

The endophytic fungus Piriformospora indica protects wheat from Fusarium crown rot disease in simulated UK autumn conditions

Article

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1 Short title: *Piriformospora indica* reduces Fusarium

2

3 **The endophytic fungus *Piriformospora indica* protects wheat from Fusarium crown rot**
4 **disease in simulated UK autumn conditions**

5

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14

15 Key words: *Piriformospora indica*, root endophytic fungus, mutualistic symbiosis, crown rot
16 disease, *Triticum aestivum*, real-time polymerase chain reaction

17

18

19 The root endophytic fungus *Piriformospora indica* (Sebacinacea) forms mutualistic
20 symbioses with a broad range of host plants, increasing their biomass production and
21 resistance to fungal pathogens. We evaluated the effect of *P. indica* on Fusarium crown rot
22 disease of wheat, under *in vitro* and glasshouse conditions. Interaction of *P. indica* and
23 Fusarium isolates under axenic culture conditions indicated no direct antagonistic activity of
24 *P. indica* against Fusarium isolates. Seedlings of wheat were inoculated with *P. indica* and
25 pathogenic *Fusarium culmorum* or *F. graminearum* and grown in sterilised soil-free medium
26 or in a non-sterilised mix of soil and sand. Fusarium alone reduced emergence and led to
27 visible browning and reduced root growth. Roots of seedlings in pots inoculated with both
28 Fusarium isolates and *P. indica* were free of visible symptoms; seed emergence and root
29 biomass were equivalent to the uninoculated. DNA was quantified by real-time polymerase
30 chain reaction (qPCR). The ratio of Fusarium DNA to wheat DNA rose rapidly in the plants
31 inoculated with Fusarium alone; isolates and species were not significantly different. *P.*
32 *indica* inoculation reduced the ratio of Fusarium to host DNA in the root systems. The
33 reduction increased with time. The ratio of *P. indica* to wheat DNA initially rose but then
34 declined in root systems without Fusarium. With Fusarium, the ratio rose throughout the
35 experiment. The absolute amount of Fusarium DNA in root systems increased in the absence
36 of *P. indica* but was static in plants co-inoculated with *P. indica*.

37

38 **Introduction**

39 Crown rot disease of wheat, primarily caused by *Fusarium culmorum* and *F. graminearum*
40 (Fernandez & Chen, 2005), damages wheat in most parts of the world. The disease reduces
41 wheat grain yield and quality and wheat straw production. Infection of seedlings and basal
42 stems leads to yield loss from damaged seedlings, pre-harvest lodging, and impaired grain
43 filling (Schilling *et al.*, 1996). In the UK these problems are largely avoided by certified seed,
44 seed treatment with fungicides and rotation (www.hgca.com), but *Fusarium spp.* remain a
45 serious concern in grain because they produce a range of mycotoxins that can lead to possible
46 human and animal health problems if they enter the food chain (Goswami & Kistler, 2004,
47 Xu *et al.*, 2008). These *Fusarium* pathogens are soil-borne and stubble-borne and can
48 survive in the soil and crop residues for several seasons (Leplat *et al.*, 2013). This long term
49 survival in plant debris or grass weeds, along with the lack of commercial cultivars with
50 resistance to *Fusarium* crown rot, makes controlling the disease difficult (Wildermuth *et al.*,
51 1997). The effects of agronomic practices on this disease are often unpredictable (Bailey *et*
52 *al.*, 2000) and depend on the causal species as well as the environmental conditions.

53 *Piriformospora indica* (sebacinales: basidiomycota) is a root endophytic fungus with a wide
54 host range that was first isolated from the rhizosphere of woody shrubs in the Thar region of
55 northwest India (Verma *et al.*, 1998). All members of the Sebacinales are involved in
56 mycorrhizal associations (Weiss *et al.*, 2004). *P. indica*, like arbuscular mycorrhizal fungi,
57 has plant growth promoting effects, but, in contrast to mycorrhizal fungi, can be cultured on
58 various synthetic media (Verma *et al.*, 1998). *P. indica* can mobilise and transport phosphorus
59 ,nitrogen and micronutrients from soil to the infected host plant via plant-fungal interfaces
60 (Malla *et al.*, 2004, Sherameti *et al.*, 2005, Varma *et al.*, 2013, Yadav *et al.*, 2010). It has also

61 been reported that *P. indica* can improve growth in a range of economically important
62 monocot and dicot hosts (Bagde et al., 2010, Varma et al., 1999, Varma et al., 2000).

63 *Piriformospora indica* has been shown to increase resistant to biotic stresses including a
64 wheat leaf disease (caused by *Blumeria graminis* f.sp. *tritici*), a wheat stem base disease
65 (caused by *Oculimacula* Spp.), wheat and barley root rot diseases (caused by *Fusarium*
66 *culmorum*, *Gaeumannomyces graminis* var. *tritici*) (Harrach et al., 2013, Serfling et al., 2007,
67 Deshmukh & Kogel, 2007), a maize root disease (caused by *F. verticillioides*) (Kumar et al.,
68 2009) and a lentil vascular wilt disease (caused by *Fusarium oxysporum* f. sp. *lentis*)
69 (Dolatabadi et al., 2012). In tomato infected with *Verticillium dahliae*, *P. indica* increased
70 leaf and fruit biomass and decreased disease severity. Also in tomato, *P. indica* reduced the
71 concentration of Pepino mosaic virus in shoots (Fakhro et al., 2010). *Piriformospora indica*
72 also increased plant tolerance to abiotic stresses including salt stress in barley (Alikhani et al.,
73 2013, Baltruschat et al., 2008), wheat (Zarea et al., 2012) and tomato (Cruz et al., 2010). The
74 fungus conferred drought tolerance in Chinese cabbage and enhanced seed production and
75 grain yield (Michal Johnson et al., 2013, Sun et al., 2010). Previous investigations, have been
76 concentrated in tropical and subtropical conditions. It remains to be shown whether *P. indica*
77 is suited to temperate climatic conditions.

78 In this investigation, we tested the hypothesis that *P. indica* would reduce damage to wheat
79 seedlings by restricting growth of *F. culmorum* and *F. graminearum* on roots in controlled
80 environmental chambers adjusted to UK autumn conditions. Pathogen progression in the
81 presence and absence of *P. indica* colonising simultaneously with or after *Fusarium* was
82 measured.

83

84

85 **Materials and methods**

86 **Fungal inoculation**

87 *Piriformospora indica* was obtained from Dr. Patrick Schafer, Warwick University, UK and
88 was grown on agar containing complex modified *Aspergillus* medium (CM medium) (Pham
89 *et al.*, 2004). To produce inoculum of *P. indica*, five plugs of 5 mm discs of 4 days old *P.*
90 *indica* culture were added to 500 mL flasks of CM medium and incubated on an orbital
91 shaker at 140 rpm at room temperature ($21\pm 1^\circ\text{C}$) for 14 days.

92 Isolates of *F. culmorum* (98/11 and UK.99) and *F. graminearum* (576 and 602.1), of UK
93 origin, were obtained from the School of Biological Science at the University of Reading and
94 Rothamsted Research Centre, UK and cultured on potato dextrose agar (PDA). Inoculum was
95 prepared by the methods described by Ghahfarokhy *et al.* (2011).

96 **Test for antagonistic activity**

97 Interactions between *P. indica* and Fusarium isolates were examined by the method described
98 by Ghahfarokhi and Goltapeh (2010). A 5 mm mycelial disc of *P. indica* was placed on one
99 side of a PDA plate and incubated at room temperature ($21 \pm 1^\circ\text{C}$). Single 5 mm discs of
100 Fusarium mycelium taken from the margins of 4 day old cultures were placed on the other
101 side of the plates, simultaneously or 3-4 days after. To see the interaction between *P. indica*
102 and Fusarium isolates microscopically, a clean glass microscope slide was placed in the
103 middle of Petri dishes and a thin layer of PDA poured onto it. Single 5 mm discs of 4 day old
104 cultures of *P. indica* and Fusarium isolates were placed at opposite ends of the slide
105 simultaneously or 3-4 days apart and incubated at room temperature ($21\pm 1^\circ\text{C}$). After 3-4
106 days, when leading hyphae of each culture met, the slides were observed microscopically
107 using a LeitzDialux 20 microscope attached to a Canon camera (EOS, 300D).

108

109 **Plant materials and glasshouse experiments**

110 Seeds of winter wheat cv. Battalion were surface disinfected by rinsing for 2 mins in a 20
111 mL/L sodium hypochlorite (Fisher scientific, UK), followed by three rinses in sterilized
112 distilled water, and germinated on damp filter paper in a Petri dish at room temperature under
113 natural indoor light for 48 hours.

114 To determine whether *P. indica* interacted with wheat to reduce Fusarium crown rot, pre-
115 germinated wheat seeds were planted into 4-inch pots (5 seeds per pot), filled with a 1:1
116 mixture of vermiculite (Medium, Sinclair, UK) and sand, steam sterilised at 121°C for 60 min
117 on two consecutive days. The pots were incubated at temperatures ranging between 15 °C
118 and 25 °C; humidity and light were not controlled. Inoculations were performed at the time of
119 sowing or 7 days later in a 3 × 3 factorial combination by mixing 4 g of *P. indica* and 6 g of
120 *F. culmorum* into the surface layer of the soil, without disturbing the seedling roots. Harvest
121 was performed at 7, 14, 21, and 30 days after inoculation (dai) and DNA concentrations of
122 the fungi in the root system determined. Each time point was independently replicated.

123 *P. indica* and *F. culmorum* interaction during the first week after inoculation was tested in the
124 glasshouse in conditions similar to the above experiment. Inoculations were done at the time
125 of sowing and roots were harvested daily for one week, DNA concentrations of the fungi and
126 wheat in the root system determined and a sample stained for microscopy. The experiment
127 had four treatments, $\pm P. indica$ and $\pm F. culmorum$, with two replications.

128 In a confirmatory experiment inoculations were done at the time of sowing in a 2×2 factorial
129 combination with 4 g of *P. indica* and 6 g of *F. culmorum*. Harvest was performed at 1, 2, 4,
130 8, 16 and 32 days after inoculation and DNA concentrations of both fungi and wheat in the
131 root system determined.

132 A further experiment was done to determine whether the interactions occurred under cooler,
133 conditions more similar to UK field environments. Germinated seeds were planted in a 1:1
134 mixture of non-sterilised soil (John Innes Composts, BHGS Ltd, UK) and sand and pots
135 were incubated in a controlled environment chamber. The experiment lasted 42 days. For the
136 first 14 d, the day-length was 12 h and temperature and humidity were 15°C, 65% ,
137 respectively, during day and 10°C, 65% during night; for the second 14 d conditions were
138 adjusted to 12°C, 70% during day and 9°C, 70% during night; and for the last 14 d the day
139 length was reduced to 10 h with conditions set at 10°C, 75% during day and 7°C, 75% during
140 night. Pots were arranged in two randomised blocks. The experiment had 10 treatments with
141 two replicates and five harvests. The treatments were based on 2 × 5 factorial combinations
142 of *±P indica* with one of the following: no amendment, *F. culmorum* 98/11, *F. culmorum*
143 UK.99, *F. graminearum* 576 or *F. graminearum* 602.1. One pot of each treatment in each
144 replicate was harvested at 7, 17, 28, 35 and 42 dai.

145 Each pot received 60 mL of fresh nutrient solution once a week. Nutrient solution was
146 prepared each week using tap water with the final concentrations given: NO₃⁻ 10 mM, PO₄²⁻
147 1 mM, K⁺ 6 mM, Ca²⁺ 1.5 mM, Mg²⁺ 1 mM, SO₄²⁻ 1.5 mM, Fe 10 µM, Mn²⁺ 1 µM, Zn²⁺ 0.01
148 µM, Cu²⁺ 0.1 µM, MoO₄²⁻ 0.07 µM and B₄O₇²⁻ 0.07 µM. Sodium metasilicate (100 mg/L)
149 included to control powdery mildew.

150 **Staining and microscopy**

151 Wheat root samples inoculated with *P. indica*, Fusarium isolates, and both fungi together
152 were stained using black ink (Pelikan Fountain Pen Ink, Niche Pens Ltd, UK) (Vierheilig et
153 al., 1998). Roots were cleared by soaking them in 10% (w/v) KOH for one hour at 80°C, then
154 rinsed 5 times with tap water. Cleared roots were covered with 2% HCl (v/v) for at least 30
155 min. Thereafter, HCl was poured off and roots were covered with 50 g/L black ink for 30

156 min at 80°C. Roots were de-stained by rinsing in tap water and viewed under a microscope
157 with 10x and 40x objectives.

158 **DNA isolation and primer development**

159 Total genomic DNA was isolated from 100 mg of harvested roots using a Qiagen DNeasy
160 plant mini kit (Qiagen, UK) following the manufacturer's instructions. Samples were eluted
161 into 100 µl of elution buffer and stored at -20°C until required. Single species genomic DNA
162 standards were obtained from roots of uninoculated plants and from mycelia of *P. indica* and
163 *Fusarium* isolates scraped off the agar. Bulk DNA concentration was measured using a
164 NanoDrop-lite spectrophotometer (Thermo Scientific, UK). The extent of shearing of DNA
165 was determined by electrophoresis of an aliquot of DNA in a 1% agarose gel.

166 Primers were designed using the Primer BLAST tool from NCBI
167 (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) to amplify fragments of *P. indica* *tef* gene
168 for EF-1-alpha (accession number: AJ249911.2; Pi-forward: TCCGTCGCGCACCAT and
169 Pi-reverse: AAATCGCCCTCTTCCACAA, 84 bp), *Fusarium* elongation factor 1 alpha
170 (EF1a) (accession number: JX534485; for *F. culmorum*, F1-forward:
171 GCCCTCTTCCCACAAACCATTC and F1-reverse: CTCGGCGGCTTCCTATTGACAG,
172 85 bp and for *F. graminearum*, F2-forward: AAGCCGAGCGTGAGCGTGGTA and F2-
173 reverse: CGGGAGCGTCTGATAGTCGTGTTA, 142 bp) and wheat translation elongation
174 factor 1 alpha-subunit (TEF1) (accession number: M90077; Wt- forward:
175 GTGCACCAAATCTTCCTGCC, Wt-reverse: GGTTATGGAATGTAGATGCTCGG, 71
176 bp). The accession numbers were obtained from <http://www.ncbi.nlm.nih.gov>. All primers
177 were supplied by Invitrogen (Life Technologies, UK). To assess specificity of the primers for
178 the targeted species and investigate any cross reactivity, genomic DNA isolated from pure
179 cultures of *P. indica* and *Fusarium* isolates and root tissue of wheat seedlings were subjected
180 to PCR using all primer sets.

181 **Quantification of *P. indica* and Fusarium in wheat roots**

182 The amount of Fusarium and *P. indica* in wheat root samples was quantified by real-time
183 PCR (qPCR). qPCR was performed in a 20 µl final reaction volume using 1X SYBR[®] Green
184 Jump Start[™] TaqReady Mix[™] (Sigma Aldrich, UK), 0.25 µM forward and reverse primers,
185 1.5 µl of sample DNA and 7.5µl molecular grade water, in a 72 tube rotor of a Rotor-Gene
186 6000 System (Corbett Life Sciences, UK). Thermal cycling was set up at one cycle of 95 °C
187 for 2 min; then 40 cycles of 95 °C for 15 s and 60 °C for 1 min followed by melt curve
188 analysis from 65 to 95 °C at the rate of 0.5 °C per second. PCR controls in every assay
189 included no template controls (NTC) and genomic DNA standards in duplicate for Fusarium
190 isolates, *P. indica* and wheat. Serial dilutions of pure genomic wheat, Fusarium and *P. indica*
191 DNA standards were initially tested in triplicate to determine a calibration curve and PCR
192 efficiencies. Data were obtained and analysed using Rotor-Gene 6000 series software Version
193 1.7. After quantification, estimates of *F. culmorum*, *F. graminearum* and *P. indica*
194 colonization of wheat tissues were obtained by dividing the concentration of fungal DNA by
195 the concentration of wheat DNA. Absolute mass of DNA of each fungus in a root system was
196 estimated by multiplying the concentration of fungal DNA by the ratio of root weight to the
197 sample weight that was taken for DNA extraction.

198 **Statistical analysis of experiments**

199 ANOVA was used to analyse all data using GenStat 16th ed, (VSN, UK) with appropriate
200 blocking. Where applicable, data were log and arcsine transformed to stabilize the residual
201 variance and aid interpretation.

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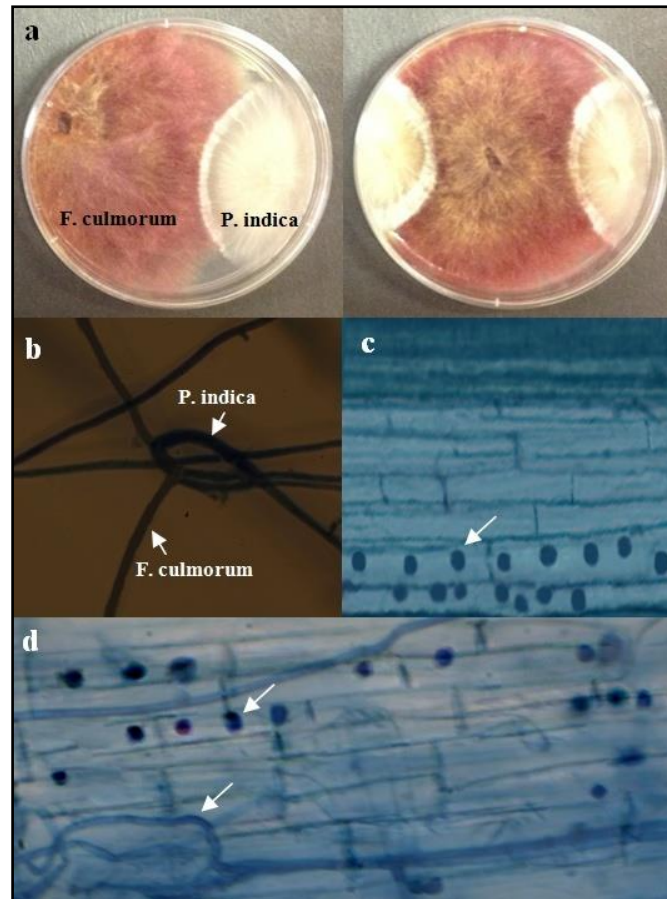
205 **Results**

206 **Interaction of *P. indica* and Fusarium**

207 Neither Fusarium isolates nor *P. indica* growth was visibly affected by the presence of the
208 other fungus under axenic culture conditions on PDA, and there was no zone of inhibition at
209 the contact point of two fungal colonies. There was occasional loose coiling of *P. indica*
210 around Fusarium hyphae but no clear evidence of mycoparasitism (Fig. 1a,b).

211 Fusarium- inoculated root samples of both species showed extensive growth of Fusarium ,
212 with the mycelium completely covering the roots by the final observation date, when brown
213 symptoms were clearly visible. In *P. indica*-Fusarium inoculated plants, Fusarium
214 colonisation was visually much less, but colonisation by *P. indica* was extensive.

215 *Piriformospora indica* colonisation started on root surfaces in the differentiation zone behind
216 the root meristem with inter- and intracellular penetration of epidermal cells, during the first
217 two to three days after inoculation, with hyphae filling up the cells. By four days after
218 inoculation coiled hyphae could occasionally be seen inside the cells.. Later, a little
219 colonisation could be observed in epidermal cells of the meristematic and elongation zones of
220 roots. *P. indica* chlamydospores were not observed until six days after inoculation (Fig. 1c,d).



221

222 Fig.1. Interaction of *Piriformospora indica* and *Fusarium* in agar plates and in wheat roots;
 223 (a). Agar plate co-cultivated with *F. culmorum* and *P. indica*; (b). Interaction of coiled hypha
 224 of *P. indica* around *F. culmorum* in agar plates at the encounter point; (c). *P.indica*
 225 chytrid spores inside wheat root cells, the fungus was not detected in endodermic and central
 226 part of the root, (d). *P.indica* hyphae and chytrid spores inside wheat root cells.

227

228 **Effect of *P. indica* on emergence rate, root weight and pathogen DNA concentration**

229 The emergence rates of seeds inoculated with *F. culmorum* and *F. graminearum* and *P.*
 230 *indica* were evaluated seven days after sowing. Seeds inoculated with *F. culmorum* and *F.*
 231 *graminearum* isolates emerged less often than the uninoculated ($p < 0.001$). Seeds inoculated
 232 with *P. indica* alone had the same emergence rate as the uninoculated. The emergence rate of
 233 seeds inoculated with both pathogen and *P. indica* was significantly higher than *Fusarium*-
 234 inoculated plants but slightly lower than the uninoculated ($p: 0.02$) (Fig. 2

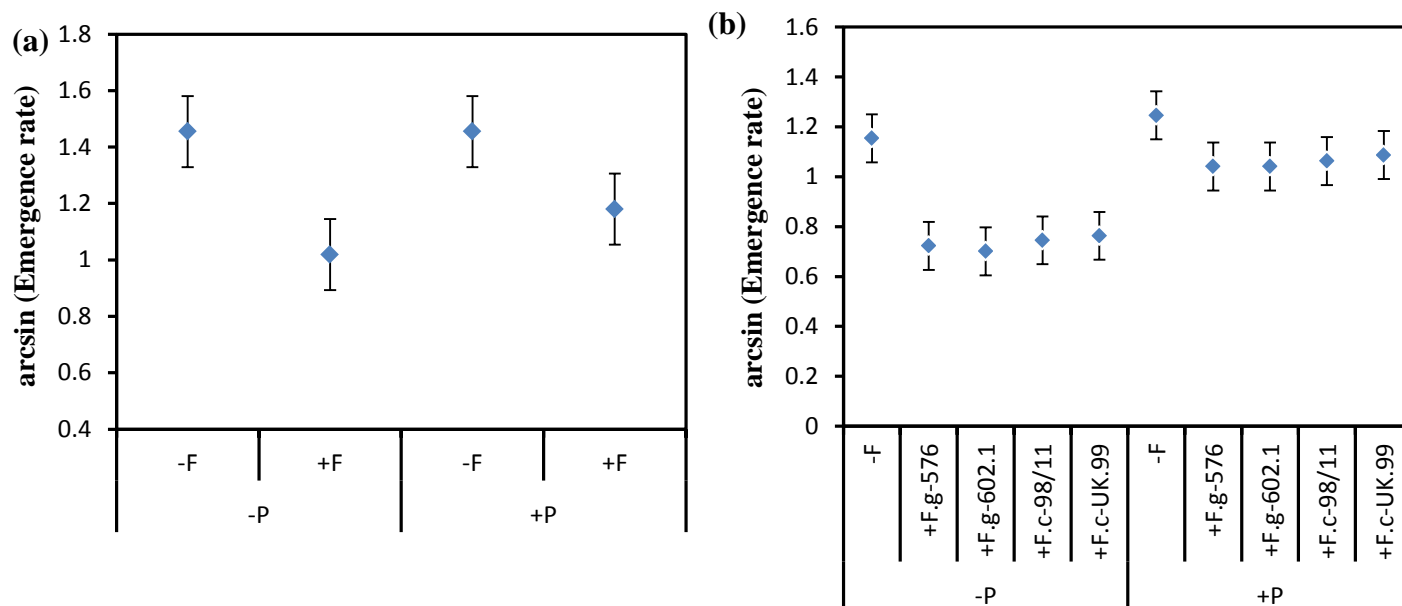


Fig.2. Emergence rates of seeds inoculated with *Fusarium* and *Piriformospora indica* evaluated 7 days after sowing; data were arcsine transformed. (a). Roots inoculated with *F. culmorum* and *P. indica* simultaneously at sowing time (s.e.d= 0.09, d.f= 57); (b). Roots inoculated with *F. culmorum* (98/11 and UK.99), *F. graminearum* (576 and 602.1) and *P.indica* simultaneously at sowing time (s.e.d= 0.07, d.f= 89). Each bar represents mean \pm 2 SEM (P:*P. indica* and F:*Fusarium*).

243 Root weights were evaluated at the final harvest. Roots of plants inoculated with *P. indica*
244 alone at sowing or 7 days later had weights equivalent to the control. Roots inoculated with
245 *F. culmorum* or *F. graminearum* had 40% lower root weight ($p < 0.001$). Roots of plants
246 inoculated with *P. indica* prior to Fusarium or simultaneously weighed roughly the same as
247 uninoculated plants and much more than the roots inoculated with Fusarium alone ($p < 0.001$).
248 *P. indica* inoculated 7 days after *F. culmorum* was less effective (Fig. 3).

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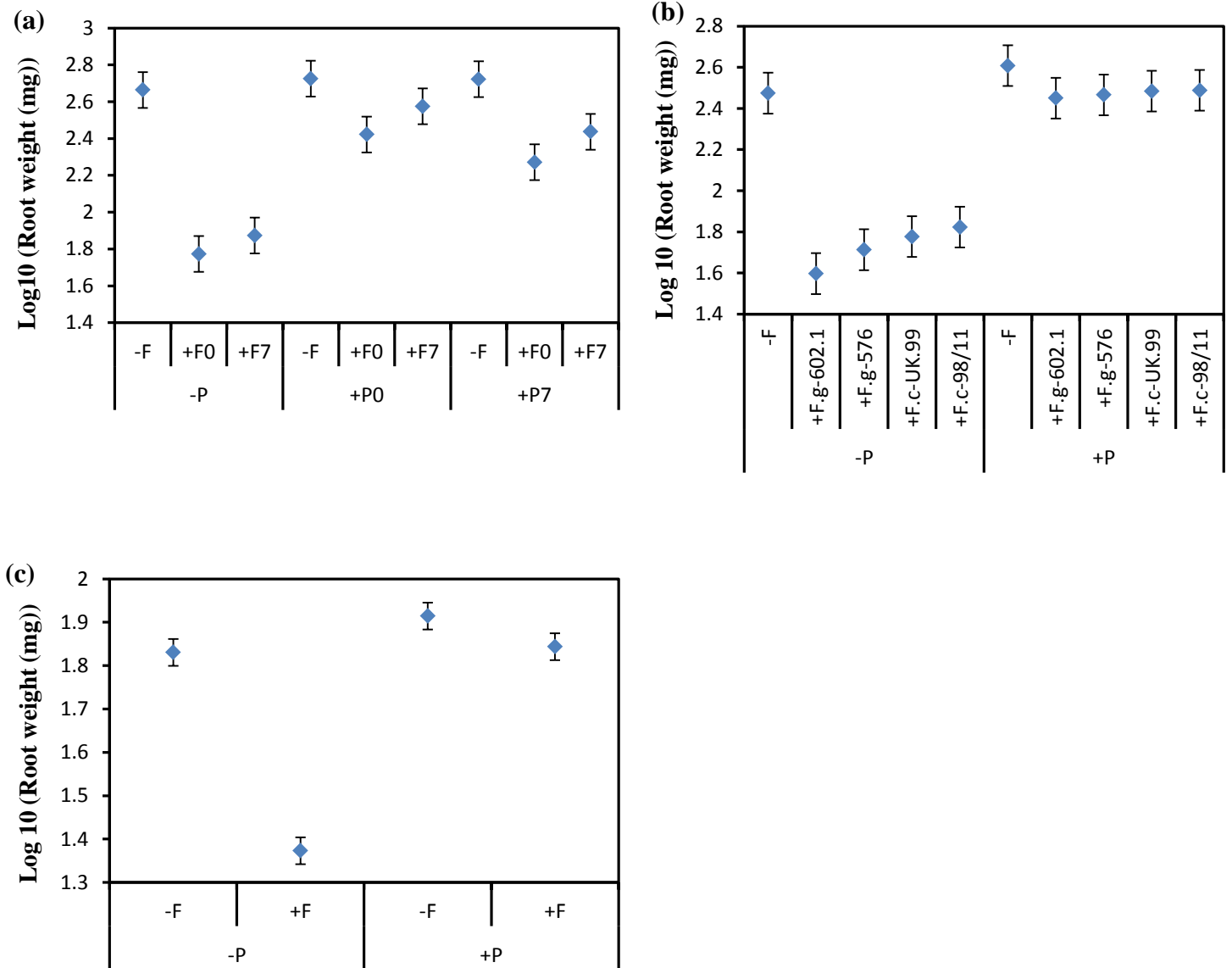


Fig.3. Root weight of samples (mg) inoculated with *Fusarium* and *Piriformospora indica* evaluated at last harvest; data were Log₁₀ transformed. (a). Roots inoculated with *F. culmorum* or *P. indica* simultaneously or 7 days after sowing, harvested at 30 dai (s.e.d= 0.07, d.f= 8); (b). Roots inoculated with *F. culmorum* (98/11 and UK.99), *F. graminearum* (576 and 602.1) and *P. indica* simultaneously at sowing time, harvested at 42 dai (s.e.d= 0.07, d.f= 9); (c). Roots inoculated with *F. culmorum* or *P. indica* simultaneously at sowing, harvested at 32 dai (s.e.d= 0.02, d.f= 3). Each bar represent mean \pm 2 SEM, (P: *P. indica*, F: *Fusarium*, P0: *P. indica* added to soil at sowing, P7: *P. indica* added to soil at 7 days after sowing, F0: *F. culmorum* added to soil at sowing and F7: *F. culmorum* added to soil at 7 days after sowing).

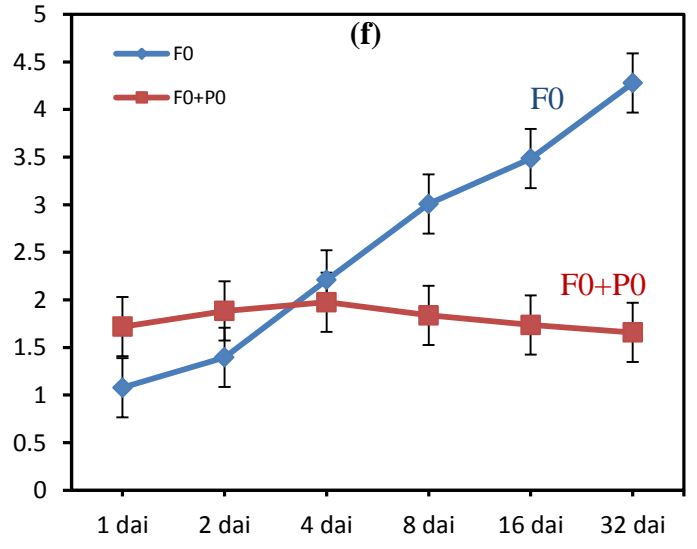
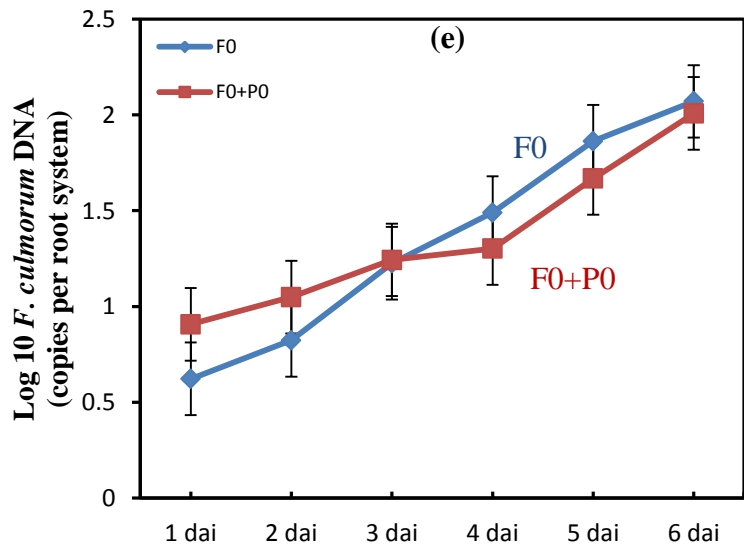
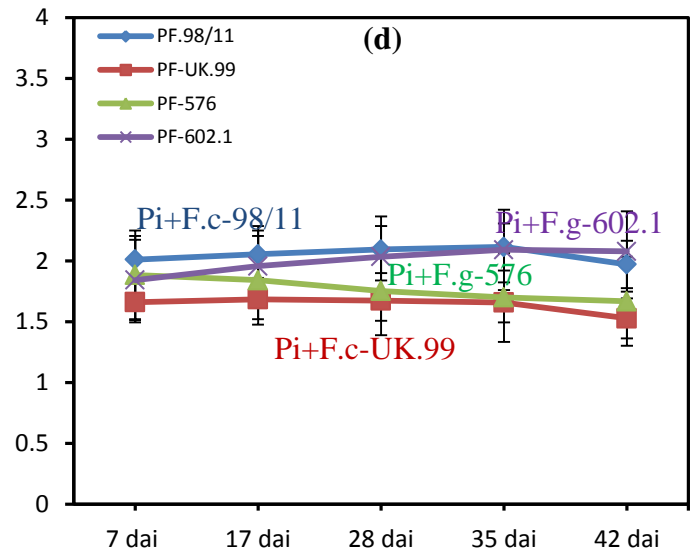
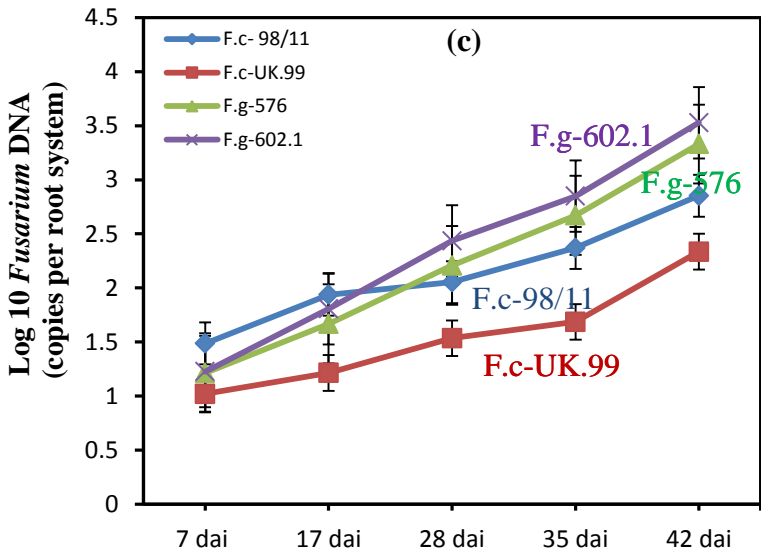
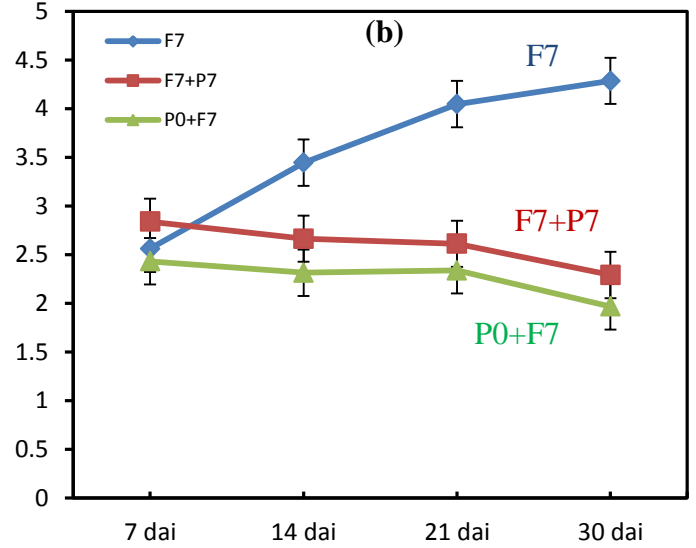
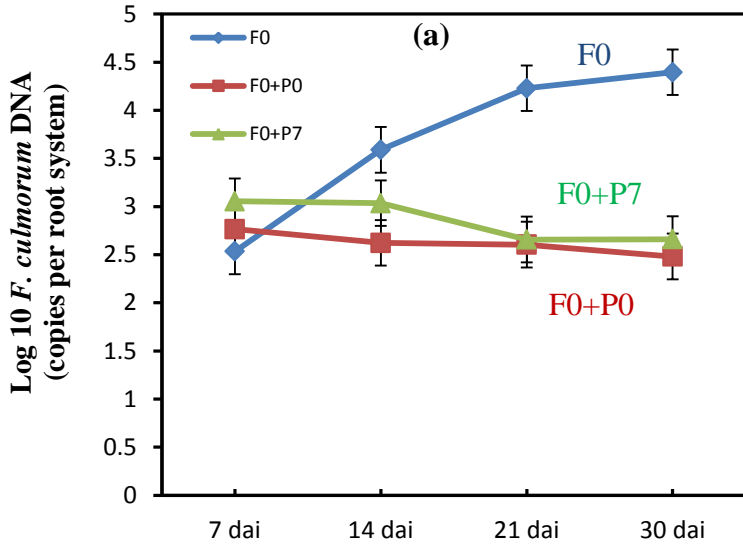
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265 The absolute quantity of Fusarium DNA in the root systems without *P. indica* grew at about
266 $10\% \text{ day}^{-1}$ throughout the experiment (Fig 4 a-c,f). The rate of growth of Fusarium inoculated
267 at 7 dai was similar to that inoculated at sowing time (Fig 4 a,b). The relative rate of increase
268 was constant for *F. graminearum* but declined in *F. culmorum* particularly in the first
269 experiment (Fig 4a-c). In co-inoculated samples, the absolute amount of pathogen was static
270 or slightly declining from 7-42 d (Fig 4a, b, d, f) after an initial period of increase (Fig. 4 e,f).

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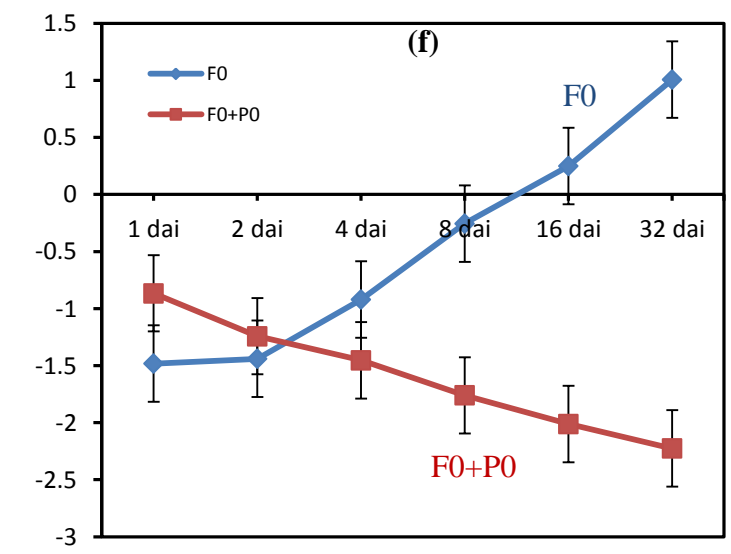
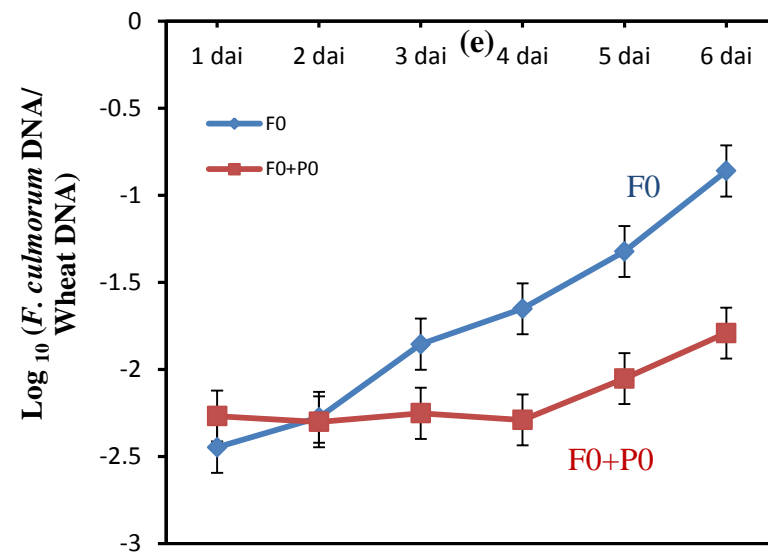
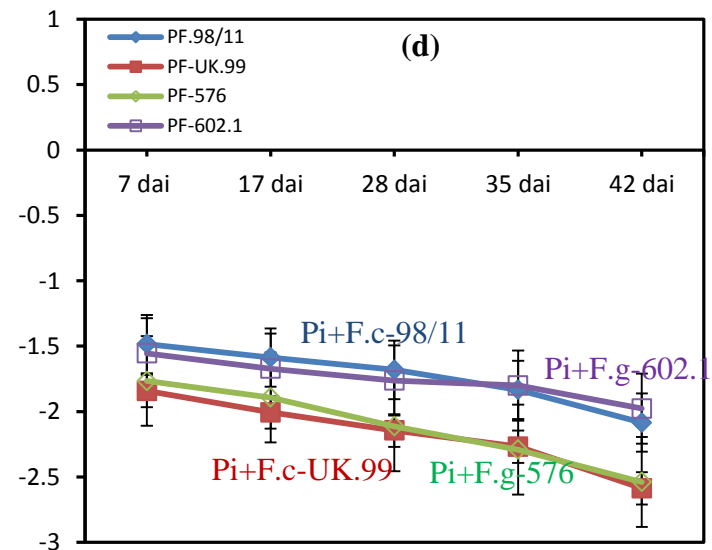
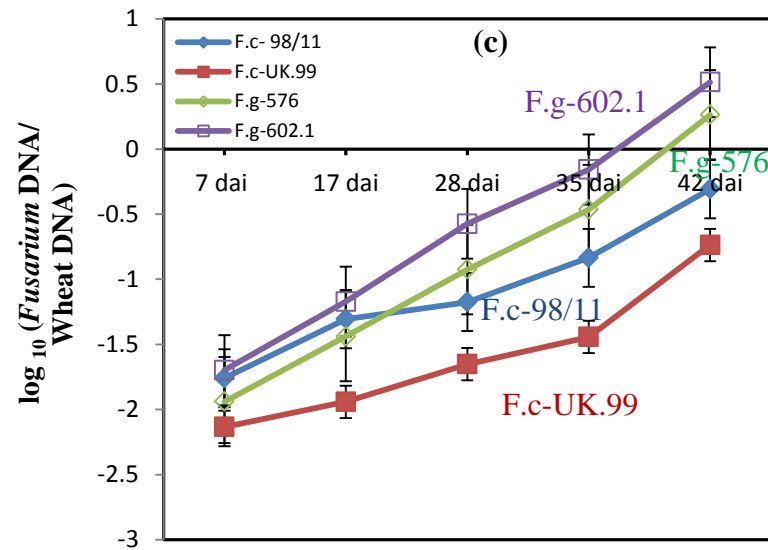
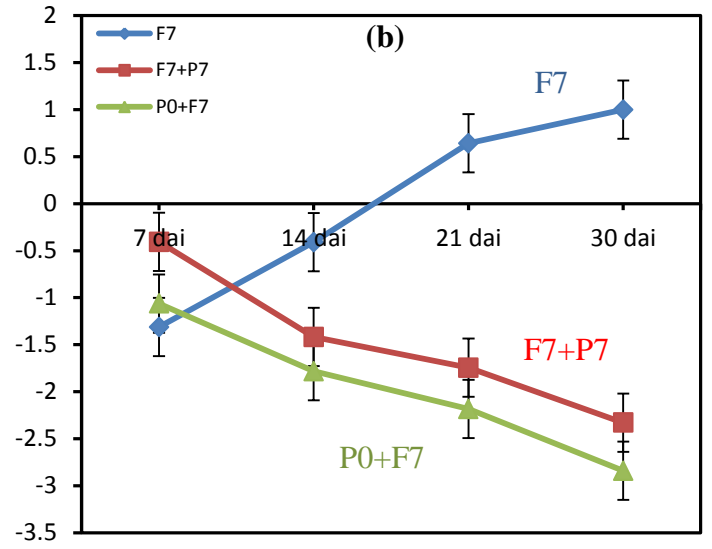
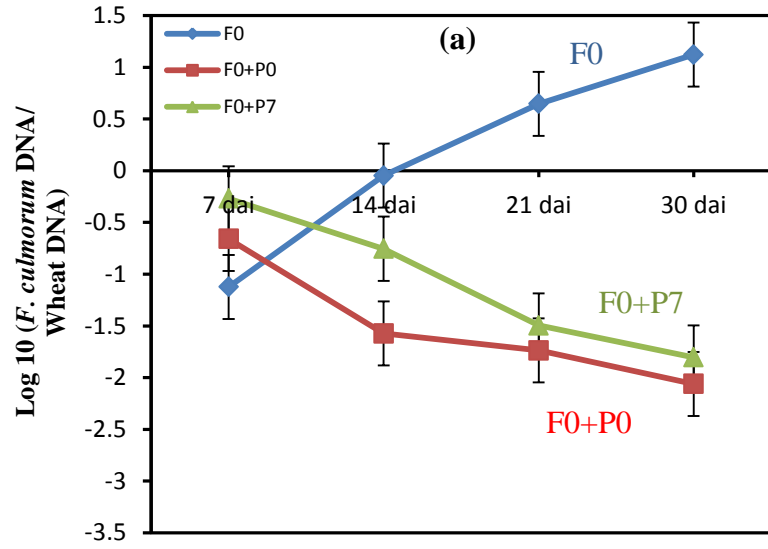


277 Fig.4. The absolute growth of Fusarium in inoculated wheat roots. The absolute amount
 278 obtained by adding \log_{10} fungal DNA to \log_{10} (root weight/sample weight in mg). (a). *F.*
 279 *culmorum* added to soil at sowing (F0); *Piriformospora indica* added simultaneously (P0) or
 280 7 days after sowing (P7); (b). *F. culmorum* added to soil 7 days after sowing (F7); *P. indica*
 281 added at sowing (P0) or simultaneously 7 days after sowing (P7); (c). *F. culmorum*98/11, *F.*
 282 *culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 added at sowing time; (d).
 283 *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 and
 284 *P. indica* added simultaneously at sowing time; (e). *F. culmorum* added to soil at sowing (F0)
 285 and *P. indica* added simultaneously (P0), during the first week of inoculation; (f). *F.*
 286 *culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0), during the
 287 first month of inoculation. Each point represent mean ± 2 SEM (for a and b; s.e.d= 0.2 and
 288 d.f= 23), (for F. c. 98/11 and PF.c. 98/11: s.e.d= 0.14 and d.f= 9; for F. c. UK.99 and PF.c.
 289 UK.99: s.e.d= 0.12 and d.f= 9; for F.g. 576 and PF.g. 576: s.e.d= 0.2 and d.f= 9; for F.g.
 290 602.1 and PF.g.602.1: s.e.d= 0.2 and d.f= 9), (for e, s.e.d= 0.13, d.f= 11) and (for f, s.e.d=
 291 0.2, d.f=11).

292

293 The ratio of *F. culmorum* or *F. graminearum* DNA to plant DNA, in the absence of *P.*
 294 *indica*, grew approximately exponentially at about 18% day⁻¹ (Fig 5 a,c,f), after the first 7
 295 days; growth of *F. culmorum* in the first week was faster (Fig 5e,f). Despite the difference in
 296 temperatures, both glasshouse and environmental chamber experiments had similar rates of
 297 fungal growth. Increase in *F. graminearum* DNA was faster than increase in *F. culmorum*
 298 DNA (Fig 5c). The rate of growth of Fusarium inoculated at 7 dai was similar to that
 299 inoculated at sowing time (Fig 5a, b). In the presence of *P. indica*, Fusarium growth was
 300 immediately reduced to the rate of growth of the root system (Fig 5 e,f) and then declined
 301 (Fig 5 b,d). *P. indica* inoculation 7 d after the pathogen reduced the rate of Fusarium growth
 302 relative to the root similarly to the reduction when inoculated simultaneously (Fig. 5b).
 303 Because of the initial period of growth alone, the *F.culmorum* to root ratio remained
 304 consistently higher when *P. indica* inoculation was delayed until 7 d after *F. culmorum*
 305 inoculation.

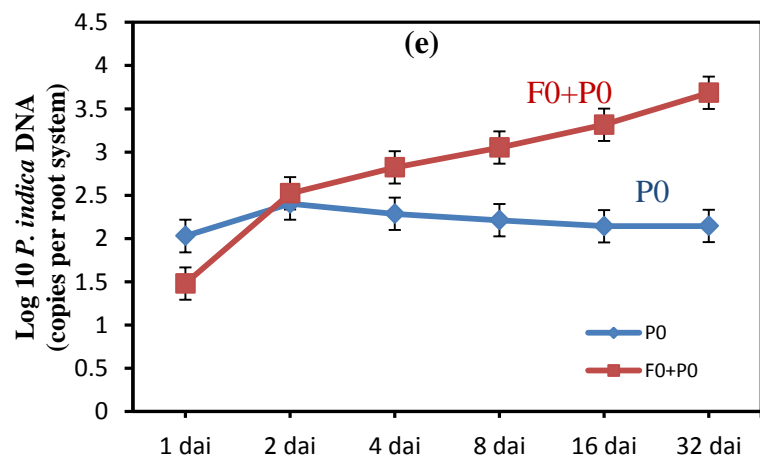
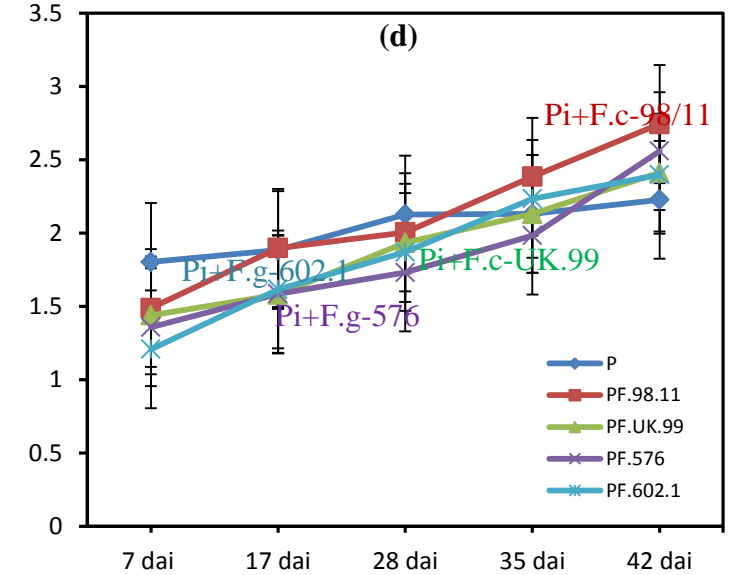
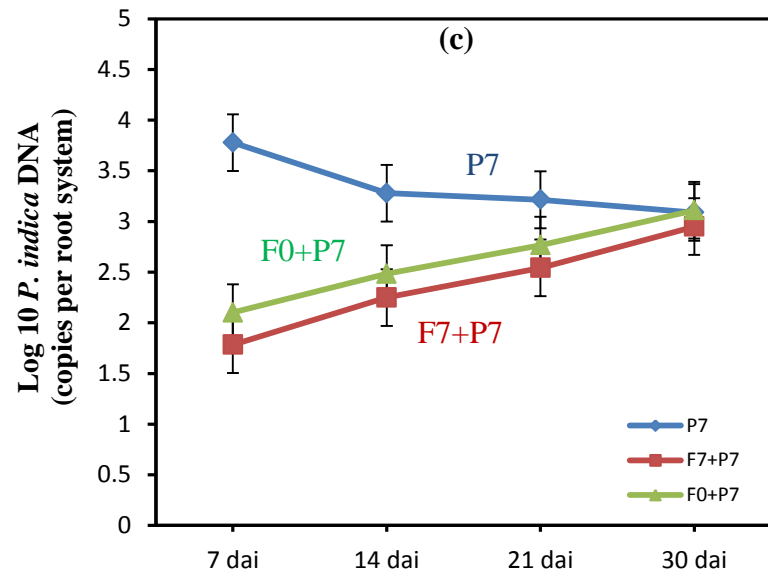
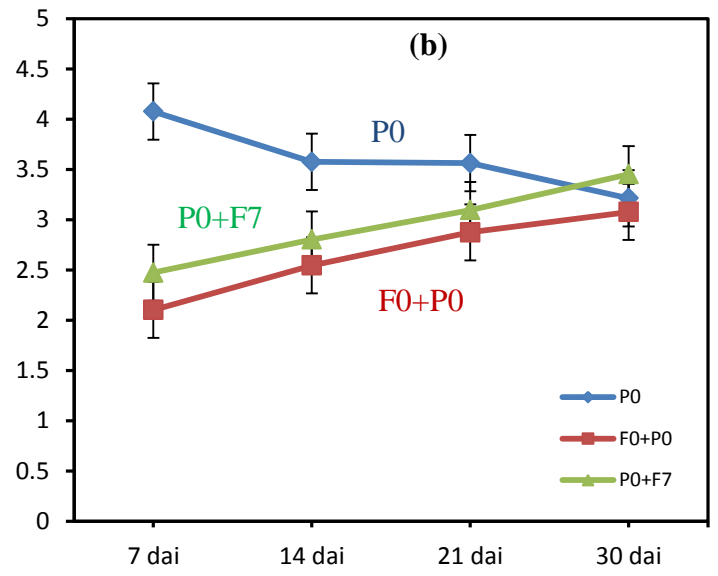
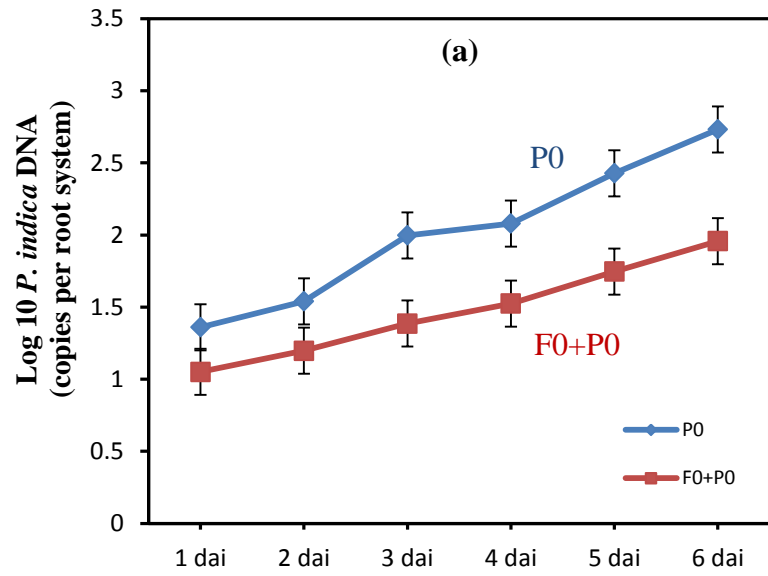
306



310 Fig.5. Development of the ratio of Fusarium DNA to wheat DNA in inoculated wheat roots.
 311 The ratio obtained by subtracting \log_{10} fungal DNA from \log_{10} wheat DNA. (a). *F. culmorum*
 312 added to soil at sowing (F0); *Piriformospora indica* added simultaneously (P0) or 7 days after
 313 sowing (P7); (b). *F. culmorum* added to soil 7 days after sowing (F7); *P. indica* added at
 314 sowing (P0) or simultaneously 7 days after sowing (P7); (c). *F. culmorum* 98/11, *F.*
 315 *culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 added at sowing time; (d).
 316 *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 and
 317 *P. indica* added simultaneously at sowing time; (e). *F. culmorum* added to soil at sowing
 318 (F0) and *P. indica* added simultaneously (P0), during the first week after inoculation; (f). *F.*
 319 *culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0), during the
 320 first month of inoculation. Each point represents mean \pm 2 SEM (for a and b; s.e.d= 0.2
 321 and d.f= 23), (for F.c. 98/11 and PF.c. 98/11: s.e.d= 0.15 and d.f= 9; for F.c. UK.99 and PF.c.
 322 UK.99: s.e.d= 0.08 and d.f= 9; for F.g. 576 and PF.g. 576: s.e.d= 0.2 and d.f= 9; for F.g.
 323 602.1 and PF.g.602.1: s.e.d= 0.2 and d.f= 9), (for e; s.e.d= 0.1, d.f= 11) and (for f, s.e.d= 0.2,
 324 d.f= 11).

325

326 The absolute quantity of *P. indica* DNA in the root systems of soil free medium, in the
 327 absence of Fusarium, increased in the first 7 days after inoculation (Fig 6a), then decreased
 328 from a peak of 10^4 copies/root system to 10^3 over the 30 days of the experiment (Fig 6b,c,e);
 329 but slightly increased, under simulated autumn conditions, by 42 days into the experiment
 330 (Fig 6d). In the presence of Fusarium, *P. indica* DNA grew gradually throughout the
 331 experiment (Fig 6a-e). The rate of growth of *P. indica* was lower under the simulated autumn
 332 conditions than under temperatures ranging between 15°C and 25°C (Fig 6b-d).

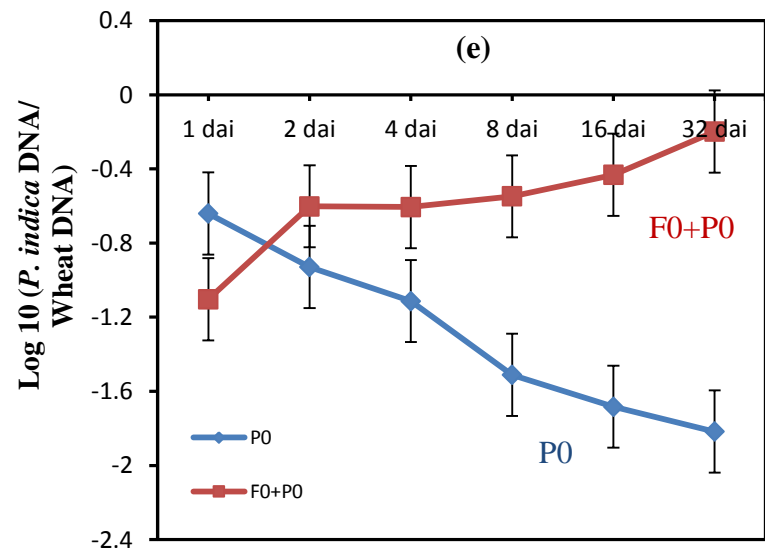
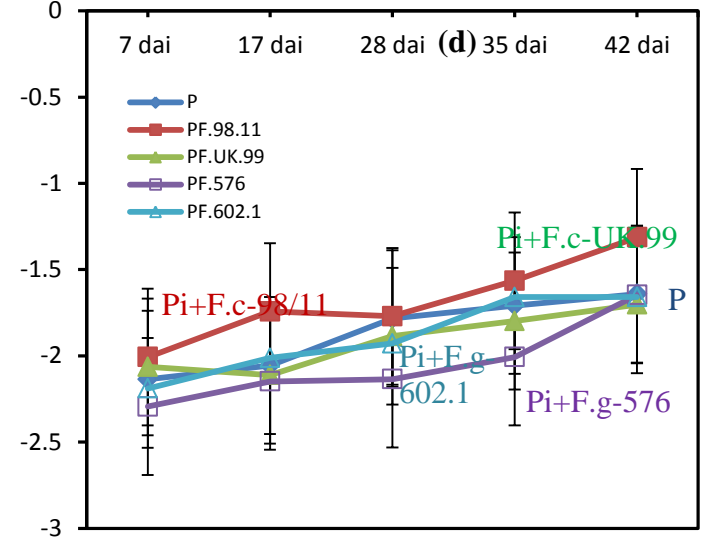
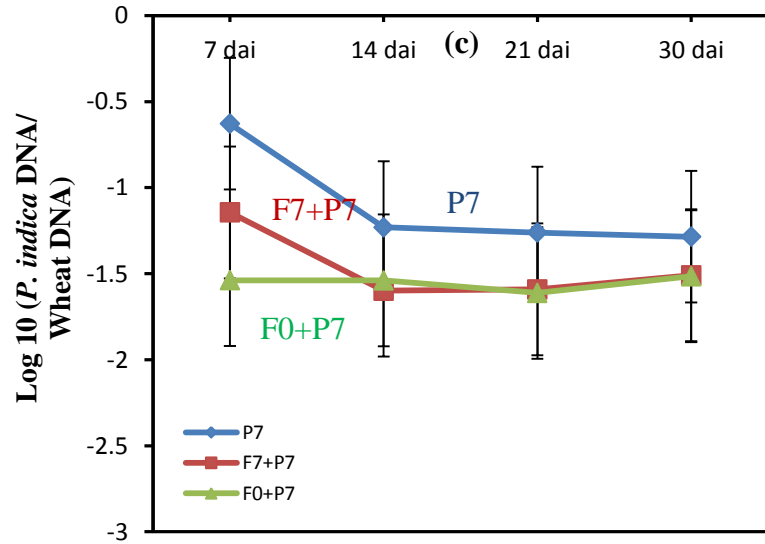
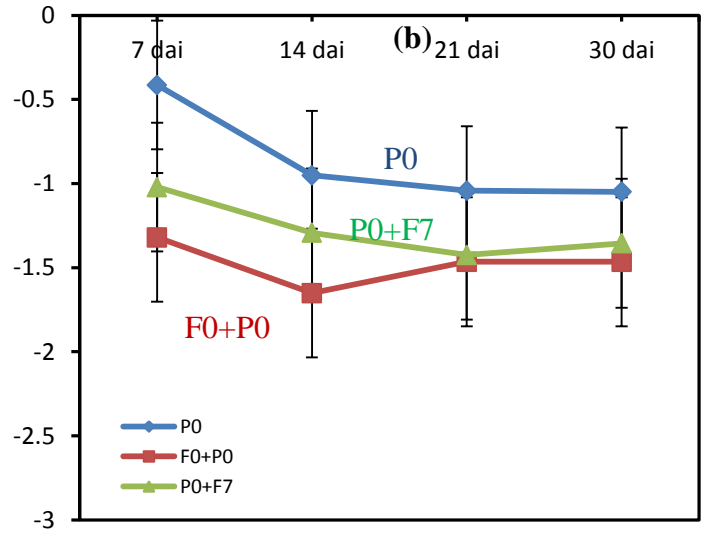
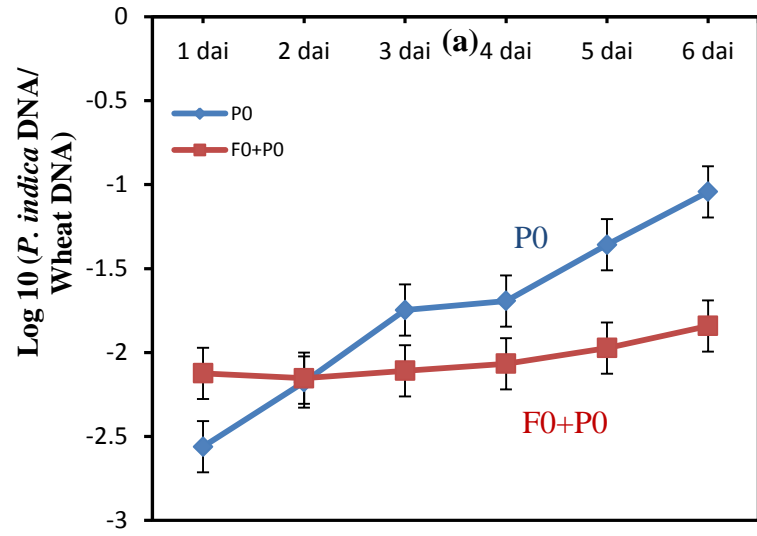


337 Fig.6. The absolute growth of *Piriformospora indica* in inoculated wheat roots. The absolute
338 amount obtained by adding \log_{10} fungal DNA to \log_{10} (root weight/sample weight in mg). (a).
339 *P. indica* added to soil at sowing (P0) and *Fusarium culmorum* added simultaneously (F0),
340 during the first week of inoculation; (b). *P. indica* added to soil at sowing (P0); *F. culmorum*
341 added simultaneously (F0) or 7 days after sowing (F7); (c). *P. indica* added to soil 7 days
342 after sowing (P7); *F. culmorum* added at sowing (F0) or simultaneously 7 days after sowing
343 (F7); (d). *P. indica*, *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F.*
344 *graminearum*602.1 added at sowing time; (e). *P. indica* added to soil at sowing (P0) and *F.*
345 *culmorum* added simultaneously (F0), during the first month of inoculation. Each point
346 represent mean \pm 2 SEM (for a; s.e.d= 0.1 and d.f=11),(for b and c; s.e.d= 0.2 and d.f= 23),
347 (for d; s.e.d= 0.3 and d.f= 24) and (for e, s.e.d= 0.1, d.f= 11).

348

349 The ratio of *P. indica* DNA to plant DNA, in the absence of *F. culmorum*, grew exponentially
350 at about 25% day⁻¹ in the first 7 days after inoculation (Fig 7a), then declined, then stayed
351 constant for the remainder of experiment from 14 to 30 dai (Fig 7b,c). However, this early
352 increase was not consistent (Fig 7e).The rate of growth of *P. indica* inoculated at 7 dai was
353 similar to that inoculated at sowing time (Fig 7b,c). In the presence of *F. culmorum*, the rate
354 growth of *P. indica* was static throughout the experiment (Fig 7a,b,c,e). In the experiment
355 under simulated autumn condition the ratio of *P. indica* DNA to wheat DNA, in the absence
356 or presence of Fusarium isolates, grew slowly at about 2% day⁻¹ throughout the experiment
357 (Fig 7d).

358



362 Fig.7. Development of the ratio of *Piriformospora indica* DNA to wheat DNA in inoculated
 363 wheat roots. The ratio obtained by subtracting \log_{10} fungal DNA from \log_{10} wheat DNA. (a).
 364 *P. indica* added to soil at sowing (P0) and *Fusarium culmorum* added simultaneously (F0),
 365 during the first week after inoculation; (b). *P. indica* added to soil at sowing (P0); *F.*
 366 *culmorum* added simultaneously (F0) or 7 days after sowing (F7); (c). *P. indica* added to soil
 367 7 days after sowing (P7); *F. culmorum* added at sowing (F0) or simultaneously 7 days after
 368 sowing (F7); (d). *P. indica*, *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or
 369 *F. graminearum* 602.1 added at sowing time; (e). *P. indica* added to soil at sowing (P0) and
 370 *F. culmorum* added simultaneously (F0), during the first month of inoculation. Each point
 371 represent mean ± 2 SEM (for a; s.e.d= 0.1 and d.f=11), (for b and c; s.e.d= 0.3 and d.f= 23),
 372 (for d; s.e.d= 0.3 and d.f= 24) and (for e, s.e.d= 0.2, d.f =11).

373

374 Discussion

375 In these experiments *P. indica* very effectively controlled *F. culmorum* and *F. graminearum*
 376 under simulated conditions similar to UK autumn, even though *P. indica* was found in the
 377 Thar region, India, which experiences extreme temperature conditions.

378 As in other *P. indica* studies, the mechanism appeared to be indirect. Dual culture tests of *P.*
 379 *indica* and *F. culmorum* or *F. graminearum* and microscopy showed no capability of either
 380 fungus to inhibit the other, with no inhibition zone at the interaction point and no other direct
 381 antagonistic activities. This is consistent with Kumar et al (2009) and Deshmukh and Kogel
 382 (2007) who reported that *P. indica* did not have any direct antagonistic effect on *F.*
 383 *graminearum* and *F. verticillioides* respectively, in-vitro. However, Ghahfarokhi and
 384 Goltapeh (2010) found a clear inhibition zone at the interaction point of *Gaeumannomyces*
 385 *graminis* var. *tritici* and *P. indica*. This could be a species difference or due to environmental
 386 effects, in particular the incubation temperature in Ghahfarokhi and Goltapeh was 28 °C, the
 387 most favourable temperature for *P. indica* growth.

388 In inoculated roots *P. indica* penetration started at the differentiation zone of the roots, with
 389 inter- and intracellular hyphae penetration during the first two to three dai. *P. indica* hyphae
 390 filled up the cortical and epidermal cells. Chlamydospores were visible from six days after

391 inoculation. Occasionally, coiled hyphae could be observed within root cells. Jacobs *et al.*
392 (2011) proposed a colonisation model for *P. indica* in Arabidopsis root, which started with
393 inter- and intracellular penetration of rhizodermal and cortical tissues and then root hair cells
394 by three days after inoculation. Fungal hyphae branched and sometimes formed whorls.
395 Finally sporulation started at seven dai; this is completely consistent with our observations.

396 Surprisingly, pathogen DNA was slightly higher than in plants inoculated with pathogen
397 alone during the first week after inoculation, in all experiments. This effect was probably due
398 to the slight extra supply of exogenous nutrients from the substrate of the *P. indica* inoculum.
399 Brown symptoms on root and crown were obvious in the Fusarium-inoculated samples,
400 which reflected the extensive invasive growth of Fusarium hyphae in the samples, which was
401 confirmed microscopically. In the presence of *P. indica*, the ratio of pathogen DNA to wheat
402 DNA increased much more slowly and then decreased by the end of the experiment. The
403 results are consistent with previous work in other host-pathogen systems. Kumar *et al.* (2009)
404 reported PCR analysis of maize samples inoculated with *P. indica* and *F. verticillioides*. They
405 showed that *P. indica* suppressed further colonization by *F. verticillioides*. Harrach *et al.*
406 (2013) reported preinoculation of barley roots with *P. indica* prior to *F. culmorum* resulted in
407 reduced colonization of roots by *F. culmorum*, which is consistent with less root rot–
408 symptom expression and a reduced loss of biomass. Deshmukh and Kogel (2007) reported a
409 decrease in the relative amount of *F. graminearum* DNA in barley roots in the presence of *P.*
410 *indica*, followed by a sharp decrease at 19 days after inoculation of *P. indica*.

411 Inoculation of plants with *P. indica* before pathogen had a greater effect on both the ratio
412 between pathogen and host DNA and the actual amount of pathogen than simultaneous or
413 delayed inoculation. In the absence of Fusarium, the absolute quantity of *P. indica* DNA and
414 the ratio of *P. indica* DNA to plant DNA decreased to a steady level after the first 7 days in
415 the warm environment, but increased slightly under cool conditions. These results are

416 consistent with a number of possible modes of action. For example *P. indica* might interfere
417 with host signalling pathways leading to oxidative burst, which are essential to successful
418 Fusarium establishment (Varma et al., 2012, Waller et al., 2005). Although qPCR is a precise
419 and reliable method to quantify DNA, caution needs to be taken in interpreting the data.
420 qPCR results must be verified by other methods and understood in the context of the
421 sampling protocol. Fusarium causes massive plant cell death, which might result in
422 overestimation by qPCR of the abundance of Fusarium DNA in root tissues that contain less
423 intact plant DNA (Harrach *et al.*, 2013). Hogg et al. (2007) found that Fusarium crown rot
424 disease severity and symptoms in wheat were often, but not always, correlated with actual
425 Fusarium colonization. Strausbaugh *et al.* (2005) did experiments in both field and
426 glasshouse. In their field study, they found no correlation between root-rot severity index and
427 Fusarium DNA quantities in root samples. However, in their glasshouse study percent
428 infected root area was correlated with Fusarium DNA quantities in both wheat and barley.
429 This contrast in their results might have various causes. It is possible that there were sampling
430 problems in the field study. For example rotting might be so fast in soil that they only ever
431 sampled nearly healthy plant tissues.

432 Our studies show that *P. indica* can protect wheat from damage by Fusarium disease at the
433 seedling stage, in simulated UK conditions. However, the ecological-side-effects of *P. indica*
434 are still unclear: how *P. indica* interact with other beneficial soil microorganisms, like
435 arbuscular mycorrhizal fungi, how it affects soil functioning, such as turnover of soil organic
436 matter, incorporation of residues, etc, and what effects *P. indica* has on other soil-borne
437 diseases. These must be considered in further studies.

438 **Acknowledgment**

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