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

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Short title: *Piriformospora indica* reduces Fusarium

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

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

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

Introduction

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

Piriformospora indica (sebacinales: basidiomycota) is a root endophytic fungus with a wide host range that was first isolated from the rhizosphere of woody shrubs in the Thar region of northwest India (Verma *et al.*, 1998). All members of the Sebacinales are involved in mycorrhizal associations (Weiss *et al.*, 2004). *P. indica*, like arbuscular mycorrhizal fungi, has plant growth promoting effects, but, in contrast to mycorrhizal fungi, can be cultured on various synthetic media (Verma et al., 1998). *P.indica* can mobilise and transport phosphorus ,nitrogen and micronutrients from soil to the infected host plant via plant-fungal interfaces (Malla et al., 2004, Sherameti et al., 2005, Varma et al., 2013, Yadav et al., 2010). It has also been reported that *P. indica* can improve growth in a range of economically important monocot and dicot hosts (Bagde et al., 2010, Varma et al., 1999, Varma et al., 2000).

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

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

Materials and methods

Fungal inoculation

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

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

Test for antagonistic activity

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

Plant materials and glasshouse experiments

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

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

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

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

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

Each pot received 60 mL of fresh nutrient solution once a week. Nutrient solution was prepared each week using tap water with the final concentrations given: NO^-_3 10 mM, PO^{2-}_4 1 mM, K^+ 6 mM, Ca^{2+} 1.5 mM, Mg^{2+} 1 mM, SO^{2-}_4 1.5 mM, Fe 10 µM, Mn^{2+} 1 µM, Zn^{2+} 0.01 µM, Cu^{2+} 0.1 µM, MoO^{2-}_4 0.07 µM and $B_4O^{2-}_7$ 0.07 µM. Sodium metasilicate (100 mg/L) included to control powdery mildew.

Staining and microscopy

Wheat root samples inoculated with *P. indica*, Fusarium isolates, and both fungi together were stained using black ink (Pelikan Fountain Pen Ink, Niche Pens Ltd, UK) (Vierheilig et al., 1998). Roots were cleared by soaking them in 10% (w/v) KOH for one hour at 80°C, then rinsed 5 times with tap water. Cleared roots were covered with 2% HCl (v/v) for at least 30 min. Thereafter, HCl was poured off and roots were covered with 50 g/L black ink for 30 min at 80°C. Roots were de-stained by rinsing in tap water and viewed under a microscope with 10x and 40x objectives.

DNA isolation and primer development

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

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

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

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

Statistical analysis of experiments

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

Results

Interaction of *P. indica* and Fusarium

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

Fusarium- inoculated root samples of both species showed extensive growth of Fusarium , with the mycelium completely covering the roots by the final observation date, when brown symptoms were clearly visible. In *P. indica*-Fusarium inoculated plants, Fusarium colonisation was visually much less, but colonisation by *P. indica* was extensive. *Piriformospora indica* colonisation started on root surfaces in the differentiation zone behind the root meristem with inter- and intracellular penetration of epidermal cells, during the first two to three days after inoculation, with hyphae filling up the cells. By four days after inoculation coiled hyphae could occasionally be seen inside the cells.. Later, a little colonisation could be observed in epidermal cells of the meristematic and elongation zones of roots. *P. indica* chlamydospores were not observed until six days after inoculation (Fig. 1c,d).

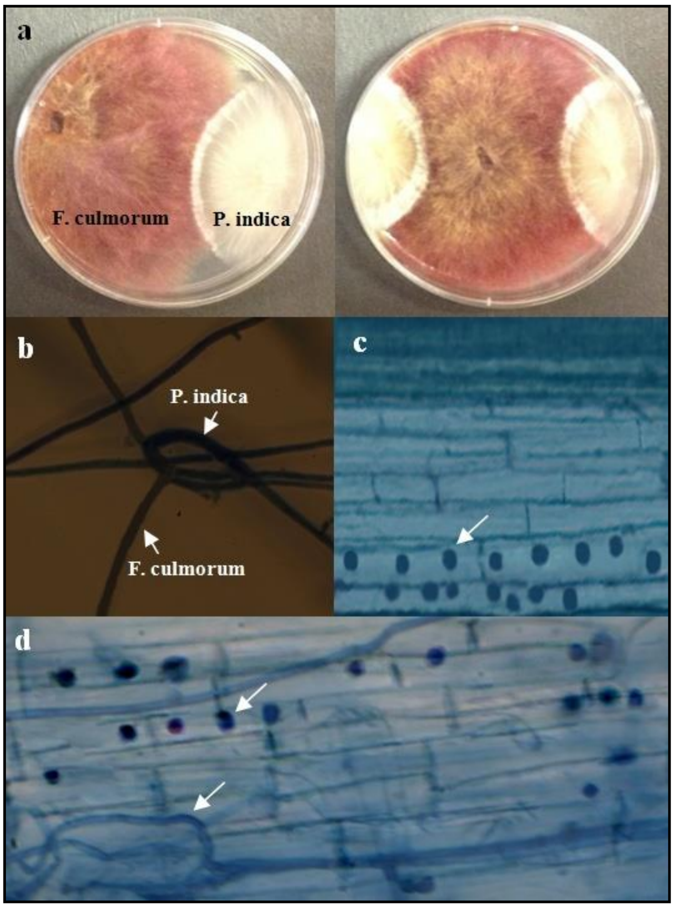

Fig.1. Interaction of *Piriformospora indica* and Fusarium in agar plates and in wheat roots; (a). Agar plate co-cultivated with *F. culmorum* and *P. indica*; (b). Interaction of coiled hypha of *P. indica* around *F. culmorum* in agar plates at the encounter point*;* (c). *P.indica* clamydospores inside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

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

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

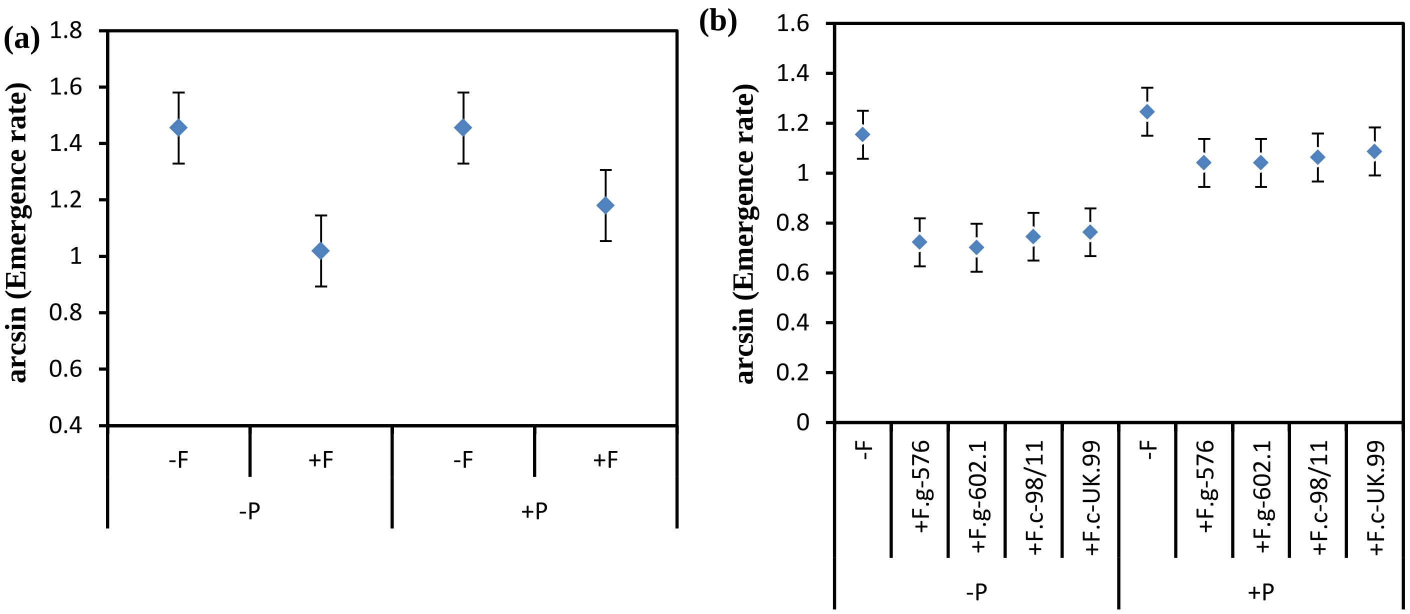

Fig.2. Emergence rates of seeds inoculated with Fusarium and *Piriformospora indica* evaluated 7 days after sowing; data were arcsine transformed. (a). Roots inoculated with *F. culmorum* and *P. indica* simultaneously at sowing time (s.e.d= 0.09, d.f= 57); (b). Roots inoculated with *F. culmorum* (98/11 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±2 SEM (P:*P. indica* and F:Fusarium).

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

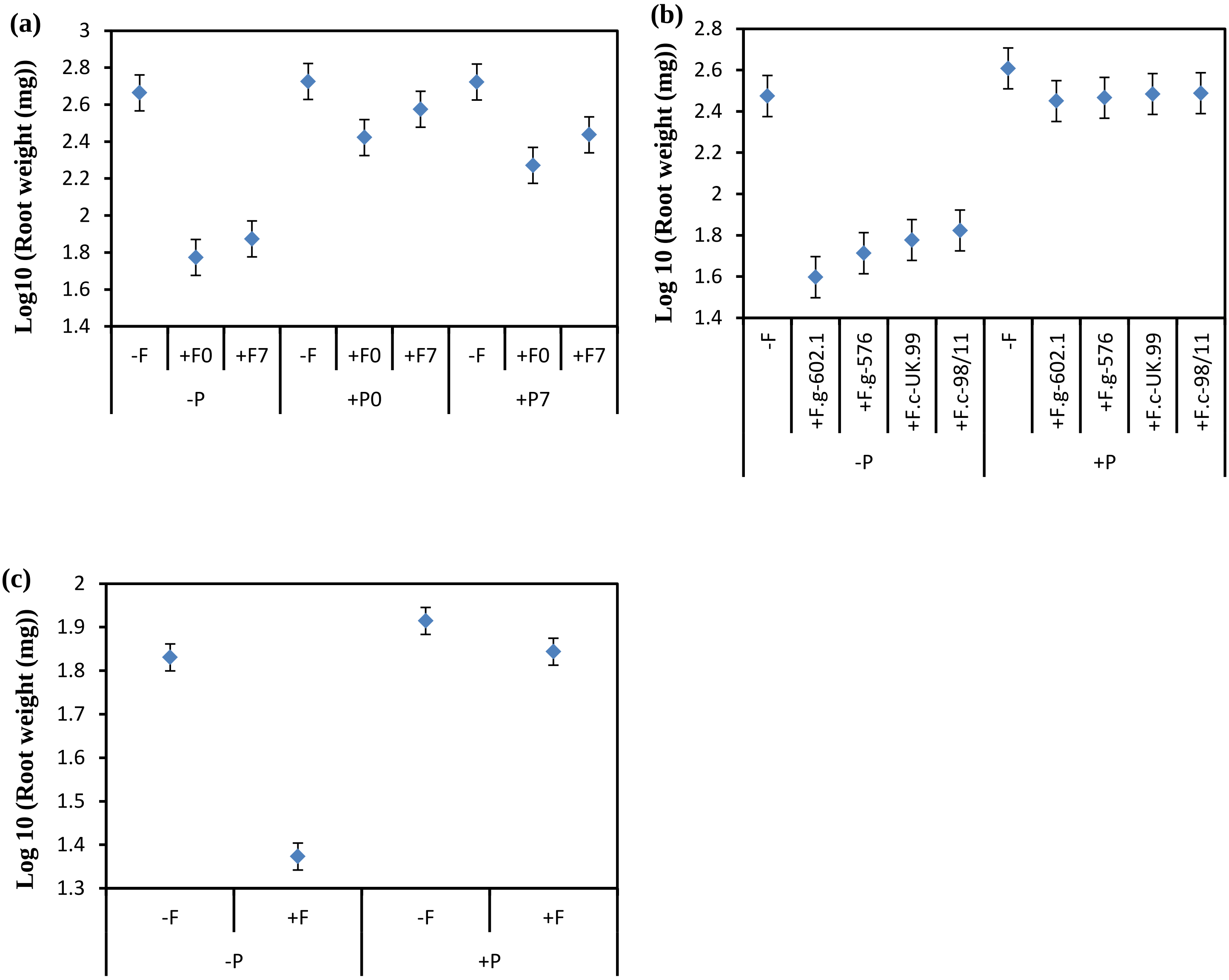

Fig.3. Root weight of samples (mg) inoculated with Fusarium and *Piriformospora indica* evaluated at last harvest; data were Log_{10} transformed. (a). Roots inoculated with *F. culmorum* or *P. indica* simultaneously or 7 days after sowing, harvested at 30 dai (s.e.d= 0.07, d.f= 8); (b). Roots inoculated 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 ±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).

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

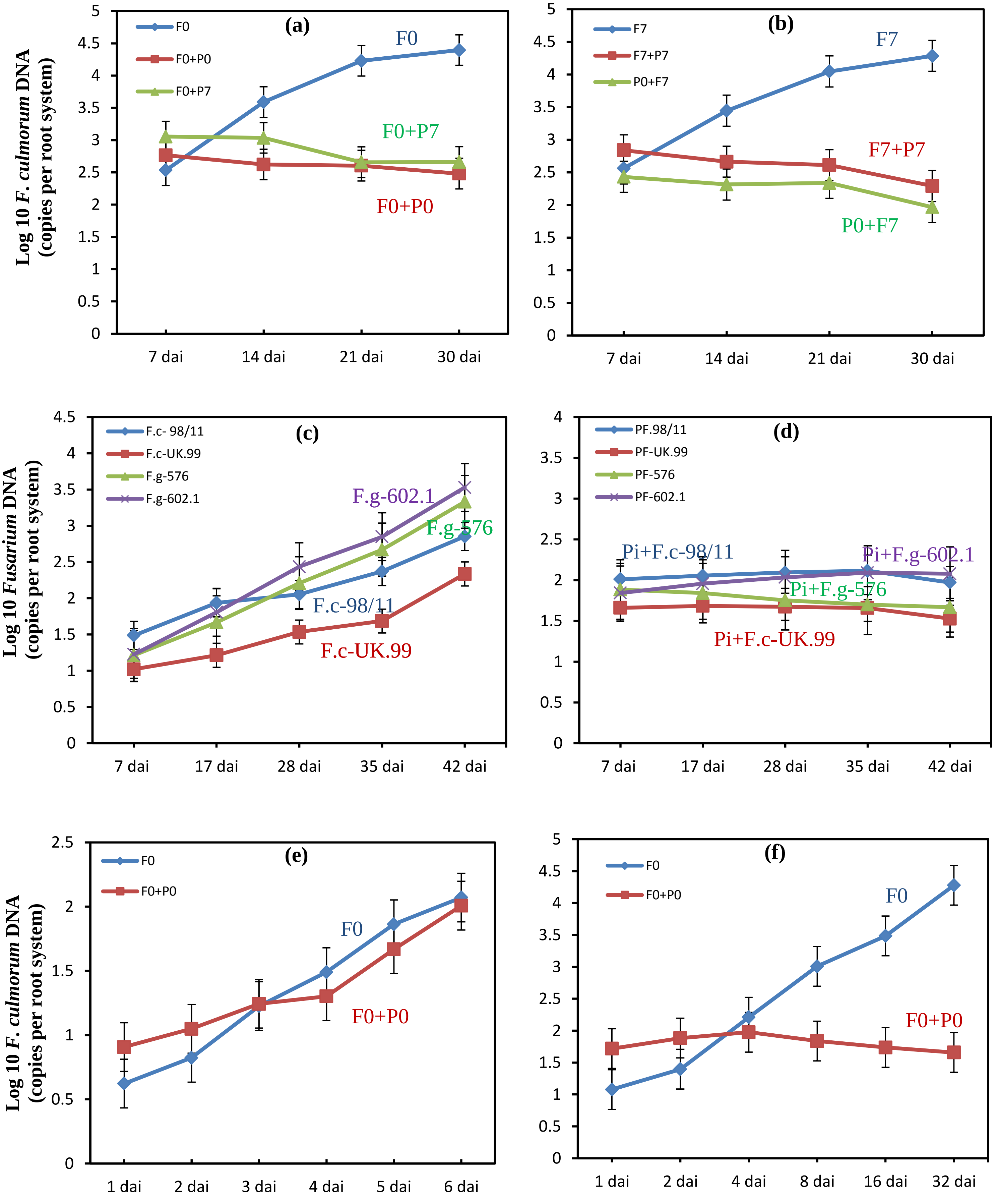
(a)
Log 10 F. culmorum DNA (copies per root system)
F0
F0+P0
F0+P7
7 dai
14 dai
21 dai
30 dai
(b)
F7
F7+P7
P0+F7
7 dai
14 dai
21 dai
30 dai
(c)
Log 10 Fusarium DNA (copies per root system)
F.c- 98/11
F.c-UK.99
F.g-576
F.g-602.1
F.c-98/11
7 dai
17 dai
28 dai
35 dai
42 dai
(d)
PF.98/11
PF-UK.99
PF-576
PF-602.1
Pi+F.c-98/11
Pi+F.g-602.1
Pi+F.g-576
Pi+F.c-UK.99
7 dai
17 dai
28 dai
35 dai
42 dai
(e)
Log 10 F. culmorum DNA (copies per root system)
F0
F0+P0
1 dai
2 dai
3 dai
4 dai
5 dai
6 dai
(f)
F0
F0+P0
1 dai
2 dai
4 dai
8 dai
16 dai
32 dai

Fig.4. The absolute growth of Fusarium in inoculated wheat roots. The absolute amount obtained by adding $\log_{10}$ fungal DNA to $\log_{10}$ (root weight/sample weight in mg). (a). *F. culmorum* added to soil at sowing (F0); *Piriformospora indica* added simultaneously (P0) or 7 days after sowing (P7); (b). *F. culmorum* added to soil 7 days after sowing (F7); *P. indica* added at sowing (P0) or simultaneously 7 days after sowing (P7); (c). *F. culmorum*98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 added at sowing time; (d). *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 and *P. indica* added simultaneously at sowing time; (e). *F. culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0), during the first week of inoculation; (f). *F. culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0), during the first month of inoculation. Each point represent mean ±2 SEM (for a and b; s.e.d= 0.2 and 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. 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. 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= 0.2, d.f=11).

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

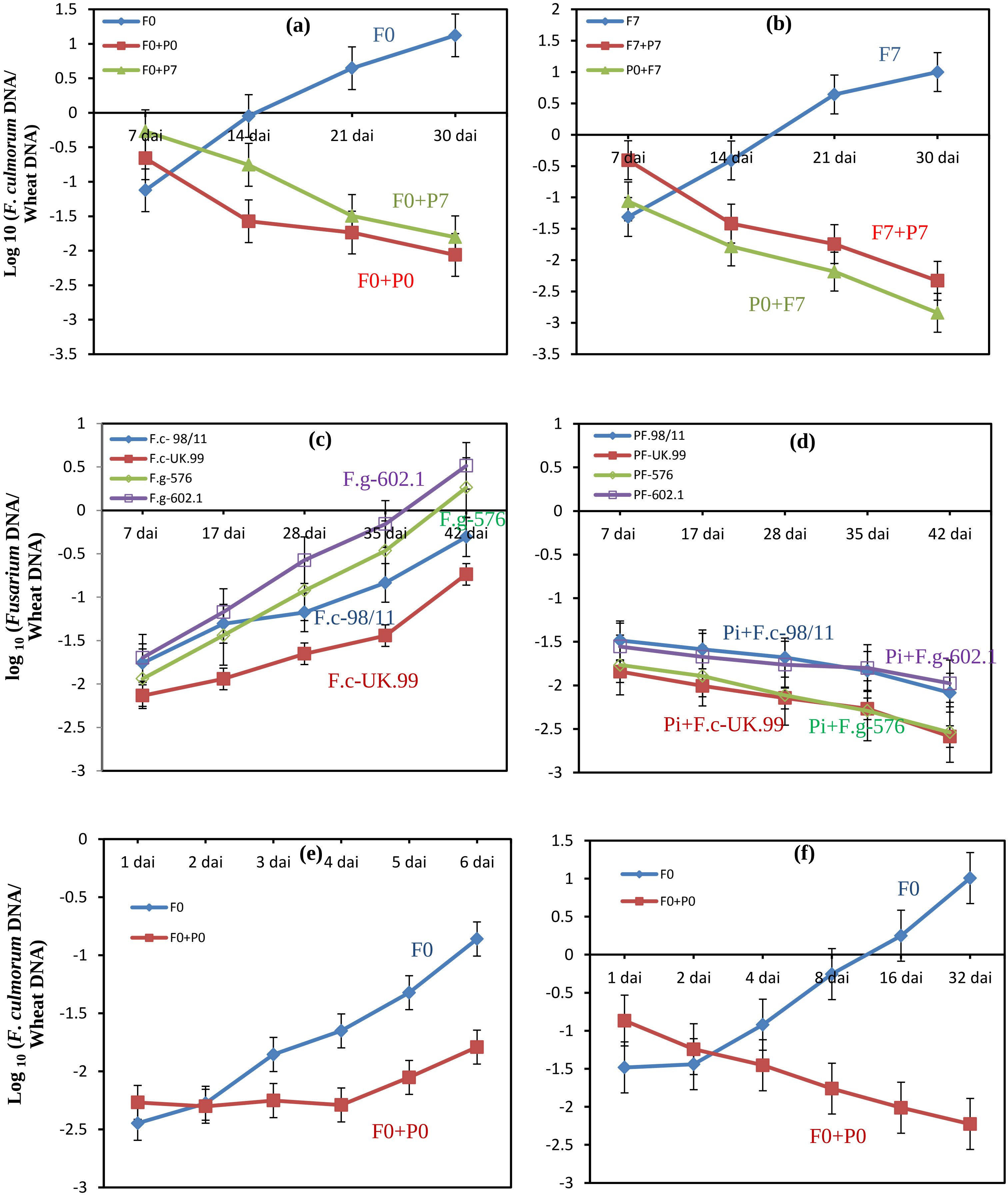
(a)
Log 10 (*F. culmorum* DNA/ Wheat DNA)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
7 dai
14 dai
21 dai
30 dai
F0
F0+P0
F0+P7
F0
F0+P7
F0+P0
(b)
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
7 dai
14 dai
21 dai
30 dai
F7
F7+P7
P0+F7
F7
F7+P7
P0+F7
(c)
log 10 (*Fusarium* DNA/ Wheat DNA)
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
7 dai
17 dai
28 dai
35 dai
42 dai
F.c- 98/11
F.c-UK.99
F.g-576
F.g-602.1
F.g-602.1
F.g-576
F.c-98/11
F.c-UK.99
(d)
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
7 dai
17 dai
28 dai
35 dai
42 dai
PF.98/11
PF-UK.99
PF-576
PF-602.1
Pi+F.c-98/11
Pi+F.g-602.1
Pi+F.c-UK.99
Pi+F.g-576
(e)
Log 10 (*F. culmorum* DNA/ Wheat DNA)
0
-0.5
-1
-1.5
-2
-2.5
-3
1 dai
2 dai
3 dai
4 dai
5 dai
6 dai
F0
F0+P0
F0
F0+P0
(f)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
1 dai
2 dai
4 dai
8 dai
16 dai
32 dai
F0
F0+P0
F0
F0+P0

Fig.5. Development of the ratio of Fusarium DNA to wheat DNA in inoculated wheat roots. The ratio obtained by subtracting $\log_{10}$ fungal DNA from $\log_{10}$ wheat DNA. (a). *F. culmorum* added to soil at sowing (F0); *Piriformospora indica* added simultaneously (P0) or 7 days after sowing (P7); (b). *F. culmorum* added to soil 7 days after sowing (F7); *P. indica* added at sowing (P0) or simultaneously 7 days after sowing (P7); (c). *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 added at sowing time; (d). *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 and *P. indica* added simultaneously at sowing time; (e). *F. culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0), during the first week after inoculation; (f). *F. culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0), during the first month of inoculation. Each point represents mean±2 SEM (for a and b; s.e.d= 0.2 andd.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. 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. 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, d.f= 11).

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

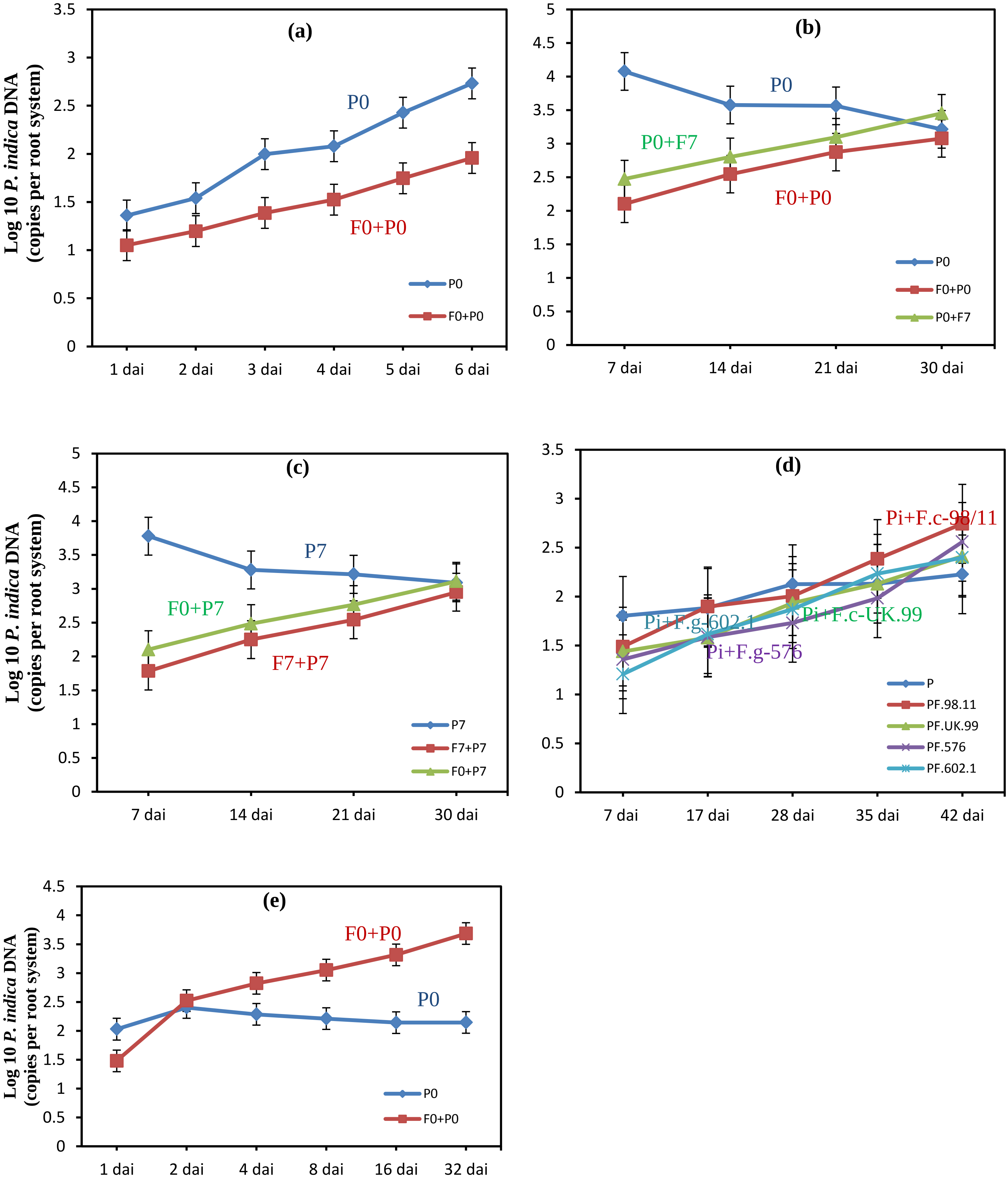
(a)
Log 10 *P. indica* DNA (copies per root system)
3.5
3
2.5
2
1.5
1
0.5
0
1 dai
2 dai
3 dai
4 dai
5 dai
6 dai
P0
F0+P0
P0
F0+P0
(b)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
7 dai
14 dai
21 dai
30 dai
P0
P0+F7
F0+P0
P0
F0+P0
P0+F7
(c)
Log 10 *P. indica* DNA (copies per root system)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
7 dai
14 dai
21 dai
30 dai
P7
F0+P7
F7+P7
P7
F7+P7
F0+P7
(d)
3.5
3
2.5
2
1.5
1
0.5
0
7 dai
17 dai
28 dai
35 dai
42 dai
Pi+F.c-98/11
Pi+F.g-602.1
Pi+F.c-UK.99
Pi+F.g-576
P
PF.98.11
PF.UK.99
PF.576
PF.602.1
(e)
Log 10 *P. indica* DNA (copies per root system)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1 dai
2 dai
4 dai
8 dai
16 dai
32 dai
F0+P0
P0
P0
F0+P0

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

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

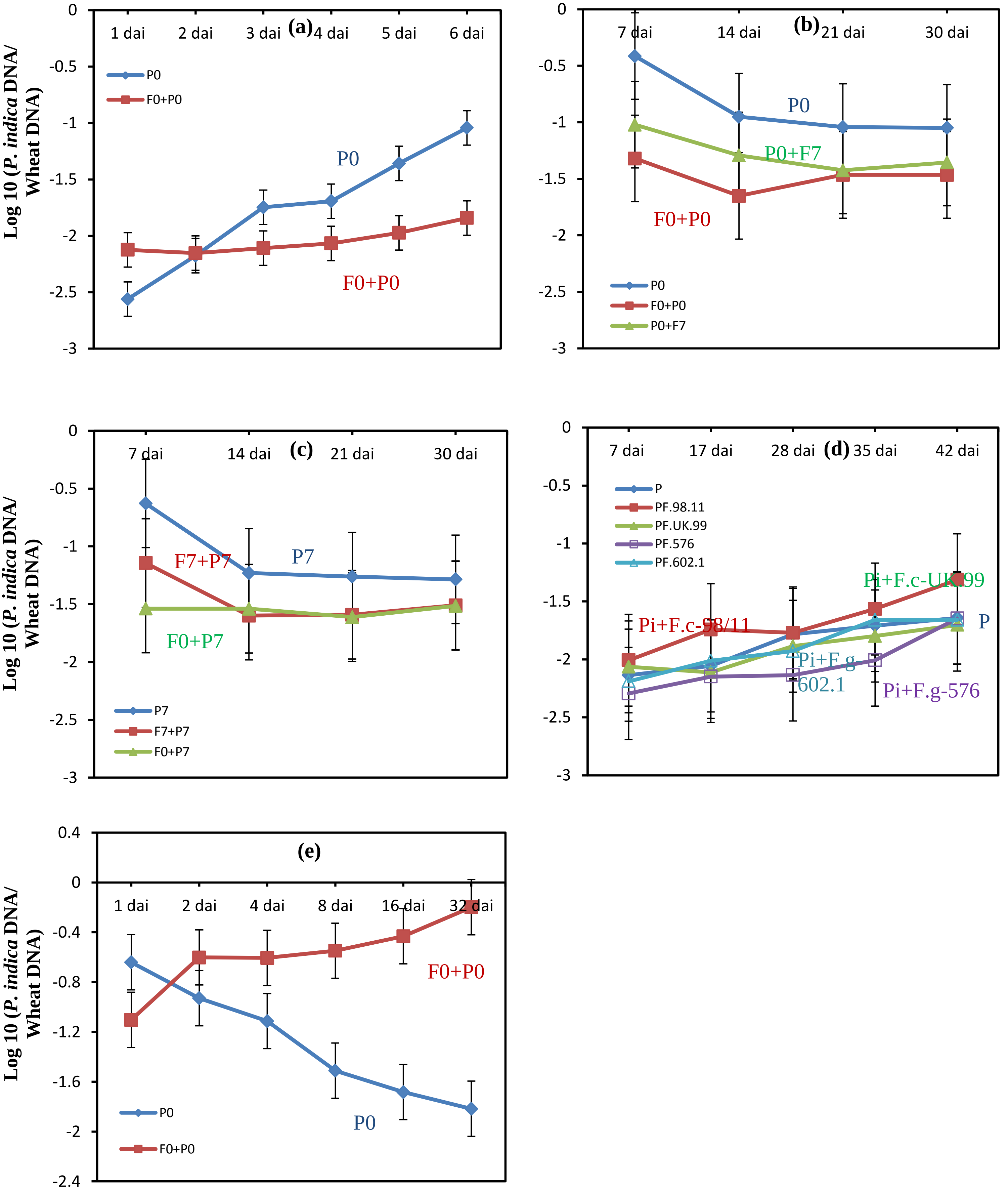
Log 10 (*P. indica* DNA/ Wheat DNA)
(a)
1 dai
2 dai
3 dai
4 dai
5 dai
6 dai
0
-0.5
-1
-1.5
-2
-2.5
-3
P0
F0+P0
P0
F0+P0
(b)
7 dai
14 dai
21 dai
30 dai
P0
P0+F7
F0+P0
P0
F0+P0
P0+F7
(c)
7 dai
14 dai
21 dai
30 dai
F7+P7
P7
F0+P7
P7
F7+P7
F0+P7
(d)
7 dai
17 dai
28 dai
35 dai
42 dai
P
PF.98.11
PF.UK.99
PF.576
PF.602.1
Pi+F.c-UK.99
Pi+F.c-98/11
P
Pi+F.g-602.1
Pi+F.g-576
(e)
1 dai
2 dai
4 dai
8 dai
16 dai
32 dai
0.4
0
-0.4
-0.8
-1.2
-1.6
-2
-2.4
F0+P0
P0
P0
F0+P0

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

Discussion

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

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

In inoculated roots *P. indica* penetration started at the differentiation zone of the roots, with inter- and intracellular hyphae penetration during the first two to three dai. *P. indica* hyphae filled up the cortical and epidermal cells. Chlamydospores were visible from six days after inoculation. Occasionally, coiled hyphae could be observed within root cells. Jacobs *et al.* (2011) proposed a colonisation model for *P. indica* in Arabidopsis root, which started with inter- and intracellular penetration of rhizodermal and cortical tissues and then root hair cells by three days after inoculation. Fungal hyphae branched and sometimes formed whorls. Finally sporulation started at seven dai; this is completely consistent with our observations.

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

Inoculation of plants with *P. indica* before pathogen had a greater effect on both the ratio between pathogen and host DNA and the actual amount of pathogen than simultaneous or delayed inoculation. In the absence of Fusarium, the absolute quantity of *P. indica* DNA and the ratio of *P. indica* DNA to plant DNA decreased to a steady level after the first 7 days in the warm environment, but increased slightly under cool conditions. These results are consistent with a number of possible modes of action. For example *P. indica* might interfere with host signalling pathways leading to oxidative burst, which are essential to successful Fusarium establishment (Varma et al., 2012, Waller et al., 2005). Although qPCR is a precise and reliable method to quantify DNA, caution needs to be taken in interpreting the data. qPCR results must be verified by other methods and understood in the context of the sampling protocol. Fusarium causes massive plant cell death, which might result in overestimation by qPCR of the abundance of Fusarium DNA in root tissues that contain less intact plant DNA (Harrach *et al.*, 2013). Hogg et al. (2007) found that Fusarium crown rot disease severity and symptoms in wheat were often, but not always, correlated with actual Fusarium colonization. Strausbaugh *et al.* (2005) did experiments in both field and glasshouse. In their field study, they found no correlation between root-rot severity index and Fusarium DNA quantities in root samples. However, in their glasshouse study percent infected root area was correlated with Fusarium DNA quantities in both wheat and barley. This contrast in their results might have various causes. It is possible that there were sampling problems in the field study. For example rotting might be so fast in soil that they only ever sampled nearly healthy plant tissues.

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

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