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| **Project title:** | Lettuce: biology and management of Fusarium wilt caused by *Fusarium oxysporum* f.sp. *lactucae* race 4 |
| **Project number:** | FV/PE 458 |
| **Project leader:** | John Clarkson, Warwick Crop Centre, University of Warwick |
| **Report:** | Final report, April 2020 |
| **Previous report:** | N/A |
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| **Date project commenced:** | 01/10/2018 |

# DISCLAIMER

*The results and conclusions in this report are based on an investigation conducted over a two-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.*

# 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.

John Clarkson

Reader

Warwick Crop Centre, School of Life Sciences, University of Warwick

Signature     John Clarkson     Date: 18/04/2020

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# GROWER SUMMARY

## Headline

Specific molecular diagnostic tests were developed for Fusarium wilt of lettuce and critical levels of pathogen inoculum and effects of temperature on disease defined. A range of disinfectants and heat treatments were identified that killed *Fusarium* spores.

## Background

Fusarium wilt of lettuce caused by the soilborne fungal pathogen *Fusarium oxysporum* f.sp. *lactucae* (FOL) results in severe losses in production areas globally. Four races occur, with race 1 (FOL1) being the most widespread in the field and protected crops particularly in warmer parts of the world such as the USA and Italy. FOL was first reported in the UK in 2017, and identified as the recently emerged FOL race 4 (FOL4) first reported in the Netherlands in 2013. So far, FOL4 has been restricted to protected lettuce and has not yet been identified as causing disease in the field. Control of FOL4 is challenging, as like all *F. oxysporum* f.spp., it produces chlamydospores which survive for long periods of time in the soil which makes management using fungicides or biological control agents difficult. Currently there is no varietal resistance widely available for the lettuce types grown under protection although breeding companies have begun the limited release of new resistant or partially resistant cultivars. Consequently, FOL4 represents a significant threat to the UK lettuce industry. Crop hygiene is therefore very important to prevent further local and regional spread. This project was undertaken in response to an AHDB research call to investigate aspects of the biology and management of FOL4 which followed an extensive literature review to identify gaps in the knowledge base (AHDB project CP17/18-1006; Taylor & Clarkson, 2018).

The main aims of this project were to i) develop molecular tools for rapid FOL4 identification and quantification to help monitoring of further outbreaks and understand key aspects of pathogen biology such as infection, colonisation and survival, ii) understand key factors affecting FOL4 disease development including inoculum level and temperature and iii) identify hygiene (heat and disinfectant) approaches to limit pathogen spread. The specific project objectives were to:

1) Develop tools for molecular detection and quantification of FOL / FOL4
2) Determine the effect of temperature and inoculum level on FOL4 disease development and the impact of non-hosts / fallow on FOL4 survival.
3) Test hygiene measures to eliminate FOL4 inoculum

# Summary

**Develop tools for molecular detection and quantification of FOL/FOL4**

Objective 1.1 Test LAMP assay for FOL / Objective 1.2 Develop and test qPCR for FOL

Genome sequencing of isolates of FOL1 and FOL4, identification of pathogenicity genes and comparison with other *F. oxysporum* genomes was carried out by NIAB-EMR to identify target genes for diagnostic tests.  Primers gene specific to FOL (both FOL1 and FOL4) were then designed for use in both LAMP and qPCR assays while further sets of primers were designed for use in a qPCR assay to specifically detect FOL4. The new LAMP assay was highly specific to FOL, unlike a previously published test which resulted in some non-specific detection of other *F. oxysporum* f.spp. The qPCR primer pairs targeting g23490 and g19968 genes were highly specific to FOL4 and FOL (FOL1 / FOL4) respectively. Both the new LAMP and FOL4 qPCR assays also successfully detected FOL4 in artificially inoculated diseased lettuce plants as well as in soil infested with the pathogen. Both tests have also since been used to confirm presence of FOL4 in lettuce samples from growers as part of ongoing monitoring of FOL4 in the UK at Warwick. These molecular tools will be valuable not only for diagnostics and soil tests, but also for determining FOL4 dynamics and understanding key aspects of pathogen biology such as infection, colonisation and survival. The LAMP assay also detected FOL4 in diseased plant material in approx. 7 min following a crude 5 min DNA extraction allowing for very rapid disease diagnosis. The Genie II LAMP machine also has the advantage of being portable and could therefore potentially be used at grower sites to quickly identify FOL4. These tests could be made available to growers through commercial companies already specialising in molecular diagnostics such as FERA Science Ltd and Eurofins.

Objective 1.3 Assess FOL viability in relation to molecular tests

In a preliminary test, DNA from dead FOL4 spores was shown to rapidly degrade in a sandy soil in just a few days, suggesting that if molecular tests such as LAMP or qPCR were used to monitor pathogen levels in soil, then they would most likely only detect live spores. DNA survival can be enhanced through binding to clay minerals, larger organic molecules, humic acids and other charged particles and compared with clays, sand has been found less effective in binding DNA. Therefore, further work would be required to determine if there are differences in FOL4 DNA survival in different soil types.

**Objective 2: Determine effect of temperature and inoculum level on FOL4 disease development and impact of non-hosts and fallow on FOL4 survival**

Objective 2.1 Determine the effect of inoculum concentration on FOL4 disease development

A clear relationship was established between concentration of FOL4 inoculum and Fusarium wilt development in lettuce grown in both compost and soil with critical levels of $1 \times 10^4$ cfu g$^{-1}$ and $1 \times 10^5$ cfu g$^{-1}$ respectively, required to cause substantial disease. The greater amount of FOL4 inoculum required to cause disease in soil could be due to some suppressive activity of the resident microbial community. Further work is now required to directly relate soil FOL4 inoculum levels and disease development with qPCR results as has been recently done for *F. oxysporum* f.sp. *cepae* on onion (AHDB project P1908312; Clarkson, 2020). This will allow results of soil tests to be directly related to risk of Fusarium wilt development in lettuce.

Objective 2.2 Determine the effect of temperature on FOL4 disease development

Although there was little difference between growth of FOL1 and FOL4 isolates on agar at different temperatures, high levels of Fusarium wilt disease developed between 12 and 28°C when lettuce plants were inoculated with FOL4 but this only occurred at 28°C for FOL1 (Figure A).
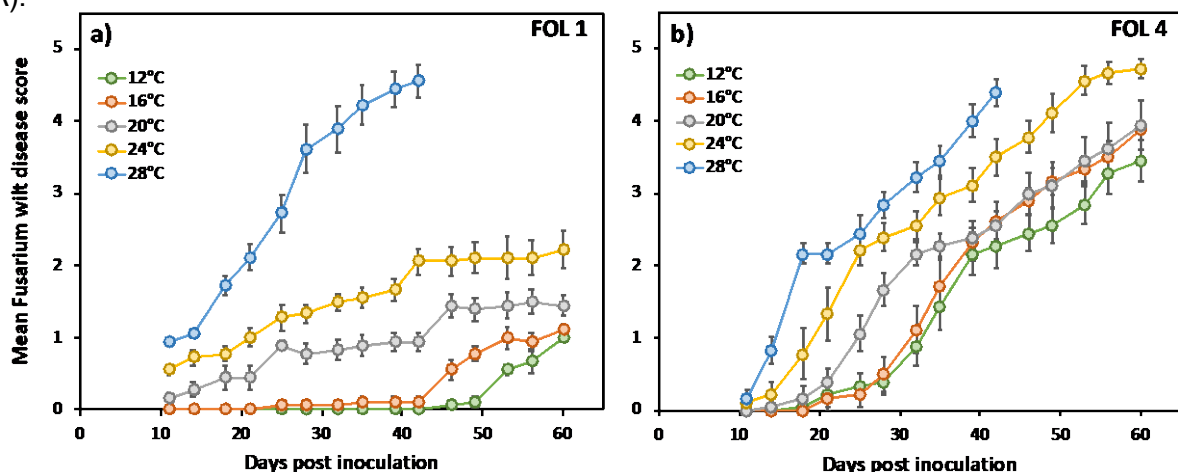


**Figure A.** Effect of temperature on Fusarium wilt disease development for lettuce plants inoculated with FOL1 and FOL4.  Error bars represent the standard error of the mean (SEM).

However, the rate of FOL4 disease development in lettuce increased at higher temperatures and anecdotal evidence from growers in the Netherlands and UK does suggest that Fusarium wilt due to FOL4 is more damaging in Summer. Hence, affected growers have resorted to growing lettuce only in the cooler months of the year. Nonetheless, the ability of FOL4 to cause disease at low temperature has been observed in UK protected lettuce grown in Lancashire in December 2017 (transplanted in October) where air temperatures were 8°C (Taylor & Clarkson, 2018). This raises the possibility that FOL4 could cause disease in outdoor lettuce,

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and hence growers should be vigilant, although the less intense production system with the rotations employed may help prevent build-up of FOL4 inoculum in the soil. Further work is required to confirm the effect of temperature on FOL1 and FOL4 disease development across a range of different isolates.

<u>Objective 2.3: Evaluate the impact of fallow and non-host cropping on FOL4 survival</u>

The survival of FOL4 in fallow soil from an artificially inoculated polytunnel was assessed. Immediately following a summer lettuce crop (Crop 1) with a high incidence of Fusarium wilt, FOL4 was detected at an elevated level in the soil by qPCR but this declined by 96% three weeks after harvest of a following winter crop (Crop 2) with low disease. Thereafter, FOL4 inoculum in the soil remained relatively stable up to 10 months and this was confirmed by parallel tests using dilution plating where levels were approx. $10^3$ cfu $g^{-1}$ soil for most of this period. This is in principal below the threshold required to cause substantial Fusarium wilt based on our experiment examining the effect of different FOL4 inoculum levels on disease development. Nonetheless, it would be anticipated that successive lettuce plantings would quickly increase this level of FOL4 inoculum allowing the critical concentration for substantial disease development to be reached. It is likely that survival of FOL chlamydospores will vary with soil type, moisture level and resident microbial community. The soil in the FOL4 infested polytunnel in our experiment was left to dry out during the fallow period which may have prolonged spore survival as it has been shown that chlamydospores of *F. oxysporum* f.sp. *melonis* can survive for 17 years in dry soil stored at 3-4 °C (McKeen & Wensley 1961).

The potential colonisation of rotation / alternative crop plants by FOL4 as assessed by qPCR was also investigated. Here, high levels of the pathogen were detected in roots of inoculated susceptible lettuce,1695-6483 picograms (pg) FOL4 DNA $mg^{-1}$ root, with reduced levels in resistant cultivars (15-467 pg FOL4 DNA $mg^{-1}$ root). Lower FOL4 levels were detected in the roots of all other crop plants tested, with mizuna, pak choi, chard and cucumber most conducive to pathogen colonisation (20-34 pg FOL4 DNA $mg^{-1}$ root). This suggests that these other crops can sustain FOL4 populations. In the Netherlands, a grower reported that lettuce grown between pak choi crops showed no reduction in Fusarium wilt disease (Taylor & Clarkson, 2018), confirming the results here that this crop can be colonised by FOL4. However, initial observations in Lancashire showed that after two crops of pak choi, Fusarium disease incidence was greatly reduced with only around 5% losses observed (Taylor & Clarkson, 2018). Further work is therefore required to understand these reported inconsistencies. Our results also indicated that even when resistant lettuce cultivars are grown, FOL4 can still colonise roots and levels were higher than for the other crop plants. Overall therefore, growers should be cautious about how useful 'break' crops and resistant lettuce cultivars can be in reducing FOL4 inoculum.

**Objective 3: Test hygiene measures to eliminate FOL4 inoculum**

<u>Objective 3.1 Test disinfectants for activity against FOL4 chlamydospores *in vitro*</u>
All the disinfectants evaluated in this project (Jet 5, Unifect G, bleach, Huwa-San, Distel, Virkon and Disolite) effectively killed FOL4 chlamydospores at 100% and 50% of the manufacturers recommended rates in as little as 1 min exposure time and are therefore all potentially useful for maintaining hygiene and eliminating the pathogen from trays, equipment work surfaces etc and for foot dips. However, the efficacy of these products in the presence of large amounts of soil or organic matter which can limit efficacy was not assessed. Nevertheless, results largely confirm those from previous studies with other *F. oxysporum* f.spp. Overall, a range of products effective against FOL4 are therefore available to growers, allowing a choice to be made based on cost and situation. However, further work is needed to confirm their efficacy in the presence of soil and organic matter which potentially contaminates propagation trays and foot dips etc. Ideally however, growers should clean all equipment and surfaces before using disinfectants. Residues might also be an issue especially for quaternary ammonium compounds and it should be noted that the limit is 0.1 mg kg$^{-1}$ for lettuce (Taylor & Clarkson, 2018).

<u>Objective 3.2 Test heat treatments against FOL4 chlamydospores *in vitro*</u>
Heat treatment is an effective way of killing FOL4 chlamydospores and our results showed that at 70˚C, spores in a suspension of water were killed within 5 min. The minimum heat treatment required to kill FOL4 spores was 60˚C for 15 mins although viability was only 4.7% after 1 min. Unlike chemical disinfection, heat treatments are unaffected by soil contamination and have no residue issues and would therefore be potentially useful for propagation trays.

<u>Objective 3.3 Test disinfectant and heat treatments against FOL4 chlamydospores on polypropylene</u>
Efficacy of all the disinfectants tested in this project at 50% of the manufacturers recommended rates was confirmed on polypropylene discs with FOL4 chlamydospores, confirming their utility for sterilising propagation trays. Heat in the form of steam was also effective at eliminating FOL4 from polypropylene but when using a jet of steam, it was demonstrated that it is important that the distance and duration of this treatment is sufficient to achieve >65°C for at least 1 min which will depend on the equipment used.

## Conclusions

- Specific LAMP and qPCR based molecular diagnostic assays were successfully developed for FOL / FOL4 and utilised to identify and quantify the pathogen in diseased

plants, roots and soil. These can now be employed to identify further FOL4 outbreaks and potentially carry out soil tests.

- Critical levels of FOL4 inoculum required to cause substantial Fusarium wilt in lettuce were determined in both compost and soil in artificially inoculated systems. Further work is now required to relate inoculum and disease levels to molecular quantification of the pathogen.

- FOL4 can cause substantial Fusarium wilt at temperatures as low as 12°C with more rapid disease development at higher temperatures up to the maximum of 28°C tested. In contrast, FOL1 only caused significant wilt at 28°C. Further work is required to confirm this across multiple isolates of FOL1 and FOL4.

- Following a lettuce crop with high levels of Fusarium wilt, there was an initial reduction in FOL4 inoculum in soil, but the pathogen then persisted for 10 months when the soil was left fallow. In this situation, it is likely that FOL4 disease would quickly increase if successive planting of lettuce crops were grown.

- A range of non-host crop roots can be colonised by FOL4 and of those tested, mizuna, pak choi, chard and cucumber were the most conducive to the pathogen. However, colonisation levels were variable.

- The disinfectants Jet 5, Unifect G, bleach, Huwa-San, Distel, Virkon and Disolite were all effective at killing FOL4 chlamydospores in solution, even at 50% of the manufacturers recommended rates and 1 min exposure time. However, efficacy in the presence of soil contamination still needs to be assessed.

- FOL4 chlamydospores in water were killed within 5 min when exposed to a temperature of 70°C and within 15 min at 60°C. Heat treatments are therefore an effective way of eliminating pathogen inoculum.

- All disinfectants applied at 50% of the manufacturers recommended rates killed FOL4 chlamydospores on polypropylene within 5 min. Steam jet treatments also eliminated FOL4 spores but temperatures >65°C for at least 1 min were required.

## Financial Benefits

- None at this time

## Action Points

- Check lettuce plants for symptoms of FOL and cut suspect plants in half to look for typical vascular browning. If this symptom is observed, send intact plant samples FAO

Alison Jackson, Warwick Crop Centre, University of Warwick, Wellesbourne, Warwick, CV35 9EF for confirmation. Early diagnosis is critical for limiting the spread of FOL.

- Remove lettuce plants with Fusarium wilt if possible as well as soil around plants, to reduce FOL inoculum levels and dispose by bagging-up and taking to land-fill or burning.

- If already affected by Fusarium wilt, avoid lettuce production in the hotter summer months and consider diversifying crops grown. Reducing the intensity of cropping and / or introducing alternative crops will reduce build-up of FOL in the soil.

- Follow hygiene procedures and wash plant trays, pallets and equipment prior to use of disinfectants. Heat treatments are an alternative approach to eliminating FOL4 spores.

# SCIENCE SECTION

## Introduction

Fusarium wilt of lettuce caused by the soilborne fungal pathogen *Fusarium oxysporum* f. sp. *lactucae* (FOL) results in severe losses in production areas globally. Four races occur, with race 1 (FOL1) being the most widespread in the field and protected crops particularly in warmer parts of the world such as the USA (Gordon & Koike 2015) and Italy (Garibaldi et al., 2002). FOL was first reported in the UK in 2017, and genetic analysis at Warwick identified that outbreaks were caused by the recently emerged FOL race 4 (FOL4; Taylor et al., 2019). FOL4 was initially reported in the Netherlands (2013) followed by Belgium (2015; Gilardi et al., 2017) and anecdotal evidence suggests it may be more aggressive than FOL1, especially at low temperatures. So far, FOL4 has been restricted to protected lettuce and has not yet been identified as causing disease in the field. Control of FOL4 is challenging, as like all *F. oxysporum* f. spp., it produces chlamydospores which survive for long periods of time in the soil (Gordon & Koike 2015) which makes management using fungicides or biological control agents difficult. Currently there is no varietal resistance widely available for the lettuce types grown under protection (butterhead / 'Little Gem' types) although breeding companies have begun the limited release of new resistant or partially resistant cultivars. Consequently, FOL4 represents a significant threat to the UK lettuce industry and those growers affected are struggling to manage the disease. Crop hygiene is therefore very important to prevent further local and regional spread (Taylor & Clarkson, 2018). This project was undertaken in response to an AHDB research call to investigate aspects of the biology and management of FOL4 which followed an extensive literature review to identify gaps in the knowledge base (Taylor & Clarkson, 2018).

The main aims of the project were to i) develop molecular tools for rapid FOL4 identification and quantification to help monitoring of further outbreaks and understand key aspects of pathogen biology such as infection, colonisation and survival, ii) understand key factors affecting FOL4 disease development including inoculum level and temperature and iii) identify hygiene (heat and disinfectant) approaches to limit pathogen spread. The specific project objectives were to:

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Additional non-contracted research was also initially carried out to understand the relationship between different FOL races and investigate if there was diversity within FOL1 and FOL4 using isolates collected prior and during the project using a DNA sequencing approach.

# Added Objective: Investigate diversity of FOL using DNA sequencing

## Materials and Methods

DNA was extracted from cultures of FOL1 and FOL4 isolates originating from UK lettuce samples with Fusarium wilt sent by growers (FOL 4 only) or obtained from other researchers from overseas. Part of the elongation factor 1 alpha (EF1) gene was then amplified by PCR and sequenced as described by Taylor et al., (2016). Sequences were aligned with those of other FOL isolates (total 39 FOL1, FOL2, FOL3, FOL4, sequences) and other *F. oxysporum* spp. publicly available on NCBI using MEGA version 10 (Kumar et al., 2018) and a phylogenetic tree constructed using the Kimura 2 method (gamma distributed) and 1000 bootstrap replicates.

## Results

The phylogenetic tree indicated that all isolates of FOL1 and FOL4, including those where sequences were obtained from NCBI, shared identical EF1 sequences, suggesting a common genetic origin (Fig. 1). This FOL1 / FOL4 clade also contained *F. oxysporum* f.spp. from statice and column stocks, showing that these pathogens are closely related. FOL races 2 and 3 formed separate clades, suggesting a different evolutionary origin. Interestingly, *F. oxysporum* isolate GP3 isolated from UK lettuce shared an identical EF1 sequence with FOL3 isolates but was non-pathogenic in preliminary tests. Isolate 0349 identified as FOL and originating from diseased lettuce in California did not group with any other FOL isolates. Selected non-pathogenic *F. oxysporum* isolates from UK lettuce plants were genetically diverse and were placed in different clades of the tree.

**Figure 1.** Maximum likelihood phylogenetic tree of *F. oxysporum* isolates based on partial sequence of the elongation factor gene. Numbers represent bootstrap values (1000 replicates). Scale bar indicates 0.01 substitutions per site. FOL4 isolates included representatives from The Netherlands, England and Ireland. FOL1 isolates included representatives from France, Spain, Norway, Israel, Italy and the USA. 'Non-path' refers to tested or suspected non-pathogenic *F. oxysporum* isolates from UK lettuce plants (negative for FOL1 or FOL4 using specific PCR testing). The tree is rooted using an isolate of *F. proliferatum* from onion.

# Objective 1: Develop tools for molecular detection and quantification of FOL/FOL4

## Objective 1.1 Test LAMP assay for FOL

### Materials and Methods

This part of the project initially tested a published LAMP assay for FOL (Franco-Ortega et al., 2018). LAMP (loop-mediated isothermal amplification) is a comparable technique to PCR in that it rapidly amplifies DNA from target genes and can therefore also be used for molecular diagnostics of plant pathogens (Aglietti et al., 2019). However, the technique is carried out at a single temperature rather than a series of thermal cycles as used in PCR and is therefore generally more rapid. Moreover, LAMP works with cruder DNA extracts and portable machines are available, opening up the possibility of in-field diagnostics (Tomlinson, 2013).

The published FOL LAMP assay was tested using DNA extracts from pure cultures of FOL1 isolate AJ520 (from Italy) and FOL4 isolate AJ516 (from Lancashire), as well as DNA extracts from FOL4 infected lettuce roots and from soil from an artificially infested polytunnel (FOL4 isolate AJ516, see Objective 2.3) as described in AHDB project CP 113 (Taylor, 2019). Next, specificity tests were carried out using DNA extracted from pure cultures of 71 different FOL, *F. oxysporum* f.spp., *Fusarium* spp. and other fungal isolates (**Error! Reference source not found.**). All LAMP reactions were set up in strip cap tubes (OP-0008, Optigene) using 15 µl isothermal mastermix ISO-001 (Optigene, UK), 5 µl of primer mix (Optigene PK-F.oxy_lac-050W kit) and 5 µl of DNA. LAMP was run on an Optigene Genie II machine at 65°C for 45 min, followed by an anneal curve analysis (95 to 70°C at increments of $0.05°C\ s^{-1}$).

Due to some non-specific amplification of DNA from other *F. oxysporum* isolates with the published FOL LAMP assay, as well as the high cost of the Optigene kit, new LAMP primers were designed based on FOL effector gene targets identified from whole genome sequences of FOL1 and FOL4 generated previously by NIAB-EMR. Primers were designed using LAMP designer software (Premier Biosoft, USA) with assistance from Matt Dickinson (University of Nottingham). Initially, these new FOL primers were tested using DNA from pure cultures of FOL1 isolate AJ520 and FOL4 isolate AJ516 as well as the soil DNA extracts from a FOL4 infested tunnel (isolate AJ516; see Objective 2.3). LAMP reactions were set up in strip cap tubes using 15 µl of isothermal mastermix, 2.5 µl of primer mastermix, 5 µl of DNA, (Table 1) and 2.5 µl of sterile distilled water (SDW) and run on the Genie II LAMP machine at 64°C for 40 mins, followed by an anneal curve analysis (98°C to 80°C for 1 min reducing by $0.05°C\ s^{-1}$).

**Table 1** Primer mix for new FOL LAMP assay

| Primer* | Conc. in 25 µl reaction (µM) | Volume for 100 reactions (µl) |
|---------|------------------------------|-------------------------------|
| F3 | 0.2 | 5 |
| B3 | 0.2 | 5 |
| FIP | 2.0 | 50 |
| BIP | 2.0 | 50 |
| LoopF | 1.0 | 25 |
| LoopR | 1.0 | 25 |
| SDW | | 90 |

The new FOL LAMP assay was then tested on diseased lettuce plant samples from three different cultivars (Skye, Amica and Temira) inoculated with FOL4 (isolate AJ516) as part of a different project. Small tissue pieces (approx. 10 x 10 x 10 mm) excised from areas of vascular browning in the lettuce tap root were placed in a screw cap tube and a crude DNA extract prepared using a plant kit (Optigene EXT-001). Following a 1 in 5 dilution in SDW, 5 µl of extract was used to set up LAMP reactions as described above. Plants with mild or more severe wilt symptoms and vascular browning were selected with three tissue samples taken from each and compared with tissue from uninoculated control plants. Specificity of the new FOL LAMP assay was then carried out by testing against the panel of 71 DNA extracts from pure cultures of FOL, *F. oxysporum* f.spp., *Fusarium* spp. and other fungal isolates as described above (**Error! Reference source not found.**).

## Results

When the published FOL LAMP assay (Franco-Ortega et al., 2018) was tested for specificity against different isolates of FOL, *F. oxysporum* f.spp., *Fusarium* spp. and other fungal species, DNA from FOL1 and FOL4 isolates was amplified as expected but there was non-target amplification of non-pathogenic *F. oxysporum* isolates from lettuce as well as pathogenic *F. oxysporum* isolates from both statice and rocket (Table 2a). In contrast, the new FOL LAMP assay was completely specific, only amplifying DNA from FOL1 and FOL4 isolates (Table 2). Both LAMP assays (published and new) resulted in amplification of FOL DNA from the infested polytunnel soil but amplification was detected earlier with the new LAMP assay, with some samples having substantially quicker detection times (e.g. samples 3 and 7; Table 3).

**Table 2a.** Specificity of LAMP and qPCR assays for *F. oxysporum* isolates

| *Fusarium oxysporum* | Host | Country | Code | LAMP assays | | qPCR assays | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Published[1] | New[2] | g18785 FOL4[3] | g23490 FOL4[4] | g19968 FOL1&4[5] | Fujinaga FOL1[6] | Pasquali FOL1[7] | Gilardi FOL4[8] |
| *Fusarium oxysporum f.sp. lactucae* race 1 | Lettuce | ITA | AJ520 | + 07:15 | + 07:00 | - | - | + | + | + | - |
| *Fusarium oxysporum f.sp. lactucae* race 1 | Lettuce | ISR | PC7984 | + 07:15 | + 07:00 | - | - | + | + | + | - |
| *Fusarium oxysporum f.sp. lactucae* race 4 | Lettuce | UK | AJ516 | + 07:00 | + 07:00 | + | + | + | + | - | + |
| *Fusarium oxysporum f.sp. apii* | Celery | CHN | HL2 | - | - | NT | - | - | - | - | - |
| *Fusarium oxysporum f.sp. apii* | Celery | USA | Fenlander 5 | - | - | NT | - | - | + | - | - |
| *Fusarium oxysporum f.sp. cepae* | Onion | UK | FUS2 | - | - | - | - | - | - | - | - |
| *Fusarium oxysporum f.sp. conglutinans* race 2 | Brassica | USA | NRRL 54008 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. cubense* | Banana | UK | E421A-3 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. dianthi* | Dianthus | UK | BX13/113 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. freesia* | Freesia | NL | NRRL26990 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. gladioli* | Gladioli | NL | NRRL 26993 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. limonii* | Statice | UK | 30A-9 | + 07:15 | - | + | - | - | + | - | - |
| *Fusarium oxysporum f.sp. lini* | Flax | UK | | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. lycopersici* race 1 | Tomato | UK | FOL R1 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. lycopersici* race 2 | Tomato | UK | FOL R2 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. lycopersici* race 3 | Tomato | USA | FOL R3 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. matthioli* | Stocks | UK | Stocks 4 | - | - | - | - | - | + | - | + |
| *Fusarium oxysporum f.sp. narcissi* | Daffodil | UK | FON63 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. phaseoli* | Bean | USA | ATCC90245 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. pisi* race 1 | Pea | UK | FOP R1 | - | - | + | - | - | + | - | - |
| *Fusarium oxysporum f.sp. pisi* race 2 | Pea | UK | FOP R2 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. pisi* race 5 | Pea | UK | FOP R5 | - | - | - | - | - | + | - | - |
| *Fusarium oxysporum f.sp. radicis-lycopersici* | Tomato | USA | NRRL 26381 | - | - | - | - | - | - | - | - |
| *Fusarium oxysporum f.sp. vasinfectum* | Cotton | CHN | NRRL 25433 | - | - | + | - | - | - | - | - |
| *Fusarium oxysporum* (pathogenic) | Rocket | UK | FR3 (77) | + 18:45 | - | NT | - | - | + | - | + |
| *Fusarium oxysporum* (non-pathogenic) | Lettuce | UK | GOP 7 | + 13:30 | - | NT | - | - | - | - | - |
| *Fusarium oxysporum* (non-pathogenic) | Lettuce | UK | FS16-1 | - | - | NT | - | - | + | - | - |
| *Fusarium oxysporum* (non-pathogenic) | Lettuce | UK | GOP14-1 | - | - | NT | - | - | + | - | - |
| *Fusarium oxysporum* (non-pathogenic) | Lettuce | UK | GOP3 | - | - | NT | - | - | + | - | - |
| *Fusarium oxysporum* (non-pathogenic) | Lettuce | UK | GOP15-1 | + 18:45 | - | NT | - | - | + | - | - |
| *Fusarium oxysporum* (non-pathogenic) | Soil | UK | Fo47 | + 19:15 | - | - | - | - | + | - | - |

**Table 2b.** Specificity of LAMP and qPCR assays for *Fusarium* spp. isolates

| *Fusarium* spp. | Host | Country | Code | LAMP assays | | qPCR assays | | | | | |
| | | | | Published[1] | New[2] | g18785 FOL4[3] | g23490 FOL4[4] | g19968 FOL1&4[5] | Fujinaga FOL1[6] | Pasquali FOL1[7] | Gilardi FOL4[8] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Fusarium avenaceum* | Leek | UK | L5 | - | - | - | - | - | - | - | - |
| *Fusarium coeruleum* | Potato | UK | F88 | - | - | - | - | - | - | - | - |
| *Fusarium commune* | Lettuce | UK | Lettuce 2 Preston | - | - | NT | - | - | + | - | - |
| *Fusarium culmorum* | Wheat | UK | Fc/01/W001 | - | - | - | - | - | - | - | - |
| *Fusarium equiseti* | Rocket | UK | NL1 | - | - | - | - | - | - | - | - |
| *Fusarium flocciferum* | Asparagus | UK | AT4 | - | - | - | - | - | - | - | - |
| *Fusarium graminearum* | Wheat | UK | WR21 | - | - | - | - | - | - | - | - |
| *Fusarium lactis* | Pepper | UK | P9 | - | - | - | - | - | + | - | - |
| *Fusarium langsethiae* | Wheat | UK | 34f.l.003.2 | - | - | - | - | - | - | - | - |
| *Fusarium poae* | Wheat | UK | Fp/01/W001 | - | - | - | - | - | - | - | - |
| *Fusarium proliferatum* | Onion | UK | A40 | - | - | - | - | - | + | - | - |
| *Fusarium redolens* | Onion | UK | NL96 | - | - | - | - | - | + | - | - |
| *Fusarium redolens* | Rocket | UK | ML3 | - | - | NT | - | - | - | - | - |
| *Fusarium sambucinum* | Potato | UK | F37 | - | - | - | - | - | - | - | - |
| *Fusarium solani* | Pea | UK | PG14 | - | - | - | - | - | - | - | - |
| *Fusarium torulsoum* | ? | UK | 102 | - | - | - | - | - | - | - | - |
| *Fusarium tricinctum* | Brassica | UK | CO | - | - | - | - | - | - | - | - |

**Table 2c.** Specificity of LAMP and qPCR assays for fungal species isolates

| Fungi | Host | Country | Code | LAMP assays | | qPCR assays | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Published[1] | New[2] | g18785 FOL4[3] | g23490 FOL4[4] | g19968 FOL1&4[5] | Fujinaga FOL1[6] | Pasquali FOL1[7] | Gilardi FOL4[8] |
| *Alternaria infectoria* | Onion | UK | AT2 | - | - | - | - | - | - | - | - |
| *Botrytis cinerea* | Onion seed | UK | WRAR-4 | - | - | - | - | - | - | - | - |
| *Botrytis cinerea* | Lettuce | UK | Lettuce 2 Preston | - | - | NT | - | - | - | - | - |
| *Cylindrocarpon destructans* | Parsnip | UK | CD10 | - | - | - | - | - | - | - | - |
| *Itersonilia perplexans* | Parsnip | UK | IP10 | - | - | - | - | - | - | - | - |
| *Microdochium majus* | Wheat | UK | MM/X/W/003 | - | - | - | - | - | - | - | - |
| *Microdochium nivale* | Wheat | UK | MN/X/W/003 | - | - | - | - | - | - | - | - |
| *Mycocentrospora acerina* | Parsnip | UK | Ma5 | - | - | - | - | - | - | - | - |
| *Phoma* sp. | ? | UK | | - | - | - | - | - | - | - | - |
| *Phoma* sp. | Lettuce | UK | Lancs Lettuce 7-1 | - | - | NT | - | - | - | - | - |
| *Phytophthora cactorum* | Strawberry | UK | P414 | - | - | - | - | - | - | - | - |
| *Plectosphaella cucuminera* | Lettuce | UK | GHO 2-1(2nd) | - | - | NT | - | - | - | - | - |
| *Pythium ultimum* | Carrot | UK | 3b/P174 | - | - | - | - | - | - | - | - |
| *Pythium violae* | Carrot | UK | 2C/P2d | - | - | - | - | - | - | - | - |
| *Rhizoctonia solani* | ? | UK | R5(A92-1) | - | - | - | - | - | - | - | - |
| *Sclerotinia minor* | Lettuce | UK | | - | - | NT | - | - | - | - | - |
| *Sclerotinia sclerotiorum* | Lettuce | UK | L6 | - | - | - | - | - | - | - | - |
| *Sclerotium cepivorum* | Onion | UK | RUG1-1 | - | - | - | - | - | - | - | - |
| *Setophoma terrestris* | Onion | UK | PQF4 | - | - | - | - | - | - | - | - |
| *Stemphylium* sp. | Onion | UK | SQ3 | | - | | | | | | |
| *Trichoderma* sp. | Onion | UK | WRLG11 | | - | - | - | - | - | - | - |
| *Verticillium albo-atrum* | Potato | UK | PD693 | - | - | - | - | - | - | - | - |
| *Verticillium tricorps* | Lettuce | UK | FS8 ( | - | - | NT | - | - | - | - | - |

[1]Published LAMP (Franco-Ortega et al., 2018); [2]New LAMP (this project); [3,4]new qPCR for FOL4 (this project); [5]new qPCR for FOL1 and FOL4 (this project); [6]published qPCR for FOL1 (Fujinaga et al., 2014); [7]published qPCR for FOL1 (Pasquali et al., 2007); [8]published qPCR assay for FOL4 (Gilardi et al., 2017).

+ = positive amplification; - = no amplification; NT = not tested. For LAMP assays, time to postive is indicated in mm:ss.

**Table 3.** Time taken to detect FOL4 in DNA extracted from artificially infested soil from a polytunnel using published and new LAMP assays.

| Soil sample | Published LAMP[1] | New LAMP[2] |
|:---:|:---:|:---:|
| | Amplification time (mm:ss) | |
| 1 | 12:15 | 10:45 |
| 2 | 13:00 | 10:30 |
| 3 | 22:45 | 13:15 |
| 4 | 11:30 | 11:00 |
| 5 | 18:00 | 10:45 |
| 6 | 17:15 | 13:45 |
| 7 | 33:45 | 12:45 |
| 8 | 13:45 | 12:00 |
| 9 | 16:30 | 12:30 |
| 10 | 11:15 | 10:45 |
| **Mean** | **17:00** | **11:48** |

[1]Published LAMP (Franco-Ortega et al., 2018); [2]New Lamp (this project)

The new LAMP assay also successfully detected FOL4 in DNA extracted from diseased lettuce plants (cvs. Skye, Amica and Temira) following artificial inoculation. Samples from plants exhibiting both mild and severe wilt symptoms resulted in LAMP amplification of FOL4 (Fig. 2) while those from healthy uninoculated control plants resulted in no amplification. The time taken for amplification was greater for cv. Amica suggesting it has lower levels of FOL4 DNA present. Plants with severe wilt symptoms resulted in slightly earlier amplification of FOL4 DNA than for those with mild symptoms (Fig. 2).
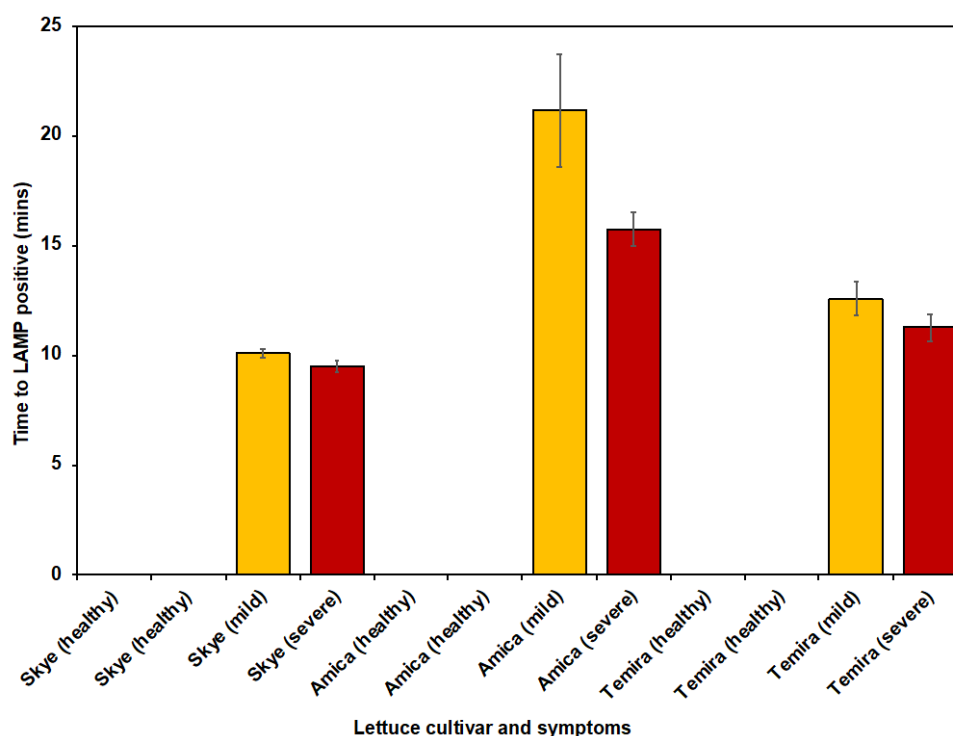


**Figure 2.** LAMP amplification of FOL4 DNA from inoculated lettuce samples showing no (healthy), mild or severe Fusarium wilt symptoms for three lettuce cultivars (Skye, Amica and Temira). Samples were replicated three times and error bars represent the SEM.

## Objective 1.2 Develop and test qPCR for FOL

**Materials and Methods**

Following FOL1 (isolate AJ520) and FOL4 (isolate AJ516) genome analysis at NIAB-EMR, putative effector gene targets for qPCR development were identified. FOL4-specific primers were designed for two gene targets g18785 and g23490, while FOL-specific (FOL1 and FOL4) primers were designed for gene target g19968 (AHDB project CP113; Taylor, 2019). The three primer pairs were tested for specificity using conventional PCR using DNA from the same range of FOL, *F. oxysporum* f.spp., *Fusarium* spp. and other fungal isolates as before (Table 2) and compared with three different sets of published conventional PCR primers reported to be specific to FOL1 (Pasquali et al., 2007; Fujinaga et al., 2014) or FOL4 (Gilardi et al., 2017). All PCR was carried out using 20 µl reactions containing both primers (final concentration 0.5 µM), 5 µl of RedTaq (Sigma, UK) and 1 µl DNA with thermal cycling conditions as listed in Table 4. PCR products were visualised using 1.2% agarose gel electrophoresis.

**Table 4.** Primer sets and PCR conditions tested for specificity to FOL1 and FOL4

| Primer set | FOL specificity | Conditions | Reference |
|---|---|---|---|
| FOL R1-F / FOLR1-RR | FOL1 | 1 cycle of 94°C for 2 mins<br>94°C for 45s, 63°C for 30s and 72°C for 30s (35 cycles)<br>1 cycle of 72°C for 5 mins | Fujinaga et al. (2014) |
| Hani3 / Hani latt3 Rev | FOL1 | 1 cycle of 94°C for 1 min<br>Touchdown PCR: 94°C for 15s and 66°C for 30s (decrease by $0.5^0C$ each cycle) for 10 cycles.<br>94°C for 15s and 61°C for 30s (25 cycles) 1 cycle of 72°C for 2 mins | Pasquali et al. (2007) |
| FPuF / FuPR | FOL4 | 1 cycle of 94°C for 2 mins<br>94°C for 45s, 60.6°C for 30s and 72°C for 30s (35 cycles)<br>1 cycle of 72°C for 5 mins | Gilardi et al., (2017) |
| g18785F / g18785R<br>g23490F / g23490R<br>g19968F / g19968R | FOL4<br>FOL4<br>FOL1 | 1 cycle of 94°C for 2 mins<br>94°C for 45s, 60°C for 30s and 72°C for 30s (35 cycles)<br>1 cycle of 72°C for 5 mins | This study |

One set of new FOL4 specific primers (g23490F / g23490R) were then tested for their utility in qPCR against a dilution series of FOL4 DNA (isolate AJ516) in a QuantStudio 5 (384-well) Real Time PCR machine (Applied Biosystems, UK). Reactions (20 µl) contained both primers (final concentration 0.4 µM), 10 µl Power SYBR™ Green PCR Master Mix (Applied Biosystems) and 1 µl of DNA. All samples were run in triplicate using the following thermocycling conditions; 95°C for 120 s, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. A melt curve analysis was then performed consisting of 95°C for 15s followed by 60°C for 60 s increasing to 95°C for 15 s by 0.075°C s$^{-1}$. Finally, the qPCR assay was also used to quantify FOL4 DNA in the same soil samples from the FOL4 infested polytunnel as well as the diseased tissue samples from the three artificially inoculated lettuce plant cultivars as used for the LAMP testing above (Objective 1.1).

## Results

When the two new qPCR primer sets designed to specifically detect FOL4 were tested, primers amplifying the g18785 gene target were not found to be specific, as there was also amplification of isolates of *F. oxysporum* f. sp. *pisi* (race 1), f.sp. *limonii* and f.sp. *vasinfectum* (Table 2a); these primers were therefore discarded. However, primers amplifying gene target g23490 resulted in specific detection of FOL4 while those amplifying gene target g19968 specifically detected both FOL1 and FOL4 as expected (Tables 2abc). In comparison, the primers developed by Fujinaga *et al.*, (2014) reported to only detect FOL1 were not at all specific, and resulted in amplification of DNA from FOL4 as well as a wide range of other *F. oxysporum* f.spp (Table 2a), non-pathogenic *F. oxysporum* as well as *F. commune, F. lactis, F. redolens* and *F. proliferatum* (Table 2c). In contrast, the primers developed by Pasquali *et al.* (2007) were specific to FOL1 as reported. Finally, primers for detection of FOL4 developed by Gilardi *et al.* (2017) were not specific as they also amplified DNA from isolates of *F. oxysporum* affecting stocks and rocket (Table 2a) although they did not amplify isolates of FOL1.

The FOL4 specific primers for g23490 developed in this project resulted in detection of the pathogen in all the infested soil samples from the polytunnel and values ranged between 15.7 and 88.2 pg pathogen DNA (Table 5). For the diseased lettuce tissue samples, those from cvs. Amica and Temira, had higher FOL4 DNA levels in plants showing severe symptoms (245 and 716 pg) compared to those with mild symptoms (37 and 26 pg FOL4 DNA) but this was not the case for Skye where plants with severe symptoms had levels of 288 pg pathogen DNA compared to 549 pg for those with mild symptoms. No FOL4 DNA was detected in

uninoculated healthy control plants. Overall, the FOL4 primers targeting g23490 were efficient and specific and were therefore used for all qPCR in further experiments reported here.

**Table 5.** Detection of FOL4 in soil and plant samples using FOL4 specific primers for gene target g23490.

| Sample No. | Date | Origin | Sample type | FOL4 DNA (pg) | SEM |
|---|---|---|---|---|---|
| 1 | 24/08/18 | Polytunnel | Soil sample | 79.2 | 0.004 |
| 2 | 24/08/18 | Polytunnel | Soil sample | 60.9 | 0.006 |
| 3 | 24/08/18 | Polytunnel | Soil sample | 15.7 | 0.001 |
| 4 | 24/08/18 | Polytunnel | Soil sample | 23.9 | 0.000 |
| 5 | 24/08/18 | Polytunnel | Soil sample | 32.7 | 0.001 |
| 6 | 24/08/18 | Polytunnel | Soil sample | 88.2 | 0.009 |
| 7 | 24/08/18 | Polytunnel | Soil sample | 20.7 | 0.003 |
| 8 | 24/08/18 | Polytunnel | Soil sample | 26.1 | 0.002 |
| 9 | 24/08/18 | Polytunnel | Soil sample | 26.4 | 0.001 |
| 10 | 24/08/18 | Polytunnel | Soil sample | 43.6 | 0.001 |
| Amica 1 | 21/03/19 | Experiment | Healthy plant tissue | ND | |
| Amica 2 | 21/03/19 | Experiment | Healthy plant tissue | ND | |
| Amica 3 | 21/03/19 | Experiment | Plant tissue / mild symptoms | 37.3 | 0.002 |
| Amica 4 | 21/03/19 | Experiment | Plant tissue / severe symptoms | 245.3 | 0.002 |
| Skye 1 | 21/03/19 | Experiment | Healthy plant tissue | ND | |
| Skye 2 | 21/03/19 | Experiment | Healthy plant tissue | ND | |
| Skye 3 | 21/03/19 | Experiment | Plant tissue / mild symptoms | 549.4 | 0.038 |
| Skye 4 | 21/03/19 | Experiment | Plant / severe symptoms | 288.4 | 0.008 |
| Temira 1 | 21/03/19 | Experiment | Healthy plant tissue | ND | |
| Temira 2 | 21/03/19 | Experiment | Healthy plant tissue | ND | |
| Temira 3 | 21/03/19 | Experiment | Plant tissue / mild symptoms | 26.8 | 0.003 |
| Temira 4 | 21/03/19 | Experiment | Plant tissue / severe symptoms | 716.8 | 0.081 |

[1] ND = not detected.

## Objective 1.3 Assess FOL viability in relation to molecular tests

### Materials and Methods

It was established previously in AHDB project CP 113 (Taylor, 2019) that FOL4 conidia were killed when treated at 60°C for 15 mins *in vitro*. To determine whether the FOL4 qPCR would detect DNA from dead spores, a soil sample was collected from a field with no history of lettuce production (sandy loam, 'Wick' series, Wharf Ground field, Wellesbourne), air-dried, sieved (2 mm mesh), mixed thoroughly and 0.5 g subsamples aliquoted into 2 ml DNA extraction tubes. Each of these soil samples was then spiked with either dead (heat treated 60°C for 15 mins) or live FOL4 conidia (isolate AJ516) in a volume of 200 µl sterile water to give a final concentration of $1 \times 10^6$ spores $g^{-1}$ soil. The effectiveness of the heat treatment in killing spores was confirmed by plating 100 µl of spore suspension onto potato dextrose agar (PDA) plates (three replicates) prior to soil spiking. Tubes containing the soil with dead or live FOL4 conidia as well as soil where no spores were added were then incubated at 20°C and DNA extracted at 0, 3, 7, 14, 21, 28 and 35 days after spiking (three replicate extractions per timepoint) using the optimised SoilSV kit protocol described previously in AHDB project CP 113 (Taylor, 2018). FOL4 qPCR was then carried out on diluted DNA (1 in 6 in TE buffer) as described for Objective 1.2.

### Results

The heat treatment (60°C, 15 mins) was confirmed as being 100% effective at killing FOL4 conidia as no growth was evident following plating of spores on PDA (Fig. 3). Under the conditions in this *in vitro* experiment, results suggested that DNA from dead FOL4 conidia was rapidly degraded in soil (Table 6) with a FOL4 DNA concentration of 0.07 pg for dead spores after 7 days compared to 8.5 pg at the start of the experiment. At all timepoints after 7 days, DNA was undetectable with the exception of a single replicate at day 21 which was at the limit of detection (0.01 pg). These values indicate a 99% reduction in the quantity of detectable FOL4 DNA for dead conidia after only 7 days. In contrast, live FOL4 spores were detectable throughout the experiment but declined from 10.4 pg FOL4 DNA on day 0 to 0.16 pg on day 35, suggesting that conidia may not survive for long periods in soil. No FOL4 DNA was detected in soil where no conidia were added (data not shown).
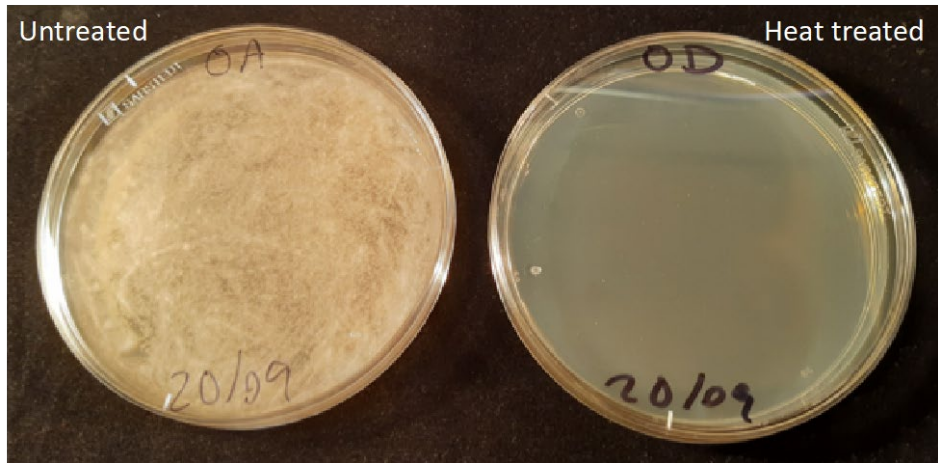
**Figure 3.** Effect of heat treatment (60°C for 15 minutes) on the viability of FOL4 conidia following plating on PDA.

**Table 6.** Detection of live and dead (heat treated) FOL4 conidia by qPCR in soil.

| Time (days) | FOL4 DNA (pg)[1] | | SEM[2] | |
|---|---|---|---|---|
| | Live Spores | Dead spores | Live Spores | Dead spores |
| 0 | 10.35 | 8.50 | 0.77 | 0.70 |
| 3 | 14.05 | 0.82 | 0.97 | 0.08 |
| 7 | 0.26 | 0.07 | 0.03 | 0.03 |
| 14 | 0.20 | ND | 0.06 | 0.00 |
| 21 | 0.23 | 0.01* | 0.11 | 0.01 |
| 28 | 0.11 | ND | 0.08 | 0.00 |
| 35 | 0.16 | ND | 0.07 | 0.00 |

[1]Colours indicate level of FOL4 detection. Green, high FOL4 level; orange, intermediate FOL4 level; red, low FOL4 level. [2]SEM = standard error of the mean (three replicates). *only detected in 1 out of 3 biological replicates.

# Objective 2: Determine effect of temperature and inoculum level on FOL4 disease development and impact of non-hosts and fallow on FOL4 survival

## Objective 2.1 Determine the effect of inoculum concentration on FOL4 disease development

### Materials and Methods

A glasshouse experiment was set up to determine the effect of FOL4 inoculum concentration on disease development in both compost and soil. A FOL4 (isolate AJ516) wheat bran / compost inoculum was prepared as described by Taylor et al. (2013) and mixed into M2 compost or a soil / vermiculite mix (air-dried, 4 mm sieved sandy soil from Soakwaters field, Wellesbourne amended with medium grade vermiculite; 4:1, v:v) to achieve a range of concentrations from $1 \times 10^2$ - $1 \times 10^6$ cfu g$^{-1}$. The FOL4 infested compost and soil was then dispensed into 9 cm pots and two-week-old lettuce plants (cv. Amica, raised in peat blocks) transplanted (one plant per pot, 27 pots per inoculum concentration). Uninoculated control treatments (M2 compost or soil only) were also set up. Pots were arranged in a randomised block design in a glasshouse set at 25°C day, 18°C night, 16 h day-length. Lettuce plants were assessed for Fusarium wilt symptoms twice weekly for seven weeks using the following scoring system: 0, healthy; 1, 1-2 leaves wilted (<10%); 2, 10-50% wilt; 3, >50% wilt; 4, 100% wilt; 5, plant dead. Lettuce plants were then cut longitudinally and internal vascular browning recorded using the following scoring system: 0, no browning; 1, mild vascular browning; 2, medium vascular browning; 3, severe vascular browning; 4, plant dead. Vascular browning data was analysed by ANOVA using Genstat.

### Results

A clear relationship between concentration of FOL4 inoculum and Fusarium wilt disease development was observed for lettuce grown in both compost and soil. Substantial disease was only observed at concentrations of $1 \times 10^5$ cfu g$^{-1}$ or above in soil, whereas $1 \times 10^4$ cfu g$^{-1}$ was sufficient to cause wilt in compost (Fig. 4). Disease development was also much more rapid in compost, with most plants dead (mean disease score = 4.5) for $1 \times 10^6$ cfu g$^{-1}$ after 20 days (Fig. 4a), compared to 50 days for the same concentration in soil (Fig. 4b).
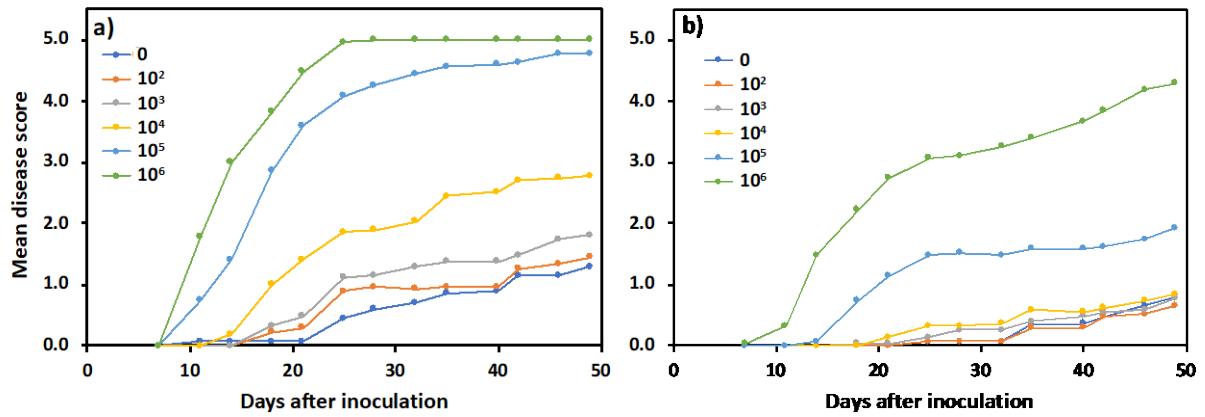
**Figure 4.** Effect of the FOL4 inoculum concentration (1 x $10^2$ - 1 x $10^6$ cfu g$^{-1}$) on development of Fusarium wilt in lettuce grown in (a) inoculated compost and (b) inoculated soil.

There were also significant differences in the lettuce vascular browning scores for both the FOL4 inoculated compost and soil ($P < 0.001$). All FOL4 concentrations in the inoculated compost resulted in significantly more browning than the uninoculated control; however, significant browning for plants in inoculated soil was only observed at 1x $10^5$ cfu g$^{-1}$ and higher (Fig. 5a). There was also a strong correlation between vascular browning score and FOL4 inoculum concentration for both compost (Fig. 5b) and soil (Fig. 5c). The correlation was greater ($R^2$ = 0.96) in compost than in soil ($R^2$ = 0.85), mainly because no vascular browning was observed in soil below a concentration of 1 x $10^5$ cfu g$^{-1}$.
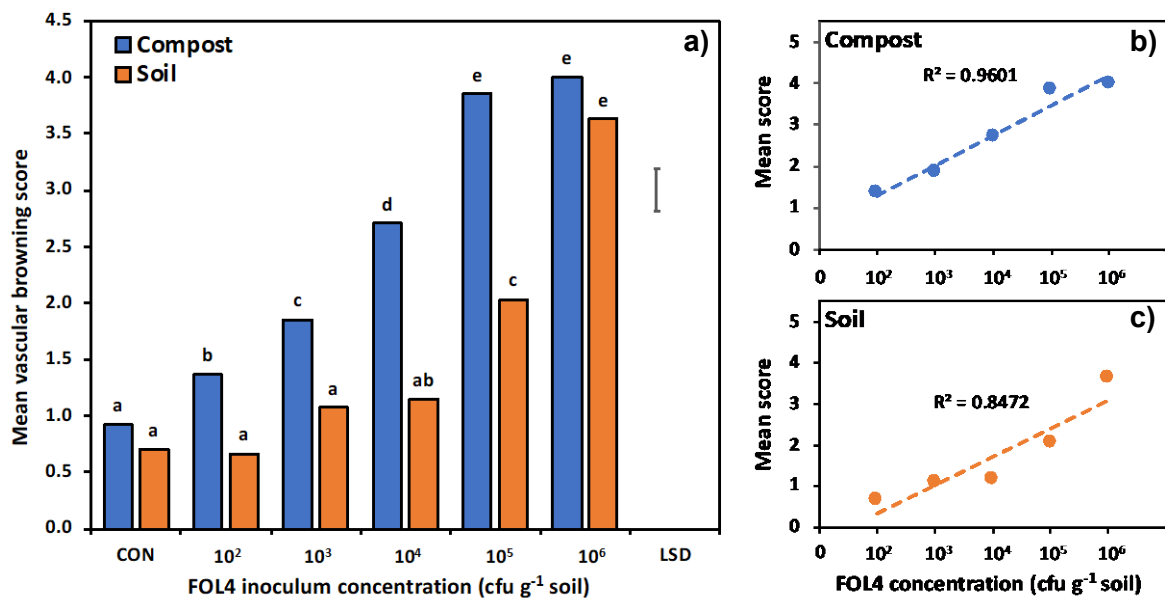


**Figure 5.** Effect of the FOL4 inoculum concentration (1 x $10^2$ - 1 x $10^6$ cfu g$^{-1}$) on vascular browning of lettuce 49 days after transplanting into inoculated compost or soil: a) mean browning score (letters indicate a significant difference between treatments based on the LSD value - 5% level - following ANOVA); correlation of mean vascular browning score and FOL4 inoculum concentration in b) compost and c) soil.

## Objective 2.2 Determine the effect of temperature on FOL4 disease development

**Materials and Methods**

<u>Effect of temperature on FOL colony growth on agar</u>

Initially, FOL4 isolate AJ516 and FOL1 isolate AJ520 were grown on PDA at 25˚C for 8 days. After this, 1 cm plugs were removed from the growing colony edge and placed face down onto PDA plates. Three replicate plates were then set up at different temperatures ranging from 5-40˚C (increments of 5˚C). The diameter of the growing colonies was measured every day for 6 days (both vertical (V) and horizontal (H) axis growth). This was used to calculate mean colony size (V+H/2) for each day. The values obtained at day 4 and day 6 were then used to calculate the mean growth rate per day using the equation: (V/H at day 6 – V/H at day 4) /2.

<u>Effect of temperature on FOL disease development in lettuce</u>

Lettuce plants (cv. Amica) were raised in peat blocks for two weeks and wheat bran / compost inoculum of FOL4 (isolate AJ516) and FOL1 (isolate AJ520) produced as described previously for Objective 2.1. Inoculum of each isolate was then mixed with M2 compost to achieve a concentration of $1 \times 10^6$ cfu $g^{-1}$ which was then dispensed into 9 cm square pots. Non-inoculated control pots were prepared using M2 compost only. Lettuce (two weeks old, raised in peat blocks) were transplanted into pots which were positioned in a randomised block design (18 replicate pots for each temperature treatment) in controlled environment cabinets set at 12, 16, 20, 24 and 28˚C with a 16-hour daylength. Symptoms of Fusarium wilt were recorded twice weekly for 60 days following transplanting, after which plants were removed from the compost and cut longitudinally to score internal vascular browning as described for Objective 2.1. These data were analysed by ANOVA using Genstat.

## Results

<u>Effect of temperature on FOL colony growth on agar</u>

FOL1 and FOL4 isolates showed similar growth rates on PDA across the range of temperatures tested (Fig. 6). FOL1 showed a very slightly increased growth rate at 25°C and 30°C with 11 mm and 10.5 mm growth per day respectively compared to FOL4 with 10.2 mm and 9 mm (Fig. 6).
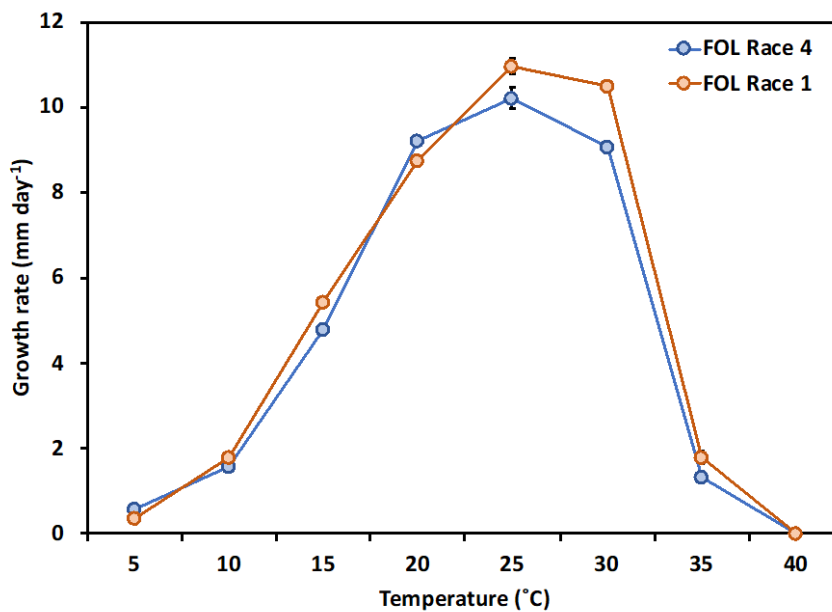


**Figure 6.** Effect of temperature on mean growth rate of FOL1 isolate AJ520 and FOL4 isolate AJ216 on PDA. Error bars represent the standard error of the mean (SEM).

<u>Effect of temperature on FOL disease development in lettuce</u>

Despite the similarities in growth rate in agar culture, it was observed that FOL4 caused disease on lettuce plants at much lower temperatures than FOL1 (Fig. 7). A minimum temperature of 24°C was required for substantial disease development for FOL1 (disease score >2; Fig. 7a) while, in contrast, Fusarium wilt was clearly observed for FOL4 at 12°C (disease score >2; Fig. 7b), although the rate of disease development was slower than at higher temperatures. Maximum, and similar rates of disease development were observed for both FOL1 and FOL4 at 28°C. No disease was observed in uninoculated control plants (data not shown).
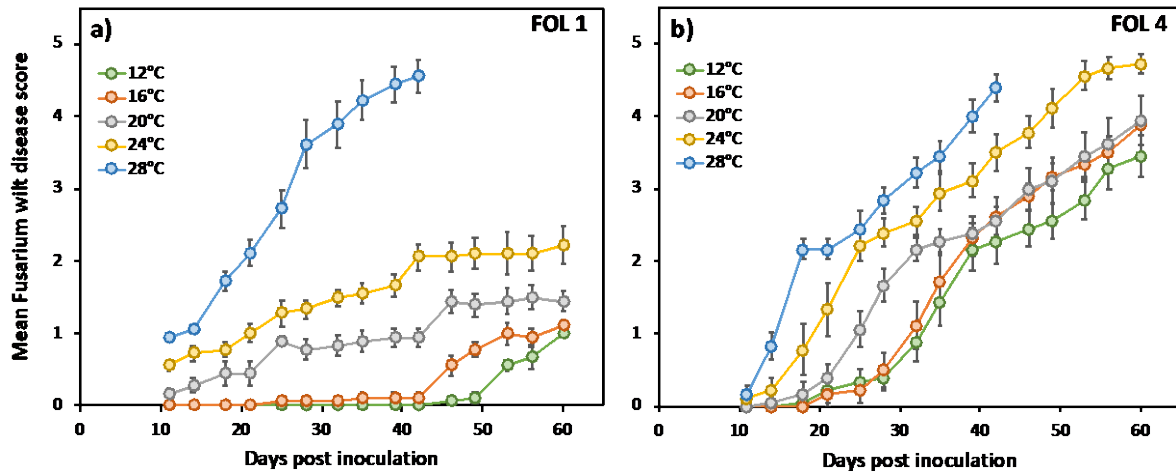
**Figure 7.** Effect of temperature on Fusarium wilt disease development for lettuce plants inoculated with FOL1 and FOL4.  Error bars represent the standard error of the mean (SEM).

Significantly increased vascular browning ($P < 0.05$) was observed in lettuce inoculated with FOL4 compared to FOL1 and the uninoculated control plants across all temperatures between 12 and 24°C (browning score > 3.5; Fig. 8). In contrast, no significant differences in browning score were observed for FOL1 and the uninoculated controls between 12 and 20°C and only a small significant increase at 24°C. However, at 28°C, there was no difference in vascular browning between FOL1 and FOL4 and both were significantly greater than the uninoculated control.
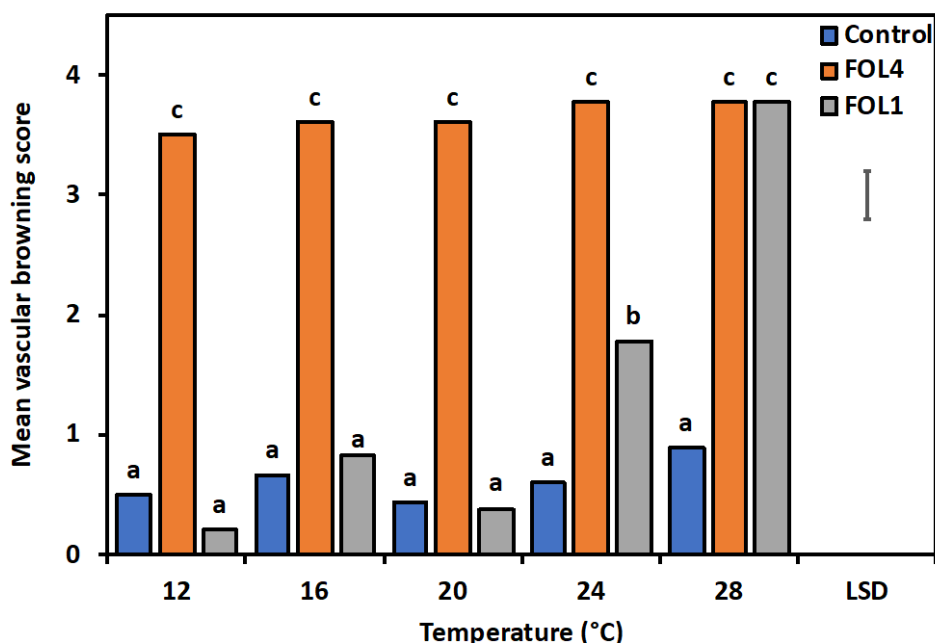


**Figure 8.** Effect of temperature on mean vascular browning score for lettuce plants inoculated with FOL1 and FOL4. Letters indicate a significant difference between treatments based on the LSD value (5% level) following ANOVA.

## Objective 2.3: Evaluate the impact of fallow and non-host cropping on FOL4 survival

**Materials and Methods**

Survival of FOL4 in fallow soil

Soil in a quarantine polytunnel at Wellesbourne (approx. 11 x 5.5 m) was inoculated with FOL4 isolate AJ516 and a lettuce crop (cv. Temira) grown between August and September 2018 to confirm disease development and increase the level of inoculum (Crop 1). Disease incidence was >80% in this crop which was then then rotavated into the soil before a second crop of lettuce (Crop 2) was grown over autumn / winter as part of the Sceptre+ project SP25 between October 2018 and January 2019. Although there were some symptoms of Fusarium wilt in this crop, disease development was slow and final vascular browning scores were low (max = 2). Again, at the end of this experiment, lettuce were rotavated in and, for this project, soil subsequently left fallow for a period of 10 months with the soil drying out during this period. Soil samples were taken from this polytunnel from 31/01/19 to 07/11/19 at intervals of two weeks initially and then monthly after eight weeks to assess levels of FOL4 inoculum either by qPCR or plating onto a semi-selective agar. On each occasion, approx. 50 g soil was collected from five positions and pooled for each of eight 1 m$^2$ areas in the polytunnel and then air-dried and sieved (2 mm mesh). Soil samples had also been collected just before the diseased Crop 1 was rotavated in (September 2019) and these therefore served as a useful baseline to compare FOL4 inoculum levels for the samples collected in the fallow period.

To assess inoculum levels of FOL4 by qPCR, DNA was extracted from each of the eight air-dried soil samples following the optimised SoilSV protocol as described in AHDB project CP 113 (Taylor, 2018) and the FOL4 qPCR (g23490 primers) carried out as previously described for Objective 1.2.

To assess inoculum levels of FOL4 by plating onto semi-selective agar, 1 g of air-dried soil from each of the eight areas in the polytunnel were pooled into a single sample and 10 ml SDW added to give a 1 in 10 dilution, which was again further diluted 1 in 5. Aliquots of each dilution (100 µl) were then spread onto three replicate PDA plates containing 2.5 mg L$^{-1}$ malachite green and 50 mg L$^{-1}$ streptomycin. Plates were incubated for 5 days at 22˚C and colonies that resembled FOL counted. To confirm these were FOL4, selected colonies were transferred onto additional PDA plates using sterile pins and incubated for 7 days at 25˚C. DNA extraction was then carried out using a crude boil prep method. This involved taking a

loop of mycelium from the growing edge of each colony and adding 25 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 using the FOL4 (g23490) specific primers as described for Objective 1.2.

<u>Colonisation of non-host crops by FOL4</u>

The ability of FOL4 to colonise roots of 11 different crop plants (Table 7) as well as two resistant (R1 and R2) and two susceptible lettuce cultivars (Amica and Temira) was evaluated in a glasshouse experiment. All plants were raised in modular trays for 2-5 weeks depending on growth rate before transplanting into M2 compost infested with FOL4 isolate AJ516 at a concentration of 1 x 10$^5$ cfu g$^{-1}$. Non-inoculated control plants were set up for lettuce cv. Amica. Plants were grown in 9 cm square pots arranged in a randomised block design (10 replicate pots per treatment) in a glasshouse set at 25°C day, 18°C night, 16 h day-length. After five weeks, plants were removed from pots and the majority of the compost removed by shaking gently. Roots were then rinsed in water and blotted dry before flash freezing in liquid N and storing at -80°C. Up to 20 mg freeze dried root tissue was used for DNA extraction following the optimised SoilSV protocol as described in AHDB project CP 113 (Taylor, 2018), with the modification that freeze dried roots were first ground in liquid nitrogen before being homogenised by adding to a tube containing a 6.38 mm ceramic bead (MP Biomedicals, Cambridge, UK) in a FastPrep-24 (MP Biomedicals, Cambridge, UK) machine set at 5.5 m s$^{-1}$ for 25 seconds. Then, 550 μl of SL buffer and 200 μl of SDW was added to the root material before repeating the FastPrep step twice. Samples were diluted 1:1 in 1 x TE buffer before carrying out FOL4 qPCR (g23490 primers) as described before in Objective 1.2. Although 10 replicate plants were included per crop type, only five plants per crop were selected for DNA extraction and qPCR initially, with the others pending analysis. FOL4 DNA concentration from qPCR results was adjusted by the amount of root tissue extracted.

**Table 7.** Lettuce cultivars and non-host crops used to assess root colonisation by FOL4

| Crop | Cultivar | Supplier |
|------|----------|----------|
| Lettuce (resistant) | R1 | UKVGB Wellesbourne, UK |
| Lettuce (resistant) | R2 | UKVGB Wellesbourne, UK |
| Lettuce (susceptible) | Amica | Enza Zaden |
| Lettuce (susceptible) | Temira | Enza Zaden |
| Baby kale | *B. napus* KX-1 | Shamrock Seeds |
| Celery | Victoria F1 | Just Seed |
| Chard | Intense | Tozer Seeds |

| Coriander | Cruiser | CN Seeds |
| Cucumber | La Diva | Johnsons Seeds |
| Lambs lettuce | Corn salad Vit | Kings Seeds |
| Land cress | Not stated | Blackdown Growers |
| Mizuna | CN Mizuna | CN seeds |
| Pak choi | Red lady F1 | CN Seeds |
| Rocket | Mugello | CN Seeds |
| Spinach | Platypus | Rijk Zwaan |

## Results

Survival of FOL4 in fallow soil

The pre-fallow soil sample from the FOL4 infested polytunnel collected in September 2018 (following Crop 1 with high levels of Fusarium wilt) contained 35.5 pg FOL4 DNA $g^{-1}$ soil, and this was reduced to 1.5 pg $g^{-1}$ soil at the start of the fallow period (date), three weeks after the overwintered Crop 2 (low disease) was incorporated (Fig. 9). Levels of DNA initially declined over the first three months that the polytunnel was left fallow and then remained at a low level (0.4-0.6 pg FOL4 DNA $g^{-1}$ soil; Fig. 9). Over this period, the soil dried out and temperatures reached a maximum of approx. 35 ˚C (Appendix, Fig. S1). This indicates that the level of FO4 inoculum declines over time in fallow soil.

The FOL4 colony counts on PDA + malachite green from the fallow polytunnel soil samples were more variable than the DNA results. FOL4 levels declined between the pre-fallow (4 x $10^3$ cfu $g^{-1}$ soil, following Crop 1 with high levels of Fusarium wilt) and the first four fallow samples taken between 31/01/19 and 18/03/19 (1.2-1.3 x $10^3$ cfu $g^{-1}$ soil;), but subsequent results were variable (with large SEM values) with an increase recorded to levels similar to those in the pre-fallow sample of up to 3.7 x $10^3$ cfu $g^{-1}$ soil on 18/07/19, and a decrease to 6.7 x $10^2$ cfu $g^{-1}$ soil by the end of the experiment on 07/11/19 (Fig. 10). Assessing FOL4 levels by plating was therefore variable and was also at the limit of detection, with plates often becoming contaminated by other fungi, which potentially masked FOL4 growth. Colonies counted were all verified through PCR using the FOL4 specific primers.
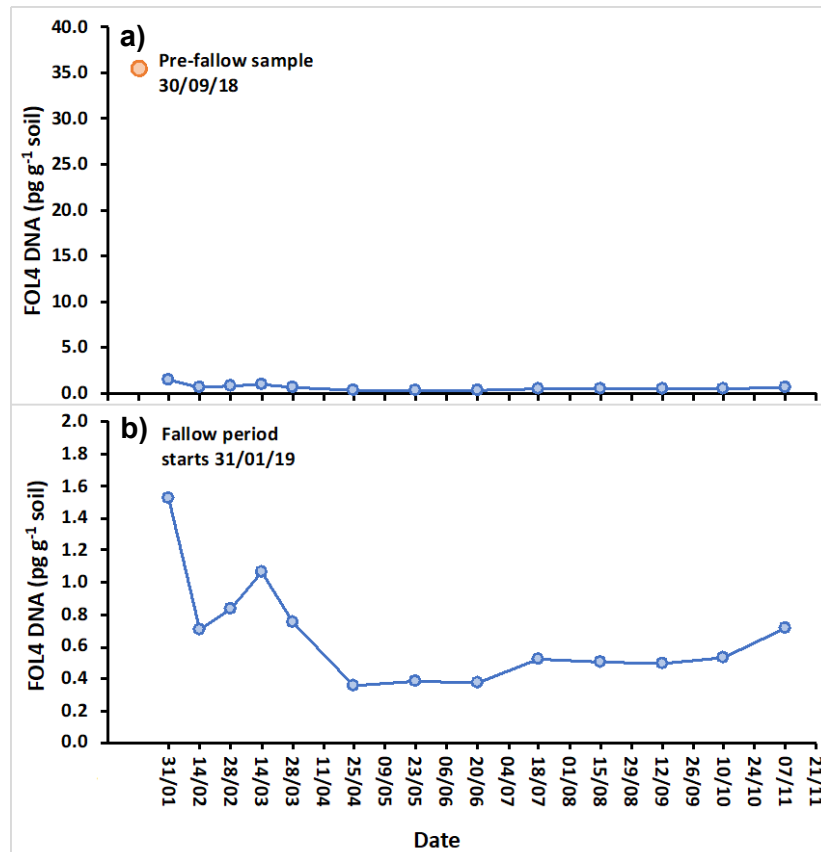
**Figure 9.** Survival of FOL4 in soil in an infested polytunnel during a 10-month fallow period as measured by qPCR: a) pre-fallow sample (orange) following lettuce Crop 1 (high disease) and fallow samples (blue) starting three weeks after overwintered Crop 2 (low disease) and b) fallow samples only. SEM values for all sample points < 0.006.



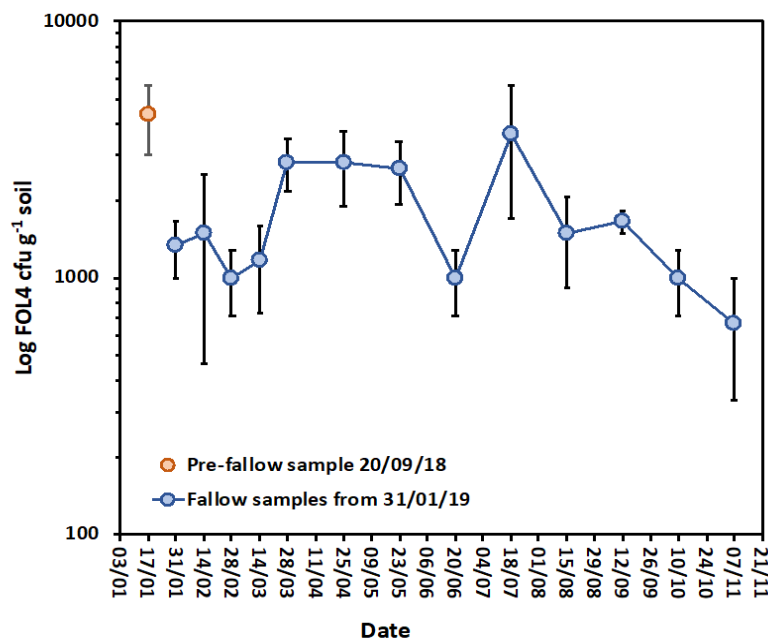**Figure 10.** Survival of FOL4 in soil in an infested polytunnel during a 10-month fallow period as measured by plating onto semi-selective agar. Pre-fallow sample (orange) following lettuce Crop 1 (high disease) and fallow samples (blue) starting three weeks after overwintered Crop 2 (low disease). Error bars indicate SEM. Colonies were verified as FOL4 using conventional PCR.

## Colonisation of non-host crops by FOL4

There were clear differences in the amount of FOL4 DNA detected in the roots of different crop hosts. Roots of the two susceptible lettuce lines (Amica and Temira) contained the most FOL4 DNA (Fig. 11; Appendix, Table S1), with mean values of 6483.6 and 1695.0 pg mg$^{-1}$ root respectively. The two resistant lettuce cultivars R1 and R2 contained 14.9 and 467.4 pg mg$^{-1}$ root; however, the latter was very variable across the five replicate plants. Levels of FOL4 DNA across the other crop plants was lower and varied between 3.5 and 34 pg mg$^{-1}$ root for celery and mizuna respectively. Pak choi, chard and cucumber also had relatively high levels of FOL4 DNA compared with the other crops with mean values of 25.3, 20.5 and 20.3 pg mg$^{-1}$ root respectively. However, these results should be interpreted with some caution as i) FOL4 DNA levels varied across the five replicate plants per crop, ii) there was also considerable variation in the amount of plant root harvested from each host (1-20 mg) and iii) some roots were more contaminated by compost than others which could have affected DNA extraction and qPCR. However, five more replicate plants are available for each host for future analysis.
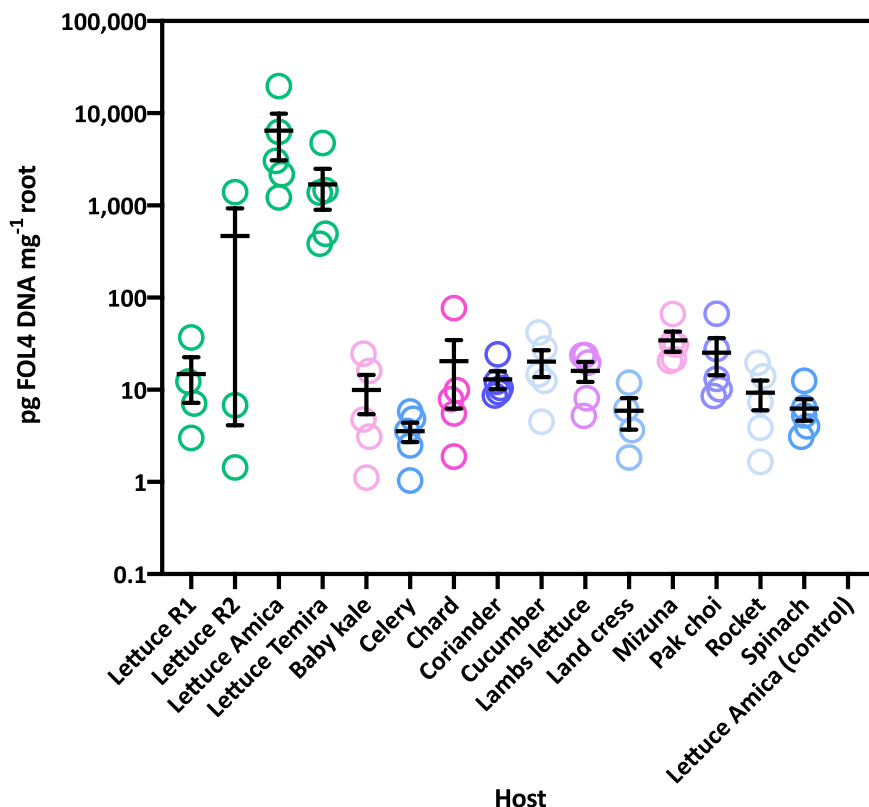


**Figure 11.** Root colonisation of lettuce and other crop plants by FOL4 as detected by qPCR. Lettuce R1 and R2 represent resistant cultivars. Individual circles represent FOL4 DNA concentrations (pg mg$^{-1}$ root) for up to five replicate root samples (samples with undetectable levels of FOL4 DNA were excluded). The mean FOL4 DNA concentration is displayed as the middle bar with associated error bars indicating the standard error (SEM).

# Objective 3: Test hygiene measures to eliminate FOL4 inoculum

## Objective 3.1 Test disinfectants for activity against FOL4 chlamydospores *in vitro*

**Materials and Methods**

Production of FOL4 chlamydospores

Preliminary tests carried out in AHDB project CP 113 (Taylor, 2019) previously evaluated four different methods for FOL4 (isolate AJ516) chlamydospore production using: i) a talc method, ii) a soil broth method (adapted from Bennett & Davis, 2013), iii) a saturated salt solution method (Navas-Cortés et al., 2007) and iv) a sterile soil method (Phytopathology lab, Ghent University). The soil method was found to be the most reliable for chlamydospore production. This involved air-drying and sieving (2 mm mesh) a sandy-loam soil ('Wick' series, Wharf Ground field, Wellesbourne) and autoclaving 40 g quantities twice in 100 ml bottles. FOL4 isolate AJ516 was grown on PDA for 14 days at 20°C and a concentrated suspension of conidia produced by adding a small volume of SDW and agitating with a sterile spreader. The conidia were then added to the soil in the bottles (8 ml at a concentration of 1 x $10^7$ spores $ml^{-1}$) which were incubated for one month at 20°C. At the end of this period, chlamydospore production was confirmed by microscopy and concentrations calculated following series dilution on PDA containing 2 ml $L^{-1}$ chlortetracycline.

Effect of disinfectants on survival of FOL4 chlamydospores *in vitro*

A total of seven disinfectants were tested for their effect on FOL4 chlamydospore survival at different rates and contact times. These were selected based on previous efficacy data from Belgium (Jenny Neukermans, PCG, personal communication) and also represented different active ingredients (Table 8). Soil containing FOL4 chlamydospores was added to 10 ml of SDW, vortexed and left to settle for 60 seconds before the supernatant containing the spores was pipetted off and retained. This suspension was then adjusted to approx. 2 x $10^4$ chlamydospores $ml^{-1}$ and 500 μl added to 500 μl of each disinfectant in a 1.5 ml tube to achieve a final concentration of 100% of the product's recommended rate and 1 x $10^4$ spores $ml^{-1}$. Disinfectants were also tested at 50% and 10% of the recommended rate ensuring that the

same overall chlamydospore concentration and volume of each disinfectant product was used. Each rate of disinfectant was tested using contact times of 1, 5, 15 and 30 mins, after which suspensions were centrifuged for 1 min at 15200 rcf to pellet the chlamydospores. The supernatant (containing the disinfectant) was then quickly removed, and spores washed by resuspending in 1 ml SDW after which they were again centrifuged for 1 min and resuspended in SDW. Spores were washed a total of three times before finally resuspending in 1 ml of SDW. Disinfectants were tested sequentially at different times but for each, untreated controls were set up comprising two tubes containing 500 µl FOL4 chlamydospore suspension and 500 µl SDW with one tube receiving the same centrifugation and washing steps as for the disinfectants. To determine chlamydospore viability, washed spores from each disinfectant treatment (100 µl, $1 \times 10^4$ spores ml$^{-1}$) as well as a 1 in 10 dilution ($1 \times 10^3$ spores ml$^{-1}$) were spread onto three replicate PDA plates containing 2 ml L$^{-1}$ chlortetracycline. Plates were incubated for 2 days at 25˚C after which FOL4 colonies were counted and percentage spore survival compared to the control (centrifuged / washed) calculated. Plates that had few or no germinated FOL4 spores after 2 days were kept at 25˚C and checked again for growth after 7 days.

**Table 8.** Disinfectants and rates tested for their effect on survival of FOL4 chlamydospores.

| Disinfectant | Class | Active ingredient(s) | 100%* | 50%* | 10%* |
|---|---|---|---|---|---|
| Disolite | Phenolics | Ortho-phenyl phenol, orthobenzyl, chlorophenol, iso-Propyl alcohol, chelating agents, biodegradable anionic detergent | 2.0% | 1.0% | 0.20% |
| Virkon S | Oxidising agent / peroxygen compounds | Potassium peroxymonosulfate, sodium dodecylbenzenesulfonate, sulfamic acid, inorganic buffers. | 1.0% | 0.5% | 0.10% |
| Jet 5 | Oxidising agent / peroxide compounds | Peroxyacetic acid, hydrogen peroxide, acetic acid | 0.8% | 0.4% | 0.08% |
| Unifect-G | Quaternary ammonium compounds | Quaternary ammonium compounds, gluteraldehyde | 4.0% | 2.0% | 0.40% |
| Huwa-San | Oxidising agent / peroxide compounds | Silver stabilised hydrogen peroxide | 6.0% | 3.0% | 0.60% |
| Distel | Quaternary ammonium compounds | Polymeric biguanide hydrochloride, didecyl dimethyl ammonium chloride, alkyldimethyl benzyl ammonium chloride | 2.0% | 1.0% | 0.20% |
| Bleach-Domestos extended power | Sodium hypochlorite | Sodium hypochlorite, non-Ionic surfactants, cationic surfactants | 3.6% (3.84 g in 50 ml) | 1.8% | 0.36% |

* Indicates percentage of manufacturers recommended rate

**Results**

All the disinfectants were completely effective at killing FOL4 chlamydospores at both 100% and 50% of recommended rates for all the contact times tested (Table 9). Unifect G and bleach were the only products that resulted in no spore survival for all rates (100%, 50% and 10% of recommended rates) and contact times (1, 5, 15, 30 min), suggesting they are the most effective disinfectants. However, all products were effective at the manufacturer's recommended rates. Treatment with Distel and Virkon resulted in some survival of FOL4 chlamydospores even after 30 min contact time at the 10% product rates with up to 12% and 10% spore viability respectively. For a number of treatments (data with asterisks, Table 9), FOL4 colonies were observed after 7 days incubation but not at 2 days, suggesting that some disinfectants at certain dilutions may delay FOL4 spore germination. Comparison between the two untreated control treatments indicated that there was a small loss of FOL4 chlamydospores when they were centrifuged / washed.

**Table 9.** Effect of different disinfectants on survival of FOL4 chlamydospores at 100%, 50% and 10% of the manufacturers recommended rates for contact times of 1, 5, 15 and 30 min. Data represents mean percentage of viable spores compared to the control (centrifuged / washed) for two independent experiments.

| Treatment | | Jet 5 | | Unifect G | | Bleach | | Huwa-San | | Distel | | Virkon | | Disolite | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time | Rate[1] | % | SEM | % | SEM | % | SEM | % | SEM | % | SEM | % | SEM | % | SEM |
| Control | Washed | 100.0 | 5.8 | 100.0 | 10.3 | 100.0 | 10.3 | 100.0 | 2.9 | 100.0 | 9.0 | 100.0 | 5.3 | 100.0 | 5.3 |
| | Not washed | 107.7 | 9.3 | 127.9 | 12.8 | 127.9 | 12.8 | 102.1 | 7.7 | 115.4 | 4.6 | 102.9 | 6.0 | 102.9 | 6.0 |
| 1 min | 100% | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 50% | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 10% | 1.7* | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4* | 0.1 | 10.4* | 2.3 | 10.3 | 0.9 | 1.5* | 0.1 |
| 5 min | 100% | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 50% | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 10% | 0.3* | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3* | 0.2 | 10.6 | 0.8 | 7.0* | 0.2 | 0.4* | 0.1 |
| 15 min | 100% | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 50% | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 10% | 0.1* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.3* | 2.0 | 4.2* | 0.3 | 0.0 | 0.0 |
| 30 min | 100% | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 50% | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 10% | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 12.1* | 0.7 | 1.1 | 0.1 | 0.0 | 0.0 |

[1] Percentage of manufacturer's recommended rate

* Values include additional germination of spores present at 7 days post treatment, not present after 2 days

## Objective 3.2 Test heat treatments against FOL4 chlamydospores *in vitro*

**Materials and Methods**

Soil containing FOL4 chlamydospores was added to 10 ml of SDW, vortexed and left to settle for 60 seconds before the supernatant containing the spores was pipetted off and retained. This suspension was adjusted to approx. $1 \times 10^4$ chlamydospores $ml^{-1}$ and 1 ml added to 2 ml tubes which were incubated at 20˚C, 40˚C, 50˚C, 60˚C and 70˚C in heating blocks for 5, 10, 15, 30 and 60 min (one tube for each time and temperature treatment). At the end of the treatment, tubes were removed and immediately placed on ice before the spore suspensions were diluted 1 in 10 in separate tubes. Each dilution was then pipetted and spread onto three replicate PDA plates containing 2 ml $L^{-1}$ chlortetracycline. Two control treatments which received no heat treatment were also set up, one of which was put on ice at the start of the experiment and the other kept at room temperature for the duration of experiment. PDA plates were incubated for 2 days at 25˚C after which the number of FOL4 colonies were counted and those that had little or no growth were checked again after 7 days of incubation. Percentage spore survival compared to the control (spores on ice) was calculated. Results were analysed by ANOVA using Genstat.

**Results**

There was a significant decrease in the number of surviving FOL4 chlamydospores with increasing temperature across all heat treatment durations (Fig. 12). At 70˚C, no spores survived for any of the treatment durations from 5 - 60 min and this was also the case at 60˚C, apart from the 5 and 10 min durations which resulted in 4.7% and 0.4% spore survival respectively (7 day incubation). At 50˚C, the number of FOL4 spores surviving decreased significantly with increasing duration from 86% after 5 min to 0.9% after 60 min. The percentage of viable spores after treatment at 20˚C and 40˚C was similar to the untreated control for durations of 5-15 min (>90% spore survival) but at 30 and 60 min, the number of spores surviving decreased at 40˚C to 79 and 63% respectively. The minimum heat treatment required to kill FOL4 spores was 60˚C for 15 mins.
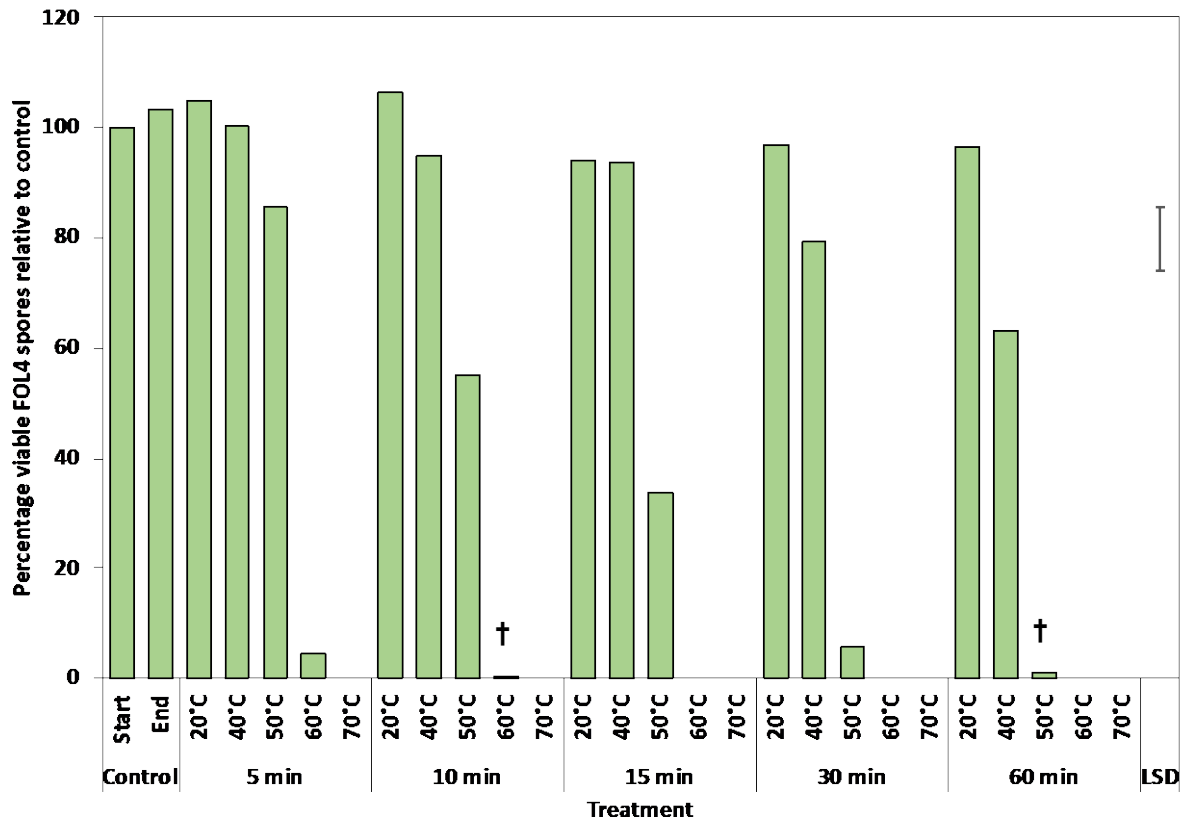
**Figure 12.** Effect of temperature and duration on survival of FOL4 chlamydospores relative to the control (spores on ice). Data is the combination of two independent experiments and the error bar represents least significant difference (LSD, P<0.05) following ANOVA. † represents treatments with additional spore germination after 7 days at 25˚C compared to 2 days.

## Objective 3.3 Test disinfectant and heat treatments against FOL4 chlamydospores on polypropylene

**Materials and Methods**

Polypropylene discs (approx. 3 cm in diameter) were cut from plant propagation trays in order to test the efficacy of disinfectants and steaming on FOL chlamydospores survival on a hard surface.

Effect of disinfectants on survival of FOL4 chlamydospores on polypropylene discs

A FOL4 chlamydospore suspension ($1 \times 10^4$ spores ml$^{-1}$) was prepared as described in Objective 3.1 and nine 5 µl drops pipetted onto sterile polypropylene discs and left to dry for 1 h at room temperature in a Class II laminar flow cabinet (Fig. 13a). The same seven disinfectants tested in Objective 3.1 were diluted with SDW to achieve 50% of the recommended rate (Table 8) and approx. 30 ml decanted into deep Petri dishes. Four polypropylene discs with FOL4 spores were then placed face down in each disinfectant for 5 min, and then washed three times in SDW. Control discs were also set up which were soaked in SDW water instead of disinfectant. Discs were dried on Whatman 3M filter paper and then placed face down onto PDA plates containing 2 ml L$^{-1}$ chlortetracycline and incubated at 25˚C. Plates were checked for FOL4 mycelial growth emerging from the discs after 3 and 7 days.

Effect of steaming on survival of FOL4 chlamydospores on polypropylene discs

A FOL4 chlamydospore suspension ($1 \times 10^4$ spores ml$^{-1}$) was prepared as described in Objective 3.1 and nine 5 µl drops pipetted onto sterile polypropylene discs and left to dry as before (Fig. 13a). SDW (300 ml) was heated in an 18 cm stainless steel kitchen food steamer (Wilko, UK) placed on a hot plate until the pan was full of steam (Fig. 13b). Four polypropylene discs with FOL4 spores were placed face up in the steamer rack for durations of 15 s, 30 s, 1, 2 and 5 min, after which they were removed and dried in the Class II cabinet. The temperature in the steamer was recorded and untreated control discs (no heat treatment) were also set up. Following treatment, discs were placed face down on PDA plates containing 2 ml L$^{-1}$ chlortetracycline and incubated for 7 days at 25˚C after which they were checked for emerging mycelial growth around.

Additionally, a hand-held steam cleaner (900W, Home Electric; Fig. 13c) was also used to investigate the effect of a jet of steam on FOL4 spores on polypropylene discs. As before, FOL4 spores were dried on discs after which were exposed to a jet of steam from a distance of 5 cm and 10 cm for 15, 30 and 60 seconds and 20 cm for 5, 15 and 30 s (three discs per treatment). Control discs which received no steam treatment were also set up. After treatment, discs were dried as before, and placed face down on PDA plates containing 2 ml L$^{-1}$ chlortetracycline and assessed for FOL4 growth after 7 days. The maximum temperature of the steam for each distance was also recorded.



**Figure 13.** a) dried FOL4 chlamydospore suspension dried on polypropylene discs; b) food steamer used to treat spores, c) handheld steam cleaner used to steam treat spores.

## Results

### Effect of disinfectants on survival of FOL4 chlamydospores on polypropylene discs

There was no mycelial growth from any of the polypropylene discs containing FOL4 spores treated with the disinfectants at 50% of recommended manufacturers rate for 5 min. After treatment with Virkon, bleach and Disolite, the dried spore suspension was no longer visible on the discs, unlike for the other disinfectants and the untreated control where all drops were still visible after treatment and washing. FOL4 growth was observed for all the untreated control discs treated with SDW only. This suggests that all the disinfectants tested were very effective at eliminating the risk of FOL4 spore contamination on plastics.

### Effect of steaming on survival of FOL4 chlamydospores on polypropylene discs

When polypropylene discs with FOL4 chlamydospores were steamed using the kitchen food steamer where the maximum temperature recorded was 100˚C, no mycelial growth was observed for any of the exposure times tested (Fig. 14). The dried spore suspension was still visible on the discs after treatment and untreated control discs resulted in growth of FOL4.

Similarly, where the handheld steamer was used, no FOL4 growth was observed for discs exposed to a jet of steam for 15 s, 30 s and 1 min and a distance of 5 cm or 10 cm where the maximum temperature recorded was 70 and 65°C respectively (Table 10). However, discs steam-treated at a distance of 20 cm where the maximum recorded temperature was 55°C resulted in mycelial growth of FOL4 after 7 days for all exposure times (Fig. 14). FOL4 mycelium was not present after 3 days, suggesting that the treatment may have delayed spore germination as untreated control discs resulted in growth after 3 days. These results are comparable with those from the heat-block experiment where FOL4 growth was still recorded when chlamydospores were exposed to temperatures of 50°C and 60°C, while no growth was observed for spores exposed to 70°C (Fig. 12).
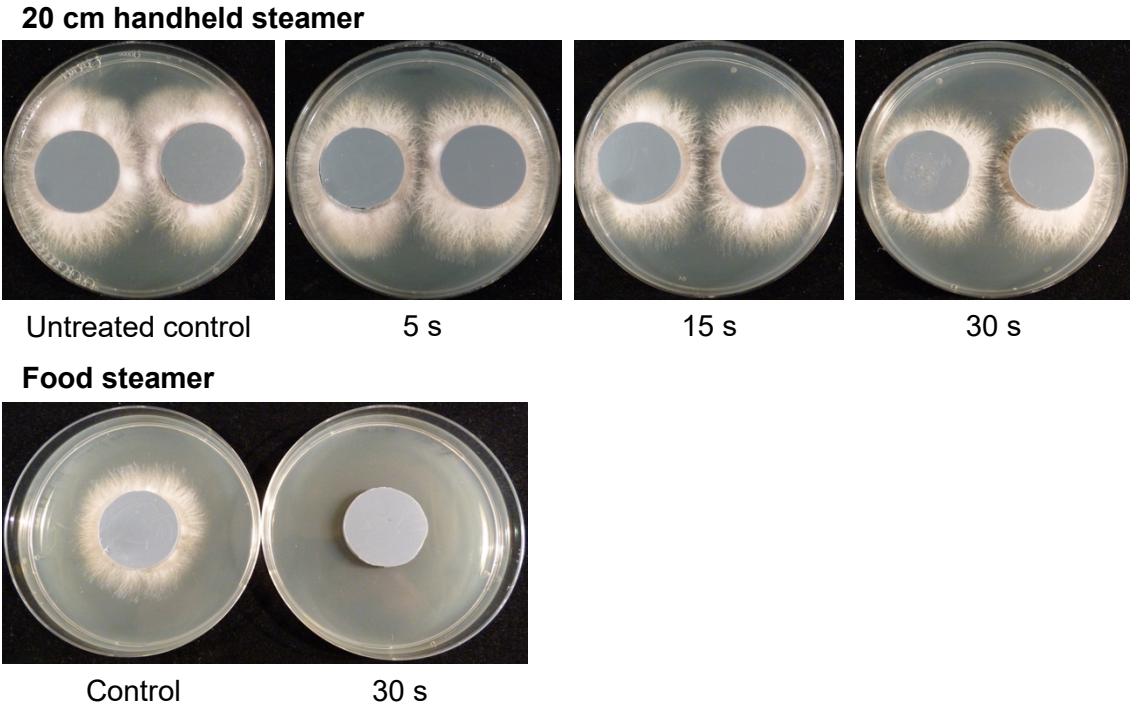
**20 cm handheld steamer**



| Untreated control | 5 s | 15 s | 30 s |

**Food steamer**



Control          30 s

**Figure 14.** Growth of mycelium from polypropylene discs on agar from surviving FOL4 chlamydospores treated with a jet of steam from a handheld steam cleaner at 20 cm for exposure times of 5, 15 and 30s (top) and no growth for spores steamed in a food steamer for 30 s (bottom).

**Table 10.** Temperatures at different distances from a held-held steam cleaner and the temperature of the metal steamer plate/steam in the vessel of a kitchen food steamer, both used to treat spores of *Fusarium oxysporum* f. sp. *lactucae* FOL4

| Treatment | Distance (cm) | Max temperature (°C) |
|---|---|---|
| Hand-held steamer | 5 | 70 |
| Hand-held steamer | 10 | 65 |
| Hand-held steamer | 20 | 55 |
| Food steamer | Steamer plate inside vessel | 100 |

# Discussion

## Objective 1: Develop tools for molecular detection and quantification of FOL/FOL4

Objective 1.1 Test LAMP assay for FOL / Objective 1.2 Develop and test qPCR for FOL

Different *F. oxysporum* f.spp. have a very high level of DNA sequence similarity in their 'core' genome meaning that they are difficult to distinguish based on sequencing of conventional 'house-keeping' genes present. Recently however, molecular diagnostic tools for *F. oxysporum* f.spp. are specifically targeting pathogenicity-related genes in the so called 'lineage-specific' areas of the genome which are important in conferring host specificity (Ma et al., 2010). This approach has been successful for identifying isolates and even different races of *F. oxysporum* f.sp. *cubense*, *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *phaseoli* affecting banana, tomato and bean respectively (Lievens et al., 2009; Fraser-Smith et al., 2014; de Sousa et al., 2015). The rapid increase in availability of genome sequences means that this approach is now feasible for an increasing number of different *F. oxysporum* pathogens.

Following genome sequencing of isolates of FOL1 and FOL4, identification of pathogenicity genes and comparison with other *F. oxysporum* genomes by NIAB-EMR, primers for a gene specific to FOL (both FOL1 and FOL4) were designed for use in both LAMP and qPCR assays while further sets of primers were designed for use in a qPCR assay to specifically detect FOL4 (Taylor, 2019). The new LAMP assay was highly specific to FOL, unlike a previously published test (Franco-Ortega et al., 2018) which resulted in some non-specific detection of other *F. oxysporum* f.spp. Similarly, the qPCR primer pairs targeting g23490 and g19968 gene targets were highly specific to FOL4 and FOL (FOL1 / FOL4) respectively. Both the new LAMP and FOL4 qPCR assays also successfully detected FOL4 in artificially inoculated diseased lettuce plants as well as in soil infested with the pathogen. Both tests have also since been used to confirm presence of FOL4 in lettuce samples from growers as part of ongoing monitoring of FOL4 in the UK at Warwick. These molecular tools will be invaluable not only for diagnostics and soil tests, but also for determining FOL4 dynamics and understanding key aspects of pathogen biology such as infection, colonisation and survival. The LAMP assay also detected FOL4 in diseased plant material in approx. 7 min following a crude 5 min DNA extraction allowing for very rapid disease diagnosis without the need for expensive reagents. The Genie II LAMP machine also has the advantage of being portable and could therefore potentially be used at grower sites to quickly identify FOL4.

Objective 1.3 Assess FOL viability in relation to molecular tests

To determine whether the FOL4 qPCR would detect DNA from dead spores, a preliminary test was carried out using spores killed by heat. DNA from the dead FOL4 conidia was shown to rapidly degrade in a sandy soil in just a few days, suggesting that if molecular tests such as LAMP or qPCR were used to monitor pathogen levels in soil, then they would likely only detect live spores. The detection of dead fungal pathogen material in soil due to the survival of DNA has often been debated although there are few studies investigating this possibility. DNA survival can be enhanced through binding to clay minerals, larger organic molecules, humic acids and other charged particles and compared with clays, sand has been found less effective in binding DNA, the primary explanation being its small surface area (Pederson et al., 2015). Therefore, further work would be required to determine if there are differences in FOL4 DNA survival in different soil types. Nonetheless, the evidence from these preliminary experiments suggests that FOL4 DNA does not survive for long periods in soil and therefore if molecular tests were employed for detection, they would most likely detect live FOL inoculum.

## Objective 2: Determine effect of temperature and inoculum level on FOL4 disease development and impact of non-hosts and fallow on FOL4 survival

Objective 2.1 Determine the effect of inoculum concentration on FOL4 disease development

A clear relationship was established between concentration of FOL4 inoculum and Fusarium wilt development in lettuce grown in both compost and soil with critical levels of $1 \times 10^4$ cfu g$^{-1}$ and $1 \times 10^5$ cfu g$^{-1}$ respectively required to cause substantial disease. These are similar to levels identified for basal rot disease development due to *F. oxysporum* f.sp. *cepae* in onion (AHDB project P1908312; Clarkson, 2020). The greater amount of FOL4 inoculum required to cause disease in soil could be due to some suppressive activity of the resident microbial community. Although there are no directly comparable studies, these results are similar to those of Hubbard & Gerrick (1993) and Scott et al., (2012) where a relationship was observed between FOL1 inoculum and disease development using a method where lettuce roots were dipped in conidial spore suspensions and grown in compost in the glasshouse. In both these studies, Fusarium wilt was evident at spore concentrations from $10^3$ spores ml$^{-1}$ with lettuce mortality increasing from $10^4$ to $10^7$ spores ml$^{-1}$. However, in a field study, soil adjacent to infected lettuce plants contained 50-300 FOL1 cfu g$^{-1}$ whereas soil adjacent to healthy plants, but close to the infected area, contained 5-27 cfu g$^{-1}$ (Gordon & Koike 2015). Further work is now required to directly relate soil FOL4 inoculum levels (as measured by cfu g$^{-1}$ through

plating) and disease development with qPCR results. This has been recently done for *F. oxysporum* f.sp. *cepae* on onion (AHDB project P1908312; Clarkson, 2020) and would allow results of soil tests to be directly related to risk of Fusarium wilt development in lettuce.

<u>Objective 2.2 Determine the effect of temperature on FOL4 disease development</u>

Although there was little difference between growth of FOL1 and FOL4 isolates on agar at different temperatures, high levels of Fusarium wilt disease developed between 12 and 28°C when lettuce plants were inoculated with FOL4 but this only occurred at 28°C for FOL1. Several studies have associated high temperatures (24-33°C) with FOL1 infection and disease development (Matheron et al., 2005; Scott et al., 2010; Paugh et al., 2019) but there have been no similar reports for FOL4. However, the rate of FOL4 disease development in lettuce in this study increased at higher temperatures and anecdotal evidence from growers in the Netherlands and UK does suggest that Fusarium wilt due to FOL4 is more damaging in Summer. Hence, affected growers have resorted to growing lettuce only in the cooler months of the year. Nonetheless the ability of FOL4 to cause disease at low temperature has been observed in UK protected lettuce grown in Lancashire in December 2017 (transplanted in October) where air temperatures were 8°C (Taylor & Clarkson, 2018). Similarly, in the Netherlands, it has been reported that losses of up to 70% can still occur in December (Taylor & Clarkson, 2018). This raises the possibility that FOL4 could cause disease in outdoor lettuce, and hence growers should be vigilant, although the less intense production system with the rotations employed may help prevent build-up of FOL4 inoculum in the soil. Further work is required to confirm the effect of temperature on FOL1 and FOL4 disease development across a range of different isolates and inoculum levels.

<u>Objective 2.3: Evaluate the impact of fallow and non-host cropping on FOL4 survival</u>

The survival of FOL4 in fallow soil from an artificially inoculated polytunnel was also assessed in this project both by qPCR and direct plating onto agar with the latter being quite variable. Nonetheless, both tests gave some indication of FOL4 inoculum levels but further more detailed work is required to link inoculum levels, qPCR results and potential for disease development. As expected, immediately following a summer lettuce crop (Crop 1) with a high incidence of Fusarium wilt, FOL4 was detected at an elevated level in the soil by qPCR but this declined by 96% three weeks after harvest of a following winter crop (Crop 2) with low disease. Thereafter, FOL4 inoculum in the soil remained relatively stable up to 10 months and this was confirmed by parallel tests using dilution plating where levels were approx. $10^3$ cfu g$^{-1}$ soil for most of this period. This is in principal below the threshold required to cause substantial Fusarium wilt based on our experiment examining the effect of different FOL4 inoculum levels on disease development. Nonetheless, it would be anticipated that successive

lettuce plantings would quickly increase this level of inoculum allowing the critical concentration for substantial disease development to be reached. These results could be explained by the presence of active mycelium, conidia and chlamydospores immediately following Crop 1 while in the fallow period after Crop 2, both mycelium and conidia potentially decline rapidly, leaving just long-lived chlamydospores. Chlamydospores are the principal means of *F. oxysporum* survival between crops and many studies have confirmed their longevity in soil (e.g. Couteaudier et al., 1990; Vakalounakis et al., 2004). A study with FOL1 in the USA showed that these spores can remain in fallow soil in the field for at least 2.5 years although the number viable decreased by 86% from 3.5 x $10^3$ to 500 cfu g$^{-1}$ after 12 months (Scott et al., 2012; Gordon & Koike 2015). However, it is likely that survival of FOL chlamydospores will vary with soil type, moisture level and resident microbial community. The soil in the FOL4 infested polytunnel in our experiment was left to dry out during the fallow period which may have prolonged spore survival as it has been shown that chlamydospores of *F. oxysporum* f.sp. *melonis* can survive for 17 years in dry soil stored at 3-4 °C (McKeen & Wensley 1961).

The potential colonisation of rotation / alternative crop plants by FOL4 as assessed by qPCR was also investigated. Here, high levels of the pathogen were detected in roots of inoculated susceptible lettuce (1695-6483 pg FOL4 DNA mg$^{-1}$ root) with reduced levels in resistant cultivars (15-467 pg FOL4 DNA mg$^{-1}$ root). Lower FOL4 levels were detected in the roots of other crop plants tested, with mizuna, pak choi, chard and cucumber most conducive to pathogen colonisation (20-34 pg FOL4 DNA mg$^{-1}$ root). This suggests that these other crops can sustain FOL4 populations. In the Netherlands, a grower reported that lettuce grown between pak choi crops showed no reduction in Fusarium wilt disease (Taylor & Clarkson, 2018), confirming the results here that this crop can be colonised by FOL4. However, initial observations in Lancashire showed that, after two crops of pak choi, Fusarium disease incidence was greatly reduced with only around 5% losses observed (Taylor & Clarkson, 2018). Further work is required to understand these reported inconsistencies. Similarly, in this study, FOL4 colonisation of spinach roots was low, while in contrast, Scott et al., (2014) reported extensive growth of FOL1 in this crop with 50% of the vascular tissue invaded compared to 7.4% for cauliflower and 0% for broccoli. Other research has also demonstrated that FOL1 can colonise the roots of tomato, melon and cotton (Hubbard & Gerik 1993). Our results also indicated that even when resistant lettuce cultivars are grown, FOL4 can still colonise roots and levels were higher than for the other crop plants. This was also demonstrated by Scott et al., (2014) where 71% of plants resistant to FOL1 still contained the pathogen in roots and vascular tissue. Overall therefore, growers should be cautious about how useful 'break' crops and resistant lettuce cultivars can be in reducing FOL4 inoculum.

## Objective 3: Test hygiene measures to eliminate FOL4 inoculum

<u>Objective 3.1 Test disinfectants for activity against FOL4 chlamydospores *in vitro*</u>

All the disinfectants evaluated in this project (Jet 5, Unifect G, bleach, Huwa-San, Distel, Virkon and Disolite) effectively killed FOL4 chlamydospores at 100% and 50% of the manufacturers recommended rates in as little as 1 min exposure time and are therefore all potentially useful for maintaining hygiene and eliminating the pathogen from trays, equipment work surfaces etc and for foot dips. However, the efficacy of these products in the presence of large amounts of soil or organic matter which can limit efficacy was not assessed. Nevertheless, results largely confirm those from previous studies with other *F. oxysporum* f.spp. (which mainly used conidia rather than the more robust chlamydospores). For instance, Disolite, Unifect G and Domestos bleach used at the manufacturers rates of 2%, 4% and 2.4% respectively and a 5 min exposure time were particularly effective at killing both conidia and mycelium of *F. oxysporum* f.sp. *matthiolae* affecting column stocks in the presence of small amounts (0.1%) of peat contamination and, with the exception of bleach, on a range of surfaces including glass, plastic, aluminium, concrete and woven ground-cover (Wedgwood 2015). Similarly, use of Sporekill® (International Chemicals), another quaternary ammonium compound similar to Unifect G, resulted in a 100% reduction in viability of *F. oxysporum* f.sp. *cubense* conidia at the manufacturers recommended rate of 1% with only a 30 s exposure time (Meldrum *et al.,* 2013). Quaternary ammonium compounds in several different disinfectant products as well as bleach were also found to be effective in another study using chlamydospores and conidia of *F. oxysporum* f.sp. *vasinfectum* affecting cotton even at 1% of the manufacturers rates and a 5 min exposure time. However, when soil contamination (10%) was introduced, only bleach was effective (Bennett *et al.,* 2011). Finally, studies using chlamydospores of *F. oxysporum* f.sp. *narcissi* affecting daffodil again indicated that Boot, another product containing quaternary ammonium compounds, was also effective even at low concentrations and with a 5 min contact time (Lillywhite, 2016). This level of control was reduced in the presence of organic matter (*Narcissus* scales as opposed to soil) but a 70-85% reduction in spore viability was still achieved at higher concentrations of Boot.

Overall, a range of products effective against FOL4 are therefore available to growers, allowing a choice to be made based on cost and situation. However, further work is needed to confirm their efficacy in the presence of soil and organic matter which potentially contaminates propagation trays and foot dips etc. Ideally however, growers should clean all equipment and surfaces before using disinfectants. Residues might also be an issue especially for quaternary ammonium compounds and it should be noted that the limit is 0.1 mg kg$^{-1}$ for lettuce (Taylor & Clarkson, 2018).

<u>Objective 3.2 Test heat treatments against FOL4 chlamydospores *in vitro*</u>

Heat treatment is an effective way of killing FOL4 chlamydospores and results showed that at 70°C, spores in a suspension of water were killed within 5 min. The minimum heat treatment required to kill FOL4 spores was 60°C for 15 mins although spore viability was only 4.7% after 1 min. This confirms results from previous research where most chlamydospores of *F. oxysporum* f.spp. *narcissi* were killed after 2 min at 60°C with all spores killed after 15 min (Lillywhite 2016). Unlike chemical disinfection, heat treatments are unaffected by soil contamination and have no residue issues and would therefore be potentially useful for propagation trays.

<u>Objective 3.3 Test disinfectant and heat treatments against FOL4 chlamydospores on polypropylene</u>

Efficacy of all the disinfectants tested in this project at 50% of the manufacturers recommended rates was confirmed on polypropylene discs with FOL4 chlamydospores, confirming their utility for sterilising propagation trays. Heat in the form of steam was also effective at eliminating FOL4 from polypropylene but when using a jet of steam, it was demonstrated that it is important that the distance and duration of this treatment is sufficient to achieve >65°C for at least 1 min which will depend on the equipment used.

# Conclusions

- Specific LAMP and qPCR based molecular diagnostic assays were successfully developed for FOL / FOL4 and utilised to identify and quantify the pathogen in diseased plants, roots and soil. These can now be employed (potentially on farm for LAMP) to identify further FOL4 outbreaks, potentially on farm and also carry out soil tests.
- Critical levels of FOL4 inoculum required to cause substantial Fusarium wilt in lettuce were determined in both compost and soil in artificially inoculated systems. Further work is now required to relate inoculum and disease levels to molecular quantification of the pathogen.
- FOL4 can cause substantial Fusarium wilt at temperatures as low as 12°C with more rapid disease development at higher temperatures up to the maximum of 28°C tested. In contrast, FOL1 only caused significant wilt at 28°C. Further work is required to confirm this across multiple isolates of FOL1 and FOL4.

- Following a lettuce crop with high levels of Fusarium wilt, there was an initial reduction in FOL4 inoculum in soil (to below threshold levels likely to cause disease?), but the pathogen then persisted for 10 months when the soil was left fallow. In this situation, it is likely that FOL4 disease would quickly increase if successive lettuce crops were grown.
- A range of non-host crop roots can be colonised by FOL4 with mizuna, pak choi, chard and cucumber the most conducive to the pathogen of those tested. However, colonisation levels were variable. Roots of lettuce varieties resistant to FOL4 were also colonised by the pathogen.
- The disinfectants Jet 5, Unifect G, bleach, Huwa-San, Distel, Virkon and Disolite were all effective at killing FOL4 chlamydospores in solution, even at 50% of the manufacturers recommended rates and 1 min exposure time. However, efficacy in the presence of soil contamination still needs to be assessed.
- FOL4 chlamydospores in water were killed within 5 min when exposed to a temperature of 70˚C and within 15 min at 60˚C. Heat treatments are therefore an effective way of eliminating pathogen inoculum.
- All disinfectants applied at 50% of the manufacturers recommended rates killed FOL4 chlamydospores on polypropylene within 5 min. Steam jet treatments also eliminated FOL4 spores but temperatures >65°C for at least 1 min were required.


## Knowledge and Technology Transfer

- Andy Taylor: Presentation 'Lettuce Fusarium wilt: potential management options' at Growing Media Developments in Vegetable Propagation meeting, Doddington, 09/10/18.
- Andy Taylor: Presentation 'Lettuce Fusarium wilt: potential management options' at BLSA meeting, Warwick Crop Centre, 16/10/18.
- Andy Taylor: Presentation 'Understanding and combating Fusarium diseases of onion and lettuce' at VeGIN meeting, Warwick Crop Centre, 27/11/18.
- Andy Taylor: Presentation 'Lettuce Fusarium wilt in the UK' at UK Brassica and Leafy Salads Conference, Peterborough, 23/01/19.
- Andy Taylor: Presentation Research on Fusarium basal rot of onion and other vegetable diseases' at Hutchinsons Veg Conference, Peterborough, 26/02/19.
- John Clarkson: Presentation 'Biology, diagnostics and control of Fusarium wilt of lettuce in the UK' at Fusarium growers meeting, PCG, Kruishoutem, Belgium, 03/10/19.

- John Clarkson: Presentation 'Biology, control and detection of Fusarium diseases in UK horticulture' at 34th Meeting of the Fusarium working group of the Koninklijke Nederlandse Planteziektenkundige Vereniging, Westerdijk Fungal Biodiversity Centre, Utrecht, The Netherlands, 30/10/19.
- John Clarkson: Presentation 'Lettuce, biology and management of Fusarium wilt at BLSA meeting, Harper Adams University, 14/11/19.
- John Clarkson: Presentation 'Biology, detection and control of Fusarium diseases in UK horticulture' at Hutchinsons Veg Conference, Boston, 21/01/20.
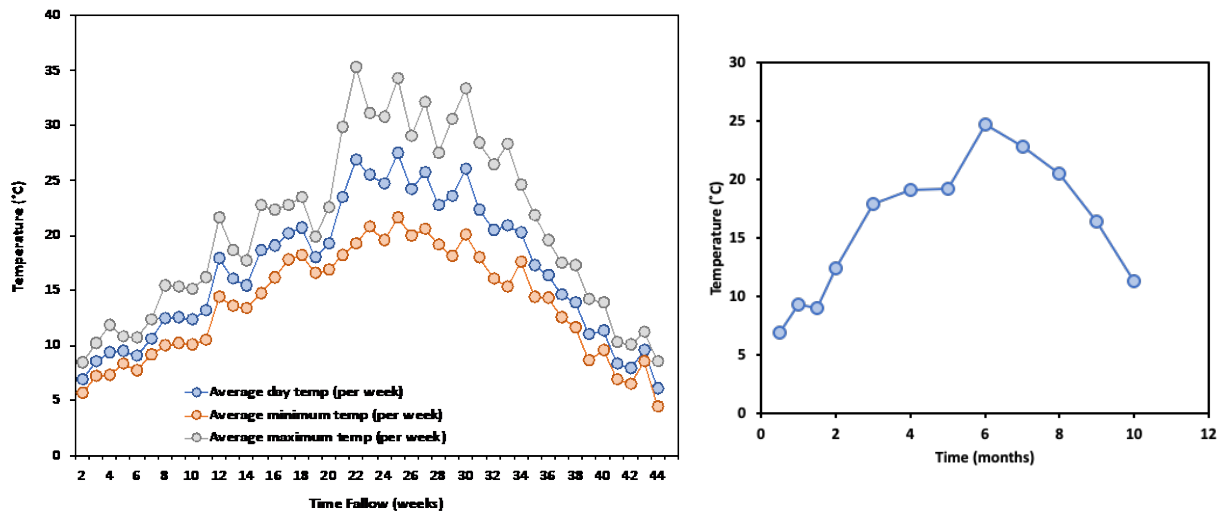
# Appendix



**Figure S1.** Temperature of soil in FOL4-infested polytunnel over the 10 month fallow period: a) mean, min and max daily temperature; b) mean temperature for the weeks that soil samples were taken for FOL4 qPCR and dilution plating.

**Table S1.** Amount of FOL4 DNA detected by qPCR in the roots of different crop hosts grown in infested compost for five replicate plants with mean and standard error of the mean (SEM). ND = not detected.

| Crop Host | FOL4 DNA pg (mg$^{-1}$ root) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Rep1 | Rep2 | Rep3 | Rep4 | Rep5 | Mean | SEM |
| Lettuce R1 | 37.1 | 7.1 | 12.3 | - | 3.0 | 14.9 | 7.6 |
| Lettuce R2 | 1393.9 | 6.8 | - | - | 1.4 | 467.4 | 463.3 |
| Lettuce Amica | 6291.0 | 1221.9 | 19688.5 | 3031.9 | 2184.9 | 6483.6 | 3409.4 |
| Lettuce Temira | 491.0 | 1377.5 | 1458.9 | 4762.8 | 384.6 | 1695.0 | 798.0 |
| Baby kale | 1.9 | 10.0 | 6.4 | 0.1 | 1.3 | 9.9 | 4.5 |
| Celery | 5.8 | 3.6 | 2.5 | 1.0 | 4.8 | 3.6 | 0.8 |
| Chard | 15.4 | 0.8 | 2.6 | 0.2 | 0.5 | 20.5 | 14.2 |
| Coriander | 3.4 | 2.4 | 4.1 | 6.6 | 0.5 | 13.0 | 2.9 |
| Cucumber | 12.5 | 41.7 | 14.6 | 4.5 | 27.9 | 20.3 | 6.5 |
| Lambs lettuce | 23.6 | 23.8 | 19.7 | 5.2 | 8.1 | 16.1 | 3.9 |
| Land cress | 1.8 | 12.0 | 3.6 | - | 6.1 | 5.9 | 2.0 |
| Mizuna | 30.8 | 20.6 | 66.6 | 21.6 | 32.0 | 34.3 | 8.4 |
| Pak choi | 27.6 | 13.2 | 8.6 | 10.2 | 67.1 | 25.3 | 11.0 |
| Rocket | 1.6 | 4.3 | 2.1 | 1.8 | 0.3 | 9.3 | 3.3 |
| Spinach | 4.1 | 0.8 | 2.1 | 0.6 | 2.5 | 6.3 | 1.7 |
| Lettuce Amica (control) | ND | ND | ND | ND | ND | - | - |

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