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Relationships between yield, rotation length, and abundance of *Olpidium brassicae* and *Pyrenochaeta* sp. in the rhizosphere of oilseed rape

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Abstract

Oilseed rape yields in the UK have been found to decline with more frequent cropping in a rotation. Previously, two soil-borne organisms (*Olpidium brassicae* (Chytridiomycota) and *Pyrenochaeta* sp. (Ascomycota)) were identified as having high relative abundance in rhizosphere fungal communities associated with oilseed rape crops where yield decline had been recorded. In order to better understand these organisms' association with the oilseed rape crop, the current study was designed to investigate the fungal rhizosphere microbiome of oilseed rape grown in a wide range of rotational frequencies. Samples collected from a long-term rotation trial site at three time points through the growing season were used to determine fungal community composition, and quantification of *O. brassicae* and *Pyrenochatea* sp. Analyses showed the combined root and rhizosphere fungal communities were similar across all oilseed rape rotations, largely due to the high relative abundance of *O. brassicae*, irrespective of cropping frequency. *Olpidium brassicae* abundance peaked in March (mid-season) in all rotations, before declining in abundance by June (pre-harvest). In contrast, *Pyrenochaeta* sp. increased in abundance throughout the season, with significantly higher

levels reached in June than earlier in the season. *Pyrenochaeta* sp. had a greater relative abundance in the rhizosphere fungal community of alternate oilseed rape (grown one year in two) than in rotations with longer gaps between oilseed rape crops. This study concludes that *O. brassicae* cannot be solely associated with yield decline of OSR observed in short rotation cropping due to its prevalence in the extended rotations examined (up to 6-year gap).

Key words

Oilseed rape, T-RFLP, qPCR, *Olpidium brassicae*, *Pyrenochaeta* sp., rotation, yield

Introduction

Monocultures and short crop rotations, where the same crop is grown once every three years or less, have been common in global agriculture in recent times due to a range of technical, economic and social drivers. However, short rotation cropping is also associated with yield declines in many crops, including oilseed rape (OSR; *Brassica napus*) (Bennett et al., 2012). Oilseed rape is a globally significant crop, grown for biofuel, production of animal feed and oil for human consumption (Weightman et al., 2010; Carré and Pouzet, 2014). In 2018/2019 70.9M metric tons of rapeseed were produced, second only to soybean in the major oilseeds market (USDA, 2019). However, frequent cropping of OSR in short rotations with wheat (*Triticum aestivum*) has been associated with reduced yields of up to 25% in the UK (Stobart and Bingham, 2013).

The cause of OSR yield decline is not straightforward and may arise in part from changes in soil biology, particularly selection of deleterious microbiomes with repeated cropping (Bennett et al., 2012). Hilton et al. (2013) demonstrated that increased cropping frequency of OSR, as part of a rotation with wheat, resulted in a shift in fungal communities associated with the

rhizosphere and roots. In particular, they found that *Olpidium brassicae* and *Pyrenochaeta* sp. increased in abundance in the rhizosphere of OSR grown for four consecutive years, compared to when grown for the first time (Hilton et al., 2013). *Olpidium brassicae* is an obligate plant parasite of brassicas, including OSR (Lay et al., 2018a, 2018b). It survives in soil for many years in the form of resting spores, which germinate in the presence of a host plant to produce infectious zoospores that infect the roots (Singh and Pavgi, 1977).

Hilton et al. (2013) determined that the *Pyrenochaeta* sp. isolated from OSR was found to have a high similarity to *P. lycopersici,* the causal organism of tomato corky rot. *Pyrenochaeta lycopersici* produces microsclerotia that can remain viable in the soil for long periods and, under favourable conditions, hyphae germinate and infect the epidermal cells of host roots (Aragona et al., 2014). Visible symptoms of tomato corky root are brown lesions in the roots and swelling of root epidermis with subsequent cracking into a corky texture (Varela et al., 2009). As similar symptoms were seen on brassica species inoculated with *Pyrenochaeta* sp. isolated from OSR (Hilton et al., 2013), the infection mechanism of this species may be similar.

The potential of *O. brassicae* and *Pyrenochaeta* sp. to impact negatively on rooting and plant productivity was demonstrated in glasshouse bioassays using the model crop species *Brassica oleracea* (Hilton et al., 2013) and, taken together, the evidence suggested that *O. brassicae* and *Pyrenochaeta* sp. are deleterious organisms associated with reduced yield of OSR grown in short rotations.

OSR is susceptible to a range of plant pathogens including *Rhizoctonia solani, Verticillium longisporum, Plasmodiophora brassicae* and *Sclerotinia sclerotiorum* (AHDB, 2014), which interact with the crop at different stages of plant growth during the season due to their differing

76 life-cycles and trophic states. For example, *R. solani* typically infects the germinating seed and
77 young seedlings of OSR to cause pre- and post-emergence damping-off, thereby impacting on
78 crop establishment (Verma, 1996; Sturrock et al., 2015). In contrast, *V. longisporum* produces
79 soil-borne resting spores (microsclerotia), which germinate to produce hyphae that penetrate
80 the root, allowing the pathogen to colonise vascular tissue (xylem). Symptoms are seen later
81 in the season as the pathogen causes premature ripening and yield losses up to 50% (Gladders,
82 2009).
83
84 However, little is known about the seasonal dynamics of *O. brassicae* and *Pyrenochaeta* sp.,
85 or about the impact of rotation gaps on the root and rhizosphere fungal community
86 compositions of OSR. This is important because extending the rotation gap between host crops
87 can be used by farmers in an attempt to manage soil-borne plant diseases, alongside other
88 cultural practices such as sanitation, changing tillage operations, altering sowing date or
89 applying soil amendments (Katan, 2017; Burnett et al., 2013).
90
91 Here we used a long-term field trial to investigate links between OSR cropping frequency, crop
92 yield and the rhizosphere abundance of *O. brassicae* and *Pyrenocheata* sp. Firstly, we used
93 Terminal Restriction Fragment Length Polymorphism (T-RFLP) of fungal internal transcribed
94 spacer regions (ITS), which gives relative species abundance, to investigate the seasonal
95 population dynamics of the rhizosphere fungal communities associated with OSR grown under
96 a wide range of cropping frequencies. Secondly, we used quantitative PCR to determine how
97 cropping frequency affected the absolute abundance of *O. brassicae* and *Pyrenochaeta* sp. in
98 the rhizosphere of OSR at three times in the growing season, and the relationships between
99 abundance of these pathogens and crop yield.
100

101 **Materials and methods**

102 The study made use of a long-term field trial in Morley, East Anglia, UK, in which OSR and
103 wheat were grown according to different rotations in randomised plots (Table 1) (Hilton et al.,
104 2013; Stobart and Bingham, 2013; Hilton et al., 2018). The trial was on a sandy-loam soil and
105 was managed according to conventional practices (Hilton et al., 2013). Plants were sampled
106 from different rotation frequencies of OSR during the eighth year following establishment of
107 the trial, as indicated in Table 1. Samples were taken as previously described (Hilton et al.,
108 2013; Hilton et al., 2018), from four replicate plots for each of the following OSR rotations: 6-
109 year gap, 4-year gap, 3-year gap, 2-year gap, 1-year gap (ie alternate years of OSR) and OSR
110 grown continuously for 8 years. Six plants were dug up and pooled to comprise one sample
111 from each replicate plot. Field sampling took place at three points in the growing season:
112 January (early growth stage; leaf development), March (mid-season; stem extension) and June
113 (pre-harvest, seed development).

114

115 OSR harvest yield data were collected and analysed as part of a related study using the same
116 plots at the long-term experimental site, with a central sub-plot (2m x 24m) of each plot
117 harvested using a plot combine (Stobart and Bingham, 2013). Yields (t/ha) are quoted as 91%
118 dry matter (Stobart and Bingham, 2013).

119

120 For samples collected in January, March and June, processing took place in the laboratory:
121 roots were shaken free of loose soil, and fine roots were cut into approximately 5mm sections.
122 Fine roots plus closely adhering soil were designated as rhizosphere samples, and sub-samples
123 (0.5g) were frozen for molecular analyses, all of which were conducted as described in Hilton
124 et al. (2013). DNA extraction, T-RFLP and quantitative PCR protocols were conducted as
125 previously described (Hilton et al., 2013). Briefly, DNA was extracted from rhizosphere

126 samples and amplified with fungal internal transcribed spacer (ITS) region primers (ITSf1 and
127 ITS4r). Terminal restriction fragments were generated with the restriction enzyme HhaI, with
128 TRF of 284 and 98 bp corresponding to *O. brassicae* and *Pyrenochaeta* sp. respectively, as
129 confirmed in Hilton et al. (2013). T-RFLP community profiles were expressed in relative
130 abundance (based on TRF peak heights). Supplemental Table S1 reports the mean number of
131 TRFs for each treatment, out of 144 different TRFs recorded in total.
132
133 Abundance of *O. brassicae* and *Pyrenochaeta* sp. was determined using quantitative PCR using
134 the species specific primers to the ITS region of the rRNA gene and the conditions reported in
135 Hilton et al. (2013): ObF (5'-TCT CCT CGT TGG GAA GAC TTG T-3') and ObR (5'-GAG
136 CTT GAA TTT TTA AGT TCG TCG TT-3'); and PyF (5'-CCG CCG GTT GGA CAC TAT
137 AA-3') and PyR (5'-TCG ATG CCA GAA CCA AGA GAT-3'). The quantities of DNA
138 obtained were converted to copy numbers of rRNA gene /µg total extracted DNA.
139
140 Statistical analyses were performed using R version 3.5.1 (R Core Team, 2016). Differences
141 in population structure were tested by analysis of similarity (ANOSIM) with 999 permutations
142 and non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarities within the
143 *vegan* package (Clarke, 1993; Oksanen et al., 2013). Quantitative PCR data were $\log_{10}$
144 transformed before correlation analysis with yield data and ANOVA, which was used to
145 compare the abundance of *O. brassicae* and *Pyrenochaeta* sp. across rotations and sampling
146 times.
147
148 **Results**
149 ANOSIM analysis showed there was no significant difference in rhizosphere fungal
150 community composition with respect to cropping frequency of OSR (p=0.798 R=-0.017).

However, there was a significant difference in fungal community composition between the three sample times (January, March, June) ($P < 0.001$, $R=0.502$), and the NMDS plot shows grouping by sampling time (Figure 1).

Olpidium brassicae increased significantly in copy number from January to March ($P<0.001$), followed by a subsequent significant decline in June ($P<0.001$), i.e. a mid-season peak occurred (Figure 2a). In contrast, *Pyrenochaeta* sp. increased in copy number over the season, with significantly higher levels being found pre-harvest in June compared to January ($P<0.001$) or March ($P<0.001$) (Figure 2b).

Quantitative PCR showed no significant differences in *O. brassicae* copy number with different OSR rotation gaps at any time point (Figure S1). In contrast, *Pyrenochaeta* sp. had a significantly higher copy number in the continuous and alternate OSR rotation in January compared to the longer rotation gaps ($P<0.01$; Figure 2c).

Linear regression confirmed a significant negative relationship between *Pyrenochaeta* sp. copy number and yield in January ($F(1,22) = 16.2$, $P < 0.001$, $R^2=0.424$) and in March ($F(1,22) = 7.1$, $P = 0.014$, $R^2=0.243$) but not in June (Figure 3). There was no relationship between *O. brassicae* copy number and yield. Yield data from the sampled year of the field site (Stobart and Bingham, 2013) is shown in Figure 4. Yields in alternate and continuously cropped OSR were reduced by between 27% and 34 % relative to rotations with between 3 and 6 year gaps.

Discussion

Previous studies have shown that more frequent cropping of OSR impacts negatively on yield, compared to longer rotation gaps and that *O. brassicae* and *Pyrenochaeta* sp. are implicated as

176 putative pathogens that may be involved in yield reductions (Stobart and Bingham, 2013;
177 Hilton et al., 2013). The results from this study indicate that the abundance of *O. brassicae*
178 was not significantly different across a range of cropping frequencies of OSR and that
179 extending the rotation gap did not reduce relative abundance of this organism associated with
180 the crop. As a yield reduction was still observed in OSR crops grown in shorter rotations in
181 this study, we conclude that *O. brassicae* cannot be solely associated with yield decline of
182 OSR. *Olpidium brassicae* spores are known to survive in soil for many years and these results
183 suggest that once OSR has been previously grown, then for at least a 6-year rotation gap *O.*
184 *brassicae* is able to infect OSR plants to a similar extent.
185
186 This study, in contrast to Hilton et al., 2013, did not include plots where OSR was grown for
187 the first time (virgin land), which would have been likely to have shown lower levels of *O.*
188 *brassicae*. Lay et al. (2018a) similarly found that *O. brassicae* dominated the fungal core
189 microbiome of oilseed rape (canola) in Canada, but that it was not significantly correlated with
190 yield. It may, however, play a role in allowing other soil-borne organisms entry to the root
191 through initial infection sites (wounds), or through weakening the root systems more generally
192 due to its biotrophic state, resulting in a less tolerant crop overall. Although not addressed in
193 this research, another consideration is the increased aggressiveness or virulence of strains with
194 continuous cropping (El-Nashaar and Stack, 1989).
195
196 *Pyrenochaeta* sp. was found in greater abundance in the rhizosphere early in the season
197 (January) in rotations where OSR was grown in close succession (continuous or alternate
198 crops). Bennett et al. (2014) showed that *Pyrenochaeta* sp. survives in mature root residues of
199 OSR, so it is likely that there is carry-over of inoculum from residues into an OSR crop that
200 follows in short succession. In this case, extended rotations would be of benefit in reducing

inoculum as crop debris breaks down over time. Although the abundance of *Pyrenochaeta* sp. was lower than that of *O. brassicae*, there was a correlation between higher absolute abundance (qPCR) found early in the season and lower yield at harvest. Accepting that correlation is not evidence of causation, it would seem possible that in situations where there is a high inoculum potential during the active stage of crop growth and seed set, this fungus may have potential to impact on OSR productivity. The mechanism for this is not confirmed from field studies with OSR, but Hilton et al. (2013) demonstrated in glasshouse studies that *Pyrenochaeta* sp. resulted in the development of root lesions in young plants of *Brassica oleracea* and, in high doses, delayed flowering and reduced seed weight, quality and quantity per pod. Additional research is required to better understand how *Pyrenochaeta* sp. interacts with OSR crops.

In the field, both *O. brassicae* and *Pyrenochaeta* sp. show strong seasonal differences in abundance: *O. brassicae* peaked in March, when the crop was actively growing, whereas *Pyrenochaeta* sp. peaked in June when the crop was senescing. Although seasonal differences are likely due to a combination of plant age, climatic and edaphic factors, the temporal differences in the abundance of these two organisms within the OSR microbiome associated with plant age is likely to be due to their trophic states. As an obligate parasite (biotroph), *O. brassicae* requires living plant cells for nutrients (Singh and Pavgi, 1977; Raaijmakers, 2009). Higher abundance of *Pyrenochaeta* sp. in rhizosphere samples obtained pre-harvest in this study corroborate the findings of Bennett et al. (2014), where *Pyrenochaeta* sp. was found to survive in high numbers on mature (field-derived) OSR root residues collected immediately after harvest.

The different times of peak abundance of the two organisms investigated in this study show that seasonal dynamics of rhizosphere microorganisms should be taken into account in trying

to better understand rhizosphere ecology and its impact on crop productivity. In assessing only one point in the growing season, there is a risk of not capturing fluctuations in population growth of key organisms. Factors involved in temporal dynamics of soil-borne pathogen epidemics include the starting inoculum potential, how quickly they can grow and reproduce, and whether they are monocyclic or polycyclic (Raaijmakers et al., 2009). Seasonal sampling time and plant development age has also been shown to influence fungal, bacterial and nematode rhizosphere communities in winter wheat and OSR (Hilton et al., 2018), and bacterial communities in the rhizosphere of crops including OSR, strawberry and potato (Smalla et al., 2001; Farina et al., 2012).

Other glasshouse bioassays carried out as part of this wider research indicated *Rhizoctonia solani* to be a virulent pathogen of OSR (data not shown), although this pathogen was not evident from T-RFLP community analysis using the primers described throughout the current work (Hilton et al., 2013; Bennett et al., 2014; Hilton et al., 2018). However, other research using qPCR primers specific to *Rhizoctonia solani* has also shown this pathogen to be widespread in commercial OSR crops in the UK (McCormack, 2018). Other molecular techniques such as high throughput sequencing are now used routinely to provide greater resolution of microbial communities, although these should be complemented with qPCR if data on absolute abundance of a pathogen are required. In order to better understand the rhizosphere microbiome, samples should be taken repeatedly throughout the growing season and a range of techniques should be utilised.

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254

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325

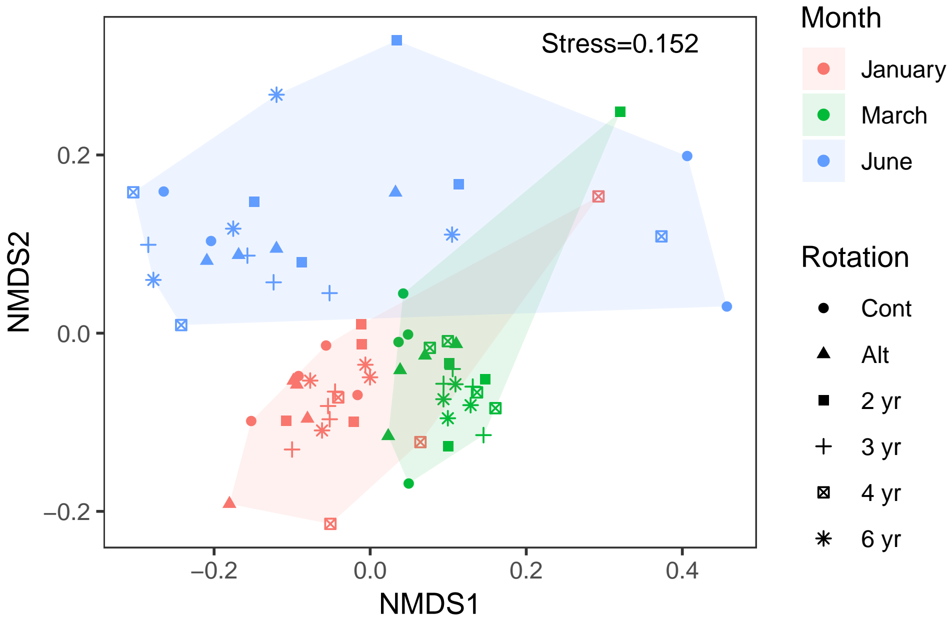
Figure 1: MDS plot of T-RFLP data indicating differences in rhizosphere fungal communities taken from oilseed rape grown at different cropping frequencies, at different sampling times throughout the season. MDS analyses were derived from a Bray-Curtis similarity matrix constructed with percentage peak height data of TRFs. Each point represents one plot (four replicate plots for each treatment).

Figure 2: Population dynamics using quantitative PCR of *Olpidium brassicae* and *Pyrenochaeta* sp. associated with oilseed rape grown in rotation with winter wheat. a) Quantification of *O. brassicae* over the growing season; b) Quantification of *Pyrenochaeta* sp. over the growing season c) Quantification of *Pyrenochaeta* sp. early season (January) in different rotational cropping frequencies. Bars represent means ± SEM taken from four replicate field plots. Different letters indicate significant differences between groups ($p<0.05$).

Figure 3: Negative correlation between oilseed rape yield and *Pyrenochaeta* sp. in the OSR rhizosphere in a) January, b) March an c) June. *Pyrenochaeta* sp. was quantified in rhizosphere samples taken from six different rotations of oilseed rape. Data are abundance data from qPCR and were $\log_{10}$ transformed before analysis.

Figure 4: Yield of oilseed rape grown in rotation with winter wheat, with six different rotation gaps between the oilseed rape crops, at a long-term experimental trial at Morley, Norfolk, UK. (from data presented in Stobart and Bingham, 2013).

Supplemental Figure S1: Population dynamics of *Olpidium brassicae* and *Pyrenochaeta* sp. associated with oilseed rape grown in different frequencies in a rotation with winter wheat, and sampled at three times in the growing season. a) Quantification of *O. brassicae*; b) Relative abundance of *O. brassicae* in the rhizosphere fungal community as determined by T-RFLP; c) Quantification of *Pyrenochaeta* sp.; d) relative abundance of *Pyrenochaeta* sp. in the rhizosphere fungal community as determined by T-RFLP. Bars indicate standard error of the mean of samples taken from four replicate field plots.



Stress=0.152
NMDS2
NMDS1
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0.0
−0.2
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Month
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Rotation
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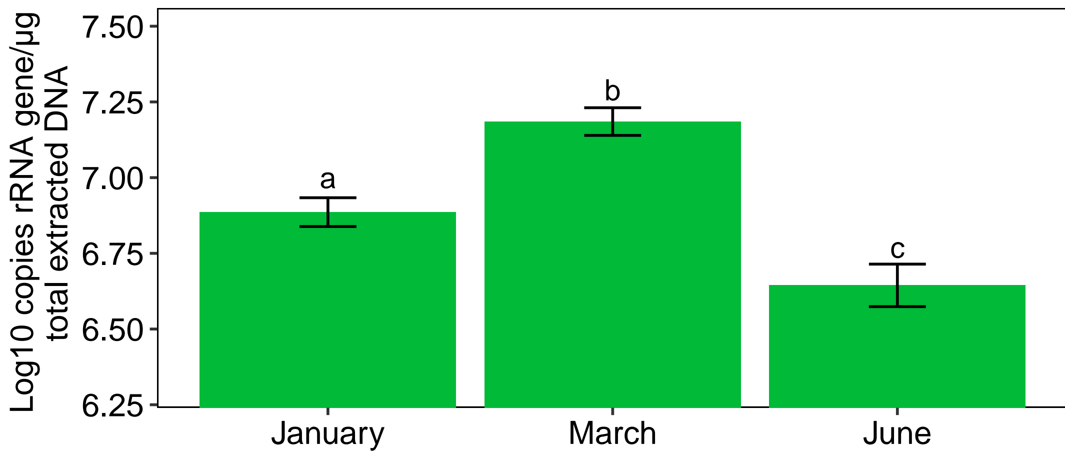
Alt
2 yr
3 yr
4 yr
6 yr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6.75
6.50
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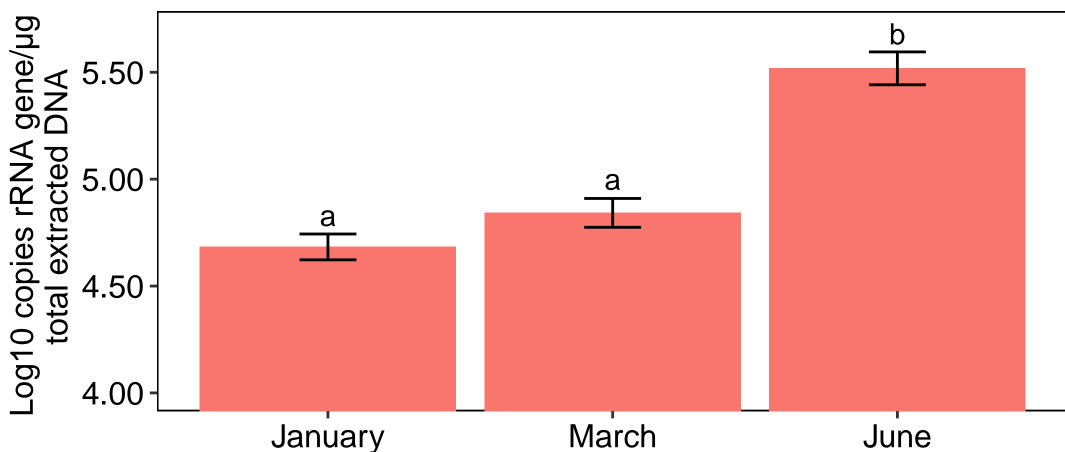
January
March
June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Log10 copies rRNA gene/µg total extracted DNA
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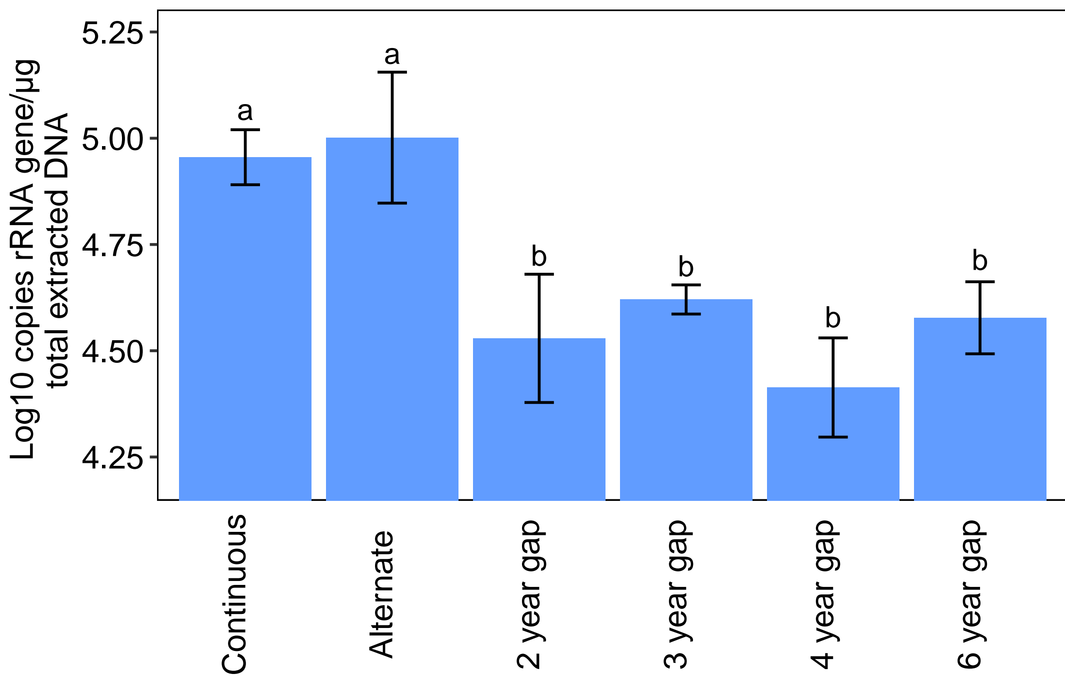
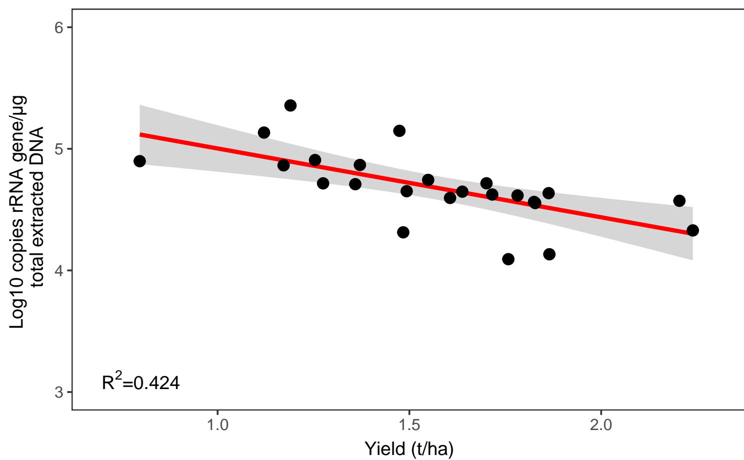
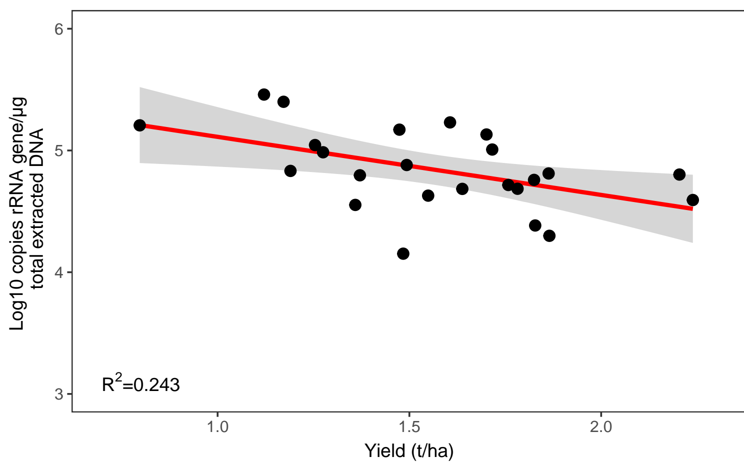
January
March
June


C
Log10 copies rRNA gene/µg total extracted DNA
5.25
5.00
4.75
4.50
4.25
a
a
b
b
b
b
Continuous
Alternate
2 year gap
3 year gap
4 year gap
6 year gap


A
Log10 copies rRNA gene/µg total extracted DNA
Yield (t/ha)
R^2=0.424
6
5
4
3
1.0
1.5
2.0


B
Log10 copies rRNA gene/µg total extracted DNA
Yield (t/ha)
R^2=0.243
6
5
4
3
1.0
1.5
2.0

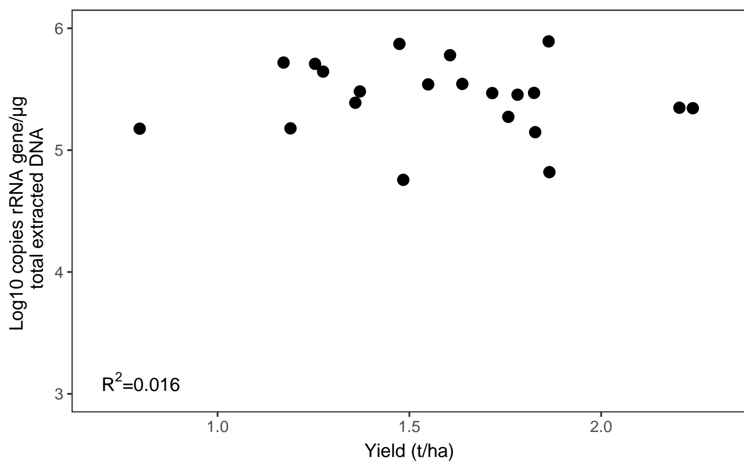

C
Log10 copies rRNA gene/µg total extracted DNA
Yield (t/ha)
R^2=0.016
6
5
4
3
1.0
1.5
2.0

Fig.4

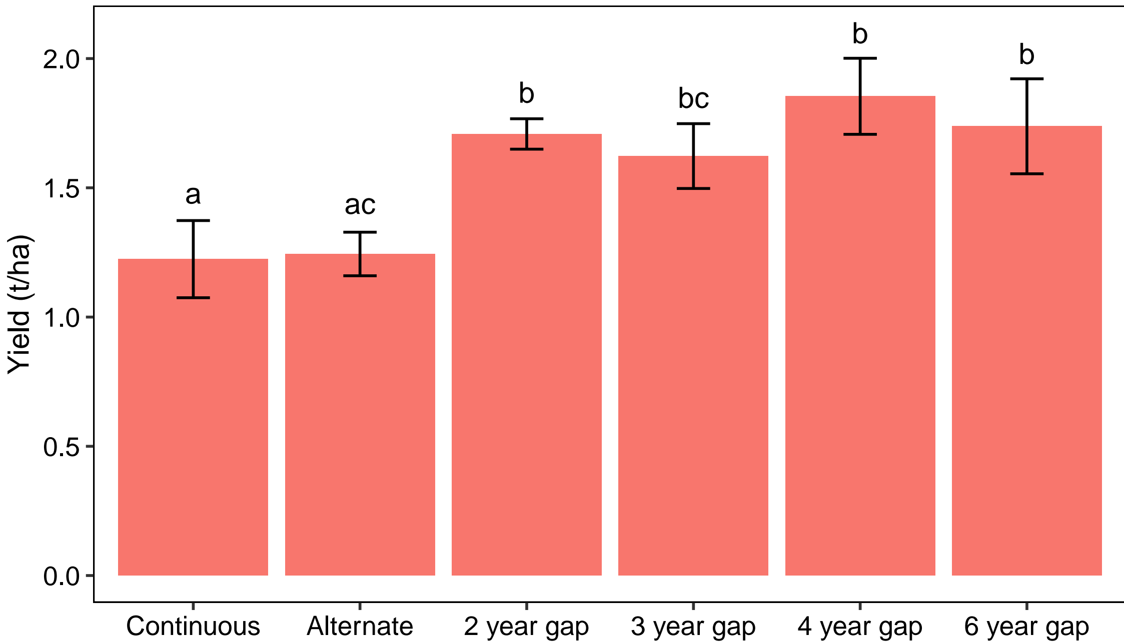


Fig.S1

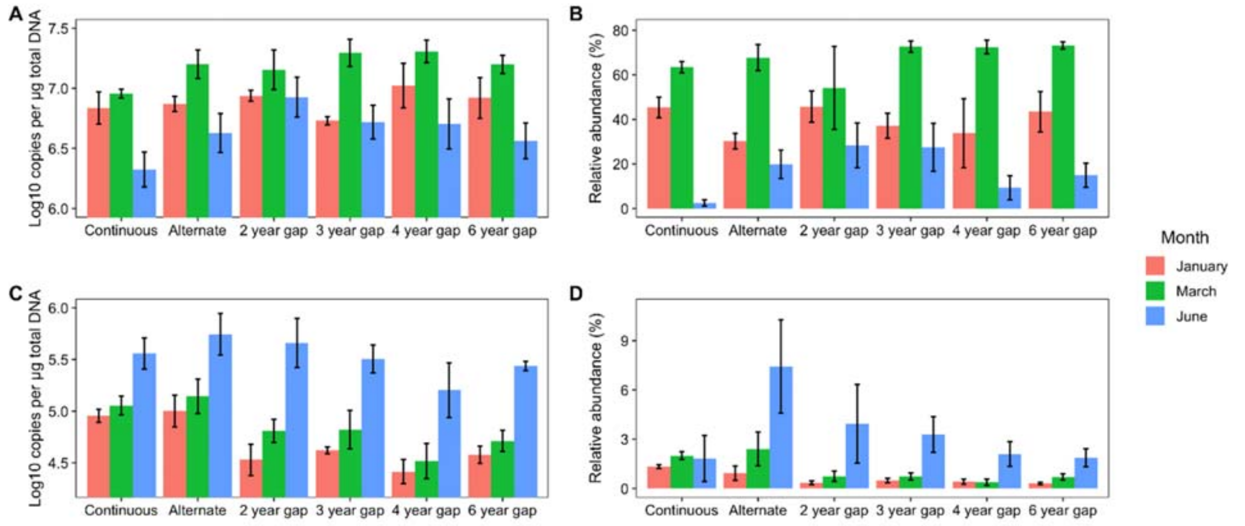


Table 1: Cropping history of rotations sampled at long-term oilseed rape rotation trial at Morley, East Anglia, UK (O = oilseed rape; W = winter wheat)

Rotation of oilseed rape	**Year of trial**							
	1	**2**	**3**	**4†**	**5**	**6**	**7**	**8‡**
Continuous	O	O	O	O	O	O	O	O
Alternate	W	O	W	O	W	O	W	O
2 year gap	W	O	W	W	O	W	W	O
3 year gap	W	W	W	O	W	W	W	O
4 year gap	W	W	O	W	W	W	W	O
6 year gap	O	W	W	W	W	W	W	O

† Oilseed rape sampled in year 4; described in Hilton et al. 2013

‡ Oilseed rape sampled in current study

Supplementary Table S1: Number of Terminal Restriction Fragments generated with the restriction enzyme HhaI, from rhizosphere samples collected from oilseed rape grown in a range of cropping frequencies (average of four replicates ± standard deviation)

	January	**March**	**June**
Continuous	137 ± 2.9	136 ± 2.2	133 ± 11.6
Alternate	137 ± 0.5	137 ± 2.2	135 ± 9.6
2 year gap	138 ± 2.2	134 ± 8.7	138 ± 0.5
3 year gap	137 ± 5.4	135 ± 1.9	140 ± 1.0
4 year gap	136 ± 3.0	135 ± 1.9	133 ± 7.5
6 year gap	139 ± 0.5	133 ± 1.0	137± 1.0