from the amended Rotorods were carried out. The average melt-curve value from the genomic DNA standards (83°C) was used to set a range of melt temperatures (82<x<84°C**)** to denote a whether the sample was specifically amplifying the target DNA and therefore "positive". Each sample had two results assigned, one from the melt curve analysis and the second an expected result which assumed that if DNA was added to the sample regardless of the concentration, it should be positive. These two results per sample were compared in ROC analysis with Youden Index as described by Nutz et al. (2011). The ROC tested a range of C_T cut-offs, starting from 0-50 C_T with intervals of 2 C_T, to find which C_T cut-off gave the least false positives and false negatives. The qPCR assay did not have a melt-curve verification step and was thus excluded from the ROC curve analysis as the methodology did not include an intercalating dye.

5.3 Detection and quantification of DNA of *B. lactucae* from Rotorods using the LAMP and qPCR assay

5.3.1 Detection limit of LAMP and qPCR assay

After 10 minutes of incubation the LAMP assay could reliably detect DNA of *B. lactucae* in samples containing 1000 sporangia equivalents, whereas the qPCR assay could reliably detect *B. lactucae* after 20 minutes from the same samples. The LAMP and qPCR diagnostic assays could detect to the lowest sporangial equivalents tested, which was 0.01 sporangial equivalents per reaction, but detection was not consistent between replicates. Using the LAMP assay, the time until detection varied between sample repeats (22.5-30 minutes). This was in part due to only half the repeats amplifying successfully (2/4 using LAMP; Figure 5-1). The reliable lower limit of detection of the LAMP assay is estimated to be between 1-10 sporangia equivalents per reaction considering a lower incidence of positive results in samples containing less DNA. This was particularly evident in samples with 0.10 sporangial equivalents, where no amplification was observed using qPCR. Neither the LAMP nor qPCR assays showed amplification of *B. lactucae* DNA when no sporangia were added to the reaction mix (false positives).

Overall, the time taken until detection increased as the number of sporangial equivalents decreased per sample. Samples in which *B. lactucae* DNA was undetectable were given the value of the maximum number of cycles for the particular assay. Samples with 0.10 sporangia added all yielded amplification curves that did not match the curves produced by either the negative or positive controls and were considered non-specific amplification when tested using qPCR. The non-specific amplification was attributed to contamination and data were removed from analysis.

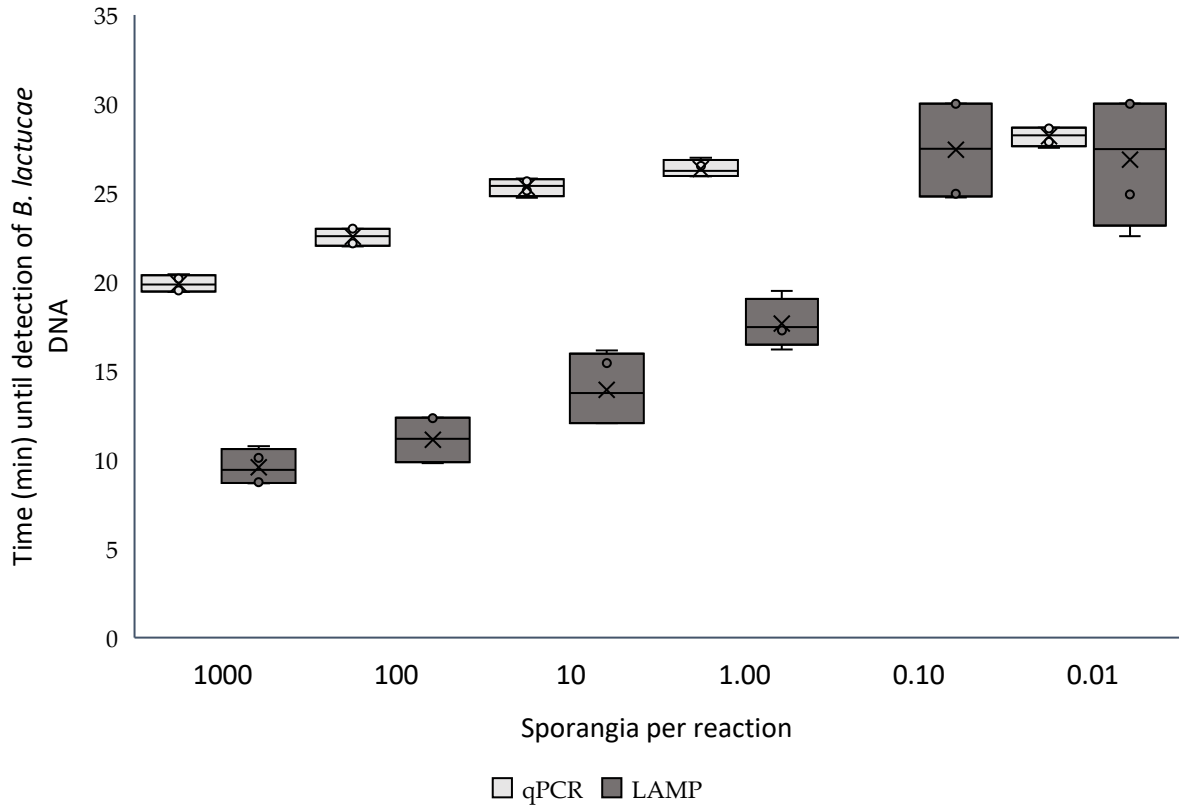


Figure 5-1 Time (min) until detection of DNA of B. lactucae in the real-time LAMP and qPCR assays. DNA concentrations were made to the equivalent of 1000, 100, 10, 10, 1, 0.1, and 0.01 sporangia per reaction, each was run in duplicate over two plates (n=4). qPCR times exclude the initial 10-minute incubation. Samples in which B. lactucae DNA was undetectable were given the max cycle in calculations, however in the case of replicates of 0.10 sporangia equivalents for qPCR all had suspected contamination from amplification curves not matching positive nor negative control amplification curves and were removed from analysis. Box and whisker plot; X is the mean, dots are each individual result, median is exclusive.

5.3.2 Detection of DNA of *B. lactucae* from Rotorods to which sporangia had been added

The detection limit for DNA of *B. lactucae* was between 10-100 sporangial equivalents using the LAMP assay when sporangia were added to Rotorods (data not shown). Following 11 minutes of incubation, the LAMP assay consistently detected DNA of *B. lactucae* from Rotorods to which 100 sporangia had been added and low levels of variation were observed (Figure 5-2).

A low incidence of amplification was observed in samples that had 10 sporangia added. The lowest concentration at which DNA was detected in all replicates was 25 sporangia per Rotorod using the LAMP assay. The results suggest that the limit of detection of the LAMP assay when samples are collected using sampling Rotorods is approximately 25 sporangia, which results in values equivalent to ~3.5 sporangia per LAMP reaction (and ~1.4 sporangia

per qPCR reaction). Variation between replicates for the time until detection of *B. lactucae* using the LAMP assay decreased with an increase in the number of sporangia added to sampling Rotorods (Figure 5-2).

Overall, the time taken for DNA of *B. lactucae* to be detected using a qPCR assay increased with a decreasing number of sporangia added to sampling rods. For example, a reaction time of ~27 minutes was recorded when 100 sporangia were added compared with ~32 minutes when 10 sporangia were added (Figure 5-2). However, the average time taken until detection of DNA of *B. lactucae* using the qPCR assay varied between replicates and did not appear to follow a trend (Figure 5-2); this was attributed to amplification not being observed in all replicates.

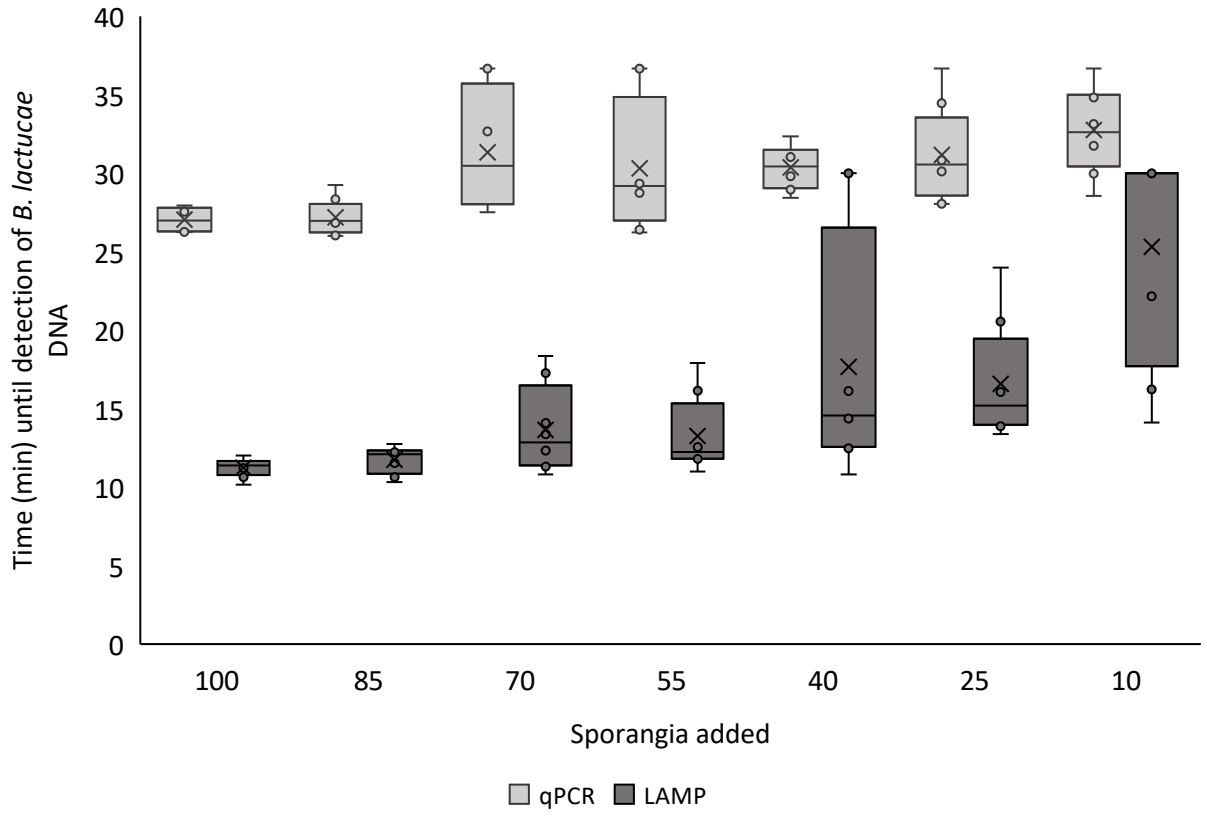


Figure 5-2 Time (minutes) until the detection of DNA of B. lactucae in LAMP and qPCR assays from DNA extracted from Rotorod samplers to which a known number of sporangia of B. lactucae had been added.

5.3.3 Comparison of *B. lactucae* detection from Rotorod samplers compared with controls

The effect of sampling method on detection efficiency was investigated by a comparison of the results from the Rotorods to which sporangia had been added and the genomic DNA standards. Overall, the Rotorod sampling method resulted in increased variation between sample replicates when compared to the genomic DNA standards, which was evident in Figure 5-1 and Figure 5-2. At 100 sporangial equivalents, genomic DNA was detected in 22.9 minutes using qPCR and 12.3 minutes using the LAMP asssay, with the difference between replicates being less than 0.01 minutes. When 100 sporangia were added to sampling Rotorods the time taken for the qPCR to detect *B. lactucae* ranged from 26.3-27.9 minutes, and when using the LAMP assay detection ranged from 10.2-11.7 minutes.

There was also an effect of diagnostic assay on detection (Figure 5-3), the relationship curve between sporangia added and time taken until detection was on average 5 minutes longer for DNA extracted from sampling rods (R^2<0.45) compared with genomic DNA(R^2<0.46) when tested using qPCR. In contrast, the difference between time until DNA of *B. lactucae* was detected between Rotorod samples and genomic DNA in the LAMP assay was not correlated (R^2<0.45) (Figure 5-3). This can be explained by greater variance for time until detection in the LAMP assay (10.8-30 minutes at 40 sporangia added) compared with the qPCR results (28.9-32.3 minutes at 40 sporangia added) and by the LAMP having a lower incidence of amplification at lower sporangial concentrations (3/8 replicates amplified of 10 sporangia added in LAMP vs 7/8 replicates in qPCR).

Overall, results showed that sampling of aerial sporangia using Rotorods would affect the detection of DNA of *B. lactucae* in the LAMP assay compared with the qPCR assay, increasing the time taken until detection, or preventing amplification (Figure 5-3). This was especially evident in samples to which low numbers of sporangia were added. A similar phenomenon was observed with the qPCR assay results. For the qPCR assay and the LAMP assay, less DNA of *B. lactucae* was detected when using sampling Rotorods than with genomic DNA; this could be a result of inhibition of the reaction due to contaminants from the collection procedure and/or a reduction in the total amount of DNA extracted from the sample.

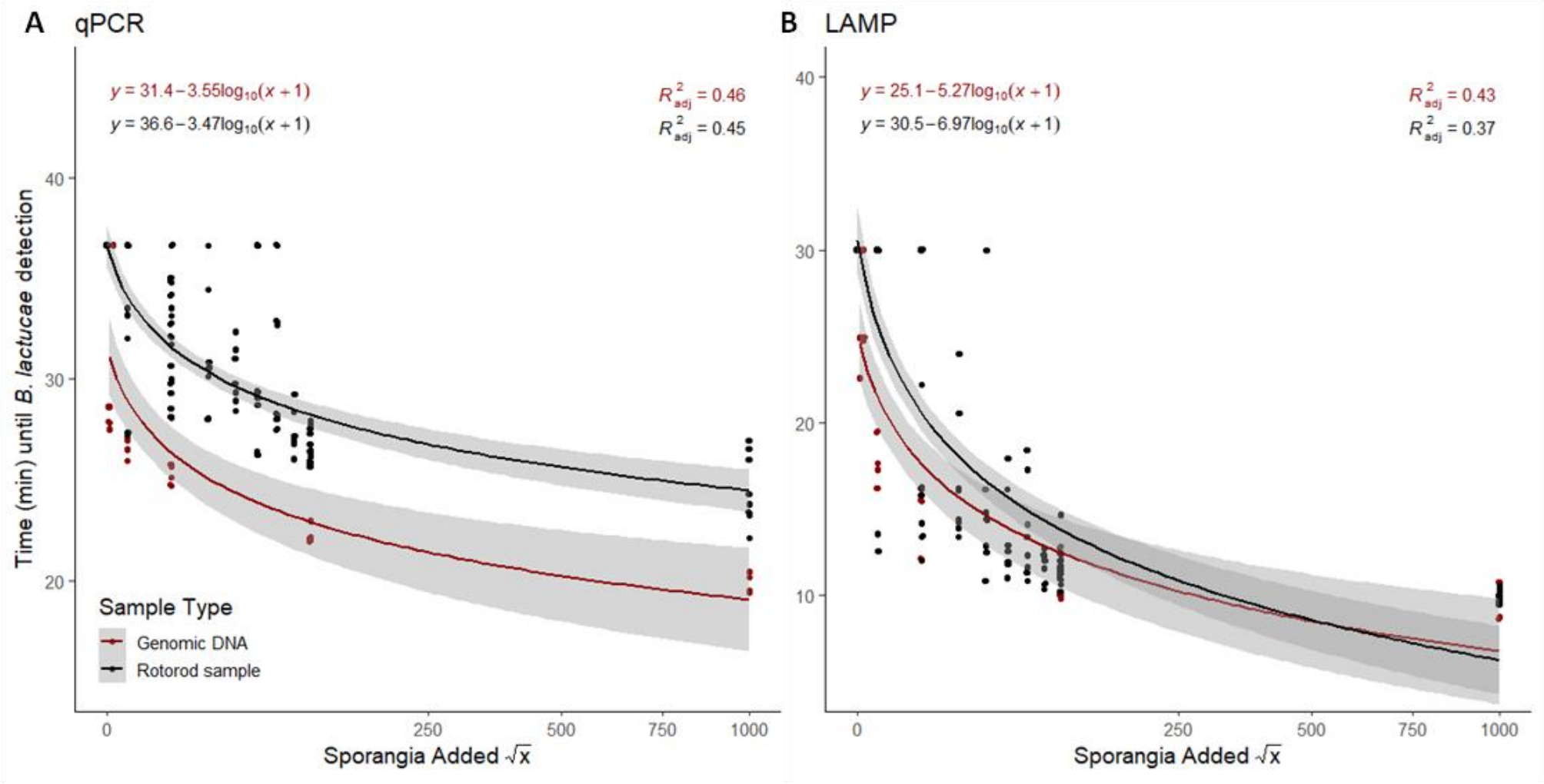


Figure 5-3 DNA of B. lactucae detectable from a dilution series of B. lactucae genomic DNA extracted from sporangia and samples obtained from Rotorods to which sporangia were added at a range of concentrations (0-1000 sporangia added or equivalents) measured as time (mins) until detection. Black line represents samples obtained from Rotorods; red line represents genomic DNA. Error range is confidence interval. X-axis was transformed using square-root function to allow visual clarity. A is qPCR assay results, and B is the LAMP assay results.

5.3.4 Assessing LAMP C_T cut-off values

LAMP results were checked for non-specific amplification using melt-curve analysis with DNA samples extracted from sampling Rotorods to which different sporangial dilutions of *B. lactucae* had been added. Amplification of *B. lactucae* was in general observed in samples which had 10 or more sporangia added to the sampling Rotorods.

To assess C_T cut-off values, DNA extracted from Rotorods to which different sporangial dilutions of *B. lactucae* were added were assigned a positive or negative score based on two different approaches and the results compared. These approaches were 1) a theoretical (expected) positive approach in which any sample that resulted in amplification following the addition of sporangia was considered positive, and 2) specific amplification observed in melt-curve analysis. These were compared at different cut-off points ranging from 0-50 C_T,

A ROC analysis to compare sensitivity (true positive rate) and specificity (false positive rate) showed a difference in results according to the method of analysis. The melt-curve approach resulted in a false positive rate not exceeding 0.7 at all C_T cut-offs, whereas the theoretical (expected results) approach exceeded this threshold at a C_T of 22 (

Figure 5-4 A:. The expected results started with a 0.2 false positive rate, whereas with the melt-curve approach the last C_T before a false positive rate of less than 0.2 was recorded was a C_T of 24 Therefore, results indicate that using a melt-curve approach would reduce false positives. The max Youden index (J=1), which is considered an ideal cut-off point (Nutz et al., 2011), was reached at a C_T of 48 for both approaches used to determine "positive" results, (Figure 5-4, B). The relevant C_T cut-off using the Youden Index was calculated to be between 42-48 cycles, as at 40 cycles a) the Youden Index starts to reach below 0.9 on the index scale and b) the two approaches used to determine positive results start to diverge, indicating higher false positive and false negative rates.

5.3.5 Assessing qPCR C_T cut-off values

Kunjeti et al. (2016) suggested a C_T cut-off of 35 cycles (23.3 minutes) for the qPCR assay. When applied to genomic DNA samples only 100 sporangial equivalents or more gave a positive result. With a subsequent application to Rotorod data, only two of eight replicate samples with 1000 sporangia added would be considered positive. No other sporangial concentration tested gave a positive result. As there was no melt-curve set for the qPCR assay a ROC curve

and Youden Index analysis could not be carried out. Instead, by observing the data collected at this point, the latest a sigmoidal curve usually reached a plateau of peak fluorescence was between 53-54 cycles.

5.3.6 Parameters for determining a positive result

Following a comparison of results obtained from genomic DNA standards and DNA extracted from sporangia added to Rotorods, and analysis of Youden index test results, the following criteria were chosen for determining a positive score. A positive score was assigned to sample replicates if they met these requirements.

For real-time LAMP:

1. Cycle threshold (C_T) = 15<x<45
2. Melting temperature (°C) = 82<x<84

For qPCR:

1. Cycle threshold (C_T) = 15<x<53.5
2. Quantity value (fg/µl) = 0<

5.3.7 Comparison of LAMP and qPCR assays

The McNemar's test compares diagnostic assays by assessing discrepancies in amplification (positive) incidence. There was no significant difference between the LAMP and qPCR assays when tested using genomic DNA (McNemar's mid-*p*, mid-*p*=0.45, *p*=0.69, n=6). However, a significant difference was observed (Mcnemar's mid-*p*, mid-*p*=0.03, *p*=0.04, n=20) when the two assays were compared using DNA extracted from Rotorods to which sporangia had been added. Discrepancies between the two assays were due to a higher incidence of positive results from the qPCR assay compared with the LAMP, which suggests that sensitivity of detection of *B. lactucae* is decreased with the LAMP assay when DNA is obtained from the Rotorod sampler.

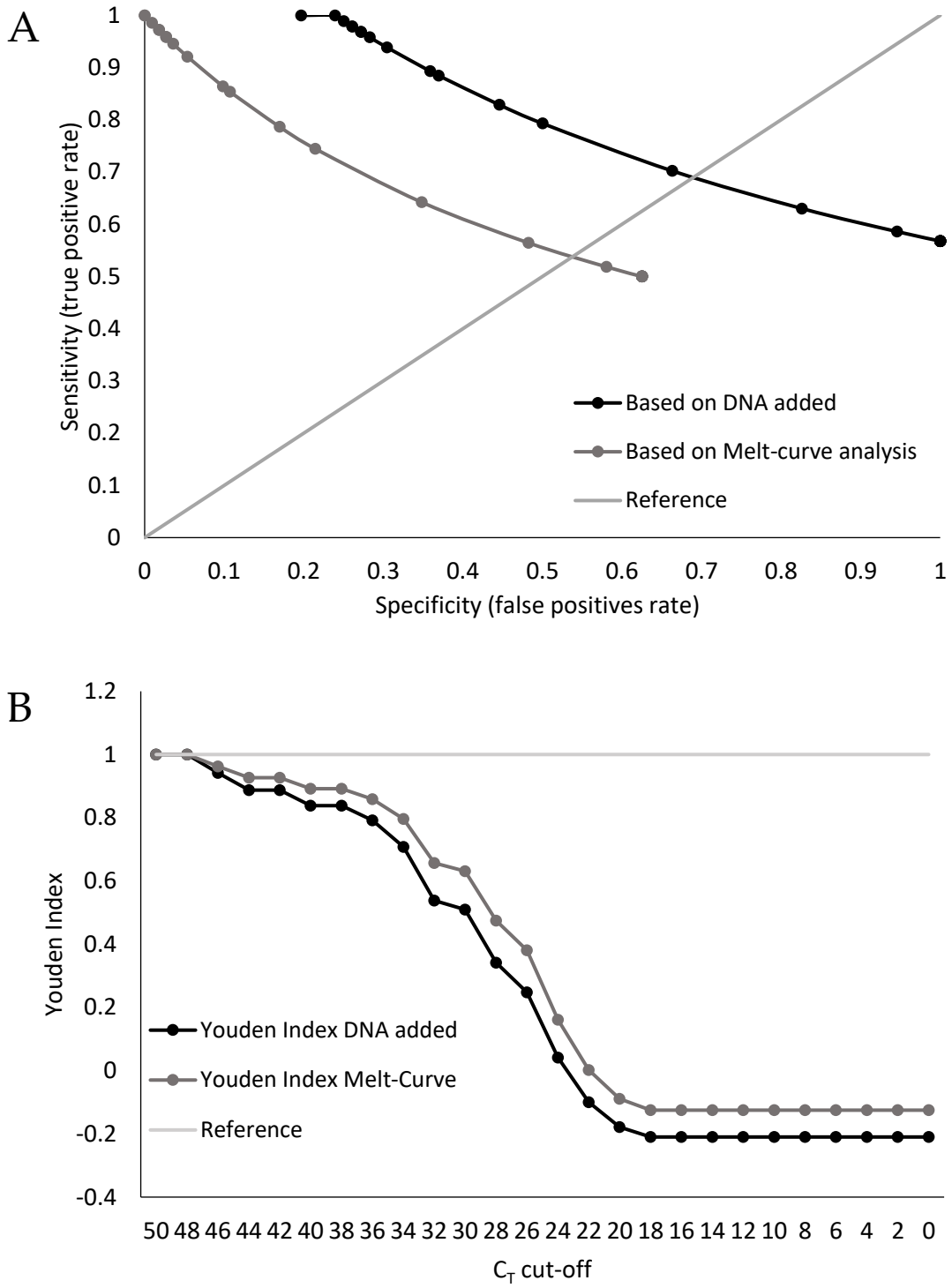


Figure 5-4 A: ROC curve analysis of real-time LAMP assay results where positives are determined by the melt-curve, or by DNA added. A reference line where y=x is included. B: corresponding Youden Index J values against different C_T cut-off points applied to real-time LAMP assay results from Rotorods with DNA of B. lactucae added and processed as field samples, a reference line for the best J value y=x was included.

5.4 Discussion

Data analysis showed that the LAMP and qPCR assays differ in sensitivity. The LAMP assay could detect DNA concentrations of *B. lactucae* equivalent to 1 sporangium when genomic DNA was tested, whereas the qPCR assay could detect DNA concentrations equivalent to 0.01 sporangium (Figure 5-1). Both the LAMP and qPCR assays could detect DNA of *B. lactucae* from three or more of four replicate samples to which 100 - 1000 sporangia had been added to Rotorod samplers (Figure 5-2). DNA of *B. lactucae* was reliably detected from Rotorod air samplers using qPCR when 10 sporangia or more were added to the sampler and 25 or more sporangia when using LAMP. Though the minimum detection limit for both assays is lower than these values, detection became more variable across technical replicates and therefore less reliable. The use of aerial Rotorod samplers for collection of sporangia increased the time until detection of DNA of *B. lactucae* when compared to genomic DNA equivalents (Figure 5-3). Accounting for the reliable limit of detection of genomic DNA mentioned above (Figure 5-1 and Figure 5-2), the Youden index value of 0.9 was lost at C_T 40 (Figure 5-4) and that time until detection is increased when sampling Rotorods are used (Figure 5-3); a C_T cut-off of 45 (equivalent to 22.5 minutes) for the LAMP assay was determined to be appropriate in avoiding false-positive results based on lab tests.

Kunjeti et al., (2016) advised a C_T cut-off of <35 cycles for the qPCR assay. However, when applied in these tests to Rotorod data, only two replicates from 8 replicates containing 1000 sporangia were detected using this cut off value. The differences in collection or processing of samples between Kunjeti et al., (2016) and our study may have contributed to the difference in DNA detection. Thus, the C_T cut-off for the qPCR assay was set at 53.5 cycles out of 55 cycles, as this allows for enough of the amplification curve to be observed to characterise correct amplification in the form of a sigmoidal curve.

Part B. Implementing the LAMP diagnostic under field conditions

5.5 Materials and methods

5.5.1 Monitoring aerial dispersal of sporangia of *B. lactucae* from an inoculum source

5.5.1.1 Set up of inoculated lettuce field

Three field trials were conducted to a) test the feasibility of in-field detection of *B. lactucae* sporangia, b) examine the effect of distance between an inoculum source and the sampler on detection efficiency and c) understand the timing of sporangial release from diseased plants. Assessing in-field detection of aerial sporangia was carried out to examine LAMP assay performance for the surveillance of inoculum of *B. lactucae* under field conditions. The dispersal gradient was monitored to give information relating to the spread of inoculum of *B. lactucae*, and the timing of release to inform prevention of the spread of airborne inoculum.

Two trials (Trial 1 and Trial 2) were conducted in 2021 and one (Trial 3) in 2022. Trials consisted of randomised blocks, each containing one 24 plant plot of each cultivar. Plants were spaced 30cm apart and surrounded by guard plants of disease resistant cultivars. Commercial cultivars were chosen, and lettuce plugs were kindly sourced and supplied by Kettle Produce. Trials 1 and 2 had four blocks of four plots of the commercial cultivars Excalibur, Coventry, Actina and Stallion. Trial 3 had five blocks of three plots of cultivars Excalibur, Coventry and Actina. Lettuce plugs were kept in a glasshouse until transfer to the field on the 11/06/2021 (Trial 1), 18/08/2021(Trial 2), and 28/07/2022 (Trial 3), after which plants were irrigated regularly.

To inoculate the field trials, 5–6-week-old lettuce plants of the universally LDM susceptible cultivar Green Towers, were grown in a north facing green house before a sporangial suspension made from an equal mixture of *B. lactucae* isolates was sprayed onto them. Isolates differed between trials (Table 5-3) as the inoculated field was additionally used to test cultivar selection (Chapter 4). After inoculation, the infector plants were kept in a dark room at high humidity overnight before being moved to a north facing greenhouse under high RH conditions.

Table 5-3 Isolates utilised in each field Trial.

Trial	Isolates
Trial 1 (2021)	2020_Bl2A, 2020_Bl4E, 2020_Bl4G
Trial 2 (2021)	2020_Bl2A, 2020_Bl4E, 2020_Bl4G, 2021_Bl1B
Trial 3 (2022)	2021_Bl3A, 2021_Bl4C, 2021_Bl8A

Infector plants were placed in the field on the 16/07/2021(Trial 1), 19/08/2021 (Trial 2), and the 26/08/2022 (Trial 3). LDM incidence and severity was scored on all the lettuce plants, except the guard plants, approximately every three days.

5.5.1.2 Aerial monitoring

An aerial sampler was placed in the centre of the lettuce trial field (position 0 m (H2)), the other five samplers were placed in a line on the axis of the prevailing wind at distances relative to the central sampler, at 5 m (H3), 30 m (H4), 60 m (H5) and 90 or 100 m (H6) in a north-easterly direction, and -5 m (H1) in a south-westerly direction (Figure 5-5). Aerial samplers were programmed to sample twice per week, 2-3 days apart, with the Rotorod spinning for 2 mins on and 2 mins off between 04:00-23:00.

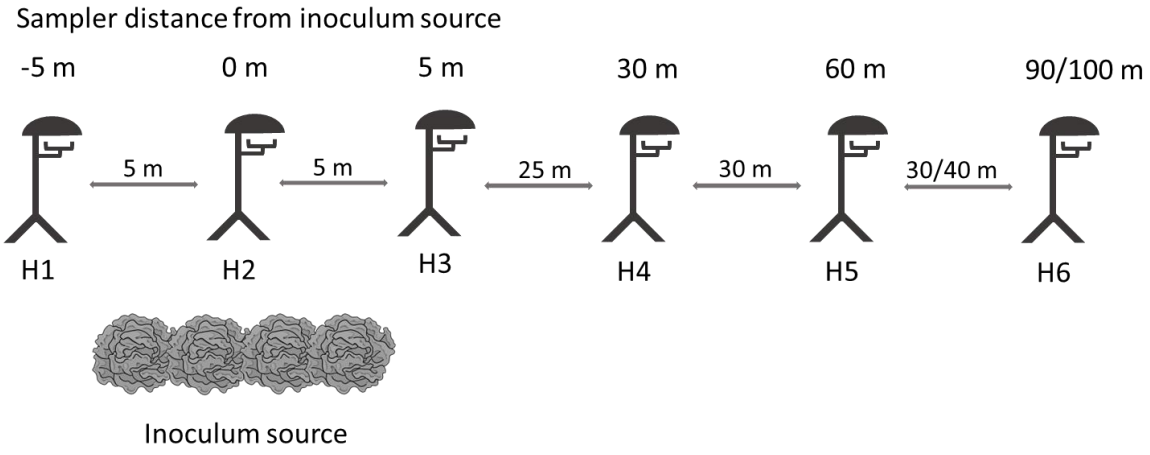


Figure 5-5 Distance of aerial samplers from inoculum source and each other. Not to scale. Inoculum source was a lettuce field inoculated by plants infected with B. lactucae.

The trials ran from 03/06/2021-18/08/2021, 19/08/2021-14/10/2021 and 28/07/2022-17/10/2022. DNA was extracted from the Perspex Rotorod using the MasterPure™ Yeast DNA Purification Kit (Lucigen/Cambio, Cambridge UK) following the manufacturer's instructions (with some modifications see appendix i) before amplification using the qPCR assay as

described by Kunjeti et al., (2016) and the real-time LAMP assay as previously described. Each sample was run in duplicate, along with a serial dilution of genomic DNA as a positive control and a HPLC grade water negative DNA template.

5.5.2 Examining timing of sporangial release from diseased plants

In a preliminary assessment into whether sporangial release time was favoured in the morning or evening in Scotland a Burkard 7-day continuous sampler (Burkard, Rickmansworth, UK), referred to as a '7-day sampler', was used to collect samples. The 7-day sampler contained a tape coated with grease in the drum which slowly turns throughout the 7-days. The 7-day sampler was positioned next to the aerial Rotorod sampler at position H3 (5m downwind from the outbreak). The drum (and tape) was changed weekly at 11am each Friday. Sampling was carried out from 28/08/2021-21/10/2021 with each day split into 12-hour blocks beginning at 11AM. Using ethanol sterilised tools, the tape was cut to represent each time block, then cut in half to provide a test sample and a back-up sample. DNA was extracted from each section of tape using the YCL kit (Qiagen) following the manufacturer's instructions, (with some modifications see appendix i), before being amplified using both the real-time LAMP and qPCR assays as previously described. Samples collected from PM 24/09/2021-AM 01/10/2021 were excluded from the analysis due to processing errors with samples. Therefore, DNA from 51 samples were tested in duplicate with both diagnostic assays.

Figure 5-6 Aerial samplers at the 5m (H3 position) from the centre of the field trial. The GRIPS-99M (H3 5 m) aerial sampler (left), the Burkard 7-day continuous samples (Tape trap) (right).

5.5.3 Commercial field site testing

Field sampling of sporangia was carried out in commercial crops between 07/07/2020-09/10/2020, 23/05/2021-27/09/2021, and 08/7/2022-29/09/2022. Field sites were sampled using Rotorod samplers (Burkard, Rickmansworth, UK), or Grips-99M (Aerobiology Research Laboratories, Nepean, Canada), in which two petroleum-jelly (Vaseline®) coated Perspex Rotorods to which sporangia adhere, were spun by a motor. Rain guards were used to protect airborne particles from being washed off the Rotorods. Two commercial lettuce growing sites were sampled in each year located in Cambridgeshire, England (courtesy of G's Growers), and Fife, Scotland (courtesy of Kettle Produce). Aerial samplers were moved periodically throughout the growing season to different lettuce fields in the same region due to the short cultivation times of lettuce crop (Table 5-4).

Figure 5-7 Rotorod sampler at commercial field sites. A: Rotorod sampler with rain guard attached. B: Scottish field site 2 (2021), and C: English field site 1 (2021).

Two Rotorod samplers were placed adjacent to a lettuce crop at a 50 m spacing according to the prevalent wind direction at each sample location, Figure 5-7. The traps were programmed to sample twice per week, 2-3 days apart, at 2 min intervals (2 mins on/ 2 mins off) between 04:00 – 23.00. Samples were retrieved twice a week, by G's Growers in England, and by JHI in Scotland, after which the rods were replaced. Samples were returned to the laboratory and DNA was extracted using method YCL kit (Qiagen) following manufacturer's instructions, before amplifying in the LAMP and qPCR as previously described in Part A. In 2020 an additional multi-vial aerial sampler (Burkard, Rickmansworth, UK) was located at the commercial site in England adjacent to a Rotorod sampler. The multi-vial sampler was programmed to sample every day according to the same schedule as the Rotorod samplers. No multi-vial samples were collected between 07/08/2020-28/08/2020 due to technical issues. The use of the multi-vial sampler was discontinued after 2020 due to high levels of introduced contaminants in the samples and therefore unreliable results.

Table 5-4 Aerial sampler locations and sample dates in commercial fields 2020-2022

Location of Commercial Field	Fields	Sampling Date Start	Sampling Date End	Trap Name	Location ~X cord.	Location ~Y cord.
Fife, Scotland	1	07/07/2020	13/08/2020	Rotorod - 1 Rotorod - 2	56.265255	-3.115123
	2	18/08/2020	09/10/2020	Rotorod - 1 Rotorod - 2	56.275747	-3.166688
Cambridgeshire, England	1	08/07/2020	30/07/2020	Rotorod - West	52.421389	0.367667
				Rotorod - East		
		08/07/2020	30/07/2020	Multi-vial		
	2	30/07/2020	28/08/2020	Rotorod - West	52.408278	0.394667
				Rotorod - East		
		30/07/2020	07/08/2020	Multi-vial		
	3	28/08/2020	28/09/2020	Rotorod - East	52.4562312	0.3936124
				Rotorod - West		
		28/08/2020	09/10/2020	Multi-vial		
Fife, Scotland	1	28/05/2021	14/06/2021	Rotorod – 1 Rotorod – 2	56.271063	-3.155168
	2	18/06/2021	26/07/2021	Rotorod – 1 Rotorod – 2	56.311348	-3.067131
	3	29/07/2021	27/09/2021	Rotorod – 1 Rotorod – 2 Rotorod – 3	56.287767	-3.200956
Cambridgeshire, England	1	23/05/2021	04/06/2021	Rotorod – West	52.464701	0.356782
				Rotorod – East		
	2	27/06/2021	17/08/2021	Rotorod – West Rotorod – East	52.414373	0.390583
	3	18/08/2021	19/09/2021	Rotorod – West Rotorod – East	52.446883	0.436014
Fife, Scotland	1	14/07/2022	21/07/2022	Grips 99M - 1 Grips 99M - 2	56.260174	-3.163644
	2	25/06/2022	08/09/2022	Grips 99M - 1 Grips 99M - 2	56.268327	-3.158698
	3	12/09/2022	22/09/2022	Grips 99M - 1 Grips 99M - 2	56.311348	-3.067131
Cambridgeshire, England[*1]	1	08/07/2022	29/08/2022	Rotorod - 1 Rotorod - 2	*~52.4*	*~0.4*
	2	01/09/2022	22/09/2022	Rotorod - 1 Rotorod - 2	*~52.4*	*~0.4*
	3	26/09/2022	29/09/2022	Rotorod - 1 Rotorod - 2	*~52.4*	*~0.4*

[*1] Locations were not given via GPS

5.5.4 Sample purification

Samples occasionally exhibited visible contaminants, such as soil and insects (Figure 5-8) which resulted in DNA pellet discolouration. To mitigate the effects of discolouration and/or inhibitors interfering with the real-time amplification, a purification step was incorporated into the DNA extraction. Selected samples were intentionally spiked with known amounts of DNA of *B. lactucae* to verify whether the inhibitors were present and had led to false negative results. For purification, Micro Bio-Spin Columns (7326204) (BioRad) had the bottom snapped off and were placed into 2ml Eppendorf tubes. Each Eppendorf had 900 µl of 10% PVPP solution (10 g in 100 ml of SDW) added, followed by centrifugation at 12.3 rpm for 1 minute. After removing the water, this step was repeated until no water appeared below the column. DNA samples were added on top of the PVPP layer and centrifuged at 13,200 rpm for one minute.

Figure 5-8 Example of Multivial sample collected, note the amount of soil contained.

This method filters contaminants along with some DNA which could potentially affect diagnostic accuracy especially at low DNA of *B. lactucae* concentrations. As discoloration was infrequent, this purification method was only applied to discoloured samples.

For assessment of potential inhibition, field-collected samples were spiked with a 1:1 ratio of genomic DNA of *B. lactucae*. Ten commercial samples including five discoloured (two purified and spiked with DNA equivalent to 700 sporangia/µl), were selected in 2020. Subsequent samples from 2021 and 2022, from fields with observed disease but no detection, were chosen. In 2021, five negative controls and ten test samples (3 Scottish, 2 English, 5 Hutton field site) were tested. In 2022, eight samples from each commercial field (LDM or no LDM) and three Trial 3 JHI inoculated samples were tested, both un-spiked and spiked with DNA of *B. lactucae* (10 ng/µl). All samples were duplicates, following previously described protocols, using genomic DNA standards, and duplicate water controls.

5.5.5 Data analysis

Each real-time assay was analysed and viewed in the StepOne Software v2.3 (Thermofisher). The baseline and detection threshold were modified per run if required. Each real-time LAMP or qPCR run was evaluated for various attributes, including the positive and negative control

results. Samples with particularly low levels of DNA amplification and abnormal amplification curves which suggested contamination was present were treated as negative samples and given the highest C_T possible. Data management and collation were performed in excel before input into R studio (ver1.2.5042) for analysis. Statistical significance was set at p value <0.05, unless otherwise stated. To ensure legitimate comparison unpaired data was omitted from the analysis. A set of criteria was employed to exclude data with abnormal melt-curves for genomic standards for the 2021 data only.

The criteria for transforming assay data into binary sample status data (positive or negative for detection of DNA of *B. lactucae*) are available in Appendix ii. This binary dataset was analysed with the McNemar's mid-p test, as described by Fagerland et al. (2013) for the assessment of clinical diagnostic assays. This analysis was chosen as the data violated assumptions for the typical Chi-squared test. There are four variations of the McNemar's test. Fagerland et al. (2013) tested each of these on pre-collected data and found the best of these that preserved statistical power whilst reducing Type 1 error rates on small to moderate sized data was the mid-p. Therefore, the McNemar's mid-p test was utilised to process the data following the R script provided by Fagerland et al. (2013). When diagnostic assays were not being directly compared, the average value of C_T, melt-curve, and quantity for all the replicates of each sample were used to determine a positive or negative result. The proportion of replicates that were positive was recorded to account for different replicates, and to indicate how reliable a positive result is relative to replicates.

A Friedman's Chi squared test was used to compare results obtained from each aerial sampler in the dispersal gradient, using the base R function. Results from each pair of samplers were checked using Nemanyi post-hoc test (sometimes referred to as Wilcoxon–Nemenyi–McDonald–Thompson test) using the R package: PMCRplus (v1.9.4, Pohlert, 2014) The following R packages were also used to aid in data visualisation: ggplots2 (v3.3.5, Wickham, 2016), qqpmisc (v0.4.0, Aphalo, 2023), ggpp (v0.4.4, Aphalo,2023), tibble (v.3.1.6, Müller & Wickham, 2023) and dplyr (v.1.0.9, Wickham et al., 2023). Data was also visualised with Excel (v2204 Build 16.0.15128.20278, Microsoft, n.d.). All R packages were installed and run with their dependencies.

5.6 Results

5.6.1 Dispersal gradient of sporangia of *B. lactucae* originating from an inoculum source

Three field trials were conducted at the James Hutton Institute to assess the dispersal gradient of sporangia of *B. lactucae* from an inoculum source, in addition to testing the efficacy of the LAMP assay with samples collected in-field. Each of the three trials showed that the further the aerial sampler from the source of inoculum, the lower the incidence of positive samples for DNA of *B. lactucae*. Each trial demonstrated that the LAMP assay was able to detect *B. lactucae* from in-field samples.

5.6.1.1 Trial 1

The epidemic in Trial 1 progressed well in the two cultivars (cv Excalibur and cv Stallion) that were susceptible to the three isolates of *B. lactucae* introduced. The weather was generally conducive to disease development throughout the trial with an average temperature range between 12.0-20.7 °C, and an average RH of 82.6%. DNA of *B. lactucae* was detected using both the LAMP assay and the qPCR assay before the infector plants were placed in Trial 1. This could be due to contamination, or prior LDM established in the area (Figure 5-9). The first detection of *B. lactucae* after infector plants were placed out was 19/07/2021, which was 9 days before disease was first observed in the non-inoculated lettuce plants, and 17 days before disease was widespread (8.5% mean foliar sporulation on susceptible lettuce plants).

In Trial 1, generally the closer the aerial sampler to inoculum source the higher the incidence of positive results in the LAMP diagnostic assay; aerial samplers H1, H2 and H3 had five or more incidences of positives each, H4 had three, whereas H5 and H6 had only two (Figure 5-9). Positive incidences of detection did not appear to be correlated with distance when using the qPCR assay, H3 and H5 had the highest number of positive incidences (eight), whereas H2 had six incidences. Quantity of DNA detected decreased as the distance from the inoculum source increased, the highest quantity for H1 was 88.1 pg/µl, whereas for H6 it was 0.5 pg/µl (Figure 5-9). An increase in the distance of sample collection from the epidemic source reduced the detection of *B. lactucae* differently in each diagnostic assay. With the LAMP assay, the incidence of positive results decreased as the distance a sample was collected increased. Whereas, when testing samples with the qPCR assay, an increase in sample distance from the

epidemic resulted in a decrease in the quantity of DNA detected but not the incidence of positive results.

The quantity of DNA detected ranged between 0.01-88 pg/µl for a positive result, which indicated the variation in the values of a positive result and lead to closer analysis of the values of a positive when samples were collected further from an inoculum source.

Subsequently, comparisons between aerial samplers were made by measuring the variation in both the C_T and proportion of replicates that had a positive outcome, and using Friedman's Chi squared to detect overall differences between samples collected from aerial samplers and Nemenyi test to identify which aerial samplers sample datasets were most different. This comparison was carried out to better understand the dispersal gradient of *B. lactucae* from an inoculum source.

In Trial 1, when comparing the sample results between aerial samplers a statistically significant difference ($p<0.05$) was obtained on evaluation of the LAMP assay C_T values (χ 2 (5) = 11.6, $p<0.05$) and similarly in the proportion of replicates that were positive (χ 2 (5) = 11.3, $p<0.05$), (Figure 5-10, C and D). When the sample qPCR results of aerial samplers were compared by which aerial sampler the sample was collected from there was no statistically significant difference between the qPCR C_T values nor the proportion of replicates that were positive (Figure 5-10, A and B).

Subsequent exploration of the data with Nemenyi post-hoc analysis did not reach statistical significance when comparing the differences in C_T value between positive results nor in the proportion of replicates with a positive result, in pairwise comparisons between each aerial sampler.

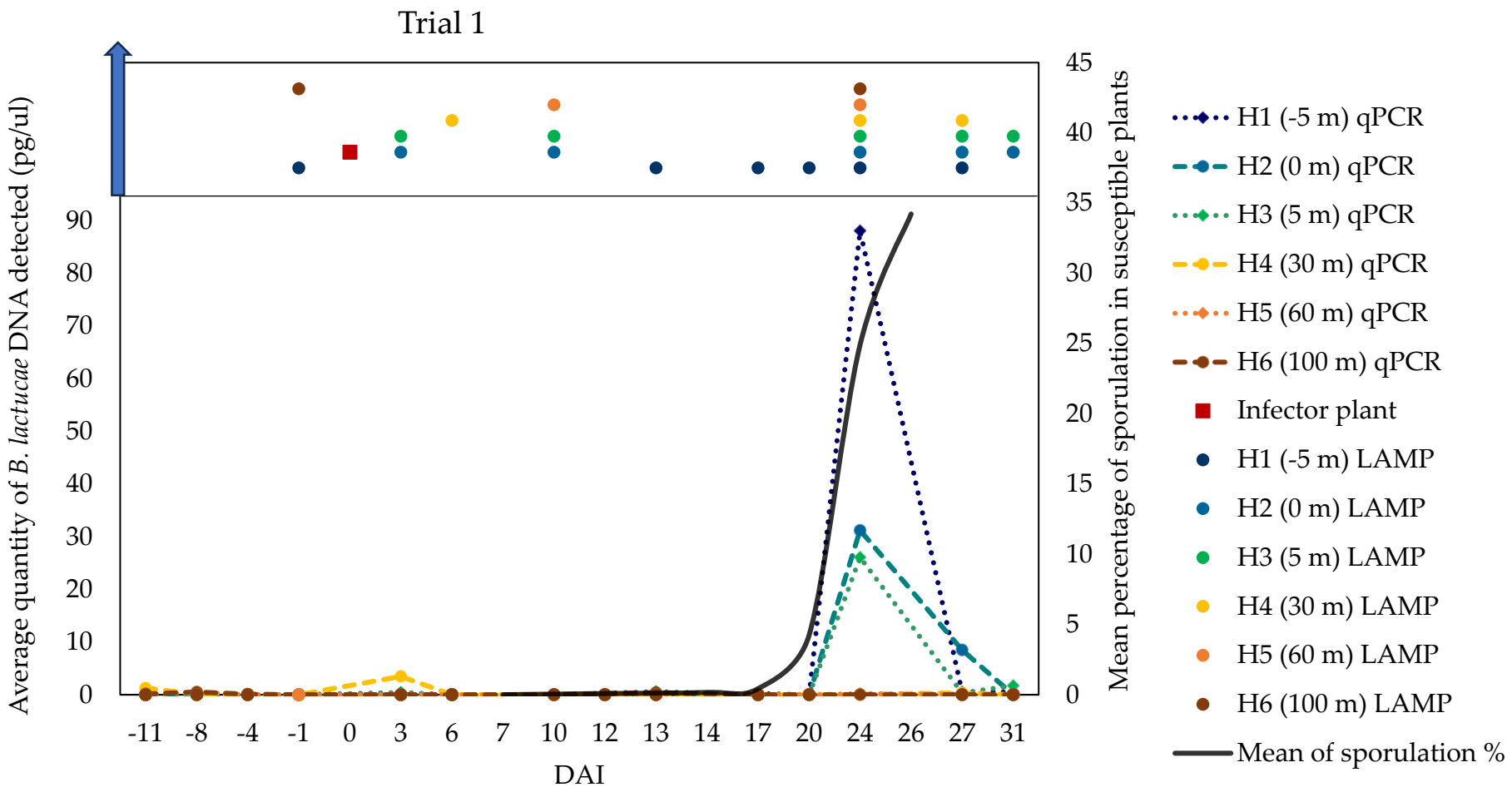


Figure 5-9 Quantitative detection of DNA of B. lactucae (pg/µl) using qPCR in samples collected at different distances from an LDM inoculum source. Aerial samples were collected between 05/07/2021– 16/08/2021), quantity of DNA as measured using qPCR assay was between ~0.01-88 pg/µl. Mean percentage of leaf area sporulating was assessed. Results from LAMP assays are shown as positive/negative results represented by dots. Arrow shows increasing distance from inoculum source where H2 = 0m from inoculated plant. Results from aerial samplers are represented in the same colour for both assays. Infector plant in the figure is positioned according to the DAI, which it was placed in field, and its relative distance compared to the other aerial samplers. DAI is days after inoculation.

A qPCR Trial 1

C_T (χ 2 (5)=3.08, p>0.05)

	H1	H2	H3	H4	H5
H2	0.99	-	-	-	-
H3	1.00	0.92	-	-	-
H4	1.00	1.00	0.98	-	-
H5	0.98	1.00	0.88	1.00	-
H6	0.98	1.00	0.86	1.00	1.00

B Positives (χ 2 (5)=7.48, p>0.05)

	H1	H2	H3	H4	H5
H2	0.90	-	-	-	-
H3	1.00	0.81	-	-	-
H4	1.00	0.98	0.99	-	-
H5	0.99	1.00	0.96	1.00	-
H6	0.78	1.00	0.65	0.94	0.98

C LAMP Trial 1

C_T (χ 2 (5)=11.63, p<0.05)

	H1	H2	H3	H4	H5
H2	1.00	-	-	-	-
H3	1.00	1.00	-	-	-
H4	0.69	0.75	0.65	-	-
H5	0.75	0.81	0.72	1.00	-
H6	0.26	0.32	0.24	0.98	0.97

D Positives (χ 2 (5)=11.25, p<0.05)

	H1	H2	H3	H4	H5
H2	1.00	-	-	-	-
H3	1.00	1.00	-	-	-
H4	0.86	0.81	0.97	-	-
H5	0.72	0.65	0.90	1.00	-
H6	0.62	0.55	0.83	1.00	1.00

Figure 5-10 Comparison of positive results in LAMP and qPCR assays for each aerial sampler from Trial 1. Friedman's chi squared test and Nemenyi post-hoc analysis on the differences between aerial samplers both C_T and proportion of replicates that were positive per sample. Relevant Friedmans test is above each Nemenyi p-value table giving p -values from pair-wise comparisons of sample results from each aerial sampler. Positives (B and D) refers to using the data of the proportion of sample replicates that were positive per sample in Friedmans and Nemenyi test. C_T (A and C) refers to using the average C_T for replicates samples for input into Friedmans test and Nemenyi test. A and B were for qPCR results, and C and D for LAMP results.

However, Nemenyi *p*-values decreased as aerial samplers increased in distance in the LAMP assay comparisons (Figure 5-10, C and D). This pattern indicated overall differences in C_T and proportion of positives, particularly evident when comparing H6 (100m) with samplers H1-H3 (-5m to 5m), (Figure 5-10, C and D). In contrast, adjacent samplers generally displayed *p*-values exceeding 0.90. This phenomenon is attributed in part to the close-to-threshold *p*-values from the Friedman test (p=0.040 and p=0.047) and the conservative nature of the Nemenyi test. Regardless, this result indicates that overall distance influenced the value of the positive results obtained with the LAMP assay. However, the difference in values of a positive between aerial samplers the effect was not always significantly different. The decrease in Nemenyi *p*-value between aerial samplers as distance increased, this suggests that during this trial sporangial dispersion of *B. lactucae* gradually declined with increasing distance from inoculum source. The sporadic occurrence of higher values of DNA detected, for example 88 pg/µl determined by a low C_T value of 35.9 in qPCR sample from sampler H1 at DAI 24, when most DNA values were below 31 pg/µl, may have led to a lack of statistical significance

between results obtained from different aerial samplers. The previous comparison between results obtained from different aerial samplers did not account for the presence of disease. DNA of *B. lactucae* was detected before inoculation of Trial 1 via infector plants and therefore the results relating to aerial sporangia concentrations may be confounded. However, a 3D regression analysis was employed to investigate potential associations between disease presence (quantified in terms of days after initial symptoms - DAS), the distance of aerial samplers from the inoculum source, and the time taken until detection of *B. lactucae*.

The incidence and severity of disease symptoms increased throughout the epidemic, as shown in Figure 5-9. Therefore, as DAS increases so does the level of disease. The orientation and slope of the 3D regression plane indicates the strength and direction of correlation between DAS, distance and the time until detection. The regression plane for Trial 1 showed that the time taken until detection of DNA of *B. lactucae* decreased for both assays when samples were collected closer to the inoculum source, and with increased levels of disease (indicated through an increase in DAS; Figure 5-11). The regression plane was steeper for LAMP than qPCR showing a stronger correlation between distance and time until detection. This also indicates greater variation in time taken for detection ranging from 11.5-30 minutes when testing samples with the LAMP assay but 24.0-36.7 minutes when using the qPCR assay.

To summarise, in Trial 1 the LAMP assay could detect *B. lactucae* in-field, but both the distance of sample collection from the inoculum source and the level of disease (therefore concentration of aerial sporangia present) was found to influence the incidence and value of positive results.

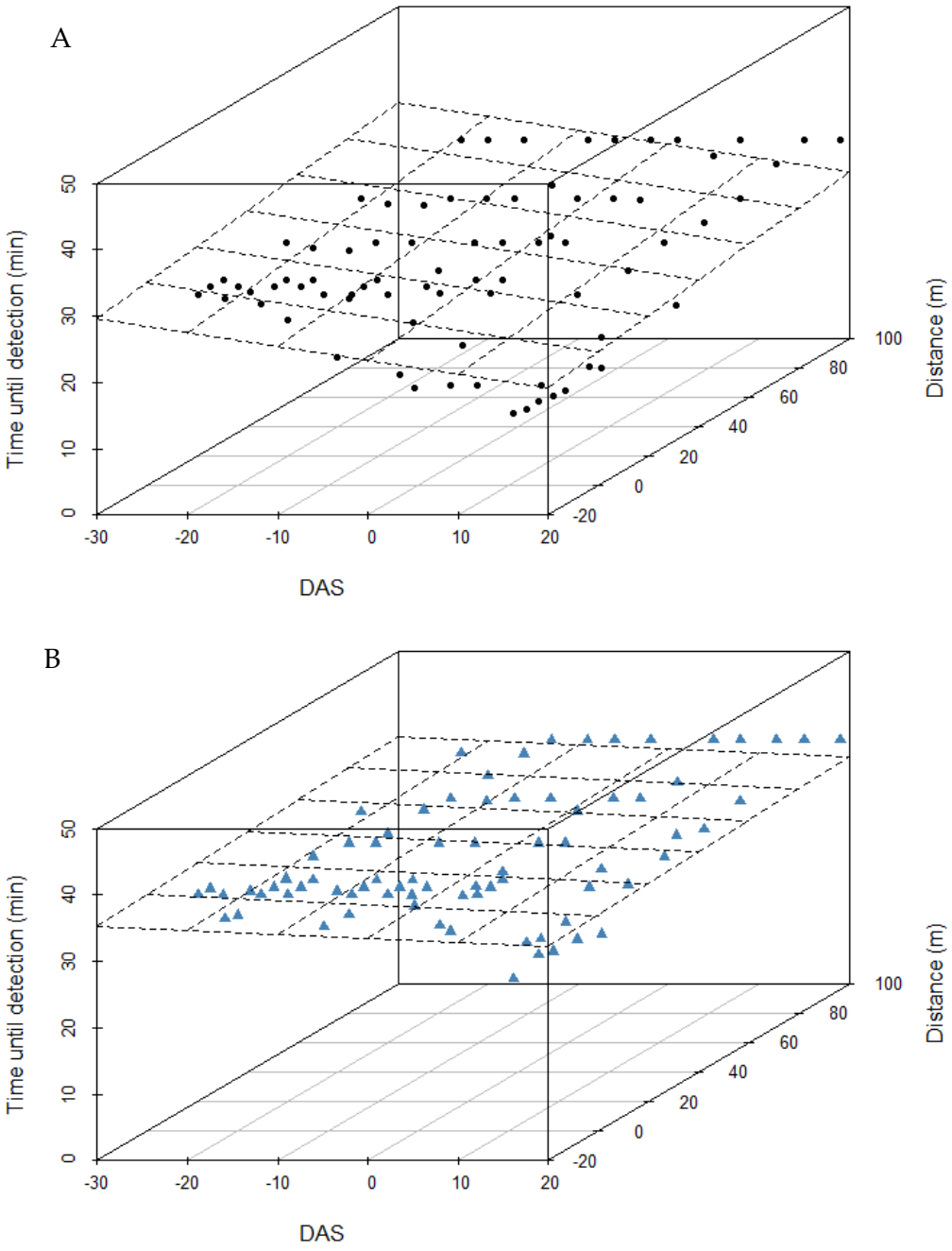


Figure 5-11 Modelling the relationship between time after first symptoms, distance of sample collection from inoculum source and time taken until B. lactucae detection when using the LAMP assay (A) and qPCR assay (B). Regression plane is the relationship between the three variables. Data from Trial 1. DAS is days after initial symptoms.

5.6.1.2 Trial 2

Trial 2 was a repeat of Trial 1 and used aerial samplers to monitor another trial lettuce field with LDM inoculated plant triggered epidemic. The epidemic progressed well as weather was conducive to LDM; with the average temperature range between 9.7-17.1 °C, and an average RH of 86.4%. Trial 2 had similar findings to Trial 1, in that an increase in distance from inoculum source decreased the incidence of samples positive for DNA of *B. lactucae.* Unlike Trial 1, this was the case for both diagnostic assays, sampler H2 had six positive outcomes in LAMP and 18 in qPCR, whereas sampler H6 had two and four respectively. The greatest quantity of DNA detected by qPCR was from a sample collected 5 m from the inoculum source (138.8 pg/µl for H2)

In general, aerial samplers in a 5 m radius of the centre of the trial had a higher incidence of positives for DNA of *B. lactucae* in both diagnostic assays compared to samples collected further away (Figure 5-12). Samples collected from H1-H3 had 19 positive incidences when tested with the LAMP assay, and 36 when tested with the qPCR. Whereas samples from H4-H6 had 9 and 31 positive incidences when tested in the LAMP and qPCR respectively. DNA of *B. lactucae* was detected in samples collected 90 m away from an inoculum source using both the LAMP assay and the qPCR assay (Figure 5-12). The incidence of positives in the LAMP assay positively correlated to the mean percentage of leaf area sporulating, with a higher incidence of positives as the trial progressed. DNA of *B. lactucae* was detected using the LAMP assay and the qPCR assay before the infector plants were placed in-field in Trial 2. This could be due to contamination, or leaf debris from Trial 1 in an adjacent area. In addition, the qPCR assay detected a high amount of DNA of *B. lactucae* four days after the infector plants were placed in field, this was presumed to be sporangial dispersal from the infector plants.

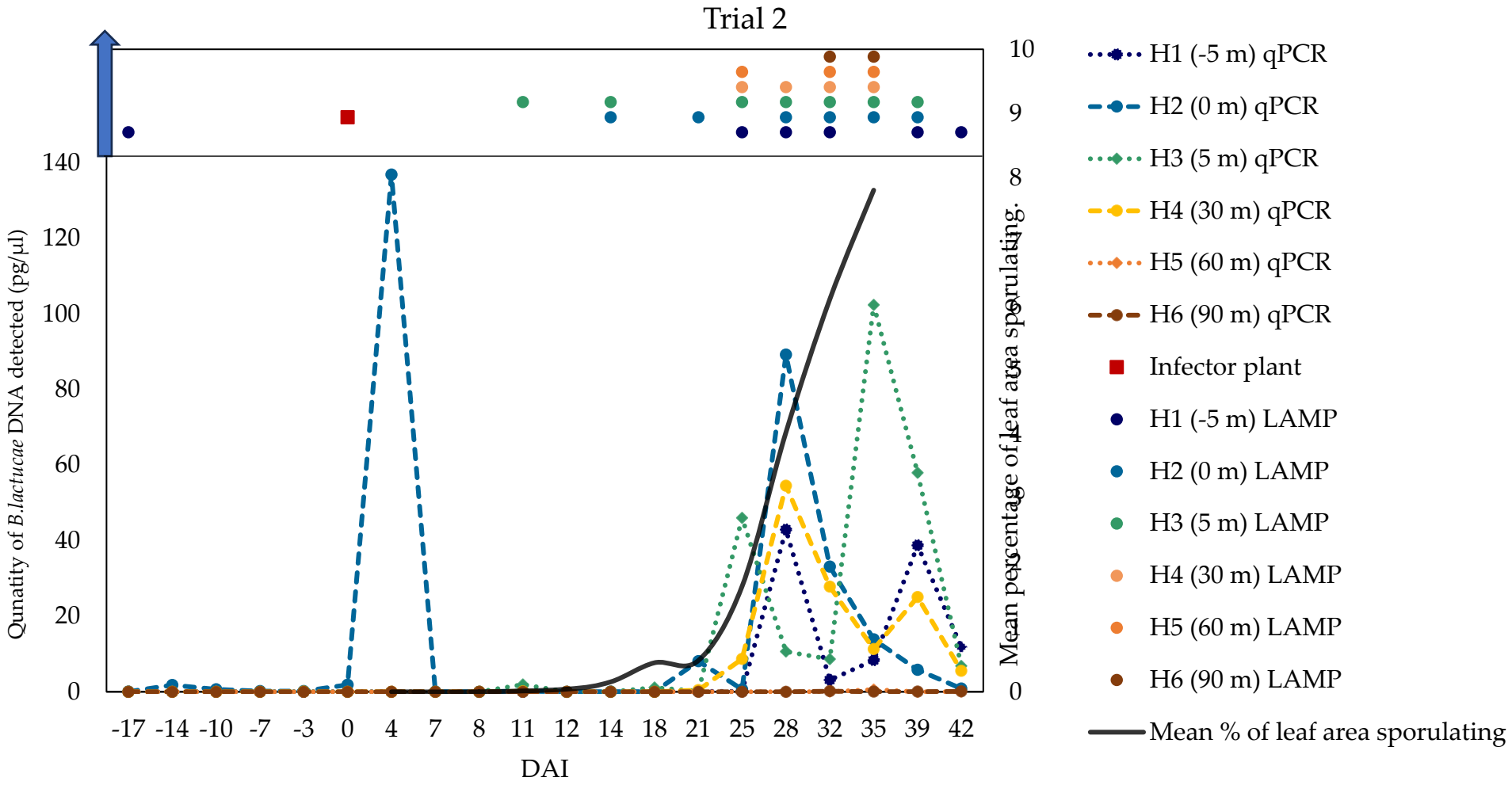


Figure 5-12 Detection of DNA of B. lactucae in samples collected at different distances from an inoculum source. Aerial samples were collected between 23/08/2021– 21/10/2021, *in Trial 2, quantity of DNA as measured using qPCR assay (lower figure) was between ~0.02-103 pg/µl. Mean percentage of leaf area sporulating was taken from living susceptible plants (≤384). Results from LAMP assay are shown as positive/negative results represented by dots. Arrow shows increasing distance from inoculum source where H2 = 0m from inoculated plant. Results from aerial samplers are represented in the same colour for both assays. Assessments of disease were carried out at approximately 3-4 day intervals. Arrow shows the increasing distance between aerial samplers. Infector plant is both the position (in line with date placed out) and H2 (0 m) aerial sampler for proximity. DAI is days after inoculation.*

Significant variation in average LAMP assay C_T between samples collected from different aerial samplers (χ 2 (5) = 14.0, p<0.02), was observed in Trial 2. The proportion of replicates that were positive per sample using the LAMP assay did not vary significantly according to sampler location (χ 2 (5) = 7.2, p>0.05; Figure 5-13, C and D). Therefore, although the incidence of positive results per sample was not significantly different when collected at different distances, the value of a positive result was. Although a Friedman's chi squared could identify differences between the average LAMP C_T results collected from the different aerial samplers in general, the Nemenyi test did not identify specifically which sampler results were significantly different when using p=0.05 as a cut-off. The lowest p value observed was between aerial samplers H3 and H5 when comparing LAMP C_T values (p = 0.15; Figure 5-13, C).

A qPCR Trial 2

C_T (χ 2 (5)=51.42, p<0.01)

	H1	H2	H3	H4	H5
H2	0.01	-	-	-	-
H3	0.71	0.37	-	-	-
H4	0.99	0.06	0.97	-	-
H5	0.51	0.00	0.02	0.17	-
H6	0.63	0.00	0.03	0.25	1.00

B Positives (χ 2 (5)=45.39, p<0.01)

	H1	H2	H3	H4	H5
H2	0.17	-	-	-	-
H3	0.98	0.57	-	-	-
H4	1.00	0.27	1.00	-	-
H5	0.48	0.00	0.13	0.34	-
H6	0.65	0.00	0.23	0.51	1.00

C LAMP Trial 2

C_T (χ 2 (5)=13.97, p<0.02)

	H1	H2	H3	H4	H5
H2	1.00	-	-	-	-
H3	0.86	0.98	-	-	-
H4	0.99	0.89	0.48	-	-
H5	0.81	0.54	0.15	0.99	-
H6	0.97	0.81	0.37	1.00	1.00

D Positives (χ 2 (5)=7.22, p>0.05)

	H1	H2	H3	H4	H5
H2	1.00	-	-	-	-
H3	1.00	0.99	-	-	-
H4	0.99	1.00	0.97	-	-
H5	0.97	0.99	0.89	1.00	-
H6	0.89	0.97	0.76	0.99	1.00

Figure 5-13 Comparison of positive results in LAMP and qPCR assay for each aerial sampler from Trial 2. Friedman's chi squared test and Nemenyi post-hoc analysis on the differences between aerial samplers both C_T and proportion of replicates that were positive per sample. Relevant Friedmans test is above each Nemenyi p-value table giving p -values from pair-wise comparisons of sample results from each aerial sampler. Positives (B and D) refers to using the data of the proportion of sample replicates that were positive per sample in Friedmans and Nemenyi test. C_T (A and C) refers to using the average C_T for sample's replicates for input into Friedmans test and Nemenyi test. A and B were for qPCR results, and C and D for LAMP results.

In Trial 1, the distance of aerial samplers from the inoculum source in the dispersal gradient appeared to be associated with lower p-values in the Nemenyi test when comparing average LAMP C_T and proportion of positive replicates (Figure 5-10, C and D). Conversely, in Trial 2 there did not seem to be any strong association of Nemenyi p-values to the distance of aerial

samplers from the inoculum source when LAMP values of a positive were analysed (Figure 5-13, C and D).

When samples from Trial 2 were tested for DNA of *B. lactucae* using the qPCR assay there were significant differences ($p<0.01$) between each aerial sampler in the average qPCR C_T, and proportion of replicates per sample that had a positive outcome (Figure 5-13, A and B). When the Nemenyi test was carried out to determine which aerial samplers were different, two aerial samplers pairwise comparisons were consistently statistically significantly different H2 (0m) to H5 (60m), and H2 (0m) to H6 (90m). H2 has a difference of 65m to H5 and 95m to H6 in the prevailing wind direction. Consequently, the significant difference obtained from sample set results suggests that in this trial distance of sample collection from inoculum source affected the value of a positive result when samples were tested with qPCR more than when tested with the LAMP assay.

A 3D regression analysis was employed to assess the correlation between the presence of disease (quantified in terms of days after initial symptoms), the distance of aerial samplers from the inoculum source, and the time taken until the detection of *B. lactucae*. LDM symptoms increased in incidence and severity throughout the epidemic (Figure 5-12). Consequently the 3D regression plane indicated that time until detection of DNA of *B. lactucae* decreased for both assays when samples were collected from closer to inoculum source, and with increased levels of disease (indicated through an increase in DAS; Figure 5-14**).** The regression plane for Trial 2 data was steeper for the LAMP assay results than qPCR showing a stronger correlation between distance and time until detection of DNA of *B. lactucae*. This was expected as the variation in time taken until detection ranged from 14.8-30 minutes when testing samples with the LAMP assay but 25.6-36.7 minutes when using the qPCR assay.

In conclusion, data from Trial 2 demonstrates that the LAMP assay could detect *B. lactucae* in-field. In addition, the distance of sample collection from inoculum source and the level of disease (therefore aerial sporangia) was found to influence the incidence of positive results.

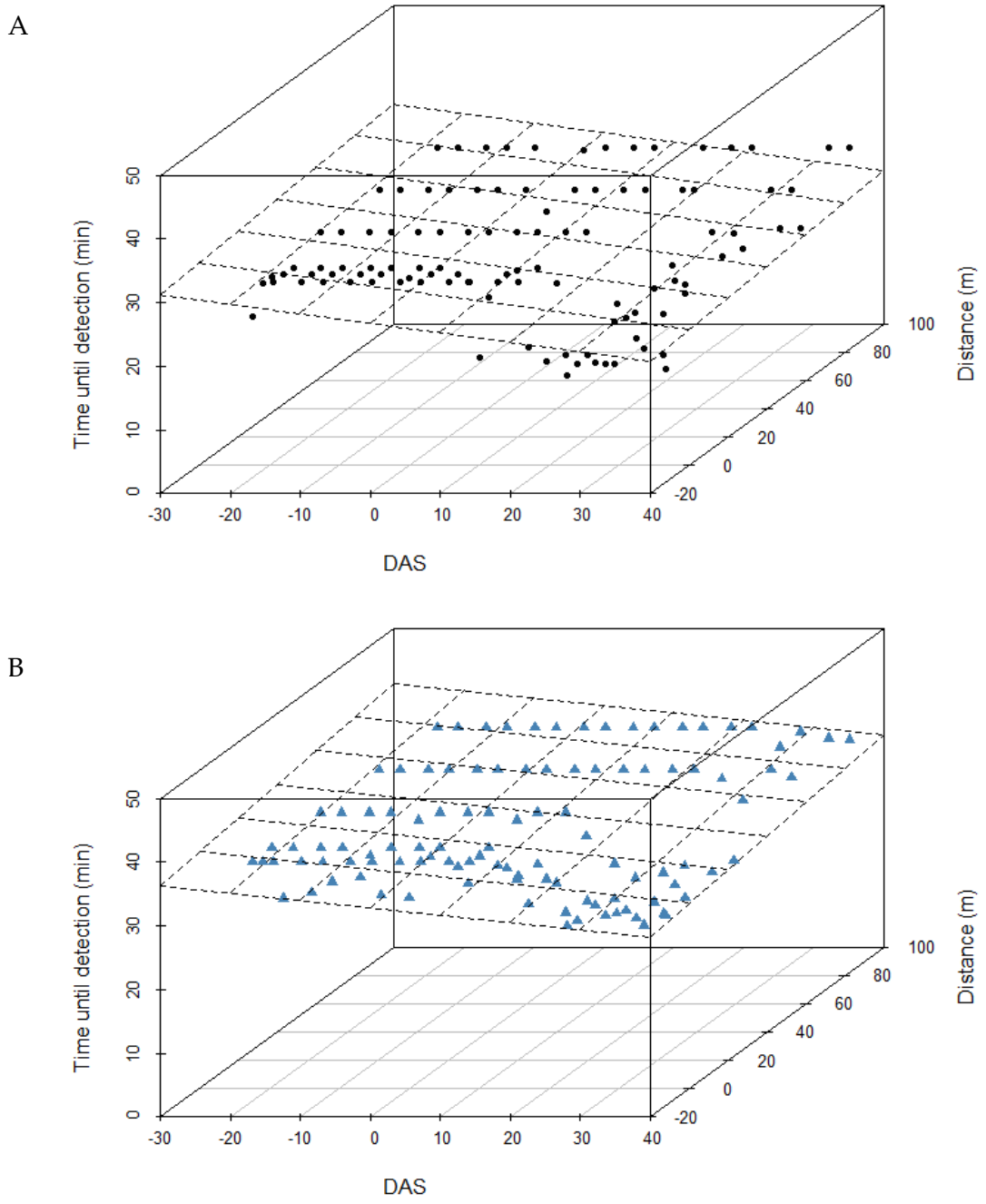


Figure 5-14 Modelling the relationship between time after first symptoms, distance of sample collection from inoculum source and time taken until B. lactucae detection when using the LAMP assay (A) and qPCR assay (B). Regression plane is the relationship between the three variables. Data from Trial 2. DAS is days after initial symptoms.

5.6.1.3 Trial 3

LDM progressed well in the inoculated lettuce field in Trial 3. Weather was conducive to LDM with the average temperature range between 8.7 and 16.6°C, and a total of 119.9 mm of rain. DNA of *B. lactucae* was detected in samples collected 90 m away from an inoculum source in both the LAMP assay and the qPCR assay (Figure 5-15). The aerial samplers in a 5 m radius of the centre of the trial had a higher incidence of positive *B. lactucae* detection when using the LAMP assay compared to samples collected further away, with a total of 25 positives for samples collected from samplers H1-H3, and 21 from samples collected from H4-H6 (Figure 5-15).

Significant differences were observed in Trial 3 samples for both LAMP C_T averages and proportional positives in sample replicates (χ 2 (5) = 17.2, p<0.01; χ 2 (5) = 14.4, p<0.05 respectively) (Figure 5-16, C and D). Nemenyi pairwise comparisons, identified that the C_T difference was significant between sampler H2 (0m) and the two furthest samplers H5 (60m) and H6 (100m). Conversely, there was no clear correlation nor statistically significant difference between aerial sampler position and the proportion of replicates with a positive outcome in pairwise comparison of aerial samplers. When testing the qPCR parameters with Friedmans Chi squared test, only the C_T (χ 2 (5) =20.6, p<0.001) and proportion of sample positives were significant (χ 2 (5) =13.9, p<0.05) (Figure 5-16, A and B). Nemenyi pairwise comparisons on qPCR results found no clear statistically significant interaction between aerial sampler location from inoculum source when comparing the proportion of replicates that were positive. However, there was a significant difference (p<0.05) for average C_T between sampler H1(-5m) and the samplers H4(30m) and H5(60m).

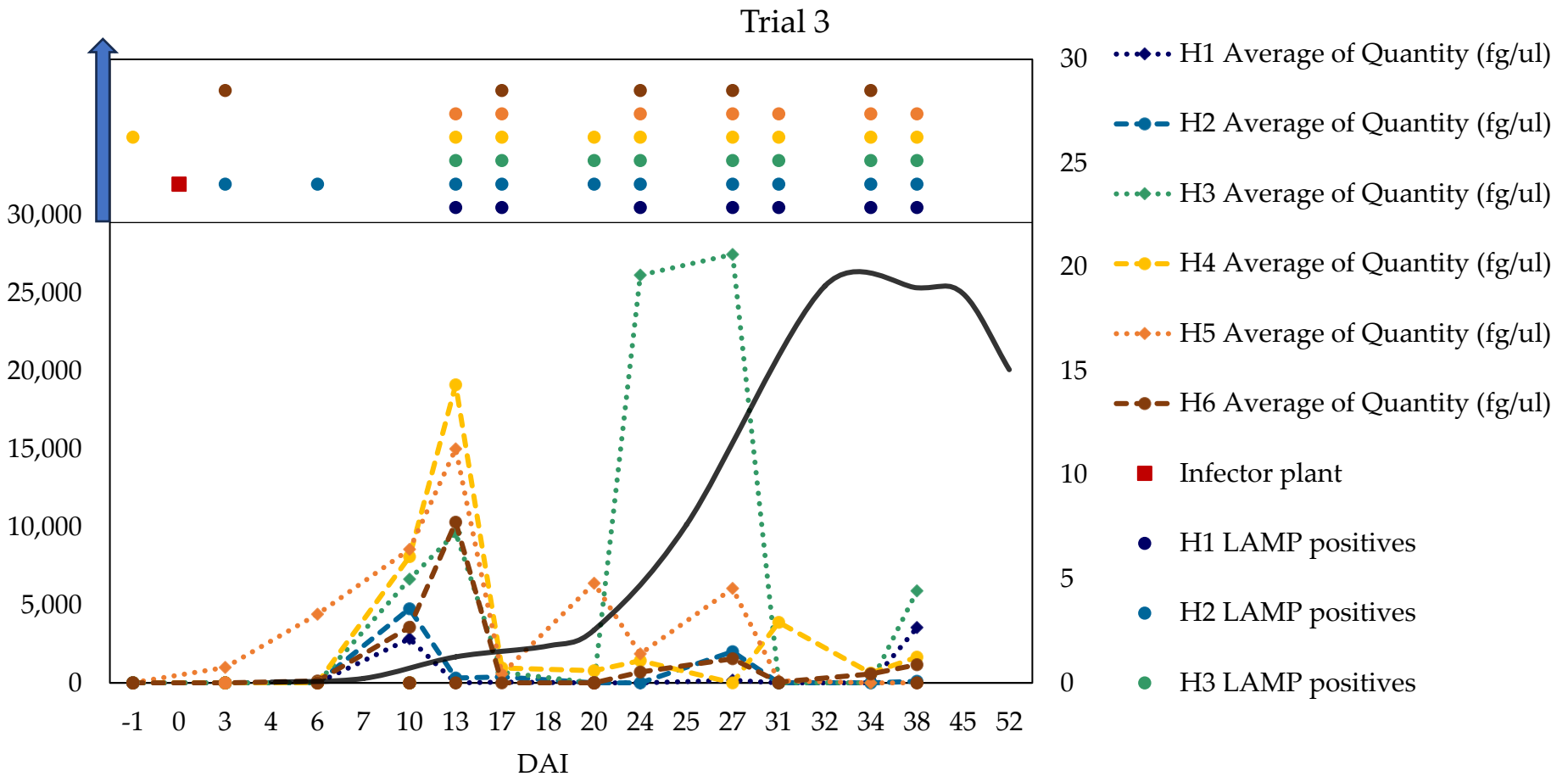


Figure 5-15 Detection of DNA of B. lactucae in samples collected from different distances from an inoculum source, in Trial 3. Aerial samples were collected between 25/08/2022–03/10/2022, quantity of DNA was between ~0.01-27 pg/µl. Mean percentage of leaf area sporulating was taken from living susceptible plants (≤360). Results from LAMP assay are shown as positive/negative results represented by dots. Arrow shows increasing distance from inoculum source where H2 = 0m from inoculated plant. Results from aerial samplers are represented in the same colour for both assays. Assessments of disease were carried out at approximately 3-4 day intervals but were matched to the closest aerial sampling data for clearer figure. Arrow shows the increasing distance between aerial samplers. Infector plant is both the position (in line with date placed out) and H2 (0 m) aerial sampler for proximity. DAI is days after inoculation.

A qPCR Trial 3

C_T (χ 2 (5)=20.62, p<0.01)

	H1	H2	H3	H4	H5
H2	1.00	-	-	-	-
H3	0.40	0.61	-	-	-
H4	0.03	0.07	0.86	-	-
H5	0.03	0.07	0.86	1.00	-
H6	0.86	0.96	0.97	0.40	0.40

B Positives (χ 2 (5)=13.93, p<0.02)

	H1	H2	H3	H4	H5
H2	1.00	-	-	-	-
H3	0.78	0.94	-	-	-
H4	0.22	0.43	0.94	-	-
H5	0.22	0.43	0.94	1.00	-
H6	0.75	0.92	1.00	0.95	0.95

C LAMP Trial 3

C_T (χ 2 (5)=17.25, p<0.01)

	H1	H2	H3	H4	H5
H2	0.09	-	-	-	-
H3	0.68	0.86	-	-	-
H4	1.00	0.27	0.92	-	-
H5	0.99	0.01	0.27	0.86	-
H6	0.99	0.01	0.27	0.86	1.00

D Positives (χ 2 (5)=14.41, p<0.02)

	H1	H2	H3	H4	H5
H2	0.75	-	-	-	-
H3	0.98	0.99	-	-	-
H4	1.00	0.86	1.00	-	-
H5	0.99	0.36	0.78	0.96	-
H6	0.91	0.16	0.50	0.81	1.00

Figure 5-16 Comparison of positive results in LAMP and qPCR assay for each aerial sampler from Trial 3. Friedman's chi squared test and Nemenyi post-hoc analysis on the differences between aerial samplers both C_T and proportion of replicates that were positive per sample. Relevant Friedmans test is above each Nemenyi p-value table giving p -values from pair-wise comparisons of sample results from each aerial sampler. Positives (B and D) refers to using the data of the proportion of sample replicates that were positive per sample in Friedmans and Nemenyi test. C_T (A and C) refers to using the average C_T for sample's replicates for input into Friedmans test and Nemenyi test. A and B were for qPCR results, and C and D for LAMP results.

Similar to previous trials, disease symptom severity increased throughout the epidemic, therefore as DAS increases so does disease, as observed in Figure 5-15. 3D regression analysis looked at the effect of distance on the detection of *B. lactucae* when accounting for disease presence (DAS). The regression plane was steeper for LAMP than qPCR showing a stronger correlation between distance and time until *B. lactucae* detection (Figure 5-17). This also indicates greater variation in time taken for detection ranging from 12.7-30 minutes when testing samples with the LAMP assay but 27.9-36.7 minutes when using the qPCR assay.

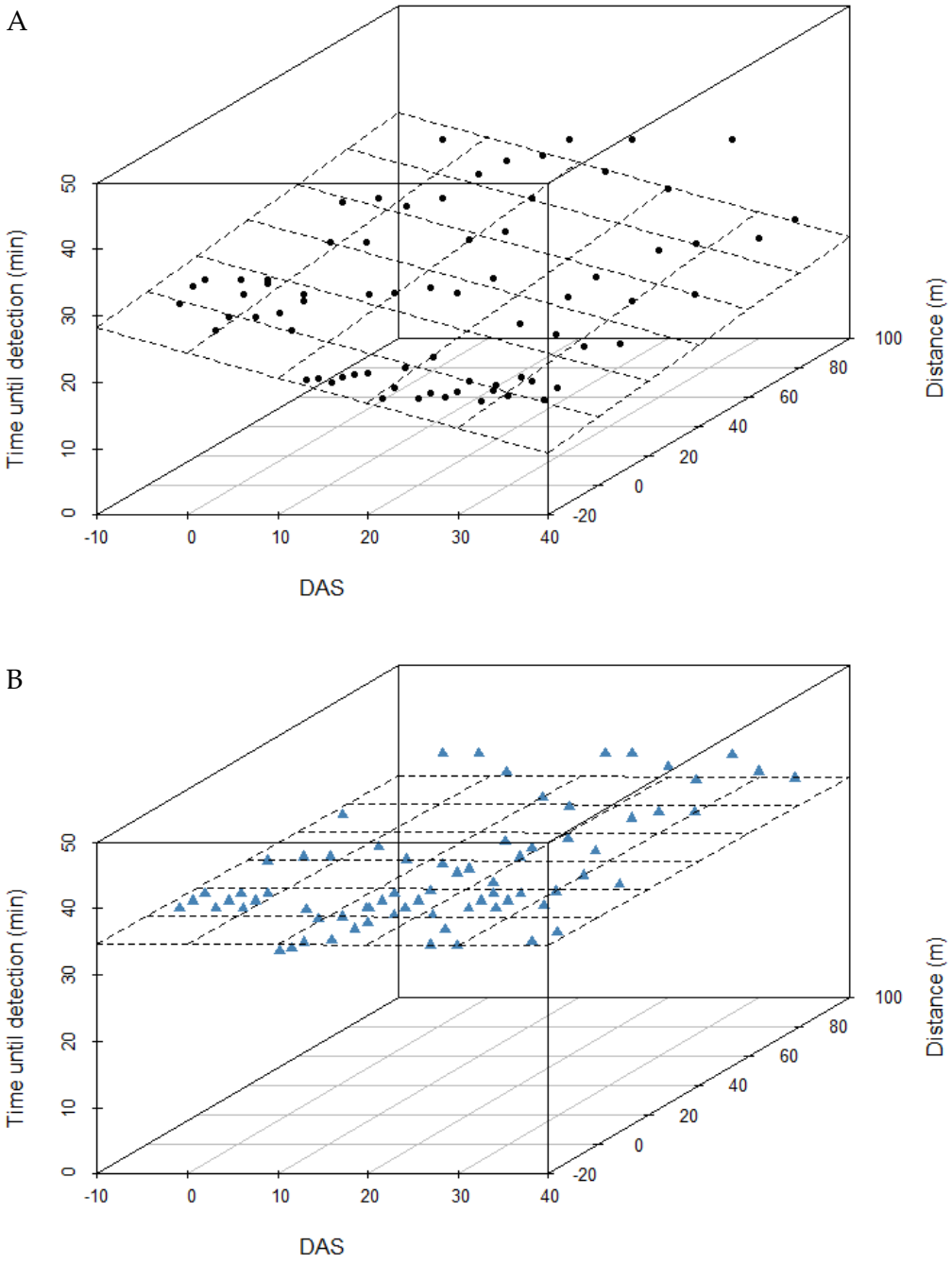


Figure 5-17, Modelling the relationship between time after first symptoms, distance of sample collection from inoculum source and time taken until B. lactucae detection when using the LAMP assay (A) and qPCR assay (B). Regression plane is the relationship between the three variables. Data from Trial 3. DAS is days after initial symptoms.

5.6.1.4 Summary

In all three trials *B. lactucae* could be detected using the LAMP assay and qPCR assay, and every aerial sampler had at least one positive result in all trials. Consequently, dispersal can be assumed to occur as far as 100 m from an inoculum source. Generally, the aerial samplers within a 5 m radius of the inoculum source had a higher incidence of *B. lactucae* detection

when using the LAMP assay. In Trial 1, the incidence of positives obtained from samples collected in a 5m radius of the inoculum source when testing with the LAMP assay was 16 out of a total of 23 positives obtained by the whole trial. In the following trials the positive LAMP outcomes obtained from samples collected in the 5m radius was 19 out of a total of 28 obtained in Trial 2 and 25 out of a total of 46 in Trial 3. In each case the samples from the 5m radius when tested in LAMP, accounted for more than half of the positives obtained in each trial (Trial 1 = 69.6%, Trial 2 = 67.9%, Trial 3 = 54.3%). The 5 m radius also had the highest individual quantity of DNA of *B. lactucae* detected in every trial, which was 88.1 pg/µl from H1 in Trial 1, 138.8 pg/µl from H2 in Trial 2 and 27.5 pg/µl from H3 in Trial 3.

Analysis showed that distance from inoculum source did generally have a statistically significant effect on positive result values (Trial 1 - Figure 5-10, C and D: $p < 0.05$; Trial 2 - Figure 5-13, A, B and C: $p < 0.05$; Trial 3 - Figure 5-16, A, B, C and D: $p < 0.05$). Overall, aerial samplers at an increased distance from inoculum source generally yielded lower *p*-values in a Nemenyi test than adjacent aerial samplers, particularly with aerial samplers at the start (H1 and H2) and the end (H5 and H6) of the dispersal gradient. This was expected as the further from an inoculum source the fewer the sporangia captured by aerial samplers.

Further checks into the effect of distance on the time taken until detection in the presence of disease (DAS) with 3D regression analysis found that the LAMP assay results were affected more than the qPCR results by disease presence and distance. This was clear by the variation in time taken until detection, which for the LAMP assay ranged from a minimum of 11.5 (Trial 1), 14.8 (Trial 2) and 12.7 (Trial 3) minutes to a maximum of 30 minutes. This was a greater difference in time until detection in comparison to the qPCR which ranged from a minimum of 24.0 (Trial 1), 25.6 (Trial 2) and 27.9 (Trial 3) minutes to a maximum of 36.7 minutes.

5.6.2 Timing of sporangial release

The Burkard 7-day continuous sampler (7-day sampler) monitored the aerial space 5m from the centre of an inoculated lettuce field from 28/08/21-21/10/21. Samples collected from the 7-day sampler tested positive for DNA of *B. lactucae* using both the LAMP and qPCR assay (Figure 5-18). Weather became increasingly conducive to disease throughout the 7-day sampler monitoring period, with an increase in rainfall (total rainfall 50 mm in September and 59.4 mm in October), and a decrease in solar radiation (9.3 MJ/m2 in September and 5.1 MJ/m2

in October) and temperature (average max temperature was 18.3°C in September and 13.6°C in October) (Figure 5-18). There was a widespread incidence of sporulating lesions in the trial 14 days after infector plants were placed, (170 of 288 susceptible plants had sporulation). The increasing incidence of sporulation matches prior reports of the days taken (14-21 days) for *B. lactucae* to complete its asexual life-cycle (Verhoeff, 1960). Though week 5 was removed from analysis it was retained and highlighted graphically.

When samples were tested using the LAMP assay, the incidence of positive results were only slightly different between AM and PM collection periods; samples collected in the AM period had 21 positives and those in the PM period had 20 positives. There was no difference in the incidence of positives detected in samples from AM and PM collection periods when tested using qPCR, with each collection period having 36 positives. For both assays the number of replicates of a sample that gave a positive result varied. Statistical analysis using McNemar's mid-*p* test identified no significant difference between incidence of positives when samples were collected in the AM or PM (LAMP McNemar's mid-*p*: mid-*p*= 0.688, *p* =1, n=5; qPCR McNemar's mid-*p*: mid-*p*= 1, *p* =1.19, n=18).

However, the quantity of DNA of *B. lactucae* detected in samples using the qPCR assay, showed differences between AM and PM, with the AM having a total of 4964.5 pg/µl detected and the PM period having a total of 3525.6 pg/µl detected. However, the difference between the quantity of DNA detected in the AM and PM periods was not significant (Kruskal-Wallis: H = 0.2474, N = 98, *p* = 0.62). Therefore, data shows that though AM period has more aerial sporangia, sporangia were still prevalent in the PM period, but in lower quantities. Interestingly, the quantity of DNA for 'PM' collected samples appeared to "echo" the 'AM' peaks at some points throughout the trial (Figure 5-18). This could have occurred by chance as the highest peak in PM (DAI 38: 17/10/21) could have been triggered by the increase in rainfall and the epidemic being further progressed.

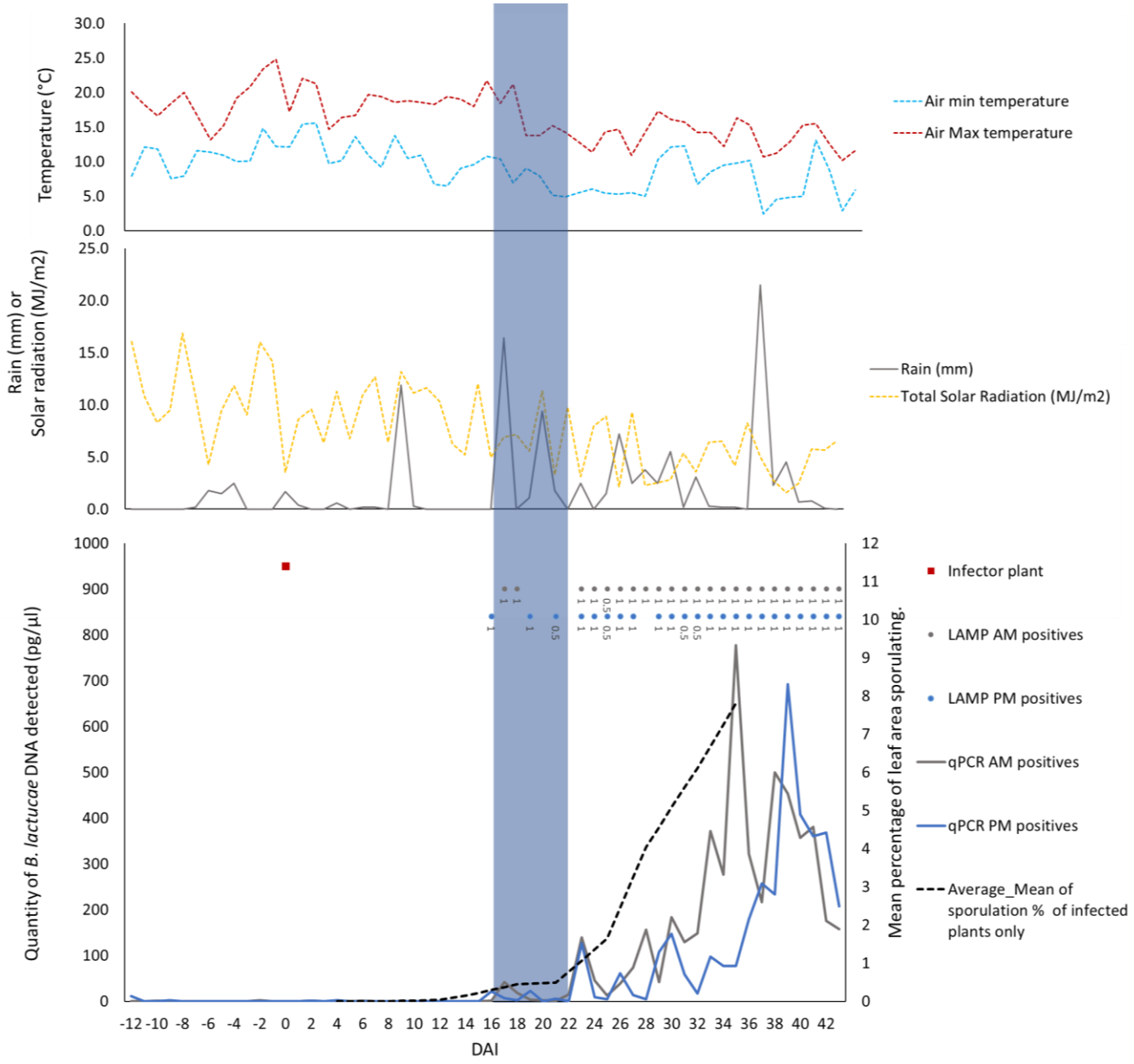


*Figure 5-18 Timeline of Burkard 7-day continuous sampler results against weather parameters. Blue box is indicative of problematic sample processing. Number labels denote proportion of the replicates that amplified (n=2). Mean percentage of leaf area sporulating was from susceptible and living plants (n≤ 384**).** DAI is days after inoculation.*

5.6.3 Aerial sampling at commercial field sites

5.6.3.1 Aerial sampling at commercial field sites in 2021

Commercial sampling in 2021 was conducted between 23/05/21-23/09/21. LDM outbreaks were reported at both commercial sampling sites. Generally, weather was conducive to disease throughout the monitoring period at both sites (data not shown.) Samples collected from the English commercial field site had complications with inhibition, possible due to inhibiting material such as peat soil and insects being present in samples collected (see section: 5.6.4.2).

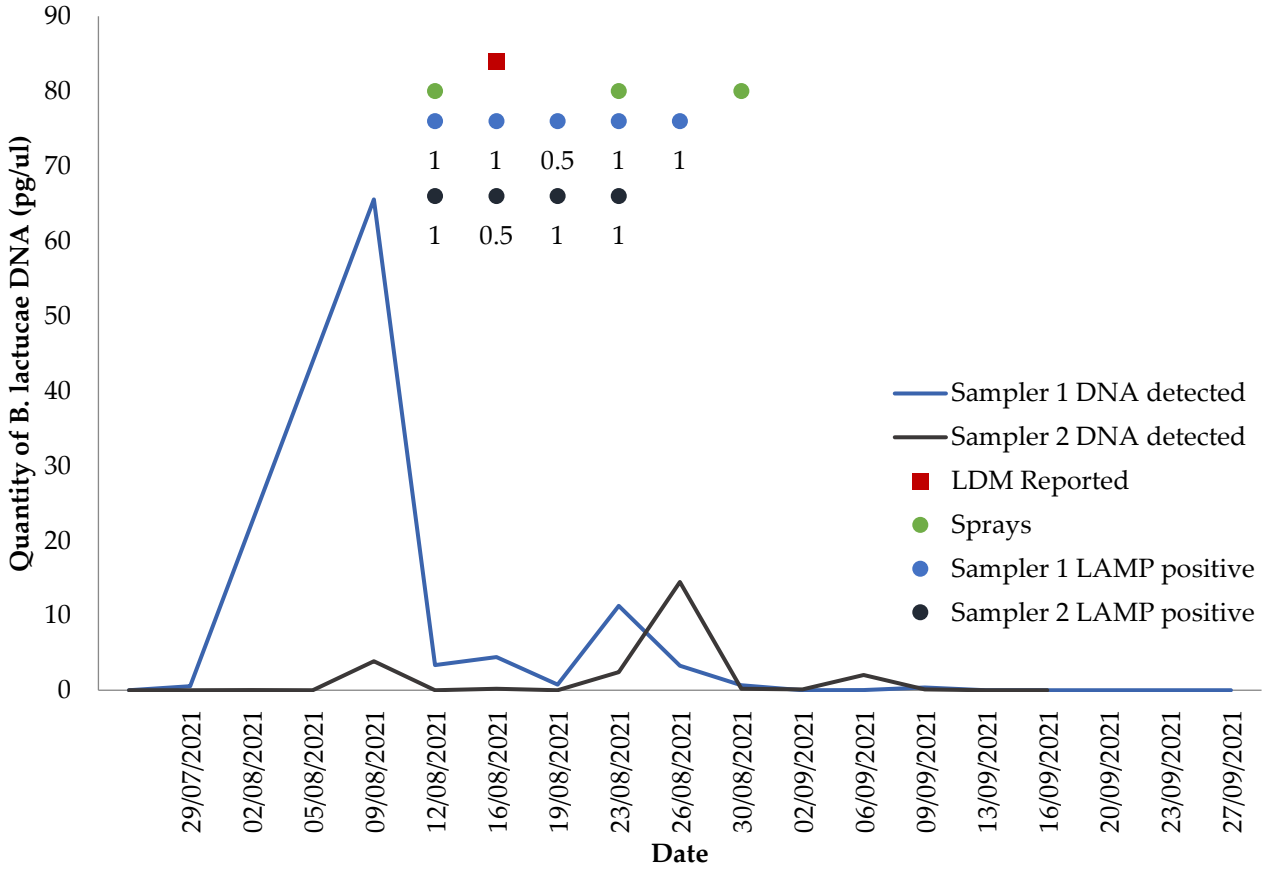


Figure 5-19 Detection of DNA of B. lactucae (pg/ µl) from aerial samples. Results from the third Scottish commercial sampling site over the 2021 growing season (29/07/21-27/09/21). Values ranged from 0.012pg/µl to >65pg/µl. Dates of fungicide applications are noted. There is missing data, 01/08/21-09/08/21 (sampler 1), and 20/09/21 (Sampler 2). Positive qPCR results are plotted as values, and positive LAMP results by binary positive/negative with the proportion of the assays (0.5 or 1.0) that tested positive when the sample was run in duplicate.

In field 1 and 2 of the Scottish commercial sampling site, positives for *B. lactucae* were detected but were sporadic in incidence and sample results from each assay did not coincide, nor was LDM reported in field (data not shown). Field 3 of the Scottish commercial sampling site was the only field with reported incidence of LDM, which was on the 16/08/2021 (Figure 5-19). The LDM outbreak in field 3 was approximately 50 m from one of the aerial samplers. Samples

from both aerial samplers in the field amplified in the LAMP assay and the qPCR assay detected DNA of *B. lactucae* prior to the LDM incidence reported in this field. Samples tested using the qPCR assay were positive for DNA of *B. lactucae* at an earlier date than when tested with the LAMP assay; with a positive result from qPCR on the 29/07/2021 followed by several positives after the 2/08/2021. The quantity of DNA of *B. lactucae* detected decreased after 9/08/2021, which may have been linked to the fungicide spray on 12/08/2021. The LAMP assay was able to detect DNA of *B. lactucae* from samples collected in field, even in the presence of fungicide sprays.

5.6.3.2 Aerial sampling at commercial field sites in 2022

In 2022, sampling of commercial crops occurred between 08/07/2022-29/09/2022. Each commercial site had at least one field with LDM symptoms reported. Samples from the English site had contaminants present. The inclusion of sample purification in DNA extraction protocol could not completely remove the contaminants that inhibited DNA amplification in both diagnostic assays (see section: 5.6.4.2).

Two fields at the Scottish site reported LDM symptoms in lettuce crops, on 24/08/2022 (field 2), and 22/09/2022 (field 3). Both outbreaks were close (~50-80m) to at least one aerial sampler. In field 2, DNA of *B. lactucae* was detected in samples collected on 18/08/2022 and 22/08/2022 using the LAMP assay, six and two days respectively prior to the observed in-field LDM symptoms (Figure 5-20).

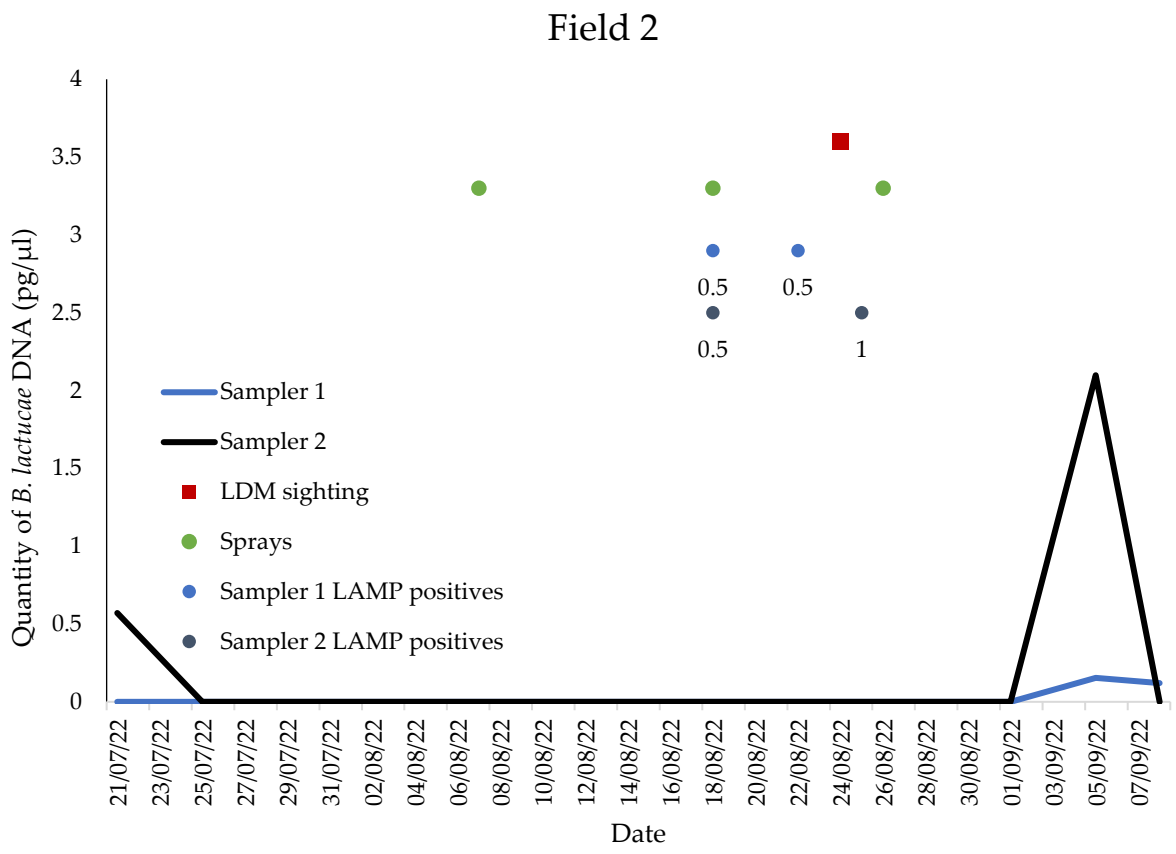


Figure 5-20, Detection of DNA of B. lactucae (pg/ µl). Results from Scottish field site, field 2 (21/07/2022-08/09/2022). Dates of fungicide applications are noted. Values range from 0.241 pg/µl to >2 pg/µl. Positive qPCR results are plotted as values, and positive LAMP results by binary positive/negative with the proportion of the assays (0.5 or 1.0) that tested positive when the sample was run in duplicate. Samplers 1 and 2 were 50 m apart in line with the prevailing wind direction.

Similarly, in field 3, samples retrieved on the 12/09/2022 and 19/09/2022 gave positive results when amplified using the LAMP assay (data not shown). In field 3 a single sample gave a positive result prior to the LDM observation using the qPCR assay (data not shown). The quantity of DNA detected at commercial sites was notably lower in 2022 than in 2021.

One sample in 2022, collected on 19/09/2022 from sampler 2, was positive in both the LAMP and qPCR. The only observed sample where both replicates were positive when tested using the LAMP assay was from sampler 2 collected on 25/08/2022. When samples were tested using qPCR it was only the samples collected on 05/09/2022 that had positive results for both replicates. Other positives were each in a single replicate of a sample.

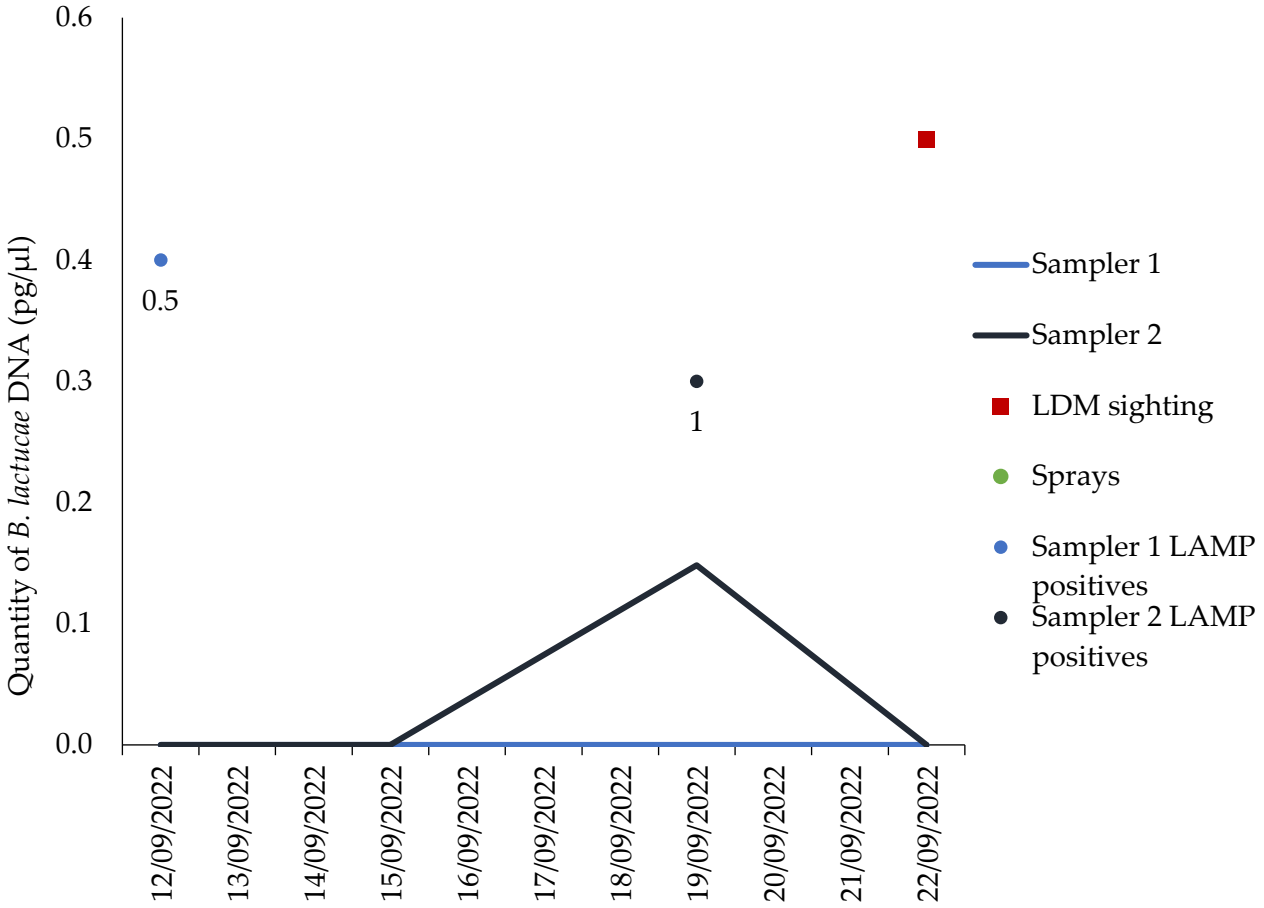


Figure 5-21, Detection of DNA of B. lactucae (pg/ µl). Results from Scottish field site, field three (12/09/2022-22/09/2022). Dates of fungicide applications are noted. Values range from 0pg/µl to >0.2 pg/µl. Positive qPCR results are plotted as values, and positive LAMP results by binary positive/negative with the proportion of the assays (0.5 or 1.0) that tested positive when the sample was run in duplicate. Samplers 1 and 2 were 50 m apart in line with the prevailing wind direction.

5.6.4 Inhibition and contamination

5.6.4.1 Exploration into the inhibiting effects of discolouration from contamination on the detection of DNA of B. lactucae

During commercial field trial data collection, it was observed that some fields with reported LDM symptoms were not represented in, or correlated with, the diagnostic assay results. Additionally, post DNA extraction certain samples displayed unusually dark DNA pellets and suspensions. Based on the 2020 results, it was determined that discolouration resulting from contaminants reduced positive results. None of the three discoloured non-purified 2020 samples produced clear amplification curves or prominent melt-curves run in the real-time LAMP assay, suggesting inhibition (Figure 5-22).

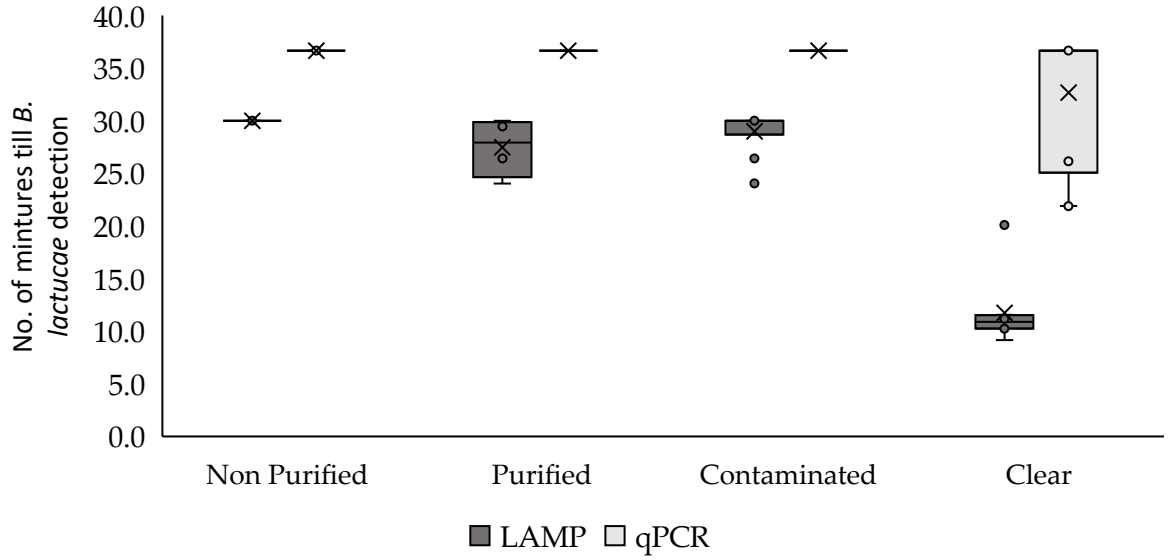


Figure 5-22 Comparison of time until B. lactucae detection when samples were contaminated, purified or non-contaminated. Contaminated includes the results of both Purified and Non-purified samples as N=N with clear samples. Spiked samples were spiked to a 1:1 ratio with DNA of B. lactucae (10ng/µl). Samples in which B. lactucae DNA was undetectable were given the max cycle in calculations, qPCR = 37 mins; LAMP = 30 minutes

For the purified discoloured samples (n=2), all but one replicate produced amplification curves with evidence of specific amplification (melt-curve analysis, Tm = 82-83°C, Figure 5-23, B). However, the amplification curves were detected in later cycles (mean time 26.6 minutes, SD=7.7, Figure 5-22) and melt-curve peaks were smaller than genomic DNA standards. The smaller melt-curve peaks suggested reduced target DNA was present and/or that there was still interference with fluorescent signal from contaminants (Figure 5-23, B). The discolouration of samples appeared to be more problematic for detection of *B. lactucae* with the qPCR assay, as none of the discoloured samples (regardless of purification) were detected, while only two of the clear samples amplified (n=5). Ultimately, although the purification did improve readings, none of the sample readings would be classified as a positive result.

5.6.4.2 Inhibition in field samples 2021-2022

Exploration of whether inhibition was present in samples collected by aerial samplers in 2021 that were near lettuce that had LDM symptoms found that inhibition was prevalent in samples collected at one commercial site (Figure 5-24). Sample sub-sets were taken from each commercial site close to when disease symptoms were confirmed in-field as putative positives and from when the field did not have disease reported as negative controls. A sub-set of samples from the Hutton trial looking at dispersal gradients was also used to compare to the commercial sites.

Scottish commercial samples, including negative controls (n=2) and samples of interest (putative positives, n=3), gave positive results when spiked with DNA, suggesting that low target DNA concentrations in the sample, rather than inhibition, was the cause of the initial negative result. Samples collected from the Hutton trial showed similar results (n=6). Only one of the sample replicates was positive for *B. lactucae* and the spiked version of this sample took less time for detection. This suggested that there was no inhibitory contamination present, but rather that the sample had a low concentration of *B. lactucae* DNA and therefore did not amplify within the C_T cut-off set. None of the English samples of interest (n=2) or their negative controls gave a positive result, even when spiked with *B. lactucae* DNA. This suggested that, at this particular site, inhibitors present in the sample were behind the negative results. This qPCR plate also included re-testing of purified samples from the English commercial site. None of these samples gave a positive result when tested using the qPCR, further suggesting the presence of inhibitors.

In 2022, samples were checked for inhibition across all fields as LDM was generally reported at least once within each field where aerial sampling was carried out, but *B. lactucae* was not always detected in the samples where LDM was reportedly present. Spiking of samples collected from the Scottish commercial site decreased the time taken until *B. lactucae* was detected using both assays (Figure 5-25, A). This suggests that if a negative result was obtained from samples at this commercial site it could be attributed to low concentrations or no DNA of *B. lactucae* present in the sample rather than occurring as a result of inhibition. However, there was an exception with field 2 of the Scottish commercial site. When spiking samples collected from field 2 fewer than half of the samples run through the qPCR came up positive (n=4/10), which could suggest the presence inhibitors, possibly at low concentrations.

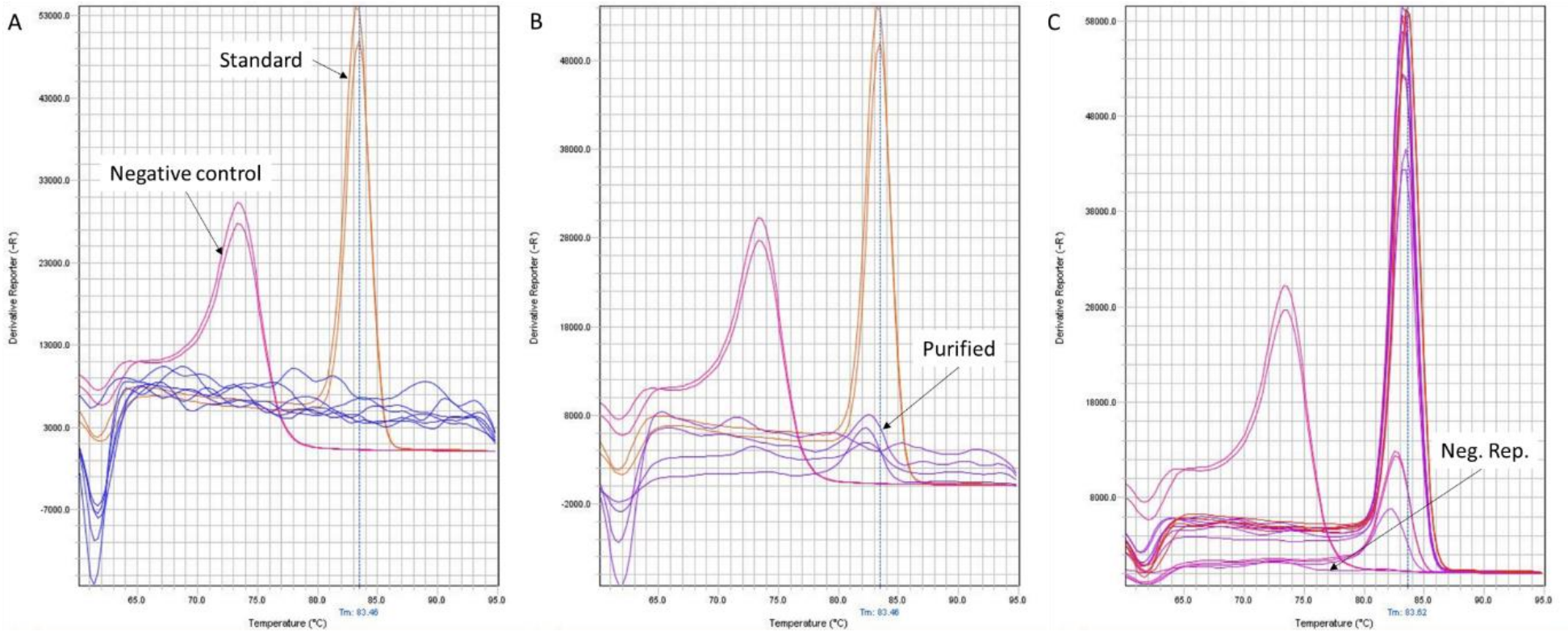


Figure 5-23 Melt curves of discoloured samples amplified using the real time LAMP assay. A) Non-purified in blue, (B) purified small purple, (C) the clear samples, magenta lines around 74°C are the negative control, red lines around 83°C are the standards. Neg. Rep. is the only clear sample replicate that had no peak that matched the temperature of the standards.

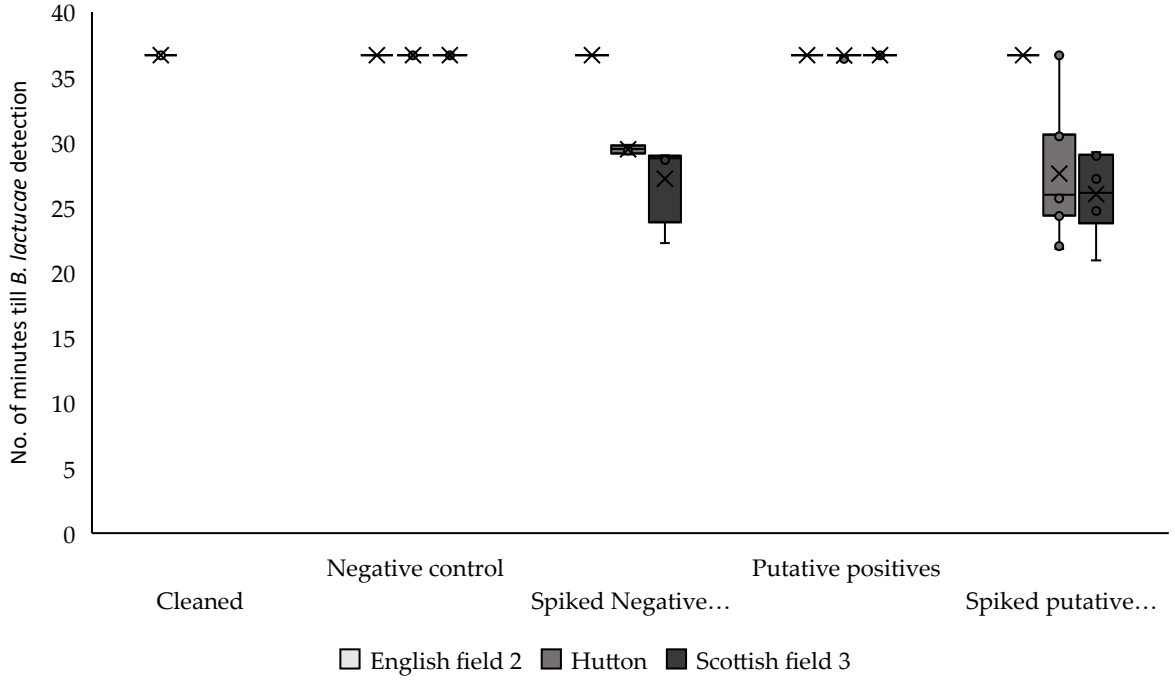


Figure 5-24 The difference in time for DNA of B. lactucae to be detected in field samples, untreated, purified and or spiked. Cleaned means sample went through soil purification protocol, putative positives are field samples of interest where with LDM was viewed in-field, or a replicate came up positive in one assay and is a putative positive result. Spiked means the samples was spiked to a 1:1 ratio with DNA of B. lactucae (10ng/µl). Samples in which B. lactucae DNA was undetectable were given the max cycle in calculations, qPCR = 37 mins.

Spiking the samples collected from field 1 and 2 of the English commercial site fields only resulted in positives when using the LAMP assay (n=10/11) (Figure 5-25, B). In field 3, both the LAMP and one replicate of qPCR gave positive results when spiked (LAMP n=4/4; qPCR n=1/4). This suggests that inhibitors were generally more prevalent at the English commercial site, confirming the findings observed with 2021 samples. The subset of samples collected from the Hutton field trials all gave positive results, with the exception of one replicate in each assay. For these samples, spiking generally reduced the time for detection of *B. lactucae.* Therefore, contamination that could inhibit DNA amplification in both diagnostic assays was not observed on this site (Figure 5-25, C).

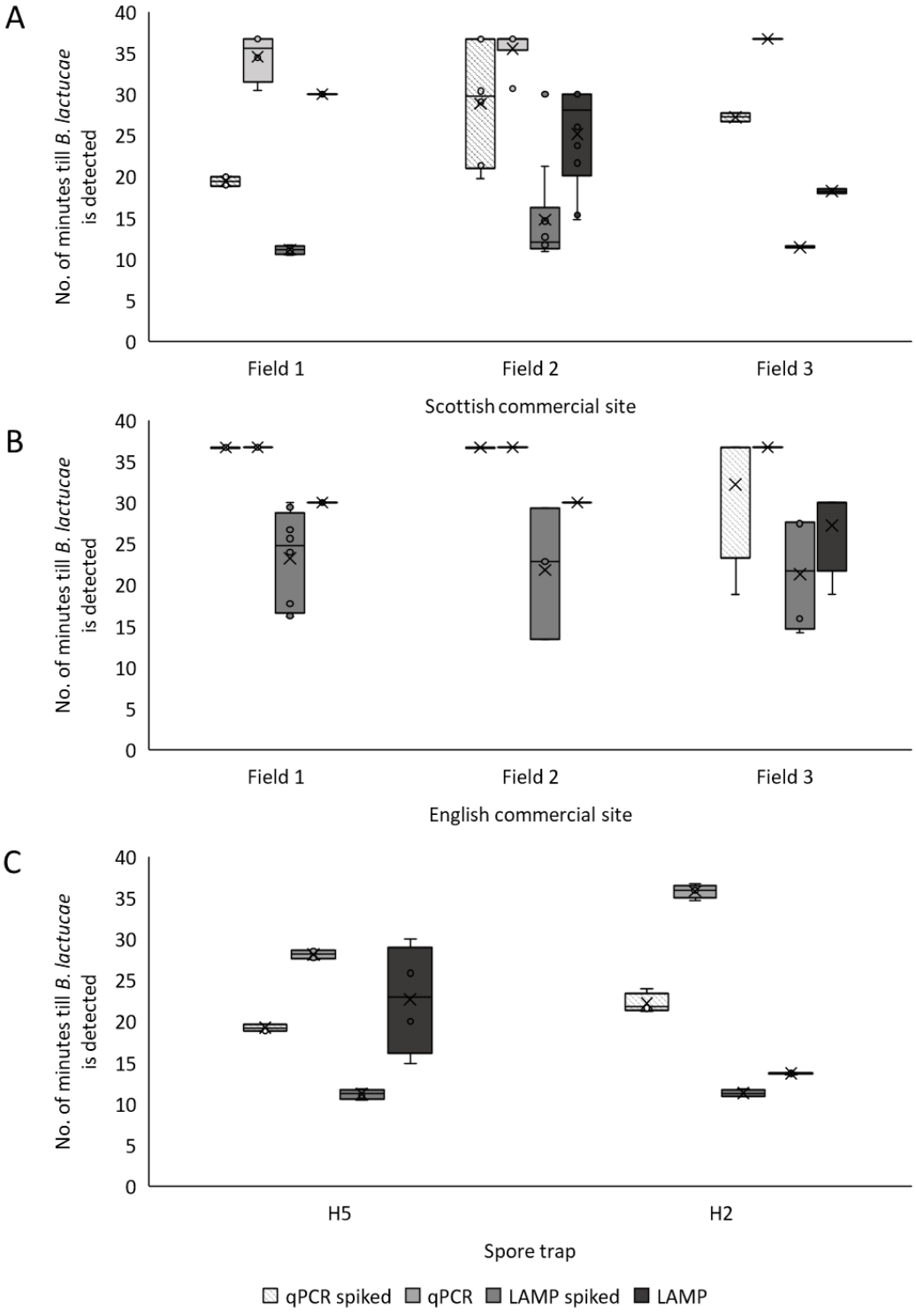


Figure 5-25 Check for inhibition in 2022 samples. Spiked means the samples was spiked to a 1:1 ratio with DNA of B. lactucae (10ng/µl). Samples in which B. lactucae DNA was undetectable were given the max cycle in calculations, qPCR = 37 mins. A, is samples from the Scottish commercial site, B, is from the English commercial site and C is from the Hutton field trial.

5.7 Discussion

In this study, a previously developed real-time LAMP assay, capable of detecting *B. lactucae* in DNA extracted from sporangial suspensions containing as few as 0.01 sporangia equivalents per reaction, was tested to determine detection limits for in-field application in combination with aerial samplers. The assay could detect 25 sporangia equivalents reliably. Real-time amplification of *B. lactucae* followed by a melt-curve analysis for confirmation of positive detection on both genomic DNA and DNA from sampling Rotorods to which sporangia were added, was used to set cycle thresholds to reduce the number of false positive results. The LAMP assay cycle threshold was set at 45 C_T as the limit for a binary (positive/negative) outcome, which was 22.5 minutes run time.

The real-time LAMP assay was used in field trials at Hutton to test detection in field environments, and to study the epidemiology of LDM, including the spore dispersal gradient which could give an indication of how wide an area can be affected from a single disease outbreak. With samples collected in JHI field trials, DNA of *B. lactucae* was detected in samples collected from an aerial sampler 100m from an inoculum source. Wu, van Bruggen, et al. (2001) estimated that sporangial dispersal was between 80 m and 3000 m. The most consistent detection of DNA of *B. lactucae* occurred directly adjacent to the inoculum source in the Hutton trials, generally within a 5 m radius. The 5 m radius was also where the highest amount of DNA of *B. lactucae* was detected in each Hutton trial. Distance had an effect on detection of *B. lactucae* when using the LAMP assay in field environments. However, detection was not consistent, the sporadic detection of *B. lactucae* can be attributed to wind dynamics which are complex and not consistent.

Preliminary analysis of the timing of sporangial release, found that sporangia were prevalent and present in the aerial space in both parts of the day sampled, with the 'AM' having slightly more sporangia released than the 'PM' period. According to Carisse & Philion, (2002), different sporangial release times were observed in Canada compared with in California (Scherm & van Bruggen, 1995b). Preventative measures may therefore be slightly more effective in the morning, but further studies into quantity of aerial sporangia and timing of fungicide applications in the UK would need to be carried out to confirm this.

The key objective of the LAMP assay development was to detect *B. lactucae* prior to a disease outbreak, preferentially prior to symptom development, in order to inform prompt action, such as altering fungicide applications to prevent further disease spread (Buja et al., 2021; Crandall et al., 2018). The LAMP assay detected DNA of *B. lactucae* two to six days before disease was reported in commercial fields in Scotland (Figure 5-20 and Figure 5-21). Sampling was carried out twice per week, approximately 3-4 days apart. An increased sampling frequency may have detected DNA of *B. lactucae* more than six days ahead of symptom development. At least one of the aerial samplers was roughly 50-80 m from LDM symptomatic lettuces when *B. lactucae* was detected. As both diagnostic assays were able to detect *B. lactucae* in these fields, this means that the LAMP assay (and qPCR assay) can detect *B. lactucae* up to 80 m from an inoculum source in commercial field environments when using Rotorod aerial samplers.

LDM symptoms were present up until harvest at the Scottish site, although lesions were more necrotic post-fungicide sprays and new growth had fewer lesions. In addition, not all commercial environments with observable LDM symptoms in lettuce crops had positive results when samples were tested using the LAMP assay. This was attributed to soil contaminants on aerial samples collected from commercial trials in England.

The LAMP assay is often used as an alternative to conventional and quantitative PCR, in part due to its high tolerance of crude DNA samples (Notomi et al., 2000; Wong et al., 2018). The LAMP assay for detecting *B. lactucae,* though not as sensitive as the qPCR assay, was found to be less sensitive to inhibition from soil contamination (see section 5.6.4). Spiking of commercial samples with relatively high quantities of DNA showed that strong inhibition had occurred at some sites (Figure 5-25), specifically at one commercial field in 2021 where no DNA of *B. lactucae* was amplified despite visual confirmation of in-field LDM adjacent to the aerial sampler. Analysis did not suggest that agrochemical applications had a notable effect on pathogen detection and both commercial sites utilised similar active ingredients in their spray programs (data not shown). However, soil did have a significant effect, preventing fluorescent readings through discolouration, whilst also introducing PCR inhibitors which are prevalent in soil (Watson & Blackwell, 2000). Soil was presumed to cause overloading, in which the adhesive surface was coated by soil preventing sporangial adhesion (Jackson &

Bayliss, 2011; West & Kimber, 2015). Soil contamination of this level was unanticipated and subsequent changes to protocols were unable to resolve the issue.

Detection of *B. lactucae* could be refined further through optimisation of timing and duration of sampling. Other studies have highlighted that sporangial release varies throughout the day in part due to conducive weather conditions (Carisse & Philion, 2002; Fall et al., 2015; Scherm & van Bruggen, 1995b). The efficacy of weather-based decision support systems has been demonstrated for informing spray programs for multiple downy mildew species (Araújo et al., 2017; Hovius et al., 2007; Kushalappa, 2001; Scherm et al., 1995). These systems could be utilised in combination with spore monitoring techniques to inform disease control decisions.

LAMP assays can be made suitable for use in in-field detection devices or adapted for less sophisticated heating apparatus (e.g. water-baths) (Notomi et al., 2015; Wong et al., 2018). The LAMP assay for *B. lactucae* could be adapted for full in-field use utilising machinery, such as the Genie® II developed by Optigene (Optigene, 2018). However, further testing and optimisation would be required, as currently the YCL DNA extraction kit utilised for DNA extraction in LAMP protocol does not lend itself to in-field preparation.

The integration of in-field devices that incorporate aerial samplers with isothermal detection assays, such as LAMP, could be a potential approach for notifying growers of disease risk periods and to facilitate disease control through the efficient use of fungicides. Villari et al. (2016) demonstrated that the use of aerial samplers capturing the airborne inoculum of *Magnaporthe oryzae* (the cause of grey leaf spot of perennial ryegrass) in conjunction with real-time LAMP assay for pathogen detection, allowed detection of *M. oryzae* up to 12 days before symptoms developed in the field. Thus, the assay they developed could be used for disease risk forecasting. A similar finding was reported by Thiessen et al., (2016), who demonstrated that using aerial sampling and a variety of LAMP assays for the detection of *Erysiphe necator* (the cause of grape powdery mildew) to determine fungicide sprays led to an average reduction of fungicide applications by 3.3 fold. Detection of *B. lactucae* using qPCR assays (Dhar et al., 2020) and a cut-off of 24 C_T did save 1-3 fungicide applications, however the assay was not developed for in field use.

In this study the LAMP assay detected *B. lactucae* before the first disease symptoms were observed, 9 days before symptoms in Trial 1 and 1 day in Trial 3. Similarly, DNA of *B. lactucae* was detected in samples 4 days (field 3 - 2021), 7 days (field 2 - 2022), and 10 days (field 3 - 2022) before disease was reported in Scottish commercial trials. When detection of *B. lactucae* using LAMP occurred after symptoms were observed, the disease symptoms were not widespread, with only 0.21% of susceptible plant foliage in Hutton Trial 2 and 0.03% in Trial 3 recorded at the time of the first instance of disease. This indicates that the LAMP assay when employed with aerial samplers is sensitive enough to detect *B. lactucae* a stage where it could be used to impact decision making regarding chemical controls.

5.8 Conclusion

Both the real-time LAMP assay and the qPCR assay are capable of detecting *B. lactucae* in field environments. Each diagnostic assay has its own strengths and weaknesses, specifically if weather stations do not measure in-field parameters. Both diagnostic assays are best employed in combination with monitoring of conditions conducive to disease development.

The qPCR was more accurate than the real-time LAMP in terms of assessing the extent of disease as the quantity of DNA of *B. lactucae* detected by the qPCR assay tended to represent the level of disease, more accurately than the time taken until detection outcome by LAMP. Better measurements of the amount of inoculum present can aid in detection of an epidemic. Epidemics are signalled by an exponential increase in DNA quantity detected. Therefore, if an assay can reliably detect low levels of DNA (as is the case for qPCR) the exponential trend becomes apparent more quickly than an assay that cannot detect low levels of DNA. However, the qPCR assay was more susceptible to inhibition from contaminants. Although the real-time LAMP assay protocol developed was carried out in laboratory, it could be fully adapted for in-field devices. For example, Lucchi et al., (2010) reported a LAMP assay reaction that was carried out and read with an ESE-Quant Tube Scanner (ESE GmbH., Stockach, Germany) which is battery operated and portable. Further modifications could be used to increase detection capabilities via methods such as "bumping", in which slight modifications to primers based on their thermodynamic predictions are carried out to reduce undesirable hairpin and dimer structures whilst retaining primer fidelity to target DNA (Meagher et al., 2018). More effective amplification and in-field devices could increase the turn-over of results which is crucial for detecting the exponential growth phase of a pathogen. Further tests would be required for fully operational in-field detection using the LAMP assay. The diagnostic assays proved to be useful in further exploratory experiments addressing *B. lactucae* epidemiology, providing more data to support reported dispersal distances with field trials and commercial case studies.

Chapter 6 General discussion, conclusions and future directions

6.1 Reiteration of research problem and aims

Lettuce is a horticultural crop grown for fresh consumption in many countries including the UK. Approximately 4000 ha of land was used to grow 95 thousand tonnes of lettuce worth £187 million in the UK in 2020 (DEFRA, 2023). This industry is threatened by Lettuce Downy Mildew (LDM) a foliar disease caused by *Bremia lactucae* that damages leaf tissue reducing marketability and has been linked to secondary pathogenic infections (Simko et al., 2015). It is estimated that LDM costs £15 million in annual crop losses in the UK (AHDB, 2019b).

Disease management is predominantly through host resistance and fungicide spray programs (Barrière et al., 2014). The risk of emergence of more complex virulence and fungicide insensitivity in the pathogen population is a threat to management. Considering the potential of *B. lactucae* to adapt to overcome host resistance (Lebeda & Zinkernagel, 2003b; Trimboli & Nieuwenhuis, 2011; van Hese et al., 2016), develop fungicide insensitivity (Brown et al., 2004; Cobelli et al., 1998; Crute et al., 1987; Schettini et al., 1991), and be transmitted aerially, a lack of knowledge on the contemporary pathogen population can be problematic for management.

This work aimed to explore several aspects of the contemporary *B. lactucae* population, including monitoring the genetic diversity, virulence, and fungicide insensitivity present in the population to aid decisions on appropriate cultivars and to elucidate the reproductive biology of *B. lactucae* in the UK (Chapter 3). An assay that could examine genetic diversity at an appropriate level was developed (Chapter 2).

Competitive interactions between *B. lactucae* isolates were studied to gain an understanding of the effect of cultivar choice on population diversity (Chapter 4). Finally, aerial transmission of *B. lactucae* was studied to determine whether sporangia could be detected in commercial field environments, at what distance from an inoculum source sporangia could be detected and the effect of time of day on sporangial release (Chapter 5).

6.2 Key results

To investigate the genetic diversity of the *B. lactucae* population, a multiplex SSR assay was developed and through analysis of variation at ten loci it could distinguish between *B. lactucae* isolates. Using the SSR multiplex assay 135 MLLs were identified from 254 pathogen samples that were sourced from 29 disease outbreaks sampled from 12 UK counties between 2019 and 2022. Population statistics, including Simpson's diversity index, expected MLG, evenness and Nei's unbiased gene diversity, were notably similar across the years 2019 to 2022 after correction for population size, indicating a degree in consistency in annual scores. Further analysis suggested that mixed reproduction was likely, with clonal reproduction and evidence of heterokaryons. Specifically, trisomy in clonal lineages that re-emerged year on year (*K* cluster group 5) was observed; trisomy has been previously linked to secondary homothallism and oospore formation (Michelmore & Sansome, 1982; Michelmore & Wong, 2008). Therefore, heterokaryon overwintering in the UK is likely and should be accounted for in LDM management. Clonal lineages appear to be widely distributed, for example *K* cluster group 2 was identified in Surrey, Lancashire, and Fife.

Virulence and fungicide insensitivity had minimal association with genotype. However, there was great diversity in virulence present in the population, with at least one UK isolate able to overcome each differential in the differential set-C. Fungicide insensitivity was not observed in the 15 *B. lactucae* samples tested with the fungicide active ingredients axoystrobin, mandipropamid and dimethomorph.

Cultivar choice greatly influenced the population diversity when trials were inoculated with isolates of various pathogen genotypes. In general, specific MLLs were associated with some cultivars. For example, only isolate 2021_Bl1B and 2021_Bl1B variants were recovered from cv. Coventry in Trial 1. Genotype variants of the original released isolates were common. At least one of the strains in each trial was a strain hypothesised to be heterokaryotic, 2020_Bl4 in Trial 1, 2021_Bl4C and 2021_Bl8A in Trial 2. Nuclear sorting in the hyphae of such heterokaryotic strains would explain part of the variation observed. Other sources could be replication slippage occurring naturally or during PCR (Dufresne et al., 2014). Although it appears that heterokaryons can provide an advantage through more complex virulence the clonal multi-allelic strains did not always perform better in simulated populations in this

study. Homokaryon isolates with virulence effective against host resistance performed better than heterokaryons with partial effective virulence (Fletcher et al., 2019) and this was observed in both trials with isolates with non-heterokaryotic indicators (e.g. T3_283) being less prevalent than heterokaryons (e.g.2021_Bl8A).

DNA of *B. lactucae* was detected up to 100 m from an inoculum source. The estimation of outbreak influence (implied maximum sporangial dispersion) was between 80 m to 3000 m (Wu, van Bruggen, et al., 2001). Aerial samplers in commercial fields detected *B. lactucae* DNA when situated up to approximately 50-80m away from plants with LDM symptoms. DNA of *B. lactucae* was detected in commercial environments using the LAMP assay as early as two days before symptomatic plants were observed or reported. LDM symptomatic plants in commercial environments were observed at a 50-80 m distance to at least one aerial sampler each monitoring year. Therefore, gene flow from aerial transmission via sporangia within field and between fields was evident and should be considered in lettuce production. Aerial transmission of sporangia, or other pathways, such as movement of lettuce plugs from specialised growers to field or crop debris rotovated back into soil, may have contributed to the widespread occurrence of certain *K* cluster groups.

6.3 Study limitations

As this PhD study coincided with Covid-19 lockdowns, travel was restricted, severely limiting the options to travel to lettuce fields and sampling from domestic gardens and allotments. The full picture of *B. lactucae* diversity in the UK population remains unknown. Lettuce grown in garden and allotments and also on native host plants and could be a reservoir for *B. lactucae* isolates initiating epidemics in commercial crops.

SSRs are appropriate for analysing genotypic diversity, with multiple alleles indicative of heterokaryosis, full sequencing for these samples to confirm this has not been carried out. NGS sequencing of one or two of the heterokaryotic-like strains would be informative both on accessory chromosomes and the full extent of the trisomy/heterokaryosis observed. Three of the ten SSR markers were relatively stable therefore less informative. The SSR assay developed has room for improvement or further modification for other studies. However, the assay developed provided sufficient distinguishing power for this study.

6.4 Recommendations for future directions

The data collected suggests that the UK population of *B. lactucae* is genetically diverse and appears to contain heterokaryotic strains. Monitoring heterokaryosis is important to understand, for example, if it has an impact in encouraging oospore formation, and therefore overwintering. The *K* group 5 heterokaryotic strains were collected from a large geographical area (Cambridge, West Sussex, Kent, Norfolk, Suffolk and Lincolnshire) and there is evidence of this clonal lineage overwintering which implies dispersal or transmission mechanisms. Deciphering whether dispersal occurs through seed, soil and/or aerial sporangial transmission will be key in understanding gene flow in the *B. lactucae* population. A potential way of investigating heterokaryosis and the link to overwintering on a smaller scale would be to carry out the mark and recapture trials, but in the same field location a year later, as mentioned in the chapter 4 discussion. Notably, phenylamine insensitive isolates from a disease outbreak in Lancashire were reported to have spread by distribution of plants from specialised propagators (Crute et al., 1987). Thus, the inclusion of more details of the host the *B. lactucae* samples are collected from, i.e. where the lettuce plugs were sourced, would also need to be accounted for, as the human factor can impact gene flow in *B. lactucae* populations.

Preliminary experiments to investigate desiccation of aerial sporangia were not continued due to difficulties in maintaining the desired controlled environmental conditions. Research into leaf wetness duration (LWD) and its effect on sporulation and germination (Scherm & van Bruggen, 1993, 1995b) has previously been conducted however there has been little research into breaking LWD periods. In several other studies, induction of desiccation at key time points halted the spread of disease in glasshouse environments (Cohen & Ben-Naim, 2016; Davidson & Krysinska-Kaczmarek, 2007; Jhorar et al., 1998). This is an additional area of integrated management that could be implemented. Drying can be introduced at certain time points under glasshouse conditions to trigger germ tube growth, exhausting the energy reserves of sporangia but preventing infection of plant tissues. As a large portion of lettuce is grown in glasshouses for the full lifecycle, or for transplanting to field, this could be a key management practise to reduce disease.

6.5 Conclusion

In summary, this study found that the UK population of *B. lactucae* is complex and shows diversity for genotype and virulence. The virulence in the sampled UK population was diverse and widespread. Knowledge of population diversity linked to type of host resistance used, (i.e. cultivar choice) is a key area of importance in disease management.

Aerial dispersal of sporangia was predominantly local to infected plants (0-5 m) but sporangia were detectable at distances of 100 m in trials and ~80 m in commercial fields. Sporangial release did not vary significantly between AM and PM periods; therefore, dispersal should be treated as generally consistent through most of the day. The LAMP assay, provides a tool to detect *B. lactucae* in commercial fields to allow for improved timing of control measures and therefore mitigation of disease.

Management of LDM should account for the genetic flexibility of heterokaryons of *B. lactucae*, which are likely to result in overwintering soil-borne inoculum, and aerial dispersal within, and potentially between fields.

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Appendix

i. DNA extraction of aerial sampler samples

DNA was extracted from field samples using the MasterPure™ Yeast DNA Purification Kit (Lucigen, Cambio), following the manufacturer's instructions with some minor modifications. If samples had high Vaseline® debris evident in the first centrifuge step, a repeat of centrifuging the sample for 2 minutes at 13,200 rpm and transferring into a new 1.5 ml Eppendorf was used to ensure as much Vaseline® as possible was removed whilst not compromising on DNA yield.

To begin the process, 300 µl YCL mixture was added to samples, vortexed, then incubated on a hot plate for 15 minutes at 65°C. The samples were vortexed briefly after the incubation before resting on ice for 5 minutes. Aerial sampler collection components (Rotorods/tape) were then removed using forceps cleaned with 70% ethanol mixture between each sample. Following this 150 µl MPC was added and vortexed briefly before centrifuging for 10 minutes at 13,200 rpm. The supernatant was transferred into a 1.5 µl Eppendorf tube.

If high Vaseline® precipitate was present, the sample was centrifuged for a further 2 minutes at 13,200rpm and transferred to another 1.5 µl Eppendorf tube. 500 µl of Isopropanol was added and gently inverted till well mixed. Samples were then centrifuged for 10 minutes at 13,200 rpm, before carefully discarding the supernatant leaving behind a pellet. The pellet was washed using 500 µl of ethanol (70%), during which the tube was flicked to suspend the pellet, so that all sides of the pellet came in contact with the ethanol. A brief spin down was used to ensure pellet was at the bottom of the Eppendorf before carefully removing the ethanol. A repeat centrifuge for 2 minutes at 13,200 rpm was used to remove excess liquid. Samples were then left to dry for 20-30 minutes at room temperature, with the lids open on the bench to allow the ethanol to evaporate. When liquid was no longer present DNA was resuspended in 35 µl 1 x TE buffer and then stored at -20°C.

ii. Binary data-set rules

Sorting out the binary data set to two values could add subjectivity and skew the data, thus a list of parameters is needed to make sure they are best represented.

(i) **Remove missing values:** this is likely a plating error or contamination during plating.

(ii) **Choose data from the best run:** (i.e., the positives controls amplify, with the majority of the standards appearing in clear distances, and the negative controls don't amplify), some runs were retained in the data set as the positive's controls did come up, but due to degradation they didn't come up as early as they should and samples stock is limited

(iii) **If multiple runs were all similar,** then the following applied:

a. If the reps are 50:50, the sample was assigned 1:1.

b. If the reps were 1:3 split, then:

i. If the 1 was a strong positive in LAMP it was considered an error 0:1

ii. If the 1 was positive in LAMP and all qPCR reps were positive, it is likely a low positive that just made the threshold to account for this 1:1.

iii. If the 1 was positive in LAMP, and the 3 were low positives (had the right melting temperature but failed the C_T threshold marginally- thus counted negative) **the sample would be marked as 1:1.**

iv. If the 1 was a negative or a low positive it was considered an error, and the samples is positive.