Studentship Project: Annual Progress Report April /2021 to April/2022

Student Name:	Alicia A. Farmer	**AHDB Project Number:**	CP 186
Project Title:	Understanding populations of the lettuce downy mildew pathogen *Bremia lactucae* to inform integrated disease management		
Lead Partner:	James Hutton Institute and University of St. Andrews		
Supervisor:	Dr Alison Lees, Dr David Cooke, Dr Tim Pettitt, Prof John Jones		
Start Date:	27 Jan 2020	**End Date:**	27 April 2023

1. Project aims and objectives

Lettuce downy mildew (LDM) is a foliar disease caused by *Bremia lactucae*, that results in reduced quality, yield and marketability of lettuce crops. This disease is more prevalent in cooler climates, such as the U.K. and is currently managed through a combination of routinely sprayed fungicides and host resistance (Barriere et al., 2014, Fall et al., 2016).

As lettuce crop value is closely related to visual appearance and LDM epidemics establish rapidly after first symptom appearance, prophylactic fungicide sprays are applied regularly regardless of pathogen presence in-field. This indiscriminate approach, along with the use of monoculture and successive plantings, have increased the risk of evolution of fungicide insensitivity and new virulences within *B. lactucae* populations (Crute et al., 1987; Crute and Harrison, 1988; Brown et al., 2004; Parra et al., 2016).

According to Crandall et al. (2018), early detection is crucial in the management of aerial oomycetes such as *B. lactucae,* along with an understanding of the contemporary pathogen population: how they migrate, and their phenotypic characteristics. Understanding the population diversity both genotypically and phenotypically would allow for more informed decisions on crop breeding along with fungicide and resistance gene stewardship.

The main aim of the project is to understand the population diversity of *B. lactucae* to help inform integrated pest management (IPM) of LDM. To achieve this aim I will examine the pathogen population with simple sequence repeat molecular markers (SSRs) to genotypically profile isolates. SSR markers are not diagnostic for specific traits but are a commonly utilised method to get a representation of population diversity. We hypothesise that isolates belonging to a common genotype profile will be more phenotypically similar than those with a different genotype. Therefore we aim to explore associations between genotype and phenotype and factors including host cultivar, aggressiveness, fungicide sensitivity and location. This information will help inform growers to allow better targeted IPM strategies when LDM is encountered. Profiling the contemporary pathogen population of *B. lactucae* will provide information to help inform IPM strategies, especially cultivar selection along with targeted chemical controls and lettuce breeding.

The project also aims to validate a diagnostic LAMP (Loop-mediated Isothermal Amplification) assay for in-field spore detection, to determine risk thresholds, to analyse whether the LAMP assay has utility in providing early warning of infection to growers for IPM strategies. Knowledge of inoculum presence and risk thresholds can provide decision support for the timing and use of chemical control.

2. Key messages emerging from the project

- ***B. lactucae* genotypes:** Molecular (SSR) markers are being used to analyse the genomic diversity of the *B. lactucae* population. Results to date show that ten candidate SSR markers used in combination were able to identify >50 Multi Locus Genotypes (MLGs) from UK samples collected from 29 different outbreaks. Genotypes were shown to vary within and between outbreaks, The markers therefore have the ability to distinguish between different *B. lactucae* strains.
- **In-field spore detection:** DNA of *B. lactucae* was detectable before the onset of disease symptoms using both the LAMP and qPCR assays in samples collected from a commercial field site in 2021.

3. Summary of results from the reporting year

SSR Marker development and testing

Simple sequence repeats (SSR) were chosen to analyse the genomic diversity of the *B. lactucae* population, as they are an accepted way of discriminating genotypes based on length variation (alleles) at a range of points in the pathogen genome (loci) (Cooke and Lees, 2004; Li et al., 2013). A combination of different markers with different allele lengths can create a unique genotypic profile for an isolate, essentially giving a 'fingerprint'.

Seventeen candidate SSR markers were identified and 12 of these were shown to be polymorphic. These included 4 new SSR markers and 8 markers obtained from collaboration with UC Davis (USA). Of the 12 polymorphic markers, 10 were selected as the final set of discriminatory SSRs following validation and optimisation of the assays with a range of *B.lactucae* isolates. Assays were multiplexed, allowing for the genotyping to be carried out in one reaction per sample. The 10 selected SSR markers were able to discriminate >50 Multi Locus Genotypes (MLGs) from UK samples collected by industry partners from 29 different UK outbreaks. Variation for genotype was observed both within and between outbreaks. Full data analysis is underway. *B. lactucae* DNA on FTA cards was kindly provided by Syngenta to examine the ability of markers to distinguish UK samples from overseas samples. These assays have been completed and the data is being analysed.

Table 1 Genotypic profiles of a selection of* B. lactucae *samples tested in 2021 and 2022. *Each row is a different* B. lactucae *sample and each column is an SSR marker (locus). Different colours at each marker represent a different length/allele. Solid line separates year of collection, dotted lines separate outbreaks. Last two rows are the reference races Reference_1 (Bl:16EU) and Reference_2 (Bl:36EU).*

DNA Template	JHI Primers												UC Davis Primers																					
	1001a		1008b			1011a			Marker 1			Marker 2		Marker 4			Marker 5		Marker 7					Marker 9		Marker 10	
	Allele 1	Allele 2	Allele 1	Allele 2		Allele 1	Allele 2	Allele 3	Allele 1	Allele 2	Allele 3	Allele 1	Allele 2	Allele 1	Allele 2	Allele 3	Allele 1	Allele 2	Allele 1	Allele 2	Allele 3			Allele 1	Allele 2	Allele 1	Allele 2
2019_Bl11E	146	146	362	362		220	223		145	165				277	277		155	157	284	284				206	209	330	330
2019_Bl11F	146	146	362	362		220	223		145	165				277	279		155	157	249	251	283	284		206	209	330	330
2019_Bl11G	146	146	362	362		220	223		142	142		272	272	275	277		155	157	249	251	281	299		206	209	330	330
2019_Bl11H	146	146	362	362		220	223		142	142		272	272	275	277		155	157	249	251	281	299		206	209	330	330
2019_Bl1G	146	146	362	362		220	223		142	142		272	272	275	275		155	157	249	251	281	299		206	209	327	330
2019_Bl1H	146	146	362	362		220	223		142	142		272	272	275	275		155	157	249	251	281	299		206	209	327	330
2019_Bl2E	146	146	362	362		220	220		142	142		272	272	275	277		157	157	247	249				206	209	327	327
2019_Bl2F	146	146	362	362		220	220		142	142		272	272	275	277		157	157	247	249				206	209	327	330
2019_Bl3E	146	146	362	362		220	220		142	142				275	277		157	157	287	287				209	209	327	330
2019_Bl3F	146	146	362	362		220	220		142	142				275	277		157	157	287	287				209	209	327	330
2019_Bl9E	140	146	359	359		220	223		142	145	148	266	266	275	277		155	157	290	292	303			209	209	327	330
2019_Bl9F	140	146	359	359		220	223		142	145	148	266	266	275	277		155	157	290	292	303			209	209	327	330
2019_Bl9G	140	146	359	359		220	223		142	145	148	266	266	275	277		155	157	290	292	303			209	209	327	330
2019_Bl9H	140	146	359	359		220	223		142	145	148	266	266	275	277		155	157	290	292	303			209	209	327	330
2020_Bl2A	140	146	359	359		223	223		142	148		266	266	275	277		157	157	277	288				209	209	330	330
2020_Bl4E	140	146	359	359		220	223		142	148		266	266	275	277		155	157	275	277	284	286		209	209	330	330
2020_Bl4G	140	146	359	359		220	223		142	145	148	266	266	275	277		155	157	275	277	286			209	209		
2021_Bl1A	146	146	359	359		220	223		142	145		272	272	275	275		157	157	284	284				209	209		
2021_Bl2E	140	146	359	359		220	223		142	145	148	266	266	275	277		155	157	290	292	301			209	209	327	327
2021_Bl2F	140	146	359	359		220	223		142	145	148	266	266	275	277		155	157	290	292	301			209	209	328	328
2021_Bl3A	146	146	359	362		215	220	223	142	142		266	266	275	277		157	157	290	292				206	209	330	330
2021_Bl4C	140	146	359	359		220	223		142	145	148	266	266	275	277		155	157	290	292	301			209	209	330	330
2021_Bl4E	140	146	359	359		220	223		142	145	148	266	266	275	277		155	157	290	292	301			209	209	330	330
2021_Bl8A	146	146	359	362		220	223		142	142		266	266	275	277		157	157	297	299	301			206	209	330	330
2021_Bl8E	146	146	362	362		220	223		142	142				276	288		157	157	299	301				206	209		
2021_Bl8F	146	146	362	362		220	223		142	142				276	288		157	157	299	301				206	209		
2022_Bl1E	146	146	359	362		220	223		142	148		272	272	275	277		155	157	251	283	291			206	209	327	330
2022_Bl1F	146	146	359	362		220	220		142	148		272	272	275	277		155	157	249	251	283	291		206	209	330	330
Reference_1	146	164	362	362		220			142	148		272	272	275	275		157	157	284	291				206	209	327	327
Reference_2	140	146	359	362		220	223		142	145		266	272	275	275		155	157	281	291	299			209	209	330	330

Field trial to investigate population dynamics

A field trial was carried out in 2021 to investigate the population dynamics of *B. lactucae* with the following questions in mind:

- Is there variation for aggressiveness between isolates of *B. lactucae*?
- Does genetic diversity in *B. lactucae* correspond with any variation for aggressiveness?
- Do *B. lactucae* populations change throughout an epidemic or in relation to host resistance?
- Is spore monitoring a suitable tool for predicting disease incidence?

Aggressiveness is often defined as a quantitative measure of ability to attack a host and the host's partial resistance. It is often a combined measure of infection efficiency/frequency, latent period, rate of lesion growth and sporulation density. This is difficult to measure in a field situation, so an alternative is to use a 'mark and recapture' trial to measure the frequency of an isolate identifiable using markers, as the measure of competitive ability relating to environmental factors including host resistance. In addition, the trial allowed the ability of the previously developed real-time- LAMP assay to detect *B. lactucae* DNA from spores to be tested on a dispersal gradient as the epidemic developed.

Two separate replicated field trials were conducted in 2021 with a range of lettuce cultivars and inoculated with mixed UK *B. lactucae* isolates of known genotype. Disease progress (Figure 1) and sporulation (Figure 2) was measured. Each experiment represented different epidemic progress according to optimal and sub-optimal conditions for disease development. Diseased leaf and FTA samples were taken from every plot in each trial on several occasions and analysed using the SSR markers able to discriminate the isolates. Data analysis is underway.

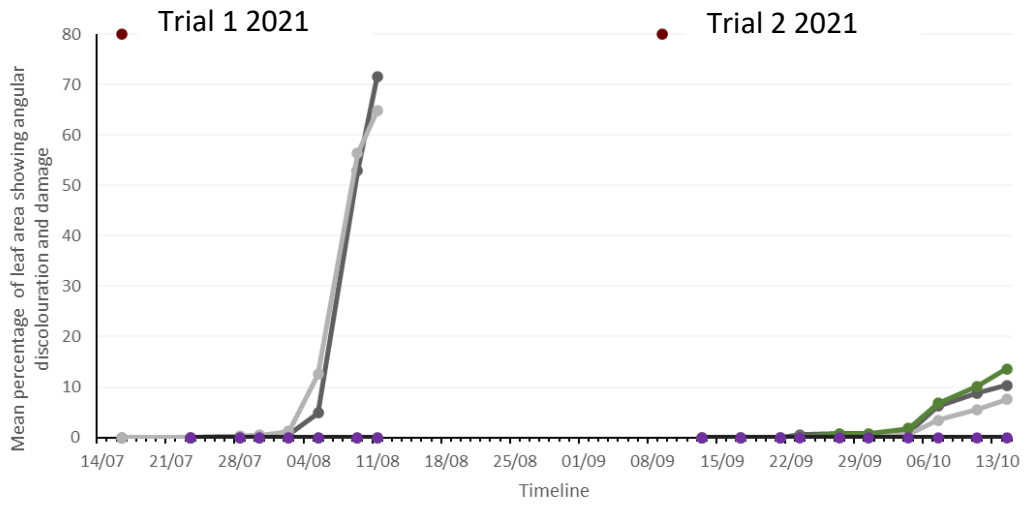

Figure 1 Mean percentage disease symptoms on 4 lettuce cultivars (solid lines) inoculated with a mixed inoculum of B. lactucae *isolates of known genotype. Red dot = date of inoculation in each case.*

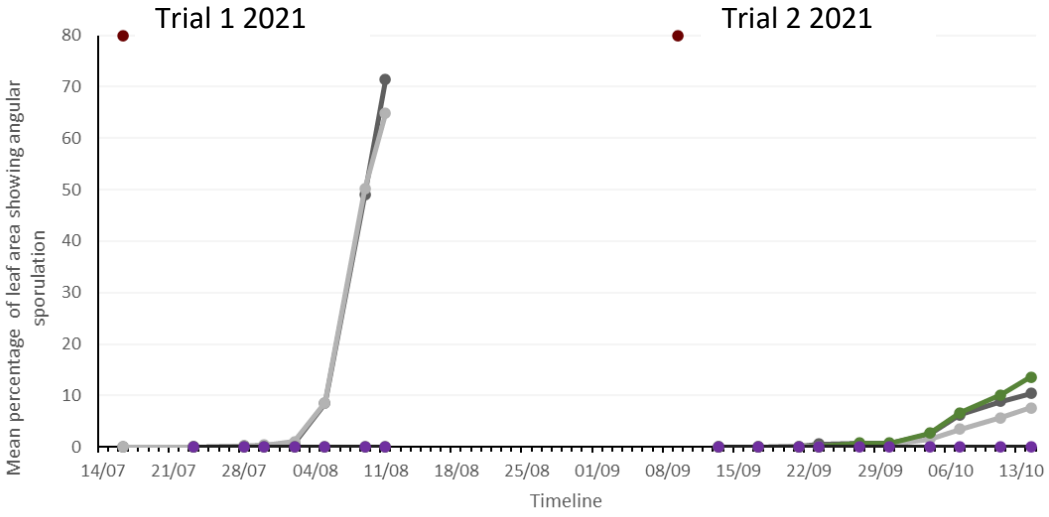

Figure 2 Mean percentage of angular discolouration/sporulation on a range of 4 lettuce cultivars (solid lines) inoculated with a mixed inoculum of B. lactucae *isolates of known genotype. Red dot = date of inoculation in each case.*

Data analysis is not complete, but in general *B. lactucae* strains seem to be associated with certain cultivars. An example is given in Figure 3. representing 3/5 sampling dates across trial 2. It can be seen that isolate 1 is unable to infect cultivar 1 and only isolate 3 causes disease on cultivar 4 later in the epidemic. A full analysis of all samples at all sampling dates will be completed to examine interactions between isolates and cultivars and associations with race testing data and existing host resistance information.

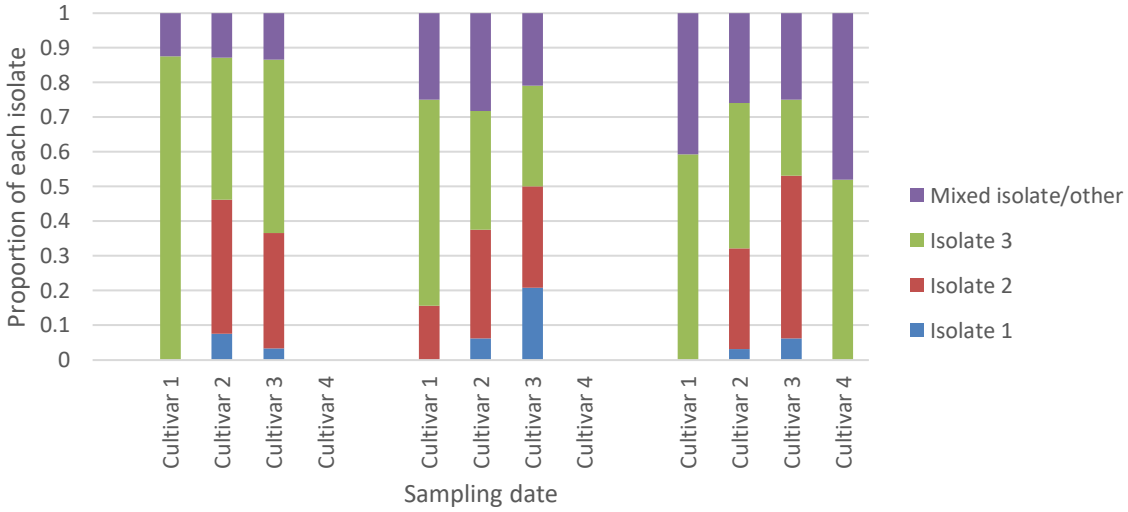

Figure 3. Proportion of each of 3 genotypically distinct B. lactucae *isolates recovered from 4 cultivars in trial 2 (2021) at 3 sampling dates during the epidemic*

Spore detection

Real-time loop-mediated isothermal amplification (real-timeLAMP) is a diagnostic assay that can amplify DNA at a constant temperature. The real-time LAMP assay is generally more robust, simple, quicker, and cheaper compared to the conventional real-time quantitative polymerase chain reaction (qPCR) previously developed for *B. lactucae* (Kunjeti et al., 2016). As the LAMP assay does not require a thermocycler like PCR procedures, it can be more readily adapted for use outside the laboratory (Notomi et al., 2015).

This diagnostic test was developed and validated under laboratory conditions as part of the wider AHDB project (CP 184) with which the studentship is aligned. The studentship's aim was to assess the procedure's performance under field conditions, determining risk threshold levels, detection levels, and testing sensitivity when exposed to the commercial field environment.

In-field trials are being conducted to assess the utility of the real-time LAMP assay for early detection for input into decision support (DS) for IPM. Currently, DS are based on meteorological data meaning that sprays may be recommended at times when LDM inoculum is absent or below risk threshold levels. However, if the assumption that aerial spores are the main means of infection and transmission of *B. lactucae* is correct, warnings from an in-field LAMP assay could reduce the number of sprays recommended by an appropriately modified DS.

Field trials 2021:

Field sampling of spores was carried out between 23/05/2021-27/09/2021 at two commercial farms, one in England (Cambridgeshire) the other in Scotland (Fife). Field samples were collected using two rotorod spore samplers (Burkard) twice a week. Spore samplers were moved to follow the field plantings of lettuce, which changed throughout the growing season at both farms.In addition, rotorod spore samplers were placed at increasing distance from an inoculum source in the previously described field experiments located at JHI in 2021 and samples taken twice weekly. DNA was extracted from field samples, purified where necessary and analysed using optimised real-time LAMP and qPCR procedures as previously described. Thresholds for the designation of positive samples were set by comparison with controls. *B. lactucae* DNA was detected at the Scottish commercial site in field 1 onthe 10th of June (field 1), field 2 on the 28th of June and 19th of July and in field 3 on the 29th of Julybefore the multiple positives following the 2nd of August (Figure 4). Both the LAMP and the qPCR assay detected B. lactucae DNA prior to the LDM incidence reported in field 3, the qPCR reporting earlier than the LAMP.

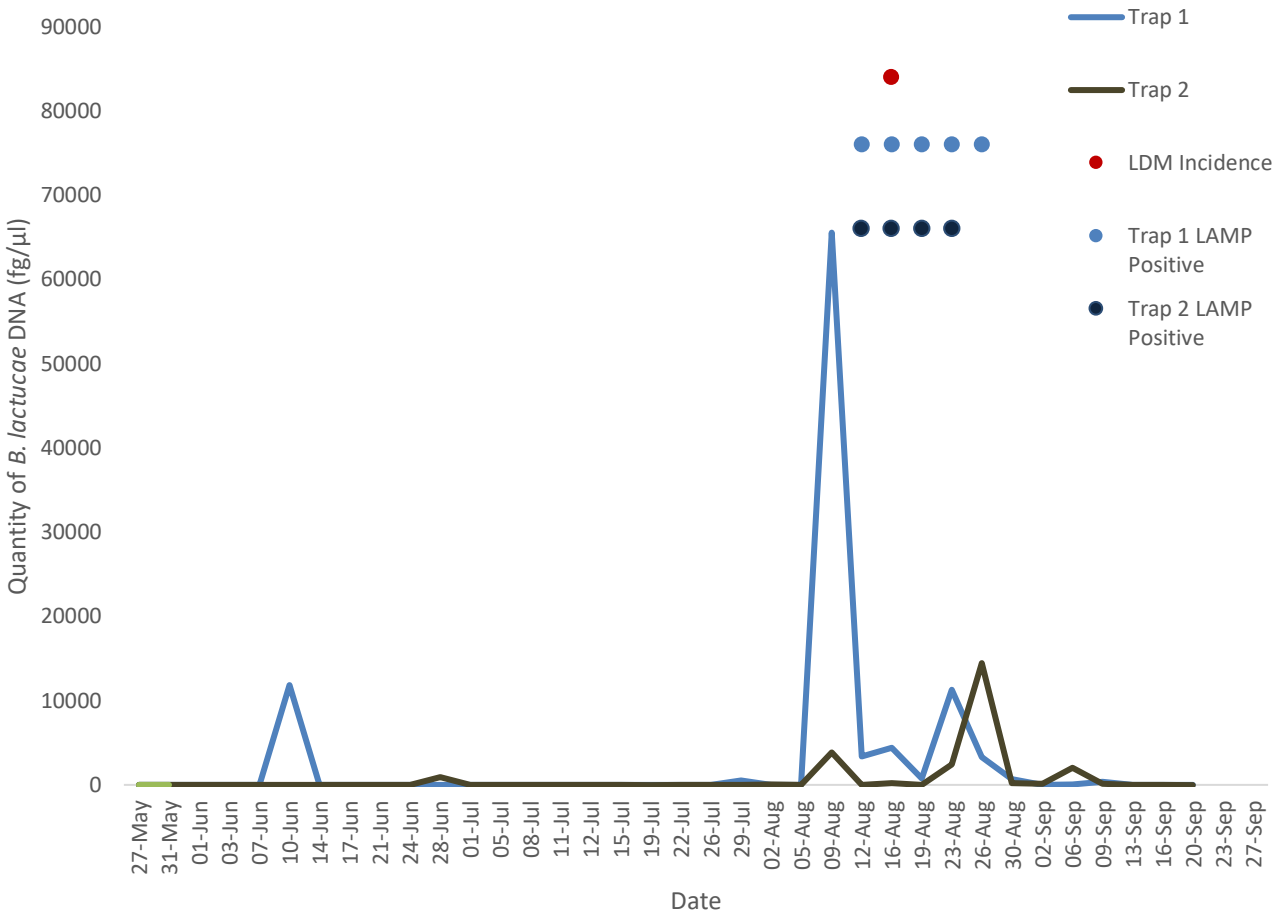

Figure 4 Detection of B. lactucae DNA from samples collected at a commercial lettuce growing site in Scotland in 2021. Solid lines represent quantitative measure of DNA collected from 2 independent spore traps using qPCR. Dots represent detection of DNA from the same samples using the real-time LAMP assay. Red dot indicates observation of disease symptoms in the field. Sampling was carried out in three fields, field one (27/05/21-14/06/21), field two (18/06/21-26/07/21) and field three (29/07/21-27/09/21). Note quantity values ranged from 9.5 fg/µl to over 65,000 fg/µl, as such lower quantity values may not be visible in this graph.

Phenotypic characteristics:

Race testing was carried out on UK *B. lactucae* samples collected in 2021. The differential assay is a standardised method for race determination set by the International Bremia Evaluation Board (IBEB) and is comprised of a standard set of lettuce cultivars with known resistance genes (*Dm*) that are inoculated with the test *B. lactucae* sample to determine which *Dm* genes it can overcome as indicated by successful infection. Infection scoring is translated into a sextet code that is used to represent the race of *B. lactucae*. Race / virulence testing was done following the IBEB protocol, using seeds supplied by Naktuinbouw (NAK, Netherlands).

The virulence assay was successfully completed (Table 2), with infection of the universally susceptible control (Cv. Green Towers) by each *B. lactucae* sample, and no obvious contamination. When checking the UK *B. lactucae* samples against prior IBEB race virulence profiles, samples 2021_Bl11A and 2021_Bl11C matched with the European race Bl:35EU. Results were reported to industry partners.

Table 2 Race profiles of a selection of B. lactucae *isolates tested in 2021 on IBEB differentials (set C) compared with officially designated races (in red).*

	Green Towers	Dandie	R4T57 D	UC DM14	NunDm15	CG Dm16	Colorado	FrRsal-1	Argeles	RYZ 2164	RYZ 910457	Bedford	Balesta	Bartoli	Design	Kibrille	C Sextet Code
		Dm3	Dm4	Dm14	Dm15	Dm16	Dm18	Rsal-1	R38								
Grid Position		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	
Sextet Value		1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	
2021_Bl1A	+	-	+	+	+	+	+	-	+	-	-	-	+	-	-	+	62-34-04
2021_Bl1B	+	-	+	+	-	+	+	-	+	-	-	-	+	-	-	-	54-34-00
2021_Bl1J	+	+	+	+	-	+	+	-	+	-	-	-	+	-	-	-	55-34-00
2021_Bl1L	+	-	+	+	-	+	+	-	+	-	-	+	+	-	-	+	54-50-04
2021_Bl2B	+	+	+	+	-	+	+	-	+	+	-	-	+	+	+	+	55-38-07
2021_Bl2D	+	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	54-38-07
2021_Bl2H	+	-	+	+	-	+	+	-	+	-	-	-	+	+	-	-	54-34-01
2021_Bl3A	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-	+	62-07-04
2021_Bl3B	+	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	62-06-00
2021_Bl3C	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	30-09-00
2021_Bl3D	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	30-09-00
2021_Bl4C	+	+	+	+	-	+	+	-	+	-	+	-	-	-	-	+	55-10-04
2021_B8A	+	-	+	+	+	+	+	-	+	+	-	-	+	-	+	+	62-38-06
2021_B8B	+	-	+	+	-	+	-	-	+	+	+	-	+	-	-	+	22-46-04
2021_B8C	+	-	+	+	+	+	+	-	+	+	-	-	+	-	+	+	62-38-06
2021_B8D	+	-	+	+	+	+	+	-	+	+	-	-	+	-	+	+	62-38-06
2021_BL9	+	-	+	+	+	+	+	+	+	+	-	-	-	+	+	-	62-07-03
BL:35EU	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	62-15-06
2021_BL11A	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	62-15-06
2021_BL11C	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	62-15-06
2021_BL11B	+	-	+	+	-	+	+	+	+	+	-	-	-	-	+	+	54-07-06
2021_BL11D	+	-	+	+	-	+	+	+	+	+	-	-	-	-	+	+	54-07-06
BL:36EU	+	+	+	+	-	+	+	+	+	+	+	-	-	+	(-)	-	55-15-01

4. Key issues to be addressed in the next year (2022/23):

- Complete genotyping of *B. lactucae* isolates using markers developed and compare with phenotypic information
- Complete testing and analysis of samples from 2021 field trials to investigate isolate competitiveness
- Repeat field trial in 2022
- Carry out controlled environment experiments as appropriate to investigate epidemiological traits of isolates
- Carry out in field spore monitoring at commercial sites in 2022 and analyse samples

5. Outputs relating to the project

(events, press articles, conference posters or presentations, scientific papers):

Output	Detail
AHDB progress meetings	Attended and presented project plans and progress to supervisors, AHDB staff and industry representatives on 1 April 2021
University progress meeting	Presented project information as an informal talk to my asseessors at the university to show project progress.
JHI Student Conference	Presented a Dragon's Den Pitch (prize winner)
BSPP Plants of our future conference	Presented project information as a poster and partook in the J. Colhoun award competition. Additionally attended the career workshop available to students.
Project Update with G's Fresh	Presented project information, including virulence profile and fungicide test results of samples obtained to our industry leads.
Upcoming – OMGN conference	Offer to present poster at an international conference specialising in oomycete pathogens and molecular methods accepted. Obtained BSPP travel grant funding to attend.
Upcoming – BSPP conference	Offer to to present project in the BSPP P. H. Gregory competition accepted

6. Partners (if applicable)

Scientific partners	UC Davis (collaborator)
Industry partners	G's Fresh, Kettle Produce,
Government sponsor	

7. References:

BARRIERE, V., LECOMPTE, F., NICOT, P. C., MAISONNEUVE, B., TCHAMITCHIAN, M. & LESCOURRET, F. 2014. Lettuce cropping with less pesticides. A review. *Agronomy for Sustainable Development,* 34**,** 175-198.

BROWN, S., KOIKE, S. T., OCHOA, O. E., LAEMMLEN, F. & MICHELMORE, R. W. 2004. Insensitivity to the fungicide fosetyl-aluminum in California isolates of the lettuce downy mildew pathogen, Bremia lactucae. *Plant Disease,* 88**,** 502-508.

COOKE, D. E. L. & LEES, A. K. 2004. Markers, old and new, for examining Phytophthora infestans diversity. *Plant Pathology,* 53**,** 692-704.

CRANDALL, S. G., RAHMAN, A., QUESADA-OCAMPO, L. M., MARTIN, F. N., BILODEAU, G. J. & MILES, T. D. 2018. Advances in Diagnostics of Downy Mildews: Lessons Learned from Other Oomycetes and Future Challenges. *Plant Disease,* 102**,** 265-275.

CRUTE, I. R. & HARRISON, J. M. 1988. Studies on the Inheritance of Resistance to Metalaxyl in Bremia-Lactucae and on the Stability and Fitness of Field Isolates. *Plant Pathology,* 37**,** 231-250.

CRUTE, I. R., NORWOOD, J. M. & GORDON, P. L. 1987. The Occurrence, Characteristics and Distribution in the United-Kingdom of Resistance to Phenylamide Fungicides in Bremia-Lactucae (Lettuce Downy Mildew). *Plant Pathology,* 36**,** 297-315.

FALL, M. L., VAN DER HEYDEN, H. & CARISSE, O. 2016. A Quantitative Dynamic Simulation of Bremia lactucae Airborne Conidia Concentration above a Lettuce Canopy. *Plos One,* 11.

KUNJETI, S. G., ANCHIETA, A., MARTIN, F. N., CHOI, Y. J., THINES, M., MICHELMORE, R. W., KOIKE, S. T., TSUCHIDA, C., MAHAFFEE, W., SUBBARAO, K. V. & KLOSTEIMAN, S. J. 2016. Detection and Quantification of Bremia lactucae by Spore Trapping and Quantitative PCR. *Phytopathology,* 106**,** 1426-1437.

LEES, A. K., ROBERTS, D. M., LYNOTT, J., SULLIVAN, L. & BRIERLEY, J. L. 2019. Real-Time PCR and LAMP Assays for the Detection of Spores of Alternaria solani and Sporangia of Phytophthora infestans to Inform Disease Risk Forecasting. 103**,** 3172-3180.

LI, Y., COOKE, D. E. L., JACOBSEN, E. & VAN DER LEE, T. 2013. Efficient multiplex simple sequence repeat genotyping of the oomycete plant pathogen Phytophthora infestans. *Journal of microbiological methods,* 92**,** 316–322.

NOTOMI, T., MORI, Y., TOMITA, N. & KANDA, H. 2015. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *Journal of Microbiology,* 53**,** 1-5.

OPTIGENE 2018. Determining the Detection Limits of a LAMP Assay .

PARRA, L., MAISONNEUVE, B., LEBEDA, A., SCHUT, J., CHRISTOPOULOU, M., JEUKEN, M., MCHALE, L., TRUCO, M. J., CRUTE, I. & MICHELMORE, R. 2016. Rationalization of genes for resistance to Bremia lactucae in lettuce. *Euphytica,* 210**,** 309-326.