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AUTHENTICATION

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

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GROWER SUMMARY

Headline

DNA-based approaches have been developed to identify and quantify major *Fusarium oxysporum* pathogens affecting key horticultural crops. Pathogen levels have been defined which result in rapid disease development in onions and column stocks.

Background

Fusarium oxysporum

F. oxysporum is the most important and economically damaging *Fusarium* species for horticulture and can be a major constraint to the production of many food crops including onion, leek, lettuce, tomato, brassicas, asparagus, cucurbits, peppers, coriander, spinach, basil, beans, peas, strawberry and watermelon as well as non-food crops such as carnation, column stocks and narcissus (Michielse et al., 2009). The *F. oxysporum* complex comprises a large array of more than 70 pathogenic *formae speciales* (f.spp.) which are adapted to infect these different crop and plant hosts as well as non-pathogenic isolates.

Control of *Fusarium*

Control of *F. oxysporum* and other species is challenging as most produce long-lived chlamydospores that survive in the soil for many years, resulting in the need for long rotations. Past approaches have also relied on the use of soil sterilisation or fumigation, fungicides or seed treatments but approval for their use in many cases has been withdrawn or threatened by further legislation. Generally, there are also no sources of plant resistance with a few notable exceptions but in these cases, the deployment of major gene resistance has often broken down as new pathogen races emerge. Other management strategies such as biological control have yet to be widely proven although there is a large amount of published literature on this approach including the use of non-pathogenic *Fusarium* species. Two microbial products in the UK (Prestop, T34 Biocontrol) are currently registered for *Fusarium* disease control.

Impact of *Fusarium oxysporum* and other species on key horticultural crops

F. oxysporum was identified as the key species in horticulture and following consultation, the f.spp. affecting onion and leek (*F. oxysporum* f.sp. *cepae*, FOC), column stocks (*F. oxysporum* f. sp. *mathiolae*, FOM) and narcissus (*F. oxysporum* f.sp. *narcissi*, FON, Narcissus) were selected as the primary focus of this project.

Fusarium basal rot of onion (FOC) and leek

FOC can affect onion crops at any stage, causing damping-off in seedlings and a root/stem rot in immature plants, but the greatest impact is generally at harvest and in store. On average, 2-6% of the bulb crop (8889 ha valued at approx. £126M in 2017; Defra, 2017) is lost each year in the field with a corresponding economic value of £7.6M but more recently, basal rot incidence of 10% or greater is becoming more common, equating to losses of approx. £12.6M. Average losses in store are 3% (Andy Richardson, personal communication), but in some years, storage can result in total failure (>10% basal rot). Although seed treatments are available for control of seedling blight (e.g. fludioxonil ± metalaxyl, thiram) and boscalid + pyraclostrobin can be applied to sets, these fungicides may not provide long-term control of FOC or protect the bulbs from basal rot. Foliar sprays of cyprodinil and fludioxonil approved for *Botrytis* control may have some activity against FOC but are unlikely to have much effect at soil level at approved application rates. Leeks, which have a value of £24M per year, are also susceptible to seedling blight, root and basal rots caused by *Fusarium* species. Although these can be caused by FOC, a range of other *Fusarium* species including *F. proliferatum*, *F. culmorum* and *F. avenaceum* have also been associated with these disease symptoms (Armengol et al., 2001; Hall et al., 2007; Koike et al., 2003; Palmero et al., 2012). These other *Fusarium* spp. are generalists and the extent to which they affect UK leeks is unknown.

Fusarium wilt of column stocks (FOM)

FOM is one of the major problems for nurseries growing column stocks with losses due to this pathogen ranging from 5 to >50% and an average of 15% which given the industry value of approx. £3.7M equates to £0.5M per annum (Lyndon Mason, personal communication). Symptoms include failure to establish and wilting symptoms progressing from the base upwards eventually resulting in plant death (Mason, 2013; O'Neill et al., 2004). Certain varieties such as Centum Deep Blue and Fedora Deep Rose are also more susceptible to *Fusarium* than other varieties (Mason, 2013). Many growers continually cultivate stocks which exacerbates *Fusarium* disease problems and control has largely relied on soil steaming or sterilisation with dazomet. Despite these treatments, problems can still occur (Mason, 2013; Graham Whitehead, personal communication) and the high cost of these inputs therefore increases the overall economic burden to growers further.

Fusarium basal rot of Narcissus (FON)

FON, affecting *Narcissus,* is a major problem for the UK daffodil industry causing a basal rot very similar to that in onion (Clarkson, 2012). The industry is estimated to be worth £45M and 10% losses are not unusual with a corresponding value of £4.5M (Hanks, 2010). Currently, control is dependent on just two active substances, thiabendazole (Storite) and chlorothalonil

(Bravo) applied as part of the hot water treatment process used to eradicate stem nematode from bulbs. However, registration for both these actives may potentially be under threat in the future and some FON isolates show resistance to thiabendazole (Clarkson, 2012). An alternative product containing cyprodinil and fludioxonil (Switch) has also just been approved, although performance has not been assessed in HWT. Despite the regular application of fungicides by *Narcissus* growers, extensive losses are still common in certain parts of the production area and the long periods of time the crop is in the ground makes it vulnerable to basal rot irrespective of initial fungicide applications.

Identification of *Fusarium* spp. and approaches for understanding *Fusarium* dynamics

Most individual *Fusarium* species can be identified by sequencing part of the translation elongation factor (TEF) gene (Geiser et al, 2004) with the exception of specific pathogenic f.spp. in the *F. oxysporum* complex. However, there has been little attempt to develop the tools and approaches required to examine the dynamics and interaction of individual *F. oxysporum* f.spp. on different crops and rotations. Standard molecular approaches including TEF sequencing, DNA fingerprinting and multi-gene sequencing fail to reliably distinguish different *F. oxysporum* f. spp., but more recent studies have identified genes associated with pathogenicity including 'Secreted in Xylem' (SIX) genes which could form the basis for diagnostics (Lievens et al., 2009; van Dam et al., 2016). As it is clear that a wide range of other *Fusarium* species can also cause disease problems in addition to *F. oxysporum*, an understanding of the dynamics of the entire *Fusarium* community which includes multiple species and pathogenic / non-pathogenic forms in soil is also required to optimise rotations, determine disease in relation to cropping patterns and develop management strategies. Therefore, a method of identifying and quantifying entire *Fusarium* communities in roots or soil would also be very useful. DNA 'barcoding' of entire microbial communities through the use of next generation sequencing of PCR amplicons (amplicon sequencing) now offers the promise of being able to identify a wide range of species at the same time. With this technology, total DNA is extracted from the sample and a gene target common to all or selected species (but with sequence differences between species) is amplified by PCR and subjected to high-throughput sequencing. This results in different DNA sequences being generated for each individual species present which are quantified and identified through comparison with a database.

Approaches, aims and objectives

In this project we initially collected and identified *Fusarium* isolates from leeks to add to our existing collections for onion, narcissus and stocks. Genomes of a pathogenic FOM isolate and also a range of FON isolates were also sequenced and comparative bioinformatics analysis carried out with genomes previously sequenced for FOC and other *F. oxysporum* f.spp to identify common and unique pathogenicity genes. These were then assessed for their suitability as potential diagnostic markers for FOC, FOM and FON and quantitative PCR (qPCR) developed for each pathogen. Based on the genome information, the feasibility of using a DNA barcoding approach based on amplicon sequencing to analyse *Fusarium* species within entire microbial communities is also being examined. The project also aimed to determine the effect of inoculum concentration of FOC, FOM and FON on disease development in onion, stocks and narcissus respectively to determine the critical levels required for significant damage to occur which could then be related to qPCR results. Finally, large scale artificial inoculations were carried out to establish a field area for FOC and a polytunnel area for FOM with high disease pressure for testing the qPCR and amplicon sequencing approaches and to provide a resource for further research on control approaches in the future.

The aims and objectives of the project are:

Aim 1: Development of molecular tools and resources for identifying and studying *Fusarium*

Objectives

1.1: Collection, identification and pathogenicity testing of different *Fusarium* spp.

1.2: Development of a specific quantitative (real-time) qPCR tests for *F. oxysporum* f.spp.

1.3: Development of a DNA barcoding approach for analysis of *Fusarium* communities

1.4: Development of disease areas for onions and stocks

Aim 2: To determine the effect of *Fusarium* inoculum concentration on disease development

Objectives

2.1: Determine the effect of *F. oxysporum* inoculum level on disease development in onions

2.2: Determine the effect of *F. oxysporum* inoculum level on disease development in stocks

2.3: Determine the effect of *F. oxysporum* inoculum level on disease development in Narcissus

2.4: Quantify colonisation of *F. oxysporum* on onions, stocks and Narcissus

Summary

Aim 1: Development of molecular tools and resources for identifying and studying *Fusarium*

Objective 1.1: Collection, identification and pathogenicity testing of different *Fusarium* spp.

In year 1, four *Fusarium* species were identified in diseased leek plant samples from commercial crops; *F. culmorum*. *F. avenaceum*, *F. proliferatum* and *F. oxysporum*. All these species have been identified previously as causing a basal rot on leek plants. However, pathogenicity testing in year 2 indicated that *F. culmorum* and *F. avenaceum* caused significant disease on inoculated leek plants, with the former causing more severe symptoms while *F. proliferatum* and *F. oxysporum* caused little or no symptoms. This suggests therefore that future detection and management approaches should focus on *F. culmorum* and *F. avenaceum.* The generalist nature of both *F. culmorum* and *F. avenaceum* means that crop rotation may not be effective and the potential for seed borne transmission of both pathogens means that growers should be vigilant regarding crop hygiene.

Objective 1.2: Development of a specific quantitative (real-time) qPCR tests for *F. oxysporum* f.spp.

In year 1, specific qPCR tests were developed for FOC, FOM and FON based on pathogenicity genes identified through comparative genome analysis and further work in year 2 have shown these tests to be accurate, sensitive and applicable for testing of soil and plant samples. Data has also been generated that has begun to relate pathogen DNA levels (as measured through qPCR) to the number of spores in a soil sample, a first step to understanding how useful these tests can be for practical diagnostics and to determine inoculum levels in the field. However, further work is required to determine how these assays can be successfully implemented for assessing disease risk following testing of soil samples and in the case of FOC, as a means of potentially assessing levels of the pathogen in onions going into store. This would include optimisation of sampling and testing strategies across multiple onion, *Narcissus* and stocks commercial field sites and monitoring of symptoms in order to build a relationship between pathogen DNA test results and disease levels.

Objective 1.3: Development of a DNA barcoding approach for analysis of *Fusarium* communities

In year 1, pathogenicity genes were identified in FOC, FOM and FON following genome analysis and subsequent comparison with other *Fusarium* spp. genomes. Several of these were present in one or more *F. oxysporum* f.spp. (but with different sequences) and hence could be used to potentially distinguish between these pathogens in an amplicon sequencing approach. Primers were developed for four of these genes (SIX13, OG13890, OG4952 OG13397) and used for PCR and amplicon sequencing to evaluate their utility in determining the presence and abundance of *F. oxysporum* f.spp. in mixed DNA 'pools' from multiple *Fusarium* spp, *F. oxysporum* f.spp and other soilborne fungal plant pathogens, as well as in soil samples from areas infested with FOC (inoculated Quarantine Field, Wellesbourne), FOM (inoculated polytunnel, Cut Flower Centre) and FON (naturally infested field soil). This approach showed promise with one locus (OG4952) being particularly effective in detecting high levels of FOC, FOM and FON in infested soils. There were however some areas that require development and optimisation relating to low numbers of sequencing reads for some gene targets. A further issue with this approach was that FOC, FOM and FON were unexpectedly detected in soils not infested with those particular pathogens. For instance, FOC and FOM were detected at higher levels in the daffodil field soil than FON, while FOM was detected in both FOC and FON field soils. While it is possible that these pathogens were also present in soil, qPCR using specific primers for FOC, FOM and FON only detected these pathogens in the onion, stocks and daffodil soils respectively (as expected) so further work is required to identify why this was this non-target detection occurred. It is possible that this is a result of sample contamination or sequencing errors, or that there are other unknown *F. oxysporum* f.sp. isolates present in the fields that share the same sequence. As well as specific gene targets for detection of *F. oxysporum* f.sp., results showed that PCR and amplicon sequencing of 16S, ITS and TEF housekeeping genes was very effective in determining the presence and abundance of bacteria, fungi and *Fusarium* spp. respectively in soil. In particular, TEF identified a range of *Fusarium* spp. in the FOC, FOM and FON infested soils and as expected a very high abundance of *F. oxysporum*. 16S and ITS have been routinely used in amplicon sequencing to define the composition of bacterial and fungal communities while TEF has been employed recently to define the composition of *Fusarium* communities associated with Fusarium head blight so we can confirm the utility of these gene targets for horticultural soils.

Finally, results of the amplicon sequencing were generally consistent across beds in FOC, FOM and FON infested areas suggesting that a realistic sampling strategy can be developed in the future to optimise detection of these pathogens. However, FOC and FOM soils were

artificially infested with the aim of spreading these pathogens evenly across these areas so further work needs to determine if distribution of *F. oxysporum* is more heterogeneous in naturally infested soils.

Overall, the use of an amplicon sequencing approach based on specific gene targets to define the presence and abundance of *Fusarium* spp. and *F. oxysporum* shows potential and is a new and novel approach. Alongside more conventional gene targets to define fungal and bacterial communities, this could be a powerful tool with which to dissect *Fusarium* disease complexes and examine dynamics in relation to the whole soil microbial community. Further work now needs to further optimise the technique and explore how it performs across multiple commercial onion and daffodil field and protected stocks cropping sites.

Objective 1.4: Development of disease areas for onions and stocks

Artificial inoculation of a field area for FOC and a polytunnel for FOM in year 1 was successful in creating high disease levels in bulb onions and stocks respectively. These areas provided a valuable resource for both validation of the specific qPCR tests for FOC and FOM as well as the amplicon sequencing. They are also being used in other AHDB projects as a means of testing new disease control products and approaches.

Aim 2: To determine the effect of Fusarium inoculum concentration on disease development

Objective 2.1-2.3: Determine the effect of *F. oxysporum* inoculum level on disease development in onions, stocks and narcissus.

Objective 2.4: Quantify colonisation of *F. oxysporum* on onions, stocks and Narcissus

In year 1, experiments determined the critical levels of FOC and FOM inoculum that are required to cause significant disease development in onions and stocks respectively and these were confirmed in year 2. The specific qPCR tests for FOC and FOM allowed root colonisation of these pathogens to be explored for the first time, and results have shown that this occurs and can be detected within a few days of the plants being introduced into infested soil, two to three weeks before symptoms begin to be observed on plants. These tests may therefore be useful not only in detecting FOC, FOM and FON in soil in advance of the crop as outlined

previously, but also in crops already planted where plants could be sampled to assess the likelihood of symptom development. Again, this approach requires testing across multiple commercial field sites.

Benefits

- The main *Fusarium* pathogens affecting leek have been identified as *F. culmorum* and *F. avenaceum*.
- Specific diagnostic tests have been developed for FOC, FOM and FON for the first time and may provide a way of assessing disease risk as a commercial service in the future.
- Critical levels of FOC, FOM and FON (experiment ongoing) inoculum required for significant disease development have been defined and related to qPCR tests, hence paving the way to relate inoculum levels detected by these assays to disease development in the field.
- An amplicon sequencing approach using new and novel gene targets has been developed that shows promise for defining the presence and identity of *Fusarium* spp. *F. oxysporum* f.spp. for the first time. Combined with more conventional gene targets used to elucidate the components of bacterial and fungal communities, this provides a tool for dissecting Fusarium disease complexes and examining dynamics in relation to the whole soil microbial community. This aligns with other projects funded by AHDB to develop tools to generally measure 'soil health'.
- The project has provided tools, resources and expertise that has been applied to other Fusarium disease problems of concern to growers including asparagus, rocket and most notably *F. oxysporum* f.sp. *lactucae* race 4 on lettuce that has recently emerged in the UK.

Action Points

- FOC and FOM can colonise roots quickly so any treatments being applied may need to be targeted at an early crop development stage.
- Growers should be aware that Fusarium inoculum can potentially build up quickly to critical levels in soil such that high levels of disease may develop in areas with apparently little Fusarium previously.

SCIENCE SECTION

Background

Fusarium

The genus *Fusarium* contains many pathogenic fungi, which can cause disease in plants, humans, and domesticated animals (Leslie et al., 2007). Pathogenic *Fusarium* strains cause some of the most devastating diseases in agriculture and horticulture with both specialist and generalist species that can affect single or multiple hosts respectively. *Fusarium* spp. are primarily soilborne and many plants have at least one *Fusarium*-associated disease resulting in an extensive range of symptoms such as crown and root rots, stalk rots, head and grain blights, and vascular wilt diseases (Summerell et al., 2010). However, many *Fusarium* strains are saprophytes or symptomless endophytes which may have beneficial effects; for instance, *F. oxysporum* Fo47 is a biological control agent which as an aggressive competitor and root coloniser has been shown to prevent invasion from some pathogenic *F. oxysporum* (Alabouvette et al., 2009). Identification of *Fusarium* species is improving and most can now be distinguished using molecular methods based on sequencing part of the translation elongation factor gene (TEF; Geiser et al, 2004). The notable exception to this however is the multiple pathogens within the *F. oxysporum* complex (see below).

Fusarium oxysporum

F. oxysporum is the most important and economically damaging *Fusarium* species for horticulture and can be a major constraint to the production of many food crops including onion, leek, lettuce, tomato, brassicas, asparagus, cucurbits, peppers, coriander, spinach, basil, beans, peas, strawberry and watermelon as well as non-food crops such as carnation, column stocks and narcissus (Michielse et al., 2009). *F. oxysporum* was recently identified as the 5th most important plant pathogenic fungus based on its economic and scientific impact (Dean et al., 2012). The *F. oxysporum* complex comprises a large array of at least 120 pathogenic *formae speciales* (f.spp.) which are adapted to infect the different crop and plant hosts as well as non-pathogenic isolates. The genetically heterogeneous nature and lack of reliable morphological characters in *F. oxysporum* means that distinguishing between pathogenic and non-pathogenic isolates, and also between the different f. spp., is very difficult and can only be done through pathogenicity testing on different hosts which is time-consuming and expensive. Molecular methods have therefore been investigated as a better means of identifying members of the *F. oxysporum* complex but standard approaches including TEF sequencing, DNA fingerprinting and multi-gene sequencing have failed to reliably distinguish the different f. spp.

The factors which determine host specificity and pathogenicity of different *F. oxysporum* f.spp. were, until recently, also poorly understood but current studies have identified multiple genes associated with pathogenicity including 'Secreted in Xylem' (SIX) genes which may also form the basis for diagnostics (Lievens et al., 2009; van Dam et al., 2016). This recent advance now offers the possibility for the first time of not only discriminating between pathogenic and non-pathogenic *F. oxysporum* isolates but also the ability to identify the host range and specificity of different *F. oxysporum* f.spp.

Control of *Fusarium*

Control of *F. oxysporum* and other species is challenging as most produce long-lived chlamydospores that survive in the soil for many years, resulting in the need for long rotations. However, pressure on production and land use means that control approaches in the UK and elsewhere have relied on the use of soil sterilisation or fumigation, fungicides or seed treatments. However, some of these methods have undesirable environmental effects or pose a potential risk to human health and hence approval for their use has been withdrawn or threatened by further legislation. Generally, there are also no sources of plant resistance to many of the pathogenic *Fusarium* spp. with a few notable exceptions for *F. oxysporum* but in these cases, the deployment of major gene resistance has often broken down as new pathogen races emerge. Other management strategies such as biological control have yet to be widely proven although there is a large amount of published literature on this approach including the use of non-pathogenic *Fusarium* species. Two microbial products in the UK (Prestop, T34 Biocontrol) are currently registered for *Fusarium* disease control.

Impact of *Fusarium oxysporum* and other species in key horticultural crops

Following a consultation by AHDB Horticulture to review *Fusarium* problems in different sectors, *F. oxysporum* was identified as a key species with most interest in the f.spp. affecting onion and leek (f.sp. *cepae*, FOC), column stocks (f. sp. *mathiolae*, FOM) and narcissus (f.sp. *narcissi*, FON, Narcissus). These pathogens are therefore the focus of this project.

Fusarium basal rot of onion (FOC) and leek

FOC can affect onion crops at any stage, causing damping-off in seedlings and a root/stem rot in immature plants but the greatest impact is generally at harvest and in store. A PhD project at Warwick showed that although FOC appears to be the predominant *Fusarium* species affecting onion, *F. proliferatum* also caused basal rot in a few cases (Vágány, 2012). Although the significance of basal rot varies between different growing operations and seasons, the impact of the disease has undoubtedly increased significantly over recent years

and represents a major threat to the industry. For instance, in recent seasons onions grown on irrigated sandy soils have seen 2-20% incidence in recent seasons (Tom Will, VCS, personal communication) and field losses also tend to be more significant when the crop is stressed by other factors such as bean seed fly attack, herbicide damage etc. On average 2-6% of the bulb crop (8889 ha valued at approx. £126M in 2017; Defra, 2017) is lost each year in the field with a corresponding economic value of £7.6M but more recently, basal rot incidence of 10% or greater is becoming more common, equating to losses of approx. £12.6M. In addition to in-field losses, further basal rot often occurs in store where apparently healthy bulbs develop the disease. Average losses in store are 3% (Andy Richardson, Allium and Brassica Centre, personal communication), but in some years, storage can result in total failure (>10% basal rot) where the entire consignment is abandoned as it is too costly to extract rotting bulbs. Overall, combined losses in field and store due to basal rot are estimated at up to £22M per annum (Tom Will, personal communication). Although seed treatments are available for control of seedling blight (e.g. fludioxonil ± metalaxyl, thiram) and boscalid + pyraclostrobin can be applied to sets, these fungicides do not provide long-term control of FOC or protect the bulbs from basal rot. Foliar sprays of cyprodinil and fludioxonil approved for *Botrytis* control may have some activity against FOC but are unlikely to have much effect at soil level at approved application rates.

Leeks, which have a value of £24M per year, are also susceptible to seedling blight, root and basal rots caused by *Fusarium* species. Although these can be caused by FOC, a range of other *Fusarium* species including *F. proliferatum*, *F. culmorum* and *F. avenaceum* have also been associated with these disease symptoms (Armengol et al., 2001; Hall et al., 2007; Koike et al., 2003; Palmero et al., 2012). Some of these have also been associated with root diseases of onion in other countries (Hall et al., 2007; Galván et al., 2008; Bayraktar et al., 2011). These other *Fusarium* spp. are generalists and the extent to which they affect UK leeks is unknown. They also have the potential to affect a range of crops. For instance, *F. avenaceum* is part of the foot rot complex affecting pea while *F. proliferatum* is a pathogen of crops as diverse as maize, pineapple and asparagus (Jurado et al., 2010).

Fusarium wilt of column stocks (FOM) and other cut flowers

In a recent survey, *Fusarium* disease of column stocks was identified as one of the major problems for nurseries with losses ranging from 5 to >50% and an average of 15%, which given the industry value of approx. £3.7M, equates to £0.5M per annum (Lyndon Mason, personal communication). Symptoms include failure to establish and wilting symptoms progressing from the base upwards eventually resulting in plant death (Mason, 2013; O'Neill

et al., 2004). Certain varieties such as Centum Deep Blue and Fedora Deep Rose are also more susceptible to *Fusarium* than other varieties (Mason, 2013). FOM was identified as causing disease in UK column stocks, based on morphology (O'Neill et al., 2004) and recent work at Warwick has confirmed this result through DNA sequencing of multiple isolates from nurseries. Many growers continually cultivate stocks which exacerbates *Fusarium* disease problems and control has largely relied on soil steaming or sterilisation with dazomet. Despite these treatments, problems can still occur (Mason, 2013; Graham Whitehead, personal communication) and the high cost of these inputs therefore increases the overall economic burden to growers further. Other cut flowers particularly affected by *F. oxysporum* include Lisianthus and Statice with the former having a value of approx. £1.5M per annum with losses due to *Fusarium* of 5-10% equating to a value of £150,000 (Lyndon Mason, personal communication). A relatively new market is emerging for Statice in similarly intensive production systems and although one grower was alternating production with stocks, *Fusarium* problems emerged in both crops with resultant losses in sales of approx. £24,000 (Graham Whitehead, personal communication, Taylor et al., 2017). The sustainability of some parts of the cut flower industry are therefore at considerable risk, to such an extent that some growers have experimented with, or have already invested in, hydroponic systems. This adds further expense and may not provide a long-term solution as growth conditions need to be optimised and there are already reports of *Fusarium* contamination of the irrigation water causing disease.

Fusarium basal rot of *Narcissus* (FON)

FON, affecting *Narcissus,* is a major problem for the UK daffodil industry causing a basal rot very similar to that in onion (Clarkson, 2012). FON appears to be the predominant *Fusarium* sp. causing basal rot following a study where the identity and pathogenicity of 30 *F. oxysporum* isolates from diseased bulbs from different locations was confirmed (Clarkson, 2012; Clarkson, 2014). The industry is estimated to be worth £45M and 10% losses are not unusual with a corresponding value of £4.5M (Hanks, 2010). Currently, control is dependent on just two active substances, thiabendazole (Storite) and chlorothalonil (Bravo) applied as part of the hot water treatment process used to eradicate stem nematode from bulbs. However, registration for both these actives may potentially be under threat in the future and some FON isolates show resistance to thiabendazole (Clarkson, 2012). However, an alternative product containing cyprodinil and fludioxonil (Switch) has also just been approved, although performance has not been assessed in HWT. Despite the regular application of fungicides by *Narcissus* growers, extensive losses are still common in certain parts of the production area

and the long periods of time the crop is in the ground makes it vulnerable to basal rot irrespective of initial fungicide applications.

Approaches for understanding *Fusarium* dynamics

Overall, *Fusarium* spp. therefore have a significant impact on a very wide range of crops with many of the disease problems occurring as a complex of species. Although most individual *Fusarium* species can be identified by sequencing part of the TEF gene (Geiser et al, 2004) with the exception of specific pathogenic f.spp. in the *F. oxysporum* complex, there has been little attempt to develop specific molecular tests required to examine the dynamics and interaction of specific *F. oxysporum* f.spp. on different crops and rotations. This is important as there is emerging evidence that specialist f.spp. within the *F. oxysporum* complex may colonise and proliferate on roots of non-host plants (e.g. Leoni et al., 2013). In addition, as there are clearly generalist species such as *F. avenaceum* that infect multiple crops, an understanding of the dynamics of the entire *Fusarium* community which includes multiple species and pathogenic / non-pathogenic forms in soil is also needed to optimise rotations, determine disease in relation to cropping patterns and develop management strategies. An approach to identify and quantify entire *Fusarium* communities in roots or soil is therefore also required. The overall aim of the project is therefore to develop qPCR tests to identify and quantify key *F. oxysporum* f.spp. and sequencing approaches to identify multiple *Fusarium* species and other members of the microbial community simultaneously, hence providing essential tools for examining pathogen dynamics and interactions.

Identification of *Fusarium* spp. using molecular methods and the potential of pathogenicity genes to distinguish *F. oxysporum* f.spp.

As stated previously, plant pathogenic *Fusarium* species are notoriously difficult to identify by conventional means such as type of symptom, culture morphology on agar plates or microscopy. *F. oxysporum* has already been identified as the main causal agent of basal rot in onions, *Narcissus* and column stocks. The situation is less certain for leek where more *Fusarium* isolates needed to be obtained to establish the range of species that commonly cause disease. Fungi in general are often now identified by sequencing parts of 'house-keeping' genes such as the internal transcribed spacer region (ITS) of the ribosomal RNA gene which has been proposed as a 'bar code' for distinguishing fungal species (Schoch et al., 2012). However, this does not distinguish between all the different *Fusarium* species, and in this case, the TEF gene has been shown to better resolve members of this genus (Geiser et al, 2004). Based on differences in this gene sequence between fungal species, qPCR has been developed to identify and quantify a range of *Fusarium* species including those causing head blight of cereals (e.g. Nicolaisen et al., 2009). In addition, as there is particular interest

in the ‘chemotypes’ of these species that produce mycotoxins in the grain, qPCR has also been developed based on the key genes involved in this process (Edwards et al., 2001).

Although differences in the sequence of the TEF gene can successfully identify different *Fusarium* species, importantly it does not distinguish between the different f.spp of *F. oxysporum* or between pathogenic and non-pathogenic isolates. As mentioned previously, recent studies in tomato (Lievens et al., 2009; van Dam et al., 2016) and in a BBSRC HAPI project at Warwick on onion (AHDB CP 116) have begun to establish the role of SIX genes in *F. oxysporum* pathogenicity and it now appears that the complement and sequence of these genes varies between the different f.spp. hence opening up opportunities for new diagnostic targets. In contrast, non-pathogenic isolates do not appear to contain any of these pathogenicity genes. In this project, we sequenced the FOC genome, and subsequent analysis and PCR tests have shown that all pathogenic FOC isolates contain seven of the fourteen known SIX genes (SIX 3, 5, 7, 9, 10, 12, 14), as well as other putative pathogenicity genes (Taylor et al., 2015). We also demonstrated that the presence of these genes was directly related to the ability of *F. oxysporum* isolates to infect and produce disease symptoms on onion seedlings and bulbs. The complement and sequence of pathogenicity genes in FOC and other *F. oxysporum* f.spp. therefore provide the potential basis for a specific diagnostic test for the first time. Direct PCR tests have also identified SIX 7, 9, 10, 12, 13 in FON but unlike in FOC, isolates (all shown to be pathogenic) had different complements of these genes ranging from one (SIX 10) to all five, with most isolates having four (SIX 7, 9, 10, 12).

DNA barcoding using next generation amplicon sequencing

It is clear that a wide range of other *Fusarium* species can also cause disease problems in addition to *F. oxysporum* and being generalists, these pathogens can affect a wide range of crops in the rotation. A method of identifying and quantifying entire *Fusarium* communities in roots or soil would therefore be very useful in understanding the relative levels, dynamics and interactions between *F. oxysporum* f.spp., other pathogenic *Fusarium* species as well as non-pathogenic and endophytic isolates. DNA ‘barcoding’ of entire microbial communities through the use of next generation sequencing of PCR amplicons now offers the promise of being able to identify a wide range of species at the same time and is being used extensively to examine microbial populations in natural terrestrial and marine environments (Hill et al., 2000). With this technology, total DNA is extracted from the sample and a gene target common to all species (but with sequence differences between species) is amplified by PCR and subjected to high-throughput sequencing. This results in different DNA sequences being generated for each individual species present which are quantified and identified through comparison with a database. Although primarily used so far in investigating bacterial communities, the

technique has also been applied to fungi (Lindahl et al., 2013) including plant pathogens. For instance, in one study, both pathogens and beneficial fungi were identified in a study of the pea root rot complex (Xu et al., 2012).

Approaches, aims and objectives

In this project we initially collected and identified *Fusarium* isolates from leeks to add to our existing collections for onion, narcissus and stocks. Genome sequences of a pathogenic FOM isolate and also a range of FON isolates were generated and comparative bioinformatics analysis carried out previously generated whole genome sequences for FOC and other *F. oxysporum* f.spp to identify common and unique pathogenicity genes. These were then assessed for their suitability as potential diagnostic markers for FOC, FOM and FON and quantitative PCR (qPCR) developed for each pathogen. Based on the genome information, the feasibility of using a DNA barcoding approach based on amplicon sequencing to analyse *Fusarium* species within entire microbial communities was also examined. The project also determined the effect of inoculum concentration of FOC, FOM and FON on disease development in onion, stocks and narcissus respectively to determine the critical levels required for significant damage to occur which can then be related to qPCR results. Finally, large scale artificial inoculations were carried out to establish a field area for FOC and a polytunnel area for FOM with high disease pressure for testing the qPCR and amplicon sequencing approaches and to provide a resource for further research on control approaches in the future.

The aims and objectives of the project were:

Aim 1: Development of molecular tools and resources for identifying and studying *Fusarium*

Objectives

1.1: Collection, identification and pathogenicity testing of different *Fusarium* spp.

1.2: Development of a specific quantitative (real-time) qPCR tests for *F. oxysporum* f.spp.

1.3: Development of a DNA barcoding approach for analysis of *Fusarium* communities

1.4: Development of disease areas for onions and stocks

Aim 2: To determine the effect of *Fusarium* inoculum concentration on disease development

Objectives

2.1: Determine the effect of *F. oxysporum* inoculum level on disease development in onions

2.2: Determine the effect of *F. oxysporum* inoculum level on disease development in stocks

2.3: Determine the effect of *F. oxysporum* inoculum level on disease development in Narcissus

2.4: Quantify colonisation of *F. oxysporum* on onions, stocks and Narcissus

Aim 1: Development of molecular tools and resources for identifying and studying *Fusarium*

Objective 1.1: Collection, identification and pathogenicity testing of *Fusarium* spp.

- Milestone 1.1a / 1.1b Obtain *Fusarium* isolates from diseased leeks and identify by TEF sequencing (completed in year 1).
- Milestone 1.1c Test pathogenicity of *Fusarium* isolates from leeks
- Milestone 1.1d Obtain key pathogenic *Fusarium* spp. from cereals and potatoes for validation of approaches to identify and quantify specific *F. oxysporum* f.spp. in 1.2 and *Fusarium* communities in 1.3 (completed in year 1).

Summary of year 1 results

- Four *Fusarium* species were identified from diseased leek samples; *F. culmorum*. *F. avenaceum*, *F. proliferatum* and *F. oxysporum*.
- *Fusarium* isolates from potatoes / cereals were obtained from culture collections including *F. coeruleum*, *F. sambucinum* (potato), *F. culmorum*, *F. langsethiae*, *F. poae* and closely related species *Microdochium majus* and *M. nivale* (wheat). TEF gene was sequenced to confirm identity and DNA from these isolates used to validate qPCR diagnostics for FOC, FOM and FON (Objective 1.2) and the DNA barcoding approach (Objective 1.3).

Materials and Methods

Pathogenicity of *Fusarium* spp. on leek

Following preliminary assays in year 1, isolates of *F. oxysporum* (FUS2 from onion and L2-1 from leek), *F. avenaceum* (L5 from leek) and *F. culmorum* (E1 from leek) were selected for pathogenicity testing against two leek cultivars (Longton F1 and Krypton F1) using two different inoculation methods. The first method involved transplanting 6-week-old leek seedlings into Levington's M2 compost infested with a solid inoculum of each *Fusarium* isolate in 7 cm pots (1×10^5 cfu g^{-1}) as described by Taylor et al., (2013), while the second method involved dipping leek roots in a spore suspension. For this latter method, compost was removed from roots of 6-week-old leek seedlings which were then rinsed in sterile water and blotted dry before dipping. *Fusarium* isolates were grown on Potato Dextrose Agar (PDA) for 14 d at 25°C and spore suspensions made by adding sterile water and gently removing conidia

with a disposable spreader. Suspensions were filtered through three layers of sterile Mira cloth (Merck, UK) and adjusted to 1 x 10^6 spores ml^{-1}. The bottom 0.5cm of leek root was removed before dipping plants in 80ml of the spore suspension for each isolate for 5 min and then transplanting into Levington's M2 compost in 7cm pots. Pots (8 replicates per isolate / cultivar combination) were positioned in the glasshouse using a randomised block design and the temperature maintained at 25°C day, 18°C night to encourage disease development which was scored twice weekly. After 15 weeks, all remaining plants were harvested, bisected and scored for disease on a 0-4 scale where 0 = healthy, 1 = less than 10% of the base with symptoms (browning), 2 = more than 10% of the base with browning symptoms and travelling up the leaves, 3 = 100% of the base with browning symptoms and travelling up the leaves, 4 = dead plant. Data were analysed using ANOVA in Genstat.

Results

Pathogenicity of *Fusarium* spp. on leek

F. culmorum was the only *Fusarium* isolate that caused any significant disease symptoms on the leeks in the plant assay (Fig. 1) while isolates of *F. avenaceum* and *F. oxysporum* caused little or no symptoms with a maximum of one of the eight replicate plants affected. The root dip method was more effective than using infested compost for *F. culmorum* with a total of 11 of 16 plants showing disease symptoms, 10 of which progressed to plant death. The leek cv. Krypton was generally more susceptible to infection by *F. culmorum* than cv. Longton (Table 1).

There was a significant difference in *Fusarium* disease levels between the different treatments 15 weeks after inoculation. For infested compost, inoculation with *F. culmorum* and *F. avenaceum* resulted in significant disease levels compared to the uninoculated control (Fig. 2). However, this was only evident for cv. Longton as there was some background infection of control plants for cv. Krypton. The root dip assay produced clearer results with a significant increase in level of disease symptoms caused by *F. culmorum* for both cv. Longton and cv. Krypton and *F. avenaceum* for cv. Longton compared to the uninoculated control (Fig. 2). The most severe disease symptoms were observed in plants inoculated with *F. culmorum*.

Table 1: Infection of two leek cultivars with *F. culmorum* using two inoculation methods.

Leek cv.	Inoculation method	% plants showing disease symptoms	% plant death
Krypton	Solid inoculum	50	13
Krypton	Root dip	88	88
Longton	Solid inoculum	13	13
Longton	Root dip	50	38

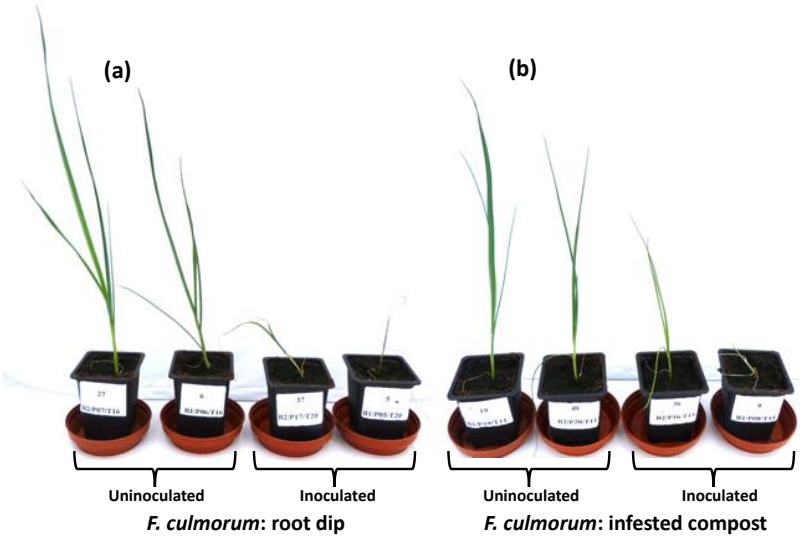

Figure 1: Disease symptoms on leek following infection by *F. culmorum* (first two pots from left 5 weeks after transplanting compared to non-inoculated control (first two pots from right). (a) root dip inoculation, (b) transplanting into infested compost.

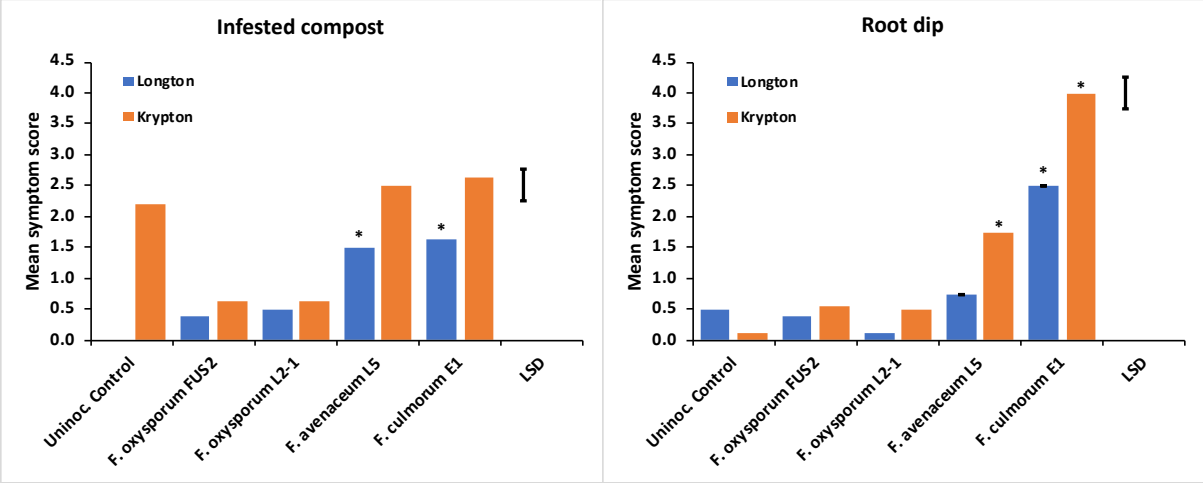

Figure 2: Mean disease symptom scores on leek (cv Longton, cv Krypton) 15 weeks after inoculation by *F. culmorum* using infested compost (left) and root dip (right) methods. Error bar indicates the LSD value (5%) following ANOVA analysis. Asterisks indicate statistically significant difference compared to the uninoculated control.

Objective 1.2: Development of specific quantitative (real-time) PCR tests for *F. oxysporum* f.spp.

- Milestone 1.2a Extract DNA from a pathogenic FOM isolate and sequence genome (completed in year 1)
- Milestone 1.2b FOM genome assembly and comparative bioinformatics analysis of FOC, FON, FOM and other available *Fusarium* genomes including identification of SIX or other pathogenicity genes (completed in year 1)
- Milestone 1.2c Confirm presence of SIX or other genes in FON and FOM isolates by PCR and sequencing (completed in year 1)
- Milestone 1.2d Develop qPCR for FOC, FON and FOM and test specificity using other key *Fusarium* spp. and common soilborne fungi (completed in year 1)
- Milestone 1.2e Determine sensitivity of FOC, FON and FOM qPCR for DNA extracted from soil spiked with different *F. oxysporum* f.spp
- Milestone 1.2f Calibrate qPCR tests to relate amount of DNA detected to pathogen inoculum concentration
- Milestone 1.2g Test qPCR on soil/root samples from FOC, FOM and FON-infested sites

Summary of year 1 results

- Following genome analysis and confirmation by PCR, FOM contained SIX1, SIX8 and SIX9, FON isolates contained between two and five SIX genes in different combinations of SIX7, 9, 10, 12, 13 and FOC was previously shown to contain SIX3, 5, 7, 9, 10, 12 and 14.
- Specific qPCR tests were developed for FOC, FOM and FON based on pathogenicity genes identified through comparative genome analysis. None of these tests resulted in amplification of any other *Fusarium* spp. or *F. oxysporum* f.spp, nor any other common soilborne fungi.

Materials and methods

Testing sensitivity of FOC qPCR using DNA

In order to test the sensitivity of the FOC qPCR assay, a series dilution of FOC (isolate FUS2) DNA was prepared ranging from 10 ng μl^{-1} – 0.5 pg μl^{-1}. qPCR (Roche Lightcycler) was carried out using these dilutions in 10 µl reactions containing primers (0.5 µM), 5 µl SensiFAST™ SYBR® No-ROX Kit mastermix and 1 µl of DNA. Conditions were as follows: 1 cycle of 95°C

for 3 mins followed by 45 cycles of 95°C for 5 s, 60°C for 10 s and 72°C for 10 s. All samples were run in triplicate and a melt curve analysis carried out.

Testing sensitivity of FOC qPCR using spiked soil samples

The sensitivity of the FOC qPCR assay was assessed using soil spiked with different concentrations of FOC conidia. FOC isolate FUS2 was grown on PDA for 14 days and spores removed by adding sterile water and gently scraping with a sterile spreader. The conidial suspension was filtered through three layers of sterile Mira cloth and quantified using a haemocytometer. A soil sample was taken from a field at Wellesbourne (Wharf ground, sandy loam inceptisol, 'Wick' series) with no history of onion, stocks or Narcissus production and sieved (4 mm mesh), air-dried for 3 days, sieved again (2 mm mesh), mixed well and stored at 5°C. Soil spiking was carried out by adding 200 µl of the FOC conidial suspension to 0.5 g of soil in a SoilSV extraction tube. A dilution series was prepared such that soil was spiked at conidial concentrations ranging from 160-1 x 10^7 spores g^{-1}. A negative control (sterile distilled water) was also included. Samples were incubated at room temperature for 24h before DNA was extracted using the SoilSV kit following the manufacturer's instructions. The whole experiment was repeated four times. qPCR (Roche Lightcycler) was then carried out on all samples (1 in 6 DNA dilution) as described in the previous section.

Testing FOC qPCR on soil and plant samples

To test the FOC qPCR assay on soil and plant samples, soil samples were taken on 06/06/17 from a quarantine field at Wellesbourne that was previously inoculated with FOC in 2015 (FOC QF). A total of 15 soil samples were collected from three beds (5 samples per bed, approx. 8 m apart) and DNA extracted using an optimised method based on the Soil SV kit (GeneAll, AHDB CP113 annual report 2018). A total of 23 plant samples (cv. Hytech) were also collected from the FOC QF, comprising onion root, basal plate and bulbs with and without disease symptoms. As a negative control, samples were also taken from three asymptomatic onion bulbs from a commercial onion store (cv. Vision, Andy Richardson, Allium and Brassica Centre). Samples were washed in sterile water, freeze dried and DNA extracted from 20 mg of tissue using a DNeasy plant mini kit (Qiagen) following the manufacturers protocol but with the addition of an extra centrifugation after the cell lysis step (13,000 rpm for 5 min). DNA quality was checked using a DeNovix DS-11 Spectrophotometer after which all DNA samples were diluted in TE (1 in 2 for plant samples, 1 in 6 for soil samples) and qPCR carried out as described above but using a PowerUp SYBR Green Master Mix and a StepOnePlus instrument (Applied Biosystems). Conditions were as follows: 1 cycle of 95°C for 2 mins 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.

Testing sensitivity of FOM qPCR using DNA

In order to test the sensitivity of the FOM qPCR assay, a series dilution of FOM (isolate Stocks 4) DNA was prepared ranging from 30ng μl^{-1} – 0.3 pg μl^{-1}. qPCR (Roche Lightcycler) was carried out using these dilutions in 10µl reactions containing primers (0.5 µM), 5 µl SensiFAST™ SYBR® No-ROX Kit mastermix and 1 µl of DNA. Conditions were as follows: 1 cycle of 95°C for 3 mins followed by 45 cycles of 95°C for 5 s, 63°C for 10 s and 72°C for 10 s. All samples were run in triplicate and a melt curve analysis carried out.

Testing sensitivity of FOM qPCR using spiked soil samples

The sensitivity of the FOM qPCR assay was assessed using soil spiked with different concentrations of FOM conidia (isolate Stocks 4; 160-1 x 10^7 spores g^{-1}) followed by DNA extraction and qPCR as described above for FOC.

Testing FOM qPCR on soil and plant samples

To test the FOM qPCR assay on plant and soil samples, a total of 18 soil samples were taken (23/07/18) from a polytunnel at the Cut Flower Centre (CFC) inoculated in 2017 (see annual report 2018) which was planted with different varieties of stocks in trials carried out by Lyndon Mason in both 2017 and 2018. Six soil samples per bed were collected at approx. 3m apart and DNA extracted as described above for FOC. Soil was also collected from plots in 2017 where non-host Lisianthus plants were grown. In addition, a total of 24 plant samples were tested, comprising root and stem sections of stocks plants showing symptoms of Fusarium infection that were part of the CFC trial in 2017 as well non-symptomatic non-host ornamental cabbage plants. Selected diseased stocks plants from the FOM-inoculated dose-response experiments were also tested (Objective 2.2). As before, plant samples were washed in sterile water and freeze dried before DNA was extracted as described above for FOC. All DNA samples were diluted in TE (1 in 2 for plant samples, 1 in 6 for soil samples) and qPCR carried out as described above for FOC using the StepOnePlus instrument. Conditions were as follows: 1 cycle of 95°C for 2 mins followed by 45 cycles of 95°C for 3 s and 63°C for 30 s. All samples were run in triplicate and a melt curve analysis carried out.

Testing sensitivity of FON qPCR using DNA

In order to test the sensitivity of the FON qPCR assay, a series dilution series of FON (isolate FON63) DNA was prepared ranging from 10 ng μl^{-1} - 1 pg μl^{-1}. qPCR (Roche Lightcycler) was carried out using these dilutions in 10 µl reactions containing primers (0.5 µM), 5 µl SensiFAST™ SYBR® No-ROX Kit mastermix and 1 µl of DNA. Conditions were as follows: 1 cycle of 95°C for 3 mins followed by 45 cycles of 95°C for 5 s, 60°C for 10 s and 72°C for 10 s. All samples were run in triplicate and a melt curve analysis carried out.

Testing sensitivity of FON qPCR using spiked soil samples

The sensitivity of the FON qPCR assay was assessed using soil spiked with different concentrations of FON conidia (isolate Stocks 4; 25 - 1 x 10^7 spores g^{-1}) followed by DNA extraction and qPCR as described above for FOC.

Testing FON qPCR on soil and plant samples

To test the FON qPCR assay on plant and soil samples, seven soil samples 25 m apart were collected from a field (TR27 5DG) which previously had a high level of Narcissus basal rot. Soil was prepared and DNA extracted as described above for FOC. A total of 21 plant samples were also tested, comprising basal plate and scale tissue from diseased bulbs (cv. Carlton, Lingarden Bulbs). As before, plant samples were washed in sterile water and freeze dried before DNA was extracted as described above for FOC. All DNA samples were diluted in TE (1 in 5 for plant samples, 1 in 6 for soil samples) and qPCR carried out as described above for FOC using the StepOnePlus instrument (Applied Biosystems). Conditions were as follows: 1 cycle of 95°C for 2 mins 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.

Results

Testing sensitivity of FOC, FOM and FON qPCR assays using DNA and spiked soil samples

Testing the FOC, FOM and FON qPCR assays on dilution series of genomic DNA, resulted in efficient amplification for all assays with minimum detection levels of 0.5 pg, 1 pg and 1 pg DNA respectively (Fig. 3). Assay efficiencies and slopes were 100% / -3.309, 97.2% / -3.463 and 97.1% / 3.472 for FOC, FOM and FON respectively. R^2 values of 0.99 for all assays indicated that they were all accurate.

When qPCR was performed on DNA from the soil samples spiked with different concentrations of conidia, both the FOC and FOM assays could effectively detect FOC / FOM spores with a limit of 4000 conidia g^{-1}. In both cases, a good correlation (FOC r^2=0.995, FOM r^2=0.975) between spore concentration and Cp value was observed, demonstrating that these assays were accurate (Fig. 3). A lower detection limit was observed for the FON qPCR assay with accurate detection down to 3200 conidia g^{-1} and detection was successful in 2 out of 4 replicates at 640 conidia g^{-1}. Again, a good correlation (r^2=0.99) was observed between spore concentration and Cp value was observed. In all the qPCR assays, the error between replicates was very low indicating that the assay and extraction methods were highly reproducible.

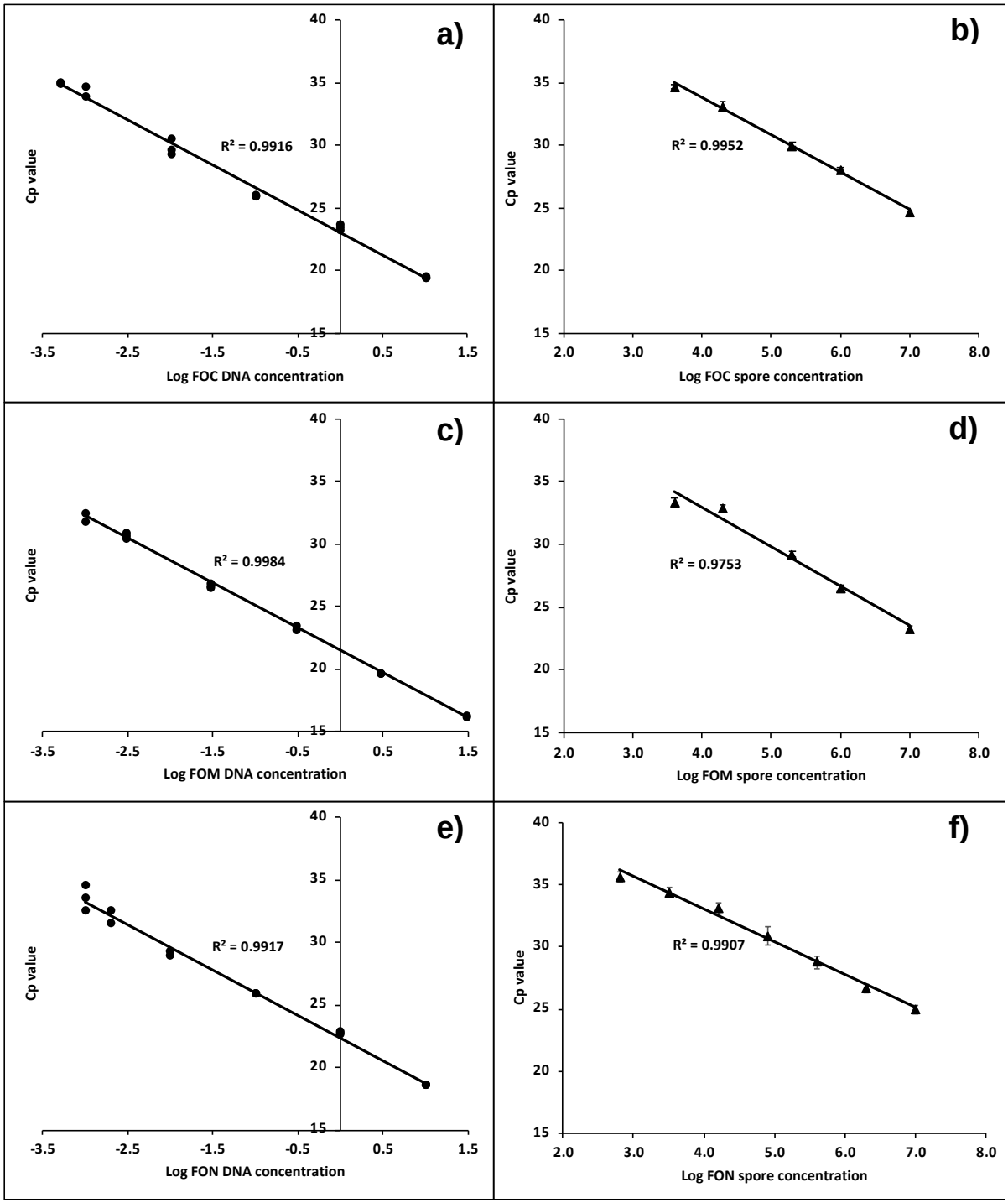

Figure 3: Sensitivity of FOC, FOM and FON qPCR assays using dilution series of pathogen DNA (a, c, e) and soil spiked with different concentrations of conidia (b, d, f). Cp = crossing point (equivalent to Ct, cycle threshold). Data are from three and four replicate samples for each dilution of DNA and conidia respectively. Error bars indicate standard error of the mean.

Testing FOC qPCR on soil and plant samples

FOC was successfully detected in all the soil samples tested from the FOC QF with Ct values ranging from 32.3 to 35.2 (Table 2; higher Ct values correspond with lower pathogen DNA levels). This is consistent with FOC levels being at their lowest level of the year (06/06/18, early in crop development) as also reported in AHDB project CP 113 (Year 5 report). However, FOC was not detected in any of the soil samples from the FOM inoculated polytunnel at CFC nor in those from the *Narcissus* field with previous high levels of basal rot. FOC was also detected in all plant samples tested from the Fusarium QF, including those with no obvious symptoms of infection. Samples with clear FOC symptoms had lower qPCR Ct values and hence higher levels of FOC DNA. No FOC was detected on asymptomatic bulbs (cv. Vision) from a commercial store.

Testing FOM qPCR on soil and plant samples

FOM was successfully detected in all soil samples tested from the CFC inoculated polytunnel with Ct values ranging from 28.1 to 34.3 (Table 3; higher Ct values correspond with lower pathogen DNA levels). This was expected as samples were taken at a time when stocks plants were in the ground and showing symptoms of infection. Comparable levels of FOM were detected in plots where non-host Lisianthus plants were growing. FOM was not detected in any of the soil samples from the Fusarium QF nor from the *Narcissus* field with previous high levels of basal rot. FOM was detected in all stem and root sections from the stocks plant samples with higher levels in the stems. No FOM was detected in the roots or stems of the non-host ornamental Brassica species.

Testing FON qPCR on soil and plant samples

FON was successfully detected in all soil samples tested from the *Narcissus* field with previous high levels of basal rot with Ct values ranging from 25.2 to 28.1 (Table 4; higher Ct values correspond with lower pathogen DNA levels). FON was not detected in any of the soil samples from the FOC QF but, a low level of FON was detected in 4 out of 18 samples from the CFC FOM inoculated polytunnel, potentially indicating some past history of *Narcissus* production in this location. FON was detected in all symptomatic *Narcissus* bulb samples tested and also some with no visible symptoms of infection. Higher levels of FON were detected in scales with severe symptoms compared to those with moderate symptoms.

Table 2: Detection of FOC in soil and plant samples using the FOC qPCR assay. Green = negative, yellow = Ct > 30, orange = Ct 25-30, red = Ct < 25. Higher Ct values correspond with lower pathogen DNA levels.

Sample No.	Date	Origin	Details	Mean Ct	SEM
1	06/06/17	FOC QF soil	Bed 1	34.1	0.18
2	06/06/17	FOC QF soil	Bed 1	33.6	0.27
3	06/06/17	FOC QF soil	Bed 1	33.4	0.22
4	06/06/17	FOC QF soil	Bed 1	34.8	0.62
5	06/06/17	FOC QF soil	Bed 1	33.6	0.12
6	06/06/17	FOC QF soil	Bed 2	33.2	0.14
7	06/06/17	FOC QF soil	Bed 2	35.2	0.64
8	06/06/17	FOC QF soil	Bed 2	34.6	0.47
9	06/06/17	FOC QF soil	Bed 2	32.9	0.27
10	06/06/17	FOC QF soil	Bed 2	33.2	0.21
11	06/06/17	FOC QF soil	Bed 3	32.6	0.43
12	06/06/17	FOC QF soil	Bed 3	34.0	0.67
13	06/06/17	FOC QF soil	Bed 3	33.8	0.26
14	06/06/17	FOC QF soil	Bed 3	33.2	0.46
15	06/06/17	FOC QF soil	Bed 3	32.3	0.01
FON1-9	20/09/18	FON field soil		Negative	n/a
FOM1-18	21/07/18	FOM PT soil		Negative	n/a
1	06/07/18	QF Onion root	No visible symptoms	26.2	0.40
2	06/07/18	QF Onion root	No visible symptoms	28.6	0.42
3	06/07/18	QF Onion basal plate	No visible symptoms	34.5	0.47
4	06/07/18	QF Onion basal plate	No visible symptoms	34.2	0.34
5	06/07/18	QF Onion bulb	No visible symptoms	32.3	0.32
6	06/07/18	QF Onion bulb	No visible symptoms	33.1	0.57
7	06/07/18	QF Onion root	Visible symptoms	23.2	0.03
8	06/07/18	QF Onion root	Visible symptoms	22.2	0.04
9	06/07/18	QF Onion root	Visible symptoms	22.6	0.33
10	06/07/18	QF Onion basal plate	Visible symptoms	20.4	0.05
11	06/07/18	QF Onion basal plate	Visible symptoms	17.2	0.21
12	06/07/18	QF Onion basal plate	Visible symptoms	20.1	0.07
13	06/07/18	QF Onion bulb	Visible symptoms	20.7	0.22
14	06/07/18	QF Onion bulb	Visible symptoms	19.9	0.09
15	06/07/18	QF Onion bulb	Visible symptoms	19.6	0.43
16	06/07/18	QF Onion basal plate	Highly infected	17.8	0.11
17	06/07/18	QF Onion basal plate	Highly infected	19.1	0.29
18	06/07/18	QF Onion basal plate	Highly infected	21.4	0.16
19	06/07/18	QF Onion basal plate	Highly infected	19.2	0.22
20	06/07/18	QF Onion bulb	Highly infected	16.8	0.12
21	06/07/18	QF Onion bulb	Highly infected	25.0	0.13
22	06/07/18	QF Onion bulb	Highly infected	18.8	0.19
23	06/07/18	QF Bulb	Highly infected	22.4	0.30
24	09/01/19	Onion basal plate, commercial store	No symptoms,	Negative	n/a
25	09/01/19	Onion basal plate, commercial store	No symptoms	Negative	n/a
26	09/01/19	Onion basal plate, commercial store	No symptoms	Negative	n/a
27	09/01/19	Onion bulb, commercial store	No symptoms	Negative	n/a
28	09/01/19	Onion bulb, commercial store	No symptoms	Negative	n/a
29	09/01/19	Onion bulb, commercial store	No symptoms	Negative	n/a

Table 3: Detection of FOM in soil and plant samples using the FOM qPCR assay. Stocks plant 5 was taken from glasshouse dose-response experiments whereas the other plants were taken from an infected polytunnel. Green = negative, yellow = Ct > 30, orange = Ct 25-30, red = Ct < 25. Higher Ct values correspond with lower pathogen DNA levels.

Sample No.	Date	Origin	Details	Mean Ct	SEM
1	21/07/18	FOM PT soil	Bed 1	28.9	0.16
2	21/07/18	FOM PT soil	Bed 1	29.6	0.18
3	21/07/18	FOM PT soil	Bed 1	31.5	0.35
4	21/07/18	FOM PT soil	Bed 1	30.2	0.04
5	21/07/18	FOM PT soil	Bed 1	30.0	0.11
6	21/07/18	FOM PT soil	Bed 1	34.3	0.29
7	21/07/18	FOM PT soil	Bed 2	30.5	0.33
8	21/07/18	FOM PT soil	Bed 2	31.1	0.06
9	21/07/18	FOM PT soil	Bed 2	28.6	0.14
10	21/07/18	FOM PT soil	Bed 2	33.2	0.31
11	21/07/18	FOM PT soil	Bed 2	30.1	0.34
12	21/07/18	FOM PT soil	Bed 2	33.3	0.05
13	21/07/18	FOM PT soil	Bed 3	28.1	0.05
14	21/07/18	FOM PT soil	Bed 3	29.6	0.07
15	21/07/18	FOM PT soil	Bed 3	30.2	0.41
16	21/07/18	FOM PT soil	Bed 3	29.0	0.33
17	21/07/18	FOM PT soil	Bed 3	28.8	0.14
18	21/07/18	FOM PT soil	Bed 3	31.8	0.27
L1	21/07/18	FOM PT soil	Lisianthus plot	32.4	0.28
L2	21/07/18	FOM PT soil	Lisianthus plot	29.3	0.19
L3	21/07/18	FOM PT soil	Lisianthus plot	30.2	0.29
L4	21/07/18	FOM PT soil	Lisianthus plot	30.6	0.23
FON1 - 9	20/09/18	FON field soil		Negative	n/a
FOC1 - 15	06/06/17	FOC QF soil		Negative	n/a
1	11/10/17	Stocks plant 1	stem section 1	29.4	0.34
2	11/10/17	Stocks plant 1	stem section 2	25.7	0.06
3	11/10/17	Stocks plant 1	stem section 3	26.9	0.15
4	11/10/17	Stocks plant 1	root	31.6	0.19
5	11/10/17	Stocks plant 2	stem section 1	29.1	0.06
6	11/10/17	Stocks plant 2	stem section 2	27.7	0.10
7	11/10/17	Stocks plant 2	stem section 3	25.9	0.28
8	11/10/17	Stocks plant 2	root	32.5	0.30
9	11/10/17	Stocks plant 3	stem section 1	24.7	0.09
10	11/10/17	Stocks plant 3	stem section 2	25.9	0.11
11	11/10/17	Stocks plant 3	stem section 3	29.1	0.32
12	11/10/17	Stocks plant 3	root	35.6	0.41
13	11/10/17	Stocks plant 4	stem section 1	21.0	0.07
14	11/10/17	Stocks plant 4	stem section 2	21.7	0.13
15	11/10/17	Stocks plant 4	stem section 3	23.4	0.34
16	11/10/17	Stocks plant 4	root	23.4	0.13
17	26/06/18	Stocks plant 5	stem section 1	23.4	0.19
18	26/06/18	Stocks plant 5	stem section 2	24.2	0.12
19	26/06/18	Stocks plant 5	stem section 3	25.0	0.36
20	26/06/18	Stocks plant 5	root	26.4	0.21
21	11/10/17	Ornamental Brassica plant	stem section 1	negative	n/a
22	11/10/17	Ornamental Brassica plant	stem section 2	negative	n/a
23	11/10/17	Ornamental Brassica plant	stem section 3	negative	n/a
24	11/10/17	Ornamental Brassica plant	root	negative	n/a

Table 4: Detection of FON in soil and plant samples using FON specific primers. *only amplified in 1 out of 3 replicates. Higher Ct values correspond with lower pathogen DNA levels.

Sample No.	Date Taken	Origin	Details	Mean Ct	SEM
1	20/09/18	FON field soil		26.7	0.38
2	20/09/18	FON field soil		25.2	0.83
3	20/09/18	FON field soil		26.0	0.48
4	20/09/18	FON field soil		28.1	0.65
5	20/09/18	FON field soil		26.0	0.53
6	20/09/18	FON field soil		26.6	0.43
7	20/09/18	FON field soil		27.6	0.36
4	21/07/18	FOM PT soil	Bed 1	32.7	0.093
5	21/07/18	FOM PT soil	Bed 1	32.7	*
11	21/07/18	FOM PT soil	Bed 2	33.5	0.20
16	21/07/18	FOM PT soil	Bed 3	33.2	0.009
FOM 1-3, 6-10, 12-15, 17-18	21/07/18	FOM PT soil		Negative	n/a
FOC 1- 5	06/06/17	FOC QF soil		Negative	n/a
1	12/12/18	Bulb 1	basal plate, high infection	24.4	0.10
2	12/12/18	Bulb 1	scale, high infection	20.1	0.14
3	12/12/18	Bulb 1	scale, moderate infection	24.8	0.63
4	12/12/18	Bulb 2	basal plate, high infection	25.2	0.19
5	12/12/18	Bulb 2	scale, high infection	22.2	0.35
6	12/12/18	Bulb 2	scale, moderate infection	26.7	0.23
7	12/12/18	Bulb 3	basal plate, high infection	20.1	0.23
8	12/12/18	Bulb 3	scale, high infection	20.5	0.71
9	12/12/18	Bulb 3	scale, moderate infection	22.4	0.32
10	12/12/18	Bulb 4	basal plate, high infection	26.7	1.68
11	12/12/18	Bulb 4	scale, high infection	27.1	1.84
12	12/12/18	Bulb 4	scale, moderate infection	27.5	1.99
13	12/12/18	Bulb 5	Basal plate, no visible symptoms	33.2	2.78
14	12/12/18	Bulb 6	Basal plate, no visible symptoms	25.9	1.37
15	12/12/18	Bulb 7	Basal plate, no visible symptoms	26.3	1.53
16	09/01/19	Bulb 8	Basal plate, no visible symptoms	Negative	n/a
17	09/01/19	Bulb 8	Scale, no visible symptoms	Negative	n/a
18	09/01/19	Bulb 9	Basal plate, no visible symptoms	Negative	n/a
19	09/01/19	Bulb 9	Scale, no visible symptoms	Negative	n/a
20	09/01/19	Bulb 10	Basal plate, no visible symptoms	Negative	n/a
21	09/01/19	Bulb 10	Scale, no visible symptoms	Negative	n/a

Objective 1.3: Development of a whole amplicon sequencing for analysis of *Fusarium* communities

- Milestone 1.3a Identify appropriate gene targets such as housekeeping, pathogenicity, mycotoxin and other functional genes for *Fusarium* genus, species and f.spp. to enable whole amplicon sequencing at different phylogenetic resolutions using bioinformatics analyses using all known *Fusarium* genome sequences (completed in year 1).
- Milestone 1.3b Carry out whole amplicon sequencing for mixed *Fusarium* DNA samples and soil spiked with different *Fusarium* communities and other soilborne fungi (including samples from 1.2e to compare with qPCR). Perform analysis of identity and relative abundance.
- Milestone 1.3c Carry out whole amplicon sequencing for soils infested with FOC, FON and FOM as collected in 1.2g.
- Milestone 1.3d Develop a database system for storage of amplicon sequence data and associated metadata (to be completed).
- Milestone 1.3e Develop statistical methodologies to assess microbial community composition.

Summary of year 1 results

- Genome sequencing of FOC, FOM and FON and subsequent analysis identified housekeeping and pathogenicity-related genes suitable for identification of Fusarium species and *F. oxysporum* f.spp. using amplicon sequencing.
- Seven genes SIX13 (T1), FOC_g17143 (T2), OG10859 (T3), OG13890 (T4), OG4927 (T5), OG4952 (T6), OG12981 (T7) were selected as targets for amplicon sequencing as they were present in FOC, FOM or FON and also showed sequence variation in other *F. oxysporum* f.spp. where they occurred.
- The ability of the primer pairs designed for each gene target to amplify DNA from selected Fusarium and other fungal species was confirmed by PCR.

Materials and Methods

Testing and selection of gene targets using DNA from *Fusarium* and other fungi

Seven gene targets selected as being present in different combinations in *F. oxysporum* f. spp. including FOC, FOM and FON were identified in year 1 as being potentially suitable for amplicon sequencing and comprised SIX13 (T1), FOC_g17143 (T2), OG10859 (T3), OG13890 (T4), OG4927 (T5), OG4952 (T6), OG12981 (T7). An additional gene that showed sequence variation between FOM and other *F. oxysporum* f. spp., OG13397 (T8) was also subsequently identified. The use of primers for all these genes in combination were predicted to allow the identification of different *Fusarium* f.spp. (Table 5). In addition, further primers for amplicon sequencing were also selected for 16S, ITS and TEF genomic regions to profile the bacterial community, fungal community and the *Fusarium* species respectively in a soil sample based upon sequence variation. Primers for all gene targets were designed with the aim of i) obtaining PCR products <500 bp (preferably around 350 bp), ensuring that there was good overlap in the reads to give improved accuracy and ii) minimising variation in amplicon product size to prevent preferential clustering of smaller amplicons. This proved challenging for some loci due to the repetitive nature of the sequences and only limited regions of homology. As described in the year 1 annual report, to confirm that primers for all the gene targets listed resulted in the expected pattern of amplification and did not result in additional non-specific products, further testing was carried out by PCR of DNA from selected *Fusarium* spp. and *F. oxysporum* f.spp. as well as DNA from selected FOC, FOM and FON infested soil. All PCR reactions were performed in a total volume of 12.5 µl using 1 ng of template DNA, primers at a final concentration of 0.4 µM using KAPA HiFi HotStart 2x Master Mix (Roche Diagnostics). PCR reactions were carried out using thermocycling conditions of 95ºC for 3 min, then 35 cycles of 95ºC 30s, 62ºC 30s, 72ºC 30s, and final extension at 72ºC for 5 min. In each case, amplification of the expected product size was confirmed by gel electrophoresis.

Table 5: Predicted identification of different *F. oxysporum* f.spp. by amplicon sequencing using different target genes. For each gene target, green boxes indicate a unique sequence for a particular *F. oxysporum* f.sp.; blue and yellow boxes indicate where one or more *F. oxysporum* f.sp. has the same sequence (same colour = same sequence). NP indicates that sequence was identical to a non-pathogenic *F. oxysporum* isolate. Last column indicates other *F. oxysporum* f.spp. amplified with unique sequences.

Target gene	**FOC**	**FOM**	**FON**	**FOP1**	**FOP2**	**FOP5**	**FOL**	**f.sp. *niveum***	**f.sp. *conglutinans***	**f.sp. *cucumerinum***	**Other *Fusarium* spp. or f.spp**
SIX13 (T1)											f.sp. *cubense, fragariae*
OG17143 (T2)											f.sp. *vasinfectum*
OG10859 (T3)		raphani									*F. avenaceum*
OG13890 (T4)		raphani						melonis			
OG4927 (T5)	FOP	raphani tulipae	NP			FOC					f.sp. *vasinfectum* f.sp. *radicis-lycopersici*
OG4952 (T6)											f.sp. *tulipae*
OG12981 (T7)											f.sp. *melonis*
OG13397 (T8)											f.sp. *raphani*

Amplicon sequencing of artificial community DNA pools from *Fusarium* and other fungi

Two artificial community DNA 'pools' were used to assess the utility / specificity of the gene targets above for amplicon-sequencing by profiling *Fusarium* communities alongside other common fungal pathogens in soil. The pools comprised Pool 1, six *F. oxysporum* f.sp. (including FOC, FOM and FON) and 14 other *Fusarium* spp. and related species; Pool 2, five *Fusarium* spp. and 15 other soil borne fungal pathogens (Table 6). In each test, isolate FON63 was included as a positive control as it should be amplified by all the primer sets for each gene target (Table 5).

Table 6: Pools of DNA from different *Fusarium* spp., *F. oxysporum* f.sp. and other soilborne fungal pathogens used to test primers for amplicon sequencing

Pool 1	**Pool 2**
F. oxysporum f.sp. cepae	*F. oxysporum* f.sp. cepae
F. oxysporum f.sp. matthioli	*F. graminearum*
F. oxysporum f.sp. narcissi	*F. avenaceum*
F. oxysporum f.sp. lycopersici	*F. solani*
F. oxysporum f.sp. pisi	*F. redolens*
F. oxysporum (non -path)	*M. nivale*
F. avenaceum	*Alternaria infectoria*
F. coeruleum	*Botrytis cinerea*
F. culmorum	*Cylindrocarpon destructans*
F. equiseti	*Itersonilia perplexans*
F. flocciferum	*Mycocentrospora acerina*
F. graminearum	*Phoma spp*
F. poae	*Phytophthora cactorum*
F. proliferatum	*Pythium ultimum*
F. redolens	*Rhizoctonia solani*
F. sambucinum	*Sclerotinia sclerotiorum*
F. solani	*Sclerotium cepivorum*
F. tricinctum	*Setophoma terrestris*
M. majus	*Trichoderma*
M. nivale	*Verticillium albo atrum*

To prepare samples for amplicon sequencing, DNA from Pool 1 or 2 (3 ng) from each of the fungal isolates was combined and 3.33 µl from this 3 ng pool (total 10 ng DNA) used as the template for the PCR reactions for each of the target genes. This gave 0.15 ng of each species template in each pool. All PCR reactions were performed in a total volume of 25 µl using 1 ng of template DNA, primers at a final concentration of 0.4 µM using KAPA HiFi HotStart 2x Master Mix. PCR reactions were carried out using thermocycling conditions of 95ºC for 3 min, then 35 cycles of 95ºC 30s, 62ºC 30s, 72ºC 30s, and final extension at 72ºC for 5 min.

DNA concentration in soil samples is expected to differ between fungal species reflecting their relative abundance but this can potentially be affected by biases for certain species during the DNA extraction and PCR steps used during amplicon sequencing. Therefore, to test this, the effect of reducing the starting DNA concentration of selected species in DNA Pools 1 and 2 before amplicon sequencing was examined, by carrying out PCR of DNA from additional pools where DNA from certain species was diluted at 0.1x (Mix B) and 0.01x (Mix C). These were FOM, FON and FOP for Pool 1, and *Alternaria infectoria*, *Sclerotinia sclerotiorum*, *Verticillium albo-atrum* and *F. avenaceum* for Pool 2.

An Illumina dual index, 2-step PCR approach was used to carry out whole amplicon sequencing of the DNA pools of mixed fungal species. Dual-indexing reduces mis-tagging events and enables a larger number of DNA libraries to be pooled for sequencing.

DNA libraries for sequencing were prepared following the '16S metagenomic workflow' protocol from Illumina. First round gene-specific PCR reactions as described above were carried out in triplicate with all PCR reactions carried out in individually capped PCR tubes rather than plates to minimise cross-sample contamination. Different multiplexing approaches were tested for the PCR reactions to examine the potential of reducing costs in the future (Fig. 4). The first approach involved carrying out a multiplex PCR reaction (MP1) in triplicate by mixing ITS and all the *F. oxysporum* f.sp. specific primers. This was designed to determine if multiplexing at the locus-specific PCR step would lead to more non-specific products. Secondly, combining the products from the first (locus-specific) PCR reactions to carry out the barcoding will also reduce costs although combining these mixed loci amplicons could lead to an increase in chimeric sequences which will reduce correct amplicon reads. Two different pooled mixes were therefore tested for the barcoding step. After bead clean-up using AmpureXP beads from Beckman-Coulter, each locus-specific PCR triplicate were combined for the barcoding step. In addition, a mix was also created by combining the PCR products from one of each of the *F. oxysporum* f.spp. specific first round PCRs together for the barcoding step. Twice as much TEF PCR product was added to this multi-locus mix (MP2) to account for the larger number of amplicons expected from the TEF locus compared to the *F. oxysporum* f.sp. loci to ensure enough reads would be generated.

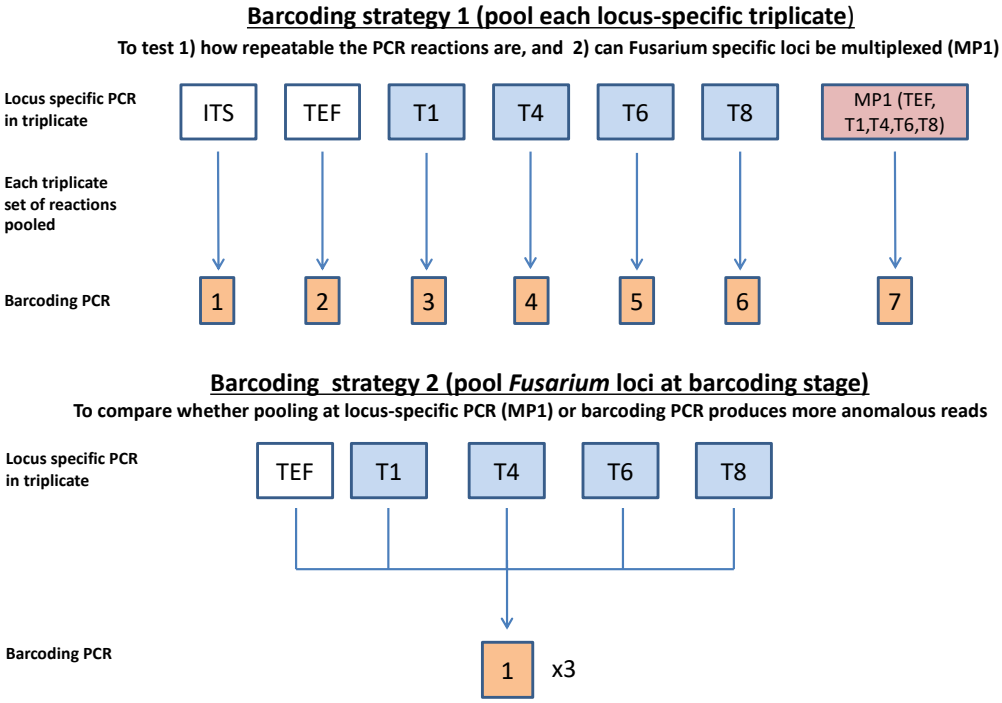

Figure 4: Multiplexing and barcoding strategy for amplicon sequencing of DNA pools of different fungal species

Amplicon sequencing of soils infested with FOC, FON and FOM

DNA was extracted from the same set of soil samples as used to test the qPCR assays comprising i) 15 soil samples across three beds from the FOC QF, ii) 18 soil samples across three beds from the CFC FOM polytunnel and iii) 7 soil samples from the field with high levels of FON. DNA libraries were prepared for sequencing from all the soil samples following the same protocol as described above but with some alterations in pooling (Fig 5). Here, separate first round PCR reactions were carried out for 16S, ITS and TEF due to the larger number of potential target amplicons expected compared to the *F. oxysporum f.sp.* specific targets. The *Fusarium oxysporum* specific target PCR reactions were carried out as two pools (MP1 and MP2). To keep reaction numbers down so that all samples could be run together on one sequencer flow cell, for each sample the two multi-copy gene targets (ITS and 16S) were combined together in one pool for barcoding (Mix A, Fig. 5) while the single copy *F. oxysporum f.sp.* specific and TEF gene targets were combined together in two pools for barcoding (MP1+TEF, Mix B; MP2+TEF, Mix C; Fig. 5). Equal volumes of the PCR reactions (Mix, A, B, C) were pooled prior to bead clean-up to mitigate variation in the pools due to differing bead clean-up efficiencies. DNA extraction efficiencies can also vary between repeat extractions

and between soil types. To account for this, the soil DNA samples were diluted to 2 ng ul^{-1} and 5 µl (10 ng) used in a 25 µl PCR reaction using a KAPA HiFi HotStart 2X master mix. After bead clean-up and quantification, the DNA libraries were normalised to 4 nM and pooled for sequencing on the MiSeq using a V3 600 cycle kit. To allow for the greater number of OTUs expected for 16S and ITS amplicons, these were pooled at twice the volume of TEF/ *F. oxysporum* f.sp. loci libraries. The final pool was loaded at 6 pM spiked with PhiX.

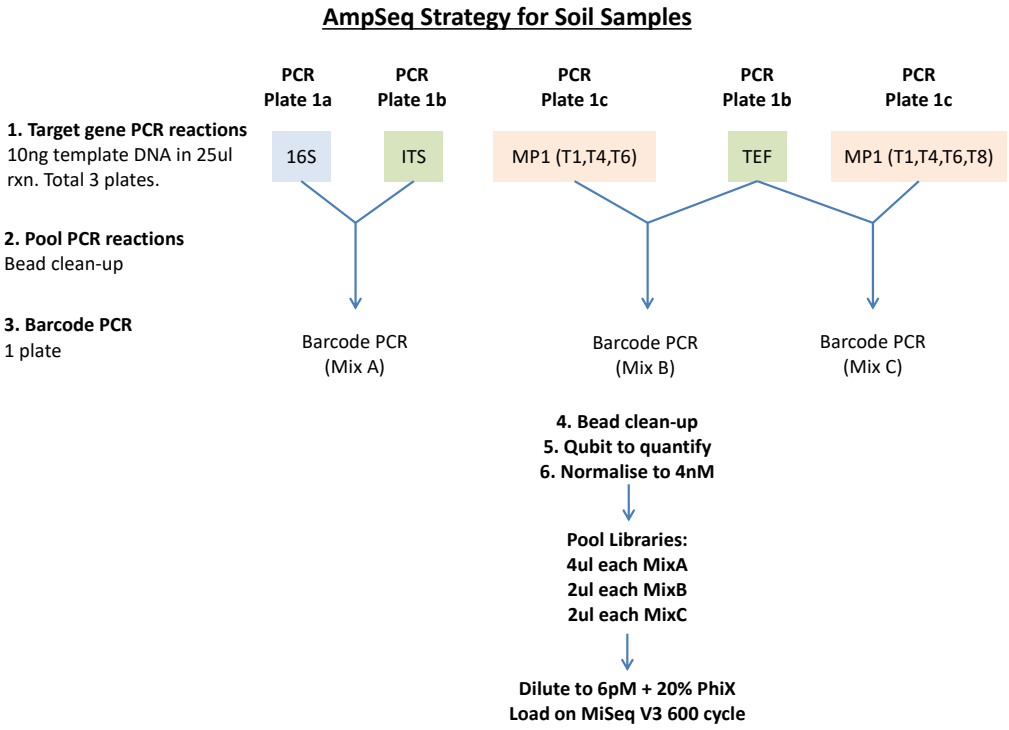

Figure 5: Amplicon sequencing workflow for DNA from soil samples.

Amplicon sequence data analysis

Illumina sequencing reads for each gene generated for the samples in a single sequencing run were separated ("demultiplexed") based upon the barcode sequence ligated present on each read (Fig. 6, Step 1). Reads for each sample were then assigned to target gene amplicons based upon the primer sequence present at the beginning/end of the paired reads (Fig. 6, Step 2). Amplicon identity was assigned if either the forward or reverse read of a sequence contained a 100% match to one of the target primer sequences. Following this, forward and reverse pairs of reads were merged into a single sequence (Fig. 6, Step 3). Although Illumina sequence data has a low error rate, this increases with increasing read length; hence by merging reads, confidence in the base calls across the entire sequence is

increased. Quality scores are retained in the merged read, allowing the chance of a base containing an error to be assessed. Merged reads with more than a 50% chance of containing an error were therefore discarded. Following this, two different approaches were used depending upon the locus analysed, a more lenient approach for analysis of 16S and ITS amplicons and a more stringent approach for analysis of TEF and *F. oxysporum* f.sp. specific amplicons.

For analysis of the 16S and ITS amplicons, reads were clustered into operational taxonomic units (OTUs) based upon sequence similarity, and these OTUs assigned an identity (Fig. 6, Step 4). This step was performed using all reads attributed to 16S or ITS in the sequencing run, thereby ensuring that the same OTUs were identified across the entire experiment. In contrast to approaches that cluster based upon a 97% similarity cut-off, we used the unoise3 option in usearch that aims to resolve different sequence types by estimating error rates around clustered reads. Prior to this, all reads were adjusted to be the same length by adding 'N' bases to the ends of the sequences, as required. Taxonomy was assigned to OTU sequences by searching against reference sequence databases using the SINTAX algorithm in usearch software. In the case of 16S, the RDP 16S database v.16 was used, and in the case of ITS the UNITE ITS database v.7.2 was used. Reads were quantified against the identified OTUs and summarised by genus/species (Fig. 6, Step 5). Quantification was performed using usearch, with mapping requiring 97% sequence identity to OTUs.

For analysis of TEF and the *F. oxysporum* f.sp. specific genes SIX13, OG4952, OG13890 and OG13397 a more stringent approach was taken. Here, many of the fungal taxa that needed to be resolved had sequence differences as low as a single base pair. Therefore, a clustering approach may cluster multiple *Fusarium* species or f.sp. into a single OTU leading to miss-identification. Also, OTUs could contain sequences that include sequencing errors. This is of less importance for ITS and 16S where the aim is to describe differences in community structure but of much greater importance for loci such as TEF and the *F. oxysporum* f.sp. specific loci, where the aim is to describe presence or absence of particular *Fusarium* spp. or *F. oxysporum* f.sp. Therefore, clustering was not performed for these loci (Step 4 in Fig. 6 skipped). Instead, reads were quantified against databases of reference sequences directly, with a requirement of 100% sequence identity during this mapping (Fig. 6, Step 5). This provided greater confidence in identification of what was present. However, this comes at the expense of not being able to identify novel species or *F. oxysporum* f.sp present in the samples. All data plots were generated in R using ggplot and to account for differences in sequencing depth between samples, counts were normalised to 1000 reads per sample. Runs with less than 1000 reads assigned to taxa were not normalised.

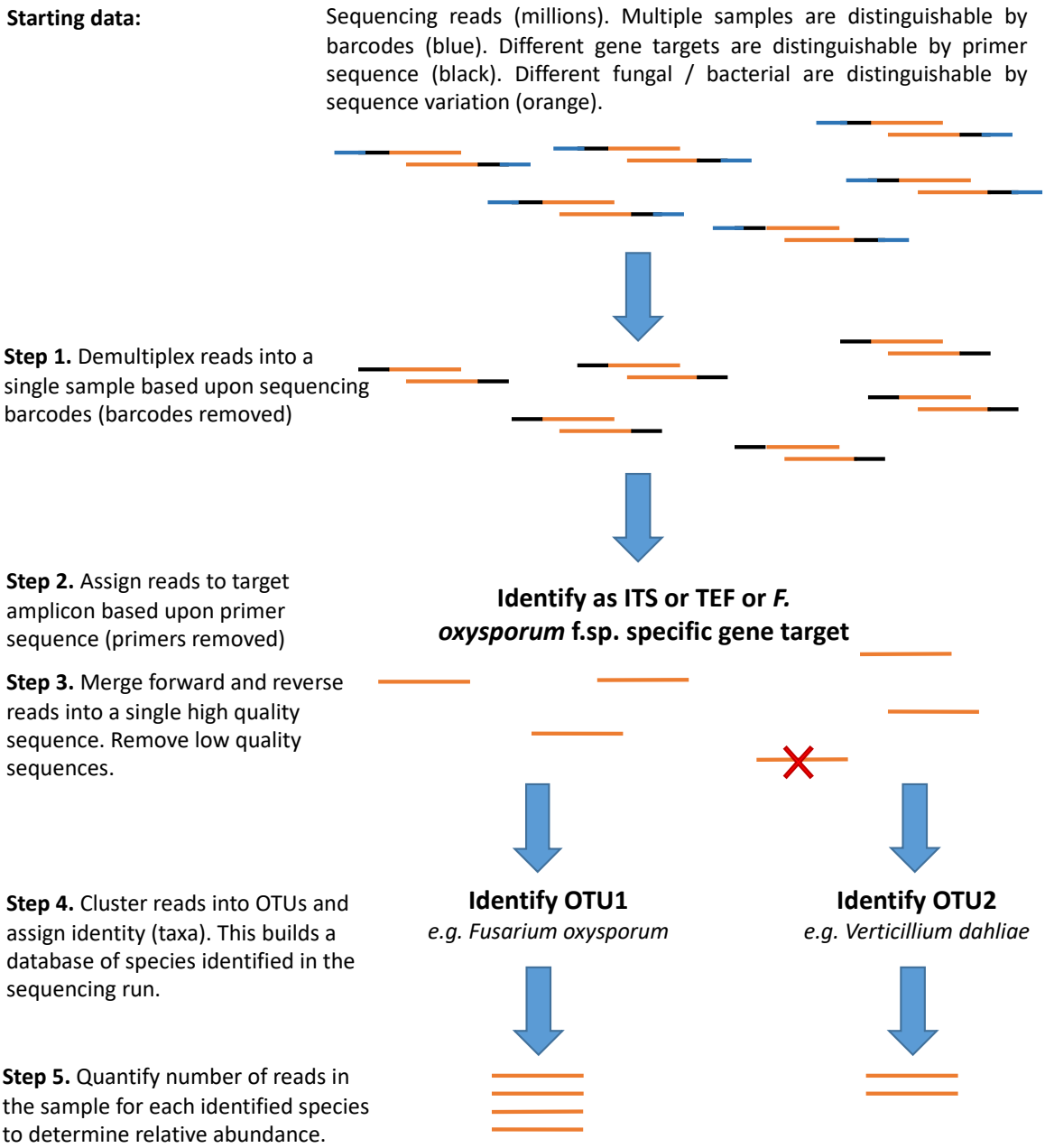

Figure 6: Summary of steps used in the analysis of amplicon sequence data.

Results

Testing and selection of gene targets using DNA from *Fusarium* and other fungi

Following primer design for amplicon sequencing, a combination of SIX13, OG13890, OG4952 or FOC_g17143 sequences were predicted to allow identification of FOC, FOM, FON and other *F. oxysporum* f.spp. Target genes OG10859 and OG4927 were discontinued as these were less informative in distinguishing some *F. oxysporum* f.spp. while OG4927 was also predicted to be present in non-pathogenic isolates. Another locus, OG13397 was identified with the aim of improving identification of FOM. Testing of primers for these target genes by PCR using DNA from selected *Fusarium* spp. and *F. oxysporum* f.spp. as well as DNA from selected FOC, FOM and FON infested soil generally showed the predicted pattern of amplification (Table 5). However, testing of FOC_g17143 primers using FOC QF soil DNA soil sample, resulted in an unacceptably high background of non-specific PCR products despite attempts to optimise the PCR reaction conditions and hence this target gene was discontinued. For the target gene OG13397, tests were less clear as it resulted in a high level of unspecific product in FOC QF and FON soil DNA samples but gave a strong specific band of the correct size when tested against the FOM soil DNA samples with a lower level of background. In summary, following this initial testing, 16S, ITS, TEF, SIX13, OG13890, OG4952 and OG13397 were selected as the target gene loci for amplicon sequencing

Amplicon sequencing of artificial community DNA pools from *Fusarium* and other fungi

Sequencing run overview

Amplicon sequencing of the DNA from Pool 1 and Pool 2 performed on the Illumina Miseq machine generated 13,895,367 paired end reads distributed between 91 samples loaded onto the run. A low level of reads was identified as contaminants in each sample as a result of index hopping (typically 0.01-0.03 % reads per sample).

Species identified by ITS sequencing

Analysis of the ITS target amplicons for DNA Pool 2 (*Fusarium* species + other common soilborne fungal pathogens) allowed identification of taxa to genus or species level. Use of the curated Unite v7 Fungal ITS database led to some discrepancy between taxon names,

typically between the different anamorph / teleomorph names for the same organism (e.g. *Fusarium* and *Giberella*), or between closely related species groups (e.g. *Alternaria orgenensis* and *Alternaria infectoria*). This is expected to improve with the continued curation of barcoding locus databases over time. Of the 20 fungal soil pathogens in Pool 1, all were amplified by the ITS primers with the exception of the two oomycete pathogens (*Phytophthora cactorum* and *Pythium ultimum*; Table 7) as expected. Of the eighteen remaining species, fifteen were correctly identified (Table 7), but *Fusarium avenaceum*, *Sclerotinia cepivorum* and *Trichoderma* were not. However, *F. proliferatum* was detected despite not being in the original DNA mixture, and therefore likely represented *F. avenaceum*. Similarly, an additional taxon identified as a *Sclerotinia* sp. was detected in the sample possibly explaining the missing *S. cepivorum*. No evidence was found for the taxon *Trichoderma* and further work is required to determine if this is a problem at library preparation, a result of PCR amplification bias or miss-labelling within the ITS database.

Table 7: Fungal species identified from artificial community DNA pools from *Fusarium* and other fungi (Pool 2). Green boxes indicate species correctly identified by amplicon sequencing of ITS and TEF, those in orange were expected to be amplified but could not be identified within the sample and those in grey were not expected to be detected in the sample. Taxa names in the publicly available UNITE ITS database differed from those used elsewhere in this report, reflecting the fungal teleomorph name, or reflecting species poorly resolved by ITS at the species level.

Species in Pool 2	**Identification by amplicon sequencing (ITS database)**	**ITS**	**TEF**
Alternaria infectoria	*Alternaria oregonensis*		
Botrytis cinerea	*Botrytis caroliniana*		
Cylindrocarpon destructans	*Ilyonectria mors-panacis*		
Fusarium avenaceum	*Fusarium avenaceum*		
Fusarium graminearum	*Gibberella zeae*		
Fusarium oxysporum f.sp. *cepae*	*Fusarium oxysporum*		
Fusarium redolens	*Gibberella tricincta*		
Fusarium solani	*Fusarium neocosmosporiellum*		
Itersonilia perplexans	*Itersonilia perplexans*		
Microdochium nivale	*Monographella nivalis*		
Mycocentrospora acerina	*Mycocentrospora acerina*		
Phoma spp.	*Didymella arachidicola*		
Phytophthora cactorum			
Pythium ultimum			
Rhizoctonia solani	*Thanatephorus cucumeris*		
Sclerotinia sclerotiorum	*Sclerotinia sclerotiorum*		
Sclerotium cepivorum	*Stromatinia rapulum*	0-3 reads only	
Setophoma terrestris	*Setophoma terrestris*		
Trichoderma			
Verticillium albo atrum	*Verticillium albo atrum*		
non-target organisms:	*F. proliferatum, Sclerotinia* sp.		

Species identified by TEF sequencing

Analysis of the TEF target amplicons for Pool 1 (*Fusarium* species and *F. oxysporum* f.spp.) and Pool 2 (*Fusarium* species + other soil pathogens) DNA demonstrated the suitability of TEF to accurately identify different *Fusarium* species and some other fungi (Table 8). Of the 20 fungal pathogens in Pool 2, TEF sequencing identified and differentiated all five *Fusarium* species (*F. avenaceum*, F. *graminearum*, *F. oxysporum*, *F. redolens* and *F. solani*) as well as *Cylindrocarpon destructans, Microdochium nivale* and *Verticillium albo atrum*. Within Pool 1, TEF sequencing identified and differentiated all 13 different *Fusarium* species as well as *Microdochium nivale* but not *M. majus* (Table 8). Sequence variation was observed between reads assigned to *F. oxysporum*, but sequence similarity and the polyphyletic nature of host adaptation within this species meant that the identification of *F. oxysporum* f.spp. could not be resolved by TEF alone.

Table 8. Fungal species identified from artificial community DNA pools from *Fusarium* spp., *F. oxysporum* f.spp. and related species (Pool 1): Green boxes indicate species correctly identified by amplicon sequencing of TEF and *F. oxysporum* f.spp. gene targets, those in orange were expected to be amplified but could not be identified within the sample and those in grey were not expected to be detected in the sample. TEF resolves all *Fusarium* spp. while the other loci identified and differentiated different *F. oxysporum* f.spp. present. Where a locus cannot differentiate f.spp. they are both listed in the cell.

Species in Pool 1	TEF	SIX13	OG13890	OG4952	OG13397
Fusarium avenaceum					
Fusarium coeruleum					
Fusarium culmorum					
Fusarium equiseti					
Fusarium flocciferum					
Fusarium graminearum					
Fusarium oxysporum					
Fusarium oxysporum f.sp. *cepae*					
Fusarium oxysporum f.sp. *lycopersici*					
Fusarium oxysporum f.sp. *matthioli*			matthiolae/raphani	matthiolae/conglutinans	matthiolae/raphani[3]
Fusarium oxysporum f.sp. *narcissi*					narcissi/pisi
Fusarium oxysporum f.sp. *pisi*		[1]			narcissi/pisi
Fusarium poae					
Fusarium proliferatum					
Fusarium redolens					
Fusarium sambucinum					
Fusarium solani					
Fusarium tricinctum					
Microdochium nivale					
Microdochium majus					
Non-target organisms:			c*onglutinans*	tulipae[2]	

[1]SIX13 primers will detect *F. oxysporum* f.sp. *pisi* races 3 and 5 but not race 1 as used in this experiment; [2] one of the two *F. oxysporum* f.sp. *tulipae* genotypes was detected; [3] one of the two *F. oxysporum* f.sp. *matthioli* genotypes was detected.

F. oxysporum f.spp. identified by sequencing of specific genes

Selective amplification of *F. oxysporum* f.spp. specific genes was successful, with the four different loci resulting in amplification of DNA from different *F. oxysporum* f.sp. present in DNA Pool 1 (Table 8). SIX13 primers selectively amplified DNA from FOL and FON and would also be expected to amplify FOP race 2 or 5; however, FOP race 1 was used in this analysis and was therefore not detected. Locus OG13890 primers selectively amplified DNA from FOC, FOM and FOP, but also detected the non-target organism *F. oxysporum* f.sp. *conglutinans*. OG4952 primers selectively amplified DNA from FOC, FOM, FON and FOP, but also identified the non-target organism *F. oxysporum* f.sp. *tulipae*. Finally, OG13397 primers selectively amplified DNA from FOM, FON and FOP. Overall, although the key *F. oxysporum* f.spp. FOC and FON can be identified through sequencing the target genes, it was not possible to distinguish between the closely related FOM, *F. oxysporum* f.sp. *raphani* and *F. oxysporum* f.sp. *conglutinans* which all infect various brassica species. This is highlighted by the results for OG1390 which led to the false positive detection of *F. oxysporum* f.sp. *conglutinans*.

Effects of reducing DNA concentration of certain fungal species

Effects of diluting *Alternaria infectoria*, *Sclerotinia sclerotiorum* and *Verticillium albo atrum* DNA on Pool 2 were observed in ITS sequencing, with dilution leading to corresponding reductions in the number of reads for these species (Fig. 7). Similar effects were observed for TEF and the *F. oxysporum* f.spp. specific target gene SIX13 with dilution of FOM, FON and FOP in Pool 1 (Fig. 8, Fig. 9). For TEF, there was a reduction in two sequence types associated with *F. oxysporum*, concomitant with the dilution of FOM, FON and FOP. For SIX13, a reduction in FON sequence reads was observed in response to DNA dilution (Fig. 9). However, the 10 and 100-fold dilutions of FON in SIX13 did not show a corresponding 10- and 100-fold reduction in reads, indicating that PCR and library preparation biases also affect the subsequent number of FON sequence reads.

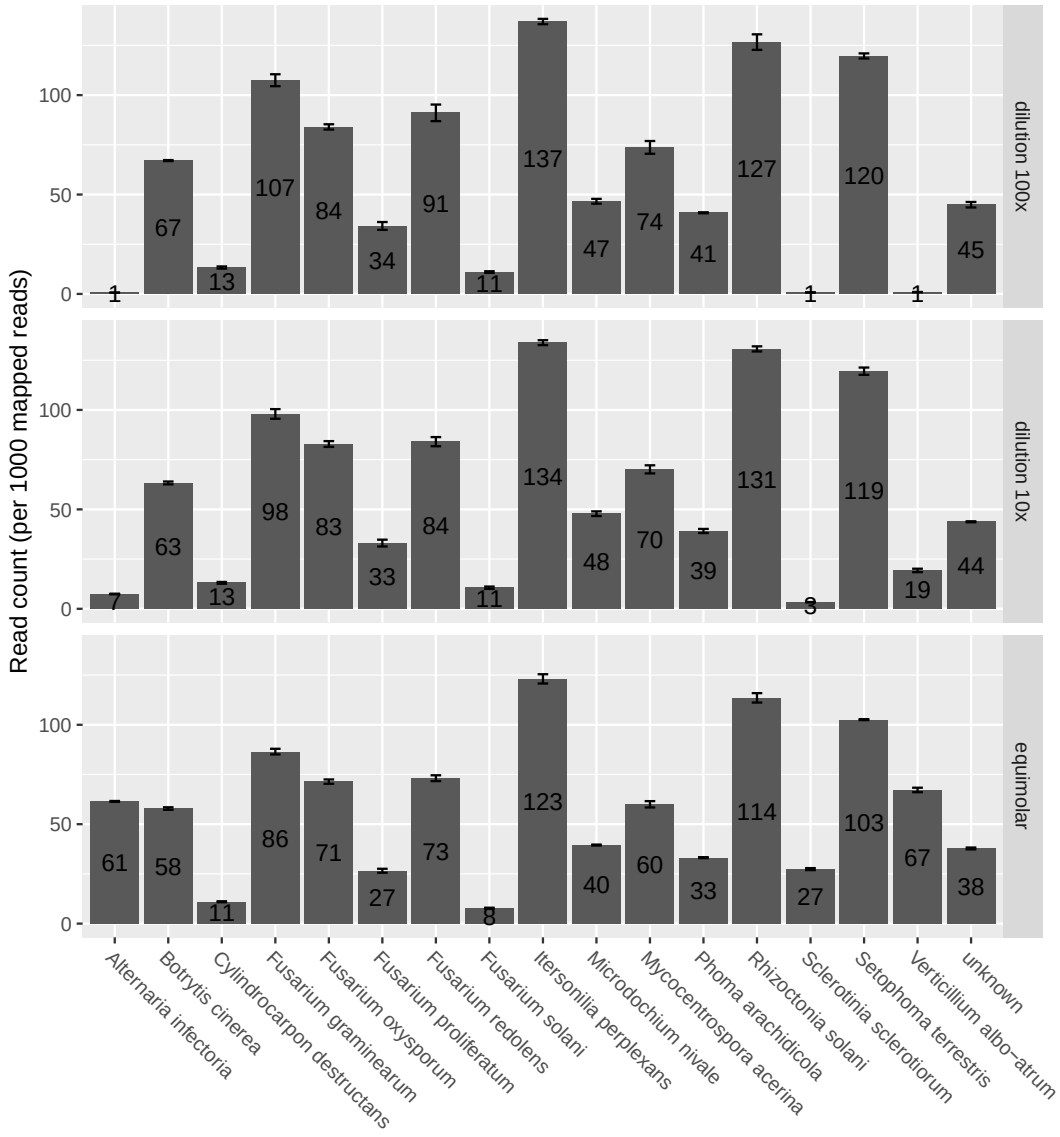

Figure 7: Effect of diluting DNA of *Alternaria infectoria*, *Sclerotinia sclerotiorum* and *Verticillium albo atrum* within DNA Pool 2 (five *Fusarium* species, 15 soilborne fungal pathogens, Table 6) prior to ITS amplicon sequencing. Data are mean read counts for each identified species with 100x (top), 10x (middle) and undiluted (bottom) of the three pathogens. Error bars represent standard error across three technical replicates of the PCR and library preparation processes.

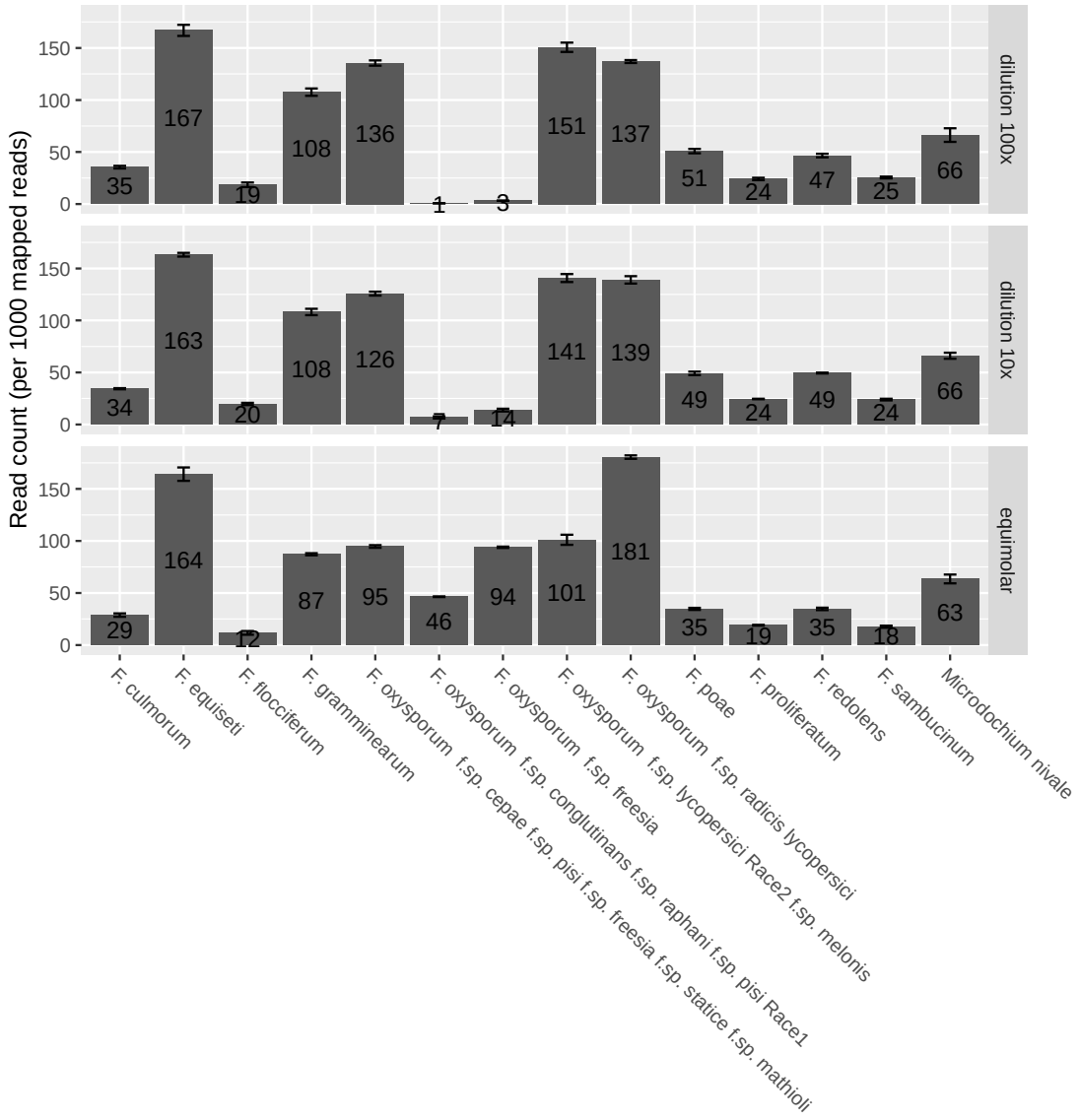

Figure 8: Effect of diluting DNA of FOM, FON and FOP within DNA Pool 1 (*Fusarium* species, F. oxysporum f.spp. and related species, Table 6) prior to TEF amplicon sequencing. Data are mean read counts for each identified species with 100x (top), 10x (middle) and undiluted (bottom) of FOM, FON and FON. Error bars represent standard error across three technical replicates of the PCR and library preparation processes. Read counts for two TEF genotypes associated with FOP and FOM decrease in abundance in response to 10x and 100x dilutions.

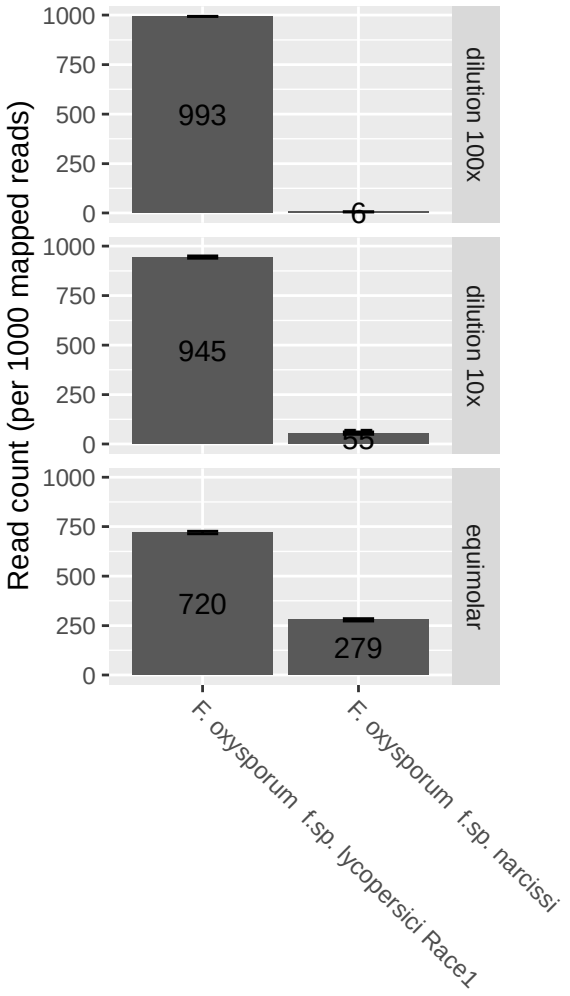

Figure 9: Effect of diluting DNA of FON within DNA Pool 1 (*Fusarium* species, *F. oxysporum* f.spp. and related species, Table 6) prior to SIX13 amplicon sequencing. Data are mean read counts for each identified species with 100x (top), 10x (middle) and undiluted (bottom) of FON. Error bars represent standard error across three technical replicates of the PCR and library preparation processes. Read counts of FON decrease in abundance in response to 10x and 100x dilutions.

Effects of multiplexing and pooling

The feasibility of multiplexing PCR reactions for target loci, or for combining amplicons after PCR was determined using the species pools. Following mixing, these samples were run under a single sequencing barcode, allowing more samples to be loaded in a single sequencing run. TEF and *F. oxysporum* f.sp. specific loci were amplified in multiplex and sequenced using the same barcode. All target loci had large numbers of reads between 13918 and 112532 (Table 9), with some bias between loci. Typically, SIX13 amplicons represented 47% of the sequencing reads, whereas TEF represented 15% and other loci each represented 8-16% of reads. Where PCR reactions were pooled for barcoding, the pooling was deliberately biased towards TEF to ensure enough coverage as more OTUs were expected to be amplified for that locus. However, this set also showed bias towards SIX13 compared to other *F. oxysporum* f.sp. loci. This shows, that multiplexing or pooling is feasible for samples, but bias

is to be expected. Multiplexing the first locus-specific PCR appeared to introduce less variability in technical replicates than combining samples at the barcoding stage. However, multiplexing the first PCR also gave a higher number of ambiguous reads

Table 9: Effects of multiplexing multiple loci during library preparation: Multiple loci were combined in a single sample through performing multiplex PCR on the DNA sample or combining PCR products at equal concentrations and performing barcoding steps on multiple loci at once. The ITS primer sets were not used for these samples, and reads attributed to this locus during this run are a result of primer hopping from other samples in the same sequencing plate (data not shown). Read distributions showed some bias, with OG13890 and OG13397 having fewer % reads than other samples.

Multiplex at step	Pool	Rep	Read counts							% Read counts						
			ITS	Tef	SIX13	OG13890 (T4)	OG4952 (T6)	OG13397 (T8)	Ambiguous	ITS	Tef	SIX13	OG13890 (T4)	OG4952 (T6)	OG13397 (T8)	Ambiguous
PCR	*Fusarium* spp.	rep1	6	23828	75588	13918	25455	17488	3936	0.00	14.87	47.18	8.69	15.89	10.92	2.46
PCR	*Fusarium* spp.	rep2	12	31694	101252	19254	34880	24248	5741	0.01	14.60	46.64	8.87	16.07	11.17	2.64
PCR	*Fusarium* spp.	rep3	15	35321	112532	20812	38634	24220	5981	0.01	14.87	47.38	8.76	16.27	10.20	2.52
PCR	*Fusarium* spp.	pool	9	23848	78752	14254	26141	18424	3618	0.01	14.45	47.72	8.64	15.84	11.16	2.19
barcoding	Soil pathogens + *Fusarium* spp.	rep1	20	75079	151568	17708	32401	20601	2495	0.01	25.04	50.54	5.91	10.80	6.87	0.83
barcoding	Soil pathogens + *Fusarium* spp.	rep2	15	59365	109492	17940	34701	23948	1231	0.01	24.06	44.38	7.27	14.07	9.71	0.50
barcoding	Soil pathogens + *Fusarium* spp.	rep3	21	75308	101344	53133	68452	5373	1809	0.01	24.66	33.18	17.40	22.41	1.76	0.59

Amplicon sequencing of soils infested with FOC, FON and FOM

Sequencing run overview

The presence and abundance of bacterial and fungal species as well as *Fusarium* species and *F. oxysporum* f.spp. were assessed by amplicon sequencing of DNA from a total of 41 soil samples from areas infested with FOC, FOM or FON. These comprised of the 15 FOC (5 samples from 3 beds), 18 FOM (6 samples from 3 beds) and 7 FON (field transect) soil samples as described above. A further FOC sample represented a combined DNA pool of all the FOC QF samples. The presence and abundance of general bacterial and fungal species was determined used 16S and ITS gene targets (41 barcoded samples 'mix A'), while the presence and abundance of *Fusarium* spp. and *F. oxysporum* f.spp. was determined using TEF, SIX13, OG13890 and OG4952 gen targets (41 barcoded samples 'mix B'). A subset of samples was also chosen to assess the use of the four target loci from 'mix B' alongside an additional amplicon OG13387 (5 barcoded samples 'mix C'). Sequencing on the Illumina MiSeq generated 10,989,383 paired-end reads across the 87 barcoded, mixed-amplicon samples. As found in the pools of DNA described above, index hopping led to contamination between barcoded samples at a low level (typically below 1%, with a maximum of 114 reads).

Presence and abundance of bacteria using 16S sequencing

In the FOC field soil samples, 384 bacterial genera were identified by 16S amplicon sequencing compared with 406 and 388 in FOM and FON soil samples respectively (the most abundant genera represented by more than 10 reads per 1000 reads in a sample, Fig. 10). No bacterial genera were particularly dominant with the greatest representing *Arthobacter* in from the daffodil field, to which 63 per 1000 reads were attributed. Despite many taxa being present in all three infested areas, some did show high abundance in one field, but were absent in others; e.g. Ktedonobacter was present in the FOC field at high levels (31 per 1000 reads), but absent in daffodil and stocks fields (Fig. 10).

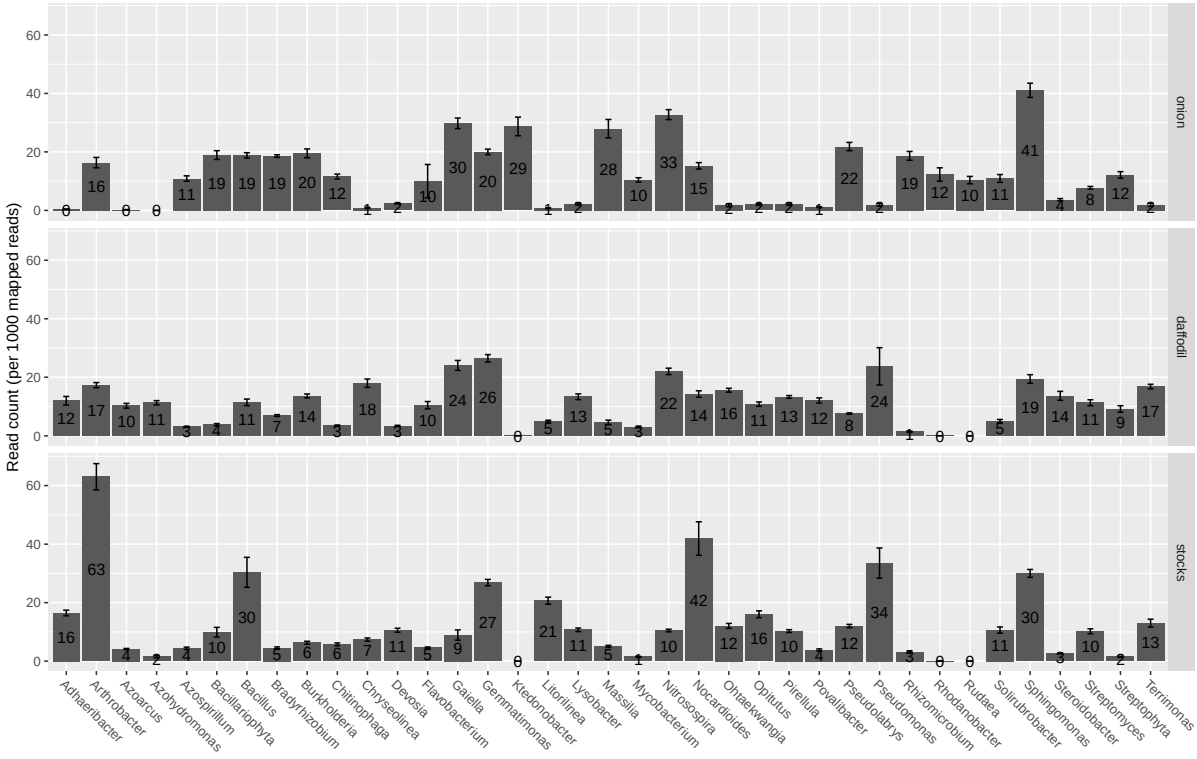

Figure 10: Presence and abundance of bacteria in fields infested with FOC (onion), FOM (stocks) and FON (daffodil). Data are mean read counts per 1000 reads for each identified genus identified by 16S amplicon sequencing with high prevalence (>10 in 1000 reads). Error bars represent standard error across individual soil samples.

Presence and abundance of fungi using ITS sequencing

Analysis of the fungal community using ITS amplicon sequencing identified a wide range of fungi but in contrast to the bacterial community, there was dominance of certain genera (Fig. 11). As expected for onion, stocks and daffodil soils infested with FOC, FOM and FON, *Fusarium* was identified in the samples and was the predominant genus in the FOM stocks and FON daffodil areas. Interestingly, the FOC QF onion field soil was dominated by the yeasts *Saitozyma* and *Solicococcozyma* while *Fusarium* was the fifth most abundant taxon, behind *Mortierella* (soil fungal saprotrophs) and *Apiotrichum* (soil yeast). Notably, *Mortierella* was the most abundant genus over all three *Fusarium* infested areas (59-101 per 1000 reads), followed by *Fusarium* (51-518 per 1000 reads).

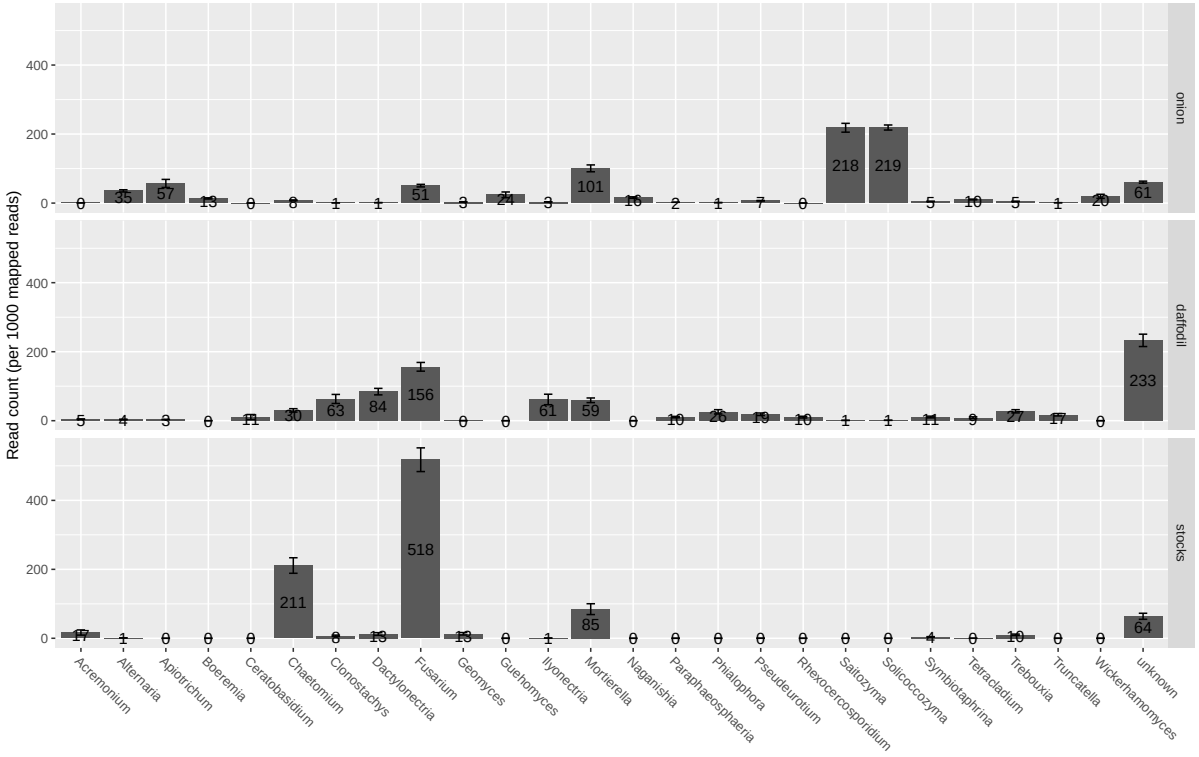

Figure 11: Presence and abundance of fungi in fields infested with FOC (onion), FOM (stocks) and FON (daffodil). Data are mean read counts per 1000 reads for each identified genus identified by ITS amplicon sequencing with high prevalence (>10 in 1000 reads). Error bars represent standard error across individual soil samples.

Presence and abundance *Fusarium* spp. using TEF sequencing

A range *of Fusarium* species were identified by TEF amplicon sequencing in the onion, stocks and daffodil soils infested with FOC, FOM and FON (Fig. 12). As expected, *Fusarium oxysporum* was the dominant species with 344-746 per 1000 sequencing reads across all the soils. In the FOC QF soil, *Fusarium* oxysporum accounted for 746 per 1000 reads, with *Fusarium redolens* the next most abundant species with 57 perf 1000 reads. Similarly, FON and FOM accounted for 404 and 344 in 1000 reads respectively in the corresponding soil samples. *F. redolens*, *F. flocciforum* and *F. culmorum* were highly abundant in all three fields, with *F. redolens* showing similar abundance (275 in 1000 reads) to *F. oxysporum* (344 in 1000 reads) in the FOM infested soil.

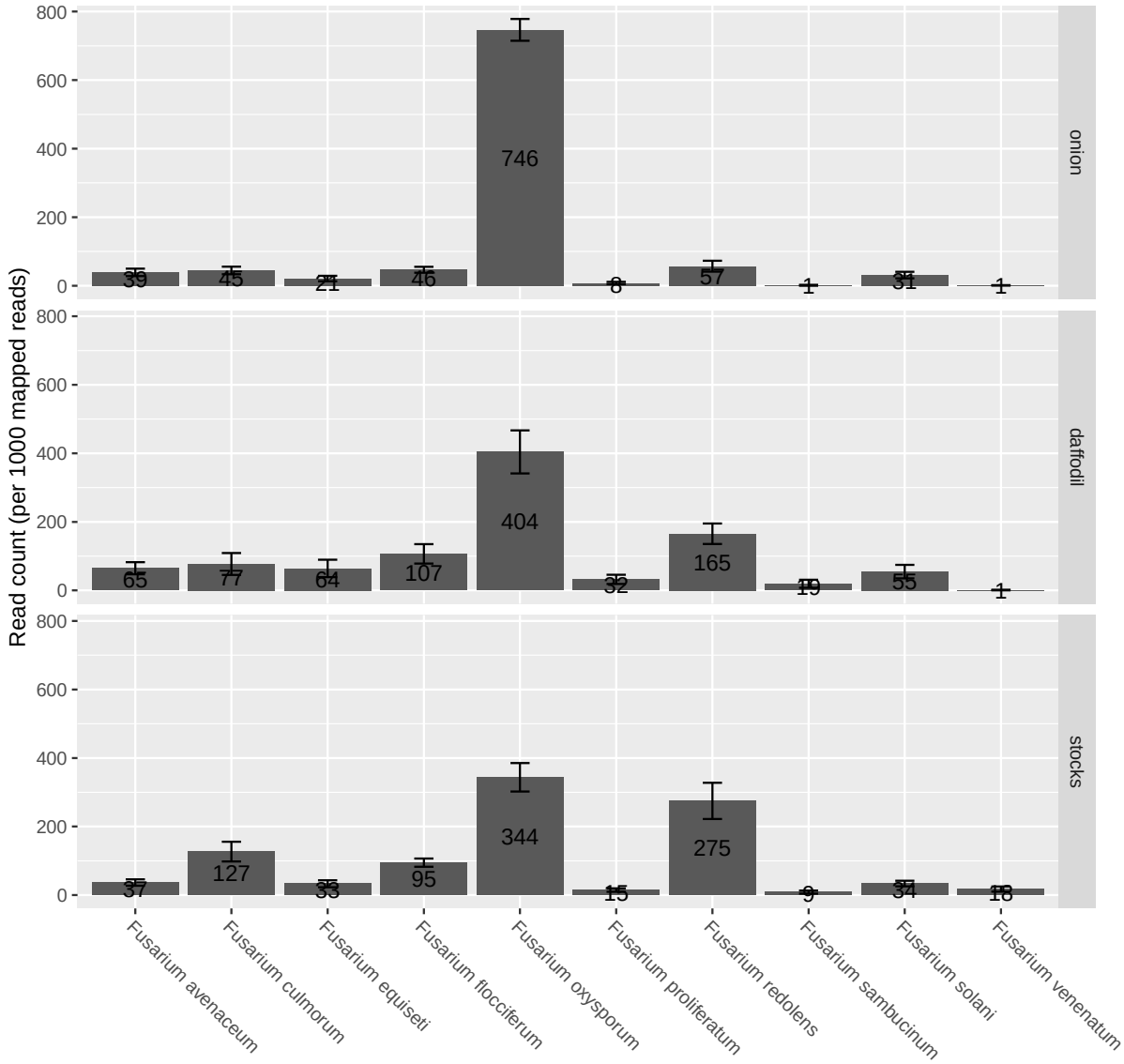

Figure 12: Presence and abundance of *Fusarium* spp. in fields infested with FOC (onion), FOM (stocks) and FON (daffodil). Data are mean read counts per 1000 reads for each *Fusarium* sp. identified by TEF amplicon sequencing with high prevalence (>10 in 1000 reads). Error bars represent standard error across individual soil samples.

Detection of FOC FON and FOM from infested soil sites using sequencing of specific genes

Amplicon sequencing of DNA from the onion, stocks and daffodil soils infested with FOC, FOM and FON using the *F. oxysporum* f.spp. specific loci resulted in considerable variation in reads between the different loci. A sufficient number of reads were generated for a robust analysis of OG4952 amplicons, but low numbers of reads were attributed to SIX13 (1-16 reads mapped across samples) and OG13890 (1-104 reads mapped across samples). A low read count for these two loci was not a result of the target *F. oxysporum* f.sp. being absent from soil samples as OG4952 correctly identified presence of each of the target species (see below). Of the few SIX13 reads present, all were mapped to FOP (race 5) which occurred at low levels in soil samples from all three infested areas (data not shown). For OG13890, FOC and FOP (race 1) were identified at very low levels but a greater number of total reads (24) were mapped to FOM (or *F. oxysporum* f.sp. raphani) in the FOM soil samples compared with the FOC and FON samples (data not shown). The low read count for SIX13 and OG13890 means that all these results should be treated with considerable caution.

Analysis of OG4952 sequences led to robust identification of FOC, FOM and FON in the onion, stocks and daffodil soil samples (Fig. 13). As expected, FOC was the predominant f.sp. in the FOC QF (698 per 1000 reads) while FOM was the predominant f.sp. in the stocks soil samples (836 per 1000 reads). However, although FON was detected in the daffodil field soil samples (138 per 1000 reads), both FOC and FOM were at higher levels (196 and 440 per 100 reads respectively). Unexpectedly, reads matching FOP (race 1) were also observed in all samples. Overall, although the expected *F. oxysporum* f.spp. were identified in the corresponding infested soil samples, the presence of FOC, FOM and FON in all samples was unexpected. Furthermore, results using the specific qPCR tests for FOC, FOM and FON indicated that they were only present in the onion, stocks and daffodil soils respectively (with the exception of FON in the FOM soil) as was originally expected.

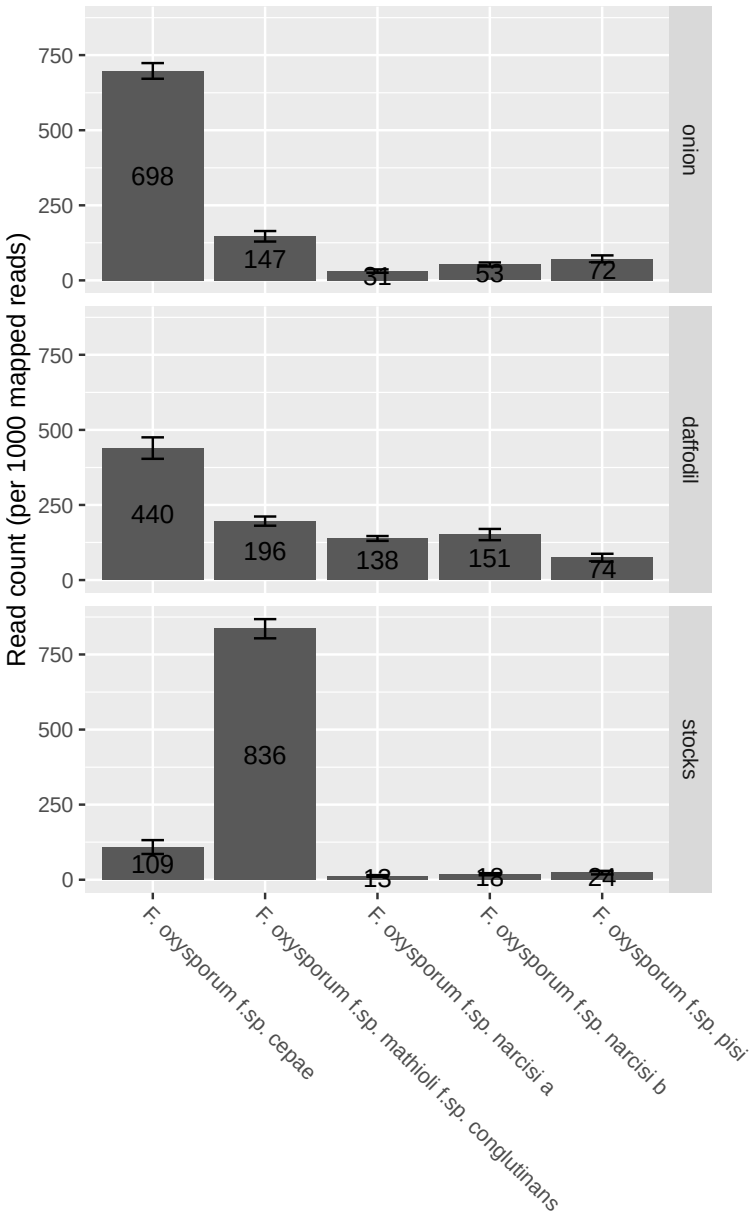

Figure 13: Presence and abundance of FOC, FON and FOM in onion, daffodil and stocks soils infested with each pathogen. Data are mean read counts per 1000 reads for each *F. oxysporum* f.sp. identified by OG4952 amplicon sequencing with high prevalence (>10 in 1000 reads). Error bars represent standard error across individual soil samples.

The additional *F. oxysporum* f.spp. specific locus OG13397 was used to sequence DNA from a limited number of soil samples from onion (two samples), daffodil (one sample) and stocks (three samples) in order to try and distinguish FOM from *F. oxysporum* f.sp. *raphani* and *F. oxysporum* f.sp. *conglutinans*. Primers for this locus were expected to amplify two different genotypes of FOM, one specific to FOM and the other found in both FOM and *F. oxysporum* f.sp. *raphani* (Table 6). In addition, the same primers should also amplify FON and FOP. Adequate numbers of reads were obtained for this locus following amplicon sequencing but FOM (or *F. oxysporum* f.sp. *raphani*) was identified in all the soil samples from the sites infested with FOC, FOM and FON with low numbers of reads for FON / FOP in all samples (Fig. 14). In addition, a similar relative abundance for FOM / *F. oxysporum* f.sp. *raphani* and

FON / FOP was observed in all samples. These observations in addition to the lack of FOM specific sequences in the FOM infested soil and the negative results from FOM in FOC and FON infested soils using specific qPCR indicates that a systematic bias must be present in how this locus is amplified which is supported by the results for the DNA pools tested previously. This locus is therefore not suitable for reliable assessment of *F. oxysporum* f.sp. present in soils.

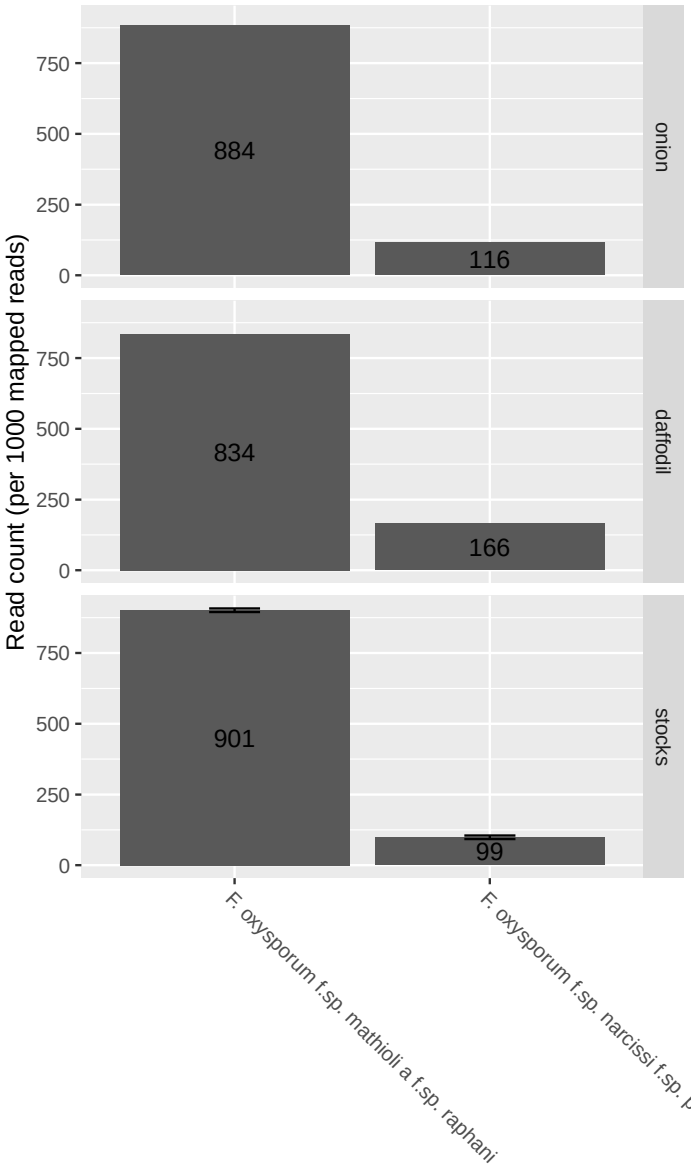

Figure 14: Presence and abundance of FOC, FON and FOM in onion, daffodil and stocks soils infested with each pathogen. Data are mean read counts per 1000 reads for each *F. oxysporum* f.sp. identified by OG13397 amplicon sequencing with high prevalence (>10 in 1000 reads). Error bars represent standard error across individual soil samples.

Variability in presence and abundance of bacteria, fungi, *Fusarium* spp. and *F. oxysporum* within onion and stocks sites infested with FOC and FOM

Assessing variability in amplicon sequencing results between individual samples within a field site informs sampling strategy for future work. Using the 15 FOC (5 samples from 3 beds) and 18 FOM (6 samples from 3 beds) soil samples, the variability in species detected between beds was evaluated for bacteria (16S), fungi (ITS), Fusarium and related spp. (TEF) and Fusarium f.spp. (OG4952).

Overall, the presence and absence of bacterial, fungal and *Fusarium* spp. in the sites infested with FOC and FOM was similar between beds within each site but individual samples within beds showed similar variation to that between sites, highlighting the need for replicate samples to be taken from the field.

The bacterial species identified by 16S sequencing generally showed similar patterns of incidence and abundance across the three beds in the FOC QF (Fig. 15a) with the exception of *Flavobacterium* where high variation in abundance was observed. Similarly, for the FOM infested area, results were also similar between samples from different planting beds but in this case, *Enterobacter* was observed at a high level in one bed, but was variable within each sample from this bed (Fig. 15b)

The fungal species identified by ITS sequencing showed very consistent patterns of incidence and abundance across the three beds for both FOC and FOM infested sites (Fig. 16a, Fig. 16b).

Fusarium spp. incidence and abundance as determined by TEF sequencing was largely consistent between beds in the FOC and FOM infested sites (Fig. 17a, Fig. 17b). However, when DNA was combined from all samples from the FOC QF, there was reduced dominance of *F. oxysporum* and *F. graminearum* was also identified as being present. This may reflect bias in the amplification of non-*F. oxysporum* taxa when present, and highlights the need for multiple samples from a field. For the FOM infested site there was some difference in abundance of *F. equiseti* and *F. culmorum* between beds (Fig. 17b).

Finally, analysis of the *F. oxysporum* f.spp. pathogen-specific locus OG4952 showed that the presence and abundance of FOC, FOM, FON and FOP was similar between the three beds at the FOC and FOM (Fig. 18a, Fig. 18b).

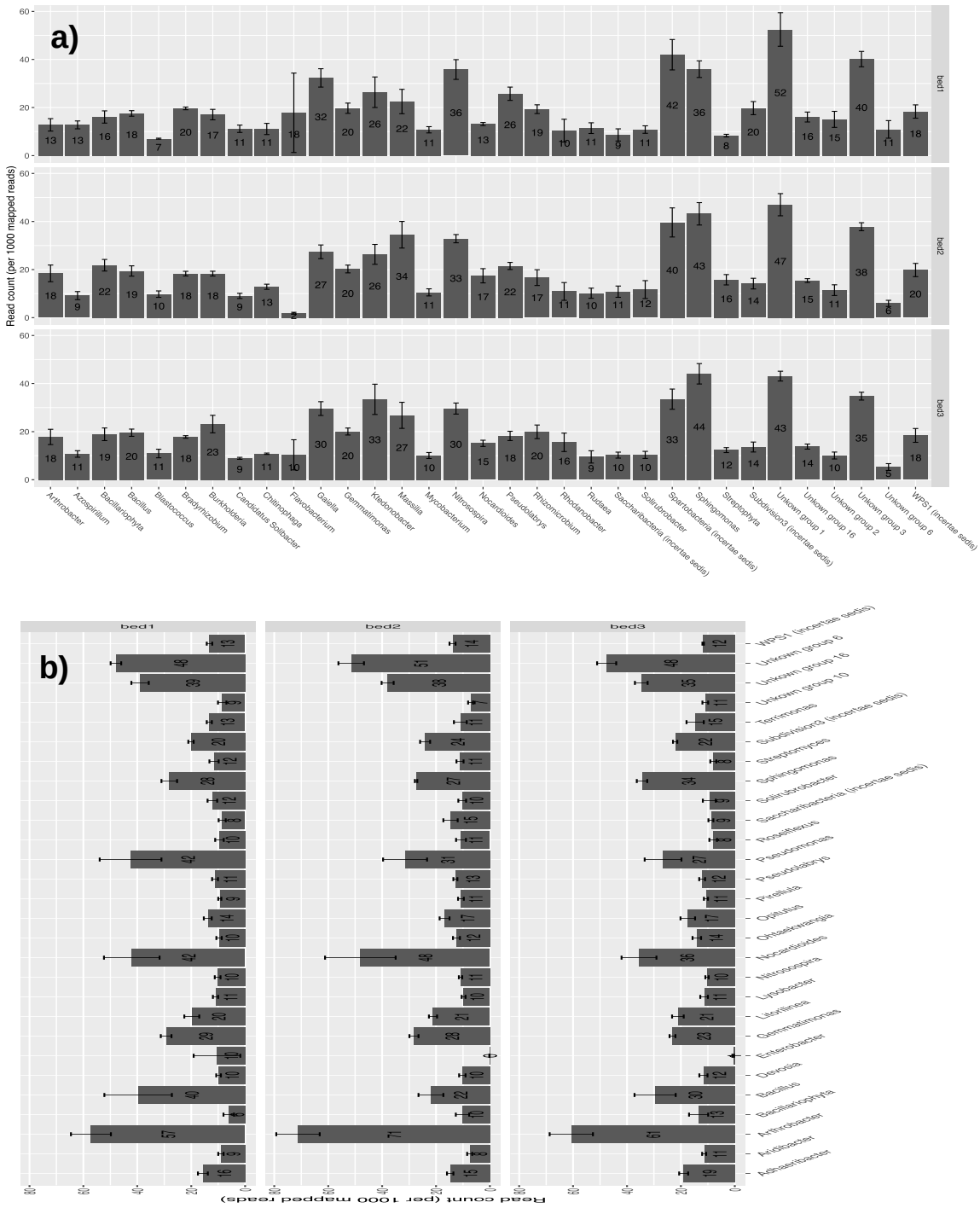

Figure 15: Presence and abundance of bacteria in three beds for soils infested with a) FOC (onion) and b) FOM (stocks). Data are mean read counts per 1000 reads for each identified genus identified by 16S amplicon sequencing with high prevalence (>10 in 1000 reads). Error bars represent standard error across individual soil samples within each bed.

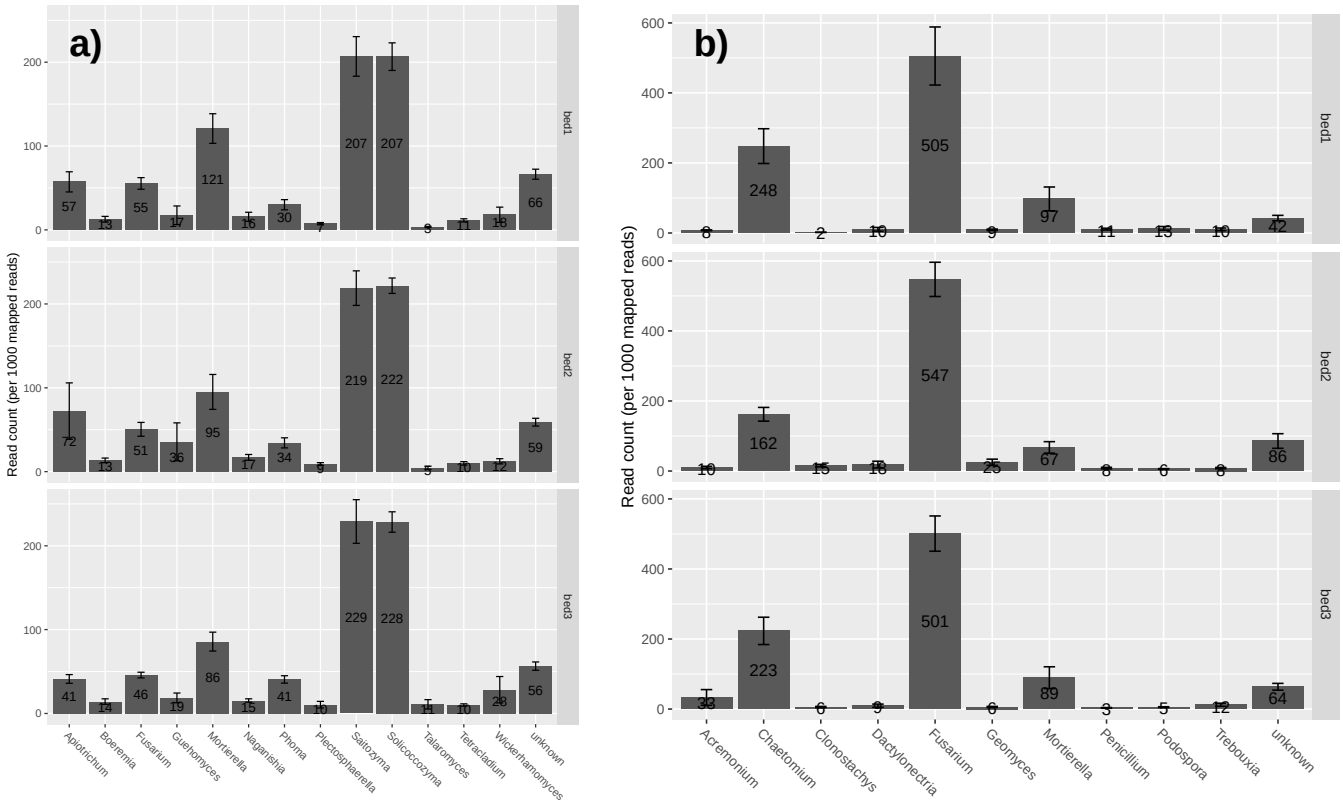

Figure 16: Presence and abundance of fungi in three beds for soils infested with a) FOC (onion) and b) FOM (stocks). Data are mean read counts per 1000 reads for each identified genus identified by ITS amplicon sequencing with high prevalence (>10 in 1000 reads). Error bars represent standard error across individual soil samples within each bed.

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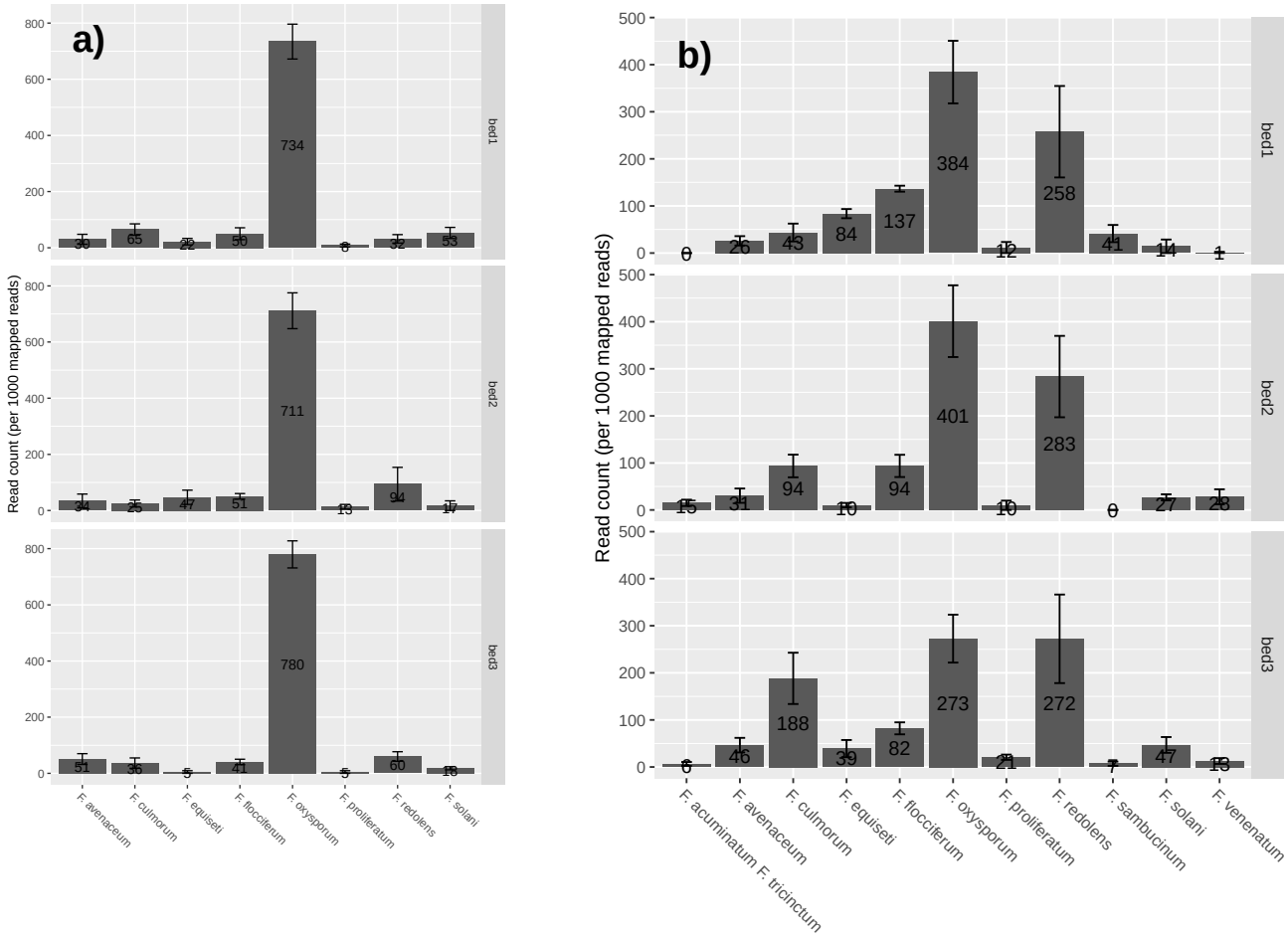


Figure 17: Presence and abundance of *Fusarium* spp. in three beds for soils infested with a) FOC (onion) and b) FOM (stocks). Data are mean read counts per 1000 reads for each identified genus identified by TEF amplicon sequencing with high prevalence (>10 in 1000 reads). Error bars represent standard error across individual soil samples within each bed.

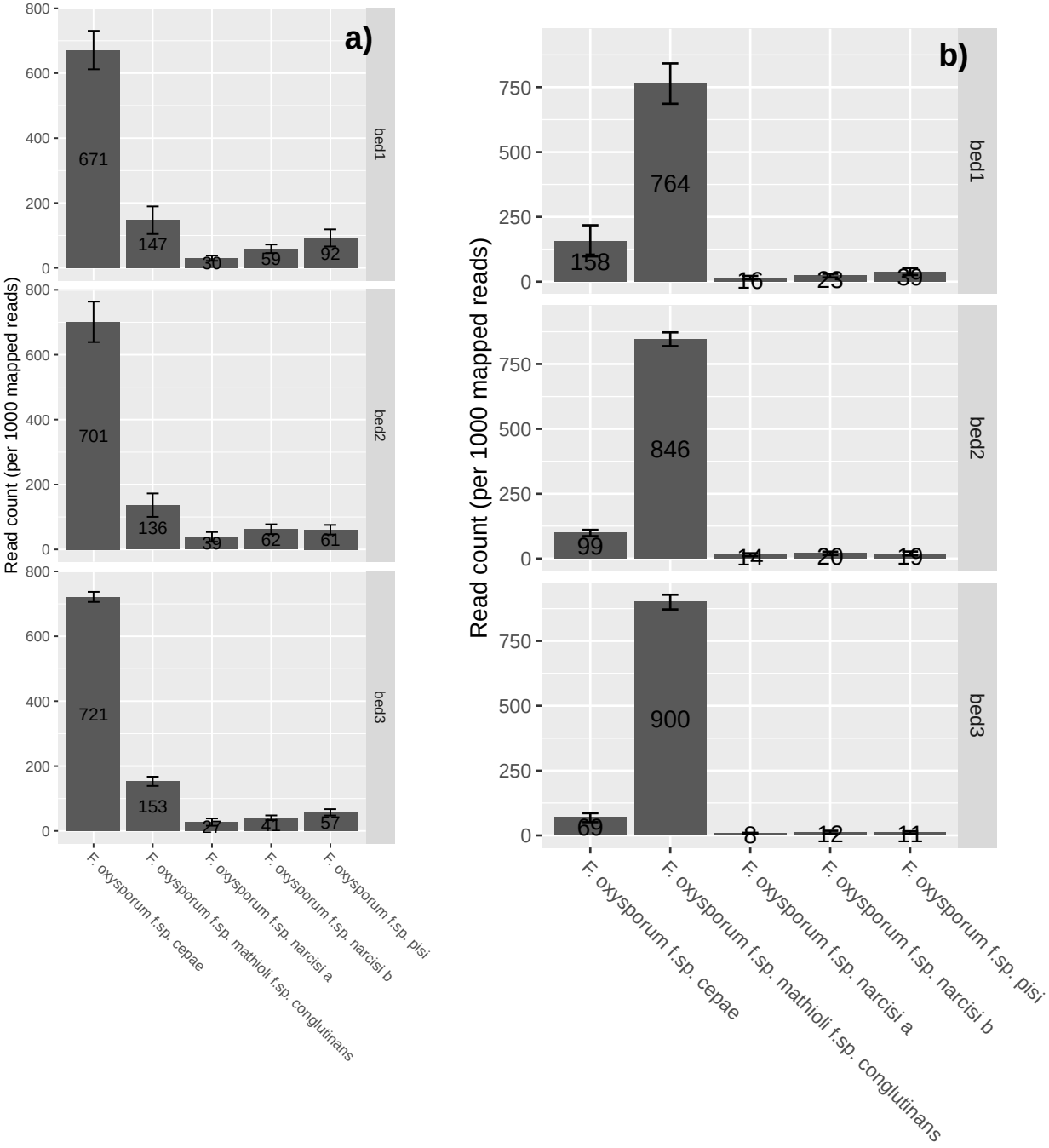


Figure 18: Presence and abundance of *F. oxysporum* f.spp. in three beds for soils infested with a) FOC (onion) and b) FOM (stocks). Data are mean read counts per 1000 reads for each identified genus identified by OG4952 amplicon sequencing with high prevalence (>10 in 1000 reads). Error bars represent standard error across individual soil samples within each bed.

Objective 1.4: Development of disease areas for onions and stocks

- Milestone 1.4a Develop FOC infested field at Wellesbourne for qPCR and DNA barcoding testing (completed in year 1).
- Milestone 1.4b Develop FOM infested field at Cut Flower Centre for qPCR and DNA barcoding testing (completed in year 1).

Summary of year 1 results

- Artificial inoculation of a field area for FOC at Wellesbourne and a polytunnel for FOM at the CFC was successful in creating high disease levels in bulb onions and stocks respectively.

Aim 2: To determine the effect of Fusarium inoculum concentration on disease development

Objectives 2.1, 2.2, 2.3: Determine the effect of *F. oxysporum* inoculum level on disease development in onions, stocks and narcissus

- Milestone 2.1 Determine the effect of different FOC inoculum levels on disease development in onion plants (completed in year 1).
- Milestone 2.2 Determine the effect of different FOM inoculum levels on disease development in stock plants (completed in year 1).
- Milestone 2.3 Determine the effect of different FON inoculum levels on disease development in *Narcissus*.

Objective 2.4: Quantify colonisation of *F. oxysporum* on onions, stocks and Narcissus

- 2.4a Use qPCR developed in 1.2 to quantify FOC, FOM and FON in selected inoculum treatments in 2.1-2.3 over time (completed with the exception on FON).

Summary of year 1 results

- Experiments using compost inoculated with different concentrations of FOC and FOM inoculum identified the critical levels required to cause significant disease development in onions and stocks respectively.

- For both FOC and FOM, few disease symptoms were observed at the lowest concentration of 1 x 10^2 cfu g^{-1} while between 1 x 10^5 and 1 x 10^6 cfu g^{-1}, disease development was at a maximum with almost all plants dead by the end of the experiment.

Materials and Methods

Effect of FON inoculum concentration on disease development

A bran/compost inoculum of FON isolate 139 was prepared as described in the Annual Report 2018 and mixed into a *Narcissus* growing medium (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})) using a cement mixer to achieve a range of concentrations from 1 x 10^2 - 1 x 10^6 cfu g^{-1} and dispensed into 20 cm, 4 L pots. Five *Narcissus* bulbs (cv. Carlton, no fungicide treatment obtained from Lingarden Bulbs Ltd) were planted approx. 10 cm deep (measured to the base of the bulb) in each pot with 28 replicate pots per treatment. An untreated control treatment was also set up with bulbs planted in clean growing medium only. Planting was carried out over two days (12/10/17-13/10/17) and all pots were placed in saucers in a frost-free glasshouse under natural light with shading and watered from below as required (Fig. 19). Pots were arranged in a randomised block design and assessments for emergence, foliar symptoms (chlorosis and leaf die-back), number of flowers and plant mortality were recorded on 06/03/18, 05/04/18 and 11/05/18. Foliage was allowed to die back naturally after which, bulbs were lifted and basal rot scored on 31/07/2018 using a 0-10 scale (Table 10).

Table 10: Scoring scheme for assessing the severity of Fusarium basal rot in *Narcissus* bulbs (from AHDB project BOF 74a).

Score	Severity	Zones affected by basal rot
0	Low	None
1	Low	Spots (up to 2mm-diameter) in base plate
2	Low	Small area of basal plate (up to 10%) but no spread to bulb scales
3	Medium	Up to 25% of basal plate area but no spread to bulb scales
4	Medium	Up to 50% of basal plate area but no spread to bulb scales
5	Medium	More than 50% of basal plate area but no spread to bulb scales
6	High	Start of spread from basal plate to bulb scales (up to 10% of scale area)
7	High	Up to 25% of bulb scale area
8	High	Up to 50% of bulb scale area
9	High	More than 50% of bulb scale area
10	High	Whole bulb (or virtually whole bulb) (includes dried, 'mummified' bulbs)

Figure 19: Glasshouse assay to determine the effect of different FON inoculum levels on disease development in *Narcissus*.

Quantifying colonisation of onion roots by FOC using qPCR

To assess root colonisation by FOC, onion plants were transplanted into compost infested with different levels of the pathogen as described in the Annual Report 2018 using the published method of Taylor et al., (2013). A bran/compost inoculum of FOC isolate FUS2 was prepared and mixed into M2 compost to achieve of concentrations of 1 x 10^3, 1 x 10^4 and 1 x 10^5 cfu g^{-1}. These concentrations were selected based on results from year 1 experiments as they resulted in different rates of disease development. Infested compost was dispensed into 7 cm pots and five-week-old onion seedlings (cv. Hytech) transplanted (one plant per pot, 48 pots per concentration). An untreated control treatment (M2 compost only) was also set up. Pots were arranged in a randomised block design in a glasshouse set at 25°C day, 18°C night, 16 h day-length. For half the onion plants in each treatment, death due to Fusarium was recorded twice weekly for nine weeks until the bulbs had fully formed, when watering was ceased and plants allowed to dry out after which they were bisected and symptoms of basal rot scored on a 0-3 scale, as described by Taylor et al., (2013). This allowed a direct comparison of disease development with the same treatments tested in Year 1. The remaining half of the plants for each treatment were harvested at 0h, 24h, 48h, 72h, 96h, 7d and 14d post-transplanting into the infested compost in order to assess FOC colonisation of onion roots. At each of these timepoints, four replicate onion plants were harvested for each inoculum concentration, roots washed to remove soil and then flash frozen in liquid N and stored at -80°C after which they were freeze-dried. For qPCR, up to 20 mg of freeze-dried onion root tissue was disrupted in a lysing matrix A tube (MPBio) by a FastPrep-24™ machine (MPBio) set at 6 ms^{-1} for 40 s. A DNeasy plant mini kit (Qiagen) was then used for DNA extraction using the manufacturers protocol but with the addition of an extra centrifugation step after the cell lysis stage (13,000 rpm for 5 min). DNA quality was checked using a DeNovix DS-11 Spectrophotometer and by running a small volume on a 1% agarose gel. Following initial optimisation, qPCR (Roche Lightcycler) was then carried out using the FOC assay as previously described using DNA diluted with an equal volume of TE.

Quantifying colonisation of stocks roots by FOM using qPCR

To assess root colonisation by FOM, stocks plants were transplanted into compost infested with different levels of the pathogen as described in the Annual Report 2018 using the published method of Taylor et al., (2013) for FOC. A bran/compost inoculum of FOM isolate Stocks 4 was prepared and mixed into M2 compost to achieve concentrations of 1 x 10^3, 1 x 10^4 and 1 x 10^5 cfu g^{-1}. As for FOC, these concentrations were selected based on results from year 1 experiments as they resulted in different rates of disease development. Plug plants of stocks (cv. Fedora Deep Rose, roots washed to remove compost) were transplanted into the

infested compost in 7 cm pots (one plant per pot, 48 pots per concentration). An untreated control treatment (M2 compost only) was also set up. Pots were arranged in a randomised block design in a glasshouse set at 25°C day, 18°C night, 16 h day-length. As for FOC, half the stocks plants in each treatment were assessed for wilting and death due to Fusarium twice weekly while the other half were harvested at 0h, 24h, 48h, 72h, 96h, 7d and 14d post-transplanting into the infested compost, in order to assess FOM colonisation of stocks roots. At each timepoint, four replicate stocks plants were harvested for each inoculum concentration and roots processed and freeze dried as described above for FOC. However, as it was challenging to remove all soil particles from the root system, DNA was extracted from up to 20 mg of tissue using a SoilSV kit and quality checked using a DeNovix DS-11 Spectrophotometer and by running a small volume on a 1% agarose gel. Following initial optimisation, qPCR (Roche Lightcycler) was then carried out using the FOM assay as previously described using undiluted DNA.

Quantifying colonisation of *Narcissus* roots by FON using qPCR

In 2017, we set an experiment to assess the effect of FON dose on disease development. Unfortunately, due to the very high background level of basal rot in the untreated controls, no dose-response was observed. In October 2018, the experiment was repeated using a batch of bulbs (cv. Carlton) from Scotland which appeared to have a low level of background infection. Bulbs were planted (5 per pot, 28 replicate pots per treatment) in infested compost ranging from 0 – 1 x 106 cfu g-1. On 4th June 2019 bulbs were bisected and the level of basal rot scored on a 1-10 scale.

Results

Effect of FON inoculum concentration on disease development

Unfortunately, the *Narcissus* bulbs used in this experiment had high background levels of FON (not obvious at planting) which confounded the results and obscured any relationship between FON inoculum levels and disease development (Fig. 20a). Based on the data from the assessment on 11/05/18 (7 months after planting), there was little difference in the percentage plants with Fusarium disease symptoms between the different inoculum concentration treatments with 60% of the uninoculated *Narcissus* control plants showing symptoms compared with 65% of plants in the highest FON concentration (1 x 10^6 cfu g^{-1}; Fig. 20a). Fusarium disease in the uninoculated control plants was also confirmed when the bulbs were subsequently lifted and bisected with no or little differences in disease score between treatments (Fig. 20b, Fig. 21).

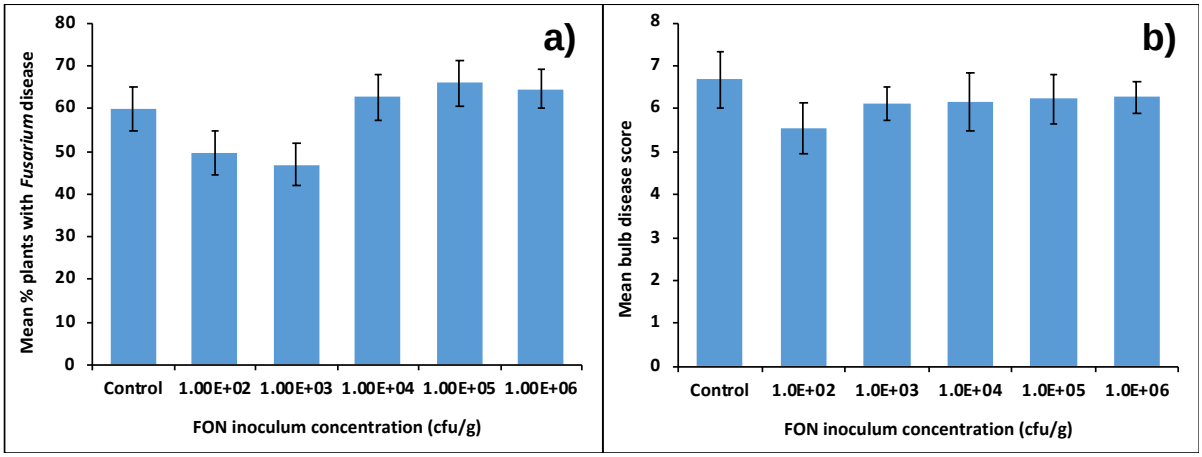
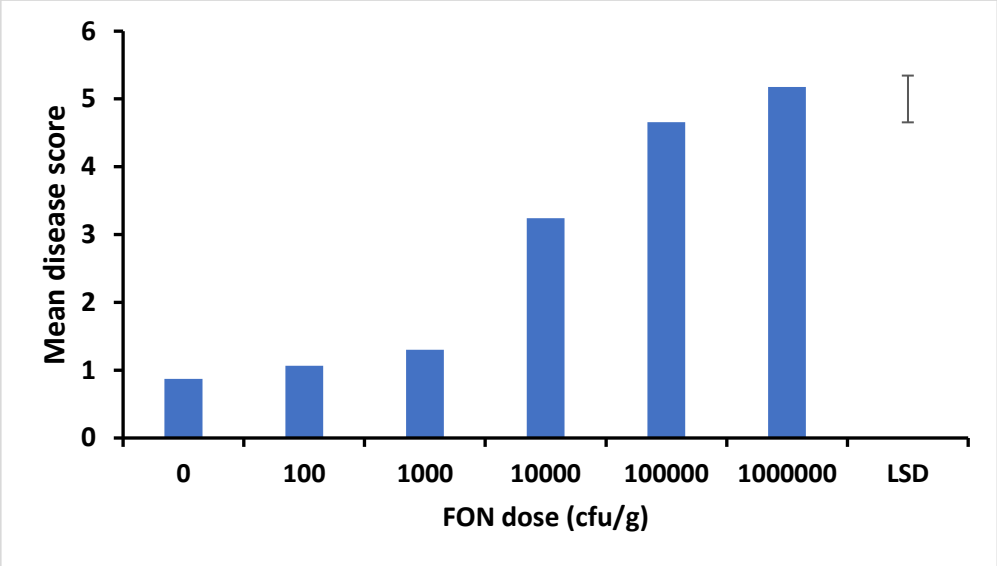


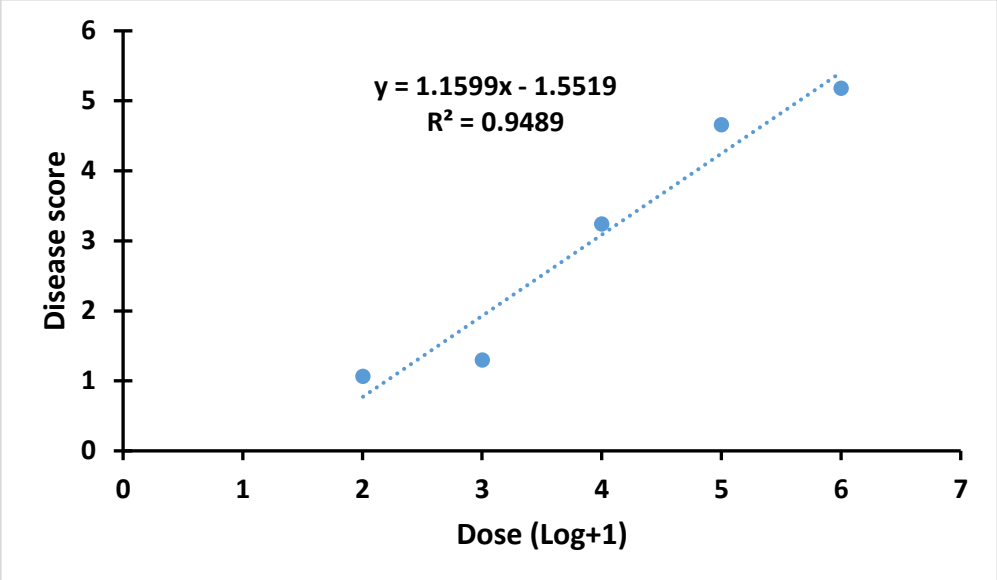
Figure 20: a) Percentage of *Narcissus* plants with basal rot symptoms after 7 months and b) mean disease score for *Narcissus* bulbs at harvest following planting in compost infested with different concentrations of FON. Error bars indicate the SEM of 28 replicate pots.

Figure 21: Fusarium basal rot symptoms on non-inoculated *Narcissus* bulbs.

Results from the repeated experiment to investigate the effect of FON concentration on disease development

A very clear dose-response was observed (see graphs below) with no disease below 10,000 cfu g^{-1}. A low level of background infection was observed in the non-inoculated controls. Soil and root samples have been taken and DNA will be extracted to further validate out FON qPCR assay.





Quantifying colonisation of onion roots by FOC and FOM using qPCR

The development of Fusarium disease on plants inoculated with different selected levels of FOC and FOM inoculum was comparable with the year 1 results with different rates of disease development at 1000, 10,000 and 100,000 cfu g^{-1} (Fig. 22 ab, Fig. 23 ab).

Despite Fusarium disease symptoms not being evident on onions until 14 days post transplanting, colonisation of roots by FOC was detectable by qPCR from 2 days post transplanting for inoculum levels of 10,000-100,000 cfu g^{-1} (Fig. 24a). The amount of FOC DNA detected in onion roots then increased rapidly for these treatments over subsequent timepoints of 4, 7 and 14 days (Fig. 24a). FOC DNA was (inconsistently) detected by qPCR at a very low level in the 1000 cfu g^{-1} FOC treatment over the entire time course.

Similarly, although Fusarium disease symptoms on stocks was not evident until 18 days post transplanting, colonisation of stocks roots by FOM was detectable by qPCR from 2 days post transplanting for inoculum levels of 10,000-100,000 cfu g^{-1} (Fig. 24b). Again, as for FOC, the amount of FOM DNA increased rapidly for these treatments over subsequent timepoints. FOM DNA was undetectable by qPCR in the 1000 cfu g^{-1} FOM treatment until the final sampling point 14 days post transplanting (Fig. 24b).

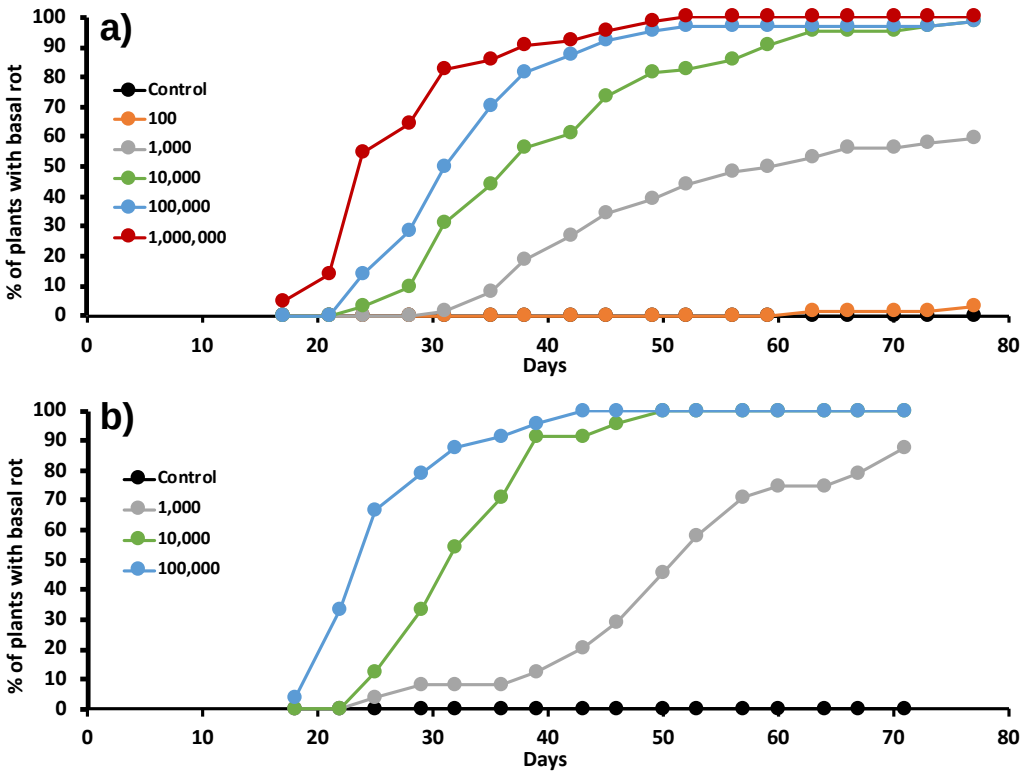


Figure 22: Fusarium disease development in onion plants for different levels of FOC inoculum (cfu g^{-1}) for experiments in a) year 1 and b) year 2.

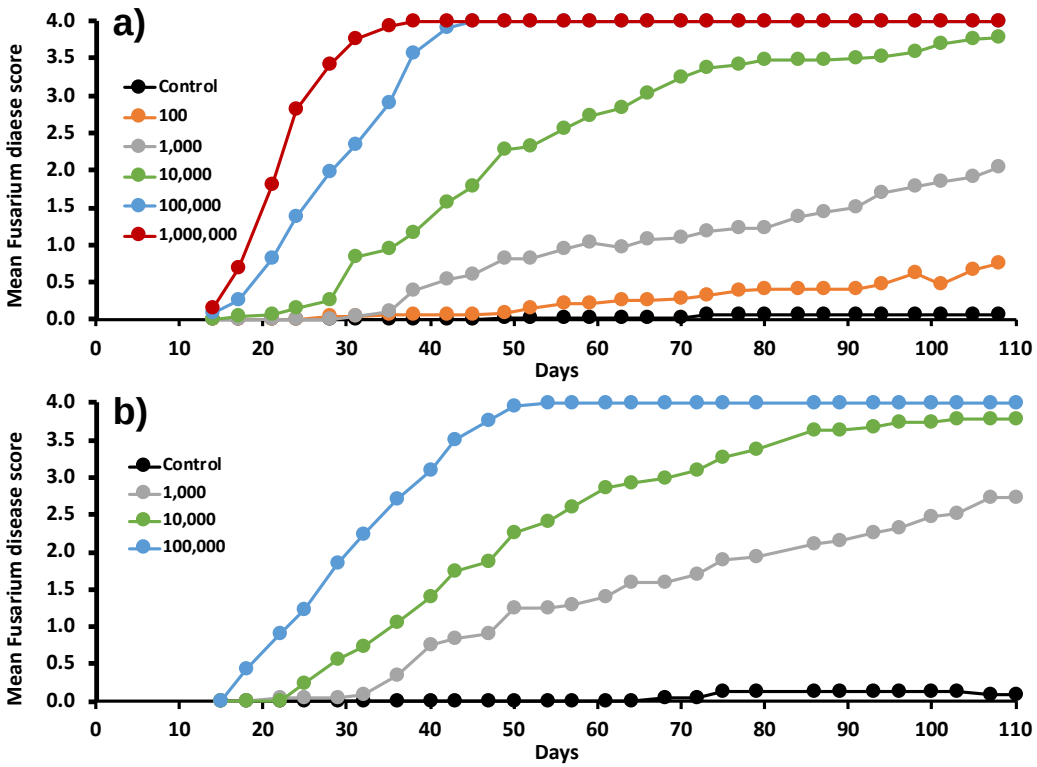


Figure 23: Fusarium disease development in stocks plants for different levels of FOM inoculum (cfu g^{-1}) for experiments in a) year 1 and b) year 2.

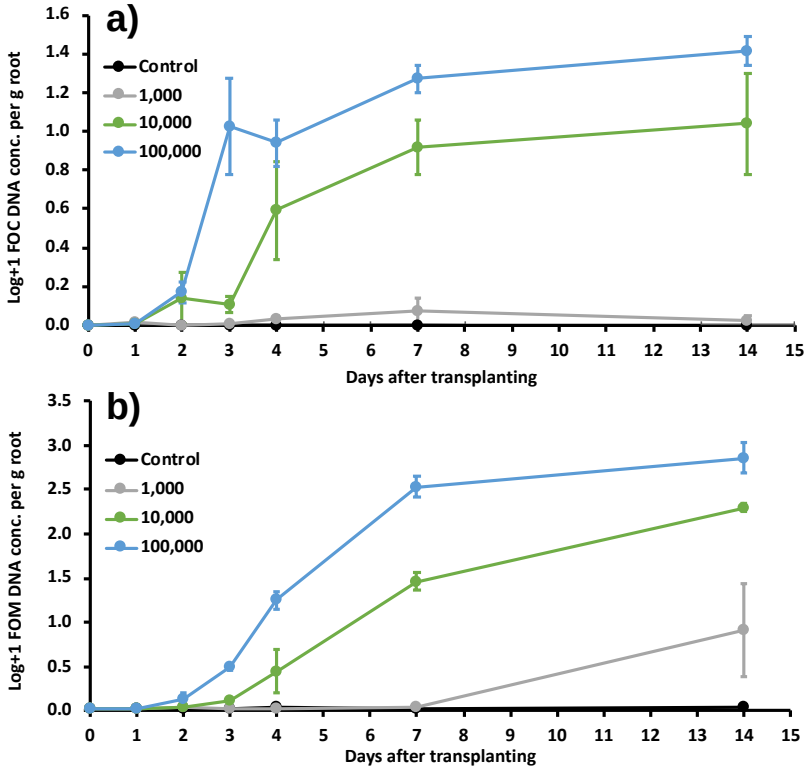


Figure 24: Fusarium root colonisation for a) onion plants inoculated with different levels of FOC and b) stocks plants inoculated with different levels of FOM using qPCR. Error bars represent the SEM of 4 biological replicates.

Discussion and conclusions

Aim 1: Development of molecular tools and resources for identifying and studying *Fusarium*

Objective 1.1: Collection, identification and pathogenicity testing of different *Fusarium* spp.

In year 1, four *Fusarium* species were identified in diseased leek plant samples from commercial crops; *F. culmorum*. *F. avenaceum*, *F. proliferatum* and *F. oxysporum*. All these species have been identified previously as causing a basal rot on leek plants (Armengol et al., 2001; Hall et al., 2007; Koike et al., 2003; Palmero et al., 2012). However, pathogenicity testing in year 2 indicated that *F. culmorum* and *F. avenaceum* caused significant disease on inoculated leek plants, with the former causing more severe symptoms while *F. proliferatum* and *F. oxysporum* caused little or no disease. This suggests therefore that future detection and management approaches should focus on *F. culmorum* and *F. avenaceum*. *F. culmorum* is more commonly known as a pathogen of small grain cereals such as wheat and barley causing foot and root rot as well as head blight although other reported hosts (in addition to leek) include sugar beet, flax, carnation, bean, pea, asparagus, red clover, hop, Norway spruce, strawberry and potato (Scherm et al., 2012). In addition, *F. culmorum* has been detected on leek transplants grown in a soilless rooting medium and on raised benches in enclosed greenhouses suggesting that the pathogen could be seedborne (Koike et al., 2003). Similarly, *F. avenaceum* also has a large host range, being reported to cause disease on more than 80 genera of plants including staple crops such as wheat, barley (again causing head blight) maize, legumes, oilseed rape and potato (Pollard and Okubara, 2018).

The generalist nature of both *F. culmorum* and *F. avenaceum* means that crop rotation may not be effective and the potential for seed borne transmission of both pathogens means that growers should be vigilant regarding crop hygiene.

Objective 1.2: Development of a specific quantitative (real-time) qPCR tests for *F. oxysporum* f.spp.

In year 1, specific qPCR tests were developed for FOC, FOM and FON based on pathogenicity genes identified through comparative genome analysis and further work in year

2 have shown these tests to be sensitive and applicable for testing of soil and plant samples. Data has also been generated that has begun to relate pathogen DNA levels (as measured through qPCR) to the number of spores in a soil sample, a first step to understanding how useful these tests can be for practical diagnostics and to determine inoculum levels in the field. However, further work is required to determine how these assays can be successfully implemented for assessing disease risk following testing of soil samples and in the case of FOC, as a means of potentially assessing levels of the pathogen in onions going into store. This would include optimisation of sampling and testing strategies across multiple onion, *Narcissus* and stocks commercial field sites and monitoring of symptoms in order to build a relationship between pathogen DNA test results and disease levels.

Objective 1.3: Development of a DNA barcoding approach for analysis of *Fusarium* communities

In year 1, pathogenicity genes were identified in FOC, FOM and FON following genome analysis and subsequent comparison with other *Fusarium* spp. genomes. Several of these were present in one or more *F. oxysporum* f.spp. (but with different sequences) and hence could be used to potentially distinguish between these pathogens in an amplicon sequencing approach. Primers were developed for four of these genes (SIX13, OG13890, OG4952 OG13397) and used for PCR and amplicon sequencing to evaluate their utility in determining the presence and abundance of *F. oxysporum* f.spp. in mixed DNA 'pools' from multiple *Fusarium* spp, *F. oxysporum* f.spp and other soilborne fungal plant pathogens as well as in soil samples from areas infested with FOC (inoculated Quarantine Field, Wellesbourne), FOM (inoculated polytunnel, CFC) and FON (naturally infested field soil). This approach showed promise with one locus (OG4952) being particularly effective in detecting high levels of FOC, FOM and FON in infested soils. There were however some areas that require development and optimisation.

One problem was the low numbers of sequencing reads observed for two of the *F. oxysporum* f.spp. gene targets (SIX13 and OG13890). This could be due to a combination of factors. Firstly, SIX13 (used primarily to detect FON, FOL and FOP) is only present in some FON isolates so it is possible that these were not present in the infested daffodil field. Secondly, the DNA from the FON daffodil field soils samples was at a much higher concentration than for the other field samples, and was therefore subject to more dilution before PCR which could have led to low read numbers. The amount of DNA and level of dilution before PCR and amplicon sequencing may therefore need to be optimised for different field samples to improve detection. The low level of reads for OG13890 across a whole range of soil samples

as well as the mixed species DNA pools could be due to PCR bias against this gene target in the multiplex reactions suggesting that it may be better not to use it in multiplex reactions with primers for other targets.

A second problem with the *F. oxysporum* f.spp. amplicon sequencing was that FOC, FOM and FON were unexpectedly detected in soils not infested with those particular pathogens. For instance, FOC and FOM were detected at higher levels in the daffodil field soil than FON, while FOM was detected in both FOC and FON field soils. While it is possible that these pathogens were also present in soil, qPCR using specific primers for FOC, FOM and FON only detected these pathogens in the onion, stocks and daffodil soils respectively (as expected) with the exception of FON detected in the FOM soil, so further work is required to identify why this non-target detection occurred. It is possible that this is a result of sample contamination or sequencing errors, or that there are other unknown *F. oxysporum* f.sp. isolates present in the fields that share the same sequence.

As well as specific gene targets for detection of *F. oxysporum* f.sp., results showed that PCR and amplicon sequencing of 16S, ITS and TEF housekeeping genes was very effective in determining the presence and abundance of bacteria, fungi and *Fusarium* spp. respectively in soil. In particular, TEF identified a range of *Fusarium* spp. in the FOC, FOM and FON infested soils and as expected a very high abundance of *F. oxysporum*. 16S and ITS have been routinely used in amplicon sequencing to define the composition of bacterial and fungal communities (Hill et al., 2000; Lindahl et al., 2013) while TEF has been employed recently to define the composition of *Fusarium* communities associated with Fusarium head blight (Edel Hermann et al., 2016) so we can confirm the utility of these gene targets for horticultural soils.

Finally, results of the amplicon sequencing were generally consistent across beds in FOC, FOM and FON infested areas suggesting that a realistic sampling strategy can be developed in the future to optimise detection of these pathogens. However, FOC and FOM soils were artificially infested with the aim of spreading these pathogens evenly across these areas so further work needs to determine if distribution of *F. oxysporum* is more heterogeneous in naturally infested soils.

Overall, therefore the use of an amplicon sequencing using specific gene targets to define the presence and abundance of *Fusarium* spp. and *F. oxysporum* shows potential and is a novel approach. Alongside more conventional gene targets to define fungal and bacterial communities, this could be a powerful tool with which to dissect *Fusarium* disease complexes and examine dynamics in relation to the whole soil microbial community. Further work is now needed in order to fully optimise the technique and explore how it performs across multiple commercial onion and daffodil field and protected stocks cropping sites.

Objective 1.4: Development of disease areas for onions and stocks

Artificial inoculation of a field area for FOC and a polytunnel for FOM in year 1 was successful in creating high disease levels in bulb onions and stocks respectively. These areas provided a valuable resource for both validation of the specific qPCR tests for FOC and FOM as well as the amplicon sequencing. They are also being used in other AHDB projects as a means of testing new disease control products and approaches.

Aim 2: To determine the effect of Fusarium inoculum concentration on disease development

Objective 2.1-2.3: Determine the effect of *F. oxysporum* inoculum level on disease development in onions, stocks and narcissus.

Objective 2.4: Quantify colonisation of *F. oxysporum* on onions, stocks and Narcissus

In year 1, experiments determined the critical levels of FOC and FOM inoculum that are required to cause significant disease development in onions and stocks respectively and these were confirmed in year 2. The specific qPCR tests for FOC and FOM allowed root colonisation of these pathogens to be explored for the first time, and results have shown that this occurs and can be detected within a few days of the plants being introduced into infested soil, a couple of weeks before symptoms begin to be observed on plants. These tests may therefore be useful not only in detecting FOC, FOM and FON in soil in advance of the crop as outlined previously, but also in crops already planted where plants could be sampled to assess the likelihood of symptom development. Again, this approach requires testing across multiple commercial field sites.

Knowledge and Technology Transfer

- Journal paper: Armitage AD, Taylor A, Sobczyk MK, Baxter L, Greenfield BPJ, Bates HJ, Wilson F, Jackson AC, Ott S, Harrison RJ, Clarkson JP, 2018. Characterisation of pathogen-specific regions and novel effector candidates in *Fusarium oxysporum* f. sp. *cepae*. *Scientific Reports* **8**, 13530.
- Journal paper: Taylor A, Jackson AC, Clarkson JP, 2018. First report of *Fusarium oxysporum* f. sp. *lactucae* race 4 causing lettuce wilt in England and Ireland. *Plant Disease*. *In Press*.
- Taylor A, Barnes, A, Jackson AC, Clarkson JP, 2018. First report of *Fusarium oxysporum* and *Fusarium redolens* causing wilting and yellowing of wild rocket (*Diplotaxis tenuifolia*) in the UK *In Press*.
- Article in AHDB grower, 'Fusarium Futures' (June 2017)
- Article in AHDB grower, 'Tools of the Trade' (Dec 2018)
- Presentation: 'Understanding the genetic control of pathogenicity and resistance for *Fusarium oxysporum* in onion'. International Horticulture Research Conference, East Malling (17-19 July 2017)
- Presentation: Detection and quantification of Fusarium diseases. National Cut Flower Centre Open Evening (8 August, 2018).
- Presentation: 'Understanding the genetic control of pathogenicity and resistance for *Fusarium oxysporum* in onion'. BSPP Presidential meeting in Nottingham (11th - 13th Sept 2017)
- Presentation: 'Root rots, bulb rots and wilts: tackling Fusarium in onion and other crops'. Carrot and Onion Conference, Nottingham, (13-14 November 2017)
- Presentation: 'Update on diagnostics for lettuce Fusarium wilt and discussion on sampling and monitoring'. Lettuce Fusarium Workshop, Skelmersdale (14 December 2017)
- Presentation: 'Update on lettuce Fusarium wilt: minimising impact on the UK lettuce industry'. Outdoor and Protected Leafy Salad Technical Day, Stockbridge House (20 March 2018).
- Presentation: 'Understanding the genetic control of pathogenicity and resistance to *Fusarium oxysporum* in onion'. 10th Australasian Soilborne Diseases Symposium, Adelaide (4-7 September 2018).
- Presentation: 'Lettuce Fusarium wilt: potential management options'. Growing media developments in vegetable propagation, Doddington. (9th October 2018).

- Presentation: ‘Lettuce Fusarium wilt: potential management options’. BLSA Protected R & D Committee meeting, Warwick (16 October 2018).
- Presentation: ‘Biology and control of Fusarium diseases across multiple crops’. British Onions & AHDB Horticulture Bulb Onion Variety Trials meeting. (1st November, 2018).
- Presentation: ‘Lettuce Fusarium wilt in the UK’. Brassicas and Leafy Salads Conference, Peterborough, (23 January 2019).

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