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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: IFO128
2. Project title: The impact of shortened rotations on rhizosphere microbial diversity
3. Contractor organisation(s): University of Warwick, Gibbett Hill Road, Coventry, Warwickshire, CV4 8UW
4. Total Defra project costs (agreed fixed price): £
5. Project: start date 01/07/2007
   end date 31/12/2011

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

The overall objective of this project was to examine the impact of shortened rotations on rhizosphere biodiversity associated with yield loss using oilseed rape (OSR) as an exemplar crop. A previously established rotational field trial, run by NIAB-TAG (Morley, Norfolk) and funded by HGCA (Project RD-2003-2922) was used to provide samples throughout this project. OSR grown frequently in rotation at this trial was suffering from yield decline compared to OSR grown for the first time or in long rotations. The trial ran for 8 seasons, from 2003-2011, with samples collected for use in the Defra project in 2007, 2008 and 2011.

Findings from the first phase of work investigating rhizosphere biodiversity highlighted several organisms of interest that had a greater relative abundance when OSR was cropped frequently. Two fungi in particular (*Olpidium brassicae* and a novel pathogen, *Pyrenochaeta* sp.) were subsequently chosen for empirical studies to investigate their potential role in yield decline.

Isolation of cultivable soil-borne plant pathogens

Initial work investigated the occurrence of soil-borne pathogens associated with OSR grown in short rotation. Numerous cultivable fungi were isolated from root lesions from field-grown OSR plants and putative pathogens identified following both cultural and molecular techniques included *Rhizoctonia solani, Pythium* sp., *Phoma* sp., *Fusarium* sp., *Verticillium* sp. and *Pyrenochaeta* sp. In addition, a virulent strain of *R. solani* was also isolated from OSR seedlings (used as bait plants), which were grown in the glasshouse using field soil that had been repeatedly cropped to OSR.

Molecular analysis of soil and rhizosphere communities associated with OSR

Microbial community analysis using molecular techniques can reveal a greater diversity of microorganisms than cultural techniques. Soil and rhizosphere samples taken from the HGCA-funded OSR rotation trial were used for molecular analysis of fungal, bacterial and nematode communities associated with OSR grown in different rotations with wheat. Samples were taken at one time point (June) in Year 4 of the trial, and three time points (November, March and June) in Year 5 of the trial. Year 4 included virgin OSR as one of the rotations, and analysis indicated that the fungi *O. brassicae* (an obligate parasite) and *Pyrenochaeta* sp. had a greater relative abundance in the rhizosphere of continuously cropped OSR compared to virgin OSR. In addition, *O. brassicae* and *Gibellulopsis* (formerly *Verticillium*) *nigrescens* had a greater relative

abundance in the bulk soil where OSR was cropped continuously.

By Year 5 of the trial, OSR had been included previously in all rotations and analysis showed that at this point *O. brassicae* dominated all OSR rhizosphere communities, irrespective of rotation. OSR cropping frequency did not have a significant effect on bacterial communities in the soil or rhizosphere. Whilst no significant differences in the bulk soil nematode community were found between rotations, continuously cropped OSR had a significantly different rhizosphere nematode community compared to other OSR rotations early in the season (November, Year 5). Seasonal data from Year 5 indicated that some members of the fungal, bacterial and nematode communities had significant seasonal shifts in abundance (an overall increase, an overall decrease or a mid-season peak). *Olpidium brassicae* was most abundant mid-season (March, Year 5).

Effect of crop residues and root exudates on OSR rhizosphere microbial populations

Organic matter incorporation can influence soil microbial populations, and OSR residues have been shown to impact on populations of soil-borne pathogens. Glasshouse experiments investigated i) the effect of incorporation of mature crop residues (shoots and roots), and ii) the effect of repeated inputs of fresh decomposing root material and root exudates, on microbial communities associated with subsequently grown OSR. In the first experiment, fungi dominant in the mature crop residues did not become dominant in the soil after incorporation, but the addition of residues to the soil did enhance the population of an indigenous soil fungus, likely a decomposer species. Irrespective of mature crop residue treatment, *O. brassicae* dominated the rhizosphere fungal community of subsequently grown OSR. Shifts in bacterial communities also occurred with the addition of mature crop residues to soil, but identification of species was not possible.

In the second glasshouse experiment, *O. brassicae* dominated the rhizosphere fungal community, with very high abundance from the second time OSR was planted in the same soil. Fresh decomposing OSR root material in the soil increased the relative abundance of *O. brassicae* in the soil fungal community. The effect of OSR exudates or fresh decomposing residues on less abundant rhizosphere fungi was not clear due to the high relative abundance of the obligate parasite. Soil bacterial communities were affected relatively similarly by the presence of fresh decomposing residues or exudates, but fresh decomposing residues had a greater impact on rhizosphere bacteria than exudates over time.

Impact of* Olpidium brassicae *and* Pyrenochaeta *sp. on plant growth and yield

Using molecular techniques, several species had been identified as being more abundant in the rhizosphere or soil when OSR was grown more frequently in rotation. A model system using *Brassica oleracea* (closely related to oilseed rape) was used to investigate the impact of two selected fungi (*O. brassicae* and *Pyrenochaeta* sp.) on early growth and seed yield in the glasshouse. In a soilless substrate, *O. brassicae* reduced plant biomass of OSR and *B. oleracea* at high doses, and also reduced branching and thus total pod production of *B. oleracea* at high doses. Plants inoculated with *Pyrenochaeta* sp. developed root lesions, and high doses of this pathogen delayed flowering and reduced seed quality of *B. oleracea* grown in a soilless substrate. In addition, *Pyrenochaeta* sp. had a small effect in reducing top growth of OSR seedlings in a soil-based experiment.

Development of quantitative PCR for* O. brassicae *and* Pyrenochaeta *sp.

PCR primers were developed and tested to quantify *O. brassicae* and *Pyrenochaeta* sp. in field and glasshouse samples. These were found to be specific to the organisms for which they had been developed, and were subsequently used to reveal important seasonal dynamics of these fungi.

Susceptibility of commercial OSR varieties

The HGCA-funded OSR rotation trial at Morley had been used to provide all field samples for use in this project. In order to determine whether results were site- or cultivar-specific, this Objective focussed on examining the microbial communities at other sites. However, no significant differences were found between rhizosphere fungal communities of eight spring OSR varieties, at different sampling times over two years. Analyses indicated that irrespective of variety, sample time or year, rhizosphere fungal communities were dominated by the high relative abundance of *O. brassicae*. Seasonal data showed that *O. brassicae* was most abundant in samples taken mid-season.

Impact of extended rotation intervals on fungal rhizosphere communities
The eighth and final season of the OSR rotation trial at Morley included rotations of OSR with a range of rotation intervals, including 6-year gap, 4-year gap, 3-year gap, 2-year gap, 1-year gap (alternate years) and continuous OSR. Rhizosphere samples were taken from all OSR rotations, at three points in the growing season (January, March, June). Molecular analysis indicated that the rhizosphere fungal communities were similar across all OSR rotations, irrespective of interval between crops. This was due to the high relative abundance of *O. brassicae* in all roots. Quantitative PCR showed that *O. brassicae* was not affected by rotation gap, and that infection was high even after a 6-year gap between OSR crops. Again it was seen that *O. brassicae* peaked in abundance mid-season (March).

Pyrenochaeta sp. increased in abundance throughout the season, with significantly higher levels reached in June than earlier in the season. Furthermore, significantly more *Pyrenochaeta* sp. was found in the alternate OSR than in the longer rotations, in both January and March, and analysis showed that levels of *Pyrenochaeta* sp. in January and March correlated negatively with yield.

Future work
Empirical work to date has focussed on *O. brassicae* and *Pyrenochaeta* sp. However, the microbial dynamics associated with yield decline in short rotation cropping of OSR are not yet fully elucidated, and the roles of other key organisms identified through molecular analysis are yet to be established. Future work should centre on characterising the pathogen complex more fully, investigating interactions between pathogens, and examining the potential to manage the patho-complex through crop genotype.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Scientific context

INTRODUCTION

Reduction in yield with continuous cropping has been recorded for numerous crops, including oilseed rape (OSR), sugar cane, wheat, maize, rice, barley, tobacco and potato (Bennett et al., 2011). These yield declines have frequently been related to a build-up of organisms deleterious to the crop including plant pathogenic fungi and nematodes, as well as changes in the microbial population in general. A further concern is the long-term effects that such monocultures may have on soil quality and biodiversity in general and whether break crops can restore both soil biodiversity and allow a return in subsequent yields.

There are significant knowledge gaps in our understanding of this phenomenon, particularly with respect to the interaction of root exudates and crop residues with soil microbial biodiversity and their influence on crop yield. This project investigated these interactions using OSR as an exemplar, chosen because it is cultivated widely, has potential for increased cultivation as a biofuel crop, but suffers significant yield decline with continuous cropping (Stobart, 2009; Stobart, 2011; Christen and Sieling, 1995). The future of OSR and other biofuel crops will depend on improved agricultural practices, as

currently OSR crops only achieve predicted yields when grown on land not previously used to grow the crop. With increased pressure to produce OSR for biofuel use, rotation intervals are likely to shorten and the loss of productive capacity will increase.

The overall objective of this project was to examine the impact of shortened rotations on rhizosphere biodiversity associated with yield loss using OSR as an exemplar crop. A previously established rotational field trial, run by NIAB-TAG (Morley, Norfolk) and funded by HGCA (Project RD-2003-2922) was used to provide samples throughout this project, as yield decline had been recorded at this site. The trial ran for 8 seasons, from 2003-2011 with samples collected for use in the current project in 2007, 2008 and 2011. The rotation plan is given in Appendix 1, and also includes yield data.

All Figures are included in Appendix 2.

OBJECTIVE 1: Investigate the potential role of plant pathogens in contributing to yield loss following continuous cropping of OSR

No obvious disease differences were found between rotational approaches at the OSR rotation trial and it was not considered that yield reductions were the result of foliar or stem diseases (Stobart 2009, 2011). However, a build-up of soil-borne plant pathogens is one of the possible causes of reduced yields in short-rotation crops (Bennett *et al*., 2011), and this was investigated here.

Methods

Two approaches were used to identify soil-borne pathogens that may have contributed to yield losses. The first investigated the presence of soil-borne pathogens at the Morley site, where yield decline was occurring, using OSR seedlings as bait plants. Field soil collected from the OSR rotation trial plots was sieved to remove stones and subsequently used in a glasshouse bioassay. OSR seed was planted and, following emergence, disease symptoms were recorded.

The second approach involved isolation of fungi from roots of field-grown OSR collected from the Morley site. Plant samples were collected from plots of continuously grown OSR, and OSR grown alternately with winter wheat. Roots were removed and, following standard aseptic techniques, root segments with visible lesions were plated onto agar media to isolate fungi.

Results

Several plants used in the first approach (baiting from soil) developed disease symptoms, including brown lesions causing a thinning of the stem at the crown (wirestem). *Rhizoctonia solani* was isolated from this diseased material and identified based on visual characteristics and subsequent molecular identification from sequence data. Further work confirmed that the isolated fungus was responsible for causing the wirestem disease symptoms seen on OSR seedlings, as well as pre-emergence damping off, thus indicating a virulent isolate of *R. solani* was present in the field soil from the OSR rotation trial. *Rhizoctonia solani* can result in poor crop establishment, which may impact on yield if establishment is severely affected.

Numerous fungi were isolated from root lesions in the second approach. Putative pathogens identified following cultural and molecular techniques included *Rhizoctonia solani, Pythium* sp., *Phoma sp., Fusarium* sp., *Verticillium* sp. and *Pyrenochaeta* sp.

The use of both approaches identified a range of soil-borne fungi, many of them already recognised as major pathogens with potential to impact on yield (*e.g. Phoma* sp.*, Verticillium* sp.). However, a lesser-known pathogen, *Pyrenochaeta* sp. was also identified. It is worth noting that techniques used here would not have picked up obligate parasites, as these are not cultivable on standard media.

Objective 1 KEY FINDINGS:

- A virulent strain of *Rhizoctonia solani* was isolated from diseased OSR seedlings used as bait plants.
- Cultural methods identified a variety of known soil-borne pathogens from root lesions of field-grown OSR.
- A lesser-known OSR pathogen, *Pyrenochaeta* sp., was also isolated from root lesions.

OBJECTIVE 2: Examine changes to soil and rhizosphere microbial populations and nematode populations associated with the development of OSR yield loss

Microbial community analysis using molecular techniques can reveal a greater diversity of microorganisms than cultural techniques, as the total community structure can be included, including facultative or obligate pathogens. Thus, molecular approaches were used in this Objective to monitor changes in the complex fungal, bacterial and nematode communities associated with OSR grown in different rotations. The aim was to determine if more frequent cropping of OSR caused a shift in microbial or nematode populations that could be related to yield decline.

Methods

Rotations for sample collection

Selected rotations from the OSR rotation trial were chosen to provide bulk soil and rhizosphere samples for molecular community analysis, and were collected at one time-point in Year 4 of the trial (June 2007) and three time-points in Year 5 (November 2007, March 2008, and June 2008). Table 1 indicates the rotations used for sample collection (see also Appendix 1).

Table 1: Rotations selected for sampling in rotation trial (indicated in bold red). O = oilseed rape (cv Winner), W = winter wheat (cv Brompton).

Rotation	Year 4				Year 5				
Virgin OSR	W	W	W	**O**	n/a				
Continuous OSR	O	O	O	**O**	O	O	O	O	**O**
2 year gap OSR	O	W	W	**O**	W	O	W	W	**O**
Alternate OSR	W	O	W	**O**	O	W	O	W	**O**
'Continuous' wheat	O	W	W	**W**	O	W	W	W	**W**
Alternate wheat	n/a				W	W	W	O	**W**

Four replicate plots were sampled for each rotation, and from each plot six bulk soil samples were collected using an auger and pooled, and six rhizosphere samples were collected and pooled. Bulk soil was sieved through a 0.3 cm sieve and sub-samples of 0.5g were taken for DNA extraction. Roots were shaken free of loose soil, and the fine roots (plus closely adhering soil) were cut into small (5 mm) sections as the rhizosphere samples (0.5g sub-samples taken for DNA extraction).

Community fingerprinting

DNA was extracted from the soil and rhizosphere samples and universal primers for fungi (ITS1 + ITS4), bacteria (63f + 1087r), and nematodes (Nem_18s_F + Nem_18S_R) were used to amplify a region of ribosomal DNA (see Appendix 2 for sequences). One of each primer pair was fluorescently labelled for Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis. Purified Polymerase Chain Reaction (PCR) products were digested with restriction enzymes *Hha*I or *Msp*I (fungi and bacteria only), and *Hae*III or *Aci*I (nematodes only).

In all cases, TRFLP analysis was carried out on an automated sequencer, and terminal restriction fragments (TRFs) generated by the sequencer were analysed using GeneMarker 1.60. Community profiles were then generated based on the percentage of the total peak height, calculated for each TRF, and analysed for resemblance using ANOSIM and non-metric multi-dimensional scaling (MDS) (PRIMER, version 6.1.12). Significance values were obtained by permutation tests and TRFs contributing >5% to the differences between community profiles were indicated using SIMPER analysis (PRIMER, version 6.1.12). Emphasis was placed on shifts in communities with more frequent cropping

of OSR, but differences were also noted between crop types (OSR compared to wheat in rotation). Data pertaining to differences in microbial communities associated with different crop types are not presented.

Cloning and sequencing

In order to identify the most dominant peaks in the TRFLP traces, DNA clone libraries were constructed. The universal primers for fungi, bacteria and nematodes were used to amplify DNA from the pooled rhizosphere samples from continuous OSR, and 'continuous' wheat. PCR products were then cloned using the Qiagen PCR Cloning Plus kit and plasmid DNA underwent TempliPhi™ amplification. Sequencing was carried out on an automated sequencer and database searches were then carried out to identify microorganisms. Finally, *in silico* restriction sites were correlated to TRFs. Where identification was possible, organisms are referred to by name with the TRF size in parentheses. Unidentified organisms are referred to by TRF size only.

Results

TRFLP analysis of fungal communities

Significant differences were found between the bulk soil and rhizosphere fungal communities for all rotations at all sample times (P = 0.03; Figures 1-2). Thus, soil and rhizosphere data are considered separately below to highlight differences resulting from crop rotation. Generally, in the OSR rotations, *Olpidium brassicae* (TRF 284) had a high relative abundance in the rhizosphere communities and *Gibellulopsis nigrescens* (formerly *Verticillium nigrescens,* TRF 125) was more abundant in the soil communities. Note that TRFs 124+125 were shown to be a single sequence (*G. nigrescens*) due to an overlapping *Hha*I restriction site.

June 2007: Year 4 harvest

Bulk soil

The continuous OSR had a significantly different bulk soil fungal community to all other rotations (P = 0.03; Figure 3). *Gibellulopsis nigrescens* (TRFs 124+125) had a greater relative abundance in the continuous OSR bulk soil than in all other OSR rotations, and *O. brassicae* (TRF 284) was more abundant in continuous OSR than in the alternate OSR or the 2 year gap OSR. TRFs that had a greater relative abundance in the longer OSR rotations included *Botryotinia fuckeliana* (TRF 327) and TRF 112 in the 2 year gap rotation, and TRF 286 in virgin OSR. Crop-associated differences were also noted.

Rhizosphere

The continuous OSR had significantly different rhizosphere fungal communities compared to the other OSR rotations (P = 0.03; Figure 4), with a greater relative abundance of *O. brassicae* (TRF 284) in the rhizosphere with more frequent cropping (Figure 5a). In addition, *Pyrenochaeta* sp. (TRF 98) had a greater relative abundance in the rhizosphere fungal community of continuous OSR than those of the 2 year gap rotation or the virgin OSR (Figure 5b). Significant differences were also seen between the 2 year gap and virgin OSR (P = 0.03; Figure 4) due to a greater relative abundance of *G. nigrescens* (TRF 124+125) in the 2 year gap OSR. Crop-associated differences were also noted.

November 2007, March 2008, June 2008: Year 5 harvest

Bulk soil

i) Effect of rotation

Each sampling time was analysed separately to assess the affect of rotation on fungal community structure. In November (early season), the continuous OSR had a significantly different soil fungal community compared to all other OSR rotations (P = 0.03; Figure 6), due to a greater relative abundance of *G. nigrescens* (TRFs 124+125) in the continuous OSR soil. However, by March no differences were found between soil fungal communities across all OSR rotations, and they remained similar in June. Crop-associated differences were also noted.

ii) Effect of sampling time

Within the OSR rotations, seasonal fluctuations were examined further. Significant differences in soil fungal communities were found at the three sample times during the season (P = 0.001; Figure 7). All OSR rotations indicated an increase in relative abundance of *B. fuckeliana* (TRF 327) between November and March and a subsequent decrease of the same fungus between March and June (*i.e*. a peak mid-season). The alternate OSR soil fungal community also had an increase in relative

abundance of TRF 383 and a decrease in relative abundance of *G. nigrescens* (TRF 125) between November and March. The 2-year gap OSR had an increase in TRF 149 between March and June.

Rhizosphere
i) Effect of rotation
No significant differences in rhizosphere fungal communities were found within the OSR rotations, with a high proportion of *O. brassicae* (TRF 284) occurring in all OSR plots at all sampling times. Crop-associated differences were noted.

ii) Effect of sampling time
Significant differences in rhizosphere fungal communities were found between the three sample times during the season (P = 0.001, November-March and March-June; Figure 8). In all OSR rotations, *O. brassicae* (TRF 284) increased from November to March, followed by a decrease in relative abundance from March to June. TRF 298 followed the opposite seasonal pattern in the continuous and alternate OSR rotations, decreasing in relative abundance from November to March before increasing again from March to June.

TRFLP analysis of bacterial communities
Significant differences were found between the bulk soil and rhizosphere bacterial communities for all rotations at all sample times (P = 0.03, with the sole exception of the continuous OSR in March 2008, P=0.057; Figures 9-10). Soil and rhizosphere data are therefore considered separately below to highlight differences resulting from crop rotation.

June 2007: Year 4 harvest
Bulk soil and rhizosphere
No significant differences in bacterial soil or rhizosphere communities were found between the different crop rotations.

November 2007, March 2008, June 2008: Year 5 harvest
Bulk soil
i) Effect of rotation
Each sampling time was analysed separately to assess the affect of rotation on bacterial community structure. However, no significant differences in bacterial soil communities were found between the different OSR rotations at any sampling time. Crop-associated differences were noted.

ii) Effect of sampling time
Within the OSR rotations, seasonal fluctuations were examined further. As no effect of OSR cropping frequency was seen (see above), data from all OSR rotations were combined for this analysis. Significant differences in soil bacterial communities were found at the three sample times during the season (P = 0.001; Figure 11). Unidentified TRFs increasing in relative abundance from November to March included TRFs 721 and 340, whilst TRFs 722, 341 and 402 decreased over this time. From March to June, *Pseudomonas fluorescens* (TRF 245) and TRF 524 increased in relative abundance, and TRFs 132 and 338 decreased somewhat during this time.

Rhizosphere
i) Effect of rotation
Frequency of OSR cropping did not affect rhizosphere bacterial diversity, and no significant differences were found with the OSR rotations. Crop-associated differences were noted, however.

ii) Effect of sampling time
Similar to the bulk soil community analysis, no effect of OSR cropping frequency was seen on bacterial rhizosphere communities (see above). Thus data from all OSR rotations were combined for this analysis. Significant differences in rhizosphere bacterial communities were found at the three sample times during the season (P = 0.001; Figure 12). *Pseudomonas fluorescens* (TRF 245) increased in relative abundance in the OSR rhizosphere from November to March, followed by a subsequent decrease from March to June. In contrast, TRFs 524 and 723 decreased in relative abundance from November to March and increased again from March to June. TRF 523 decreased in relative abundance throughout the season. Other peaks only contributed >5% to the differences between communities on single occasions, and were also unidentified.

TRFLP analysis of nematode communities

Nematode communities were analysed during Year 5 of the trial. TRFLP data showed a significant difference between soil and rhizosphere communities at all sampling times (*P* = 0.03; Figure 13), due mainly to the higher levels in the rhizosphere of *Pratylenchus neglectus* (TRFs 304 and 303) and *Chiloplacus propinquus* (TRF 413). Soil and rhizosphere data are therefore considered separately below to highlight differences resulting from crop rotation.

November 2007, March 2008, June 2008: Year 5 harvest

Bulk soil

i) Effect of rotation

There was no significant difference between any samples in the bulk soil nematode community.

ii) Effect of sampling time

The bulk soil communities were significantly different at each time point (*P* = 0.001; Figure 14). These were mainly due to an increase in TRF 145 (Plectidae family) throughout the growing season and a decrease in *C. propinquus* (TRF 413) between March and June .

Rhizosphere

i) Effect of rotation

The continuous OSR rhizosphere had a significantly different nematode community to all other OSR rotations in November (*P* = 0.03; Figure 15), but not in March or June. This was due mainly to an increase in TRFs 610 and 611 (94 % identification match to members of the family Monhysteridae), and a decrease in *P. neglectus* (TRFs 303, 304) and TRF 298 in the rhizosphere of continuous OSR compared with OSR grown after wheat. Crop-associated differences were also noted.

ii) Effect of sampling time

The rhizosphere communities were significantly different at each time point (*P* = 0.001; Figure 16). In the OSR rhizosphere these were mainly due to an increase in *P. neglectus* (TRFs 303, 304) and decrease in TRFs 610 and 611 in March relative to November.

Objective 2 KEY FINDINGS:

- Crop type significantly altered rhizosphere and soil microbial diversity (OSR compared to wheat).
- Rhizosphere communities were significantly different to bulk soil communities for fungi, bacteria and nematodes.
- Limitations of database entries impede identification of species, particularly bacteria.
- FUNGI
  - *Gibellulopsis nigrescens* and *Olpidium brassicae* were abundant in the bulk soil where OSR was cropped continuously.
  - *Olpidium brassicae* and *Pyrenochaeta* sp. had a greater relative abundance in the rhizosphere of continuously cropped OSR compared to virgin OSR (Year 4 harvest).
  - *Olpidium brassicae* was dominant in all rhizosphere communities associated with OSR, and was most abundant in March (Year 5 harvest).
  - Some members of the fungal communities had significant seasonal shifts in abundance (overall increase, overall decrease or a mid-season peak).
- BACTERIA
  - OSR cropping frequency did not have a significant effect on bacterial communities in the soil or rhizosphere.
  - Some members of the bacterial communities had significant seasonal shifts in abundance (overall increase, overall decrease or a mid-season peak).
- NEMATODES
  - There was no significant difference between any samples in the bulk soil nematode community.
  - There was a significant difference in the continuous OSR rhizosphere to all other rotations at one sampling time, due mainly to an increase in TRFs 610 and 611 which were unidentified nematodes possibly of the family Monhysteridea.
  - Some members of the nematode communities experienced significant seasonal shifts in abundance (overall increase, overall decrease or a mid-season peak).

OBJECTIVE 3: Determine the effect of crop residues and root exudates on microbial populations in the rhizosphere of OSR

Organic matter incorporation can influence soil microbial populations, and OSR residues have been shown to impact on populations of soil-borne pathogens due to biofumigation effects. Repeated inputs of the same crop residues and exudates to the soil through short-rotation cropping may also exert a selective pressure on soil and rhizosphere microbial communities, which in turn may impact on yield. It is not known whether root exudates and mature crop residues exert the same influence on rhizosphere microbial communities associated with OSR. Thus work in this Objective aimed to examine the relative significance of crop residues and root exudates on microbial population development in the rhizosphere of OSR, in two separate glasshouse pot bioassays. Due to previous findings from Objective 1 and 2, cultivation-independent analysis alone was used to assess microbial communities.

3.1 The effect of mature crop residues

Methods

This experiment examined whether mature OSR crop residues (incorporated into soil) influenced the rhizosphere microbial communities of subsequently grown OSR plants. In 2008, mature crop residues (shoots and roots) were collected after harvest from plots that had been cropped continuously with OSR for 5 years at the HGCA-funded OSR rotation trial. Shoot residues included small stem sections, pod husks and above ground material left after combine harvesting. Residues from four replicate plots were pooled and mixed. Root residues consisted of the root system remaining in the ground after harvest, and were collected by pulling up the stalk remnants with the roots attached. Roots were cut into pieces 2-3cm long and samples from four replicate plots were pooled and mixed. Soil was also collected from the OSR rotation trial, from under wheat (wheat for 4 years), and sieved to remove

stones. Sub-samples of soil, root and shoot residues were taken for TRFLP analysis of the associated microbial communities (start; 5 replicates of each), as previously described. Where necessary for identification of TRFs, clone libraries were also constructed as previously described.

A pot experiment was set up in the glasshouse with 10 replicates of each of the following treatments: soil only; 2% roots (w:w); 2% shoots (w:w); and 1% roots + 1% shoots (w:w). Pots were left unplanted for 1 month in the glasshouse and maintained at 15% moisture content. After this time, the pot contents were individually tipped into a bag and mixed, and sub-samples of soil were taken for TRFLP analysis (pre-planting). The remaining soil and residues were returned to the pots and winter OSR seed (*Brassica napus* cv. Winner) was planted in each pot. Plants were grown for six months, after which time soil and rhizosphere samples were taken for TRFLP analysis (harvest). At harvest, fresh and dry weight of top growth was also recorded as biomass yield.

Results

Plant harvest data

There were no significant differences between treatments in top growth fresh or dry weight at harvest. These plants were not taken to seed-yield.

TRFLP analysis of fungal communities

i) Effect of residues

Fungal communities were significantly different in the starting material (soil, root residues and shoot residues)(P = 0.008; Figure 17). The dominant fungi associated with root residues included *Verticillium dahliae* (TRF 308) and *Pyrenochaeta* sp. (TRF 98).

At pre-planting (one month after residue incorporation), soil fungal communities in all treatments were significantly different to one another (P = 0.001; Figure 18), suggesting that the incorporation of residues into the soil had caused a shift in the fungal communities. The differences were largely due to an increase in abundance of TRF 485. This peak was not abundant in any of the residue samples, indicating the shift in soil fungal communities after one month was more likely due to the proliferation of an indigenous fungus in the soil community, rather than the introduction of a specific fungus to the soil along with the residues.

After six months of OSR growth, the soil fungal communities remained significantly different between the soil control and those treatments where shoot residues had been incorporated, and also between all the different residue-amended treatments (P = 0.001; Figure 19). Differences were still largely related to the increased abundance of TRF 485 in the soil of residue-amended treatments at this time. At harvest, rhizosphere fungal communities were similar for all treatments due to the abundance of *O. brassicae* (TRF 284) in all samples, irrespective of residue addition to the soil. Infection with *O. brassicae* resulted from germination of naturally-occurring resting spores present in the soil, as no inoculum had been added.

ii) Effect over time

As well as treatments being compared at the different time points separately, changes over time were also assessed within each treatment. Significant differences were found in soil fungal communities over time, in all treatments (P < 0.01; Figure 20). For the soil control (no residues added), the main differences in soil fungal communities were due to a decrease in TRFs 335 and 336 in the month that the pots were in the glasshouse before planting, and an increase in *O. brassicae* (TRF 284) in the soil between pre-planting and harvest sample times. *Olpidium brassicae* in the soil was likely to have come from secondary cycling of the obligate parasite following high levels of infection in the rhizosphere.

In all residue-amended treatments, the differences in soil fungal communities over time were affected by shifts in the relative abundance of TRF 485, which increased during the month from the start of the experiment to pre-planting, and subsequently decreased from pre-planting to harvest. The biggest increase in relative abundance of TRF 485 during this time was seen following incorporation of shoot residues. Other shifts contributing to the significant differences seen included an increase in relative abundance of *O. brassicae* (TRF 284) from pre-planting to harvest in the soil amended solely with root residues, as well as that amended solely with shoot residues. The shoot residue treatment also had a significant increase in relative abundance of *G. nigrescens* (TRF 124+125), and TRFs 333 and 334 in the soil from pre-planting to harvest. The combined roots and shoots residue treatment similarly

showed a significant increase in relative abundance of *G. nigrescens* (TRF 124+125) from pre-planting to harvest. The extent of changes to the soil fungal community over time varied depending on residue composition.

TRFLP analysis of bacterial communities

i) Effect of residues

Bacterial communities were significantly different in the starting material (soil, root residues and shoot residues) (P = 0.008; Figure 21), due to differences in relative abundance of a number of unidentified TRFs in the residue samples. *Pseudomonas fluorescens* (TRF 245) was more abundant in the residue material than in the soil.

Soil bacterial communities were also significantly different between some treatments one month after the incorporation of the residue material, including between the soil control and Shoot residue treatment (P = 0.015), between the soil control and combined Root and Shoot residue treatment (P = 0.001) and between the Root residue treatment and combined Root and Shoot residue treatment (P = 0.022) (Figure 22). These differences were again due to shifts in relative abundance of a number of unidentified TRFs in the samples.

At harvest, soil bacterial communities were significantly different between all treatments (P < 0.05; Figure 23), with the exception of the soil control and root residue treatment. Again, differences were due to marginal shifts in relative abundance of a number of unidentified TRFs.

Rhizosphere bacterial communities were similar across the residue treatments, with the exception of the soil control and combined Root and Shoot residue treatment (P = 0.021; Figure 24). *Pseudomonas fluorescens* (TRF 245) had a greater relative abundance in the soil control than in the Root and Shoot treatment, and other unidentified TRFs also contributed to differences between the treatments.

ii) Effect over time

As well as treatments being compared at the different time points, changes over time were also assessed within each treatment. Significant differences were found in soil bacterial communities over time, in all treatments (P < 0.01; Figure 25). Shifts in relative abundance of a number of unidentified TRFs were responsible for differences, including, in general, an increase in TRFs 338 and 721 between the start and pre-planting sample times; an increase in TRFs 135 and 243 between the pre-planting and harvest sample times; and a decrease in TRF 132 over the course of the experiment.

Although fingerprinting techniques have demonstrated differences in community structure, it has not been possible to identify most bacterial species due to limited information available on databases. No clear selection for individual TRFs in the soil or rhizosphere was shown following incorporation of mature OSR residues into soil.

3.2. The effect of root exudates

Methods

This experiment examined whether OSR root exudates influenced the rhizosphere microbial communities of subsequently grown OSR plants. The work was conducted as a glasshouse experiment to allow for a rapid cycle of successive plantings of OSR (*Brassica napus* cv. Winner), in order to mimic the effect of repeated cultivation of OSR. Furthermore, leaving the roots in the soil or removing them at harvest (for each successive planting) allowed differentiation of the influence of root exudates *per se* and the combination of exudates and fresh decomposing roots on microbial population development.

There were three main treatments, consisting of an unplanted control (soil only), OSR seedlings grown and the root material removed from the soil at each of 6 replanting Rounds (roots-removed; determining the effect of exudates), and OSR seedlings grown and the root material retained in the soil at each of 6 replanting Rounds (roots-retained; determining the effect of exudates and fresh decomposing roots). Enough pots were set up initially to allow for an individual pot to be sampled at each of 6 sampling times for each treatment. Seedlings were grown for 4 weeks in each Round, after which time one pot per treatment was removed for sample collection, with the remaining pots being processed following a replanting procedure. Four complete replicates were set up.

For all three treatments, at each sampling time sub-samples of soil (0.5g) were taken and for the two treatments where OSR had been grown, sub-samples of rhizosphere material (roots and closely

adhering soil particles; 0.5g) were also taken. Soil and rhizosphere samples taken throughout the experiment were used for TRFLP analysis of the microbial communities, as previously described.

Results

TRFLP analysis of fungal communities

In both the planted treatments *O. brassicae* quickly infected roots and proliferated, thus contributing highly to both the rhizosphere fungal communities and the soil fungal communities through the release of zoospores back into the soil. The high abundance of *O. brassicae* in the roots also resulted in a significant difference between rhizosphere and soil fungal communities in the planted treatments. The relative abundance of *O. brassicae* in samples was the main contributory factor to differences in fungal community structure between treatments, as well as changes in fungal community structure over time.
Each planting Round was initially analysed separately to examine differences between treatments. Soil fungal communities were similar between the unplanted control and the roots-removed treatment at all Rounds. They were also similar between the roots-removed and roots-retained treatments at all Rounds, with the exception of Round 4 (P = 0.03; Figure 26) where differences were due to greater abundance of *O. brassicae* (TRF 284) in the roots-retained soil fungal community at this time. In addition, the roots-retained treatment had a significantly different soil fungal community to that of the unplanted control in Rounds 2, 3, 4 and 6 (P = 0.03; Figure 26). This was also due to a higher relative abundance of *O. brassicae* (TRF 284) in the roots-retained treatment, and a higher relative abundance of *G. nigrescens* (TRF 125) in the unplanted control at these times. The greater abundance of *O. brassicae* in the soil in the roots-retained treatment was likely due to the presence of previously infected root material remaining in the soil. Thus, soil fungal communities were affected more by the presence of fresh decomposing residues than exudates *per se*, due to the presence of the obligate parasite in the root residue material.

There were no significant differences in rhizosphere fungal communities between the two planted treatments at any Round, and the rhizosphere communities were dominated by *O. brassicae* (TRF 284) throughout the experiment.

As well as considering each planting Round separately, comparisons were made for fungal communities over time within each treatment. The soil fungal community profiles for the unplanted control and for the roots-removed treatment showed no significant differences over time. However, significant differences were found in the soil fungal communities in the roots-retained treatment over time (P = 0.03; Figure 27a), largely due to shifts in the abundance of *O. brassicae* (TRF 284). From Round 1 to Round 2 an increase in relative abundance of *O. brassicae* (TRF 284) occurred, and this fungus then remained in high abundance from Rounds 2-4. An unexplained decrease in relative abundance of *O. brassicae* (TRF 284) occurred in Round 5, resulting in a significant difference in the soil fungal communities between Rounds 4 and 5, and between Rounds 5 and 6. However, in general, the analysis again showed that the fresh decomposing residues retained in the soil had a greater influence on the soil fungal communities over time than exudates.

The rhizosphere fungal community was significantly different between Rounds 1 and 2 for both the roots-removed and roots-retained treatments (*P* = 0.03; Figure 27b), due to an increase in relative abundance of *O. brassicae* (TRF284) after the first Round of growing OSR. After this time, the rhizosphere fungal communities were generally similar throughout the experiment, although further significant differences were found between Rounds 3 and 4 in the roots-retained treatment as *O. brassicae* decreased slightly in relative abundance in Round 4 for this treatment. Due to the high abundance of *O. brassicae* (TRF 284) in the rhizosphere, it was not possible to determine whether less abundant rhizosphere fungi were affected by the OSR exudates or fresh decomposing residues incorporated into the soil.

TRFLP analysis of bacterial communities

Differences in bacterial community profiles between treatments, and over time, were related to shifts in abundance of several TRFs, many of which remain unidentified due to limitations of database entries.
Each planting Round was initially analysed separately to examine differences between treatments. Soil bacterial communities were significantly different between the unplanted control and the roots-removed treatment in Rounds 2, 3, 4 and 6 (*P* = 0.03; Figure 28), and between the unplanted control and the roots-retained treatment in Rounds 3, 4, 5 and 6 (*P* = 0.03; Figure 28). In general, differences were due to a greater relative abundance of TRF 338 in the unplanted control and a greater relative

abundance of TRFs 722 and 522 in the roots-removed and roots-retained treatments. Soil bacterial communities remained similar between the roots-removed and roots-retained treatments at all Rounds, with the exception of Round 4 (P = 0.03; Figure 28), due to a greater relative abundance of TRF 722 in the roots-retained treatment at this time. These results indicate that soil bacterial communities were affected relatively similarly by the presence of fresh decomposing residues and exudates, as the planted treatments had a similar effect on the soil bacterial community, and both were different to the unplanted control.

There were no significant differences in rhizosphere bacterial communities between the two planted treatments at any Round.

As well as considering each planting Round separately, comparisons were made for bacterial communities over time within each treatment. Soil bacterial communities in the unplanted control were significantly different between Rounds 2-5 (P = 0.03; Figure 29a). These shifts may have been due to changes in nutrient availability over time, and included minor changes in relative abundance of TRFs 132, 135, 242, 243 and 338. Where roots were removed from the soil at each replanting round, the soil bacterial communities were generally similar between consecutive rounds, with an exception between Rounds 3 and 4 (P = 0.03; Figure 29a) due to an increase in relative abundance of TRF 135 and a decrease in relative abundance of TRF 522 at this time. Where roots were retained in the soil at each replanting round, significant changes in the soil bacterial communities were found between Rounds 2-4 (P = 0.03; Figure 29a), largely due to an increase in relative abundance of TRF 722. TRFs 135 and 130 also increased in relative abundance from Round 3-4.

Objective 3 KEY FINDINGS:

3.1 The effect of mature residues

- Mature OSR root and shoot residues have distinct fungal and bacterial communities, different to those found in the bulk soil.
- Fungi dominant in the residues did not become dominant in the soil after incorporation for one month, but the addition of residues to the soil did enhance the population of an indigenous soil fungus, likely a decomposer species (TRF 485).
- TRF 485 contributed significantly to the differences seen in soil fungal communities between residue treatments, but did not become dominant in the rhizosphere of the growing OSR plants (although it did contribute more to the rhizosphere community in treatments where residues had been incorporated into the soil).
- *Olpidium brassicae* dominated the rhizosphere fungal community, irrespective of soil residue treatment.
- Shifts in bacterial communities occurred with the addition of residues to soil, but detailed identification of species was not possible.

Objective 3 KEY FINDINGS:

3.2 The effect of root exudates

- Soil fungal communities were affected more by the presence of fresh decomposing residues than exudates *per se*, due to the presence of *O. brassicae* in the root residue material, *ie* fresh decomposing residues in the soil increased the relative abundance of *O. brassicae* in the soil fungal community.
- *Olpidium brassicae* dominated the rhizosphere fungal community, with very high abundance from the second time OSR was planted in the same soil.
- The effect of OSR exudates or fresh decomposing residues on less abundant rhizosphere fungi was not clear.
- Shifts in soil and rhizosphere bacterial communities were demonstrated with repeated planting of OSR.
- Soil bacterial communities were affected relatively similarly by the presence of fresh decomposing residues or exudates.
- Rhizosphere bacterial communities were similar between the two planted treatments at each sample time, but fresh decomposing residues had a greater impact on rhizosphere bacteria than exudates over time.

General

- Fresh decomposing residues and mature crop residues had different effects on the soil fungal communities, and TRF 485 was only identified in samples from the mature crop residue experiment.
- *O. brassicae* as a component of the soil fungal community was enhanced by fresh decomposing residues, but not by mature residues. Abundance of other soil fungi following incorporation of mature residues reduced the relative abundance of *O. brassicae* in the soil.
- Soil fungal communities do not appear to impact on the infection of OSR by *O. brassicae,* irrespective of the incorporation of fresh or mature residues.
- Residue incorporation had a greater affect on soil fungal communities than soil bacterial communities. Exudates had a greater impact on soil bacterial communities.
- Fresh decomposing residues had a greater impact on rhizosphere bacterial communities than exudates over time.
- Bacteria associated with OSR soil and rhizosphere were not able to be identified using TRFLP and clone libraries. Key bacterial TRFs associated with OSR include TRFs 722, 522 and 135.

The rhizosphere bacterial community was significantly different between Rounds 1 and 2 for both the roots-removed and roots-retained treatments (P = 0.03; Figure 29b), due to a greater relative abundance of TRF 338 in Round 1, and a greater relative abundance of TRFs 722 and 522 in Round 2.

A greater relative abundance of TRF 135 in Round 2 also contributed to the differences seen in the roots-retained treatment. No further significant differences were found between Rounds for the rhizosphere bacterial community in the roots-removed treatment. However, in the roots-retained treatment, significant differences were also found between Rounds 2-5 (P = 0.03; Figure 29b). These differences were mainly due to shifts in abundance of TRFs 722, 522, 135, 338, 721, 130 and 245 (Figure 30). Results indicate that, over time, fresh decomposing residues impacted on the rhizosphere bacterial community to a greater extent than exudates *per se*.

Shannon diversity indices

Shannon diversity indices calculated for each treatment at each replanting Round indicated that repeated growth of OSR decreased soil and rhizosphere microbial diversity (Figure 31).

OBJECTIVE 4: Investigate the contribution of specific microorganisms (identified through Objective 2) to yield decline in continuously cropped OSR

Using molecular techniques, several species had been identified as being more abundant in the rhizosphere or soil when OSR was grown more frequently in rotation (Objective 2). To investigate the impact of these organisms on plant growth and productivity, cultures were required for empirical studies. Once isolated, identities of the selected fungi were confirmed by DNA sequencing of the pure cultures, and TRFLP analysis showed they produced peaks at the same TRF as had been found in the field trial analysis. Two fungi in particular were selected for further study, namely *Olpidium brassicae* and *Pyrenochaeta* sp.

Methods

Isolation of cultures and inoculum preparation

A culture of *O. brassicae* was established through isolation of zoosporangia from infected plant material, and stock cultures were subsequently maintained on oilseed rape seedlings grown in a soilless substrate (sand:terragreen; 50:50 v:v) in the glasshouse. Zoospores were found to survive and infect plants better if released into a solution of GS (0.05 M glycine, 1 % sucrose). Zoospores were amplified in OSR plants, extracted in GS, counted and diluted to provide a range of doses in the glasshouse experiments. Seedlings were grown for 10 days before inoculation with *O. brassicae*, and at this time 1 ml of the required zoospore dilution was pipetted onto the base of each seedling.

Pyrenochaeta sp. was isolated onto tap water agar from a root lesion found on an OSR plant collected at the OSR rotation trial (Morley). It was subsequently found to grow best on an agar medium containing OSR leaf extracts. The pure culture of *Pyrenochaeta* sp. would not sporulate in the laboratory and therefore mycelium was used as inoculum for the glasshouse experiments. Mycelium was grown on autoclaved wheat grain for 7 weeks before being macerated in water. Half of the resulting liquid inoculum was autoclaved for use as negative controls. Dilutions of the liquid inoculum were used to provide a range of doses in the glasshouse experiments, and were incorporated into the growing medium at the time of planting.

Glasshouse experiments using model system

Brassica oleracea was used as a model Brassica for seed yield experiments, as it is a rapid-cycling Brassica suited to glasshouse conditions. The effect of *O. brassicae* and *Pyrenochaeta* sp. on early growth of OSR and *B. oleracea,* and seed yield of *B. oleracea,* was initially assessed in a soilless substrate using a sand-terragreen mix as a growing medium. This system allowed the candidate microorganisms to be tested in isolation, without the presence of other soil microorganisms. Experiments were subsequently repeated in soil (collected from Morley, Norfolk, from a site with no previous Brassica cropping history), which was sieved to remove stones prior to use.

In a first series of experiments (early growth experiments), OSR (*Brassica napus* cv. Winner) and *Brassica oleracea* (DHSL150) plants were inoculated independently with the selected fungi in a range of doses. Seedlings were grown for 6-7 weeks before top growth and root biomass were recorded, and root material (0.4g) was taken for DNA extraction and analysis of the inoculated fungi using quantitative PCR (qPCR; see Objective 5 for details). Two pots per sample were used with 3 plants in each pot to maximise root material for DNA analysis without impacting on overcrowding in the pots. Four complete replicates were set up.

In a second series of experiments (seed yield experiments), *B. oleracea* plants were again inoculated independently with the selected fungi in a range of doses and were grown to seed set. At harvest, assessments were made on various components of yield, including number of branches, number of pods, number of seed-containing pods, and number and weight of normal or deformed seeds. Rhizosphere samples (0.4g) were also taken at harvest for DNA extraction and quantification of inoculated fungi using qPCR. One pot per treatment was used, with a single plant per pot, and ten complete replicates were set up.

Results

a) Olpidium brassicae

a-i) Early growth experiment (soilless substrate: sand-terragreen)

The three doses in this experiment were 'High' = 1 x 10^7 zoospores seedling^{-1}, 'Medium' = 4 x 10^4 zoospores seedling^{-1} and 'Low' = 2.5 x 10^2 zoospores seedling^{-1}. A control was also set up using GS alone. After 6-7 weeks growth, a significant reduction in top growth and root biomass occurred in plants inoculated with the High dose, for both OSR and *B. oleracea* ($P \leq 0.01$; Figure 32). Quantitative PCR confirmed the presence of *O. brassicae* in the roots of all inoculated samples, with the highest amount associated with the High dose treatments. There was no difference in top growth or root weight between the control plants and the Low or Medium dose treatments.

a-ii) Early growth experiment (soil)

As 'Low' and 'Medium' doses in the sand-terragreen experiments were found to produce similar results, two doses were set up in the soil experiment: 'High' = 1 x 10^7 zoospores seedling^{-1} and 'Low' = 4 x 10^4 zoospores seedling^{-1}. A control was also set up using GS alone. After 6-7 weeks growth no significant differences were found in the top growth or root weight between treatments. However, qPCR indicated that little infection with *O. brassicae* had occurred with the zoospore inoculum in either of the inoculated treatments, suggesting that inoculum failure may be responsible for these results. No time was available to repeat these experiments with an alternative inoculum source, such as resting spores or dried infected root material.

a-iii) Seed yield experiment (soilless substrate: sand-terragreen)

The three doses in this experiment were 'High' = 1 x 10^7 zoospores seedling^{-1}, 'Medium' = 4 x 10^4 zoospores seedling^{-1} and 'Low' = 2.5 x 10^2 zoospores seedling^{-1}. At harvest, there was a significant reduction in total number of pods produced in the plants inoculated with the High dose of *O. brassicae* ($P = 0.017$; Figure 33a). This was found to be due to a reduced potential for pod production in this treatment as these plants also produced fewer primary branches than the uninoculated control (Figure 33b). Quantitative PCR confirmed the presence of *O. brassicae* in the roots of all inoculated samples, with the highest amount associated with the High dose treatments. There was no difference between the control plants and the Low or Medium dose treatments.

a-iv) Seed yield experiment (soil)

As 'Low' and 'Medium' doses in the sand-terragreen experiments were found to produce similar results, two doses were set up in the soil experiment: 'High' = 1 x 10^7 zoospores seedling^{-1} and 'Low' = 4 x 10^4 zoospores seedling^{-1}. A control was also set up using GS alone. Unlike the equivalent experiment conducted in sand-terragreen, no significant differences were seen in the number of branches or total number of pods produced. However, qPCR also indicated little infection by *O. brassicae* in this experiment, suggesting that inoculum failure may be responsible for these results. Seed components were thus not analysed further. No time was available to repeat these experiments with an alternative inoculum source, such as resting spores or dried infected root material, and the impact of *O. brassicae* on brassica growth and yield remains unclear in soil.

b) Pyrenochaeta sp.

b-i) Early growth experiment (soilless substrate: sand-terragreen)

The three doses in this experiment were 'High' = 4.7 x 10^4 (± 1.0 x 10^4) cfu g^{-1} substrate, 'Medium' = 4.7 x 10^2 (± 1.0 x 10^2) cfu g^{-1} substrate and 'Low' = 4.7 (± 1.0) cfu g^{-1} substrate, each of which had a corresponding autoclaved control. After 6-7 weeks growth, there was no effect of *Pyrenochaeta* sp. on the top growth or root biomass at any dose, although the High dose autoclaved inoculum was found to have a phytotoxic effect on *B. oleracea*. Lesions were found on the roots of inoculated plants, and qPCR also confirmed the presence of *Pyrenochaeta* sp. in the roots.

b-ii) Early growth experiment (soil)

As 'Low' and 'Medium' doses in the sand-terragreen experiments were found to produce similar results, two doses were set up in the soil experiment: 'High' = 9.1 x 10^4 (± 1.8 x 10^4) cfu g^{-1} and 'Low' = 1 x 10^2 (± 1.8 x 10^2) cfu g$^{-1,}$ each of which had a corresponding autoclaved control. An uninoculated control was also set up. The High dose caused a significant reduction in OSR leaf dry weight compared to its autoclaved control ($P = 0.04$; Figure 34). A similar trend was noted for the *B. oleracea* seedlings, but the effect was not statistically significant. No effects of *Pyrenochaeta* sp. was seen on root biomass for either plant species.

b-iii) Seed yield experiment (soilless substrate: sand-terragreen)
The three doses in this experiment were 'High' = 4.9 x 10^4 (± 0.97 x 10^4) cfu g^{-1} substrate, 'Medium' = 4.9 x 10^2 (± 0.97 x 10^2) cfu g^{-1} substrate and 'Low' = 4.9 (± 0.97) cfu g^{-1} substrate, each of which had a corresponding autoclaved control. In all treatments, less than 50% of pods contained seed (combined main and primary branches). Plants inoculated with a High dose of *Pyrenochaeta* sp. had a delay in time to first flower compared to the equivalent control (Figure 35).

Subsequent analyses of the seed yield components revealed that the High dose had a negative impact on seed quantity and quality. The High dose resulted in significantly fewer normal seeds per pod compared to its control (P=0.044; Figure 36a), and a correspondingly greater number of deformed seed in total compared to the autoclaved control (P = 0.016). As a result of the reduction in number of normal seeds at the High dose, the seed weight per pod was also significantly less compared to the autoclaved control (P=0.019; Figure 36b). Not only were fewer normal seeds produced per pod, but those that were produced also weighed less per seed (P=0.009; Figure 37). The Medium dose resulted in significantly fewer total number of seeds (including all normal and deformed seeds) compared to its autoclaved control (P = 0.028; Figure 38). However, the individual component analysis did not reveal further significant differences in number and weight of seed per pod for the Medium dose. There were no significant effects of the low dose of *Pyrenochaeta* sp. on any yield component assessed. Quantitative PCR confirmed the presence of *Pyrenochaeta* sp. in the roots in the non-autoclaved treatments, which was highest in the 'High' inoculated plants.

b-iv) Seed yield experiment (soil)
As 'Low' and 'Medium' doses in the sand-terragreen experiments were found to produce similar results, two doses were set up in the soil experiment: 'High' = 3.0 x 10^4 (± 0.4 x 10^4) cfu g^{-1} and 'Low' = 3.0 x 10^2 (± 0.4 x 10^2) cfu g^{-1}, each of which had a corresponding autoclaved control. An uninoculated control was also set up. No delay in time to first flower was noted in this experiment. In all treatments, less than 20% of pods contained seed (combined main and primary branches). However, no significant effect on seed yield components was found with treatments inoculated with the different doses of *Pyrenochaeta* sp. Quantitative PCR confirmed the presence of *Pyrenochaeta* sp. in the roots in the non-autoclaved treatments, which was highest in the 'High' inoculated plants. Despite high levels being found in the roots, no significant effect was seen on the seed yield.

Objective 4 KEY FINDINGS

- The model system was useful in testing the effects of individual fungal isolates on brassica growth and yield, but care must be taken in extrapolating results to field-grown OSR.
- *Olpidium brassicae* reduced plant biomass of oilseed rape and *B. oleracea* at high doses, in a soilless substrate.
- *Olpidium brassicae* reduced branching and thus total pod production of *B. oleracea* at high doses, in a soilless substrate.
- *Olpidium brassicae* results were not able to be confirmed in soil-based experiments due to inoculum failure.
- Inoculation with *Pyrenochaeta* sp. resulted in the development of root lesions.
- *Pyrenochaeta* sp. delayed flowering and reduced seed quality of *B. oleracea* at high doses, in a soilless substrate.
- *Pyrenochaeta* sp. had a small effect in reducing top growth of oilseed rape seedlings in a soil-based experiment.

OBJECTIVE 5: Develop quantitative PCR for two fungal pathogens
The ITS regions of all of the fungal organisms from the clone libraries produced during this project were aligned and unique DNA sequences were identified for *Olpidium brassicae* and *Pyrenochaeta* sp. Primers were then designed within these regions using Primer Express 2.0 (Applied Biosystems). Two sets of potential primers were identified for each organism and the DNA sequences of the primers were used in a BLAST search (NCBI) to determine if they were unique within the DNA database. The most specific primer pair was chosen for each organism (Appendix 2).

The *O. brassicae* primers (forward and reverse) only matched *O. brassicae* sequences and four uncultured Olpidiaceae sequences that had been isolated from oilseed rape. The forward *Pyrenochaeta* sp. primer only matched a clade of uncultured environmental *Pyrenochaeta* sp. sequences on the database. It did not match *Pyrenochaeata lycopersici* (a related tomato pathogen) and so was specific to this organism. The reverse primer was less specific, but in combination with the specific forward primer was expected to only amplify *Pyrenochaeta* sp.

To confirm the specificity of the primers, the primers were used to amplify DNA from rhizosphere samples which had been shown by TRFLP to contain approximately 1% of the total fungal population of either *O. brassicae* or *Pyrenochaeta* sp. The PCR was carried out using the quantitative PCR conditions (below) and the products were cloned and sequenced. All of the clones (48) had the correct sequences with no other organisms being amplified demonstrating the specificity of the primers.

A standard curve of a fivefold dilution of either cloned *O. brassicae* or *Pyrenochaeta* sp. ITS was made from 32 pg/ml down to 4.1 x 10^{-3} pg/ml. Total rhizosphere or bulk soil DNA (1 ng) was used in the real-time PCR using the SYBR Green PCR master mix (Applied Biosystems). This gave plasmid DNA concentrations within the standard curve. Each reaction was set up in triplicate in a 384-wellplate with the following components: 2 X SYBR Green PCR mastermix (10 µl), 1 mM forward primer, 1 mM reverse primer, standard curve DNA or 1 ng total sample DNA, 8 µg non-acetylated BSA and water added to 20 µl. Real-time PCR was carried out using the ABI Prism 7900 HT sequence detection system (Applied Biosystems) with standard Taqman cycling conditions (40 cycles of 95 ^{o}C for 15 s followed by 1 min at 60 ^{o}C). An average of the triplicate results was taken. The quantities of DNA obtained were converted to copy number/µg total DNA.

Objective 5 KEY FINDINGS

- Primers were designed to amplify a unique DNA sequence within the ITS region for *Olpidium brassicae* and *Pyrenochaeta* sp. Following testing and optimisation, these were shown to be specific to the organisms to which they were designed.

OBJECTIVE 6: Determine the susceptibility of commercial OSR varieties to the development of yield decline and the microbial basis underlying differences

The HGCA-funded OSR rotation trial at Morley, Norfolk, had been used to provide field samples for all previous project Objectives. In order to determine whether results were site- or cultivar-specific, this Objective focussed on examining the microbial communities at other sites. Initially a small survey was carried out in November 2008 to compared fungal diversity of winter OSR grown in short rotation or grown for the first time, at four farms across England (one in Bedfordshire, one in Suffolk and two in Warwickshire). The farms varied in soil type, as well as cultivar choice and management practices. However, analysis of the fungal rhizosphere communities indicated the presence of *O. brassicae* and *Pyrenochaeta* sp. at all sites. There was a trend that these fungi were found in greater relative abundance in the short rotation samples than the virgin OSR, although not in all cases.

Further investigations into the susceptibility of commercial cultivars were carried out at a replicated field trial site in Bunny, Nottinghamshire, described below.

Methods

A field trial based at, and managed by, the University of Nottingham (funded as part of Defra project WQ0119), was used to provide samples for this Objective. Plots were established in 2008 and each plot was scheduled to receive the same variety and P treatment in 2009 and 2010. The crops were cultivated and managed in accordance with conventional commercial practices, with insecticides and fungicides applied as necessary. Samples were taken to assess the effects of OSR variety on microbial diversity at two sampling times, each over two seasons.

Rhizosphere samples were collected from the P fertilised plots of 8 commercially available varieties of spring OSR: Ability, Gladiator, Heros, Hunter, Oban, Ortego, Palladium and Seven. Samples were taken from Site A in May 2009 (early) and July 2009 (late) (OSR grown in 2009 only; fallow in 2010)

and from Site B in June 2010 (early) and July 2010 (late) (two years continuous OSR). At each sampling time, six plants were pooled from each of three replicate plots for each of the eight OSR varieties. In addition, replicated soil samples were taken from Sites A and B in July 2010. It was not possible to sample from the winter OSR varieties as originally planned due to failed crop establishment. DNA was extracted from the samples, and TRFLP analysis of the microbial communities was performed as previously described.

Results

TRFLP analysis of fungal communities

No significant differences were found between rhizosphere fungal communities of the eight varieties, at any of the sample times. Analyses indicated that irrespective of variety, sample time or year, fungal communities were dominated by the high relative abundance of *O. brassicae* (TRF 284).

On average across varieties, differences were found in the rhizosphere fungal communities between years (*P* = 0.007; Figure 39) and also between early and late sampling times (P = 0.001; Figure 39), largely due to shifts in relative abundance of *O. brassicae* (TRF 284). *Olpidium brassicae* had a lower relative abundance in 2010 compared to 2009, and a lower relative abundance in the late samples compared to the early samples. Other less obvious shifts in community structure may have also contributed in part to the overall significant differences seen, but other dynamics were difficult to ascertain due to the prevalence of *O. brassicae* in the samples.

Further analyses of each variety separately indicated that the variety Oban had significantly different fungal communities between early and late sample times (P = 0.028), largely due to an increase in the relative abundance of TRF 298 in the late samples for this variety.

Soil samples taken from sites A and B in 2010 had similar fungal communities.

TRFLP analysis of bacterial communities

Significant differences were found between rhizosphere bacterial communities of the eight varieties, in the late 2010 samples only (P = 0.003; Figure 40). Changes in the relative abundance of TRFs 722, 522, 523, 135, 400, 484 and 245 contributed to the differences between the varieties at this sampling time, with small increases or decreases in relative abundance of specific TRFs depending on the variety.

On average across varieties, differences were found in the rhizosphere bacterial communities between years (*P* = 0.001; Figure 41). TRFs contributing >5% to the differences were TRFs 245, 523, and 722, which all increased in abundance in 2010. Bacterial communities were also significantly different between early and late sample times (P = 0.001; Figure 41), largely due to shifts in relative abundance of TRFs 245, 523 and 270, which decreased in relative abundance in the late samples compared to the early samples, and TRFs 722 and 135, which increased in relative abundance in the late samples.

Bacterial communities were also analysed separately for each variety. There were differences in community profiles between 2009 and 2010 for varieties Heros (*P* = 0.009), Oban (*P* = 0.024), and Gladiator (*P* = 0.032). TRFs contributing >5% to these differences included, in general, an increase in relative abundance of TRFs 722, 245, 524, 484 and 523; and a decrease in relative abundance of TRFs 270, 135, 522. In addition, for all varieties except Palladium, the bacterial communities in early season samples were different to those in late season samples (P<0.05). TRFS contributing >5% to these differences included, in general, an increase in relative abundance of TRFs 722 and 135; and a decrease in relative abundance of TRFs 245, 523, 270, 524 and 484.

Soil bacterial communities were similar at Site A and site B.

Objective 6 KEY FINDINGS:

- All tested varieties of spring OSR were infected to a high degree by *O. brassicae.*
- *Olpidium brassicae* was most abundant in samples taken earlier in the season (May/June).
- Other differences in fungal community structure were difficult to ascertain.
- Bacterial communities were similar across varieties, with the exception of the July 2010 sample, where differences were noted. This was the second season of OSR cultivation on the same plots.

OBJECTIVE 7: Investigate the impact of extended rotation intervals on fungal communities in the rhizosphere of OSR

The 2010-2011 season of the HGCA-funded OSR rotation trial was an important one in understanding the impact of rotation interval on microbial communities in relation to yield decline. Work in this Objective focussed on assessing the role of longer rotations on microbial community structure, as well as how longer rotations affected the abundance of two selected fungi, *O. brassicae* and *Pyrenochaeta* sp., in the rhizosphere of OSR.

Methods

This eighth season of the trial included rotations of OSR as follows: 6 year gap, 4 year gap, 3 year gap, 2 year gap, 1 year gap (alternate years) and continuous OSR (Appendix 1). Rhizosphere samples were taken from all OSR rotations, at three points in the growing season, January (early-season), March (mid-season) and June (late-season). DNA was extracted from the samples and TRFLP analysis was carried out as previously described to determine the effect of rotation interval on fungal community diversity. In addition, qPCR was done for *O. brassicae* and *Pyrenochaeta* sp. to monitor population levels throughout the growing season and to determine whether there was a correlation between these fungi and reduced yields seen with more frequent cropping.

TRFLP analysis of fungal communities

i) Effect of rotation

Each sampling time was analysed separately to assess the affect of rotation on fungal community structure. There were no significant differences in the rhizosphere fungal communities, irrespective of the rotation gap, at any time point in the growing season. This was due to the high abundance of *O. brassicae* (TRF 284) in all rhizosphere samples. These findings are similar to those from Year 5 of the trial (see Objective 2).

ii) Effect of sampling time

As no effect of OSR cropping frequency was seen (see above), data from all OSR rotations were combined for this analysis. Averaging across all rotations, ANOSIM analysis indicated a significant difference in fungal communities between the three sample times (January, March, June 2011) ($P<0.05$; Figure 42). Between January and March there was an increase in relative abundance of *O. brassicae* (TRF 284) and a decrease in TRF 130 in the rhizosphere fungal community. Between March and June there was a decrease in relative abundance of *O. brassicae* (TRF 284), but an increase in relative abundance of TRF 270.

TRFLP indicated that *O. brassicae* (TRF 284) peaked in relative abundance in March (Figure 43), whereas *Pyrenochaeta* sp. peaked in relative abundance in June (Figure 44).

iii) Quantitative PCR

Quantitative PCR was used to monitor levels of *O. brassicae* and *Pyrenochaeta* sp. in the OSR rhizoshere at three time points during the growing season. This confirmed that *O. brassicae* reached its highest levels in March, followed by a decline in June, for all rotations assessed (Figure 45). However, there was no significant difference between rotation gaps. These data show that even a rotation gap of 6 years between OSR crops did not reduce the amount of infection by this obligate parasite. Note however that the role of this organism in yield decline of OSR is not yet clear.

In contrast, *Pyrenochaeta* sp. increased in abundance over the season, with significantly higher levels being found in June compared to earlier in the season (P <0.001; Figure 46), confirming findings shown by TRFLP analysis. Also, significantly higher levels of *Pyrenochaeta* sp. were found in the alternate OSR than in the longer rotation gaps in January (P = 0.012) and March (P = 0.013) (Figure 47). Further analyses demonstrated a significant negative correlation between the levels of *Pyrenochaeta* sp. in January and yield (P <0.001), as well as a significant negative correlation between the levels of *Pyrenochaeta* sp. in March and yield (P = 0.014) (Figure 48). Note however, it cannot be confirmed from this that *Pyrenochaeta* sp. is responsible for the reduced yield, and it may be that the yield reduction was a result of other factors that also affected *Pyrenochaeta* sp. populations.

Key findings:

- TRFLP analysis indicated the rhizosphere fungal communities were similar across all OSR rotations, largely due to the high relative abundance of *Olpidium brassicae* in all roots.
- *O. brassicae* peaked in abundance in March, but was not affected by rotation gap.
- *Pyrenochaeta* sp. increased in abundance throughout the season, with significantly high levels reached in June than earlier in the season.
- Significantly more *Pyrenochaeta* sp. was found in the alternate OSR than in the longer rotations, in both January and March.
- Levels of *Pyrenochaeta* sp. in January and March correlated negatively with yield.

CONCLUSIONS

- Yield decline of frequently cropped OSR was demonstrated at the HGCA-funded OSR rotation trial. Community fingerprinting, carried out on bulk soil and rhizosphere samples collected from this trial, demonstrated differences in fungal, bacterial and nematode communities associated with OSR cropped in different rotational frequencies with wheat.

- Molecular analysis showed in particular that the obligate parasite *O. brassicae* was highly abundant in the rhizosphere of OSR. Infection levels in virgin OSR were found to be significantly lower than for continuously cropped OSR, but once land had been cropped to OSR and the fungus was present in the soil, future crops became infected to a similar level regardless of rotation gap. In general, the high abundance of *O. brassicae* in the roots of OSR made it difficult to assess the population dynamics of other rhizosphere fungi using TRFLP.

- Glasshouse experiments confirmed that *O. brassicae* quickly became abundant in the rhizosphere of OSR, and levels remained high once infection had occurred. The incorporation of mature crop residues into soil did not affect root infection by *O. brassicae*, but infected young root material did increase the abundance of this fungus in the soil fungal community. In a model system using a soilless substrate, it was demonstrated that *O. brassicae* significantly reduced both top growth and root biomass of young OSR and *B. oleracea* seedlings, and impacted on *B. oleracea* yield through reducing seed production potential.

- *Olpidium brassicae* has been found to be associated with both winter and spring varieties of OSR, at different field sites across the UK. Furthermore, a field trial at Nottingham showed that there was little difference in the structure of fungal communities in the rhizosphere of commercial OSR varieties, indicating little difference in resistance to *O. brassicae* in the varieties tested.

- Molecular analysis also revealed that increased cropping frequency of OSR in rotation was associated with increased relative abundance of *Pyrenochaeta* sp. in the rhizosphere. *Pyrenochaeta* sp. also increased in abundance over the course of the growing season, and was also found to be associated with root residues after harvest. Experimental work demonstrated that *Pyrenochaeta* sp. grew well on agar amended with OSR extracts, and also confirmed that *Pyrenochaeta* sp. produced lesions on OSR roots. In a model system using a soilless substrate, this fungus caused a delay in *B. oleracea* flowering at high doses, and also affected seed quality. Further characterisation of this novel OSR pathogen is needed.

- Quantitative PCR procedures were developed to accurately monitor the effect of rotation gap on the dynamics of *O. brassicae* and *Pyrenochaeta* sp. Field sampling throughout the season proved informative in determining that *O. brassicae* peaked in abundance mid-season. Although *Pyrenochaeta* sp. peaked in abundance late-season (June), it was also found that the levels of *Pyrenochaeta* sp. early-season (January and March) correlated negatively with yield.

- Other organisms were highlighted as potential components of the complex disease system, but were unable to be studied further in the timeframe of this project. For example, *Gibellulopsis* (formerly *Verticillium*) *nigrescens* also had a greater relative abundance in the bulk soil of continuously cropped OSR compared to virgin OSR. Whilst identification of bacteria was very limited, and cultures were not obtained in this project, specific TRFs were also found to be associated with frequent cropping of OSR. The parasitic nematode *Pratylenchus neglectus* was associated with OSR, as were other unidentified nematodes. Cultural techniques also highlighted the presence of a virulent damping-off pathogen, *Rhizoctonia solani*, in the soil. However, the contribution of these organisms to yield decline has not yet been investigated, and interactions among organisms in different trophic levels remains unknown.

- Whilst the use of TRFLP was useful in examining overall soil and rhizosphere community structures with frequent cropping of OSR, the technique does have limitations and may not distinguish all species. Furthermore, in the analysis of the TRFLP peaks, the percentage cut-off used to decide significant contribution (>5%) to community differences is relatively arbitrary and there may be organisms of biological significance whose abundance falls below this cut-off value. In the case of rhizosphere fungal analysis, the dominance of *O. brassicae* in samples may have masked the important contribution of less abundant fungi (that may have high biological activity).

- Identification of organisms of interest was also challenging, and it was only possible to identify highly abundant or well-spaced peaks in the TRFLP profiles. In addition, database entries limited identification further, particularly for the bacteria.

FUTURE RESEARCH

1) Characterisation of the pathogen complex

The current project has identified a range of fungal and nematode pathogens which increase in abundance during growth of OSR. Further work is needed to characterise the complex to understand how it develops and functions in space and time. In addition there is a need to integrate understanding of the fungal and nematode components of the complex.

For fungi, work to date has focussed on *O. brassicae* and *Pyrenochaeta*. Further work is needed to understand development of *G. nigrescens* so a picture of the structure and dynamics of the entire fungal pathogen complex can be developed (e.g. development of the complex as the crop matures to identify when each part of the complex is active; the effect of extended rotation intervals on the stability of the complex).

For nematodes, only part of the pathogen complex has been identified. Work is needed to identify the other components which are favoured by increased cropping frequency of OSR. There is also a need to match understanding of the nematode pathogen complex with that of the fungal complex e.g. development and activity of the nematode complex during crop growth and the effect of rotation interval on structure and survival of the complex.

Lastly, molecular studies in related work at Warwick have identified *Plasmodiophora brassicae,* which causes clubroot, in OSR samples from Morely, although no clubroot symptoms were seen in samples collected from the field. The possibility that *P. brassicae* is part of the patho-complex should be investigated.

High throughput sequencing approaches, including 454 and Illumina platforms, which have become available recently at low cost, provide the potential to elucidate both the structure of the rhizosphere patho-complex and its functional interactions with the plant with great clarity and depth. Material

gathered and characterised in the current project (including DNA extracts and pathogen pure cultures) would be suitable for such analysis.

2) Interactions between pathogens

Experiments have shown that at high doses, two of the fungal components of the pathogen complex inoculated separately onto OSR and the model species *B. oleracea* reduce growth and seed yield. The relative importance of the other pathogens needs to be established. Furthermore the interactions between the pathogens need to be determined e.g are some of the components greater contributors to yield decline, and hence more important, than others; are there interactive or synergistic effects between the pathogens on yield decline?

There is also scope to investigate interactions between pathogens and other rhizosphere organisms, particularly bacteria, which can be either deleterious or plant growth promoting, depending on population dynamics, environmental factors, and other interactions in the rhizosphere. It is not known whether the bacteria associated with OSR in short-rotation cropping are detrimental or beneficial. Furthermore, taxonomic identification does not necessarily provide an indication of function. Understanding of function is important in a yield decline situation to determine if a high relative abundance of any particular organism is indicative that it is in itself having a negative effect on the crop, or whether it is a saprotroph or a biocontrol agent responding to increased populations of other deleterious microorganisms.

3) Potential to manage the patho-complex through crop genotype

Experiments have investigated whether current commercial cultivars of OSR differ in their levels of *O. brassicae* infection in the field, and little difference was seen in the varieties tested. More directed experiments are needed to establish whether there is resistance to key components of the pathogen complex which could be used in breeding approaches to generate lines with reduced susceptibility to yield decline. Furthermore there is a need to use appropriate and diverse genetic material. This could be achieved using OSR mapping populations and related Brassica accessions which contain far greater genetic diversity, and potential resistance to the pathogens, than commercial lines. Experiments with this material could provide a mean of identifying genetic loci associated with resistance to yield decline pathogens which could be used to breed resistant lines.

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REFERENCES

Bennett, A. J., Hilton, S., Bending, G., Chandler, D. and Mills, P. (2011) Meeting the demand for crop production: The challenge of yield decline in crops grown in short rotations. *Biological Reviews* doi: 10.1111/j.1469-185X.2011.00184.x

Christen, O. and Sieling, K. (1995) Effect of different preceding crops and crop rotations on yield of winter oil-seed rape (*Brassica napus* L.). Journal of Agronomy & Crop Science 174, 265-271.

Stobart, R. (2009) Identifying changes in crop performance and microbial populations under frequent cropping with oilseed rape. *Aspects of Applied Biology 91, Crop Protection in Southern Britain,* pp 1-5.

Stobart, R. (2011) The impact of close rotation systems on the performance of oilseed rape. *Aspects of Applied Biology 106, Crop Protection in Southern Britain,* pp 209-212.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Peer-reviewed publications

Bennett, A. J., Hilton, S., Bending, G., Chandler, D. and Mills, P. (2011) Meeting the demand for crop production: The challenge of yield decline in crops grown in short rotations. *Biological Reviews,* doi:10.1111/j.1469-185X.2011.00184.x

Articles

Mills, P. Oilseed rape and yield decline: Understanding the interactions. TAG Research Bulletin, Issue 19, December 2008.

Publications in preparation

Hilton, S., Bennett, A. J., Bending, G. D., Chandler, D., Stobart, R. and Mills, P. (*in preparation)* Impact of shortened crop rotation of oilseed rape on soil and rhizosphere microbial diversity in relation to yield decline

Hilton, S., Bennett, A. J., Bending, G., Chandler, D. and Mills, P. (*in preparation)* Seasonal variation in fungal, bacterial, and nematode communities associated with a range of oilseed rape-wheat rotations

Bennett, A. J., Hilton, S., Bending, G., Chandler, D. and Mills, P. (*in preparation)* Influence of mature crop residues, fresh decomposing residues and root exudates on winter oilseed rape rhizosphere microbial populations

Bennett, A. J., Hilton, S., Bending, G., Chandler, D. and Mills, P. (*in preparation)* Impact of extended rotation intervals on oilseed rape rhizosphere fungi

Conference proceedings

Mills P, Hilton, S., Bennett, A., Atwood, R., Bending G. and Chandler D. (2009) Impact of shortened rotations on rhizosphere microbial populations on oilseed rape. *Aspects of Applied Biology* 91, 29-32

Bennett, A. J., Hilton, S., Bending, G., Chandler, D. and Mills, P. (2011) Soil microbial community changes under oilseed rape grown in shortened rotations. *IOBC Working Group "Multitrophic Interactions in Soil", Proceedings of the meeting at Uppsala (Sweden), 10-13th June 2009, IOBC/wprs Bulletin* Vol. 63, pp 7-11.

Bennett, A. J., Hilton, S., Bending, G., Chandler, D. and Mills, P. (*in press*) Fungal community changes under oilseed rape grown in shortened rotations: implications for yield decline. *IOBC Working Group "Multitrophic Interactions in Soil", Proceedings of the meeting at Cordoba (Spain), 4-7th April 2011*.

Hilton, S., Bennett, A. J., Bending, G., Chandler, D. and Mills, P. (*in press*) Detection and diversity of nematodes in the rhizosphere and bulk soil of oilseed rape and wheat grown in shortened rotations. *IOBC Working Group "Multitrophic Interactions in Soil", Proceedings of the meeting at Cordoba (Spain), 4-7th April 2011.*

Conference proceedings published as abstracts

Hilton, S., Bennett, A., Bending, G., Chandler, D., Whipps, J. and Mills, P. (2008) The impact of shortened rotations of oilseed rape on the microbial diversity of the rhizosphere. Meeting Abstract. *British Society for Plant Pathology Break Crops Workshop*. PGRO, Peterborough, 24th June 2008.

Hilton, S., Bennett, A., Bending, G., Whipps, J. and Mills, P. (2008) The impact of shortened rotations of oilseed rape on the microbial diversity of the rhizosphere. Meeting abstract. *14th Meeting of the Molecular Microbial Ecology Group.* University of Warwick, 3rd-4th July 2008.

Hilton, S., Bennett, A., Bending, G., Chandler, D., Whipps, J. and Mills, P. (2008) The impact of shortened rotations of oilseed rape on the microbial diversity of the rhizosphere. Meeting Abstract. *Autumn Meeting of the British Society of Soil Science: "Something old, something new: Integrating molecular biology with other techniques in soil science".* Rothamsted Research, 17th September 2008.

Bennett, A. J., Hilton, S., Bending, G., Chandler, D. and Mills, P. (2009) Effect of mature crop residues on microbial communities associated with oilseed rape grown in short rotation. Meeting abstract. *15th Meeting of the Molecular Microbial Ecology Group.* University of Aberdeen, 29-30th July 2009.

Bending, G. D., Hilton, S., Bennett, A., and Mills, P. (2011) Shifts in rhizosphere diversity associated with the onset of yield decline in oilseed rape. *Rhizosphere 3.* Perth, Australia, 25-30th September 2011.

Conference and workshop presentations

Bennett, A. J**.** (2008) Yield decline in continuous cropping. *Pulse yield decline – a brainstorming workshop.* PGRO, Peterborough, 5th March 2008.

Hilton, S. L., Bennett, A., Bending, G., Chandler, D., Whipps, J. & Mills, P. (2008). Microbial populations associated with yield decline in continuous oilseed rape. UK-Brassica Research Community meeting, Wellesbourne, Warwick, UK, May 21st 2008.

Hilton, S., Bennett, A., Bending, G., Chandler, D., Whipps, J. and Mills, P. (2008) The impact of shortened rotations of oilseed rape on the microbial diversity of the rhizosphere. *British Society for Plant Pathology Break Crops Workshop*. PGRO, Peterborough, 24th June 2008.

Hilton, S**.,** Bennett, A., Bending, G., Whipps, J. and Mills, P. (2008) The impact of shortened rotations of oilseed rape on the microbial diversity of the rhizosphere. *14th Meeting of the Molecular Microbial Ecology Group*. University of Warwick, 3rd-4th July 2008.

Hilton, S**.,** Bennett, A., Bending, G., Chandler, D., Whipps, J. and Mills, P. (2008) The impact of shortened rotations of oilseed rape on the microbial diversity of the rhizosphere. *Autumn Meeting of the British Society of Soil Science: "Something old, something new: Integrating molecular biology with other techniques in soil science".* Rothamsted Research, 17th September 2008.

Mills, P. and Bennett, A. J**.** (2008) HGCA Oilseed rape disease and pest management workshops. Wellesbourne, 11th November 2008
Newmarket, 12th November 2008
Winchester, 20th November 2008
York, 2nd December 2008
Grantham, 3rd December 2008

Mills P**.,** Hilton, S., Bennett, A., Atwood, R., Bending G. and Chandler D. (2009) Impact of shortened rotations on rhizosphere microbial populations on oilseed rape. *Crop protection in Southern Britain, Peterborough, 10-11th February 2009.*

Bennett, A. J**.,** Hilton, S., Bending, G., Chandler, D. and Mills, P. (2009) Soil microbial community changes under oilseed rape grown in shortened rotations. *5th Meeting of the IOBC/wprs Working Group "Multitrophic Interactions in Soil".* Uppsala, Sweden, 10-13th June 2009.

Bennett, A. J., Hilton, S., Bending, G., Chandler, D. and Mills, P. (2009) Effect of mature crop residues on microbial communities associated with oilseed rape grown in short rotation. *15th Meeting of the Molecular Microbial Ecology Group*. University of Aberdeen, 29-30th July 2009.

Hilton, S**.,** Bennett, A. J., Bending, G., Chandler, D. and Mills, P. (2011) Detection and diversity of nematodes in the rhizosphere and bulk soil of oilseed rape and wheat grown in shortened rotations. *6th Meeting of the IOBC/wprs Working Group "Multitrophic Interactions in Soil".* Cordoba, Spain, 4-7th April 2011.

Bennett, A. J., Hilton, S., Bending, G., Chandler, D. and Mills, P. (2011) Fungal community changes under oilseed rape grown in shortened rotations: implications for yield decline. *6th Meeting of the IOBC/wprs Working Group "Multitrophic Interactions in Soil".* Cordoba, Spain, 4-7th April 2011.

Bending, G. D**.,** Hilton, S., Bennett, A., and Mills, P. (2011) Shifts in rhizosphere diversity associated with the onset of yield decline in oilseed rape. *Rhizosphere 3.* Perth, Australia, 25-30th September 2011.