Grower Summary

Diagnostic tests to assess Fusarium disease risk, select rotation crops and monitor microbial communities

CP 204

Final report

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The results and conclusions in this report include investigations 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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AUTHENTICATION

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

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GROWER SUMMARY

Headline

DNA based tests were developed to identify and quantify *Fusarium oxysporum* pathogens causing Fusarium disease for three important horticultural crops; onion, lettuce and Narcissus. In onion, detection of Fusarium DNA was enhanced in roots compared with soil but was improved in the latter samples by baiting / enriching the pathogen through growing onion seedlings. *F. oxysporum* could also be detected in asymptomatic onion bulbs before symptoms developed. After refinements, these tests could be used to determine the risk of Fusarium disease developing in all three crops through analysis of soil samples collected in advance. In the case of onion, the molecular test could also be used to assess risk of basal rot developing in store. In more fundamental work the build-up of *F. oxysporum* in sterile / non-sterile soil through successive rounds of lettuce growth was quantified as was the dynamics of the entire microbial community through mass (amplicon) sequencing. This approach allowed measurement of the relative abundance of fungi, bacteria, the entire *Fusarium* spp. community as well as applied biocontrol agents and in the future could be used to understand the dynamics of pathogens in relation to the microbiome. Finally products evaluated for control of Fusarium basal rot in onion in field trials were largely ineffective although Rudis increased the number of healthy bulbs.

Background

Fusarium oxysporum is a soil borne plant pathogen with a worldwide distribution and causes vascular wilt, root rot and bulb rot diseases in many economically important crops. *F. oxysporum* is a species complex comprised of over 150 *formae speciales* (f. spp.) which are adapted to infect specific crops such as onion, lettuce, leek, banana, tomato peas, brassicas and also narcissus and column stocks.

Fusarium wilt disease of lettuce

Fusarium wilt of lettuce, which is caused by *F. oxysporum* f. sp. *lactucae* (FOL), has spread to most production areas globally. There are four cultivar specific races, with race 1 and race 4 being the most widespread which cause severe economic losses in both field and protected crops respectively. In the UK, FOL race 4 (FOL4) was first identified in 2017 in Lancashire and Ireland, but has since spread to Cambridgeshire and Yorkshire, as well as locally within each area; so far FOL4 occurrence has been restricted to lettuce grown under protection. Disease symptoms include yellowing and necrosis of leaves, stunting and wilting of plants and reddish-brown/black necrosis of vascular tissue. There are currently no commercially

available resistant cultivars and therefore rapid spread between growers is being prevented through hygiene measures such as rigorous cleaning of equipment and glasshouses and by using foot dips/containment procedures for people moving from infected to clean areas. Growers have been mitigating disease impact through occasional use of the soil fumigant dazomet (Basamid), removal of contaminated soil or by abandoning affected growing areas.

Fusarium disease of onion

Fusarium basal rot of onion, caused by *F. oxysporum* f.sp. *cepae* (FOC), represents a major threat to the industry, with incidence levels increasing over the last few years. Recently basal rot losses have increased from 2-6% to over 10%, resulting in economic losses of approx. £13M. Many of these losses have been the result of disease developing in storage, where apparently healthy bulbs develop disease, which can result in worse cases to the whole consignment being abandoned as it becomes too costly to extract affected bulbs. Fungicides may have limited effect as FOC, like other f. spp. is soilborne, therefore it can be difficult to control once an area becomes infected. Early detection is key to prevent crops being grown in affected soils to reduce disease incidence as much as possible, and prevent bulbs being contaminated before going into storage. Hence in this project we will investigate the use of molecular diagnostics as a tool to assess disease risk for bulb onions pre-planting and also during the cropping period.

Fusarium disease of daffodil

Fusarium basal rot of *Narcissus* is caused by *F. oxysporum* f. sp. *narcissi* (FON). It infects the roots or damages basal plates resulting in soft and rotting bulbs, which leads to bulbs not sprouting or producing short lived or early senescing foliage with few or no flowers (Taylor et al., 2019a). It is a problem for UK growers as bulbs are lifted after 2 years and used as replanting stock, therefore maintaining inoculum levels or allowing it to spread (Hanks, 2013; Taylor et al., 2019a).

Control of Fusarium diseases

As *F. oxysporum* is a soil borne plant pathogen, control is fundamentally difficult as it invades plants via the roots, and produces long lived chlamydospores which can survive in the soil for many years. Crop rotations are one of the most successful ways to avoid build-up of inoculum in the soil to levels capable of producing disease. However, there is increasing evidence to suggest that *F. oxysporum* can proliferate on non-host crops, therefore maintaining levels of inoculum which continue to increase when the host is again grown in the rotation. Fungicides usually have little effect; however, soil sterilisation or chemical fumigation is often used in protected crops to try and prevent disease occurring. These have been shown to reduce the

levels of inoculum in the soil to below the required level for disease to occur, therefore reducing incidence and preventing losses. Unfortunately, they also negatively impact the microbial communities in soil which often act to suppress diseases and can therefore lead to *F. oxysporum* inoculum building up after fewer cropping cycles. This is a particular problem with crops grown under protection, such as lettuce, and multiple crops are often sown in the same location every year without rotation, therefore facilitating *F. oxysporum* proliferation. This project aims to investigate the potential of soil sterilisation techniques to suppress disease, but also to determine their effect on microbial communities and how this interaction relates to levels of disease. One approach to mitigate the negative effects of soil sterilisation is to introduce biological control agents, such as Trianum and T34, or Calcium cyanamide (Perlka) to encourage the recovery of microbial communities and suppress inoculum build-up; therefore the use of these treatments will be investigated in the project.

Project aims and objectives

In this project we will utilise molecular diagnostics developed at Warwick Crop Centre in a previous AHBD project FV POBOF 452 to determine the risk of Fusarium disease with a focus on FOC causing basal rot in onion but also with some testing of samples from Narcissus and lettuce. Initially, the relationship between Fusarium inoculum concentration, disease development and the amount of DNA detectable in the soil will be established through glasshouse experiments involving lettuce and narcissus grown in inoculated soils (this has been done previously for onion). We will then use these diagnostic techniques to detect the different *F. oxysporum* pathogens in soil samples to establish whether molecular diagnostics can accurately detect and predict Fusarium diseases in the field. In addition, large scale artificial inoculations will be used to screen multiple non-host plants to determine the extent of colonisation by *F. oxysporum*, as crops which enable proliferation of the pathogen should be avoided in rotations. Finally, microbial communities have been shown to be important for suppression of disease; therefore, we will investigate how to establish healthy suppressive microbial communities through soil amendments and biological control agents. Amplicon sequencing will enable quantification of microbial communities in comparison with *F. oxysporum* populations.

The overall aim of the project is:

To use molecular methods to determine the risk of Fusarium disease, select effective rotation crops and monitor *F. oxysporum* pathogens and associated microbial communities.

This will be achieved through the following objectives:

1. Define a relationship between the amount of Fusarium DNA, Fusarium inoculum and disease development in soil
2. Evaluate the use of molecular diagnostics to detect and assess the risk of Fusarium disease in onion, lettuce and Narcissus
3. Evaluate the use of molecular diagnostics to determine the presence of FOC in harvested onion bulbs to assess the risk of disease development in store or pre-planting
4. Determine the colonisation of non-host plants by *F. oxysporum* pathogens to identify suitable rotation crops
5. Investigate the feasibility of establishing Fusarium-suppressive microbial communities and biological control agents in protected cropping systems
6. Employ amplicon sequencing to quantify *F. oxysporum* pathogens alongside suppressive components of the soil microbial community (Year 1 report, current report).
7. Evaluate products for control of FOC in field experiments

A summary of the main research findings over the three years of the project is presented in the section below. Experiments conducted in this final year of the project are reported in full in the Science Section, alongside a summary of results from Years 2 and 3 which can be found in full detail in the annual reports for Year 1 (2021) and Year 2 (2022)

Summary

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

Pot experiments were carried out in the glasshouse where different soils were inoculated with varying levels of FOL or FON inoculum, lettuce / Narcissus grown, and disease development recorded. At the start of the experiments, the amount of *Fusarium* pathogen present was also quantified using specific DNA-based qPCR assays developed previously. In both experiments, there was a clear relationship between levels of pathogen inoculum, disease development and *Fusarium* DNA concentrations detected in soil. These quantitative molecular diagnostic assays were therefore successful in quantifying FON and FOL in field soils and with further development could potentially be used to assess risk of disease in

Narcissus and lettuce. This would involve testing the approach on field samples from growers and comparing with observed levels of disease as done for the diagnostics for FOC in Objective 2.

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

Soil and onion root samples were collected from grower fields across three growing seasons, between 2020-2022, to evaluate the use of the FOC specific qPCR assay for disease prediction. Onions were also assessed for disease over the growing season, at harvest and after a period of storage, so FOC detection could be related to the amount of disease at each site. Two approaches were taken: i) analysis of soil samples pre-drilling across multiple commercial onion field sites and ii) analysis of both soil and onion root samples at different timepoints during the growing season at two intensively sampled commercial onion field sites.

Over the project it became clear that FOC DNA was very rarely detected in pre-planting soil samples and hence a different onion seedling 'baiting' approach was examined (see below). For the intensively sampled onion field sites, FOC detection in soil was variable over time and was generally at low levels. In contrast, FOC detection was more frequent in onion roots and in much higher quantities over the growing season. Generally, an increase in frequency of FOC detection in soil and root samples was related to higher basal rot disease levels at harvest or in store. However, detection of FOC during the season is of limited value to growers and a pre-planting soil test would be more practical. As direct DNA extraction and qPCR was not sensitive enough to detect FOC in pre-planting soil samples from onion fields, we investigated growing onion seedlings in these soil samples as a means of 'baiting' out and allowing FOC to proliferate, hence potentially increasing the sensitivity of detection. Here, onion seedlings were grown for 7-8 weeks in soils from onion fields and maintained at 20°C. Soil was tested for FOC by qPCR at sowing while both soil and roots were tested when plants were harvested. Here it was found that there was a significant increase in FOC detection between sowing and harvest with higher levels of DNA detected and more onion field sites testing positive in the latter. Onion root samples also resulted in enhanced FOC detection, with DNA levels around 100-fold higher than in soils post-harvest and yet more onion field sites testing positive using this approach. This plant 'enrichment' for FOC could therefore be further refined to predict the risk of basal rot disease in onion using pre-planting soil samples from growers.

Objective 3: Evaluate the use of molecular diagnostics to determine the presence of FOC in harvested onion bulbs to assess the risk of disease development in store

Detection of FOC in onion bulbs by qPCR diagnostics is a potentially useful approach for assessing the risk of basal rot developing in store. Initially, we confirmed that qPCR could detect FOC in the basal plates of onion bulbs with different levels of basal rot symptoms but also in some asymptomatic bulbs. In follow up work we focussed on FOC detection in asymptomatic apparently healthy onion bulbs sourced from the *Fusarium*-inoculated quarantine field at Wellesbourne. Here, FOC was detected in 38% of asymptomatic bulbs while 51% of bulbs from the same batch went on to develop basal rot when incubated under conditions conducive to disease development. In one sample of apparently healthy onions from a high-risk grower site, around 10% of bulbs tested positive for FOC with 24% of the same batch developing basal rot after incubation. Overall therefore, qPCR diagnostics to detect FOC in onion basal plates is a promising approach to predict the likelihood of basal rot developing in storage which is a particular issue for growers, as only relatively low levels of disease are required for complete store loss. As DNA-based PCR based diagnostics was particularly useful for FOC detection in onion bulbs, we also evaluated a LAMP assay (a quicker version of PCR which uses crude DNA extracts) previously developed at Warwick for assessing presence of FOC in artificially inoculated onion bulbs. Here, results showed that we could detect FOC in basal plates as early as 8 days after inoculation before any symptoms appeared, which suggests that LAMP can detect FOC in asymptomatic onion bulbs as well as qPCR. In future work, the LAMP assay would need to be tested using apparently healthy onion bulbs from a highly infected commercial site to determine if detection was reliable and related to subsequent basal rot development. If successful, this approach provides a far more rapid method of testing bulbs for FOC as it uses a simple DNA extraction method from basal plate tissue and could also be done using a portable machine on site.

Objective 4: Determine the colonisation of non-host plants by *F. oxysporum* pathogens (FOC) to identify suitable rotation crops

F. oxysporum f.spp. spores can survive in the soil for many years, even in the absence of a host and hence lengthy crop rotations are usually required to allow inoculum levels to decrease to low enough levels for production. However, it has been noted recently by onion growers that even with long crop rotations, disease levels can remain high; therefore, it was hypothesised that FOC may be able to survive and even proliferate on non-host crops in the rotation. To test this, 12 plants of non-host crop species were grown in FOC inoculated compost and colonisation of roots examined by qPCR. FOC was found to colonise the roots

of all the crops tested to varying degrees. For instance, FOC was detected in roots of 12 and 10 plants respectively for pea and maize compared to only three plants for oilseed rape. Roots of all 12 plants for a susceptible and resistant onion cultivar were also colonised. However, concentrations of FOC DNA in roots also varied but were far lower for non-host crops (on average >100-fold lower) compared with roots of the susceptible and resistant onion cultivars. Nonetheless, even some root colonisation of non-host crops over multiple years could maintain FOC levels at high enough concentrations in the soil to cause disease on onions next time they featured in the rotation. Therefore, it may be important to include crops where FOC colonisation was lower such as oil seed rape, barley and wheat, as opposed to crops where colonisation was more consistent (pea and maize).

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

A method was developed to examine FOL4 inoculum build up and disease development in sterilised and non-sterilised soil with successive rounds of lettuce plant growth in pots. This system was used to determine whether FOL4 build up could be reduced or prevented by products such as Perlka, Trianum G and T34 Biocontrol using soil from a UK lettuce grower. All these treatments had some previous published evidence that they might have some activity against *Fusarium* disease. Over the three rounds of lettuce growth, it was clear that FOL4 inoculum build-up was much quicker in sterile soil compared to non-sterile soil but none of the treatments prevented this occurring or reduced disease development compared with a FOL4 only control. In the non-sterile soil at the end of the third round of lettuce growth, only mild to moderate Fusarium disease was observed in the lettuce compared with much more severe levels of disease for plants grown in the sterile soil. This was concomitant with the finding that there was a general reduction in microbial community diversity in sterilised soil and a predominance of the pathogen FOL4 as measured by amplicon sequencing (Objective 6). Quantification of FOL4 inoculum using qPCR revealed that FOL4 could be detected at harvest in sterilised soils only for the crop 1 and 2, with only very low levels detected in the non-sterilised soils by the end of crop 3, again confirming that that the pathogen proliferated rapidly in sterilised soil.

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

Amplicon sequencing was successfully used to quantify the relative abundance of bacterial (16S gene target), fungal (ITS gene target) and *Fusarium* spp. (TEF1a gene target) communities in both FON and FOL4 experiments which examined effects of inoculum concentration in different soils (Objective 1). Moreover, the novel gene target OG4952 was also effective in identifying the presence of FON in soils at inoculation levels of $2x10^4$ spores g^{-1} soil and above in both soils tested. This technique is therefore useful in understanding how the microbial community reacts to the presence of *F. oxysporum* pathogens. The use of novel gene targets (such as OG4952) to specifically identify a range of different *F. oxysporum* f.spp. is challenging as often the sequences of these targets are identical for several *F. oxysporum* f.spp. or they result in unexpected positive detection even when it is known that the pathogen is not present. The presence of two paralogues for the OG4952 target in FON makes it a good identifier as both targets need to be present. In contrast, for the gene target (g19096) used to identify FOL4, it was found that high levels of endogenous *F. oxysporum* in some soils gave false positives. What is clear however is that the TEF1a is an excellent gene target for identifying *F. oxysporum* in soils with multiple *Fusarium* species present. In inoculated systems as investigated in this project, the abundance of a particular *F. oxysporum* f.sp. used in experiments can often be effectively quantified by TEF1a amplicon sequencing but only if the background level of other *F. oxysporum* isolates is low (as this target cannot distinguish f.spp) and inoculum levels are greater than $2x10^3$ cfu g^{-1} soil. Similarly, in some cases the ITS gene target can also be used as proxy for *F. oxysporum* abundance but only if levels of other *Fusarium* spp. are low (as this target cannot distinguish different *Fusarium* species). The establishment of a novel system to examine build up of FOL4 inoculum over three rounds of lettuce growth in both sterilised and non-sterilised soil with and without *Trichoderma* / Perlka treatments (Objective 5) gave us the opportunity in the final year of the project to examine the microbial and *Fusarium* communities over time. This generated a lot of data but there were some clear conclusions; firstly, the amplicon sequencing approach clearly identified big differences in the fungal / *Fusarium* spp. community between sterilised and non-sterilised soil and critically demonstrated that FOL4 inoculum builds up more rapidly in sterilised soil over the three cycles of lettuce growth which was supported by the much higher levels of disease in the final lettuce growth cycle. It was also demonstrated that the two *Trichoderma* isolates (T22 and T34) could effectively be detected by amplicon sequencing and that T34 and to a lesser extent T22 also proliferated more in the sterilised soil. As no disease control was observed for these treatments in

Objective 5, this suggests that they were ineffective against FOL4 in this particular situation where they were added to the soil two weeks before transplanting for each round of lettuce growth. Another approach for the future might to be try and establish these organisms on the lettuce transplants before they are planted in FOL4 infested soil as they are both known to be efficient root colonisers. Nonetheless, the ability to track the relative abundance of bacteria, fungi, *Fusarium* spp. as well as *F. oxysporum* pathogens and specific *Trichoderma* biocontrol agents is a valuable tool to understand the complex interactions and dynamics of these different soilborne organisms.

Objective 7: Evaluate products for control of FOC in field experiments

Products were tested for their ability to prevent Fusarium basal rot of onion in four field trials carried out by ABC and VCS. A range of biological and chemical treatments were tested either as an in-furrow treatment at drilling or as a foliar spray. Application of Rudis in-furrow was the only product that appeared to consistently increase the number of healthy onion bulbs compared to an untreated control in fields with (generally) high Fusarium levels across several trials but this increase was not statistically significant.

Conclusions

- **Objective 1:** A clear relationship between inoculum concentration, *Fusarium* pathogen DNA levels in soil (measured by specific qPCR assays) and Fusarium wilt disease development was established for FOL4 in lettuce and FON in *Narcissus*. These molecular diagnostics could be used to determine the level of *Fusarium* inoculum in soil and determine the risk of severe disease.
- **Objective 2:** FOC was rarely detected by qPCR molecular diagnostics in soil samples collected pre-planting of onion crops but was more reliably detected in onion roots during the season. As this has limited practical value for growers, a system whereby onion seedlings were grown in pre-planting soil samples was developed that allowed FOC to proliferate and hence improved qPCR detection in both soil and roots. Further development of such a *Fusarium* 'enrichment' is likely to be a better approach for detection in soil and assessing disease risk.
- **Objective 3:** qPCR and LAMP based molecular diagnostics was effective at detecting FOC in asymptomatic onion bulbs. This could therefore be used as a method to detect FOC in harvested onion bulbs to assess the risk of disease in store.

- **Objective 4:** FOC was found to colonise the roots of all non-host crops tested with a higher incidence of the pathogen in pea and maize compared to the other crops. However, the amount of FOC DNA was much lower in all non-host crops compared with onions. This suggests that non-host plants may sustain FOC populations between crops of onions to different degrees.
- **Objective 5:** FOL4 proliferated more rapidly in sterilised soil compared to non-sterilised soil over three successive rounds of lettuce planting (in pots) in the same soil. Several products tested did not reduce this build-up of inoculum or reduce disease development.
- **Objective 6:** Amplicon sequencing successfully identified and quantified the relative abundance of the microbial community including bacteria, fungi and *Fusarium* spp. in soils inoculated with FOL4 and FON. Gene targets to identify multiple *F. oxysporum* f.spp. were identified but were not always reliable. In inoculated FOL4 and FON systems, the identification of *F. oxysporum* using TEF1a sequencing amongst other *Fusarium* spp. was generally a good indicator of the abundance of these pathogens.
- **Objective 7:** Rudis was the only product that consistently increased the number of healthy onion bulbs in Fusarium-affected fields but the effect was not statistically significant.

Financial Benefits

This project has considerable future potential financial benefits for growers if quantitative diagnostic tests for FOC, FOL and FON can be refined, validated and commercialised. In onion Approx. 450,000 tonnes of onions are produced annually in the UK by around 90 growers and in 2022, 8,800 ha were grown with around 3 /4 being direct drilled, where a pre-planting soil test would be most useful. FOC is becoming increasingly widespread, and losses in affected fields generally range from 2-12% with badly affected fields >30% equating to approx. ~£20M in lost crop. A diagnostic for FOC could help growers avoid cropping in fields with high levels of Fusarium and could also identify crops with a greater risk of developing disease in store, thus informing which crops will store best. If this approach could reduce losses by 10%, then this would still result in a return of £2M per annum to the industry. Other benefits include

- Reduced waste due to infected bulbs or over estimation of disease
- Reduction in imports to replace lost produce (370,000 tonnes of onions imported in 2022)

- Energy savings due to better disease management and control of storage crop
- Less CO2 and other inputs (fertilisers, crop protection products, diesel, labour) being wasted through loss of crop at harvest.
- A more sustainable onion producing sector in the UK leading to retention of jobs in the agricultural industry, which currently employs over 400,000 people in the UK.
- Increases in resilience of UK onion production, including reversing the contraction of the sector, to move towards self-sufficiency in production.

This work has led to the funding of a Defra / Innovate project which aims to further develop molecular diagnostics to assess risk of Fusarium basal rot of onion in field or store to achieve these financial benefits. Similar benefits could be realised by both lettuce (farm gate value £220M) and Narcissus growers (farmgate value £30M, with exports worth over £20M).

Action Points

Growers should note that crops used in rotations may sustain populations of FOC in soil. Of the crop plants tested, peas and maize were particularly highly colonised by FOC.

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