Grower Summary

FV POBOF 452

Fusarium: Investigations into the control of basal rots in crops

Annual report, March 2018

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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.]

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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of soil sterilisation or fumigation, fungicides or seed treatments but approval for their use in many cases has been withdrawn or threatened by further legislation.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.