Studentship Project: Annual Progress Report
September 2020 to June 2021

Student Name:	**Andrew D. Legg**	**AHDB Project Number:**	**CP 198**
Project Title:	Exploiting pathogenomics and resistance for the control of Fusarium wilt of lettuce		
Lead Partner:	University of Warwick		
Supervisor:	John Clarkson		
Start Date:	23/09/2019	**End Date:**	23/09/2023

1. Project aims and objectives

Fusarium wilt of lettuce

This project focuses on Fusarium wilt of lettuce caused by *F. oxysporum* f. sp. *lactucae* (FOL) which was first identified in Japan in 1967 (Matuo & Motohashi, 1967) and has since been found in multiple lettuce producing countries worldwide. Four races (1, 2, 3 and 4) of FOL have been identified so far with race 1 being the most prominent globally, having been reported in the USA (Hubbard & Gerik, 1993), Europe (Garibaldi *et al.*, 2002), Iran (Millani *et al.*, 1999), Taiwan (Huang & Lo, 1998) and South America (Ventura & Costa, 2008; Malbrán *et al.*, 2014). FOL races 2 and 3 are currently confined to Asia (Fujinaga *et al.*, 2005; Lin *et al.*, 2014) while race 4 has only recently emerged and was first identified in the Netherlands in 2013 (Gilardi *et al.*, 2017a). FOL4 has since spread and has been reported in Belgium (Claerbout *et al.*, 2017) and the UK and Ireland (Taylor *et al.*, 2018). So far, in contrast to FOL1, FOL4 has only affected protected lettuce crops. In mainland Europe and the USA, FOL is considered as one of the main limiting factors for commercial production of lettuce during the summer season (Taylor & Clarkson, 2018). Reports from France (FOL1 or FOL4) and the Netherlands (FOL4) have commonly observed 50% yield losses (Gilardi *et al.*, 2017a; Gilardi *et al.*, 2017b) while in Italy up to 70% losses of field lettuce have been observed (AHDB, 2018).

Symptoms of Fusarium wilt of lettuce

Symptoms of Fusarium wilt of lettuce include stunting, wilting and leaf yellowing (often at leaf margins), but the key characteristic symptom of the disease is a brown, black, or red discolouration of the vascular tissue of the stem/taproot which can be observed upon longitudinal dissection of infected plants (Taylor & Clarkson, 2018; Figure 1). FOL travels through the xylem and blocks the vascular tissue, causing wilt symptoms, ultimately resulting in plant death. One of the main modes of FOL transmission appears to be spread via infested soil on farming equipment, trays, pallets and footwear.

FOL resistant lettuce cultivars and distribution of FOL in the UK

All FOL isolates identified in UK protected lettuce thus far have been identified to be FOL4 (Taylor *et al.*, 2018) and as previously mentioned, all outbreaks of FOL4 within the UK, Belgium and the Netherlands (from where it was first identified) have been confined to protected lettuce with none identified in outdoor production. However, there is particular concern that FOL4 may begin to affect field grown crops despite current measures in place to limit pathogen spread. Arguably the best option for control of *F. oxysporum* pathogens is the cultivation of resistant varieties (Okungbowa & Shittu, 2012). As part of the Defra-funded Vegetable Genetic Improvement Network (VeGIN) project a FOL resistance screening experiment was carried out where 54 accessions from the Warwick lettuce diversity set were screened against FOL1 and FOL4. This succeeded in identifying resistant lettuce lines that have been used as parents of mapping populations in a collaboration with Enza Zaden, with the aim of discerning the genetic nature of the resistance. Development of FOL4 resistant lettuce cultivars would be of great benefit to UK growers and consumers by reducing losses, decreasing the need for less environmentally and more costly interventions such as soil steaming / sterilisation and application of fungicides, therefore enabling year-round production.


Figure 1. Vascular browning in lettuce caused by FOL4

Aims and Objectives
The main aims of this project are to identify and characterise new sources of FOL resistance in lettuce and to compare the genetics and biology of FOL1 and FOL4, with a focus on identifying and characterising virulence genes and studying interactions between the pathogen and susceptible/resistant lettuce lines. Of particular interest are *Secreted in Xylem* (*SIX*) genes (Rep et al., 2004) first identified in *F. oxysporum* f. sp. *lycopersici* (Houterman et al., 2007; Schmidt et al., 2013) and homologs of these have been identified in a wide range of *F. oxysporum* f. spp. Current objectives are:

1. Characterise FOL isolates from different European locations
2. Identify pathogenicity genes expressed during infection and confirm their roles in virulence

2. Key messages emerging from the project

Characterise FOL isolates from different European locations

- FOL1 and FOL4 isolates from different European locations were obtained. Sequencing of the 'housekeeping' *TEF* gene revealed identical sequences for FOL1 and FOL4 indicating they are closely related while FOL2 and FOL3 TEF sequences were different. TEF sequence therefore cannot distinguish between FOL1 and FOL4 isolates
- Screening of FOL1 and FOL4 isolates for presence of SIX genes showed that both races contain identical sequences of SIX9 while FOL4 isolates contained two variants of SIX8 indicating some genetic variability within this race.

Identify FOL pathogenicity genes expressed during infection and confirm their roles in virulence

- An *in vitro* infection system was developed for growing lettuce seedlings on agar in square petri dishes with FOL inoculated directly onto roots using spore suspensions. This will allow detailed gene expression studies to be carried out for both lettuce and FOL during infection.
- Clear phenotypic differences were seen between selected resistant and susceptible lettuce lines using this system when inoculated with FOL4, confirming the phenotypes of these lines and allowing the selection of a resistant and a susceptible line for future gene expression studies. Additionally, these same lines will be screened in a separate glasshouse experiment to confirm results observed in the *in vitro* assay.
- RNA was extracted from the root material at different timepoints for one susceptible lettuce line and qPCR wil be carried out to examine if *SIX* genes are expressed during early infection and to identify the best timepoint for an RNAseq experiment which will examine total gene expression.

3. Summary of results from the reporting year

Characterisation of FOL isolates from different European locations

The translation elongation factor 1-a (*TEF*) gene has become the marker of choice as a single-locus identification tool in *Fusarium*. This is due to it being consistently single-copy in *Fusarium*, and it showing a high level of sequence polymorphism among closely related species (Geiser *et al.*, 2004). *TEF* sequencing was therefore used to compare sequence similarity between FOL1 and FOL4 isolates as well as sequence similarity within races. *TEF* was sequenced for 20 FOL1 and FOL4 isolates from different European locations and all isolates had identical sequences indicating a

common monophyletic origin for FOL1 and FOL4 (Figure 2). Moreover, FOL2 and FOL3 TEF sequences (from online database) differed both from FOL1 / FOL4 and each other. TEF sequencing does therefore not distinguish between FOL1 and FOL4 isolates.

Genome sequences of FOL1 (isolate AJ520) and FOL4 (isolate AJ516) generated in a previous AHDB project were used to identify presence / absence of *SIX* genes in these two isolates. Here it was found that FOL4 contained SIX8 and SIX9 whilst FOL1 only contained SIX9. To confirm this result, primers for the 14 known *SIX* genes were then used to screen all 20 FOL isolates and it was confirmed that all FOL1 isolates were positive for SIX9 amplification only whilst FOL4 isolates were positive for both SIX8 and SIX9 amplification. Furthermore, all FOL isolates were tested with FOL, FOL1 and FOL4 specific diagnostic primers (FOL1 and FOL4 specific, G19968; FOL4 specific, G23490; FOL1 specific, (Pasquali *et al.*, 2007). Results showed that FOL1 specific primers identified all isolates containing SIX9 only while FOL4 specific primers identified all isolates containing both SIX8 and SIX9, further confirming the allocation of race based on SIX gene presence / absence. Sequence similarity of SIX8 within FOL4, and sequence similarity of SIX9 within each race and between races was then compared, and phylogenetic trees constructed. This showed that there were two sequence variants within SIX8 in FOL4 isolates (Figure 3) while all SIX9 sequences in both FOL1 and FOL4 were identical (Figure 4).

Table 1 Results from screening European FOL isolates for presence of SIX genes (SIX1-14) and using FOL4 specific (G23490), FOL specific (G19968), FOL1 specific (Pasquali et al, 2007) primers. + symbol denotes a positive PCR result, whilst – symbol denotes a negative PCR results.

FOL isolate	Race	Country	SIX8	SIX9	G23490	G19968	Pasquali *et al.* (2007)	Other SIX genes
AD035	FOL4	NL	+	+	+	+	-	-
AN072	FOL4	IE	+	+	+	+	-	-
AN190	FOL4	BE	+	+	+	+	-	-
AP001	FOL4	IE	+	+	+	+	-	-
AP002	FOL4	IE	+	+	+	+	-	-
AP004	FOL4	IT	+	+	+	+	-	-
AR002	FOL4	IT	+	+	+	+	-	-
AR069	FOL4	IT	+	+	+	+	-	-
AR106	FOL4	IT	+	+	+	+	-	-
AS027	FOL4	IT	+	+	+	+	-	-
AS063	FOL4	IT	+	+	+	+	-	-
AT021	FOL4	IT	+	+	+	+	-	-
AU122	FOL4	IT	+	+	+	+	-	-
AU153	FOL4	IT	+	+	+	+	-	-
AT131	FOL4	UK	+	+	+	+	-	-
AU069	FOL4	UK	+	+	+	+	-	-
AU079	FOL4	IT	+	+	+	+	-	-
AT105	FOL1	IT	-	+	-	+	+	-
AT106	FOL1	IT	-	+	-	+	+	-
P142	FOL1	SP	-	+	-	+	+	-
AJ516	FOL4	UK	+	+	+	+	-	-
AJ520	FOL1	IT	-	+	-	+	+	-

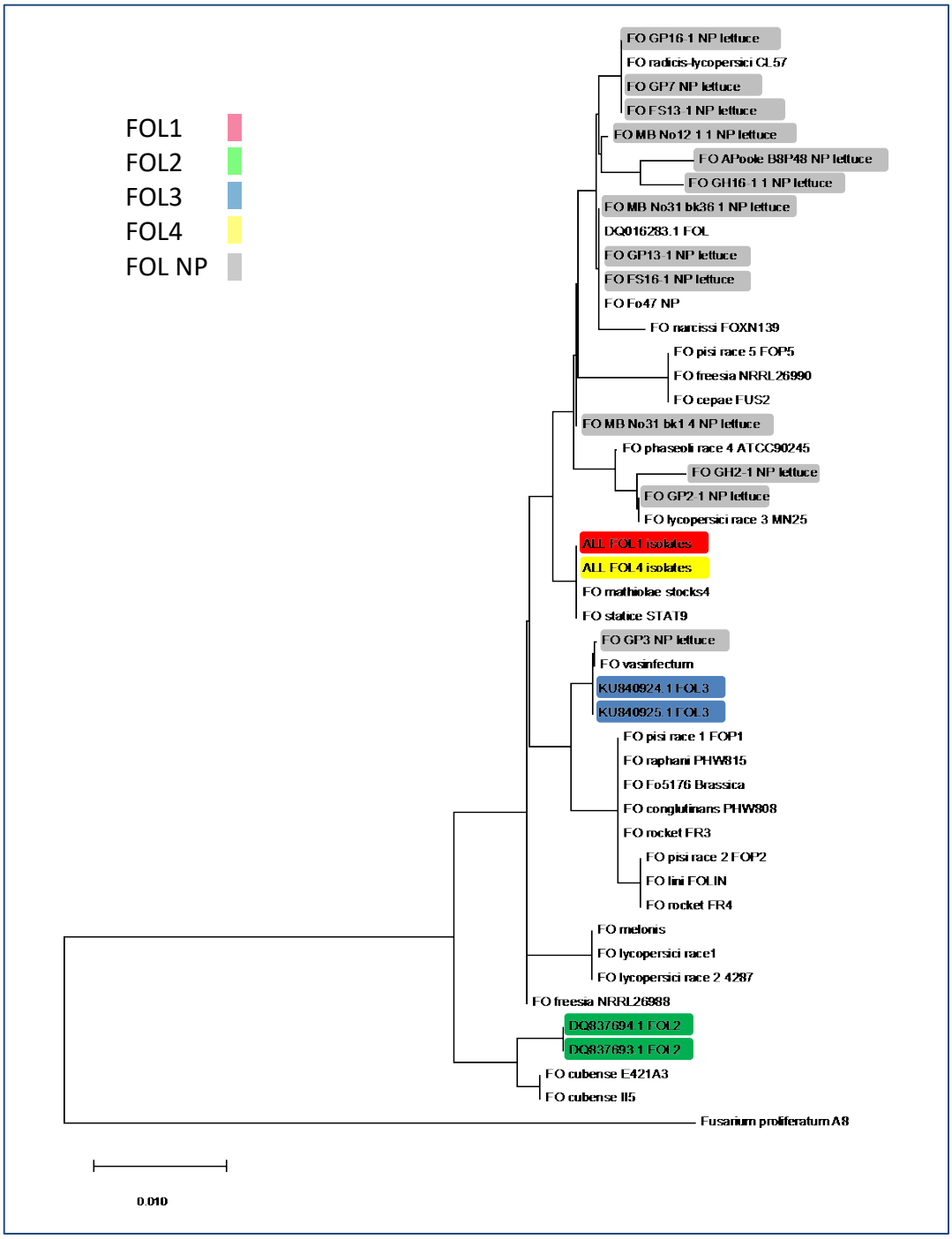

Figure 2 TEF phylogenetic tree for FOL isolates and other *F. oxysporum* f. spp.. Evolutionary history was inferred using the Minimum Evolution method. FOL1, FOL2, FOL3, FOL4, and FOL non pathogenic isolates are denoted by the colours red, green, blue, yellow, and grey respectively. The tree is rooted with a TEF sequence from *F proliferatum*.

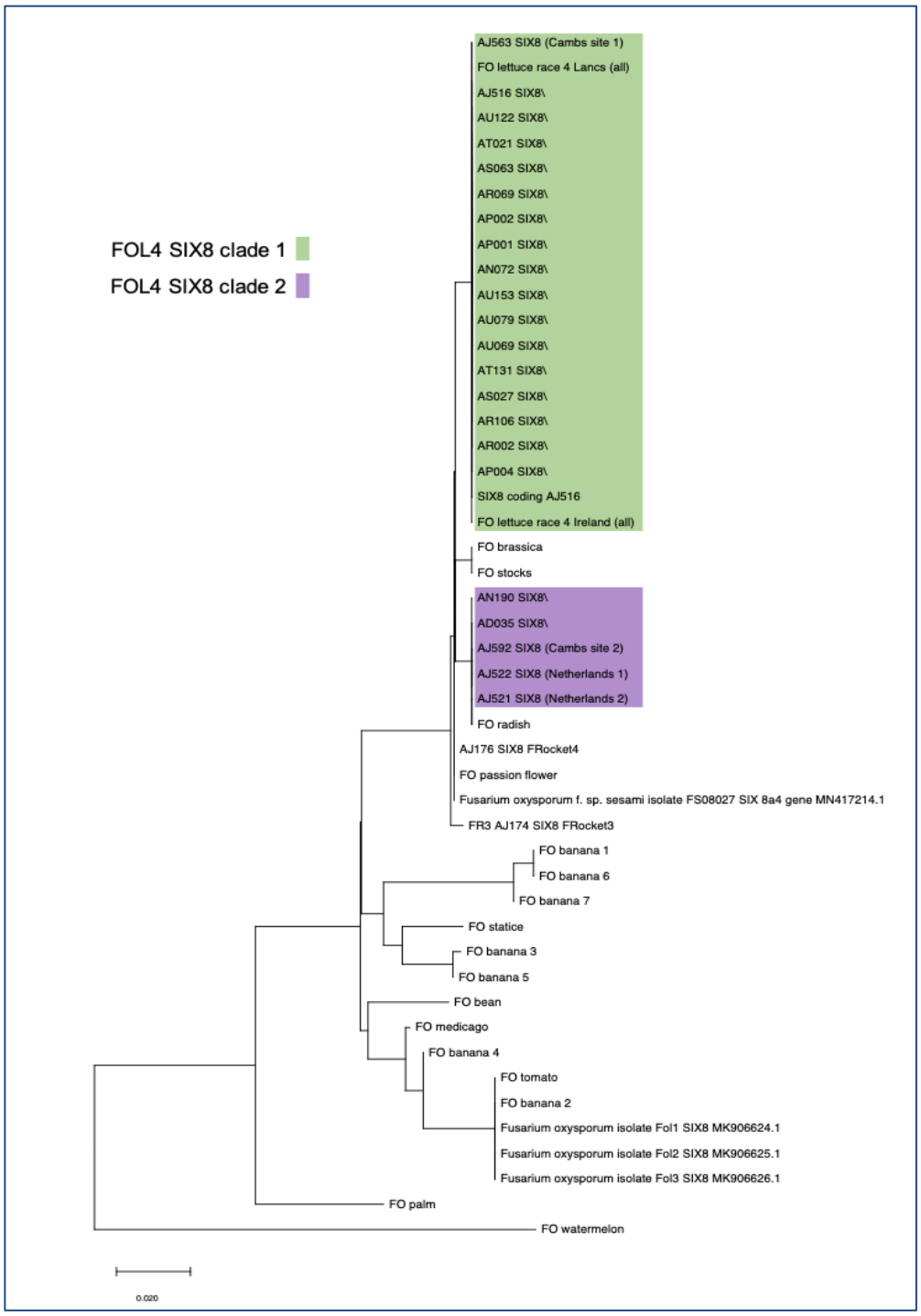

Figure 3 SIX8 phylogenetic tree for FOL4 isolates and other *F. oxysporum* f. spp.. Evolutionary history was inferred using the Minimum Evolution method. Two separate FOL4 SIX 8 clades are highlighted in different colours.

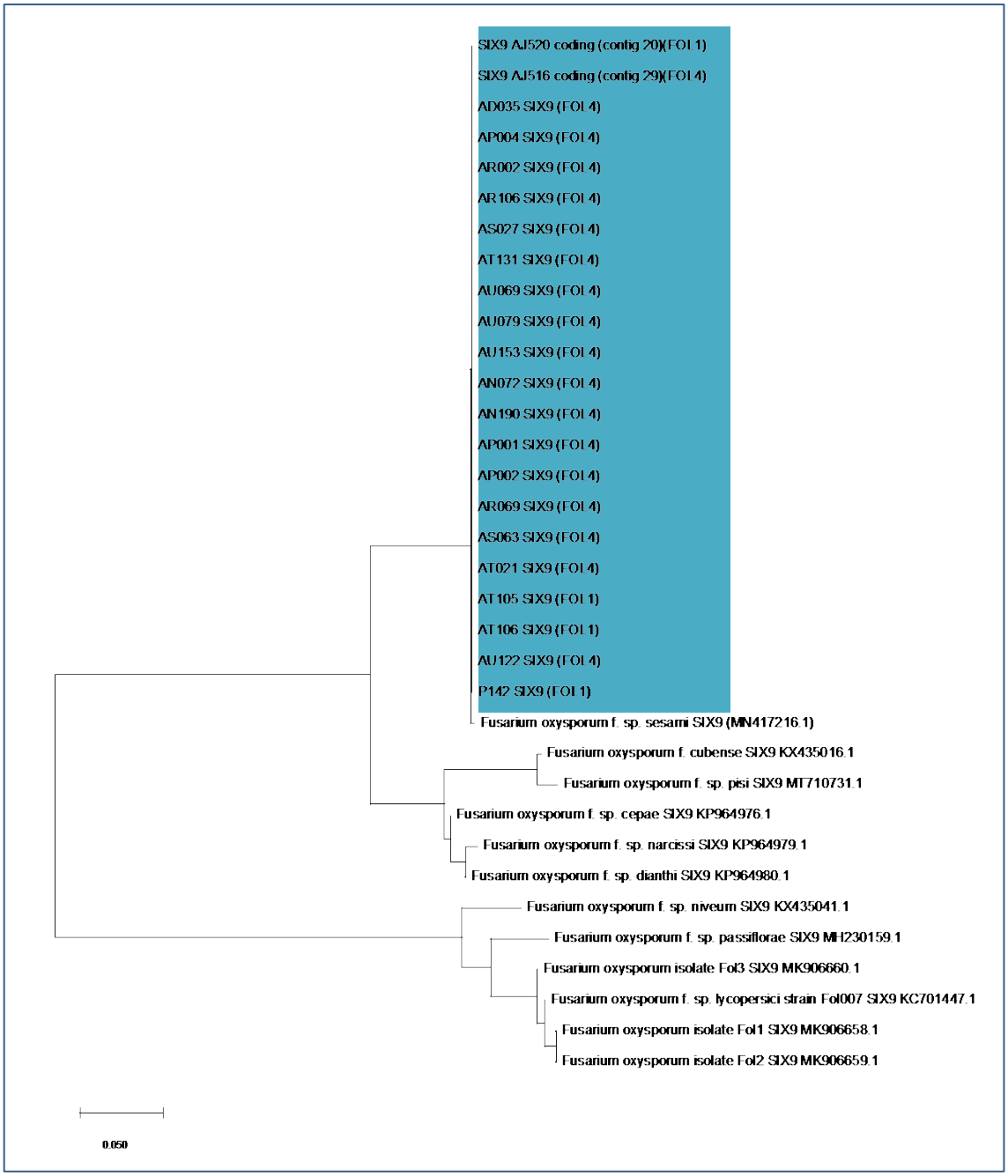

Figure 4 SIX9 phylogenetic tree for FOL1 and FOL4 isolates and other *F. oxysporum* f. spp.. Evolutionary history was inferred using the Minimum Evolution method. FOL sequences are highlighted in blue.

Identify FOL pathogenicity genes expressed during infection and confirm their roles in virulence

An *in vitro* lettuce system was developed where by lettuce seedlings were grown in large square Petri dishes and infected with FOL4 spore suspensions. This system was used to confirm the susceptible / resistant phenotype of 12 lettuce lines identified previously through glasshouse tests carried out in the Defra VeGIN project or through our project collaborator Enza Zaden; Table 2).These lines are also parents of mapping populations being developed by Enza Zaden. Disease development was scored over a period of 38 days using two metrics; a root browning score based on the percentage of total roots affected and a browning score based on percentage of the main tap root only. This was following an initial test where an interesting phenotype was observed where some lines exhibited vascular browning of only the lateral roots, with FOL4 being unable to penetrate into the main tap root (Figure 5). Results indicated that in this system, the susceptible lettuce lines 12 and 11 were very susceptible to FOL4 (high browning scores), whilst the susceptible lines 9 and 10 were not very different from some of the resistant lines (Figures 6a, 6b). Of the resistant lines 1, 2, 3, and 5 showed the lowest root browning scores whilst also being more consistent amongst replicates, as indicated by the low value for standard error of the mean (Figures 6a, 6b). These results have therefore identified some clear

phenotypic differences between selected resistant and susceptible lines and these will be used in future experiments to examine gene expression in both host and pathogen.

Table 2 Summary table of resistant and susceptible parental lines used in in vitro tests for suseptibility to FOL4, along with their phenotypes as observed in glasshouse screening tests

Lettuce line ID	Predicted phenotype
1	Resistant
2	Resistant
3	Resistant
4	Resistant
5	Resistant
6	Resistant
7	Resistant
8	Resistant
9	Susceptible
10	Susceptible
11	Susceptible
12	Susceptible

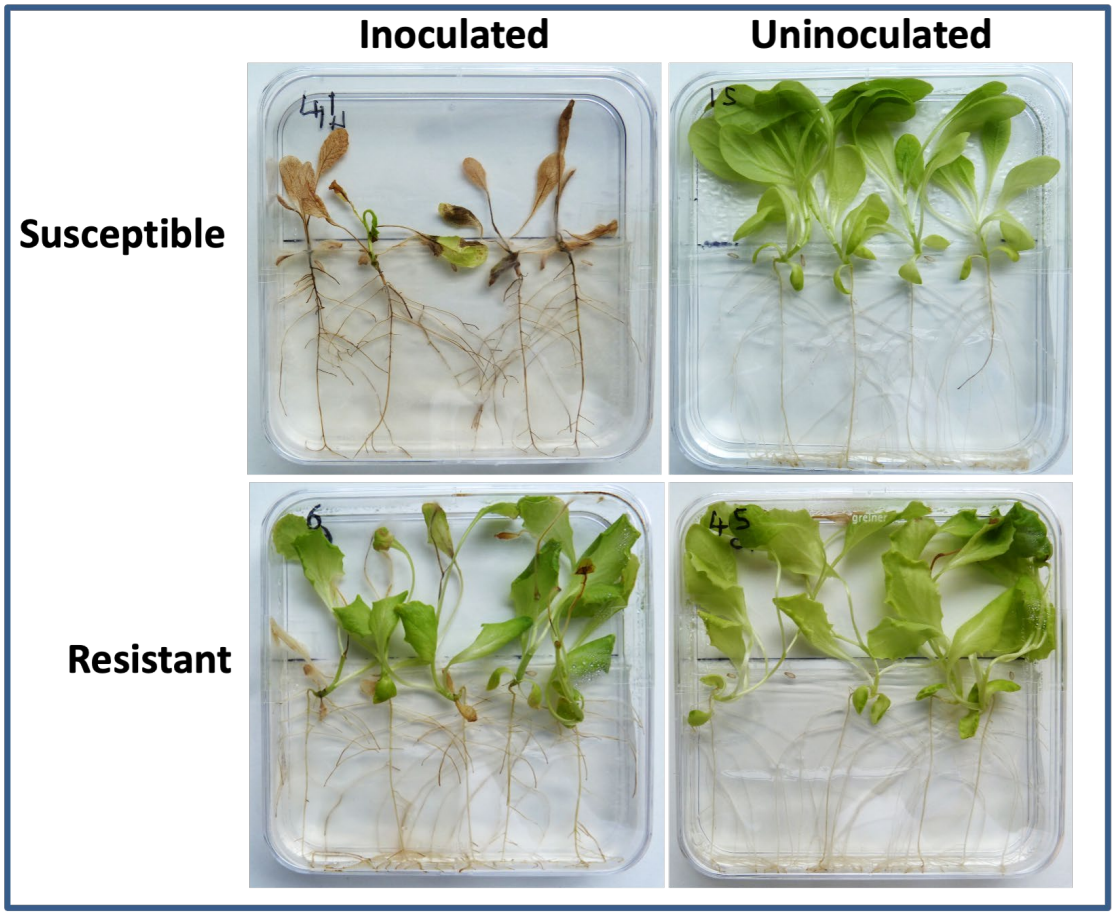

Figure 5 Comparison of FOL4 disease symptoms for two lettuce lines, line 12 (susceptible) and line 1 (resistant)) in the in vitro plate system 5 weeks post infection. Uninoculated control plants are also shown.

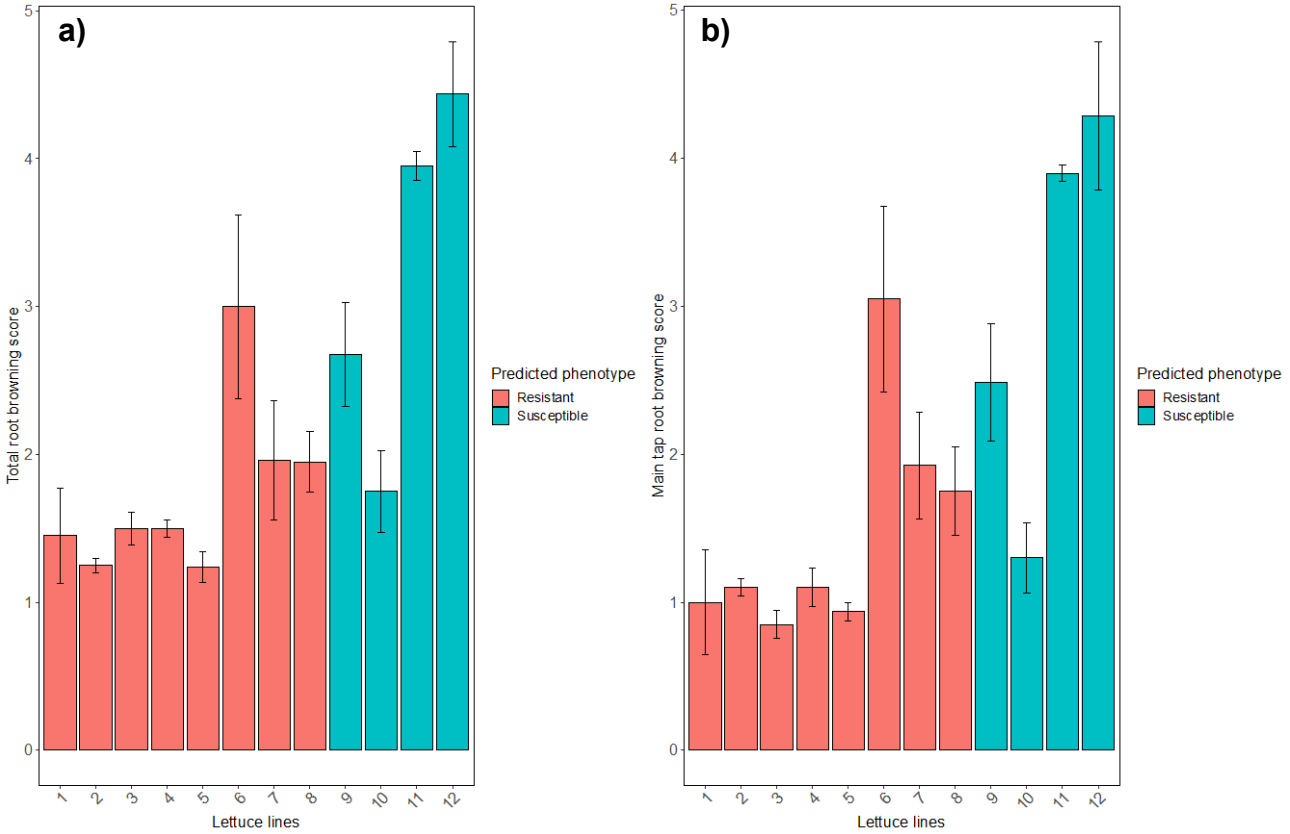

Figure 6 Mean root browning scores for a) whole root systems and b) main tap root in resistant and susceptible lettuce lines 38 days post infection with FOL4. Error bars indicate standard error of the mean. Root browning scores 1,2,3 and 4 denote % browning ranges of 1-25%, 26-50%, 51-75%, and greater than 75% respecticvely. A score of 5 would indicate complete plant death.

4. Key issues to be addressed in the next year

Identify FOL4 pathogenicity genes expressed during infection and confirm their roles in virulence

- Using the *in vitro* inoculation system and a lettuce susceptible line, the expression of SIX8 and SIX9 genes will be determined over time following RNA extraction and qPCR using primers already developed. Expression of these genes will indicate if they may be potentially involved in pathogenicity. This experiment will also help determine the best timepoint for an RNAseq experiment where total RNA will be extracted, sequenced and all upregulated genes in both lettuce and FOL4 identfied during infection.
- A 'knockout system' will be developed and used to generate FOL mutants where putative pathogenicity genes (SIX8 / SIX9 and others identified through RNAseq) are individually deleted and tested on lettuce plants to determine if there is a reduction in virulence, hence proving their function.

Investigate a new lettuce mapping population and identify potential markers for FOL resistance

- A glasshouse experiment where FOL4 resistant and susceptible lettuce lines used in the in vitro system will be inoculated with FOL4 will confirm the phenotypes under more natural condtions and with more replication than has been possible previously.
- FOL4 resistant lettuce lines have been crossed with susceptible lettuce lines by Enza Zaden in order to create 'mapping populations' which should segregate for resistance. These populations should consist of both resistant and susceptible individuals and these will be phenotyped in glasshouse or polytunnel experiments. Following genotyping and analysis this will potentially allow areas of the genome associated with resistance to be identified.

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 17th December 2020
AHDB Crops PhD conference Jan 2020	A 5-minute in person presentation introducing my PhD project to conference attendees
AHDB Crops PhD conference Jan 2021	Poster was prepared describing the PhD project aims, and results to date
MIBTP student symposium April 2021	Poster was prepared describing the PhD project aims, and results to date
PACTS seminar series presentation May 2021	20-minute presentation describing project aims and touching on results gathered during first year of research.

6. Partners (if applicable)

Scientific partners	
Industry partners	Enza Zaden
Government sponsor	BBSRC (MIBTP iCASE studentship)

7. References

AHDB 2018. Lettuce fusarium wilt and root rot.

Claerbout J, Venneman S, Vandevelde I, Decombel A, Bleyaert P, Volckaert A, Neukermans J, Höfte M. 2017. First Report of Fusarium oxysporum f. sp. lactucae Race 4 on Lettuce in Belgium. *Plant Disease* **102**(5): 1037.

Fujinaga M, Ogiso H, Shinohara H, Tsushima S, Nishimura N, Togawa M, Saito H, Nozue M. 2005. Phylogenetic relationships between the lettuce root rot pathogen Fusarium oxysporum f. sp. lactucae races 1, 2, and 3 based on the sequence of the intergenic spacer region of its ribosomal DNA. *Journal of General Plant Pathology* **71**(6): 402-407.

Garibaldi A, Gilardi G, Gullino ML. 2002. First report of Fusarium oxysporum on lettuce in Europe. *Plant Disease* **86**: 1052.

Geiser DM, del Mar Jiménez-Gasco M, Kang S, Makalowska I, Veeraraghavan N, Ward TJ, Zhang N, Kuldau GA, O'Donnell K. 2004. FUSARIUM-ID v. 1.0: A DNA Sequence Database for Identifying Fusarium. *European Journal of Plant Pathology* **110**(5): 473-479.

Gilardi G, Franco Ortega S, van Rijswick PCJ, Ortu G, Gullino ML, Garibaldi A. 2017a. A new race of Fusarium oxysporum f. sp. lactucae of lettuce. *Plant Pathology* **66**(4): 677-688.

Gilardi G, Pons C, Gard B, Franco-Ortega S, Gullino ML. 2017b. Presence of Fusarium Wilt, Incited by Fusarium oxysporum f.sp. lactucae, on Lettuce in France. *Plant Disease* **101**(6): 1053.

Huang JH, Lo CT. 1998. Wilt of lettuce caused by Fusarium oxysporum
in Taiwan. *Plant Pathology Bulletin* **7**: 150–153.

Hubbard JC, Gerik JS. 1993. New wilt disease of lettuce incited by Fusarium oxysporum f. sp. lactucum forma specialis nov. *Plant Disease* **77**: 750-755.

Lin Y-H, Lai P-J, Chang T-H, Wan Y-L, Huang J-W, Huang J-H, Chang P-FL. 2014. Genetic diversity and identification of race 3 of Fusarium oxysporum f. sp. lactucae in Taiwan. *European Journal of Plant Pathology* **140**(4): 721-733.

Malbrán I, Mourelos CA, Mitidieri MS, Ronco BL, Lori GA. 2014. Fusarium Wilt of Lettuce Caused by Fusarium oxysporum f. sp. lactucae in Argentina. *Plant Disease* **98**(9): 1281-1281.

Matuo T, Motohashi S. 1967. On Fusarium oxysporum f. sp. lactucae n. f. causing root rot of lettuce. *Trans. Mycol. Soc. Jpn.*(32): 13-15.

Millani MJ, Etebarian HR, Alizadeh A. 1999. Occurrence of Fusarium wilt of lettuce in Shahre-Ray, Varamin and Karaj areas. *Iranian Journal of Plant Pathology* **35**: 44–45.

Okungbowa F, Shittu H. 2012. Fusarium wilts: An overview. *Environ. Res. J* **6**(2): 83-102.

Pasquali M, Dematheis F, Gullino ML, Garibaldi A. 2007. Identification of race 1 of Fusarium oxysporum f. sp. lactucae on lettuce by inter-retrotransposon sequence-characterized amplified region technique. *Phytopathology* **97**(8): 987-996.

Taylor A, Clarkson JP. 2018. Technical review on lettuce Fusarium wilt, caused by Fusarium oxysporum f. sp. lactucae: Agriculture and Horticulture Development Board.

Taylor A, Jackson AC, Clarkson JP. 2018. First Report of Fusarium oxysporum f. sp. lactucae Race 4 Causing Lettuce Wilt in England and Ireland. *Plant Disease* **103**(5): 1033-1033.

Ventura JA, Costa H. 2008. Fusarium Wilt Caused by Fusarium oxysporum on Lettuce in Espirito Santo, Brazil. *Plant Disease* **92**(6): 976-976.