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

AUTHENTICATION

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

John Clarkson

Reader

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Signature:  Date: 23/03/2018

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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 oxysporum*

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. Approaches have also relied in the past 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of soil sterilisation or fumigation, fungicides or seed treatments but approval for their use in many cases has been withdrawn or threatened by further legislation.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 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 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 (9159 ha valued at approx. £106.1M in 2015; Defra, 2016) is lost each year in the field with a corresponding economic value of £6.4M but more recently, basal rot incidence of 10% or greater is becoming more common, equating to losses of approx. £10.6M. 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 £37M 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 disease 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). 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.

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 aims 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 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 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*

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

Leeks with symptoms of basal rot were collected from Lincolnshire, Norfolk and Cambridgeshire and *Fusarium* spp. isolated. DNA was then extracted from each isolate and identity determined through PCR and sequencing of part of the TEF gene. A total of four *Fusarium* species were identified; *F. avenaceum*, *F. culmorum*, and *F. oxysporum* and *F. proliferatum*. There was little within-species diversity based on the TEF sequences except for *F. oxysporum* where there were two groups, one of which corresponded to that containing FOC isolates pathogenic on onion. In additional work, six *Fusarium* species were also identified from diseased asparagus samples; *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. flocciferum, F. oxysporum* and *F. proliferatum*. Pathogenicity tests were also developed for leek and ongoing work will evaluate the relative ability of representatives of the four identified species to cause disease.

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

The FOM isolate stocks 4 as well as FON isolates FON63, 77, 89, 129, 139 were sequenced using both MinION and high-accuracy Illumina technologies to add to existing genome data for FOC. FOC, FOM and FON genomes were then compared with 133 publically available *Fusarium* genomes and the presence of SIX and other pathogenicity genes identified. A total of 13 genes in FOM, 48 genes in FON and 24 in FOC were identified as potential targets for specific PCR-based diagnostic tests as well as for identification of a range of different f.spp. using amplicon sequencing. FOM contained SIX1, SIX8 and SIX9 while FON isolates contained between two and five SIX genes in different combinations of SIX7, 9, 10, 12, 13. By comparison, FOC was previously shown to contain SIX3, 5, 7, 9, 10, 12 and 14 (Taylor et al., 2016). These results were all confirmed by PCR. SIX5 was identified as a potential PCR

diagnostic target for FOC (only otherwise present in FOL) while one gene (Ortho_g153) and two genes (Ortho_g16122, Ortho_g17178) were identified for FOM and FON respectively as diagnostic targets, as they were unique to these *F. oxysporum* f.spp. Primers designed for these genes (Ortho_g17178 for FON) resulted in specific amplification of FOC, FOM and FON respectively with no amplification of DNA from 62 *F. oxysporum* f.spp., *Fusarium* spp., and other fungi / oomycetes that were tested. Although these qPCR assays need to be further validated using soil / plant samples containing FOC, FOM or FON, they should provide effective tools for studying the dynamics of the individual *F. oxysporum* f.spp. and a means of examining the colonisation of both host and non-host plants.

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

Following the assessment of the distribution of pathogenicity-related genes throughout a total of 164 *Fusarium* genomes, seven genes (SIX13, FOC_g17143, Ortho_g10859, Ortho_g13890, Ortho_g4927, Ortho_g4952, Ortho_g12981) were selected as potential targets for amplicon sequencing as they were predicted to amplify FOC, FOM or FON as well as some other *F. oxysporum* f.spp where they occurred with sequence variation that would allow each of these different pathogens to be distinguished. Primers were designed for these gene targets as well as ITS and TEF. Initial testing of PCR primers for these genes showed that various combinations of these target genes in amplicon sequencing have potential to identify common fungi (both pathogens and non-pathogens), different *Fusarium* spp. and *F. oxysporum* f.spp. in a single DNA sample extracted from soil or roots. However, this needs to be validated using soil / samples containing FOC, FOM and FOC.

1.4: Development of *Fusarium* disease areas for onions and stocks

Artificial inoculation of a field area for FOC and a polytunnel for FOM using inoculum of each pathogen grown on sterile compost / bran was successful in creating high disease levels in bulb onions and stocks respectively. These areas provide a valuable resource for both validation of the specific qPCR tests for FOC and FON as well as the amplicon sequencing and will also provide a means of testing new disease control products and approaches in the future.

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

2.1-2.3 Determine the effect of different FOC, FOM and FON inoculum levels on disease development in onions, stocks and narcissus plants.

A bran/compost inoculum for FOC, FOM and FON was prepared and dispensed into pots to achieve a range of concentrations from 1 x 10^2 - 1 x 10^6 cfu g^{-1}. Onions, stocks (transplants) or narcissus (bulbs) were then planted and disease recorded over time. For onions and stocks, levels of between 1 x 10^4 and 1 x 10^6 cfu g^{-1} resulted in rapid disease development but few disease symptoms observed at 1 x 10^2 cfu g^{-1}. Hence, critical levels of FOC and FOM inoculum required to cause significant disease development have been identified and further work will now utilise the specific qPCR tests for FOC and FON in repeat experiments to relate qPCR values to inoculum rate and disease development. This will be an important first step in assessing the utility of the PCR tests for assessing disease risk in the field. Experiments with FON are still ongoing.

Benefits

- The molecular diagnostic tests developed for FOC, FOM and FON may provide a way of assessing disease risk as a commercial service in the future.
- Critical levels of FOC and FOM inoculum required for significant disease development.

Action Points

None at this time.

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* 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* 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 more than 70 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 in the past 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). 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, 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 (9159 ha valued at approx. £106.1M in 2015; Defra, 2016) is lost each year in the field with a corresponding economic value of £6.4M but more recently, basal rot incidence of 10% or greater is becoming more common, equating to losses of approx. £10.6M. 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, 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 £13.9M (Hort stats 2016, 2015 data), 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 disease 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). 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 (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 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 a BBSRC HAPI project (AHDB CP 116), 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. 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 aims 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 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 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

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).
- Milestone 1.1c Test pathogenicity of *Fusarium* isolates from leeks (ongoing)
- 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).

Materials and Methods

Isolation and identification of *Fusarium* spp. from leeks

Leek samples with Fusarium basal rot disease symptoms were obtained from Lincolnshire, Norfolk and Cambridgeshire (Fig. 1) through the British Leek Growers Association with the help of David Norman. To isolate *Fusarium* spp., leeks were dissected longitudinally and 0.5 cm sections of internal tissue removed which were then surface sterilised by immersing in 70% ethanol for 1 min before rinsing twice in sterile distilled water (SDW) and placing onto potato dextrose agar (PDA) containing 20 µg ml^{-1} chlortetracycline. Plates were incubated at 20°C and colonies with *Fusarium* morphology further sub-cultured onto PDA. DNA was then extracted from mycelium from each culture using a rapid method developed by Stephen Rehner (USDA-ARS, Beltsville, USA) and PCR amplification of part of the TEF gene carried out using conditions and primers published previously (Taylor et al., 2016). PCR amplification was confirmed by gel electrophoresis and the remaining PCR product purified (QIAquick PCR Purification Kit, Qiagen) and sequenced (GATC Biotech). Sequences were identified through BLAST analysis, aligned with additional sequences from *Fusarium* spp. isolated from leek previously at Warwick and phylogenetic trees constructed using the methods described by Taylor et al., (2016).

Figure 1: Symptoms of leek basal rot caused by *Fusarium* spp.

Isolation and identification of *Fusarium* spp. from asparagus

In additional work, samples of asparagus plants with Fusarium disease symptoms (stem yellowing / browning a and discolouration of the vascular system; Fig. 2) were obtained from Tiddington, Warwickshire (three fields), Luddington, Warwickshire (samples from polytunnel) and Birlingham (near Pershore, Worcestershire). Isolations were carried out as described for leeks except that surface sterilisations were carried out by soaking stem pieces in 5% sodium hypochlorite for 1 min. DNA extraction, PCR and sequencing were carried out as described previously.

Figure 2: Typical symptoms of *Fusarium* spp. infection on asparagus: 1) vascular browning, b) yellowing of stems.

Development of *Fusarium* spp. pathogenicity tests for leek

Two *Fusarium* species isolated from leek (*F. avenaceum* L5, *F. oxysporum* L2-1) were selected for initial pathogenicity testing. Isolates were grown on PDA for 14 d at 20°C and

spore suspensions made by adding sterile water to plates 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 (L2-1) and 1 x10^5 (L5) spores ml^{-1}. Glasshouse grown leek plants (nine weeks old, cv. Musselborough) were removed from raising modules, rinsed with sterile water, blotted dry and then either left intact or one third of the root system cut off with scissors. Plant roots (eight plants per treatment) were then soaked in 80 ml of spore suspension from each *Fusarium* isolate for 5 min before transplanting into M2 compost in 7 cm pots. Plants were kept well-watered, incubated at 25°C with a 16 h day length and monitored for disease symptoms.

Results

Isolation and identification of *Fusarium* spp. from leeks

Four *Fusarium* species were identified from the diseased leek samples (Table 1); *F. culmorum* (three sites), *F. avenaceum* (four sites), *F. proliferatum* (two sites) and *F. oxysporum* (three sites). The phylogenetic tree indicated that the isolates of *F. proliferatum* were very closely related whilst two distinct groups of *F. oxysporum* isolates were observed (*F. oxysporum* clade 1 / clade 2; Fig. 3). One of these groups (*F. oxysporum* clade 2) corresponded to the same clade that contains *F. oxysporum* f.sp. *cepae* isolates pathogenic on onion (Taylor *et al*., 2016). All *F. culmorum* isolates shared identical sequences as did all *F. avenaceum* isolates, with the exception of isolate UK 7-3 (Norfolk; Fig. 3).

Table 1: *Fusarium* isolates identified from infected leek samples

Isolate	No. isolates	Location	Year isolated	Fusarium species / frequency
E1-E10	10	Lincs	2017	*F. avenaceum* (3) *F. culmorum* (7)
M1-1, UK3-1, UK3-2, UK6-1, UK7-1, UK7-3, UK8-1	7	Norfolk	2017	*F. avenaceum* (1) *F. oxysporum* (1) *F. proliferatum* (5)
AP2, AP5, AP7, AP8, AP10, AP11-AP14	9	Cambs	2017	*F. oxysporum* (6) *F. proliferatum* (3)
6-1, 8, 10-1, 13-1, 15	5	Unknown	2014	*F. culmorum* (5)
Leek 1 - Leek 5	5	Lancashire	2014	*F. oxysporum* (5)
L2-1, L2-3, L5, L9-1, L9-2, L8	6	Unknown	2011	*F. avenaceum* (1) *F. oxysporum* (5)
LB8, LB9, LLI1	3	Cambs	2010	*F. avenaceum* (2) *F. culmorum* (1)

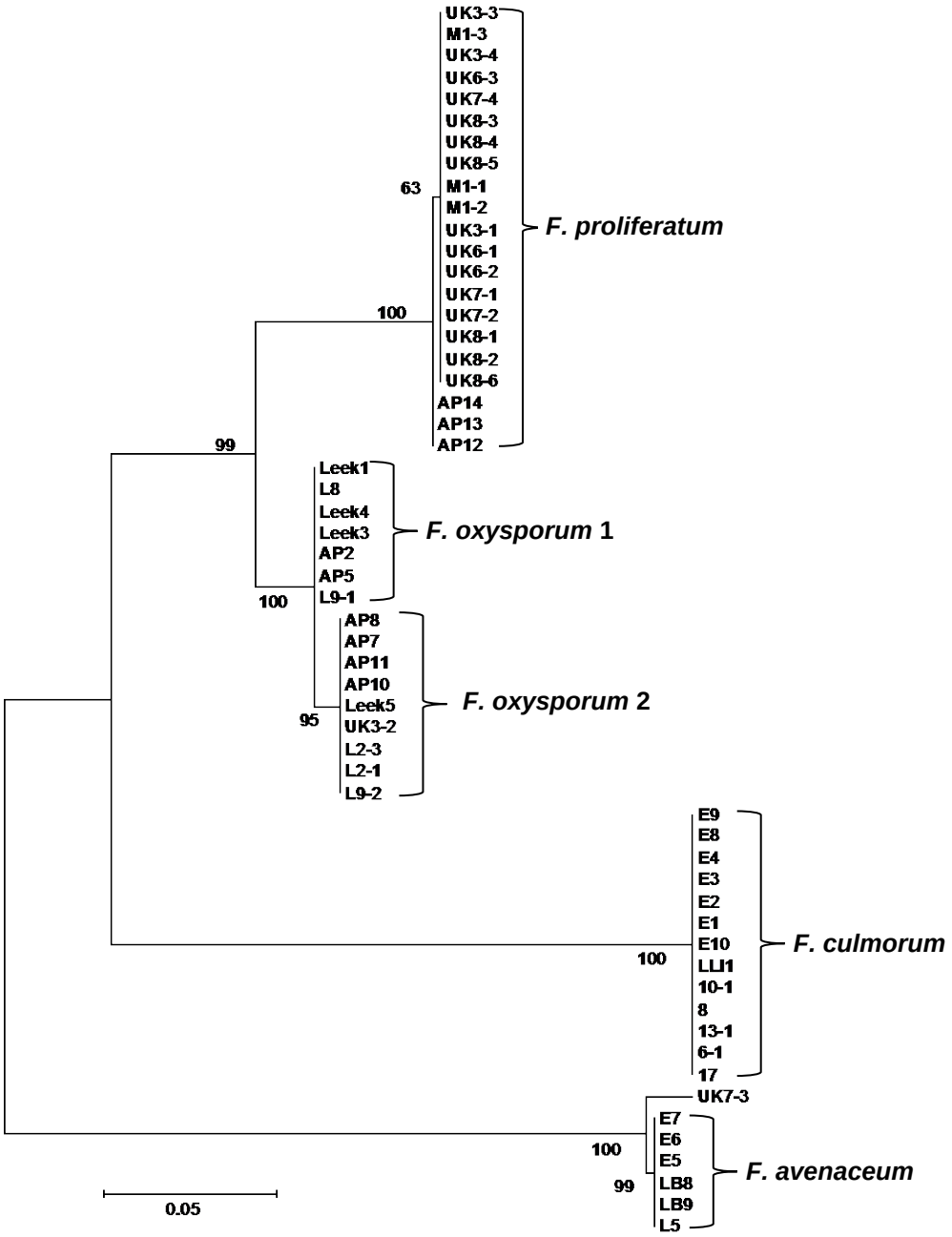

Figure 3: Phylogenetic tree (maximum likelihood method) showing diversity of *Fusarium* isolates from leek based on TEF sequence. Numbers represent bootstrap values (1000 replicates). *F. oxysporum* 2 clade has the same sequence as *F. oxysporum* f.sp. *cepae* infecting onion.

Isolation and identification of *Fusarium* spp. from asparagus

Five *Fusarium* species were identified from the diseased asparagus samples (Table 2); *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. oxysporum* and *F. proliferatum*. In addition, two isolates provided previously by ADAS were identified as *F. oxysporum* and *F. flocciferum*. None of these have been tested for pathogenicity.

Table 2: *Fusarium* isolates identified from infected asparagus samples

Isolate	No. isolates	Location	Year isolated	Fusarium species / frequency
ASP1-21	13	Tiddington, Warwickshire (3 fields)	2017	*F. culmorum* (4) *F. equiseti* (3) *F. oxysporum* (4) *F. proliferatum* (2)
PT1-4	3	Luddington, Warwickshire	2017	*F. avenaceum* (1) *F. culmorum* (1) *F. proliferatum* (1)
ASPJ1-5	1	Birlingham, Worcestershire	2017	*F. equiseti* (1)
BX17/90A & BX17/90B	2	From ADAS	Unknown	*F. oxysporum* (1) *F. flocciferum* (1)

Development of *Fusarium* spp. pathogenicity tests for leek

Initial results suggested that the root dip test was an effective assay for evaluating pathogenicity of *Fusarium* spp. on leek. First wilt symptoms were observed for *F. avenaceum* L5 9 days after inoculation (Fig. 4) both for plants with intact and trimmed roots while initial symptoms for *F. oxysporum* L2-1 appeared 14 d after inoculation. This experiment is still in progress.

Figure 4: Symptoms of infection by *F. avenaceum* (left) on leek 9 days post inoculation compared to non-inoculated control (right)

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

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

Materials and methods

Genome sequencing of FOM and FON and comparative genomics

The FOM isolate stocks4 as well as FON isolates FON63, 77, 89, 129, 139 were selected for genome sequencing with the aim of generating reference-quality assemblies. To do this, MinION sequencing was performed, which benefits from long read lengths which in conjunction with high-accuracy Illumina sequencing data allowed assembly of these genomes into 34 (FOM stocks 4) and 40 contigs (FON63) (chromosome fragments), which was comparable to a reference FOC genome (isolate FUS2) generated previously (34 contigs). Using bioinformatics pipelines, ‘core’ and ‘lineage specific’ (LS) genome regions were identified and functional annotation performed. Core regions generally contain genes required for general growth and reproduction in common to all *F. oxysporum* f.spp. while LS regions often contain pathogenicity-related genes associated with host specificity such as SIX genes. These pathogenicity genes are most often associated with so called ‘miniature impala’ (MIMP) short sequence inverted-repeat transposable elements, allowing MIMPs to be used as a means of identifying these genes in the genome (van Dam et al., 2016).

Identification of SIX and other pathogenicity genes for diagnostics

As described previously, it has been shown that focusing on pathogenicity genes (including SIX genes) is a good approach for DNA-based diagnostic tests for *F. oxysporum* f.spp and potentially other *Fusarium* spp. Potential targets for this approach were therefore identified through determining the presence / absence of all the pathogenicity genes identified from the multi-genome analyses at four levels; i) within *F. oxysporum* isolates of the same f. spp., ii) across multiple *F. oxysporum* f.spp., iii) non-pathogenic *F. oxysporum* isolates and iv) in other species of the *Fusarium* genus. Sequencing as part of this project and the previous BBSRC HAPI project (AHDB CP 116) has generated a total of 31 genomes including *F. oxysporum* pathogens of onion (FOC), stocks (FOM), narcissus (FON), pea (*F. oxysporum* f.sp. *pisi* ; FOP), strawberry (*F. oxysporum* f.sp. *fragariae*; FOF), *F. oxysporum* non-pathogens as well as other species such as *F. avenaceum* and *F. proliferatum*. These data along with 133 publically available *Fusarium* genomes were searched for presence of all pathogenicity genes identified from FON, FOM and FOC. The presence / absence of specific SIX genes (also generally in LS regions and associated with MIMPs) previously identified as being associated with pathogenicity of FOC in onion and other f.spp. was also determined for FOM and FON and confirmed using PCR as described by Taylor et al., (2016).

Development of qPCR for FOC

Putative FOC-specific primers based on SIX5 were designed manually following alignment of the FOC isolate FUS2 SIX5 gene with available sequences (from whole genomes and GenBank) using MEGA version 7 (Kumar et al., 2015). Primers were initially tested using conventional PCR carried out in 20 µl reactions containing primers (final concentration 0.5 µM), 5 µl of RedTaq (Sigma, UK) and 1 µl of DNA with the following thermocycling conditions: 1 cycle of 94°C for 2 mins followed by 35 cycles of 94°C for 45s, 60°C for 30s and 72°C for 30s followed by 1 cycle of 72°C for 5 mins. PCR amplification was confirmed by gel electrophoresis and the remaining PCR product purified and sequenced to confirm identity. To confirm that the specific primers were effective for a range of FOC isolates, PCR was carried out as described above using DNA from a collection of 30 diverse isolates from onion (Taylor et al., 2016). Next, to test the suitability of primers for a quantitative assay, qPCR was carried out using a dilution series of FOC FUS2 DNA ranging from 10 ng μl^{-1} -10 fg μl^{-1}. qPCRs were carried out in 10 µl reactions containing primers (final concentration 0.5 µM), 5 µl SensiFAST™ SYBR® No-ROX Kit mastermix and 1 µl of DNA with the following thermocycling conditions: 1 cycle of 95°C for 3 min 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. Finally,

the specificity of the new SIX5 primers was tested by carrying out PCR and qPCR against a panel of 62 DNA samples from a range of different *F. oxysporum* f.spp., *Fusarium* species, other common soilborne oomycetes / fungi as well as other selected onion pathogens (Table 3).

Development of qPCR for FOM

FOM specific primers were designed based on a putative effector gene identified by the presence of a MIMP motif. PCR and qPCR testing were carried out as described for the FOC primers with identical PCR reaction and thermocycling conditions with the exception of the annealing temperature (62°C) and primer concentration (0.35 µM). To confirm that primers were effective for a range of FOM isolates, they were tested against 19 additional FOM isolates from the Warwick culture collection.

Development of qPCR for FON

FON specific primers were designed based on putative effector genes identified by the presence of a MIMP motif. PCR and qPCR testing were carried out as described for the FOC primers with identical PCR reaction and thermocycling conditions with the exception of the annealing temperature (62°C). To confirm that primers were effective for a range of FON isolates, they were tested against 30 isolates which represented different SIX gene complements (see results).

Table 3: Fungal and oomycete isolates used to test specificity of qPCR assays for FOC, FOM and FON.

No.	Species	Isolated from	Country	Name
1	*Fusarium oxysporum f.sp. cepae*	onion	UK	FUS2
2	*Fusarium oxysporum conglutinans (Race 2)*	Brassica/Arabidopsis	USA	NRRL 54008
3	*Fusarium oxysporum cubense*	banana	UK	E421A-3
4	*Fusarium oxysporum dianthi*	dianthus	UK	BX13/113
5	*Fusarium oxysporum freesia*	freesia	NL	NRRL26990
6	*Fusarium oxysporum gladioli*	gladioli	NL	NRRL 26993
7	*Fusarium oxysporum lini*	flax	UK	FOLIN
8	*Fusarium oxysporum lycopersici*	tomato	UK	FOL R1
9	*Fusarium oxysporum lycopersici*	tomato	UK	FOL R2
10	*Fusarium oxysporum lycopersici*	tomato	USA	FOL R3 (MN25)-NRRL 54003
11	*Fusarium oxysporum mathioli*	stocks	UK	Stocks 4 (10A-4)
12	*Fusarium oxysporum melonis*	melon	Mexico	NRRL 26406
13	*Fusarium oxysporum narcissi*	daffodil	UK	FON63
14	*Fusarium oxysporum phaseoli*	bean	USA	ATCC90245
15	*Fusarium oxysporum pisi*	pea	UK	FOP R1
16	*Fusarium oxysporum pisi*	pea	UK	FOP R2
17	*Fusarium oxysporum pisi*	pea	UK	FOP R5
18	*Fusarium oxysporum radicis-lycopesici*	Lycopersicum	USA	NRRL 26381
19	*Fusarium oxysporum statice*	statice	UK	30A-9

20	*Fusarium oxysporum vasinfectum*	cotton	China	NRRL 25433
21	*Fusarium oxysporum*	rocket	UK	FR3
22	*Fusarium oxysporum*	leek	UK	L2-1
23	*Fusarium oxysporum*	onion	UK	Fo47
24	*Fusarium avenaceum*	leek	UK	L5
25	*Fusarium begoniae*	begonia	UK	775
26	*Fusarium cerealis*	?	UK	831
27	*Fusarium coeruleum*	potato	UK	F88
28	*Fusarium culmorum*	wheat	UK	Fc/01/W001
29	*Fusarium equiseti*	rocket	UK	NL1
30	*Fusarium flocciferum*	Asparagus	UK	AT4
31	*Fusarium graminerum*		UK	WR21
32	*Fusarium lactis*	pepper	UK	P9
33	*Fusarium langsethiae*	wheat	UK	34f.l.003.2
34	*Fusarium poae*	wheat	UK	Fp/01/W/001
35	*Fusarium proliferatum*	onion	UK	A40
36	*Fusarium pseudocircinatum*	banana	UK	
37	*Fusarium redolens*	onion	UK	NL96
38	*Fusarium sacchari*	banana	UK	18RFB 2015
39	*Fusarium sambucinum*	potato	UK	F37
40	*Fusarium solani*	pea	UK	PG14
41	*Fusarium torulsoum*	?	UK	102
42	*Fusarium tricinctum*	Brassica	UK	CO
43	*Alternaria infectoria*	onion	UK	AT2
44	*Botrytis cinerea*	onion seed	UK	WRAR-4
45	*Botrytis aclada*	onion	UK	9736
46	*Botrytis allii*	onion	UK	9745
47	*Cylindrocarpon destructans*	parsnip	UK	CD10
48	*Itersonilia perplexans*	parsnip	UK	IP10
49	*Microdochium majus*	wheat	UK	MM/X/W/003
50	*Microdochium nivale*	wheat	UK	MN/X/W/003
51	*Mycocentrospora acerina*	parsnip	UK	Ma5
52	*Phoma sp.*		UK	
53	*Pythium ultimum*	carrot	UK	3b/P174
54	*Pythium violae*	carrot	UK	2C/P2d
55	*Rhizoctonia solani*		UK	R5(A92-1)
56	*Sclerotinia sclerotiorum*	lettuce	UK	L6
57	*Sclerotium cepivorum*	onion	UK	RUG1-1
58	*Setophoma terrestris*	onion	UK	PQF4
59	*Stemphylium sp.*	onion	UK	SQ3
60	*Trichoderma sp.*	onion	UK	WRLG11
61	*Verticillium albo atrum*	potato	UK	PD693
62	*Phytophthora cactorum*	??	UK	

Results

Genome sequencing of FOM and FON, comparative genomics

Mapping of orthologous genes known to be on core chromosomes from a reference *F. oxysporum* f.sp. *lycopersici* (FOL) genome with FON and FOM contigs identified those representing both core and pathogenicity-related LS regions of each genome (Fig. 5). Both the FON and FOM genomes contained high numbers of MIMPs in LS regions and genes that were close to these sequences (within 2kb), which also encoded secreted proteins, were identified as being potentially involved in pathogenicity. This totaled 48 genes in FON and 13 genes in FOM which, along with the 24 previously identified in FOC, represented potential targets for specific PCR-based diagnostic tests for FOC, FOM and FON as well as for identification of a range of different *F. oxysporum* f.spp. using amplicon sequencing (Fig. 6).

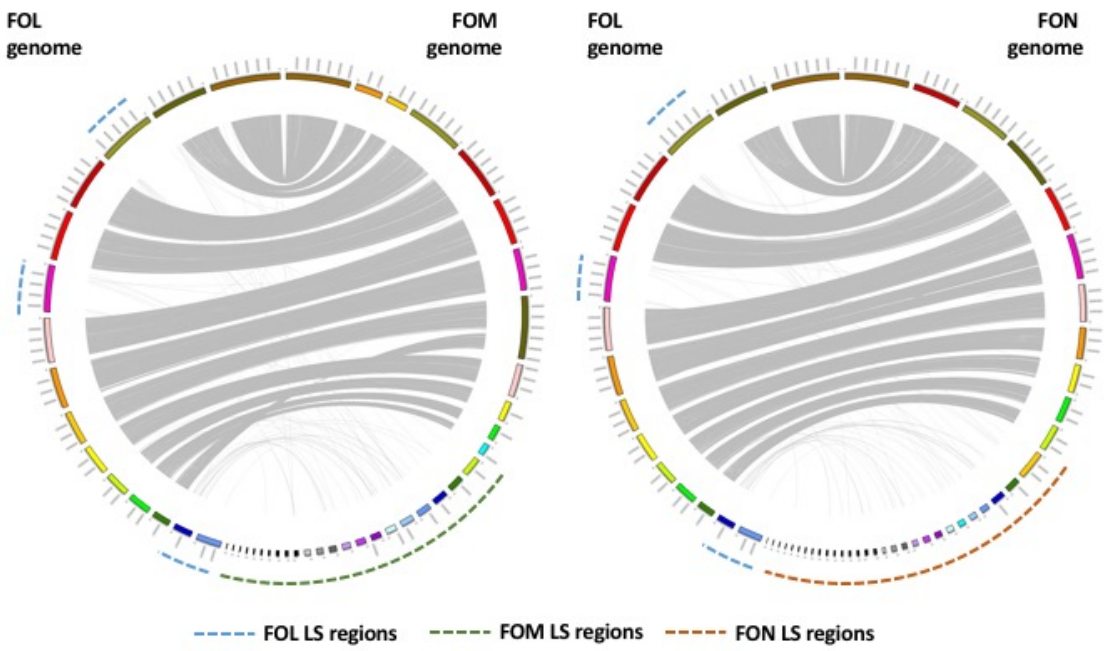

Figure 5: Generation of reference genomes for FOM (stocks4) and FON (63) using MinION sequencing technology. Core chromosomes in these genomes were identified through identification of orthologous genes with the FOL genome (grey lines). Regions showing reduced orthology are identified as lineage specific (LS) regions of FOM and FON harboring putative pathogenicity genes.

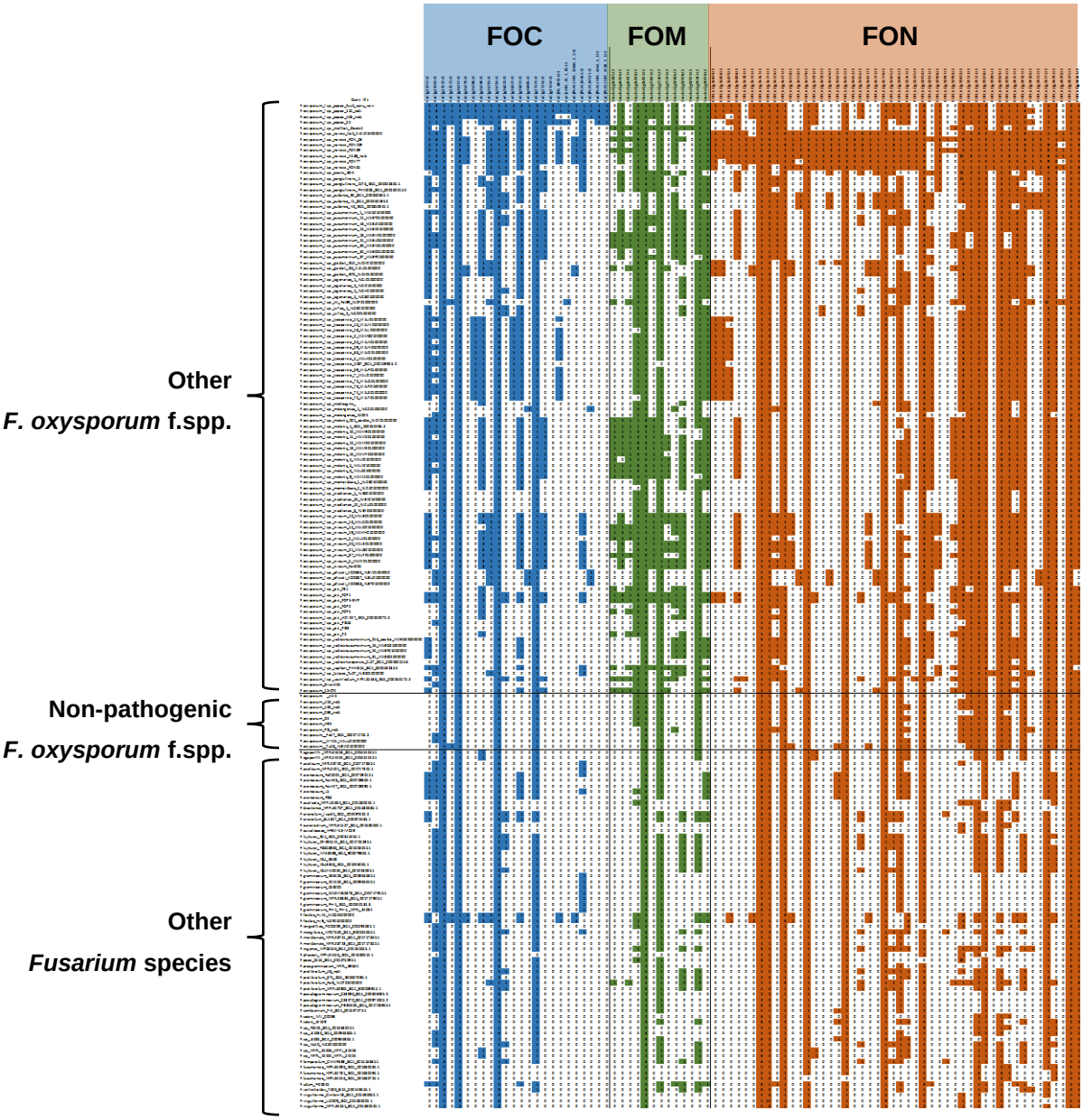

Figure 6: Distribution of 85 LS pathogenicity-associated genes in FOC (FUS2), FOM (stocks4) and FON (63) and their presence/absence in 164 *Fusarium* genomes. This presence absence matrix, along with an assessment of sequence variation between candidate genes, allowed selection of genes with the potential to identify and differentiate a range of *F. oxysporum* f.spp.

Identification of SIX and other pathogenicity genes for diagnostics

Following the genome analysis, FON and FOM LS regions were noted to contain some of the SIX genes previously identified in FOL, and these, as expected, showed varied presence/absence and sequence amongst all the other the different *F. oxysporum* f.spp. FOM

contained SIX1 (three copies), SIX8 and SIX9 (2 copies) while FON isolates contained between two and five SIX genes in different combinations of SIX7, 9, 10, 12, 13 (Table 4). These results were confirmed by PCR which was hence in agreement with the whole genome sequence analyses. By comparison, FOC was previously shown to contain SIX3, 5, 7, 9, 10, 12 and 14 (Table 4). SIX5 was identified as a potential PCR diagnostic target for FOC (only otherwise present in FOL, Table 4) while one gene (Ortho_g153) and two genes (Ortho_g16122, Ortho_g17178) were identified as diagnostic targets for FOM and FON respectively, as they were unique to these *F. oxysporum* f.spp.

Table 4: Presence / absence of SIX genes in FOL, FOC, FOM and FON isolates (blue shading indicates presence of a SIX gene).

	FOL	**FOC (FUS2)**	**FOM (stocks 4)**	**FON (63)**	**FON (77)**	**FON (89)**	**FON (129)**	**FON (139)**
SIX1								
SIX2								
SIX3								
SIX4								
SIX5								
SIX6								
SIX7								
SIX8								
SIX9								
SIX10								
SIX11								
SIX12								
SIX13								
SIX14								

Development of qPCR for FOC

PCR using the newly developed FOC primers targeting SIX5 resulted in specific amplification of all 30 FOC isolates tested with no amplification of DNA from FOM, FON or any of the other 62 fungi / oomycetes tested with the exception of an *F. oxysporum* isolate from leek which

was subsequently shown to be pathogenic on onion and hence designated as FOC (Fig. 7). Results from the qPCR showed the same level of specificity (Fig. 8) and resulted in a clear single product on the melt curve analysis indicating that that there were no other amplification products. (Fig. 9). In terms of sensitivity, the assay was able to accurately quantify samples containing as little as 100 fg (0.0001 ng) of FOC DNA.

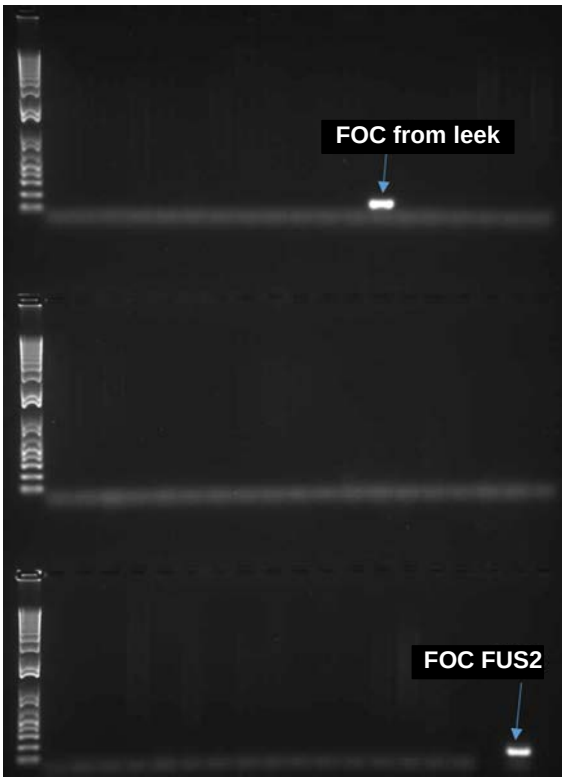

Figure 7: Gel electrophoresis of PCR products using FOC SIX5 primers showing amplification of DNA from FOC isolates from leek and onion (FUS2) and no amplification for DNA from a range of fungi / oomycetes as listed in Table 3.

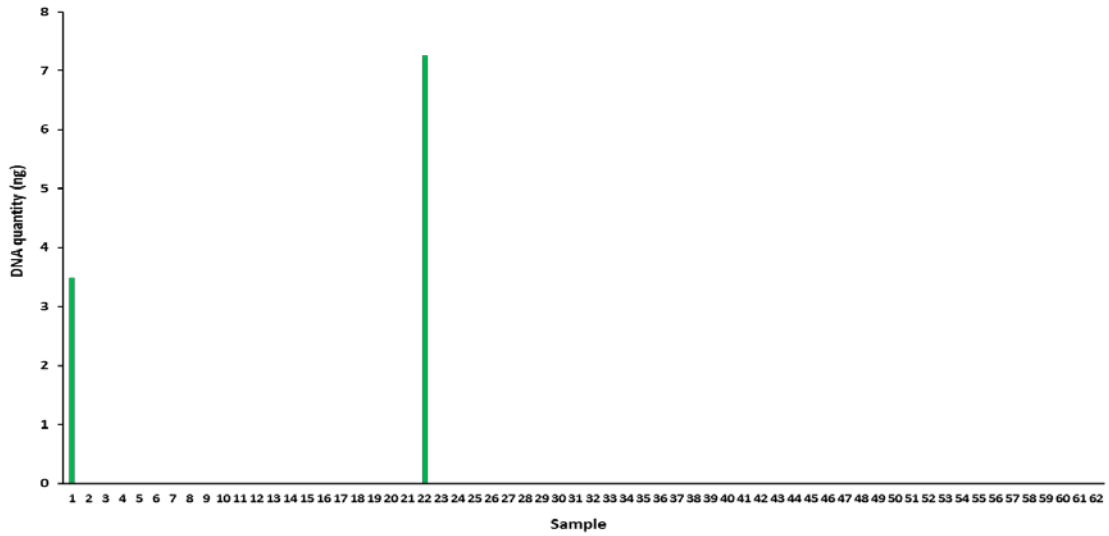

Figure 8: Results of qPCR using FOC SIX5 primers showing amplification of DNA from FOC isolates from leek (sample 1) and onion (FUS2, sample 22) and no amplification for DNA from a range of fungi / oomycetes as listed in Table 3.

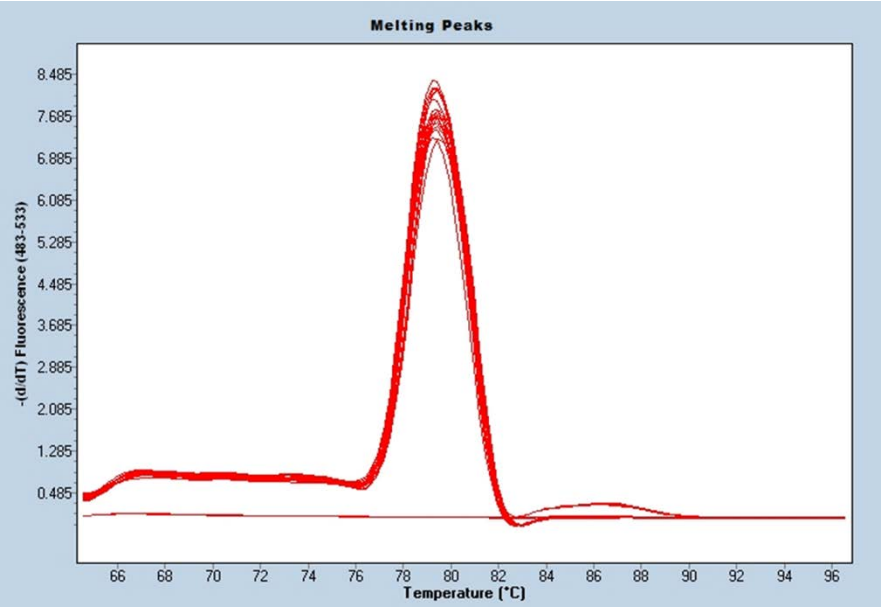

Figure 9: Melt curve analysis of PCR amplicon produced using FOC specific primers.

Development of qPCR for FOM

qPCR using the newly developed FOM primers targeting the putative pathogenicity gene Orth_g153 resulted in specific amplification of all FOM isolates tested with no amplification of DNA from FOC, FON or any of the other 62 fungi / oomycetes tested (Fig. 10). The qPCR assay also resulted in a clear single product on the melt curve analysis indicating that that

there were no other amplification products (Fig. 11). In terms of sensitivity, the assay was able to accurately quantify samples containing as little as 100 fg (0.0001 ng) of FOM DNA.

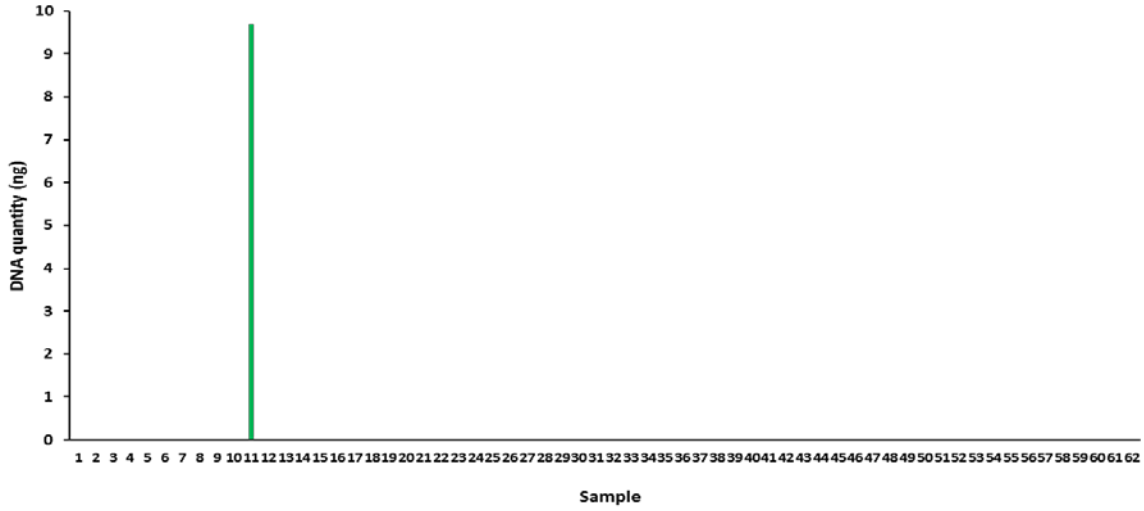

Figure 10: Results of qPCR using FOM primers showing amplification of DNA from FOM isolate (sample 11) and no amplification for DNA from a range of fungi / oomycetes as listed in Table 3.

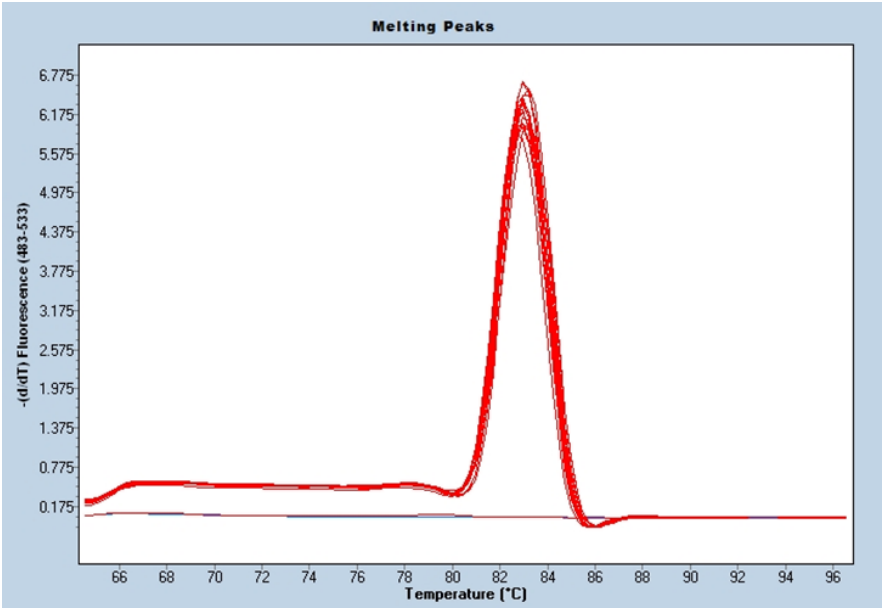

Figure 11: melt curve analysis of amplicon produced using FOM-specific primers

Development of qPCR for FON

Several primer pairs potentially specific for FON were initially designed but only one pair targeting the putative pathogenicity gene Orth_g17178 was found to amplify all 30 FON isolates tested (Fig. 12). qPCR using these primers resulted in specific amplification of all FON isolates tested with no amplification of DNA from any of the 62 other fungi / oomycetes tested

and also resulted in a clear single product on the melt curve analysis indicating that that there were no other amplification products. In terms on sensitivity, despite attempts at optimisation, the assay was not as good as for FOC and FOM with accurate quantification down to samples containing 1000 fg (0.001 ng) of FON DNA.

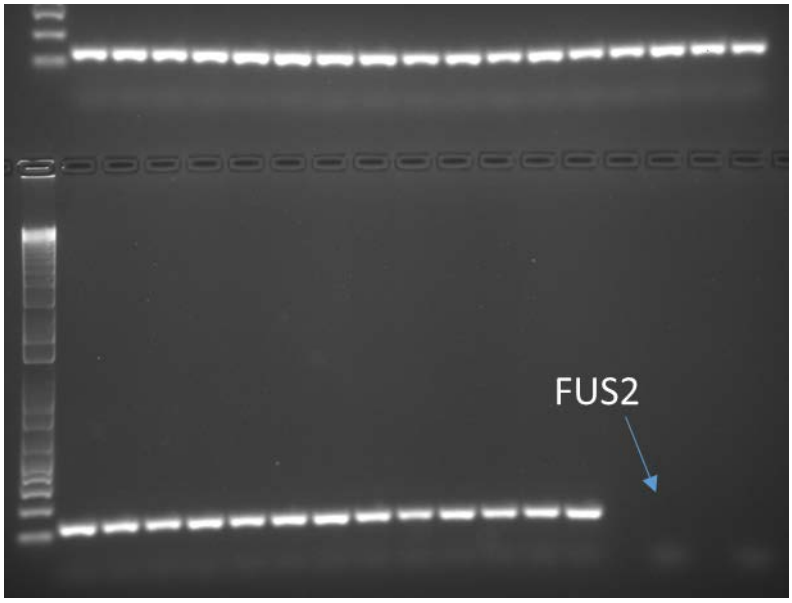

Figure 12: Gel electrophoresis of PCR products using FON primers showing amplification of DNA from FON isolates compared to no amplification for DNA from FOC isolate FUS2.

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).
- 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 (to be completed).
- Milestone 1.3c Carry out whole amplicon sequencing for soils infested with FOC, FON and FOM as collected in 1.2g (to be completed).
- 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 (to be completed).

Materials and Methods

Identification of pathogenicity genes for amplicon sequencing

Following the assessment of the distribution of pathogenicity-related genes throughout the 164 *Fusarium* genomes (Fig. 6), seven loci (SIX13, FOC_g17143, Orth_g10859, Orth_g13890, Orth_g4927, Orth_g4952, Orth_g12981) were selected as potential 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. Primers were designed for these genes which in combination should allow the identification of different f.spp. Further primers for amplicon sequencing were also designed for ITS and TEF genomic regions (present in all fungi) to allow identification of different *Fusarium* species and other common soilborne fungi based on sequence variation.

Testing primers for amplicon sequencing using mixed DNA from different *Fusarium* species and *F. oxysporum* f.spp.

To determine the utility of four of the selected primers for amplicon sequencing (ITS, TEF, SIX13, FOC_g17143 and Orth_g1298), PCR reactions were carried out using a mix of DNA (10 ng total, 0.5 ng / species) from two different fungal species pools for each primer set: Pool 1; six *F. oxysporum* f.sp. (including FOC, FOM and FON) and 14 other Fusarium spp. and Pool 2; five *Fusarium* spp and 14 other soil borne fungi (Table 5). In each test, FON63 was included as a positive control as it should be amplified by all the primers (Table 6).

Table 5: Pools of DNA from different *Fusarium* spp, *F. oxysporum* f.sp. and other soilborne fungi 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	*Pythiumultimum*
F. proliferatum	*Rhizoctonia solani*
F. redolens	*Sclerotinia sclerotiorum*
F. sambucinum	*Sclerotium cepivorum*
F. solani	*Setophoma terrestris*
F. tricinctum	*Trichoderma*
M. majus	*Verticillium albo atrum*
M. nivale	

Results

Identification of pathogenicity genes for diagnostics and amplicon sequencing

Following primer design for amplicon sequencing, a combination of SIX13, FOC_g17143 and Orth_g4952 or Orth_g12981 gene sequences were predicted to allow identification of FOC, FOM, FON and other *F. oxysporum* f.spp. f.sp. (Table 6). The target gene Orth_g4927 was discontinued as the amplicon was less informative compared to others.

Testing primers for amplicon sequencing using mixed DNA from different *Fusarium* species and *F. oxysporum* f.spp.

PCR of DNA from species Pool 1 with primers for four of the selected target genes (ITS, TEF, SIX13, FOC_g17143 and Orth_g12981) resulted in the expected pattern of amplification for the *F. oxysporum* f.spp. as indicated in Table 6 with no visible non-specific products, although the SIX13 amplicon was not as strongly amplified as the others. PCR of DNA from species Pool 2 also gave the predicted results with amplicons of the expected product size. PCR with primers developed for ITS and TEF regions also successfully amplified for all the *Fusarium* spp., *F. oxysporum* f.spp. and other fungi in both Pool 1 and 2 indicating their utility for identifying all the species tested.

Table 6: 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.sp; blue and yellow boxes indicate where one or more 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***	**f.sp. *melonis***	**f.sp. *raphani***	**f.sp. *tulipae***	**Other *Fusarium* spp. or f.spp**
SIX13														*f.sp cubense, f.sp. fragariae*
FOC_g17143														*f.sp. vasinfectum*
Orth_g10859														*F. avenaceum*
Orth_g13890														
Orth_g4927			NP											*f.sp vasinfectum* *f.sp. radicis-lycopersici*
Orth_g4952														*f.sp. tulipae*
Orth_g12981														

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).
- Milestone 1.4b Develop FOM infested field at Cut Flower Centre for qPCR and DNA barcoding testing (completed).

Materials and Methods

Inoculation of field with FOC

A large field plot at Wellesbourne (53 x 33 m) was inoculated with FOC in April 2015. Initially, approx. 2.5 tonnes of bulbs from a failed onion store with very high levels of Fusarium basal rot were crushed, spread over the entire area and incorporated into the soil. Subsequently, the plot was artificially inoculated with FOC isolate FUS2 using a sterile wheat bran / compost inoculum in April 2015 and 2016 which was raked into the top 10 cm of soil to achieve a concentration of 6 x 10^3 cfu ml^{-1}. This was calculated to be equivalent to a standard rate of FOC inoculum used in pot trials (see 2.1) of 2 x10^4 cfu g^{-1} compost. Bulb onions were grown over the area in 2015-2017 and disease symptoms recorded.

Inoculation of polytunnel with FOM

A polytunnel (8 x 36 m) at the National Cut Flower Centre (Holbeach St Johns, Lincs) was inoculated with FOM isolate stocks4 in July 2017. A sterile wheat bran / compost inoculum was prepared as for FOC and raked into the top 10 cm of soil to achieve a concentration of 6 x 10^4 cfu ml^{-1}, equivalent to a rate of 2 x 10^5 cfu g^{-1} in pot tests (see 2.2). Stocks plants of different varieties were then planted over the area by Lyndon Mason in 3 m plots.

Results

Inoculation of field with FOC

Severe symptoms of FOC were observed in bulb onions in 2015, 2016 and 2017 with a high proportion of plants wilting and dying before bulbs were formed while the majority of bulbs that did form showed typical basal rot symptoms when dissected (Fig. 13). Overall, total basal rot disease incidence was consistently greater than 70% plants / bulbs affected in each year.

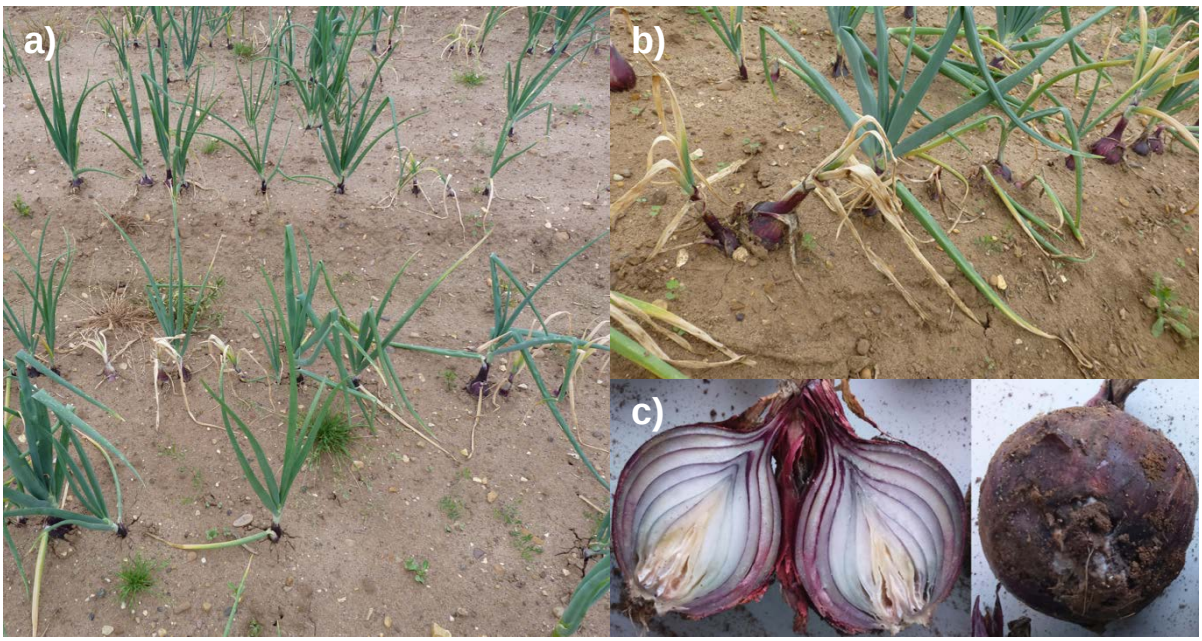

Figure 13: Symptoms of Fusarium basal rot on onion plants (cv. Red Baron) in the quarantine field at Wellesbourne; a) wilting and death pre-bulbing; wilting and death at bulb stage, basal rot in mature bulbs.

Inoculation of polytunnel with FOM

Severe Fusarium wilt symptoms were observed in stocks across the polytunnel inoculated with FOM with incidence up to 70% (Fig. 14). Marketable yield for each plot was also assessed (Lyndon Mason) and ranged between 7 and 166 marketable stems depending on variety.

Figure 14: Symptoms of Fusarium wilt of stocks observed in the inoculated polytunnel at the National Cut Flower Centre.

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).
- Milestone 2.2 Determine the effect of different FOM inoculum levels on disease development in stock plants (completed).
- Milestone 2.3 Determine the effect of different FON inoculum levels on disease development in narcissus (ongoing).

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 (to be completed).

Materials and Methods

Effect of FOC inoculum dose on disease development

A bran/compost inoculum of FOC isolate FUS2 was prepared as described earlier and mixed into M2 compost to achieve a range of concentrations from 1 x 10^2 - 1 x 10^6 cfu g^{-1}. FOC infested compost was then dispensed into 7 cm pots and five-week-old onion seedlings (cv. Hytech) transplanted (one plant per pot, 64 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. Plant death was recorded twice weekly for nine weeks until the end of bulbing, after which watering was then ceased and plants allowed to dry out. Bulbs were then bisected and symptoms of basal rot scored on a 0-3 scale, as described by Taylor et al., (2012).

Effect of FOM inoculum dose on disease development

A bran/compost inoculum of FOM isolate stocks4 was prepared as described earlier, mixed into M2 compost to achieve a range of concentrations from 1 x 10^2 - 1 x 10^6 cfu g^{-1} and

dispensed into 9 cm pots. Plug plants (cv. Fedora deep rose) were then transplanted (one plant per pot, 32 pots per concentration). As before, pots were arranged in a randomised block design in a glasshouse set at 25°C day, 18°C night, 16 h day-length. Symptoms of wilt were then recorded twice weekly for 15 weeks and scored on a scale of 0-4 where: 0=no symptoms, 1=first visible wilt symptoms (generally on 1 or 2 leaves), 2=50% of leaves wilted, 3=100% of leaves wilted, 4=completely dead plant.

Effect of FON inoculum dose on disease development

A bran/compost inoculum of FON isolate 139 was prepared as described earlier 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 magnesium 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) were planted approx. 10 cm deep (measured to the base of the bulb) in each pot with twenty replicate pots per treatment. An untreated control treatment was set up with bulbs planted in Narcissus 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. Pots were arranged in a randomised block design and assessments will be carried out from mid-March 2018.

Results

Effect of FOC inoculum dose on disease development

There was a very clear relationship between FOC inoculum concentration and disease development in onions (Fig. 15a). Almost no disease symptoms were observed at the lowest concentration of 1 x 10^2 cfu g^{-1} while between 1 x 10^4 and 1 x 10^6 cfu g^{-1} disease development was very rapid with 100% of plants affected by the end of the experiment.

Effect of FOM inoculum dose on disease development

There was also a very clear relationship between FOM inoculum concentration and disease development in stocks (Fig. 15b, Fig. 16). Again, 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 very rapid with almost all plants dead by the end of the experiment.

Effect of FON inoculum dose on disease development

This experiment is still in progress.

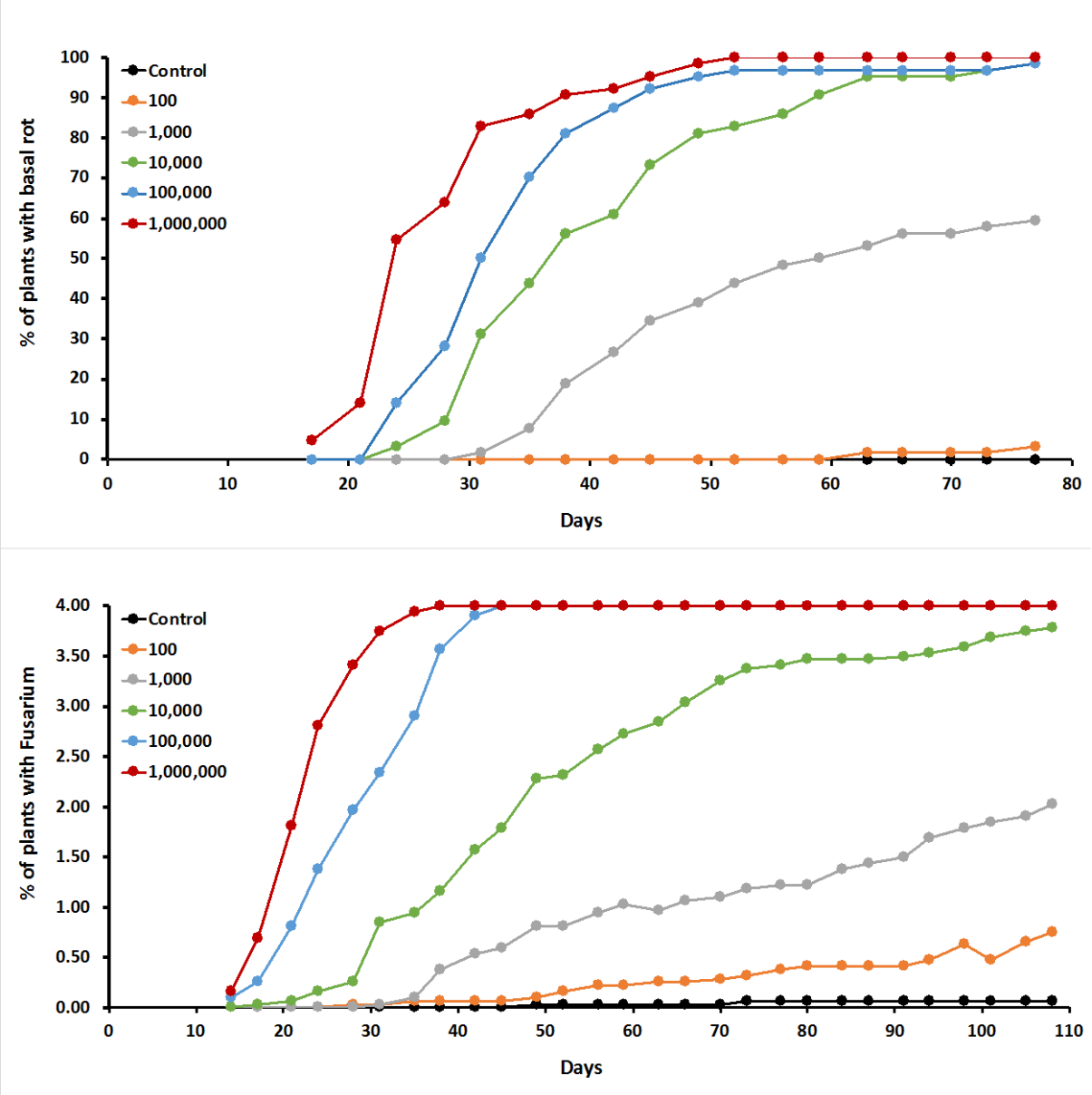

Figure 15: Effect of inoculum concentration (cfu g^{-1}) for a) FOC and b) FOM on disease development in onion and stocks plants.

Figure 16: Fusarium wilt symptoms in stocks plants for different concentrations of FOM inoculum from 0 (far left) to 1 x 10^6 cfu/g (far right).

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.

Four *Fusarium* species were identified within the isolates obtained from diseased leek samples; *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) and these results suggest that the disease may be caused by a complex of *Fusarium* species in the UK. However, the relative importance and pathogenicity of these different species on leek has yet to be assessed and this is currently being investigated following the success of initial experiments where leek roots were inoculated with *Fusarium* spp. spore suspensions. It is also possible that combinations of different *Fusarium* spp. enhance disease development further.

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

Specific qPCR tests have been developed for FOC, FOM and FON based on pathogenicity genes identified through comparative genome analysis, confirming the utility of this approach for distinguishing very closely related *F. oxysporum* f.spp. Although these assays need to be

further validated using soil / plant samples containing each pathogen, they should provide effective tools for studying the dynamics of the individual *F. oxysporum* f.spp. and provide a means of examining the colonisation of both host and non-host plants.

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

Identification of pathogenicity genes in FOC, FOM and FON following genome analysis and subsequent comparison with 131 other *Fusarium* spp. genomes identified several that were present in one or more *F. oxysporum* f.spp (but with different sequences) which could therefore potentially distinguish between these pathogens using amplicon sequencing. Initial testing of PCR primers for these pathogenicity genes as well as ITS and TEF housekeeping genes suggest that various combinations of these targets in amplicon sequencing could be used to identify common fungi (both pathogens and non-pathogens), different *Fusarium* spp. and *F. oxysporum* f.spp. in a single DNA sample extracted from soil or roots. However, this needs to be validated using soil / samples containing FOC, FOM and FOC to ensure that these can be identified amongst the other *Fusarium* or fungal species that may also be present. This will be achieved through sampling from the disease areas created for FOC and FOM (1.4). The amplicon sequencing approach developed here is novel in terms of identifying both *Fusarium* spp. and *F. oxysporum* f.spp. simultaneously and should allow dynamics of *Fusarium* disease complexes such as those occurring on leek and asparagus to be investigated in the future.

Objective 1.4: Development of disease areas for onions and stocks

Artificial inoculation of a field area for FOC and a polytunnel for FOM was successful in creating high disease levels in bulb onions and stocks respectively. These areas provide a valuable resource for both validation of the specific qPCR tests for FOC and FON as well as the amplicon sequencing and will also provide a means of testing new disease control products and approaches in the future.

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.

Experiments have determined the critical levels of FOC and FOM inoculum that are required to cause significant disease development in onions and stocks respectively (with an experiment with FON still in progress). Further work will now utilise the specific qPCR tests for FOC and FON in repeat experiments to relate qPCR values to inoculum rate and disease development. This will be an important first step in assessing the utility of the PCR tests for assessing disease risk in the field.

Knowledge and Technology Transfer

- 'Fusarium Futures' article in AHDB Grower, June 2017
- 'Understanding the genetic control of pathogenicity and resistance for *Fusarium oxysporum* in onion' presentation at the International Horticulture Research Conference, East Malling, UK, 17-19 July 2017
- 'Root rots, bulb rots and wilts: tackling Fusarium in onion and other crops' presentation at the Carrot and Onion Conference, Nottingham, UK, 13-14 November 2017

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