AHDB

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Project leader:	John Clarkson, University of Warwick
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Key staff:	Sascha Jenkins Sarah van Amsterdam Helen Bates (NIAB)
Location of project:	Warwick Crop Centre, School of Life Sciences, University of Warwick Wellesbourne Campus
Industry Representative:	Sam Rix
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The results and conclusions in this report include investigations conducted over a one-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.

Sascha Jenkins

Research Fellow

University of Warwick

Signature: *Sascha Jenkins* Date: 23/11/2023

Helen Bates

Research Scientist

University of Warwick

Signature: *Helen Bates* Date: 23/11/2023

Report authorised by:

John Clarkson

Professor

University of Warwick

Signature: *John Clarkson* Date: 23/11/2023

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GROWER SUMMARY

Headline

DNA based tests were developed to identify and quantify *Fusarium oxysporum* pathogens causing Fusarium disease for three important horticultural crops; onion, lettuce and Narcissus. In onion, detection of Fusarium DNA was enhanced in roots compared with soil but was improved in the latter samples by baiting / enriching the pathogen through growing onion seedlings. *F. oxysporum* could also be detected in asymptomatic onion bulbs before symptoms developed. After refinements, these tests could be used to determine the risk of Fusarium disease developing in all three crops through analysis of soil samples collected in advance. In the case of onion, the molecular test could also be used to assess risk of basal rot developing in store. In more fundamental work the build-up of *F. oxysporum* in sterile / non-sterile soil through successive rounds of lettuce growth was quantified as was the dynamics of the entire microbial community through mass (amplicon) sequencing. This approach allowed measurement of the relative abundance of fungi, bacteria, the entire *Fusarium* spp. community as well as applied biocontrol agents and in the future could be used to understand the dynamics of pathogens in relation to the microbiome. Finally products evaluated for control of Fusarium basal rot in onion in field trials were largely ineffective although Rudis increased the number of healthy bulbs.

Background

Fusarium oxysporum is a soil borne plant pathogen with a worldwide distribution and causes vascular wilt, root rot and bulb rot diseases in many economically important crops. *F. oxysporum* is a species complex comprised of over 150 *formae speciales* (f. spp.) which are adapted to infect specific crops such as onion, lettuce, leek, banana, tomato peas, brassicas and also narcissus and column stocks.

Fusarium wilt disease of lettuce

Fusarium wilt of lettuce, which is caused by *F. oxysporum* f. sp. *lactucae* (FOL), has spread to most production areas globally. There are four cultivar specific races, with race 1 and race 4 being the most widespread which cause severe economic losses in both field and protected crops respectively. In the UK, FOL race 4 (FOL4) was first identified in 2017 in Lancashire and Ireland, but has since spread to Cambridgeshire and Yorkshire, as well as locally within each area; so far FOL4 occurrence has been restricted to lettuce grown under protection. Disease symptoms include yellowing and necrosis of leaves, stunting and wilting of plants and reddish-brown/black necrosis of vascular tissue. There are currently no commercially

available resistant cultivars and therefore rapid spread between growers is being prevented through hygiene measures such as rigorous cleaning of equipment and glasshouses and by using foot dips/containment procedures for people moving from infected to clean areas. Growers have been mitigating disease impact through occasional use of the soil fumigant dazomet (Basamid), removal of contaminated soil or by abandoning affected growing areas.

Fusarium disease of onion

Fusarium basal rot of onion, caused by *F. oxysporum* f.sp. *cepae* (FOC), represents a major threat to the industry, with incidence levels increasing over the last few years. Recently basal rot losses have increased from 2-6% to over 10%, resulting in economic losses of approx. £13M. Many of these losses have been the result of disease developing in storage, where apparently healthy bulbs develop disease, which can result in worse cases to the whole consignment being abandoned as it becomes too costly to extract affected bulbs. Fungicides may have limited effect as FOC, like other f. spp. is soilborne, therefore it can be difficult to control once an area becomes infected. Early detection is key to prevent crops being grown in affected soils to reduce disease incidence as much as possible, and prevent bulbs being contaminated before going into storage. Hence in this project we will investigate the use of molecular diagnostics as a tool to assess disease risk for bulb onions pre-planting and also during the cropping period.

Fusarium disease of daffodil

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

Control of Fusarium diseases

As *F. oxysporum* is a soil borne plant pathogen, control is fundamentally difficult as it invades plants via the roots, and produces long lived chlamydospores which can survive in the soil for many years. Crop rotations are one of the most successful ways to avoid build-up of inoculum in the soil to levels capable of producing disease. However, there is increasing evidence to suggest that *F. oxysporum* can proliferate on non-host crops, therefore maintaining levels of inoculum which continue to increase when the host is again grown in the rotation. Fungicides usually have little effect; however, soil sterilisation or chemical fumigation is often used in protected crops to try and prevent disease occurring. These have been shown to reduce the

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levels of inoculum in the soil to below the required level for disease to occur, therefore reducing incidence and preventing losses. Unfortunately, they also negatively impact the microbial communities in soil which often act to suppress diseases and can therefore lead to *F. oxysporum* inoculum building up after fewer cropping cycles. This is a particular problem with crops grown under protection, such as lettuce, and multiple crops are often sown in the same location every year without rotation, therefore facilitating *F. oxysporum* proliferation. This project aims to investigate the potential of soil sterilisation techniques to suppress disease, but also to determine their effect on microbial communities and how this interaction relates to levels of disease. One approach to mitigate the negative effects of soil sterilisation is to introduce biological control agents, such as Trianum and T34, or Calcium cyanamide (Perlka) to encourage the recovery of microbial communities and suppress inoculum build-up; therefore the use of these treatments will be investigated in the project.

Project aims and objectives

In this project we will utilise molecular diagnostics developed at Warwick Crop Centre in a previous AHBD project FV POBOF 452 to determine the risk of Fusarium disease with a focus on FOC causing basal rot in onion but also with some testing of samples from Narcissus and lettuce. Initially, the relationship between Fusarium inoculum concentration, disease development and the amount of DNA detectable in the soil will be established through glasshouse experiments involving lettuce and narcissus grown in inoculated soils (this has been done previously for onion). We will then use these diagnostic techniques to detect the different *F. oxysporum* pathogens in soil samples to establish whether molecular diagnostics can accurately detect and predict Fusarium diseases in the field. In addition, large scale artificial inoculations will be used to screen multiple non-host plants to determine the extent of colonisation by *F. oxysporum*, as crops which enable proliferation of the pathogen should be avoided in rotations. Finally, microbial communities have been shown to be important for suppression of disease; therefore, we will investigate how to establish healthy suppressive microbial communities through soil amendments and biological control agents. Amplicon sequencing will enable quantification of microbial communities in comparison with *F. oxysporum* populations.

The overall aim of the project is:

To use molecular methods to determine the risk of Fusarium disease, select effective rotation crops and monitor *F. oxysporum* pathogens and associated microbial communities.

This will be achieved through the following objectives:

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1. Define a relationship between the amount of Fusarium DNA, Fusarium inoculum and disease development in soil
2. Evaluate the use of molecular diagnostics to detect and assess the risk of Fusarium disease in onion, lettuce and Narcissus
3. Evaluate the use of molecular diagnostics to determine the presence of FOC in harvested onion bulbs to assess the risk of disease development in store or pre-planting
4. Determine the colonisation of non-host plants by *F. oxysporum* pathogens to identify suitable rotation crops
5. Investigate the feasibility of establishing Fusarium-suppressive microbial communities and biological control agents in protected cropping systems
6. Employ amplicon sequencing to quantify *F. oxysporum* pathogens alongside suppressive components of the soil microbial community (Year 1 report, current report).
7. Evaluate products for control of FOC in field experiments

A summary of the main research findings over the three years of the project is presented in the section below. Experiments conducted in this final year of the project are reported in full in the Science Section, alongside a summary of results from Years 2 and 3 which can be found in full detail in the annual reports for Year 1 (2021) and Year 2 (2022)

Summary

Objective 1: Define a relationship between the amount of Fusarium DNA, Fusarium inoculum and disease development in field soil (dose response)

Pot experiments were carried out in the glasshouse where different soils were inoculated with varying levels of FOL or FON inoculum, lettuce / Narcissus grown, and disease development recorded. At the start of the experiments, the amount of *Fusarium* pathogen present was also quantified using specific DNA-based qPCR assays developed previously. In both experiments, there was a clear relationship between levels of pathogen inoculum, disease development and *Fusarium* DNA concentrations detected in soil. These quantitative molecular diagnostic assays were therefore successful in quantifying FON and FOL in field soils and with further development could potentially be used to assess risk of disease in Narcissus and

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lettuce. This would involve testing the approach on field samples from growers and comparing with observed levels of disease as done for the diagnostics for FOC in Objective 2.

Objective 2: Evaluate the use of molecular diagnostics to detect and assess the risk of Fusarium disease in onion

Soil and onion root samples were collected from grower fields across three growing seasons, between 2020-2022, to evaluate the use of the FOC specific qPCR assay for disease prediction. Onions were also assessed for disease over the growing season, at harvest and after a period of storage, so FOC detection could be related to the amount of disease at each site. Two approaches were taken: i) analysis of soil samples pre-drilling across multiple commercial onion field sites and ii) analysis of both soil and onion root samples at different timepoints during the growing season at two intensively sampled commercial onion field sites.

Over the project it became clear that FOC DNA was very rarely detected in pre-planting soil samples and hence a different onion seedling 'baiting' approach was examined (see below). For the intensively sampled onion field sites, FOC detection in soil was variable over time and was generally at low levels. In contrast, FOC detection was more frequent in onion roots and in much higher quantities over the growing season. Generally, an increase in frequency of FOC detection in soil and root samples was related to higher basal rot disease levels at harvest or in store. However, detection of FOC during the season is of limited value to growers and a pre-planting soil test would be more practical. As direct DNA extraction and qPCR was not sensitive enough to detect FOC in pre-planting soil samples from onion fields, we investigated growing onion seedlings in these soil samples as a means of 'baiting' out and allowing FOC to proliferate, hence potentially increasing the sensitivity of detection. Here, onion seedlings were grown for 7-8 weeks in soils from onion fields and maintained at 20°C. Soil was tested for FOC by qPCR at sowing while both soil and roots were tested when plants were harvested. Here it was found that there was a significant increase in FOC detection between sowing and harvest with higher levels of DNA detected and more onion field sites testing positive in the latter. Onion root samples also resulted in enhanced FOC detection, with DNA levels around 100-fold higher than in soils post-harvest and yet more onion field sites testing positive using this approach. This plant 'enrichment' for FOC could therefore be further refined to predict the risk of basal rot disease in onion using pre-planting soil samples from growers.

Objective 3: Evaluate the use of molecular diagnostics to determine the presence of FOC in harvested onion bulbs to assess the risk of disease development in store

Detection of FOC in onion bulbs by qPCR diagnostics is a potentially useful approach for assessing the risk of basal rot developing in store. Initially, we confirmed that qPCR could detect FOC in the basal plates of onion bulbs with different levels of basal rot symptoms but also in some asymptomatic bulbs. In follow up work we focussed on FOC detection in asymptomatic apparently healthy onion bulbs sourced from the *Fusarium*-inoculated quarantine field at Wellesbourne. Here, FOC was detected in 38% of asymptomatic bulbs while 51% of bulbs from the same batch went on to develop basal rot when incubated under conditions conducive to disease development. In one sample of apparently healthy onions from a high-risk grower site, around 10% of bulbs tested positive for FOC with 24% of the same batch developing basal rot after incubation. Overall therefore, qPCR diagnostics to detect FOC in onion basal plates is a promising approach to predict the likelihood of basal rot developing in storage which is a particular issue for growers, as only relatively low levels of disease are required for complete store loss. As DNA-based PCR based diagnostics was particularly useful for FOC detection in onion bulbs, we also evaluated a LAMP assay (a quicker version of PCR which uses crude DNA extracts) previously developed at Warwick for assessing presence of FOC in artificially inoculated onion bulbs. Here, results showed that we could detect FOC in basal plates as early as 8 days after inoculation before any symptoms appeared, which suggests that LAMP can detect FOC in asymptomatic onion bulbs as well as qPCR. In future work, the LAMP assay would need to be tested using apparently healthy onion bulbs from a highly infected commercial site to determine if detection was reliable and related to subsequent basal rot development. If successful, this approach provides a far more rapid method of testing bulbs for FOC as it uses a simple DNA extraction method from basal plate tissue and could also be done using a portable machine on site.

Objective 4: Determine the colonisation of non-host plants by *F. oxysporum* pathogens (FOC) to identify suitable rotation crops

F. oxysporum f.spp. spores can survive in the soil for many years, even in the absence of a host and hence lengthy crop rotations are usually required to allow inoculum levels to decrease to low enough levels for production. However, it has been noted recently by onion growers that even with long crop rotations, disease levels can remain high; therefore, it was hypothesised that FOC may be able to survive and even proliferate on non-host crops in the rotation. To test this, 12 plants of non-host crop species were grown in FOC inoculated compost and colonisation of roots examined by qPCR. FOC was found to colonise the roots

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of all the crops tested to varying degrees. For instance, FOC was detected in roots of 12 and 10 plants respectively for pea and maize compared to only three plants for oilseed rape. Roots of all 12 plants for a susceptible and resistant onion cultivar were also colonised. However, concentrations of FOC DNA in roots also varied but were far lower for non-host crops (on average >100-fold lower) compared with roots of the susceptible and resistant onion cultivars. Nonetheless, even some root colonisation of non-host crops over multiple years could maintain FOC levels at high enough concentrations in the soil to cause disease on onions next time they featured in the rotation. Therefore, it may be important to include crops where FOC colonisation was lower such as oil seed rape, barley and wheat, as opposed to crops where colonisation was more consistent (pea and maize).

Objective 5: Investigate the feasibility of establishing Fusarium-suppressive microbial communities and biological control agents in protected cropping systems (sterilised soil)

A method was developed to examine FOL4 inoculum build up and disease development in sterilised and non-sterilised soil with successive rounds of lettuce plant growth in pots. This system was used to determine whether FOL4 build up could be reduced or prevented by products such as Perlka, Trianum G and T34 Biocontrol using soil from a UK lettuce grower. All these treatments had some previous published evidence that they might have some activity against *Fusarium* disease. Over the three rounds of lettuce growth, it was clear that FOL4 inoculum build-up was much quicker in sterile soil compared to non-sterile soil but none of the treatments prevented this occurring or reduced disease development compared with a FOL4 only control. In the non-sterile soil at the end of the third round of lettuce growth, only mild to moderate Fusarium disease was observed in the lettuce compared with much more severe levels of disease for plants grown in the sterile soil. This was concomitant with the finding that there was a general reduction in microbial community diversity in sterilised soil and a predominance of the pathogen FOL4 as measured by amplicon sequencing (Objective 6). Quantification of FOL4 inoculum using qPCR revealed that FOL4 could be detected at harvest in sterilised soils only for the crop 1 and 2, with only very low levels detected in the non-sterilised soils by the end of crop 3, again confirming that that the pathogen proliferated rapidly in sterilised soil.

Objective 6: Employ amplicon sequencing to quantify *F. oxysporum* pathogens alongside suppressive components of the soil microbial community

Amplicon sequencing was successfully used to quantify the relative abundance of bacterial (16S gene target), fungal (ITS gene target) and *Fusarium* spp. (TEF1a gene target) communities in both FON and FOL4 experiments which examined effects of inoculum

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concentration in different soils (Objective 1). Moreover, the novel gene target OG4952 was also effective in identifying the presence of FON in soils at inoculation levels of $2x10^4$ spores g^{-1} soil and above in both soils tested. This technique is therefore useful in understanding how the microbial community reacts to the presence of *F. oxysporum* pathogens. The use of novel gene targets (such as OG4952) to specifically identify a range of different *F. oxysporum* f.spp. is challenging as often the sequences of these targets are identical for several *F. oxysporum* f.spp. or they result in unexpected positive detection even when it is known that the pathogen is not present. The presence of two paralogues for the OG4952 target in FON makes it a good identifier as both targets need to be present. In contrast, for the gene target (g19096) used to identify FOL4, it was found that high levels of endogenous *F. oxysporum* in some soils gave false positives. What is clear however is that the TEF1a is an excellent gene target for identifying *F. oxysporum* in soils with multiple *Fusarium* species present. In inoculated systems as investigated in this project, the abundance of a particular *F. oxysporum* f.sp. used in experiments can often be effectively quantified by TEF1a amplicon sequencing but only if the background level of other *F. oxysporum* isolates is low (as this target cannot distinguish f.spp) and inoculum levels are greater than $2x10^3$ cfu g^{-1} soil. Similarly, in some cases the ITS gene target can also be used as proxy for *F. oxysporum* abundance but only if levels of other *Fusarium* spp. are low (as this target cannot distinguish different *Fusarium* species). The establishment of a novel system to examine build up of FOL4 inoculum over three rounds of lettuce growth in both sterilised and non-sterilised soil with and without *Trichoderma* / Perlka treatments (Objective 5) gave us the opportunity in the final year of the project to examine the microbial and *Fusarium* communities over time. This generated a lot of data but there were some clear conclusions; firstly, the amplicon sequencing approach clearly identified big differences in the fungal / *Fusarium* spp. community between sterilised and non-sterilised soil and critically demonstrated that FOL4 inoculum builds up more rapidly in sterilised soil over the three cycles of lettuce growth which was supported by the much higher levels of disease in the final lettuce growth cycle. It was also demonstrated that the two *Trichoderma* isolates (T22 and T34) could effectively be detected by amplicon sequencing and that T34 and to a lesser extent T22 also proliferated more in the sterilised soil. As no disease control was observed for these treatments in Objective 5, this suggests that they were ineffective against FOL4 in this particular situation where they were added to the soil two weeks before transplanting for each round of lettuce growth. Another approach for the future might to be try and establish these organisms on the lettuce transplants before they are planted in FOL4 infested soil as they are both known to be efficient root colonisers. Nonetheless, the ability to track the relative abundance of bacteria, fungi, *Fusarium* spp. as well as *F. oxysporum* pathogens and specific *Trichoderma* biocontrol agents is a

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valuable tool to understand the complex interactions and dynamics of these different soilborne organisms.

Objective 7: Evaluate products for control of FOC in field experiments

Products were tested for their ability to prevent Fusarium basal rot of onion in four field trials carried out by ABC and VCS. A range of biological and chemical treatments were tested either as an in-furrow treatment at drilling or as a foliar spray. Application of Rudis in-furrow was the only product that appeared to consistently increase the number of healthy onion bulbs compared to an untreated control in fields with (generally) high Fusarium levels across several trials but this increase was not statistically significant.

Conclusions

- **Objective 1:** A clear relationship between inoculum concentration, *Fusarium* pathogen DNA levels in soil (measured by specific qPCR assays) and Fusarium wilt disease development was established for FOL4 in lettuce and FON in *Narcissus*. These molecular diagnostics could be used to determine the level of *Fusarium* inoculum in soil and determine the risk of severe disease.
- **Objective 2:** FOC was rarely detected by qPCR molecular diagnostics in soil samples collected pre-planting of onion crops but was more reliably detected in onion roots during the season. As this has limited practical value for growers, a system whereby onion seedlings were grown in pre-planting soil samples was developed that allowed FOC to proliferate and hence improved qPCR detection in both soil and roots. Further development of such a *Fusarium* 'enrichment' is likely to be a better approach for detection in soil and assessing disease risk.
- **Objective 3:** qPCR and LAMP based molecular diagnostics was effective at detecting FOC in asymptomatic onion bulbs. This could therefore be used as a method to detect FOC in harvested onion bulbs to assess the risk of disease in store.
- **Objective 4:** FOC was found to colonise the roots of all non-host crops tested with a higher incidence of the pathogen in pea and maize compared to the other crops. However, the amount of FOC DNA was much lower in all non-host crops compared with onions. This suggests that non-host plants may sustain FOC populations between crops of onions to different degrees.
- **Objective 5:** FOL4 proliferated more rapidly in sterilised soil compared to non-sterilised soil over three successive rounds of lettuce planting (in pots) in the same

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soil. Several products tested did not reduce this build-up of inoculum or reduce disease development.

- **Objective 6:** Amplicon sequencing successfully identified and quantified the relative abundance of the microbial community including bacteria, fungi and *Fusarium* spp. in soils inoculated with FOL4 and FON. Gene targets to identify multiple *F. oxysporum* f.spp. were identified but were not always reliable. In inoculated FOL4 and FON systems, the identification of *F. oxysporum* using TEF1a sequencing amongst other *Fusarium* spp. was generally a good indicator of the abundance of these pathogens.
- **Objective 7:** Rudis was the only product that consistently increased the number of healthy onion bulbs in Fusarium-affected fields but the effect was not statistically significant.

Financial Benefits

This project has considerable future potential financial benefits for growers if quantitative diagnostic tests for FOC, FOL and FON can be refined, validated and commercialised. In onion Approx. 450,000 tonnes of onions are produced annually in the UK by around 90 growers and in 2022, 8,800 ha were grown with around 3 /4 being direct drilled, where a pre-planting soil test would be most useful. FOC is becoming increasingly widespread, and losses in affected fields generally range from 2-12% with badly affected fields >30% equating to approx. ~£20M in lost crop. A diagnostic for FOC could help growers avoid cropping in fields with high levels of Fusarium and could also identify crops with a greater risk of developing disease in store, thus informing which crops will store best. If this approach could reduce losses by 10%, then this would still result in a return of £2M per annum to the industry. Other benefits include

- Reduced waste due to infected bulbs or over estimation of disease
- Reduction in imports to replace lost produce (370,000 tonnes of onions imported in 2022)
- Energy savings due to better disease management and control of storage crop
- Less CO2 and other inputs (fertilisers, crop protection products, diesel, labour) being wasted through loss of crop at harvest.
- A more sustainable onion producing sector in the UK leading to retention of jobs in the agricultural industry, which currently employs over 400,000 people in the UK.

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- Increases in resilience of UK onion production, including reversing the contraction of the sector, to move towards self-sufficiency in production.

This work has led to the funding of a Defra / Innovate project which aims to further develop molecular diagnostics to assess risk of Fusarium basal rot of onion in field or store to achieve these financial benefits. Similar benefits could be realised by both lettuce (farm gate value £220M) and Narcissus growers (farmgate value £30M, with exports worth over £20M).

Action Points

Growers should note that crops used in rotations may sustain populations of FOC in soil. Of the crop plants tested, peas and maize were particularly highly colonised by FOC.

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We would like to acknowledge the huge support from UK lettuce, onion and Narcissus growers as well as advisors and agronomists for supporting this project. We would particularly like to thank Andy Richardson (Allium and Brassica Centre) and Tom Will (Vegetable Consultancy Services) for all their support including supplying all the onion field soil / root and bulb samples and carrying out the field trials. We also thank Elsoms for providing onion seed and AHDB for funding.

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SCIENCE SECTION

Introduction

Fusarium oxysporum is the most widely dispersed and economically important plant pathogenic species in the *Fusarium* genus as it infects numerous hosts and causes extensive crop losses (Leslie and Summerell, 2006). In 2012 it was identified as 5th in a list of the top 10 fungal plant pathogens in terms of scientific and economic importance (Dean et al., 2012). *F. oxysporum* is responsible for a wide range of plant diseases, usually causing a vascular wilt but also causes root and bulb rots (Edel-Hermann and Lecomte, 2019). *F. oxysporum* is a species complex with over 150 'special forms' (*formae speciales*; f. spp.), adapted to infect specific hosts plants which affects many agriculturally important crops such as lettuce, onion, tomato, banana, leek, peas, melon and narcissus (Edel-Hermann and Lecomte, 2019). The complex also includes non-pathogenic *F. oxysporum* isolates. As *F. oxysporum f.spp.* are polyphyletic (Fourie et al., 2011; van Dam et al., 2018), isolates from one f. spp. may be more closely related to isolates infecting other hosts than to each other (O'Donnell et al., 1998) resulting in them being difficult to distinguish. The need for pathogenicity tests to determine host range and pathogenicity is very costly and time consuming. *TEF* is reliably used to identify *F. oxysporum* (Geiser et al., 2004) but cannot distinguish between f. spp.; therefore molecular approaches using effector gene targets have been investigated. Genes associated with pathogenicity in *F. oxysporum* f. spp., such as *Secreted In Xylem* (*SIX*) genes and other putative effectors have been the focus for developing molecular diagnostics due to their presence / absence or difference in sequence between different f. spp. (Lievens et al., 2009; van Dam et al., 2016).

Fusarium disease of lettuce

Fusarium wilt of lettuce is caused by *F. oxysporum* f. sp. *lactucae* (FOL) and affects lettuce production across many countries (Gordon and Koike, 2015). Symptoms of Fusarium wilt in lettuce are yellowing and necrosis of leaves, stunting and wilting of plants and reddish-brown/black necrosis of vascular tissue (Matheron, 2015). There are four cultivar specific races, with races 1 and 4 being the most widespread and the only two identified in Europe (Gilardi et al., 2017). FOL race 4 was first characterised in the Netherlands (Gilardi et al., 2017) and was subsequently reported in Belgium (Claerbout et al., 2018), the UK and Ireland (Taylor et al., 2019b) and very recently in Italy (Gilardi et al., 2019). So far FOL4 has been restricted to protected lettuce (grown under cover: polytunnels or glasshouses) and therefore mainly affects varieties such as butterhead and Little Gem. Due to this there are currently no commercially available resistant varieties, and this makes control of disease difficult. FOL, like

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many other *F. oxysporum* f. spp. produces long lived chlamydospores (Gordon and Koike, 2015), enabling inoculum levels to increase over time, especially in the case of FOL4 as protected lettuce production can be intensive with up to six crops a year in the same soil (Taylor et al., 2019b). Generally, FOL is managed with good crop hygiene procedures to prevent spread between fields and farms, crop rotations and soil treatments (chemical and heat/steam treatments) to try to reduce the pathogen pressure in the soil (Matheron, 2015).

Fusarium disease of onion

Fusarium oxysporum f. sp. *cepae* (FOC) is the causal agent of Fusarium disease in onion and symptoms can include seedling damping off, root/stem rot in immature plants and most importantly basal rot of bulbs resulting in significant losses (Entwistle, 1990; Taylor et al., 2013). In the UK, 2-6% of the onion bulb crop (8779 ha valued at approx. £132M in 2018; Defra, 2019) is lost each year in the field with a corresponding economic value of £7.9M. In addition, basal rot also occurs in storage as onions which appear healthy when harvested in the field go onto develop disease (Cramer, 2000). As with FOL, Fusarium disease in onion is very difficult to control, and although there are fungicide treatments available as a seed treatment (Cramer, 2000), they do not provide long lasting efficacy resulting in bulbs developing disease later in the season. Onion diversity sets have been developed to improve future onion breeding by identifying accessions with high levels of basal rot resistance (Taylor et al., 2019c), however, currently, there are no commercial onion varieties resistant to basal rot. Previous work at Warwick Crop Centre has developed a reliable diagnostic tool for identifying FOC in soil (Clarkson, 2018).

Fusarium disease of daffodil

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

Control of *F. oxysporum*

As mentioned previously, control of *F. oxysporum* is challenging, requiring long crop rotations and good hygiene practices to reduce inoculum concentration and prevent spread. However, even crop rotations are becoming difficult to manage as there is increasing evidence that *F.*

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oxysporum f. spp. can proliferate on non-host reservoir crops, enabling inoculum levels to remain high (Leoni et al., 2013; Scott et al., 2014). Black bean and corn (maize) were among the highest of 13 crops to enable FOC proliferation (Leoni et al., 2013), whereas broccoli, cauliflower and spinach were all found to support colonisation of FOL, in addition to resistant lettuce cultivars (Scott et al., 2014). Fungicide treatment can help control some level of disease, especially seed borne infection or seedling blight, however, their use for control in soil is very limited (Cramer, 2000; Gilardi et al., 2005). For FOC, seed treatments such as fludioxonil ± metalaxyl are available, but they do not provide long term control of FOC and offer no protection for bulbs from basal rot (Clarkson, 2018). In lettuce, azoxystrobin and fosetyl-aluminium have been shown to offer some reduction in disease severity (Gilardi et al., 2016), however, once FOL is present in the soil, the likelihood of complete control with fungicides is very low and they are only really useful as a preventative measure.

Managing soil health and a diverse microbial community has been shown to be important in combating *F. oxysporum*. Soils with high organic matter content were found to be more suppressive to flax wilt (caused by *F. oxsporum* f. sp. *lini*) due to the higher microbial biomass' resilience against disturbances, enabling it to maintain a more stable environment and suppress pathogens from proliferating (van Bruggen et al., 2015). Therefore, it has been suggested that organic amendments could be applied to soils to stimulate a diverse community of bacteria and fungi which aid in disease suppression (Baum et al., 2015).

Soil disinfection such as steaming/heat treatments and chemical fumigation can use used in protective cropping systems like glasshouses/polytunnels to help lower the inoculum levels of pathogens (Gullino et al., 2003). However, steam sterilisation is very energy intensive making it a potentially expensive and unappealing to most growers (Panth et al., 2020). However, an advantage of steam sterilising is that cropping can occur soon after treatment, unlike with chemical fumigation. It has been shown previously to reduce levels of fungi in soils more than with equivalent chemical treatments (Tanaka et al., 2003), however, it is also known to decrease microbial communities and cause an increase in nitrogen in the soil (Tanaka et al., 2003; Minuto et al., 2004). For control of soil borne pathogens, including *Fusarium*, this technique has been shown to be effective in controlling Fusraium wilt of tomato (Luvisi et al., 2008) and *F. oxysporum* f. sp. *basilici* and Rhizoctonia solani disease in basil (Minuto et al., 2004). Different methods of steaming have also been shown to be effective in reducing *F. oxysporum* f. spp. *raphani*, *conglutinans* and *basilici* under laboratory settings in artifically inoculated soils (Garibaldi et al., 2014). Steam needs to be applied regularly, every three or so cropping cycles (or less) as pathogens can re-establish quickly in sterilised soil due to the lack of a diverse microbial community which are known to naturally suppress disease development (O'Neill et al., 2005). A combination of soil sterilisation followed by the

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application of organic amendments could provide a solution to lower disease pressure and prevent *F. oxysporum* re-establishing rapidly.

There are a number of biological control agents which have been shown to be effective at controlling Fusarium diseases in different crops. One Trichoderma product containing *T. harzianum* strain T22 (Trianum G) has been shown to have a variable but significant effect on reducing disease severity in lettuces inoculated with FOL race 1 (Gilardi et al., 2007; Innocenti et al., 2015). Alternative strains of *T. harzianum* (JF419706) have also been shown to be effective in reducing disease severity of *F. oxysporum* infecting lettuces by 61%, and improved seed germination in inoculated compost from 40% (FOL only) to 71% (FOL + T. harzianum) (Alamri et al., 2019). In addition *T. asperellum* strain T34 (product name: T34 Biocontrol) has shown to be effective against *F. oxysporum* f. sp. *lycopersici* affecting tomatoes (Cotxarrera et al., 2002) and *F. oxysporum* f. sp. *dianthi* affecting carnations (Sant et al., 2010). Calcium cyanamide (Perlka) is applied as a fertiliser, but has been shown to have potential anti-fungal properties including against *F. oxysporum* infecting cucumber (Shi et al., 2009), although it had little effect in suppressing *F. oxysporum* infecting spinach (McDonald et al., 2021).

Project aims and objectives

In this project we will utilise molecular diagnostics developed at Warwick Crop Centre in a previous AHBD project FV POBOF 452 to determine the risk of Fusarium disease with a focus on FOC causing basal rot in onion but also with some testing of samples from Narcissus and lettuce. Initially, the relationship between Fusarium inoculum concentration, disease development and the amount of DNA detectable in the soil will be established through glasshouse experiments involving lettuce and narcissus grown in inoculated soils (this has been done previously for onion). We will then use these diagnostic techniques to detect the different *F. oxysporum* pathogens in soil samples to establish whether molecular diagnostics can accurately detect and predict Fusarium diseases in the field. In addition, large scale artificial inoculations will be used to screen multiple non-host plants to determine the extent of colonisation by *F. oxysporum*, as crops which enable proliferation of the pathogen should be avoided in rotations. Finally, microbial communities have been shown to be important for suppression of disease; therefore we will investigate how to establish healthy suppressive microbial communities through soil amendments and biological control agents. Amplicon sequencing will enable quantification of microbial communities in comparison with *F. oxysporum* populations.

The overall aim of the project is:

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To use molecular methods to determine the risk of Fusarium disease, select effective rotation crops and monitor *F. oxysporum* pathogens and associated microbial communities.

This will be achieved through the following objectives:

1. Define a relationship between the amount of Fusarium DNA, Fusarium inoculum and disease development in soil.
2. Evaluate the use of molecular diagnostics to detect and assess the risk of Fusarium disease in onion.
3. Evaluate the use of molecular diagnostics to determine the presence of FOC in harvested onion bulbs to assess the risk of disease development in store or pre-planting.
4. Determine the colonisation of non-host plants by *F. oxysporum* pathogens to identify suitable rotation crops.
5. Investigate the feasibility of establishing Fusarium-suppressive microbial communities and biological control agents in protected cropping systems.
6. Employ amplicon sequencing to quantify *F. oxysporum* pathogens alongside suppressive components of the soil microbial community.
7. Evaluate products for control of FOC in field experiments.

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Materials and methods

Objective 1: Define a relationship between the amount of Fusarium DNA, Fusarium inoculum and disease development in field soil (dose response)

Effect of *Fusarium* inoculum concentration on disease development in lettuce and Narcissus and pathogen detection

Glasshouse experiments were conducted to determine the relationship between the concentration of FOL4 and FON inoculum on disease development in lettuce and narcissus respectively, and the amount of DNA detected in different soils and in the roots post inoculation.

FON and FOL inoculum were used to inoculate soils at different concentrations and dispensed into pots before narcissus bulbs or lettuce seedlings were transplanted. Plants were assessed for symptoms and root samples were collected and frozen at multiple time points post inoculation to assess pathogen root colonisation with qPCR. DNA was also extracted from soils for comparison with inoculum levels and disease development. For full details see previous annual reports (Clarkson, 2021; 2022).

Statistical analysis was carried out on the average wilt and vascular browning score (lettuce) or bulb rot score (narcissus) using Analysis of Variance (ANOVA) at the final time point. Means were compared using the least significant difference (LSD) at 5% level. DNA quantity detected in soils and roots was log transformed before using in ANOVA.

Objective 2: Evaluate the use of molecular diagnostics to detect and assess the risk of Fusarium disease in onion

In order to determine the utility of a molecular qPCR test for FOC in assessing disease risk in the field as well as to examine pathogen dynamics, soil samples were collected from different commercial onion fields pre-planting while at two intensively sampled sites both soil and root samples were collected pre-planting and at seven different timepoints during the season for two years (2020 and 2021). Soil and root samples were used for DNA extraction and FOC specific qPCR as described previously in CP204 annual report March 2021 and CP204 annual report March 2022 (Clarkson, 2021; 2022). Fusarium disease was also assessed during the season and in harvested bulbs (carried out by Vegetable Consultancy Services - VCS and the Allium and Brassica centre - ABC). In this final year of the project, a reduced selection of field

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sites and time points were sampled for roots and soil in 2022, with a total of four sites (ABC: FP1 and PGR L/A; VCS: TUN and HAR) being sampled at three time points throughout the season (FP1 and PGR L/A were only sampled twice). As before, soil and root samples were tested for presence of FOC following DNA extraction qPCR assay and compared to disease levels observed in the field and after storage.

Onion plant baiting to improve FOC detection in soil

In 2021, soil samples were collected pre-planting from commercial onion field sites and used to test whether growing onion seedlings and performing DNA extraction and qPCR on both soil and onion roots after 7-8 weeks would result in better detection of FOC than if initial soil samples were used. The full methods for this experiment are described in CP204 annual report March 2022 (Clarkson, 2022). Briefly, soil from 14 commercial onion field sites was dispensed into pots, before four onion seeds were sown per pot. A FOC free soil (Soakwaters field, Wellesbourne, UK) and a heavily FOC infested soil (inoculated area of Quarantine Field at Wellesbourne, UK) were included as negative and positive controls respectively. Roots were harvested and pooled before using for DNA extraction and qPCR. In 2022, soil samples were collected pre-planting from 13 commercial onion field sites. The soil from each field site was sieved (4 mm mesh, and mixed with medium grade vermiculite in a ratio of 4:1 (soil:vermiculite). Soil was dispensed into 7 cm pots and watered before sowing ten onion seeds per pot. Three additional treatments were included using course loamy soil from the Wick series from the University of Warwick Wellesbourne campus (Soakwaters field): uninoculated soil as a FOC free control, soil from the FOC infested Quarantine Field at Wellesbourne (QF) and a 1:1 mix of the uninoculated soil and the QF soil. Pots were maintained at 20˚C, 16 hr light, for 7.5 weeks. Roots were then harvested and pooled together from each pot before being washed, blotted dry and flash frozen in liquid nitrogen. Soil samples were also collected before and after growing the onion seedlings. Twelve pots of onions (up to four pooled plants per pot) were used for each soil from onion fields for DNA extraction (including all the control samples: uninoculated soil and QF). Root material was ground in liquid nitrogen and DNA extraction performed using a Qiagen DNeasy plant mini kit as above. DNA was used in qPCR reactions (20 µL reactions) set up as above with q*SIX*5 primers (Taylor et al., 2016) to determine whether FOC present in the soil had colonised the roots. For samples where DNA was detected, a three-way contingency table was used to determine whether baiting with onions improves DNA detection in soil. This was defined by soil type, whether DNA was detected or not and when the sample was taken and was analysed using a log-linear model.

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Objective 3: Evaluate the use of molecular diagnostics to determine the presence of FOC in harvested onion bulbs to assess the risk of disease development in store

Determining the presence of FOC in onion bulbs with different levels of Fusarium basal rot symptoms

In year 1 onion bulbs were collected from commercial fields or stores and assessed for presence of basal rot. Bulbs were cut open, scored for symptoms and then samples excised from the basal plate and scales of all bulbs, regardless of disease presence. Samples taken from each half were used either for agar plating or qPCR to determine the presence of FOC. Remaining apparently healthy bulbs were incubated at 20˚C for 8 weeks to determine if bulbs developed basal rot. In year 2, bulbs were collected fresh from grower fields before being dried or stored. Bulbs were cut in half and basal plate samples were collected and frozen for DNA extraction and qPCR from healthy bulbs only (infected bulbs were scored but discarded). The remaining bulbs were dried then incubated as above to assess the development of basal rot in storage.

Determining the presence of FOC in healthy onion bulbs from *Fusarium* affected fields

This year the approach was to compare levels of FOC detected in apparently healthy bulbs from a high-risk grower site to onions grown in a known heavily infected QF field at Warwick Crop Centre (see Objective 2). As with last year, bulbs were cut in half, photographed and basal plate samples were collected from healthy bulbs only, flash frozen and used for DNA extraction using the Qiagen DNeasy plant mini kit and qPCR using FOC specific primers (Clarkson, 2021). One hundred bulbs were cut open from each site and samples taken from those assessed to be healthy. A further one hundred bulbs (assessed externally as healthy) from each site were incubated at 25˚C for approximately 7 weeks to determine whether Fusarium basal rot would develop.

Evaluating LAMP for detection of FOC in inoculated onion bulbs

In this new milestone, onion bulbs (cv. unknown, from wholesaler) were inoculated with pathogenic FOC isolate FUS2 and the ability of a LAMP assay (a rapid form of PCR using crude DNA extracts) evaluated for detecting FOC at different timepoints post-inoculation. To inoculate the bulbs, 2-3 mm of basal plate was cut off and the whole bulbs sprayed with 70% ethanol before being left to dry. An 8 mm agar plug containing the actively growing edge of isolate FUS2 was placed inverted on the cut surface of the basal plate. Control bulbs were cut

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and sterilised but had no agar plug applied. Bulbs were placed in a damp box inside a sealed bag at 20˚C for 48 hours before being wrapped individually in cling film. They were replaced in the box/bag and incubated at 20˚C until removed for sampling. Five inoculated and three control bulbs were harvested at 4, 8, 11, 15, 18, 22, 25, 29, 32 and 36 days post inoculation (dpi). Onion bulbs were cut in half, photographed, and one half of the bulb sampled for LAMP. Here, two tissue samples were used for analysis; i) the mid / upper portion of the basal plate – to determine colonisation of the basal plate by FOC (bottom part with agar plug removed) and ii) a piece of the onion scale excised from a set position on one half of the bulb – to determine successful colonisation of onion tissue by FOC from the basal plate. Crude DNA extractions were carried out in 1 mL of KOH extraction buffer (60g PEG 200, 0.93 mL 2M KOH and 39 mL water, pH adjusted to 13.3-13.5) and homogenised twice in a FastPrep-24™ machine set at 6.5 ms^{-1} for 1 min, with a ceramic bead. Extracts were diluted 1 in 100 in sterile water using a sterile 10 µL loop.

Initially a calibration curve was conducted using genomic DNA extracted from a culture of FOC isolate FUS2. DNA was diluted to 10, 1, 0.1, 0.005, 0.001, 0.0005 and 0.0001 ng μL^{-1} and 5 µL used to set up LAMP reactions. The extracted DNA from onion bulbs was then used in LAMP reactions containing 5 µL of DNA, 2.5 µL of primer mastermix (2 µM F3/B3, 4 µM LoopF/LoopR, 16 µM FIP/BIP primers made up in sterile water), 15 µL of isothermal mastermix ISO-001 (Optigene) and 2.5 µL of sterile water in strip cap tubes (OP-0008, Optigene). Strips were placed in a Genie II LAMP machine and run at 64°C for 40 mins followed by the anneal curve analysis (98°C for 1 min reducing by 0.05°C/s to 80°C).

Objective 4: Determine the colonisation of non-host plants by *F. oxysporum* pathogens to identify suitable rotation crops

Different crops which may regularly be included in rotation with onion were grown in FOC inoculated compost to determine the ability of FOC to colonise the roots of non-host plants. FOC inoculum (isolate FUS2) was prepared in a compost/bran mix as described by Taylor et al. (2013) and incorporated into John Innes No.3 compost (Bathgate Horticulture) to achieve a concentration of 1 x 10^5 cfu g^{-1}. Seven non-hosts of FOC (barley, brown mustard, maize, oil seed rape, pea, sugarbeet and wheat) as well as both a susceptible and resistant cultivar of onion were grown for 1-3 weeks to a similar plant growth stage (approximately two true leaves) before being transplanted into the inoculated compost. A non-inoculated susceptible onion cultivar was also included as a control. Twelve replicate pots were set up for each plant type including the control. Additional pots of compost were included which contained no plant to

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examine FOC proliferation in the absence of a plant. Inoculated plants were placed in a glasshouse compartment at 25˚C/18˚C day/night with a 16hr photoperiod and monitored for any symptoms for approx. 4 weeks. At harvest, the roots were cut away, washed and flash frozen in liquid nitrogen. Compost from each plant host was combined from all pots, mixed and dried before a sample was collected for FOC detection. Roots were freeze fried and used for DNA extraction using a DNeasy plant mini kit (QIAgen) as described in the first year annual report (Clarkson, 2021). Compost samples were used for DNA extraction using the GeneAll Exgene Soil SV kit (Cambio), as described in Clarkson, (2021). Root and soil DNA were used for qPCR with FOC specific primers (Clarkson 2021 and 2022) to determine the amount of FOC DNA present.

Objective 5: Investigate the feasibility of establishing Fusarium-suppressive microbial communities and biological control agents in protected cropping systems (sterilised soil)

A system was developed to examine the build-up of FOL4 inoculum and disease development in successive rounds of lettuce growth in sterilised and non-sterilised soil (CP204 annual report March 2021; Clarkson 2021). After successful method development an experiment was set up this year to examine if products could slow down or prevent FOL4 proliferation in three successive rounds of lettuce growth.

Effect of treatments on FOL4 inoculum build up and disease development

FOL4 free soil ((loamy sand) was obtained from a lettuce growing site in the UK which was dried and sieved through 4 mm and 2 mm mesh. The moisture content of the soil was determined, and water was added so the soil held together when compressed by hand (approx. 75% field capacity). Some of the soil was sterilised in a Camplex Plantcare Soil Steriliser (Cat no, HD5116, Thermoforce Ltd, Essex, UK) following the manufacturers operational instructions. The soil was heated to 71˚C (in approx. 1.25 hours), then switched off where it remained sealed until the temperature reached 82˚C (approx. 1.5 hours). Sterilised and non-sterilised soil was then mixed 4:1 with medium grade vermiculite. FOL4 inoculum was prepared as described by (Taylor et al., 2013) and used to inoculate sterilised/non-sterilised soil to achieve a final concentration of 1 x 10^2 cfu g^{-1} (a level previously identified as not causing disease symptoms in lettuce). Three treatments (Perlka, T34 and Trianum G (T22)) were applied separately to both the sterilised and non-sterilised soil (Table 1), with the

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addition of a FOL4 only treatment and a non-inoculated treatment (control) for each soil with ten replicate pots for each treatment. Soil was used to fill ten replicate 9 cm pots which were minimally watered and left to stand for two weeks at 25˚C (16 hr light), to allow the calcium cyanamide (Perlka) treatment to convert to urea in order to prevent any phytotoxicity to transplanted lettuce. Two to three week old lettuce plants (cv. Amica) were then transplanted into the different soils and were scored for Fusarium wilt symptoms, and vascular browning at harvest (Table 2).

After harvest of each round of lettuce, soil from each treatment was combined and mixed in a 1:1 ratio with fresh sterilised or non-sterilised soil. The treatments of Perlka, T34 and T22 were reapplied, and the soil used to fill 11 cm pots (increased size due to the amount of root material now present in the soil mix). Lettuce seedlings were transplanted as above, and wilt symptoms scored until harvest where internal vascular browning was assessed (Table 2). Three rounds of lettuce were grown in total, with the dilution step and application of treatments between each crop.

Soil samples were collected during each round at pot set up (treatment application), lettuce transplant and lettuce harvest. DNA was extracted from the soil samples (as above; Clarkson, 2021) and used for qPCR with FOL4 specific primers g23490 F3/R to determine changes in pathogen in the different treatments.

Table 1. Soil treatments used to prevent or slow down the proliferation of FOL4 in sterilised and non-sterilised soils.

Treatment	Active ingredient	Supplier	Application rate	Product applied per pot (mg)
Perlka	Calcium cyanamide	Alzchem group	300 kg h^{-1}	201.72
T34	*Trichoderma asperellum*, strain T34	Fargro	10 g m^{-1}	6.72
Trianum-G (T22)	*Trichoderma harzianum T-22*	Koppert Biological Systems	375 g m^{-1}	252.15

Statistical analysis was carried out on the average wilt and vascular browning score using Analysis of Variance (ANOVA) at harvest for each lettuce crop. Means were compared using the least significant difference (LSD) at 5% level.

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

Score	Disease/ wilt scale		Score	Vascular browning scale
0	Healthy		0	No browning
1	1 – 2 leaves wilted		1	Mild vascular browning
2	10% wilt (3 leaves or more)		2	Vascular browning
3	10 – 25% wilt		3	Severe vascular browning (SVB)
4	25 – 50% wilt		4	Dead
5	50 – 75% wilt			
6	75 – 99% wilt			
7	100% wilt			
8	Dead			

Objective 6: Employ amplicon sequencing to quantify *F. oxysporum* pathogens alongside suppressive components of the soil microbial community

Amplicon sequencing is a technique used to identify all components of the microbial community by mass sequencing a common gene target. We used this approach to try and identify the presence of *Fusarium oxysporum* f. sp. *cepae*, *narcissi* and *matthiolae* (FOC, FON and FOM) in soil samples from diseased fields as part of a previous AHDB project FV POBOF 452 (Clarkson, 2019) using novel gene targets while at the same time identifying the abundance of bacteria (using 16S gene target), fungi (using ITS gene target) and *Fusarium* spp. (using TEF1α gene target). While use of these standard gene targets allowed good identification of different members of the bacterial, fungal and *Fusarium* spp. community, the use of the novel gene targets to specifically target FOC, FON and FOM was more problematic as in some cases false positives for presence of these *Fusarium* pathogens in some samples, likely due to amplification of other unknown f.spp. or non-pathogenic *F. oxysporum*.

In this project we aimed to use the same approach to identify bacterial, fungal and *Fusarium* spp. communities in soils artificially inoculated with different amounts of FOL4 or FON inoculum. Here, the artificially inoculated soils used for these analyses were sourced from the experiments set up to relate the amount of FOL4 / FON inoculum to disease development and the amount of pathogen DNA detected by pathogen-specific qPCR assays (Objective 1). We previously demonstrated that the bacterial, fungal and *Fusarium* spp. communities could be effectively identified in three different soils artificially inoculated with different inoculum levels of FOL4 and also that we could successfully identify the abundance of FOL4 itself using a newly designed amplicon target (g19096) in two of the soils (Soils 1 and 3; see previous

annual reports 2021, 2022). However, a high background of *F. oxysporum* in the soil fungal community in Soil 2 (as shown by ITS and TEF1α amplicon sequencing) meant that cross-reactivity with endogenous members of the soil community masked the presence of the FOL inoculum in this particular soil.

The remaining work in this objective aimed to use the same approach to:

1) Identify the bacterial, fungal, *Fusarium* spp. communities as well as FON in two soils inoculated with different levels of the pathogen (see Objective 1).
2) Identify the bacterial, fungal, *Fusarium* spp. communities as well as FOL4 in an experiment testing the ability of fungal biocontrol agents to reduce the build of the pathogen in both sterile and non-sterile soil (see Objective 5).

<u>Amplicon sequencing of soils inoculated with FON</u>

Amplicon sequencing was carried out for the two soils from Objective 1 (S1 = Soil 1, S2 = Soil 2) which were infested with different concentrations of FON isolate 139 (D1=0, D2=$2x10^2$, D3=$2x10^3$, D4=$2x10^4$, D5=$2x10^5$, D6=$2x10^6$ cfu g^{-1} soil) and where disease levels were recorded over time.

<u>Soil DNA Extractions</u>

Soil DNA extractions were carried out as described in previous annual reports (2021, 2022) using the Soil SV kit (GeneAll, UK). Briefly, extractions were carried out in triplicate for each soil sample (total 1.5g soil) and pooled. To remove PCR inhibitors and further clean up the template DNA, a clean-up step using 0.4x volume Mag-Bind Total Pure NGS (Omega BioTek) was carried out. DNA was then diluted to 2ng/μl for use in amplicon sequencing PCR reactions.

<u>DNA library preparation, PCR and amplicon sequencing</u>

Illumina dual-index DNA library preparation, PCR and amplicon sequencing was carried out as described in previous annual reports (2021, 2022). Briefly, first round target PCR reactions were carried out using 10ng DNA in a 25μl reaction using KAPA HiFi-HS 2x master mix (Roche, UK). Libraries were pooled for barcoding as described previously (ITS with 16S, TEF with OG4952 and g19096). Barcoded libraries were bead-cleaned and normalised to 4nM before pooling and loading on the MiSeq at 8ρM on a V3 2x300bp flow cell.

<u>Amplicon sequencing data analysis pipeline</u>

The sequence data generated was analysed as described previously in AHDB project FV PO BOF 452 (Clarkson, 2019) Illumina reads are first demultiplexed by barcode and each read

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assigned to a target amplicon based on a 100% match to one of the primer sequences for that target. Forward and reverse reads of a sequence were merged and barcodes removed. Reads are quality filtered and assigned to Operational Taxonomic Unit (OTU, clustered sequences based on shared similarity threshold). Reads were quantified against identified OTUs and summarised by genus, species or *F. oxysporum* f. spp. Reads were mapped with USEARCH at 97% sequence identity for 16S and ITS and 100% identity for mapping to TEF1α, OG4952 and g19096. Reads were normalised to 1000 reads per sample to account for differences in sequencing depth (total read number) between samples. Counts from three technical replicates were combined to produce a mean and standard error of the mean was also calculated.

Amplicon sequencing of sterilised and non-sterilised soils inoculated with FOL4 with and without the addition of control treatments

Amplicon sequencing was also carried out for the soil samples from Objective 1 where the build-up of FOL4 inoculum and corresponding disease levels were examined over three rounds of lettuce growth in pot experiments in both sterilised and non-sterilised soil with and without treatments of Perlka (calcium cyanamide), and *Trichoderma* biocontrol products Trianum G (*T. harzianum* T22) and T34 Biocontrol (*T. asperellum* isolate T34). Soil samples were taken at three time points in the experiment for each of the three rounds of lettuce grown in sterilised / non-sterilised soil for each treatment; at set up (post-sterilisation of soil) and after introducing FOL4 and the control treatments, at lettuce transplanting (2 weeks later) and at harvest. All soil DNA extractions, PCR and Illumina sequencing were then carried out as described above. Soil DNA extractions were carried out in triplicate and pooled, while PCR reactions of each sample were carried out in triplicate and pooled prior to barcode labelling to reduce the cost and number of sequencing flow cells required. To simplify presentation of results below, some data are pooled and specific sets of data used to determine the overall effects of soil sterilisation on bacteria (16S gene target), fungi (ITS gene target) and *Fusarium* spp. (TEF1α gene target) with some comparisons only made between the communities present at the setup of the first round of lettuce plant growth and harvest of the 3rd round of lettuce growing. In addition, the relative abundance of *Fusarium* spp. and the *Trichoderma* isolates in both sterilised and non-sterilised soil was examined. Finally attempts were made to specifically identify FOL4 within the microbial communities using novel gene targets identified previously (see annual report; Clarkson, 2022) but these were unsuccessful as they lacked specificity (data not shown).

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Objective 7: Evaluate products for control of FOC in field experiments

Nine crop treatments were tested for their ability to control Fusarium basal rot in commercial onion fields by subcontractors VCS and Allium and Brassica Centre. They were applied in-furrow at drilling or as a foliar spray or a mixture of both (Table 3) at manufacturer recommended rates. Rudis was included as an in-furrow treatment, foliar application and both together. For each treatment (and an untreated control), five replicate plots were included. Disease assessments were carried out, and then the total number of bulbs at harvest recorded. These bulbs were then stored and assessed for disease again after approx. 8 weeks.

Statistical analysis was carried out on the number of healthy bulbs harvested from the field and the number of healthy bulbs remaining after storage using Analysis of Variance (ANOVA). Means were compared using the least significant difference (LSD) at 5% level.

Table 3 Crop protection / fertiliser treatments and the methods/rates of application when used in field experiments to evaluate their use for control of Fusarium basal rot in onions.

Product	Application method and rate
Control	Untreated
Frutogard	Infurrow at drilling (4.5 L ha^{-1}) + 2 x foliar applications (4.5 L ha^{-1})
Perlka	100 kg ha^{-1} pre-drilling + 2 x 100 kg ha^{-1} post emergence
Prestop	Infurrow at drilling (12 kg ha^{-1}) + 2 x foliar applications (6.0 kg ha^{-1})
Rudis	2 x foliar applications (0.4 L ha^{-1})
Rudis	Infurrow at drilling (0.6 L ha^{-1})
Rudis	Infurrow at drilling (0.6 L ha^{-1}) + 2 x foliar applications (0.4 L ha^{-1})
Serenade	Infurrow at drilling (10 L ha^{-1}) + 2 x foliar applications (10 L ha^{-1})
Serifel	Infurrow at drilling (1.0 kg ha^{-1}) + 2 x foliar applications (1.0 kg ha^{-1})
T34 Biocontrol	Infurrow at drilling (10 kg ha^{-1}) + 2 x foliar applications (5.0 kg ha^{-1})
Toledo	Infurrow at drilling (0.6 L ha^{-1}) + 1 x foliar application (0.6 L ha^{-1})
Velum Prime	Infurrow at drilling (0.625 L ha^{-1})

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Results

Objective 1: Define a relationship between the amount of Fusarium DNA, Fusarium inoculum and disease development in field soil (dose response)

Effect of FOL4 inoculum concentration on disease development in lettuce and pathogen detection

The full results for the experiment testing the effect of different FOL4 inoculum concentrations on disease levels in lettuce can be found in CP204 annual report March 2021 and CP204 annual report March 2022 (Clarkson, 2021; 2022) while a summary of the results and the statistical analysis is reported below.

FOL4 caused significant levels of disease of lettuce in terms of lettuce wilt and vascular browning scores ($p<0.001$), with inoculum concentration, soil type and the interaction between both being highly significant, especially at high FOL4 concentrations. Disease symptoms significantly increased with inoculum level; however, Soil 3 generally had less severe symptoms than the other soils (Figure 1 A and B). FOL4 infested compost (Soil 4) resulted in significantly more vascular browning and wilt (compared with 5% LSD) than the soils (Figure 1 A and B).

There were significantly different quantities of DNA detected in soil between the three higher concentrations (D4-D6) of FOL4 inoculum ($p<0.001$) over all the soils, but there was little effect of pathogen DNA quantity detected between the different soil types (Table 4). For two soils, FOL4 was detected down to inoculum levels of 2×10^3 cfu g^{-1} (D3; Figure 1 C). FOL4 DNA was also detected consistently in lettuce roots grown at the two highest inoculum concentrations (D5 and D6) (Figure 1 D), with significant differences observed between levels (Table 4). FOL4 DNA quantities were significantly higher in roots grown in compost compared to soils at inoculum concentration D6, which correlates with the greater disease levels observed with this treatment. Soil 1 resulted in significantly less FOL4 DNA detected in lettuce roots compared to all other treatments at D6, with no significant differences observed between Soils 2 and 3 (Table 4).

There was a clear correlation between the concentration of FOL4 inoculum, the concentration of FOL4 DNA detected in the soil and the disease scores (both wilt and vascular browning (Figure 1 E).

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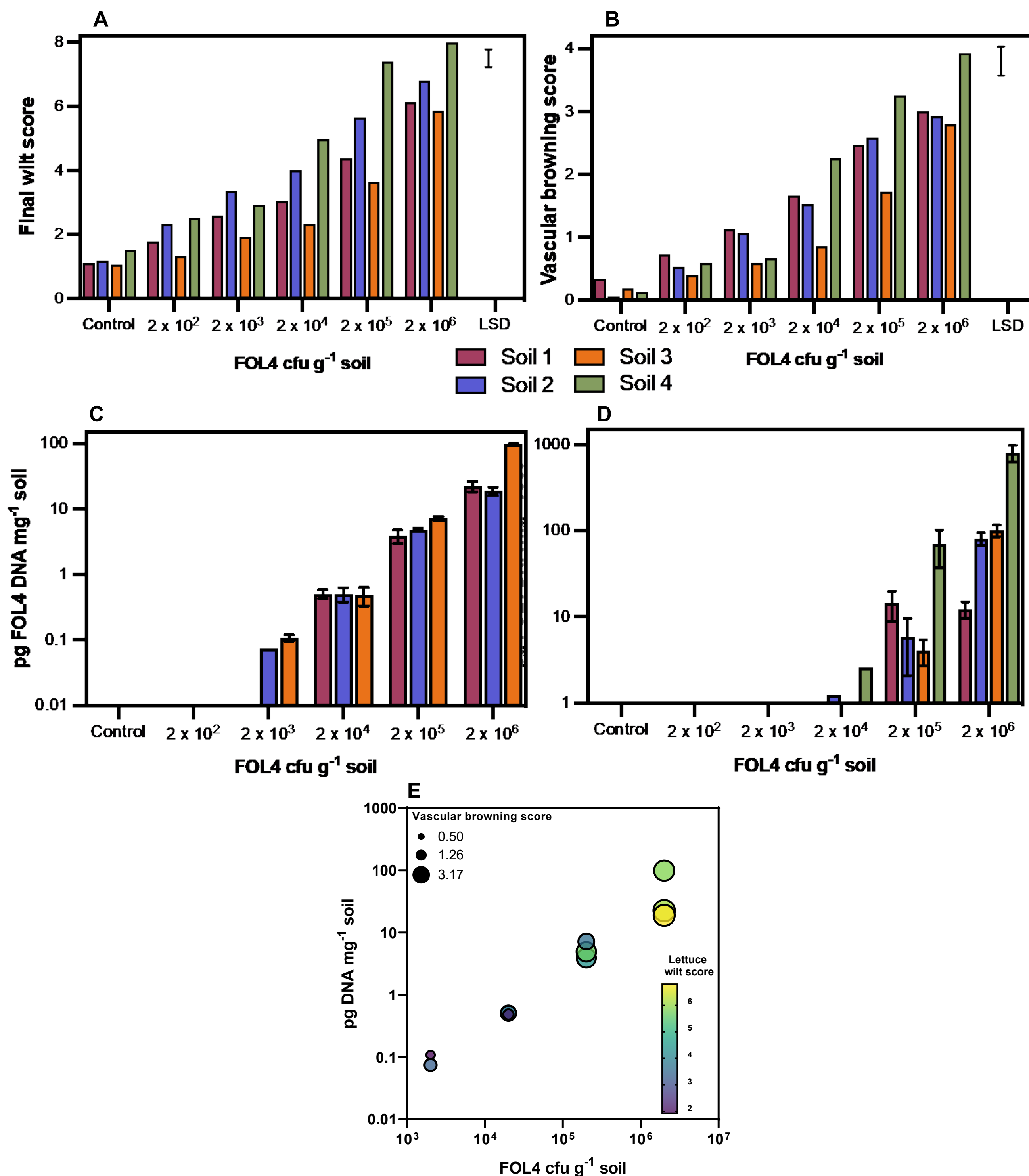

Figure 1. Effect of different *Fusarium oxysporum* f. sp. *lactucae* race 4 (FOL4) inoculum levels (D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil) on lettuce grown in three soil types and compost. A) ANOVA means of final lettuce wilt scores in three soils (Soil 1-3) and compost (Soil 4); B) ANOVA means of final vascular browning scores; C) Amount of FOL4 DNA detected (pg mg^{-1} soil) in inoculated soil; D) Amount of FOL4 DNA detected (pg mg^{-1} root) in lettuce roots; E) Correlation between FOL4 inoculum concentration, FOL4 DNA detected in soil and the disease severity scores (wilt and vascular browning score), with circles representing each soil type (Soil 1-3). Error bars represent least significant difference (5% LSD; A and B) and SEM (C and D)).

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Table 4 ANOVA means for log transformed qPCR data of FOL DNA detected in soil and root extractions. Analysis conducted on highest doses only.

	Soil 1	Soil 2	Soil 3	Soil 4	LSD
Soil qPCR					
2 x 10^5 cfu g^{-1} (D5)	1.31	1.58	1.96		1.26
2 x 10^6 cfu g^{-1} (D6)	3.08	2.92	4.59		
Root qPCR					
2 x 10^5 cfu g^{-1} (D5)	2.61	1.33	1.30	3.70	0.51
2 x 10^6 cfu g^{-1} (D6)	2.46	4.36	4.59	6.64	

Effect of FON inoculum concentration on disease development in Narcissus and pathogen detection

The full results for the experiment testing the effect of different FON inoculum concentrations on disease levels in narcissus can be found in CP204 annual report March 2022 (Clarkson, 2022) while a summary of the results and the statistical analysis is reported below.

An experiment was conducted to determine the relationship between different inoculum concentrations of FON inoculum, amount of pathogen DNA detected and disease development in narcissus bulbs grown in three soil types. As reported previously, bulbs grown in Soil 3 (compost mixture) had significantly more disease development at all inoculum concentrations compared to Soils 1 and 2 (5% LSD, Figure 2 A). Basal rot symptoms increased significantly in Soil 3 from inoculum concentrations D1 to D2, and also from D2 to D3, but higher concentrations did not result in significantly greater disease scores as they were close to the maximum on the scale. In Soil 1, only inoculum concentration D6 caused significantly more disease than observed in the uninoculated control (Figure 2 A). In Soil 2, inoculum concentration D5 resulted in significantly more disease than all lower concentrations (no differences between D1-D4), with D6 causing significantly more disease than D5 (Figure 2 A).

FON DNA was extracted from narcissus roots at two time points (TP2 results shown), and generally more pathogen DNA was detected in the roots of plants grown in Soil 3 from D3 to D6 than in the roots of plants grown in Soils 1 and 2 (Figure 2 B). FON DNA in soil was only detected from inoculum concentrations D4-D6 (Figure 2 C), and there was significantly more DNA detected in soils at D6 compared to all other concentrations (Table 5).

There was a positive correlation between the amount of FON inoculum, the pathogen DNA concentration detected in soil and roots and basal rot disease development in the narcissus

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bulbs (Figure 2 D). There was a positive correlation between FON inoculum concentration and pathogen DNA detected in both Soils 1 and 2 in addition to DNA detection in roots (size of circles represents an increase in FON DNA in the roots) and disease development (green and yellow coloured circles represent higher disease scores, Figure 2 D).

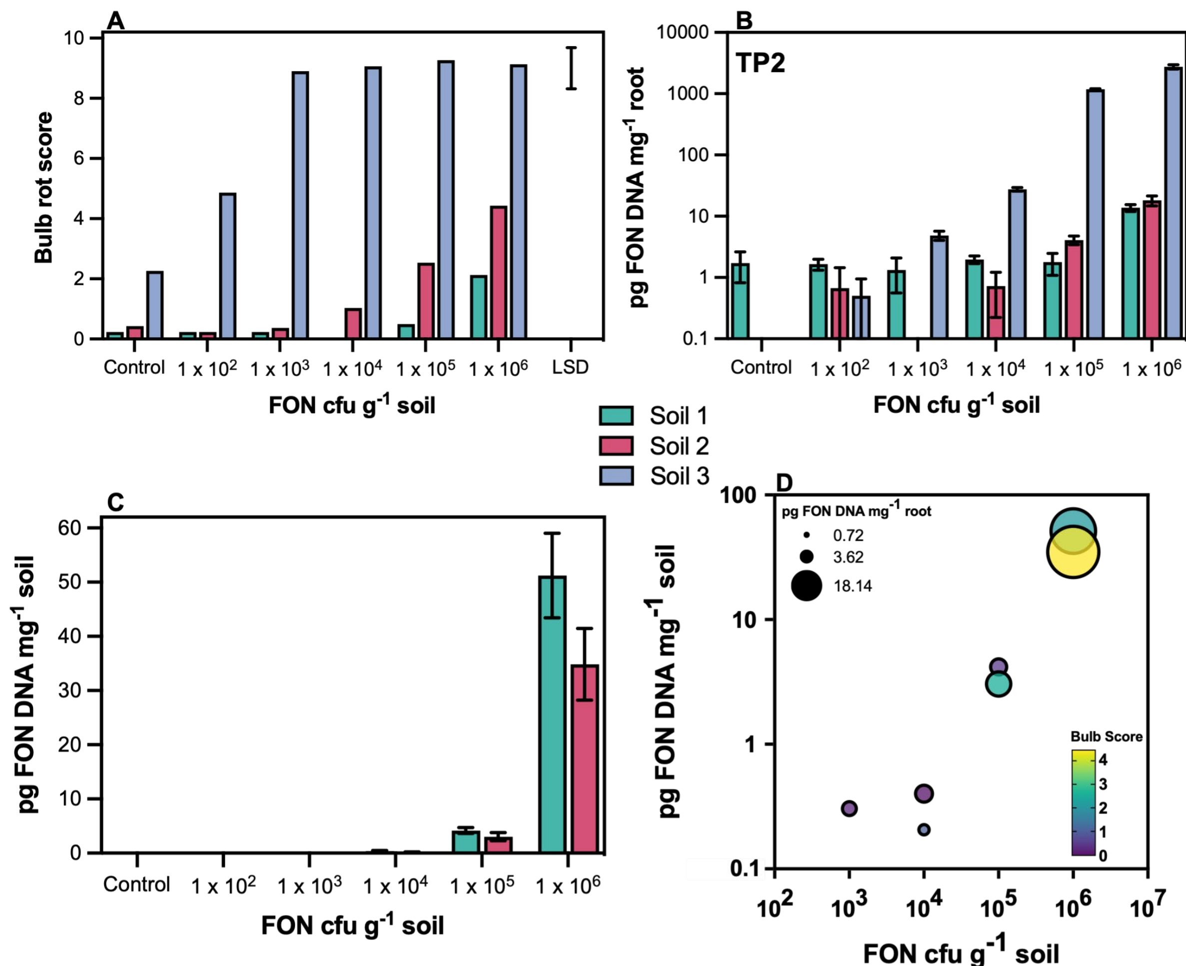

Figure 2. Effect of different *Fusarium oxysporum* f. sp. *narcissi* (FON) inoculum levels D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil) on narcissus grown in two soil types and compost. A) ANOVA means of bulb rot disease score (Soil 1 and 2) and compost (Soil 3); B) Amount of FON DNA detected (pg mg^{-1} root) in narcissus roots grown in infested soils; C) Amount of FON DNA detected (pg mg^{-1} soil) in Soils 1 and 2; D) Correlation between FON inoculum concentration, FON DNA detected in soil and roots and the disease severity scores (basal rot score), circles representing each soil type (Soil 1 and 2). Error bars represent least significant difference (5% LSD (A) and SEM (B and C)).

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Table 5 ANOVA means for log transformed qPCR data of FOL DNA detected in soil and root extractions.

	Soil 1	Soil 2	LSD
Soil qPCR			
1 x 10^4 cfu g^{-1} (D4)	-0.95	-1.59	
1 x 10^5 cfu g^{-1} (D5)	1.41	1.04	0.58
1 x 10^6 cfu g^{-1} (D6)	3.91	3.51	

Objective 2: Evaluate the use of molecular diagnostics to detect and assess the risk of Fusarium disease in onion

Onion fields were monitored for disease symptoms of Fusarium basal rot (VCS / Allium and Brassica Centre) over three growing seasons and disease levels recorded for onions in the field, at harvest and after a period of storage. For soil samples that were collected pre-planting at each site, FOC DNA quantity was too low to be detected and hence the potential of baiting with onion plants was subsequently examined. In addition, two fields per year were intensively examined with soil and onion root samples being collected throughout the growing season, which were again used to measure the quantity of FOC DNA detected using qPCR. Here, detection in roots was generally more consistent than in soil (see previous annual reports (Clarkson, 2021; 2022). In this final year, sampling was conducted for 2-3 timepoints during the season but from four sites instead of two. A summary of the results for field sites examined from each year can be found below, with full details reported in previous annual reports (Clarkson, 2021; 2022).

Testing of soil and onion root samples for FOC 2020

FOC DNA levels were low in soil samples from both intensively sampled sites FP1 and WRE and varied in quantity throughout the season (Figure 3 A and B). FOC DNA was detected in soil at all time points for site FP1 (Figure 3 A), but only in three timepoints mid-late season at the WRE site (Figure 3 B). FOC DNA was detected at a much higher level in onion roots than in soil and for every sample from all time points from both sites (except for 18.05.20 at WRE site); Figure 3 A and B). Generally, FOC DNA levels decreased in onion roots over the growing season, with the peak at both sites being in June 2020, and decreasing towards the end of the season (Figure 3 A and B). Disease levels at site FP1 were low during the season and at harvest, with average levels of >4.5%, whereas disease levels at harvest at the WRE site were much greater at 23% (Figure 3 C and D). After bulbs had been stored there were high levels

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of basal rot recorded in bulbs from both sites (FP1 = 28.5%, WRE = 47.2%; Figure 3 C and D). The slightly higher disease levels at WRE, correlate reasonably with the higher quantities of FOC DNA detected in onion roots from this site, compared to FP1.

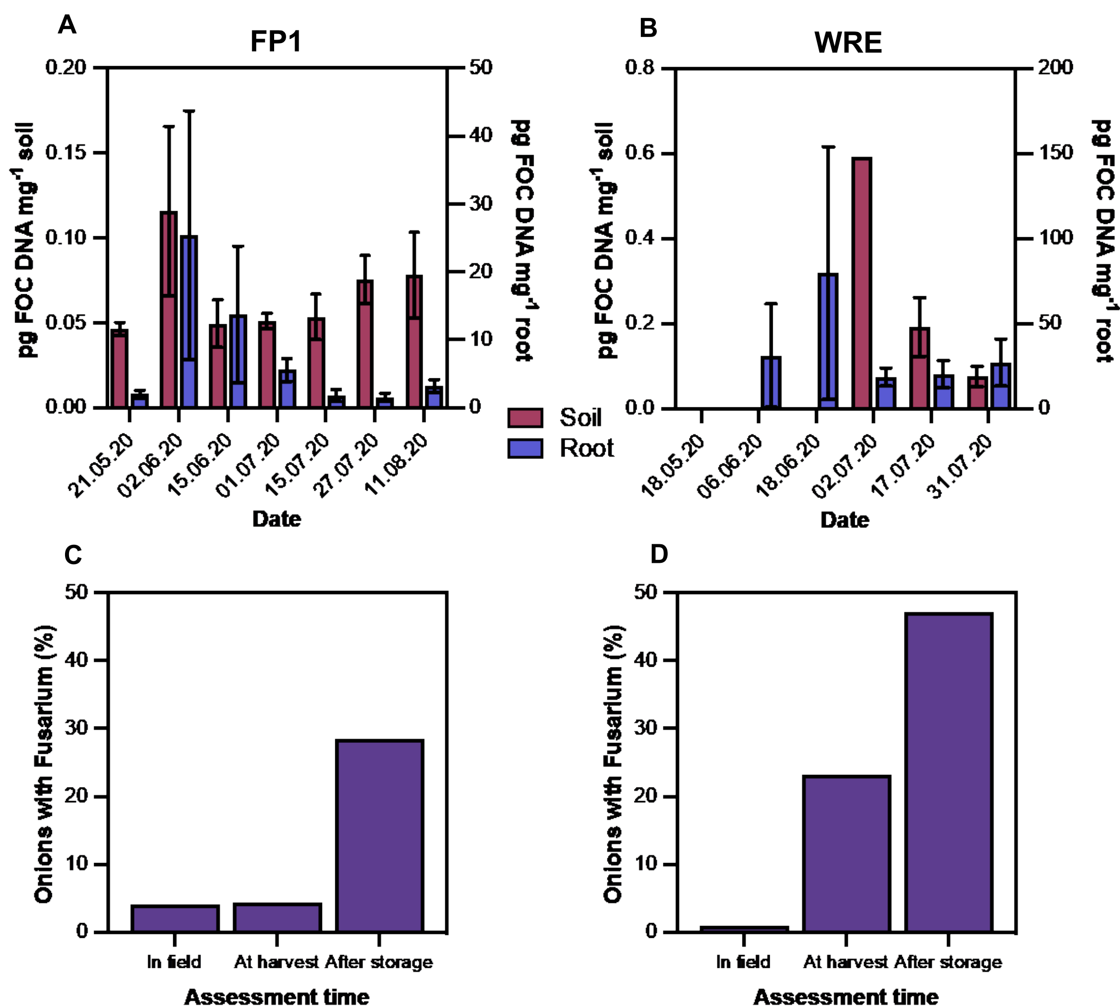

Figure 3. Concentration of FOC DNA in onion soil samples (left axis) and onion root samples (right axis) for A) ABC FP1 field site and B) VCS WRE field site, from the 2020 growing season. Error bars represent standard error of the mean (SEM). Percentage of onions with Fusarium symptoms in the field, at harvest and after storage for C) ABC FP1 field site and D) VCS WRE field site.

Testing of soil and onion root samples for FOC 2021

In year 2 (2021) different fields were chosen to be intensively sampled based on their risk factor for Fusarium disease that year. However, both sites had low levels of disease in the field and in bulbs post storage (Figure 4 C and D). Correspondingly there were also very few onion root samples from these sites where FOC DNA was detected. Of the 24 samples that were collected for each time point (see CP204 annual report March 2021; Clarkson, 2021 for full sampling procedure) only four samples across the whole growing season were positive for

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FOC DNA at site PGR2, and only five samples for THO. FOC DNA levels were also considerably lower than for 2020, with >3 pg FOC DNA mg^{-1} root (Figure 4 A, B) compared to up to 150 pg FOC DNA mg^{-1} root in 2020 (Figure 3 A and B). As DNA levels in root samples were low or not detectable, and disease levels in the field and post storage were also very low, the corresponding soil samples were not tested.

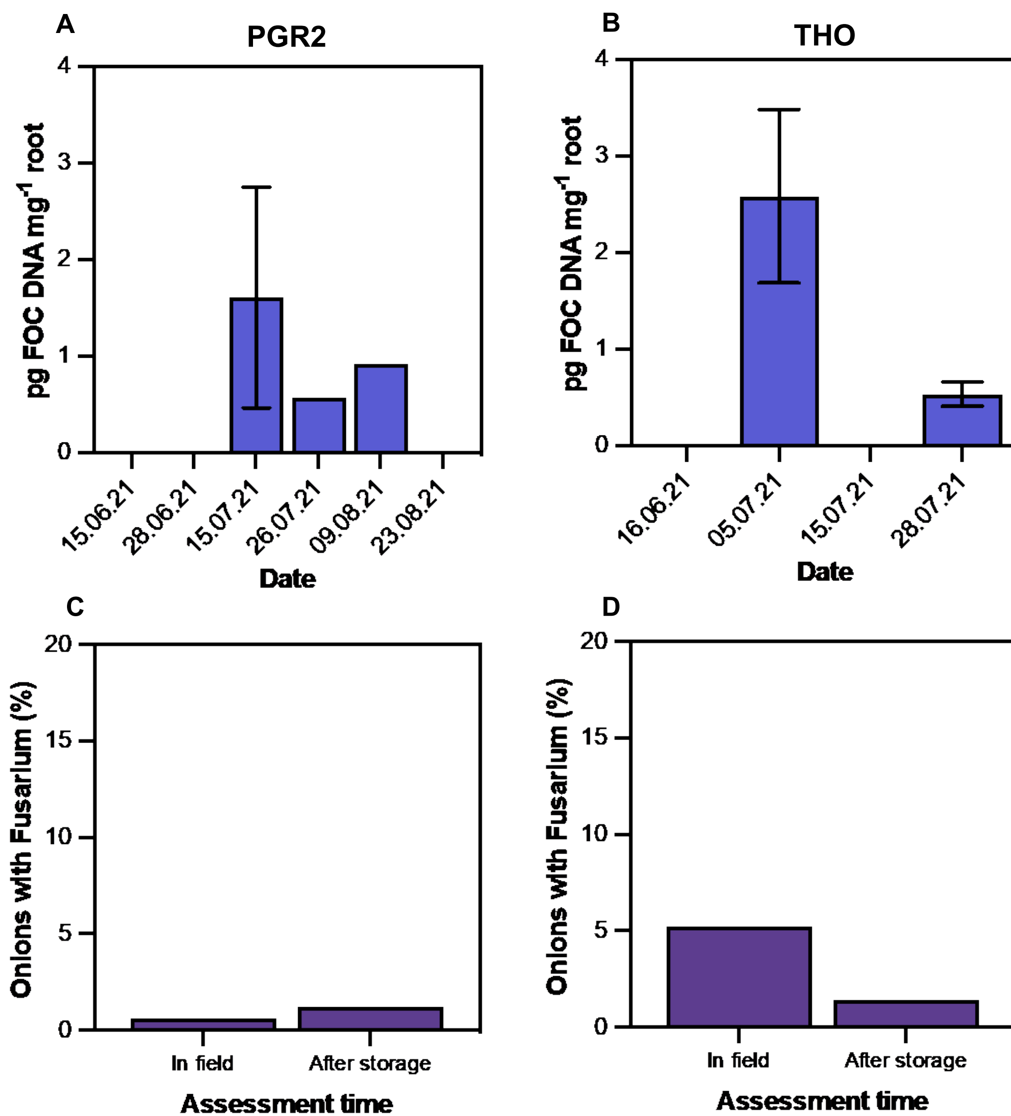

Figure 4. Concentration of FOC DNA in onion root samples for A) ABC PGR2 field site and B) VCS THO field site, from the 2021 growing season. Error bars represent standard error or the mean (SEM). Percentage of onions with Fusarium symptoms in the field and after storage for C) ABC PGR2 field site and D) VCS THO field site.

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Testing of soil and onion root samples for FOC 2022

As stated previously, four commercial onion fields were intensively sampled in 2022 (FP1 and PGR L/A, both ABC; TUN and HAR, both VCS) at 2 timepoints and with fewer samples than in 2020 / 2021 (six per timepoint, compared to 24). As before, soil and root samples from each timepoint were collected and used for FOC DNA extraction and qPCR. Disease assessments were also conducted for the same sites. However, field site FP1 was the only location where FOC DNA was detected in soil (although only at the second timepoint), while DNA was detected in onion roots from two field sites at both time points (FP1 and PGR L/A, Figure 5 A and B). Greater quantities of FOC DNA were detected in onion roots from site FP1, with up to 65.3 pg FOC DNA mg^{-1} root compared to 20.8 pg FOC DNA mg^{-1} root at the PGR L/A site (Figure 5 A and B). This correlated with the amount of disease observed, with more disease being recorded in the field and after storage at FP1 than at PGR L/A (Figure 5 C), with post storage disease at 14.9% and 5.0% respectively. Low levels of disease were also observed at the two VCS field sites which were intensively sampled, with post storage disease levels of 10.9% (TUN) and 2.0% (HAR, Figure 5D); however, no FOC DNA was detected in any of the soil or roots samples from these sites (data not shown).

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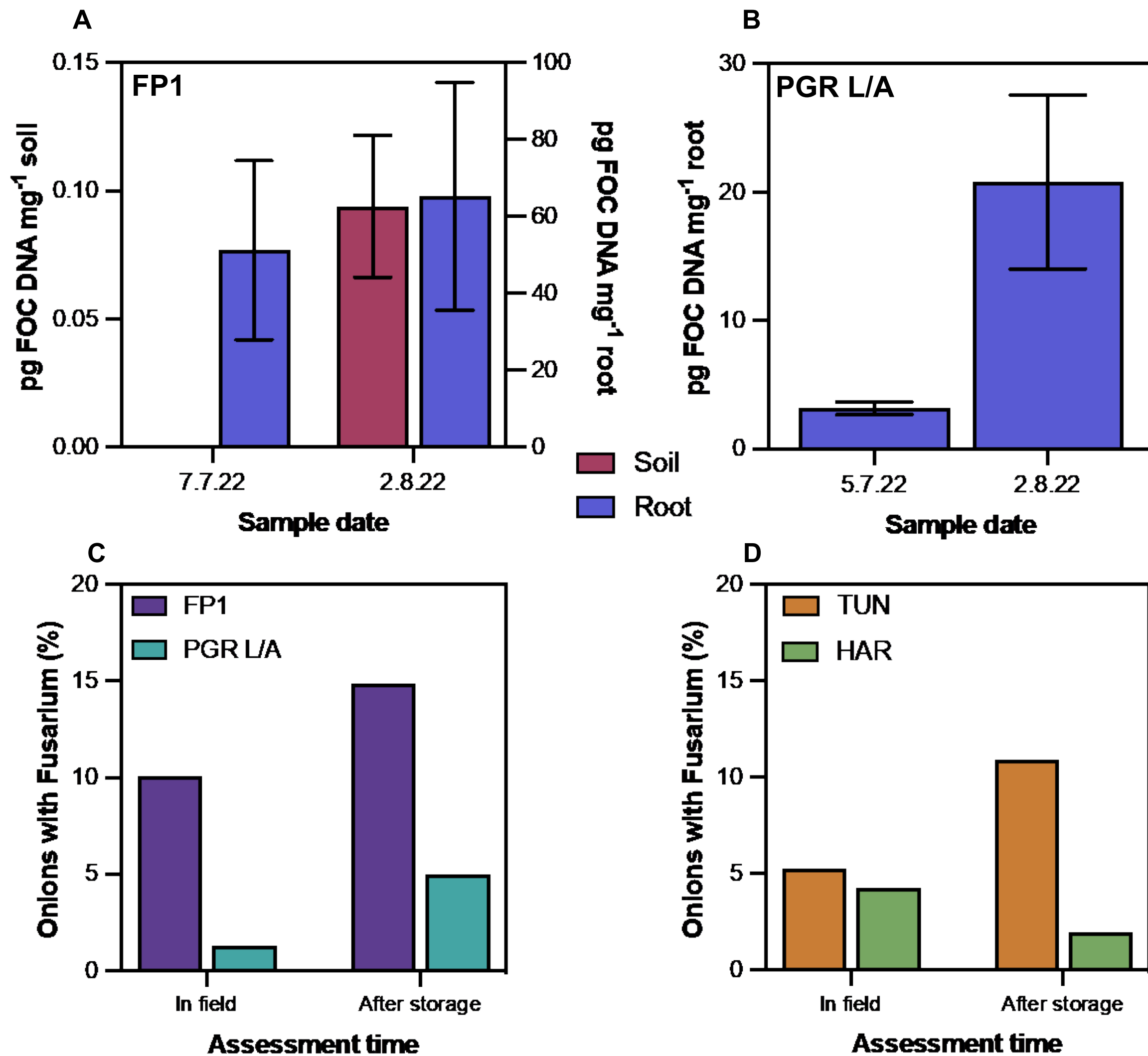

Figure 5 Levels of FOC DNA in onion soil samples (left axis) and onion root samples (right axis) for A) ABC FP1 field site and B) ABC PGR L/A field site, from the 2022 growing season. Error bars represent standard error of the mean (SEM). Percentage of onions with Fusarium symptoms in the field and after storage for C) ABC FP1 and PGR L/A field sites and D) VCS TUN and HAR field sites.

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Onion plant baiting to improve FOC detection in soil

Onion plant baiting using 2021 soil samples

Full details of onion plant baiting results can be found in CP204 annual report March 2022 (Clarkson, 2022) while an overall summary is reported below.

Onion seedlings were grown at 20°C in a total of 14 soils collected from commercial onion field sites and two from Wellesbourne, and the roots harvested for qPCR analysis. Seed germination and seedling damping off was also recorded, which varied between soils, with only 41% of seeds germinating in Wellesbourne QF soil (high prevalence of FOC, Figure 6 A). The highest germination of seeds grown in field soils were from FP3 and PR3 at 94%, whereas, field site TUN had the lowest percentage germination at 41% (Figure 6 A). Roots were also tested for the presence of FOC using qPCR; however FOC DNA was only detected in onion roots from seedlings grown in FP1 soil and QF soil (Figure 6 B).with levels much lower for FP1 roots than for QF roots (1.8 pg DNA mg^{-1} root compared to 13.2 pg DNA mg^{-1} root; Figure 6 B).

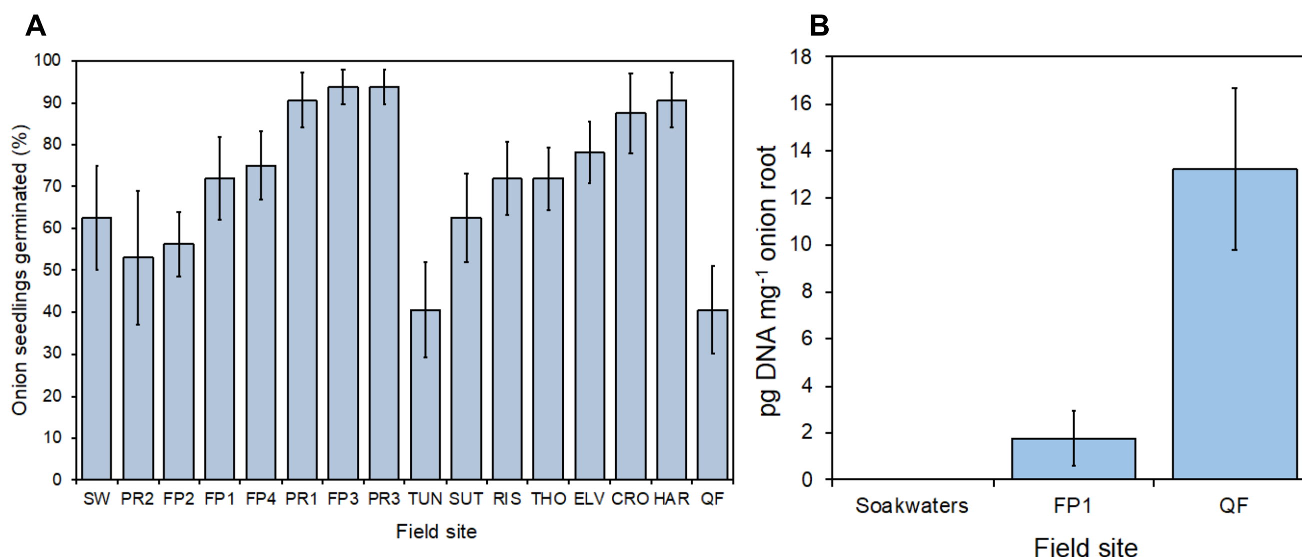

Figure 6. A) Percentage of onion seeds which germinated when grown in soil from 14 onion field sites, with a positive (QF = FOC infested soil from Warwick Crop Centre quarantine field) and negative control (SW = FOC negative Soakwaters field soil). B) Concentration of FOC DNA in onion seedling roots (pg mg^{-1} onion root) for FP1 and QF (the only FOC positive field sites). Error bars represent standard error of the mean.

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Onion plant baiting using 2022 soil samples

Onion seedlings were grown in a total of 15 soils collected from commercial onion field sites in 2022 with two from the Wellesbourne QF (diluted 50% v/v and undiluted), and this time DNA from both soil (pre-sowing and after seedling harvest) and roots (at harvest) extracted for qPCR analysis. The percentage of onion seed which germinated when grown in these soils was relatively high, with all >74% (Figure 7 A). The average percentage germination in field soils (not including QF and negative control) ranged from 76.6% (FP1) to 94.2% (HIA), with germination in the negative control being relatively low at 84.2% compared to some of the field soils (Figure 7 A). FOC DNA was only detected at low levels in the diluted QF soil and the undiluted QF soil before onion seeds were sown and not in any of the commercial field soils (Figure 7 B). After onions had been grown in the soil, the amount of FOC DNA detected significantly increased in the QF soil from 0.13 to 0.77 pg FOC DNA mg^{-1} soil and in the diluted QF soil from 0.05 to 0.36 pg FOC DNA mg^{-1} soil (Figure 7 B). FOC was also detected by PCR in soil after seedling harvest from two of the commercial field sites, FP1 and FP2 (Figure 7 C), whereas it was not detected pre-planting (Figure 7 B). In onion roots, FOC DNA was detected at much higher levels than in soil with 131.4 pg FOC DNA mg^{-1} found in the onion roots from the QF soil (Figure 7 D). FOC DNA was also detected in roots grown in soil from five of the commercial sites (FP1, FP2, FP3, RIX L/H and LAK, Figure 7 D) compared with only two sites for DNA extracted from soil (Figure 7 C).

Overall therefore, baiting with onion seedlings significantly improves FOC DNA detection in both post-harvest soil and roots compared with pre-planting soil.

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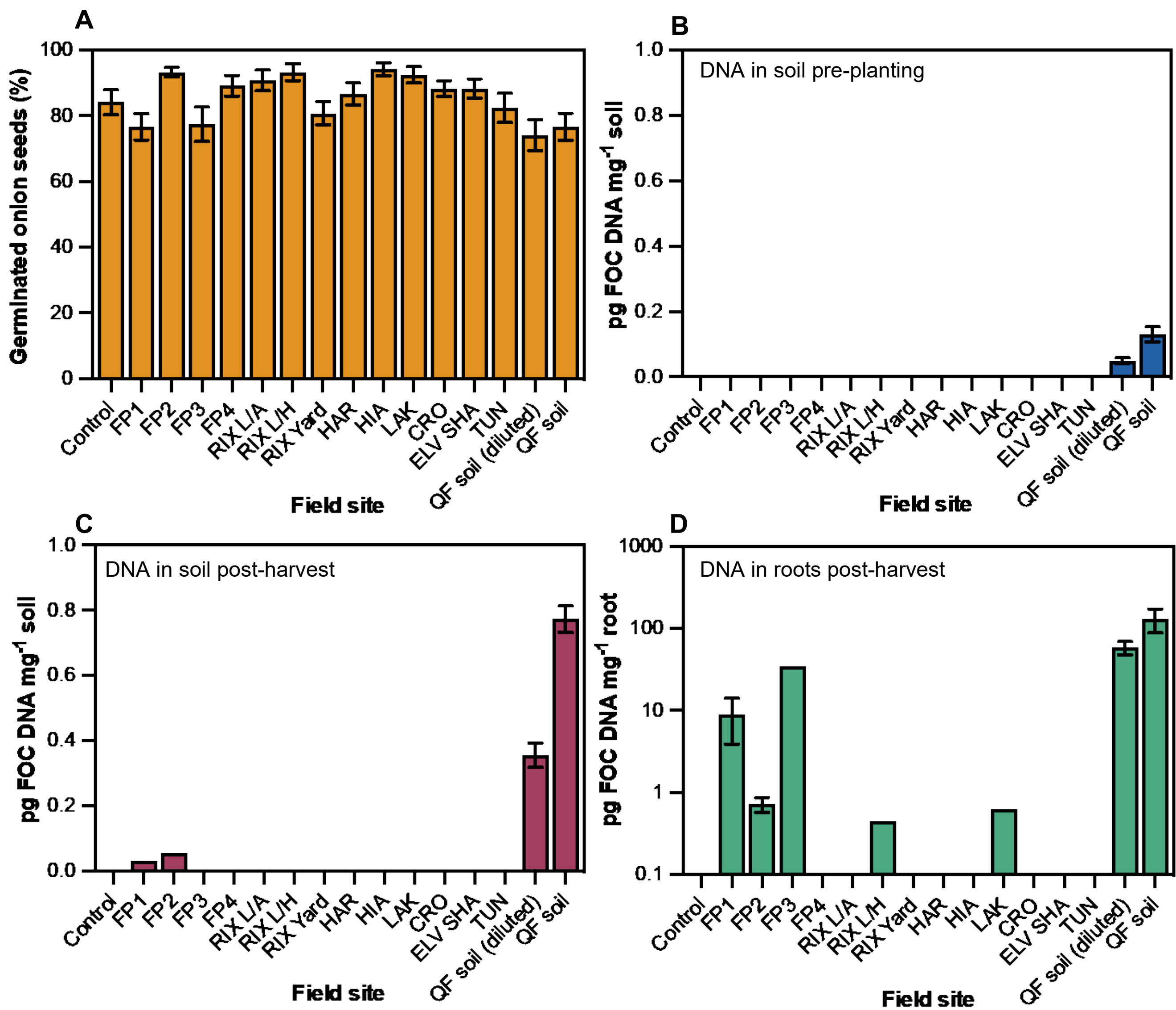

Figure 7. Baiting using onion seedlings for improved detection of FOC in soil from 13 commercial onion field sites, the FOC-infested Quarantine Field (QF) and a FOC free soil (negative control). A) Percentage of germinated onion seeds B) Concentration of FOC DNA (pg mg^{-1}) in soil before planting onions. C) Concentration of FOC DNA (pg mg^{-1}) in soil post-harvest of onion seedlings. D) Concentration of FOC DNA (pg mg^{-1}) in onion roots at harvest. Error bars represent standard error of the mean.

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Objective 3: Evaluate the use of molecular diagnostics to determine the presence of FOC in harvested onion bulbs to assess the risk of disease development in store

Determining the presence of FOC in onion bulbs with different levels of Fusarium basal rot symptoms 2020

The full results from Year 1 can be found in CP204 annual report March 2021 (Clarkson, 2021) but a brief summary is outlined below.

When detection of FOC was examined by plating tissue samples from onion bulbs with different levels of basal rot symptoms onto PDA, it was possible to isolate *Fusarium* from almost 100% of both basal plates and scales for those bulbs with severe and intermediate symptoms (Figure 8). The percentage of positive *Fusarium* isolations from basal plates of bulbs exhibiting 'corky' symptoms was also relatively high at 68% but for these bulbs only 2.3% of scales resulted in pathogen isolation indicating that for these samples, infection was restricted to the basal plates (Figure 8). *Fusarium* was also isolated from bulbs which appeared healthy, with positive isolations from the basal plates of 10% of bulbs (Figure 8). This suggested that FOC may be present in asymptomatic onion bulbs which could then potentially develop symptoms in store.

A smaller number of other bulbs from each basal rot symptom category was also used for FOC detection using both isolation and qPCR. In this case, FOC was detected in 100% of bulbs with severe basal rot symptoms, 32% of bulbs with corky symptoms and also in 18.8% of healthy bulbs with no symptoms using qPCR (Table 6). For corky and healthy bulbs this was lower than the percentage of *Fusarium* isolated (68% and 38% respectively, Table 6); however it was not known if this was FOC or another *Fusarium* species.

When further onion bulbs from the healthy and corky bulb categories (assessed externally) were incubated to promote FOC disease development, basal rot developed in 55 and 75% of these bulbs respectively indicating that as also shown by the results from isolations and qPCR that FOC infection can arise form asymptomatic bulbs and that the corky basal plate symptom is also strongly associated with FOC infection.

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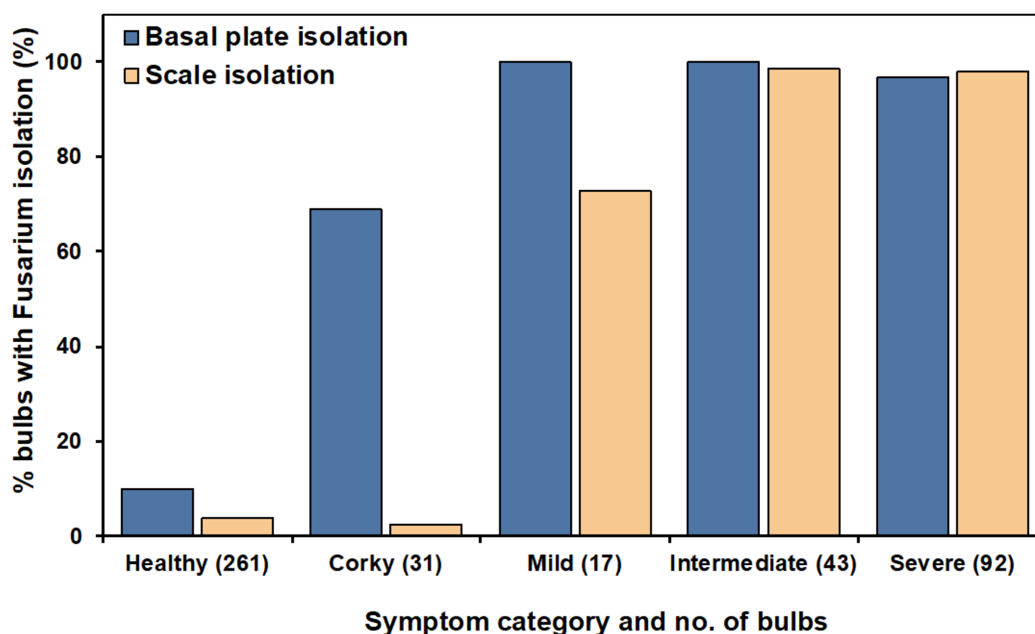

Figure 8. Percentage of onion bulbs where *Fusarium* spp. were isolated from basal plates or scales for different categories of Fusarium basal rot symptoms from grower field sites. Number in brackets is the number of bulbs examined for each symptom category.

Table 6. Detection of FOC by PCR and isolation of *Fusarium* spp. from onion bulbs from different symptom categories

Symptom	No. bulbs for qPCR	% Fusarium isolated (basal plate)	% positive FOC qPCR	No. bulbs hotboxed	% Fusarium developed
Healthy	48	37.5	18.8	231	55
Corky	31	67.7	32.3	94	75
Mild / Intermediate	28	100.0	96.4	0	-
Severe	17	100.0	100.0	0	-

Determining the presence of FOC in onion bulbs with different levels of Fusarium basal rot symptoms 2021

Onion bulbs were obtained from commercial onion fields with a high and low risk of developing Fusarium disease and were tested for presence of FOC using qPCR. In addition, ABC/VCS also dried and stored bulbs from the same sites, to determine if basal rot developed in storage. Unfortunately, the bulbs from all sites (2 each from ABC and VCS) did not develop symptoms after storage, with little or no Fusarium recorded (<5%).

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We received 100 onion bulbs from each high and low risk site (400 in total), half of which were used for FOC detection using qPCR while the other half were left intact, dried and subsequently incubated at 20˚C for 5-6 weeks to determine if Fusarium basal rot would develop in optimal conditions. Of the 50 onion bulbs per field site (four sites), 25 were randomly selected for initial DNA extraction and qPCR but none of these exhibited any basal rot symptoms when dissected or were positive for FOC using qPCR; therefore, the remaining samples were not tested. Of the bulbs that were incubated, less than 10% from each site developed basal rot symptoms, but many bulbs did have darkened or black spots in the basal plates, which could not be directly attributed to FOC, but was noteworthy as it was also observed in the stored bulbs (Figure 9). Bulbs from the higher risk sites had a higher percentage of symptomatic bulbs, and also bulbs with dark basal plates (Figure 9). These sites also had considerably fewer completely healthy bulbs, although many bulbs from ABC sites did also develop bacterial neck rot.

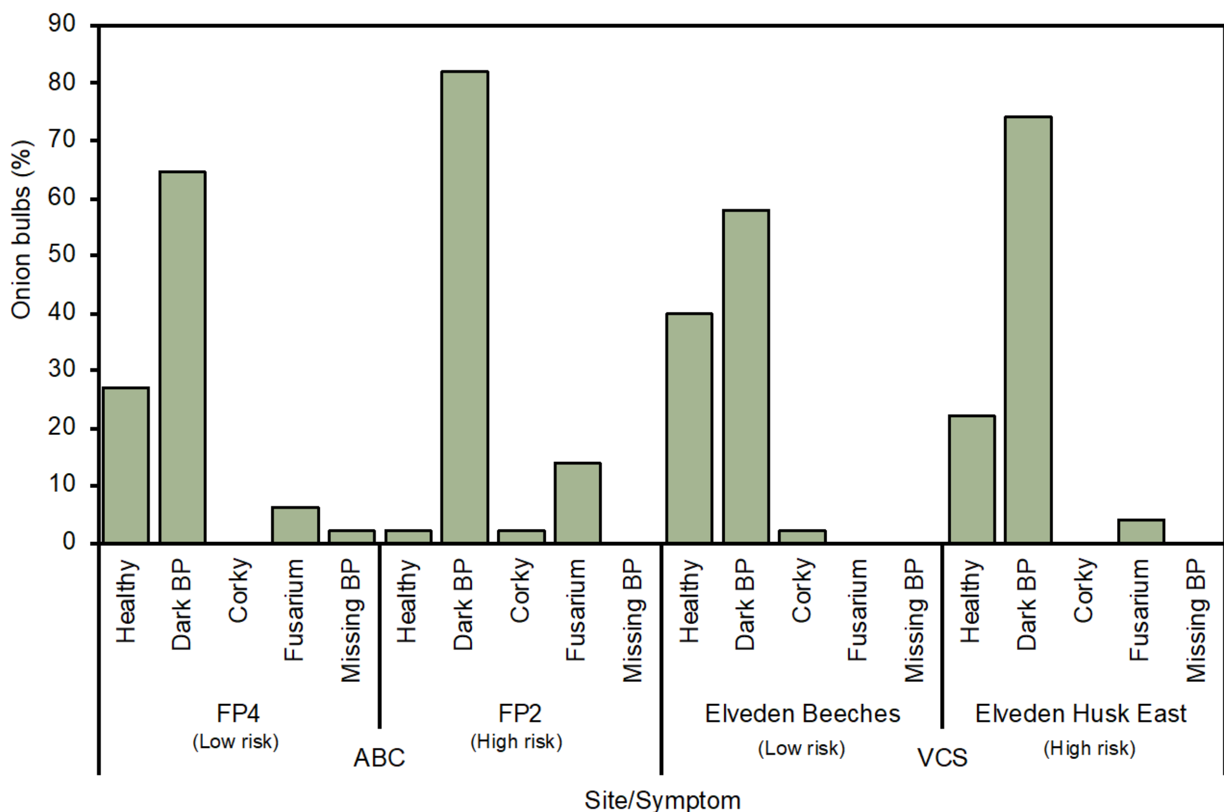

Figure 9. Percentage of onion bulbs which were healthy or showed signs of Fusarium disease after being incubated for 5-6 weeks at 20˚C.

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Determining the presence of FOC in healthy onion bulbs from *Fusarium* affected fields 2022

In the third year we focussed on the detection of FOC in the basal plates of healthy (asymptomatic) onions, which were obtained from a crop in the FOC infested Quarantine Filed site at Wellesbourne and also from a commercial field site which was a high risk for development of Fusarium basal rot. Of the 100 bulbs dissected and examined from the QF field, 10% were considered to have mild Fusarium symptoms, with the remaining 90% considered healthy. 34 of these 90 healthy bulbs tested positive for FOC with qPCR (38%), with DNA levels ranging from 0.36 to 10.60 pg FOC DNA mg^{-1} basal plate (Figure 10 A). For bulbs obtained from the commercial site, 82 of the 100 bulbs were scored as being healthy, with only 8 of those testing positive for FOC with qPCR (9.8%, Figure 10 B). When additional healthy bulbs from both the QF field and the commercial site were incubated to determine whether basal rot would develop, 51.4% of those from the QF field, and 24.2% of those from the commercial site developed Fusarium basal rot. These results therefore suggested that detection of FOC in basal plates by PCR may be useful as a predictor of Fusarium basal rot development in store.

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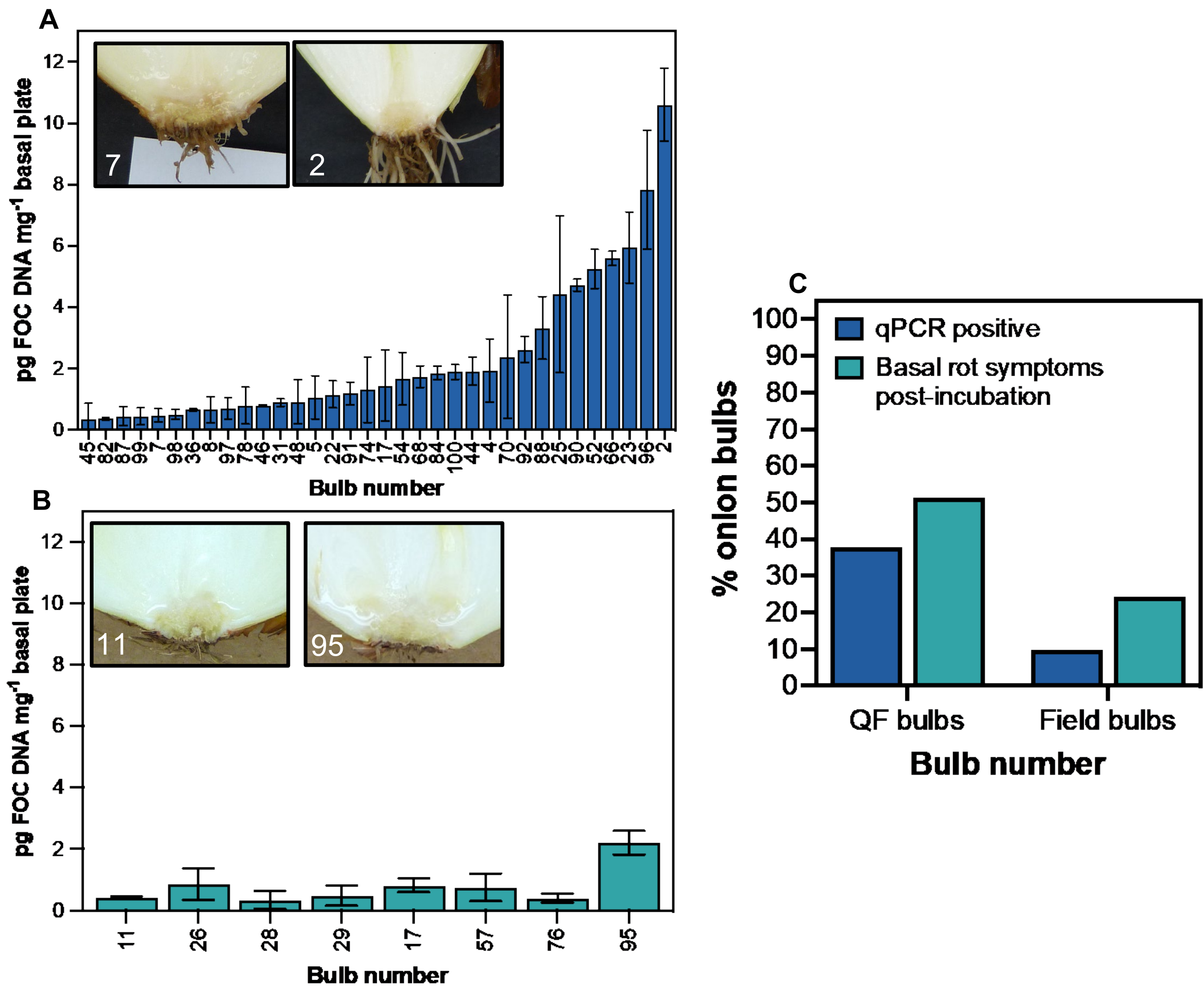

Figure 10 Quantity of FOC DNA detected in basal plates (pg mg^{-1}) of apparently healthy onion bulbs from infested QF field at Wellesbourne (A), and from a commercial onion field site (B), with example photos of asymptomatic basal plates. C) % of onion bulbs testing positive for FOC with qPCR and % onions with basal rot symptoms after incubation.

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Evaluating LAMP for detection of FOC in inoculated onion bulbs

The calibration curve using known concentrations of FOC isolate FUS2 genomic DNA revealed that LAMP detected all dilutions down to 0.0005 ng DNA μL^{-1} (Figure 11). A shorter time for DNA amplification is indicative of a higher DNA concentration and hence for the most concentrated sample, 10 ng μL^{-1}, was 6:45 mins and increased as the concentration of DNA reduced (Table 7). Based on this, the 0.1 ng μL^{-1} sample was chosen as the positive control for the LAMP assays with inoculated onion bulbs.

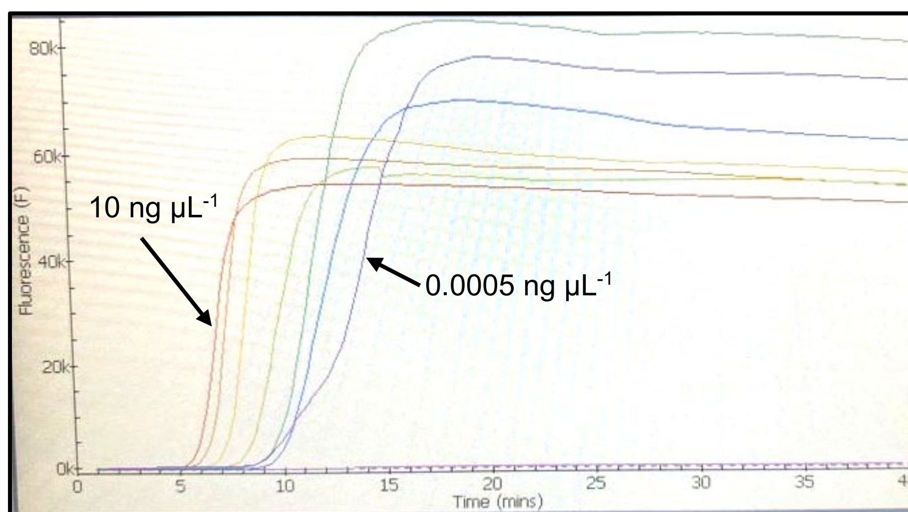

Figure 11 DNA amplification plot for the calibration curve of genomic FOC isolate FUS2 DNA using LAMP.

Table 7 Amplification times for FOC isolate FUS2 DNA samples at concentrations ranging from 0.0001 – 10 ng μL^{-1} using LAMP.

DNA concentration (ng μL^{-1})	Amplification time (mm:ss)
10	6:45
1	7:15
0.1	8:15
0.01	9:30
0.005	11:00
0.001	11:15
0.0005	13:45
0.0001	Negative

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Onion bulbs were inoculated with agar plugs of FOC isolate FUS2 and then destructively sampled twice a week commencing 4 days post inoculation (dpi), whereby sections of basal plate and onion scale tissue were used for FOC detection using the LAMP assay.

After 4dpi, none of the bulb tissue samples gave a positive result for FOC, but at 8 dpi, two of the five inoculated onion bulb basal plate samples resulted in positive FOC amplification at 10:30 and 23:00 min respectively. At 11 dpi, all five of the onion basal plate samples were positive for FOC DNA and all had short times to amplification, with values between 10-14 min (positive FOC DNA control, 9.5 minutes; Table 8), but were still asymptomatic (Figure 12). Symptoms appeared in bulbs from 15 dpi, in those which tested positive with LAMP (Table 8, Figure 12). With later timepoints, all basal plate samples continued to test positive for FOC amplification with the scale samples beginning to test positive from 18-22 days (1 of 5 bulbs and 2 of 5 bulbs respectively) as the bulbs began to develop more severe disease symptoms (Table 8 Figure 12). At 25 dpi, basal plates and scales from all 5 onions were positive for FOC with amplification times ranging from 8 to 15:15 min. All samples, other than the uninoculated control bulbs, were also positive at all subsequent time points, with amplification times generally getting shorter the more diseased the bulbs became (Table 8). Amplification times for positive DNA samples were generally very consistent with times ranging from 8:15 to 9:45 minutes (Table 8) and could therefore be related to the calibration curve (Figure 11, Table 7). Melt curves and amplification plots were consistent for all samples indicating that FOC DNA was being amplified (Figure 13).

Table 8 LAMP amplification times for FOC DNA extracted from onion basal plates (BP) and scales (S) at different timepoints for FOC isolate FUS2 inoculated onion bulbs.

Bulb no.	Sample location	Days post inoculation and amplification time (min:s)									
		4	8	11	15	18	22	25	29	32	36
Bulb 1	BP	-	10:30	10:00	-	10:30	11:15	9:45	8:15	10:00	9:30
Bulb 1	S	-	-	-	-	15:15	11:00	10:00	9:15	8:30	8:30
Bulb 2	BP	-	23:00	14:00	15:15	10:15	10:15	8:30	9:00	10:30	9:15
Bulb 2	S	-	-	-	-	-	11:45	15:15	9:30	9:15	8:15
Bulb 3	BP	-	-	11:45	11:45	10:00	11:00	10:30	9:15	10:15	9:00
Bulb 3	S	-	-	-	-	-	-	11:15	9:15	8:30	9:15
Bulb 4	BP	-	-	10:15	-	9:00	10:15	9:30	8:15	13:00	10:00
Bulb 4	S	-	-	-	-	-	-	9:00	8:15	10:15	8:15
Bulb 5	BP	-	-	10:15	11:15	9:30	10:00	10:30	8:45	9:30	10:15
Bulb 5	S	-	-	-	-	-	-	8:00	8:45	8:30	9:00
Con.1	BP	-	-	-	-	-	-	-	-	-	-
Con.2	BP	-	-	-	-	-	-	-	-	-	-
Con.3	BP	-	-	-	-	-	-	-	-	-	-
DNA 0.1 ng μL^{-1}		9:45	8:15	9:30	8:30	8:30	8:45	8:30	8:45	8:30	8:30

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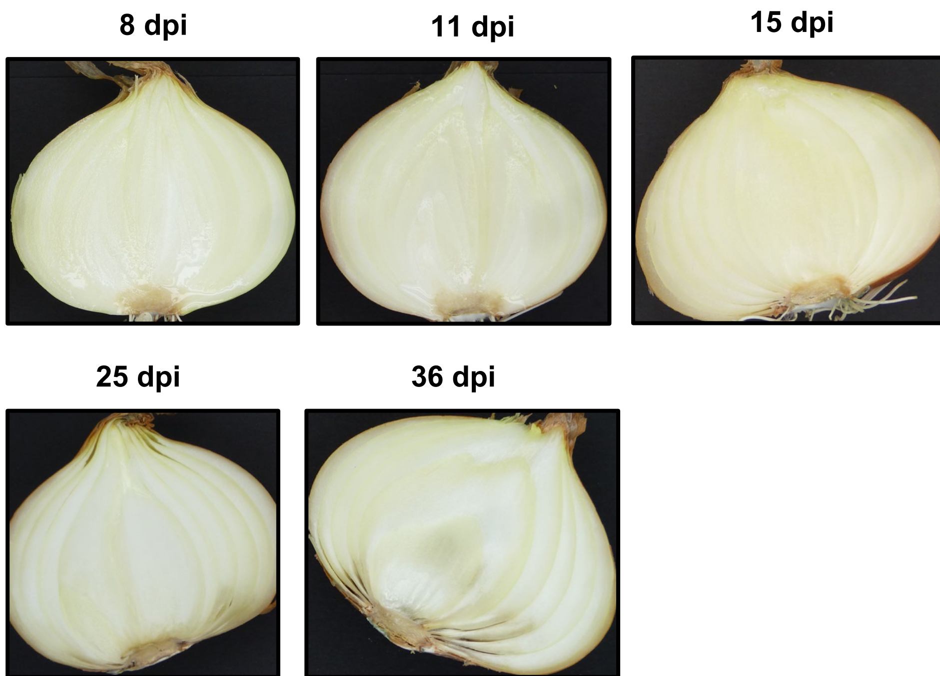

Figure 12 Onion bulbs inoculated with FOC isolate FUS2 at increasing time points post-inoculation. Symptoms developed from 15 dpi in the basal plate.

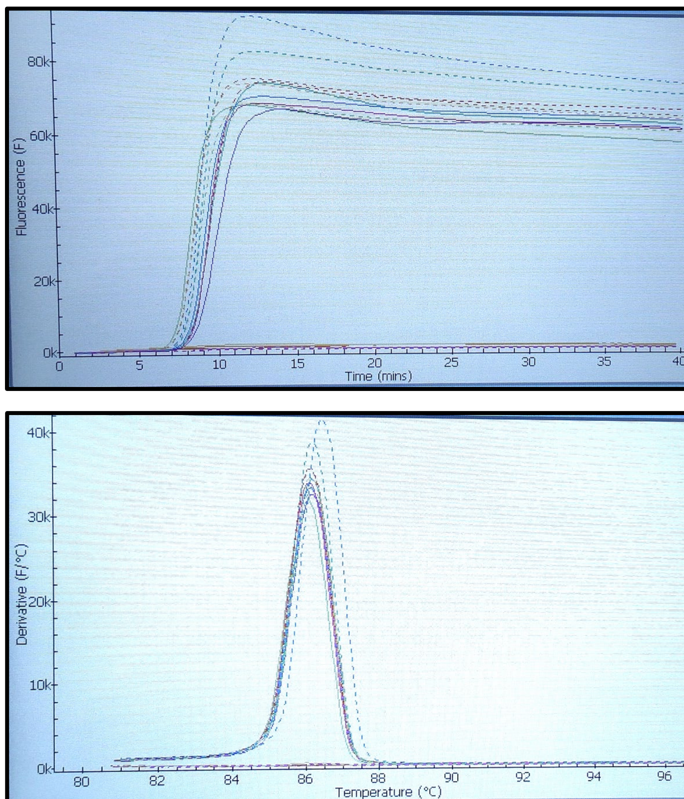

Figure 13. Amplification plot (top) and melt curve (bottom) for inoculated onion basal plates and scales positive for FOC DNA using LAMP.

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Objective 4: Determine colonisation of non-host plants by FOC to identify suitable rotation crops

FOC DNA was detected in the roots of all the non-host crops grown in FOC (isolate FUS2) inoculated compost (Figure 14). However, the number of replicate plants where FOC DNA could be detected varied between the non-host crops, with greater values for pea, maize, sugarbeet and barley and (12, 10, 8 and 7 plants respectively (Table 9) compared to only 3 plants for oilseed rape. The roots of all 12 plants were colonised by FOC for both resistant and susceptible onions plants. When the amount of FOC DNA was quantified for those plants testing positive for the pathogen, more was detected in roots in the susceptible onion cultivar (mean 2639 pg DNA mg^{-1} root), compared to the resistant onion cultivar (mean 829 pg DNA mg^{-1} root), and both contained considerably more FOC DNA than roots of the non-host crops (Figure 14, Table 9). Mean FOC DNA detected in non-host crops ranged from 2.35 pg DNA mg^{-1} root in sugarbeet to 7.13 pg DNA mg^{-1} root in maize (Figure 14, Table 9). Therefore, FOC was able to colonise some non-host roots better than others. One sample of the non-inoculated control had a low level of FOC DNA, which could be explained by contamination of this sample post-harvest.

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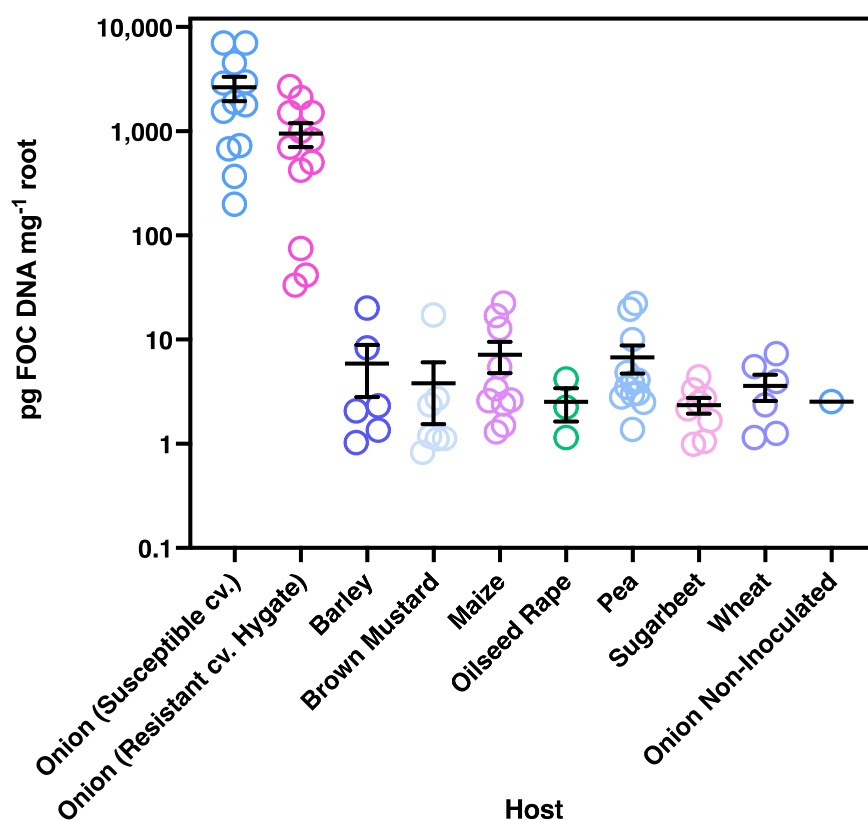

Figure 14 Concentration of FOC DNA (pg mg^{-1}) detected in the roots of non-host plants when grown in FOC inoculated compost for 5 weeks. Bars represent the average DNA detected per crop and the standard error of the mean.

Table 9 Incidence of FOC colonisation of non-host crops compared to onion, including the number of replicate pots where root samples were positive for FOC DNA detection using qPCR, the average DNA detected across replicates and the range of DNA detected in pg FOC DNA mg^{-1} root.

Plant host	Number of positive replicates out of 12	Average DNA detected (pg DNA mg^{-1} root)	Range of DNA detected (pg DNA mg^{-1} root)
Onion (Susceptible cv.)	12	2639.04	198.83 - 7040.00
Onion (Resistant cv. Hygate)	12	950.16	33.40 - 2688.17
Barley	6	5.86	1.03 - 20.09
Brown Mustard	7	3.79	0.82 - 17.22
Maize	10	7.13	1.30 - 22.34
Oilseed Rape	3	2.53	1.15 - 4.19
Pea	12	6.73	1.37 - 22.28
Sugarbeet	8	2.35	0.99 - 1.39
Wheat	6	3.59	1.15 - 7.32
Onion Non-Inoculated	1	2.54	0

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Objective 5: Investigate the feasibility of establishing Fusarium-suppressive microbial communities and biological control agents in protected cropping systems (sterilised soil)

Effect of treatments on FOL4 inoculum build up and disease development

The effect of biological control agents (*Trichoderma asperellum* T34 and *T. harzianum* T22) and Perlka on the build-up of FOL4 in successively grown lettuce plants was evaluated in sterilised and non-sterilised soil.

After the first round of lettuce growth, highly significant results for the interactions between all treatments for both Fusarium wilt and vascular browning scores were observed ($p>0.001$). There was mild wilting and vascular browning symptoms observed in the lettuce plants for all the treatments which was most pronounced for the Perlka treatment (Figure 15 A and B). The *Trichoderma* biological control agents resulted in a minor reduction in Fusarium wilt and browning symptoms, but this was not statistically significant (5% LSD, Figure 15 A and B). FOL4 DNA was only detected in the sterilised soil treatments at harvest, but at low and variable levels (Figure 15 C).

Fusarium disease levels increased in the second round of lettuce growth and the effect of the treatments and the interaction between the two were all highly significant ($p<0.001$) irrespective of whether soil was sterilised or not (Figure 15). For both Fusarium wilt and vascular browning scores, the sterilised soil resulted in significantly increased disease symptoms compared with the non-sterilised soil across all treatments (FOL4 only, T34, T22 and Perlka). None of the products applied resulted in any significant reduction in disease score (either wilt or browning) compared to FOL4 only in the non-sterilised soil but application of Perlka resulted in significantly more disease compared to the uninoculated control and the FOL4 only treatment (5% LSD, Figure 15 D and E). In the non-sterilised soil, very mild Fusarium wilt and browning was observed in the FOL4 only, T22 and T34 treatments but disease scores were not significantly different from the uninoculated control or FOL4 only treatments (5% LSD, Figure 15 D and E). FOL DNA was again only detected in the sterilised soils at harvest, but at low levels (Figure 15 F). As levels vary, it is difficult to compare DNA detection levels between crops.

In the third round of lettuce growth, Fusarium disease was observed in all treatments apart from the non-inoculated control, with treatment (sterilised and non-sterilised soils, with / without all soil applications) having a significant effect on disease ($p<0.001$). Again, in all treatments, there was significantly more wilt and browning for lettuce grown in the sterilised soil (5% LSD, Figure 15 G and H). As in the previous round, none of the treatments (T22, T34

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or Perlka) reduced disease incidence compared to the FOL4 only treatment (Figure 15 G and H). Fusarium disease overall increased in the non-sterilised soil between lettuce rounds 2 and 3 (Figure 15 E and H) but as for the sterilised soil, none of the soil applications reduced disease. FOL4 DNA was detected in the FOL4-inoculated sterilised soil as for the previous rounds of lettuce growth, but also at very low levels in the non-sterilised soils in soils treated with T22, T34 and Perlka (Figure 15 I).

Overall, there was therefore a distinct increase in Fusarium disease with successive rounds of lettuce growth in both non-sterilised and sterilised soil but this was not affected by the application of T34, T22 and Perlka in comparison with the FOL4 only treatment. Disease increase clearly occurred more rapidly in the sterilised soil, with severe Fusarium disease observed in round 2. Build-up of Fusarium disease in the non-sterilised soil was slower, with only moderate disease levels by crop 3. Overall therefore, sterilisation of the soil allows FOL4 to proliferate more rapidly.

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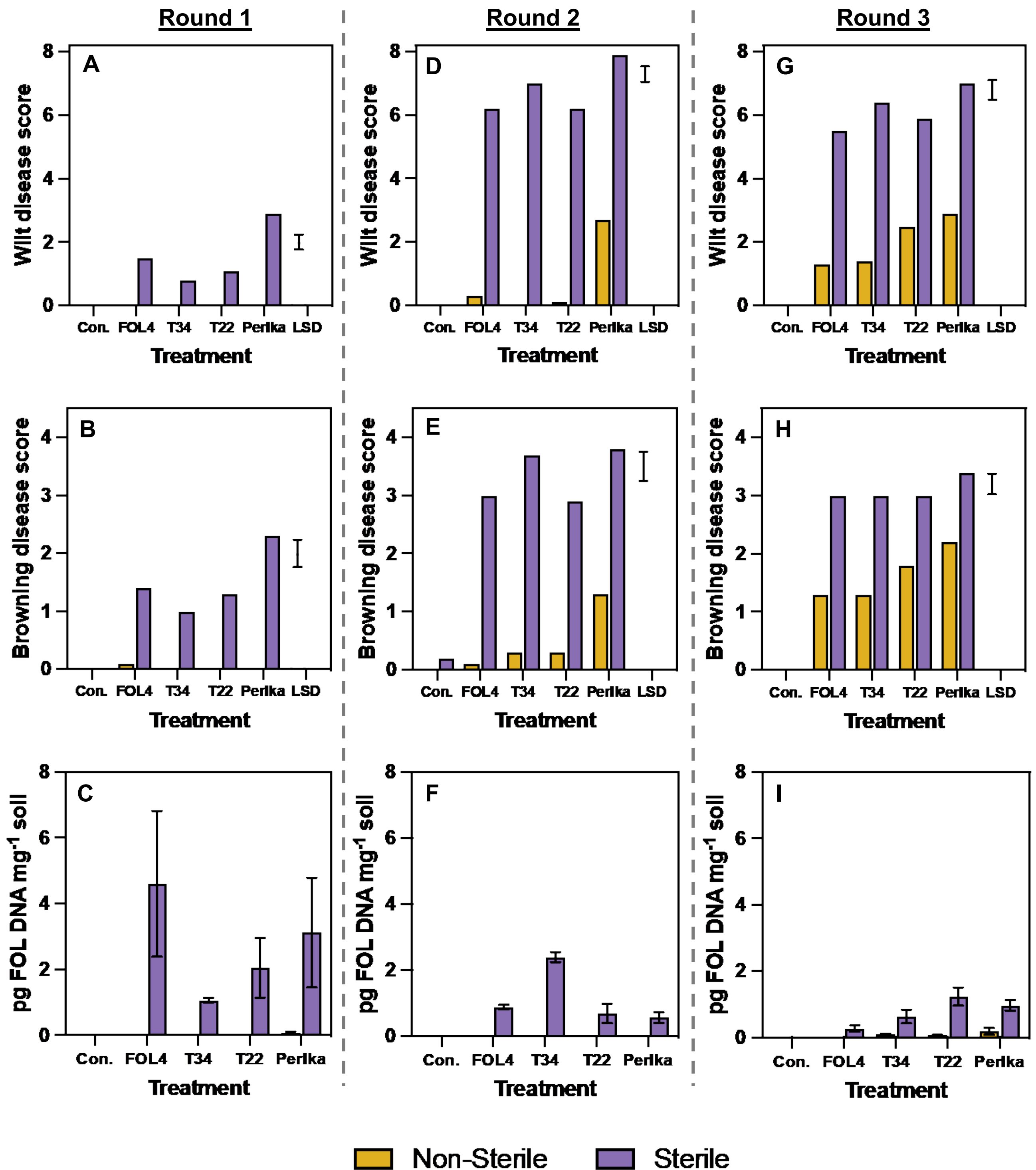

Figure 15. Average Fusarium wilt scores (A, D, G), vascular browning scores (B, E, H) and FOL4 DNA detected (in soil; C, F, I) for three rounds of lettuce grown successively in FOL4 infested sterilised or non-sterilised soil with treatments of Perlka, T22 (Trianum G) or T34 compared with no treatment (FOL4 only) and uninoculated control (Con). Error bars in graphs of wilt and vascular browning represent least significant difference (LSD), and in graphs of FOL DNA detection error bars represent standard error of the mean.

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Objective 6: Employ amplicon sequencing to quantify *F. oxysporum* pathogens alongside suppressive components of the soil microbial community

Amplicon sequencing of soils inoculated with FON

Analysis of the bacterial soil communities based on ITS amplicon sequencing

Diversity of the bacterial community in FON inoculated Soil 1 and 2 using 16S amplicon sequencing

Alpha diversity plots indicated that the bacterial community in Soil 2 was more diverse than Soil 1 when considering species richness measured by Chao 1 (Figure 16 a), while the Simpson index indicated that Soil 1 tends to be more diverse in those species that are more abundant (Figure 16 c). The Shannon index takes account of both species richness and dominance (Figure 16 b), and indicated that both soils had approximately equal diversity. Overall Soil 2 therefore has a greater variety of bacterial genera present at low levels compared with Soil 1.

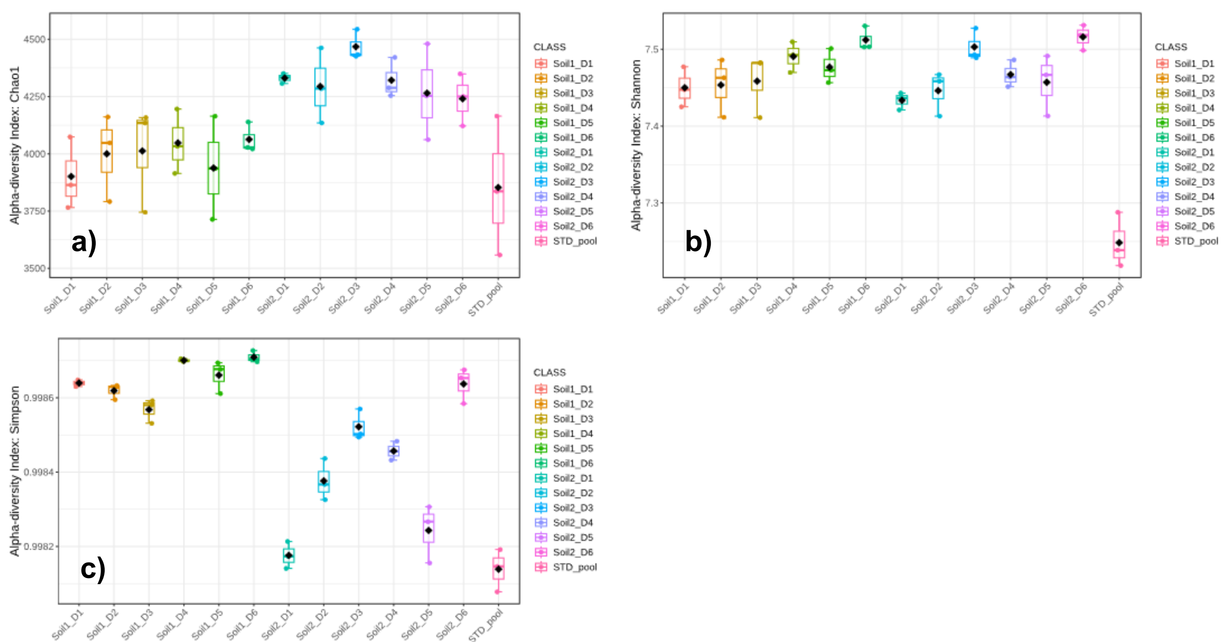

Figure 16 a) species richness (Chao 1 index), b) diversity (Shannon index) and c) dominance (Simpson index) for the bacterial community in Soil 1 and 2 inoculated with FON. All values represent an average of three replicates ± standard deviation.

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Identity of bacteria in FON inoculated Soil 1 and 2 using 16S amplicon sequencing

There were similar and diverse bacterial communities in Soil 1 and Soil 2 with no FON (D1) as measured at the phylum level, while adding FON at increasing levels (D2-D6) resulted in no obvious changes in the community structure and abundance (Figure 17).

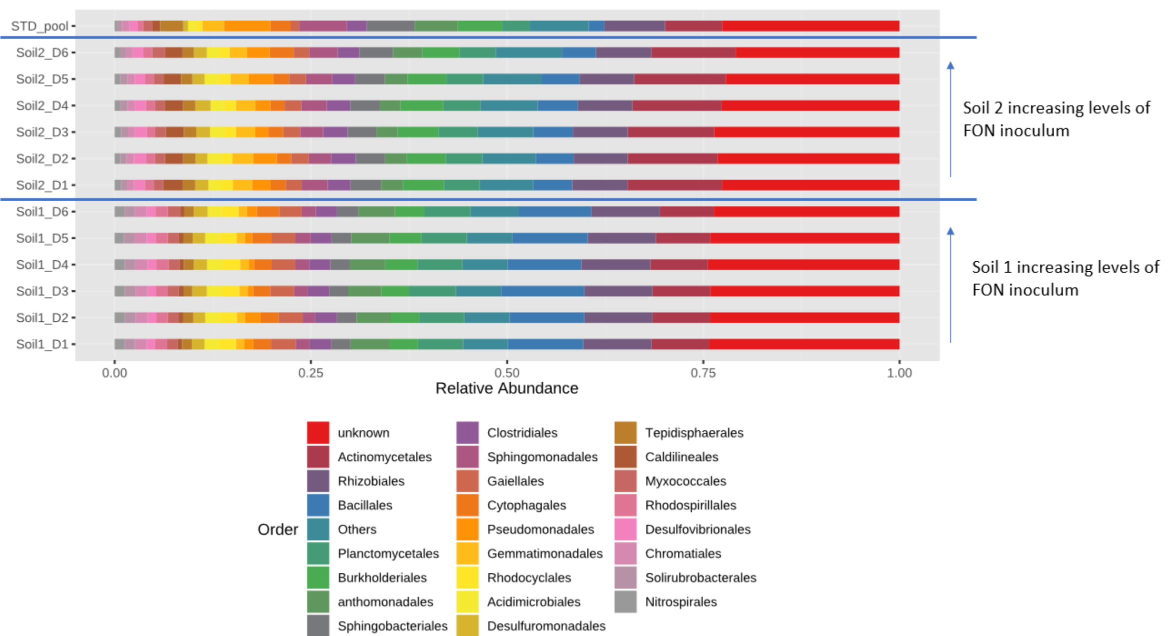

Figure 17 Abundance of bacterial genera in Soil 1 and Soil 2 for different inoculum concentrations of FON (D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil).

Analysis of the fungal soil communities based on ITS amplicon sequencing

Diversity of the fungal community in Soil 1 and Soil 2 with and without FON using ITS amplicon sequencing

In both soils there was a clear trend for a decrease in diversity of the fungal community with increasing levels of FON inoculum (Figure 18). This was particularly noticable in both the Shannon and Simpson diversity indices for the higher inoculum levels D5 and D6.

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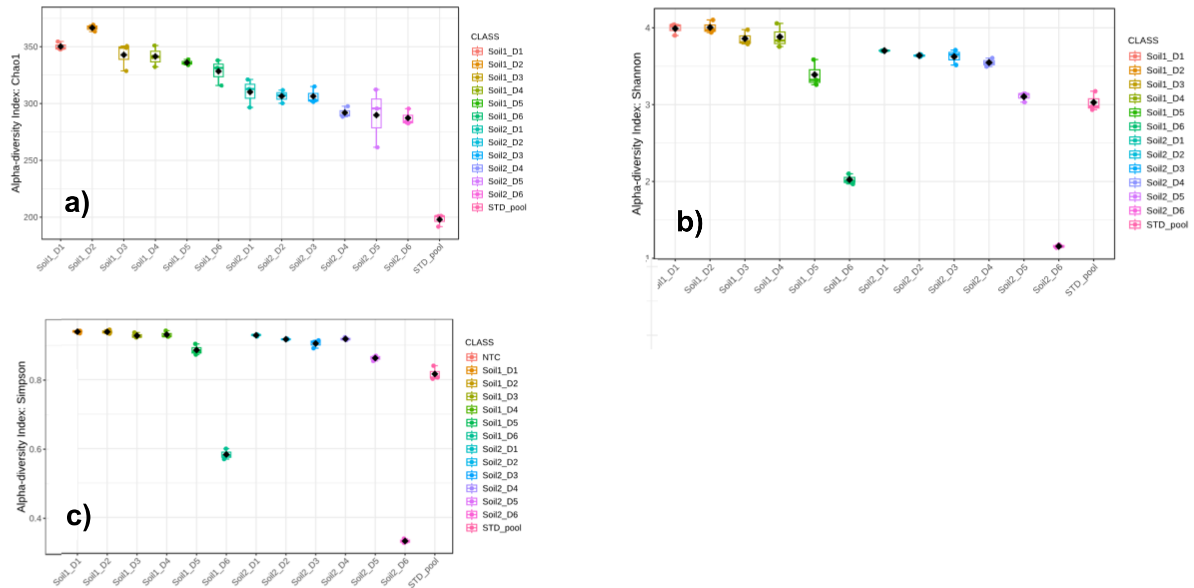

Figure 18 a) species richness (Chao 1 index), b) diversity (Shannon index) and c) dominance (Simpson index) for the fungal community in Soil 1 and 2 inoculated with FON. All values represent an average of three replicates ± standard deviation.

Identity of fungal genera in Soil 1 and Soil 2 without FON using ITS amplicon sequencing

There were some differences in the fungal populations between Soil 1 and Soil 2 in the absence of FON (D1) at the genus level. (Figure 19 a and b). Although both soils had a high proportion of *Gibellulopsis* spp. (23-24%), it was notable that Soil 2 had a high abundance of *Fusarium* spp. present (14%) compared with Soil 1 (5%). Other high abundance genera included *Podia* and *Linnemannia* spp. (10-14%) in both soils. In addition, Soil 1 had a higher abundance of Mortierella spp. (8%) compared with Soil 2 (1%).

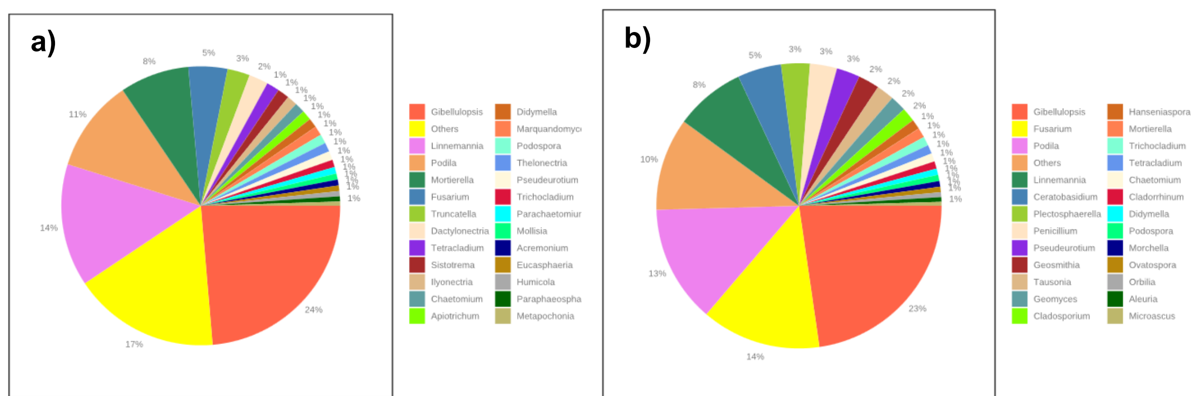

Figure 19. Relative abundance of fungal genera in Soil 1 and Soil 2 in the absence of FON.

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Identity of fungal genera in Soil 1 and Soil 2 inoculated with FON using ITS amplicon sequencing

When Soil 1 and Soil 2 fungal communities were examined with increasing levels of FON inoculum, the abundance of *Fusarium* spp. increased above the background levels in the samples without FON (D1) for inoculum levels of D4 and above (Figure 20). This indicates that use of the ITS amplicon target detects the increasing amount of FON inoculum. *Gibellulopsis* spp. continued to be at relatively high abundance up to FON concentration treatments D4 / D5.

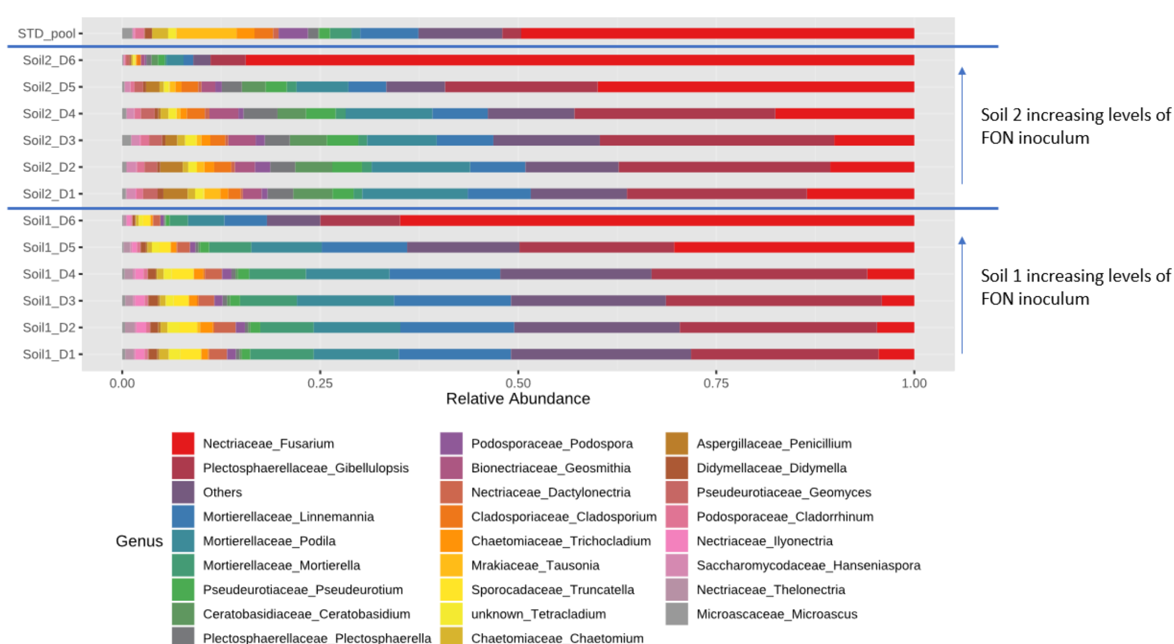

Figure 20. Relative abundance of fungal genera in Soil 1 and Soil 2 using ITS sequencing for different inoculum concentrations of FON (D1=0, D2=$2x10^2$, D3=$2x10^3$, D4=$2x10^4$, D5=$2x10^5$, D6=$2x10^6$ cfu g^{-1} soil).

Identification of *Fusarium* species in Soil 1 and 2 without FON using TEF1α amplicon sequencing

The community profile of *Fusarium* spp. and closely related species appeared to differ between Soil 1 and Soil 2 in the absence of FON inoculum (Figure 21). In Soil 1, *Ilyonectria radicicola (syn. Cyclindrocarpon destructans*), *Metarhizium majus* and *F. solani* were at high abundance while *F. culmorum*, *F. proliferatum*, *F. coeruleum*, *F. redolens* were also detected. In contrast, Soil 2 also had high abundance of *M. majus* but was dominated by *F. equisiti*. Other species also present at significant levels in Soil 2 were *F. culmorum, F. poae, F. avenaceum, F. coeruleum* and *Ilyonectria radicicola* while *F. flocciferum, F. oxysporum, F.*

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proliferatum and *F. venenatum* were detected at lower levels. Most of these species are plant pathogens causing root rots across a broad host range of plants with the exception of *M. majus* which is an entomopathogen. Only Soil 2 had detectable 'background' levels of *F. oxysporum*.

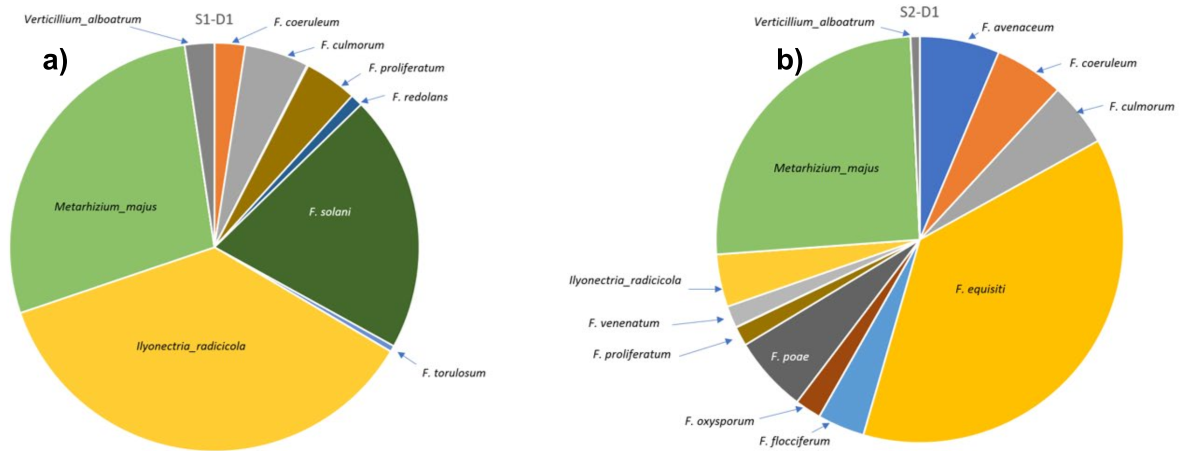

Figure 21. Relative abundance of *Fusarium* and related species identified by TEF1α amplicon sequencing in the absence of FON inoculum.

<u>Identification of *Fusarium* species in Soil 1 and 2 inoculated with FON using TEF1α amplicon sequencing</u>

When *Fusarium* spp. communities in Soil 1 and Soil 2 were examined with increasing levels of FON inoculum, the relative abundance of *F. oxysporum* also increased as expected such that by inoculum levels of D4, D5 and D6 it becomes the predominant species detected (Figure 22). This indicates that use of the TEF1α amplicon target detects the increasing amount of FON inoculum.

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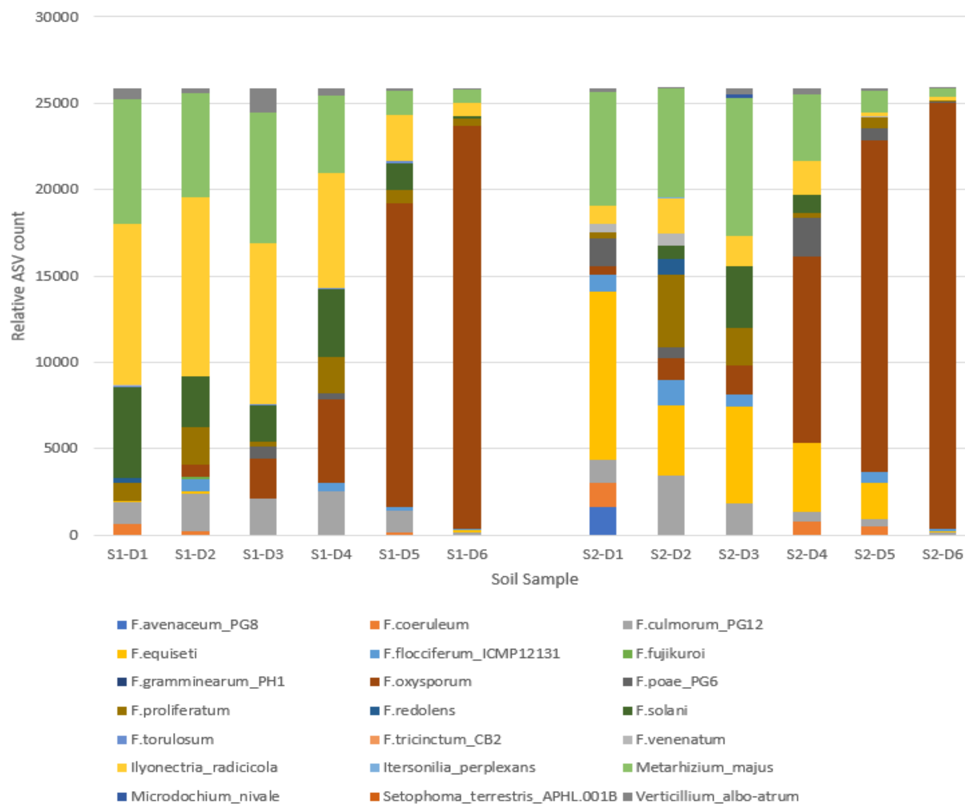

Figure 22. Relative abundance of *Fusarium* and related species identified by TEF1α amplicon sequencing at increasing levels of FON inoculum.

<u>Analysis of the *F. oxysporum* f. spp population in Soil 1 and Soil 2 using OG4952 amplicon sequencing</u>

OG4952 target gene has two paralogues in FON making it a good target for FON identification. OG4952 successfully amplified gene targets in both Soil 1 and 2 (Figure 23). No amplification was detected in S1 D1. S1 D2 showed the potential presence of FOC and *Fo conglutinans* at very low and highly variable levels. In Soil 1 FON is first detected at D3 but this is only one of the paralogues and is highly variable, making it unreliable. From D4 both paralogues of this target gene are being identified with increasing reliability. In Soil 2 the two paralogues of this gene target associated with FON are first identified at D4 ($2x10^4$ spores/g soil) with increasing reliability at higher inoculum levels.

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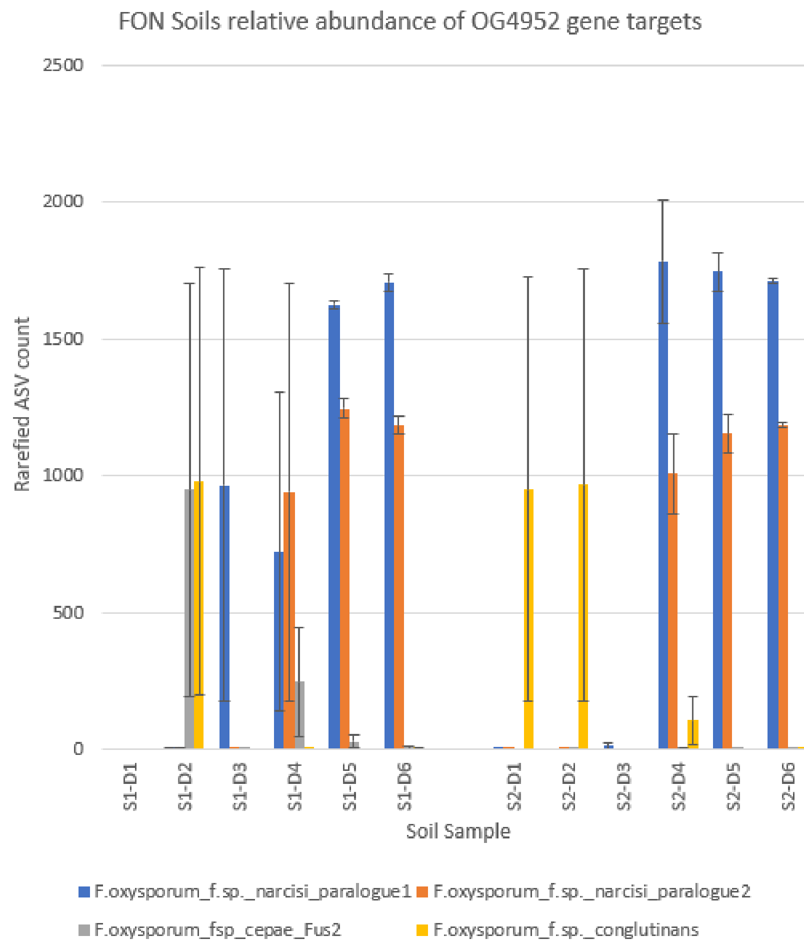

Figure 23. Relative abundance of *F. oxysporum* f.spp. using OG4952 amplicon sequencing in Soil 1 and 2 for different inoculum concentrations of FON (D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil).

<u>Analysis of the *F. oxysporum* f. spp population in Soil 1 and Soil 2 using g19096 amplicon sequencing</u>

Amplicon sequencing of g19096 identified the presence of *F. oxysporum* f. spp. in all soil samples except S1 D1 and S1 D3. Detection of the SNP pattern associated with FOC and *F. oxysporum* f.sp. *pisi* / *F. oxysporum* f.sp. *conglutinans* in S1 D2 was highly variable indicating the presence of this gene target at very low levels. In Soil 1, g19096 detected the SNP pattern associated with FON from inoculum levels of D4 upwards. In Soil 2, FON was detected at highly variable levels in D1 and D3 with reliable detection only at levels of D4 (2x10^4 spores g^{-1} soil) upwards (Figure 24).

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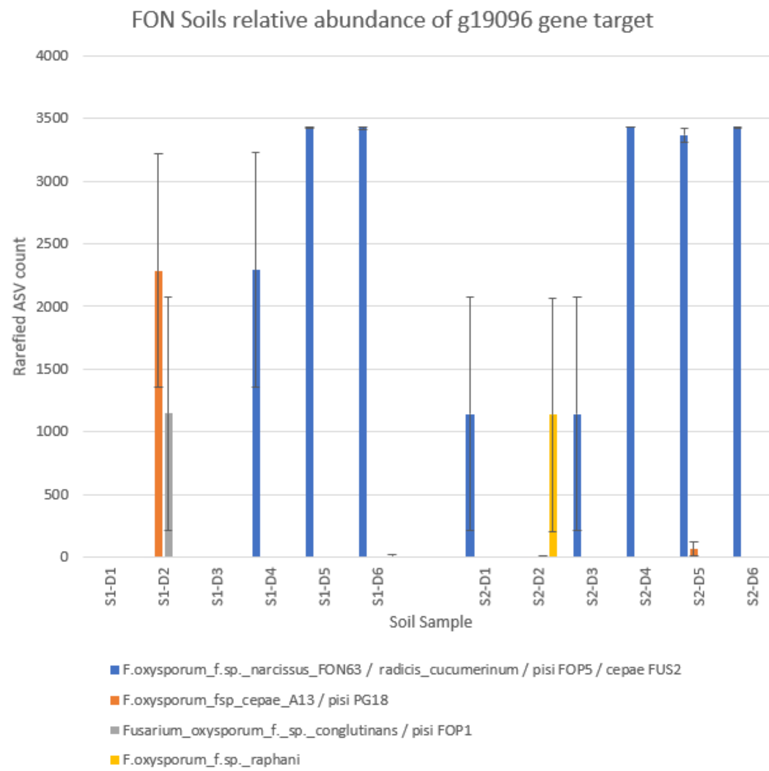

Figure 24. Relative abundance of *F. oxysporum* f.spp. using g19096 amplicon sequencing in Soil 1 and Soil 2 for different inoculum concentrations of FON (D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil).

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Amplicon sequencing of sterilised and non-sterilised soils inoculated with FOL4 with and without the addition of control treatments

Diversity of the bacterial community in sterilised / non-sterilised soil in FOL4 inoculated soil using 16S amplicon sequencing

Alpha diversity analysis was used to examine the overall effect of soil sterilisation compared with non-sterilised soil on the bacterial community over all the control treatments. Here it was evident that soil sterilisation caused a decrease in bacterial diversity as measured by 16S amplicon sequencing (Figure 25). When considering species richness (Chao, Figure 25 a) and both species richness and dominance (Shannon, Figure 25 b), there were clear reductions in bacterial diversity in the sterilised soil. This was slightly less evident for the Simpson index (Figure 25 c) where there is more emphasis on relative abundance. This implies that bacteria that are relatively abundant in the soil may recover better post-sterilisation compared with those that are less abundant.

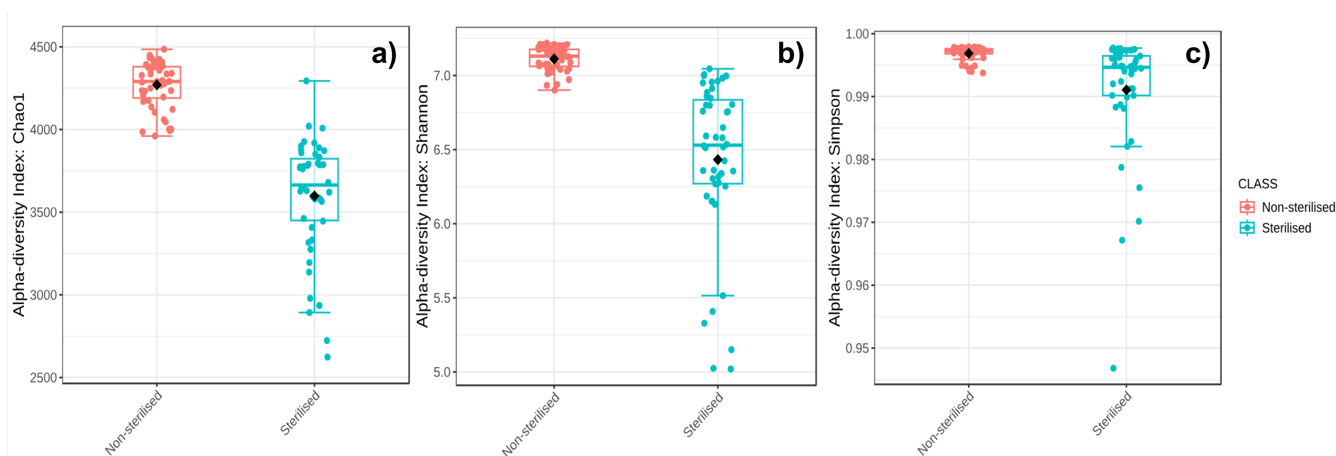

Figure 25 a) species richness (Chao 1 index), b) diversity (Shannon index) and c) dominance (Simpson index) for the bacterial community in sterilised / non-sterilised soil inoculated with FOL4 over all treatments using 16S amplicon sequencing.

Identity of bacteria in sterilised / non-sterilised soil using 16S amplicon sequencing

Overall, the general structure and relative abundance of the different components of the bacterial community did not differ much between sterilised and non-sterilised soil over all the treatments (Figure 26). At the Phylum level, sterilising the soil did however decrease the abundance of Acidobacteria with a concomitant increase in Actinobacteria (Figure 26 a) that was also evident at the Class level (Figure 26 b). At the Order level of taxonomic identification, the dominant bacteria present in both sterilised and non-sterilised soil were identified as

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Actinomycetales (Actinobacteria) and Rhizobiales (Alphaproteobacteria). At the end of the three rounds of lettuce growth the top 27 taxa of the bacterial community in the sterilised samples closely resembled that of the non-sterilised samples at all taxon levels.

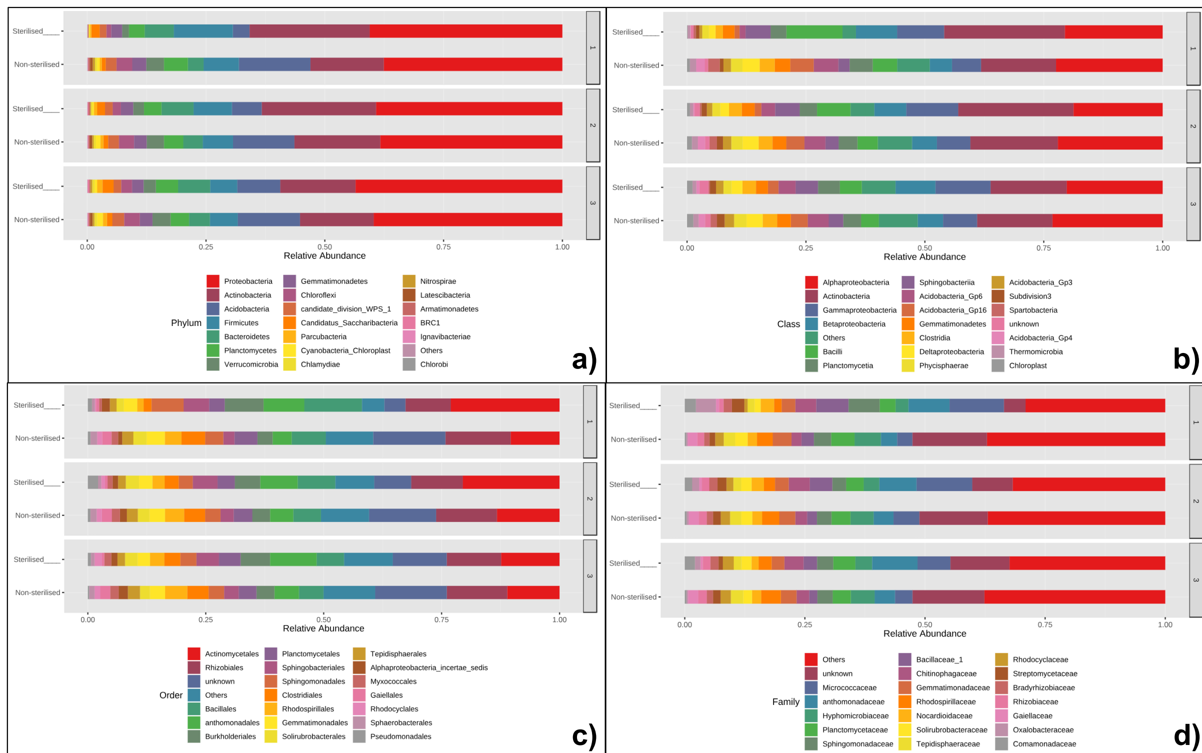

Figure 26 Relative abundance of bacterial genera at a) Phylum, b) Class, c) Order and d) Family levels of taxon for three rounds of lettuce growth in sterilised / non-sterilised soil using 16S amplicon sequencing.

Diversity of the fungal community in sterilised / non-sterilised soil in FOL4 inoculated soil using ITS amplicon sequencing

Alpha diversity analysis was used to examine the overall effect of soil sterilisation compared with non-sterilised soil on the fungal community over all the control treatments. Here as for the bacteria, it was evident that soil sterilisation caused a decrease in bacterial diversity as measured by ITS amplicon sequencing (Figure 27) in particular for species richness (Chao, Figure 27 a) and both species richness and dominance (Shannon, Figure 27 b). Again, this was slightly less evident for the Simpson index (Figure 27 c) where there is more emphasis on relative abundance and again implies that fungi that are relatively abundant in the soil may recover better post-sterilisation compared with those that are less abundant.

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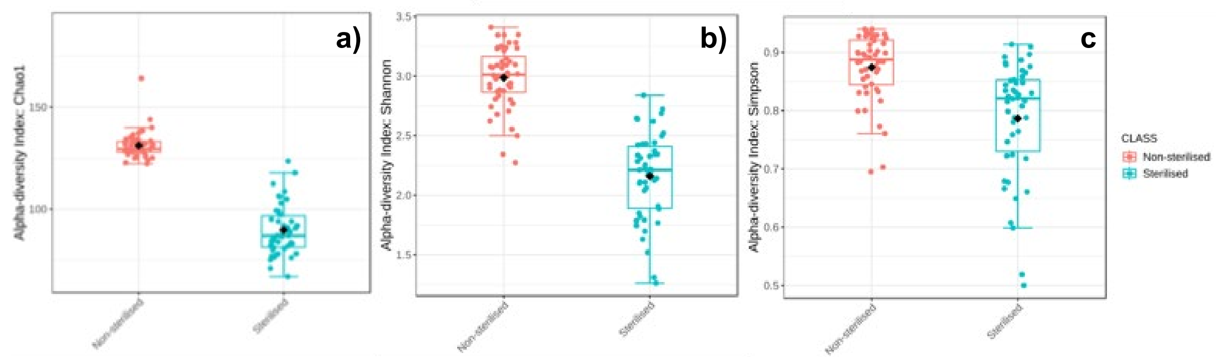

Figure 27 a) species richness (Chao 1 index), b) diversity (Shannon index) and c) dominance (Simpson index) for the fungal community in sterilised / non-sterilised soil inoculated with FOL4 over all treatments using ITS amplicon sequencing.

Identity of fungi in sterilised / non-sterilised soil in FOL4 inoculated soil using ITS amplicon sequencing

Firstly, the effect of soil sterilisation on the relative abundance of different components of the fungal community was examined at the order level by pooling the data over all the treatments and rounds of lettuce growth. Here, the Mortierellales decreased in abundance from 37% to 6% in the sterilised soil treatments while the Hypocreales were present at high abundance in both sterilised (30%) and non-sterilised (22%) soil, and in contrast therefore remained relatively unaffected by the treatment (Figure 28 ab). Next, to investigate this in more detail for each of the three rounds of lettuce growth, comparison of the fungal community was made at different taxonomic levels (Figure 28 cdef). At the Phylum level, the relative abundance of the Mucoromycota and Chytridiomycota was reduced in the sterilised soil while the Ascomycota was in much higher abundance compared to the non-sterilised soil and this was consistent over the three rounds of lettuce growth. At the Class and Order level of the Ascomycota, the Sodariomycetes and Hypocreales were also all at higher abundance in the sterilised soil compared with the non-sterilised soil with elevated levels in the second round of lettuce growth (Figure 28 de). These are also the taxonomic groups to which *Fusarium* spp. belong which was also at higher abundance at the Genus level in the sterilised soil compared to the non-sterilised soil with levels again greatest for the second round of lettuce growth (Figure 28 f). These observations are most likely explained by the greater proliferation of FOL4 in the sterilised soil (as observed for the qPCR results in Objective 5) compared with the non-sterilised soil over all the treatments. The Mucoromycota phylum which was reduced by soil sterilisation includes the classes Mortierellomycetes, Glomeromycetes and Mucoromycotina and consists mainly of mycorrhizal fungi, root endophytes and decomposers of plant material. Further examining the order and genus data suggests that the majority of the Mortierellomycetes were in the Mortieralles Order and Genus *Linnemania* (synonym of *Mortierella*). There are over 120 species of *Mortierella* which live as saprotrophs in soil, on

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decaying leaves and other organic material and they are clearly at high abundance in the non-sterilised soil but much reduced by the sterilisation process.

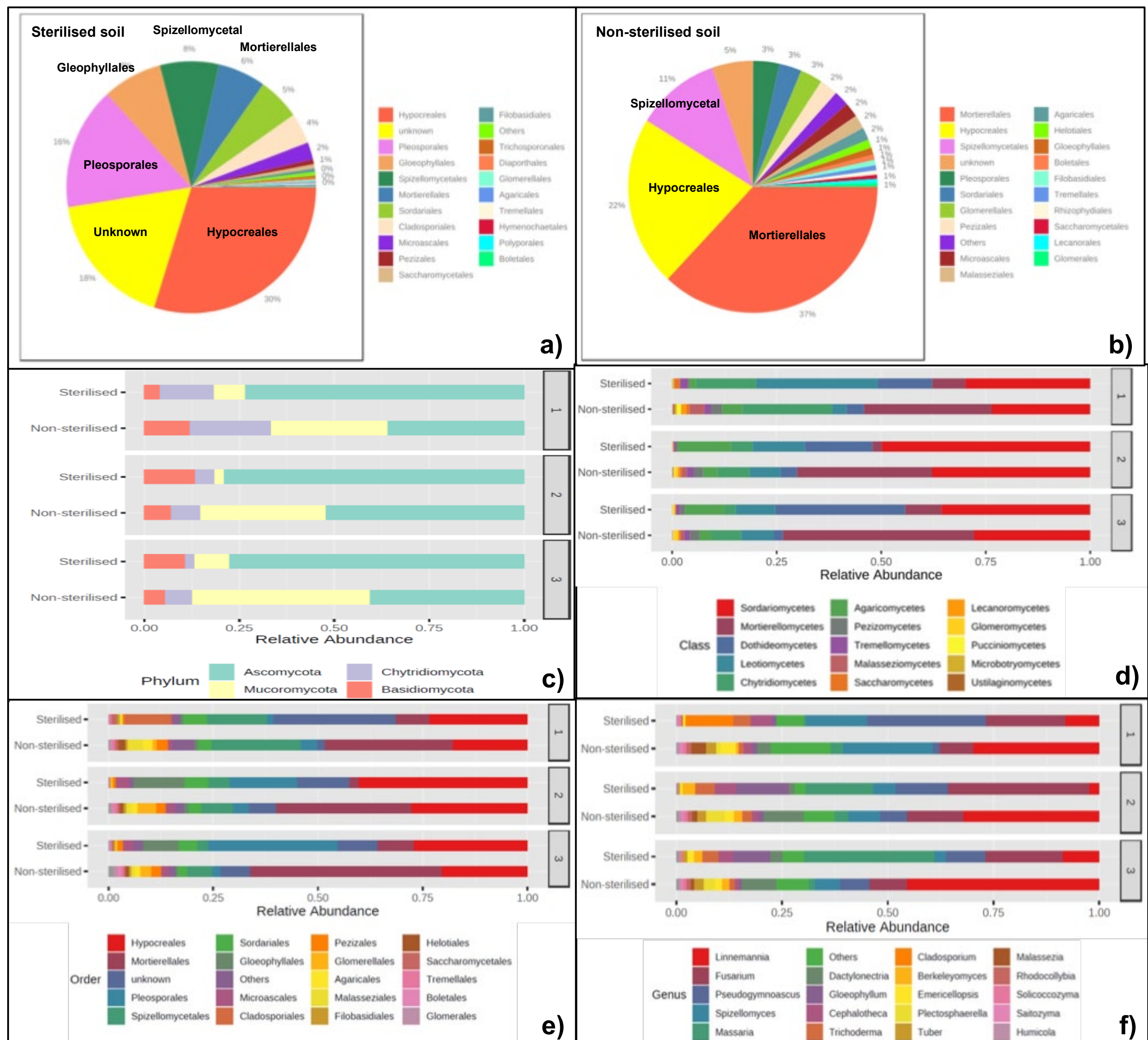

Figure 28 Relative abundance of different fungi in sterilised and non-sterilised soil over a), b) all rounds of lettuce growth at Order level and for each round of lettuce growth for c) Phylum, d) Class, e) Order and f) Genus using ITS amplicon sequencing.

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Comparison of *Fusarium* spp. abundance in sterilised / non-sterilised soil in FOL4 inoculated soil using TEF1α amplicon sequencing over three rounds of lettuce growth

To understand how the overall *Fusarium* community changed over the three rounds of lettuce growth, data for the soil samples taken at the start (round 1 set-up) and end (round 3 harvest) of the experiment were pooled for sterilised and non-sterilised soils across all the treatments (Figure 29). This showed that *F. oxysporum* spp. dominates the *Fusarium* community in both sterilised and non-sterilised soil samples which would be expected due to the FOL4 inoculations in the majority of samples. In the sterilised soil, *F. oxysporum* doubles in abundance as does *F. solani* and *Ilyonectria radicola* while both *Metarhizium majus* and *Verticillium alboatrum* both decrease in abundance from round 1 pot set-up to round 3 harvest (Figure 29 a). In the non-sterilised soil, there is a smaller increase in the abundance of *F. oxysporum* by the end of the experiment and a large increase in *Ilyonectria radicola* (Figure 29 b). Overall, these data suggest that *F. oxysporum* abundance increases more in sterilised soil than in non-sterilised soil samples. This is supported by observations in the experiment that Fusarium disease developed more rapidly in the sterilised soil (Objective 5).

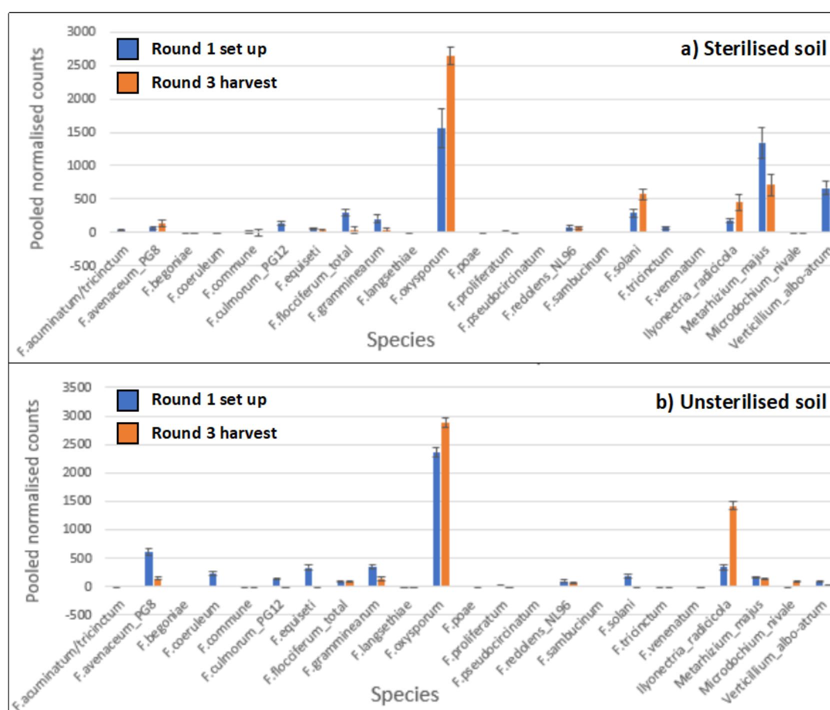

Figure 29 Comparison of relative abundance of *Fusarium* and related species at the beginning of the first round of lettuce growth compared to at harvest of the third round of lettuce growth for a) sterilised and b) non-sterilised soil using TEF1α amplicon sequencing.

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Comparison of *Fusarium* spp. abundance in sterilised / non-sterilised soil in FOL4 inoculated soil and uninoculated soil using TEF1α amplicon sequencing over three rounds of lettuce growth

To compare how the overall Fusarium community changed in response to FOL4 inoculation over the three rounds of lettuce growth, data for the soil samples taken at the start (round 1 set-up) and end (round 3 harvest) of the experiment for sterilised / non-sterilised and for FOL4 inoculated / uninoculated soils were examined across FOL4 only / no FOL4 control treatments (Figure 30). At the start of the experiment (set up of round 1), sterilised and non-sterilised soils contained a high abundance of *F. oxysporum* in both FOL4 inoculated and uninoculated samples. This suggests that the soil used for experiments had a relatively high proportion of background *F. oxysporum* (most likely non-pathogenic) isolates. In the sterilised soil in the absence of FOL4 (no inoculation) there was a modest increase in the abundance of *F. oxysporum* in the community by the end of the experiment (round 3 harvest) from 282 to 419 reads per 1000 (Figure 30 ac). In contrast, for the FOL4 only inoculated treatment, there was a substantial increase in *F. oxysporum* reads from 179 to 681 per 1000 (Figure 30 ac). This suggests that the large increase in *F. oxysporum* in the sterilised soil is most likely due to FOL4 inoculation rather than an increase in the background *F. oxysporum* community. This was supported by the results over all treatments (described above) and the more rapid increase in Fusarium disease observed in the sterile soil in the experiment (Objective 5). In contrast in the non-sterilised soil there was little change in the *F. oxysporum* abundance in either uninoculated (slight decrease 550 to 419 reads per 1000 reads) or FOL4 only inoculated treatments (slight increase 431 to 523 reads per 1000; Figure 30 bd). This suggests that post-inoculation FOL4 did not increase over the three rounds of lettuce growth in the experiment. However in contrast, a modest increase in Fusarium disease was observed in the experiment for non-sterilised soil suggesting that some proliferation, but perhaps below the threshold that could be detected by amplicon sequencing. Other *Fusarium* species were also identified in both sterilised and non-sterilised soil, with several species present in the former at set-up (Figure 30 a). Generally, these tended to decline in abundance by harvest of the final round 3 of lettuce growth (Figure 30 c).

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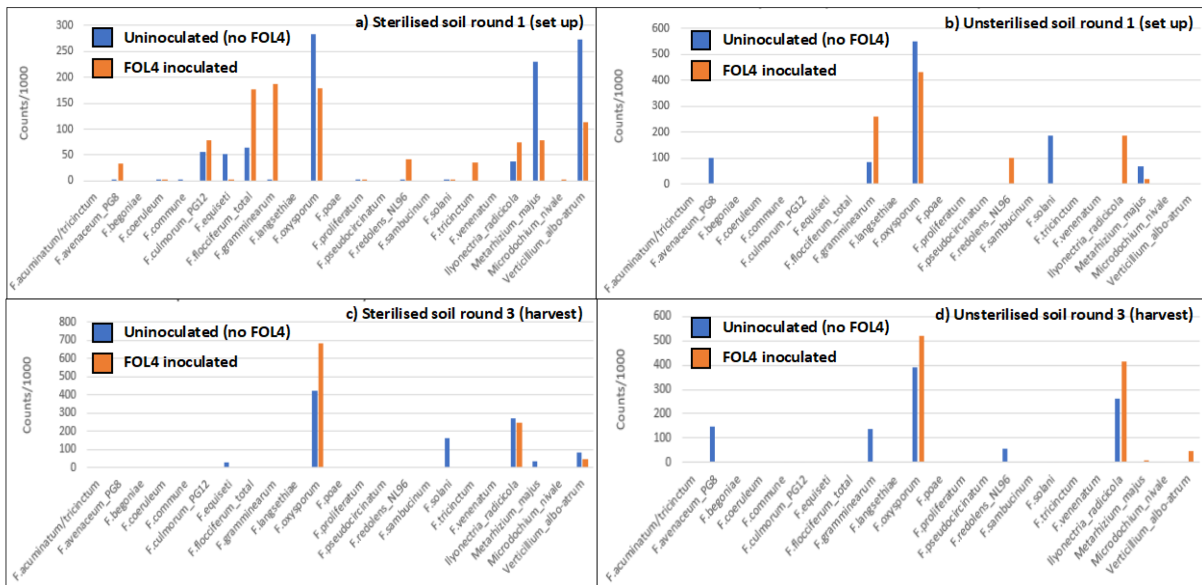

Figure 30 Relative abundance of *Fusarium* and related species at the beginning of the first round of lettuce growth compared to at harvest of the third round of lettuce growth for a), c) sterilised and b), d) non-sterilised soil using TEF1α amplicon sequencing.

<u>Comparison of *Fusarium* spp. abundance in sterilised / non-sterilised soil in FOL4 inoculated soil using ITS amplicon sequencing over three rounds of lettuce growth</u>

Having identified the changes in bacterial, fungal and Fusarium communities over the three rounds of lettuce growth it was clear that FOL4 greatly increased in abundance in the sterilised soil but much less so in non-sterilised soil. This was reflected in the increasing amounts of disease observed in the experiment in all the sterilised soil treatments (see Objective 5). This suggested that build-up of FOL4 is much slower in non-sterilised soil but also that the two *Trichoderma* products Trianum G (*T. harzianum* isolate T22) and T34 Biocontrol (*T. asperellum* isolate T34) that were tested as soil applications did not suppress FOL4. To determine the relative abundance of both FOL4 and the *Trichoderma* spp. at setup of round 1 and at harvest of round 3 lettuce growth the ITS amplicon sequencing data was again examined at the Genus level to determine the % reads for each organism. The ITS region for both T22 and T34 were sequenced which enabled these particular isolates to be distinguished from any background *Trichoderma* spp. in the soil. Unlike TEF1α, ITS sequence does not identify individual *Fusarium* spp. but as outlined in the TEF1α analysis above, the majority of *Fusarium* reads were identified as due to FOL4 and hence ITS sequence reads are likely to be a good proxy for the abundance of FOL4.

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For the FOL4 + T34 treatment, relative abundance at set up for round 1 was low for both fungi in both sterilised and non-sterilised soil (Figure 31 ac; <6% of reads) but after harvest of round 3 of lettuce growth the percentage reads for both Fusarium and Trichoderma has increased to 17% (Figure 31 b) but abundance for both fungi remained relatively unchanged for the non-sterilised soil (Figure 31 d). In all cases, reads for T34 accounted for more than 82% of total *Trichoderma* spp. reads. These results suggest that FOL4 proliferated in the sterile soil over the three rounds of lettuce growth even in the presence of T34 which also increased in abundance. However, both populations remained static for the non-sterilised soil.

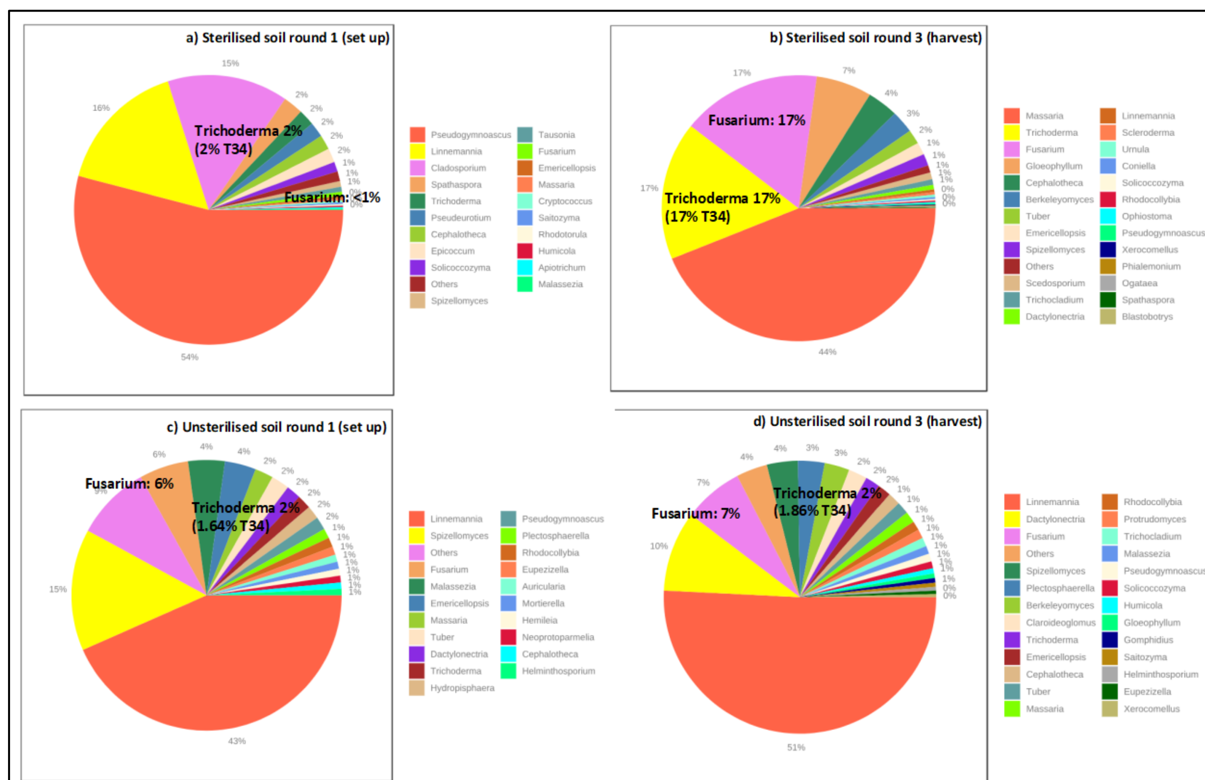

Figure 31 Relative abundance of *Fusarium* (majority FOL4) and *Trichoderma* (majority *T. asperellum* T34) spp. in sterilised (a, b) and non-sterilised soil (c, d) at the beginning of the first round of lettuce growth (a, c) compared to at harvest of the third round of lettuce growth (b, d).

For the FOL4 + T22 treatment, relative abundance at set up for round 1 was low for both fungi in both sterilised and non-sterilised soil (Figure 32 ac; <5% of reads) but after harvest of round 3 of lettuce growth the percentage reads for both *Fusarium* and *Trichoderma* had increased to 19% and 8% respectively (Figure 32 b) while for the non-sterilised soil *Fusarium* had increased slightly from 5% to 10% and *Trichoderma* remained relatively unchanged at 2% of reads (Figure 32 d). In all cases, reads for T22 accounted for more than 94% of total *Trichoderma* spp. reads. These results suggest that FOL4 proliferated rapidly in the sterile soil

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and also to a certain extent in the non-sterilised soil even in the presence of T22, and that T22 was less able to colonise sterile soil.

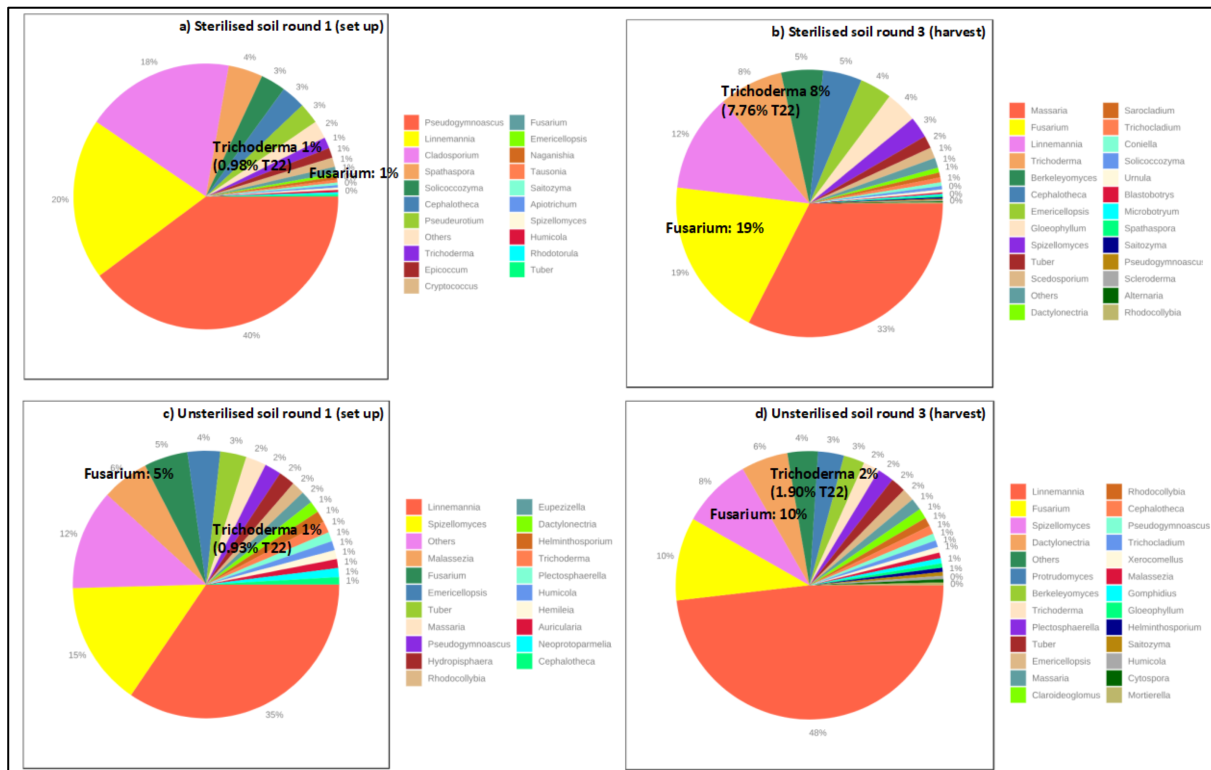

Figure 32 Relative abundance of *Fusarium* (majority FOL4) and *Trichoderma* (majority *T. harzianum* T34) spp. in sterilised (a, b) and non-sterilised soil (c, d) at the beginning of the first round of lettuce growth (a, c) compared to at harvest of the third round of lettuce growth (b, d).

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Objective 7: Evaluate products for control of FOC in field experiments

A selection of treatments was tested for their efficacy in controlling Fusarium disease on onion in the field. Four sites in total (two each managed by ABC and VCS) were used to test products as either a foliar application or as an in-furrow treatment (Table 3).

In the ABC trials, none of the tretaments significantly increased the number of onion bulbs which remained healthy at harvest. For site FP1 at harvest, there was a significant overall effect of treatment ($p = 0.036$) in the ANOVA, but the only significant difference was that Perlka resulted in fewer healthy bulbs at harvest compared to the control (and other treatments, Figure 33 A and B). However, Rudis applied as an infurrow treatment or in-furrow+foliar resulted in the greatest number of healthy bulbs at harvest and after storage. Similarly at field site RIX, although there was no overall significant effect of the treatments, Rudis applied as an in-furrow treatment (but not in-furrow+foliar) resulted in more healthy bulbs at harvest and after storage (Figure 33 C and D).

In the VCS trials, there were no overall significant effects from treatments in the VCS 2021 trial (Figure 34 A and B), but as above Perlka reduced the number of healthy bulbs at harvest and after storage. In addition, Rudis applied as an in-furrow treatment or in-furrow+foliar resulted in the greatest number of healthy bulbs at harvest and after storage.

Although there was a significant overall effect of the treatments in the VCS 2022 site ($p<0.001$ at harvest and after storage), the only significantly different treatment compared to the control again was Perlka, which reduced the number of healthy bulbs (Figure 34 C and D). No other treatment had any significant effects to improve the number of healthy bulbs compared to the control, but disease pressure was low for this site given that all treatments had a high number of healthy bulbs with little difference between treatments.

Overall therefore, Rudis appears to consistently increase the number of healthy bulbs in onion fields with Fusarium compared to untreated plants but in these trials the effect was not statistically significant.

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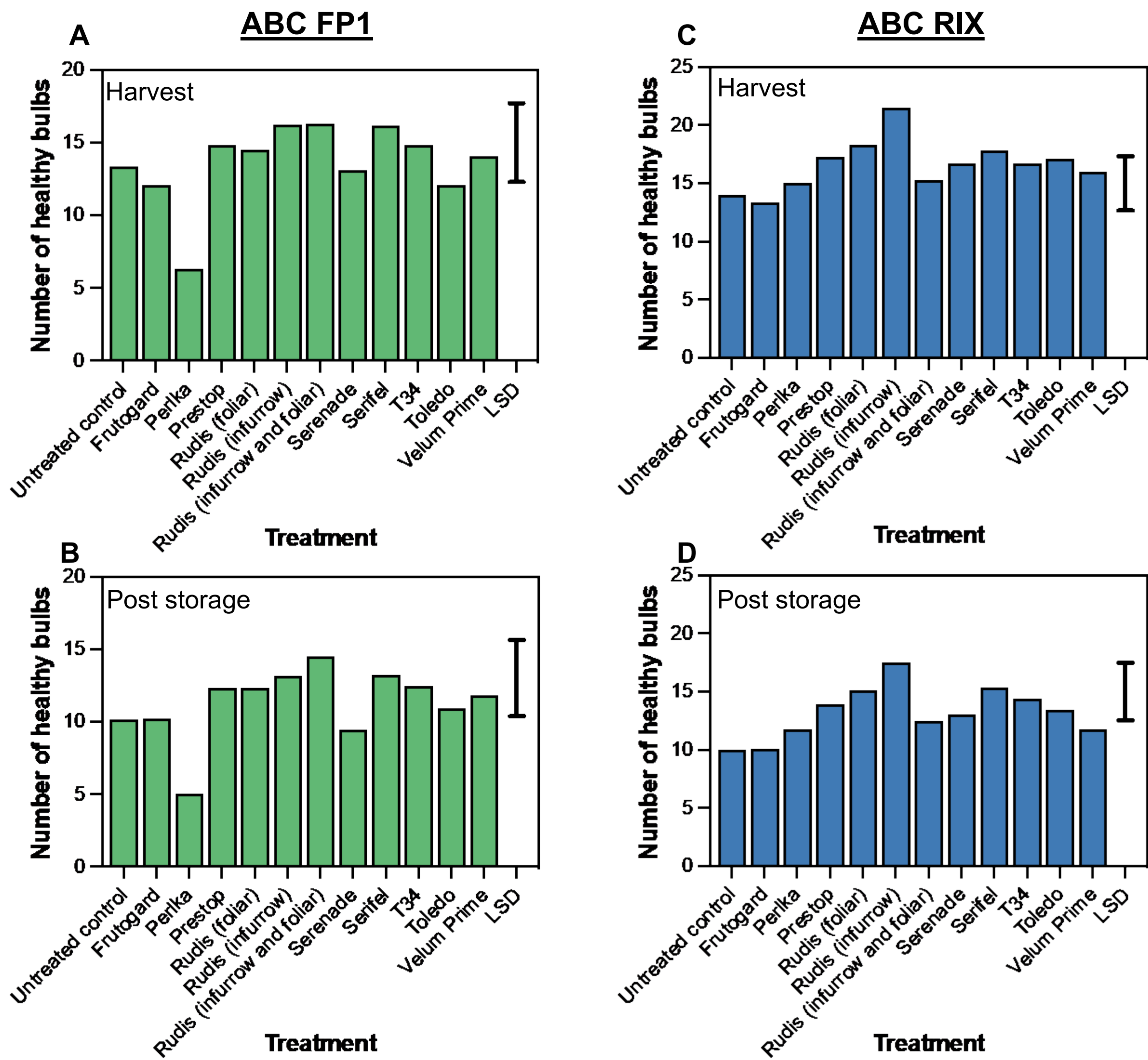

Figure 33. Number of healthy onion bulbs at harvest and post-storage for two ABC field sites with Fusarium (FP1 – A and B; RIX – C and D) treated with various products. Error bars represent least significant difference (LSD at 5%).

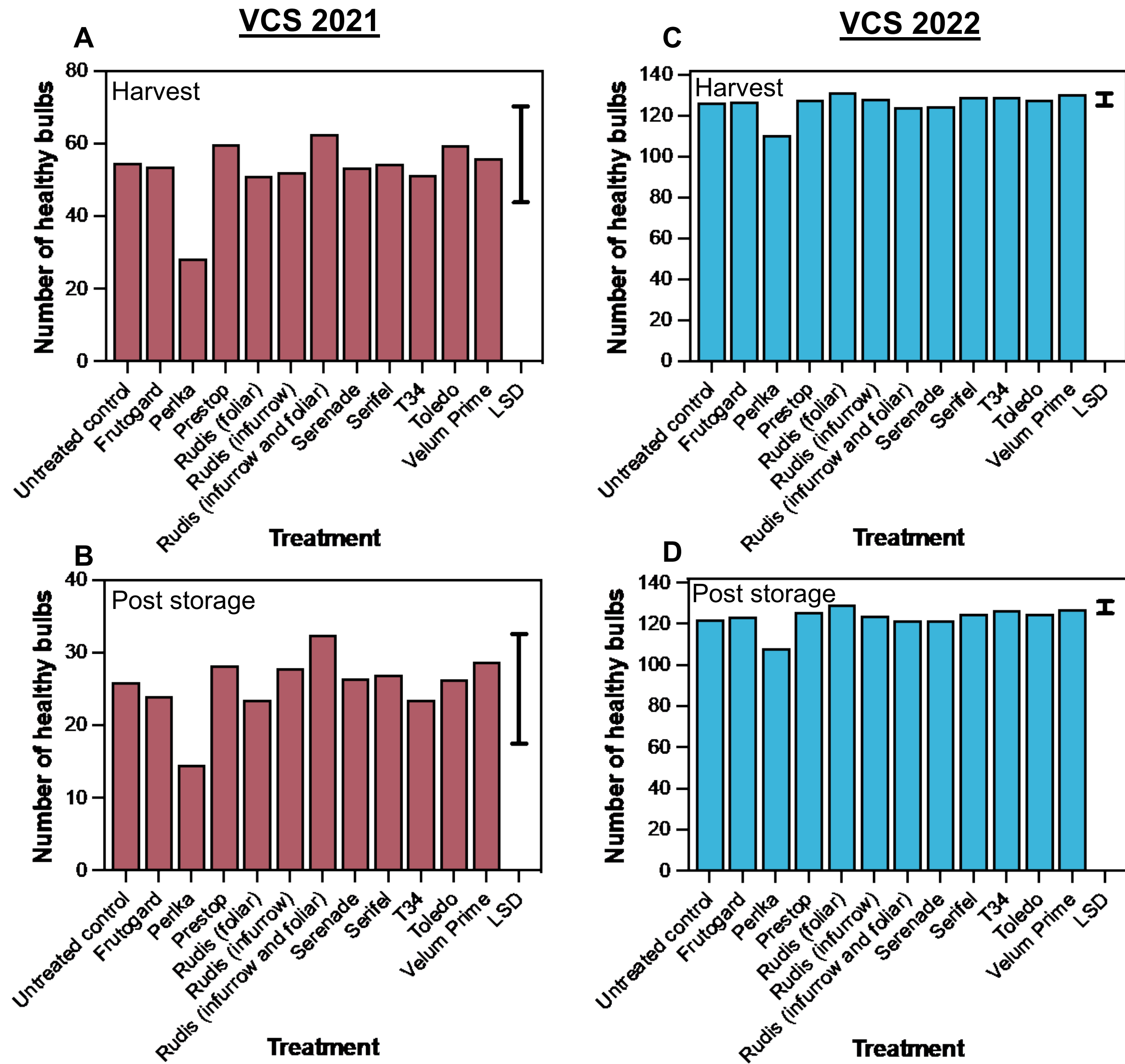

Figure 34. Number of healthy onion bulbs at harvest and post-storage for two VCS field sites with Fusarium (2021 – A and B; 2022 – C and D) treated with various products. Error bars represent least significant difference (LSD at 5%).

Discussion

Objective 1: Define a relationship between the amount of Fusarium DNA, Fusarium inoculum and disease development in field soil (dose response)

Effect of FOL4 inoculum concentration on disease development in lettuce and pathogen detection

The results from the experiment where lettuce was grown in FOL4 inoculated soils at different concentrations revealed a clear relationship between FOL4 inoculum concentration, disease development and the amount of FOL DNA detected in the soil. In two soils, FOL4 was detected at quite a low level of inoculum (2×10^2 cfu g^{-1} soil). This suggests that qPCR could be used to quantify FOL4 in soil samples and identify those with high levels and hence determine risk of Fusarium wilt disease. In contrast, FOL4 DNA detection was only possible in roots grown at higher inoculum doses. The two soils obtained from growers for this experiment varied in their physical composition, with Soil 1 having a much higher organic matter content compared with Soil 2 which in turn was greater than Soil 3 (from Wellesbourne). Nonetheless, high levels of disease were observed in all three soils suggesting that organic matter did not have a major impact on disease development. However, Fusarium disease levels in lettuce grown in Soil 1 were slightly lower than in those in Soil 2, but higher than those in Soil 3 and this was reflected in the amount of DNA detected in lettuce roots, where those in Soil 1 had less FOL4 DNA than the other soils at the highest concentration of FOL4 inoculum.

Effect of FON inoculum concentration on disease development in Narcissus and pathogen detection

Two different field soil types were inoculated with FON, along with a compost mix suitable for growing daffodils. As observed in the experiment with FOL4 and lettuce, there was a strong positive correlation between FON inoculum concentration, basal rot disease, FON DNA in soil and FON DNA in roots suggesting that qPCR diagnostics could be used to estimate the concentration of FON in field soils and predict disease risk. A small quantity of FON DNA was detected in the roots of Narcissus bulbs grown in the control (non-inoculated) treatment for Soil 1. This was likely due to some FON already being present in this soil at a low level. It was also noted that levels of basal rot were lower for the Narcissus bulbs grown in the two soils obtained from growers which were classified as clay and sandy silt loam respectively. In contrast to the Narcissus compost, these soils had much lower amounts of peat which might be a factor explaining the lower disease levels in the soils.

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Overall, in this objective we have successfully demonstrated that quantitative molecular diagnostics tools quantifying FON and FOL in field soils can potentially be used to assess risk of disease in Narcissus and lettuce.

Objective 2: Evaluate the use of molecular diagnostics to detect and assess the risk of Fusarium disease in onion

Soil and onion root samples were collected from grower fields across three growing seasons, between 2020-2022, to evaluate the use of a FOC specific qPCR assay for disease prediction. Onions were also assessed for disease over the growing season, at harvest and after a period of storage, so FOC detection could be related to the amount of disease in at each site. Two approaches were taken: i) analysis of soil samples pre-drilling across multiple commercial onion field sites and ii) analysis of both soil and onion root samples at different timepoints during the growing season at two intensively sampled commercial onion field sites.

Over the project it became clear that FOC DNA was very rarely detected in pre-planting soil samples and hence a different onion seedling 'baiting' approach was examined (see below). For the intensively sampled field sites with a history of Fusarium disease, soil collected in 2020 over the season had low levels of FOC DNA, and the pathogen was detected sporadically (not in all plots at each site). In contrast, FOC DNA was detected in higher quantities in roots from onions grown at the same sites across the season. Disease levels, especially post storage, were high, which corresponded quite well with FOC DNA detection in onion roots. Of the sites tested in 2021, FOC DNA was detected in very few onion root samples across the whole season, but it was still evident that FOC was more reliably detected in roots compared with soil. Fusarium basal rot disease levels in 2021 were correspondingly low. Fewer timepoint samples were collected in 2022, but from more field sites and two of these had detectable levels of FOC DNA (PGR L/A and FP1). For FP1, all root samples at each timepoint were positive and FOC DNA concentrations were greater. These results were reflected by a higher level of disease at FP1 compared with PGR L/A. Overall, detection in onion roots was therefore shown to be a more reliable way to detect FOC in the field compared with soil samples but this has limited practical application for growers.

The most useful solution for growers is to have a pre-planting soil test for FOC. As noted above, direct detection by soil DNA extraction and qPCR was not sensitive enough to detect FOC and hence we investigated growing onion seedlings in the soil samples as a means of 'baiting' out and allowing FOC to proliferate, hence increasing the sensitivity of detection. This hypothesis was initially tested using soils collected pre-drilling in 2021 but only one site had detectable levels of FOC DNA in onion roots, and very little disease was recorded in those

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fields that year. The approach was repeated for pre-planting soil samples from onion fields in 2022. Here, the soil was again tested directly but also after onion seedlings had been grown for 7-8 weeks with the roots also assessed for presence of FOC at harvest. Here it was found that there was a significant increase in FOC detection between the soils at sowing and at harvest with higher levels of DNA detected and more sites with positive detection in the latter. As had been shown from the field sampling, onion root samples resulted in enhanced FOC detection, with DNA levels around 100-fold higher in roots than in soils post-harvest and yet more sites then tested positive using this approach. This plant ‘enrichment’ for FOC could therefore be further refined to predict the risk of basal rot disease in onion using pre-planting soil samples.

Objective 3: Evaluate the use of molecular diagnostics to determine the presence of FOC in harvested onion bulbs to assess the risk of disease development in store

Detection of FOC in onion bulbs by PCR diagnostics is a potentially useful approach for assessing the risk of basal rot developing in store. In year 1 of the project (2020), we confirmed that PCR could detect FOC in the basal plates of onion bulbs with different levels of basal rot symptoms but also in some asymptomatic bulbs. In 2021, we focussed on FOC detection in asymptomatic healthy onion bulbs but all the bulbs tested negative and only a few bulbs from the same batch of onions developed symptoms when incubated at temperatures favourable to FOC. In 2022, apparently healthy onion bulbs from the inoculated quarantine field at Wellesbourne were tested. Here FOC was detected in 38% of asymptomatic bulbs while 51% of bulbs from the same batch went onto develop basal rot when incubated under conditions conducive to disease development. In one sample of apparently healthy onions from a high-risk grower site, around 10% of bulbs tested positive for FOC with 24% of the same batch developing basal rot after incubation. Overall therefore, PCR diagnostics to detect FOC in onion basal plates is a promising approach to predict the likelihood of basal rot developing in storage which is a particular issue for growers, as only relatively low levels of disease are required for complete store loss.

As DNA-based PCR based diagnostics was particularly useful for FOC detection in onion bulbs, we also evaluated a LAMP assay (a quicker version of PCR which uses crude DNA extracts and a portable machine) previously developed at Warwick for assessing presence of FOC in artificially inoculated onion bulbs. Here, results showed that we could detect FOC in the basal plates as early as 8 days after inoculation before any symptoms appeared, which suggests that FOC can also be detected in asymptomatic onion bulbs using LAMP, as well as

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qPCR. Basal plates which tested positive for FOC from 15 days onwards all had the first symptoms of Fusarium basal rot. In future work, the LAMP assay would need to be tested using apparently healthy onion bulbs from commercial sites with a history of Fusarium (as was done with qPCR), to determine if detection in bulbs is reliable and can be related to subsequent basal rot development. If successful, LAMP provides a far more rapid method of testing bulbs for FOC and also has the advantage of using a simple DNA extraction method from fresh basal plate tissue and takes only a few hours from start to finish to complete. Therefore, LAMP could be used as a rapid FOC detection method for onion bulbs before they go into storage.

Objective 4: Determine colonisation of non-host plants by FOC to identify suitable rotation crops

As is well known for many *F. oxysporum* f.spp., spores can survive in the soil for many years, even in the absence of a host. Due to this, lengthy crop rotations are usually required to allow inoculum levels to decrease to low enough levels as to not cause disease next time the host grown in that soil. However, it has been noted recently by onion growers that even with long crop rotations, disease levels can remain high; therefore, it was hypothesised that FOC may be able to survive and even proliferate on non-host crops in the rotation. To test this, non-host crop species were grown in FOC inoculated compost and colonisation of roots examined by qPCR. FOC was found to colonise the roots of all the crops tested to varying degrees. For instance, FOC was detected in 12 and 10 plants for pea and maize respectively compared to only three plants for oilseed rape. Concentrations of FOC DNA in roots also varied but were far lower for non-host crops (on average >100-fold lower) compared with roots of susceptible and resistant onion cultivars. Nonetheless, even some root colonisation of non-host crops over multiple years could maintain FOC levels at high enough concentrations in the soil to cause disease on onions next time they featured in the rotation. Therefore, it may be important to include crops where FOC colonisation was lower such as oil seed rape, barley and wheat, as opposed to crops where colonisation was more consistent (pea and maize).

Objective 5: Investigate the feasibility of establishing Fusarium-suppressive microbial communities and biological control agents in protected cropping systems (sterilised soil)

During Year 1 of the project (2020) a method was developed to examine FOL4 inoculum build up and disease development in sterilised and non-sterilised soils with successive rounds of

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lettuce plant growth in pots (Clarkson, 2021). In year 3 of the project (2022) this system was used to determine whether FOL4 build up could be reduced or prevented by products such as Perlka, Trianum G and T34 Biocontrol using soil from a UK lettuce grower. All these treatments had some previous published evidence that they might have some activity against *Fusarium* disease. Calcium cyanamide (Perlka) has fungicidal activity when it is converted to hydrogen cyanamide in the soil (McDonald et al., 2021), *T. harzianum* T22 (Trianum G) reduced Fusarium disease in lettuce (FOL race 1) (Gilardi et al., 2007) and *T. asperellum* T34 has been shown to reduce disease caused by *F. oxysporum* f. sp. *lycopersici* in tomato (Cotxarrera et al., 2002). Although soil sterilisation has been used to reduce FOL4 inoculum in indoor lettuce cultivation in Europe, it has also been noted that disease can return quickly due to a decrease or change in the microbial community (O'Neill et al., 2005); therefore finding products to mitigate this is important. Over the three rounds of lettuce growth, it was clear that FOL4 inoculum build-up was much quicker in sterile soil compared to non-sterile soil but unfortunately, none of the treatments prevented this occurring or reduced disease development compared with a FOL4 only control. In the non-sterile soil at the end of the third round of lettuce growth, only mild to moderate Fusarium disease was observed in the lettuce compared with much more severe levels of disease for plants grown in the sterile soil. This was concomitant with the finding that there was a general reduction in microbial community diversity in sterilised soil and a predominance of the pathogen FOL4 (Objective 6). Quantification of FOL4 inoculum using qPCR revealed that FOL could be detected at harvest in sterilised soils only for the crop 1 and 2, with only very low levels detected in the non-sterilised soils by the end of crop 3, again confirming that that the pathogen proliferated rapidly in sterilised soil. As the amount of FOL4 inoculum used to infest the soil was low, it was difficult to detect FOC DNA accurately and reliably and levels did not appear to increase as with each crop of lettuce. This could be due to low levels of inoculum and also difficulties in reliably detecting *F. oxysporum* in soils, as we observed for FOC in onion in Objective 2.

Objective 6: Employ amplicon sequencing to quantify *F. oxysporum* pathogens alongside suppressive components of the soil microbial community

Overall, amplicon sequencing was successfully used to quantify the relative abundance of bacterial (16S), fungal (ITS) and *Fusarium* spp. (TEF1a) communities in both FON and FOL4 experiments which examined effects of inoculum concentration in different soils (Objective 1). Moreover, the novel gene target OG4952 was also effective in identifying the presence of FON in soils at inoculation levels of $2x10^4$ spores g^{-1} soil and above in both soils tested. This technique is therefore useful in understanding how the microbial community reacts to the

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presence of *F. oxysporum* pathogens. The use of novel gene targets (such as OG4952) to specifically identify a range of different *F. oxysporum* f.spp. is challenging as often the sequences of these targets are identical for several *F. oxysporum* f.spp. or they result in unexpected positive detection even when it is known that the pathogen is not present. The presence of two paralogues for the OG4952 target in FON makes it a good identifier as both targets need to be present. In contrast, for the gene target (g19096) used to identify FOL4 (see annual report 2022), it was found that high levels of endogenous *F. oxysporum* in some soils gave false positives. What is clear however is that the TEF1a is an excellent gene target for identifying *F. oxysporum* in soils with multiple *Fusarium* species present. In inoculated systems as investigated in this project, the abundance of a particular *F. oxysporum* f.sp. used in experiments can often be effectively quantified by TEF1α amplicon sequencing but only if the background level of other *F. oxysporum* isolates is low (as this target cannot distinguish f.spp) and inoculum levels are greater than $2x10^3$ cfu g^{-1} soil. Similarly, in some cases the ITS gene target can also be used a proxy for *F. oxysporum* abundance but only if levels of other *Fusarium* spp. are low (as this target cannot distinguish different *Fusarium* species).

The establishment of a novel system to examine build up of FOL4 inoculum over three rounds of lettuce growth in both sterilised and non-sterilised soil with and without *Trichoderma* / Perlka treatments gave us the opportunity in the final year of the project to examine the microbial and *Fusarium* communities over time. This generated a lot of data but there were some clear conclusions; firstly, the amplicon sequencing approach clearly identified big differences in the fungal / *Fusarium* spp. between sterilised and non-sterilised soil and critically demonstrated that FOL4 inoculum builds up more rapidly in sterilised soil over the three cycles of lettuce growth which was supported by the much higher levels of disease in the final lettuce growth cycle. It was also demonstrated that the two *Trichoderma* isolates (T22 and T34) could effectively be detected by amplicon sequencing and that for T34 and to a lesser extent T22 also proliferated more in the sterilised soil. As no disease control was observed for these treatments in Objective 5, this suggests that they were ineffective against FOL4 in this particular situation where they were added to the soil two weeks before transplanting for each round of lettuce growth. Another approach for the future might to be try and establish these organisms on the lettuce transplants before they are planted in FOL4 infested soil as they are both known to be efficient root colonisers. Nonetheless the ability to track the relative abundance of bacteria, fungi, *Fusarium* spp. as well as *F. oxysporum* pathogens and specific *Trichoderma* biocontrol agents is a valuable tool to understand the complex interactions and dynamics of these different soilborne organisms.

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Objective 7: Evaluate products for control of FOC in field experiments

Products were tested for their ability to prevent Fusarium basal rot of onion in four field trials carried out by ABC and VCS. A range of biological and chemical treatments were tested either as an in-furrow treatment at drilling or as a foliar spray. Application of Rudis in-furrow was the only product that appeared to consistently increase the number of healthy onion bulbs compared to an untreated control in fields with (generally) high Fusarium levels across several trials but this increase was not statistically significant.

Conclusions

- **Objective 1:** A clear relationship between inoculum concentration, *Fusarium* pathogen DNA levels in soil (measured by specific qPCR assays) and Fusarium wilt disease development was established for FOL4 in lettuce and FON in *Narcissus*. These molecular diagnostics could be used to determine the level of *Fusarium* inoculum in soil and determine the risk of severe disease following further validation using samples from commercial field sites.
- **Objective 2:** FOC was rarely detected by qPCR molecular diagnostics in soil samples collected pre-planting of onion crops but was more reliably detected in onion roots during the season. As this has limited practical value for growers, a system whereby onion seedlings were grown in pre-planting soil samples was developed that allowed FOC to proliferate and hence improved qPCR detection in both soil and roots. Further development of such a *Fusarium* 'enrichment' is likely to be a better approach for detection in soil and assessing disease risk.
- **Objective 3:** qPCR and LAMP based molecular diagnostics was effective at detecting FOC in asymptomatic onion bulbs. This could therefore be used as a method to detect FOC in harvested onion bulbs to assess the risk of disease in store.
- **Objective 4:** FOC was found to colonise the roots of all non-host crops tested with a higher incidence of the pathogen in pea and maize compared to the other crops. However, the amount of FOC DNA was much lower in all non-host crops compared with onions. This suggests that non-host plants may sustain FOC populations between crops of onions to different degrees.
- **Objective 5:** FOL4 proliferated more rapidly in sterilised soil compared to non-sterilised soil over three successive lettuce rounds of lettuce planting (in pots) in the same soil. Several products tested did not reduce this build-up of inoculum or reduce disease development.

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- **Objective 6:** Amplicon sequencing successfully identified and quantified the relative abundance of the microbial community including bacteria, fungi and *Fusarium* spp. in soils inoculated with FOL4 and FON. Gene targets to identify multiple *F. oxysporum* f.spp. were identified but were not always reliable. In inoculated FOL4 and FON systems, the identification of *F. oxysporum* using TEF1a sequencing amongst other *Fusarium* spp. was generally a good indicator of the abundance of these pathogens.
- **Objective 7:** Rudis was the only product that consistently increased the number of healthy onion bulbs in Fusarium-affected fields, but the effect was not statistically significant.

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Knowledge and Technology Transfer

- Presentation at Plant and Crop Theme Seminar (PACTS), School of Life Sciences, University of Warwick (Sascha Jenkins; 3/03/21)
- Presentation to ABC / VCS (Sascha Jenkins; 15/09/21)
- Presentation at AAB Meeting: Thinking Differently about Soil-borne Disease Management' (Helen Bates, 10/11/21)
- Presentation to Hutchinsons Vegetable Conference (John Clarkson 07/03/22)
- Presentation to ABC / VCS (Sascha Jenkins; 10/02/22)
- Poster at British Society of Plant Pathology (BSPP) conference (Sascha Jenkins 6.9.22)
- Presentation at Onion and Carrot Conference, Peterborough (John Clarkson, December 2022)
- Presentation at Warwick Crop Centre Seminar (Sascha Jenkins 15.02.23)
- Presentation at 16th European Fusarium Seminar (EFS16) Rome (Sascha Jenkins 13.06.23)

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