Project title:	Diagnostic tests to assess Fusarium disease risk, select rotation crops and monitor microbial communities
Project number:	CP204
Project leader:	John Clarkson, University of Warwick
Report:	Annual report, March 2022 (Year 2)
Previous report:	March 2021
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Location of project:	Warwick Crop Centre, School of Life Sciences, University of Warwick Wellesbourne Campus
Industry Representative:	Sam Rix
Date project commenced:	01/04/2020

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The results and conclusions in this report are based on an investigation 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.

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GROWER SUMMARY

Headline

Fusarium oxysporum DNA concentration correlates highly with disease development and inoculum concentration in *formae speciales* affecting lettuce and Narcissus. DNA of *F. oxysporum* affecting onion was detected in field soil, onion roots and basal plates using molecular diagnostics.

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 are investigating 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 damage**s** 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 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 are utilising 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:

1. Define a relationship between the amount of Fusarium DNA, Fusarium inoculum and disease development in soil (Year 1 report, current report; objective complete).
2. Evaluate the use of molecular diagnostics to detect and assess the risk of Fusarium disease in onion, lettuce and Narcissus (Year 1 report, current report).
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 (Year 1 report, current report).
4. Determine the colonisation of non-host plants by *F. oxysporum* pathogens to identify suitable rotation crops (To be carried out in 2022 - Year 3).
5. Investigate the feasibility of establishing Fusarium-suppressive microbial communities and biological control agents in protected cropping systems (Year 1 report, current report).

6. Employ amplicon sequencing to quantify *F. oxysporum* pathogens alongside suppressive components of the soil microbial community (Year 1 report, current report).
7. Evaluate crop protection products for control of FOC in field experiments (to report in 2023; one field trial carried out in Year 2, remaining 2 field trials to be carried out in 2022).

Summary

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

In the last report, we demonstrated a strong positive correlation between FOL4 inoculum concentration, disease symptoms in lettuce and the amount of DNA detected in the soil. This has been further confirmed by extracting DNA from the roots of lettuces grown in the inoculated soils, which after 13 days, contained approximately 100 pg pathogen DNA mg^{-1} root dry weight for the highest dose.

A dose response experiment was also conducted to determine the relationship between FON inoculum, disease development in Narcissus and DNA concentration. Bulbs were planted into two FON inoculated soils and a compost mix suitable for growing daffodils at different concentrations between 1 x 10^2 - 1 x 10^6 cfu g^{-1} soil. Currently, daffodils have just finished flowering and therefore disease development in the bulbs has yet to be assessed. However, there was a strong positive correlation between the concentration of FON inoculum and the amount of pathogen DNA present in the soil and in Narcissus roots.

This work enables us to determine the critical level of FOL4 / FON inoculum needed to cause disease in different soils, and to be able to reliably quantify these concentrations using molecular diagnostics.

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

Soil / onion root sampling 2020 and detection of FOC

Onion root samples were collected from two intensively sampled commercial fields at different timepoints during the 2020 growing season with moderate to high levels of Fusarium. DNA was extracted from each sample and used for qPCR analysis to determine the level of FOC in the roots. This was compared with the quantity of FOC DNA in soil samples taken at the same timepoints (see 2021 report) and to Fusarium disease assessments conducted throughout the season, at harvest and after storage. FOC DNA was detected in all root samples from different time points using molecular diagnostics and it was concluded that detection of the pathogen in onion roots was more informative and reliable than detection in soil. This will be further tested using 2021 samples.

Soil and onion root sampling 2021 and detection of FOC

Bulb onions in fields selected for sampling in 2021 developed very low levels of Fusarium basal rot and only a small percentage of bulbs went onto develop symptoms post store. The onion roots and soils sampled from this season are still being processed, but it is predicted that low levels of FOC DNA may be detected due to the low levels of disease observed.

Onion root baiting – 2021 soils

Growing onions in soil sampled pre-drilling was another new approach used to try and improve detection of FOC in soil. The rationale was that detection of FOC could be improved by 'baiting' the pathogen from soil as it quickly colonises and multiplies on onion roots. Hence, onion seeds were sown in soil collected from each of the same fields used for sampling above (mixed with vermiculite), and after 5-6 weeks were plants were harvested, roots washed and then frozen before extracting DNA and performing qPCR with the FOC specific diagnostic assay. Although, onion roots from only one field had detectable levels of FOC DNA, this could be due to there being very little disease pressure for any of the field sites and the method will be improved and refined in 2022.

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

In Year 1, apparently healthy onion bulbs and others with different levels of basal rot were used for FOC detection to establish the utility of the FOC specific PCR assay. This year, the focus was only on apparently healthy bulbs to investigate if PCR could detect FOC in symptomless bulbs to determine the risk of Fusarium development in store. Onion bulbs with no symptoms of Fusarium disease were obtained from 4 field sites (2 high and 2 low risk of developing basal rot) and the basal plate excised and frozen. These were used for DNA extraction and qPCR with the FOC specific diagnostic assay. Additional bulbs were incubated at 20˚C for 5-6 weeks (conditions favourable for Fusarium development) to determine if any disease would develop in apparently healthy bulbs. None of the bulbs tested positive for FOC infection with qPCR, and only a maximum of 14% of bulbs from one field site displayed symptoms after incubation (other sites ranged from 0-6%). This approach therefore requires additional work as we were unable to correlate qPCR detection with symptoms in storage due to the lack of bulbs developing symptoms.

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

An experiment was set up to determine how FOL inoculum builds up in successive lettuce crops grown in sterilised and non-sterilised FOL inoculated soil and if the addition of soil treatments (Perlka, Trianum G and T34 Biocontrol) can slow or halt this process. Steam sterilised and non-sterilised soil was inoculated with FOL at 1 x 10^2 cfu g^{-1} soil, dispensed into pots and lettuce seedlings transplanted. Mature lettuce were harvested, assessed for disease (internal vascular browning) and the soil diluted 1 in 2 with fresh sterilised/non-sterilised soil. This soil was then used to grow a second crop of lettuce (currently underway). Soil samples were collected at product application, lettuce transplanting (2 weeks later to allow activity of treatments) and at lettuce harvest. Some Fusarium wilt disease developed in lettuces grown in all the FOL-inoculated sterilised soil treatments in the first crop, with those treated with Perlka having the most severe symptoms. There was therefore no indication that Trianum or T34 reduced FOL inoculum level. FOL DNA was also consistently detected in inoculated

sterilised soils at lettuce harvest. In contrast, no FOL disease symptoms were observed in non-sterilised soils, suggesting that FOL is able to establish far more quickly in sterilised soil than non-sterilised soil, even in the presence of soil treatments such as Perlka, Trianum G and T34.

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. This approach was used successfully used to quantify the relative abundance of bacterial and fungal communities using 16S and ITS gene targets in two different soils (from lettuce growers) artificially inoculated with FOL4. TEF1a amplicon sequencing also enabled identification of different *Fusarium* spp. present in soils and showed that there was a high level of *F. oxysporum* naturally present in Soil 2 (not FOL4) while Soil 3 contained a variety of different *Fusarium* spp. Several gene targets were also identified to potentially enable multiple *F. oxysporum* f.spp. to be identified concurrently with a focus on FOL, FOC and FON. The gene target, g19096, successfully enabled identification of FOL4 in inoculated soils and a clear increase in the abundance of FOL4 in Soil 3 with increasing inoculum levels. However, the high naturally occurring level of *F. oxysporum* in Soil 2 masked the ability to clearly identify FOL4 using g19096 and also affected the usefulness of another gene target OG4952 for cross-identification. Further work will investigate the effectiveness of an additional gene target for FOL identification and will use cross-referencing of existing targets to more accurately identify different *F. oxysporum* f. spp. present in soil samples.

Conclusions

- **Objective 1:** A clear relationship between FOL4 inoculum concentration, FOL DNA levels in soil and lettuce roots and Fusarium wilt disease development in lettuce was established. In addition, a strong relationship was also found for *Narcissus*, between FON inoculum concentration and the quantity of DNA recovered in inoculated soils and colonised roots. These molecular diagnostics could be used to predict the level of inoculum in fields and determine the risk of severe disease.

- **Objective 2:** FOC is more reliably detected in onion roots than directly from soil for commercial field sites. Therefore, testing onion roots using a plant baiting approach or from field sampling is likely to be a better approach for assessing disease risk.
- **Objective 3:** Molecular qPCR diagnostics was effective at detecting FOC in onion bulbs even in the absence of visible symptoms from bulbs in 2020, however, in healthy bulbs collected in 2021, FOC was undetectable in basal plate tissue. This approach requires further investigation and bulbs from infected sites need to be used to ensure development of disease in store.
- **Objective 5:** The methodology developed previously to examine FOL4 inoculum build up in sterilised and non-sterilised soil after successive lettuce crops was successfully used to determine the effect of products (Perlka, Trianum G (T22) and T34) on disease development. Further lettuce crops will be grown successively to determine their effect in an intensive cropping situation.
- **Objective 6:** Amplicon sequencing successfully identified and quantified the relative abundance of bacteria, fungi and *Fusarium* spp. in soils inoculated with FOL4. Gene targets to identify multiple *F. oxysporum* f.spp. have also been identified but some technical challenges need to be solved for these to be used reliably.

Financial Benefits

None at this time.

Action Points

None at this time.

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. *lactuace* (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 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 on to 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.*

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

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 mostly as a fertiliser, but it has been shown to have anti-fungal properties as it breaks down to hydrogen cyanamide in the soil (Shi et al., 2009; McDonald et al., 2021). It has been shown to supress *F. oxysporum* infecting cucumber (Shi et al., 2009), but had little effect 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:

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 (Year 1 report, current report; objective complete).
2. Evaluate the use of molecular diagnostics to detect and assess the risk of Fusarium disease in onion, lettuce and Narcissus (Year 1 report, current report).
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 (Year 1 report, current report).
4. Determine the colonisation of non-host plants by *F. oxysporum* pathogens to identify suitable rotation crops (To be carried out in 2022 - Year 3).
5. Investigate the feasibility of establishing Fusarium-suppressive microbial communities and biological control agents in protected cropping systems (Year 1 report, current report).
6. Employ amplicon sequencing to quantify *F. oxysporum* pathogens alongside suppressive components of the soil microbial community (Year 1 report, current report)..
7. Evaluate crop protection products for control of FOC in field experiments (to report in 2023; one field trial carried out in Year 2, remaining 2 field trials to be carried out in 2022).

Materials and methods

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

Glasshouse experiments were set up 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.

Lettuce response to FOL4 inoculum level

A glasshouse experiment was set up to determine the relationship between FOL4 inoculum levels, disease development and soil FOL4 DNA concentrations as reported in CP204 annual report March 2021 (Clarkson, 2021). Briefly, three soil types (Table 1) and also M2 compost were mixed with medium grade vermiculite in a ratio of 4:1 (soil:vermiculite) and moistened to aid inoculum mixing. A bran/compost inoculum of FOL4 isolate AJ516 was prepared as described by Taylor et al. (2013) and mixed into each of the three soil types as well as M2 compost to achieve five ten-fold increases in concentrations from 2 x 10^2 - 2 x 10^6 cfu g^{-1} soil/compost. The infested soil/compost was dispensed into 7 cm pots and 2-3 week old lettuce seedlings (cv. Temira) transplanted into each pot. An untreated control (non-inoculated soil/compost) was included for each soil type and compost. Pots were arranged in a randomised block design in a glasshouse compartment set at 25°C day, 18°C night, 16 h day-length. Plants were assessed twice-weekly for Fusarium wilt and then for vascular browning at harvest. Soil samples were also taken at the start of the experiment and following DNA extraction FOL4 was quantified using a specific qPCR assay (method described in CP204 annual report March 2021 (Clarkson, 2021)). Additional plants were harvested at 3, 6 and 13 dpi, where the roots were washed, blotted dry and flash frozen in liquid nitrogen for quantification of FOL4 by qPCR (see below). Three replicate lettuce roots were harvested for each soil/dose at each time point.

Table 1. Locations of soils from UK lettuce growers (1 and 2) and from Warwick Crop Centre

Soil No.	Location
1	Tarleton, Lancashire
2	Selby, North Yorkshire
3	Warwick Crop Centre, Warwickshire

Narcissus response to FON inoculum level and soil / root sampling

An experiment was set up to determine the relationship between FON inoculum levels, disease development and soil FON DNA concentrations. Two soils were collected from Narcissus growing regions in the UK: Cornwall (Soil 1) Lincolnshire (Soil 2) and a FON-specific qPCR assay (described below) used to determine if they were FON-free. Soils were then dried for 3-4 days and sieved (4 mm mesh) before being amended with a medium grade vermiculite in a ratio of 4:1 (soil:vermiculite) and moistened (to aid inoculum mixing). Samples of each soil were sent to NRM laboratories (Bracknell, UK) for analysis to determine the pH; N, P, K, Mg, organic matter content, and CO_2 respiration (Solvita test). An additional Narcissus growing medium was also included, consisting of a blend of sphagnum peat / horticultural sand (3:1, v/v) mixed with John Innes No.1 compost (1:1, v/v) and amended with ammonium nitrate (0.40 kg m-3), potassium nitrate (0.75 kg m^{-3}), single super-phosphate (1.50 kg m^{-3}), ground chalk (2.25 kg m^{-3}), ground magnesian limestone (2.25 kg m^{-3}) and fritted trace elements WM 255 (0.40 kg m^{-3}). A bran/compost inoculum of FON isolate FON139 was prepared as described by Taylor et al. (2013) and mixed into each of the two soil types as well as the Narcissus growing medium to achieve five ten-fold increases in concentrations from 1 x 10^2 - 1 x 10^6 cfu g^{-1} soil/compost. The infested soil/compost was dispensed into 4 L pots (up to inner rim) and five narcissus bulbs sown so the base of the bulb was at a 10 cm depth from the soil surface. Additional 3 L pots were filled and two narcissus bulbs were placed in each pot as before. Untreated controls pots were included for all soils. Pots were arranged in a randomised design in a frost-free glasshouse compartment. Plants will be maintained until late Spring 2022 when bulbs will be dissected and scored for disease development.

After 12 and 26 dpi, the 3 L pots containing two Narcissus bulbs were harvested to determine the level of root colonisation by FON. The roots from each bulb were washed and blotted dry before being frozen in liquid nitrogen. The soil was also retained, dried and passed through 4 mm and 2 mm sieves for DNA extraction and qPCR analysis to determine FON DNA levels in the soil.

DNA extraction and qPCR analysis to determine levels of FOL and FON

For the lettuce roots, DNA was extracted from lyophilised and ground root tissue using the Qiagen DNeasy Plant mini kit (Qiagen, UK) in accordance with manufacturer's protocol with a minor modification whereby the sample was first homogenised in a in a FastPrep-24™ machine set at 6 ms^{-1} for 40 s. DNA was used to perform qPCR with FOL4 specific primers g23490 F3/R (AHDB project FV/PE 458). qPCR was carried out using a QuantStudio 5 (384-

well) machine (Applied Biosystems) using 20 µL reactions containing both primers (final concentration 0.4 µM), 10 µl Power SYBR™ Green PCR Master Mix (Applied Biosystems) and 1 µl of DNA. Conditions were as follows: 1 cycle of 95°C for 120s followed by 45 cycles of 95°C for 3 s, and 60°C for 30 s. All samples were run in triplicate and a melt curve analysis carried out. The concentration of FOL4 DNA in each root sample was calculated as pg mg^{-1} of root.

For Narcissus, the lyophilized roots were ground and extracted in the same way as for the lettuce roots above (1 harvested bulb). For the qPCR reaction, specific FON primers g16122 F/R2 (Clarkson, 2018) were used in the same way as above but at a final concentration of 0.5 µM. The concentration of FON DNA was in each root sample was calculated as pg mg^{-1} of root.

For the Narcissus soils inoculated with FON, DNA was extracted using the GeneAll Exgene Soil SV kit (Cambio, Cambridge, UK) following the manufacturers protocol, with the following modifications: 1) 550 µL of SL buffer and 200µl of SDW was added to 500 mg of soil, which was homogenised in a Powerbead tube using a FastPrep-24 (MP Biomedicals, Cambridge, UK) machine set at 5.5 m s^{-1} for three cycles of 25 seconds; 2) spin columns were incubated for 5 min after the addition of buffer EB before elution. Three replicate soil samples were extracted. Following extraction, DNA diluted was 1:2 with TE Buffer and used for qPCR analysis with FON specific primers as above.

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 and both soil and root samples collected from two field sites pre-planting and at seven different timepoints during the season (intensively sampled sites). Fusarium disease was assessed during the season and in harvested bulbs (carried out by Vegetable Consultancy Services - VCS and the Allium and Brassica centre - ABC).

Soil and onion root sampling 2020

Soil samples from fields where disease was high (post storage) were used for DNA extraction and qPCR using FOC specific primers to determine the level of FOC DNA in the soil. Field sites RX2 and FP1 (intensively sampled) were selected from ABC and field sites RIS and

WRE (intensively sampled) were chosen from VCS. Full methods for DNA extraction and qPCR can be found in CP204 annual report March 2021 (Clarkson, 2021).

In addition to soils, onion root samples were collected from the two intensively sampled onion fields with high Fusarium disease risk by VCS (WRE) and ABC (FP1) at seven timepoints during the season (Table 2). For each field site, onions were sampled from four rows (labelled A-D, Figure 1) from a total of six beds per field, which was the same sampling method used for the soil.

DNA extraction and qPCR onion roots

Onion root samples (from six pooled plants) were washed and dissected from any remaining bulb tissue before freeze drying for 48 hours. Roots were ground in liquid nitrogen and the DNA extracted using the DNeasy plant mini kit (as in Objective 1). DNA was used in FOC specific qPCR reactions (20 µL reactions) set up as above but with q*SIX*5 primers (Taylor et al., 2016) at a final concentration of 0.6 µM. All samples were run in triplicate and a melt curve analysis carried out. The concentration of FOC DNA in each sample was calculated as pg mg^{-1} of root.

Table 2. Onion root samples collected over the 2020 growing season from sites at high risk of developing Fusarium disease. Sampled collected by Allium and Brassica Centre (ABC) and Vegetable Consultancy Services (VCS).

Company	Site code	Sample date
ABC	FP1	10/04/2020
ABC	FP1	21/05/2020
ABC	FP1	02/06/2020
ABC	FP1	15/06/2020
ABC	FP1	01/07/2020
ABC	FP1	15/07/2020
ABC	FP1	27/07/2020
ABC	FP1	11/08/2020
VCS	WRE	17/04/2020
VCS	WRE	23/05/2020
VCS	WRE	04/06/2020
VCS	WRE	18/06/2020
VCS	WRE	02/07/2020
VCS	WRE	17/07/2020
VCS	WRE	31/07/2020

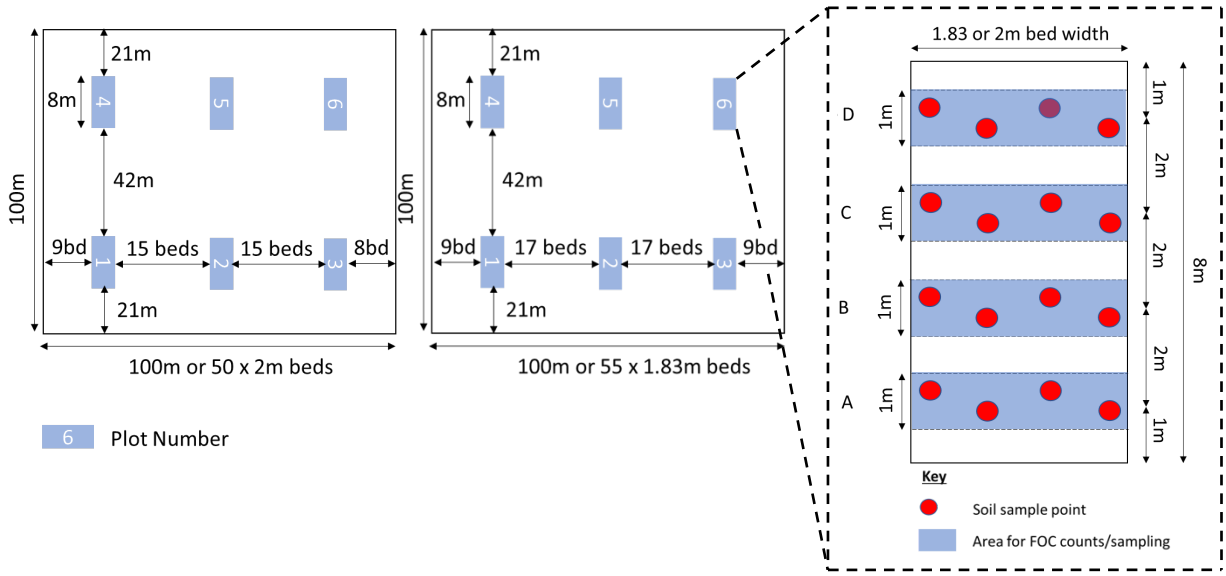

Figure 1. Layout of the 6 plots in each field site (1 ha area) for soil/onion sampling and the positions of the 4 sampling areas within each of the 6 plots. Red circles represent the locations in which samples were collected. Samples were pooled across each area A-D.

Soil and onion root sampling 2021 and onion plant baiting

Soil sampling and onion root sampling as described above was repeated during the 2021 growing season using different field sites to those in 2020. Two sites were again intensively sampled over the season, whereas only pre-planting soil samples were collected from the remaining 12 sites (Table 3). Disease assessments were carried out by VCS and ABC during the season and after the onions had been harvested and stored for six weeks. These soil and root samples are currently being processed for FOC quantification by qPCR.

Table 3. Sites of soil and onion root sampling in 2021 completed by Allium and Brassica Centre (ABC) and Vegetable Consultancy Services (VCS). PR2 and THO were intensively sampled across the season.

Company	Site code
ABC	PR2- intensive
ABC	PR3
ABC	FP2
ABC	PR1
ABC	FP1
ABC	FP3
ABC	FP4
VCS	THO- intensive
VCS	SUT
VCS	RIS
VCS	HAR
VCS	CRO
VCS	TUN
VCS	ELV

Additional soil samples were also collected pre-planting from the 14 field sites (Table 3) which were then used to test whether sowing onion seeds and performing DNA extraction and qPCR on onion roots (plant baiting) would result in better detection of FOC than from soil as previously the pathogen could not be detected in soil pre-planting.

This additional 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 four onion seeds per pot. Two additional soils were included from the Wellesbourne campus: Soakwaters soil (known to be FOC free) and soil from a heavily FOC infested quarantine field (QF). Pots were maintained at 22˚C, 16 hr light, for five weeks. Roots were then harvested and pooled together from each pot before being washed, blotted dry and flash frozen in liquid nitrogen.

Four pots of onions (up to four pooled plants per pot) per were selected from each soil for DNA extraction (including all the control samples: soakwaters 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.

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

This year, the approach of FOC detection in onion basal plates focused on apparently healthy bulbs only, instead of bulbs showing different levels of disease symptoms as in the previous report (Clarkson, 2021). Bulbs were also fresh from the field (not dried), to see if FOC could be detected before bulbs went into storage to assess the risk of disease development.

Onion bulbs were obtained from two sites from both ABC and VCS, with one site having Fusarium present and one being of low disease risk. Bulbs were assessed to be healthy by visual inspection of the outer surface and pressing the basal plate firmly to look for disease symptoms. 50 healthy bulbs per site were cut in half, photographed and the basal plate excised and frozen in liquid nitrogen. The other 50 bulbs per site were dried in an ambient glasshouse then placed at 20˚C for approx. 6 weeks.

Frozen basal plates were ground in liquid nitrogen and 25 bulbs per site were randomly selected for DNA extraction using the Qiagen DNeasy plant mini kit as above. DNA was used in FOC specific qPCR reactions (20 µL reactions) set up as above with q*SIX*5 primers (Taylor et al., 2016) to determine whether FOC was present in the basal plates of visually healthy bulbs.

Incubated bulbs were cut open after 6 weeks, photographed and scored for Fusarium disease symptoms (Table 4). This was conducted to determine whether FOC was present in any bulbs and whether disease symptoms would develop after incubation at a temperature favourable for Fusarium growth.

Table 4. Fusarium basal rot disease score scale in incubated onions.

Score	Description
0	Healthy
1	Symptoms in basal plate only
2	Mild- basal plate and spreading into scales
3	Fusarium basal rot – basal plate and significant amount of scale affected

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 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 the build-up of FOL4 in soils after successive crops of lettuce but with the addition of products to determine if they could slow down or prevent FOL proliferation.

Treatments to prevent FOL4 inoculum build up

FOL4 free soil 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. 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 is heated to 71˚C (in approx. 1.25 hours), then is switched off and remains sealed until the temperature reaches 82˚C (approx. 1.5 hours). Sterilised and non-sterilised soil was then mixed 4:1 with medium grade vermiculite. FOL4 inoculum was prepared as before (Taylor et al., 2013) and used to prepare a serial dilution in soil in order to inoculate sterilised/non-sterilised soil at the final concentration of 1 x 10^2 cfu g^{-1}. Three treatments (Perlka, T34, Trianum G (T22)) were applied separately to both sterilised and non-sterilised soil (Table 5), with the addition of a FOL4 only treatment and a non-inoculated treatment for each soil. Soil was used to fill 9 cm pots which were minimally watered and left to stand for two weeks at 25˚C (16 hr light), due to the calcium cyanamide (Perlka) treatment needing time to convert through hydrogen cyanamide (toxic to soil microbes) to urea in order to be safe for transplanting lettuces. Two to three week old lettuces (cv. Amica) were then transplanted into the different soils and were scored for Fusarium wilt symptoms, and vascular browning at harvest.

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

Table 5. 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

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

Targeted amplicon sequencing has been used previously to identify the presence of *F. oxysporum* f.sp. *cepae*, *narcissi* and *matthioli* (FOC, FON and FOM) in soil samples from diseased sites as part of AHDB project FV POBOF 452 (Clarkson, 2019). We also demonstrated the ability to identify levels of FOC inoculum in soil samples at or just below disease causing levels as part of AHDB project CP 196 using amplicon target OG4952 (Table 6). Initial data from this current project showed that we could successfully identify *F. oxysporum* f.sp. *lactucae* (FOL) from inoculated soil samples of one soil type (Soil 1) using a newly designed amplicon target g19096 (see CP 204 annual report 2020; Clarkson, 2020). This continuing work investigates using these amplicon targets as listed in Table 6 to identify multiple *F. oxysporum* f.spp. in two additional soils inoculated with FOL4 and the development of additional target genes to give more robust *F. oxysporum* f.spp. identification across different soils.

Table 6. List of *F. oxysporum* f.spp. potentially identified from amplicon sequencing of different gene targets

	Gene target			
Sequence variant	**OG4952**	**OG13890**	**g19096**	**g19482**
1	f.sp. conglutinans, f.sp matthiolae	f.sp. conglutinans, f.sp. raphani, f.sp. matthiolae	f.sp. lactucae (FOL), f.sp. matthiolae, f.sp. tulipae	f.sp. lactucae, f.sp. pisi (race 1; FOP1)
2	f.sp. niveum	f.sp. pisi (race 1; FOP1)	f.sp. narcissi, f.sp. nicotianae, f.sp. radicis-cucumerinin f.sp. cepae, f.sp. fragariae, f.sp. pisi (race 5; FOP5)	f.sp. lactucae
3	f.sp. narcissi	f.sp. cepae	f.sp. limonii (statice)	f.sp. conglutinans, f.sp. matthiolae,

4	f.sp. narcissi	f.sp. cucumerinum, f.sp. melonis, f.sp. niveum	f.sp. physalis, f.sp. lycopersici	f.sp. conglutinans, f.sp. matthiolae
5	f.sp. cepae		f.sp. melonis, f.sp.niveum, f.sp. pisi (race 2; FOP2)	f.sp. narcissi
6	f.sp. pisi (race 1; FOP1)		f.sp. conglutinans, f.sp. pisi (race 1; FOP1)	f.sp. narcissi
7	f.sp. tulipae		f.sp. cepae, F. oxysporum pea foot rot (PG18)	f.sp. lycopersici
8				f.sp. pisi (race 1; FOP1)
9				f.sp. lycopersici

Identification of additional gene targets for FOL identification

As described previously, targets suitable for amplicon sequencing need to be present in multiple *F. oxysporum* f.spp. but have differences along their sequence to enable identification of multiple targets in a single sample. Potential pathogenicity genes are likely to make good targets as they are usually only present in pathogenic isolates and show a lot of sequence diversity. However, this sequence diversity also makes it difficult to find suitable regions to amplify across many *F. oxysporum* f.spp. After analysing the initial potential target genes and testing against *F. oxysporum* f.spp. DNA and soil samples only g19096 was found to be suitable. However, after careful analysis of the FOL genomes by eye (rather than using bioinformatic approaches) an additional potential gene target has been identified for testing using both purified gDNA from a selection of *F. oxysporum* f.spp. as well as soil samples.

Soil DNA Extractions

Soil DNA extractions were carried out as previously described 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/ml for use in amplicon sequencing PCR reactions.

DNA library preparation, PCR and amplicon sequencing

Illumina dual-index DNA library preparation, PCR and amplicon sequencing was carried out as described previously. Briefly, first round target PCR reactions were carried out using 10ng DNA in a 25ml 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 8rM on a V3 2x300bp flow cell.

Amplicon sequencing data analysis pipeline

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 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 are 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 TEF1a, 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.

Results

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

Lettuce response to FOL4 inoculum level

The results for the lettuce wilt and vascular browning disease symptoms can be found in CP204 annual report March 2021 (Clarkson, 2021) while a summary of the qPCR results from soil extractions and the correlation to wilt and vascular browning scores is reported below.

The quantification of FOL4 DNA by qPCR analysis in the three soil types (excluding compost) revealed an increase in FOL4 DNA in the soils with increasing FOL4 inoculum concentrations (Figure 2 A). There was clear correlation between the concentration of FOL inoculum, the concentration of FOL4 DNA detected in the soil and the disease severity scores (wilt and vascular browning (Figure 2 B).

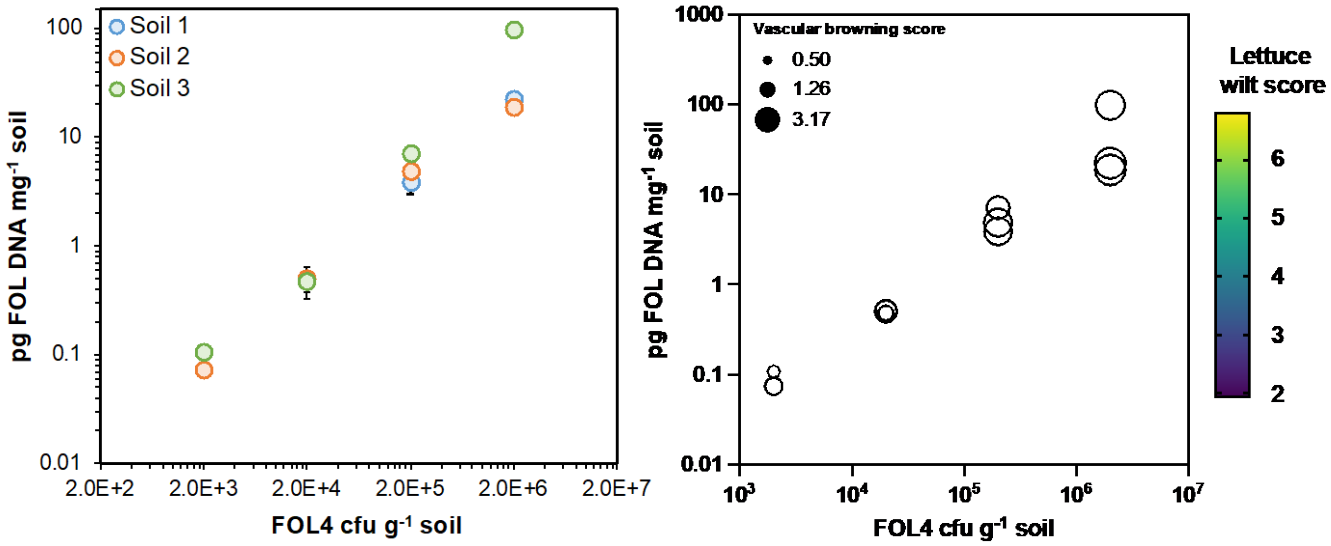

Figure 2. A) Amount of FOL4 DNA detected (pg mg^{-1} dry soil) in three inoculated soils at concentrations ranging from 2 x 10^2 to 2 x 10^6 cfu g-1 soil. No FOL4 DNA was detected below 2 x 10^3 cfu g^{-1}. Error bars represent SEM. B) Correlation between FOL4 inoculum concentration, FOL4 DNA detected in soil and the disease severity scores (wilt and vascular browning score).

Root samples were collected from lettuces grown in all soil types (including compost) at all six doses at 3, 6 and 13 dpi. At 3 dpi, only lettuce roots grown in soil inoculated at 2 x 10^6 cfu g^{-1} (dose 6, D6) had detectable levels of FOL DNA (Figure 3, A) although levels were low at <7 pg DNA mg^{-1} soil and detection was patchy with only one or two of the replicate root samples displaying positive amplification on the qPCR. Levels of FOL DNA detected in root samples at 6 dpi were greater between 4 and 24 pg DNA mg^{-1} soil and the pathogen was detected in all samples from the D6 inoculated soils (Figure 3, B). At this timepoint, FOL DNA could also be detected in some replicates of D5 (2 x 10^5 cfu g^{-1}) in soil 1 and 2 (Figure 3, B). Finally at 13 dpi, DNA could be detected in all (except for one replicate from soil 1) samples from D6 and D5 at significantly higher quantities than after 6 days. Roots from soil 4 contained the greatest quantity of DNA in D6 and D5, with quantities of 70 and 808 pg DNA mg^{-1} soil respectively (Figure 3, C). DNA was also detected in individual replicates of roots from soil 2 and 4 at 2 x 10^4 cfu g^{-1} (D4), although at lower levels than for the other doses (Figure 3, C). The results from TP3 (13 dpi) correlate well with the wilt/vascular browning scores and the amount of DNA detected in the soil, in that the amount of DNA detected in the roots increases with inoculum concentration (Figure 2, Figure 3)

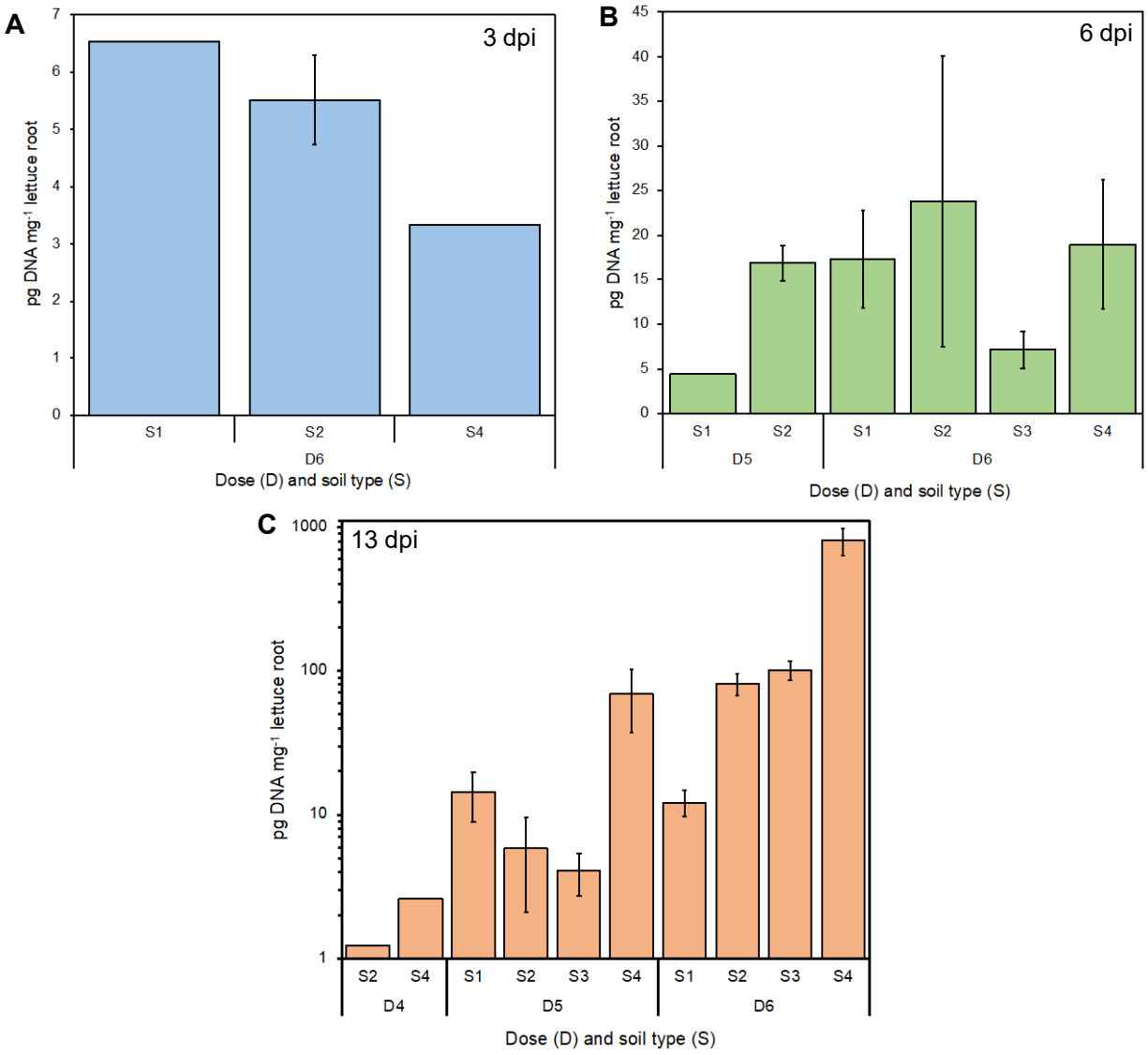

Figure 3 Levels of *Fusarium oxysporum* f. sp. *lactucae* race 4 (FOL4) DNA extracted from lettuce root samples at three time points post inoculation (3, 6 and 13 dpi). Error bars represent standard error of the mean of two or three replicate root samples. Where error bars are missing, DNA from only one replicate sample amplified using qPCR.

Narcissus response to FON inoculum level

There were some notable differences in the physical properties between the two soil types (Table 7). Soil 1 was found to be mostly clay, but contained nearly equal amounts of sand, silt and clay. Soil 2 was classed as sandy silt loam due to the higher proportions of sand and silt compared to clay (Table 7). Soil 1 contained the most organic matter, 6.1%, compared to only 2.9% in soil 2, however both are still relatively low. Both soils had a similar pH, P and K and with Mg index greater for Soil 2 (Table 6).

Table 7. Physical properties of UK Narcissus growing soils.

	Soil 1 (Cornwall)	**Soil 2 (Lincolnshire)**
Major soil classification	Heavy	Medium
Textural Classification	**Clay** *Sand 31%* *Silt 32%* *Clay 37%*	**Sandy Silt Loam** *Sand 42%* *Silt 40%* *Clay 18%*
Soil pH	7.8	7.9
P index	3	3
K index	2-	2-
Mg index	1	3
P (mg/L)	36.2	42.4
K (mg/L)	128	141
Mg (mg/L)	48.8	138
Nitrate N (mg/kg)	21.57	36.86
Ammonium N (mg/kg)	0.07	0.06
Available N (kg N/ha)	81.2	138.5
CO2 respiration (mg/kg)	59	32
OM (%)	6.1	2.9

An experiment was then conducted to determine a relationship between levels of FON inoculum in the different soils with pathogen DNA and disease development. Soil (at planting) and root samples (at 12 and 26 dpi) were analysed to determine the amount of FON DNA at each inoculum dose. FON was successfully detected at 1 x 10^3 cfu g^{-1} in soil 1 and 1 x 10^4 cfu g^{-1} in soil 2. However, only one of the triplicate samples was positive for FON at 1 x 10^3 cfu g^{-1}; therefore, this value might be less accurate. The quantity of FON DNA significantly increased in D5 and D6 samples in both soils, with the highest quantity recorded in soil 1 (D6) with 50 pg DNA mg^{-1} soil (Figure 4).

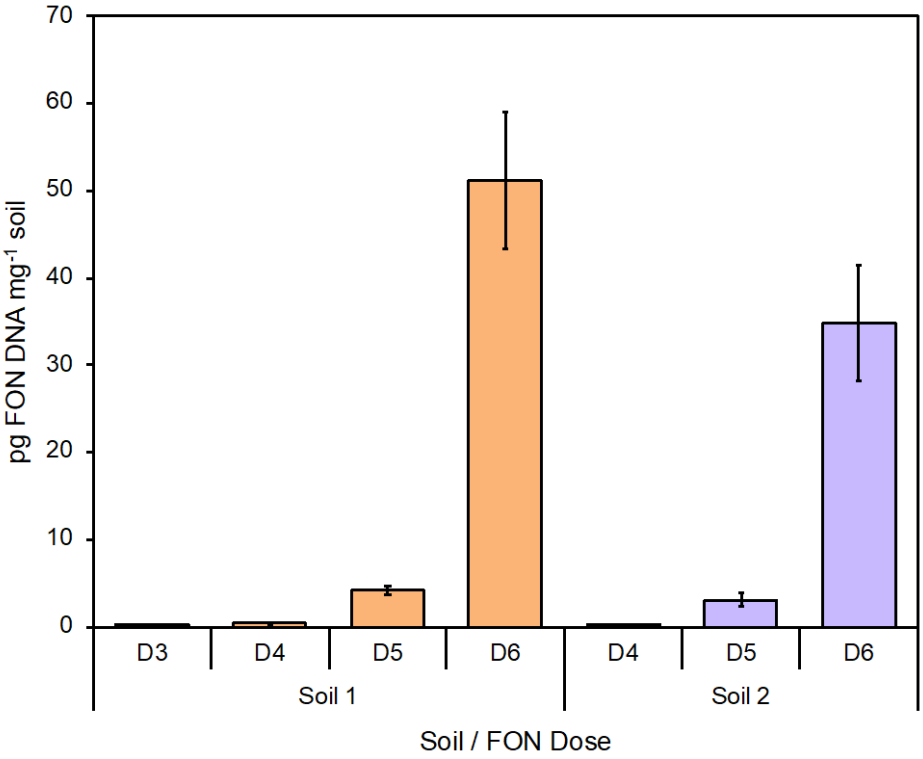

Figure 4. Amount of FON DNA detected (pg mg^{-1} dry soil) in two inoculated soils at concentrations ranging from 1 x 10^2 (D2) to 1 x 10^6 (D6) cfu g^{-1} soil. No FON DNA was detected for D2 or in the control. Error bars represent SEM from three replicate extractions.

The roots of the daffodils grown in each inoculated soil were tested for the presence of FON DNA, to determine root colonisation after two time points (12 and 26 dpi, TP1 and TP2). The quantity of FON DNA detected in roots grown in soil 1 was the lowest, with maximum levels at 14 pg mg^{-1} root (Figure 5). Levels generally increased between TP1 and TP2 in soil 1 apart from at lower doses. Roots grown in the control soil (no FON added) also resulted in positive amplification using qPCR (Figure 5), consistent with earlier soil tests showing low levels of FON in the soil (data not shown). In soil 2, FON was detected reliably in roots from plants grown in FON doses of D4 and above, and for D5 and D6, the quantity of FON DNA decreased between time points (Figure 5). Finally, the levels of DNA detected in roots of daffodils grown in the compost mix were significantly higher than in the other soils, with up to 2754 pg DNA mg^{-1} root (Figure 5). In all doses (D3-D5), there was considerably more DNA detected at TP2 than TP1.

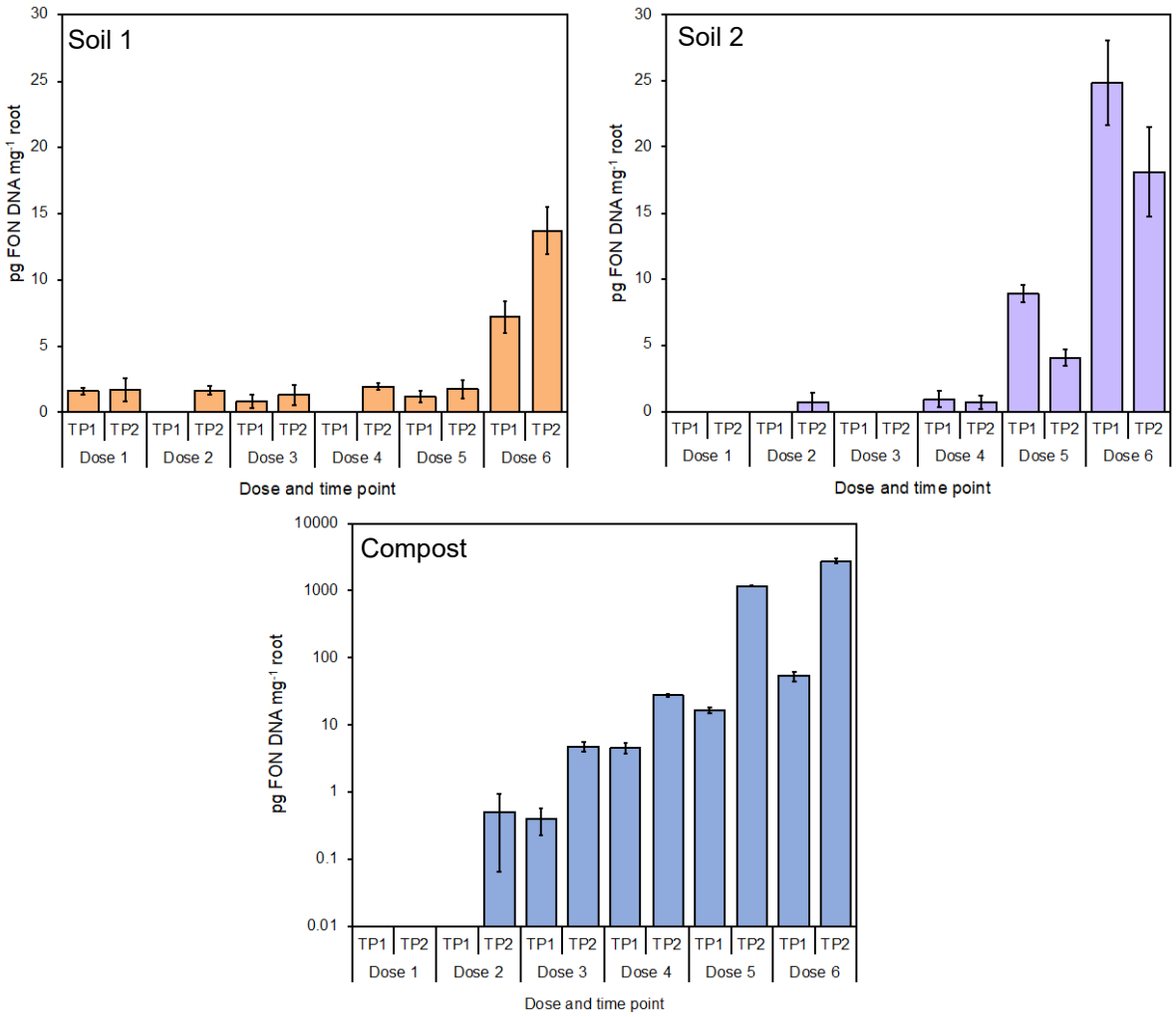

Figure 5. Amount of FON DNA detected (pg mg^{-1} dry root) in roots grown in two inoculated soils and compost mix at concentrations ranging from 1 x 10^2 (Dose 2) to 1 x 10^6 (Dose 6) cfu g^{-1} soil. Error bars represent standard deviation of qPCR triplicates. Concentrations of DNA in compost roots were significantly higher and displayed on a logarithmic axis. TP1 = 12 dpi, TP2 = 26 dpi

There was a positive relationship between the amount of FON inoculum added to the soil, the pg DNA detected in the soils and the pg DNA detected in the roots (Figure 6). Apart from D3 from soil 1, as described earlier as an outlier due to the low number of positive replicates, both soils have a proportional positive correlation (size of circles represents an increase in FON DNA in the roots). These data will be compared with disease development data when the experiment concludes in June 2022.

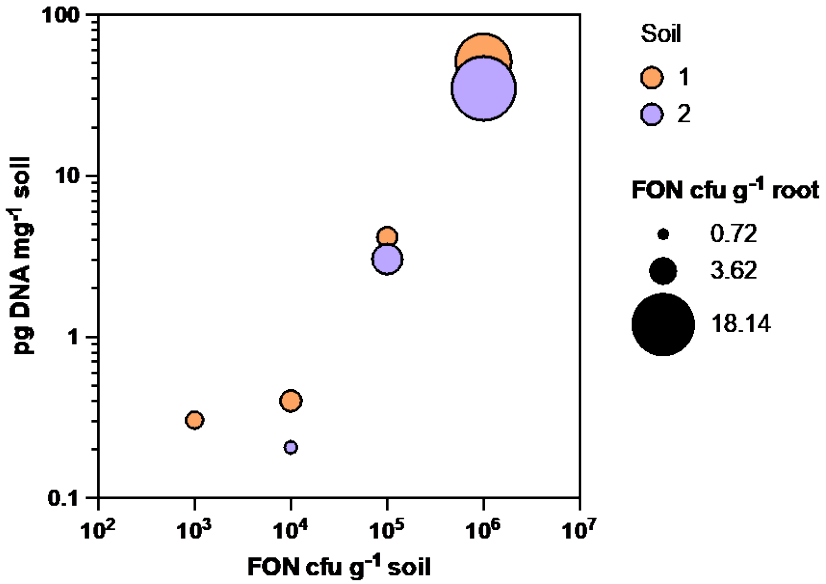

Figure 6. Correlation between FON inoculum concentration, FON DNA detected in soil and FON DNA detected in roots (circle size) for two soil types.

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

Soil and onion root sampling 2020

Field sites monitored by ABC, RX2 and FP1 had relatively low levels of disease during the growing season with approximately 5% of onion plants showing symptoms of disease (apart from plot 4 from RX2 which had 27% disease). Again, disease levels were low in onion bulbs at harvest, but rapidly increased up to 70% in bulbs from some plots from RX2 and up to 40% in bulbs from FP1 post storage (see CP204 annual report March 2021; Clarkson, 2021 for full results).

Disease levels were lower at VCS sites RIS and WRE with 2-2.5% disease in onions plants across the growing season. However, disease level increased in harvested bulbs up to 20% (RIS) and 30% (WRE), and increased further after bulbs had been stored with up to 35% of bulbs from RIS and up to 60% of bulbs from WRE displaying symptoms (see CP204 annual report March 2021; Clarkson, 2021 for full results).

The qPCR analysis of the soil samples revealed no detection of FOC DNA in the two sites with only pre-planting samples (RX2 and RIS) as previously reported for all other sites where just pre-planting soil samples were analysed. However, there were low levels of DNA detected across the growing season from the intensively sampled sites FP1 and WRE (Figure 7) and DNA was detected pre-planting in two out of six plots at FP1 with levels reaching a maximum in early June (Figure 7 A). In contrast, FOC DNA was not detectable until early June in the soil collected from plots in WRE (Figure 7 B).

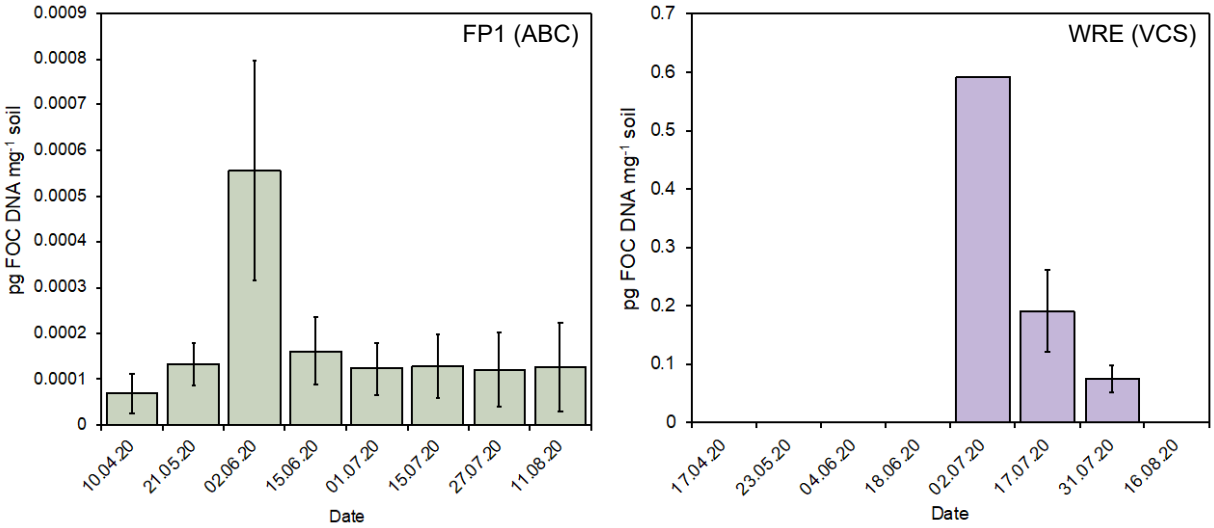

Figure 7. Levels of FOC DNA in onion soil samples, averaged across 6 plots in the field sites FP1 (ABC site) and WRE (VCS site) for eight time points in the 2020 onion growing season. Error bars represent the SEM across the six plots from each time point.

Onion roots from ABC and VCS intensively sampled sites, FP1 and WRE respectively, were also analysed using qPCR to determine whether FOC could be detected.

The concentration of FOC DNA mg^{-1} root was relatively consistent across the time course of sampling, from May to August 2020 for the 6 plots from FP1 (Figure 8). There were some unusually high levels detected e.g. plot 6 on 02.06.20 and plot 4 on 15.06.20; however, there was unreliable detection at sampling sites A-D for these samples either meaning FOC was detected in one sample (plot 6) or that FOC detection was highly variable in samples (plot 4). Levels of FOC were generally low in roots from all other plots with <12 pg DNA mg^{-1} root (Figure 8).

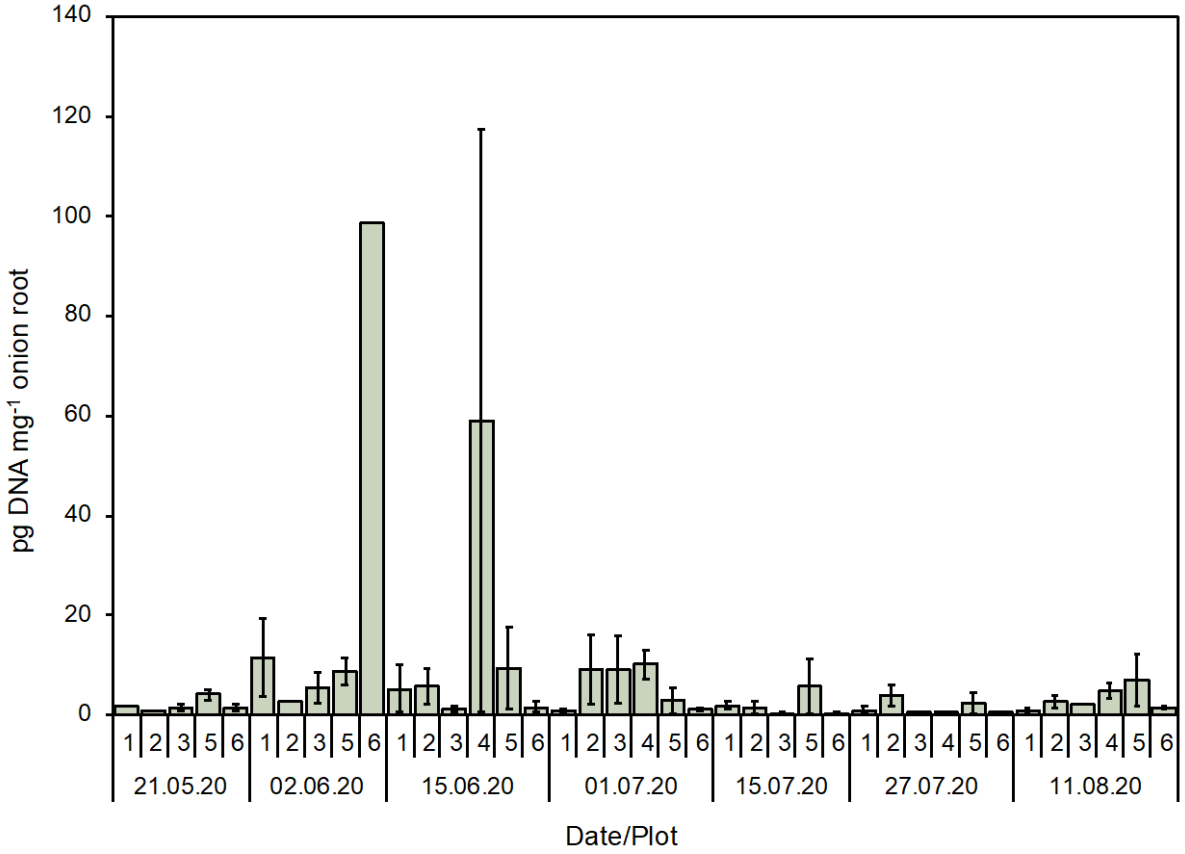

Figure 8. Levels of FOC DNA in onion roots samples from 6 plots in the field site FP1 at seven time points in the 2020 onion growing season. Samples were obtained by the Allium and Brassica Centre (ABC). Error bars represent the SEM across detected samples A-D sampled from each plot.

In the onion root samples from WRE (VCS) there were greater levels of DNA detected compared with FP1, with up to 450 pg DNA mg^{-1} root, but most samples contained less than 91 pg DNA mg^{-1} root (Figure 9). Plot 3 contained high (but variable between samples) levels of DNA when sampled at both time points in June with 183 and then 450 pg DNA mg^{-1} root, however, levels then reduced to 36 pg DNA mg^{-1} root in the later samples from July (Figure 9). The sample for plot 3 was missing from sampling date 02.07.20. The level of DNA in the other plots remained relatively stable across the sampling dates apart from plot 6 which consistently increased with every sampling point (Figure 9).

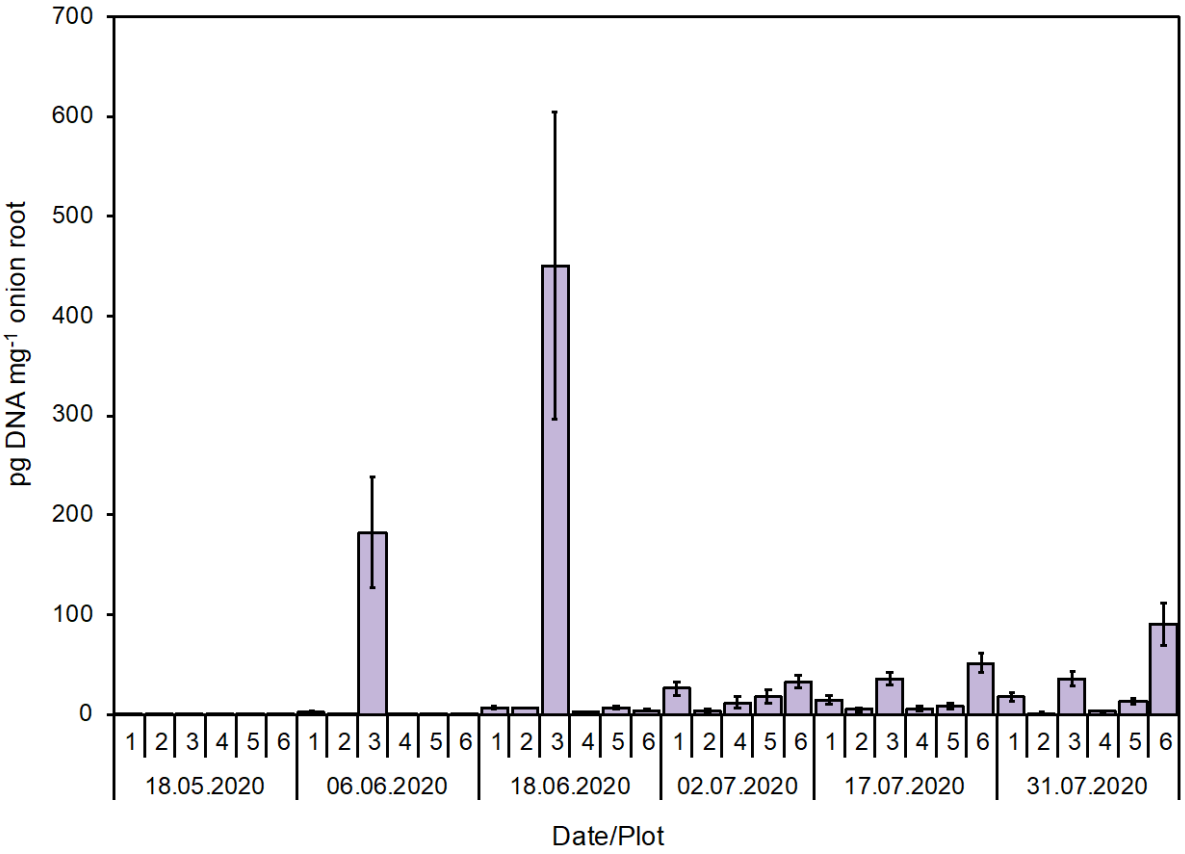

Figure 9. Levels of FOC DNA in onion roots samples from 6 plots in the field site WRE at six time points in the 2020 onion growing season. Samples were obtained by the Vegetable Consultancy Services (VCS). Error bars represent the SEM across detected samples A-D sampled from each plot.

Overall, the quantity of FOC DNA detected in onion roots was significantly higher than in the soil from both sites and was also more consistent (Figure 10). DNA was detected a month earlier in onion roots (06.06.20) from WRE than in the soil samples (02.07.20), with the highest quantity detected on 18.06.20 for roots and 02.07.20 in the soil samples (Figure 10). Therefore, FOC DNA detection is more reliable in onion roots than in soil samples.

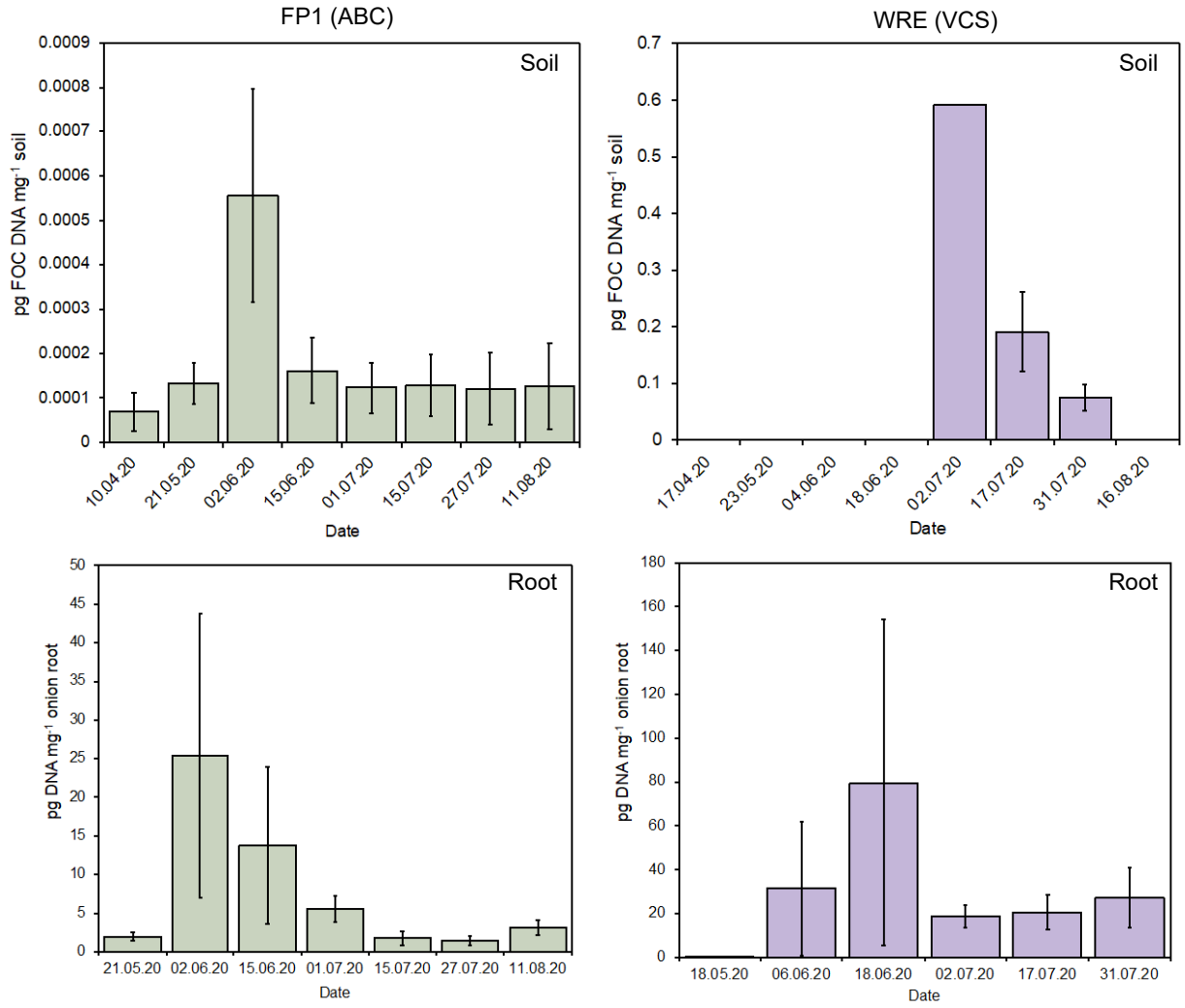

Figure 10. Comparison between quantity of FOC DNA detected in soils and onion roots (mean values of samples from 6 plots) for field sites FP1 and WRE in the 2020 growing season. Error bars represent standard error of the mean.

Soil and onion root sampling 2021

As with the 2020 field assessments, there were very low levels of Fusarium disease recorded in onions plants over the growing season in the 2021 field sites. All ABC fields had less than 4% Fusarium disease in onion bulbs in the field over the season (Figure 11), apart from plot 5 in field FP2 which had 14% of bulbs displaying disease. In contrast to 2020 disease levels were also low after the bulbs had been harvested and stored for six weeks in 2021, with a maximum of 8.7% of bulbs showing clear Fusarium basal rot symptoms (ABC field FP2, plot 5), and most of the other sites having less than 3% disease (Figure 12) although some had higher levels of 'corky base' plate symptoms which may be associated with FOC infection. However, in site PGR3 (ABC), onions from most plots had disease levels between 5-8% (Figure 12).

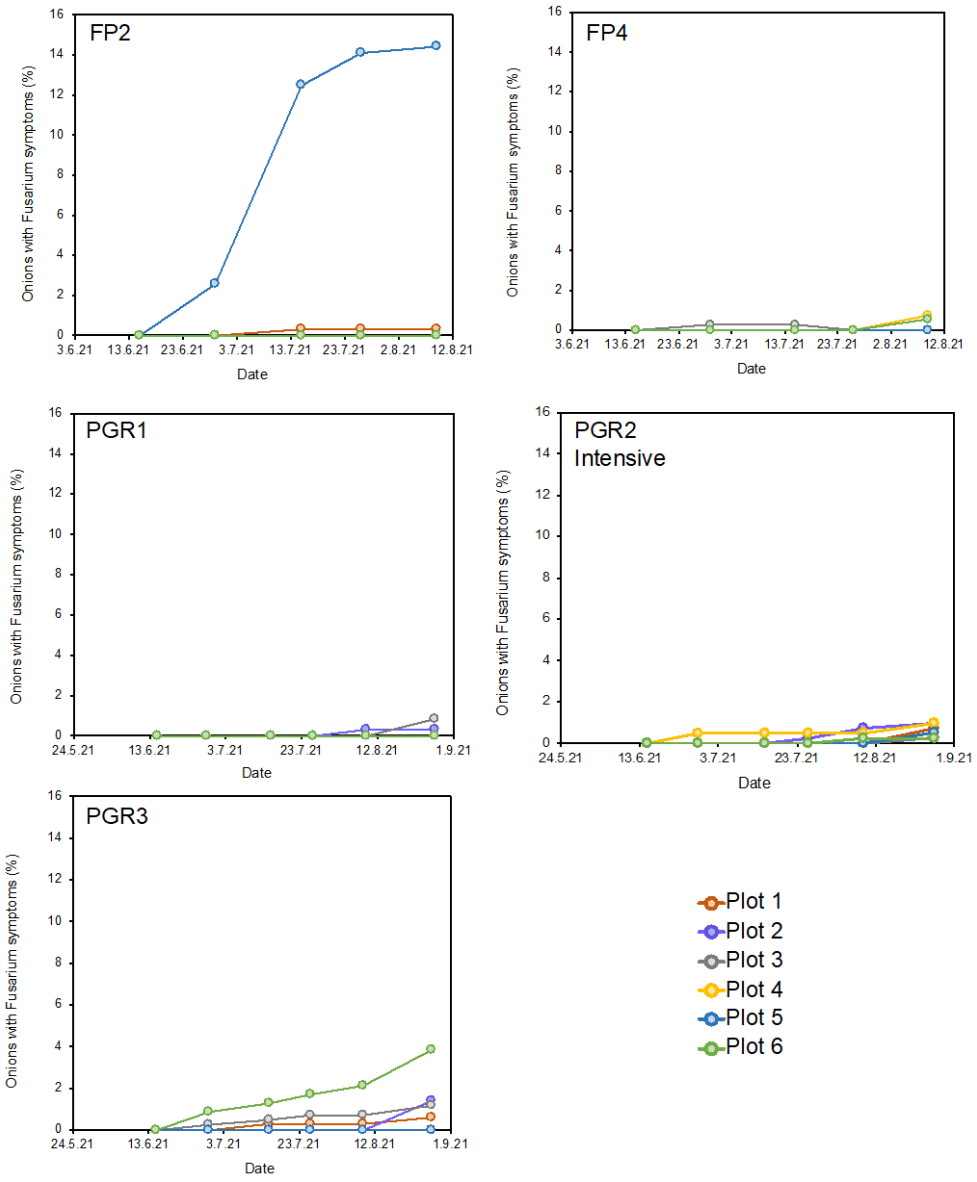

Figure 11. Fusarium disease development (percentage onions affected) in onion plants during the growing season for five of the six field sites monitored by the Allium and Brassica Centre (ABC). Site FP3 had no disease recorded therefore was excluded. Site PGR2 was the intensively sampled site.

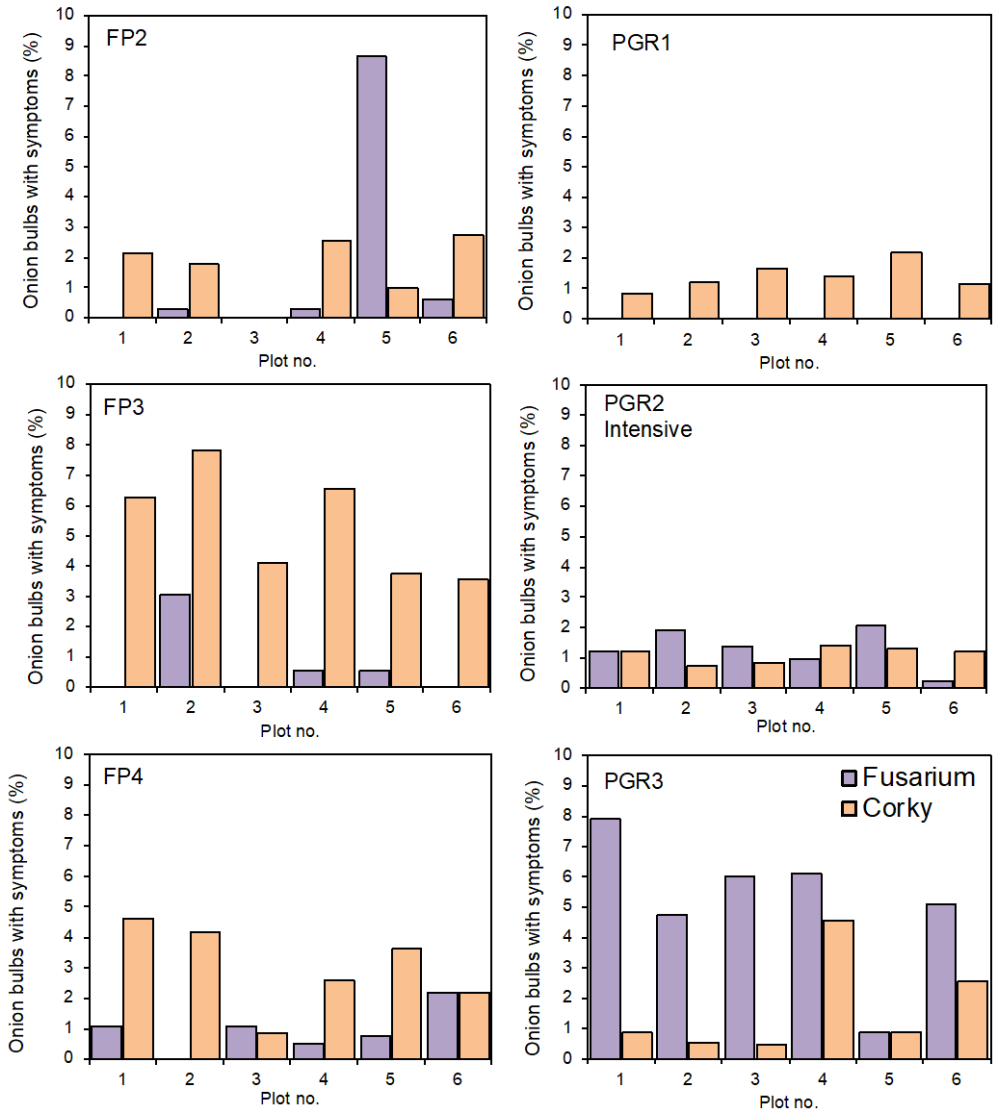

Figure 12. Fusarium disease development (percentage onion bulbs affected) after six weeks of storage for onion bulbs from six field sites monitored by the Allium and Brassica Centre (ABC). The percentage of bulbs with corky and active Fusarium symptoms are displayed separately. Site PGR2 was the intensively sampled site.

As with the ABC sites, all VCS fields had low levels of Fusarium with less than 7% disease in onion bulbs in the field over the season (Figure 13). The intensively sampled field had the highest level of disease (7%), but this was still relatively low. As for the ABS sites, disease levels were also low after the bulbs had been harvested and stored for six weeks, with a

maximum of 6% of bulbs showing basal rot symptoms (VCS field TUN, plot 4), and most of the other sites having less than 5% disease (Figure 14). However, onions did develop corky symptoms after storage, with site HAR having 24% of the bulbs post storage displaying this symptom (Figure 14). It is not clear if the corky basal plate symptom is directly associated with Fusarium basal rot.

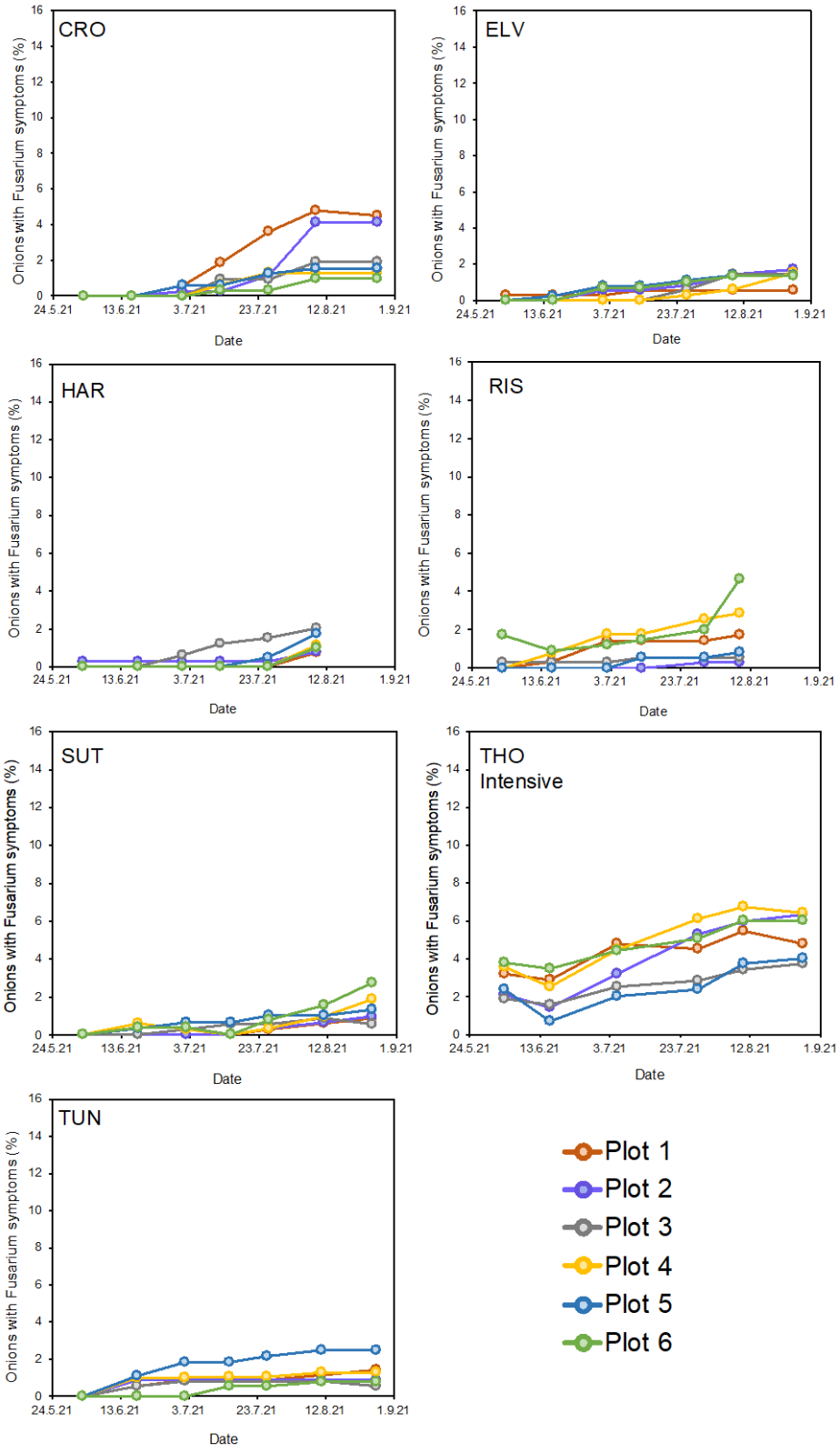

Figure 13. Fusarium disease development in onion plants during the growing season for seven field sites monitored by the Vegetable Consultancy Services (VCS). Site THO was the intensively sampled site.

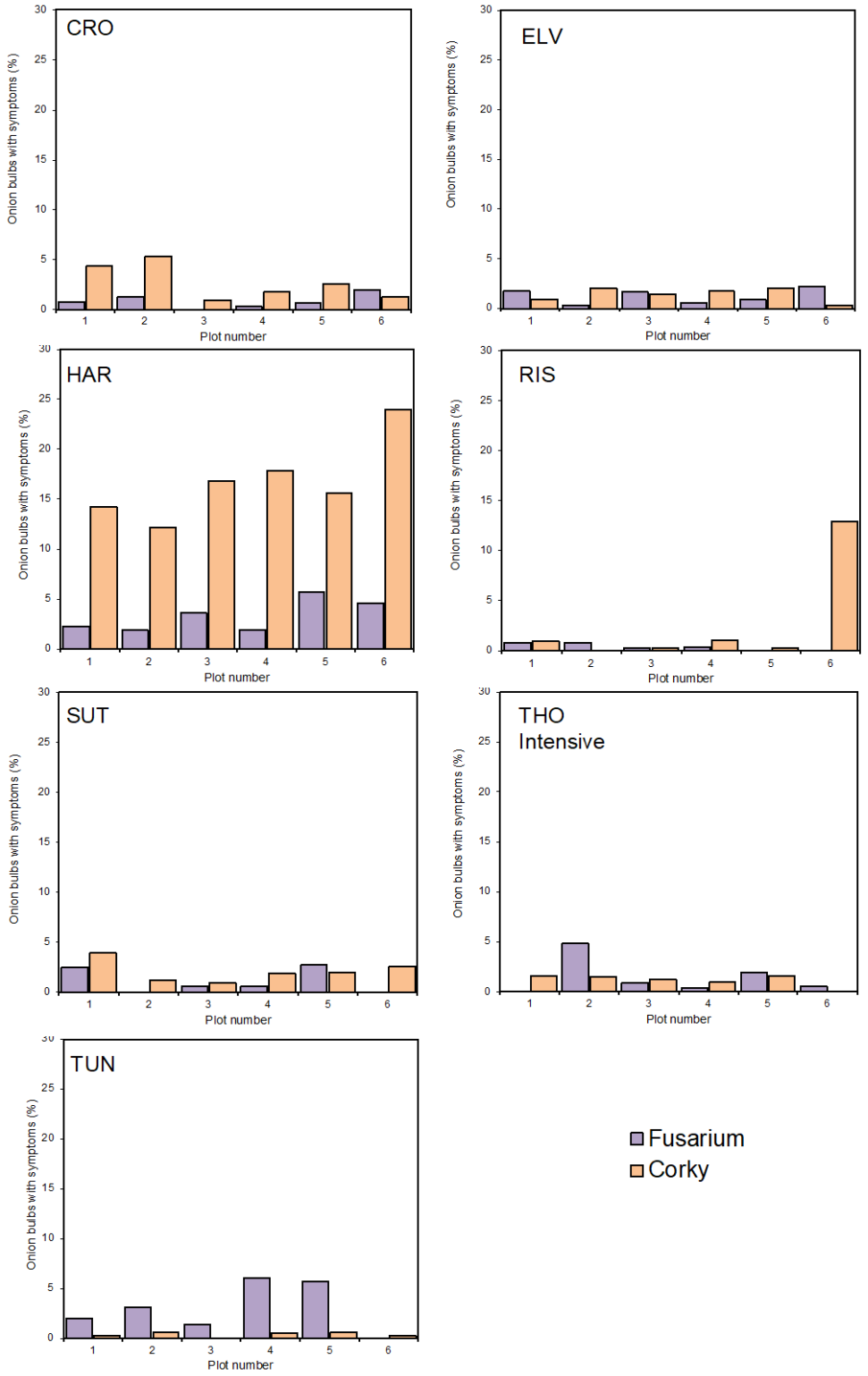

Figure 14. Fusarium disease development after six weeks of storage in onion bulbs from six field sites monitored by the Vegetable Consultancy Services (VCS). The percentage of bulbs with corky and active Fusarium symptoms are displayed separately. Site THO was the intensively sampled site.

Onion plant baiting using 2021 soils

Onion seedlings were grown in 14 soils collected from grower sites and two from Wellesbourne (Table 3), and the roots harvested for qPCR analysis. Seed germination and seedling damping off was also recorded, which varied between soils, with 63% of seeds germinating in soakwaters soil (SW, no FOC, Wellesbourne) and only 41% of seeds germinating in Wellesbourne QF soil (high prevalence FOC, Figure 15 A). The highest germination was in seeds grown in field soils FP3 and PR3 with 94%, followed by PR1 and HAR with 91% (Figure 15 A). The seeds with the lowest percentage germination were grown in field soil TUN with only 41%. Relatively little post emergence damping off was observed with only a small percentage of seeds from FP2, FP1, PR1, THO and QF not surviving until the seeds were harvested (Figure 15 A).

Roots were also tested for the presence of FOC using qPCR, however, only roots grown in FP1 soil and the QF soil had detectable levels of DNA (Figure 15 B), with FP1 roots being considerably lower than QF roots with 1.8 pg DNA mg-1 root compared to 13.2 pg DNA mg-1 root (Figure 15 B).

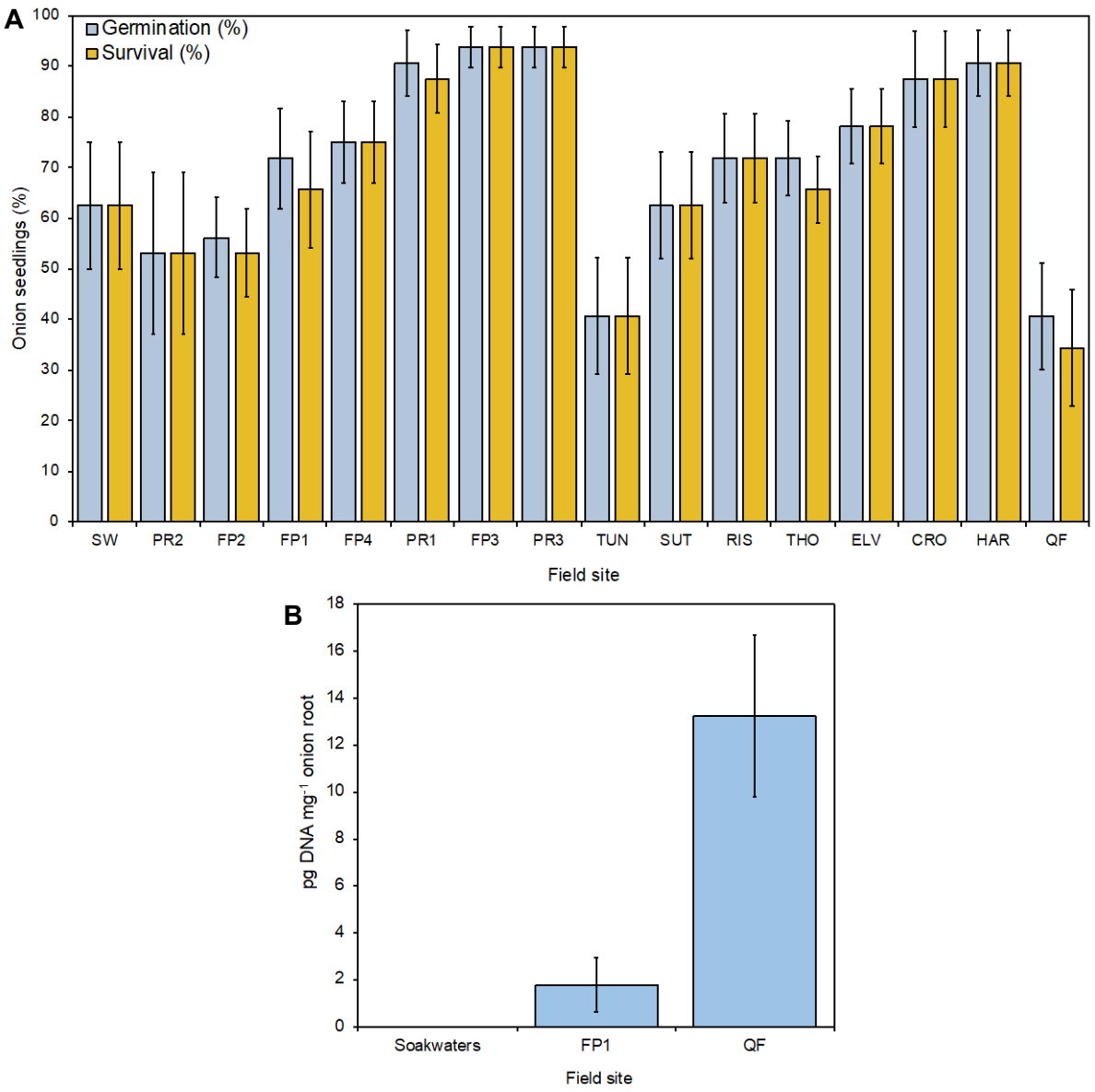

Figure 15. A) Percentage of onion seeds which germinated and the percentage of germinated that survived grown in soil from 14 onion field sites, with a positive (QF = FOC infested soil from Warwick Crop Centre quarantine field) and negative control (SW = FOC negative soakwaters soil). B) pg FOC DNA mg^{-1} onion root from (A). FP1 was the only positive field site. Error bars represent standard error of the mean.

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

Onion bulbs were obtained from 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%).

We received 100 bulbs from each site (400 in total), half of which were used for FOC detection using qPCR and the other half were dried and subsequently incubated at 20˚C for 5-6 weeks in controlled environment to determine if Fusarium basal rot would develop in optimal conditions.

Of the 50 onion bulbs per site (four sites), 25 were randomly selected for initial DNA extraction and qPCR. None of the samples tested were positive using qPCR and therefore contained no detectable FOC DNA, therefore the remaining samples were not tested.

Of the other 50 bulbs that were incubated in controlled environment, less than 15% from each sample developed Fusarium symptoms; however, many bulbs had darkened or black spots in the basal plates, which could not be directly attributed to Fusarium disease, but was noteworthy as it was also observed in the stored bulbs (Figure 16). Bulbs from the higher risk sites had a higher percentage of symptomatic bulbs, and also bulbs with dark basal plates (Figure 16). These sites also had considerably fewer completely healthy bulbs, although many bulbs from ABC sites did also develop bacterial neck rot.

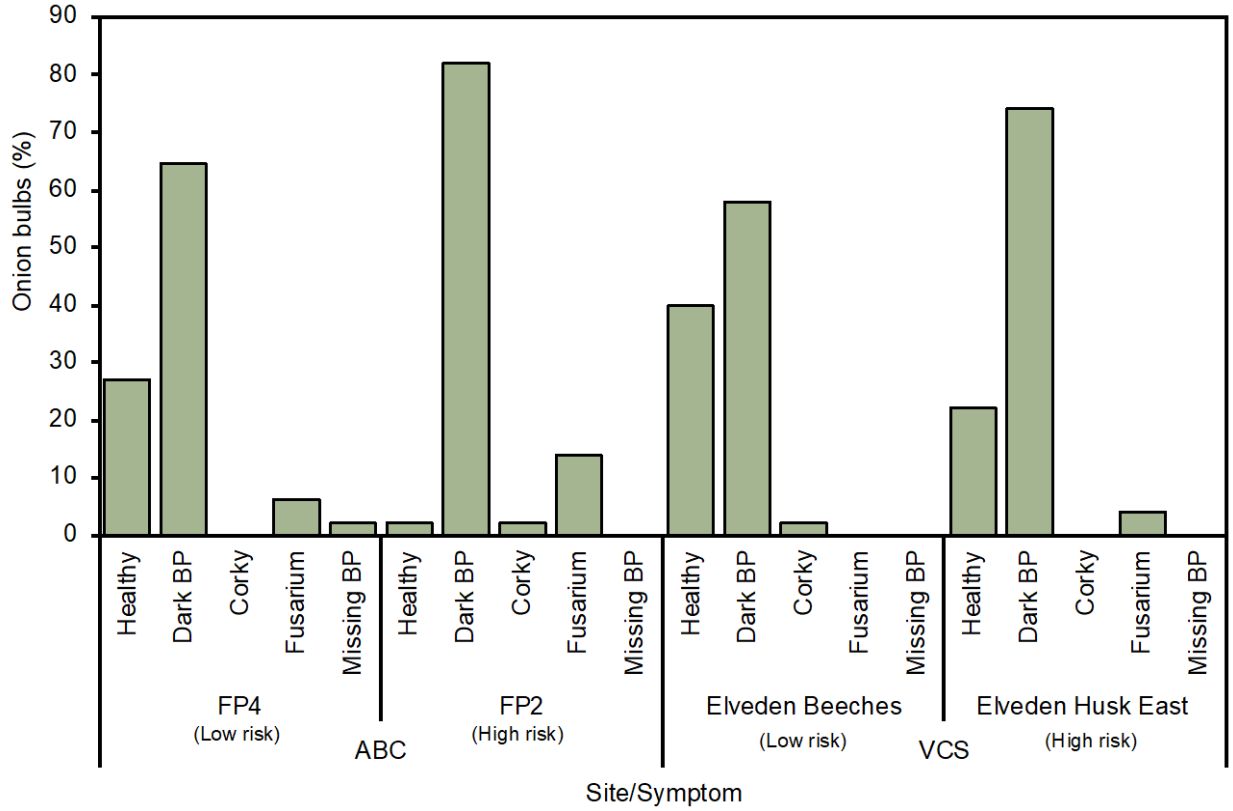

Figure 16. Percentage of onion bulbs which were healthy or showed signs of Fusarium disease after being incubated for 5-6 weeks at 20˚C.

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

The effects of biological control agents and Perlka were determined in sterilised and non-sterilised soil containing FOL, to see if they prevented the build-up of FOL in successively grown lettuce plants. So far, only one round of lettuce plants have been grown, however, the effects of FOL build up will continue to be examined in three crops.

In the sterilised soil after just one lettuce crop, mild wilting due to Fusarium was observed in the lettuce plants across all treatments, with the highest wilt observed in soil treated with Perlka (Figure 17 A), (higher than the FOL4 only treatment). The biological control agents resulted in a minor reduction in wilt symptoms, with lettuces in T34 treated soils having the least wilt (Figure 17 A). Vascular browning due to FOL4 was also observed at harvest in all treatments in sterile soil and again the lettuce grown in the Perlka treated soil had more severe symptoms (Figure 17 B, D, E).

In non-sterile soil, there were no symptoms of Fusarium either as wilt or vascular browning.

In all treatments, apart from Perlka, the lettuces were bigger (higher dry weights) in sterilised soil compared to non-sterilised soil (Figure 17 C).

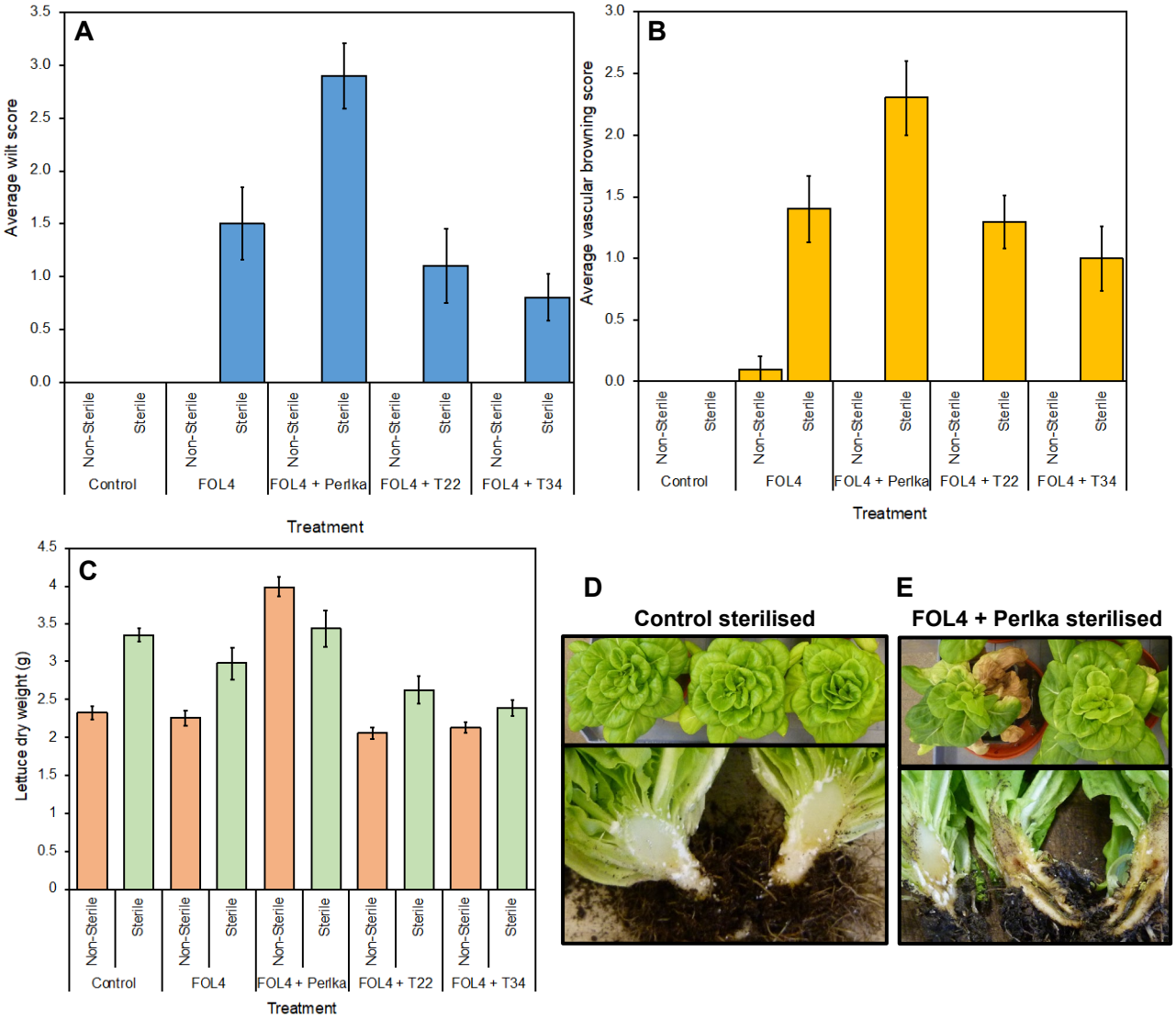

Figure 17. A) mean Fusarium wilt score, B) mean vascular browning score and C) mean lettuce head dry weight (g) for lettuce plants grown in FOL4 infested soil, which was either steam sterilised or not sterilised, with the addition of three treatments: Perlka, T22 (Trianum G) or T34. Error bars represent standard error of the mean. Symptoms of wilt and vascular browning in D) Control sterilised and E) FOL4 with Perlka sterilised.

Soil samples were collected from all of the soils at treatment application, two weeks later when lettuces were transplanted into pots and at lettuce harvest. Three replicate DNA extractions and subsequent qPCR assays were conducted for each soil sample and for all sterilised treatments the amount of FOL4 DNA detected at lettuce harvest had increased significantly compared with the levels at treatment application/lettuce transplant (Figure 18). At harvest, the Trianum-G (T22) and T34 treatments resulted in lower levels of FOL4 DNA detected suggesting a small effect in preventing FOL proliferation as significantly less FOL

DNA was detected than the FOL4 only treatment (Figure 18) and less wilting/vascular browning was observed after one lettuce crop (Figure 17).

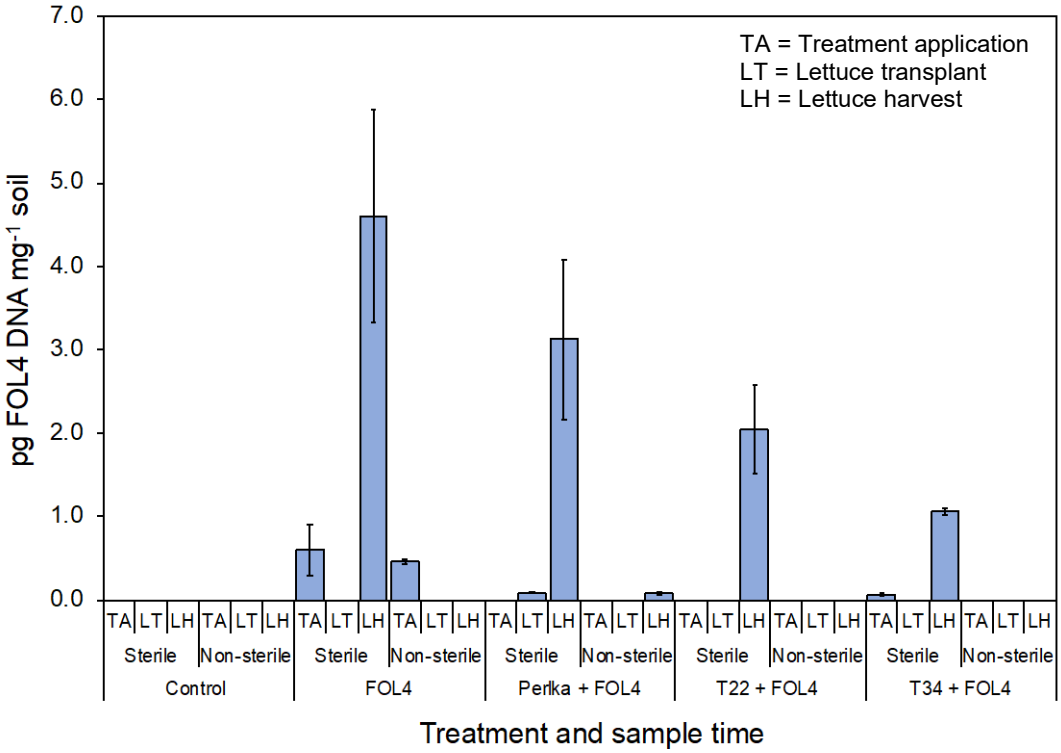

Figure 18. Quantity (pg) of FOL4 DNA mg^{-1} soil for sterilised and non-sterilised soil inoculated with 1 x 10^2 cfu g^{-1} of FOL4. Soils were treated with Perlka, T22 (Trianum G) or T34, or untreated. A non-inoculated control was also included. Soil samples were taken at treatment application (TA), lettuce transplant two weeks later (LT) and at lettuce harvest (LT). Error bars represent standard error of the mean for three replicate DNA extractions.

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

Amplicon sequencing of soils inoculated with FOL

Amplicon sequencing was successful for all three of the soils from Objective 1 (S1 = Soil 1, S2 = Soil 2, S3 = Soil 3) inoculated with different concentrations of FOL4 (D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil). The results from S1 were presented in the project annual report 2021 (Clarkson, 2021) and were similar to the results presented here for S2 and S3.

Identity of bacteria using 16S amplicon sequencing in Soil 2 and Soil 3

Clear differences were observed between bacterial community populations of S2 and S3 and between different FOL4 inoculum levels (D1-D6) within each soil (Fig 19). The two most common bacterial taxa in S2, *Gemmatimonas* and *Pseudomonas* were ranked 5th and 6th most abundant in S3, whereas the most abundant in S3, *Spartobacteria*, was ranked 11th in S2. Out of the 25 most abundant taxa in S1 and S2, only 16 were shared and these differed in relative abundance between soils. Nine of the top 25 bacterial genera were distinct for each soil type. Changes across FOL4 inoculum levels in the soils showed a marked decrease in *Pseudomonas* abundance at D5 and D6 for S2, whereas in S3 there was marked variation in the abundance of *Acinetobacter*.

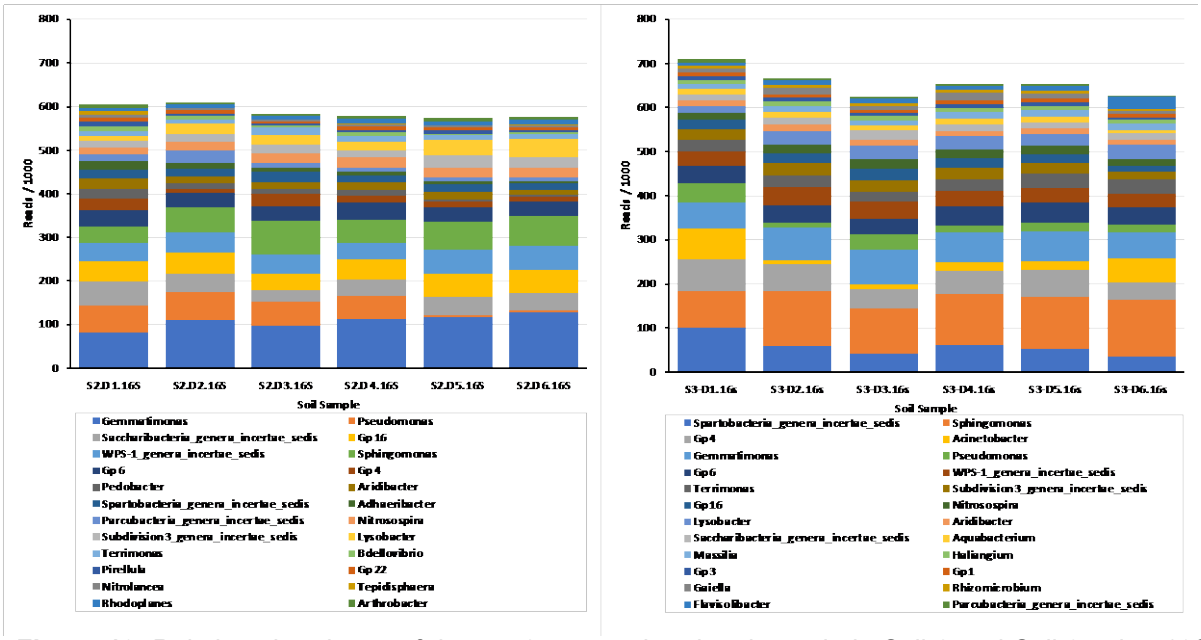

Figure 19. Relative abundance of the top 25 most abundant bacteria in Soil 2 and Soil 3 using 16S sequencing for different inoculum concentrations of FOL4. The key shows the genera listed by abundance from top left to right going down. D1 (no FOL) for each soil is used as the reference. Where reads / 1000 are below a total of 1000 the remainder consist of numerous genera of low abundance.

Identity of fungal genera using ITS amplicon sequencing in Soil 2 and Soil 3

ITS sequencing of S2 and S3 showed a diverse range of fungi between soils with only a few shared genera. As might be expected, *F. oxysporum* was identified in high abundance in all the FOL4 inoculated samples for both soils (D2-D6) but was also prevalent in the uninoculated samples (D1). This indicates a high background presence of *F. oxysporum* in the two soils but these could be common non-pathogenic isolates. The two most abundant genera in S3, *Cryptococcus* and *Cladosporium* were not represented in the top 24 most abundant genera in S2. S2 was not dominated by any one taxa but shows the two most abundant genera, *Fusarium* and *Mortierella* were almost equally abundant, closely followed by *Archaeorhizomyces* and *Spizellomyces*. It is noticeable that there is a sudden abundance of *Fusarium* dominating sample S3D6. Although an increase would be expected, such a dramatic increase is likely to be a result of sampling due to unevenly mixed soil or PCR bias.

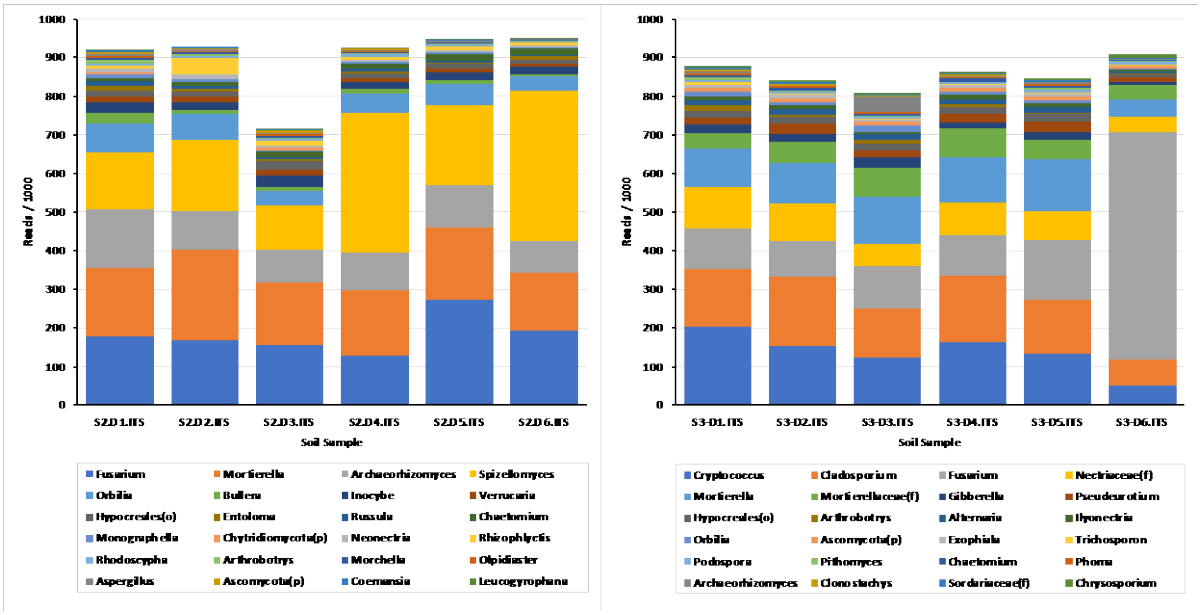

Figure 20. Relative abundance of the top 24 most abundant fungal genera in Soil 2 and Soil 3 using ITS sequencing for different inoculum concentrations of FOL4 (D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil).

Identification of *Trichoderma* spp. with the ITS amplicon

The ITS primers used also amplified sequences associated with *Trichoderma* spp. from both soil samples although these were in low abundance (1.44 reads / 1000 in Soil 2 and 2.64 reads / 1000 in Soil 3). As part of Objective 5, we will be quantifying the different components of the fungal community in sterile and unsterile soil inoculated with FOL4 in the presence of treatments including the biocontrol agents *Trichoderma harzianum* T22 (Trianum G) and *Trichoderma asperellum* T34 (T34 Biocontrol). However, to ensure we can identify and

monitor levels of these biocontrol strains though amplicon sequencing, it was necessary to establish that the ITS primers resulted in successful amplification of DNA from these particular *Trichoderma* spp. This was confirmed for both species following PCR of extracted DNA which resulted in amplification of single products. The sequences for these will be added to the database to enable species level identification for these biocontrol strains.

Abundance of *Fusarium* species found in Soil 2 and 3 samples using TEF amplicon sequencing

Sequencing the TEF1a amplicon enables identification of different *Fusarium* species. As might be expected, *F. oxysporum* was identified in high abundance in all the FOL4 inoculated samples for both soils (D2-D6). The data also showed that the *Fusarium* genera present in S2 in the absence of FOL4 was dominated by *Fusarium oxysporum* with only minimal presence of other members of the genus (Fig. 21) which confirms the results from ITS sequencing (Fig. 20). In contrast, in the absence of FOL4, S3 contained a mix of *Fusarium* genera including several other known pathogenic species (*F. culmorum*, *F. redolens*). The large error bars for abundance of *F. oxysporum* in S3 D1-D5 and other *Fusarium* spp. for all S3 samples indicated that these species were present at low background levels and the stochastic nature of PCR on rare templates is causing this variation. The smaller error bar size for *F. oxysporum* for all S2 samples and S3D6 indicated a more reliable detection of *F. oxysporum* in the sample as this species out-competes other *Fusarium* spp. in the PCR reactions.

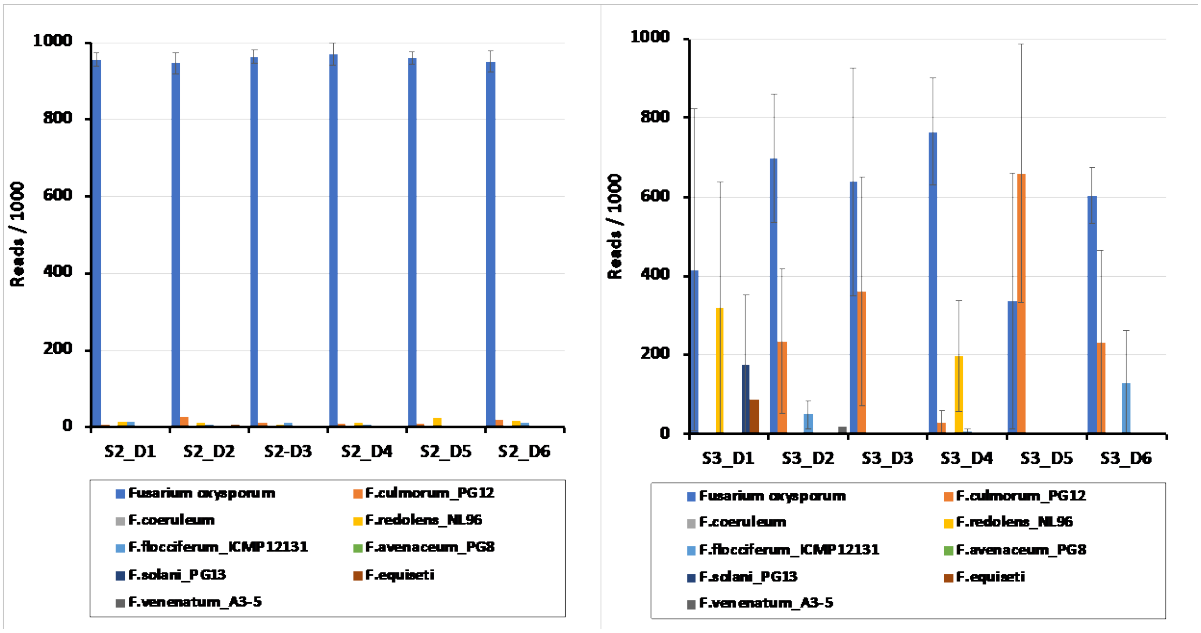

Figure 21. Relative abundance (number of reads / 1000) of *Fusarium* spp. using TEF sequencing in a) Soil 2 and b) Soil 3 for different inoculum concentrations of FOL (D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil).

Abundance of *F. oxysporum* f.spp. in Soil 2 and 3 samples using OG4952 amplicon sequencing

Sequencing the amplicon gene target OG4952 does not identify FOL4 but in principle will distinguish *F. oxysporum* f.sp. *cepae* from other combinations of fspp. including f.sp. *conglutinans*, f.sp *matthiolae* (FOM), f.sp. *niveum*, some f.sp. *narcissi* (FON) isolates, f.sp. *cepae* (FOC), f.sp. *pisi* (race 1; FOP1) and f.sp. *tulipae* (Table 6).

OG4952 PCR products failed to amplify to any significant level in Soil 3 despite two attempts even though positive controls did amplify as expected. This indicates that although *Fusarium oxysporum* is present in Soil 3 these isolates do not carry this target gene.

Sequencing OG4952 was successful for Soil 2 and the dominant amplicon sequence variant present was associated with FOC although FOC specific PCR would be required to confirm this (Fig. 22). Alternatively, it is possible that this indicates the presence of another unknown isolate carrying the same OG4952 sequence variant.

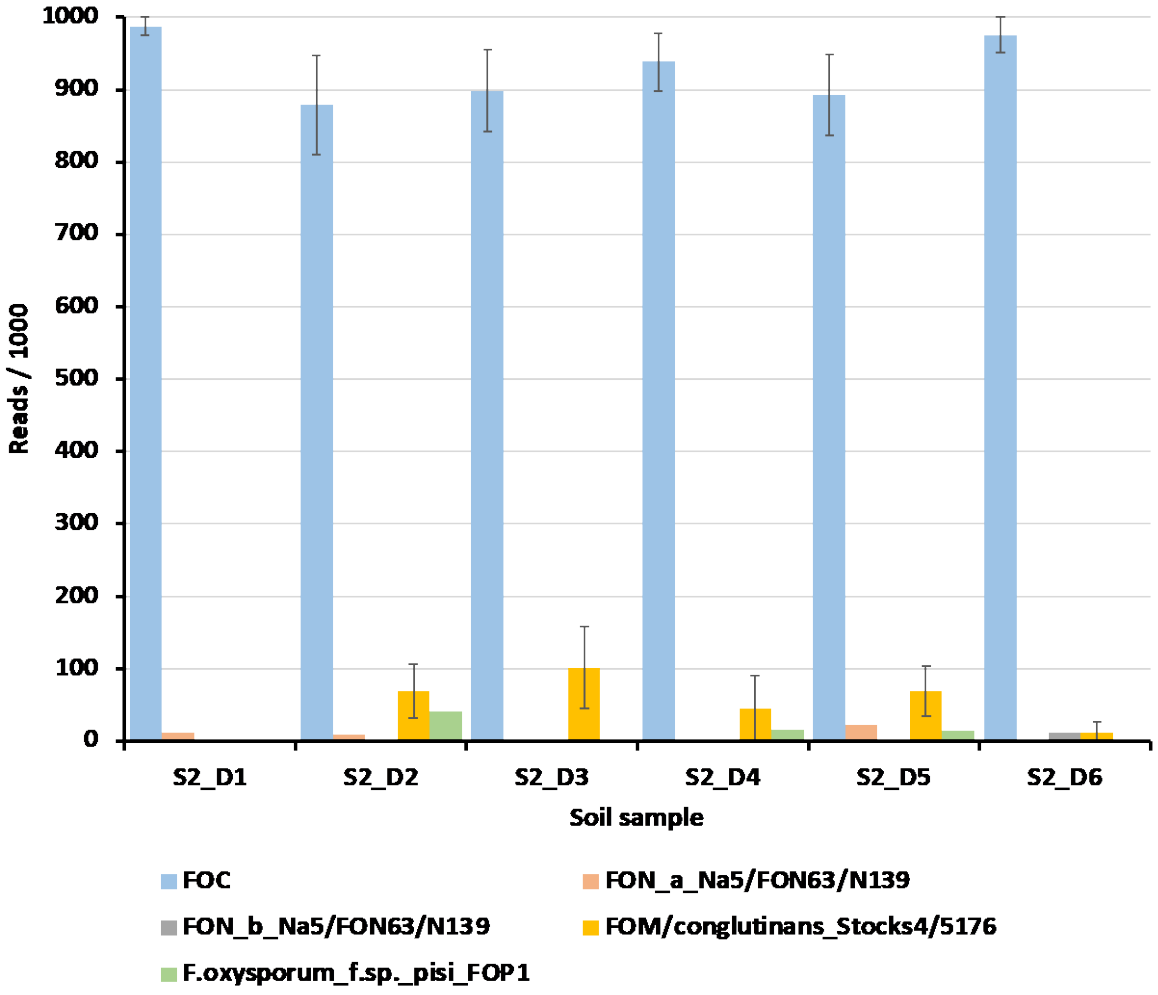

Figure 22. Relative abundance of *Fusarium* spp. using OG4952 amplicon sequencing in Soil 2 for different inoculum concentrations of FOL4 (D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil).

Abundance of *F. oxysporum* f.spp. in Soil 2 and Soil 3 samples using g19096 amplicon sequencing

Sequencing the amplicon gene target g19096 identifies *F. oxysporum* f.sp. *lactucae* (FOL), alongside other f.spp. including f.sp. *matthiolae*, f.sp. *tulipae*, some isolates of f.sp. *narcissi*, f.sp. *nicotianae*, f.sp. *radicis-cucumerinin* f.sp. *cepae*, (FOC), f.sp. *fragariae*, f.sp. *pisi* (race 5; FOP5), f.sp. *limonii* (statice), f.sp. *physalis*, f.sp. *lycopersici*, f.sp. *melonis*, f.sp. *niveum*, f.sp. *pisi* (race 2; FOP2), f.sp. *conglutinans*, f.sp. *pisi* (race 1; FOP1), *F. oxysporum* pea foot rot (PG18) (Table 6).

Amplicon sequencing of g19096 identified the presence of *F. oxysporum* f.spp. in all soil samples. Soil 2 showed a high abundance of two different g19096 amplicon sequence variants associated with different *F. oxysporum* f. spp. The first sequence variant is associated with several *F. oxysporum* f.spp. including f.spp. *nicotiana*, *radicis-lycopersici*, *radicis-cucumerin*, *narcissi*, *cepae*, *pisi* (FOP; race 5), and *fragariae* and was most abundant in Soil 2 in the absence of FOL4 in sample D1 but also in FOL4 inoculated samples D2-D4. The second amplicon sequence variant is associated with FOL and *F. oxysporum* f.spp. *tulipae* and *matthioli* and was present at almost equal abundance in all samples including the uninoculated control D1 and was the most abundant in samples D5 and D6 which were those inoculated with highest levels of FOL4. A third amplicon variant associated with pea foot rotting isolates *F. oxysporum* PG18, PG3 and another form of f.sp *cepae* was also present at low levels in Soil 2. The presence of FOL4 was not detected in S2 D1 and D2 samples using FOL4 specific PCR (see Objective 1) suggesting that the high level of the g19096 amplicon variant in these samples is more likely associated with one of the other associated *F. oxysporum* f.spp.

In contrast, amplicon sequencing of g19096 for Soil3 resulted in high abundance of the amplicon variant associated with pea foot rotting isolates *F. oxysporum* PG18, PG3 and another form of f.sp *cepae* in samples D1-D4. However, the amplicon sequence variant associated with FOL, *F. oxysporum* f.spp. *tulipae* and *matthioli* was the most abundant for the higher FOL4 inoculum levels D5 and D6.

Overall, the large numbers of different *F. oxysporum* f. spp. grouping together within the different g19096 amplicon variants means that this target gene is not ideal for f.spp. discrimination even when used in combination with other target genes such as OG4952. It

would be preferential to have another target gene with a wider range of variants across f. spp. to enable better identification.

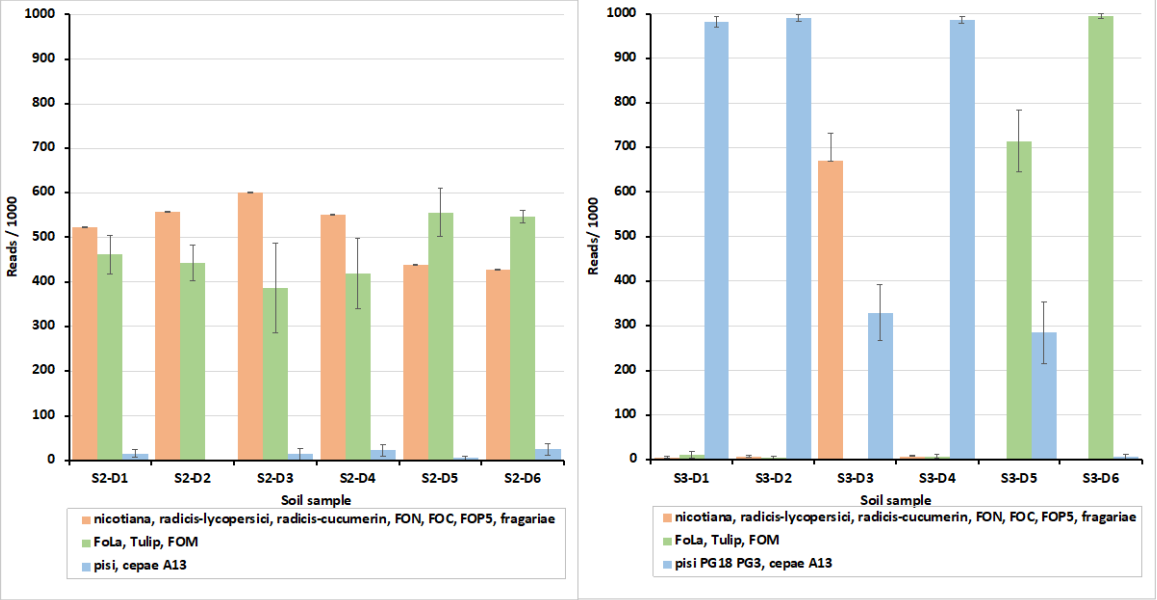

Figure 23. Relative abundance of *F. oxysporum* spp. using g19096 amplicon sequencing in Soil 2 and Soil 3 for different inoculum concentrations of FOL4 (D1=0, D2=2x10^2, D3=2x10^3, D4=2x10^4, D5=2x10^5, D6=2x10^6 cfu g^{-1} soil).

Design and testing of additional gene targets for amplicon sequencing of FOL

Further comparative analysis of FOL genomes identified an additional target gene, g19482, that would potentially enable identification of both FOL and FON amongst other f. spp (Table 6). The presence of at least two variants of the amplicon for each f.sp. will help to improve identification. If successful, this amplicon will also improve identification of FON which previously could only be positively identified by target OG4952. Primer sets were designed to test against a panel of soils and isolates and testing is currently on-going.

Discussion

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

Lettuce response to FOL4 inoculum level

The results from the dose response experiment with lettuce 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 soils suggesting that quantitative molecular diagnostics could be used to identify soils with a risk of high levels of Fusarium wilt disease in lettuce. The final aspect of this work was to determine the colonisation of FOL4 in the lettuce roots. Samples were collected at 3, 6 and 13 dpi, and DNA was detected in the higher doses in all soils by the final sampling point. There was a strong positive relationship between the pathogen DNA detected in the roots at 13 dpi, pathogen DNA detected in soil, disease development and inoculum concentration. DNA detection was more reliable in soil than in root samples, and hence this could be a reliable way to detect and predict disease in commercial production.

Narcissus response to FON inoculum level

Two different soil types were inoculated with FON, along with a compost mix suitable for growing daffodils. Soils were collected from daffodil growing regions in the UK (Cornwall and Lincolnshire) and were classified as clay and sandy silt loam respectively. Silts or fine sandy loams are ideal Narcissus growing soils, however, in the UK, they are successfully grown in silt, silt loam, brick-earth and peat soils (Hanks, 2013). Peat and heavy silt soils have been reported to make bulbs larger and less dense, however were more susceptible to basal rot (Hanks, 2013). The strong positive correlation between FON inoculum, FON DNA in soil and FON DNA in roots suggests that qPCR diagnostics could be used to estimate the concentration of FON in field soils. Following scoring of Narcissus bulbs for basal rot, it should be possible to relate disease development with inoculum concentration and FON DNA levels. Overall, we have successfully demonstrated that quantitative molecular diagnostics tools for both FON and FOL can potentially be used to predict disease levels in field soils, especially if high levels of pathogen are present.

There was a small quantity of FON DNA detected in the roots of Narcissus bulbs growing in the non-inoculated treatment (Dose 1) for Soil 1. This was likely a background level of the

pathogen as small amounts were detected when Soil 1 was initially tested to determine if it was FON free. This was a possibility as the soil was collected from a field which has previously grown daffodils, and FON, like other f.spp. can survive in the soil for many years (Di Pietro et al., 2003).

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

The soil samples from onion field collected in 2020 had low or undetectable levels of FOC DNA, and the pathogen was only detected sporadically (not in all plots at each site) over the season for the intensively sampled sites. The roots from onions grown in the same sites had higher quantities of FOC, with all samples from both intensively sampled sites containing detectable levels of DNA across the season. Detection in roots is therefore a more reliable way to detect FOC in the field, however, testing in this way could not be carried out before the fields were drilled, and therefore it would only be a predictive tool for disease levels at harvest or after storage. One solution to this is to use soils collected pre-drilling to 'bait' out FOC by growing onion seedlings to increase the sensitivity of detection. This hypothesis was initially tested using soils collected pre-drilling from the 2021 field sites; however, only one site had detectable levels of FOC DNA in onion roots. This could be explained by the fact that there was very little disease in the fields over the season, at harvest or after bulb storage, and therefore, FOC levels were likely to be too low at all these sites for qPCR detection. Additional evidence for this was the high level of FOC DNA detected in roots grown in soil collection from a highly infested site at Warwick Crop Centre, which demonstrates that the baiting approach is possible and this will be investigated further in year 3 of project. This approach has previously been shown to be successful, as FOC has been detected using qPCR in the roots of onions grown in heavily infested field sites (Sasaki et al., 2015).

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

Previously we found that we could detect FOC in the basal plates of onion bulbs with different levels of basal rot symptoms but also in some asymptomatic bulbs using qPCR. Therefore, in 2021, only healthy onion bulbs with no symptoms of Fusarium disease were used to try and predict whether bulbs would later develop Fusarium disease in store. However, all the bulbs tested negative for FOC in the basal plates, and only a small number of bulbs developed

symptoms when incubated at temperatures favourable to Fusarium development. Additional bulbs from the same site were also dried and stored by the industry partners, and very few bulbs developed disease. Therefore, we were not able to predict disease development using these batches of onions. This will be repeated in year 3 to try to use diagnostic techniques for predicting Fusarium disease in storage. Sites with high levels of Fusarium disease are required where bulbs will go onto develop symptoms later in store even if they appear healthy when harvested. In a previous study, FOC was not detected in the basal plates of onions grown in heavy infested field sites 77 days post-transplant (Sasaki et al., 2015). It has been reported that FOC can be detected in onion basal plate samples using qPCR with only 1 infected bulb in 50 healthy bulbs being present (Latvala et al., 2020). However, we have found that FOC is easily detectable in symptomatic bulbs, even in the early stages of infection (Clarkson, 2021). However, detection in asymptomatic bulbs is difficult as the amount of FOC at this stage will be very low, and possibly below the limit of detection for the qPCR assay.

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 a method was developed to examine FOL4 inoculum build up in sterilised and non-sterilised soils with successive lettuce plants (Clarkson, 2021). Using field soil from a UK lettuce grower, this system was used to determine whether pathogen build up could be reduced or prevented by products such as Perlka, T22 (Trianum G) and T34. Calcium cyanamide (Perlka) has fungicidal activity when it is converted to hydrogen cyanamide in the soil (McDonald et al., 2021). T22 (Trianum G) has been reported to reduce disease severity by FOL race 1 in lettuces previously (Gilardi et al., 2007) and T34 has been shown to reduce disease caused by *F. oxysporum* f. sp. *lycopersici* in tomato (Cotxarrera et al., 2002). As lettuce sterilisation can be used to reduce FOL4 inoculum in indoor lettuce cultivation, it has been noted that disease can return quickly due to the lack of a rich microbial community (O'Neill et al., 2005); therefore finding products to mitigate this is important. The initial phases of this experiment have already demonstrated differences in the build-up of FOL4 inoculum and disease between treatments and so far, only one lettuce crop has been grown and therefore these results may change as successive plants are grown. It was clear from the disease data that FOL4 inoculum build-up was much quicker in sterile soil but none of the treatments seem to have slowed or prevented this occurring or reduced disease development. Perlka, also had a fertiliser effect on the lettuces grown in non-sterilised soil, as have been shown previously in field grown spinach, where it was also shown to have little

effect of Fusarium disease reduction (McDonald et al., 2021). However, Perlka has been shown to supress *F. oxysporum* infecting cucumber (Shi et al., 2009). Quantification of FOL4 inoculum using qPCR revealed that FOL could be detected at harvest of lettuce crop 1 in sterilised soil again confirming that that the pathogen proliferated rapidly. As the amount of FOL4 inoculum used to infest the soil was low, it was difficult to detect DNA accurately and reliably in the samples at the time of treatment application (also time of inoculation) or two weeks later after lettuce transplant. T34 and T22 (Trianum G) did appear however to slightly reduce detectable FOL4 DNA at harvest, therefore suggesting these products may have slowed FOL proliferation compared to the FOL4 only treatment.

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. This approach was used successfully used to quantify the relative abundance of bacterial and fungal communities using 16S and ITS gene targets in two different soils (from lettuce growers) artificially inoculated with FOL4. TEF1a amplicon sequencing also enabled identification of different *Fusarium* spp. present in soils and showed that there was a high level of *F. oxysporum* naturally present in Soil 2 (not FOL4) while Soil 3 contained a variety of different *Fusarium* spp. Several gene targets were also identified to potentially enable multiple *F. oxysporum* f.spp. to be identified concurrently with a focus on FOL, FOC and FON. The gene target, g19096, successfully enabled identification of FOL4 in inoculated soils and a clear increase in the abundance of FOL4 in Soil 3 with increasing inoculum levels. However, the high naturally occurring level of *F. oxysporum* in Soil 2 masked the ability to clearly identify FOL4 using g19096 and also affected the usefulness of another gene target OG4952 for cross-identification. Further work will investigate the effectiveness of an additional gene target for FOL identification and will use cross-referencing of existing targets to more accurately identify different *F. oxysporum* f. spp. present in soil samples.

Conclusions

- **Objective 1:** A clear relationship between FOL4 inoculum concentration, FOL DNA levels in soil and lettuce roots and Fusarium wilt disease development in lettuce was established. In addition, a strong relationship was also found for *Narcissus*, between FON inoculum concentration and the quantity of DNA recovered in inoculated soils and colonised roots. These molecular diagnostics could be used to predict the level of inoculum in fields and determine the risk of severe disease.
- **Objective 2:** FOC is more reliably detected in onion roots than directly from soil for commercial field sites. Therefore, testing onion roots using a plant baiting approach or from field sampling is likely to be a better approach for assessing disease risk.
- **Objective 3:** Molecular qPCR diagnostics was effective at detecting FOC in onion bulbs even in the absence of visible symptoms from bulbs in 2020, however, in healthy bulbs collected in 2021, FOC was undetectable in basal plate tissue. This approach requires further investigation and bulbs from infected sites need to be used to ensure development of disease in store.
- **Objective 5:** The methodology developed previously to examine FOL4 inoculum build up in sterilised and non-sterilised soil after successive lettuce crops was successfully used to determine the effect of products (Perlka, Trianum G (T22) and T34) on disease development. Further lettuce crops will be grown successively to determine their effect in an intensive cropping situation.
- **Objective 6:** Amplicon sequencing successfully identified and quantified the relative abundance of bacteria, fungi and *Fusarium* spp. in soils inoculated with FOL4. Gene targets to identify multiple *F. oxysporum* f.spp. have also been identified but some technical challenges need to be solved for these to be used reliably.

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)

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