Grower Summary

FV POBOF 452

Fusarium: Investigations into the control of basal rots in crops

Final 2019

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GROWER SUMMARY

Headline

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

Background

Fusarium oxysporum

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

Control of Fusarium

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of soil sterilisation or fumigation, fungicides or seed treatments but approval for their use in many cases has been withdrawn or threatened by further legislation.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

Impact of Fusarium oxysporum and other species on key horticultural crops

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

Fusarium basal rot of onion (FOC) and leek

FOC can affect onion crops at any stage, causing damping-off in seedlings and a root/stem rot in immature plants, but the greatest impact is generally at harvest and in store. On

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

Fusarium wilt of column stocks (FOM)

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

Fusarium basal rot of Narcissus (FON)

FON, affecting *Narcissus,* is a major problem for the UK daffodil industry causing a basal rot very similar to that in onion (Clarkson, 2012). The industry is estimated to be worth £45M and 10% losses are not unusual with a corresponding value of £4.5M (Hanks, 2010). Currently, control is dependent on just two active substances, thiabendazole (Storite) and chlorothalonil (Bravo) applied as part of the hot water treatment process used to eradicate stem nematode from bulbs. However, registration for both these actives may potentially be under threat in the future and some FON isolates show resistance to thiabendazole (Clarkson, 2012). An alternative product containing cyprodinil and fludioxonil (Switch) has

also just been approved, although performance has not been assessed in HWT. Despite the regular application of fungicides by *Narcissus* growers, extensive losses are still common in certain parts of the production area and the long periods of time the crop is in the ground makes it vulnerable to basal rot irrespective of initial fungicide applications.

Identification of Fusarium spp. and approaches for understanding Fusarium dynamics

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

Approaches, aims and objectives

In this project we initially collected and identified *Fusarium* isolates from leeks to add to our existing collections for onion, narcissus and stocks. Genomes of a pathogenic FOM isolate and also a range of FON isolates were also sequenced and comparative bioinformatics analysis carried out with genomes previously sequenced for FOC and other *F. oxysporum* f.spp to identify common and unique pathogenicity genes. These were then assessed for their suitability as potential diagnostic markers for FOC, FOM and FON and quantitative PCR (qPCR) developed for each pathogen. Based on the genome information, the feasibility of using a DNA barcoding approach based on amplicon sequencing to analyse *Fusarium*

species within entire microbial communities is also being examined. The project also aimed to determine the effect of inoculum concentration of FOC, FOM and FON on disease development in onion, stocks and narcissus respectively to determine the critical levels required for significant damage to occur which could then be related to qPCR results. Finally, large scale artificial inoculations were carried out to establish a field area for FOC and a polytunnel area for FOM with high disease pressure for testing the qPCR and amplicon sequencing approaches and to provide a resource for further research on control approaches in the future.

The aims and objectives of the project are:

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

Objectives

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

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

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

1.4: Development of disease areas for onions and stocks

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

Objectives

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

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

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

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

Summary

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

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

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

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

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

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

In year 1, pathogenicity genes were identified in FOC, FOM and FON following genome analysis and subsequent comparison with other *Fusarium* spp. genomes. Several of these were present in one or more *F. oxysporum* f.spp. (but with different sequences) and hence

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

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

Overall, the use of an amplicon sequencing approach based on specific gene targets to define the presence and abundance of *Fusarium* spp. and *F. oxysporum* shows potential and is a new and novel approach. Alongside more conventional gene targets to define

fungal and bacterial communities, this could be a powerful tool with which to dissect *Fusarium* disease complexes and examine dynamics in relation to the whole soil microbial community. Further work now needs to further optimise the technique and explore how it performs across multiple commercial onion and daffodil field and protected stocks cropping sites.

Objective 1.4: Development of disease areas for onions and stocks

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

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

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

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

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

Benefits

- The main *Fusarium* pathogens affecting leek have been identified as *F. culmorum* and *F. avenaceum*.
- Specific diagnostic tests have been developed for FOC, FOM and FON for the first time and may provide a way of assessing disease risk as a commercial service in the future.

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

Action Points

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