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

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1 **Potential ecological effects of *Piriformospora indica*, a possible biocontrol agent, in**

2 **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.

45 If applied to field crops in the UK, *P. indica* would be persistent for up to 15 months

46 and likely to alter competitive relations within vegetation. Increased soil microbial

47 diversity during the first eight weeks after inoculation, although usually desirable, could

48 alter soil composition or functioning.

49 **Keywords**: denaturing gradient gel electrophoresis, microbial diversity, reverses

50 transcription-PCR, root endophytic fungus, soil microorganisms, sustainable

51 agriculture.

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53 **1. Introduction**

54 *Piriformospora indica*, a root endophytic fungus (Sebacinales: Basidiomycota), was

55 first found in the Thar dessert of India (Verma et al., 1998), an arid region which

56 experiences extreme day-time heat and diurnal temperature fluctuations as well as

57 extended drought. *P. indica* promotes plant growth, increases root and above ground

58 biomass and final yield during its mutualistic relationship with a wide variety of plants

59 (Shrivastava & Varma, 2014). It increases resistance of several hosts from diverse

60 families to many biotic stresses under glasshouse and field conditions (Waller et al.,

61 2005, Deshmukh & Kogel, 2007, Ghahfarokhy et al., 2011, Harrach et al., 2013).

62 Tolerance to abiotic stresses is also increased in a variety of hosts (Zarea et al., 2012,

63 Alikhani et al., 2013, Ghabooli et al., 2013, Varma et al., 2013). Our previous studies

64 showed that *P. indica* protected wheat from Fusarium crown rot in pot experiments

65 (Rabiey et al., 2015). Our work also suggests that Fusarium head blight and mycotoxin

66 deoxynivalenol contamination are reduced in pot experiments under UK weather

67 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

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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

Culture, strain number DSM 11827) and mycelia was grown on complex modified *Aspergillus* liquid medium (CM medium) (Pham *et al.,* 2004) and incubated on an orbital shaker at 140 rpm at room temperature (21 ± 1 ^{o}C) for two weeks. Samples were then kept at 80 ^{o}C in a hot water bath for 6 hours, then stored at -80 ^{o}C for 6 hours, one and four weeks. After storage, separate samples of mycelia were transferred to potato dextrose agar to check whether they would grow and used for RNA and DNA extraction followed by RT-PCR and PCR respectively. This experiment was repeated to confirm the results.

P. indica survival in the soil under UK weather conditions was tested in different soil types based on either the soil series or textural classification and each soil was under a different crop management. The soils were collected from the Reading University Farm at Sonning (grid ref: SU76187547). These were (1) a Clay Loam (CL) of the Neville series, from an area under winter barley which had previously been under winter wheat; (2) a Sandy Clay Loam (SCL) of the Sonning series from an area under ryegrass at the time and for the previous two years; (3) a Loamy Sand (LSO) of the Rowland series, under organic management, from an area under faba bean cultivation; (4) a Loamy Sand (LS) of the Rowland series, under non-organic management, from an area under ryegrass cultivation. The experiment was carried out between December 2013 and March 2015 at the University of Reading, under outdoor weather conditions. Six pots (3 L, top diameter: 18 cm, bottom diameter: 14 cm, depth: 15 cm) were filled with each soil. Five out of six pots received 4 g of liquid culture of *P. indica* inoculum containing an unquantified mixture of chlamydospores and mycelium and mixed thoroughly with 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 $^{\circ}$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

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

161   described above.

162   **2.2.1. DNA and RNA isolation**

163   Total genomic DNA from *P. indica* and root samples was isolated using a DNeasy plant

164   mini kit (QIAGEN, UK), and from soil samples by using a PowerLyzer™ PowerSoil®

165   DNA Isolation kit (CAMBIO Ltd, UK) following the manufacturer's instructions. Total

166   RNA from *P. indica* was isolated using a RNeasy Plant Mini Kit (QIAGEN, UK), and

167   from soil samples by using a RNA PowerSoil® Total RNA Isolation kit (CAMBIO Ltd,

168   UK). Samples were stored at -20 °C until required. Bulk DNA concentration was

169   measured using a NanoDrop-lite spectrophotometer (Thermo Scientific, Life

170   Technologies Ltd, UK). The extent of shearing of DNA and RNA was determined by

171   electrophoresis of an aliquot of DNA in a 1 % agarose gel in 1x TAE buffer.

172   **2.2.2. Primer development and PCR condition for RT-PCR study**

173   The gene-specific primer for the RT-PCR study was designed using the PRIMER

174   BLAST tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast) to amplify

175   fragments of the *P. indica* mRNA for EF-1-alpha (*TEF* gene, forward: 5-

176   CCACCATCACTGAAGTCCCTC-3 and reverse: 5-TCAATGCCACCGCACTTGTA-

177   3, 148 bp, accession number AJ249912.1, http://www.ncbi.nlm.nih.gov). The primers

178   were supplied by Invitrogen (Thermo Scientific, Life Technologies Ltd, UK). To assess

179   specificity of the primers for the targeted gene, RT-PCR was done using RNA isolated

180   from a pure culture of *P. indica.* The PCR products of the selected primer were sent to

181   Source Bioscience (http://www.sourcebioscience.com/) for sequencing to verify their

182   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 CGCCCGCCGCGCCCGCGCCCGGCCCGCCGCCCCGCCCCATTCCCCGTTAC

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).

227 For the fungal primers, amplification was set at 94°C for 2 min, 30 cycles of 94°C for

228 15 s, 50 °C for 30 s and 68 °C for 30 s with a final extension of 72 °C for 5 minutes

229 (Hoshino & Morimoto, 2008).

230 **2.2.5. Denaturing gradient gel electrophoresis of fungi and bacteria**

231 Denaturing gradient gel electrophoresis was performed according to the method

232 described by Muyzer et al. (1993) (for bacterial study) and Hoshino & Morimoto (2008)

233 (for fungal study) using the Bio-Rad DCode™ Universal Mutation Detection System.

234 PCR samples (20 µL+loading dye) were applied directly onto 8 % (wt/vol)

235 polyacrylamide gels (40 % acrylamide 37.5:1) with denaturing gradients of 40-60 %

236 (for bacteria) and denaturing gradients of 20-40 % (for fungi), where 60 % denaturant

237 compromised 24 mL 100 mL^{-1} Formamide and 25.2 g 100 mL^{-1} Urea (Sigma Aldrich

238 Company Ltd, UK). Electrophoresis was performed at a constant voltage of 75 V and a

239 temperature of 60 °C for 17 hours for bacteria and voltage of 50 V and a temperature of

240 60 °C for 20 hours for fungi. After electrophoresis, the gels were fixed (0.5 % glacial

241 acetic acid and 10 % ethanol) and silver-stained (1 g L^{-1} silver nitrate), scanned, and the

242 images analysed.

243 *2.2.5.1. Statistical analysis of DGGE banding patterns*

244 The DNA bands that migrated within each gel to the same relative distance were each

245 ascribed the same label. In each lane, corresponding to a sample, the presence of a band

246 with that label was scored 1 and absence scored 0. The band corresponding to *P. indica*

247 band (which had the same position in all *P. indica*-inoculated samples) was not included

248 in the scoring. These data were then analysed by two methods:

249   (i) Canonical variates analysis (CVA, GenStat 17th ed, VSN) was used to evaluate

250   differences in community structure and allow the comparison of community profiles

251   between groups of samples. CVA differentiate between groups variation, using a trace

252   statistic as a summary of differentiation. CVA will produce a visualization of the data

253   that shows groups as clearly separated, whether the differences are genuine or the result

254   of chance sampling effects. The natural measure of how separate the groups found are is

255   the trace of the matrix ratio $W^{-1}B$, where B is the matrix of between-group sums of

256   squares and products and W is the matrix of within-group sums of squares and products.

257   This measure and a randomization test (10,000 replicates) were used. The significance

258   of the observed separation between groups, to determine whether groups were more

259   distinct than expected by chance, was assessed by randomisation tests of 10,000

260   replicates (Rajaguru & Shaw, 2010).

261   (ii) Shannon-Wiener diversity index (H', GenStat 17th ed, VSN) was used to quantify

262   the diversity of species (bands) present in a group of samples. This index was calculated

263   by the following equation:

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

265   where Ri is the total number of occurrences of band i in a group of observations, and R

266   is total number of bands of any type observed in the group. Confidence intervals for the

267   index were obtained by randomly re-sampling band abundances from a multinomial

268   with the observed probabilities of each band type, and re-calculating the index.

269   **2.3. *P. indica* interaction with weeds**

270   Black-grass (*Alopecurus myosuroides*, 16 seeds per pot), wild-oat (*Avena fatua*, 6 seeds

271   per pot), cleavers (*Galium aparine*, 3 seeds per pot) with and without wheat (6 seeds per

272   pot) were planted in 5 L pots (top diameter: 22.5 cm, bottom diameter: 16.5 cm, depth:

273   17.5 cm) at a depth of 1 cm in one part non-sterilised vermiculite (Medium, Sinclair,

274   UK) and one part sand, mixed with 4 g L^{-1} of slow release fertiliser (3-4 months,

275   Osmocote® Pro), with and without 4 g pot^{-1} of liquid *P. indica* inoculum mixed into the

276   soil. Four replicates, distributed in four randomised blocks, were used with the

277   following factorial combinations of treatments: ± *P. indica*, ± wheat, and three weed

278   species. Wheat alone with and without *P. indica* was included as a control.

279   The pots were placed outside under natural conditions in the first two weeks of

280   November/2014 for vernalisation, and then incubated in the glasshouse at temperatures

281   ranging between 5 °C and 18 °C; humidity and light were not controlled. All pots were

282   harvested, when wheat flag leaf was fully emerged (Zadoks Growth Stage (GS) 39;

283   Zadoks et al. (1974)), and roots teased apart, washed and separated from the above

284   ground parts before drying and weighing. This experiment was repeated to confirm the

285   results.

286   In a separate experiment, to confirm the colonisation of weed roots with *P. indica*

287   microscopically, seeds of black-grass, wild-oat and cleavers were planted separately in

288   1 L pots (top diameter: 13 cm, bottom diameter: 10 cm, depth: 11 cm) in one part non-

289   sterilised vermiculite (Medium; Sinclair) and one part sand, and inoculated with *P.*

290   *indica* at sowing. The roots were harvested at one and four weeks after inoculation,

291   stained according to the method described in Rabiey et al., 2015, and viewed under a

292   microscope with 10x and 40x objectives.

293   Competitiveness of each weed species with wheat was quantified as log (wheat

294   biomass/weed biomass).

2.4. Statistical analysis of pot experiments

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

3. Results

3.1. Weather conditions during 2013-15

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

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

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

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

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

14

318 after 6 hours exposure to 80 ^{o}C then 6 hours at -80 ^{o}C, but did not detect *P. indica*

319 mRNA after exposure to 80 ^{o}C followed by one or four weeks storage at -80 ^{o}C. PCR

320 detected DNA in all treatments (Table 1).

321 RNA and DNA of *P. indica* were successfully isolated from all four soil after winter

322 2013 (collected mid March 2014) (Table 2). DNA of *P. indica* was successfully isolated

323 from all different soil types following a UK spring and summer (collected end of July

324 2014), but RNA could be detected in only six of the pots. After 15 months (collected

325 mid March 2015), neither RNA, nor DNA of *P. indica* could be detected from any of

326 the soils (Table 2). *P. indica* could not be detected in the controls that was not

327 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

330 Canonical variate analysis was used to differentiation between groups variation, using a

331 trace statistic as a summary of differentiation. Canonical variate analysis of band

332 patterns (Fig. 2), including both bacteria and fungi separated the four different harvested

333 time points (trace: 1.9, P<0.0001), mainly because the first sample was distinct (Fig. 3

334 a). Root samples were clearly distinguishable from soil samples (trace: 3.9, P<0.0001,

335 Fig. 3 b), and soil types were clearly distinct (trace: 1.6, P<0.0001, Fig. 3 c). *P. indica*-

336 inoculated and non-inoculated samples were distinct (trace: 0.6, P=0.001, Fig. 3 d), *P.*

337 *indica*-inoculated were distinguishable from non-inoculated samples by CVA when

338 restricted to either fungal (trace: 1.1, P<0.03, Fig. 3 e), or bacterial primers (trace: 1.2,

339 P<0.02, Fig. 3 f) or soil samples (trace: 2.9, P<0.0001, Fig. 3 g) but not root samples

340 (trace: 0.6, P=0.6, Fig. 3 h).

341 To check the interaction between the effects of *P. indica* and soil-root zones combined

342 factors were created. CVA of groups of samples classified by both *P. indica* inoculation

343 and root-soil zone, including both bacterial and fungal bands, separated *P. indica*-

344 inoculated from non-inoculated samples (trace: 5.5, P<0.0001, Fig. 3 i).

3.3.2. Shannon-Wiener diversity index

346 Samples harvested at different time points did not differ in diversity. Rowland series

347 soils (LSO) had more fungal and bacterial band diversity than Sonning series (SCL).

348 Both types of soil had more fungal and bacterial band diversity in the presence of *P.*

349 *indica* (Fig. 4) and samples inoculated with *P. indica* had more bands of all types than

350 non-inoculated samples. Root samples had more fungal species diversity when *P. indica*

351 was present, but slightly fewer bacterial species diversity (Fig. 5).

3.4. *P. indica* interaction with weeds

353 Two *Avena fatua* root samples out of 10 were colonised by *P. indica* at two wai and

354 three out of ten at four wai. Two *Alopecurus myosuroides* root samples out of ten were

355 colonised at four wai. No *Galium aparine* root samples (of ten samples) were colonised.

356 *P. indica* application at sowing time increased wheat shoot and root biomass by 33 %

357 (main effect P=0.05) and 100 % (main effect P=0.02) respectively, as expected (Table

358 3; supporting information Table 1).

359 *P. indica* increased root biomass, averaged over *Avena fatua, Alopecurus myosuroides*

360 and *G. aparine*, by 35 % (P=0.04). As expected, competition reduced root biomass (by

361 about 26 %, P=0.05) and there were differences between species (P=0.03; *A. fatua* was

362 about 50 % heavier than the other two species). All interactions were non-significant

363 (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).

387   If it were used in field applications in England, *P. indica* would probably remain active

388   in the soil for up to 15 months and there might be no need to re-apply it within season.

389   However, in the event of adverse side-effects, it would be hard or impossible to

390   eradicate. The longevity of *P. indica* inoculum in the soil, coupled with its strong

391   growth promotional effects on some species might alter the competitive relations

392   between existing native species. It also might affect other methods of disease

393   management as the altered soil microflora could influence crop physiology in

394   undetermined ways. The longevity of inoculum in soil might be specifically due to the

395   mild weather of 2013-15 compared with the climatic average. However, the UK is

396   predicted to experience milder winter conditions over the next decades (UKCIP;

397   www.ukcip.org.uk/).

398   Exposure of *P. indica* to heat (80 $^{\circ}$C) then immediately to -80 $^{\circ}$C, killed the mycelia

399   (Table 1). mRNA can be used as an indicator of *P. indica* viability, as it could not be

400   detected a few hours after mycelia of *P. indica* were killed, while DNA of *P. indica*

401   could be detected even four weeks after mycelium was killed (Table 1). This agrees

402   with other studies. Herdina et al. (2004) concluded that mycelium of *Gaeumannomyces*

403   *graminis* var. *tritici* killed by heating to 55 $^{\circ}$C for 1 hour and DNA could still be

404   detected by PCR after eight days. Chimento et al. (2012) killed *Phytophthora ramorum*

405   mycelia by rapid lyophilisation and could detect DNA three months later while mRNA

406   was only detected up to one week after the treatment, despite its relatively mild nature.

407   The DGGE analysis showed detectable changes in the microbial community structure

408   and increased diversity in the fungal and bacterial community of both root and soil

409   samples inoculated with *P. indica,* which are reflected in increases in Shannon diversity

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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

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648 fungal (e), or bacterial primers (f) or to soil samples (g) or root samples (h); (i) both *P.*

649 *indica* inoculation and root or soil source. In (a) and (i) the circles indicate 95%

650 confidence intervals for the centre of the group in the canonical variate space under

651 normality assumptions.

652

653 **Fig 4.** Shannon-Weiner diversity index for Sonning (SCL) and Rowland series (LSO)

654 soil samples inoculated or not with *Piriformospora indica*. Based on denaturing gel

655 electrophoresis of DNA extracts amplified using universal fungal and bacterial primers.

656 Each bar shows mean ± 95% bootstrap confidence interval.

657

658 **Fig 5.** Shannon-Weiner diversity index for wheat root and soil samples inoculated or

659 not with *Piriformospora indica* (Pi), based on denaturing gel electrophoresis of DNA

660 extracts amplified using universal fungal and bacterial primers. Both soil types

661 (Sonning series (SCL) and Rowland series (LSO)) are combined. Each bar shows mean

662 ± 95% bootstrap confidence interval.

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665 **Table Legends**

666 **Table 1.** Recovery of *Piriformospora indica* DNA and RNA after the mycelia were

667 killed by exposure to heat and cold or grown in covered petri dishes of potato

668 dextrose agar (n=3 for each condition).

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670 **Table 2.** Recovery of *Piriformospora indica* DNA and RNA from four soil types, left in

671 pots under prevailing weather conditions without plant roots present from December

672 2013 with sample collections at mid-March 2014, end-July 2014 and mid-March 2015,

673 n=5 (S.e.d.: Standard error of the difference of means).

674

675 **Table 3.** Dry weights (g) of root and shoot of *Alopecuris myosuroides*, *Avena fatua* and

676 *Galium aparine* alone and in competition with wheat, with and without inoculation with

677 *Piriformospora indica* (error d.f.: 33; S.e.d.: Standard error of the difference of means).

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

Supporting Information

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

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

Table 3a. 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) (second repeat).

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

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

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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