Horticultural Fellowship Awards

Final Report Form

Project title:	Maintaining and developing capability in vegetable crop pathology
Project number:	CP 113
Project leader:	Prof. John Clarkson, Warwick Crop Centre, University of Warwick
Report:	Final Report, September 2021
Fellowship staff: ("Trainees")	Prof. John Clarkson & Dr Sascha Jenkins
Location of project:	Warwick Crop Centre
Industry Representative:	n/a
Date project commenced:	1st November 2013
Date project completed (or expected completion date):	31st September 2021

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Dr Sascha Jenkins
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Signature  Date: 14.10.21

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Objectives:

***Fusarium oxysporum* f. sp. *lactucae* affecting lettuce**

- Identify whether *F. oxysporum* is causing infection of lettuces in the UK
- Develop methods to evaluate build-up of *F. oxysporum* f. sp. *lactucae* inoculum in sterilised/ non-sterilised soil after consecutive lettuce crops
- Test resistant cultivars of lettuce against FOL4
- Determine whether FOL4 can be isolated from the xylem sap of different cultivars of lettuce
- Complete a mini review of biological control agents which could suppress FOL

***Fusarium oxysporum* f. sp. *narcissi* (FON) affecting daffodils**

- Identify whether *F. oxysporum* is causing infection of narcissus in the UK, and whether it can be detected in soil

***F. oxysporum* f. sp. *cepae* affecting onions**

- Identification of *Fusarium* isolates from infected onions

Molecular developments

- Test methods to improve DNA quality for qPCR
- Construct a *TEF* sequence database of *Fusarium* sequences from the isolate collection at Warwick Crop Centre

Training and personal development

Plant pathogens

During this fellowship I have gained experience working with new plant pathogens, such as *Sclerotium cepivorum*, the causal agent of Allium white rot disease in onion. I have gained new skills in culturing sclerotia and monitoring germination in response to garlic products. In addition, I can perform viability tests on non-germinated sclerotia from these experiments and assess for viability/contamination.

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Plant and Crop Theme Seminars

Since September 2020 I have been actively involved in organising a Plant and Crop Theme Seminar (PACTS) series, which is a collaboration of post-docs and PhD students from both Warwick campuses to produce an intergraded seminar series. Previously, each campus had their own seminar series, but we combined them when they were required to move online to encourage students and post-docs to engage with plant and crop research from across the school. My role has been to assist with organising the schedule of seminars, actively chair/moderate seminar sessions, and produce the events on Microsoft Teams. Additional responsibilities are to obtain talk titles from speakers before the event, update the schedule and make sure they are updated on the PACTS dedicated website.

Expertise gained by trainees

- Improved understanding of the lettuce/onion industries
- Improved understanding of plant pathology
- Fungal isolations from a wider range of plant hosts
- Improved molecular skills with magnetic beads and qPCR
- New skills gained working with Allium white rot

Other achievements

- Paper entitled: Identification and expression of *Secreted In Xylem* (*SIX*) pathogenicity genes in *Fusarium oxysporum* f. sp. *pisi*
- Identification of *Fusarium* isolates from infected onions as part of additional commercial contracts

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GROWER SUMMARY

Background

Fusarium wilt of lettuce

Fusarium wilt of lettuce, caused by *Fusarium oxysporum* f.sp. *lactucae* (FOL), has spread to most production areas globally, causing severe economic losses mostly in protected crops. Several races exist, with race 4 (FOL4) being the predominant race affecting lettuce in the UK. Although hygiene measures are preventing rapid spread, it is spreading to new areas across the UK, evidenced by the fact that more growers have been sending samples for testing.

Fusarium disease in daffodil

Fusarium basal rot of *Narcissus* is caused by *F. oxysporum* f. sp. *narcissi* (FON). It infects the roots or damages basal plates resulting in soft and rotting bulbs, which leads to bulbs not sprouting or producing short lived or early senescing foliage with few or no flowers (Taylor et al., 2019a). It is a problem for UK growers as bulbs are lifted after 2-3 years and used as replanting stock, therefore maintaining inoculum levels or allowing it to spread (Hanks, 2013; Taylor et al., 2019a).

Fusarium disease in onion

Fusarium basal rot of onion, caused by *F. oxysporum* f.sp. *cepae* (FOC), represents a major threat to the industry, with incidence levels increasing over the last few years. Recently basal rot losses have increased from 2-6% to over 10%, resulting in economic losses of approx. £13M annually. Many of these losses have been the result of disease developing in storage, where apparently healthy bulbs develop disease, which can result in worse cases to the whole consignment being abandoned as it becomes too costly to extract affected bulbs. Early detection is key to prevent crops being grown in affected soils to reduce disease incidence as much as possible, and prevent bulbs being contaminated before going into storage.

Molecular identification of Fusarium

Molecular techniques for identification of Fusarium species are now common practice, using sequencing and phylogeny of housekeeping genes. The *translation elongation factor 1*α (*TEF*) gene, which encodes an essential part of the protein translation machinery, resolves different *Fusarium* spp. and is therefore the marker of choice for identification. The gene has been shown to be consistently single copy in *Fusarium* and shows a high level of sequence polymorphism between closely related species (Geiser et al., 2004), which makes it particularly useful for Fusarium species identification.

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However, *TEF* is unable to distinguish between different *formae speciales* (f. spp.) of *F. oxysporum* and therefore the presence and absence of effector genes known as *Secreted In Xylem* (*SIX*) genes can be useful for identifying *F. oxysporum* f. spp. and even specific races. Molecular tests have previously been developed at Warwick Crop Centre to help distinguish between f. sp. affecting lettuce onion and daffodils (Clarkson, 2018).

Summary

- FOL4 was identified in lettuces and soil samples from UK growers using the specific molecular test developed previously as part of the fellowship

- Methods were developed to assess the build-up of FOL4 inoculum in soil which had been steam sterilised, compared to non-sterilised soil, over three successive lettuce crops. Disease developed more rapidly in sterilised soil.

- Lettuce cultivars were tested for resistance to FOL4 by transplanting seedlings into inoculated soil in a polytunnel. Cultivars were identified which showed high levels of resistance. Some lettuces showed little wilt symptoms but high levels of vascular browning, showing the need for multiple disease assessments.

- Lettuce xylem sap was collected to determine whether FOL can be isolated from the stem xylem sap of resistant cultivars. FOL was successfully recovered from the xylem sap of resistant cultivars.

- A short literature review was conducted of biological control agents and soil disinfestation methods which could potentially be used to inform which treatments could be used after soil sterilisation to reduce to rapid build-up of FOL. We will test some of these methods with steam sterilised and non-sterilised soil

- FON was identified in soil samples taken from grower sites using a molecular qPCR assay previously developed at Warwick Crop Centre. Levels of FON varied across the field and between field sites.

- FOC was identified in onions from Sweden and Spain, with most containing the FOC specific complement of *SIX* genes.

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- *F. oxysporum* was the most commonly identified species isolated from diseased parsley roots from UK growers. Additional tests are required to determine their pathogenicity.

- Magnetic beads were used to try and improve DNA quality to try and increase the sensitivity of qPCR assays so DNA could be detected at lower levels in soil. Initially, no improvement was observed but further tests are required to confirm this.

- A *TEF* sequence database was constructed of *Fusarium* sequences for the collection of isolates at Warwick Crop Centre to improve access and availability of sequence information for all users.

Financial Benefits

None to report

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SCIENCE SECTION

1. Introduction

Fusarium oxysporum is the most widely dispersed and economically important plant pathogenic species in the *Fusarium* genus as it infects numerous hosts and causes extensive crop losses (Leslie and Summerell, 2006). In 2012 it was identified as 5th in a list of the top 10 fungal plant pathogens in terms of scientific and economic importance (Dean et al., 2012). *F. oxysporum* is responsible for a wide range of plant diseases, usually causing a vascular wilt but also causes root and bulb rots (Edel-Hermann and Lecomte, 2019). *F. oxysporum* is a species complex with over 150 'special forms' (*formae speciales*; f. spp.), adapted to infect specific hosts plants which affects many agriculturally important crops such as lettuce, onion, tomato, banana, leek, peas, melon and narcissus (Edel-Hermann and Lecomte, 2019). The complex also includes non-pathogenic *F. oxysporum* isolates.

Fusarium disease of lettuce

Fusarium wilt of lettuce is caused by *F. oxysporum* f. sp. *lactucae* (FOL) and affects lettuce production across many countries (Gordon and Koike, 2015). Symptoms of Fusarium wilt in lettuce are yellowing and necrosis of leaves, stunting and wilting of plants and reddish-brown/black necrosis of vascular tissue (Matheron, 2015). There are four cultivar specific races, with races 1 and 4 being the most widespread and the only two identified in Europe (Gilardi et al., 2017). FOL race 4 was first characterised in the Netherlands (Gilardi et al., 2017) and was subsequently reported in Belgium (Claerbout et al., 2018), the UK and Ireland (Taylor et al., 2019b) and very recently in Italy (Gilardi et al., 2019a). So far FOL4 has been restricted to protected lettuce (grown under cover: polytunnels or glasshouses) and therefore mainly affects varieties such as butterhead and Little Gem. FOL, like many other *F. oxysporum* f. spp. produces long lived chlamydospores (Gordon and Koike, 2015), enabling inoculum levels to increase over time, especially in the case of FOL4 as protected lettuce production can be intensive with up to six crops a year in the same soil (Taylor et al., 2019b). Although hygiene measures are preventing rapid spread, it is spreading to new areas across the UK, evidenced by the fact that more growers have been sending samples for testing.

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Fusarium disease of onion

Fusarium oxysporum f. sp. *cepae* (FOC) is the causal agent of Fusarium disease in onion and symptoms can include seedling damping off, root/stem rot in immature plants and most importantly basal rot of bulbs resulting in significant losses (Entwistle, 1990; Taylor et al., 2013). In the UK, 2-6% of the onion bulb crop (8779 ha valued at approx. £132M in 2018; Defra, 2019) is lost each year in the field with a corresponding economic value of £7.9M. In addition, basal rot also occurs in storage as onions which appear healthy when harvested in the field go onto develop disease (Cramer, 2000). As with FOL, Fusarium disease in onion is very difficult to control, and although there are fungicide treatments available as a seed treatment (Cramer, 2000), they do not provide long lasting efficacy resulting in bulbs developing disease later in the season. Previous work at Warwick Crop Centre has developed a reliable diagnostic tool for identifying FOC in soil (Clarkson, 2018).

Fusarium disease of daffodil

Fusarium basal rot of *Narcissus* is caused by *F. oxysporum* f. sp. *narcissi* (FON). It infects the roots or damaged basal plates resulting in soft and rotting bulbs, which leads to bulbs not sprouting or producing short lived or early senescing foliage with few or no flowers (Taylor et al., 2019a). It is a problem for UK growers as bulbs are lifted after 2 years and used as replanting stock, therefore maintaining inoculum levels or allowing it to spread (Hanks, 2013; Taylor et al., 2019a).

Identifying Fusarium species

Molecular techniques for identification of Fusarium species are now common practice, using sequencing and phylogeny of housekeeping genes. In fungi, the internal transcribed spacer (*ITS*) is universally used for identification, but it has been shown to be inadequate for distinguishing different *Fusarium* species due to non-orthologous sequence types found within *Fusarium* species and within isolates (O'Donnell and Cigelnik, 1997; Summerell and Leslie, 2011). However, the *translation elongation factor 1*α (*TEF*) gene, which encodes an essential part of the protein translation machinery, resolves different *Fusarium* spp. and is therefore the marker of choice for identification. The gene has been shown to be consistently single copy in *Fusarium* and shows a high level of sequence polymorphism between closely related species (Geiser et al., 2004).

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The presence and absence of effector genes known as *Secreted In Xylem* (*SIX*) genes have also been useful for identifying *F. oxysporum* f. spp. and even specific races, due to their sequence differences (Lievens et al., 2009; Fraser-Smith et al., 2014; van Dam et al., 2016). Molecular tests have previously been developed at Warwick Crop Centre to help distinguish between f. spp. affecting lettuce, onion and daffodils (Clarkson, 2018).

Control of *F. oxysporum*

Soil disinfection such as steaming/heat treatments and chemical fumigation can be used in protected cropping systems like glasshouses/polytunnels to help lower the inoculum levels of pathogens (Gullino et al., 2003). Steam sterilisation can be expensive as it's an energy intensive process, however, it has proven an effective method of controlling Fusarium wilt of tomato (Luvisi et al., 2008a). However, an advantage of steam sterilising is that cropping can occur soon after treatment, unlike with chemical fumigation. Microbial communities in soil play a significant role in disease suppression, and therefore these approaches have been reported to enable pathogens to establish quickly in the absence of a biologically rich environment (O'Neill et al., 2005). A combination of soil sterilisation followed by the application of organic amendments or biological control agents could provide a solution to lower disease pressure and prevent *F. oxysporum* re-establishing rapidly.

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2. Materials and methods

2.1 *Fusarium oxysporum* f. sp. *lactucae* affecting lettuce

2.1.1 Identification of *F. oxysporum* f. sp. *lactucae* from infected lettuce from UK growers

Lettuce samples showing symptoms of *Fusarium* wilt were obtained from lettuce growers (Table 1). Three small samples of internal vascular tissue with browning symptoms were taken from each lettuce plant using a sterile scalpel and forceps and surface sterilised in 70% ethanol for 30 s after which they were washed twice in sterile water. Tissue pieces were then placed on PDA plates with the addition of chlortetracycline (2 mL L^{-1}) and incubated at room temperature for 3-4 days. Fungal colonies emerging with *Fusarium* type morphology were sub-cultured onto clean PDA plates. To confirm whether these colonies were FOL4, DNA extraction was carried out using a crude boil prep method by taking a loop of mycelium from the growing edge of each colony and adding 200 µL alkaline lysis buffer (25 mM NaOH / 0.2 mM EDTA-Na_2; pH 12). Samples were then heated for 30 mins at 100˚C and chilled on ice for 5 mins. Finally, 25 µL of neutralisation buffer (40 mM Tris-HCl; pH5) was added and then 2 µL of each DNA sample used to carry out conventional PCR in 20 µL reactions. PCR was conducted on the DNA samples using *TEF* (Taylor et al., 2016) and FOL4 (g23490 F/R) specific primers from AHDB project FV PE 458 (Clarkson et al., 2019). Thermocycling conditions were: 1 cycle of 94°C for 2 mins, followed by 35 cycles of 94°C for 45s, 64˚C (*TEF*)/60°C (FOL4) for 30s and 72°C for 1 min (*TEF*)/30s (FOL4); then 1 cycle of 72°C for 5 mins.

In addition to lettuce samples, soil from around the plants was also obtained and also subjected to testing for FOL4, for sites 1-3 only. Soil was sieved (4 mm mesh) and air dried before being sieved again (2mm mesh). 500 mg soil samples were used for DNA extraction using the GeneAll Exgene Soil SV kit (Cambio, Cambridge, UK) following the manufacturers protocol, with the following modifications: 1) 550 µL of SL buffer and 200µl of SDW was added to 500 mg of soil, which was homogenised in a Powerbead tube using a FastPrep-24 (MP Biomedicals, Cambridge, UK) machine set at 5.5 m s^{-1} for three cycles of 25 seconds; 2) spin columns were incubated for 5 min after the addition of buffer EB before elution. Following extraction, DNA yield and quality was determined using a Denovix DS-11 FX spectrophotometer (Wilmington, USA), before being diluted 1:6 with TE Buffer and stored at 4°C. DNA from each sample was then used for qPCR analysis to determine the quantity of FOL in the soil using the FOL4 specific primers as above (g23490 F3/R). qPCR was carried out using a QuantStudio 5 (384-well) machine (Applied Biosystems) using 20 µL reactions

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containing both primers (final concentration 0.4 µM), 10 µl Power SYBR™ Green PCR Master Mix (Applied Biosystems) and 1 µl of DNA. Thermocycling conditions were as follows: 1 cycle of 95°C for 120s followed by 45 cycles of 95°C for 3 s, and 60°C for 30 s. All samples were run in triplicate and a melt curve analysis carried out. The concentration of DNA was calculated as pg mg^{-1} of dry soil.

Table 1 Lettuce samples with *Fusarium* wilt symptoms and associated soil samples from UK growers

Site	Sample name	Additional information
Site 1	Lettuce and soil TL-1	Sample taken Top Left of polytunnel
	Lettuce and soil BL-1	Sample taken Bottom Left of polytunnel
	Lettuce and soil TR-1	Sample taken Top Right of polytunnel
	Lettuce and soil BR-1	Sample taken Bottom Right of polytunnel
Site 2	Lettuce 1	Cultivars Almay and Amica
	Lettuce 2	Cultivars Almay and Amica
Site 3	Lettuce 1	Cultivar: Almay
	Lettuce 2	Cultivar: Almay
Site 4	Lettuce 1 - 6	Coventry

2.1.2 Development of methods to evaluate build-up of FOL inoculum in sterilised/ non-sterilised soil after consecutive lettuce crops

This preliminary work aimed to develop a system to examine the build-up of FOL inoculum in sterile and non-sterile soil in order to allow testing of treatments to mitigate this in the future.

A system was developed under controlled conditions to investigate FOL inoculum build up in sterilised and non-sterilised soil. Soil from a commercial site was sieved (4 mm mesh) and air dried for 2 days. The moisture content of the soil was determined, and water was added so the soil held together when compressed by hand (around 12% moisture content). Soil was sterilised in a Camplex Plantcare Soil Steriliser (Cat no, HD5116, Thermoforce Ltd, Essex, UK) following the manufacturer's operational instructions. The soil is heated to 71˚C (in approx. 1.25 hours), then is switched off and remains sealed until the temperature reaches 82˚C (approx. 1.5 hours). Soil was mixed 4:1 with medium grade vermiculite for both the sterilised and non-sterilised soil. FOL4 inoculum was prepared (Taylor et al., 2013) and used

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to produce a serial dilution in soil in order to inoculate sterilised/non-sterilised soil at the final concentrations of 2 x 10^2 and 2 x 10^3 cfu g^{-1}. These concentrations of the pathogen were shown previously to cause little or no disease. Non-inoculated soil was also included for both sterilised and non-sterilised treatments. Soil was used to fill 9 cm pots and 2-3 week old lettuce plants (cv. Temira) transplanted into each pot (12 pots per treatment). Pots were arranged in a randomised block design in a controlled environment cabinet set at 25˚C with 16 hr light.

After around 10 weeks, the lettuce were removed and assessed for FOL4 vascular browning symptoms (Table 2). The heads of the lettuce were then discarded, and the remaining roots chopped and broken up in the soil. Additional soil was sterilised (as above), mixed 4:1 with vermiculite and used to dilute (1:1) the remaining soil from the first experiment. Non-sterilised soil was also added to the previously non-sterilised soil in the same 1:1 ratio, and all treatments used to fill 11 cm pots, to take into account the extra root material in the soil. Again, 2-3 week old lettuce (cv. Temira) plants were transplanted into each pot (12 pots per treatment, apart from the sterilised control which had 11). After around 8 weeks, the lettuce were harvested as before, and the soil used for a third round of lettuce inoculations.

Table 2 Scoring scales for lettuce *Fusarium* wilt and internal vascular browning

Score	Disease/ wilt scale
0	Healthy
1	1 – 2 leaves wilted
2	10% wilt (3 leaves or more)
3	10 – 25% wilt
4	25 – 50% wilt
5	50 – 75% wilt
6	75 – 99% wilt
7	100% wilt
8	Dead

Score	Vascular browning scale
0	No browning
1	Mild vascular browning
2	Vascular browning
3	Severe vascular browning (SVB)
4	Dead

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2.1.3 Testing resistant cultivars of lettuce against FOL4

A polytunnel infested with FOL4 (isolate AJ516) was used to assess the resistance of 16 lettuce cultivars. Seeds of each line were raised in M2 compost and maintained in a glasshouse for two – three weeks before being transplanted into the previously inoculated polytunnel. They were arranged in four blocks with 4 plots per cultivar, each containing 8 plants. Lettuces were assessed for wilt symptoms twice per week (Table 2) and after 6 weeks they were harvested, cut open and scored for vascular browning (Table 2).

2.1.4 Sap collection

Lettuce plants inoculated with FOL4 (isolate AJ516) were used for xylem sap collection to test whether FOL spores are present in the sap and can be extracted.

Seeds of five lettuce cultivars (one susceptible, two partially resistant and 2 resistant) were sown into M2 compost and grown for 2 – 3 weeks. FOL4 inoculum was prepared as before and mixed with M2 at a concentration of 1 x 10^6 cfu g^{-1} and used to fill 9 cm pots, into which the lettuce seedlings were transplanted (one pot per plant). Uninoculated controls (M2 compost only) were also set up. Pots were arranged in a controlled environment room at 22˚C with 16 h of light. After 5 weeks plants were transferred to water filled propagator trays and covered completely in a plastic bag which was tied so it was inflated with air. They were left overnight to allow the humidity to build up. Lettuce stems were cut horizontally just above the cotyledons then the remaining stem washed with sterile water and blotted until the latex stopped exuding. A 2 mL tube containing sterile absorbent cotton wool and a sterile piece of straw was turned upside down onto the cut stem so the cotton wool was in contact with the stem (Kan et al., 2019). A larger 7 mL tube was placed over the top to keep the 2 mL tube in contact with the stem. Plants were covered with propagator lids (vents sealed) and left overnight so the sap could exude and be absorbed by the cotton wool. Tubes were centrifuged at 16,000 x g for 10 mins to release the sap from the wool. Some were also squeezed with sterile tweezers to remove remaining sap. Up to 100 µL of sap was plated onto PDA + chlortetracycline (2 mL L^{-1}) plates and incubated at room temperature to check for the presence/absence of FOL colonies. The remaining root sections were also harvested and three small samples of internal vascular tissue were taken using a sterile scalpel and forceps. They were surface sterilised in 70% ethanol for 45 s, after which they were washed twice in sterile water. Tissue pieces were then placed on PDA plates + chlortetracycline (2 mL L^{-1}) and incubated at room temperature for up to 1 week to check for the presence of FOL.

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A selection of colonies from sap and root sections were subbed onto PDA and used for DNA extraction and PCR as described in section 2.1.1, to check that it was positive for FOL4.

2.1.5 Mini review of biological control agents to supress FOL

A literature search was conducted to determine whether biological control agents and soil amendments could be used to reduce the build-up of FOL in soil. Initially the search focused on treatments which had previously been used to supress FOL in lettuce, but in instances where this had not been tested, other Fusarium species and crops were examined. It was hoped that this would inform us of possible treatments to use to prevent the build-up of FOL in sterilised and non-sterilised soils after successive rounds of lettuce cropping (section 2.1.2).

2.2 *Fusarium oxysporum* f. sp. *narcissi* (FON) affecting daffodils

2.2.1 Identification of FON from soil samples from infected UK fields

Soil samples were obtained from Narcissus fields in the UK where basal rot had been identified in bulbs or subsequently in store (**Table 3**), air dried and used for DNA extractions using the GeneAll Exgene Soil SV kit (Cambio, Cambridge, UK) as above. DNA from each sample was then used for qPCR analysis to determine the quantity of FON in the soil using primers g16122 F/R2 (Clarkson, 2018). qPCR was carried out using a QuantStudio 5 (384-well) machine (Applied Biosystems) using 20 µL reactions containing both primers (final concentration 0.5 µM), 10 µl Power SYBR™ Green PCR Master Mix (Applied Biosystems) and 1 µl of DNA. Conditions were as follows: 1 cycle of 95°C for 120s followed by 45 cycles of 95°C for 3 s, and 60°C for 30 s. All samples were run in triplicate and a melt curve analysis carried out. The concentration of FON in each sample was calculated as pg DNA mg^{-1} of dry soil.

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Table 3 Locations of soil samples from Narcissus fields with symptoms of basal rot

Location	Field	Sample	Year
Cornwall	12	1	2020
	12	2	2020
	12	3	2020
	12	4	2020
	12	5	2020
	12	6	2020
	12	7	2020
Cornwall	BV1		2021
	KA4		2021
	PK12		2021
Cornwall	PK12	N1	2018
		N2	2018
		N3	2018
		N4	2018
		N5	2018
		N6	2018
		N7	2018

2.3 *F. oxysporum* f. sp. *cepae* affecting onions

2.3.1 Identification of *Fusarium* isolates from infected onions

Fusarium Isolates from infected onions were sent from commercial companies in Spain and Sweden for identification by *TEF* gene sequencing and *SIX* gene characterisation.

Fusarium isolates from onions sent from Sweden were sub-cultured onto PDA or PDA + chlortetracycline (if bacterial contamination was present in the original culture) and grown at room temperature for around 5 days. Isolates were then grown in liquid culture by removing three 5 mm agar plugs from the growing edge of each actively growing culture, which were used to inoculate 20 mL of 50% potato dextrose broth (PDB) in a sterile Petri dish and subsequently incubated at 20˚C for 5 days. PDB was then removed by centrifugation (3000 rpm for 15 minutes) and the mycelium from each isolate was rinsed twice with sterile water (centrifugation at 3000 rpm for 15 min each time). The mycelium was freeze-dried for 3 days after which DNA extraction was performed using a DNeasy plant mini kit (Qiagen) in accordance with manufacturer's protocol with a minor modification whereby the sample was first homogenised in a lysing matrix A tube (MP Biomedicals) in a FastPrep-24™ machine set at 6 ms^{-1} for 40 s.

Fusarium isolates from Spain were sub-cultured onto PDA and grown for 7-10 days at room temperature. DNA was extracted using a rapid method (Acme) developed by Stephen Rehner

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(USDA-ARS, Beltsville, USA). Fresh mycelium for each culture was transferred into 2 mL tubes containing 10 glass beads and an Eppendorf tube cap of silica beads (0.1mm, BioSpec Products) and ground three times in a FastPrep-24™ machine set at 5.5 ms^{-1} for 20 s. Acme DNA extraction reagent (300 µL of sodium metasilicate 2.1 g, citric acid 0.5 g, 2-butoxy ethanol 2.64 mL, 1M Tris-HCl pH 7.0 13.6 mL) was added, tubes heated for 10 minutes at 100˚C, and centrifuged at 16,000 g for 5 minutes, rotated 180˚ and centrifuged again for 5 minutes. The supernatant (175 µL) was transferred to a clean tube and diluted 1:10 in TE buffer for use in PCR

Molecular identification of all the *Fusarium* DNA samples was carried out by PCR amplification of part of the *TEF* gene using published primers (Taylor *et al*., 2016). A 20 µL PCR reaction was set up, which contained 10 µL of REDTaq® ReadyMix® (Sigma-Aldrich), 1 µL of DNA, 1 µL of each primer (10 µM) and 7 µL sterile water. Thermocycling conditions were: one cycle of 2 min at 94˚C; 35 cycles of 45 s at 94˚C, 30 s at 64˚C and 1.5 mins at 72˚C, followed by one cycle of 5 min at 72˚C.

For the Spanish isolates, additional PCR reactions were also set up (as above) with published primers for the 14 *SIX* genes (Taylor et al., 2016). The isolates from Sweden were screened for the presence of *SIX*5 only (Q*SIX*5, Taylor et al., 2016), as this has been shown previously to be present in pathogenic *F. oxysporum* f. sp. *cepae* isolates causing basal rot of onion (Taylor *et al*., 2016).

All PCR amplicons (*TEF* and *SIX* genes) were visualised using gel electrophoresis (1.2% agarose gel containing GelRed™ at 2 µL per 100 mL of gel) and *TEF* products purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the forward primer sequence. Sequences were subjected to BLAST searches (Basic Local Alignment Search Tool) using the National Centre of Biotechnology Information (NCBI) database to identify species.

2.4 Fusarium isolations from Parsley

Parsley plants showing symptoms of disease were obtained for testing. Roots were washed well in water to remove any soil debris, and sections of root excised. Sections were taken from the upper roots (and further dissected lengthways) and from lower roots where they had brown discolouration. Sections were surface sterilised in 70% ethanol for 45 s after which they were washed twice in sterile water, before being placed on PDA plates with the addition of chlortetracycline (2 mL L^{-1}) and incubated at room temperature for up to 7 days. Fungal

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colonies emerging with *Fusarium* type morphology were sub-cultured onto clean PDA plates. DNA extraction and PCR were performed as in section 2.1.1 using *TEF* PCR primers, and then sequenced as described by Taylor et al. (2016) Sequences were used to conduct BLAST searches (Boratyn et al., 2013) to determine isolate identity.

2.5 Molecular developments

2.5.1 Testing methods to improve DNA quality

To improve the sensitivity of a qPCR assay to detect FOL DNA in soil, DNA clean-up was conducted on half of the samples and compared to those which hadn't been purified. DNA was extracted using the GeneAll Exgene Soil SV kit (Cambio, Cambridge, UK) as above. The extracted DNA was divided into two samples, one of which was diluted 1 in 2 and the other half was cleaned using Mag-Bind® TotalPure NGS beads (Omega BioTek, US). 1.2x the volume of beads was added to the DNA, which was mixed on a rotary mixer for 5 mins at room temperature. After a brief centrifugation, samples were placed in the magnetic rack (DynaMag™-2 Magnet, ThermoFisher, UK). The supernatant was removed and samples washed twice with 70% ethanol, then allowed to dry for 30s – 1 min in a flow cabinet to remove remaining traces of ethanol. DNA was resuspended in 50 µL TE and left at room temperature for 10 mins. They were then placed back in the magnetic rack for 5 mins and the DNA recovered in the supernatant. This was then diluted 1 in 2 as with the untreated samples.
DNA was then used to perform qPCR as described in section 2.1.1.

2.5.2 Construction of a *TEF* sequence database of *Fusarium* sequences for the collection of isolates at Warwick Crop Centre

TEF sequences of *Fusarium* isolates for multiple crop hosts have been obtained over several years from different locations in the UK and abroad through AHDB and BBSRC projects. An accessible database was created to consolidate all the data associated with these isolates and allow quick access to information including crop host, identification, sequence ID and the type of DNA preparation to cross reference with their unique storage code.

TEF sequences (from previous work) of *Fusarium* isolates were collected and entered into an excel database. Over 430 *TEF* sequences were consolidated into one database, with the corresponding *Fusarium* ID, information of the lab book record, sample name and origin (if

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known), the storage code, the sequence file code, primer information (forward of reverse) and the type of DNA preparation used for extraction. Additional isolates will be added over time as they are collected.

A small selection of the *TEF* sequences in the database were used to create a phylogenetic tree to test whether the database functioned and whether the sequences could be easily found. Sequences from 14 isolates of different *F. oxysporum* f. spp. (including two *F. proliferatum* sequences to root the tree) were aligned and trimmed in MEGAX: Molecular Evolutionary Genetics Analysis version 10 (Kumar et al., 2018) and used to construct a maximum-likelihood tree using a Kimura 2-parameter model (Kimura, 1980). Bootstrap values were inferred from 1000 replicates (Felsenstein, 1985) and percentages displayed next to the relevant branch.

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3. Results

3.1 *Fusarium oxysporum* f. sp. *lactucae* affecting lettuce

3.1.1 Identification of F. oxysporum f. sp. lactucae from infected lettuce from UK growers

All lettuce samples were positive for FOL4 following PCR with specific primers confirming the presence of the pathogen. All soil samples also tested positive for FOL4 and results presented here are expressed as pg FOL4 DNA mg^{-1} dry soil. From the four samples sent from different areas of the polytunnel from Preston Site 1, the sample from the bottom right of the polytunnel had the highest levels of FOL4 with 0.93 pg DNA mg^{-1} soil, with the top right being the second highest (Figure 1). The bottom left sample had the lowest levels of FOL4 with 0.15 pg DNA mg^{-1} soil (Figure 1). Samples from Preston Site 2 and Site 3 had relatively low levels of FOL4 in the soil compared to Preston Site 1, with 0.25 and 0.42 pg DNA mg^{-1} in soil respectively (Figure 1).

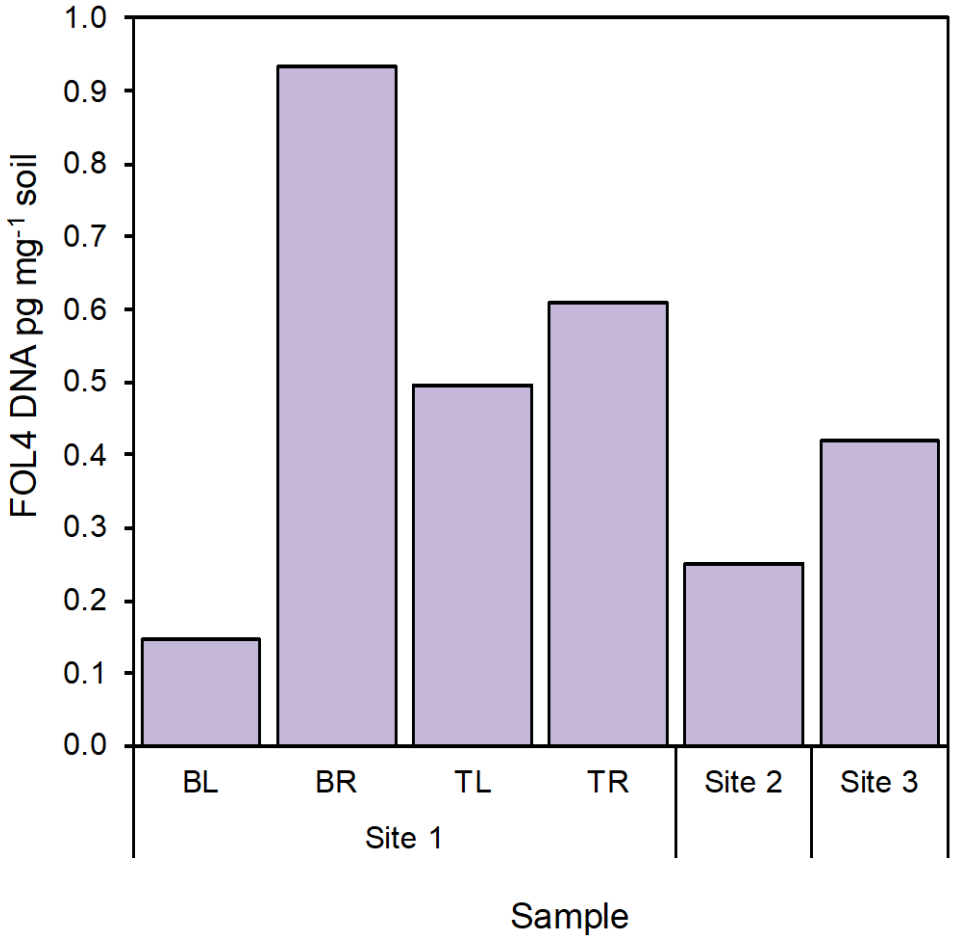

Figure 1 Levels (pg) of FOL4 DNA mg^{-1} soil as determined using qPCR for six soil samples from UK lettuce growers.

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3.1.2 Development of methods to evaluate build-up of FOL inoculum in sterilised/ non-sterilised soil after consecutive lettuce crops

In the first lettuce planting as expected, there were no visible *Fusarium* wilt symptoms in the lettuce but early signs of vascular browning (score >1) were present at harvest, especially in the sterilised soil at 2 x 10^3 cfu g^{-1} (Figure 2).

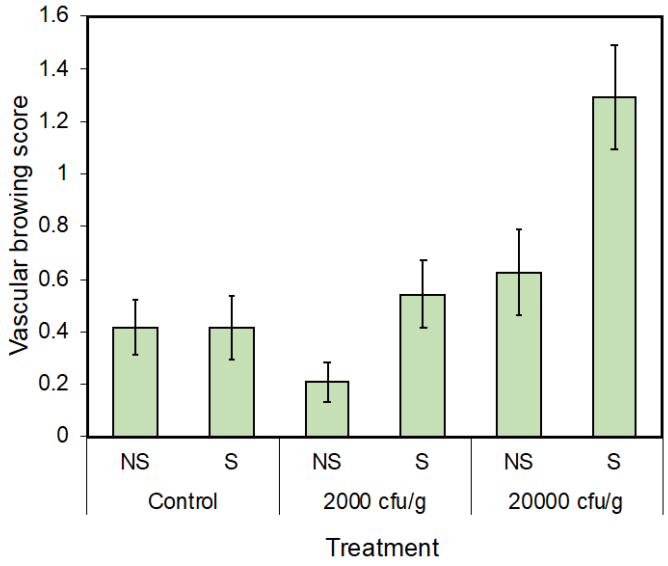

Figure 2. Mean Fusarium wilt vascular browning score for lettuce grown in FOL4 inoculated soil (2 x10^2 and 2 x 10^3 cfu g^{-1}) which was either non-sterilised (NS) or steam sterilised (S) before inoculation.

In the second lettuce crop, visible wilt symptoms were present early on, and resulted in high Fusarium wilt scores after eight weeks suggesting that the level of inoculum in the soil had increased for both initial FOL4 concentrations with a successive planting (Figure 3 A). This was particularly evident for the sterilised soil which resulted in considerably more wilt than in the non-sterilised soils at the same concentration, especially for 2 x 10^2 cfu g^{-1} (Figure 3 A). There was only a small difference in the wilt score in lettuce grown in the 2 x 10^3 cfu g^{-1} between the sterilised and non-sterilised soils. It was also noted that the lettuce grown in sterilised soil without FOL inoculum in the second lettuce planting were consistently larger than those in the unsterilised soil (Figure 3 D and E). However, this could be due some mild wilt symptoms observed in some of the uninoculated control lettuces in the non-sterilised soil by the end of the experiment.

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The third lettuce crop resulted in similar results to the second crop, and may have even resulted in less severe wilt (Figure 3 A). There was more wilt in the 2 x 10^2 cfu g^{-1} treatment in the non-sterilised soils, but less wilt than crop two for the 2 x 10^3 cfu g^{-1} treatment.

Unfortunately, there were unexpectedly high levels of Fusarium wilt disease in lettuce from the non-sterilised uninoculated control treatment, possibly due to contamination when setting up/harvesting the experiment.

When vascular browning symptoms were assessed in the second lettuce planting, there was a greater severity of disease in lettuce grown in the 2 x10^2 cfu g^{-1} inoculated sterilised soil compared with the non-sterilised soil (Figure 3 B), suggesting that FOL4 inoculum levels built up more quickly in the sterilised soil. Unexpectedly, vascular browning decreased in the 2 x10^2 cfu g^{-1} sterilised and 2 x 10^3 cfu g^{-1} non-sterilised treatments between crops 2 and 3. However, there were high browning scores observed in the non-sterilised (2 x10^2) and sterilised (2 x 10^3) treatments in crop 3 compared to crop 2 (Figure 3 B)

Plant weight was generally greater in crop 3 than crop 2 (Figure 3 C), but lower in the inoculated sterilised soil compared to the non-sterilised soils in both crops. The lettuces were generally bigger in the sterilised soils (non-inoculated) compared to the non-sterilised (Figure 3 C, D and E).

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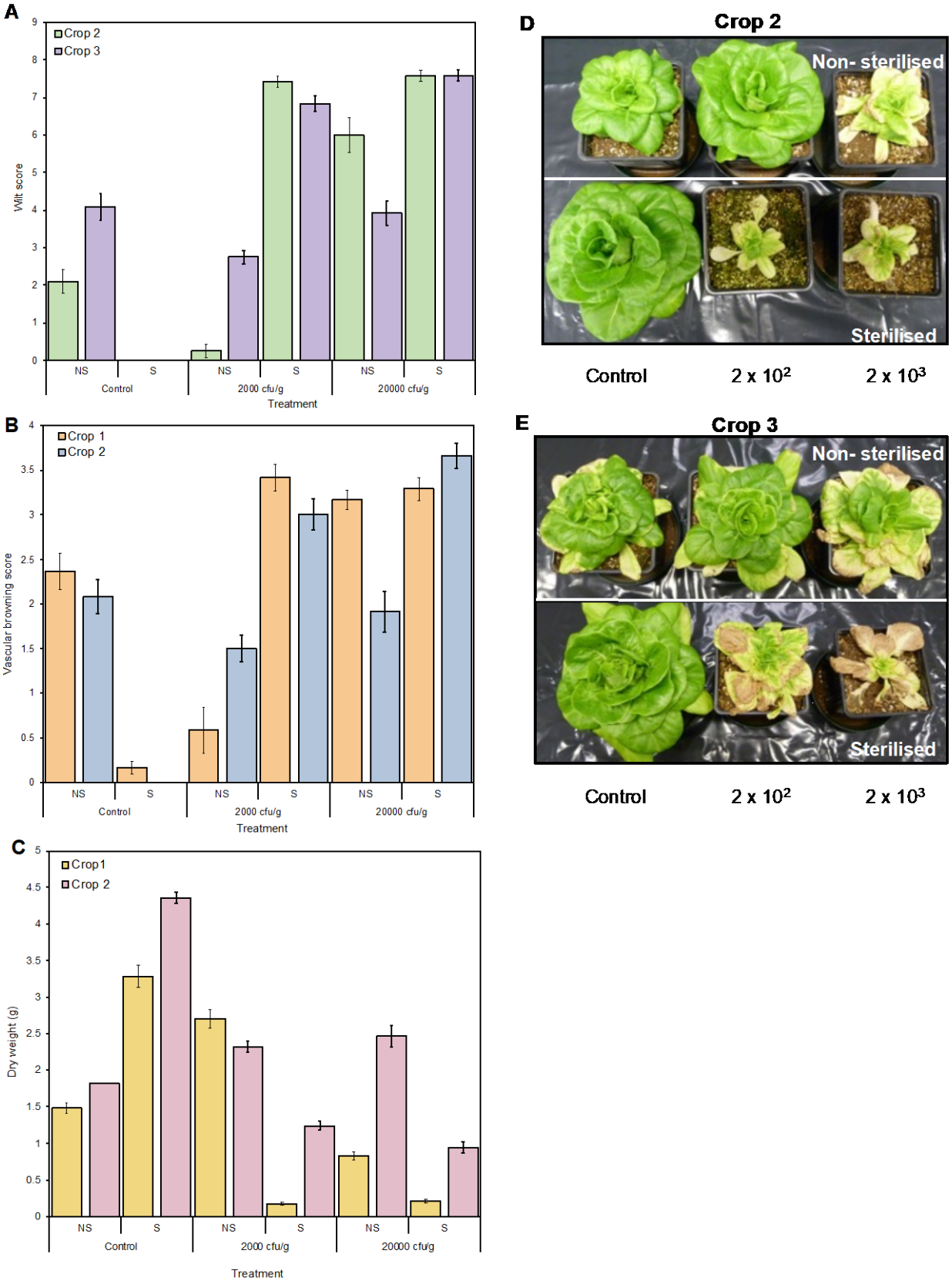

Figure 3 A) Mean Fusarium wilt score, B) Vascular browning score and C) lettuce dry weights for successive crops of lettuce grown in FOL4 inoculated soil at initial concentrations of 2 x10^2 and 2 x 10^3 cfu g^{-1}, which was either non-sterilised (NS) or had been steam sterilised (S) before inoculation. Images show lettuce size from different treatments, and the wilt symptoms observed in D) crop 2 and E) crop 3.

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3.1.3 Testing resistant cultivars of lettuce against FOL4

Lettuce cultivars were tested for resistance to FOL4 in an inoculated polytunnel. 15 lettuce cultivars were included, along with a known susceptible cultivar (cv. Amica, cultivar 16). Cultivars 7 and 13 were susceptible to FOL4, with wilt symptoms appearing rapidly and resulting in death of most plants. Cultivars 1 and 2 appeared to have partial resistance with wilt scores of 4 and 3 respectively (Figure 4 A), however, both cultivars had significant vascular browning at harvest with similar scores to the most susceptible cultivars (Figure 4 B). Most cultivars displayed high levels of resistance, with wilt scores below 2 (Figure 4 A) and vascular browning scores below 1.5 (Figure 4 B).

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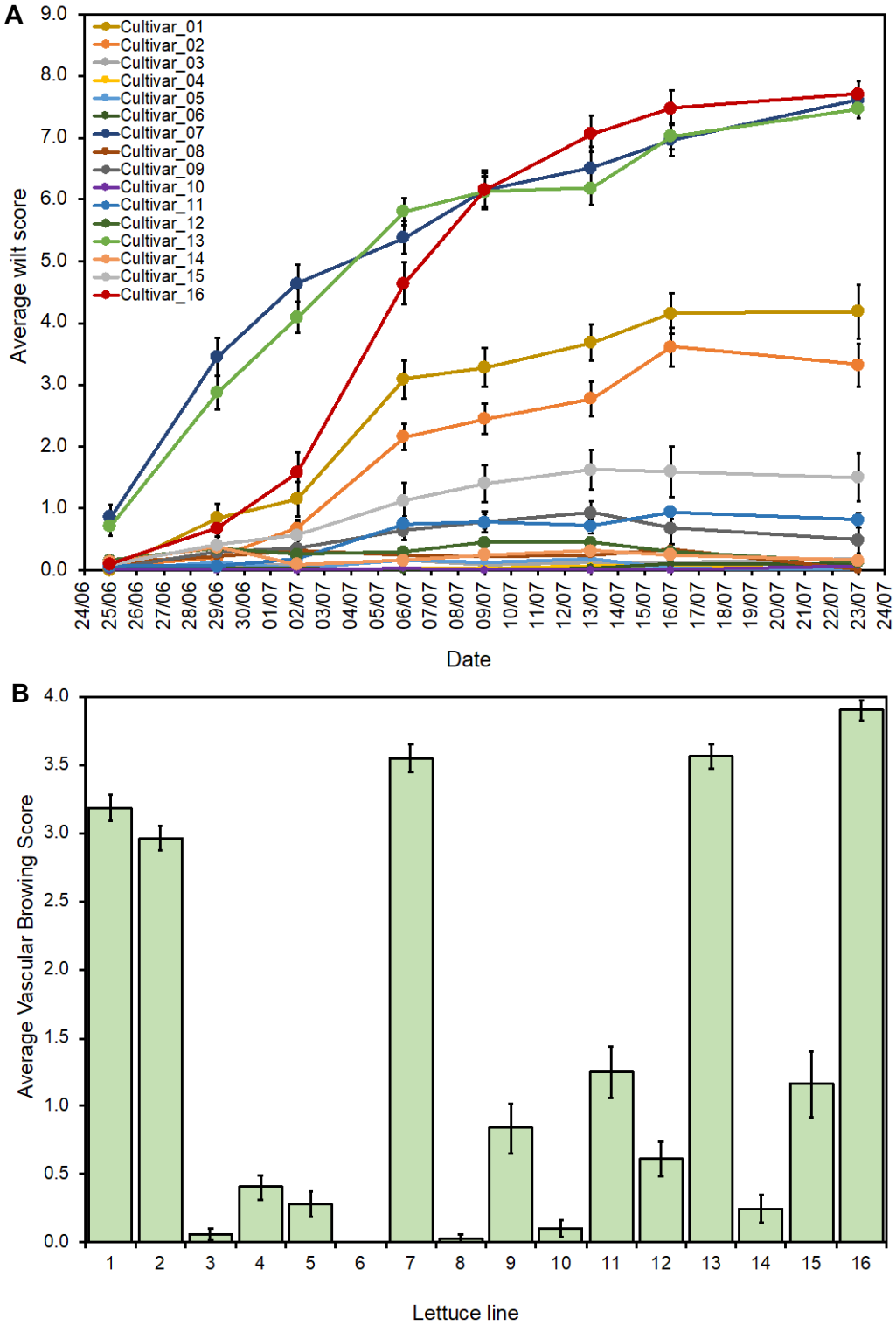

Figure 4 A) Average wilt score and B) average vascular browning of lettuce cultivars inoculated with FOL4

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3.1.4 Sap collection

The sap of lettuce plants was collected from five cultivars with different levels of resistance to FOL4. The purpose of this preliminary experiment was to determine if spores of FOL could be isolated from the sap of resistant and susceptible cultivars. It was hypothesised that FOL might not reach the vascular tissue of the plant even if it can colonise the roots. Unfortunately, some of the plants were stressed while growing and were unable to harvest for sap collection. However, the majority of plants which were harvested were used for sap collection and root isolations. Colonies of FOL4 were observed to be growing in the sap from every cultivar, however, some cultivars had higher instances of FOL in the sap e.g. resistant cultivar 1 (Table 4)

Overall, it is possible to isolate FOL from the xylem sap of resistant and susceptible lettuce cultivars. This experiment would need repeating with more replications and in more favourable conditions, so the plants did not get too stressed.

Table 4 Cultivars of inoculated lettuce where FOL4 had been isolated from xylem sap and roots tissue

Cultivar	Number plants isolated from	Number plants FOL isolated from sap	Number plants FOL isolated from roots
Resistant 1	10	9	3
Resistant 2	5	2	2
Partially resistant 1	10	1	4
Partially resistant 2	9	3	5
Susceptible	9	5	2

3.1.5 Mini review of biological control agents to supress FOL

Biological control agents for reducing FOL

Trichoderma species have been widely tested as biological controls agents for disease protection and growth enhancers in many crop types. One Trichoderma product containing *T. harzianum* strain T22 (Trianum) has been shown to have a variable but significant effect on reducing disease severity in lettuces inoculated with FOL race 1 (Gilardi et al., 2007;

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Innocenti et al., 2015). Alternative strains of *T. harzianum* (JF419706) have also been shown to be effective in reducing disease severity of *F. oxysporum* infecting lettuces by 61%, and improved seed germination in inoculated compost from 40% (FOL only) to 71% (FOL + T. harzianum) (Alamri et al., 2019). *T. harzianum* significantly increased the expression of defence genes in lettuces up to 50 days post inoculation, compared to lettuces inoculated with *F. oxysporum* alone (Alamri et al., 2019).

Other Trichoderma species have also resulted in significant decreases in disease severity (44% reduction compared to the control) when used as a pre-planting treatment for lettuces grown in a FOL infected polytunnel (Cucu et al., 2019). The authors also found that Remedier (T. asperellum and T. gamsii) resulted in an even greater reduction in disease severity of 54% (Cucu et al., 2019) as was also found by Gilardi et al. (2007); (2019b).

Another species widely studied is *Trichoderma asperellum*, with multiple strains marketed as biological control agents. *T. asperellum* strain T34 (product name: T34 Biocontrol) has shown to be effective against *F. oxysporum* f. sp*. lycopersici* affecting tomatoes (Cotxarrera et al., 2002) and *F. oxysporum* f. sp. *dianthi* affecting carnations (Sant et al., 2010). However, there are currently no studies investigating the effectiveness of this product against FOL.

In addition to Trichoderma products there are a number of other biological control formulations available which have all been shown to be effective across different crops. Prestop (*Gliocladium catenulatum*) was effective against *F. oxysporum* on pepper (Cerkauskas, 2017) and cucumber (Rose et al., 2003). There were initial reports that Prestop had a positive effect on root development and lettuce weight when applied to young plants (Vandevelde et al., 2017) and it has been shown to reduce disease severity of *Rhizoctonia solani* in field grown lettuces (Grosch et al., 2004). Serende (*Bacillus subtilis* strain QST713) has recently been shown to reduce disease caused by FOL by 60% and also resulted in a reduction of FOL abundance in the rhizosphere and bulk soil (Cucu et al., 2019). This supports findings from Gilardi et al. (2016); (2019b) where B. subtilis resulted in significant disease reductions of FOL infected lettuces.

Soil amendments and biofumigation

Poultry manure

Soil amendments which contain high levels of organic nitrogen have been shown to kill a wide range of plant pathogenic fungi. Nitrogen is converted into ammonia in the soil which is lethal

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to many pathogens (Lazarovits, 2001; Melero-Vara et al., 2011). As an example, poultry manure has been shown to reduce disease severity in FOL infected lettuces by up to 50% compared to the untreated inoculated control, however, after a second crop of lettuce this efficacy reduced to a 35% reduction in disease severity (Gilardi et al., 2016). Similar results show that poultry manure significantly reduced the levels of Fusarium wilt caused by *F. oxysporum* f. sp. *dianthi* in carnation plants under glasshouse conditions (Melero-Vara 2011).

Biofumigation is the process by which plants containing high levels of glucosinolate compounds are crushed and broken up into the soil, where they release isothiocyanates which are toxic to many plant pathogens (Meng et al., 2018). This approach has been incorporated into ready to apply products, thereby eliminating the time to grow a cover crop of a suitable species (such as mustard). One such product, Biofence (*Brassica carinata* pellets), resulted in a 56% reduction of FOL induced wilt (Gilardi et al., 2016). However, it is important to apply this product 14 days before lettuce transplanting, as when tested by applying only 7 days before transplanting there was no effect on the severity of FOL wilt (Gilardi et al., 2016). A subsequent study revealed significant reductions in disease severity (up to 80%) when applied 30 days prior to lettuce transplanting (Gilardi et al., 2018).

Soil disinfestation by steaming

Soil steaming can used to disinfect soil to reduce the burden from pathogens and weeds, and is especially useful in tunnels and greenhouses. However, it is very energy intensive making it a potentially expensive and unappealing to most growers (Panth et al., 2020). Steam sterilisation has been shown previously to reduce levels of fungi in soils more than with equivalent chemical treatments (Tanaka et al., 2003), however, it is also known to decrease microbial communities and cause an increase in nitrogen in the soil (Tanaka et al., 2003; Minuto et al., 2004). For control of soil borne pathogens, including Fusarium, this technique has been shown to be effective in controlling Fusraium wilt of tomato (Luvisi et al., 2008b) and F. oxysporum f. sp. basilici and Rhizoctonia solani disease in basil (Minuto et al., 2004). Different methods of steaming have also been shown to be effective in reducing *F. oxysporum* f. spp. *raphani*, *conglutinans* and *basilici* under laboratory settings in artifically inoculated soils (Garibaldi et al., 2014). However, it has been reported that pathogens can re-establish quickly in sterilised soil due to the lack of a diverse microbial community which are known to naturally suppress disease development (O'Neill et al., 2005).

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3.2 *Fusarium oxysporum* f. sp. *narcissi* (FON) affecting daffodils

3.2.1 Identification of FON from soil samples from infected UK fields

All soil samples form Narcissus fields tested positive for FON by qPCR except for sample 1 from the Catchall12 site (Figure 4). Soil sample 5 at Catchall12 contained 2.9 pg mg^{-1} DNA, with all other samples containing 0.7-1.5 pg mg^{-1} DNA. These samples were taken from different locations within the same field, and the variation in the concentrations of FON DNA across the field, suggests a patchy distribution of the. At the Hayle site, Field BV1, which had Narcissus plants in it, contained the highest levels of FON. KA4, which had bulbs removed in 2019, had a much lower levels of pathogen present. PK12 samples N1-N7 were taken from across a field in 2018 where harvested Narcissus bulbs had previously had severe symptoms of FON infection. Here, there was clear variation in FON levels across the field (Figure 5) while a follow up sample from 2021, suggested that levels of inoculum have since decreased.

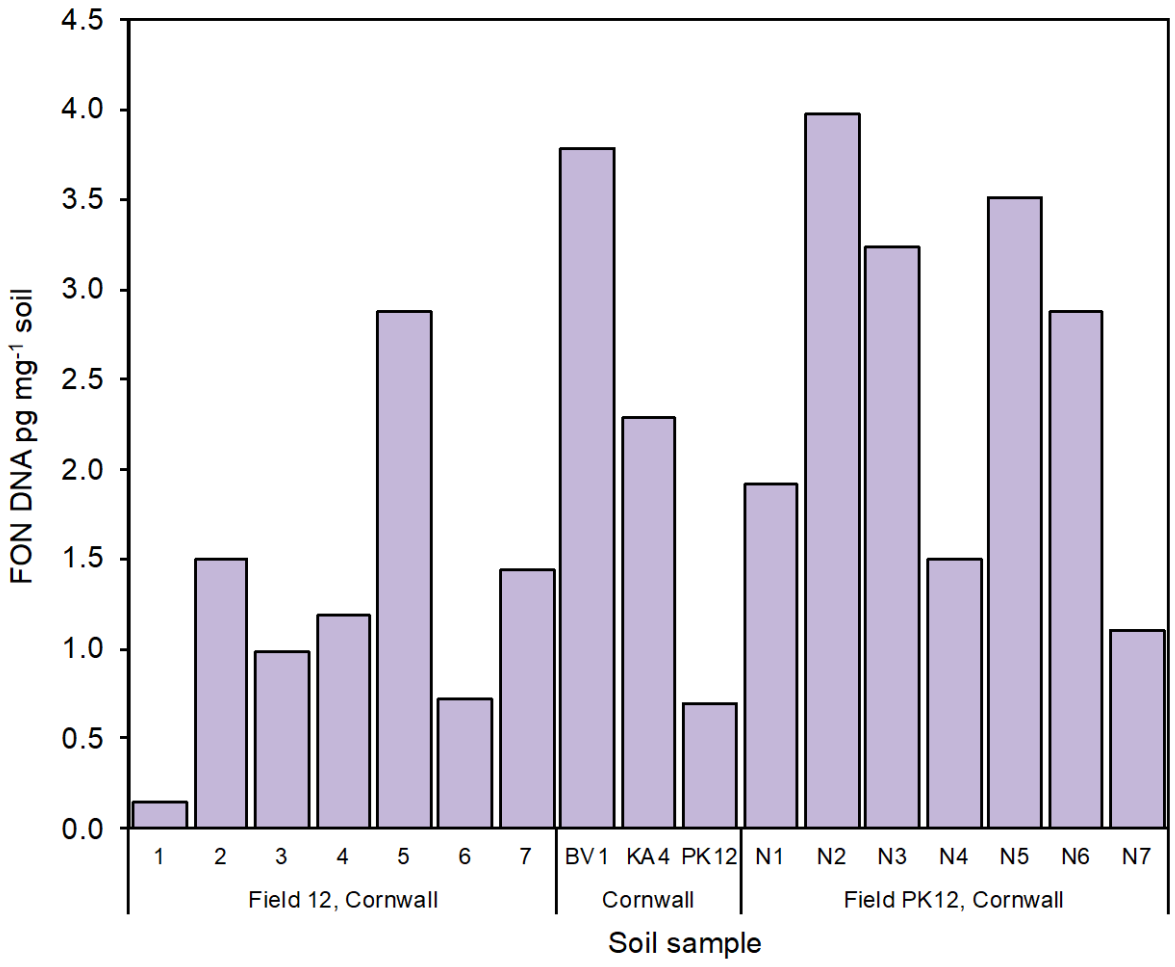

Figure 5 FON DNA levels determined using qPCR for soil samples obtained from UK Narcissus growers. ND= not detected.

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3.3 *F. oxysporum* f. sp. *cepae* affecting onions

3.3.1 Identification of *Fusarium* isolates from infected onions

Of the nine isolates sent for identification from Sweden, five were found to be *F. oxysporum*, two were identified as *F. redolens* and one for each of *F. avenaceum* and *F. culmorum* / *F. graminearum* (Table 5). Three out of the five *F. oxysporum* isolates tested positive for the presence of *SIX*5, suggesting that these are pathogenic towards onion. This was confirmed by the pathogenicity test carried out by the Swedish company.

Table 5 Identification of *Fusarium* species for isolates sent from Sweden, using *TEF* gene sequencing. Presence of *SIX5* refers to the number of isolates with a positive PCR result

Isolate ID *TEF* (Sweden)	Number	Presence of *SIX5*
F. oxysporum	5	3
F. redolens	2	0
F. culmorum / F. graminearum	1	0
F. avenaceum	1	0

All of the 46 isolates (except one) from Spain were identified as *F. oxysporum* with the additional isolate being identified as *F. proliferatum* (Table 5). All of the *F. oxysporum* isolates were found to contain the full complement of seven *SIX* genes previously identified in pathogenic onion isolates (Taylor *et al*., 2016) which were *SIX*3, 5, 7, 9, 10, 12 and 14 (Table 6).

Table 6 Identification of *Fusarium* species for isolates sent from Spain, using *TEF* gene sequencing, and the presence/absence of *SIX* genes.

		***SIX* gene presence (+) / absence (-)**						
Isolate ID *TEF* (Spain)	**Number**	**3**	**5**	**7**	**9**	**10**	**12**	**14**
F. oxysporum	45	+	+	+	+	+	+	+
F. proliferatum	1	-	-	-	-	-	-	-

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3.4 Fusarium isolations from Parsley

Root sections from 18 parsley plants potentially infected with Fusarium were taken and plated onto PDA to determine if Fusarium could be isolated. All but one plant had Fusarium like cultures growing from the root sections, which were identified by *TEF* gene sequencing. of the 21 Fusarium isolates obtained from the roots, 15 were found to be *F. oxysporum* (Table 7). The remaining isolates were *F. culmorum*, *F. flocciferum*, *F. venenatum*, *F. solani* and *F. avenaceum* (Table 7)

Table 7 Identification of Fusarium isolates from parsley roots

Plant	Isolations	Isolate ID
1	P1 (parsley 1)	*F. oxysporum*
2	No isolations	
3	P3	*F. oxysporum*
4	P4	*F. oxysporum*
5	P5	*F. oxysporum*
6	P6	*F. oxysporum*
7	P7 A (section 1)	*F. oxysporum*
	P7 B (section 2)	*F. culmorum*
8	P8	*F. oxysporum*
9	P9 A (section 1)	*F. oxysporum*
	P9 B (section 2)	*F. oxysporum*
10	P10 A (section 1)	*F. flocciferum*
	P10 B (section 2)	*F. venenatum*
11	P11	No DNA prep
12	P12	*F. oxysporum*
13	P13	*F. flocciferum*
14	P14	*F. oxysporum*
15	P15	*F. oxysporum*
16	P16	*F. oxysporum*
17	P17 A (section 1)	*F. oxysporum*
	P17 B (section 2)	*F. solani*
	P17 C (section 3)	*F. avenaceum*
18	P18	*F. oxysporum*

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3.5 Molecular developments

3.5.1 Testing methods to improve DNA quality

The effectiveness of magnetic beads was tested to determine whether it could improve the sensitivity of a qPCR assay.

The quality of the DNA did not improve the sensitivity of the assay as it did not improve the Ct value of each sample or the amount of DNA detected (Table 8). As this was only a small test on a small number of samples, it could still be a useful technique when trying to determine the quantity of DNA in samples whereby it is close to the limit of detection by the assay. Additional samples will be tested, including more soil samples, root samples and tissue samples from other plant types.

Table 8 Cycle number (Ct) and amount of FOL DNA detected in samples treated with magnetic bead clean up, compared to non-treated samples.

	Average Ct		DNA quantity (ng)	
Sample Name	**Extracted DNA**	**Magbind cleaned DNA**	**Extracted DNA**	**Magbind cleaned DNA**
D1_A				
D1_B				
D2_A	34.5		0.0001	
D2_B				
D3_A	34.0	33.4	0.0001	0.0002
D3_B				
D4_A	31.9	32.4	0.0007	0.0004
D4_B	31.3	31.4	0.0009	0.0009
D5_A	27.3	27.4	0.0136	0.0122
D5_B	27.2	26.8	0.0148	0.0188
D6_A	23.0	23.1	0.2518	0.2236
D6_B	22.7	23.2	0.4495	0.2204

3.5.2 Construction of a *TEF* sequence database of *Fusarium* sequences for the collection of isolates at Warwick Crop Centre

The database was used to easily construct a phylogenetic tree of horticulturally important *F. oxysporum* f. spp. including, f. sp. *lycopersici* (tomato), *narcissi* (daffodil), *cepae* (onion), *lactucae* (lettuce), *mathioli* (stocks) and *cubense* (banana). Two isolates per f. sp. were included which consistently grouped together (Figure 6), with the non-pathogenic isolate FO47 being separate from the other clades.

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This database will be used in future work to quickly find sequences and isolate information and will benefit many people in the group.

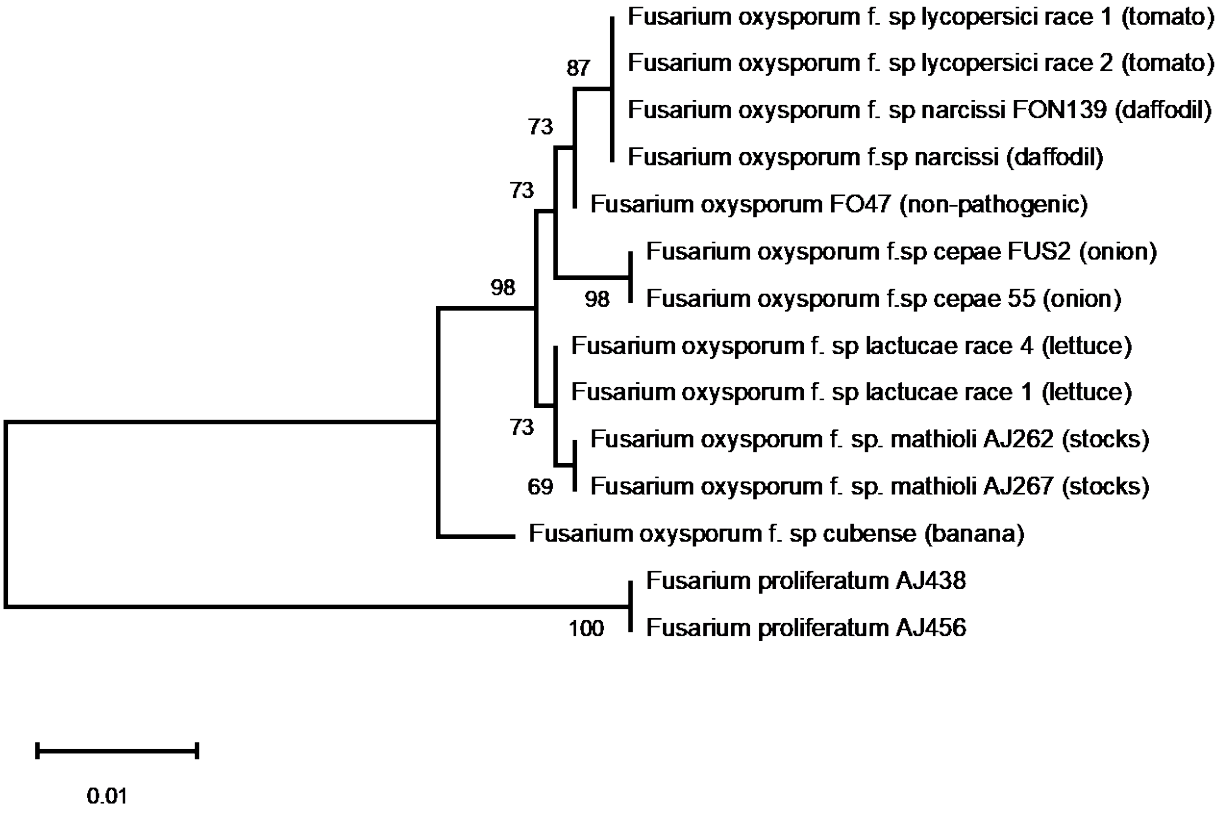

Figure 6 Maximum likelihood phylogenetic tree of horticulturally important *F. oxysporum* f. spp. based on an alignment of translation elongation factor 1a (*TEF*) sequences. Numbers represent bootstrap percentage values from 1000 replicates. Scale bar indicates 0.01 substitutions per site. The tree is rooted through two *F. proliferatum* isolates

4. Discussion

4.1 *Fusarium oxysporum* f. sp. *lactucae* affecting lettuce

4.1.1 Identification of *F. oxysporum* f. sp. *lactucae* from infected lettuce from UK growers

Lettuce growers in the UK have continued to send samples of lettuce that they are concerned about being infected with FOL. The method of isolation has a high success rate and mostly results in isolation of the pathogen from inside the infected plant, rather than capturing general root colonisers. A FOL specific PCR assay has been designed previous at Wellesbourne

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which allows rapid identification of FOL from other Fusarium species which might be isolated, which saves sequencing time and costs, which wouldn't be able to identify Fusarium f. spp. by race. The qPCR assay also allows detection of FOL in the soil, which has been extensively tested (Clarkson, 2018) and can indicate how extensive an infection problem is in a polytunnel or field.

4.1.2 Development of methods to evaluate build-up of FOL inoculum in sterilised/ non-sterilised soil after consecutive lettuce crops

In preliminary experiments to develop a system to examine FOL4 inoculum build up in sterilised and non-sterilised soils there were clear differences in the amount of disease observed. Even though low concentrations of FOL4 were used in the first round (2 $x10^2$ and 2 x 10^3 cfu g^{-1}) which caused no visible wilt, there was mild vascular browning in the root system. However, after the second crop there was visible wilt in plants with a dramatic difference in the sterilised and non-sterilised soil at 2 $x10^2$ cfu g^{-1} (initial dose). This suggests that FOL builds up in sterilised soil far more quickly than in non-sterilised soil due to the lack of a biologically rich microbial community (O'Neill et al., 2005). The amount of disease was higher in crop 2 than crop 3 which was unexpected, however, could have been due to the plants being smaller than transplanted for crop 2 which meant some of them died very quickly. We rectified that for the third crop but this meant the plants were able to establish better and therefore might not have succumb to disease in the way as in crop two. This will need to be standardised for future experiments.

This system is therefore an effective way of examining treatments to prevent the build-up of FOL4. The system will be further refined using different soils in Year 2 and begin to test different treatments in Year 3. The information in the mini review of biological control agents to supress FOL (section 3.1.3) will aid in the decision of which products to test.

4.1.3 Testing resistant cultivars of lettuce against FOL4

Although there are no commercial FOL4 resistant cultivars of lettuce, there are efforts to develop and screen more resistant cultivars. As part of this, 15 lettuce cultivars were screened for resistance in a polytunnel inoculated with a high concentration of FOL4 and monitored for wilt symptoms and vascular browning. Many of the cultivars tested showed high levels of resistance, with low wilt scores and low vascular browning scores. However, some cultivars which appeared to be partially resistant due to low wilt scores actually had moderate to high

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vascular browning scores. Therefore, it was determined that it is vital to conduct vascular browning inspections of lettuces when screening for resistance and not to solely rely on wilt scores. It is promising that resistant cultivars are available which could be used to improve commercial varieties to help reduce the issues faced by growers due to Fusarium wilt.

4.1.4 Sap collection

It was unclear whether resistant or partially resistant lettuce cultivars prevented FOL from entering the xylem sap, or whether it was able to penetrate to the sap and was inhibited in another way. Therefore, xylem sap was collected from potentially resistant and susceptible cultivars to determine if FOL could be recovered from the sap. It was clear that FOL was present in the xylem sap of resistant, partially resistant and susceptible cultivars, which suggests additional mechanisms of resistance are present. However, some of the plants in this experiment became stressed and started to snap at the base of the stem or became restricted which minimised the flow of sap. Therefore, insufficient xylem sap was collected to be plated out onto agar for some of the plants. As this was a preliminary experiment, it would need repeating with cultivars proven to be resistant/susceptible (possibly using lines from 3.1.3) and in conditions that did not induce too much stress. However, it has shown that FOL can be isolated from the xylem sap of lettuce as seen in other plants such as tomato (van der Does et al., 2019), common bean (Garcés-Fiallos et al., 2017) and banana (Guo et al., 2015).

4.2 *Fusarium oxysporum* f. sp. *narcissi* (FON) affecting daffodils

4.2.1 Identification of FON from soil samples from infected UK fields

FON was detected in soil samples from grower sites, which validates the success of this qPCR assay (developed previously at Warwick Crop Centre, Clarkson (2018)). It is sensitive enough to detect FON in small samples of soil taken from different areas of the field. It would also be able to quantify the levels of FON in each area of the fields and between fields, to help growers choose field sites. The results show that levels of FON varied across the field, potentially leading to patches in the field with more severe Fusarium symptoms as is known to be the case for many *F. oxysporum* f. spp.

4.3 *F. oxysporum* f. sp. *cepae* affecting onions

4.3.1 Identification of *Fusarium* isolates from infected onions

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It was found in previous work at Warwick Crop Centre that *SIX5* could be used to identify FOC from other f. spp. and therefore could be used as a molecular diagnostic tool. This has been shown for many other f. spp., for example, *SIX6* sequence differences have been shown to distinguish FOL from other f. spp. such as f. sp. *melonis*, f. sp. *vasinfectum*, f. sp. *cucumerinum* and f. sp. *niveum* (Lievens et al., 2009; van Dam et al., 2016). Lievens et al. (2009) showed that *SIX4* was only found in FOL race 1 and therefore subsequent races evolved to evade host detection through a loss of this gene. FOL race 3 also acquired mutations in *SIX3*, allowing it to evade host detection and therefore can be used to distinguish this race from races 1 and 2. (Lievens et al., 2009). Sequence differences in *SIX8* have also been used to distinguish races in *F. oxysporum* f. sp. *cubense* (wilt pathogen of banana causing Panama disease) allowing race 4 to be distinguished from races 1 and 2, as well as differences between tropical and sub-tropical race 4 isolates (Fraser-Smith et al., 2014). Molecular diagnostics is desired by growers and scientists as it dramatically reduced the time taken for pathogen identification and reduces the risk of incorrect identification.

The majority of isolates found were identified as FOC, which is the main cause of basal rot in onions (Taylor et al., 2013; Taylor et al., 2016). However, a few other generalist *Fusarium* species were identified (Taylor et al., 2016), which could be causing a secondary infection or just present on the bulb at the time of isolation.

4.4 Fusarium isolations from Parsley

Cultures of *Fusarium oxysporum*, *F. culmorum*, *F. flocciferum*, *F. venenatum*, *F. solani* and *F. avenaceum* were isolated from the roots of parsley plants obtained from growers. *F. oxysporum*, *F. culmorum* and *F. avenaceum* have been isolated previously from parsley (Nawrocki et al., 2002) and *F. oxysporum* has been shown to cause infection in different cultivars of parsley (Marthe et al., 2003). Although *F. oxysporum* was the most common species isolated from the roots, further pathogenicity tests would be required to establish if it was the disease causing pathogen.

4.5 Molecular developments

4.5.1 Testing methods to improve DNA quality

Mag-Bind Beads are used to improve the outcome of next generation sequencing, to size select DNA fragments (Wang et al., 2021) and also remove impurities to leave a pure sample of good quality of DNA. As soil samples can contain relatively low quantities of the target

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DNA, it can result, even after many PCR cycles, in quantities below the limit of detection by the assay. It was hypothesised that by cleaning up the samples to remove poor quality strands and ensuring it contained as few impurities as possible that it might increase the sensitivity of the qPCR assay. However, initial results showed that there was no difference between samples which had been cleaned compared to those straight from the kit. Only a small test was conducted, so additional work will be required to fully understand the benefit of purifying samples prior to qPCR.

4.5.2 Construction of a *TEF* sequence database of *Fusarium* sequences for the collection of isolates at Warwick Crop Centre

TEF (*translation elongation factor 1*α) has been found to be the most accurate way to identify Fusarium species due to it being single copy (O'Donnell et al., 2010), compared to *ITS* (internal transcribed spacer region) which is more commonly used for many fungi. *TEF* has been shown to be consistently single copy in *Fusarium* and shows a high level of sequence polymorphism between closely related species (Geiser et al., 2004). As there have been many *Fusarium* isolates and sequences collected over the year at Warwick Crop Centre, it was important to collate these into one easy accessible document, containing information from many lab books and BLAST searches which saves time when searching for sequences.

Conclusions

Identification of *F. oxysporum* f. sp. *lactucae* from infected lettuce from UK growers

- The FOL4 specific qPCR test was effective in detecting the pathogen in multiple plant and soil samples
- All lettuce samples with Fusarium wilt symptoms were positive for FOL4 by PCR
- FOL4 was also present at varying levels in soil samples obtained from UK lettuce growers as measured by qPCR

Development of methods to evaluate build-up of *F. oxysporum* f. sp. *lactucae* inoculum in sterilised/ non-sterilised soil after consecutive lettuce crops

- A system has been developed to examine build-up of FOL inoculum in sterile and non-sterile soil

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- This approach will allow testing of different biological and organic amendment treatments on build-up of FOL inoculum. Analysis of the microbial community will also allow us to understand how these treatments may result in an increase in the proportion of beneficial microorganisms.

Testing resistant cultivars of lettuce against FOL4

- Cultivars displaying high levels of resistance to FOL4 were identified
- Some cultivars displayed low wilt symptoms but had high levels of internal vascular browning, reinforcing the need for multiple methods of disease assessment.

Sap collection

- Colonies of *Fusarium* were isolated from the xylem sap of lettuce plants
- Fusarium colonies were grown from xylem sap from resistant and susceptible lettuce cultivars suggesting that resistance still allows the pathogen to penetrate into the xylem vessels.

Identification of FON from soil samples from infected UK fields

- The FON specific qPCR test was effective in detecting the pathogen in multiple soil samples
- The concentration of FON DNA found in soil samples taken across single fields was variable at two field sites tested.
- Levels of FON detected may be greater in fields containing Narcissus plants

Identification of *Fusarium* isolates from infected onions

- *F. oxysporum* was confirmed a being the main pathogen causing basal rot of onion for isolates from both Sweden and Spain as found in the UK
- *F. oxysporum* isolates from onion all contained *SIX5*, suggesting that they are all pathogenic

Fusarium isolations from Parsley

- The most common species of Fusarium isolated from diseased parsley plants was *F. oxysporum*.
- Further tests are needed to determine if these isolates are pathogenic

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Testing methods to improve DNA quality

- Initial results to clean up DNA did not improve the sensitivity of the qPCR assay
- Further samples will need to be tested to determine if this result is consistent

Construction of a *TEF* sequence database of *Fusarium* sequences for the collection of isolates at Warwick Crop Centre

- *Fusarium* TEF sequences were collated together in an easy to manage system, to allow information to be quickly found, without searching through lab books.
- This will benefit all members of the group and prevent work being repeated in the future.

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