School of Agriculture, Policy and Development

Biological Control of Fusarium Diseases of Wheat by *Piriformospora indica*

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for the degree of Doctor of Philosophy

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Mojgan Rabiey

Abstract

The threat to UK food security due to cereal diseases is serious. Diseases can affect crops and have a serious impact on the economic output of a farm and on food. Among cereal diseases, Fusarium Head Blight (FHB) and Fusarium Crown Rot (FCR) disease are two of the most widespread and damaging diseases of cereal crops. This thesis reports the effect of *Piriformospora indica* on Fusarium diseases of wheat, both head blight and crown rot, with the purpose of developing a solution to control crop diseases by using natural microorganisms.

Piriformospora indica is a root endophyte belonging to the Sebacinaceae (Sebacinales, Basidiomycota). It was originally found in the Thar desert of Rajasthan, in India. *P. indica* forms mutualistic symbioses with a broad range of host plants, increasing their biomass production and resistance to fungal pathogens. Glasshouse experiments and controlled environmental chambers with conditions adjusted to UK autumn conditions were used to determine the effect of *P. indica* on FCR disease of wheat, both *Fusarium culmorum* and *F. graminearum*. *P. indica* reduced damage to wheat seedlings by restricting growth of pathogen in the root. The effect of *P. indica* on FHB disease of winter (cv. Battalion, NABIM group 2) and spring (cv. Paragon, Mulika, Zircon (NABIM group 1), Granary, KWS Willow (NABIM group 2) and KWS Kilburn (NABIM group 4)) hard wheat and subsequent contamination by the mycotoxin deoxynivalenol (DON) were examined in the pots under UK weather conditions. *P. indica* application reduced FHB disease severity and incidence and mycotoxin DON concentration of inoculated winter and spring wheat samples. *P. indica* also increased above-ground biomass, thousand grain weight and total grain weight. The effects were similar at different fertiliser levels. The effect of *P. indica* was compatible with the arbuscular mycorrhizal fungus *Funneliformis mosseae* and foliar fungicide Aviator Xpro (Bayer CropScience, UK; with active ingredients of prothioconazole and bixafen) application. *P. indica* reduced severity and incidence of naturally arising infection by Septoria leaf blotch (caused by *Zymoseptoria tritici*), yellow rust (caused by *Puccinia striiformis* f. sp. *tritici*) and powdery mildew (caused by *Blumeria*

graminis f.sp. *tritici*). The nutrient analysis of soil and plant tissue samples showed that *P. indica* did not have any effects on phosphorus, nitrogen and potassium status and uptake were not significantly affected by *P. indica* inoculation.

P. indica mRNA for the elongation factor (*TEF* gene) was used as an indicator of *P. indica* viability in soil. *P. indica* was still alive after four and eight months in pots of soil from the Reading area, which had been left open to winter-summer weather conditions without host plants, but not after 15 months. PCR-denaturing gradient gel electrophoresis of DNA extracted from root zone or from bulk soil, in which *P. indica*-infected wheat had been grown, showed *P. indica* increased the root and soil fungal and bacterial species diversity. Test on arable weeds, black-grass, wild-oat and cleavers, showed that on average over species *P. indica* increased root biomass by 35 %; but above-ground biomass was not significantly affected by *P. indica*. The average above-ground competitiveness of the weeds with wheat was slightly decreased.

My results suggest that *P. indica* could be used to control wheat diseases in field settings in the UK. However, extensive data would be needed to determine ecological and agronomical safety and persistence, before release on a field scale was commercialised.

List of Publications arising from this work

Rabiey M, Ullah I, and Shaw MW, 2013. The effect of *Piriformospora indica*, an endophytic fungus, on wheat resistance to Fusarium disease. Positive Plant Microbial Interactions: Their role in maintaining sustainable and natural ecosystems. Aspect of Applied Biology, 120: 91-94.

Rabiey M, Ullah I, and Shaw MW, 2015. The endophytic fungus *Piriformospora indica* protects wheat from Fusarium crown rot disease under simulated UK autumn conditions. Plant Pathology, 64: 1029–1040. Doi: 10.1111/ppa.12335.

Rabiey M, and Shaw MW, 2015. *Piriformospora indica* reduces Fusarium head blight disease severity and mycotoxin DON contamination in wheat under UK weather conditions. Plant Pathology. Doi: 10.1111/ppa.12483.

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List of Abbreviations

ABA	Abscisic aicd
AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
B	Boron
BLAST	Basic Local Alignment Search Tool
BP	Before Present (1950)
Ca	Calcium
cDNA	compelimentray deoxyribonucleic acid
CM	Complex modified *Aspergillus* medium
CMC	carboxyl methyl celloluse
Ct	Cycle threshold
Cu	Copper
dai	days after inoculation
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DGGE	Denaturing Gradient Gel Electrophoresis
dGTP	deoxyguanosine triphosphate
d.f.	degree of freedom
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxy nucleotide triphosphate
DON	deoxynivalenol
EF	elongation factor
F. c.	*Fusarium culmorum*
FCR	Fusarium Crown Rot
Fe	Iron
F. g.	*Fusarium graminearum*
FHB	Fusarium Head Blight
Fun. m.	*Funneliformis mosseae*
gDNA	genomic deoxyribonucleic acid
GS	Growth Stage Hydrogen peroxide
H_2O_2	Hydrogen peroxide
JA	Jasmonic acid
JA-Ile	Jasmonic acid isoleucine
K	Potassium
Mg	Magnesium
MMN	Modified Melin-Norkrans

mRNA	messenger ribonucleic acid
N	Nitrogen
NABIM	National Association of British and Irish Flour Millers
NaClO	Sodium hypochlorite
NCBI	National Centre for Biotechnology Information
NH_4	Ammonium
NO_3	Nitrate
NTC	No template controls
OPDA	OXO-phytodienoic acid
PCR	Polymerase Chain Reaction
P	Phosphorus
Pi	*Piriformospora indica*
qPCR	quantitative real-time Polymearse Chain Reaction
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	rounds per minute
rt-PCR	reverse transcription Polymerase Change Reaction
SA	Salicylic acid
S.E.D.	Standard Error of the Difference
SEM	Standard Error of the Means
TEF	Translation elongation factor 1 alpha
TAE	Tris-acetate-EDTA
TGW	thousand grain weight
wai	weeks after inoculation
Zn	Zinc

CHAPTER 1- Literature Review

1.1. Wheat

Wheat is a major food resource globally and is the most important agricultural commodity in international trade. World wheat production is approximately 715 million tons, which is second to maize (1 billion tons) and higher than rice (480 million tons) and is currently grown on more land area (220 million hectares) than maize and rice (185 and 165 million hectares, respectively) (FAOSTAT, 2015). Wheat is one of the most common staple food crops for more than one-third of the world's population. It provides on average one-fifth of the total calorific input of the world's population (FAO, 2015). Wheat has a higher protein, fat and fiber content, compared with other grains. It is also rich in vitamins and minerals such as manganese, phosphorus, potassium, zinc, vitamin B6, folate, thiamin, riboflavin and niacin (Sramkovaa et al., 2009). Wheat flour is used to make a wide variety of foods such as bread, biscuit, cakes, breakfast cereal, pasta, noodles, and couscous (McMullen et al., 1997, Pena, 2002). Wheat can be grown within a wide range of locations having diverse environmental conditions. Therefore, for thousands of years, wheat has been one of the most prominent food sources for humans and livestock (Shewry, 2009). World wheat production is almost entirely based on just two wheat species: common wheat or bread wheat (*Triticum aestivum* L.) for about 95 % of the world production and durum wheat (*T. turgidum* L. ssp. *durum* (Desf.) Husn) for the remaining 5 % (Shewry, 2009).

Grain hardness is a key cultivar trait for milling that refers to the texture of the kernel, that is, whether the endosperm is physically hard or soft (Giroux & Morris,

1998). Hard and soft wheats have different processing requirements and end-uses. Generally, hard wheat is used for bread making whereas soft wheat is used for cookies, cakes, and pastries (Morris & Rose, 1996).

NABIM categorises UK wheat cultivars into one of four groups in order to give farmers an indication of the likely use of the grain and how much it is likely to be worth (NABIM, 2015): Group one: these are the cultivars that produce consistent milling and baking performance; Group 2: this group comprises cultivars that exhibit bread-making potential, but are not suited to all grists; Group 3: this Group contains soft cultivars for biscuit, cake and other flours where the main requirement is for soft milling characteristics, low protein, good extraction rates, and an extensible but not elastic gluten; Group 4: these cultivars are grown mainly as feed wheats for animals (NABIM, 2015).

Wheat is believed to have originated in south-western Asia over 10,000 years ago and is related to wild species that still can be found in Lebanon, Syria, northern Israel, Iraq, and eastern Turkey (Sleper & Poehlman, 2006). The spread of wheat from its site of origin across the world is summarized by Shewry (2009). The main route into Europe was via Anatolia to Greece (8000 BP) and then across to Italy, France and Iberia (7000 BP), finally reaching the British Isles and Scandinavia by about 5000 BP. Similarly, wheat spread via Iran into central Asia reaching China by about 3000 BP and to Africa, initially via Egypt. It was then introduced to Mexico in 1529 and to Australia in 1788.

The UK is one of the largest producers of cereal crops in the EU. Cereals have long been produced in the UK, to a current annual value of over £2.5 billion (Rossides,

2015). Within UK agriculture, cereal crops account for about 15 % of total UK agricultural land, but over 65 % of total cropping (DEFRA, 2015). The planted area of cereals is currently 3 million hectares, of which around 2 million hectares are under wheat cultivation. UK wheat production, in 2013, was around 12 million tons, 39 % less than 2014 production which was around 16 million tonnes (FAOSTAT, 2015). The reduced production in 2013 was probably due to prolonged wet weather leading to difficult planting conditions and a lack of sunshine during the key grain filling period leading to poor harvest including high levels of disease (Twining & Wynn, 2013).

This illustrates how wheat production can be severely limited by both biotic and abiotic constraints. Approximately 200 diseases have been reported in wheat, 50 of which cause economic losses, varying according to region and climate (Wiese et al., 2000). Among all pathogens, fungi are the main and most common agents of disease (Wiese, 1987, Bockus et al., 2010).

Among fungal diseases Fusarium Head Blight (FHB) and Fusarium Crown Rot (FCR) disease are two of the most widespread and damaging diseases of cereal crops, including both hexaploid/bread wheat and durum wheat. They are present in most parts of the world (Parry et al., 1995, Bailey et al., 2000, Fernandez et al., 2009).

1.2. *Fusarium* spp.

Fusarium spp. belong to anamorphic Hypocreaceous Ascomycetes (Ascomycota: Hypocreales: Nectriaceae) in the sexual genera Gibberella and Nectria (Liddell, 2003, Moretti, 2009). Members of the genus Fusarium are considered to be some

of the most economically important fungi causing disease in most species of plants, produces mycotoxins, with modes of genetic change with broad evolutionary implications and can be consumed in a processed food (Ma et al., 2010, Geiser et al., 2013). *Fusarium* spp. can cause a wide range of diseases such as ear rot in corn, bakane in rice, *Fusarium* head blight and crown rot in wheat and *Fusarium* patches on many species of cultivated plants other than small grains. Some species of *Fusarium* appear to be ubiquitous, while others are limited to specialized habitats as saprophytes or parasites (Leslie & Summerell, 2006).

The genus Fusarium was first described by Link, a German mycologist, in 1809, as a large, common group of fungi that could grow on many substrates such as soil, water and either living or dead organic substrates (Stack, 2003). More than 1000 *Fusarium* species had been described by the end of the 19th century and it was difficult to differentiate species within the genus. Wollenweber and Reinking (1935) work reduced the 1000 species to about a 100 taxonomic entities with 65 species and 55 varieties. Since then, the number of defined taxa has ranged from the nine species described by Snyder and Hansen (1945), to 44 species and seven varieties described by Booth (1971); and more than 70 species and 55 varieties described by Gerlach and Nirenberg (1982). Leslie and Summerell (2006) recognised 70 species based on morphological, biological and phylogenetic criteria. This instability in nomenclature and classification of *Fusarium* species has made it difficult to identify species. Currently, Fusarium comprises 300 phylogenetically distinct species that have been discovered via molecular phylogenetics; however, most of these species have not yet been described formally (Aoki et al., 2014).

1.2.1. Fusarium Crown Rot and Head Blight

1.2.1.1. History and biology of Fusarium Crown Rot

Crown rot is known by a variety of names including dryland foot rot, dryland root rot, foot rot, Fusarium crown rot, Fusarium root rot and common root rot (Paulitz et al., 2002). The disease is caused by several pathogens. Different pathogens are dominant in different areas or even by different pathogens during successive growing seasons in individual fields (Paulitz et al., 2002, Cook, 2010, Backhouse, 2014). The disease is primarily caused by *F. culmorum* and *F. graminearum* (Fernandez & Chen, 2005). Although crown rot has received less attention than FHB worldwide, it occurs in most cereal producing regions of the world including Europe, Australia, North America, South America, West Asia, South Africa, and North Africa. *Fusarium* species limit yield by rotting seed, seedlings, roots, crowns, basal stems, or heads (Smiley et al., 1996, Paulitz et al., 2002, Smiley et al., 2003). Infection of seedlings and basal stems leads to yield loss from damaged seedlings, pre-harvest lodging, and impaired grain filling (Schilling et al., 1996).

The symptoms of FCR disease are well characterized (Fig. 1.1). Typical symptoms of crown rot include a honey-brown discoloration (with an occasional pink tinge) of the subcrown internode (one, two and sometimes three internodes) extending up into the crown, and the basal leaf sheaths and stem show a brown necrosis (Scherm et al., 2013). Infection of the crown region leads to destruction of the vascular system and disruption of water movement and prevents recovery of infected plants from water stress, resulting in premature death of the tiller and the subsequent formation of 'white heads' containing little to no seed (Matny, 2015). There are two

types of infection on the roots: the most common is directly associated with the sub-crown internode; rarely, other lesions occur as discrete entities on seminal and secondary roots (Fig. 1.1) (Burgess et al., 2001, Nicol et al., 2007).

Fig. 1.1. The symptoms of Fusarium Crown Rot disease of wheat. The symptoms first appear as a honey-brown discoloration on the subcrown internode extending up into the crown, then brown necrosis on the basal leaf sheaths and stem (Source: http://www.agricentre.basf.co.uk/BASF-Disease-Encyclopedia).

1.2.1.2. History and biology of Fusarium Head Blight

Fusarium head blight (FHB), also called scab, is a common fungal disease of wheat, barley, oats and maize. The disease is an economically important disease that results in reduced grain quality and yield and straw production (Parry et al., 1995). FHB was first described by W.G. Smith in England in 1884 as wheat scab and *Fusisporium culmorum* later described as the causal agent (McInnes & Fogelman, 1923). Chester (1890) gave the first detailed description of FHB. Later in the same century, Arthur (1891) and Detmers (1892) both reported that scab was an important disease of wheat. Atanasoff (1920) argued that scab was not a suitable common name and used the term Fusarium blight. Dounin (1926) again changed

the common name to 'fusariosis'. The disease is currently known as scab or FHB (Stack, 2003).

Since the late 1930s, severe FHB epidemics have been documented in Australia (1978 and 1983), Canada (1939-1943, 1980, 1993 and 1994) (Sutton, 1982, Fernando et al., 1997, Stack, 2003), China, Brazil, Argentina, Central Europe, Kenya, USA, UK and several other countries (Windels, 1999, Muthomi & Mutitu, 2003, Goswami & Kistler, 2004, Muthomi et al., 2008, Xu et al., 2008b, Madden & Paul, 2009, HGCA, 2015b).

Several species of Fusarium have been identified in association with FHB (Liddell, 2003). The number of species causing disease is at least 17, of which *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. langsethiae*, *F. poae*, *Microdochium nivale* and *M. majus* are the most regularly important species (Parry et al., 1995, Ruckenbauer et al., 2001, Xu et al., 2005).

In the UK, *F. culmorum* and *F. graminearum* are more important because they are the major causes of deoxynivalenol (DON) mycotoxin contamination of wheat grain. The distribution of *F. graminearum* and *F. culmorum* is most likely linked to climate as several studies suggest that *F. culmorum* is the dominant pathogen in cooler/wetter climates (Backhouse & Burgess, 2002, Strausbaugh et al., 2004, Smiley et al., 2005, Xu et al., 2005). However, in the UK, there appears to be no trend associated with mean temperature for years when *F. graminearum* has predominated over *F. culmorum* and vice versa (West et al., 2012). Since 1998, when monitoring of pathogen incidence began, significant changes in the level of occurrence and distribution of both *F. culmorum* and *F. graminearum* have

occurred. Overall, there has been a downward trend in the prevalence of *F. culmorum*. Conversely, *F. graminearum* has increased in prevalence. Between 1998 and 2002, isolations of *F. graminearum* were primarily from crops in the south-west and south-east of England. Since 2002 the distribution in occurrence of *F. graminearum* has spread northwards. *F. graminearum* is generally regarded as producing larger losses in yield and more mycotoxin than *F. culmorum* (Jennings & Humphries, 2009, CropMonitor, 2015). Microdochium species, both *M. nivale* and *M. majus*, can be part of the Fusarium species complex and are associated with regions of relatively cool/moderate temperatures and frequent rainfalls of short duration. It is believed that both Microdochium species do not produce mycotoxins (Xu et al., 2008a).

The first symptoms of FHB infection are characterised by the appearance of water-soaked brown-coloured lesions of 2-3 mm in length (Fig. 1.2) (Xu, 2003). The symptoms appear within 2-4 days after infection under favourable conditions, mostly at the base of the middle spikelets in the middle of the head (Stack, 2003). Infections can occur as early as spike emergence, but the flowering stage or shortly after is considered the most vulnerable stage for *Fusarium* infection. Soon after the water soaking appears, symptoms spread to the rachis. Through the rachis the fungus can rapidly spread up, down and horizontally in the spike (Goswami & Kistler, 2004, Madgwick et al., 2011). Frequently, salmon to pink coloured fungal growth and orange coloured sporodochia can be seen at the base of the spikelets or along the edge of glumes (Nicholson et al., 2007). In most cases, in susceptible cultivars of wheat, fungal growth in the rachis causes vascular occlusion cutting off

the nutrient and water supply to spikelets above the point of infection, causing the entire head to be bleached (Fig. 1.2). Bleached spikelets are sterile or contain kernels that are shrivelled and/or appear chalky white or pink; those are often referred to as *Fusarium* damaged kernels, scabby kernels, or tomb-stones. Apparently, healthy kernels may also be infected, especially if infection occurred late in kernel development (Shaner, 2003, Steffenson, 2003).

Fig. 1.2. The symptoms of Fusarium Head Blight disease of wheat. The symptoms first appear at the base of the middle spikelets in the middle of the head as water-soaked brown-coloured lesions with salmon to pink coloured fungal growth. The fungal growth causes vascular occlusion cutting off the nutrient and water supply to spikelets, causing the entire head to be bleached.

1.2.1.3. Life cycles of Fusarium Crown Rot and Head Blight

Different sources of inoculum for the development of FCR and FHB are known. These sources are crop residues of various plants from previous seasons, such as wheat, maize, barley, soybean and rice (Parry et al., 1995, Champeil et al., 2004, Osborne & Stein, 2007). Fusarium species overwinter in soil and crop residues and can survive for several seasons as saprophytes on dead host tissues, especially if susceptible crops are planted in successive years (Fig. 1.3) (Shaner, 2003, Leplat et al., 2013). The common survival structures of FCR in the soil, in dead organic matter and in crop residues are chlamydospores, macroconidia, and mycelium (Cook, 1981, Paulitz et al., 2002). *F. culmorum* survives most commonly as thick-walled chlamydospores in the soil embedded in organic matter or formed within macroconidia, while *F. graminearum* survives most commonly as mycelium inside non-decayed plant residues. Chlamydospores have the potential for long-term survival in soil and plant debris. They can form from macroconidia (endoconidial chlamydospores) or hyphae (mycelial chlamydospores) (Pisi & Innocenti, 2001). The most important sources of inoculum for FHB are ascospores from the sexual stage and macroconidia from the anamorph stage (Bai & Shaner, 1994, Leplat et al., 2013). The dispersal of inoculum from residue, especially maize, from previous seasons to the wheat heads is a critical event in the disease cycle (Fig. 1.3) (Blandino et al., 2010).

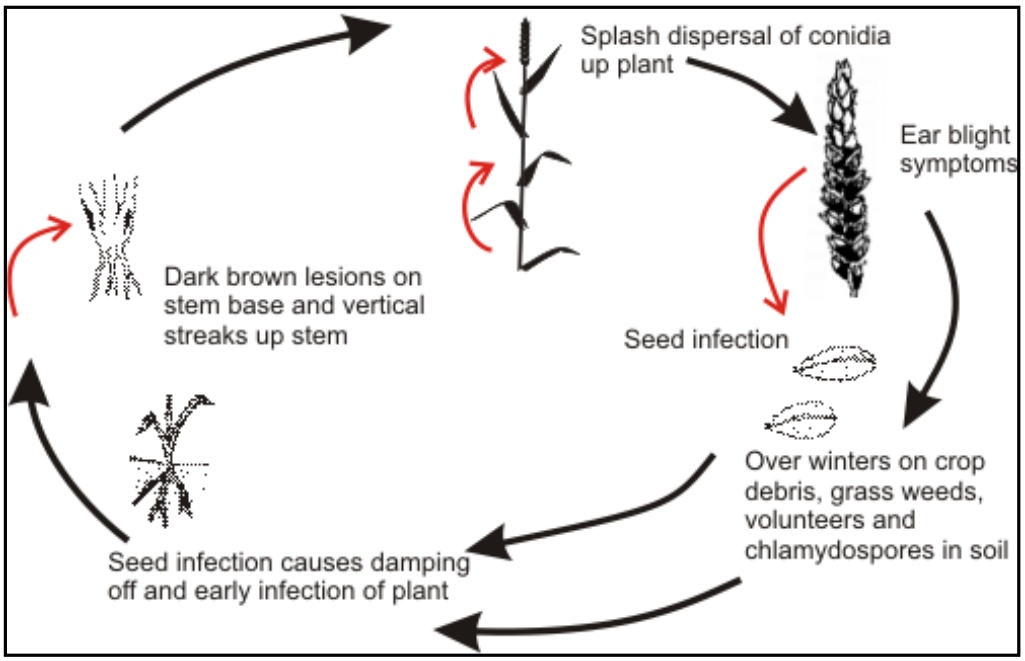

Fig. 1.3. Life cycles of Fusarium Crown Rot and Head Blight diseases of wheat (source: www.HGCA.com).

Environmental factors such as temperature, moisture and wind have an impact on FHB inoculum production and release and dispersal of spores (Shaner, 2003, Goswami & Kistler, 2005, Madgwick et al., 2011). During warm, moist and windy environmental conditions the ascospores or macroconidia are dispersed by water-splash or air currents onto wheat heads and initiate germination on wheat spikes within three hours of inoculation at an optimal 20-30 °C and by the end of six hours most of these spores will be completely germinated (Shaner, 2003, McMullen et al., 2008, Trail, 2009).

1.2.1.4. Management of Fusarium Crown Rot and Head Blight

In the UK, FCR and FHB problems are largely avoided by certified seed, seed treatment with fungicides, rotation and fungicide application- which has to be almost precise (HGCA, 2015), 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). Different Fusarium species produce different mycotoxins under different environmental conditions (Kokkonen et al., 2010). The long-term survival of the pathogen in plant debris or grass weeds, along with the lack of commercial cultivars with resistance to Fusarium, makes controlling the diseases difficult (Wildermuth et al., 1997). The effects of agronomic practices on these diseases are often unpredictable (Bailey et al., 2000) and depend on the causal species as well as the environmental conditions (Parry et al., 1995, Champeil et al., 2004). Control strategies of FCR and FHB have relied on breaking the disease cycle through management strategies such as crop rotation, stubble management, tillage practice, planting date, biological control, protective fungicides and cultivar resistance (Tinline & Spurr, 1991, Bailey et al., 2000, McMullen et al., 2008, Gilbert & Tekauz, 2011). It appears that Fusarium disease cannot be controlled by any single one of the management strategies mentioned, but may be achieved by combining multiple changes in the agronomic system (McMullen et al., 1997, Yuen & Schoneweis, 2007, McMullen et al., 2012).

Crop rotation with non-host crops, stubble management and tillage practices are environmentally friendly approaches which can be used to reduce the risk of diseases epidemics, because they reduce the amount of inoculum in the crop residue (Parry et al., 1995, Dill-Macky & Jones, 2000, Burgess et al., 2001). Crop rotation leads to a reduction in seedling and in root rot symptoms (Stein, 2010). Crown rot infection of wheat in Australia was reduced by using crop rotation management

with chickpea, canola, and mustard (Kirkegaard et al., 2004). However, about half of the inoculum of *F. culmorum* present after harvest is functional a year later, and about 10 % can survive for nearly two years (Wiese, 1991). The longevity of chlamydospore inoculum of *F. culmorum* makes use of rotation more challenging, as evidenced by experiments that showed a two-year break did not provide effective control of this species (Strausbaugh et al., 2005, Cook, 2010). FHB pathogens have wind-borne ascospores which may be transported for kilometers from a source of inoculum. Therefore, rotation alone is not sufficient to prevent the disease (McMullen, 2002).

The severity of crown rot was less when stubble was burned (Dodman & Wildermuth, 1989, Simpfendorfer et al., 2005), but burning decreases soil organic carbon, soil water storage, and the activity of soil biota, while at the same time increasing the risk of soil erosion by wind and rain. Also burning stubble does not guarantee freedom from FCR. Burning removes only above ground inoculum; the FCR fungus still survives in crown tissue below ground (Simpfendorfer et al., 2005).

The *Fusarium* fungus is stubble-borne, so in a no-till system inoculum becomes concentrated in the previous winter's cereal rows. Use of no-till and conservation tillage system practices in a wheat-fallow production system has been associated with higher levels of Fusarium infections (Smiley et al., 1996, Bailey et al., 2000). Mouldboard or chisel ploughing inverts the soil layer, burying crop residues at the soil surface, caused a significant but small decrease in FHB disease incidence, severity and DON accumulation compared to no-till plots (Dill-Macky & Jones,

2000, Krebs et al., 2000, Pereyra & Dill-Macky, 2008). Tillage does not bury all residues, and seeding operations can bring buried residues to the surface; when in contact with the moist soil surface, such *Fusarium*-infested residues will produce inoculum (Inch & Gilbert, 2003). But none of these treatments has been demonstrated to provide sufficient control to be effective against FHB (McMullen et al., 2012).

Crop planting dates or sowing several cultivars with different heading dates or maturity may help reducing the risk of FHB severity and incidence (McMullen, 2002), but as the weather during flowering cannot be predicted, early or late planting is not an assured option to protect crops (Fernandez et al., 2005).

Biological control also appears to be an environmentally friendly and a possible method to control the Fusarium disease (Schisler et al., 2002). There have been only a few studies of biological control of crown rot disease of wheat so far. Biological control of *F. pseudograminearum* by Trichoderma species (*Trichoderma koningii* and *T. harzianum*) was tested successfully in laboratory conditions (Wong et al., 2002). *F. graminearum* was controlled by the bacterium *Burkholderia cepacia* under laboratory and glasshouse conditions (Huang & Wong, 1998). Several microorganisms including bacteria (*Bacillus* spp., *Kluyvera cryocrescens, Lysobactor* spp., *Paenibacillus fluorescens, Pantoea agglomerans*, and *Pseudomonas fluorescens*), yeasts (*Cryptococcus* spp., *Rhodotorula spp*., and *Sporobolomyces roseus*) and filamentous fungi (*Trichoderma harzianum* and *T. virens*) have shown potential for the control of *F. graminearum* (Corio da Luz et al., 2003, Jochum et al., 2006, Bacon & Hinton, 2007). Musyimi et al. (2012)

indicated that Fusarium disease severity increased over time when antagonistic fungi *Alternaria* spp., *Epicoccum* spp were applied against *F. graminearum* and *F. poae* and associated T-2 toxin. They concluded that antagonists cannot solely be relied on in managing FHB and toxin accumulation. Problems encountered in using biocontrol agents include maintaining their viability, developing delivery mechanisms, incompatibility with fungicides, and inconsistent results (Yuen et al., 2007, Yuen, 2008).

Fungicide application during relevant wheat growing stages can reduce the risk of FHB and mycotoxin contamination (Paul et al., 2008, Edwards & Godley, 2010). However, inconsistent control of FHB disease with fungicide has been found in several experiments (McMullen, 1994, Horsley et al., 2006, Gaurilcikiene et al., 2011). This inconsistency has been attributed in part to fungicide timing and efficacy, cultivar resistance, and application technology, which limits the use of fungicides for FHB management (McMullen et al., 1997, Mesterhazy et al., 2003, Wegulo et al., 2010). Yoshida et al. (2012) indicated that the timing of fungicide application differentially affected FHB disease and mycotoxin concentration, considering anthesis as the crucial stage for fungicide application.

It appears therefore that the development and use of resistant hosts would be the most effective, economical and environmentally safe strategy for Fusarium disease management (Ruckenbauer et al., 2001). There are three types of resistance to FHB in wheat: resistance to initial infection (Type 1), resistance to spread within the head (Type 2) and resistance to mycotoxin degradation (Type 3) (Nicholson et al., 2008, Niwa et al., 2014). Type 2 resistance is perhaps of greatest importance against

DON-producing isolates of *F. culmorum* and *F. graminearum* (Yan et al., 2011). Most current wheat cultivars in the UK possess little Type 2 resistance. Considerable effort has been expended by wheat breeders and researchers to identify and characterise sources of Type 1 resistance in wheat, as this form of resistance should be relevant to protecting against all species of Fusarium, whatever trichothecene compounds they produce, along with the non-toxin producing Microdochium species (Nicholson et al., 2008). Several studies have focused on transgenic wheat made resistant by incorporating plant defense antifungal proteins such as thaumatine-like proteins (Chen et al., 1999, Mackintosh et al., 2007). Though the results of some of these studies have been promising in a glasshouse experiment, they have failed in field environments (Anand et al., 2003). Wheat cultivars with partial resistance are available for commercial cultivation, but immune cultivars are lacking. Breeding for commercial wheat cultivars with high levels of Fusarium resistance with all the other desired agronomic traits is a huge challenge (Bai & Shaner, 2004). Because of the polygenic nature of Fusarium resistance, the variability associated with phenotyping, the effect of environment on resistance phenotype, the complex disease evaluation procedures and an incomplete understanding of the nature of the resistance genetics make the breeding process complicated (Bai & Shaner, 2004, Herde et al., 2008).

1.2.2. Mycotoxins

Mycotoxins are natural toxic substances produced by fungi. The most common Fusarium mycotoxins of concern in UK cereals are trichothecenes: nivalenol (NIV), deoxynivalenol (DON) and its derivatives 3- and 15-acetyldeoxynivalenol (3-

ADON, 15-ADON), T-2 toxin (T2), HT-2 toxin (HT2), and non-trichothecenes: zearalenone (ZON) (Edwards, 2009). These are produced on cereal crops whilst in the field. During the infection of wheat by FCR, DON is produced in the wheat stem base. DON is an inhibitor of protein synthesis, thus may suppress the production of host defense enzymes (Mudge et al., 2006). They exist in our diet as a result of the presence of specific fungi on food crops, either in the field or in store. Mycotoxins can be hazardous to the health of humans and animals even at low concentrations. Mycotoxins cause reduced feed intake, reduced grain weight and vomiting in farm animals, while high levels of mycotoxins have been shown to adversely affect growth and immune systems in animal studies. Nausea, vomiting, diarrhea, abdominal pain, headache, dizziness and fever have been reported when high concentrations of mycotoxin were consumed by humans (Antonissen et al., 2014). The major sources of dietary intake of Fusarium mycotoxin are products made from cereals, in particular wheat and maize. European Union legislation has set a legal limit for DON of 1250 µg kg^{-1} and ZON of 100 µg kg^{-1} for cereals intended for human consumption (Anon, 2006), but even a low level contamination of grain can reduce market prices or cause the grain to be rejected entirely (Parry et al., 1995, Fernandez & Chen, 2005). Mycotoxin levels vary from year to year, so the risk is greater in some years than others, depending on weather conditions and intensity of host crops present within a region (Bai & Shaner, 1994, Häggblom & Nordkvist, 2015).

Weather is an important risk factor in increasing mycotoxin concentration. Cereals are particularly susceptible to infection if there is rain when they are in flower. Once

infection has occurred further rainfall during the summer, particularly once the crop has ripened, allows secondary infections to occur on exterior of seeds, glumes and rachis (West et al., 2012, Xu et al., 2013). Although the risk factors of weather and regional factors cannot be controlled, there are a number of other agronomic factors which can be modified to reduce the risk of exceeding legal limits for the occurrence of Fusarium mycotoxins. Good agricultural practice in the UK, based on current knowledge, includes specific practices in rotation design, crop residue management, cultivar choice, weed control, insect control, fertiliser use, fungicide use, harvest and drying of grain. The benefits of each component are cumulative so that by combining as many of the components as possible the risk of exceeding legal limits may be minimised. The risk cannot be completely removed. For example, even moderately resistant cultivars sown into moderate to high levels of crown rot inoculum are at risk of yield losses; and moisture stress during grain filling produces significant yield loss regardless of resistance level (Food Standards Agency, 2007). HGCA (2015c) published a risk assessment for Fusarium mycotoxins in wheat to ensure the wheat grain is safe for human consumption. HGCA risk assessment score is required on the grain passport.

1.3. Root symbiosis

The term symbiosis (from the Greek: sym, "with"; and biosis, "living") commonly describes close and often long-term interactions between different biological species. The term was first used in 1879 by the German mycologist, Heinrich Anton de Bary, who defined it as: "the living together of unlike organisms". The definition of symbiosis is in flux and the term has been applied to a wide range of biological

interactions (Parniske, 2004). Symbiotic relationships include those associations in which one organism lives on another (ectosymbiosis), or where one partner lives inside the other (endosymbiosis). Among all endosymbioses in natural ecosystems, the most widespread symbiotic interactions are formed between plants and fungi (Garcia-Garrido & Ocampo, 2002, Harrison, 2005, Brachmann & Parniske, 2006). Among the best studied symbioses between plant roots and fungi are mycorrhizas, but non-mycorrhizal association are increasingly of interest (Weiss et al., 2011).

1.3.1. Endophytic fungi

Non-mycorrhizal fungi associated with plants are highly diverse; some of them are endophytes (Dutta et al., 2014). Endophytes are defined as microorganisms that accomplish parts of their life cycle within living host tissues without causing apparent damage to the plant (Schulz & Boyle, 2005, Sun et al., 2014). In all ecosystems, many plant parts are colonized by fungal endophytes (Brundrett, 2002, Sieber, 2002, Mandyam & Jumpponen, 2005). Depending on the invader and the interaction, endophytes may be located in roots, leaves or needles, roots and shoots, or adapted to growth within the bark (Sokolski et al., 2007, Verma et al., 2007, Grunig et al., 2008, Rodriguez et al., 2009). Fungal endophytes may grow inter– and intra–cellulary as well as endo– and epi–phytically (Schulz & Boyle, 2005, Zhang et al., 2006). The behaviour of fungal endophytes can range from mutualistic (Usuki & Narisawa, 2007, White & Torres, 2010) to pathogenic (Tellenbach et al., 2011) and endophytes can switch their behaviour depending on environmental factors. This variation in relationship is described as the endophytic continuum (Schulz & Boyle, 2005).

Plant growth promotional effects of endophytes have received increasing attention in the hope that they will provide a consistent and effective increase in the productivity of crops. Endophytic fungi may increase plant resistance to biotic stresses, including microbial infections (Lewis, 2004, Rodriguez et al., 2004, Waller et al., 2005, Waqas et al., 2012, Dutta et al., 2014), insect pests (Breen, 1994, Vázquez et al., 2004, Kumar et al., 2008, Lopez & Sword, 2015) and herbivore attack (Schardl & Phillips, 1997, Mandyam & Jumpponen, 2005, Gange et al., 2012, Hammer & Van Bael, 2015). They may also increase plant tolerance to abiotic stresses such as drought (Cheplick et al., 2000, Hubbard et al., 2014, Khan et al., 2015), heavy metals (Monneta et al., 2001, Khan & Lee, 2013, Dourado et al., 2015), culture medium pH lower than optimal (Lewis, 2004), and high salinity (Waller et al., 2005, Halo et al., 2015). They also improve the absorption of nitrogen (Lyons et al., 1990, White et al., 2012, Dourado et al., 2015) and phosphorus (Gasoni & deGurfinkel, 1997, Malinowski et al., 1999, Dourado et al., 2015) and as a consequence produce improved yield (Schulz & Boyle, 2005, Colla et al., 2015, Murphy et al., 2015a).

1.3.2. Arbuscular mycorrhizal fungi

Mycorrhizal refers to Greek “mycos” meaning fungus and “rhiza” meaning root. Arbuscular mycorrhizas (AM) are named from the treelike structures formed inside root cortical cells, called arbuscules (Mosse, 1957, Gerdemann, 1965, Mosse & Hayman, 1971, Parniske, 2008, Jung et al., 2012). A symbiosis with AM is formed by 70-90 % of land plant species, and is thought to be the most widespread terrestrial symbiosis (Fitter, 2005, Smith & Read, 2008, Griffis et al., 2014, Walder

et al., 2015). Such symbioses are generally regarded as mutualistic, with a bidirectional transfer of nutrients (Smith & Read, 2008, Smith et al., 2011, Martínez-García et al., 2015). The fungi obtain fixed carbon compounds from host plants, while plants benefit from increased nutrient supply (e.g. phosphorus), or water supply, or enhanced stress tolerance and resistance (Solaiman & Saito, 1997, Bago et al., 2003, Finlay, 2008, Martínez-García et al., 2015). Bago et al. (2000) estimated that up to 20 % of the photosynthetic products of terrestrial plants are consumed by AM fungi. Therefore, AM symbiosis is thought to significantly contribute to global phosphate and carbon cycling and to affect productivity in land ecosystems (Fitter, 2005, van der Heijden et al., 2015). As AM fungi are obligate symbionts, they are not yet successfully cultured in the absence of plant root (Johnson et al., 1997, Buscot, 2015). Axenic fungal biomass can be obtained only from cultures on transformed plant roots, but only a small number of species are available in culture (Redecker & Raab, 2006).

1.3.2.1. Taxonomy

Fossil records suggest that the AM symbiosis dates back to the Ordovician age, 460 million years ago (Redecker et al., 2000). Based on small subunit (SSU) rDNA sequences and their symbiotic lifestyle, the AM fungi were placed in the phylum Glomeromycota (Schüβler et al., 2001). The Glomeromycota is divided into five orders, 14 families and 29 genera and approximately 230 species (Oehl et al., 2011a, Oehl et al., 2011b, Palenzuela et al., 2011, Redecker et al., 2013).

1.3.2.2. Colonization strategy of arbuscular mycorrhizal fungi

Spores of AM fungi are usually formed on the extraradical hyphae, but some species also may form spores inside the roots. During the formation of the symbiosis, AM hyphae approach the roots and form swollen appressoria. Then the hyphae grow between the root cortical cells, penetrate the cell walls, and form highly branched (arbuscules) or coil shaped hyphal structures. This creates a very large surface area between the two symbionts, across which metabolic exchange can take place (Rodrigues & Rodrigues, 2015). Once the plant root is colonised, the AM fungus produces runner hyphae, forming the extraradical mycelium, which is used by the fungus to explore the soil for resource several centimetres from the colonised roots (Jakobsen et al., 1992, Cano & Bago, 2005, Mensah et al., 2015). Colonisation of roots by AM fungi can arise from spores, infected root fragments and/or hyphae. The absorbing hyphae develop from the runner hyphae and form a network of thin hyphae extending into the soil. These hyphae appear to be the component of the fungus that absorbs nutrients from the soil for transport to the host (Gadkar et al., 2001, Varela-Cervero et al., 2015).

1.3.2.3. Beneficial effect of arbuscular mycorrhizal fungi symbiosis on host plants

In a mutualistic symbiosis, both partners (fungus and plant) gain from the symbiosis. Carbon from the photosynthesis is used by the fungus and the plant makes use of the extended soil volume (Finlay, 2008). In return for the carbon, the mycorrhizal plant obtains nutrients. Phosphorus, which occurs in inorganic or organic forms in soil, is in many ecosystems the most important nutrient whose

uptake is mediated by AM fungi. Inorganic phosphate, as well as other inorganic nutrients such as zinc, is relatively immobile in the soil, which leads to the formation of zones depleted in inorganic phosphorus around the roots (Hart & Forsythe, 2012). These depletion zones effectively limit phosphorus uptake in non-mycorrhizal plants. The symbiotic association with AM fungi allows the plant to access phosphorus beyond the depletion zone through the extraradical fungal hyphae, in addition to the root uptake. AM fungi hyphae can also absorb nitrogen in the forms of ammonium and nitrate, and contribute to the uptake of micronutrients, such as zinc (Jansa et al., 2013, Meng et al., 2015). Another fundamental factor for plant growth is water availability and AM symbiosis increases plant tolerance to drought (Auge, 2004, Auge et al., 2008, Ortiz et al., 2015). AM fungi also increase plant resistance to pathogens and heavy metals (Davies et al., 2001, Tonin et al., 2001, Rivera-Becerril et al., 2002, Krishnamoorthy et al., 2015, Nair et al., 2015).

1.3.3. Sebacinales

The members of order Sebacinales are involved in mycorrhizal associations. They occur worldwide and encompasses a great multitude of ericoid, orchid, cavendishoid (ectendomycorrhizas colonising the Andean clade of Ericaceae) and jungermannioid mycorrhizae (the symbiotic fungal associations in leafy liverworts) and ectomycorrhizae, which are associated with the roots of a wide variety of plant species (Weiss et al., 2004, Setaro et al., 2006, Selosse et al., 2007). The order was first described by Weiss et al. (2004). Sebacinales are a taxonomically, ecologically, and physiologically diverse group of fungi in the Basidiomycota. This order

includes fungi with longitudinally septate basidia and imperforate parenthesomes (or septal pore caps; these are parenthesis-shaped structures on either side of pores in the dolipore septum which separates cells within a hypha). They also lack cystedia (a relatively large cell found on the hymenium of a basidiomycete, used for identification) and clamp connexions (a structure formed by growing hyphal cells to ensure each cell, or segment of hypha separated by septa, receives a set of differing nuclei, to create genetic variation within the hypha) (Weiss et al., 2004).

This order is monotypic, containing a single family, the Sebacinaceae, which was described by Wells and Oberwinkler (1982). Based on the ultrastructural and microscopic characters, Bandoni (1984) placed the Sebacinaceae family in the order Auriculariales, a group of wood-decaying fungi. However, molecular phylogenetic studies by Weiss and Oberwinkler (2001) have proved that the family Sebacinaceae does not belong to the Auriculariales and it belongs to the new described order Sebacinales (Weiss et al., 2004). This is interesting, since species of the Sebacinaceae are morphologically very similar to members of the Auriculariales, sharing characters like the longitudinally septate basidia. There are eight genera and 29 species in the family collected from Germany, Switzerland, France, Italy, Austria, Slovenia, Great Britain, the United States, Ecuador, Ethiopia, Namibia, North Africa, South Africa, and Iceland with no geographical or host patterns. DNA sequences derived from plant roots showed that members of this family are involved in a wide spectrum of mycorrhizal types (Weiss et al., 2011). It is possible that a mycorrhizal life strategy, which was transformed into a saprotrophic strategy

several times, is a character for the Sebacinales, as more basal taxa of basidiomycetes consist of predominantly mycoparasitic and phytoparasitic fungi (Weiss et al., 2004).

Phylogenetic analyses based on nuclear sequences of the large ribosomal subunit distinguish two subgroups A and B within the order Sebacinales. These groups differ in their ecology (Weiss et al., 2004). Orchid mycorrhizas and ectomycorrhizas belong to subgroup A. The second subgroup is more diverse and contains ericoid, cavendishoid and jungermannioid mycorrhiza, *Sebacina vermifera*, the endophytic *Piriformospora indica* and some multinucleate Rhizoctonia (Weiss et al., 2004).

1.3.3.1. *Piriformospora indica*

1.3.3.1.1. *P. indica* classification

The root-colonizing endophytic fungus *Piriformospora indica* was first isolated as a contaminant of cultures of the AM fungus *Funneliformis* (=*Glomus*) *mosseae* from the rhizosphere of the woody shrubs *Prosopsis juliflora* and *Zizyphus nummularia* in the sandy desert soils of the Thar region of northwest India in 1997 by Ajit Varma and his collaborators (Verma et al., 1998). Based on ultrastructural analyses of hyphae, 18S-rRNA gene sequences and rRNA sequence at the 5´-terminal domain of the ribosomal large subunit (nucLSU), *P. indica* was grouped in class B of the order Sebacinales.

P. indica, within the Sebacinales, has a close genetic similarity to *Sebacina vermifera sensu* Warcup & Talbot and *Rhizoctonia zeae* and *R. solani* (Fig. 1.4) (Warcup, 1988, Milligan & Williams, 1998, Weiss et al., 2004).

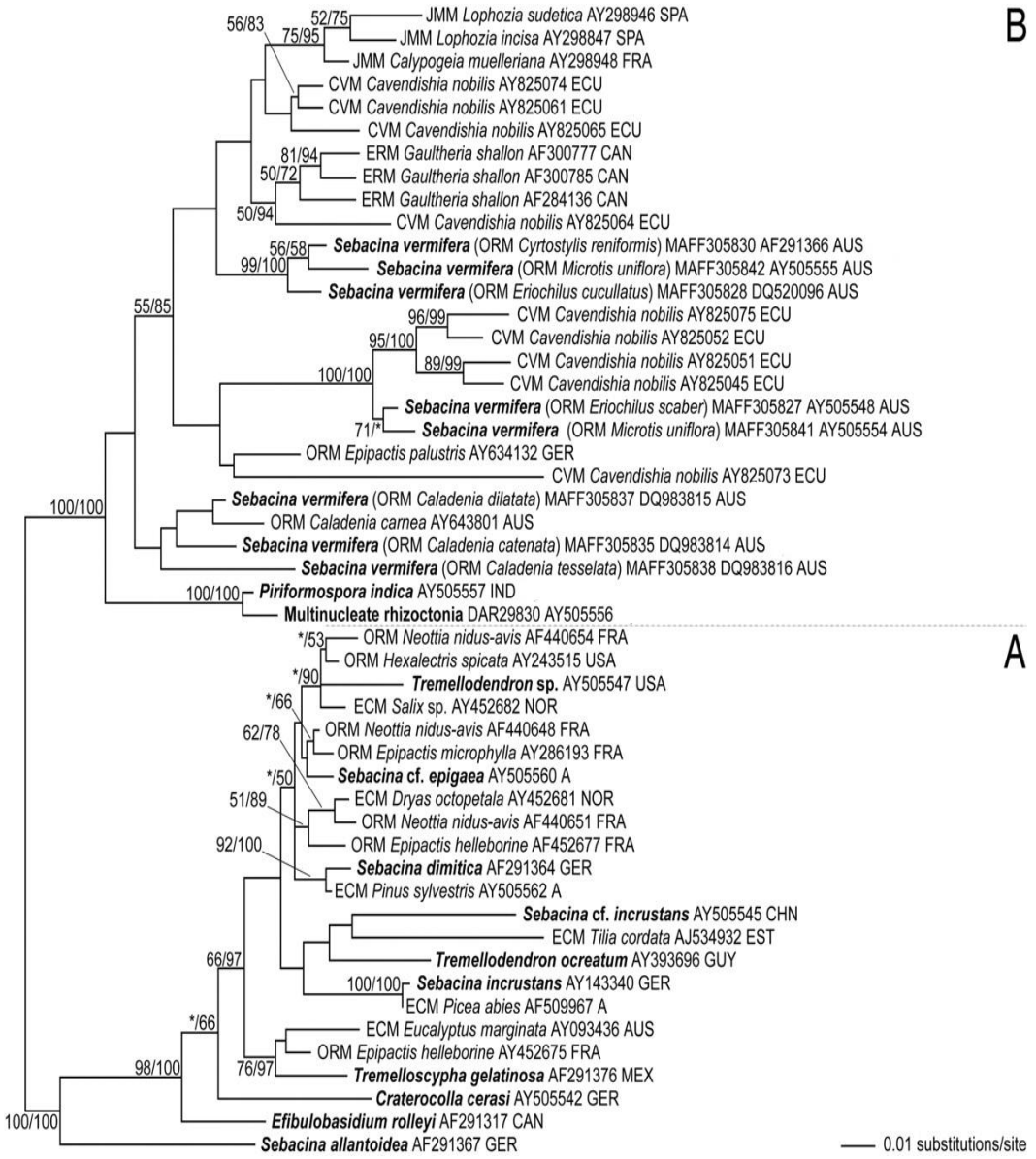

Fig. 1.4. Phylogenetic placement of *Piriformospora indica*, *Sebacina vermifera* and Rhizoctonia within Sebacinales group B, estimated by maximum likelihood from an alignment of nuclear rDNA coding for the 5' terminal domain of the ribosomal large subunit (Source: Deshmukh et al. (2006)).

1.3.3.1.2. Colonization method by *P. indica*

Morphologically, the hyphal cells of *P. indica* are thin walled, hyaline and not pigmented. Hyphae are irregularly septate and 0.7 to 3.5 μm in diameter. Septate hyphae often show anastomosis (Fig. 1.5 a). Each hyphal segment is multinucleate with variable numbers of nuclei. Hyphal tips differentiate into chlamydospores of 16-25 μm in length and 10-17 μm in width, which emerge individually or in clusters. Each spore contains 8-25 nuclei (Fig. 1.5 b). So far, neither clamp connexions nor sexual structures have been observed. Most of the mycelium of *P. indica* grows under the surface of agar media. Using solid culture media, only a few aerial hyphae are formed. The mycelium grows concentrically and covers agar media homogenously. Sometimes the mycelium forms rhythmic rings in the Petri dishes. Young mycelium cultures are white but with age the colour turns to cream yellow (Varma et al., 2001, Kost & Rexer, 2013).

The colonization procedure of *P. indica* starts with the germination of chlamydospores on the root surface. The growing hyphae form an extracellular net, then enter the root cortex and form inter- and intra-cellular hyphae. Within the cortical cells and rhizodermal cells, the fungus often forms dense hyphal coils or branched structures intra-cellularly (Fig. 1.5 c). This phase seems to be associated with host cell death. *P. indica* also forms spore- or vesicle-like structures within or between the cortical cells. Nevertheless, the fungus is never observed to traverse the endodermis and vascular tissue. It predominantly colonizes the root maturation zone. Likewise, it does not invade the plant meristematic zone or the aerial portion

of the plant. Fungal colonization results in extracellular and intracellular formation of chlamydospores (Fig. 1.5 d) (Deshmukh et al., 2006, Schäfer et al., 2009).

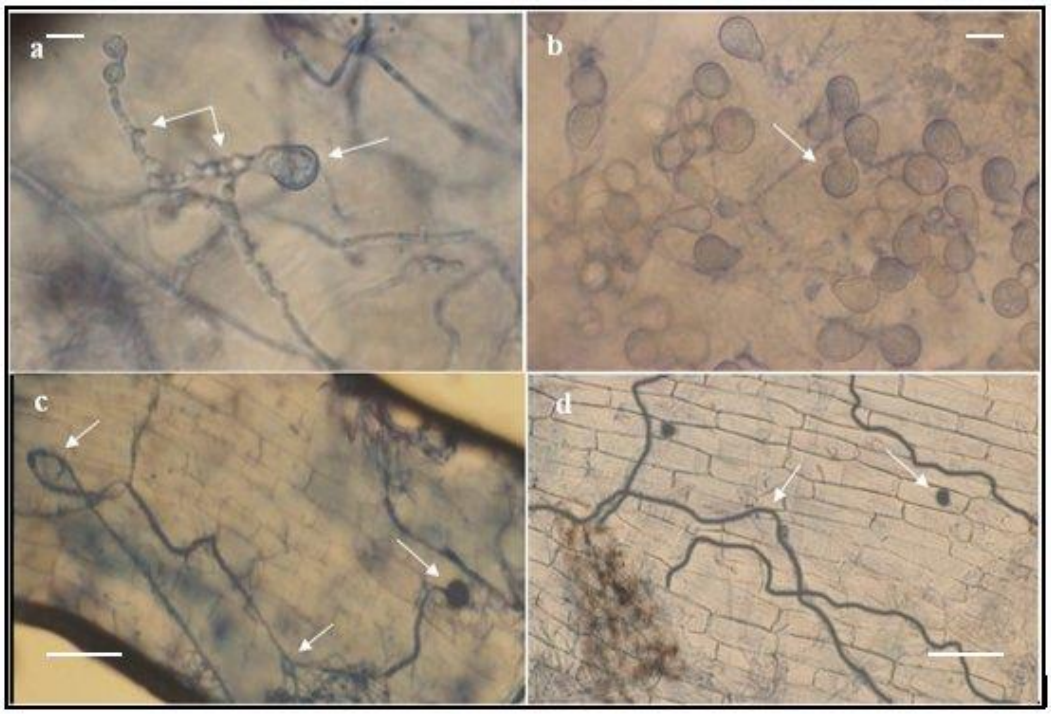

Fig. 1.5. *Piriformospora indica* hyphae and chlamydospores in agar plates (a,b; scale bar: 10 µm) and in wheat roots (c,d; scale bar: 20 µm). The fungus often forms dense hyphal coils or branched structures intracellularly and was not detected in endodermic and central parts of the root. Arrows indicate *P. indica* clamydospors and hyphae.

1.3.3.1.3. Beneficial effects of *P. indica* symbiosis on host plants

P. indica, like AM fungi, has plant growth promoting effects. In contrast to AM fungi, it can be cultured axenically on various media (Varma et al., 1999). *P. indica* has been shown to form mutualistic symbioses with a broad range of host plants including major crop plants, model organisms like Arabidopsis, tobacco and barley, and a range of economically important monocot and dicot hosts (Table 1.1) (Weiss et al., 2004, Waller et al., 2005, Deshmukh et al., 2006). The ability of *P. indica* to improve the growth rate of various host plants is well documented (Varma et al., 1999, Pham et al., 2004, Waller et al., 2005). For barley, an increase in plant biomass and final grain yield was demonstrated under greenhouse as well as out-door conditions (Waller et al., 2005, Achatz et al., 2010 a). Tomato plants that were grown in hydroponic culture and inoculated with *P. indica* showed an increase in fruit biomass and dry weight per plant (Fakhro et al., 2010). In Chinese cabbage, *P. indica* promoted shoot and root growth and lateral root development and increased plant tolerance against drought stress (Sun et al., 2010). Also, *P. indica* increased wheat tolerance under drought stress (Yaghoubian et al., 2014). The growth parameters (root and shoot lengths, fresh and dry weights) of rice seedlings were enhanced in *P. indica*-inoculated rice seedlings under high salt stress (Jogawat et al., 2013). Similarly *P. indica* could induce tolerance to salt stress in barley (Waller et al., 2005). *P. indica* also confers increased resistance to various plant pathogens in several hosts. Recent studies have shown that *P. indica* is able to increase resistance in barley against the necrotrophic root pathogens *F. culmorum* and *Cochliobolus sativus* (Waller et al., 2005, Deshmukh & Kogel, 2007) and to induce

systemic resistance in leaves of barley and *Arabidopsis thaliana* against the powdery mildew fungi *Blumeria graminis* f.sp. *hordei* and *Golovinomyces orontii*, respectively (Waller et al., 2005, Stein et al., 2008). Data collected from both greenhouse and out-door experiments showed reductions in symptom severity caused by stem rot (*Pseudocercosporella herpotrichoides*), root rot (*Fusarium culmorum*) and soil-borne take-all disease (*Gaeumannomyces graminis* var. *tritici*) in wheat (Serfling et al., 2007, Ghahfarokhy et al., 2011). This evidence makes *P. indica* a promising candidate for biological control of plant diseases (Table 1.1).

Table 1.1. Effects of *Piriformospora indica* on a range of economically important crops**.**

***P. indica* Effects**	**Crop**	**Reference**
Increased growth and yield Increased resistance against pathogens: -root diseases caused by: *F. culmorum*, *F. graminearum*, *Cochliobolus sativus*; -leaf diseases caused by: *Blumeria graminis* f.sp. *hordei.* Increased tolerance against abiotic stress: salt stress Improved nitrogen and phosphorus uptake	Barley	Waller et al. (2005, 2008) Deshmukh & Kogel, (2007) Achatz et al. (2010) Harrach et al. (2013)
Increased growth and yield Increased resistance against pathogens: -stem disease caused by *Pseudocercosporella herpotrichoides*); -root disease caused by *F. culmorum* and *Gaeumannomyces graminis* var. *tritici*; -leaf diseases caused by: *Blumeria graminis* f.sp. *tritici.* Increased tolerance against abiotic stress: salt and drought stresses	Wheat	Serfling et al. (2007) Ghahfarokhy et al. (2011) Yaghoubian et al. (2014)
Increased yield Increased resistance against pathogens: -root disease caused by: *F. verticillioides*	Maize	Kumar et al. (2009)
Increased yield Increased phosphorus uptake Increased tolerance against abiotic stress: salt stress	Rice	Jogawat et al. (2013) Das et al. (2014)
Increased fruit growth and fruit biomass Increased resistance against fungal pathogens: -*Verticillium dahliae* and F. *oxysporum* Increased resistance against viral pathogens: -virus: Pepino mosaic virus & Tomato yellow leaf curl virus Increased tolerance against abiotic stress: salt stress	Tomato	Fakhro et al. (2010) Cruz et al. (2010) Sarma et al. (2011) Wang et al. (2015)
Increased yield	Potato	Upadhyaya et al. (2013)
Increased growth and yield Increased resistance against pathogens: *F. oxysporum*	Lentil	Dolatabadi et al. (2012)

1.3.3.1.4. Mechanism of interaction of *P. indica* with plants

The mechanism by which *P. indica* confers physiological benefits to its host plants is unclear (Ansari et al., 2014). Some research has been done to find out the mechanisms behind the effects of *P. indica* on different hosts:

Growth promotion and production of higher yields as well as stress tolerance may be attributed to the production of phytohormones (like auxins and cytokinins) by the fungus itself, as well as to modulation of the host phytohormones. The growth and reproduction stimulation of Arabidopsis by *P. indica* was due to a diffusible factor that could be the auxin Indole-3 Acetic Acid (IAA), as *P. indica* produces IAA in culture filtrate. It has been suggested that auxin production affecting root growth was responsible, for or at least contributed to, the beneficial effect of *P. indica* on its host plants (Sirrenberg et al., 2007, Vadassery et al., 2008, Dong et al., 2013, Hilbert et al., 2013).

Molitor et al. (2011) demonstrated that colonization of barley roots with *P. indica* induces systemic resistance against the biotrophic leaf pathogen *Blumeria graminis* f.sp. *hordei*. *P. indica* affects the jasmonic acid (JA), ethylene, abscisic acid (ABA) and salicylic acid (SA) plant signalling hormones which regulate the plant's defence system against stresses (Stein et al., 2008, Molitor & Kogel, 2009, Camehl et al., 2010, Molitor et al., 2011, Khatabi et al., 2012, Camehl et al., 2013, Peskan-Berghofer et al., 2015, Vahabi et al., 2015). *P. indica* may also target a not yet identified signalling pathway to induce systemic resistance.

Also in Arabidopsis, it was observed that cell wall extract from *P. indica* promoted growth of seedlings and elevated intracellular calcium (Ca) in roots. The extract

and the fungus activated a set of genes in Arabidopsis roots including some with Ca^{2+} signalling related functions. Ca^{2+} is a ubiquitous intracellular second messenger molecules (Vadassery & Oelmueller, 2009).

Vadassery et al. (2009) demonstrated that ascorbate, monodehydroascorbate reductase and dehydroascorbate reductase mRNA levels were upregulated in Arabidopsis roots colonized by *P. indica*. Also, *P. indica* elevates the concentration of antioxidant enzymes in barley and maize, which may contribute to plant defence against pathogen stresses such as *Fusarium culmoum* and *F. verticillioides* (Kumar et al., 2009, Harrach et al., 2013). *P. indica* increased barley tolerance to salt stress, and conferred resistance against root and leaf pathogens, including the necrotrophic root fungus *F. culmorum* and the biotrophic fungus *Blumeria gramini.* This tolerance to salinity and resistance to pathogens was as a result of higher antioxidant enzyme levels including ascorbate, dehydroascorbate reductase, glutathione (Waller et al., 2005, Baltruschat et al., 2008). The elevation of antioxidant enzyme concentrations by *P. indica* is also reported in other host plants (Prasad et al., 2013). Additionally, Chinese cabbage showed a higher tolerance to drought stress when *P. indica* was present. The enhanced drought tolerance was due to the activation of antioxidant enzymes (peroxidases, catalases and superoxide dismutases) and drought related genes (*DREB2A, CBL1, ANAC072 and RD29A*) and Ca^{2+}-sensing regulator protein by *P. indica* (Sun et al., 2010).

Vahabi et al. (2015) indicated that *P. indica* induced stomata closure, stimulated reactive oxygen species (ROS) production, stress related phytohormone accumulation (JA and its active form JA isoleucine (JA-Ile), 12-oxo-phytodienoic

acid (OPDA), ABA and SA) and activated defense and stress genes (*ALCOHOL DEHYDROGENASE1*, which is up-regulated in roots by osmotic stress), the ethylene-responsive transcription factor gene *ERF105* (which responds to chitin treatment), *INDOLE GLUCOSINOLATE O-METHYLTRANSFERASE1* (which is involved in hydroxylation reactions of the glucosinolate indole ring), the NAC domain transcription factor gene *JUNGBRUNNEN1* (which is induced by hydrogen peroxide (H_2O_2)), *GDSL LIPASE1* (which plays an important role in plant immunity), *ERD11* and the *GLUTHATIONE S-TRANSFERASE TAU10* (which are induced by oxidative stress and bacterial infections), and *ACIREDUCTONE DIOXYGENASE3* (which is involved in systemic acquired resistance) in the Arabidopsis roots and shoots before the two partners were in physical contact. Once a physical contact was established, the stomata re-opened, ROS and phytohormone levels declined, and the number and expression level of defense/stress-related genes decreased. NRT2.5 (belongs to the nitrate transporter family which plays an essential role in plant growth promotion) was expressed in Arabidopsis roots and leaves at two and six days after inoculation (dai), respectively.

Zuccaro et al. (2011) showed that about 10 % of *P. indica* genes induced during the biotrophic colonization encoded putative small secreted proteins, including several lectin-like proteins and members of a *P. indica*-specific gene family with a conserved novel seven-amino acid motif at the C-terminus. They found 579 genes in the prepenetration phase (36–48 hours after inoculation), 397 genes in the early colonization phase (3 dai), and 641 genes at 5 dai that were differentially regulated compared to autoclaved roots.

Pedrotti et al. (2013) demonstrated that initial *P. indica* colonization triggered a local, transient response of several defense-related transcripts, of which some were also induced in shoots and in distal, non-colonized roots of the same plant. SA-responsive CBP60 (calmodulinbinding protein 60-like G), SA-regulated PR1 (pathogenesis-related protein 1), JA-regulated VSP2, gibberellin-regulated ExpPT1 (phosphatidylinositol N-acetylglucosaminyltransferase subunit P-related), ethylene responsive ERF1 transcripts, OXI1 (oxidative signal inducible1), MYB51 (indicative for glucosinolate production), mitogen-activated protein kinase 3 (MPK3) were all elevated in the root and/or shoots within one to seven days after inoculation with *P. indica*. Faster and stronger induction of defense-related transcripts during secondary inoculation revealed that a *P. indica* pretreatment triggered root-wide priming of defense responses, which could cause the observed reduction of secondary colonization levels. Secondary *P. indica* colonization also induced defense responses in distant, already colonized parts of the root.

Nitrogen, phosphorus and potassium uptake by plants were found to be increased in *Cicer arietinum*-inoculated with *P. indica* as compared with un-inoculated control plants (Nautiyal et al., 2010). In barley, *P. indica* increased final grain yield independently of fertilisation level. Grain yields were higher when phosphorus and nitrogen supply were high, indicating that *P. indica* induced yield increase was independent of low phosphorus and nitrogen supply (Achatz et al., 2010).

Malla et al. (2004) and Yadav et al. (2010) reported that *P. indica* contains substantial amounts of an acid phosphatase which has the potential to solubilise phosphate in the soil and deliver it to the plant. It was also demonstrated that growth

promotion of Arabidopsis seedlings by *P. indica,* in Petri dishes containing MMN culture medium, was associated with a massive uptake of phosphate from the growth medium to the aerial parts of the seedlings (Shahollari et al., 2005). *P. indica* also significantly enhanced activity of acid phosphatase and alkaline phosphatase in the rhizosphere soil of rice plants, contributing to higher phosphorus uptake (Das et al., 2014).

P. indica activates nitrate reductase in tobacco and Arabidopsis roots *in vitro* and *in vivo,* which plays a major role in nitrate acquisition and mediate nitrate uptake from the soil (Sherameti et al., 2005).

However, Sharma et al. (2008) indicated that *P. indica* may not be the origin of beneficial interaction as different bacterial species have been identified as closely associated with several fungi of the Sebacinales order. For example, the *Rhizobium radiobacter* strain PABac-DSM (which lacks the virulence genes causing the crown gall disease) was shown to be intimately associated with *P. indica* spores and hyphae. PABac-DSM induced growth promotion and systemic resistance against powdery mildew in barley seedlings comparable with the *P. indica*-induced phenotype.

1.3.3.1.5. *P. indica* mass production for commercialization

Laboratory, glasshouse and field trial data have shown that *P. indica* can be applied on farm-scales to increase plant growth and yield (Varma et al., 2013a). To commercialise and produce *P. indica* in large scale, so that the fungus could be used by farmers, it was formulated with talcum powder as a humectant and carrier. In India the formulated inoculum is sold as 'Rootonic'. For this, *P. indica* is grown in

liquid culture. Inoculum is then prepared by separating the *P. indica* biomass from the culture medium by filtration. On a commercial scale, a suspension of 250 g fresh weight of *P. indica* per L of 0.1 g L^{-1} carboxymethyl cellulose (CMC) is absorbed into talcum powder at 3 kg talc L^{-1} of suspension. CMC is used as an adhesive so that the inoculum sticks to the powder. Seed treatment is done by mixing Rootonic with seeds before sowing. The quantity of this *P. indica* formulation for wheat seeds has been estimated as 2.5 kg ha^{-1} (Chadha et al., 2014), and tested in different fields on different crops in India (Varma et al., 2013a, Varma et al., 2014).

1.4. Objectives

The evidence so far suggests that *P. indica* has tremendous potential as a biofertilizer and biocontrol agent in numerous crops. So far, little research on the symbiosis of *P. indica* and wheat has been done. The overall aim of the present work is to study the effect of *P. indica* on wheat productivity, especially on tolerance to Fusarium diseases, both crown rot and head blight. The targets were chosen because wheat is an important crop, and Fusarium is a difficult disease to manage. Specific objectives are described below:

1- Like other mutualistic endophytes, *P. indica* colonises roots in an asymptomatic manner. Information on colonization patterns of these endophytes is very limited. It is not yet clear how the fungus penetrates plant roots and how roots are eventually colonized. Therefore, in Chapter 2 the fungal development in a mutualistic symbiosis of the root endophytic *P. indica* and wheat will be analysed.

2- The hypothesis that *P. indica* can protect wheat from damage caused by *Fusarium spp.* under UK climate conditions will be studied in Chapters 2 and 3. This will include study of *P. indica* effects on visible disease, mycotoxin concentration, grain quality and total biomass.

3- Fungicides are widely used to control foliar and ear diseases of wheat, including *Fusarium* disease. Therefore, the compatibility of *P. indica* with fungicide and their joint effect on Fusarium diseases will be tested in Chapter 3.

4- It has long been recognised that AM fungi have an influence on plant nutrition and growth. *P. indica* is similar to AM fungi in terms of plant growth promoting effects. Therefore, the effect of both fungi on Fusarium diseases of wheat and, the interaction between them, will be compared in Chapter 3.

5- It has been shown that *P. indica* association improves plant mineral nutrient acquisition from the soil. This may or may not be the way *P. indica* improves growth. The effect of *P. indica* on soil and plant tissue nutrients will be reported in Chapter 3.

6- The hypothesis that *P. indica* can protect wheat from damage caused by foliar diseases will be studied in Chapter 4.

7- If *P. indica* is going to be applied to crops, a clear picture of its ecological effects and persistence would be needed. How *P. indica* affects other soil microorganisms in different soil types, how *P. indica* affects and interacts with weeds, and how long *P. indica* can persist in soil under UK weather conditions will be considered in Chapter 5.

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Chapter 2- The endophytic fungus *Piriformospora indica* protects wheat from Fusarium crown rot disease in simulated UK autumn conditions

M. Rabiey, I. Ullah and M. W. Shaw

M. Rabiey: did all the experiments;

I.Ullah: helped develop the molecular methods;

M. W. Shaw: advised on design, analysis and interpretation.

2.1. Summary

This study 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 sterilized soil-free medium or in a non-sterilized 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 control. 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. *Piriformospora 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*.

2.2. 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, 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., 2008b). 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 FCR, 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, Yadav et al., 2010, Varma et al., 2013b). It has also been reported that *P. indica* can improve growth in a range of economically important monocot and dicot hosts (Varma et al., 1999, Varma et al., 2000, Bagde et al., 2010).

P. 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*) (Deshmukh & Kogel, 2007, Serfling et al., 2007, Harrach et al., 2013), 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). *P. indica* also increased plant tolerance

to abiotic stresses including salt stress in barley (Baltruschat et al., 2008, Alikhani et al., 2013) , 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 (Sun et al., 2010, Michal Johnson et al., 2013). Previous investigations, have been concentrated in tropical and sub-tropical conditions. It remains to be shown whether *P. indica* is suited to temperate climatic conditions.

Hypothesis tested in this chapter: Previous investigations have been concentrated in tropical and sub-tropical conditions. It remains to be shown whether *P. indica* is suited to temperate climatic conditions.

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

2.3. Materials and Methods

2.3.1. Cultivation of fungi

2.3.1.1. Fusarium culture

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, Oxoid LTD, England). Inoculum was prepared by the methods described by Ghahfarokhy et al. (2011).

Discs (5 mm) of 4-day-old PDA cultures of Fusarium isolates were added to 500 mL Erlenmeyer flasks of wheat grains that had been boiled for 20 min, strained to remove excess water and sterilized twice at 121 ºC for 20 min on two consecutive days. For this purpose, the flasks were incubated at room temperature (21±1 °C) until all grains were fully colonised with mycelium.

2.3.1.2. Piriformospora indica *culture*

P. 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-day-old *P. indica* culture were added to 500 mL flasks of CM medium and incubated on an orbital shaker (Stuart SLL1, Bibby Scientific Ltd, UK) at 140 rpm at room temperature (21±1 °C) for 14 days. The liquid culture was then used for inoculation mixed with soil at sowing.

2.3.2. Laboratory experiments

2.3.2.1. Microscopical examination

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 after 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).

2.3.2.2. Dual culture tests

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.

2.3.2.3. Volatile metabolites

The production of volatile metabolites by *P. indica* and Fusarium isolates was examined following the method described by Dennis and Webster (1971) and Goyal et al. (1994) with slight modifications. A 5 mm mycelia disc of *Fusarium* isolates was placed at the centre of a PDA plate and incubated at room temperature (21 ± 1 ºC). After 4 days, when some mycelium growth had occurred, the lid was removed and the plate inverted over on another PDA plate containing a 5 mm mycelia disc of *P. indica.* The two were sealed together by adhesive tape. The control was the same except that *P. indica* was omitted. All of the plates were incubated at room temperature (21 ± 1 ºC) for 7 days. Inhibition was recorded daily by comparing growth of *Fusarium* isolates in the presence and absence of *P. indica*.

In another experiment, a single 5 mm disc of 4-day-old cultures of *P. indica* and Fusarium isolates were placed at opposite ends of a PDA plate simultaneously; a 1 cm strip across the centre of PDA was removed. In the control, *P. indica* and *Fusarium* isolates were cultured separately.

2.3.3. Glasshouse and growth chamber experiments

2.3.3.1. Interaction between P. indica *and* F. culmorum *during seedling growth of wheat*

Seeds of winter wheat cv. Battalion were surface disinfected by rinsing for 2 min in 20 mL L^{-1} (2 % v/v) sodium hypochlorite (Fisher Scientific UK Ltd, UK), followed by three rinses in sterile distilled water, and germinated on damp filter paper in a Petri dish at room temperature under natural indoor light for 48 hours. No micro-organisms grew from a sample of seeds so treated and placed on PDA plates for one week.

To determine whether *P. indica* interacted with wheat to reduce FCR, pre-germinated wheat seeds were planted into 10 cm diameter pots (5 seeds per pot), filled with a 1:1 mixture of vermiculite (Medium, Sinclair, UK) and sand, steam sterilised at 121 °C for 1h on two consecutive days. The pots were incubated in the glasshouse where humidity, light and temperature were not controlled; temperature ranged between 15 °C and 25 °C. 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 per pot. The treatments were: no amendment, P0, F0, P0+F0, P7, F7, P7+F7, P0+F7 and F0+P7 (P0 or F0: *P. indica* or *F. culmorum*

incoualtion at sowing, and P7 or F7: *P. indica* or *F. culmorum* incoualtion at seven days after sowing).

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 were determined and a sample stained for microscopy. The experiment had four treatments, ±*P indica* and ±*F. culmorum*, with two replications. The treatments were: no amendment, *P. indica, F. culmorum*, and *P. indica+F. culmorum*.

In a confirmatory experiment inoculations were done at the time of sowing in a 2×2 factorial combinations with 4 g of *P. indica* and 6 g of *F. culmorum*. Harvest was performed at 1, 2, 4, 8, 16 and 32 dai and DNA concentrations of both fungi and wheat in the root system determined. The treatments were: no amendment, *P. indica, F. culmorum*, and *P. indica+F. culmorum*.

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 days, the day-length was 12 hours and temperature and humidity were 15 °C, 65 %, respectively, during day and 10 °C, 65 % during night; for the second 14 days conditions were adjusted to 12 °C, 70 % during day and 9 °C, 70 % during night; and for the last 14 days the day length

was reduced to 10 hours with conditions set at 10 °C, 75 % during day and 7 °C, 75 % during night (www.met.reading.ac.uk/weatherdata). Pots were arranged in two randomised blocks. The experiment had 10 treatments with two replicates and five harvest times. The treatments were based on 2 × 5 factorial combinations of: no amendment, *P. indica*, *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576, *F. graminearum* 602.1, *P. indica*+*F. culmorum* 98/11, *P. indica*+*F. culmorum* UK.99, *P. indica*+*F. graminearum* 576, or *P. indica*+*F. graminearum* 602.1. One pot of each treatment in each replicate was harvested at 7, 17, 28, 35 and 42 dai. Each time point was independently replicated per pot.

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_4^{2-} 1 mM, K^+ 6 mM, Ca^{2+} 1.5 mM, Mg^{2+} 1 mM, SO_4^{2-} 1.5 mM, Fe 10 µM, Mn^{2+} 1 µM, Zn^{2+} 0.01 µM, Cu^{2+} 0.1 µM, MoO_4^{2-} 0.07 µM and $B_4O_7^{2-}$ 0.07 µM (Chandramohan & Shaw, 2013). Sodium metasilicate (100 mg L^{-1}) was included to control powdery mildew (Rodgers-Gray & Shaw, 2004).

2.3.3.2. 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 five 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^{-1} black ink for 30 min at 80 °C. Roots were

de-stained by rinsing in cold tap water for 3 min and viewed under a microscope with 10x and 40x objectives.

2.3.4. Molecular experiments

2.3.4.1. DNA isolation

Total genomic DNA was isolated from 100 mg of harvested roots using a DNeasy Plant Mini kit (QIAGEN, UK) following the manufacturer's instructions. Samples were eluted into 100 µL 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, Life Technologies Ltd, UK). The extent of shearing of DNA was determined by electrophoresis of an aliquot of DNA in a 1 % agarose gel.

2.3.4.2. Primer development and optimization of PCR conditions

Primers were designed using the PRIMER BLAST tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast) to amplify fragments of the *P. indica TEF* gene for elongation factor 1α, (EF-1α; accession number: AJ249911.2, Pi-forward: 5-TCCGTCGCGCACCATT-3 and Pi-reverse:5-AAATCGCCCTCTTTCCACAA-3, 84 bp), *Fusarium* EF-1α (accession number JX534485, for *F. culmorum,* F1-forward: 5-GCCCTCTTCCCACAAACCATT CC-3 and F1-reverse: 5-CTCGGCGGCTTCCTATTGACAG-3, 85 bp and for *F. graminearum,* F2-forward: 5-AAGCCGAGCGTGAGCGTGGTA-3 and F2-reverse: 5-CGGGAGCGTCTGATAGTCGTGTTA-3, 142 bp) and wheat

translation elongation factor 1α-subunit (accession number: M90077, Wt-forward: 5-GTGCACCAAATCTTCCTGCC-3, Wt-reverse: 5-GGTTATGGAATGTAGATGCTCGG-3, 71 bp). The accession numbers were obtained from http://www.ncbi.nlm.nih.gov. All primers were supplied by Invitrogen (Thermo Scientific, Life Technologies Ltd, UK).

Translation elongation factor 1 alpha (*TEF*) gene was used because it encodes an abundant and highly conserved protein which plays an important role in the elongation cycle of protein synthesis in eukaryotic cells (Merrick, 1992). *TEF* is the second most profuse protein after actin, combining 1–2 % of the total protein in normal growing cells (Condeelis, 1995). It binds charged tRNA molecules and transports them to the acceptor site on the ribosome adjacent to a growing polypeptide chain. *TEF* can also regulate other processes by interaction with cytoskeleton and mitotic apparatus (Ichi-Ishi & Inoue, 1995). *TEF* gene can be present in multiple copies in some Ascomycota and Zygomycota, whereas in many of the analyzed Basidiomycota genomes it proved to be in single copy (Basiewicz et al., 2012).

To assess specificity of the primers in this experiments 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.

Polymerase chain reaction (PCR) was performed in 0.2 mL PCR tubes (Fisher Scientific, Life Thechnologies Ltd, UK) with 20 µL final reaction volume

containing 2x Biomix PCR master mix (Life Thechnologies Ltd, UK), 0.25 µM forward and reverse primers, and varying quantities of template genomic DNA. Amplification was performed in a thermal cycler (Applied Biosystems® GeneAmp® PCR System 9700, ThermoFisher Scientific, Life Thechnologies Ltd, UK) programmed as: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, followed by incubation at 72 °C for 5 min. Amplification was confirmed by electrophoresis of an aliquot of the PCR products in 2 % agarose gel in 1x TAE buffer.

2.3.4.3. Real-time PCR

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 1×SYBR Green Jump Start *Taq*Ready Mix (Sigma Aldrich Company Ltd, UK), 0.25 µM forward and reverse primers, 1.5 µL sample DNA and 7.5 µL 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 s^{-1}. 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 v. 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 biomass 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.

2.3.5. 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.

2.4. Results

2.4.1. 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. 2.1 a,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 reduced, but colonisation by *P. indica* was extensive. *P. indica* colonisation started on root

surfaces in the differentiation zone behind the root meristem with inter- and intra-cellular penetration of epidermal cells, during the first 2-3 dai, with hyphae filling up the cells. By 4 dai 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 6 dai (Fig. 2.1 c,d).

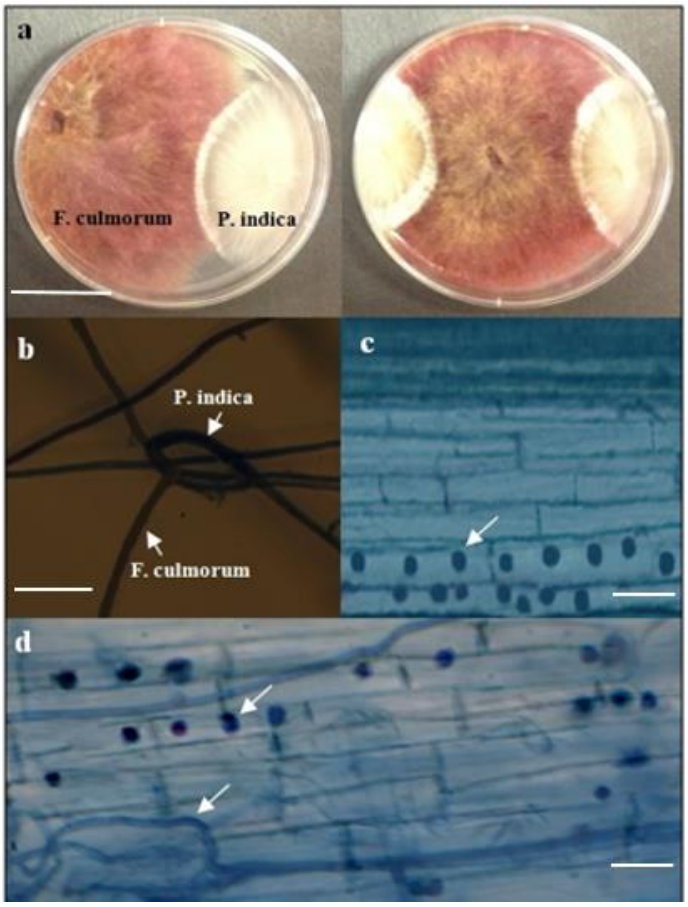

Fig. 2.1. Interaction of *Piriformospora indica* and *Fusarium* in agar plates and in the wheat roots; (a). Agar plate co-cultivated with *F. culmorum* and *P. indica*; (b). Interaction of coiled hypha of *P. indica* around *F. culmorum* in agar plates at the encounter point*;* (c). *P. indica* clamydospores 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. Arrows indicate *P. indica* clamydospores and hyphae (scale bar for a: 3 cm, b: 40 µm, c and d: 20 µm).

2.4.2. 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 7 days after sowing (Fig. 2.2). 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. 2.2).

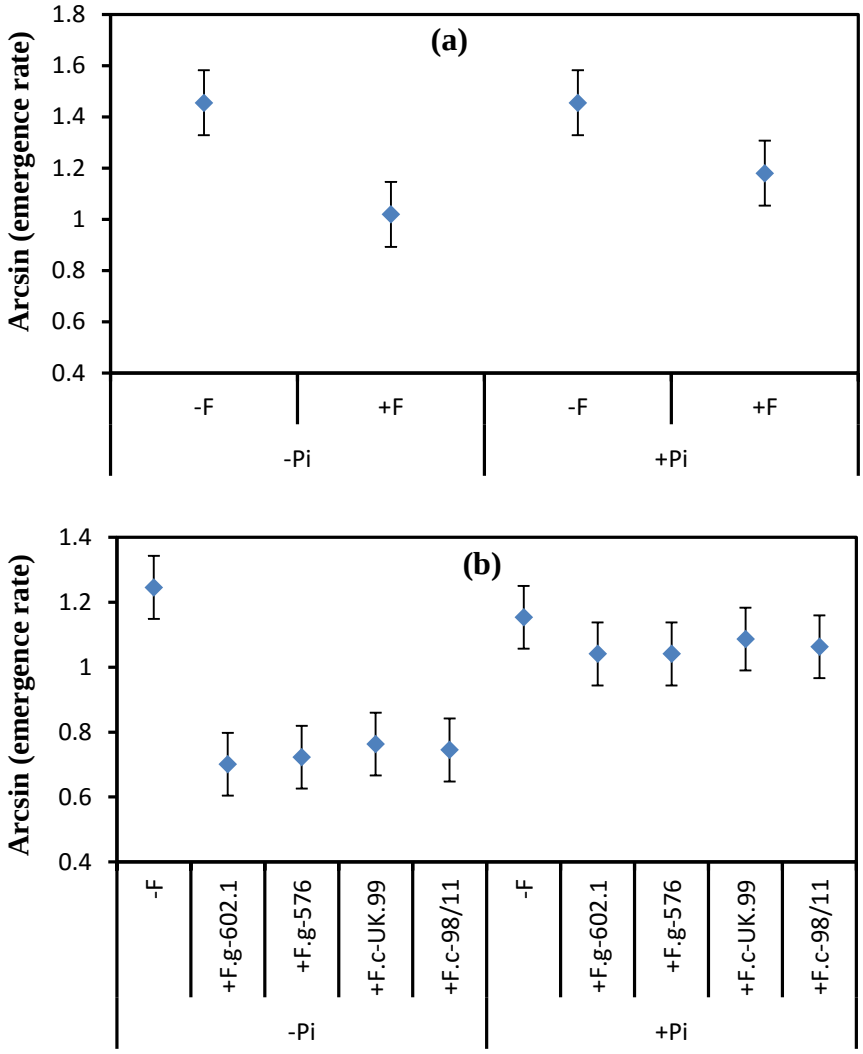

Fig. 2.2. Emergence rates of seeds inoculated with *Fusarium* (F) and *Piriformospora indica* (Pi) evaluated 7 days after sowing**;** data were arcsine transformed. (a). Roots inoculated with *F. culmorum* and *P. indica* simultaneously at sowing time (s.e.d. = 0.09, d.f. = 57); (b). Roots inoculated 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.

Root weights were evaluated at the final harvest (Fig. 2.3). Roots of plants inoculated with *P. indica* alone at sowing or 7 days later had weights equivalent to the control (Fig. 2.3 a). Roots inoculated with *F. culmorum* or *F. graminearum* had 40 % lower root weight ($P<0.001$; Fig. 2.3 b). Roots of plants inoculated with *P. indica* prior to *Fusarium* or simultaneously weighed roughly the same as uninoculated plants and much more than the root inoculated with Fusarium alone ($P<0.001$, Fig. 2.3 a,b,c). *P. indica* inoculated 7 days after *F. culmorum* was less effective (Fig. 2.3 a).

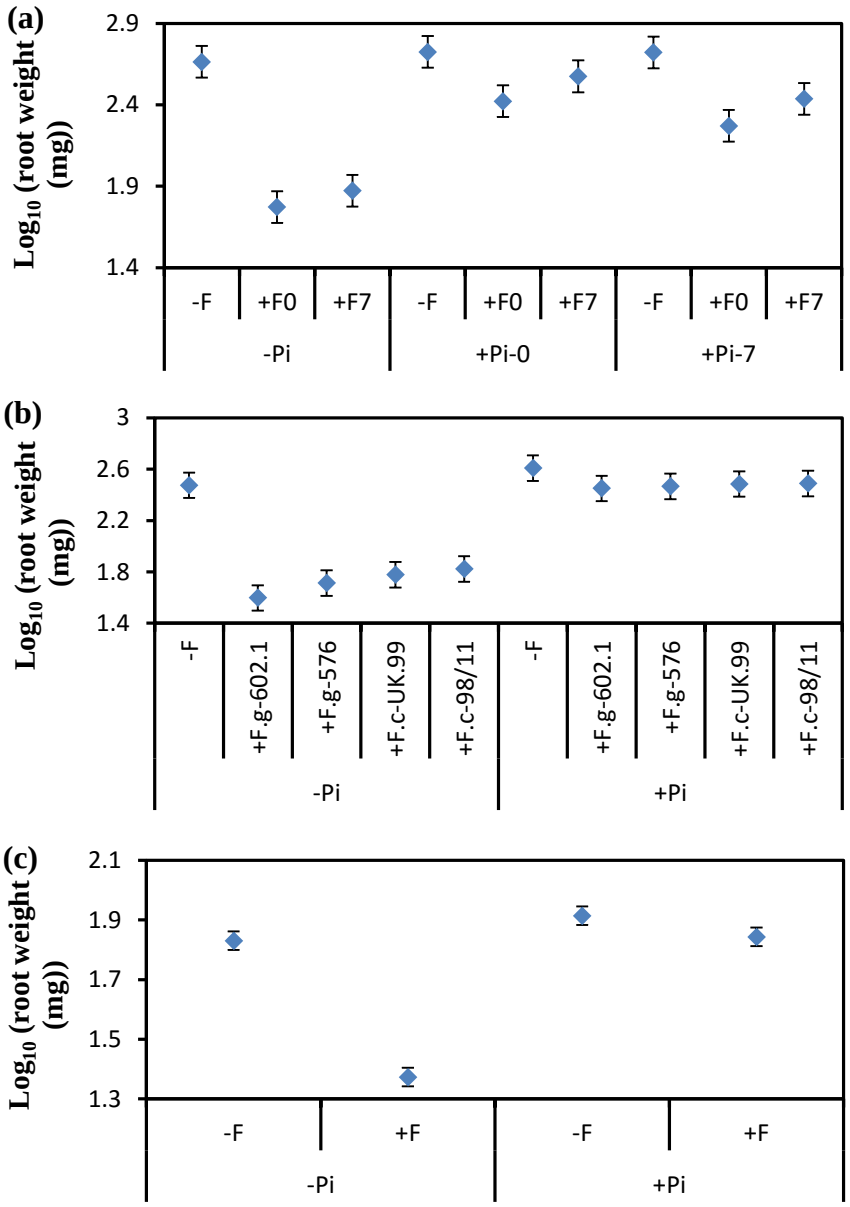

Fig. 2.3. Root weights of samples (mg) inoculated with *Fusarium* (F) and *Piriformospora indica* (Pi) evaluated at last harvest; data were Log_{10} transformed. (a). Roots inoculated with *F. culmorum* or *P. indica* simultaneously or 7 days after sowing, harvested at 30 dai (s.e.d. = 0.07, d.f. = 8); (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 represents mean ± 2 SEM, (P: *P. indica*, F: Fusarium, Pi-0: *P. indica* added to soil at sowing, Pi-7: *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 % per day throughout the experiment (Fig. 2.4 a-c,f). The rate of growth of Fusarium inoculated at 7 dai was similar to that inoculated at sowing time (Fig. 2.4 a,b). The relative rate of increase was constant for *F. graminearum* but declined in *F. culmorum* particularly in the first experiment (Fig. 2.4 a-c). In co-inoculated samples, the absolute amount of pathogen was static or slightly declining from 7-42 days (Fig. 2.4 a,b,d,f) after an initial period of increase (Fig. 2.4 e,f).

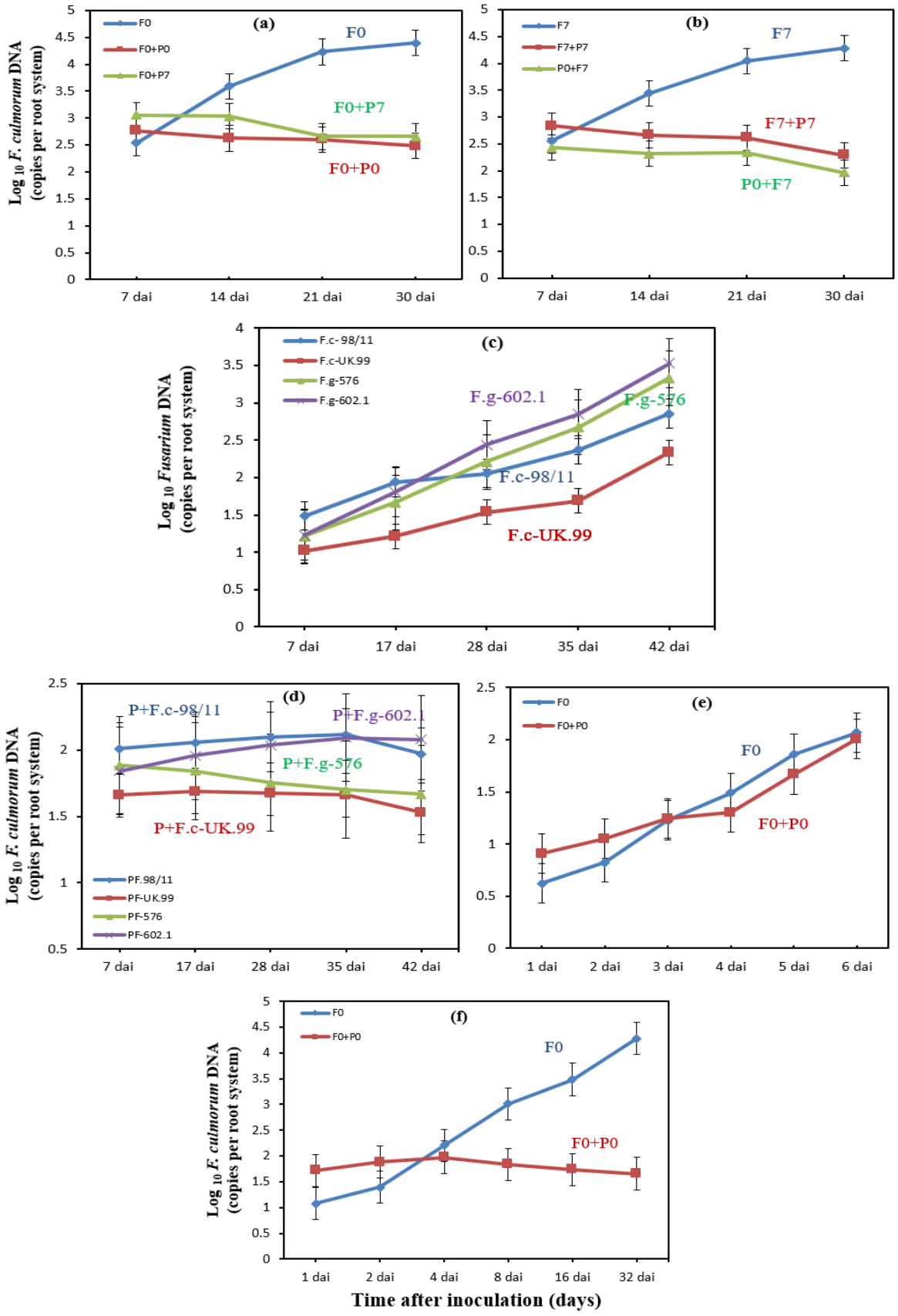
(a)
F0
F0+P0
F0+P7
Log 10 F. culmorum DNA (copies per root system)
7 dai
14 dai
21 dai
30 dai
(b)
F7
F7+P7
P0+F7
(c)
F.c-98/11
F.c-UK.99
F.g-576
F.g-602.1
Log 10 Fusarium DNA (copies per root system)
7 dai
17 dai
28 dai
35 dai
42 dai
(d)
P+F.c-98/11
P+F.g-602.1
P+F.g-576
P+F.c-UK.99
PF.98/11
PF-UK.99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

Fig. 2.4. The growth of *Fusarium* in inoculated wheat roots. The 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) (incubated in the glasshouse); (b). *F. culmorum* added to soil 7 days after sowing (F7); *P. indica* added at sowing (P0) or simultaneously 7 days after sowing (P7) (incubated in the glasshouse); (c). *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 added at sowing time (incubated in the controlled environment chamber); (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 (incubated in the controlled environment chamber); (e). *F. culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0), during the first week of inoculation (incubated in the glasshouse); (f). *F. culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0), during the first month of inoculation (incubated in the glasshouse). Each point represents 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 % per day (Fig. 2.5 a,c,f), after the first 7 days; growth of *F. culmorum* in the first week was faster (Fig 5 e,f). Despite the difference in temperatures, both glasshouse (Fig. 2.5 a,b,d,f) and environmental chamber (Fig. 2.5 c,d) experiments had similar rates of fungal growth. Increase in *F. graminearum* DNA was faster than increase in *F. culmorum* DNA (Fig. 2.5 c). The rate of growth of *Fusarium* inoculated at 7 dai was similar to that inoculated at sowing time (Fig. 2.5 a,b). In the presence of *P. indica*, *Fusarium* growth was immediately reduced to the rate of growth of the root system (Fig. 2.5 e,f) and then declined (Fig. 2.5 b,d). *P. indica* inoculation 7 days after the pathogen reduced the rate of *Fusarium* growth relative to the root similarly to the reduction when inoculated simultaneously (Fig. 2.5 b). 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 days after *F. culmorum* inoculation.

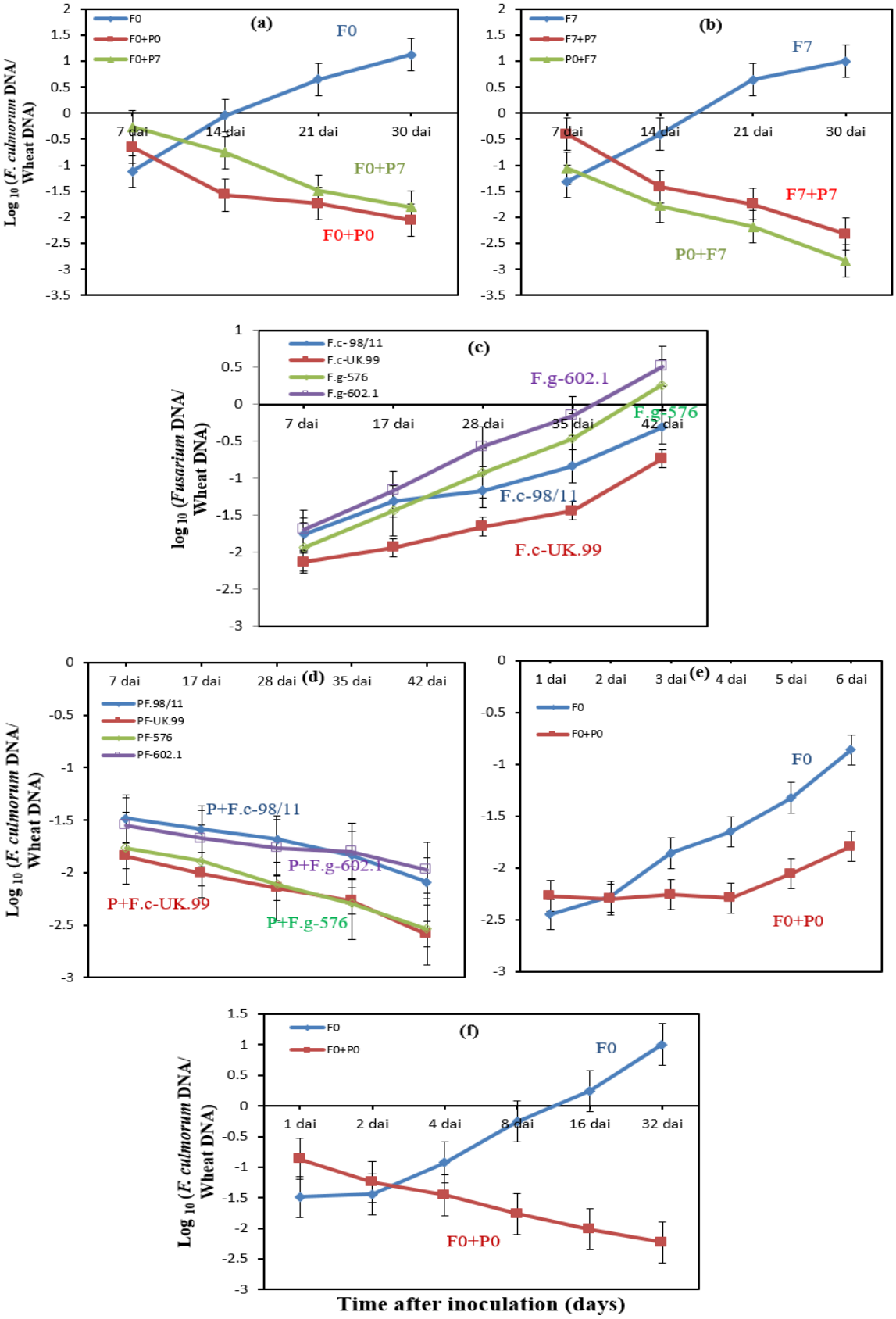
(a)
F0
F0+P0
F0+P7
Log 10 (F. culmorum DNA/ Wheat DNA)
7 dai
14 dai
21 dai
30 dai
(b)
F7
F7+P7
P0+F7
(c)
F.c-98/11
F.c-UK.99
F.g-576
F.g-602.1
log 10 (Fusarium DNA/ Wheat DNA)
7 dai
17 dai
28 dai
35 dai
42 dai
(d)
PF.98/11
PF-UK.99
PF-576
PF-602.1
P+F.c-98/11
P+F.g-602.1
P+F.c-UK.99
P+F.g-576
(e)
1 dai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

Fig. 2.5. 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) (incubated in the glasshouse); (b). *F. culmorum* added to soil 7 days after sowing (F7); *P. indica* added at sowing (P0) or simultaneously 7 days after sowing (P7) (incubated in the glasshouse); (c). *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 added at sowing time (incubated in the controlled environment chamber); (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 (incubated in the controlled environment chamber); (e). *F. culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0), during the first week after inoculation (incubated in the glasshouse); (f). *F. culmorum* added to soil at sowing (F0) and *P. indica* added simultaneously (P0) (incubated in the glasshouse), during the first month of inoculation. Each point represents 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. = 0.15 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 dai (Fig. 2.6 a), then decreased from a peak of 10^4 copies/root system to 10^3 over the 30 days of the experiment (Fig. 2.6 b,c,e); but slightly increased, under simulated autumn conditions, by 42 days into the experiment (Fig. 2.6 d). In the presence of *Fusarium*, *P. indica* DNA grew gradually throughout the experiment (Fig. 2.6 a-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. 2.6 b-d).

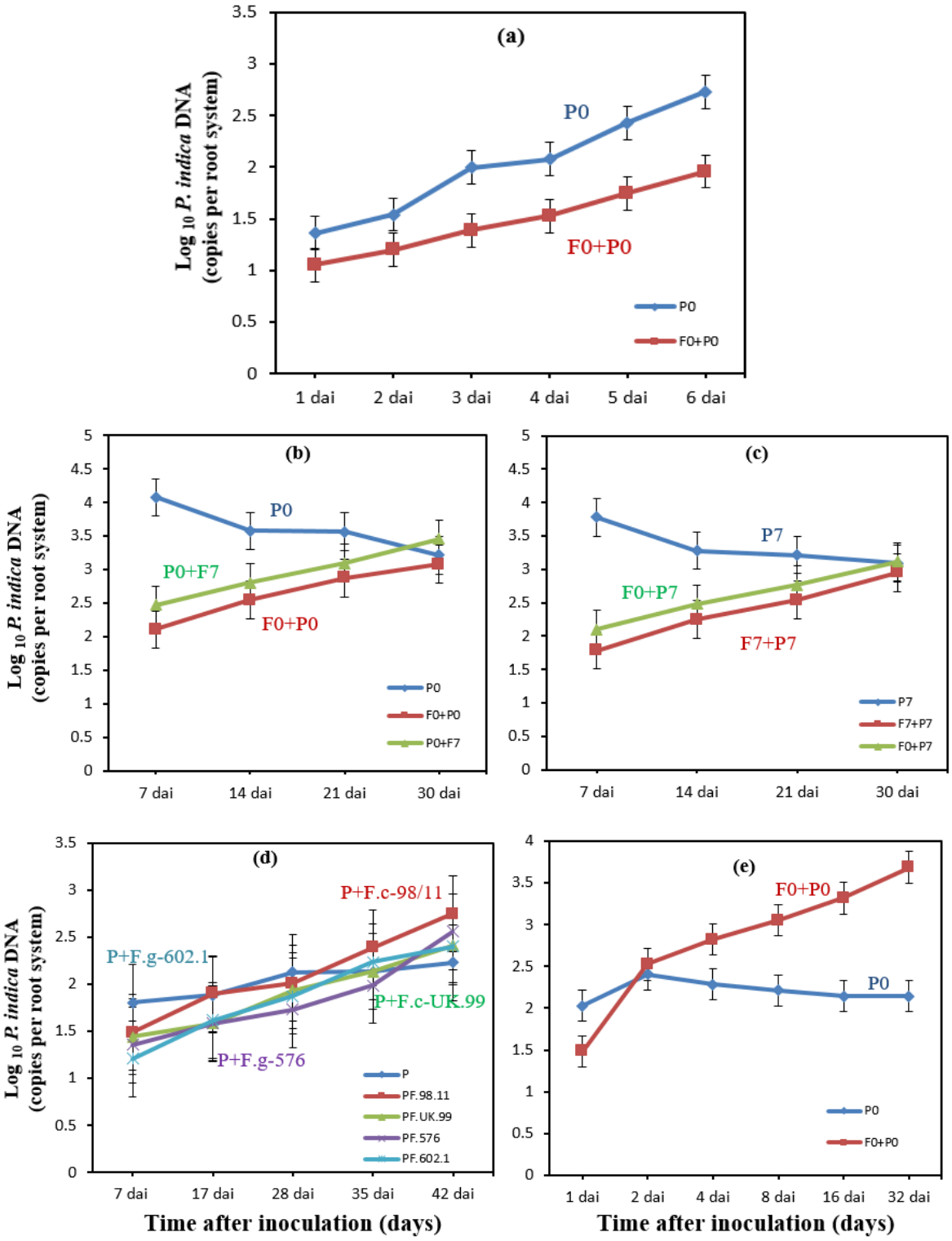
(a)
Log 10 P. indica DNA (copies per root system)
P0
F0+P0
1 dai
2 dai
3 dai
4 dai
5 dai
6 dai
(b)
P0
P0+F7
F0+P0
7 dai
14 dai
21 dai
30 dai
(c)
P7
F0+P7
F7+P7
(d)
P+F.c-98/11
P+F.g-602.1
P+F.c-UK.99
P+F.g-576
PF.98.11
PF.UK.99
PF.576
PF.602.1
7 dai
17 dai
28 dai
35 dai
42 dai
Time after inoculation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

Fig. 2.6. The 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 (incubated in the glasshouse); (b). *P. indica* added to soil at sowing (P0); *F. culmorum* added simultaneously (F0) or 7 days after sowing (F7) (incubated in the glasshouse); (c). *P. indica* added to soil 7 days after sowing (P7); *F. culmorum* added at sowing (F0) or simultaneously 7 days after sowing (F7) (incubated in the glasshouse); (d). *P. indica*, *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 added at sowing time (incubated in the controlled environment chamber); (e). *P. indica* added to soil at sowing (P0) and *F. culmorum* added simultaneously (F0), during the first month of inoculation (incubated in the glasshouse). Each point represents 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 % per day in the first 7 dai (Fig. 2.7 a), then the rate declined, then stayed constant rate for the remainder of experiment from 14 to 30 dai (Fig. 2.7 b,c). However, this early increase was not consistent (Fig. 2.7 e). The rate of growth of *P. indica* inoculated at 7 dai was similar to that inoculated at sowing time (Fig. 2.7 b,c). In the presence of *F. culmorum*, the rate of growth of *P. indica* was static throughout the experiment (Fig. 2.7 a,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 % per day throughout the experiment (Fig. 2.7 d).

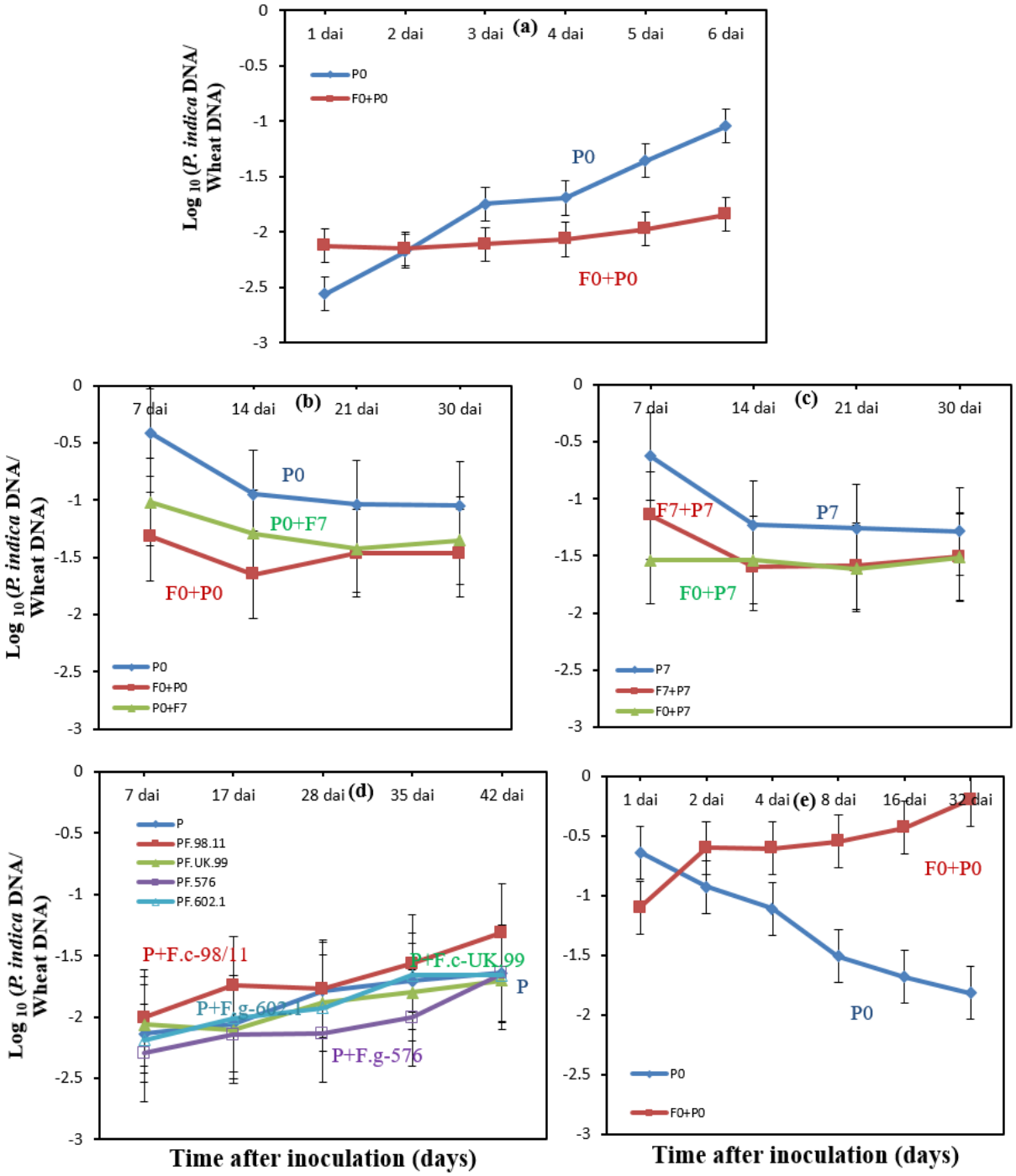
(a)
1 dai
2 dai
3 dai
4 dai
5 dai
6 dai
P0
F0+P0
Log 10 (P. indica DNA/ Wheat DNA)
(b)
7 dai
14 dai
21 dai
30 dai
P0
P0+F7
F0+P0
(c)
F7+P7
P7
F0+P7
(d)
7 dai
17 dai
28 dai
35 dai
42 dai
P
PF.98.11
PF.UK.99
PF.576
PF.602.1
P+F.c-98/11
P+F.c-UK.99
P+F.g-602.1
P+F.g-576
(e)
1 dai
2 dai
4 dai
8 dai
16 dai
32 dai
Time after inoculation (days)

Fig. 2.7. 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 (incubated in the glasshouse); (b). *P. indica* added to soil at sowing (P0); *F. culmorum* added simultaneously (F0) or 7 days after sowing (F7) (incubated in the glasshouse); (c). *P. indica* added to soil 7 days after sowing (P7); *F. culmorum* added at sowing (F0) or simultaneously 7 days after sowing (F7) (incubated in the glasshouse); (d). *P. indica*, *F. culmorum* 98/11, *F. culmorum* UK.99, *F. graminearum* 576 or *F. graminearum* 602.1 added at sowing time (incubated in the controlled environment chamber); (e). *P. indica* added to soil at sowing (P0) and *F. culmorum* added simultaneously (F0), during the first month of inoculation (incubated in the glasshouse). Each point represents 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).

2.5. 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 and volatile metabolite 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 (2010) was 28 °C, the optimum temperature for *P. indica* growth (Justice, 2014).

In inoculated roots, *P. indica* penetration started at the differentiation zone of the roots, with inter- and intra-cellular hyphae penetration during the first two to three dai. *P. indica* hyphae filled up the cortical and epidermal cells. Chlamydospores were visible from 6 dai. Occasionally, coiled hyphae could be observed within root cells. Jacobs et al. (2011) proposed a colonisation model for *P. indica* in

Arabidopsis roots, which started with inter- and intra-cellular penetration of rhizodermal and cortical tissues and then root hair cells by 3 dai. Fungal hyphae branched and sometimes formed whorls. Finally, sporulation started at 7 dai; this is completely consistent with observations (Fig. 2.1).

The pathogen DNA was slightly higher than in plants inoculated with pathogen alone during the first week after inoculation, in all experiments (Fig. 2.4 and 2.5). This effect was possibly due to the additional exogenous nutrients from the substrate of the *P. indica* inoculum. It also could be due to the fact that *P. indica* induced susceptibility in the root system as reported by Pedrotti et al. (2013), showing that *P. indica* triggered a local, transient response of several defense-related transcripts in Arabidopsis root and shoot. 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 (Fig. 2.6 and 2.7). 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 dai of *P. indica.*

Inoculation of plants with *P. indica* before the pathogen inoculation had a greater effect on both the ratio between pathogen and host DNA and the actual amount of pathogen than simultaneous or delayed inoculation (Fig. 2.4 and 2.5). 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 (under glasshouse conditions), but increased slightly under cool conditions (in the controlled environmental chamber adjusted to UK autumn 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 an oxidative burst, which is essential to successful *Fusarium* establishment (Waller et al., 2005, Varma et al., 2012). 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 over-estimation 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 FCR 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 and 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.

This study shows 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 will *P. indica* interact with other beneficial soil microorganisms, like arbuscular mycorrhizal fungi? How will *P. indica* interact with other soil-borne pathogens? How will it affect soil functioning, such as turnover of soil organic matter, incorporation of residues, etc? What effects will *P. indica* have on other soil-borne diseases? These must be considered in further studies.

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CHAPTER 3- *Piriformospora indica* reduces Fusarium head blight disease severity and mycotoxin DON contamination in wheat under UK weather conditions

M. Rabiey, and M. W. Shaw

M. Rabiey: did all the experiments;

M. W. Shaw: advised on design, analysis and interpretation.

3.1. Summary

The effect of *P. indica* on Fusarium head blight (FHB) disease of winter (cv. Battalion) and spring (cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn) hard wheat and consequent contamination by the mycotoxin deoxynivalenol (DON) was evaluated under UK weather conditions. Interactions of *P. indica* with an arbuscular mycorrhizal fungus (*Funneliformis mosseae*), fungicide application (Aviator Xpro, Bayer CropScience, UK; with active ingredients of prothioconazole and bixafen) and low and high fertiliser levels (Osmocote® Pro, the Scott Company, UK) were also considered. *P. indica* application reduced FHB disease severity and incidence by 70 %. It decreased mycotoxin DON concentrations in winter and spring wheat samples by 70 % and 80 % respectively. *P. indica* also increased above ground biomass, thousand grain weight and total grain weight. *P. indica* reduced FHB disease severity and increased yield in both high and low fertiliser levels. The effect of *P. indica* was compatible with *Fun. mosseae* and foliar fungicide application. *P. indica* did not have any

effects on soil and plant tissue nutrients. These results suggest that *P. indica* might be useful in biological control of Fusarium diseases of wheat.

3.2. Introduction

Fusarium crown rot (FCR) and head blight (FHB) are two of the most important diseases of wheat globally. The two most prevalent causal organisms are *Fusarium culmorum* and *F. graminearum* (Fernandez & Chen, 2005). *Fusarium* spp. produce a range of mycotoxins that can accumulate in the grain and, if they enter the food chain, can cause a risk to human and animal health (Xu et al., 2008b). The mycotoxin deoxynivalenol (DON), which is produced during head infection, has been identified as the most frequent contaminant associated with FHB in wheat (Bai & Shaner, 2004). European Union legislation has set a legal limit for DON of 1250 µg kg^{-1} for cereals intended for human consumption (Anon, 2006), but even low level contamination of grain can reduce market prices or cause the grain to be rejected entirely (Bai & Shaner, 2004). *Fusarium* species overwinter in soil and crop residues for several seasons. They survive as saprophytes on dead host tissues, especially if susceptible crops are planted in successive years. The most important sources of inoculum are ascospores from the sexual stage and macroconidia from the anamorph stage but chlamydospores and hyphal fragments can also act as sources of inoculum (Leplat et al., 2013). During warm, moist and windy environmental conditions the ascospores or macroconidia are dispersed by water-splash or air currents onto wheat heads and initiate infection of wheat spikes. Infections can occur as early as spike emergence, but the flowering stage or shortly

after is considered the most vulnerable stage for *Fusarium* infection (Madgwick et al., 2011). No highly resistant commercial cultivars are yet available. Agronomic practices intended to reduce these diseases are only partially effective, because the necessary actions depend on the causal species and the environmental conditions, and the results are often unpredictable (Paulitz et al., 2002). Currently, control of Fusarium diseases relies on high inputs of fungicide in FHB-endemic regions (Mesterházy, 2003). Two factors are currently increasing the *Fusarium* problem in the UK. First, the UK is predicted to experience more often weather (UKCIP; www.ukcip.org.uk/) which will increase the risks of infection, colonisation, reproduction and dispersal of Fusarium diseases (West et al., 2012) leading to increased severity and incidence. Second, maize cultivation is increasing, leading to increased populations of *F. graminearum*; as maize debris is a potent source of inoculum of *Fusarium* (West et al., 2012).

Plant roots are associated with beneficial fungi in the majority of soils. For example, arbuscular mycorrhizal fungi (AMF), such as *Funneliformis mosseae* (=*Glomus mosseae)*, are important soil microorganisms forming beneficial symbiotic associations with most land plants. AMF are obligate biotrophs which provide mineral nutrients, specifically phosphate and nitrogen, to their host plant in exchange for carbohydrates and therefore stimulate plant growth (Bucher, 2007, Schalamuk et al., 2011).

Piriformospora indica is a root endophyte with a wide host range belonging to the Sebacinaceae (Sebacinales, Basidiomycota). It was originally found in the Thar

desert of Rajasthan, an arid region in India (Verma et al., 1998), which experiences extreme day-time heat and diurnal temperature fluctuations as well as extended drought. *P. indica* promotes plant growth, increases root and above ground biomass and final yield of a broad range of host plants, including many plants of economic importance (Shrivastava & Varma, 2014) and helps plants to grow under temperature, water and physical stresses (Alikhani et al., 2013, Ghabooli et al., 2013). Evidence suggests that *P. indica* protects plants against pathogens of roots (caused by *Fusarium culmorum*, *F. graminearum*, *Gaeumannomyces graminis* var. *tritici*), stems (caused by *Oculimacula* Spp.) and leaves (caused by *Blumeria graminis* f.sp. *tritici* and *B. graminis* f.sp. *hordei*) under glasshouse and field conditions (Waller et al., 2005, Deshmukh & Kogel, 2007, Ghahfarokhy et al., 2011, Harrach et al., 2013). Our previous work shows that *P. indica* association protected wheat seedlings from FCR damage in simulated UK autumn conditions (Rabiey et al., 2015).

The effect of some root associated fungi is to improve plant nutrient uptake (Miransari, 2010, Wu et al., 2011). For instance, AMF obtain fixed carbon compounds from host plants, while plants benefit from increased nutrient supply (Finlay, 2008). Research so far suggests that *P. indica* association improves plant mineral nutrient acquisition from the soil. It can mobilise and transport phosphate, nitrogen and micronutrients from soil to the infected host plant via plant-fungal interfaces (Sherameti et al., 2005, Yadav et al., 2010). However, it is not yet clear if *P. indica* can increase nutrient uptake in all of its hosts.

Hypotheses tested in this chapter: the present study investigated the effect of *P. indica* on Fusarium infection of parts of the host not directly colonised by *P. indica*. The following hypotheses were tested: *P. indica* would reduce damage to wheat grains caused by FHB and mycotoxin contamination; any effect of *P. indica* on FHB would be greater at low soil fertility levels like AMF, such as *Fun. mosseae* (Nouri et al., 2015); *P. indica* application would be as effective as fungicide application and *P. indica* would improve plant nutrient uptake, shown by altered foliar nutrient status and the effects of *P. indica* on disease were caused by changes in nutrient status alone. FHB disease severity and incidence, mycotoxin DON, and yield parameters were determined in pots with factorial combinations of inoculation with *F. culmorum*, *F. graminearum*, *P. indica*, or *Fun. mosseae*, foliar fungicide and low and high fertiliser application rates. Plants were grown outdoors.

3.3. Materials and Methods

3.3.1. Fungal inoculation

3.3.1.1. Piriformospora indica

P. indica was grown on agar containing CM medium. Inoculum of *P. indica* was prepared by the methods described in chapter 2.

3.3.1.2. Fusarium isolates

Inoculum of *F. culmorum* was prepared by the methods described in chapter 2.

Conidia of *F. graminearum* 576 and *F. graminearum* 602.1were harvested from the surface of sporulating PDA cultures in sterile distilled water so that the resulting

suspension contained $1x10^6$ spores mL^{-1}. The spore concentration was determined using a haemocytometer (Weber Scientific International Ltd, England).

3.3.1.3. Funneliformis mosseae *culture*

Funneliformis mosseae was obtained from Prof. Alan Gange, Royal Holloway/University of London. The fungus (mixture of spores, mycelia and sands) was propagated on maize plants grown in a 3:1 mixture of steam sterilised compost (John Innes Composts, BHGS Ltd, UK) and sand. After 3 months, the contents of each pot (including compost and roots) were chopped on a sterilised surface and transferred into a zip-lock bag and stored at 4 °C until required.

3.3.2. Plant materials and pot experiments

3.3.2.1. Fusarium Crown Rot and Fusarium Head Blight of winter wheat

Winter wheat seeds, cv. Battalion (NABIM group 2), were surface disinfected as described in chapter 2 and pre-germinated at room temperature under natural indoor light for 48 hours. Eight seeds per pot were planted in 12 L pots (top diameter: 28 cm, bottom diameter: 23 cm, depth: 25 cm) at a depth of two cm in two parts non-sterilised compost (No 2, John Innes Compost, BHGS Ltd, UK) and one part sand, mixed with 1 g L^{-1} or 4 g L^{-1} of slow release fertiliser (8-9 months, Osmocote® Pro, the Scott Company, UK, contains 16 % nitrogen, 11 % phosphorus, 10 % potassium, 2 % magnesium oxide, 0.01 % boron, 0.042 % copper, 0.3 % iron, 0.04 % manganese, 0.015 % molybdenum and 0.01 % zinc) to provide wheat macro- and micro-nutrients during the experiment. Non-sterilised compost and sand were used to simulated field soil conditions. Seeds were planted in two rows at a distance of

11 cm apart and two cm between each seed to simulate field spacing. In all experiments, pots were watered as necessary to maintain the compost moist, and the experimental area was surrounded by pots filled with sand to reduce edge effects on microclimate.

The experiment was carried out in 2013-14 growing season at the University of Reading (grid ref: SU733719), under outdoor condition. The experiment had 32 treatments (giving 32 df for error), with two replicates, distributed in two randomised blocks, with the following factorial combinations of treatments= ±*P. indica*, ±*Fun. mosseae*, ±*F. culmorum* (FCR), ±*F. graminearum* (FHB) and ±fertiliser (1 g L^{-1} or 4 g L^{-1}). The treatments were:

1 g L^{-1} fertiliser, 4 g L^{-1} fertiliser, and the following treatments were either mixed with 1 g L^{-1} or 4 g L^{-1} fertiliser: *P. indica*, *Fun. mosseae*, *F. culmorum*, *F. graminearum*, *P. indica+Fun. mosseae*, *P. indica+F. culmorum*, *P. indica+F. graminearum*, *Fun. mosseae+F. culmorum*, *Fun. mosseae+F. graminearum*, *F. culmorum+F. graminearum*, *Fun. mosseae+F. culmorum+F. graminearum*, *P. indica+Fun. mosseae+F. culmorum*, *P. indica+Fun. mosseae+F. graminearum*, *P. indica+F. culmorum+F. graminearum*, and *P. indica+Fun. mosseae+F. culmorum+F. graminearum.*

Inoculations with *P. indica* (6 g liquid culture mixed with soil) and *Fun. mosseae* (50 g, 20 spores per g mixed with soil) and *F. culmorum* (6 g prepared inocula mixed with soil) were performed at sowing and *F. graminearum* was applied at

flowering. All disease symptoms, whether from inoculations or natural infections were recorded, including Septoria leaf blotch and yellow rust.

In this experiment, extra nitrogen and sulphur fertiliser were applied in two split applications, with the first dose applied in late March and the second in late April, including 1.4 g N pot^{-1} (over 2 splits) and 28 mg S pot^{-1} (in one application). The first dose was made up of ammonium nitrate (34.5 % N) and ammonium sulphate (27 % N, 30 % SO4). The second dose was ammonium nitrate (34.5 % N).

3.3.2.2. Fusarium Head Blight of spring wheat cv. Paragon

Spring wheat seeds, cv. Paragon (NABIM group 1), were surface disinfected and pre-germinated. Eight seeds per pot were planted in 12 L pots at a depth of two cm in two parts non-sterilised compost and one part sand, mixed with 4 g L^{-1} of slow release fertiliser as for winter wheat.

The experiment was carried out in 2014 growing season at the University of Reading, under outdoor conditions. The experiment had 16 treatments with three replicates, distributed in three randomised blocks, with the following combination: ±*P. indica*, ±*Fun. mosseae*, ±*F. graminearum* (FHB) and ±fungicide. The treatments were: no amendment, *P. indica*, *Fun. mosseae*, *F. graminearum*, fungicide, *P. indica*+*Fun. mosseae*, *P. indica*+*F. graminearum*, *P. indica*+fungicide, *Fun. mosseae*+fungicide, *F. graminearum*+fungicide, *Fun. mosseae*+*F. graminearum*, *P. indica*+*Fun. mosseae*+*F. graminearum*, *P. indica*+*F. graminearum*+fungicide, *P. indica*+*Fun. mosseae*+fungicide, *Fun. mosseae*+*F. graminearum*+fungicide, *P. indica*+*Fun. mosseae*+*F. graminearum*+fungicide.

Inoculations with *P. indica* (6g liquid culture mixed with soil) and *Fun. mosseae* (50 g, 20 spores per g mixed with soil) were performed at sowing. The fungicide Aviator Xpro (Bayer CropScience, UK) with active ingredients of prothioconazole (15.84 %) and bixafen (7.43 %) was applied at the concentration of 2 ml L^{-1}, diluted with water, when the flag leaf was fully emerged (Zadoks Growth Stage (GS) 39; Zadoks et al. (1974)) and also 72 hours after plants were artificially sprayed with spore suspension of *F. graminearum* (GS 65) for the selected treatments only. The fungicide Aviator Xpro exhibits both translaminar (within and across the leaf) and systemic movement (around the plant). Prothioconazole-based sprays have been proven to reduce FHB disease severity significantly (HGCA, 2015a).

3.3.2.3. Fusarium Head Blight of different cultivars of spring wheat

It is possible that some wheat cultivars benefit more than others from association with *P. indica*. In another experiment, the effect of *P. indica* on Fusarium head blight of spring hard wheat was assessed on six different spring wheat cultivars: Paragon, Mulika, Zircon (NABIM group 1), Granary, KWS Willow (NABIM group 2) and KWS Kilburn (NABIM group 4), chosen from HGCA recommended list for spring sowing and were supplied by KWS UK Ltd, UK. Eight germinated seeds per pot were planted in 12 L pots at a depth of two cm in a mixture of two parts non-sterilised compost and one part sand, mixed with 4 g L^{-1} of slow release fertiliser (3-4 months, Osmocote® Pro).

The experiment was carried out in 2015 growing season at the University of Reading, under outdoor conditions. The experiment had 24 treatments with three

replicates, distributed in three randomised blocks, with the following factorial combinations of treatments: ±*P indica*, ±*F. graminearum* (FHB), and six cultivars of spring wheat. Inoculations with *P. indica* (6 g liquid culture mixed with soil) were performed at sowing and *F. graminearum* was applied at flowering. All disease symptoms, whether from inoculations or natural infections, were recorded when appropriate.

The pots were sprayed with a mix of Cortez (Makhteshim-Agan (UK) Ltd), with active ingredient of epoxiconazole (12.1 % w/w), for the yellow rust (BASF, 2015) and Flexity (BASF, UK), with active ingredient of metrafenone (25.2 % w/w), for the powdery mildew at GS 70 (milk development) at the concentration of 2 ml L^{-1}, diluted with water (Opalski et al., 2006).

3.3.2.4. Fusarium ear inoculation

When most tillers of each pot were at mid-anthesis stage (GS 65), all tillers of a pot were inoculated with 1 mL of a 50:50 mixed conidia suspension of *F. graminearum* 576 and *F. graminearum* 602.1. In all expeiments inoculation was done in a cloudy evening with rain afterward.

3.3.2.5. Fusarium Head Blight visual disease assessment and yield determination

Visual disease assessment, based on the percentage of infected spikelets per ear, was made two weeks after artificial inoculation on each of the treated ears from each pot. *F. graminearum* disease symptoms were recognized as pink fungal growth, brown-colored lesions and premature bleaching of spikelets (Stack & McMullen, 2011).

Plants were hand harvested. The total above ground dry weight, total grain weight at 15 % moisture content, thousand grain weight (TGW), harvest index (total grain weight/total above grain weight), number of ears, plant height and root dry weight were measured.

3.3.2.6. Mycotoxin analysis

Determination of mycotoxin DON in all samples from the winter and spring experiments was performed using ELISA testing by Romer Labs (Romer Labs Ltd, UK).

3.3.2.7. The effect of **P. indica** *and* **Fun. mosseae** *on soil and plant tissue nutrients*

An experiment was carried out during 2014-15 growing season to test the effect of *P. indica* on soil and leaf tissue nutrients. Winter wheat seeds, cv. Battalion, were surface disinfected and pre-germinated. Eight seeds per pot were planted in 12 L pots at a depth of two cm in two parts non-sterilised compost and one part sand, mixed with 1 g L^{-1} or 4 g L^{-1} of slow release fertiliser (8-9 months, Osmocote® Pro). The experiment had 8 treatments with three replicates, distributed in three randomised blocks, with the following factorial combinations of treatments: ±*P indica*, ±*Fun. mosseae*, and ±fertiliser (1 g L^{-1} or 4 g L^{-1}). Inoculations with *P. indica* (6 g) and *Fun. mosseae* (50 g, 20 spores per g) were done at the time of sowing. Around 500g of soils and 200g leaf materials of each treatment at GS 27-29 were sent for analysis in the first week of April/2015. The soil analysis included pH, phosphorus (P), potassium (K), magnesium (Mg), nitrate (NO_3), ammonium

(NH_4), and available nitrogen (N). The plant tissue analysis included total N and sulphur (S) with N:S ratio, total P, K, Mg, calcium (Ca), copper (Cu), zinc (Zn), Iron (Fe) and Boron (B).

3.3.3. Statistical analysis of experiments

ANOVA was used to analyse all data using Genstat 17th ed, (VSN, UK) with appropriate blocking. Where applicable, data were log_{10} or square root transformed to stabilize the residual variance and aid interpretation.

3.4. Results

3.4.1. Effect of *P. indica* on emergence rate

The emergence rate of cv. Battalion (winter 2013), cv. Paragon (spring 2014) and the average of six cultivars of spring wheat seedlings (spring 2015) from control treatments 14 days after sowing was 90 %, 98 % and 95 % respectively. *F. culmorum* application at sowing time reduced the emergence rate by 10 % (P=0.04). There were no other significant differences between treatments.

3.4.2. Effect of *P. indica* on Fusarium Head Blight disease severity and incidence

FHB disease severity of winter wheat cv. Battalion was assessed two weeks after artificial inoculation at GS65. The main effects of fungicide and inoculation were large and significant, but interactions between them and with *P. indica* were also important. Third- and fourth-order interactions were not significant (Appendix Table 1, Chapter 8). Inoculation of ears with *Fusarium* increased the disease severity and incidence significantly (P<0.001) compared to non-inoculated

samples, but there was also some natural background infection of *Fusarium* spp. present (Fig. 3.1 a,b). *F. culmorum* application at the time of sowing did not have a significant effect on FHB disease severity or incidence. FHB severity and incidence in pots inoculated with *P. indica* (at sowing) and *F. graminearum* (at flowering) were reduced by 70 % (severity interaction P=0.004; incidence interaction P=0.005), compared to *F. graminearum* inoculated pots (Fig. 3.1 a,b). Disease severity and incidence were higher in the low fertilisation level than the high level (main effect P<0.001). *Fun. mosseae* reduced severity and incidence of FHB, but this effect was not additive to that of *P. indica*, so *Fun. mosseae* in co-inoculation with *P. indica* gave no extra advantage (Fig. 3.1 a,b).

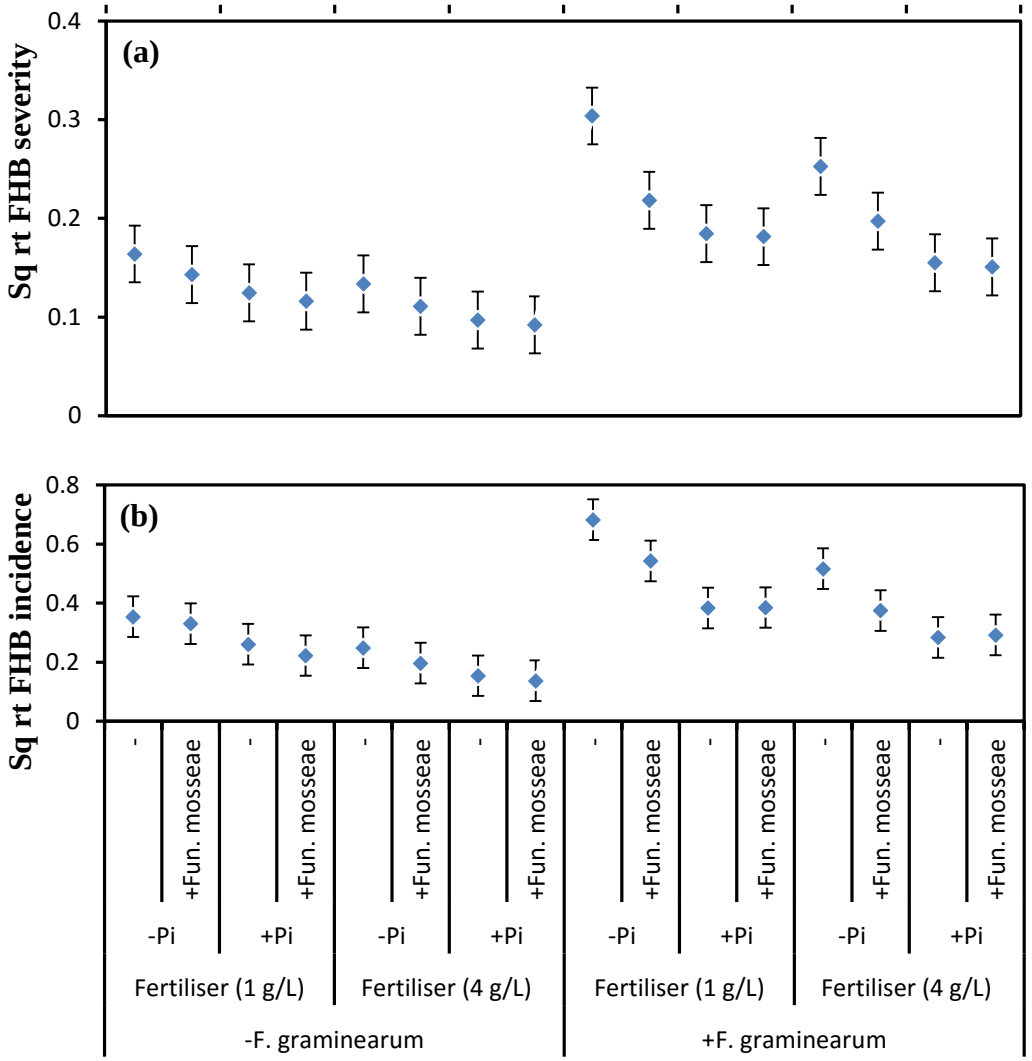


Fig. 3.1. The effect of *Piriformospora indica* (Pi) and *Funneliformis mosseae* under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels on Fusarium head blight (FHB) disease severity and incidence of winter wheat (cv. Battalion), recorded at two weeks after artificial inoculation with *Fusarium graminearum*. (a) FHB disease severity, s.e.d. = 0.02; d.f. = 31 (data were square root transformed); (b) FHB disease incidence s.e.d. = 0.05; d.f. = 31; Each point represents mean ± 2 SEM; (fertiliser: Osmocote® Pro slow release fertiliser).

In spring wheat cv. Paragon, inoculation of ears with Fusarium spores significantly increased the disease severity and incidence of FHB (main effect of inoculation $P<0.001$), but there was also some natural background infection of *Fusarium* spp. (Fig. 3.2 a,b). The application of fungicide following *F. graminearum* inoculation reduced FHB severity by 80 % (fungicide.FHB interaction $P=0.04$). *P. indica* soil inoculation resulted in a reduction in FHB severity, but the effect was only marginally significant (*P. indica* main effect $P=0.07$; Fig. 3.2 a,b; Appendix Table 2, Chapter 8).

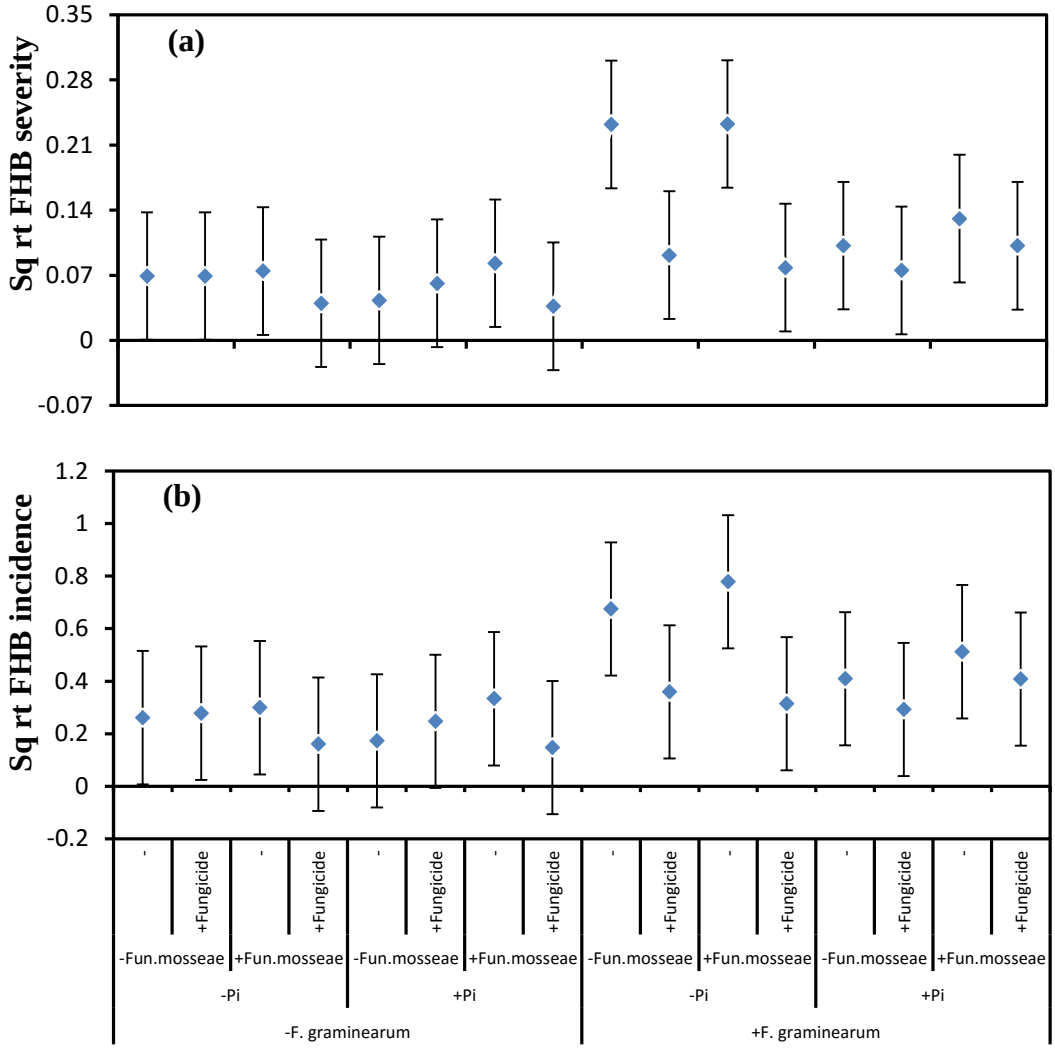


Fig. 3.2. The effect of *Piriformospora indica*, *Funneliformis mosseae* and fungicide Aviator Xpro on Fusarium head blight (FHB) disease severity and incidence of spring wheat (cv. Paragon), recorded at two weeks after artificial inoculation with *Fusarium graminearum* (a) FHB disease severity, s.e.d. = 0.05, d.f. = 30 (data were square root transformed); (b) FHB disease incidence, s.e.d. = 0.18, d.f. = 30, (data were square root transformed); Each point represents mean ± 2 SEM; (Pi = *P. indica* and fungicide: Aviator Xpro).

Ear inoculation of six cultivars of spring wheat with *F. graminearum* spores significantly increased the disease severity and incidence of FHB (main effect of inoculation $P<0.001$), but there was also some natural background infection of *Fusarium* spp. (Fig. 3.3 a,b). FHB severity and incidence in pots inoculated with *P. indica* (at sowing) and *F. graminearum* (at flowering) was reduced by around 80 % (severity *P. indica*. FHB interaction $P<0.001$; incidence interaction $P=0.02$), compared to *F. graminearum* inoculated pots (Fig. 3.3 a,b; Appendix Table 3, Chapter 8).

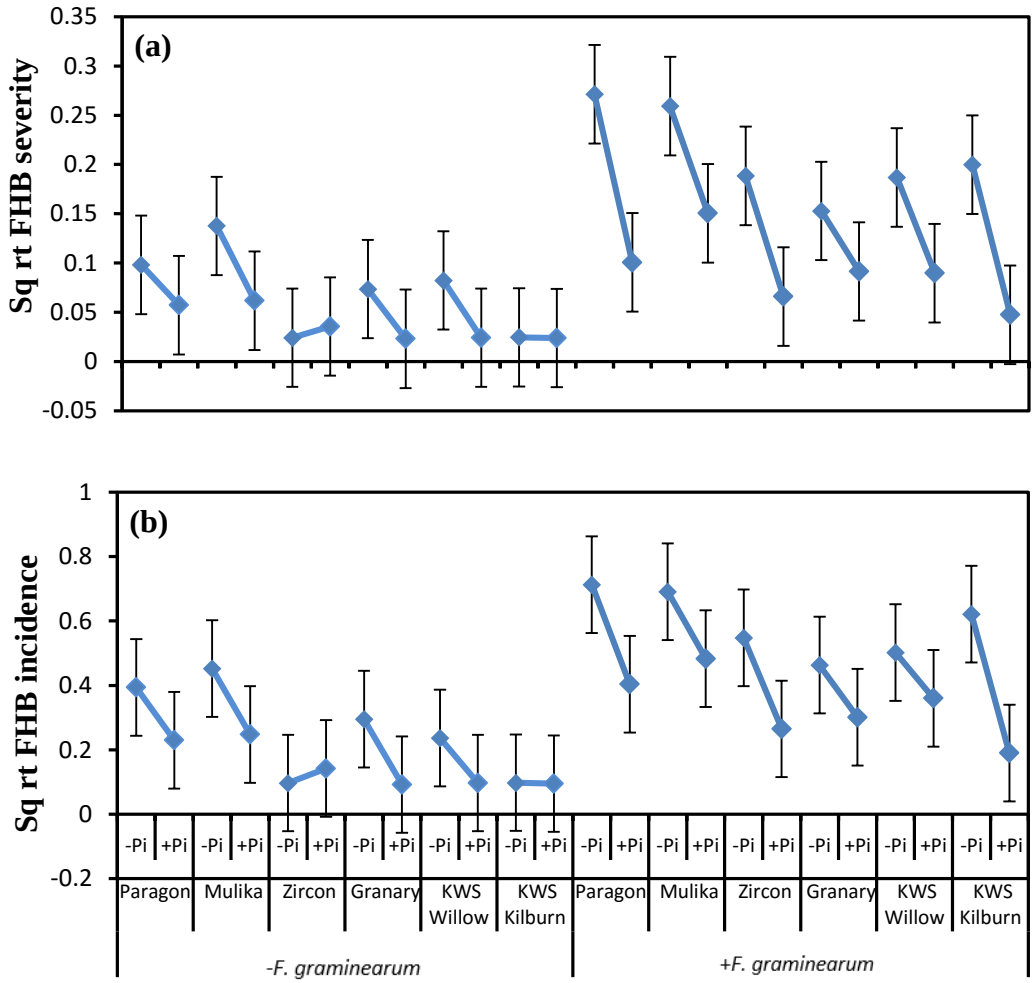


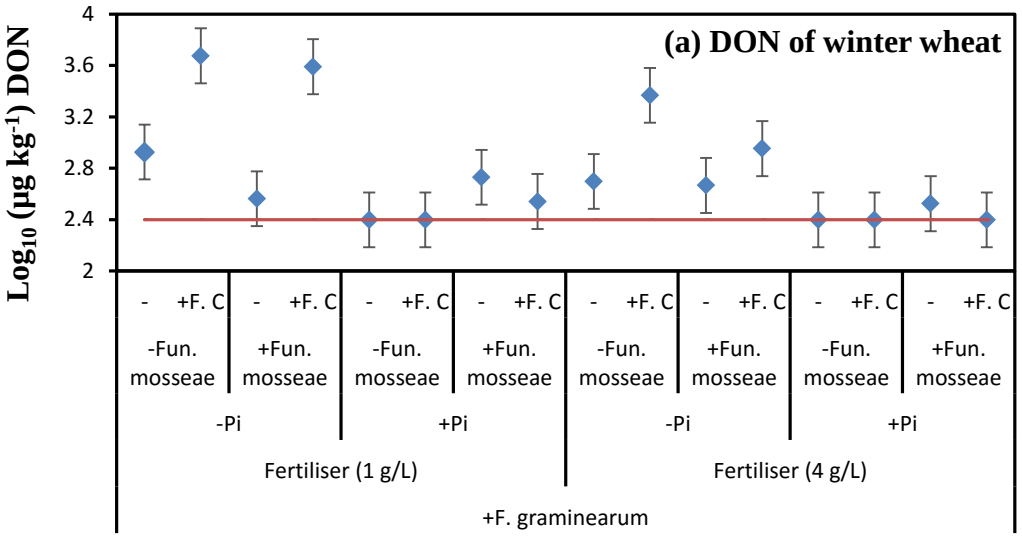
Fig. 3.3. The effect of *Piriformospora indica* (Pi) on Fusarium head blight (FHB) disease severity and incidence of six cultivars of spring wheat (cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn), recorded at two weeks after artificial inoculation with *Fusarium graminearum*. (a) FHB disease severity, s.e.d. = 0.04; d.f. = 46; (b) FHB disease incidence s.e.d. = 0.1; d.f. = 46; (data were square root transformed). Each point represents mean ± 2 SEM.

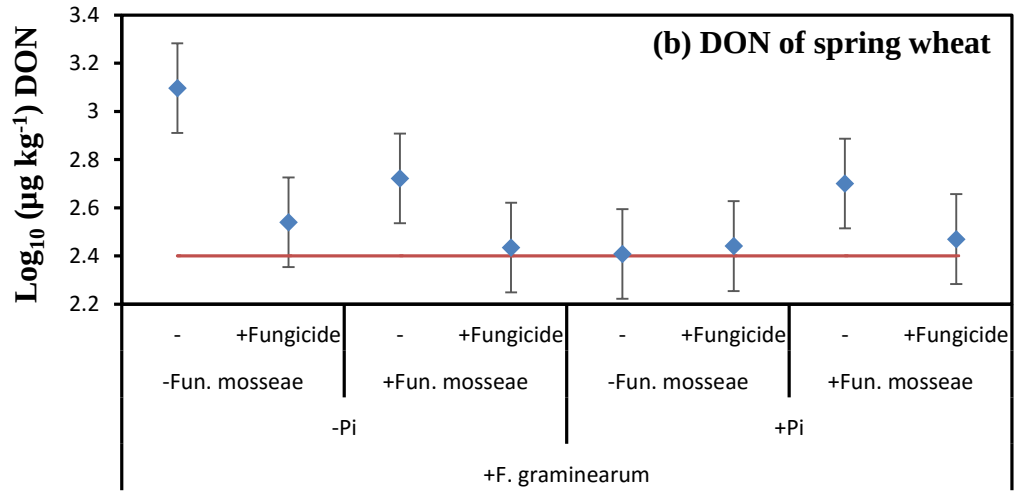
3.4.3. Mycotoxin DON analysis

For both winter and spring wheat samples with no Fusarium head inoculation, DON concentrations were below the limit of detection (<250 µg kg^{-1}). Consequently, analysis was restricted to those samples from plants which were artificially inoculated with *F. graminearum* and considered those lower than the limit of detection as 250 µg kg^{-1}. The following results concern *F. graminearum*-inoculated samples only, in the cv. Battalion in 2014: DON concentrations were 70 % higher at low fertilisation (fertiliser main effect P=0.005) than high fertilisation. *P. indica* application reduced DON concentrations by 70 % at low fertilisation and 50 % at high fertilisation (Fig. 3.4 a; *P. indica*. fertiliser interaction P<0.001), to levels close to the limit of detection, compared to non-inoculated *P. indica* samples. DON concentrations were higher in the samples inoculated at sowing with *F. culmorum* (P<0.001); however, *P. indica* reduced DON concentrations in these samples to below the limit of detection (P<0.001). *Fun. mosseae* had no main effect (P=0.5) and no significant interactions (Fig. 3.4 a; Appendix Table 4, Chapter 8).

In the cv. Paragon spring wheat samples in 2014, inoculation with *F. graminearum* significantly increased DON concentrations (main effect P<0.001, Fig. 3.4 b, Appendix Table 5, Chapter 8). The following results concern *F. graminearum*-inoculated samples only: *P. indica* application (main effect P=0.01) reduced DON concentrations by 80 % (Fig. 3.4 b). Fungicide application (main effect P=0.001) also reduced the mycotoxin concentrations by 70 %, but the effect was not additional to that of *P. indica* (interaction P=0.03). *Fun. mosseae* had no effect on

average (main effect, P=0.5) but had a significant interaction with *P. indica* (P=0.009): without *P. indica*, *Fun. mosseae* reduced DON by roughly 50 %, but in the presence of *P. indica*, *Fun. mosseae* increased DON by about 50 % (Fig. 3.4 b). In 2015, inoculation of six cultivars of spring wheat samples with *F. graminearum* significantly increased DON concentrations (main effect P<0.001, Fig. 3.4 c; Appendix Table 6, Chapter 8); No positive samples were found in the uninoculated pots. The following results concern *F. graminearum*-inoculated samples only: The cultivars differed in mycotoxin DON concentration (P<0.001). *P. indica* application reduced DON concentration by around 90 % (main effect P<0.001). *P. indica* reduced DON concentration in all cultivars, with an interaction arising because cv. KWS Willow and cv. Granary had low concentrations of DON even in non-*P. indica* treated pots (interaction P=0.002, Fig. 3.4 c).


(a) DON of winter wheat
Log10 (µg kg-1) DON
4
3.6
3.2
2.8
2.4
2
- +F. C - +F. C - +F. C - +F. C - +F. C - +F. C - +F. C - +F. C
-Fun. mosseae
+Fun. mosseae
-Pi
+Pi
Fertiliser (1 g/L)
Fertiliser (4 g/L)
+F. graminearum


(b) DON of spring wheat
Log10 (µg kg-1) DON
3.4
3.2
3
2.8
2.6
2.4
2.2
- +Fungicide - +Fungicide - +Fungicide - +Fungicide
-Fun. mosseae
+Fun. mosseae
-Pi
+Pi
+F. graminearum

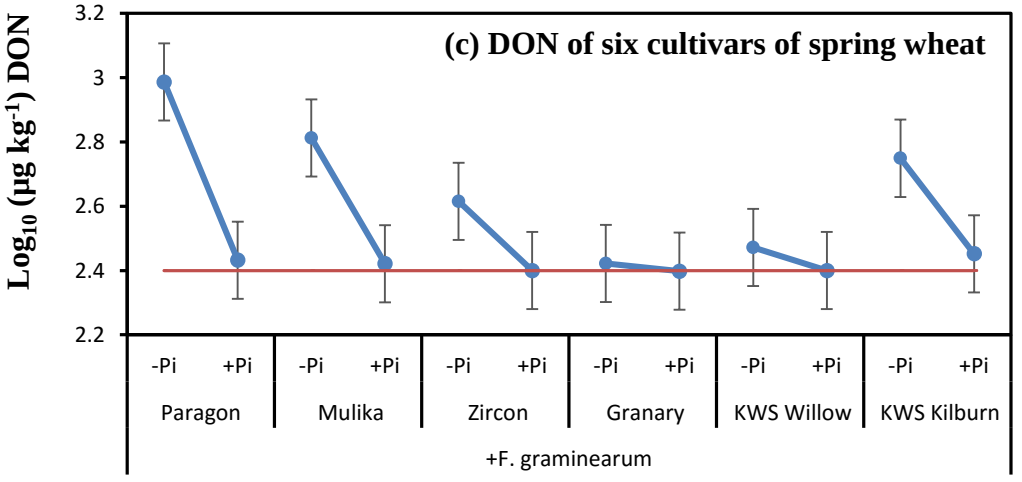

(c) DON of six cultivars of spring wheat
Log10 (µg kg-1) DON
3.2
3
2.8
2.6
2.4
2.2
-Pi +Pi -Pi +Pi -Pi +Pi -Pi +Pi -Pi +Pi -Pi +Pi
Paragon
Mulika
Zircon
Granary
KWS Willow
KWS Kilburn
+F. graminearum

Fig. 3.4. The effect of *Piriformospora indica* (Pi), *Funneliformis mosseae*, fungicide Aviator Xpro, under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels on Fusarium mycotoxin deoxynivalenol (DON) on winter and spring wheat grain samples. (a) DON in winter wheat samples (cv. Battalion), s.e.d. = 0.15, d.f. = 15; (b) DON in spring wheat samples (cv. Paragon), s.e.d. = 0.1, d.f. = 30; (c) DON of six cultivars in spring wheat samples (cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn), s.e.d. = 0.08, d.f. = 22 (data were Log_{10} transformed); Each point represents mean ± 2 SEM; (fungicide: Aviator Xpro and fertiliser: Osmocote® Pro slow release fertiliser, red line: DON limit of detection).

FHB severity was well correlated to DON (r = 0.7, data not shown). Both FHB severity and DON were weakly related to yield, but not to root-shoot ratio, above ground biomass or root biomass.

3.4.4. Harvest results

3.4.4.1. Winter wheat cv. Battalion, 2013-14

Above ground biomass*: Fun. mosseae* increased the above ground biomass in the presence of *F. culmorum* by 17 % at high fertilisation and by 10 % at low fertilisation, compared to *F. culmorum*-inoculated samples (*Fun. mosseae*. *F. culmorum* interaction P<0.001, Table 3.1; Appendix Table 1, Chapter 8). *P. indica* inoculation increased biomass on average (main effect P=0.06). Its combination with *Fun. mosseae* increased the above ground biomass in the presence of *F. graminearum* by 25 % at low fertilisation (*P. indica. Fun. mosseae. F. graminearum* interaction *P* =0.008), compared to samples inoculated with *F. graminearum* alone. The co-inoculation increased biomass also in plants inoculated with *F. culmorum*, by 15 % at low fertilisation and 34 % at high fertilisation (*P. indica. Fun. mosseae. F. culmorum* interaction P=0.07). At low fertilisation, in the

presence of *F. graminearum*, *Fun. mosseae* increased the above ground biomass by 30 % (*Fun. mosseae*. fertiliser. *F. graminearum* interaction P=0.001), compared to *F. graminearum*-inoculated samples at low fertilisation. *F. culmorum* application at sowing time reduced the above ground weight by 7 %, but the effect could have been chance (P=0.09, Table 3.1).

Root biomass: Roots were heavier at high fertilisation than low fertilisation (main effect P<0.001, Table 3.1; Appendix Table 1, Chapter 8). *P. indica* application increased the root weight by 55 % at both low and high fertilisation (main effect P<0.001), compared to non-*P. indica* inoculated samples. The co-inoculation of *Fun. mosseae* with *P. indica* also increased the root weight by 52 % at low fertilisation and 37 % at high fertilisation (*P. indica*. *Fun. mosseae* P<0.001). *F. culmorum* reduced the root weight by 40 % at both low and high fertilisation (interaction P<0.001). This reduction was smaller when *P. indica* (P=0.01) or *Fun. mosseae* (P=0.01) were also applied (Table 3.1).

Yield: *Fun. mosseae* at low fertilisation increased the total grain weight by 5 %, but at high fertilisation it decreased the weight by 20 % (*Fun. mosseae*. fertiliser interaction P=0.03, Table 3.1; Appendix Table 1, Chapter 8), compared to non-*Fun. mosseae*-inoculated samples. The combination of *P. indica* and *Fun. mosseae* increased the total grain weight by 60 % in the presence of *F. graminearum* (*P. indica. Fun. mosseae. F. graminearum* interaction P=0.09) at low fertilisation level, compared to *F. graminearum*-inoculated samples. The combination of *P. indica* and *Fun. mosseae* increased the total grain weight in the presence of *F. culmorum*

at both low and high fertilisation (*P. indica. Fun. mosseae. F. culmorum* interaction P=0.05, Table 3.1; Appendix Table 1, Chapter 8).

TGW*: P. indica* application increased thousand grain weight (TGW) by 8 % at low fertility (main effect P=0.02, Table 3.1; Appendix Table 1, Chapter 8). The application of *F. graminearum* reduced TGW by 10 % (P=0.06) at both low and high fertilisation. However, *P. indica* maintained TGW in the presence of *F. graminearum* at low fertilisation (*P. indica. F. graminearum* interaction P=0.04). The combination of *P. indica* and *Fun. mosseae* increased TGW at high fertilisation, but not at low fertilisation (*P. indica*. *Fun. mosseae.* fertiliser interaction P=0.008, Table 3.1).

Harvest index: There were no significant differences among treatments for harvest index (Appendix Table 1, Chapter 8).

Ears: Fertilisation increased the number of ears per pot (main effect P<0.001). The combination of *P. indica* and *Fun. mosseae* increased the number of ears at both low and high fertilisation (*P. indica*. *Fun. mosseae*. fertiliser interaction P=0.02), compared to non-*P. indica*-inoculated samples (Table 3.1; Appendix Table 1, Chapter 8).

Table 3.1. Harvest results of winter wheat samples (cv. Battalion), inoculated with *Piriformospora indica*, *Funneliformis mosseae*, *Fusarium culmorum* (at sowing time) and *F. graminearum* (F. g; at flowering time) under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels (F.c: *F. culmorum* and fertiliser: Osmocote® Pro slow release fertiliser). Harvest index: total grain weight (g)/total above grain weight (g).

Fertiliser	*P. indica*	F.g	*Fun. mosseae*	F.c	Total above ground weight (g)	Root weight (g)	Total grain weight per pot (g)	1000 grain weight (g)	Harvest index	no of ears per pot (Log_{10})
1 g L^{-1}	-	-	-	-	243	23	78	68	0.3	1.4
				+	227	16	77	66	0.3	1.4
			+	-	264	21	82	71	0.3	1.4
				+	251	27	84	70	0.3	1.4
		+	-	-	204	21	57	60	0.3	1.4
				+	195	17	62	63	0.3	1.4
			+	-	266	27	83	69	0.3	1.4
				+	274	33	79	67	0.3	1.4
				mean	**241**	**23**	**75**	**67**	**0.3**	**1.4**
	+	-	-	-	272	34	85	73	0.3	1.4
				+	217	38	63	68	0.3	1.3
			+	-	257	35	83	67	0.3	1.4
				+	261	34	90	68	0.3	1.4
		+	-	-	247	28	77	66	0.3	1.3
				+	221	35	65	73	0.3	1.3
			+	-	257	32	92	68	0.4	1.4
				+	276	32	88	69	0.3	1.4
	-			**mean**	**251**	**34**	**80**	**69**	**0.3**	**1.3**

Fertiliser	*P. indica*	F.g	*Fun. mosseae*	F.c	Total above ground weight (g)	Root weight (g)	Total grain weight per pot (g)	1000 grain weight (g)	Harvest index	no of ears per pot (Log_{10})
			-	-	336	27	120	69	0.4	1.7
				+	276	19	95	67	0.3	1.6
		-	+	-	303	38	96	71	0.3	1.7
				+	326	34	94	68	0.3	1.7
			-	-	307	31	89	64	0.3	1.6
				+	277	18	93	69	0.3	1.6
		+	+	-	305	38	110	65	0.4	1.7
				+	298	32	92	67	0.3	1.7
4 g L^{-1}				**mean**	**304**	**30**	**99**	**68**	**0.3**	**1.8**
			-	-	317	42	125	68	0.4	1.7
				+	281	37	94	69	0.3	1.6
		-	+	-	301	37	102	71	0.3	1.6
	+			+	372	37	129	71	0.3	1.7
			-	-	380	41	122	65	0.3	1.6
				+	316	38	97	69	0.3	1.6
		+	+	-	266	37	81	70	0.3	1.6
				+	297	39	92	68	0.3	1.6
				mean	**316**	**39**	**105**	**69**	**0.3**	**1.7**
				s.e.d.	24	3.09	17.3	3.07	0.05	0.05

3.4.4.2. Spring wheat cv. Paragon, 2014

The application of *P. indica* increased total above ground weight by 16 % (main effect P=0.05), root weight by 20 % (main effect P=0.02), total grain weight by 23 % (main effect P=0.02), TGW by 23 % (main effect P=0.08), harvest index by 8 % (main effect P=0.07), and number of ears by 12 % (main effect P=0.003), compared to samples without *P. indica* (Table 3.2; Appendix Table 2, Chapter 8). The interaction of *P. indica* with *F. graminearum* increased total grain weight of *F. graminearum*-inoculated samples by 54 % (P=0.08) and harvest index by 13 % (P=0.07), compared to samples inoculated with *F. graminearum* alone. Also, the combination of *P. indica*, *Fun. mosseae* and fungicide increased total above ground weight (P=0.03), total grain weight (P=0.003), TGW (P=0.01), harvest index (P=0.009) and number of ears (P=0.003) (Table 3.2), compared to the control (no-amendment) samples.

Table 3.2. Harvest results of spring wheat samples (cv. Paragon**),** inoculated with *Piriformospora indica*, *Funneliformis mosseae* (at sowing time), *Fusarium graminearum* (F. g; at flowering time) and fungicide Aviator Xpro (at growth stage 39 and 72 hours after artificial inoculation at flowering time). Harvest index: total grain weight (g)/total above grain weight (g).

P. indica	F.g	*Fun. mosseae*	Fungicide	Total above ground weight (g)	Root weight (g)	Total grain weight per pot (g)	1000 grain weight (g)	Harvest index	no of ears per pot (Log_{10})
-	-	-	-	193	23	73	43	0.4	39
			+	229	28	103	52	0.5	41
		+	-	212	24	98	50	0.5	39
			+	201	24	79	46	0.4	35
	+	-	-	183	21	62	38	0.3	36
			+	199	22	83	45	0.4	38
		+	-	213	29	86	50	0.4	38
			+	214	30	90	45	0.4	35
			mean	**206**	**25**	**84**	**46**	**0.4**	**38**
+	-	-	-	225	28	89	53	0.4	44
			+	205	28	91	47	0.5	40
		+	-	205	29	82	46	0.4	39
			+	232	28	102	47	0.4	41
	+	-	-	217	28	96	51	0.4	40
			+	204	28	91	47	0.4	37
		+	-	236	28	95	51	0.4	40
			+	226	25	108	48	0.5	39
			mean	**219**	**28**	**94**	**49**	**0.4**	**40**
			s.e.d.	18.5	2.8	10.9	4.1	0.04	2.01

3.4.4.3. Six cultivars of spring wheat, 2015

Averaged over other treatments, the cultivars of spring wheat differed in above ground biomass (P=0.02), root weight (P=0.09), total grain weight (P=0.001), and the number of ears per pot (P<0.001, Table 3.3; Appendix Table 3, Chapter 8). Averaged over cultivars, *P. indica* inoculation increased the above ground biomass (P<0.002), root weight (P= 0.002), total grain weight (P<0.001), TGW (P<0.001), harvest index (P<0.001) and the number of ears per pot (P=0.002), compared to the control (no-amendment) samples. *F. graminearum* application at flowering reduced the above ground biomass (P=0.06), total grain weight (P<0.001), and harvest index (P=0.03) of all cultivars (Table 3.3; Appendix Table 3, Chapter 8). In the presence of *F. gramineraum, P. indica* inoculation increased the above ground biomass and TGW (*P. indica*.*F. graminearum* interaction P=0.04 and P=0.03, respectively), compared to *F. graminearum*-inoculated samples. There was no interaction between *P. indica* or *F. graminearum* with cultivars (Table 3.3).

Table 3.3. Harvest results of six cultivars of spring wheat samples (cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn), inoculated with *Piriformospora indica* (at sowing time) and *F. graminearum* (F. g; at flowering time). Harvest index: total grain weight (g)/total above grain weight (g).

P. indica	F. g	Spring wheat cultivars	Total above ground weight (g)	Root weight (g)	Total grain weight per pot (g)	1000 grain weight (g)	Harvest index	No of ears
		Paragon	267	18.6	82	45	0.3	51
		Mulika	267	15.3	94	47	0.4	52
		Zircon	289	17.9	103	48	0.4	66
	-	Granary	250	16.2	87	46	0.4	60
		KWS Willow	283	14.8	105	45	0.4	59
		KWS Kilburn	257	16.1	93	44	0.4	62
-		**mean**	269	16.5	94	46	0.4	58
		Paragon	201	17.2	61	39	0.3	54
		Mulika	228	16.8	72	43	0.3	53
		Zircon	245	17.4	88	45	0.4	61
	+	Granary	219	15.7	74	44	0.3	60
		KWS Willow	257	17.4	71	41	0.3	65
		KWS Kilburn	251	17.1	74	41	0.3	58
		mean	234	16.9	73	42	0.3	59
		Paragon	223	27.4	102	65	0.5	56
		Mulika	284	20.1	127	65	0.4	57
		Zircon	338	22.8	154	62	0.5	74
	-	Granary	257	20.8	111	61	0.4	68
		KWS Willow	302	22.4	97	61	0.3	70
		KWS Kilburn	269	21.3	97	55	0.4	61
+		**mean**	279	22.5	115	62	0.4	64
		Paragon	280	21.7	89	60	0.3	61
		Mulika	273	23.01	108	65	0.4	58
		Zircon	269	24.6	115	60	0.4	69
	+	Granary	269	22.7	105	59	0.4	65
		KWS Willow	325	22.9	102	64	0.3	62
		KWS Kilburn	268	21.1	103	66	0.4	61
		mean	281	22.7	104	62	0.4	63
		s.e.d.	30.9	2.1	13.4	3.6	0.05	5.3

3.4.5. Soil and leaf tissue nutrients analysis, 2014-15

Soils were more acidic at high fertilisation ($P<0.001$, (Table 3.4.; Appnedix Table 7, Chapter 8). The concentrations of soil P, NO_3, NH_4 and available N and percentage wet weight were higher at high fertilisation, compared to low fertilisation (all main effects $P<0.001$). The concentration of soil Mg was 34 % higher at the low fertilisation level (main effect $P<0.001$). *P. indica* and *Fun. mosseae* did not have any effect on any of the soil nutrients. The combination of *P. indica* and *Fun. mosseae* at high fertilisation increased the amount of soil NO_3, NH_4 and available N, compared to low fertilisation (*P. indica*, *Fun. mosseae* and fertiliser interaction $P=0.02$), but on their own, each decreased these levels (Table 3.4.).

The amount of leaf total N, P, K, Ca, Mg, S, Mn, Cu, Zn and B were all higher at high fertilisation (main effect $P<0.001$, Table 3.5; Appnedix Table 8, Chapter 8). However, the concentration of Fe was higher at low fertilisation (main effect $P=0.002$). At high fertility, the concentration of B in the leaves was lower in the presence of *P. indica* (main effect $P=0.01$), relative to non-*P. indica* inoculated samples. The combination of *P. indica* and *Fun. mosseae*, at high fertilisation, increased the total amount of N in the leaves (*P. indica*, *Fun. mosseae* and fertiliser interaction $P=0.04$), but on their own, each decreased leaf N concentration (Table 3.5).

Table 3.4. Soil nutrient analysis results of winter wheat samples inoculated or not with *Piriformospora indica* and *Funneliformis mosseae* at sowing time. The experiment carried out in the 2014-15 growing season (fertiliser: Osmocote® Pro slow release fertiliser, P: phosphorus, K: potassium, Mg: magnesium, N: Nitrogen, Nitrate: NO_3, Ammonium: NH_4; d.f. = 14).

Fertiliser	*P. indica*	*Fun. mosseae*	Soil pH	P mg L^{-1}	K mg L^{-1}	Mg mg L^{-1}	NO_3 mg kg^{-1}	NH_4 mg kg^{-1}	Available N kg N ha^{-1}	Dry Matter %w/w
1 g/L	-	-	6.4	34	95	122	5	6	40	81
		+	6.4	26	95	117	3	4	25	82
		Mean	6.4	30	95	120	4	5	33	82
	+	-	6.2	31	103	120	4	5	32	80
		+	6.5	25	83	113	1	1	9	81
		Mean	6.4	28	93	117	3	3	21	81
4 g/L	-	-	5.2	53	92	82	12	20	121	88
		+	5.4	46	87	90	7	10	66	87
		Mean	5.3	49	90	86	10	15	94	88
	+	-	5.3	47	94	90	9	11	77	85
		+	5.2	51	114	91	18	23	153	84
		Mean	5.3	49	104	91	14	17	115	85
		s.e.d.	0.2	5	12	8	3	4	26	0.9

Table 3.5. Leaf tissue nutrient analysis results of winter wheat samples inoculated or not with *Piriformospora indica* and *Funneliformis mosseae* at sowing time. The experiment carried out in the 2014-15 growing season (fertiliser: Osmocote® Pro slow release fertiliser, N: Nitrogen, P: phosphorus, K: potassium, Ca: calcium, Mg: magnesium, S: sulphur, Mn: manganese, Cu: copper, Zn: zinc, Fe: Iron, B: boron; d.f. = 14).

Fertiliser	*P. indica*	*Fun. mosseae*	Total N %w/w	Total P g kg^{-1}	Total K g kg^{-1}	Total Ca g kg^{-1}	Total Mg g kg^{-1}	Total S g kg^{-1}	Total Mn g kg^{-1}	Total Cu g kg^{-1}	Total Zn g kg^{-1}	Total Fe g kg^{-1}	Total B g kg^{-1}
1 g/L	-	-	3	4.5	35.8	2.8	0.9	2.6	0.12	4	29	517	3
		+	3	5.2	40.5	2.8	0.9	3.6	0.14	4	32	192	3
		Mean	3	4.8	38.2	2.8	0.9	3.1	0.13	4	31	355	3
	+	-	3	4.9	39.8	2.8	1.02	3.4	0.15	5	31	214	3
		+	3	4.9	38	2.7	1	3.1	0.14	4	31	173	3
		Mean	3	4.9	38.9	2.7	1.01	3.3	0.15	5	31	194	3
4 g/L	-	-	5	7.8	52.6	4.1	1.5	7.4	0.22	8	60	157	4
		+	4	7.5	51	3.6	1.4	6.5	0.21	6	53	121	4
		Mean	5	7.6	51.8	3.9	1.5	6.9	0.21	7	57	139	4
	+	-	4	7.9	52.8	3.7	1.4	6.8	0.2	7	56	135	3
		+	5	7.1	52.6	4.1	1.5	6.01	0.2	6	54	121	3
		Mean	5	7.5	52.7	3.9	1.5	6.4	0.2	7	55	128	3
		s.e.d.	0.3	0.63	3.5	0.45	0.13	0.7	0.02	0.6	5	76	0.3

3.5. Discussion

P. indica effectively reduced FHB disease severity and incidence, and also grain DON contamination. It was as effective as fungicide applied 72 hours after *F. graminearum* inoculation, and the effect was consistent across years and cultivars. *P. indica* also increased yield in both high and low fertilisation, suggesting *P. indica* application is compatible with low-input systems. However, unlike mycorrhizal fungi, its effect was greater at the high fertilisation level. *P. indica* application was compatible with *Fun. mosseae* and fungicide, but effects of these were not additive. Collectively, these results suggest that *P. indica* application could be useful in the long-term. *P. indica* reduced FCR at sowing, FHB at flowering and grain DON contamination, suggesting there would be fewer spores, hyphae and macroconidia overwintering in soil and crop residues; as a result, there would be less inoculum available for the disease to occur in the next season. The results of soil and leaf tissue analysis suggest that *P. indica* does not have any effect on soil and plant tissue nutrients in the winter wheat cv. Battalion at the overall fertility levels tested. Fungicide application during wheat growing stages can reduce the risk of FHB and mycotoxin contamination (Paul et al., 2008, Edwards & Godley, 2010). However, inconsistent control of FHB disease with fungicide has been found in several reports (McMullen, 1994, Horsley et al., 2006). Yoshida et al. (2012) indicated that the timing of fungicide application differentially affected FHB disease and mycotoxin concentration, considering anthesis as the crucial stage for fungicide application. The application of fungicide, in the experiment, at GS 39 (when flag leaf was fully

emerged), and then at anthesis GS 65 (72 hours after *Fusarium* inoculation), reduced both FHB and DON concentration. In the spring wheat experiments, *P. indica* application at sowing also reduced FHB severity and incidence as effectively as fungicide (Fig. 3.2; Appendix Table 2, Chapter 8). The application of *P. indica* might not only reduce the use of fungicide and any environmental damage from fungicide use, but also increase plant resistance against other pathogens (Bagde et al., 2010, Franken, 2012).

The fungicide Aviator Xpro is systemic and it might have inhibitory effect on the colonasation of roots by both *P. indica* and *Fun. mosseae*. Both *P. indica* and *Fun. mosseae* were applied at sowing and the colonisation of the roots were confirmed microscopically. The fungicide was applied at flowering. Diedhiou et al. (2004) showed that foliar applications of fungicide did not have negative effects on established mycorrhizal colonization of maize plants. Hernández-Dorrego and Parés (2010) also demonstrated that there was no direct relationship between the application of systemic foliar fungicides and a detrimental effect on mycorrhizal symbiosis, and there was no evidence either that the foliar application of fungicides were inoquous for the mycorrhizal fungi.

The DON concentration in samples inoculated at sowing with *F. culmorum* and then at heading with *F. graminearum* was much higher than in samples inoculated only with *F. graminearum* (Fig. 3.4 a; Appendix Table 4, Chapter 8). This suggests that when *Fusarium* is already present in the plant, there is an increased risk of mycotoxin production in the grains by FHB. *F. culmorum* might have produced

DON that moved from lower parts of the plants to the heads, consistent with the results of Moretti et al. (2014) and Covarelli et al. (2012) who demonstrated that although *F. graminearum* and *F. culmorum* could not be detected beyond the third internode, a low concentration of DON was found in the kernels beyond those tissues colonized by the fungus; suggesting that DON can be moved from lower parts of the plants to the heads. This is probably due to its water solubility, which can cause a reduction in concentration at late harvest, but in this case led to transfer upwards. Alternatively, Mudge et al. (2006) isolated *F. graminearum* and DON from wheat heads and flag leaf nodes following inoculation of the stem base. Xu et al. (2007) indicated that the mycotoxin productivity of *F. graminearum* in the co-inoculation with *F. culmorum* and *F. poae* was higher than that in the single-isolate inoculations. However, in the present case DON concentrations in the ear were not detectably increased by root infection with *F. culmorum* in the absence of *F. graminearum* inoculation.

In the winter wheat experiment, *P. indica* increased the above ground weight, total grain weight and thousand grain weight by similar amounts under both low and high fertilisation, suggesting that the *P. indica* effect on grain yield was independent of fertiliser levels (Tables 3.1; Appendix Table 1, Chapter 8). Similarly Achatz et al. (2010) found that increased grain yield in *P. indica* inoculated barley was independent of the fertilisation level. Murphy et al. (2014b) found that *P. indica*-inoculated barley had greater grain weight in higher nutrient input. These indicates that *P. indica*-induced yield increase does not result from relief of low phosphorus

or nitrogen supply. By contrast, both my results and those of Achatz et al. suggest that the increase in the above ground weight caused by *Fun. mosseae* only occurred under low fertility. The difference in response to high fertility shows that the beneficial effects of *P. indica* are based on different mechanisms from mycorrhizal fungi. The effect of *P. indica* under low and high fertilisation levels on final yield of winter wheat was confirmed on a small scale experiment (see chapter 4, page 132).

Consistent with these results, Shahabivand et al. (2012) and Yaghoubian et al. (2014) reported that *P. indica* increased wheat growth more than *Fun. mosseae* and that their co-inoculation improved the defence mechanisms, drought resistance, and growth of wheat plants, suggesting *P. indica* application was compatible with *Fun. mosseae* application.

During these experiments, the severity of any air-borne diseases which occurred naturally was scored (data shown in chapter 4). *P. indica* reduced disease severity and incidence of Septoria leaf blotch at GS 22 (tillering stage) and yellow rust at GS 35-37 (stem elongation, 5th node detectable to flag leaf just visible) for the winter wheat cv. Battalion, and yellow rust and powdery mildew at GS 70 (milk development) for six different cultivars of spring wheat. In a small-scale experiment the effect of *P. indica* on Septoria leaf blotch was confirmed at seedling stage; this is consistent with *P. indica* producing a generalised increase in resistance to a wide class of fungi.

These results show that *P. indica* colonised and increased shoot and final yield of the winter wheat (cv. Battalion) and six cultivars of spring wheat. *P. indica* reduced disease severity and incidence of FHB, and other foliar diseases and DON concentration of all cultivars. It is consistent with Deshmukh et al. (2006) and Deshmukh and Kogel (2007)'s study. They inoculated different barley cultivars seedlings with *P. indica* and different isolates of *Sebacina vermifera* (member of Sebacinaceae, genetically close to *P. indica*). Despite considerable variation of the fungal activity of the different isolates, they found increase in shoot and root biomass with consistent resistance-inducing activity of all strains of the *S. vermifera* against powdery mildew (caused by *Blumeria graminis* f.sp. *hordei*) as with *P. indica.* In contrast, Gravouil (2012) showed that different barley cultivars had different rates of colonisation by *P. indica*. Some barley cultivars had the highest rate of *P. indica* colonisation and the best increase in shoot biomass and protection against pathogens such as *Rhynchosporium commune.*

The results of the nutrient experiment showed that the soil was wetter at high fertilisation, presumably because roots were growing better. *P. indica* did not have any effect on either soil or more importantly leaf nutrients, suggesting that at least in the case of this experiment, *P. indica* effects on growth and yield were not due to better nutrient uptake. These results are inconsistent with others that suggest *P. indica* increased the uptake of micro- and macro-nutrients and so leads to growth promotion (Varma et al., 2013b, Bajaj et al., 2014, Shrivastava & Varma, 2014). Gosal et al. (2010) reported that *P. indica* increased the amount of Cu, Zn and Mn

in *Chlorophytum sp*. and promoted plant growth and biomass. *P. indica* increased the amount of Zn in Turmeric (*Curcuma longa* L.) and enhanced the growth, yield and active ingredients (Bajaj et al., 2014). The inconsistency with their results might have various causes. It might be due to the host differences, the methods of plant cultivations and inoculations, environmental effects or differences in the fertilisers and their concentrations. However, *Fun. mosseae* also did not have any effects on soil and leaf nutrients, suggesting no effect of *P. indica* and/or *Fun. mosseae* might be because of the experimental conditions. However, as *P. indica* protected wheat seedlings from FCR and reduced FHB severity and the mycotoxin DON concentration in the previous experiments, it is possible to reject the hypothesis that *P. indica* mode of action is due to nutrient uptake and the effects are not simply nutritional. Therefore, more work is needed to understand the issue; this is beyond the scope of this thesis.

These results suggest that *P. indica* could be useful in control of FCR and FHB, mycotoxin contamination and other air-borne diseases. However, *P. indica* is probably an alien species in many parts of the world including the UK, so its releases into the open environments in these regions, to confirm its beneficial effects, requires consideration also of physiological trade-offs and ecological and agronomical side-effects. The wider effects of *P. indica* and similar organisms also need to be better understood before agricultural deployment. A search for native organisms with similar characteristics might be a safer direction to go in.

CHAPTER 4- *Piriformospora indica* effect on foliar diseases

M. Rabiey, and M. W. Shaw

M. Rabiey: did all the experiments;

M. W. Shaw: advised on design, analysis and interpretation.

4.1. Summary

The effect of *P. indica* on air-borne diseases of winter and spring wheat, including Septoria leaf blotch, yellow rust and powdery mildew, was assessed under outdoor conditions. *P. indica* reduced Septoria leaf blotch severity and incidence of winter wheat (cv. Battalion), naturally and/or artificaly infected with *Zymoseptoria tritici*, at early growth stage. *P. indica* also reduced yellow rust, naturally infected with *Puccinia striiformis* f.sp. *tritici*, and powdery mildew, naturally infected with *Blumeria graminis* f.sp. *tritici*, disease severity and incidence of winter (cv. Battalion) and six cultivars of spring wheat (cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn). These results suggest that *P. indica* might be a useful in biocontrol of air-borne diseases of wheat.

4.2. Introduction

Wheat is subject to many foliar diseases during its growing season, such as Septoria leaf blotch, yellow (stripe) rust and powdery mildew (Wiese et al., 2000, Bockus et al., 2010).

Septoria leaf blotch is caused by the fungus *Zymoseptoria tritici* (Quaedvlieg et al., 2011) (also known as *Mycosphaerella graminicola* and *Septoria tritici*) and is the most significant and major threat to wheat yields in the UK, much of the rest of

Europe, and many other wheat growing regions. In developed agriculture, problems are increasing as currently available fungicides become less effective against resistant strains of the disease (Cools & Fraaije, 2008, Anon, 2009, Torriani et al., 2009, DEFRA, 2013). The disease can cause serious yield losses ranging up to 50 % (Goodwin et al., 2011). A key feature of Septoria leaf blotch is the long symptomless growth of the fungus, which can nonetheless affect the host plant's cells, before it switches to the visible disease phase that eventually destroys the plant's leaves (Duncan & Howard, 2000). The disease is characterized by necrotic lesions on leaves and stems that develop after infected cells collapse, and is more prevalent during cool and wet weather. The disease is common on wheat in the tillering stages but causes little damage because leaf production outpaces leaf death due to the pathogen. After ear emergence the disease becomes quite severe on the upper leaves. Infection of the flag, second and third leaf can cause significant losses (Shaw & Royle, 1993, Jørgensen et al., 2014).

Yellow (stripe) rust is caused by the fungus *Puccinia striiformis* f.sp. *tritici*, and is a serious disease of wheat occurring in the UK and most wheat areas with cool and moist weather conditions during the growing season (Wellings, 2011, Chen et al., 2014). Severe epidemics are usually associated with very susceptible cultivars, mild winters and cool moist summers. Yield losses of 40-50 % have often been recorded in susceptible cultivars (Wellings, 2011). The disease is characterized by mass of yellow to orange urediniospores erupting from pustules arranged in long, narrow stripes on leaves (usually between veins), leaf sheaths, glumes and awns on

susceptible plants (Hovmøller et al., 2010, Hovmøller et al., 2011). The disease is common at seedling stage but also after ear emergence on the upper leaves. The disease has a very short latent period and can be found before leaves have fully expanded (Dedryver et al., 2009, de Vallavieille-Pope et al., 2011).

Powdery mildew is caused by the fungus *Blumeria graminis* f.sp. *tritici* and is widely distributed throughout the world, particularly in warm, breezy conditions with short periods of high humidity (Oberhaensli et al., 2011, Asad et al., 2014). Powdery mildew is characterized by white, cottony patches of mycelium and conidia on the surface of the plant. They can occur on all aerial parts of the plant including stems and heads, but are most conspicuous on the upper surfaces of lower leaves. As the growing season progresses, sexual fruiting structures (cleistothecia) appear as distinct brown-black dots within aging colonies on maturing plants (Li et al., 2011, Li et al., 2012, Piarulli et al., 2012).

To control all foliar diseases, growers are recommended to monitor the crop and, depending on cultivar susceptibility, disease presence and/or rain or irrigation status, apply fungicides (Hershman, 2012, Stewart et al., 2014). There are currently no fully resistant cultivars available for these diseases and use of fungicide has led to fungicide resistance and environmental pollution (Arraiano et al., 2009, Hershman, 2012). Any measure which reduces rate of development, will make resistance, fungicide and sowing date changes more effective. Casual observations from previous expeiments motivated me to do more expeiments on the effect of *P. indica*

on foliar diseases. The experiments were performed on a small scale as the main aim of this research was to examine the effect of *P. indica* on Fusarium diseases.

Hypothesis tested in this chapter: In this chapter the hypothesis that *P. indica* would reduce severity and incidence of any naturally infected foliar diseases is tested.

4.3. Materials and Methods

4.3.1. Plant materials and pot experiments

4.3.1.1. The effect of P. indica on naturally infecting foliar diseases

An experiment was set up to examine the effect of *P. indica* on foliar diseases arising from natural infections, such as powdery mildew, rust, Septoria leaf blotch and aphids. Winter wheat seeds, cv. Battalion, were surface disinfected and pre-germinated. Eight seeds per pot were planted in 12 L pots at a depth of two cm in two parts non-sterilised compost and one part sand, mixed with 4 g L^{-1} of slow release fertiliser (8-9 months, Osmocote® Pro).

The experiment was carried out in the 2014-15 growing season at the University of Reading, under natural conditions. The experiment had four treatments with five replicates, distributed in five randomised blocks, with the following factorial combinations of treatments: ±*P. indica*, and ±fertiliser (1 g L^{-1} or 4 g L^{-1}). Inoculation with *P. indica* (6 g liquid culture mixed with soil) was done at sowing.

4.3.1.2. The effect of **P. indica** *on artificially infected* **Z. tritici** *at seedling growth stage*

To confirm the effect of *P. indica* on *Z. tritici* an experiment was conducted at seedling growth stages under low and high fertiliser levels. The experiment was carried out in the spring-summer 2014 at the University of Reading, under natural conditions. The experiment had eight treatments with four replicates, distributed in four randomised blocks, with the following factorial combinations of treatments: ±*P. indica*, ±*Z. tritici*, and ± fertiliser (1 g L^{-1} or 4 g L^{-1}). Four winter wheat seeds, cv. Battalion, were sown in 1 L pots (top diameter: 13 cm, bottom diameter: 10 cm, depth: 11 cm) in two parts non-sterilised compost and one part sand, mixed with 1 g L^{-1} or 4 g L^{-1} of slow release fertiliser (3-4 months, Osmocote® Pro). Inoculation with *P. indica* (4 g) was done at the time of sowing. The spore suspension of *Z. tritici* contained $1x10^6$ spore mL^{-1}. The first and second leaf of each pot, when fully emerged at GS 12, were tagged and sprayed with 1 mL of *Z. tritici* spore suspension. Later at GS 22 the disease severity and incidence was scored visually on a percentage scale (Bazot et al., 2011).

4.3.2. Statistical analysis of experiments

ANOVA was used to analyse all data using Genstat 17th ed, (VSN, UK) with appropriate blocking. Where applicable, data were $\log_{10}$ or square root transformed to stabilize the residual variance and aid interpretation.

4.4. Results

4.4.1. Effect of *P. indica* on *Z. tritici*

Septoria leaf blotch, naturally infected with *Z. tritici* was recorded at GS 24-26 (tillering stage, main shoot with 4-6 tillers). *P. indica* reduced Septoria disease severity ($P<0.001$) and incidence ($P=0.005$) by 65 % and 46 %, respectively (Fig. 4.1 a,b; Appendix Table 9, Chapter 8). Disease severity ($P<0.001$) and incidence ($P<0.001$) were 83 % and 60 % higher at low fertilisation, respectively (Fig. 4.1 a,b), compared to high fertilisation. *P. indica* reduced Septoria disease severity at high fertility (*P. indica*. fertiliser $P=0.002$).

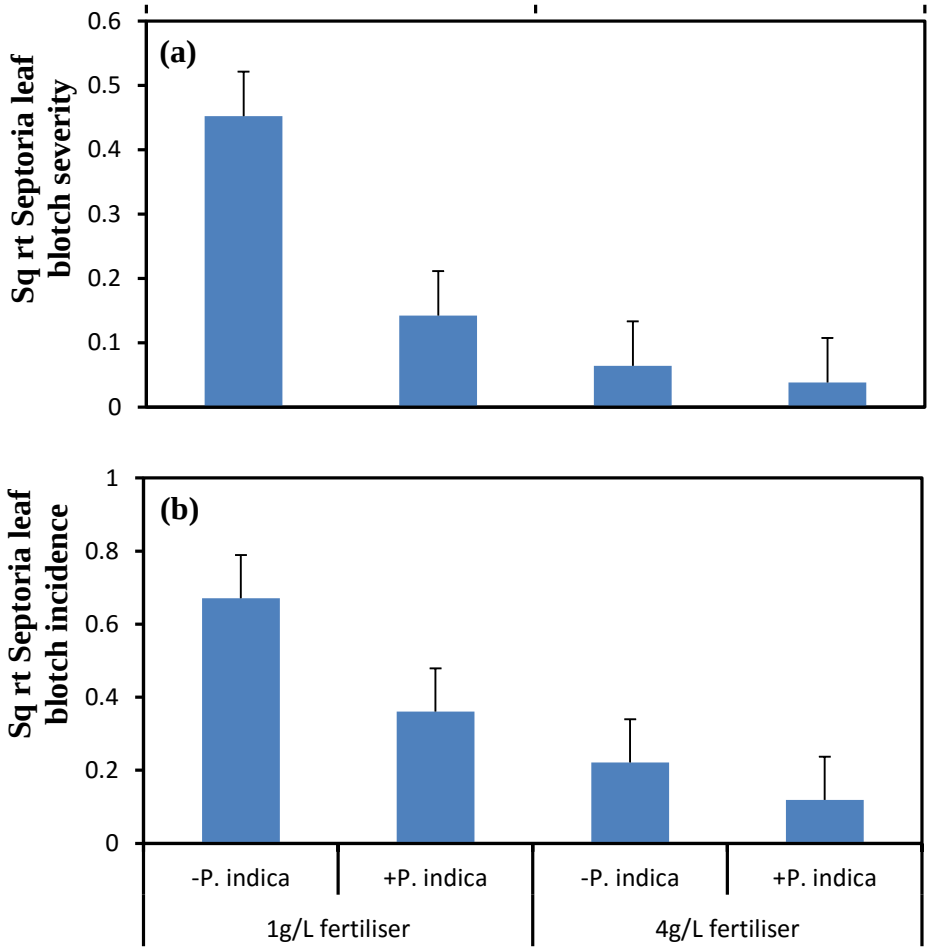


Fig. 4.1. The effect of *Piriformospora indica* under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels on Septoria leaf blotch disease severity and incidence of winter wheat (cv. Battalion), naturally infected with *Zymoseptoria tritici* at growth stage 24-26. (a). *Z. tritici* severity s.e.d. = 0.05, d.f. = 12; (b). *Z. tritici* incidence s.e.d. = 0.08, d.f. = 12) (data were square root transformed). Each point represents mean ± 2 SEM; (fertiliser: Osmocote® Pro slow release fertiliser).

Septoria disease severity and incidence was also recorded at GS 24-26 in the experiment grown for soil and plant tissues nutrient analysis, carried out in the 2014-15 growing season (chapter 3, page 84). *P. indica* reduced disease severity ($P=0.05$) and incidence ($P=0.003$) by 50 % and 65 % respectively. Disease severity ($P<0.001$) and incidence ($P=0.001$) were much higher at the low fertilisation level. *Fun. mosseae* increased the disease severity ($P=0.01$) and incidence ($P=0.08$; Fig. 4.2 a,b; Appendix Table 10, Chapter 8). The interaction between *P. indica* and fertiliser was not significant.

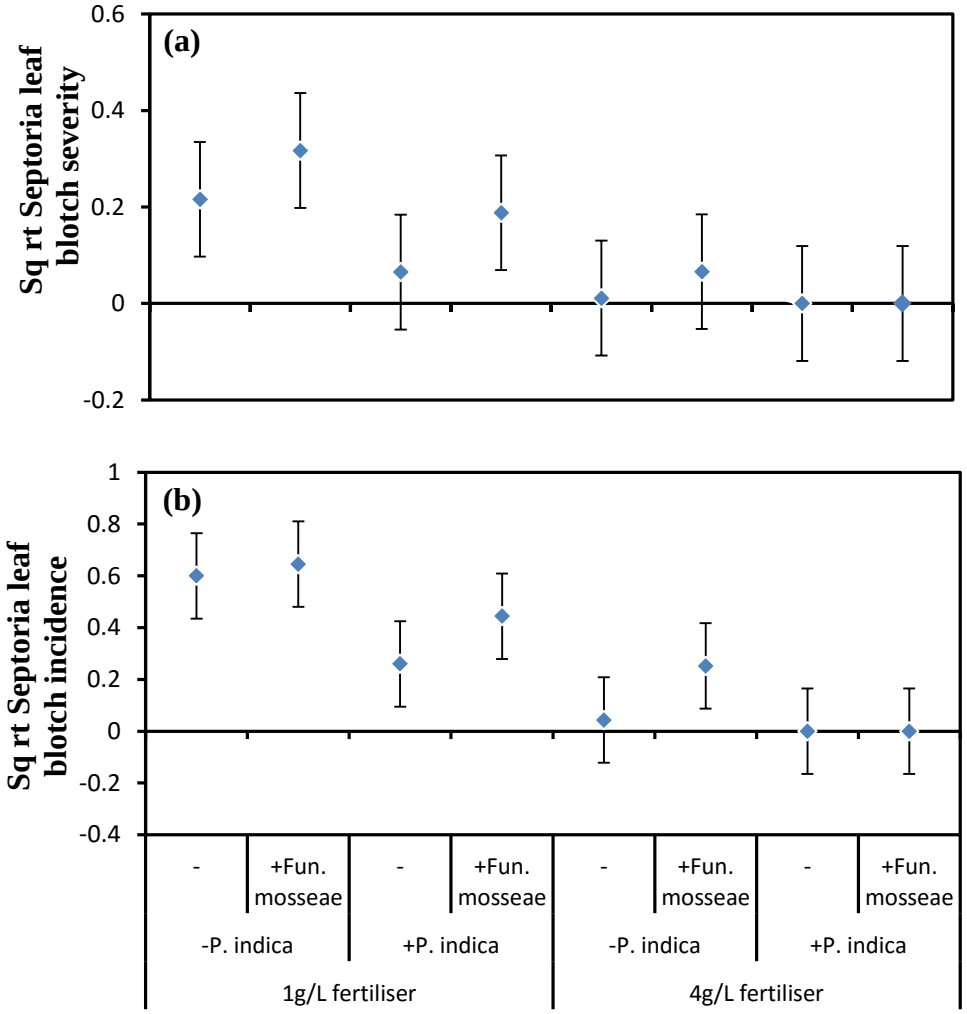


Fig. 4.2. The effect of *Piriformospora indica* and *Funneliformis mosseae* under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels on Septoria leaf blotch disease severity and incidence of winter wheat (cv. Battalion), naturally infected with *Zymoseptoria tritici* at growth stage 24-26. (a). *Z. tritici* severity s.e.d. = 0.08, d.f. = 14; (b). *Z. tritici* incidence s.e.d. = 0.1, d.f. = 14; (data were square root transformed). Each point represents mean ± 2 SEM; (Pi: *P. indica* and fertiliser: Osmocote® Pro slow release fertiliser).

Septoria leaf blotch, caused by natural background infection was recorded at GS 22-24 (tillering stage, main shoot with 2-4 tillers) in the winter wheat experiment grown for Fusarium experiment carried out in the 2013-14 growing season (chapter 3, page 79). At high fertility, *P. indica* reduced the disease severity by 85 % (*P. indica*. Fertiliser interaction P=0.002). *P. indica* (P<0.001) and *Fun. mosseae* (P<0.001) inoculation alone or in combination (*P. indica*. *Fun. mosseae* interaction P<0.001) reduced Septoria disease severity by 70 %, 16 % and 67 % respectively, compared to low fertility. Disease was much lower at high fertility (Main effect of fertiliser P<0.001; Fig. 4.3 a,b; Appendix Table 11, Chapter 8). Very little disease was apparent on the leaves at GS 39 and subsequently.

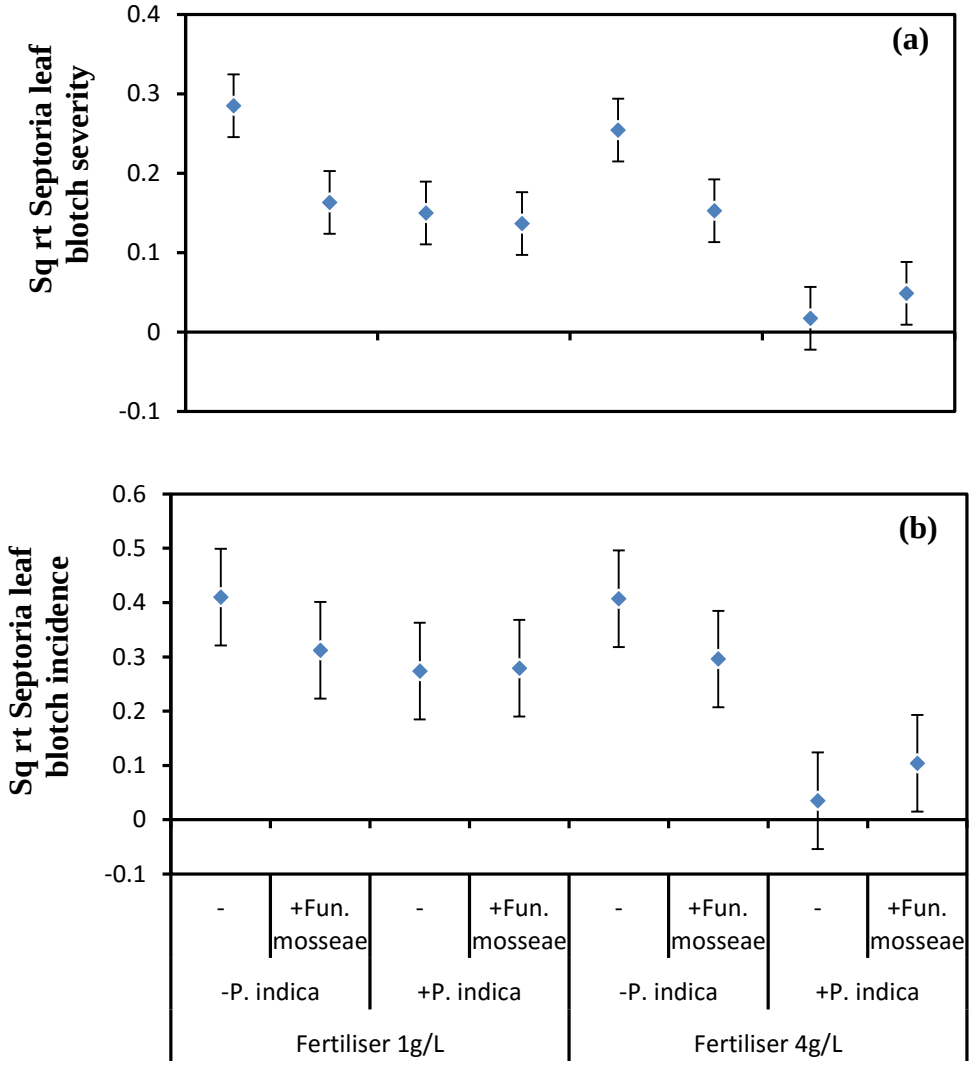


Fig. 4.3. The effect of *Piriformospora indica* and *Funneliformis mosseae* under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels on Septoria leaf blotch disease severity and incidence of winter wheat (cv. Battalion), naturally infected with *Zymoseptoria tritici*, recorded at growth stage 22-24 (tillering stage, main shoot with 2-4 tillers). (a). *Z. tritici* severity, s.e.d. = 0.03; d.f. = 47; (b). *Z. tritici* incidence, s.e.d. = 0.06, d.f. = 47; (data were sqrt transformed); Each point represents mean ± 2 SEM; (fertiliser: Osmocote® Pro slow release fertiliser).

Septoria leaf blotch, artificially infected with *Z. tritici* was recorded at GS 22 (tillering main shoot and two tillers). *P. indica* reduced *Z. tritici* severity and incidence by 90 % ($P<0.001$) at both high and low fertility. *P. indica* reduced the disease severity more at high fertilisation ($P=0.03$). The disease severity was lower at low fertiliser level, compared to high fertiliser level ($P=0.05$; Fig. 4.4 a,b).

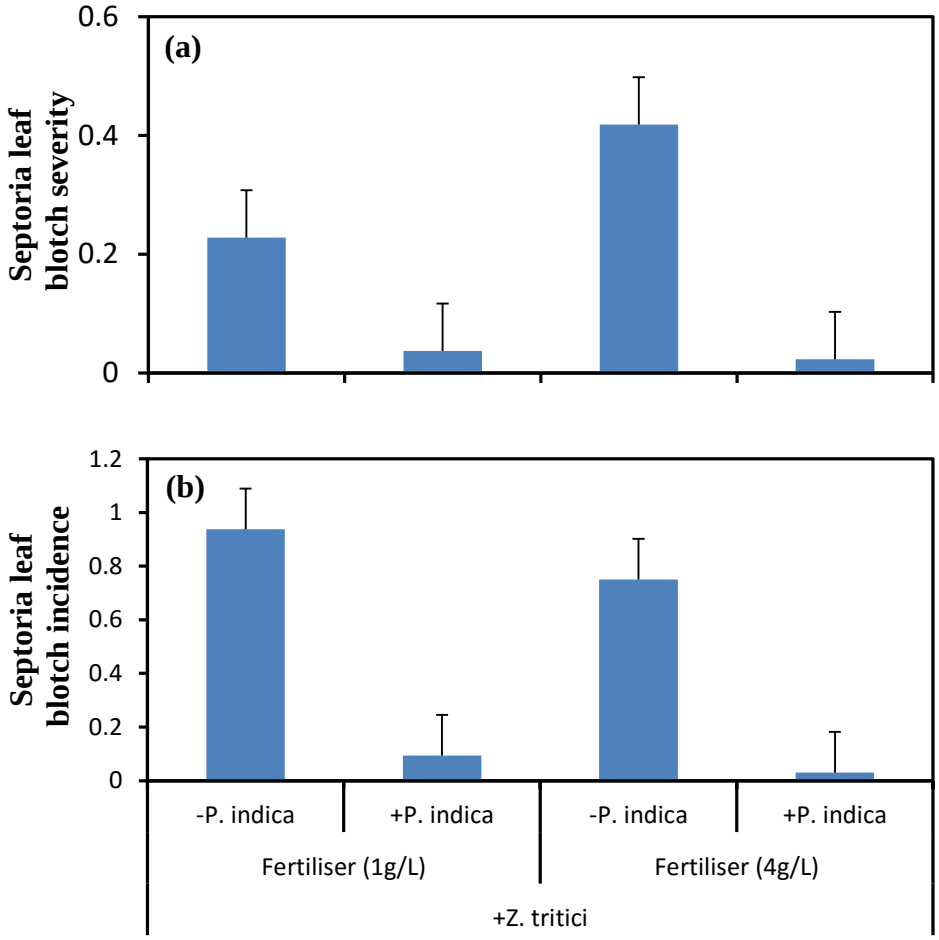


Fig. 4.4. The effect of *Piriformospora indica* under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels on Septoria leaf blotch disease severity and incidence of winter wheat (cv. Battalion), recorded at 3 weeks after artificial inoculation with *Zymoseptoria tritici* (a). *Z. tritici* severity s.e.d. = 0.06, d.f. = 9; (b). *Z. tritici* incidence s.e.d. = 0.1, d.f. = 9. Each point represents mean ± 2 SEM; (Pi: *P. indica* and fertiliser: Osmocote® Pro slow release fertiliser).

4.4.2. Effect of *P. indica* on aphids

Number of Grain aphid (*Sitobion avenae*) was also recorded at GS 65 (flowering stage) on leaf 4 and 5 for the winter wheat experiment grown for assessing *P. indica* effect on air-borne diseases, carried out in the 2014-15 growing season (page 106). *P. indica* did not reduced the number of aphids (P=0.7). Fertiliser did not have any effect on aphids either (Fig. 4.5).

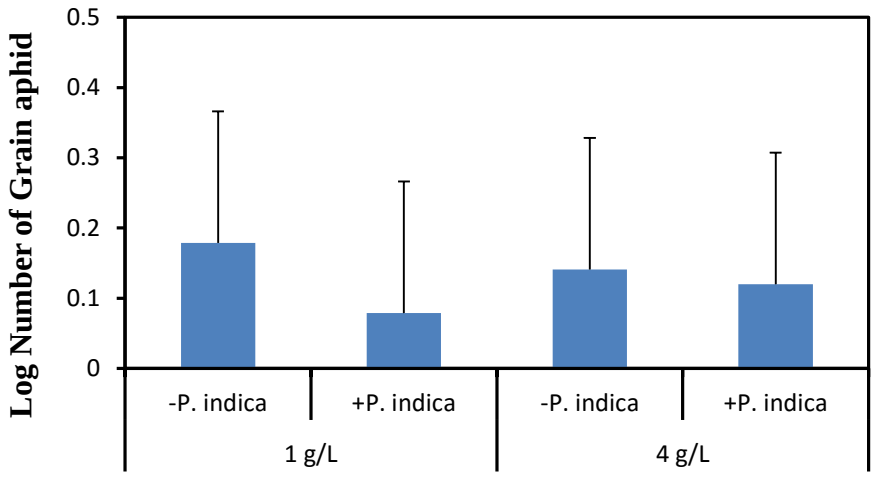


Fig. 4.5. The effect of *Piriformospora indica* under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels on Grain aphid (*Sitobion avenae*), of winter wheat (cv. Battalion), recorded at growth stage 65 (flowering). s.e.d. = 0.1; d.f. = 12; Each point represents mean ± 2 SEM; (fertiliser: Osmocote® Pro slow release fertiliser).

4.4.3. Effect of *P. indica* on yellow rust disease

Yellow rust, caused by natural background infection with *P. striiformis* f.sp. *tritici*, was recorded at growth stage 35-37 (stem elongation, 5th node detectable to flag leaf just visible) for the winter wheat experiment grown for Fusarium experiment carried out in the 2013-14 growing season (chapter 3, page 79). *P. indica* application at sowing reduced the yellow rust disease severity by 29 % (main effect $P=0.005$) and incidence (main effect $P<0.001$). Disease severity and incidence were much lower at the low fertiliser level (main effect $P<0.001$) (Fig. 4.6 a,b; Appendix Table 12, Chapter 8).

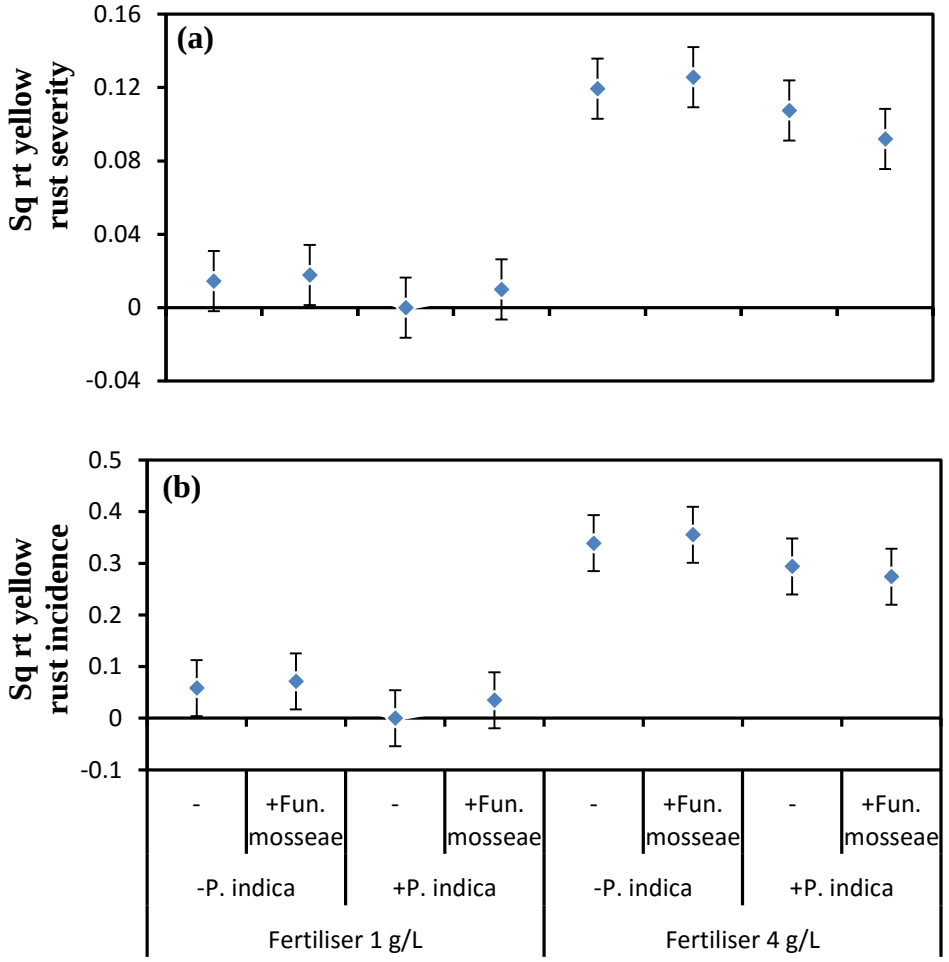


Fig. 4.6. The effect of *Piriformospora indica* and *Funneliformis mosseae* under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels on yellow rust disease severity and incidence of winter wheat (cv. Battalion), naturally infected with *Puccinia striiformis* f.sp. *tritici*, recorded at growth stage 35-37. (a). yellow rust severity, s.e.d. = 0.02; d.f. = 47; (b). yellow rust incidence, s.e.d. = 0.04; d.f. =47, (data were sqrt transformed). Each point represents mean ± 2 SEM; (fertiliser: Osmocote® Pro slow release fertiliser).

Yellow rust, caused by natural background infection, was recorded at GS 70 (milk development) on the flag and sub-flag leaf of the six different cultivars of spring wheat grown for the Fusarium experiment carried out in the 2015 growing season (chapter 3, page 82). Yellow rust severity (main effect $P<0.001$) and incidence (main effect $P<0.001$) differed between varities. Granary was the most and Zircon the least susceptible cultivar. *P. indica* application at sowing reduced the yellow rust disease severity by 55 % (main effect $P<0.001$) and incidence by 25 % on average over all cultivars (main effect $P<0.001$). Although it was apparently most effective on Granary and Paragon, the interaction between *P. indica* and cultivars was not significant (Fig. 4.7 a,b; Appendix Table 13, Chapter 8).

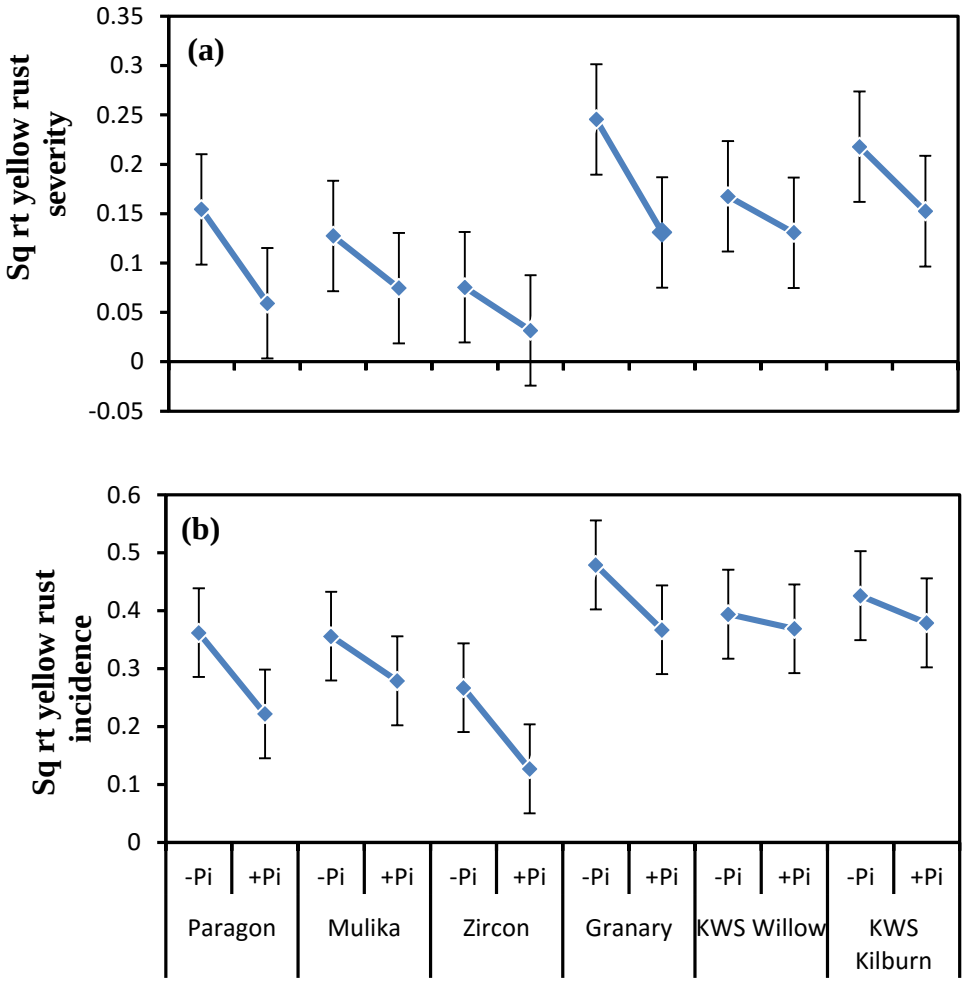


Fig. 4.7. The effect of *Piriformospora indica* (Pi) on yellow rust disease severity and incidence of six cultivars of spring wheat (cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn), naturally infected with *Puccinia striiformis* f.sp. *tritici*, recorded at growth stage 70. (a). yellow rust severity, s.e.d. = 0.04; d.f. = 58; (b). yellow rust incidence, s.e.d. = 0.05; d.f. =58, (data were sqrt transformed). Each point represents mean ± 2 SEM.

4.4.4. Effect of *P. indica* on powdery mildew disease

Powdery mildew, caused by natural background infection with *Blumeria graminis* f.sp. *tritici*, was recorded at growth stage 70, on the flag and sub-flag leaf of the six different cultivars of spring wheat grown for the Fusarium experiment carried out in the 2015 growing season (chapter 3, page 82). The six cultivars of spring wheat were differently susceptible to powdery mildew severity (main effect $P<0.001$) or incidence (main effect $P<0.001$). Granary was the most and KSW Willow the least susceptible cultivar. *P. indica* application at sowing reduced the powdery mildew disease severity and incidence by 63 % (main effect $P=0.01$). *P. indica* reduced powdery mildew severity and incidence in all cultivars, and was most effective on Granary. However, the interaction between *P. indica* and cultivars was not significant (Fig. 4.8 a,b; Appendix Table 14, Chapter 8).

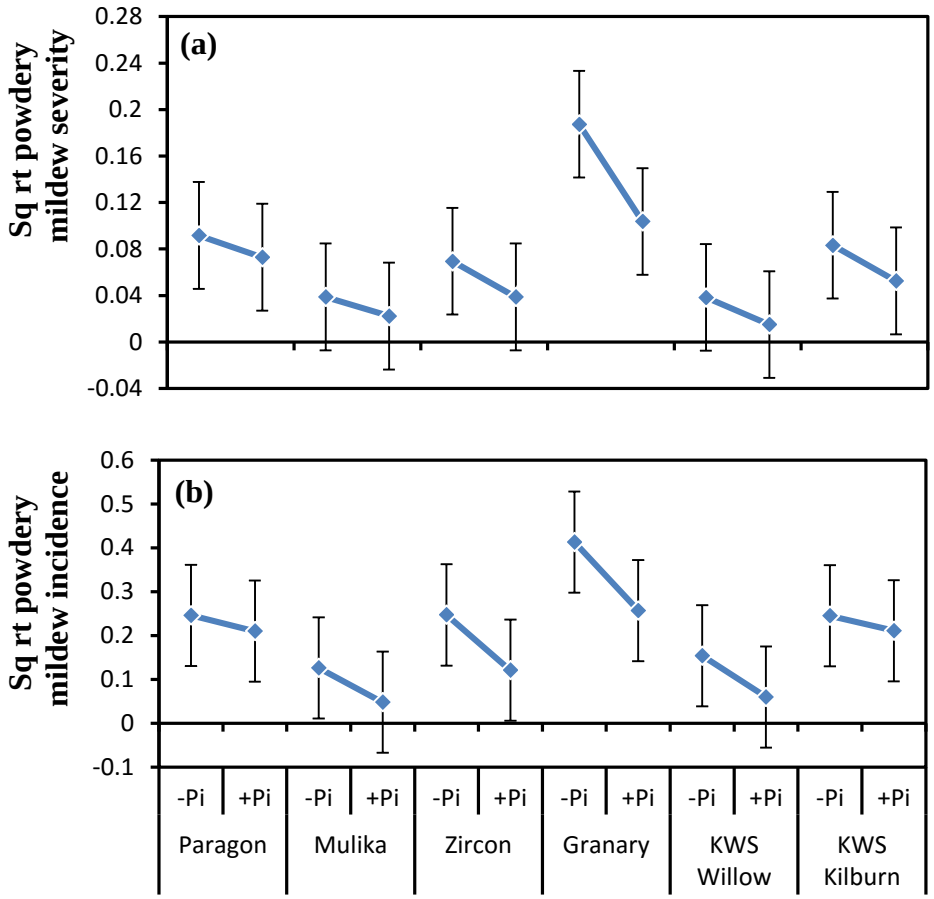


Fig. 4.8. The effect of *Piriformospora indica* (Pi) on powdery mildew disease severity and incidence of six cultivars of spring wheat (cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn), naturally infected with *Blumeria graminis* f.sp. *tritici*, recorded at growth stage 70. (a). powdery mildew severity, s.e.d. = 0.03; d.f. = 58; (b). powdery mildew incidence, s.e.d. = 0.08; d.f. =58, (data were sqrt transformed). Each point represents mean ± 2 SEM.

4.5. Harvest results

Final harvest results of the winter wheat expeiments grown for assessing *P. indica* effect on air-borne diseases (chapter 5, page 106) showed that high fertilisation increased above ground biomass by 48 % ($P<0.001$), root weight by 112 % ($P=0.002$), total grain weight by 27 % ($P=0.002$) and the number of ears per pot by 56 % ($P<0.001$, Table 4.1; Appendix Table 9, Chapter 8). *P. indica* inoculation increased the above ground biomass by 26 % at low fertilisation and by 8 % at high fertilisation (main effect $P=0.007$), root weight by 117 % at low fertilisation and by 17 % at high fertilisation (main effect $P=0.001$), total grain weight by around 35 % at both low and high fertilisation level (main effect $P<0.001$), TGW by 25 % at low fertilisation and by 12 % at high fertilisation level ($P=0.003$) and the number of ears per pot by 10 % at both low and high fertilisation level (main effect $P=0.05$). There were no significant differences among treatments for harvest index (table 4.1).

Table 4.1. Harvest results of winter wheat samples (cv. Battalion), inoculated with *Piriformospora indica*, (at sowing time) under low (1 g L^{-1}) and high (4 g L^{-1}) fertiliser levels (Osmocote® Pro slow release fertiliser). Harvest index: total grain weight (g)/total above grain weight (g).

Fertiliser	*P. indica*	Total above ground weight (g)	Root weight (g)	Total grain weight per pot (g)	1000 grain weight (g)	Harvest index	No of ears
1 g L^{-1}	-	184	8.9	69	48	0.4	30
	+	232	19.4	92	60	0.4	33
4 g L^{-1}	-	273	18.9	88	54	0.3	47
	+	296	22.1	122	61	0.4	52
	s.e.d.	15.2	2.3	8.7	3.5	0.05	2.3

4.5. Discussion

Septoria leaf blotch, yellow rust and powdery mildew are among the most significant threats to wheat yields in the UK and Europe, and most other wheat growing regions, as currently available fungicides become less effective against resistant strains of the disease and new pathogens appear (Orton et al., 2011, Dean et al., 2012, Lee et al., 2014). Here, these results show that *P. indica* reduced Septoria, yellow rust and powdery mildew disease severity and incidence. This is consistent with previous results which showed that *P. indica* reduced powdery mildew disease severity in wheat and barley (Waller et al., 2005, Deshmukh et al., 2006, Serfling et al., 2007, Molitor et al., 2011). *P. indica* reduced yellow rust and powdery mildew on six cultivars of spring wheat, despite Gravouil (2012) findings suggesting that some barley cultivars might benefit more than others from interaction with *P. indica*.

P. indica might have regulated the wheat defence response and induced systemic resistance against the pathogens causing foliar diseases (Waller et al., 2005, Deshmukh et al., 2006, Felle et al., 2009, Molitor & Kogel, 2009). Waller et al. (2005) reported that *P. indica* induced systemic resistance in barley plants against the necrotrophic fungus *F. culmorum* (root rot) and the biotrophic fungus *B. graminis* (powdery mildew), by elevating antioxidative capacity. Stein et al. (2008) indicated that *P. indica* induced systemic resistance in Arabidopsis plants against powdery mildew (caused by *Golovinomyces orontii*) by regulating jasmonic acid signalling pathway. Vahabi et al. (2015) demonstrated that *P. indica* up-regulated

the defense-related phytohormones such as jasmonic acid, ABA and SA in Arabidopsis. These hormones are involved in plant responces to pathogen attacks. *P. indica* induced a local, transient response of several defense-related transcripts, of which some were also induced in shoots of colonized plant (Zuccaro et al., 2011, Pedrotti et al., 2013).

Biological Control, accepted (2016)

CHAPTER 5- *Piriformospora indica* viability in different soil types under UK weather conditions and its interaction with other soil microorganisms

M. Rabiey, I. Ullah, E. J. Shaw, and M. W. Shaw

M. Rabiey: did all the experiments;

I. Ullah: helped develop the molecular methods;

E. J. Shaw: helped develop the DGGE methods

M. W. Shaw: advised on design, analysis and interpretation.

5.1. Summary

P. indica mRNA detection was used as an indicator of *P. indica* viability. 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.

P. indica-mRNA could still be detected by RT-PCR after four and eight months in different soil types, but was not detectable after 15 months. Samples of DNA extracted from the root zone or from bulk soil in pots in which wheat had been grown indicated that pots inoculated with *P. indica* had fungal and bacterial species communities which were distinct from and more diverse than non-inoculated controls.

Tests on arable weeds showed that *P. indica*-infected roots of *Alopecurus myosuroides* and *Avena fatua* but not *Galium aparine*. Averaged over the weed species, *P. indica* increased root biomass by 35 % (P=0.045). On average, above-ground biomass of weed species was not significantly affected by *P. indica* (P=0.5). The average above-ground competitiveness of the weeds with wheat, assessed by the log of the ratio of dry weights in co-cultured pots, was slightly decreased (P=0.02).

In the case of field application, *P. indica* would probably remain active in the soil within season. *P. indica* increased root and soil fungal and bacterial diversity. Although usually desirable, this indicates substantial effects on soil composition or functioning. 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.

5.2. Introduction

How *P. indica* interacts with other soil microorganisms is still unclear. Endophytic fungal symbionts can have profound effects on plant ecology, fitness, and evolution

(Brundrett, 2006), shaping plant communities (Clay & Holah, 1999), increasing plant tolerance to abiotic stresses (Murphy et al., 2015c), increasing plant resistance to pathogens (Rodriguez et al., 2009, Murphy et al., 2014a) and manifesting strong effects on the community structure and diversity of associated organisms (e.g. bacteria, nematodes and insects; Omacini et al. (2001)). Studies on the effects of arbuscular mycorrhizal fungi (AMF) on rhizosphere bacteria have shown variable results, with both negative (decreasing the population of bacteria) (Christensen & Jakobsen, 1993, Amora-Lazcano et al., 1998) and positive (increasing the population of bacteria) (Andrade et al., 1997, Abdel-Fattah & Mohamedin, 2000) effects. The variable results could be due to the fact that some bacteria are being stimulated and others being repressed by AMF (Wamberg et al., 2003). Söderberg et al. (2002) suggested that the effect of AMF differed between plant species; the strength of the effect on the bacterial community in the rizosphere depended more on the plant species than on AMF colonisation. If *P. indica* is going to be applied to crops, a clear picture of how it affects other soil microorganisms would be needed, as the soil microflora plays a major role in the availability of nutrients to plants and has a strong influence on plant health and productivity.

P. indica viability in UK arable soils was assessed using DNA and RNA from soil. PCR based on DNA does not distinguish between living and dead organisms (Josephson et al., 1993, Wolffs et al., 2005). So, RNA extraction and reverse transcription-PCR (RT-PCR) were also carried out, using mRNA as a viability

marker. mRNA is less stable than DNA, is turned over rapidly in living cells, and will be degraded quickly in dead cells (Mendum et al., 1998, Vettraino et al., 2010). Although culture-dependent methods are a traditional method for assessment of microbial diversity, they reflect the total diversity of microbial community very poorly (Dunbar et al., 2000, Fakruddin & Mannan, 2013). The effects of *P. indica* on other soil microorganisms by the culture-independent genetic fingerprinting method PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was tested. This compared the composition and structure of microbial communities associated with rhizosphere and roots of wheat with and without *P. indica* inoculation. PCR-DGGE is used to study bacterial and fungal community structures in rhizosphere and soil samples. The method is reliable, reproducible, rapid and affordable (Kowalchuk & Smith, 2004). It is suitable for an overview of total genetic diversity of a soil microbial community and enables comparisons among many samples (Smalla et al., 2001, Marschner et al., 2002, Garbeva et al., 2004, O'Callaghan et al., 2008).

Weed competition can threaten crop quality and quantity and ultimately the farmer's profitability (Bockus et al., 2010); it is usually managed by herbicide application. 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.

The key herbicide-resistant weed species of arable crops in the UK are: black-grass (*Alopecurus myosuroides*), wild-oats (*Avena fatua*), cleavers (*Gallium aparine*), Italian rye-grass (*Lolium multiflorum*), common poppy *(Papaver rhoeas*), common chickweed (*Stellaria media*), and scentless mayweed (*Tripleurospermum inodorum*) (Bond et al., 2007, Moss et al., 2011, Hull et al., 2014). These are also important world-wide and in other crops (Yu et al., 2013). The first three were selected to study the effect of *P. indica* in pot experiments, growing them alone and in competition with wheat.

Hypothesis tested in this chapter: 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.

5.3. Materials and methods

5.3.1. *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, mycelia of *P. indica* were grown in CM medium 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.

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 prepared as described in Chapter 2 and mixed thoroughly with the soil. The sixth (control) pot only received sterilised water. The pots were placed in holes with the tops level with the surrounding soil level to make temperature fluctuations realistic. Around 50 g of each soil type was collected, with a small core from the middle of pots, at three and half months (mid-March 2014), 8 months (end of July 2014) and 15 months (end of March 2015) after inoculation

with *P. indica*. When collecting the samples, they were kept in a cool box on ice and transferred immediately to -20 ºC before DNA and RNA were extracted and PCR or RT-PCR performed. Maximum and minimum temperatures of soil in the pots were recorded every 2 days by a digital thermometer placed in the centre of one of the pots.

5.3.2. Soil community composition

To examine whether *P. indica* affects other soil microorganisms, wheat was grown in 3 L pots containing one of two soil types, SCL or LSO, as above. Winter wheat seeds, cv. Battalion, were surface disinfected by rinsing for 2 mins in 20 mL L^{-1} sodium hypochlorite (Fisher Scientific UK Ltd, UK), followed by three rinses in sterilized distilled water, and germinated on damp filter paper in a Petri dish at room temperature (21 ± 1 °C) under natural indoor light for 48 hours. Pre-germinated seeds were planted into 3 L pots (one seed per pot). This experiment had a 2×2×4 factorial combinations of ±*P. indica* × two soil types × four harvesting points, with two replications completely randomised. The pots were incubated at temperatures ranging between 15 and 25 °C; humidity and light were not controlled. Inoculation with 4 g liquid culture of *P. indica* mixed with soil was done at the time of sowing. Root and soil samples were collected at 2, 4, 6 and 8 weeks after inoculation (wai) for DNA extraction, PCR and DGGE analysis, as below. Samples were transferred and stored as described above.

5.3.2.1. DNA and RNA isolation

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

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

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

EF (EF-1-alpha (*TEF* gene)) primer amplified cDNA of 148 bp and gDNA of 227 bp. The PCR amplicon sequence corresponded to genomic sequence from 1547 to 1756 bp of the *P. indica TEF* gene, GenBank: accession number AJ249911.2, as expected.

PCR was performed in 0.2 mL PCR tubes (Fisher Scientific UK Ltd, UK) with 20 µL final reaction volume containing 2x Biomix PCR master mix, 0.25 µM forward and reverse primer, and template genomic DNA. Amplification was performed in a thermal cycler (Applied Biosystems® GeneAmp® PCR System 9700, Thermo Scientific, Life Technologies Ltd, UK) programmed as: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 45s and 72 °C for 30 s, followed by incubation at 72 °C for 7 min. Amplification was confirmed by electrophoresis of an aliquot of the PCR products in 2 % agarose gel in 1x TAE buffer.

5.3.2.3. Reverse Transcription-PCR (RT-PCR)

RT-PCR for *P. indica* was performed by using Invitrogen SuperScript® III First-Strand Synthesis SuperMix (Life Technologies Ltd, UK) in a 20 µL final reaction volume using 10 µL 2× RT Reaction Mix, 2 µL RT Enzyme Mix, RNase-free water and 4 µL *P. indica* RNA. Reverse transcription was done in a thermal cycler. Samples were first incubated at 50 ºC for 30 minutes, then held at 85 ºC for 5 minutes and then chilled on ice for 5 min. Thereafter, 1 µL *E. coli* RNase H was added to the tube which was then incubated at 37 ºC for 20 minutes. PCR was then performed using the complementary DNA (cDNA) obtained from the reverse transcription.

RT-PCR for soil samples was performed by using a One-Step RT-PCR Kit (QIAGEN, UK), in a 25 µL final reaction volume using 5 µL 5x QIAGEN OneStep RT-PCR Buffer, 1 µL dNTP Mix, 1 µL of Enzyme Mix, 0.6 µM of each primer, RNase-free water and 4 µL *P. indica* and samples RNA. Thermal cycler was set up at 30 min 50 °C, 15 min 95 °C, 35 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 30 s, followed by incubation at 72 °C for 7 min.

5.3.2.4. Primer and PCR condition for DGGE study

Bacterial 16S rRNA genes, from the extracted DNA, were amplified using the primer 341F-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG and 534R-ATTACCGCGGCTGCTGG (Muyzer et al., 1993). Fungal 18S rRNA genes were amplified using the primer NS1F-GTAGTCATATGCTTGTCTC and GCFung-R-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTACCCGTTG (Hoshino & Morimoto, 2008).

The PCR was performed in a 20 µL final reaction volume using 2× Biomix PCR master mix, 50 pmol μL^{-1} (for bacterial study) and 0.3 pmol μL^{-1} (for fungal study) of forward and reverse primer, and sample DNA. Touchdown PCR for the bacterial study was performed in a thermal cycler set up at 94 °C for 10 min, denaturation at 94 °C for 1 min, an annealing temperature which was set at 65 °C initially, then decreased by 1 °C after each 2 cycles until it reached 55 °C. Primer extension was performed at 72 °C for 2 min. The above reaction was performed for 20 cycles,

followed by 15 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. A final extension step was performed for 10 min at 72 °C (Sasaki et al., 2009).

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

5.3.2.5. Denaturing gradient gel electrophoresis of fungi and bacteria

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

5.3.2.5.1. Statistical analysis of DGGE banding patterns

The DNA bands that migrated within each gel to the same relative distance were each ascribed the same label. In each lane, corresponding to a sample, the presence of a band with that label was scored 1 and absence scored 0. The band

corresponding to *P. indica* band (which had the same position in all *P. indica*-inoculated samples) was not included in the scoring. These data were then analysed by two methods:

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

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

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

where *Ri* is the total number of occurrences of band *i* in a group of observations, and *R* is total number of bands of any type observed in the group. Confidence

intervals for the index were obtained by randomly re-sampling band abundances from a multinomial with the observed probabilities of each band type, and re-calculating the index.

5.3.3. *P. indica* interaction with weeds

Black-grass (*Alopecurus myosuroides*, 16 seeds per pot), wild-oat (*Avena fatua*, 6 seeds per pot), cleavers (*Galium aparine*, 3 seeds per pot) with and without wheat (6 seeds per pot) were planted in 5 L pots (top diameter: 22.5 cm, bottom diameter: 16.5 cm, depth: 17.5 cm) at a depth of 1 cm in one part non-sterilised vermiculite (Medium, Sinclair, UK) and one part sand, mixed with 4 g L^{-1} of slow release fertiliser (3-4 months, Osmocote® Pro), with and without 4 g pot^{-1} of liquid *P. indica* inoculum mixed into the soil. Four replicates, distributed in four randomised blocks, were used with the following factorial combinations of treatments: ± *P. indica*, ± wheat, and three weed species. Wheat alone with and without *P. indica* was included as a control.

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

In a separate experiment, to confirm the colonisation of weed roots with *P. indica* microscopically, seeds of black-grass, wild-oat and cleavers were planted separately in 1 L pots (top diameter: 13 cm, bottom diameter: 10 cm, depth: 11 cm) in one part non-sterilised vermiculite (Medium; Sinclair) and one part sand, and inoculated with *P. indica* at sowing. The roots were harvested at one and four weeks after inoculation, stained according to the method described in chapter 2, and viewed under a microscope with 10x and 40x objectives.

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

5.3.4. Statistical analysis of pot experiments

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

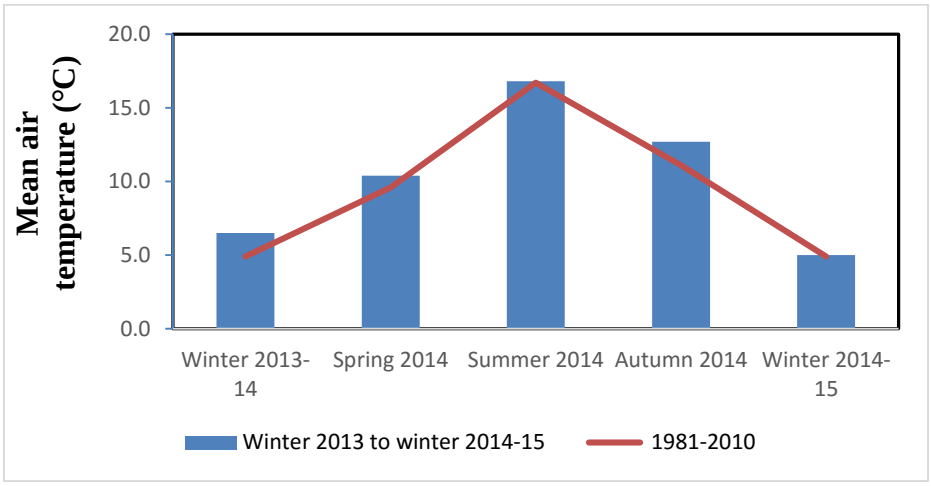
5.5. Results

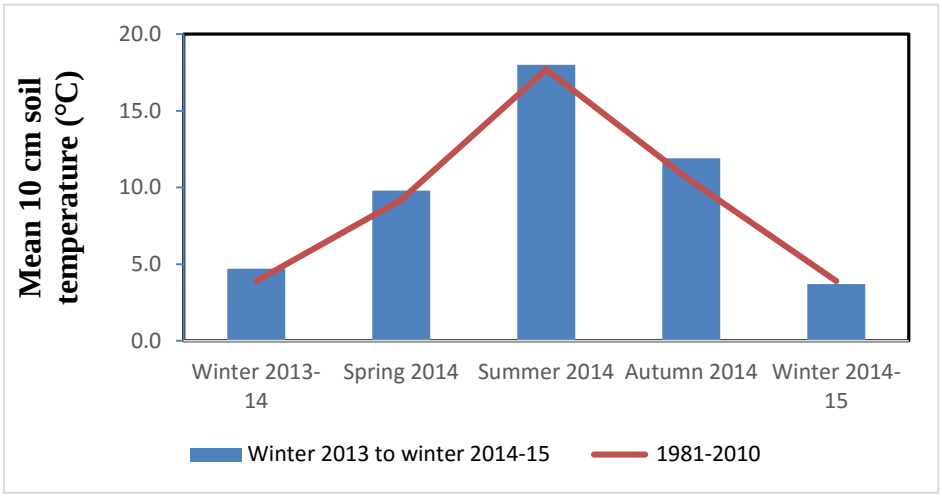
5.5.1. Weather conditions during 2013-15

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. Soil temperature was 1 °C above average. Soil froze on only five occasions (Fig. 5.1).

Following this, the mean air and soil temperature of spring and summer 2014 was near the average; total rainfall was, 55 mm and 31 mm respectively, above the long term average (Fig. 5.1).

The weather of autumn 2014 was warm, 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 and soil temperature near average. Soil froze on 20 occasions. Rainfall totals were 13 mm below average (Fig. 5.1. www.met.reading.ac.uk/weatherdata).





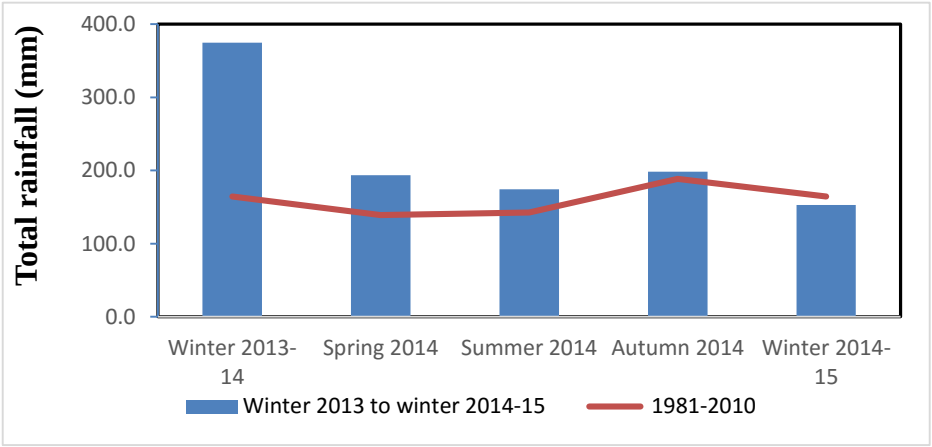


Fig. 5.1. Reading mean air temperature, mean 10 cm soil temperature, and total rainfall between winter 2013-14 and winter 2014-15, compared with 1981-2010 average (source: www.met.reading.ac.uk/weatherdata).

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

The viability of *P. indica* mycelia was tested under laboratory conditions. Exposure of mycelia to 80 ºC for 6 hours, then to -80 ºC for 6 hours, one or four weeks killed them: plates showed no growth of fungus after one month. RT-PCR detected *P. indica* mRNA after 6 hours exposure to 80 ºC then 6 hours at -80 ºC, but did not detect *P. indica* mRNA after exposure to 80 ºC followed by one or four weeks storage at -80 ºC. PCR detected DNA in all treatments (Table 5.1).

Table 5.1. Recovery of *Piriformospora indica* DNA and RNA after the mycelia were killed by exposure to heat and cold or grown in covered petri dishes of potato dextrose agar (n=3 for each condition).

Conditions	*P. indica* DNA	*P. indica* RNA	Culture
1 week at 21±1 ºC	3	3	3
1 month at 21±1 ºC	3	3	3
6 h at 80 ºC + 6h at -80 ºC	3	3	0
6 h at 80 ºC + one week at -80 ºC	3	0	0
6 h at 80 ºC + four weeks at -80 ºC	3	0	0

RNA and DNA of *P. indica* were successfully isolated from all four soils after winter 2013 (collected mid March 2014) (Table 5.2). DNA of *P. indica* was successfully isolated from all different soil types following a UK spring and summer (collected end of July 2014), but RNA could be detected in only six of the pots. After 15 months (collected mid March 2015), neither RNA, nor DNA of *P. indica* could be detected from any of the soils (Table 5.2). *P. indica* could not be detected in the controls that was not inoculated with *P. indica*, which shows the primers could only detect *P. indica* mRNA.

Table 5.2. Recovery of *Piriformospora indica* DNA and RNA from four soil types, left in pots under prevailing weather conditions without plant roots present from December 2013 with sample collections at mid March 2014, end-July 2014 and mid-March 2015, n=5.

	P. indica DNA			*P. indica* RNA		
Soil type	Mid March/2014	End July/2014	Mid March/2015	Mid March/2014	End July/2014	Mid March/2015
Neville series	5	5	0	5	0	0
Sonning series	5	5	0	5	1	0
Rowland series, under organic management	5	5	0	5	3	0
Rowland series, non-organic management	5	5	0	4	2	0

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

5.5.3.1. Canonical variate analysis

Canonical variate analysis was used to differentiation between groups variation, using a trace statistic as a summary of differentiation. Canonical variate analysis of band patterns (Fig. 5.2), including both bacteria and fungi separated the four different harvested time points (trace: 1.9, P<0.0001), mainly because the first sample was distinct (Fig. 5.3 a). Root samples were clearly distinguishable from soil samples (trace: 3.9, P<0.0001, Fig. 5.3 b), and soil types were clearly distinct (trace: 1.6, P<0.0001, Fig. 5.3 c). *P. indica*-inoculated and non-inoculated samples were distinct (trace: 0.6, P=0.001, Fig. 5.3 d), *P. indica*-inoculated were distinguishable from non-inoculated samples by CVA when restricted to either fungal (trace: 1.1, P<0.03, Fig. 5.3 e), or bacterial primers (trace: 1.2, P<0.02, Fig. 5.3 f) or soil samples (trace: 2.9, P<0.0001, Fig. 5.3 g) but not root samples (trace: 0.6, P=0.6, Fig. 5.3 h).

To check the interaction between the effects of *P. indica* and soil-root zones combined factors were created. CVA of groups of samples classified by both *P. indica* inoculation and root-soil zone, including both bacterial and fungal bands, separated *P. indica*-inoculated from non-inoculated samples (trace: 5.5, P<0.0001, Fig. 5.3 i).

5.5.3.2. Shannon-Wiener diversity index

Samples harvested at different time points did not differ in diversity. Rowland series soils (LSO) had more fungal and bacterial band diversity than Sonning series (SCL). Both types of soil had more fungal and bacterial band diversity in the presence of *P. indica* (Fig. 5.4) and samples inoculated with *P. indica* had more bands of all types than non-inoculated samples. Root samples had more fungal species diversity when *P. indica* was present, but slightly fewer bacterial species diversity (Fig. 5.5).

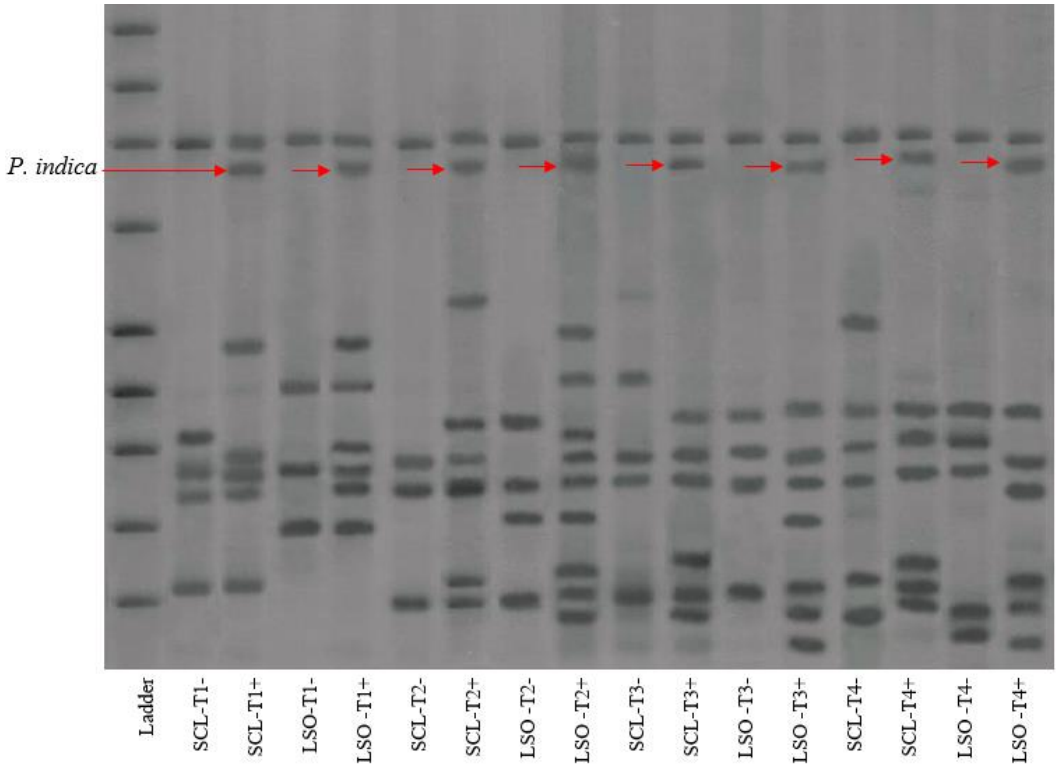
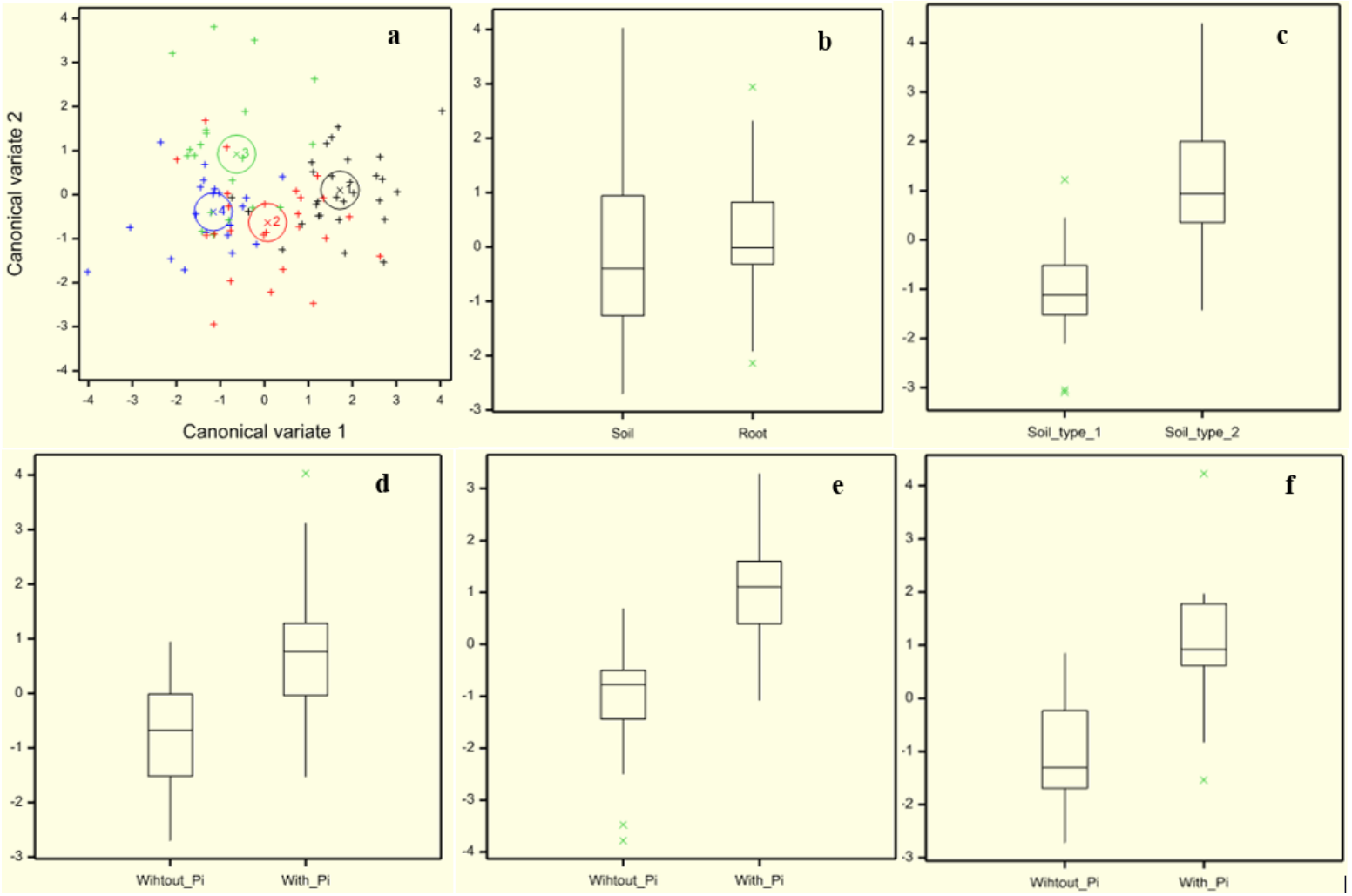


Fig. 5.2. Denaturing gradient gel electrophoresis profiles of the wheat root fungal community in Sonning series (SCL) or Rowland series (LSO) soil inoculated with (+) or without (-) *Piriformospora indica*, harvested at 2 weeks after inoculation (wai) (T1), 4 wai (T2), 6 wai (T3) and 8 wai (T4), (first lane: Hyper Ladder I-100 lanes (Bioline)).


a
Canonical variate 2
Canonical variate 1
b
Soil
Root
c
Soil_type_1
Soil_type_2
d
Wihtout_Pi
With_Pi
e
Wihtout_Pi
With_Pi
f
Wihtout_Pi
With_Pi

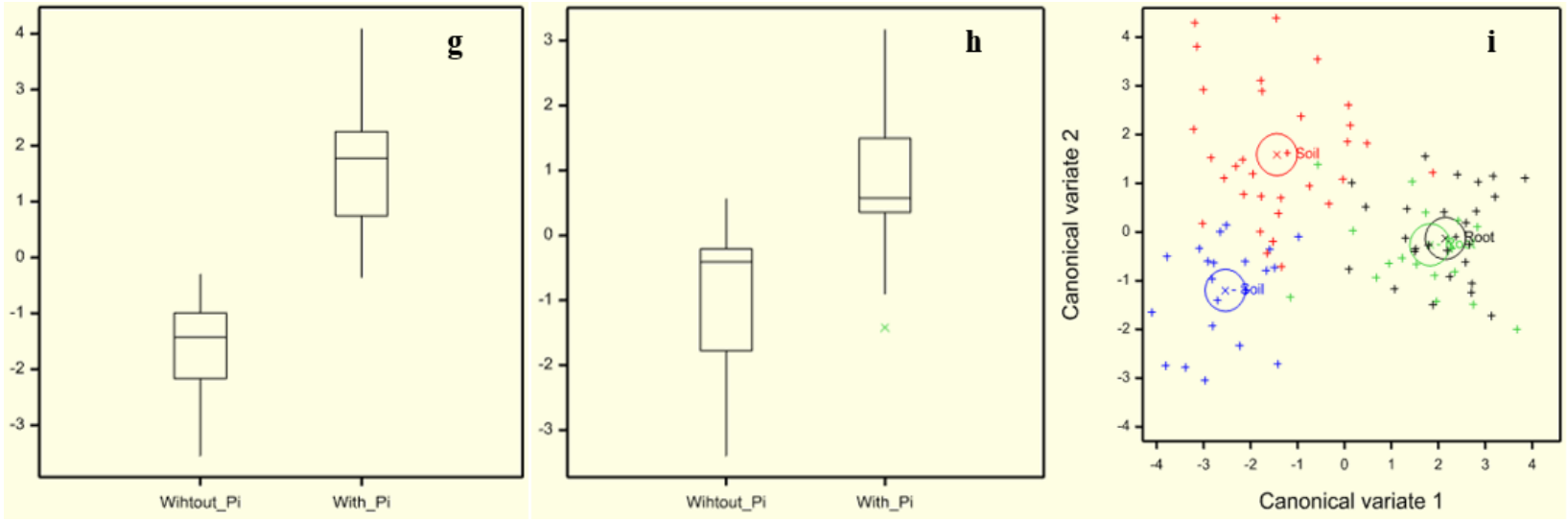


Fig. 5.3. Canonical variates analysis of bands from denaturing gradient gel electrophoresis using universal fungal and bacterial primers for wheat root samples grown in Sonning series (SCL) or Rowland series (LSO) soils, inoculated with/without *Piriformospora indica*, (Pi). First or first and second canonical axes are shown for data classified by (a) the four time points of harvest; (b) Root and soil source; (c) soil types; (d) *P. indica*-inoculation status; (e-h) *P. indica*-inoculation status using but restricted to fungal (e), or bacterial primers (f) or to soil samples (g) or root samples (h); (i) both *P. indica* inoculation and root or soil source**.**

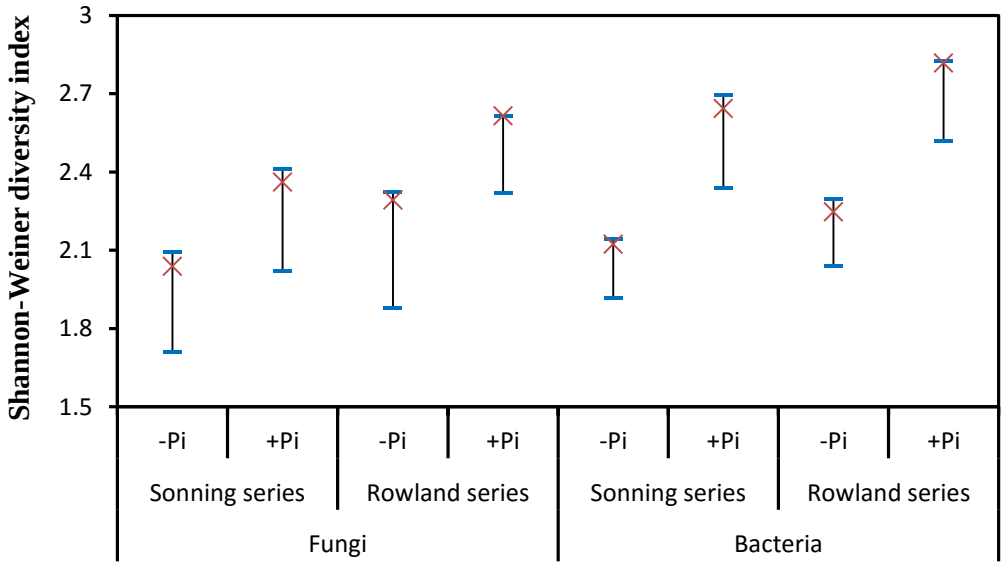


Fig. 5.4. Shannon-Weiner diversity index for Sonning (SCL) and Rowland series (LSO) soil samples inoculated or not with *Piriformospora indica* (Pi). Based on denaturing gel electrophoresis of DNA extracts amplified using universal fungal and bacterial primers. Each bar represents mean ± 95% bootstrap confidence interval.

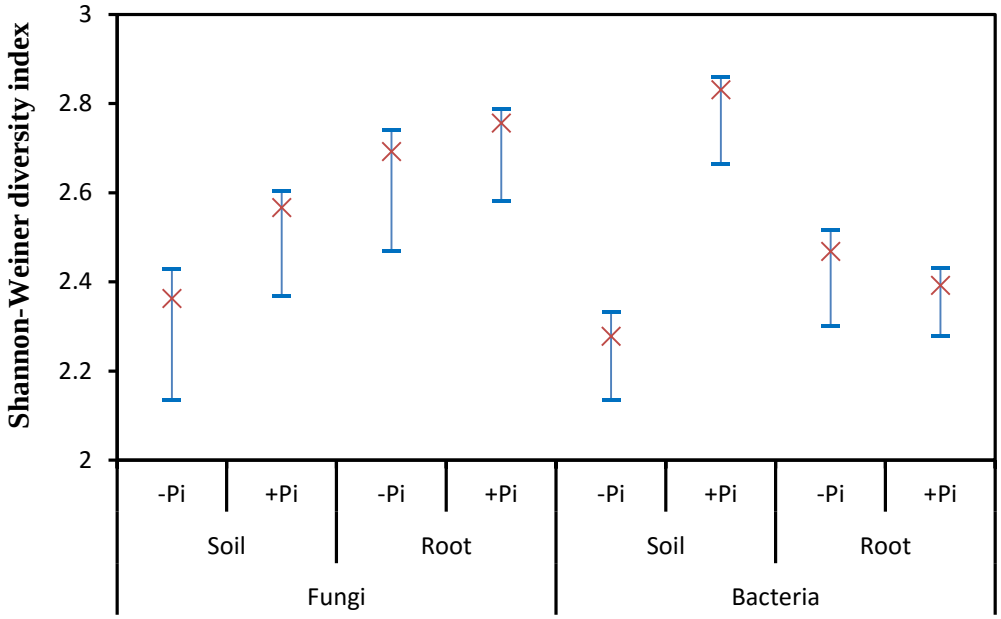


Fig 5.5. Shannon-Weiner diversity index for wheat root and soil samples inoculated or not with *Piriformospora indica* (Pi), based on denaturing gel electrophoresis of DNA extracts amplified using universal fungal and bacterial primers. Both soil types (Sonning series (SCL) and Rowland series (LSO)) are combined. Each bar shows mean ± 95% bootstrap confidence interval.

5.5.4. *P. indica* interaction with weeds

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

P. indica application at sowing time increased wheat shoot and root biomass by 33 % (main effect P=0.05) and 100 % (main effect P=0.02) respectively, as expected (Table 5.3; Appendix Table 15, Chapter 8).

P. indica increased root biomass, averaged over *Avena fatua, Alopecurus myosuroides* and *G. aparine*, by 35 % (P=0.04). As expected, competition reduced root biomass (by about 26 %, P=0.05) and there were differences between species (P=0.03; *A. fatua* was about 50 % heavier than the other two species). All interactions were non-significant (P>0.4). In particular, the effect of inoculation did not differ between weed species, and the effect of inoculation did not differ in the competition pots (Table 5.3).

Shoot biomass of all plants was decreased about 24 % (P=0.005) by competition and differed greatly between the species (P=0.001) because *G. aparine* had a lower biomass. The effect of *P. indica* was slight (a 12 % increase; P=0.2) and no interactions were significant (P>0.2 in all cases) (Table 5.3; Appendix Table 15, Chapter 8).

The average competitiveness between wheat and *Avena fatua, Alopecurus myosuroides* and *G. aparine*, measured by the ratio of shoot weights, was reduced

by 40 % (backtransformed from the $\log_{10}$ scale; P=0.02) when *P. indica* was present in the soil (Table 5.4; Appendix Table 16, Chapter 8). Although the competitiveness differed significantly between species, no interaction terms were significant (P>0.5). There were no significant differences in competitiveness measured by the ratio of root weights (P>0.13 for all main and interaction terms).

Table 5.3. Dry weights (g) of root and shoot of *Alopecuris myosuroides*, *Avena fatua* and *Galium aparine* alone and in competition with wheat, with and without inoculation with *Piriformospora indica* (error d.f.: 33).

		Weed dry weight (g)		Wheat dry weight (g)	
Weed	*P. indica*	Shoot	Root	Shoot	Root
-	-			3.3	0.5
	+			4.4	1.1
Alopecurus myosuroides	-	3.6	0.32		
		2.9	0.26	2.2	0.3
	+	4.5	0.44		
		3.2	0.35	3.4	0.6
Avena fatua	-	2.9	0.48		
		2.5	0.29	1.5	0.3
	+	4.2	0.72		
		2.6	0.47	3.5	0.6
Galium aparine	-	1.3	0.35		
		1.2	0.26	2.3	0.4
	+	0.99	0.34		
		0.88	0.34	2.5	0.8
	s.e.d.	0.54	0.13	0.7	0.2

Table 5.4. 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)

	$\log_{10}$ (shoot weight weed/shoot weight wheat)			$\log_{10}$ (root weight weed/root weight wheat)		
P. indica inoculation	*A. myosuroides*	*A. fatua*	*G. aparine*	*A. myosuroides*	*A. fatua*	*G. aparine*
-	0.14	0.21	-0.3	-0.09	0.01	-0.24
+	-0.05	-0.13	-0.46	-0.26	-0.08	-0.48
s.e.d.	0.2			0.3		

5.6. Discussion

This study demonstrates (1) that *P. indica* can survive the UK weather and soil conditions for a period of months, even when there is no host present (Table 5.2); (2) that the inoculation of *P. indica* to soil has a substantial effect on soil and wheat root-associated microflora (Fig. 5.3, 5.4, and 5.5); (3) that *P. indica* affects at least two of three tested native arable weeds, and alters their competitive relations with wheat, and with each other (Table 5.3 and 5.4).

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

Exposure of *P. indica* to heat (80 ^{o}C) then immediately to -80 ^{o}C, killed the mycelia (Table 5.1). mRNA can be used as an indicator of *P. indica* viability, as it could not be detected a few hours after mycelia of *P. indica* were killed, while DNA of *P. indica* could be detected even four weeks after mycelium was killed (Table 5.1).

This agrees with other studies. Herdina et al. (2004) concluded that mycelium of *Gaeumannomyces graminis* var. *tritici* killed by heating to 55 ^{o}C for 1 hour and DNA could still be detected by PCR after eight days. Chimento et al. (2012) killed *Phytophthora ramorum* mycelia by rapid lyophilisation and could detect DNA three months later while mRNA was only detected up to one week after the treatment, despite its relatively mild nature.

The DGGE analysis showed detectable changes in the microbial community structure and increased diversity in the fungal and bacterial community of both root and soil samples inoculated with *P. indica,* which are reflected in increases in Shannon diversity indices (Fig. 5.3, 5.4, and 5.5). How this might affect soil function is unknown. There is lots of debate about the importance of microbial community structure and diversity for soil function, plant productivity, resilience and stability. Changes in the composition of the soil microbial community can change ecosystem process rates, specifically decomposition, and affect plant productivity (positively, negatively or not at all) depending on the composition of the initial microbial community (McGuire & Treseder, 2010, Gera Hol et al., 2015). The two soils tested differed in their initial diversity, but responded similarly to inoculation with *P. indica*. The increase in microbial diversity might be due to *P. indica* causing changes in root exudate (composition and quantity) patterns, or directly through fungal exudates, as reported for AMF (Barea, 2000, Gryndler, 2000, Jeffries et al., 2003).

The primer sets 341/534 and NS1/GCFung for the bacterial and fungal community study were used as Muyzer et al. (1993) and Hoshino and Morimoto (2008) suggested these primer sets could most clearly discriminate bacterial and fungal communities in the soil. To obtain more specific results from DGGE, PCR primers must amplify only specific groups of fungi and bacteria (Jumpponen, 2007, Hoshino, 2012). The DGGE gave an overview of *P. indica*-induced changes in bacterial and fungal community structure but next generation sequencing approaches could be employed in the future for in depth study of the effects of *P. indica* on community structure and composition (Rincon-Florez et al., 2013).

P. indica has a very wide host range, and may be able to interact with and improve growth of economically-damaging weeds as well as crops. The effect of *P. indica* on *Alopecurus myosuroides*, *Avena fatua* and *Galium aparine*, three of the most important weeds in UK wheat production were evaluated. As expected, the weeds reduced wheat's root and shoot biomass significantly. *P. indica* did not colonise *G. aparine*, but did colonise *A. fatua* and *A. myosuroides*, though less than wheat (Table 5.3,4; Appendix Table 15,16, Chapter 8). The average root biomass of the three species was nonetheless increased by inoculation with *P. indica*, but less than that of wheat. The ratio of wheat shoot biomass to weed shoot biomas was increased in pots inoculated with *P. indica* so the effect on wheat had outweighed the effect on the weeds. This suggests that wheat might be a favourable host for *P. indica* and that field application of *P. indica* might not make weed control more difficult. However, since only three species were tested, on a small scale, the main conclusion

is that the fungus can alter competitive relations among both host and non-host species. The survival time and wide host range suggests that the fungus would escape into natural communities and might alter their composition or functioning. Changes would not necessarily be detrimental but these results do imply a need for extensive assessments on an ecosystem scale.

Previous studies (Rabiey et al., 2015) show that *P. indica* could be extremely useful in stabilising and increasing wheat yields and quality in the UK; other studies in northern Europe suggest it might benefit other crops also (Achatz et al., 2010, Fakhro et al., 2010, Sun et al., 2010). The present results suggest *P. indica* effect on both weeds and soil function should be studied further. A search for native organisms with similar characteristics might be a better direction to go in (Hodkinson & Murphy, 2015).

Chapter 6. General discussion

Plant diseases need to be controlled to maintain the quality and abundance of food produced by growers around the world. Growers often rely heavily on chemical fertilisers and fungicides and excessive use has led to the fungicide resistance and environmental pollution (Anon, 2009, DEFRA, 2013). There is therefore a need to develop alternative inputs to control pests and diseases. Among these alternatives are natural microorganisms. Plants are naturally found in association with many beneficial microorganisms, including several types of mycorrhizal fungi. Members of the order Sebacinales such as *P. indica* appear often to form mycorrhizal associations. This thesis focused on biological control of diseases of wheat, a crop of high economic value worldwide, by the root endophytic fungus *P. indica*. The ecological interactions of *P. indica* under UK weather conditions were also studied. However, there are several questions yet to be answered before release of *P. indica* on a wide scale: do most plants have the beneficial association with Sebacinales? Can Sebacinaceous be found from most soil types and/or fields? Has agriculture disrupted them? Is *P. indica* application compatible with fungicide seed treatments, tillage practice, crop rotation and stubble management? Can *P. indica* be used as part of integrated pest management? and if a plant can show the apparently beneficial reactions it does when infected with *P. indica*, why does it not do it all the time?

6.1. Are Sebacinales everywhere?

The order Sebacinales are known to be involved in a variety of mutualistic plant-fungal symbioses, with the ability to enhance plant growth and to increase resistance of their host plants against abiotic stress and fungal pathogens (Weiss et al., 2011). Weiss et al. (2011) collected Sebacinales from 128 root samples from 27 families from 4 continents in field specimens of bryophytes, pteridophytes and all families of herbaceous angiosperms including wheat, maize, and the non-mycorrhizal model plant *Arabidopsis thaliana*. Sebacinales were present in all habitats on four continents from Germany, Switzerland, France, Italy, Austria, Slovenia, Great Britain, the United States, Ecuador, Ethiopia, Namibia, North Africa, South Africa, and Iceland with no geographical or host patterns. Sebacinales were already found from India and Australia as well (Warcup & Talbot, 1967, Verma et al., 1998). Weiss et al. (2011)'s study showed that Sebacinales are almost universally present. Considering their proven beneficial influence on plant growth, endophytic Sebacinales may be a previously unrecognized universal hidden force in plant ecosystems.

Weiss et al. (2011) revealed that *P. indica* belongs to a group of closely related endophytic species from Western European (Germany and France) and Namibian Fabaceae, Poaceae, or Araceae. So it is possible that *P. indica* might be present in Eurepean soils or even UK.

Soil studies must be carried on to determine how common and widespread Sebacinales are in the UK; what their range of hosts is; and what effects they have on their hosts?

6.2. How does *P. indica* improve plant growth and yield?

The ability of *P. indica* to improve the growth and final yield of various host plants is well studied (see Chapter 1). But how does *P. indica* do this? Increases in nutrient uptake? Increases in photosynthesis? Phytohormone production by itself or the host? Or regulation of plant defence systems and antioxidant enzymes? Why do *P. indica* modes of action differ in different hosts? Are *P. indica* modes of action similar in different cultivars of a host? What is the plant cost in return for all the beneficial effects?

In the nutrient analysis experiment, *P. indica* did not have any effects on soil and plant tissue nutrients, but neither did *Fun. mosseae*, so these might be because of either the experimental conditions or the experimental factors as nutritional levels were too high (Table 3.4 and 3.5). More experiments are needed to confirm this.

The beneficial effects of *P. indica* have been observed on different barley cultivars including: Ingrid (Waller et al., 2005; Baltruschat et al., 2008), Annabell (Waller et al., 2005), California Mariout (Baltruschat et al., 2008), Golden Promise and Maresi (Deshmukh et al., 2006, 2007), Bowman and Optic (Gravouil, 2012). Gravouil (2012) showed that different barley cultivars had different rates of colonisation by *P. indica*. Some barley cultivars had the highest rate of *P. indica* colonisation and the best increase in shoot biomass and protection against pathogens such as

Rhynchosporium commune. Deshmuck et al. (2006 and 2007) inoculated different barley cultivar seedlings with *P. indica* and different isolates of *S. vermifera*. Despite considerable variation in the fungal activity of the different isolates, they found increases in shoot and root biomass with consistent resistance-inducing activity of all strains of the *S. vermifera* against powdery mildew (caused by *B. graminis* f.sp. *hordei*) as with *P. indica.*

In this thesis, *P. indica* colonised and increased shoot and final yield of the winter wheat (cv. Battalion, Table 3.1) and six cultivars of spring wheat (cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn, Table 3.2 and 3.3). *P. indica* reduced disease severity and incidence of FCR (Fig. 2.4-.7), FHB (Fig. 3.1-.3), and other foliar diseases including Septoria leaf blotch (Fig. 4.1-.4), yellow rust (Fig. 4.6 and 4.7) and powdery mildew (Fig. 4.8) of all cultivars.

However, more experiments need to be done to confirm if *P. indica* has continued effects on Fusarium and other air-borne diseases of different cultivars of wheat under field conditions.

6.3. *Piriformospora indica* survival under UK weather conditions

Although *P. indica* was found in the hot desert of India, with daytime temperature ranging between +40 to +50 ^{o}C, it promoted seed germination under extreme low temperatures, at temperatures ranging between –30 and 4 ^{o}C (Varma et al. 2014). The seed germination of 12 leafy vegetable plants inoculated with *P. indica* was observed to be 100 % in case of cabbage, endive, radish and onion within 25 days, carrot and cauliflower within 21 days, beetroot within 20 days, and pea within 15

days of sowing. Although germination, of *P. indica*-inoculated seeds, at the extreme low temperature was slow, no seed germination was noticed in the untreated controls. Significant increases in growth rate of cabbage, cauliflower heads and beetroot bulbs was recorded in the fungus treated plants (Varma et al., 2014). This shows that *P. indica* is not climatically limited and it is universal. As shown here, *P. indica* also delivered its beneficial effects under UK weather conditions.

Soil results show that *P. indica* survived in the soil, in the absence of any host, under winter and summer weather conditions in UK (Table 5.2), suggesting that *P. indica* might be suitable to use in the field under UK climatic weather conditions. However, more experiments need to be done under field conditions, in the absence and presence of hosts, to examine for how long *P. indica* can stay alive in the soil and how, in the event of adverse side-effects and widespread release, it can be eradicated.

6.4. *Piriformospora indica* effect on other soil microorganisms

Most plants form symbioses with fungi and bacteria, many of which function as mutualists (Bacon & White, 2000, Smith & Read, 2008). In plant communities, mutualists could change the structure of community composition, by either enhancing (Wagg et al., 2011, Murphy et al., 2015b) or reducing plant species coexistence (Clay et al., 1993). Endophytic fungal symbionts can have profound effects on plant ecology, fitness, and evolution (Brundrett, 2006), shaping plant communities (Clay & Holah, 1999), increasing plant tolerance to abiotic stresses (Murphy et al., 2015c), increasing plant resistance to pathogens (Rodriguez et al.,

2009, Murphy et al., 2014a) and manifesting strong effects on the community structure and diversity of associated organisms (e.g. bacteria, nematodes and insects; Omacini et al. (2001)). Endophyte presence may affect other community members such as herbivores (Rudgers & Clay, 2008) or mycorrhizal fungi (Mack & Rudgers, 2008), and have the potential to affect communities in both positive and negative ways (Stachowicz, 2001, Afkhami et al., 2014). The presence of a mutualist endophyte may cause net increases in community diversity. For example, losses of mutualists caused cascading declines in diversity in a plant–animal interaction web (Rodriguez-Cabal et al., 2013). In contrast Rudgers et al. (2015) drew attention to circumstances where mutualisms reduce species diversity. This can occur when a mutualist preferentially increases the competitive ability of its partner, thereby promoting competitive exclusion. For example, in tall grass prairies, nutritional mutualisms with AMF increased the competitive supremacy of the dominant grass species (Hartnett & Wilson, 2002).

Gravouil (2012) examined the overall structure of the phyllosphere of *P. indica*-inoculated and non-inoculated barley plants, but no significant difference was detected in richness, diversities and evenness of epiphytic populations or endophytic communities. The results presented here are in contrast with Gravoil, 2012, indicating that *P. indica* increased fungal and bacterial diversity in the soil and root microflora of wheat (Fig. 5.3-5). This might be because *P. indica* is a root endophytic fungus which does not colonise the shoot. Where *P. indica* is present in the soil and root, it interacts directly with other microorganisms.

However, more development work is necessary to confirm the effect of *P. indica* on other soil microorganisms including mycorrhizal fungi, plant growth promoting rhizobacteria, nematodes, and biotrophic fungi.

6.5. *Piriformospora indica* effect on weeds

P. indica has a wide range of hosts including monocots and dicots. Experiments were conducted to establish if common arable weeds can also benefit from *P. indica* interaction. When both wheat and weed species were present, the effect of *P. indica* on wheat was stronger, so competiveness was improved (Table 5.3 and 5.4; Appendix Table 15,16, Chapter 8). This suggests that wheat might be a favoured host for *P. indica*. However, the term 'weed' is not a biological category and has no botanical significance, because a plant that is a weed in one context is not a weed when growing in a situation where it is in fact wanted, and where one species of plant is a valuable crop plant, another species in the same genus might be a serious weed. Although *P. indica* might increase weed root biomass, its desirable beneficial effects on its host, such as increases in above ground biomass, final yield, and plant resistance against pathogens, and also its wide range hosts are much more attractive and useful. Growers have been using herbicide to control weeds for many years, even when they used other plant growth promoters and fertilisers in the fields. So if *P. indica* is going to be applied in the field, herbicide could still be used to control the weed problem.

However, the results presented here are based on a small scale experiment. More experiments need to be carried out to determine *P. indica* interaction with weeds, its host range and preferance.

6.6. *Piriformospora indica* application in agricultural industry

P. indica can be easily mass multiplied, its production is easy and application is cheap (Chadha et al., 2014, Varma et al., 2014). Based on data from other countries, it is likely to be useful in many crops, if it can be shown to be safe. The model of action is not via antibiotic or other toxin production and the fungus appears not to pose a health hazard that would need management. Potential sales are large and would intensify production of wheat and maybe other crops in a sustainable way. So concern is over irreversible ecological effects and the build-up of other microorganisms that decline *P. indica* population if it is widely used. Different soil types have different microorganism communities, as also shown in the experiments presented here that both Rowland series and Sonning series were clearly distinct in their fungal and bacterial diversity (Fig. 5.4). It suggests that the build-up of microorganisms would differ in different *P. indica*-inoculated soils, which might cause a decline in *P. indica* or alter its behaviour throughout time. For an example of the type of phenomenon which might occur, take-all decline in wheat monoculture is associated with build-up of root colonising antagonists in the soil that suppress the take-all pathogen in the soil in later years of monoculture.

6.6.1. Who might benefit from *Piriformospora indica* application?

Biological/crop protection science: In searching for biocontrol agents, biological control suppliers are looking for an agent which is adaptable to different environmental conditions, can be synchronised with its host and protect its host against biotic and abiotic stresses and at the same time improves host growth and productivity. With concerns over environmental side-effects and increasing fungicide resistance, the use of natural microorganisms to control crop diseases and enhance plant nutrient uptake is attractive, in product development for commercial biological control. *P. indica* application might be a bicontrol agent for the integrated pest management industry or those who sell microbial growth promoters such as plant growth promoting rhizobacteria.

Farmers and growers: When trying to control crop diseases, farmers and growers are looking for something that is economically affordable, easy to apply, with other aspects of the growing system, and controls multiple diseases. *P. indica* might be an attractive biocontrol agent because its production and application is cheap and easy, it is compatible with other foliar fungicide and it controls many diseases. Farmers would benefit by more stable production, reduced agrochemical costs and reduced disease pressure.

General public: Fungicide application to control diseases can lead to fungicide resistance (leading to increases doses) and environmental pollution. Misuse of agrochemicals and their entry in to the food chain can pose a risk to animal and human health. *P. indica* can protect its host against diseases and would minimise

the use of fungicide application, as a result minimising the risk of fungicide resistance and environmental pollution. Indirectly everyone benefits through more stable staple food prices and cleaner environment.

The fungus is out of patent in Europe, so the remaining research and development needed to establish efficacy and safety may be initially unattractive commercially and public or farmer-cooperative funding will be needed to establish a market.

6.7. Future research

Fungi of the order Sebacinales occur worldwide and encompass a great multitude of mycorrhizal associations, which are associated with the roots of a huge variety of plant species. There is no information available on Sebacinaceous fungi in the UK. More research needs to be done to understand the role of generalist sebacinaceous endophytes forming mycorrhizal associations, including the possible presence of *P. indica*, in the UK. Understanding the role of Sebacinaceous mycorrhizal fungi will help to gain more knowledge about their beneficial effects in the soil ecosystem and root-host symbiosis:

1- Develop an understanding of Sebacinales fungi, to determine how common and widespread they are; what is their range of hosts, and what effects they have on their hosts;

2- Determine whether Sebacinales fungi are actually ubiquitous, their range of environmental conditions, soil types, and their correlation with other soil microorganisms;

3- Test the effect of *P. indica* on the build-up of antagonists in soils where the fungi are permanently present; and also *P. indica*'s effect on other biotrophic fungi, insects, viruses, nematodes and wild plants.

4- Test if *P. indica* controls the root, foliar and head diseases consistently;

5- Check *P. indica* compatibility with foliar and ear fungicides, cultivar differences, and soil types, while trying to find other examples of Sebacinales and determine if all members have the same characteristics.

6.8. Conclusions

-*P. indica* protected wheat from Fusarium crown rot damage at seedling growth stages, by reducing the pathogen growth in the root system;

-*P. indica* reduced Fusarium head blight disease severity and incidence and mycotoxin DON contamination of grains contaminated wheat at flowering stage;

-*P. indica* reduced Septoria leaf blotch, yellow rust, and powdery mildew disease severity and incidence of wheat;

-*P. indica* did not have any effect on soil and leaf nutrient concentrations, but neither did *Fun. mosseae*, so this might be because of the experimental conditions;

-*P. indica* in soil survived the UK weather conditions;

-*P. indica* increased soil and root fungal and bacterial diversity;

-*P. indica* might be used to control crop diseases, but extensive data would be needed before release on a wide scale in areas where it is not native.

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Chapter 8. Annex

8.1. Complementary Statistical Data

8.1.1. Chapter 3- ANOVA P-value for figures and tables

Table 1. For Fig. 3.1. & Table 3.1. ANOVA P-value for Fusarium head blight disease severity and incidence and final harvest results measured in pots of winter wheat cv. Battalion, treated in a full factorial design with the factors shown. The experiment carried out in the 2013-14 growing season.

	P value							
	FHB severity	FHB incidence	Total above ground weight	Root weight	Total grain weight	1000 grain weight	Harvest index	No of ears
Main effect								
P. indica	**<.001**	**<.001**	**0.06**	**<.001**	0.2	**0.02**	0.6	0.2
Fun. mosseae	**0.001**	**0.006**	**0.01**	**<.001**	0.3	**0.05**	0.9	**0.02**
Fertiliser	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	0.6	0.3	**<.001**
F. graminearum	**<.001**	**<.001**	0.2	0.9	0.09	**0.06**	0.2	0.8
F. culmorum	0.09	0.1	0.09	**0.05**	0.2	0.8	0.6	0.9
2nd order interaction								
P. indica.*Fun. mosseae*	**0.008**	**0.03**	**0.06**	**<.001**	0.7	0.2	0.6	0.3
P. indica.Fertiliser	0.7	0.2	0.9	0.3	0.8	0.5	0.9	0.7
Fun. mosseae.Fertiliser	0.6	0.9	**0.004**	0.4	**0.03**	0.8	0.2	0.7
P. indica.*F. graminearum*	**0.004**	**0.005**	0.4	**0.03**	0.9	**0.04**	0.8	0.1

Fun. mosseae.*F. graminearum*	0.1	0.3	0.4	0.2	0.6	0.7	0.4	0.7
Fertiliser.*F. graminearum*	0.7	0.5	0.9	0.8	0.6	0.5	0.7	0.3
P. indica.*F. culmorum*	0.2	0.1	0.6	**0.01**	0.9	0.8	0.5	0.7
Fun. mosseae.*F. culmorum*	**0.03**	**0.01**	**<.001**	**0.01**	**0.07**	0.6	0.7	0.5
Fertiliser.*F. culmorum*	0.7	0.9	0.8	**<.001**	0.7	0.7	0.6	0.3
F. graminearum.*F. culmorum*	0.4	0.5	0.9	0.6	0.9	**0.02**	0.8	0.9
3rd order interaction								
P. indica.*Fun. mosseae*.Fertiliser	0.6	0.7	0.9	**0.05**	0.5	**0.008**	0.4	**0.02**
P. indica.*Fun. mosseae*.*F. graminearum*	0.08	**0.05**	**0.008**	0.7	0.09	0.7	0.5	0.9
P. indica.Fertiliser.*F. graminearum*	0.9	0.5	0.9	0.04	0.2	0.8	0.1	0.6
Fun. mosseae.Fertiliser.*F. graminearum*	0.6	0.9	**0.001**	0.1	0.4	0.09	0.5	0.7
P. indica.*Fun. mosseae*.*F. culmorum*	0.4	0.7	**0.07**	**0.008**	**0.05**	0.4	0.2	0.3
P. indica.Fertiliser.*F. culmorum*	0.6	0.7	0.3	0.2	0.4	0.3	0.5	0.8
Fun. mosseae.Fertiliser.*F. culmorum*	0.6	0.4	0.06	0.4	0.3	0.7	0.9	0.4
P. indica.*F. graminearum*.*F. culmorum*	0.8	0.2	0.6	0.3	0.7	0.9	0.9	0.3
Fun. mosseae.*F. graminearum*.*F. culmorum*	0.6	0.3	0.4	0.9	0.1	**0.04**	0.2	0.7
Fertiliser.*F. graminearum*.*F. culmorum*	0.07	**0.04**	0.1	0.5	0.9	0.8	0.4	0.5
4th order interaction								
P. indica.*Fun. mosseae*.Fertiliser.*F. graminearum*	0.5	0.7	0.1	0.2	0.2	0.7	0.6	0.9
P. indica.*Fun. mosseae*.Fertiliser.*F. culmorum*	0.2	0.03	0.9	**0.008**	0.4	0.7	0.6	0.8

P. indica.*Fun. mosseae*.*F. graminearum*.*F. culmorum*	**0.05**	**0.01**	0.8	0.8	0.8	0.4	0.7	0.09
P. indica.Fertiliser. *F. graminearum*.*F. culmorum*	0.06	0.04	0.4	0.4	0.7	0.1	0.8	0.9
Fun. mosseae.Fertiliser.*F. graminearum*.*F. culmorum*	0.4	0.3	0.4	0.5	0.6	0.3	0.9	0.6
5th order interaction								
P. indica.*Fun. mosseae*.Fertiliser.*F. graminearum*.*F. culmorum*	0.7	0.9	0.4	0.8	0.6	0.8	0.8	0.5

Table 2. For Fig. 3.2. & Table 3.2. ANOVA P-value for Fusarium head blight disease severity and incidence and final harvest results measured in pots of spring wheat cv. Paragon, treated in a full factorial design with the factors shown. The experiment carried out in the 2014 growing season.

	P value							
Main effect	FHB severity	FHB incidence	Total above ground weight	Root weight	Total grain weight	1000 grain weight	Harvest index	No of ears
P. indica	0.07	0.2	**0.05**	**0.02**	**0.02**	0.08	0.07	**0.003**
Fun. mosseae	0.8	0.6	0.1	0.2	0.1	0.5	0.5	0.1
F. graminearum	**<.001**	**<.001**	0.8	0.8	0.8	0.4	0.7	**0.03**
Fungicide	**0.005**	**0.02**	0.6	0.7	0.05	0.7	**0.03**	0.12
2nd order interaction								
P. indica.*Fun. mosseae*	0.4	0.5	0.8	**0.03**	0.7	0.1	0.3	0.4
P. indica.*F. graminearum*	0.2	0.4	0.4	0.6	0.08	0.1	0.07	0.9
Fun. mosseae.*F. graminearum*	0.7	0.6	0.09	**0.05**	0.2	0.1	0.7	**0.06**
P. indica.Fungicide	0.1	0.3	0.3	0.2	0.8	0.1	0.4	0.6
Fun. mosseae.Fungicide	0.4	0.3	0.8	0.3	0.3	0.2	0.1	0.8
Fungicide.*F. graminearum*	**0.04**	0.1	0.5	0.4	0.9	0.8	0.4	0.9
3rd order interaction								
P. indica.*Fun. mosseae*. *F. graminearum*	0.8	0.9	0.7	**0.01**	0.6	0.6	0.7	0.7
P. indica.*Fun. mosseae*.Fungicide	0.9	0.9	**0.03**	0.9	**0.003**	**0.01**	**0.009**	**0.003**
P. indica.*F. graminearum* . Fungicide	0.1	0.3	0.7	0.8	0.4	0.9	0.3	0.7
Fun. mosseae.*F. graminearum*.Fungicide	0.5	0.6	0.8	0.6	0.4	0.8	0.1	0.4

4th order interaction								
P. indica.*Fun. mosseae*.*F. graminearum*.Fungicide	0.7	0.6	0.2	0.3	0.3	0.5	0.9	0.5

Table 3. For Fig. 3.3. & Table 3.3. ANOVA P-value for Fusarium head blight disease severity and incidence and harvest results measured in pots of six cultivars of spring wheat cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn, treated in a full factorial design with the factors shown. The experiment carried out in the 2015 growing season.

	P value							
Main effect	FHB severity	FHB incidence	Total above ground weight (g)	Root weight (g)	Total grain weight per pot (g)	1000 grain weight (g)	Harvest index	No of ears
P. indica	**<.001**	**<.001**	**0.002**	**<.001**	**<.001**	**<.001**	**<.001**	**0.002**
F. graminearum	**<.001**	**<.001**	**0.06**	0.6	**<.001**	0.201	**0.034**	0.604
Wheat cultivars	**<.001**	**<.001**	**0.02**	0.09	**0.001**	0.102	0.119	**<.001**
2nd order interaction								
P. indica. *F. graminearum*	**<.001**	**0.02**	**0.04**	0.8	0.2	0.03	0.6	0.6
P. indica.Wheat cultivars	0.68	0.87	0.9	0.9	0.3	0.4	0.6	0.8
FHB.wheat cultivars	0.93	0.9	0.5	0.1	0.7	0.8	0.9	0.7
3rd order interaction								
P. indica. *F. graminearum*.Wheat cultivars	0.21	0.16	0.3	0.5	0.3	0.5	0.2	0.6

Table 4. For Fig. 3.4.a. ANOVA P-value for mycotoxin DON measured in pots of winter wheat cv. Battalion, treated in a full factorial design with the factors shown. The experiment carried out in the 2013-14 growing season.

	P value
main effect	mycotoxin DON
P. indica	**<.001**
F. culmorum	**<.001**
Fertiliser	**0.005**
Fun. mosseae	0.5
2rd order interaction	
P. indica.*F. culmorum*	**<.001**
P. indica.Fertiliser	0.1
Fertiliser.*F. culmorum*	0.09
P. indica.*Fun. mosseae*	**0.003**
Fun. mosseae.*F. culmorum*	0.3
Fun. mosseae.Fertiliser	0.4
3rd order interaction	
P. indica.Fertiliser. *F. culmorum*	**0.05**
P. indica.*Fun. mosseae*. *F. culmorum*	0.6
P. indica. *Fun. mosseae*.Fertiliser	0.4
Fun. mosseae.Fertiliser.*F. culmorum*	0.2
4th order interaction	
P. indica.*Fun. mosseae*.Fertiliser. *F. culmorum*	0.1

Table 5. For Fig. 3.4.b. ANOVA P-value for mycotoxin DON measured in pots of spring wheat cv. Paragon, treated in a full factorial design with the factors shown. The experiment carried out in the 2014 growing season.

	P value
main effect	Mycotoxin DON
P. indica	**0.01**
Fun. mosseae	0.5
Fungicide	**0.001**
2nd way interaction	
P. indica.*Fun. mosseae*	**0.009**
P. indica.Fungicide	**0.03**
Fun. mosseae.Fungicide	0.9
3rd way interaction	
P. indica.*Fun. mosseae*.Fungicide	0.06

Table 6. For Fig. 3.4.c. ANOVA P-value for mycotoxin DON measured in pots of six cultivars of spring wheat cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn, treated in a full factorial design with the factors shown. The experiment carried out in the 2015 growing season.

	P value
Main effect	Mycotoxin DON
P. indica	**<.001**
Wheat cultivars	**<.001**
2nd order interaction	
P. indica.Wheat cultivars	**0.002**

Table 7. For Table 3.4. ANOVA P-value for soil nutrients measured in pots of winter wheat cv. Battalion, treated in a full factorial design with the factors shown. The experiment carried out in the 2014-15 growing season.

	P value							
Main effect	Soil pH	P	K	Mg	NO_3	NH_4	Available N	Dry Matter
P. indica	0.8	0.6	0.3	0.8	0.4	0.9	0.7	**<.001**
Fun. mosseae	0.08	0.09	0.8	0.9	0.9	0.6	0.8	0.8
Fertliser	**<.001**	**<.001**	**0.6**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**
2nd order interaction								
P. indica.*Fun. mosseae*	0.9	0.2	0.8	0.6	**0.05**	0.03	**0.04**	0.5
P. indica.Fertliser	0.9	0.7	0.2	0.4	0.09	0.4	0.2	0.06
Fun. mosseae.Fertliser	0.5	0.4	0.2	0.3	0.2	0.4	0.3	0.1
3rd order interaction								
P. indica.*Fun. mosseae*.Fertliser	**0.04**	0.4	0.07	0.7	**0.02**	**0.02**	**0.02**	0.8

Table 8. For Table 3.5. ANOVA P-value for leaf tissue nutrients measured in pots of winter wheat cv. Battalion, treated in a full factorial design with the factors shown. The experiment carried out in the 2014-15 growing season.

	P value										
Main effect	Total N	Total P	Total K	Ttal Ca	Total Mg	Total S	Total Mn	Total Cu	Total Fe	Total Zn	Total B
P. indica	0.6	0.9	0.6	0.8	0.6	0.6	0.7	0.7	**0.03**	0.9	**0.01**
Fun. mosseae	0.7	0.6	0.9	0.8	0.8	0.4	0.4	0.3	0.02	0.5	0.2
Fertliser	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**0.002**	**<.001**	**<.001**
2nd order interaction											
P. indica.*Fun. mosseae*	0.4	0.3	0.5	0.4	0.5	0.5	0.1	0.7	0.06	0.9	1
P. indica.Fertliser	0.6	0.7	0.9	0.8	0.8	0.3	**0.03**	0.5	0.06	0.6	**0.02**
Fun. mosseae.Fertliser	0.8	0.2	0.5	0.9	0.7	0.1	0.2	0.3	**0.05**	0.3	0.7
3rd order interaction											
P. indica.*Fun. mosseae*.Fertliser	0.04	0.9	0.3	0.3	0.1	0.3	0.9	0.2	0.1	0.4	0.3

8.1.2. Chapter 4- ANOVA P-value for figures and tables

Table 9. For Fig. 4.1 & Table 4.1. ANOVA P-value for final harvest results measured in pots of winter wheat cv. Battalion, grown for assessing *P. indica* effect on air-borne diseases, treated in a full factorial design with the factors shown. The experiment carried out in the 2014-15 growing season.

Main effect	Septoria severity	Septoria incidence	Total above ground weight (g)	Root weight (g)	Total grain weight per pot (g)	1000 grain weight (g)	Harvest index	No of ears
P.inidca	**<.001**	**0.01**	**0.007**	**0.001**	**<.001**	**0.003**	0.2	**0.05**
Fertliser	**<.001**	**<.001**	**<.001**	**0.002**	**0.002**	0.2	0.6	**<.001**
2nd order interaction								
P. inidca.Fertliser	**0.002**	0.1	0.3	**0.04**	0.5	0.3	0.4	0.7

Table 10. For Fig. 4.2. ANOVA P-value for Septoria leaf blotch disease severity and incidence measured in pots of winter wheat cv. Battalion, grown for soil and plant tissue nutrient analysis, treated in a full factorial design with the factors shown. The experiment carried out in the 2014-15 growing season.

	P value	
Main effect	Severity	Incidence
P. indica	**0.05**	**0.003**
Fun. mosseae	0.1	0.08
Fertliser	**<.001**	**<.001**
2nd order interaction		
P. indica.*Fun. mosseae*	0.8	0.8
P. indica.Fertliser	0.2	0.3
Fun. mosseae.Fertliser	0.3	0.9
3rd order interaction		
P. indica.*Fun. mosseae*.Fertliser	0.7	0.2

Table 11. For Fig. 4.3. ANOVA P-value for Septoria leaf blotch disease severity and incidence measured in pots of winter wheat cv. Battalion, grown for Fusarium experiment, treated in a full factorial design with the factors shown. The experiment carried out in the 2013-14 growing season.

	P value	
Main effect	Severity	Incidence
P. indica	**<.001**	**<.001**
Fun. mosseae	**<.001**	0.1
Fertiliser	**<.001**	**<.001**
F. culmorum	0.094	0.1
2nd order interaction		
P. indica.*Fun. mosseae*	**<.001**	**0.003**
P. indica.Fertiliser	**0.002**	**<.001**
Fun. mosseae.Fertiliser	0.2	0.6
P. indica.*F. culmorum*	0.7	0.8
Fun. mosseae.*F. culmorum*	0.9	0.9
Fertiliser.*F. culmorum*	0.6	0.5
3rd order interaction		
P. indica.*Fun. mosseae*.Fertiliser	0.7	0.4
P. indica.*Fun. mosseae*.*F. culmorum*	0.6	**0.05**
P. indica.Fertiliser.*F. culmorum*	0.8	0.6
Fun. mosseae.Fertiliser.*F. culmorum*	0.2	0.5
4th order interaction		
P. indica.*Fun. mosseae*.Fertiliser.*F. culmorum*	0.1	0.3

Table 12. For Fig. 4.6. ANOVA P-value for yellow rust disease severity and incidence measured in pots of winter wheat cv. Battalion, grown for Fusarium experiment, treated in a full factorial design with the factors shown. The experiment carried out in the 2013-14 growing season.

	P value	
Main effect	Severity	Incidence
P. indica	**0.005**	**<.001**
Fun. mosseae	0.9	0.4
Fertiliser	**<.001**	**<.001**
F. culmorum	0.2	0.08
2nd order interaction		
P. indica.*Fun. mosseae*	0.5	0.7
P. indica.Fertiliser	0.3	0.5
Fun. mosseae.Fertiliser	0.3	0.4
P. indica.*F. culmorum*	0.8	0.4
Fun. mosseae.*F. culmorum*	0.8	0.9
Fertiliser.*F. culmorum*	0.7	0.2
3rd order interaction		
P. indica.*Fun. mosseae*.Fertiliser	0.2	0.3
P. indica.*Fun. mosseae*.*F. culmorum*	0.7	0.6
P. indica.Fertiliser.*F. culmorum*	0.1	0.2
Fun. mosseae.Fertiliser.*F. culmorum*	0.9	0.6
4th order interaction		
P. indica.*Fun. mosseae*.Fertiliser. *F. culmorum*	0.8	0.9

Table 13. For Fig. 4.7. ANOVA P-value for yellow rust disease severity and incidence measured in pots of six cultivars of spring wheat cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn, treated in a full factorial design with the factors shown. The experiment carried out in the 2015 growing season.

	P value	
main effect	Severity	Incidence
P. indica	**<.001**	**<.001**
Spring wheat cultivars	**<.001**	**<.001**
2nd order interaction		
P. indica.Spring wheat cultivars	0.7	0.5

Table 14. For Fig. 4.8. ANOVA P-value for powdery mildew disease severity and incidence measured in pots of six cultivars of spring wheat cv. Paragon, Mulika, Zircon, Granary, KWS Willow and KWS Kilburn, treated in a full factorial design with the factors shown. The experiment carried out in the 2015 growing season.

	P value	
main effect	Severity	Incidence
P. indica	**0.01**	**0.01**
Spring wheat cultivars	**<.001**	**<.001**
2nd order interaction		
P. indica.Spring wheat cultivars	0.7	0.9

8.1.3. Chapter 5- ANOVA P-value for tables

Table 15. For Table 5.3. ANOVA P-value 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.*

	P value	
Main effect	weed shoot	weed root
Mix with wheat or solo (Mix-solo)	**0.005**	**0.05**
P. indica	0.2	**0.05**
Species (weeds and wheat)	**<.001**	**0.03**
2nd order interaction		
Mix-solo.*P. indica*	0.2	0.1
Mix-solo.Species	0.2	0.4
P. indica.Species	0.2	0.5
3rd order interaction		
Mix-solo.*P. indica*.Species	0.6	0.9

Table 16. For Table 5.4. ANOVA P-value 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.

	P value	
Main effect	**Shoot competition (log_{10}(weedshoot/wheatshoot)**	**Root competition (log_{10}(weedroot/wheat root)**
P. indica	**0.02**	0.3
Species (weeds and wheat)	**0.002**	0.2
2nd order intrecation		
P. indica.Species (weeds and wheat)	0.7	0.9