Potential ecological effects of Piriformospora indica, a possible biocontrol agent, in UK agricultural systems

Potential ecological effects of *Piriformospora indica*, a possible biocontrol agent, in UK agricultural systems

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Abstract

Piriformospora indica (Sebacinaceae), a root endophytic fungus, was originally isolated from an arid sub-tropical soil. *P. indica* forms mutualistic symbioses with a broad range of host plants, increases biomass production, resistance and tolerance to fungal pathogens and abiotic stresses. These characteristics make it a very attractive component of more sustainable agriculture. So, it is desirable to understand its wider ecosystem effects. We determined how long *P. indica* could survive in the soil and how it interacts with other soil microorganisms and some important arable weeds.

Survival of *P. indica* in the soil, under winter and summer conditions in the UK was tested by isolating DNA and RNA of *P. indica* from pots of soil which had been left open to winter-summer weather conditions without host plants, followed by PCR and reverse transcription-PCR (RT-PCR) with *P. indica*-specific primers. *P. indica* effects on other soil and root microorganisms were tested by PCR-denaturing gradient gel electrophoresis analysis of DNA extracted from soil and roots from pots in which *P. indica*-infected wheat had been grown. The effect of *P. indica* on growth of black-grass (*Alopecuris myosuroides*), wild-oat (*Avena fatua*) and cleavers (*Galium aparine*) was tested alone and in competition with wheat.

In soil *P. indica*-mRNA and DNA could still be detected after eight months, but not after 15 months. Soils from *P. indica*-inoculated pots had distinct fungal and bacterial species communities which were more diverse than non-inoculated controls. *P. indica* infected *A. myosuroides* and *A. fatua* but was not detected in *G. aparine*. The average above-ground competitiveness of the weeds with wheat was decreased.

If applied to field crops in the UK, *P. indica* would be persistent for up to 15 months and likely to alter competitive relations within vegetation. Increased soil microbial diversity during the first eight weeks after inoculation, although usually desirable, could alter soil composition or functioning.

Keywords: denaturing gradient gel electrophoresis, microbial diversity, reverses transcription-PCR, root endophytic fungus, soil microorganisms, sustainable agriculture.

1. Introduction

Piriformospora indica, a root endophytic fungus (Sebacinales: Basidiomycota), was first found in the Thar dessert of India (Verma et al., 1998), an arid region which experiences extreme day-time heat and diurnal temperature fluctuations as well as extended drought. *P. indica* promotes plant growth, increases root and above ground biomass and final yield during its mutualistic relationship with a wide variety of plants (Shrivastava & Varma, 2014). It increases resistance of several hosts from diverse families to many biotic stresses under glasshouse and field conditions (Waller et al., 2005, Deshmukh & Kogel, 2007, Ghahfarokhy et al., 2011, Harrach et al., 2013). Tolerance to abiotic stresses is also increased in a variety of hosts (Zarea et al., 2012, Alikhani et al., 2013, Ghabooli et al., 2013, Varma et al., 2013). Our previous studies showed that *P. indica* protected wheat from Fusarium crown rot in pot experiments (Rabiey et al., 2015). Our work also suggests that Fusarium head blight and mycotoxin deoxynivalenol contamination are reduced in pot experiments under UK weather conditions (Rabiey & Shaw, 2015). Tests on a field scale are therefore attractive, but as

68 the organism is an alien, it would first be necessary to understand its ecosystem effects
69 and persistence.
70 How *P. indica* interacts with other soil microorganisms is still unclear. Endophytic
71 fungal symbionts can have profound effects on plant ecology, fitness, and evolution
72 (Brundrett, 2006), shaping plant communities (Clay & Holah, 1999), increasing plant
73 tolerance to abiotic stresses (Murphy et al., 2015), increasing plant resistance to
74 pathogens (Rodriguez et al., 2009, Murphy et al., 2014) and manifesting strong effects
75 on the community structure and diversity of associated organisms (e.g. bacteria,
76 nematodes and insects; Omacini et al. (2001)). Studies on the effects of arbuscular
77 mycorrhizal fungi (AMF) on rhizosphere bacteria have shown variable results, with
78 both negative (decreasing the population of bacteria) (Christensen & Jakobsen, 1993,
79 Amora-Lazcano et al., 1998) and positive (increasing the population of bacteria)
80 (Andrade et al., 1997, Abdel-Fattah & Mohamedin, 2000) effects. The variable results
81 could be due to the fact that some bacteria are being stimulated and others being
82 repressed by AMF (Wamberg et al., 2003). Söderberg et al. (2002) suggested that the
83 effect of AMF differed between plant species; the strength of the effect on the bacterial
84 community in the rizosphere depended more on the plant species than on AMF
85 colonisation. If *P. indica* is going to be applied to crops, a clear picture of how it affects
86 other soil microorganisms would be needed, as the soil microflora plays a major role in
87 the availability of nutrients to plants and has a strong influence on plant health and
88 productivity.
89 Weed competition can threaten crop quality and quantity and ultimately the farmer's
90 profitability (Bockus et al., 2010); it is usually managed by herbicide application. The

key herbicide-resistant weed species of arable crops in the UK are: black-grass (*Alopecurus myosuroides*), wild-oats (*Avena fatua*), cleavers (*Gallium aparine*), Italian rye-grass (*Lolium multiflorum*), common poppy *(Papaver rhoeas*), common chickweed (*Stellaria media*), and scentless mayweed (*Tripleurospermum inodorum*) (Bond et al., 2007, Moss et al., 2011, Hull et al., 2014). These are also important world-wide and in other crops (Yu et al., 2013). Herbicide resistance in the UK is an important and increasing problem, as in other parts of the world including western, central and northern Europe (Mennan & Isik, 2004, Moss et al., 2007, Bertholdsson, 2012). *P. indica* has a wide range of hosts which might include weeds as well. If *P. indica* was as beneficial to weeds as to wheat, it could make weed control more difficult, or increase the damage done by weeds; alternatively, it might increase the competitiveness of wheat against some species or in some settings, which would be useful in managing herbicide resistant weeds. Also, the spread of *P. indica* might have side-effects outside arable fields.

In this study the following hypotheses were tested: *P. indica* would survive the UK weather and soil conditions; *P. indica* would not affect the composition of the bulk soil or root-zone microflora; and *P. indica* would be as beneficial to weeds as to wheat.

2. Materials and methods

2.1. *P. indica* survival and viability experiment

The utility of mRNA and DNA measurements as indicators of viability of *P. indica* was determined by performing RT-PCR and PCR on heat and cold treated pure cultures of *P. indica*. For this purpose, *P. indica* was obtained from Dr. Patrick Schafer, Warwick University, UK (originally from German Collection of Microorganisms and Cell

114 Culture, strain number DSM 11827) and mycelia was grown on complex
115 modified *Aspergillus* liquid medium (CM medium) (Pham *et al.*, 2004) and incubated
116 on an orbital shaker at 140 rpm at room temperature (21 ± 1 ^{o}C) for two weeks. Samples
117 were then kept at 80 ^{o}C in a hot water bath for 6 hours, then stored at -80 ^{o}C for 6 hours,
118 one and four weeks. After storage, separate samples of mycelia were transferred to
119 potato dextrose agar to check whether they would grow and used for RNA and DNA
120 extraction followed by RT-PCR and PCR respectively. This experiment was repeated to
121 confirm the results.
122 *P. indica* survival in the soil under UK weather conditions was tested in different soil
123 types based on either the soil series or textural classification and each soil was under a
124 different crop management. The soils were collected from the Reading University Farm
125 at Sonning (grid ref: SU76187547). These were (1) a Clay Loam (CL) of the Neville
126 series, from an area under winter barley which had previously been under winter wheat;
127 (2) a Sandy Clay Loam (SCL) of the Sonning series from an area under ryegrass at the
128 time and for the previous two years; (3) a Loamy Sand (LSO) of the Rowland series,
129 under organic management, from an area under faba bean cultivation; (4) a Loamy Sand
130 (LS) of the Rowland series, under non-organic management, from an area under
131 ryegrass cultivation. The experiment was carried out between December 2013 and
132 March 2015 at the University of Reading, under outdoor weather conditions. Six pots (3
133 L, top diameter: 18 cm, bottom diameter: 14 cm, depth: 15 cm) were filled with each
134 soil. Five out of six pots received 4 g of liquid culture of *P. indica* inoculum containing
135 an unquantified mixture of chlamydospores and mycelium and mixed thoroughly with
136 the soil. The control pot received 4 g of sterilised liquid culture of *P. indica* inoculum.

137 The pots were placed in holes with the tops level with the surrounding soil level to
138 make temperature fluctuations realistic. Around 50 g of each soil type was collected,
139 with a small core (diameter: 12 mm, depth: 8 cm) from the middle of pots, at three and
140 half months (mid-March 2014), 8 months (end of July 2014) and 15 months (end of
141 March 2015) after inoculation with *P. indica*. When collecting the samples, they were
142 kept in a cool box on ice and transferred immediately to -20 ^{o}C before DNA and RNA
143 were extracted and PCR or RT-PCR performed. Maximum and minimum temperatures
144 of soil in the pots were recorded every 2 days by a digital thermometer placed in the
145 centre of one of the pots.

146 **2.2. Soil community composition**

147 To examine whether *P. indica* affects other soil microorganisms, wheat was grown in 3
148 L pots containing one of two soil types, SCL or LSO, as above. Winter wheat seeds, cv.
149 Battalion, were surface disinfected by rinsing for 2 mins in 20 mL L^{-1} sodium
150 hypochlorite (Fisher Scientific UK Ltd, UK), followed by three rinses in sterilized
151 distilled water, and germinated on damp filter paper in a Petri dish at room temperature
152 (21 ± 1 °C) under natural indoor light for 48 hours. No micro-organisms grew from a
153 sample of seeds so treated and placed on PDA plates for one week. Pre-germinated
154 seeds were planted into 3 L pots (one seed per pot). This experiment had a 2×2×4
155 factorial combinations of ±*P. indica* × two soil types × four harvesting points, with two
156 replications completely randomised. The pots were incubated at temperatures ranging
157 between 15 and 25 °C; humidity and light were not controlled. Inoculation with 4 g
158 liquid culture of *P. indica* mixed with soil was done at the time of sowing. Root and soil
159 samples were collected at 2, 4, 6 and 8 weeks after inoculation (wai) for DNA

extraction, PCR and DGGE analysis, as below. Samples were transferred and stored as described above.

2.2.1. DNA and RNA isolation

Total genomic DNA from *P. indica* and root samples was isolated using a DNeasy plant mini kit (QIAGEN, UK), and from soil samples by using a PowerLyzer™ PowerSoil® DNA Isolation kit (CAMBIO Ltd, UK) following the manufacturer's instructions. Total RNA from *P. indica* was isolated using a RNeasy Plant Mini Kit (QIAGEN, UK), and from soil samples by using a RNA PowerSoil® Total RNA Isolation kit (CAMBIO Ltd, UK). Samples were stored at -20 °C until required. Bulk DNA concentration was measured using a NanoDrop-lite spectrophotometer (Thermo Scientific, Life Technologies Ltd, UK). The extent of shearing of DNA and RNA was determined by electrophoresis of an aliquot of DNA in a 1 % agarose gel in 1x TAE buffer.

2.2.2. Primer development and PCR condition for RT-PCR study

The gene-specific primer for the RT-PCR study was designed using the PRIMER BLAST tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast) to amplify fragments of the *P. indica* mRNA for EF-1-alpha (*TEF* gene, forward: 5-CCACCATCACTGAAGTCCCTC-3 and reverse: 5-TCAATGCCACCGCACTTGTA-3, 148 bp, accession number AJ249912.1, http://www.ncbi.nlm.nih.gov). The primers were supplied by Invitrogen (Thermo Scientific, Life Technologies Ltd, UK). To assess specificity of the primers for the targeted gene, RT-PCR was done using RNA isolated from a pure culture of *P. indica*. The PCR products of the selected primer were sent to Source Bioscience (http://www.sourcebioscience.com/) for sequencing to verify their specificity.

183 EF (EF-1-alpha (*TEF* gene)) primer amplified cDNA of 148 bp and gDNA of 227 bp.
184 The PCR amplicon sequence corresponded to genomic sequence from 1547 to 1756 bp
185 of the *P. indica TEF* gene, GenBank: accession number AJ249911.2, as expected.
186 PCR was performed in 0.2 mL PCR tubes (Fisher Scientific UK Ltd, UK) with 20 µL
187 final reaction volume containing 2x Biomix PCR master mix, 0.25 µM forward and
188 reverse primer, and template genomic DNA. Amplification was performed in a thermal
189 cycler (Applied Biosystems® GeneAmp® PCR System 9700, Thermo Scientific, Life
190 Technologies Ltd, UK) programmed as: 94 °C for 5 min followed by 35 cycles of 94 °C
191 for 30 s, 56 °C for 45s and 72 °C for 30 s, followed by incubation at 72 °C for 7 min.
192 Amplification was confirmed by electrophoresis of an aliquot of the PCR products in 2
193 % agarose gel in 1x TAE buffer.

194 **2.2.3. Reverse Transcription-PCR (RT-PCR)**

195 RT-PCR for *P. indica* was performed by using Invitrogen SuperScript® III First-Strand
196 Synthesis SuperMix (Life Technologies Ltd, UK) in a 20 µL final reaction volume
197 using 10 µL 2× RT Reaction Mix, 2 µL RT Enzyme Mix, RNase-free water and 4 µL *P.*
198 *indica* RNA. Reverse transcription was done in a thermal cycler. Samples were first
199 incubated at 50 ºC for 30 minutes, then held at 85 ºC for 5 minutes and then chilled on
200 ice for 5 min. Thereafter, 1 µL *E. coli* RNase H was added to the tube which was then
201 incubated at 37 ºC for 20 minutes. PCR was then performed using the complementary
202 DNA (cDNA) obtained from the reverse transcription.
203 RT-PCR for soil samples was performed by using a One-Step RT-PCR Kit (QIAGEN,
204 UK), in a 25 µL final reaction volume using 5 µL 5x QIAGEN OneStep RT-PCR
205 Buffer, 1 µL dNTP Mix, 1 µL of Enzyme Mix, 0.6 µM of each primer, RNase-free

206 water and 4 µL *P. indica* and samples RNA. Thermal cycler was set up at 30 min 50 °C,
207 15 min 95 °C, 35 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 30 s, followed by
208 incubation at 72 °C for 7 min.

209 **2.2.4. Primer and PCR condition for DGGE study**

210 Bacterial 16S rRNA genes, from the extracted DNA, were amplified using the primer
211 341F-
212 CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG
213 GCAGCAG and 534R-ATTACCGCGGCTGCTGG (Muyzer et al., 1993). Fungal 18S
214 rRNA genes were amplified using the primer NS1F-GTAGTCATATGCTTGTCTC and
215 GCFung-R-
216 CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTAC
217 CCGTTG (Hoshino & Morimoto, 2008).
218 The PCR was performed in a 20 µL final reaction volume using 2× Biomix PCR master
219 mix, 50 pmol μL^{-1} (for bacterial study) and 0.3 pmol μL^{-1} (for fungal study) of forward
220 and reverse primer, and sample DNA. Touchdown PCR for the bacterial study was
221 performed in a thermal cycler set up at 94 °C for 10 min, denaturation at 94 °C for 1
222 min, an annealing temperature which was set at 65 °C initially, then decreased by 1 °C
223 after each 2 cycles until it reached 55 °C. Primer extension was performed at 72 °C for
224 2 min. The above reaction was performed for 20 cycles, followed by 15 cycles of 94 °C
225 for 1 min, 55 °C for 1 min and 72 °C for 2 min. A final extension step was performed
226 for 10 min at 72 °C (Sasaki et al., 2009).

For the fungal primers, amplification was set at 94°C for 2 min, 30 cycles of 94°C for 15 s, 50 °C for 30 s and 68 °C for 30 s with a final extension of 72 °C for 5 minutes (Hoshino & Morimoto, 2008).

2.2.5. Denaturing gradient gel electrophoresis of fungi and bacteria

Denaturing gradient gel electrophoresis was performed according to the method described by Muyzer et al. (1993) (for bacterial study) and Hoshino & Morimoto (2008) (for fungal study) using the Bio-Rad DCode™ Universal Mutation Detection System. PCR samples (20 µL+loading dye) were applied directly onto 8 % (wt/vol) polyacrylamide gels (40 % acrylamide 37.5:1) with denaturing gradients of 40-60 % (for bacteria) and denaturing gradients of 20-40 % (for fungi), where 60 % denaturant compromised 24 mL 100 mL^{-1} Formamide and 25.2 g 100 mL^{-1} Urea (Sigma Aldrich Company Ltd, UK). Electrophoresis was performed at a constant voltage of 75 V and a temperature of 60 °C for 17 hours for bacteria and voltage of 50 V and a temperature of 60 °C for 20 hours for fungi. After electrophoresis, the gels were fixed (0.5 % glacial acetic acid and 10 % ethanol) and silver-stained (1 g L^{-1} silver nitrate), scanned, and the images analysed.

2.2.5.1. Statistical analysis of DGGE banding patterns

The DNA bands that migrated within each gel to the same relative distance were each ascribed the same label. In each lane, corresponding to a sample, the presence of a band with that label was scored 1 and absence scored 0. The band corresponding to *P. indica* band (which had the same position in all *P. indica*-inoculated samples) was not included in the scoring. These data were then analysed by two methods:

(i) Canonical variates analysis (CVA, GenStat 17th ed, VSN) was used to evaluate differences in community structure and allow the comparison of community profiles between groups of samples. CVA differentiate between groups variation, using a trace statistic as a summary of differentiation. CVA will produce a visualization of the data that shows groups as clearly separated, whether the differences are genuine or the result of chance sampling effects. The natural measure of how separate the groups found are is the trace of the matrix ratio $W^{-1}B$, where B is the matrix of between-group sums of squares and products and W is the matrix of within-group sums of squares and products. This measure and a randomization test (10,000 replicates) were used. The significance of the observed separation between groups, to determine whether groups were more distinct than expected by chance, was assessed by randomisation tests of 10,000 replicates (Rajaguru & Shaw, 2010).

(ii) Shannon-Wiener diversity index (*H′*, GenStat 17th ed, VSN) was used to quantify the diversity of species (bands) present in a group of samples. This index was calculated by the following equation:

$$H' = - \sum_i (Ri / R) \times \log (Ri / R)$$

where *Ri* is the total number of occurrences of band *i* in a group of observations, and *R* is total number of bands of any type observed in the group. Confidence intervals for the index were obtained by randomly re-sampling band abundances from a multinomial with the observed probabilities of each band type, and re-calculating the index.

2.3. *P. indica* interaction with weeds

Black-grass (*Alopecurus myosuroides*, 16 seeds per pot), wild-oat (*Avena fatua*, 6 seeds per pot), cleavers (*Galium aparine*, 3 seeds per pot) with and without wheat (6 seeds per

pot) were planted in 5 L pots (top diameter: 22.5 cm, bottom diameter: 16.5 cm, depth: 17.5 cm) at a depth of 1 cm in one part non-sterilised vermiculite (Medium, Sinclair, UK) and one part sand, mixed with 4 g L^{-1} of slow release fertiliser (3-4 months, Osmocote® Pro), with and without 4 g pot^{-1} of liquid *P. indica* inoculum mixed into the soil. Four replicates, distributed in four randomised blocks, were used with the following factorial combinations of treatments: ± *P. indica*, ± wheat, and three weed species. Wheat alone with and without *P. indica* was included as a control.

The pots were placed outside under natural conditions in the first two weeks of November/2014 for vernalisation, and then incubated in the glasshouse at temperatures ranging between 5 °C and 18 °C; humidity and light were not controlled. All pots were harvested, when wheat flag leaf was fully emerged (Zadoks Growth Stage (GS) 39; Zadoks et al. (1974)), and roots teased apart, washed and separated from the above ground parts before drying and weighing. This experiment was repeated to confirm the results.

In a separate experiment, to confirm the colonisation of weed roots with *P. indica* microscopically, seeds of black-grass, wild-oat and cleavers were planted separately in 1 L pots (top diameter: 13 cm, bottom diameter: 10 cm, depth: 11 cm) in one part non-sterilised vermiculite (Medium; Sinclair) and one part sand, and inoculated with *P. indica* at sowing. The roots were harvested at one and four weeks after inoculation, stained according to the method described in Rabiey et al., 2015, and viewed under a microscope with 10x and 40x objectives.

Competitiveness of each weed species with wheat was quantified as log (wheat biomass/weed biomass).

295 **2.4. Statistical analysis of pot experiments**

296 ANOVA was used to analyse all data using Genstat 17th ed, (VSN, UK) with
297 appropriate blocking.

298 **3. Results**

299 **3.1. Weather conditions during 2013-15**

300 Winter 2013-14 was an "exceptionally" stormy season, with at least 12 major winter
301 storms affecting the UK. Mean temperatures and total rainfall were 2 °C and 211 mm
302 respectively, above the long-term average over Reading (Nov-Mar 2013-14 average 7.1
303 °C). Soil temperature was 1 °C above average. Soil froze on only five occasions (Fig.
304 1).

305 Following this, the mean air and soil temperature of spring and summer 2014 was near
306 the average (April-June 2014 average 13.5 °C); total rainfall was, 55 mm and 31 mm
307 respectively, above the long term average (Fig. 1).

308 The weather of autumn 2014 was warm (Sep-Nov 2014 average 12.7 °C), 1.6 °C above
309 the average with the number of air frosts well below average. Rainfall totals and soil
310 temperature were above average, 11 mm and 1.5 °C respectively. Winter 2014-15 was
311 sunny with mean air (Dec-Mar 2014/15 average 5.6 °C) and soil temperature near
312 average. Soil froze on 20 occasions. Rainfall totals were 13 mm below average (Fig. 1.
313 www.met.reading.ac.uk/weatherdata).

314 **3.2. *P. indica* viability under UK winter weather conditions**

315 The viability of *P. indica* mycelia was tested under laboratory conditions. Exposure of
316 mycelia to 80 ^{o}C for 6 hours, then to -80 ^{o}C for 6 hours, one or four weeks killed them:
317 plates showed no growth of fungus after one month. RT-PCR detected *P. indica* mRNA

after 6 hours exposure to 80 ^{o}C then 6 hours at -80 ^{o}C, but did not detect *P. indica* mRNA after exposure to 80 ^{o}C followed by one or four weeks storage at -80 ^{o}C. PCR detected DNA in all treatments (Table 1).

RNA and DNA of *P. indica* were successfully isolated from all four soil after winter 2013 (collected mid March 2014) (Table 2). DNA of *P. indica* was successfully isolated from all different soil types following a UK spring and summer (collected end of July 2014), but RNA could be detected in only six of the pots. After 15 months (collected mid March 2015), neither RNA, nor DNA of *P. indica* could be detected from any of the soils (Table 2). *P. indica* could not be detected in the controls that was not inoculated with *P. indica*, which shows the primers could only detect *P. indica* mRNA.

3.3. *P. indica* effect on other soil microorganisms

3.3.1. Canonical variate analysis

Canonical variate analysis was used to differentiation between groups variation, using a trace statistic as a summary of differentiation. Canonical variate analysis of band patterns (Fig. 2), including both bacteria and fungi separated the four different harvested time points (trace: 1.9, $P<0.0001$), mainly because the first sample was distinct (Fig. 3 a). Root samples were clearly distinguishable from soil samples (trace: 3.9, $P<0.0001$, Fig. 3 b), and soil types were clearly distinct (trace: 1.6, $P<0.0001$, Fig. 3 c). *P. indica*-inoculated and non-inoculated samples were distinct (trace: 0.6, $P=0.001$, Fig. 3 d), *P. indica*-inoculated were distinguishable from non-inoculated samples by CVA when restricted to either fungal (trace: 1.1, $P<0.03$, Fig. 3 e), or bacterial primers (trace: 1.2, $P<0.02$, Fig. 3 f) or soil samples (trace: 2.9, $P<0.0001$, Fig. 3 g) but not root samples (trace: 0.6, $P=0.6$, Fig. 3 h).

To check the interaction between the effects of *P. indica* and soil-root zones combined factors were created. CVA of groups of samples classified by both *P. indica* inoculation and root-soil zone, including both bacterial and fungal bands, separated *P. indica*-inoculated from non-inoculated samples (trace: 5.5, P<0.0001, Fig. 3 i).

3.3.2. Shannon-Wiener diversity index

Samples harvested at different time points did not differ in diversity. Rowland series soils (LSO) had more fungal and bacterial band diversity than Sonning series (SCL). Both types of soil had more fungal and bacterial band diversity in the presence of *P. indica* (Fig. 4) and samples inoculated with *P. indica* had more bands of all types than non-inoculated samples. Root samples had more fungal species diversity when *P. indica* was present, but slightly fewer bacterial species diversity (Fig. 5).

3.4. *P. indica* interaction with weeds

Two *Avena fatua* root samples out of 10 were colonised by *P. indica* at two wai and three out of ten at four wai. Two *Alopecurus myosuroides* root samples out of ten were colonised at four wai. No *Galium aparine* root samples (of ten samples) were colonised.

P. indica application at sowing time increased wheat shoot and root biomass by 33 % (main effect P=0.05) and 100 % (main effect P=0.02) respectively, as expected (Table 3; supporting information Table 1).

P. indica increased root biomass, averaged over *Avena fatua*, *Alopecurus myosuroides* and *G. aparine*, by 35 % (P=0.04). As expected, competition reduced root biomass (by about 26 %, P=0.05) and there were differences between species (P=0.03; *A. fatua* was about 50 % heavier than the other two species). All interactions were non-significant (P>0.4). In particular, the effect of inoculation did not differ between weed species, and

364 the effect of inoculation did not differ in the competition pots (Table 3; supporting
365 information Table 1).
366 Shoot biomass of all plants was decreased about 24 % (P=0.005) by competition and
367 differed greatly between the species (P=0.001) because *G. aparine* had a lower biomass.
368 The effect of *P. indica* was slight (a 12 % increase; P=0.2) and no interactions were
369 significant (P>0.2 in all cases) (Table 3; supporting information Table 1).
370 The average competitiveness with wheat of *Avena fatua, Alopecurus myosuroides* and
371 *G. aparine,* measured by the ratio of shoot weights, was reduced by 40 %
372 (backtransformed from the $\log_{10}$ scale; P=0.02) when *P. indica* was present in the soil
373 (Table 4; supporting information Table 2). Although the competitiveness differed
374 significantly between species, no interaction terms were significant (P>0.5). There were
375 no significant differences in competitiveness measured by the ratio of root weights
376 (P>0.13 for all main and interaction terms). This experiment was repeated (supporting
377 information Table 3, 4) and the results were confirmed.
378

379 **4. Discussion**

380 This study indicates (1) that *P. indica* can survive the UK weather and soil conditions
381 for period of months, even when there is no host present (Table 2); (2) that the
382 inoculation of *P. indica* to soil has a substantial effect on soil and wheat root-associated
383 microflora at least during the first eight weeks (Fig. 3,4,5); (3) that *P. indica* affects at
384 least two of three tested native arable weeds, and alters their competitive relations with
385 wheat, and with each other and at least some other species (Table 3,4; and supporting
386 information Table 3,4).

If it were used in field applications in England, *P. indica* would probably remain active in the soil for up to 15 months and there might be no need to re-apply it within season. However, in the event of adverse side-effects, it would be hard or impossible to eradicate. The longevity of *P. indica* inoculum in the soil, coupled with its strong growth promotional effects on some species might alter the competitive relations between existing native species. It also might affect other methods of disease management as the altered soil microflora could influence crop physiology in undetermined ways. The longevity of inoculum in soil might be specifically due to the mild weather of 2013-15 compared with the climatic average. However, the UK is predicted to experience milder winter conditions over the next decades (UKCIP; www.ukcip.org.uk/).

Exposure of *P. indica* to heat (80 ^{o}C) then immediately to -80 ^{o}C, killed the mycelia (Table 1). mRNA can be used as an indicator of *P. indica* viability, as it could not be detected a few hours after mycelia of *P. indica* were killed, while DNA of *P. indica* could be detected even four weeks after mycelium was killed (Table 1). This agrees with other studies. Herdina et al. (2004) concluded that mycelium of *Gaeumannomyces graminis* var. *tritici* killed by heating to 55 ^{o}C for 1 hour and DNA could still be detected by PCR after eight days. Chimento et al. (2012) killed *Phytophthora ramorum* mycelia by rapid lyophilisation and could detect DNA three months later while mRNA was only detected up to one week after the treatment, despite its relatively mild nature.

The DGGE analysis showed detectable changes in the microbial community structure and increased diversity in the fungal and bacterial community of both root and soil samples inoculated with *P. indica,* which are reflected in increases in Shannon diversity

410 indices (Fig. 3,4,5). How this might affect soil function is unknown. There is lots of
411 debate about the importance of microbial community structure and diversity for soil
412 function, plant productivity, resilience and stability. Changes in the composition of the
413 soil microbial community can change ecosystem process rates, specifically
414 decomposition, and affect plant productivity (positively, negatively or not at all)
415 depending on the composition of the initial microbial community (McGuire & Treseder,
416 2010, Gera Hol et al., 2015). The two soils tested differed in their initial diversity, but
417 responded similarly to inoculation with *P. indica*. The increase in microbial diversity
418 might be due to *P. indica* causing changes in root exudate (composition and quantity)
419 patterns, or directly through fungal exudates, as reported for AMF (Barea, 2000,
420 Gryndler, 2000, Jeffries et al., 2003).
421 The primer sets 341/534 and NS1/GCFung for the bacterial and fungal community
422 study were used as Muyzer et al. (1993) and Hoshino and Morimoto (2008) suggested
423 these primer sets could most clearly discriminate bacterial and fungal communities in
424 the soil. To obtain more specific results from DGGE, PCR primers must amplify only
425 specific groups of fungi and bacteria (Jumpponen, 2007, Hoshino, 2012). The DGGE
426 gave an overview of *P. indica*-induced changes in bacterial and fungal community
427 structure but next generation sequencing approaches could be employed in the future for
428 in depth study of the effects of *P. indica* on community structure and composition
429 (Rincon-Florez et al., 2013).
430 *P. indica* has a very wide host range, and may be able to interact with and improve
431 growth of economically-damaging weeds as well as crops. The effect of *P. indica* on
432 *Alopecurus myosuroides*, *Avena fatua* and *Galium aparine*, three of the most important

433 weeds in UK wheat production was evaluated. As expected, the weeds reduced wheat's
434 root and shoot biomass significantly. *P. indica* did not colonise *G. aparine*, but did
435 colonise *A. fatua* and *A. myosuroides*, though less than wheat (Table 3,4; supporting
436 information Table 3,4). The average root biomass of the three species was nonetheless
437 increased by inoculation with *P. indica*, but less than that of wheat. The ratio of wheat
438 shoot biomass to weed shoot biomas was increased in pots inoculated with *P. indica* so
439 the effect on wheat had outweighed the effect on the weeds. This suggests that wheat
440 might be a favourable host for *P. indica* and that field application of *P. indica* might not
441 make weed control more difficult. However, since only three species were tested, on a
442 small scale, the main conclusion is that the fungus can alter competitive relations among
443 both host and non-host species. The survival time and wide host range suggests that the
444 fungus would escape into natural communities and might alter their composition or
445 functioning. Changes would not necessarily be detrimental but these results do imply a
446 need for extensive assessments on an ecosystem scale.
447 Previous studies (Rabiey & Shaw, 2015, Rabiey et al., 2015) show that *P. indica* could
448 be extremely useful in stabilising and increasing wheat yields and quality in the UK;
449 other studies in northern Europe suggest it might also benefit other crops (Achatz et al.,
450 2010, Fakhro et al., 2010, Sun et al., 2010). The present results suggest *P. indica* effect
451 on both weeds and soil function should be studied further. Considering a close relative
452 of *P. indica*, *Serendipita herbamans*, has recently been discovered in Germany (Riess et
453 al 2014), a search for native organisms with similar characteristics might be a better
454 direction to go in (Hodkinson & Murphy, 2015).

4.1. Conclusion

In the case of field application, *P. indica* would probably remain active in the soil within season for up to 15 months. *P. indica* increased root and soil fungal and bacterial diversity. Although usually desirable, this indicates substantial effects on soil composition or functioning during the first eight weeks after inoculation. The organism would be likely to alter competitive relations among both host and non-host species. The wider effects of *P. indica* and similar organisms need to be better understood before agricultural deployment.

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Figure Legends

Fig 1. Reading mean air temperature, mean 10 cm soil temperature, and total rainfall between winter 2013-14 and winter 2014-15, compared with 1981-2010 average (source: www.met.reading.ac.uk/weatherdata).

Fig 2. Denaturing gradient gel electrophoresis profiles of the wheat (a) root fungal and (b) bacterial community in Sonning series (SCL) or Rowland series (LSO) soil inoculated with (+) or without (-) *Piriformospora indica*, harvested at 2 weeks after inoculation (wai) (T1), 4 wai (T2), 6 wai (T3) and 8 wai (T4), (first lane: Hyper Ladder I-100 lanes (Bioline)).

Fig 3. Canonical variates analysis of bands from denaturing gradient gel electrophoresis using universal fungal and bacterial primers for wheat root samples grown in Sonning series (SCL) or Rowland series (LSO) soils, inoculated with/without *Piriformospora indica*, (Pi: *P. indica*). First or first and second canonical axes are shown for data classified by (a) the four time points of harvest; (b) Root and soil source; (c) soil types; (d) *P. indica*-inoculation status; (e-h) *P. indica*-inoculation status using but restricted to

fungal (e), or bacterial primers (f) or to soil samples (g) or root samples (h); (i) both *P. indica* inoculation and root or soil source. In (a) and (i) the circles indicate 95% confidence intervals for the centre of the group in the canonical variate space under normality assumptions.

Fig 4. Shannon-Weiner diversity index for Sonning (SCL) and Rowland series (LSO) soil samples inoculated or not with *Piriformospora indica*. Based on denaturing gel electrophoresis of DNA extracts amplified using universal fungal and bacterial primers. Each bar shows mean ± 95% bootstrap confidence interval.

Fig 5. Shannon-Weiner diversity index for wheat root and soil samples inoculated or not with *Piriformospora indica* (Pi), based on denaturing gel electrophoresis of DNA extracts amplified using universal fungal and bacterial primers. Both soil types (Sonning series (SCL) and Rowland series (LSO)) are combined. Each bar shows mean ± 95% bootstrap confidence interval.

Table Legends

Table 1. Recovery of *Piriformospora indica* DNA and RNA after the mycelia were killed by exposure to heat and cold or grown in covered petri dishes of potato dextrose agar (n=3 for each condition).

Table 2. Recovery of *Piriformospora indica* DNA and RNA from four soil types, left in pots under prevailing weather conditions without plant roots present from December 2013 with sample collections at mid-March 2014, end-July 2014 and mid-March 2015, n=5 (S.e.d.: Standard error of the difference of means).

Table 3. Dry weights (g) of root and shoot of *Alopecuris myosuroides*, *Avena fatua* and *Galium aparine* alone and in competition with wheat, with and without inoculation with *Piriformospora indica* (error d.f.: 33; S.e.d.: Standard error of the difference of means).

678

679 **Table 4.** Competitiveness of *Alopecuris myosuroides*, *Avena fatua*, *and Galium aparine*
680 with wheat, measured as $\log_{10}$ (weed dry weight/wheat dry weight), in the presence and
681 absence of inoculum of *Piriformospora indica* in the soil (d.f.: 15).

682

683

684 **Supporting Information**

685 **Table 1.** ANOVA P-value for Table 3. for dry weights (g) of root and shoot of weed
686 species (*Alopecuris myosuroides*, *Avena fatua* and *Galium aparine*) alone and in
687 competition with wheat, with and without inoculation with *Piriformospora indica.*

688

689

690 **Table 2.** ANOVA P-value for Table 4. for competitiveness of weed species (*Alopecuris*
691 *myosuroides*, *Avena fatua*, *and Galium aparine*) with wheat, in the presence and
692 absence of inoculum of *Piriformospora indica* in the soil.

693

694 **Table 3a.** Dry weights (g) of root and shoot of *Alopecuris myosuroides*, *Avena fatua*
695 and *Galium aparine* alone and in competition with wheat, with and without inoculation
696 with *Piriformospora indica* (error d.f.: 33; S.e.d.: Standard error of the difference of
697 means) (second repeat).

698

699 **Table 3b.** ANOVA P-value for Table 3a. for dry weights (g) of root and shoot of weed
700 species (*Alopecuris myosuroides*, *Avena fatua* and *Galium aparine*) alone and in
701 competition with wheat, with and without inoculation with *Piriformospora indica.*

702

703 **Table 4a.** Competitiveness of *Alopecuris myosuroides*, *Avena fatua*, *and Galium*
704 *aparine* with wheat, measured as $\log_{10}$ (weed dry weight/wheat dry weight), in the
705 presence and absence of inoculum of *Piriformospora indica* in the soil (d.f.:15; S.e.d.:
706 Standard error of the difference of means) (second repeat).

707

708 **Table 4b.** ANOVA P-value for Table 4a. for competitiveness of weed species
709 (*Alopecuris myosuroides*, *Avena fatua*, *and Galium aparine*) with wheat, in the presence
710 and absence of inoculum of *Piriformospora indica* in the soil.
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713