Project title:	Defining relationships between *F. oxysporum* inoculum level, quantitative molecular diagnostics, microbial community composition and basal rot development in different soils to enable disease prediction in bulb onions
Project number:	CP 196
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Report:	Final report
Previous report:	N/A
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Location of project:	University of Warwick and NIAB-EMR
Industry Representative:	N/A
Date project commenced:	14/10/2019

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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

University of Warwick

Signature: John Clarkson Date: 27/02/2020

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GROWER SUMMARY

Headline

Detection of *Fusarium oxysporum* f.sp. *cepae* (FOC) causing onion basal rot using qPCR was related to levels of pathogen inoculum and disease development in artificially inoculated soils. A similar threshold of detection for FOC was evident when amplicon sequencing was employed as an alternative approach to quantify the pathogen at the same time as characterising the wider microbial community.

Background

This project builds on previous AHDB and BBSRC supported work where whole genome sequencing, assembly and bioinformatic analyses enabled the development of specific DNA-based quantitative PCR (qPCR) diagnostic tests for some of the most significant and economically damaging *Fusarium* pathogens in UK horticulture: *F. oxysporum* f.sp. *cepae* (FOC, onion), *F. oxysporum* f.sp. *matthiolae* (FOM, stocks) and *F. oxysporum* f.sp. *narcissi* (FON, *Narcissus*). Furthermore, using similar bioinformatic analyses, an innovative amplicon sequencing approach was developed which allowed the abundance and identity of different components of the microbial community to be identified simultaneously from the same DNA sample. This approach specifically identifies pathogenic and non-pathogenic *Fusarium* species, as well as individual pathogenic *F. oxysporum* f.spp. including FOC, alongside members of the wider fungal and bacterial microbiota. Although amplicon sequencing is now used extensively in research, its practical application to assess soil health or determine the presence of pathogens in soil is largely unproven.

The approach in this project was to focus specifically on FOC which continues to cause ever increasing concern to growers as basal rot disease levels in bulb onions continue to escalate year on year. Having developed a specific DNA-based qPCR assay for this pathogen previously, one of the main objectives of the project was to determine if this test could accurately quantify different levels of FOC inoculum in soil and hence predict the risk of disease development as a practical tool for growers. This would indicate whether pathogen-free or low pathogen field sites could be identified with confidence and hence whether a pre-planting soil test to assess basal rot disease risk was feasible. Another objective was to assess whether FOC root colonisation could be detected and quantified early in crop development as

another way of potentially assessing risk of disease. In practice, detecting FOC in seedlings or young plants before the expression of symptoms in the field would allow onion growers to manage their crop appropriately and make decisions about harvest, post-harvest curing / drying and storage.

A final objective was to use the amplicon sequencing method developed previously to determine the abundance and identity of *Fusarium* spp., fungi and bacteria that were present in the different soils and also examine whether this approach was sensitive enough to effectively distinguish between the different FOC inoculum levels. This would determine whether in the future, amplicon sequencing could be used to accurately determine pathogen levels at the same time as assessing soil health based on microbial community analysis.

In summary, the main aim of the project was to define relationships between FOC inoculum level, the amount of FOC DNA as measured by a quantitative qPCR test and onion basal rot disease development for different soils. A secondary aim was to examine onion root colonisation by FOC and determine if different soils reduced the rate of FOC disease development and if so, identify the components of the microbial community that may be responsible.

The main project objectives were to:

1) Determine the effect of different FOC inoculum levels on onion basal rot disease development for different soils
2) Quantify the amount of FOC DNA for different inoculum levels using qPCR in different soils and define relationships with onion basal rot disease development
3) Determine the rate of onion root colonisation by FOC for different soils
4) Establish the potential of amplicon sequencing to quantify levels of FOC inoculum for different soils and identify components of the microbial community that may reduce the rate of onion basal rot disease development

Summary

Four different onion growing soils (NOTTS 1, NOTTS 2, CAMBS and LINCS) including two of the same type with and without previous (grower) application of poultry manure (NOTTS1 and NOTTS2 respectively) were shown to result in significantly lower levels of basal rot disease development in onions compared to a peat-based growing compost when inoculated with different concentrations of FOC inoculum in a glasshouse experiment. However, disease development in compost was lower than expected most likely due to suboptimal environmental

conditions in the glasshouse at the time of year it was necessary to carry out the experiment (November-January). Nonetheless, the results suggested that the physical and / or biological properties of the soils were less conducive to disease than compost. Basal rot development in NOTTS 1 and NOTTS 2 soils (sandy loam) was similar (although slightly higher for NOTTS 1) and only extensively developed for the highest FOC inoculum levels (2 x 10^5 and 2 x 10^6 cfu g^{-1} soil). This indicated that the previous (grower) addition of poultry manure for NOTTS1 which resulted in relatively greater levels of NPK, organic matter and microbial biomass than NOTTS 2 did not suppress basal rot. Much higher levels of organic matter and microbial biomass were found in the CAMBS (clay) and LINCS (sandy, silt loam) soils with the former having much higher levels of N and K compared to the others. The CAMBS soil was also the most suppressive to basal rot disease development but it was not clear if this was related to these factors.

As well as differing in their physical properties, the four soils could also be distinguished based on the structure, diversity and identity of components of the bacterial, fungal and *Fusarium* spp., communities as measured by amplicon sequencing. Overall bacterial diversity was greater in NOTTS1 than for the other soils but fungal diversity was similar. The main components of the bacterial and fungal communities comprised of genera commonly found in soil. Analysis of *Fusarium* spp. and related species present identified pathogenic species including *Ilyonectria. radicicola* (*Cylindrocarpon destructans*), *F. redolens*, *F. solani*, *F. equiseti* and *F. proliferatum*. *F. redolens* and *F. proliferatum* have previously been associated with onion basal rot in addition to FOC. Given that basal rot disease development was generally similar between soils, then no specific components of microbial communities could be identified as being associated with basal rot disease suppression. Ideally a much wider range of soils and onion production systems would need to be examined with more detailed sampling to determine physical or microbial factors associated with differing levels of basal rot.

Using the qPCR, it was clear that detection of FOC in soil was only consistent for inoculum levels ≥1x10^4 cfu g^{-1} soil across all the four soils. However, basal rot disease development in soil only occurred at inoculum levels ≥1x10^5 cfu g^{-1} so for this experiment at least, this suggests that the qPCR would be able to predict disease in a pre-planting test. As conditions for disease development in this experiment were sub-optimal due to the time of year the experiment was carried out, a different disease outcome might have occurred if the experiment was repeated in better light / higher temperatures in the summer. In addition, disease development in an inoculated pot system may differ from that under commercial field conditions.

Despite lower disease levels than expected in the experiment, with first disease symptoms observed after 20-30 days, colonisation of onion roots by FOC was detected by qPCR as early as 3 days and more consistently after 13 days at inoculum levels ≥1x10^5 cfu g^{-1} soil. FOC was also less consistently detected on onion roots at 1x10^4 cfu g^{-1} and overall therefore the threshold for detection was similar as for soil. However, the ability to detect FOC at an early stage in plant development before symptoms occur suggests that using the qPCR to identify the pathogen on onion seedling roots in the field may be another approach to predict disease risk in growing crops. Moreover, if onion seedlings could be used to 'bait' FOC from soil in pre-planting tests, this might with some further development increase the sensitivity of the qPCR detection.

As expected, the amplicon sequencing approach was appropriate for characterising and defining the relative abundance of bacteria and fungi in the different soils. Targeting the TEF gene was particularly effective for identification of *Fusarium* and related spp. and background levels of a number of potentially different pathogenic spp. (including *F. oxysporum*) were identified across the different soil types. When FOC was introduced into these soils, the pathogen was successfully detected using different gene targets including ITS (to genus level), TEF (to species level) and OG4952 (to *F. oxysporum* f.sp. level) with a similar detection threshold as for the qPCR (≥1x10^4 cfu g^{-1} soil) across all the four soils.

In summary, this small project was successful in establishing the level of FOC detection achievable by qPCR and relating this to inoculum concentration and basal rot disease development. Furthermore, it showed that the amplicon sequencing approach has potential to detect FOC at a similar detection threshold and provide further information of other *Fusarium* pathogens and the wider microbial community.

Financial Benefits

The implementation of an effective pre-planting qPCR test for onion basal rot would allow growers to select disease-free or low-disease fields resulting in reduction in losses and concomitant economic benefits. However, further work is required to establish the utility of the qPCR in a range of commercial situations.

Action Points

None at this time.

SCIENCE SECTION

Introduction

This project builds on initial work in an AHDB supported BBSRC HAPI project (BB/K020870/01, AHDB CP 116) and the recently completed AHDB project FV POBOF 452 (Clarkson et al., 2019) where whole genome sequencing, assembly and bioinformatic analyses (Armitage et al., 2018) for the first time enabled the development of specific DNA-based quantitative PCR (qPCR) diagnostic tests for some of the most significant and economically damaging *Fusarium* pathogens in UK horticulture: *F. oxysporum* f.sp. *cepae* (FOC, onion), *F. oxysporum* f.sp. *matthiolae* (FOM, stocks) and *F. oxysporum* f.sp. *narcissi* (FON, *Narcissus*). Furthermore, using similar bioinformatic analyses, an innovative amplicon sequencing approach was developed which allowed the abundance and identity of different components of the microbial community to be identified simultaneously from the same DNA sample. This approach specifically identifies pathogenic and non-pathogenic *Fusarium* species, as well as individual pathogenic *F. oxysporum* f.spp. including FOC, alongside members of the wider fungal and bacterial microbiota. Although amplicon sequencing is now used extensively in research, its practical application to assess general soil health or determine the presence of pathogens in soil is largely unproven, despite its promise and potential role in precision diagnostics.

Following development of these advanced tools, one of the next steps was to explore their practical use to assess disease risk for growers. Furthermore, beginning to understand the components of the microbial community in both diseased and *Fusarium* 'suppressive' soils could also allow us to understand how microbial communities might be manipulated, through varietal choice, organic amendments and application of microbial biocontrol agents to minimise disease incidence and ameliorate pathogen infested soils in the future.

The approach in this project was to focus specifically on FOC which continues to cause ever increasing concern to growers as basal rot disease levels in bulb onions continue to escalate year on year. Having developed a specific DNA-based qPCR assay for this pathogen, we wanted to determine if this test could accurately quantify different levels of FOC inoculum in soil and hence predict the risk of disease development so that it might be of practical use to growers. This would indicate whether pathogen-free or low pathogen field sites could potentially be identified with confidence and hence whether a pre-planting soil test to assess

basal rot disease risk was feasible. In addition, we also wanted to assess whether FOC root colonisation could be detected and quantified early in crop development as another way of potentially assessing risk of disease. In practice, detecting FOC in seedlings or young plants before the expression of symptoms in the field would allow onion growers to manage their crop appropriately and make decisions about harvest, post-harvest curing / drying and storage.

In AHDB project FV PO BOF 452 (Clarkson et al., 2019) we demonstrated a clear dose response relationship between FOC inoculum level and basal rot development in onion using an artificially inoculated peat-based compost growing medium (selected to be optimal for FOC infection) and identified the critical level of inoculum required for disease occurrence. We also established that although first symptoms of FOC appeared after two weeks at high inoculum levels, colonisation of onion roots by the pathogen could be detected even earlier after just two days. However, peat compost is not representative of onion growing soils and is not conducive to extraction of high-quality DNA.

The approach in this project was to artificially inoculate four different soils with varying levels of FOC and monitor subsequent basal rot disease development in a large glasshouse pot experiment. DNA was extracted from each inoculated soil and qPCR used to quantify FOC at each level of inoculum for each soil. This allowed relationships to be established between FOC inoculum level, FOC DNA as measured by qPCR and basal rot disease development for the different soils (Objectives 1 and 2). In addition, we also assessed the rate of onion plant root colonisation by FOC in the different soils again using qPCR (Objective 3).

We also hypothesised that the rate of FOC root colonisation and disease development would be slower in soil compared with the peat compost and would also vary between soil types due to differences in the physical properties or microbial community structure. We therefore measured soil properties and identified key groups of organisms associated with each soil using the amplicon sequencing method developed previously to determine the abundance and identity of *Fusarium* spp., fungi and bacteria that were present (Objective 4). At the same time, we examined whether amplicon sequencing was sensitive enough to effectively distinguish between the different FOC inoculum levels using unique gene targets that simultaneously identify different *F. oxysporum* f.spp. including FOC. This was to determine whether in the future, amplicon sequencing could be used to accurately determine pathogen levels at the same time as assessing soil health based on microbial community analysis.

In summary the main aim of the project was to define relationships between FOC inoculum level, the amount of FOC DNA as measured by a quantitative (q)PCR test and onion basal rot disease development for different soils. A secondary aim was to examine onion root colonisation by FOC and determine if different soils reduced the rate of FOC disease development and if so, identify the components of the microbial community that may be responsible.

The main project objectives were to:

1) Determine the effect of different FOC inoculum levels on onion basal rot disease development for different soils.
2) Quantify the amount of FOC DNA for different inoculum levels using qPCR in different soils and define relationships with onion basal rot disease development.
3) Determine the rate of onion root colonisation by FOC for different soils.
4) Establish the potential of amplicon sequencing to quantify levels of FOC inoculum for different soils and identify components of the microbial community that may reduce the rate of onion basal rot disease development.

Objective 1: Determine the effect of different FOC inoculum levels on onion basal rot disease development for different soils

Materials and Methods

Soil selection and analyses

Four fields representing different soil types were selected by Andy Richardson (Brassica and Allium Centre) and Tom Will (VCS) and comprised sandy loam soil with and without previous (grower) addition of poultry manure, a clay and a sandy silt loam (Table 1). Soils were dried for two weeks before sieving (4 mm mesh) to remove large particles.

Table 1. Locations and descriptions of soil types from UK onion growing fields

Soil Code	Farm	Field	Soil type	Co-ordinates
NOTTS1	Naish Farms	Kingstone	Sandy loam (added poultry manure)	53°14'02.0"N 1°05'24.9"W
NOTTS2	Joseph Camm Farms	Blacksmiths	Sandy loam	53°17'31.2"N 1°00'30.4"W
CAMBS	Russell Smith Farms	Blakelands	Clay	52°05'15.8"N 0°09'03.4"E
LINCS	Willow Tree Farm	Field Lane Newton	Sandy silt loam	52°42'48.4"N 0°07'37.6"E

Soil physical and chemical analysis

Sieved soil from each soil type (Table 1) was dispensed into 11 cm pots, watered (to promote microbial community development) and after six days subjected to physical and chemical analyses by NRM laboratories (Bracknell, UK) to determine the pH; N, P, K, Mg, organic matter content, and CO2 respiration (Solvita test).

Measurement of soil microbial biomass carbon

A chloroform fumigation method was performed to determine microbial biomass carbon in each of the soil samples. This was performed following a protocol adapted from (Joergensen, 1996). 10 g soil was added to glass beakers and placed in a glass desiccator containing one beaker with 30 mL ethanol-free chloroform. Air was evacuated from the desiccator using a

diaphragm pump (KNF Neuberger, UK) until the chloroform boiled, at which point the air was vented and the process was repeated. On the third evacuation, the air was not vented and the vacuumed desiccator was then stored at room temperature for 3 days in the dark (to prevent breakdown of chloroform) to allow full lysis of microbial cells. After 3 days, 40 mL 0.5M potassium sulphate was added to fumigated and unfumigated (10 g of the same soil samples not incubated for 3 days with chloroform) soil samples, which were shaken at 125 rpm for 1 hour. Samples were then passed through type 1 Whatman filter paper prewashed with 0.5M potassium sulphate and the extracts collected in 50 mL falcon tubes and frozen until analysis. Total carbon content of potassium sulphate extracts were then determined using a TOC-L Shimadzu analyser. Potassium sulphate extracts were briefly centrifuged after defrosting and were diluted 1:10 with ultrapure water. A standard curve for Non-Purgeable Organic Carbon (NPOC) was generated from standard solutions made up of potassium hydrogen phthalate. For each diluted potassium sulphate extract, five 100 µl subsamples, alongside 0.5M potassium sulphate and ultrapure water controls, were injected into the TOC-L analyser and NPOC concentrations determined using a standard curve. Control values were subtracted from each sample, and microbial NPOC concentrations per gram of soil calculated by subtracting unfumigated concentrations from fumigated concentrations (Beck et al., 1997).

Basal rot disease development bioassay in different soils

Medium grade vermiculite was added at a ratio 4:1 (soil:vermiculite) to each of the four soils to help maintain structure. A bran/compost inoculum of FOC isolate FUS2 was prepared as described by Taylor et al*.,* (2013) and mixed into each of the four soil types as well as a standard Levington M2 compost to achieve five ten-fold increases in concentrations from $2x10^2$ - $2x10^6$ cfu g^{-1} soil (total of 30 treatments). The FOC infested soils and compost were then dispensed into 7 cm pots and four-week-old onion seedlings (cv. Hytech) transplanted (one plant per pot, 30 pots per treatment arranged in pairs within 15 blocks). An untreated control treatment (no FOC inoculum) was also set up for each soil type and the compost. 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 due to FOC infection was recorded twice weekly. Analysis of the final proportion of plants with basal rot was conducted using a Generalised Linear Model (GLM), assuming a binomial distribution and a logit link function. The uninoculated compost standard was used as the reference factor.

Results

Soil analyses

There were some differences in physical properties between the four different onion growing soils (Table 2). Notably the sandy loam NOTTS 1 which had received multiple applications of poultry manure had elevated levels of NPK, organic matter and microbial biomass compared to the same soil type NOTTS 2. However, much higher levels of organic matter (3.3-4.4%) and microbial biomass (17.9-21.9 µg g^{-1}) were evident in CAMBS (clay) and LINCS (sandy, silt loam) soils. The CAMBS soil notably had much higher levels of N and K compared to the others.

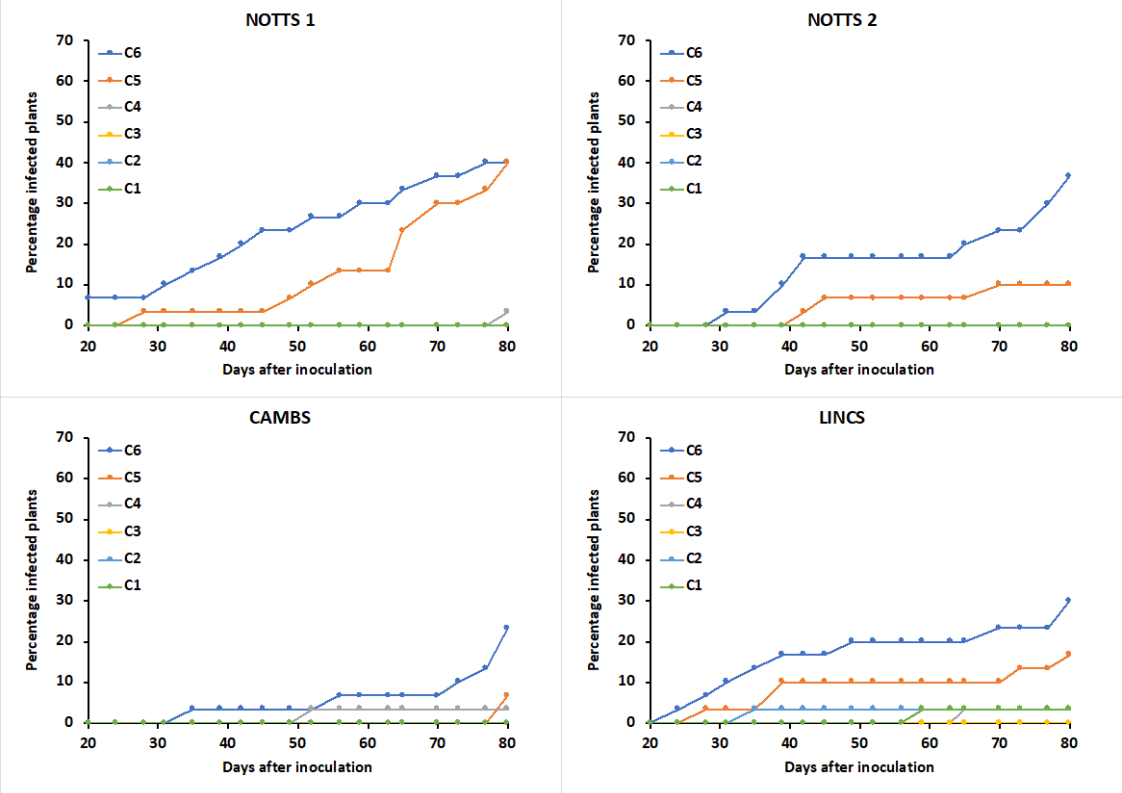
Table 2. Physical properties of four UK onion growing soils

Soil	**NOTTS 1**	**NOTTS 2**	**CAMBS**	**LINCS**
Textural Classification	Sandy Loam	Sandy Loam	Clay	Sandy Silt Loam
Soil pH	7.5	7.3	8.5	8
P index	4	3	4	3
K index	3	2	4	3
Mg index	2	3	2	3
P (mg/L)	64.0	34.8	55.8	37.0
K (mg/L)	271	143	597	257
Mg (mg/L)	91	123	80	167
Nitrate N (mg/kg)	6.74	2.51	18.13	1.89
Ammonium N (mg/kg)	0.68	1.19	0.07	1.17
Available N (kg N/ha)	27.8	13.9	68.3	11.5
CO2 respiration (mg/kg)	38	62	48	14
OM (%)	2.8	1.7	4.4	3.3
Biomass C (µg/g)	9.7	3.2	17.9	21.9

Basal rot disease development bioassay in different soils

Fusarium basal rot disease development progressed very slowly across the different soil and compost treatments compared to previous experiments (in compost; AHDB project FV POBOF 452, Clarkson et al., 2019) with first symptoms only appearing 20-30 days after inoculation. This was most likely due to sub-optimal growing conditions in the glasshouse when the experiments were carried out in November, December and January 2019/20 despite

supplemental lighting. Nevertheless, it was clear that the rate of disease development was greater in compost where final disease levels after 80 days ranged from 13% plants infected at a FOC concentration of $2x10^2$ cfu g^{-1} soil to 53-67% for FOC concentrations of $2x10^4$, $2x10^5$ and $2x10^6$ cfu g^{-1} soil (Fig. 1). In contrast, a good level of Fusarium disease only developed for NOTTS 1, NOTTS 2 and LINCS soils at FOC concentrations of $2x10^5$ and $2x10^6$ cfu g^{-1} soil with between 10 and 40% plants infected. Little disease developed in the CAMBS soil with only 23% plants infected at the highest FOC concentration of $2x10^6$ cfu g^{-1} (Fig. 1). This soil was characterised by much higher levels of available N and K compared to the other soils. Following statistical analysis, all the field soils resulted in significantly lower levels of basal rot than for the compost ($p<0.001$) with no differences between soils. Over all the soils and



compost, there was also a significant effect of FOC inoculum concentration on the final proportion of diseased onions when compared with the uninoculated control (compost). This was evident for the higher FOC concentrations of $2x10^4$ - $2x10^6$ cfu g^{-1} soil.

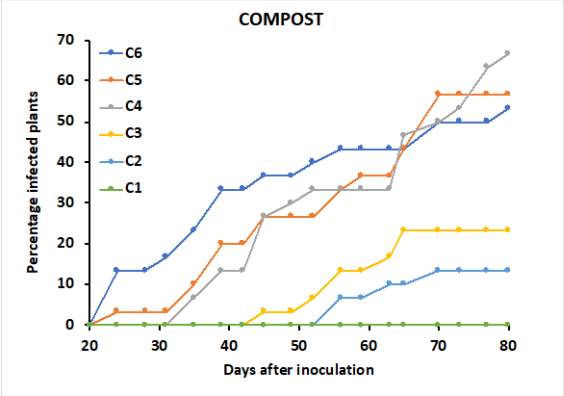


Figure 1. Effect of FOC inoculum level (C2-C6; $2x10^2$ cfu g^{-1} to $2x10^6$ cfu g^{-1} soil; C1 = uninoculated control) on onion basal rot disease development for four different soils and compost.

Objective 2: Quantify the amount of FOC DNA for different inoculum levels using qPCR in different soils and define relationships with onion basal rot disease development

Material and Methods

Soils infested with the different levels of FOC inoculum were also used to fill 11 cm pots and four onion seedlings transplanted and watered. After six days, seedlings were removed and the soil dried at room temperature for 3-5 days before sieving (2 mm mesh), mixing and storage at 4˚C. DNA was then extracted from three independent soil samples (0.5 g) for each treatment (total of 30 treatments) using the GeneAll Exgene Soil SV kit (Cambio, Cambridge, UK) following the manufacturers protocol, with the following modifications: 1) 550 µL of SL buffer and 200µl of SDW was added to 500 mg of soil, which was homogenised in a Powerbead tube using a FastPrep-24 (MP Biomedicals, Cambridge, UK) machine set at 5.5 m s^{-1} for three cycles of 25 seconds; 2) spin columns were incubated for 5 min after the addition of buffer EB before elution. Following extraction, DNA yield and quality was determined using a Denovix DS-11 FX spectrophotometer (Wilmington, USA), before being diluted 1:6 with TE Buffer and stored at -20°C. DNA from each sample was then used for qPCR analysis to determine the quantity of FOC in the soil using specific primers developed previously in AHDB project FV POBOF 452 (Clarkson et al., 2019). qPCR was carried out using a QuantStudio 5 (384-well) machine (Applied Biosystems) using 20 µL reactions containing both primers (final concentration 0.5 µM), 10 µl Power SYBR™ Green PCR Master Mix (Applied Biosystems) and 1 µl of DNA. Conditions were as follows: 1 cycle of 95°C for 120s followed by 45 cycles of 95°C for 3 s, and 60°C for 30 s. All samples were run in triplicate and a melt curve analysis carried out.

Results

In all four soils, the qPCR resulted in detection of FOC only for inoculum concentrations of $1x10^4$, $1x10^5$ and $1x10^6$ cfu g^{-1} soil but there were clear relationships between (log) inoculum

concentration and cycle threshold (Ct; the cycle number where fluorescence of FOC PCR product can be detected above the background signal) linear, R^2 value >0.99; Fig. 1a) or FOC DNA concentration (exponential; Fig. 1b).

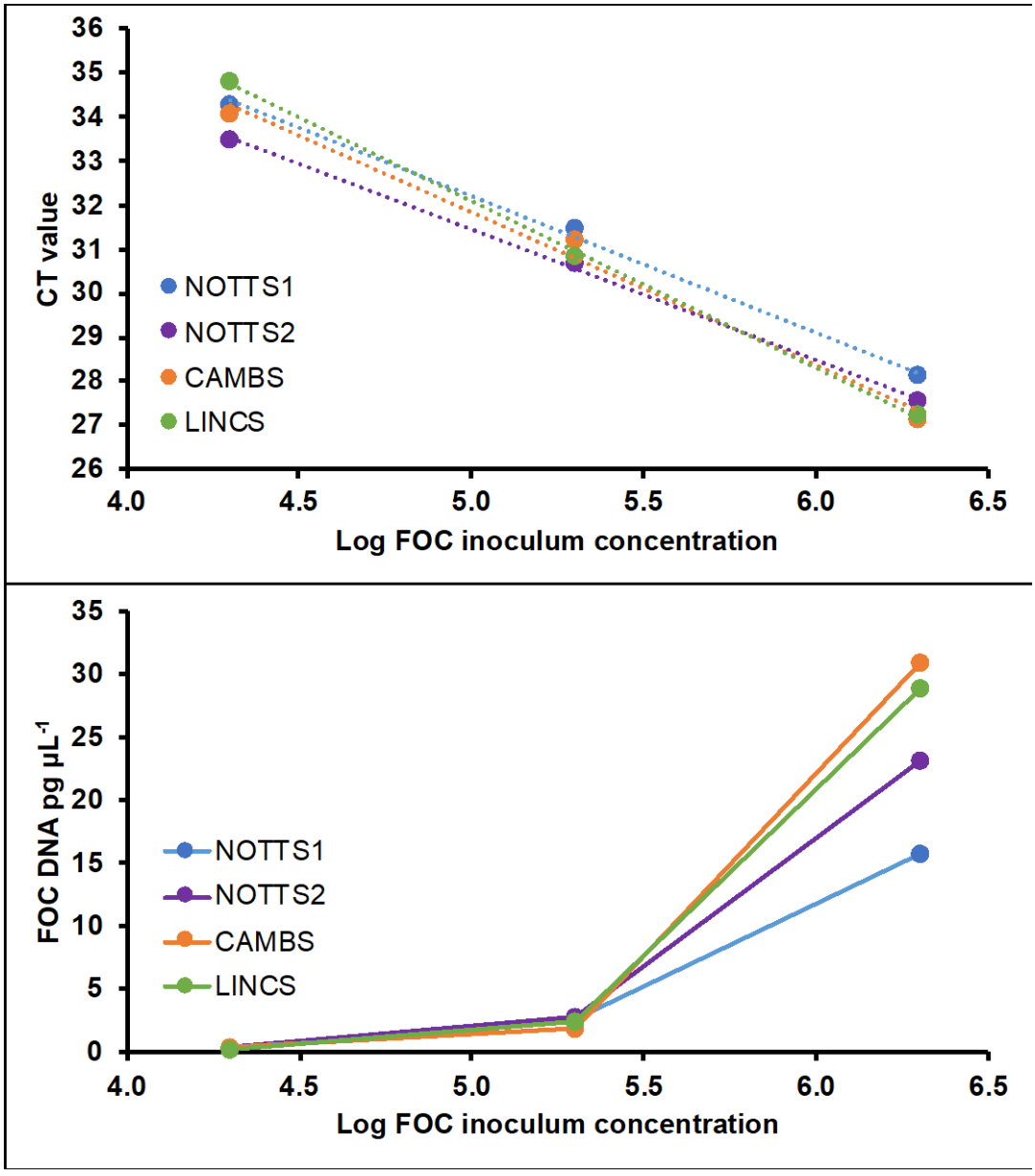


Figure 2. Relationships between log FOC inoculum concentration and a) Ct value or b) FOC DNA concentration for four different soils.

Objective 3: Determine the rate of onion plant root colonisation by FOC for different soils

Material and Methods

Additional pots (7cm, three per treatment) containing the same soil types and FOC concentrations as before were set up and onion seedlings transplanted and watered. Roots of each onion plant were then harvested at 3, 6 and 13 days after transplanting into the infested soils, washed briefly before blotting dry and flash freezing in liquid nitrogen. Root samples were initially stored at -80˚C before being freeze dried for 2 days. DNA was then extracted using a DNeasy plant mini kit (Qiagen, Germany) in accordance with manufacturer's protocol with a minor modification, whereby the roots were homogenised in a lysing matrix-A tube (MP Biomedicals, CA, USA) in a FastPrep-24™ (MP Biomedicals, Cambridge, UK) machine set at 6 ms^{-1} for 40 s. qPCR using the FOC specific primers was then carried out using the extracted DNA from each sample as described above.

Results

Despite rather slow onion basal rot disease development with first symptoms not appearing until 20-30 days, consistent FOC colonisation of onion roots as measured by qPCR was evident after just 3 days following inoculation at FOC concentrations of $2x10^5$ and $2x10^6$ cfu g^{-1} soil across all the four soils and compost (Table 3). There was also occasional detection at the $2x10^4$ cfu g^{-1} inoculum level in one or two of the three replicate samples. This same pattern was evident for the root samples taken after 6 days. After 13 days there was consistent detection of FOC in NOTTS 1 soil and in compost for FOC inoculum levels of $2x10^4$ cfu g^{-1} soil but not for NOTTS2, CAMS or LINCS. However, detection of FOC across all soils and compost was again consistent at FOC inoculum levels of $2x10^5$ and $2x10^6$ cfu g^{-1} (Table 3).

Table 3. Detection of FOC DNA by qPCR for different FOC inoculum concentrations added to four different soils and compost.

3 days		NOTTS 1	NOTTS 2	CAMBS	LINCS	COMPOST
Treatment	**FOC conc cfu g^{-1}**	**pg FOC DNA mg^{-1} root[1]**				
C1	0	ND	ND	ND	ND	ND
C2	$2x10^2$	ND	ND	ND	ND	ND
C3	$2x10^3$	ND	ND	ND	ND	ND
C4	$2x10^4$	1.27 ±0.91	0.21	ND	0.09	0.59 ±0.46
C5	$2x10^5$	0.96 ±0.50	0.88	0.12 ±0.02	0.53 ±0.35	8.46 ±8.18
C6	$2x10^6$	4.35 ±0.40	5.52 ±1.04	2.75 ±1.12	18.6 ±15.18	7.28 ±3.40

6 days		NOTTS 1	NOTTS 2	CAMBS	LINCS	COMPOST
Treatment	**FOC conc cfu g^{-1}**	**pg FOC DNA mg^{-1} root[1]**				
C1	0	ND	ND	ND	ND	ND
C2	$2x10^2$	ND	ND	ND	ND	0.04
C3	$2x10^3$	ND	ND	0.08	ND	ND
C4	$2x10^4$	ND	ND	0.37 ±0.01	ND	0.19 ±0.09
C5	$2x10^5$	0.83 ±0.28	0.81 ±0.39	1.23 ±0.85	0.74 ±0.26	7.17 ±2.5
C6	$2x10^6$	8.19 ±2.18	4.61 ±1.45	6.8 ±2.53	2.18 ±0.24	53.68 ±26.42

13 days		NOTTS 1	NOTTS 2	CAMBS	LINCS	COMPOST
Treatment	**FOC conc cfu g^{-1}**	**pg FOC DNA mg^{-1} root[1]**				
C1	0	ND	ND	ND	ND	ND
C2	$2x10^2$	ND	ND	ND	ND	0.07
C3	$2x10^3$	0.03	ND	ND	0.02	1.33 ±1.32
C4	$2x10^4$	0.06 ±0.03	0.32	ND	0.03	9.12 ±7.74
C5	$2x10^5$	0.52 ±0.22	0.67 ±0.56	1.15 ±0.96	0.67 ±0.42	16.7 ±15.48
C6	$2x10^6$	26.34 ±17.58	2.29 ±0.05	3.43 ±1.58	6.93 ±3.05	39.74 ±8.85

[1] ± indicates standard error of the mean FOC DNA concentration detected in three replicate samples. ND indicates FOC DNA not detected or below detection limit. Data highlighted in green / red indicates treatments where one or two replicate samples respectively were negative for FOC.

Objective 4: Establish the potential of amplicon sequencing to quantify levels of FOC inoculum for different soils and identify components of the microbial community that may reduce the rate of onion basal rot disease development

Material and Methods

Overview

The potential of amplicon sequencing to detect the different levels of FOC introduced into the four different soils, as well as to distinguish differences in microbial community composition was assessed using different gene targets. These were 16S for bacteria, ITS for fungi, TEF for different *Fusarium* spp. and related spp. (including *F. oxysporum*) as well as two amplicons identified previously with potential to identify FOC and several other *F. oxysporum* f.spp. (OG4952 and OG13890). Being a semi-quantitative assay, amplicon sequencing allowed assessment of relative abundance of different taxa within the soil community within each soil type as well as FOC. The analysis was performed as a multiplex assay, taking advantage of bioinformatic tools to separate multiple target loci sequenced under the same Illumina barcode. Target amplicons and analysis pipelines were based upon those reported in AHDB project FV PO BOF 452 (Clarkson et al., 2019).

DNA Extraction

DNA was extracted at NIAB-EMR from the same samples representing each soil / FOC inoculum concentration as used for qPCR detection at Warwick in Objective 2. This was done using the Soil SV kit (GeneAll, UK) as described above. As DNA extraction efficiencies can vary between repeat extractions even within the same soil type, this effect was minimised by carrying out three extractions for each soil sample and pooling before diluting to 2 ng μl^{-1} for use in amplicon sequencing PCR reactions.

Library preparation

Library preparation was as described in AHDB project FV PO BOF 452 (Clarkson et al., 2019) as outlined in Fig. 3. In the previous study, sequencing reads for amplicon OG13890 targeting FOC were low, potentially due to PCR bias and different primer efficiencies when multiplexing

during the first round target gene PCRs. Therefore, to avoid this problem in this set of experiments, the first round PCR reactions were carried out separately for each of the different gene targets (16S, ITS, TEF, OG4952 and OG13890). For each reaction, 5 µl (10 ng) of soil template DNA was used in a 25 µl PCR reaction using a KAPA HiFi HotStart 2X master mix (Roche, UK).

Prior to magnetic bead clean-up (MagBind TotalPure NGS, M1378-01, VWR) and the barcoding (index) reaction, equal volumes (12.5 µl) of the 16S and ITS PCR products were mixed to create one multiplex, while 13 µl TEF, and 4 µl each of OG4952 and OG13890 were mixed for a second multiplex barcoding reaction. After barcoding using dual index barcodes, the libraries were bead cleaned again, quantified and normalised to 4 nM. All libraries were prepared in triplicate. The 4 nM libraries were pooled using 4 µl of each 16S / ITS barcode and 2 µl each TEF/ OG4952 / OG13890 barcode. This was done to allow for enough reads for the greater number of OTUs expected for both 16S and ITS reactions. Libraries were loaded at 6 pM on the Illumina MiSeq V3 600 cycle flow cell with 20% PhiX spiked in.

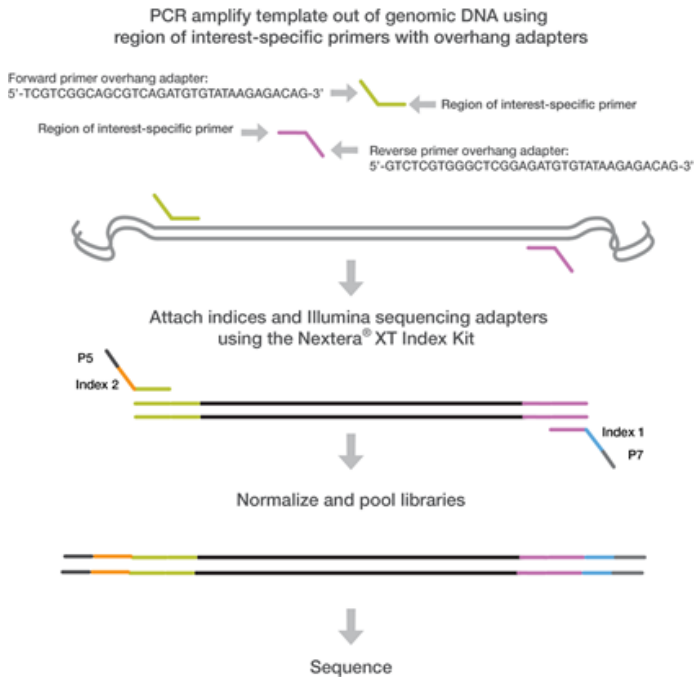


Figure 3. Illumina dual-index, 2-step PCR approach used to carry out amplicon sequencing. Dual-indexing reduces mis-tagging events and enables up to 96 libraries to be pooled for sequencing on a single flow cell.

Amplicon sequence data analysis pipeline

Amplicon sequence data was analysed using the same approach reported in AHDB project FV PO BOF 452 (Clarkson et al., 2019; Fig. 4). Illumina sequencing reads were generated for 126 samples across two sequencing plates, each containing 63 samples for this experiment and a further 18 samples for an associated experiment to assess sample consistency between plates. Illumina sequence reads were separated ("demultiplexed") into samples based upon the duel-index barcode sequence present on each read (Fig. 4, Step 1). This was done by the Illumina sequencing machine as part of the run. Reads from each sample were assigned to target amplicons based upon the primer sequence present at the beginning / end of the paired reads (Fig. 4, Step 2). Amplicon identity was assigned if either the forward or reverse read of a sequence contained a 100% match to one of the target primer sequences. Forward and reverse pairs of reads were merged into a single sequence with a maximum difference in overlap of 20 bases and the forward and reverse primers were removed (Fig. 4, Step 3). Illumina sequence data has a low error rate, but this increases over the length of the read. By merging reads, confidence in the base calls across the entire sequence are increased. Quality scores are retained in the merged read, allowing the chance of a base containing an error to be assessed. Merged reads with more than a 50% chance of one nucleotide in the entire sequence containing an error were discarded as were those containing Illumina adapter contamination or fewer than 150 nucleotides. For preparation of operational taxonomic unit (OTU) generation, merged sequences were filtered with stringent criteria. Remaining merged reads were filtered for quality with a maximum expected error threshold of 0.5 per sequence (Edgar and Flyvbjerg 2015). Sequences were de-replicated and unique sequences with fewer than 8 reads were discarded, then all unique sequence reads were sorted by their respective frequencies. Unfiltered reads were retained for OTU identification and quantification. All operational taxonomic unit (OTU) processing was carried out with the USEARCH 11.0 OTU clustering pipeline (Edgar, 2013) unless specified otherwise.

For analysis of all amplicons, reads were clustered into OTUs based upon sequence similarity, and these OTUs were assigned an identity (Fig. 4, Step 4). This step was performed using all filtered reads attributed to a locus (16S, ITS, TEF, OG4952, OG13890) in the experiment, thereby ensuring that the same OTU were identified across the entire experiment. As was performed in our previous project, we clustered reads using the UNOISE3 option in USEARCH, rather than clustering at an arbitrary threshold 97% similarity. This aims to resolve different sequence haplotypes by estimating error rates around clustered reads. Prior to this, all reads were adjusted to be the same length by adding 'N' bases to the ends of the sequences, as required. The clustering algorithm also removed chimeras during the clustering

process. Taxonomy was assigned to OTU sequences by searching against reference sequence databases using the SINTAX algorithm in USEARCH software (Edgar 2016). In the case of 16S the RDP 16S database v.16 (Cole et al. 2014) was used, and in the case of ITS the UNITE ITS database v.7.2 (Koljalg et al. 2013) was used. Unfiltered merged reads were quantified against the identified OTUs and summarised by genus, species or *F. oxysporum* f.spp. (Fig. 4, Step 5). Quantification was performed using USEARCH, using different mapping stringency based on the amplicon analysed. Mapping to 16S and ITS amplicons was less stringent to accommodate the variability of these loci at the genus/species level, and used the default 97% sequence identity. In contrast, reads required 100% sequence identity when mapping to OG4952 and OG13890 to reduce chance of false positive identification of OTUs assigned to *F. oxysporum* f.spp.

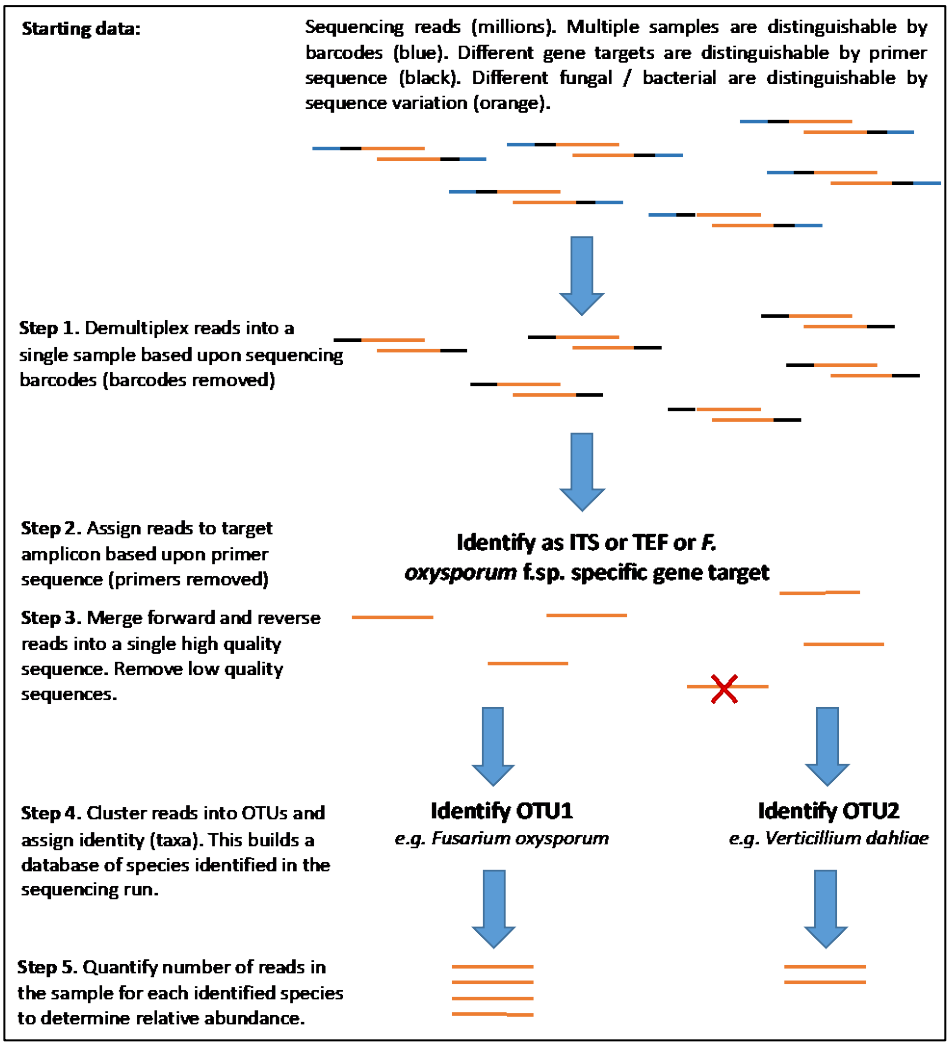


Figure 4. Summary of steps used in the analysis of amplicon sequence data.

Graphical data plots were generated in R using ggplot2. Counts from the three technical replicates were combined to produce a single sample per treatment. To account for differences in sequencing depth between samples, sequence counts for 16S, ITS and TEF were normalised to 1000 reads per sample. Runs with less than 1000 reads assigned to taxa were not normalised. For diversity analyses, library sizes were normalised using the median-of-ratios method implemented in DESeq2 (Anders and Huber 2010; Love et al., 2014). For Principal Component Analysis (PCA), counts were first transformed using the DESeq2 variance stabilisation transformation (VST). PCA was applied to the VST-transformed OTU counts, to produce principal component (PC) scores per sample. Permutation Multivariate ANOVA (PERMANOVA) as implemented in the VEGAN 2.3-1 package (Dixon, 2003) was carried out on the Bray-Curtis dissimilarity matrix of OTU abundance to assess the overall contribution of study factors to the observed differences in microbial community structures. Alpha (α) diversity (Chao1, Shannon and Simpson) indices were calculated using VEGAN 2.3-1. Chao1 estimates species richness based on their abundance, while Shannon and Simpson indices estimate species richness and evenness with more weight on richness and evenness respectively. The rank of (α) diversity indices were subjected to ANOVA to assess the differences between soil type and FOC inoculum concentration.

Results

Diversity of bacteria using 16S amplicon sequencing

Alpha diversity plots of the 16S sequence data showed that there was high bacterial diversity with an even distribution and low dominance in all soils with the highest diversity in NOTTS 1 soil (S1) (Fig. 5). ANOVA of the three diversity measures indicated that there was a statistically significantly difference in diversity between soils (Chaol1, $p=0.042$; Shannon, $p < 2 \times 10^{-16}$; Simpson, $p < 2 \times 10^{-16}$). A PCA plot based on bacterial (16S) β-diversity clearly distinguished the different soils with samples representing different levels of FOC inoculum within each soil clustered together (Fig. 7). CAMBS (S3) and LINCS (S4) soils grouped together for PC1 but were separated by PC2, while NOTTS 1 (S1) and NOTTS 2 (S2) as expected were more similar to each other than they were to the other two soils with CAMBS (S3) showing the biggest difference to S2. The results therefore indicated clear differences in bacterial community between each of the four soils. PERMANOVA indicated there were significant effects of soil ($F_{3,14}=53.30$, $p>0.001$) and also a small but significant effect of FOC inoculum

concentration ($F_{5,14}$=1.97, p=0.031) on overall community structure. The PCA plot also clearly showed that the S3_D1 sample (CAMBS, no FOC inoculum) clustered with NOTTS 2 (S2) and this was attributed to the incorrect sample being used for DNA extraction or PCR. This sample was therefore not included in the 16S diversity analyses or subsequent analyses with other gene amplicon targets.

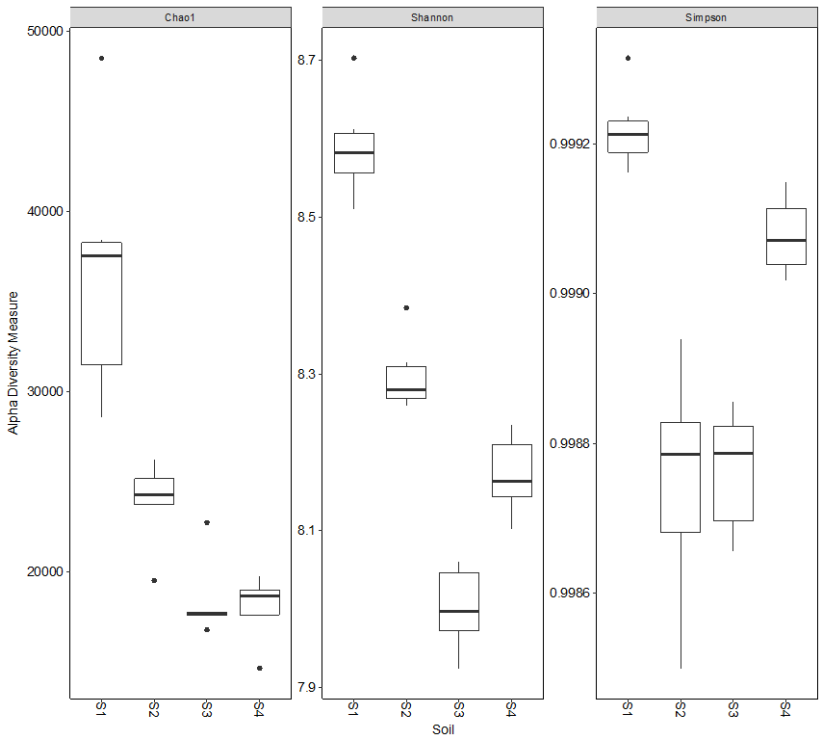


Figure 5. Alpha diversity plots for 16S sequence data showing high bacterial diversity, with even distribution and low dominance for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS).

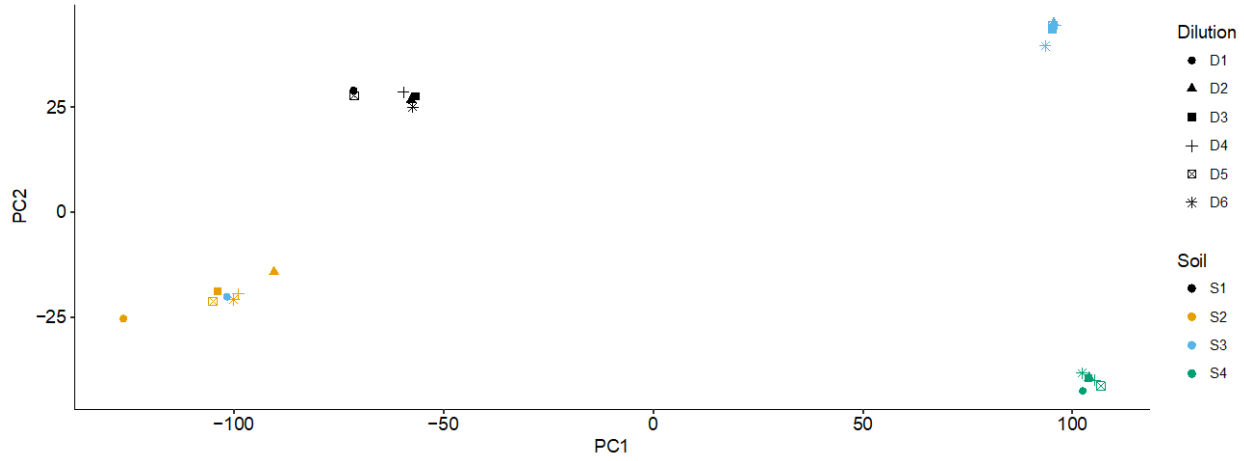


Figure 6. PCA plot of bacterial diversity based on 16S sequence data for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS) infested with different concentrations of FOC inoculum (D1, no FOC inoculum; D1, $2x10^2$ cfu g^{-1}, D2, $2x10^3$ cfu g^{-1}; D3, $2x10^3$ cfu g^{-1}, D4, $2x10^4$ cfu g^{-1}; D5, $2x10^5$ cfu g^{-1}, D6, $2x10^6$ cfu g^{-1} soil).

Identity of bacteria using 16S amplicon sequencing

Analysis of the bacterial community by 16S amplicon sequencing showed that many bacterial genera were present in the soil samples with the most prevalent representing up to 0.06% of reads (60 in 1000). Looking more closely at the 29 most abundant taxa (Fig. 7), *Flavobacterium*, *Gemmatimonas*, *Lysobacter* and *Sphingomonas* spp. were abundant across all four soils while CAMBS (S3) and LINCS (S4) showed reduced levels of *Pseudomonas* and *Arthrobacter* compared to NOTTS 1 and NOTTS 2 soils. The bacterial community was largely consistent across the different FOC levels suggesting that the addition of the inoculum did not impact upon the bacterial soil community when sampled six days later.

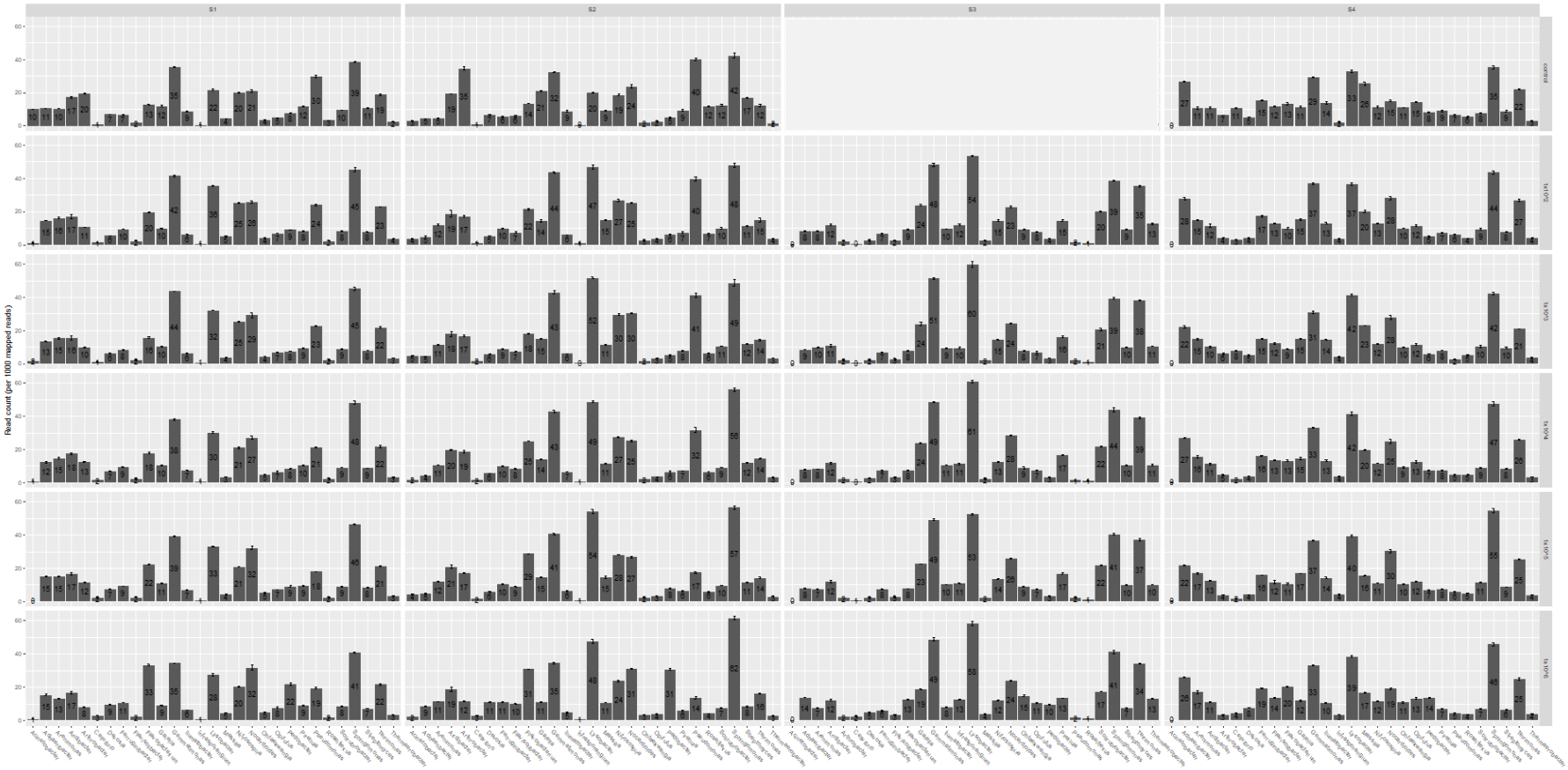


Figure 7. Bacterial community structure for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS) infested with different concentrations of FOC inoculum (10^2-10^6 cfu g^{-1} soil). Plots represent normalised read counts of most abundant bacterial genera following 16S amplicon sequencing. Standard errors represent three technical replicates of the PCR and library preparation process.

Diversity of fungi using ITS amplicon sequencing

Alpha diversity plots of the ITS data indicated high fungal diversity with an even distribution and low dominance in all soils with similar diversity across the four soils (Fig. 8). PERMANOVA indicated small but significant difference between both soil types ($p=0.049$) and FOC inoculum concentration ($p=0.024$) for the Shannon diversity measure. A PCA plot based on fungal (ITS) β-diversity again showed that as for 16S, the four soils could be clearly distinguished from each other, with samples representing different levels of FOC inoculum within soil type clustered together (Fig. 9). CAMBS (S3) and LINCS (S4) again grouped together for PC1 but were separated by PC2, while NOTTS 1 (S1) and NOTTS 2 (S2) were more similar to each other than CAMBS (S3) and LINCS (S4). The results therefore indicated clear differences in fungal community structure between each of the four soils with no effect (in the first two PCs) of adding FOC inoculum for these samples taken six days later. However, PERMANOVA of the whole community structure did indicate a significant effect of soil ($F_{3,14}=51.96$, $p<0.001$) and a small but significant effect of FOC inoculum concentration ($F_{5,14}=11.93$, $p<0.001$). As for the 16S data, the PCA plot again showed that the S3_D1 sample (CAMBS, no FOC inoculum) clustered with NOTTS 2 (S2).

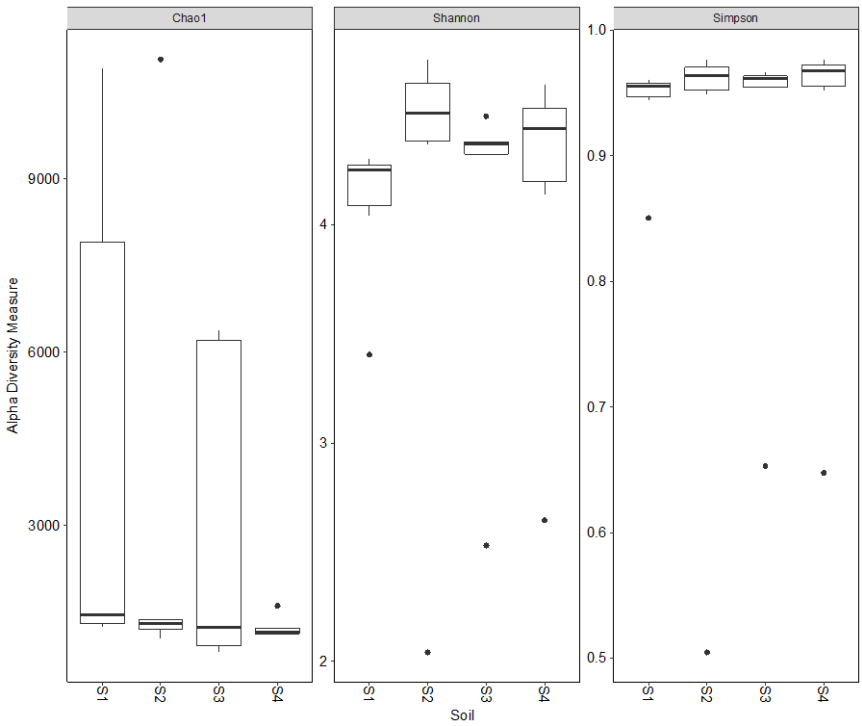


Figure 8. Alpha diversity plots for ITS sequence data showing high fungal diversity with even distribution and low dominance in each soil type (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS).

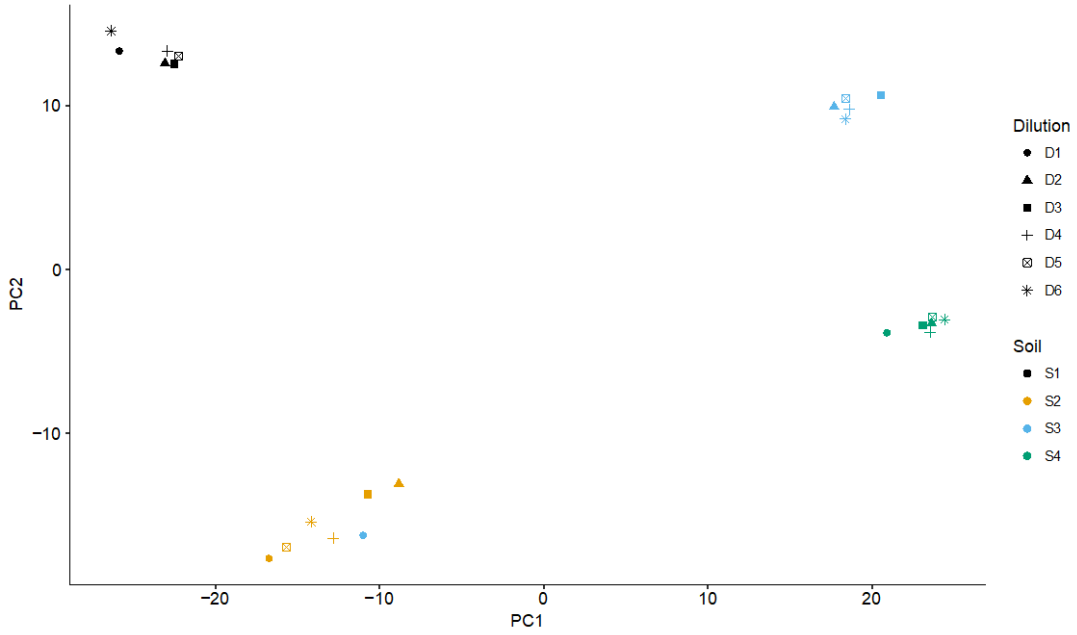


Figure 9. PCA plot of fungal diversity based on ITS sequence data for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS) infested with different concentrations of FOC inoculum (D1, no FOC inoculum; D1, $2x10^2$ cfu g^{-1}, D2, $2x10^3$ cfu g^{-1}; D3, $2x10^3$ cfu g^{-1}, D4, $2x10^4$ cfu g^{-1}; D5, $2x10^5$ cfu g^{-1}, D6, $2x10^6$ cfu g^{-1} soil).

Identity of fungi using ITS amplicon sequencing

Analysis of the fungal community by ITS amplicon sequencing showed a high diversity of genera in soils with many present at low levels (Fig. 10; only those with >0.1% reads shown). NOTTS 1 (S1) had high abundance of *Guehomyces, Mortierella* and *Podospora* and similarly NOTTS 2 (S2) had high abundance of *Guehomyces* and *Mortierella*. CAMBS (S3) had high abundance of *Chaetomium* and *Pseudeurotium* while LINCS (S4) had high abundance of *Chaetomium, Mortierella* and *Podospora.* Most of these fungi are common soil saprophytes while *Guehomyces* has been found in a very wide variety of habitats. *Fusarium* spp. were also present in high abundance in all four soils and as expected the ITS reads assigned to this genus increased with the higher levels of FOC inoculum (Fig. 10). However, this was only clearly observed for FOC inoculum levels of $2x10^5$ cfu g^{-1} and $2x10^6$ cfu g^{-1} soil and this also suggested that background levels of *Fusarium* spp. were $<2x10^5$ cfu g^{-1} soil. *Fusarium* spp. read counts per 1000 reads only increased by a factor of between 3 and 4 as FOC inoculum levels increased 10-fold; this could be due to loss of this dominant OTU during the bead clean-up steps of library preparation. Overall, four ITS OTUs were assigned to *F. oxysporum* (combined reads shown in Fig. 10) but only one of these showed a trend of increasing reads with increasing levels of FOC inoculum added (Fig. 11) indicating that this OTU was specifically associated with the FOC isolate used in this study. For this OTU, ITS sequencing reliably identified the presence of *F. oxysporum* at FOC inoculum levels of $\geq 1x10^4$ spores g^{-1} soil. The other *F. oxysporum* OTUs did not correlate with FOC inoculum level and one was associated only with CAMBS soil (data not shown).

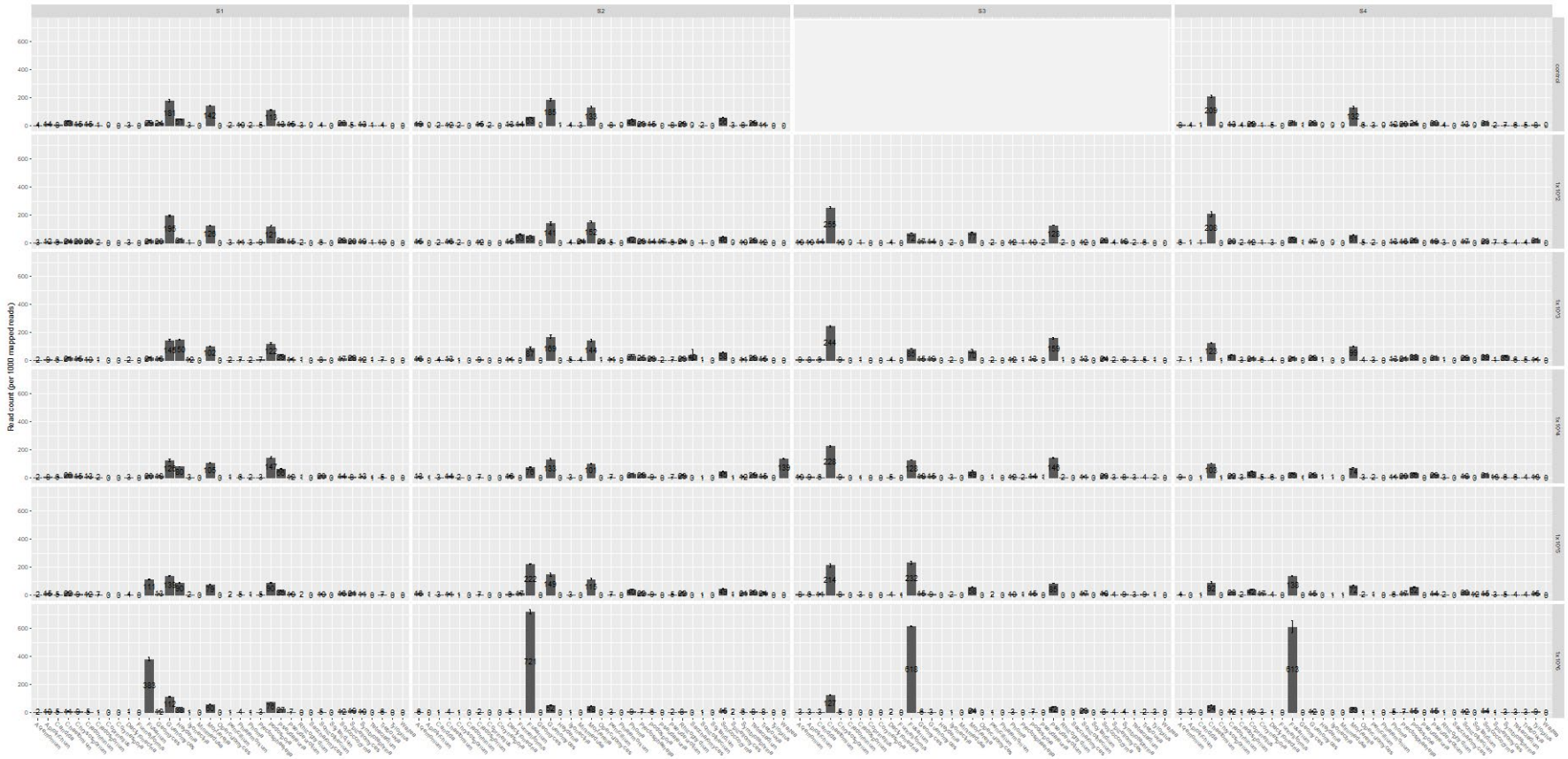


Figure 10. Fungal community structure for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS) infested with different concentrations of FOC inoculum (10^2-10^6 cfu g^{-1} soil). Plots represent normalised read counts of most abundant fungal genera following ITS amplicon sequencing. Standard errors represent three technical replicates of the PCR and library preparation process

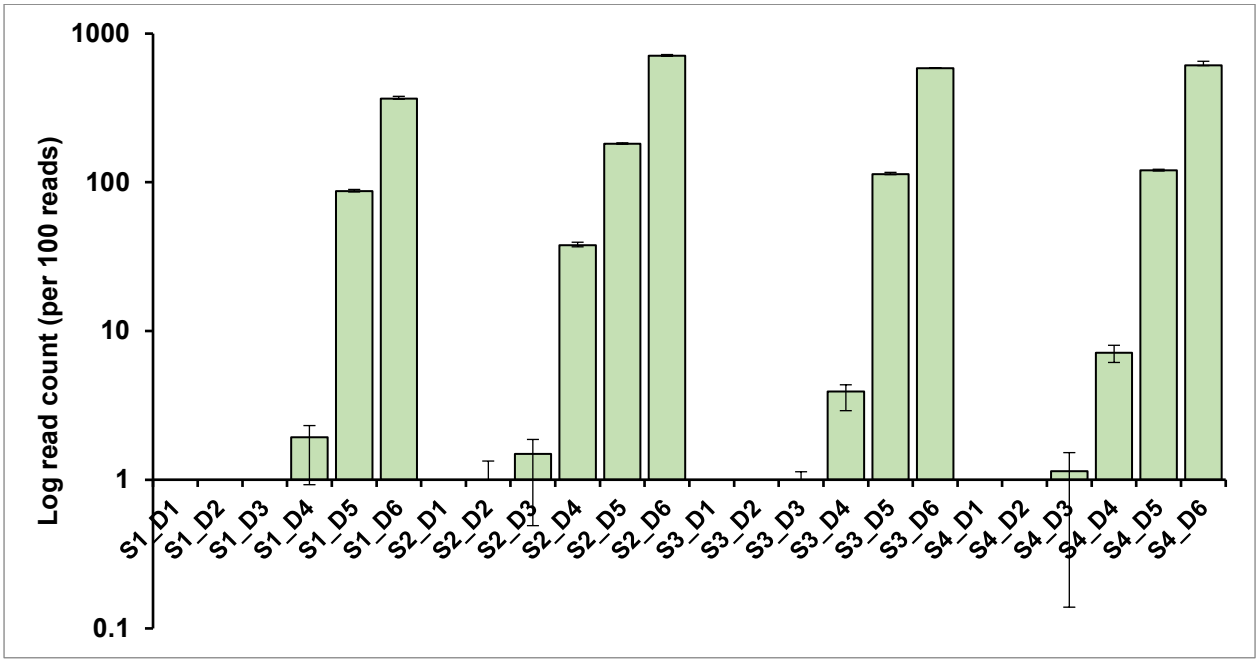


Figure 11. Log normalised read counts for *F. oxysporum* OTU SH488911 for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS) infested with different concentrations of FOC inoculum (D1, no FOC inoculum; D1, $2x10^2$ cfu g^{-1}, D2, $2x10^3$ cfu g^{-1}; D3, $2x10^3$ cfu g^{-1}, D4, $2x10^4$ cfu g^{-1}; D5, $2x10^5$ cfu g^{-1}, D6, $2x10^6$ cfu g^{-1}) showing increasing reads with increasing inoculum levels.

Diversity of *Fusarium* spp. using TEF amplicon sequencing

A PCA plot based on *Fusarium* spp. (TEF) β-diversity showed that the four soils could be clearly distinguished and had distinct fungal communities at the level of *Fusarium* species (Fig. 12). As for the 16S and ITS data, the PCA plot again showed that the S3_D1 sample (CAMBS, no FOC inoculum) clustered with NOTTS 2 (S2).

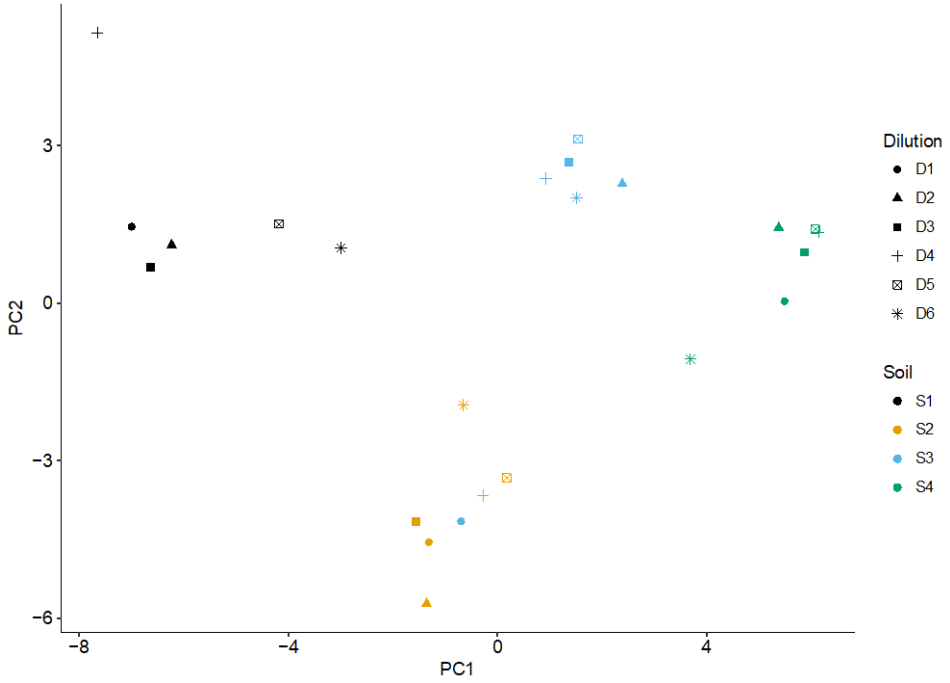


Figure 12. PCA plot of *Fusarium* spp. fungal diversity based on TEF sequence data for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS) infested with different concentrations of FOC inoculum (D1, no FOC inoculum; D1, $2x10^2$ cfu g^{-1}, D2, $2x10^3$ cfu g^{-1}; D3, $2x10^3$ cfu g^{-1}, D4, $2x10^4$ cfu g^{-1}; D5, $2x10^5$ cfu g^{-1}, D6, $2x10^6$ cfu g^{-1} soil).

Identity of *Fusarium* spp. using TEF amplicon sequencing

Analysis of *Fusarium* and related spp. identity by TEF sequencing indicated a diverse and distinct the *Fusarium* community present in each soil (Fig. 13). *F. oxysporum* was present but not the dominant *Fusarium* sp. in each of the soils before addition of FOC inoculum. All four soils contained *Metarhyzium majus,* a characterised coleopteran biocontrol as well as fungal pathogenic species. NOTTS 1 (S1) and NOTTS 2 (S2) also had high abundance of the fungal pathogens *Ilyonectria radicicola* (*Cylindrocarpon destructans*) and *F. flocciferum* while the latter also had high abundance of the pathogenic *F. redolens* and *F. proliferatum*. The CAMBS (S3) had high levels of *I. radicicola* and *Fusarium solani* while the LINCS (S4) soil contained pathogenic *F. culmorum*, *F. flocciferum, F. equiseti* and *F. proliferatum* as well as *I. radicicola*. Fewer TEF reference sequences are available for the wider fungal community, so this may limit accuracy and variety of the *Fusarium* related spp. that were identified. After addition of FOC inoculum, there was little change to the *Fusarium* community (Fig. 13) although there was a slight increase in *I. radicicola* in NOTTS 2 after addition of FOC at $2x10^2$ cfu g^{-1} soil. As expected, the TEF reads assigned to *F. oxysporum* increased with higher levels of FOC inoculum (Fig. 13). However, this was only clearly observed for FOC inoculum levels ≥$2x10^4$ cfu g^{-1} soil although smaller increases were evident at $2x10^3$ cfu g^{-1} soil for NOTTS 1 (S1) and NOTTS 2 (S2).

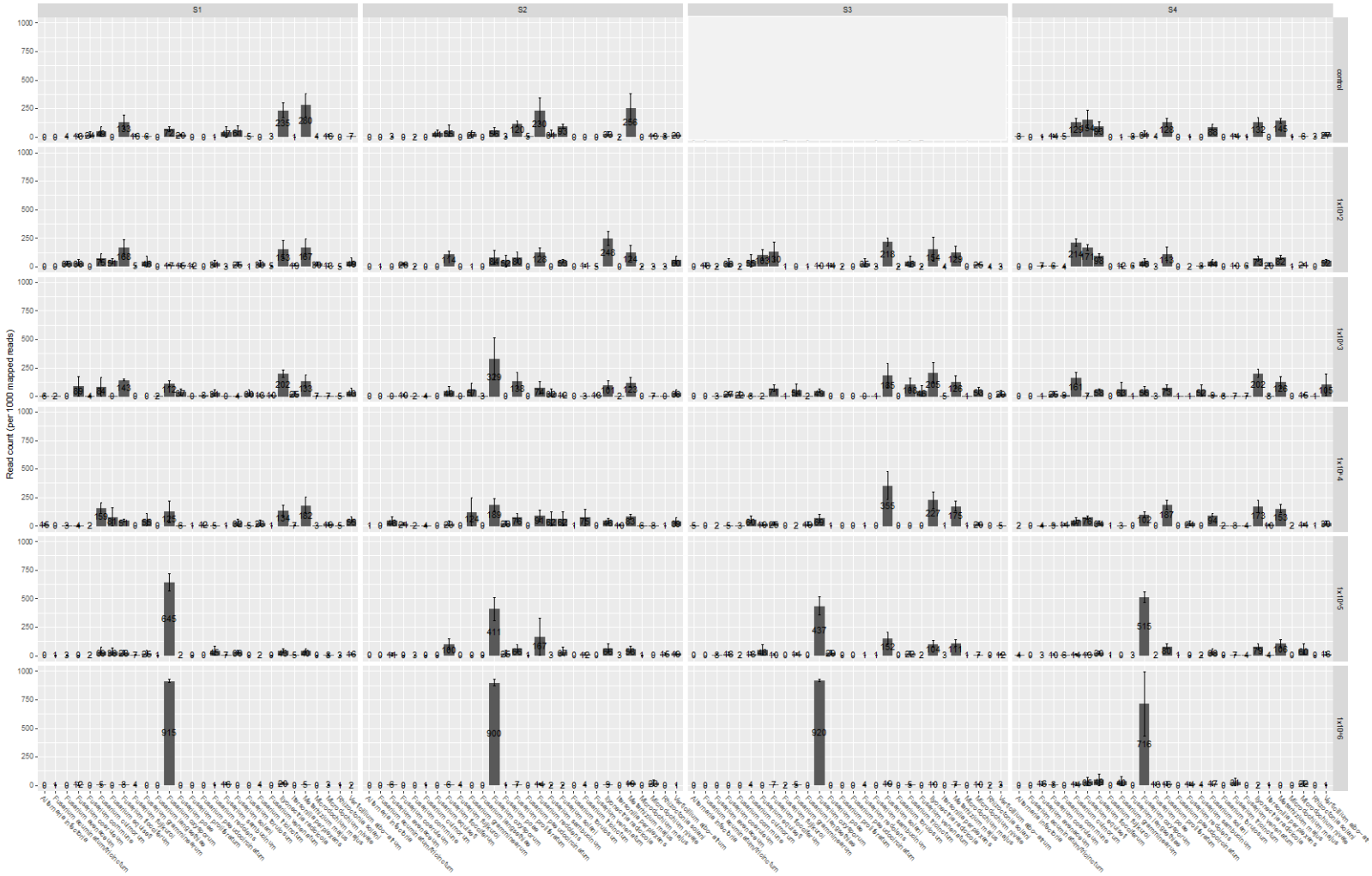


Figure 13. *Fusarium*

spp. community structure for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS) infested with different concentrations of FOC inoculum (10^2-10^6 cfu g^{-1} soil). Plots represent normalised read counts of most abundant fungal genera following TEF amplicon sequencing. Standard errors represent three technical replicates of the PCR and library preparation process.

Detection of FOC using OG4952 amplicon sequencing

Sequencing of the OG4952 locus was predicted to specifically detect FOC, *F. oxysporum* f.sp. *matthiolae*, *F. oxysporum* f. sp. *conglutinans*, *F. oxysporum* f.sp. *narcissi* and *F. oxysporum* f.sp. *pisi*. Read counts for FOC were generally very high (especially at high FOC inoculum concentrations), and when considering total read counts (Fig. 14) there was reliable detection of FOC at inoculum concentrations ≥2x10^5 cfu g^{-1} soil; however, there was some variation between soils with detection in NOTTS 1 (S1) and NOTTS 2 (S2) soil at ≥2x10^3 cfu g^{-1} and ≥2x10^4 cfu g^{-1} soil respectively. Reads associated with the other *F. oxysporum* f.spp. were detected across the different soils, but their presence was inconsistent (Fig. 14). However, as FOC levels increased to 1x10^5 and 1x10^6 cfu g^{-1} soil, these reads became negligible. The variability in detection of these other *F. oxysporum* f.spp. supports their presence at very low levels due to stochastic sampling at early cycles of PCR.

Detection of FOC using OG13890 amplicon sequencing

Sequencing of the OG13890 locus was also predicted to specifically detect FOC, *F. oxysporum* f.sp. *matthiolae*, *F. oxysporum* f. sp. *conglutinans*, *F. oxysporum* f.sp. *narcissi* and *F. oxysporum* f.sp. *pisi*. High read counts were only evident for FOC concentrations ≥2x10^5 cfu g^{-1} soil for NOTTS 1 (S1) NOTTS 2 (S2) and LINCS (S4) and 1x10^6 cfu g^{-1} soil for CAMBS (S3) (Fig. 15). In all other samples read counts were very low (<1000 reads). This indicates that although OG13890 primers are able to detect FOC in soil samples, they are much less efficient than other primers used and higher template concentrations are required for reliable detection.

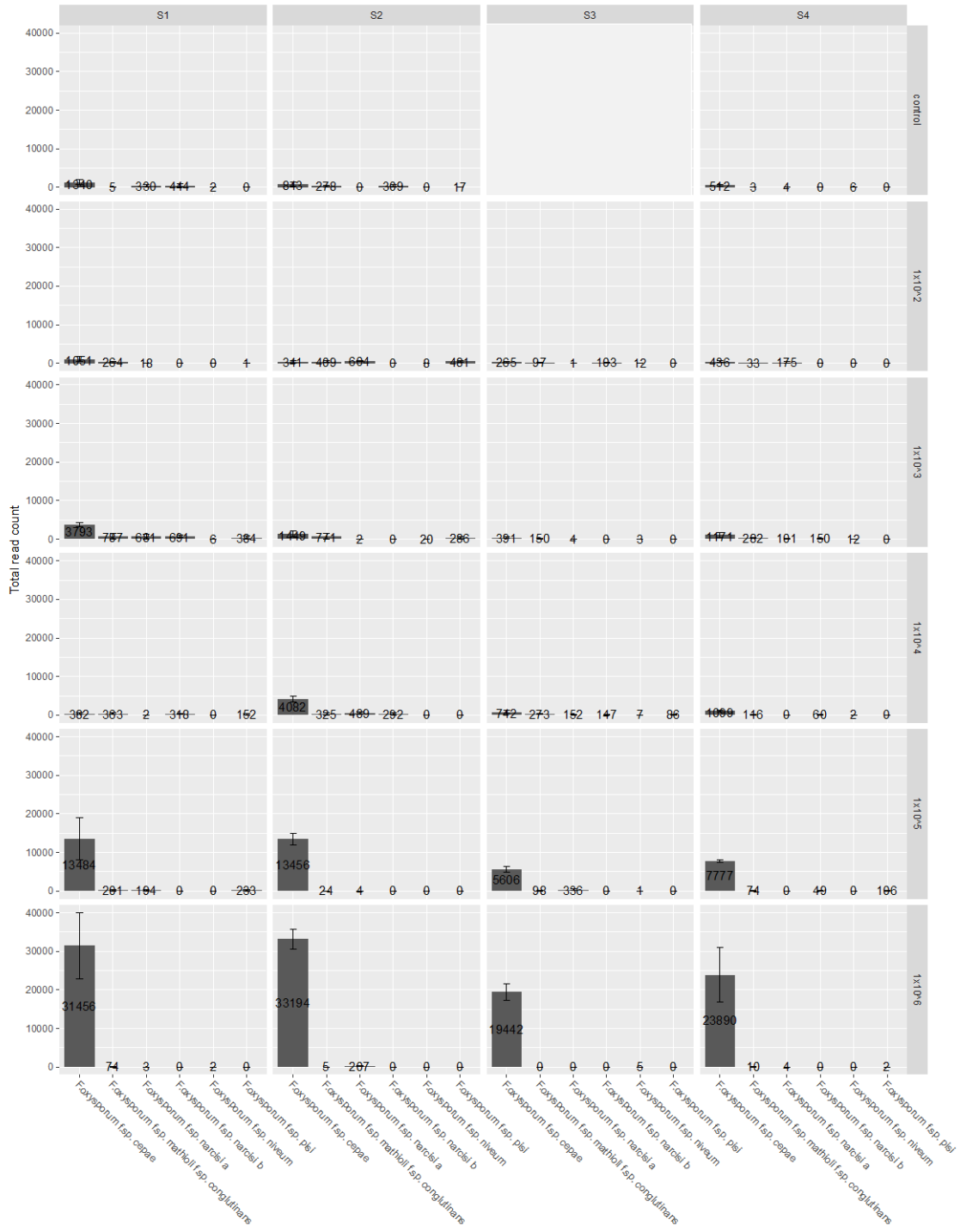


Figure 14. Total read counts of FOC and other *F. oxysporum* f.spp. following amplicon sequencing of the OG4952 locus for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS) infested with different concentrations of FOC inoculum (10^2-10^6 cfu g^{-1} soil).

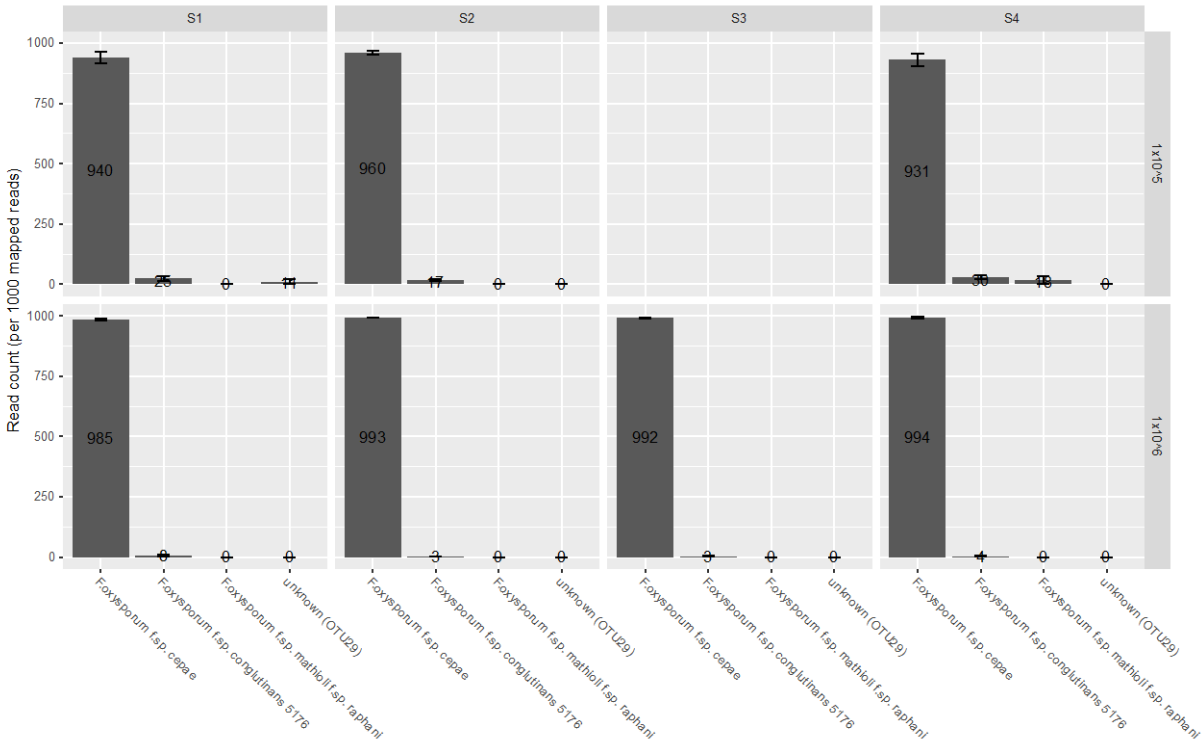


Figure 15. Total read counts of FOC and other *F. oxysporum* f.spp. following amplicon sequencing of the OG13890 locus for four soils (S1, NOTTS 1; S2, NOTTS 2; S3, CAMBS; S4, LINCS) infested with different concentrations of FOC inoculum (10^5-10^6 cfu g^{-1} soil).

Discussion and Conclusions

Four different onion growing soils (NOTTS 1, NOTTS 2, CAMBS and LINCS) including two of the same type with and without previous application of poultry manure (NOTTS1 and NOTTS 2 respectively) resulted in significantly lower levels of basal rot disease development compared to a peat-based growing compost when inoculated with different concentrations of FOC inoculum in a glasshouse experiment. This suggests that the physical and / or biological properties of these soils were less conducive to disease. Basal rot development in NOTTS 1 and NOTTS 2 soils was similar (although slightly higher for NOTTS 1) and only extensively developed for FOC inoculum levels of 2 x 10^5 and 2 x 10^6 cfu g^{-1} soil. This indicates that the previous (grower) addition of poultry manure for NOTTS1 which resulted in relatively greater levels of NPK, organic matter and microbial biomass than NOTTS 2 did not suppress disease. Much higher levels of organic matter and microbial biomass were found in the CAMBS (clay) and LINCS (sandy, silt loam) soils with the former having much higher levels of N and K compared to the others. The CAMBS soil was also the most suppressive to basal rot disease development but it was not clear if this was related to these factors.

As well as differing in their physical properties, the four soils could also be distinguished based on the structure, diversity and identity of components of the bacterial, fungal and *Fusarium* spp., communities as determined by PCA of the 16S, ITS and TEF amplicon sequencing data. Overall bacterial diversity was greater in NOTTS1 than for the other soils but fungal diversity was similar. The main components of the bacterial and fungal communities comprised of genera commonly found in soil. Analysis of *Fusarium* spp. and related species present identified pathogenic species including *I. radicicola* (*Cylindrocarpon destructans*), *F. redolens*, *F. solani*, *F. equiseti* and *F. proliferatum*. *F. redolens* and *F. proliferatum* have previously been associated with onion basal rot in addition to FOC (Haapalainen et al., 2016). Given that basal rot disease development was generally similar between soils, no components of microbial communities could be identified as being associated with basal rot disease suppression. Ideally a much wider range of soils and onion production systems would need to be examined with more detailed sampling to determine physical or microbial factors associated with differing levels of basal rot.

The major aim of this project was to establish a relationship between detection of FOC in soil by qPCR and onion basal rot disease development and understand the minimum level of FOC inoculum that could be detected. Similarly, we also wanted to establish if an amplicon sequencing approach could be used to identify and quantify FOC and / or other Fusarium pathogens within the microbial community and again understand what the threshold of detection might be. For the FOC specific qPCR, it was clear that pathogen detection was only consistent for inoculum levels ≥1x10^4 cfu g^{-1} soil across all the four soils. However, basal rot

disease development in soil only occurred at inoculum levels ≥1x10^5 cfu g^{-1} soil so for this experiment at least, this suggests that the qPCR would be able to predict disease in a pre-planting test. However, as stated previously, conditions for disease development were probably sub-optimal due to the time of year the experiment was carried out and therefore a different disease outcome might have occurred if the experiment was repeated in summer with better light levels and increased temperature. In addition, disease development in an inoculated pot system may differ from that under commercial field conditions.

Despite lower disease levels than expected in the experiment, with first disease symptoms observed after 20-30 days, colonisation of onion roots by FOC was detected by qPCR as early as 3 days and more consistently after 13 days at inoculum levels ≥1x10^5 cfu g^{-1} soil. There was also some detection of FOC on onion roots for 1x10^4 cfu g^{-1} soil and overall therefore the threshold for detection was similar as for soil. However, the ability to detect FOC at an early stage in plant development before symptoms occur suggests that using the qPCR to identify the pathogen on onion seedling roots in the field may be another approach to predict disease risk. Moreover, if onion seedlings could be used to 'bait' FOC from soil in pre-planting tests, this might with some further development increase the sensitivity of qPCR detection.

As expected, the amplicon sequencing approach was appropriate for characterising and defining the relative abundance of bacteria and fungi in the different soils using 16S and ITS gene targets respectively. Targeting TEF was also particularly effective for identification of *Fusarium* and related spp. and background levels of a number of potentially different pathogenic spp. (including *F. oxysporum*) were identified across the different soil types. When FOC was introduced into these soils, the pathogen was successfully detected through sequencing ITS (to genus level), TEF (to species level) and OG4952 (to *F. oxysporum* f.sp. level). By focussing on the appropriate ITS OTU, FOC was consistently detected at inoculum levels ≥1x10^4 cfu g^{-1} soil across all the four soils (with some detection at 1x10^3 cfu g^{-1} soil). The same level of FOC detection was also apparent when using TEF or OG4952 sequencing and this also corresponded with that achievable by qPCR. The fewer number of ITS / TEF sequencing reads associated with other *Fusarium* pathogens compared to FOC when introduced at 1x10^4 cfu g^{-1} soil suggests that they were present at below this concentration.

This study highlights the challenges associated with identifying gene targets suitable for amplicon sequencing of several specific *F. oxysporum* f.spp. including FOC. Unlike 16S and ITS, these more specific targets are not multicopy genes which often results in lower sensitivity, as was the case for OG13890 where FOC was only detected at concentrations of ≥1x10^5 cfu g^{-1} soil. Furthermore, OG4952 sequencing potentially identified other *F. oxysporum* f.spp. as well as FOC in soil samples where no inoculum was added. While

background levels of these pathogens may indeed be present (although FOC was not detected in any of these soils by qPCR), it is possible that other unknown and unsequenced *F. oxysporum* are present in soil with the same amplicon sequence, so it is not certain that FOC was actually present at these low levels (below $1x10^3$ or $1x10^4$ cfu g^{-1} soil), only that this amplicon was detected.

Another alternative approach to amplicon sequencing would be to use high coverage metagenomic sequencing to determine whether there is a background community of *F. oxysporum* f.spp. present in the four soils and also if there are other *F. oxysporum* which provide similar or identical target gene sequence; this would us to assess how reliable different gene targets are for predicting presence of FOC and other ff. spp. Using long read nanopore sequencing and WIMP (What's In My Pot) analysis could also enable the full spectrum of taxa present to be identified. For these approaches to work, a more comprehensive sequence database would be required to allow accurate identification of *F. oxysporum* f.spp.

In summary, this small project has been successful in establishing levels of FOC detection achievable by qPCR and relating this to inoculum concentration and basal rot disease development. Furthermore, it showed that the amplicon sequencing approach has potential to detect FOC at a similar detection threshold and provide further information of other *Fusarium* pathogens and the wider microbial community.

Knowledge and Technology Transfer

N/A

Acknowledgements

This work was funded by the Biotechnology and Biological Sciences Research Council, a part of UK Research and Innovation, through a joint initiative with the Agriculture and Horticulture Development Board "AHDB/BBSRC Pilot Initiative: Rapid response to high priority pests and diseases in UK Horticulture".

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