Studentship Project: Annual Progress Report
October 2021 to March 2023

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 mainly 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

FOL isolates in UK protected lettuce thus far have all been identified to be FOL4 (Taylor *et al.*, 2018) with one excpetion of FOL1 reported in Northern Ireland in 2022 (unpublished). 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 to each other 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 and SIX14 while only 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 allows 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 were screened in a separate polytunnel experiment which confirmed results observed in the *in vitro* assay.
- RNA was extracted from the root material at different timepoints for one susceptible lettuce line and qPCR showed that all three *SIX* genes (SIX8, SIX9, SIX14) were expressed during early infection.
- An RNAseq experiment identified key putative pathogenicity genes expressed during early infection of lettuce, hence providing candidates for knock out studies to prove function of these genes.

Investigate the extent of root colonisation of resistant and susceptible lines by FOL4

- Preliminary work done prior by a masters student suggested that FOL4 was able to colonise resistant lettuce plants although to a lesser extent than on susceptible plants.

- A glass house experiment with inoculated resistant, intermediate resistant and susceptible lettuce plants showed that FOL4 can colonise the vascular system of all types to some degree, including resistant lettuce where no symptoms are present.
- Further tests will look at the extent of FOL4 colonisation of the whole root mass of resistant and susceptible lines.

Use a new lettuce mapping population to identify potential genes associated with FOL4 resistance

- Preliminary screens on different lettuce varieties done in VEGIN succeeded in finding varieties that were resistant to both FOL1 and FOL4.
- Collaboration with Enza Zaden was established in order to investigate further the potential underlying genetics associated with resistance.
- An additional polytunnel experiment where soil has been infected with FOL4 confirmed the phenotypes of resistant and susceptible parent lines.
- Another polytunnel experiment tested lettuce from one of the lettuce mapping populations to further investigate the genetic nature of resistance.

3. Summary of results from the reporting year

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

An *in vitro* lettuce system was developed whereby lettuce seedlings were grown in large square Petri dishes and infected with FOL4 spore suspensions. This system was used to carry out a qPCR timecourse of FOL4 infection overtime. A susceptible lettuce line (cv. Temira) was inoculated with FOL4 conidia and root tissue harvested at 0,6,12,24,48,72, and 96 hours post infection. RNA extracted from tissue samples was used to monitor expression of putative effectors SIX8 and SIX9 over time. Relative expression of SIX8 and SIX9 was plotted and results indicate that expression of both SIX8 and SIX9 peak at 96 hours post infection (Figure 2).

An RNAseq experiment was then carried out at the 96 hour timepoint in order to find other putative effectors expressed during infection. RNAseq followed by differential expression analysis identified a range of highly expressed putative effectors including SIX8, SIX9, SIX14 and homologues of some previously identified in *F. oxysporum* f.sp. *apii* affecting celery. Figure 3 shows the pipeline used to identify these differentially expressed genes. Selected genes are being targeted for knockout studies (Table 1) which will be done during a 3-month placement at the University of Amsterdam.

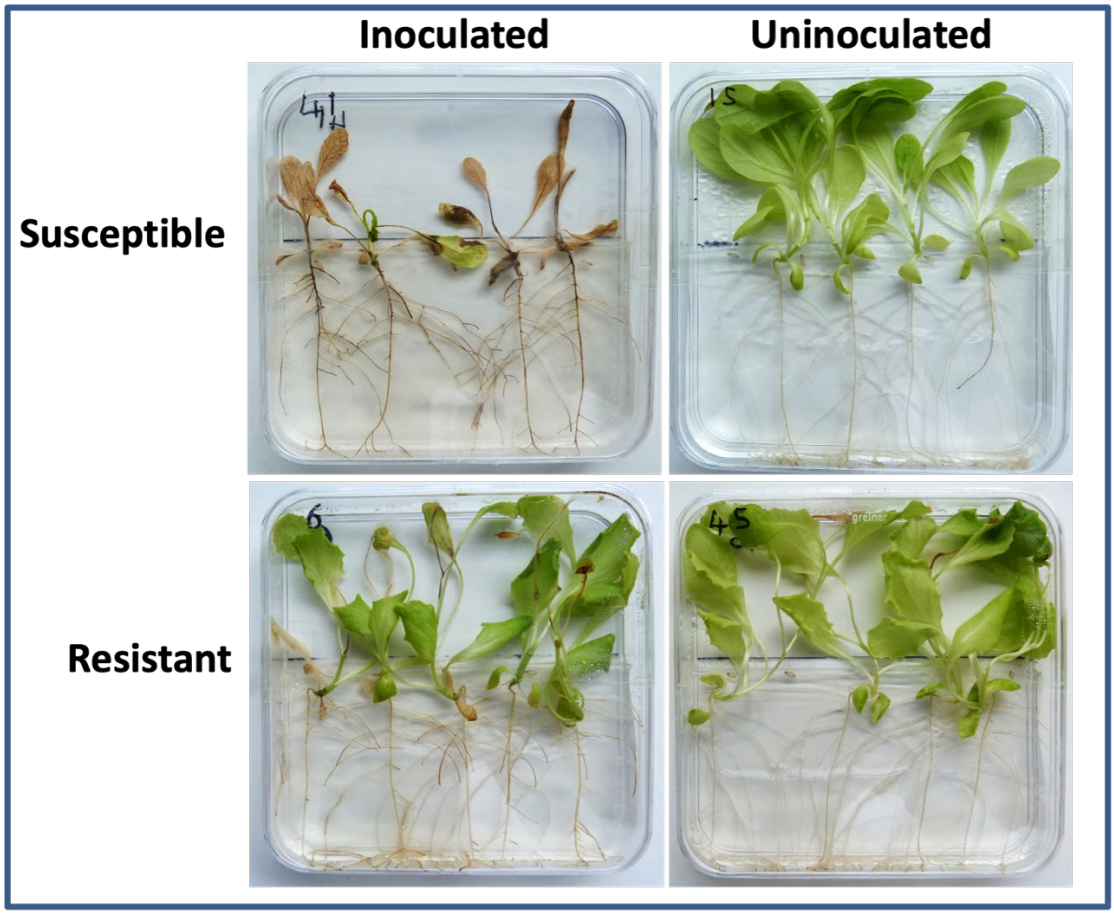

Figure 2 Comparison of FOL4 disease symptoms for susceptible (line 12) and resistant (line 1) lettuce lines in the *in vitro* plate system 5 weeks post infection. Uninoculated control plants are also shown.

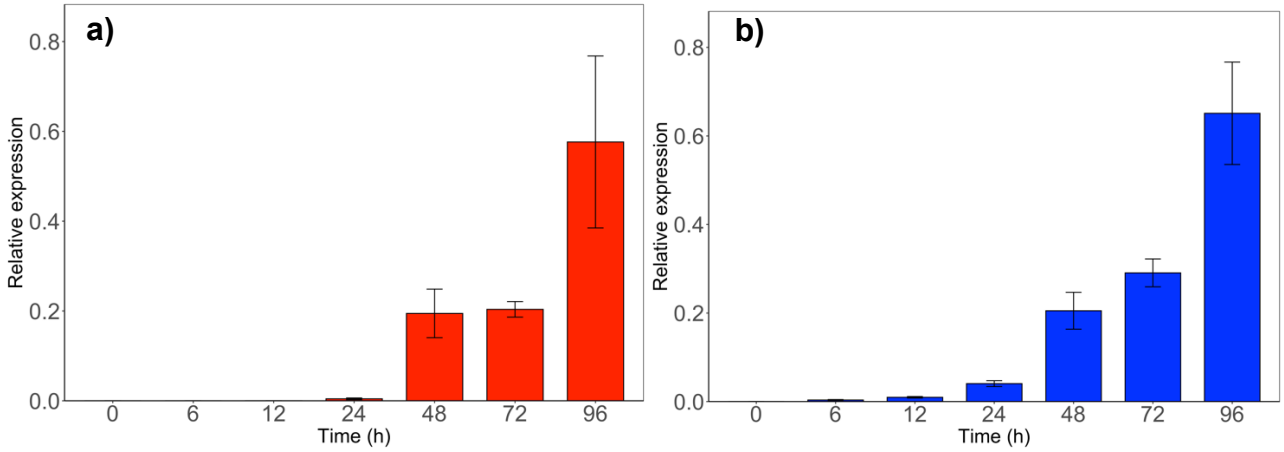

Figure 3 Expression of a) SIX8 and b) SIX9 over time relative to the house keeping gene TEF. Error bars indicate standard error of the mean.

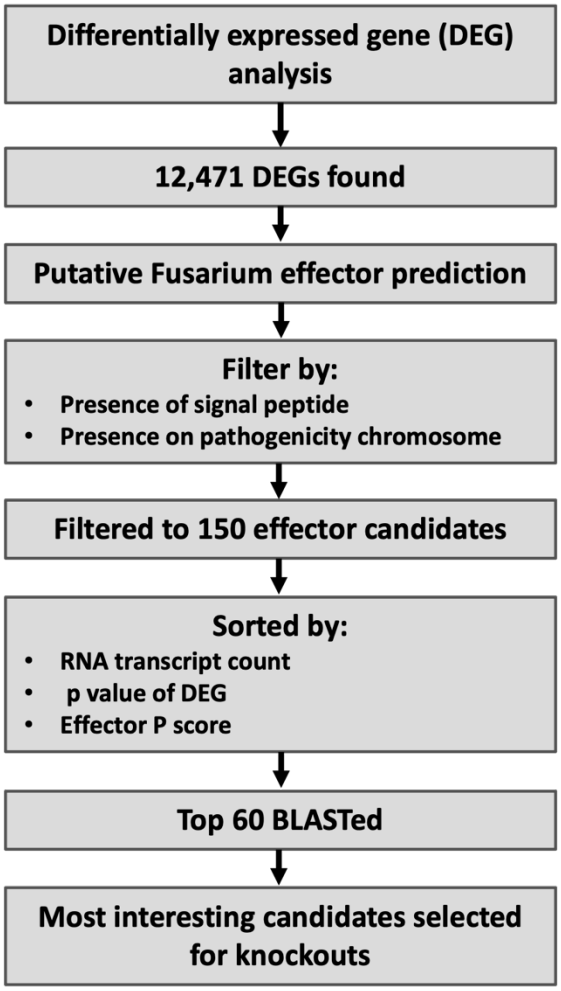

Figure 4 Flowchart outlining methods used to select candidate putative FOL4 effectors for knockout studies.

Table 1 Table outlining the most promising putative effector candidates for future knockout studies

Gene ID	Transcript count	pvalue	Putative identification
g8918	30790.4262	2.38E-70	FaEL-2
g8911	30763.7656	2.10E-70	FaEL-2
g21124	29601.0639	3.99E-62	
g24453	29594.9522	3.47E-62	
g24452	29580.1017	3.73E-62	
g21125	29580.0622	3.43E-62	
g8912	19892.0823	2.63E-141	FaEL-3
g8917	18989.1072	1.34E-141	FaEL-4
g12530	14500.4224	1.01E-60	SIX9
g9012	14485.5248	1.74E-60	SIX9
g12532	6199.90006	1.68E-83	
g9017	6191.94709	1.75E-83	
g20675	3518.75993	4.12E-70	
g12454	3460.27284	3.85E-65	

g9993	3436.47316	2.60E-65	
g10013	2463.33145	9.42E-85	
g9602	2013.16426	1.27E-15	PSE1
g9601	1091.90448	1.29E-22	SIX8
g9989	962.80038	1.25E-100	
g18830	860.689957	9.81E-09	
g12862	465.27506	5.43E-37	SIX14
g9460	379.579605	6.98E-44	
g20708	337.381593	1.87E-55	
g12538	335.451257	9.49E-56	
g9023	335.212853	4.55E-56	
g20057	327.762919	1.19E-65	
g9977	303.924501	9.86E-61	
g9484	210.813587	1.23E-23	SIX15-Like2
g18764	92.0317336	5.58E-15	
g18849	61.6440271	2.15E-14	
g18788	53.452378	3.41E-17	
g23935	32.7064657	5.40E-22	
g8937	30.1176066	2.24E-11	
g12559	29.4193965	7.60E-41	
g9617	29.3640031	1.19E-40	
g4778	22.2429523	2.60E-05	
g23863	17.9828482	1.48E-23	FaEL-1
g9612	16.4581639	8.64E-20	FaEL-1
g9669	9.13750274	1.93E-07	
g20748	3.82935437	5.73E-15	SIX15-Like1
g10014	0.70957561	2.85E-05	
g9475	1109.55502	6.76E-14	FaEL-5
g19656	444.720542	1.62E-130	
g9964	437.792967	1.48E-128	
g23733	283.220978	1.42E-85	
g8965	189.004951	1.88E-13	
g12492	188.708751	2.19E-13	
g8955	166.80979	3.10E-60	
g12478	166.53005	3.31E-60	
NA	137.87914	9.94E-29	
NA	100.831443	0.01844516	
NA	100.794175	0.01956395	
g19655	37.573372	8.52E-54	
g9956	34.8381261	2.04E-51	
g9429	29.1513707	3.11E-46	
g12560	28.3932661	4.35E-30	
g8930	11.8936203	2.04E-32	
g23845	2595.83293	3.25E-67	

Use a new lettuce mapping population to identify potential genes associated with FOL4 resistance

An experiment in the FOL4 inoculated polytunnel at Wellesbourne was set up to test 16 lettuce lines in order to more robustly confirm the susceptible / resistant phenotype identified previously through glasshouse tests carried out in the Defra VeGIN project (Table 2). These lines are also parents of mapping populations developed by Enza Zaden. Disease development was scored over a period of 35 days using two metrics; a leaf wilting score and a vascular browning score taken at harvest (Figures 5, 6). Results indicated that the susceptible lettuce lines 12 and 11 were very susceptible to FOL4 (high browning and wilt scores) although not as susceptible as our standard susceptible line 16, whilst the susceptible lines 9 and 10 were not very different from some of the resistant lines (Figures 5, 6). Of the resistant lines the majority displayed low levels of vascular browning and showing consistency amongst replicates as indicated by the low value for standard error of the mean (Figures 5, 6). However, predicted resistant lines 8, 13 and 14 showed low levels of disease indicating mild susceptibility to FOL4 (Figures 5, 6). 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 and also provide focus for which mapping populations to concentrate on in further studies.

An Enza lettuce mapping population produced from a cross between resistant line 5 and susceptible line 11 was investigated. Enza produced F2 seed which was used to raise 654 individual plants which were transplanted into the FOL4 infested poly tunnel along with 24 plants of the resistant parent and 24 plants of the susceptible parent. A single leaf from all individuals was then sampled about one week after transplanting for future DNA analysis. All individuals in the tunnel were scored for wilt over the course of 8 weeks and after harvest at 10 weeks post transplanting, vascular browning scores were recorded. Figure 7 shows the number of individuals that exhibited different vascular browning scores. Individuals that scored a vascular browning score >1 were considered susceptible. Individuals with a vascular browning score of below 1 were said to be resistant. These criteria produced a segregation ratio of 2.3:1.

Table 2 Summary table of resistant and susceptible parental lines used in FOL4 inoculated poly tunnel trial, along with their phenotypes as observed in previous 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
13	Resistant
14	Resistant
15	Resistant
16	Susceptible

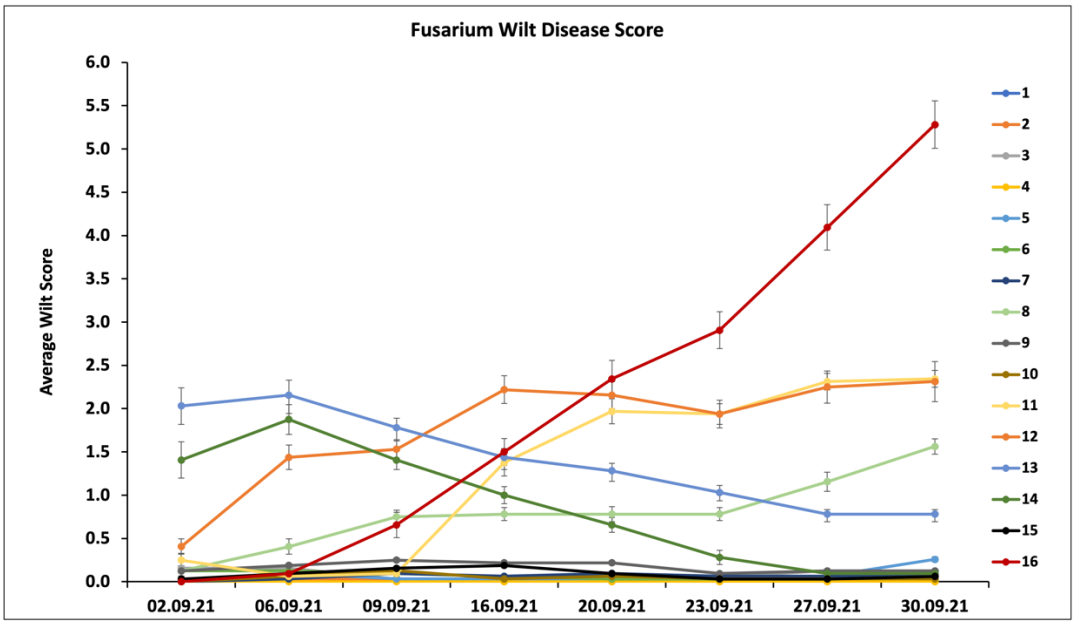

Figure 5 Average wilt scores in resistant and susceptible lettuce lines infected with FOL4 over 28 days. Error bars indicate standard error of the mean. A wilt score of 1 denotes wilting of 1-2 leaves, wilt scores of 2,3,4,5,6 and 7 denote % wilting ranges of <10%, 10-25%, 25-50%, 50-75%, 75-99%, and 100% respectively. Error bars indicate standard error of the mean.

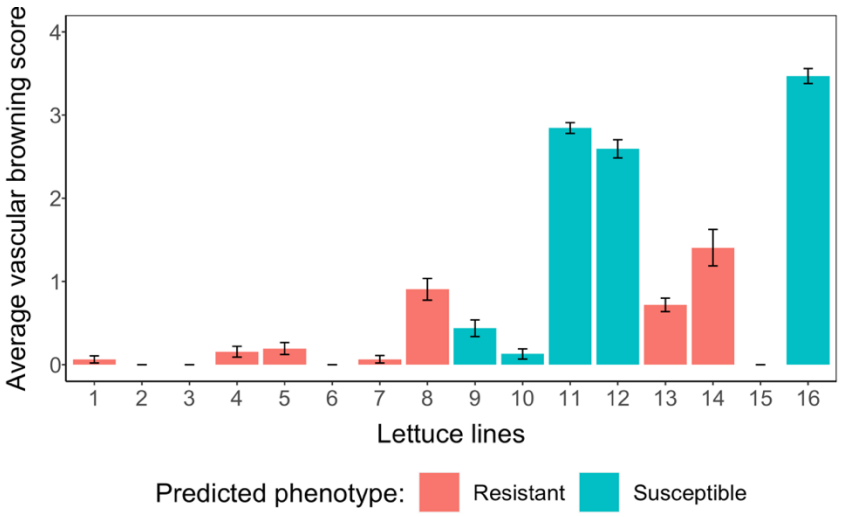

Figure 6 Average vascular browning scores in resistant and susceptible lettuce lines 35 days post infection with FOL4. Error bars indicate standard error of the mean. Vascular browning scores 0,1,2,3 and 4 denote the categories of no symptoms, mild vascular browning, vascular browning, severe vascular browning, and plant death respectively. Red bars indicate lines predicted to be resistant and blue bars indicate lines predicted to be susceptible.

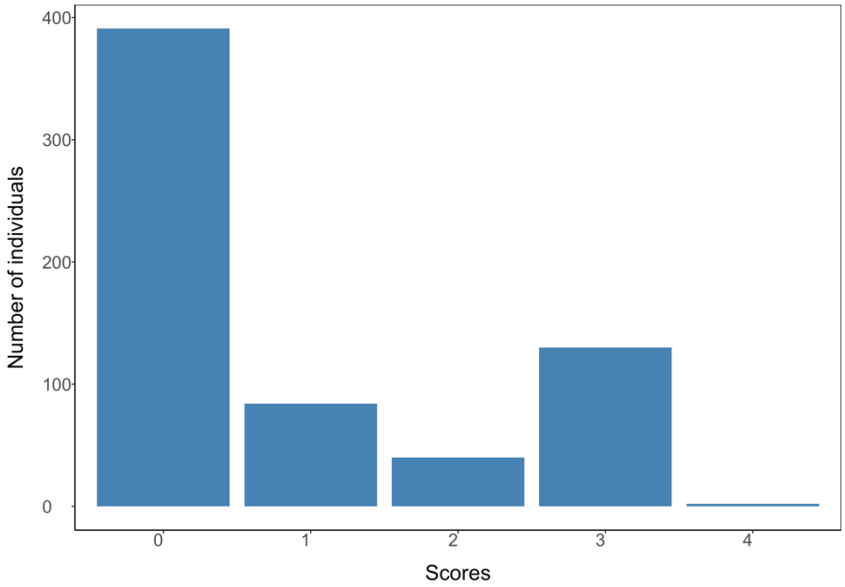

Figure 7 Number of mapping population individuals exhibiting respective vascular browning scores. Vascular browning scores 0,1,2,3 and 4 denote the categories of no symptoms, mild vascular browning, moderate vascular browning, severe vascular browning, and plant death respectively.

Investigate the extent of root colonisation of resistant and susceptible lines by FOL4

A glass house experiment was set up to investigate the extent of root colonisation of resistant, intermediate resistant and susceptible lettuce lines by FOL4 (Table 3). This experiment aimed to investigate two metrics of colonisation. Firstly how far up the tap root vascular tissue FOL4 is able to penetrate and secondly to what extent is the whole root mass is colonised by FOL4 for each lettuce type. Results for the first metric are outlined here however experiments regarding the second are ongoing. Plants were harvested either when they exhibited wilt scores of 3-4 or 4-5 weeks after infection. Prior to tap root isolations, lettuce lines were scored for vascular browning (Figure 8). These results indicate that the resistant lines exhibited low vascular browning scores and were not statistically significantly different from the uninoculated susceptible control (treatment 9) except for line 4. The intermediate resistant line exhibited more vascular browning and significantly differed from the uninoculated susceptible control and the susceptible lines exhibited the highest vascular browning score. For root isolations 3 separate locations within the tap root were sampled (Bottom, Middle, Top) as shown in Figure 9a and percentage recovery of FOL4 was recorded (Figure 9b). These results indicated that FOL4 can be isolated from the bottom and middle locations in all lettuce lines although resistant lines (with the exception of line 2) showed lower recovery rates of the pathogen from these locations. Isolation of of FOL4 was observed in all susceptible lines and in the intermediate line in the top location. Interestingly FOL4 also colonised the top location in resistant lines 2 and 4 but was not isolated from the top location in resistant lines 1 and 3. The extent of recovery of FOL4 in resistant line 2 seems comparable to that of the susceptibles in the middle and bottom locations; however recovery of FOL4 from the top location in line 2 was much lower than that of the susceptibles. PCR detection of FOL4 will be used to confirm all growth from the isolations are FOL4.

Table 3 Summary table of resistant, intermediate, and susceptible lines used in glass house trial investigating root colonisation by FOL4

Lettuce line ID	Phenotype
1	Resistant
2	Resistant
3	Resistant
4	Resistant
5	Intermediate
6	Susceptible
7	Susceptible
8	Susceptible

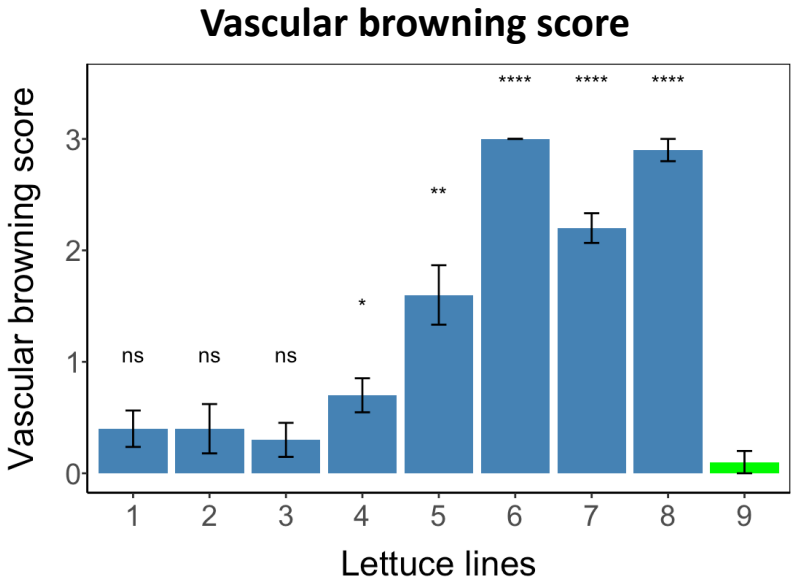

Figure 8 Average vascular browning scores in resistant, intermediate resistant and susceptible lettuce lines recorded either when the majority of individuals of a line reached a wilt score of 3-4 or if latter wilt scores were not achieved 4-5 weeks post infection with FOL4. Error bars indicate standard error of the mean. Vascular browning scores 0,1,2,3 and 4 denote the categories of no symptoms, mild vascular browning, vascular browning, severe vascular browning, and plant death respectively.

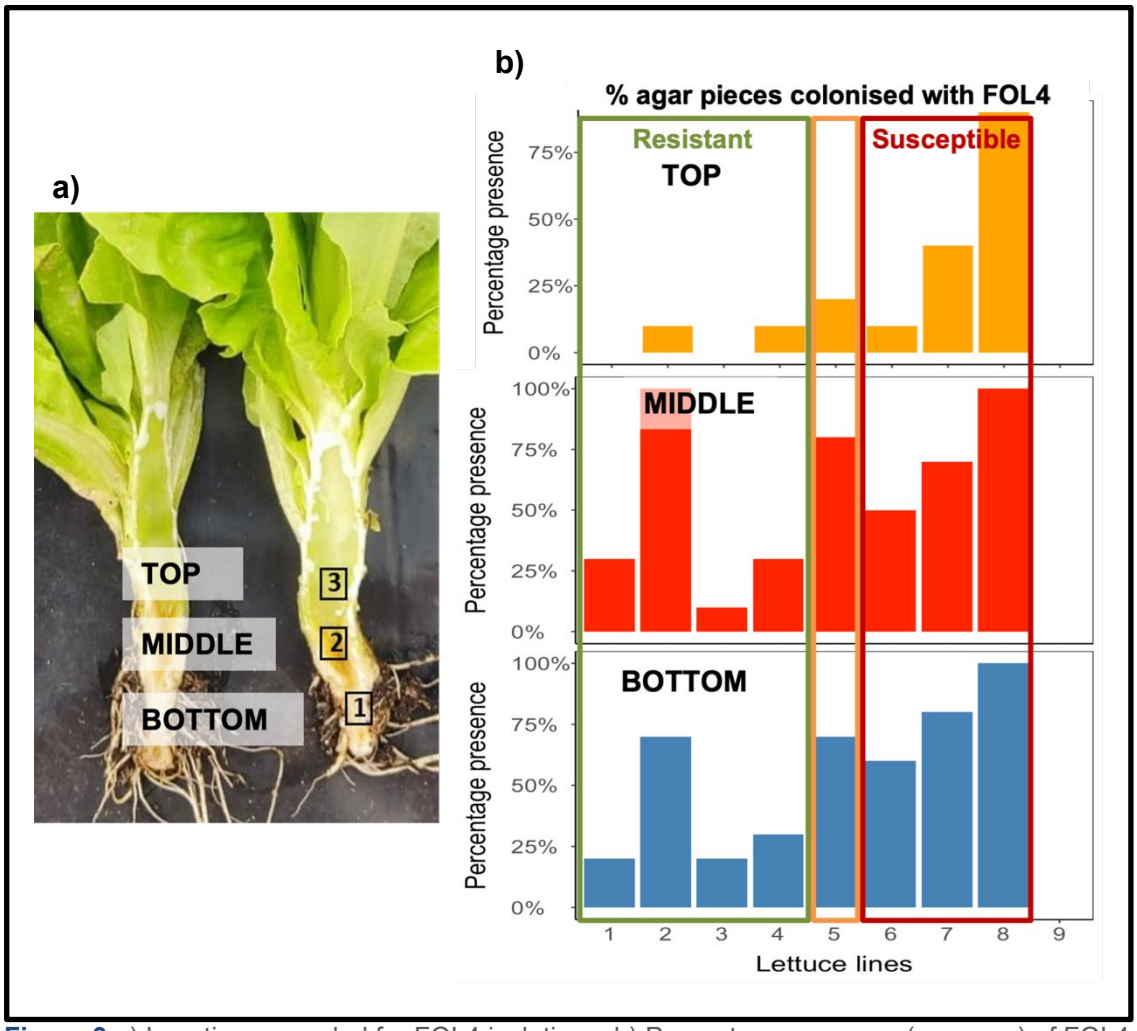

Figure 9 a) Locations sampled for FOL4 isolations. b) Percentage presence (recovery) of FOL4 from different isolation locations across resistant, intermediate resistant and susceptible lettuce lines.

4. Key issues to be addressed in the next year

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

- A current visit to Martijn Reps laboratory (University of Amsterdam) will enable a CRISPR Cas9 'knockout system' to be used to generate FOL4 mutants where putative pathogenicity genes (SIX8 / SIX9 / SIX14 and others identified through RNAseq) will be individually deleted. Mutants will be 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

- 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. A single mapping population was selected and the individuals screened in a FOL4 infested poly tunnel trial. Following genotyping and analysis this will potentially allow areas of the genome associated with resistance to be identified.

Investigate the extent of root colonisation of resistant and susceptible lines by FOL4

- FOL4 quantification will be carried out by qPCR for the three different tap root locations for resistant, intermediate resistant and susceptible lettuce lines to more robustly measure root colonisation of FOL4 and compare with the isolation data.
- FOL4 quantification by qPCR will also be carried out for the whole lettuce root mass (lateral roots) of resistant, intermediate resistant and susceptible lettuce lines investigate the extent of colonisation of the entire root system.

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.
PH Gregory presentation at annual BSPP conference Sept 2022	15-minute presentation describing project aims and touching on results gathered during research.
Presentation to Molecular plant pathology group at the UvA 2023	15-minute presentation describing project aims and touching on results gathered during research.

6. Partners (if applicable)

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

7. References

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