Performance of the Biocontrol Fungus *Piriformospora indica* on Wheat Under Greenhouse and Field Conditions

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ABSTRACT

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The endophyte *Piriformospora indica* colonizes roots of a range of host plants and increases biomass production and resistance to fungal pathogens and, thus has been considered a biocontrol fungus. However, the field performance of this fungus has not yet been tested in temperate climates. Therefore, we evaluated the performance of this fungus in different substrata under greenhouse and practical field conditions. Roots of winter wheat were colonized efficiently, and biomass was particularly increased on poor substrata. In greenhouse experiments, symptom severity of a typical leaf (*Blumeria graminis* f. sp. *tritici*), stem base (*Pseudocercospora herpotrichoides*), and root (*Fusarium culmorum*) pathogen was reduced significantly. However, in field experiments,

symptoms caused by the leaf pathogen did not differ in *Piriformospora indica*-colonized compared with control plants. In the field, *Pseudocercospora herpotrichoides* disease severity was significantly reduced in plants colonized by the endophyte. Increased numbers of sheath layers and hydrogen peroxide concentrations after *B. graminis* attack were detected in *Piriformospora indica*-colonized plants, suggesting that root colonization causes induction of systemic resistance or priming of the host plant. Although the endophyte is not well suited for growth at Central European temperature conditions, it remains to be shown whether *P. indica* is more suitable for tropical or subtropical farming.

Additional keywords: arbuscular mycorrhizal fungi, powdery mildew, systemic acquired resistance.

Traditionally, fungi have been regarded as pathogens by agronomists. However, in recent years, symbiotic fungi providing benefits to crop plants have become an additional focus of research. In addition to the arbuscular mycorrhizal fungi (AMF) that constitute a distinct fungal phylum, the Glomeromycota (22), endophytes that mainly belonging to the Ascomycota or Basidiomycota, have been shown to improve the vigor of their hosts (5,9,20,26). One particular endophyte, *Piriformospora indica*, a member of the Sebaciales order of the Basidiomycota, recently has received some attention. Originally, this fungus was recovered from the rhizosphere of shrubs growing in the Thar desert of Rajasthan, India (24,27). In contrast to the obligate biotrophic AMF, *P. indica* can be cultivated easily on synthetic media, where it forms typical pear-shaped chlamydospores. Earlier work established that *P. indica* increased the biomass of several host plants belonging to a wide range of taxa (24,26,28). This fungus even colonized mustard, cabbage, and spinach that belong to the Brassicaceae, which do not form a symbiosis with AMF (14). The enhanced biomass of the host apparently results from improved nutrient supply. In particular, *P. indica* seems to mediate phosphorous and nitrogen uptake from the soil (23,25). Recent work suggested that *P. indica* stimulates NADH-dependent nitrate reductase activity in roots of *Arabidopsis* and tobacco (23). Enhanced enzymatic activity correlated with an increased transcription of the corresponding plant gene. Thus, it seems that *P. indica* mediates nitrate uptake from the soil, which is in contrast

to AMF, where nitrogen is preferentially absorbed as ammonium. Beyond the stimulating effect on biomass production, *P. indica* apparently supports its host by protecting it from pathogenic fungi (28). It was suggested that *P. indica* may target an as-yet-unidentified signaling pathway to induce systemic resistance.

The beneficial effects promoted by *P. indica* have been determined mostly in small-scale greenhouse or growth-chamber experiments. Recently, the effect of *P. indica* on barley was assessed under seminatural conditions using Mitscherlich pots (28). In this work, the interaction between the plant and the fungus had been established in growth chambers, followed by incubation outdoors. Under these conditions, *P. indica* acted as both a biofertilizer and a biocontrol agent. However, there is no information available yet on whether or not the benefits described from greenhouse experiments also would occur in fields managed under regular farming practices. Here, we investigate whether *P. indica* also stimulates the production of biomass in wheat. Another objective of this study was to determine whether the inoculation of wheat with *P. indica* may subsequently reduce the symptoms caused by several fungal pathogens on leaves, stems, and roots. These objectives were addressed under greenhouse and field conditions.

MATERIALS AND METHODS

Cultivation of fungi. *P. indica* strain DSM 11827 (obtained from P. Franken, Leibniz Institute of Vegetable and Ornamental Crops, Grossbeeren, Germany) and *Fusarium culmorum* strain GU75 (obtained from U. Sperling, Landesanstalt für Landwirtschaft, Forsten und Gartenbau, Magdeburg, Germany) were grown on complete medium agar at ambient temperature (15).

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Pseudocercospora herpotrichoides strain Phh31/3 (obtained from V. Lind, BAFZ Aschersleben, Germany) was grown on 1.5% water agar at 23°C. *Blumeria graminis* f. sp. *tritici* strain KF01 (obtained from S. Reimann, Martin Luther Universität, Halle, Germany) was propagated in the greenhouse at ambient temperature on wheat cv. Kanzler.

To produce inoculum for infection assays, *P. indica* and *F. culmorum* were grown without shaking at room temperature in a volume of 1.5 liter of liquid complete medium (15). After 21 days of growth, the fresh weight of the mycelial fraction accounted for ≈220 g. The mycelia were minced for 5 min using a rotor-stator homogenizer (Ultra Turrax, model T8; IKA Labortechnik GmbH, Staufen, Germany) at the lowest speed setting and then immediately used for inoculation. For field experiments, the suspension of *P. indica* was diluted twofold with tap water prior to usage. To produce inoculum of *Pseudocercospora herpotrichoides*, the fungus was grown on 1.5% water agar under longwave UV light for 28 days at 23°C (Incubator CIR 350; UniEquip GmbH, Martinsried, Germany). Agar blocks were crushed with a pestle in sterile distilled water. Spores then were passed through a nylon filter (mesh width, 0.5 mm) and adjusted to a final concentration of $1 \times 10^4 \text{ mL}^{-1}$.

Mycelial growth rates of *Piriformospora indica* and *Pseudocercospora herpotrichoides* were assayed in the dark on complete medium agar (15) using three replicated cultures for each temperature. These ranged from 5 to 35°C at intervals of 5°C. Colony diameters were recorded every other day for 20 days in total. The respective data of the triplicates were combined and averaged.

DNA isolation and PCR to detect *P. indica* in infected plants. Wheat roots inoculated with *Piriformospora indica* were harvested from pot cultures and total genomic DNA was extracted from ≈100 mg of tissue, following the instructions provided by the manufacturer of a commercial kit (DNA Plant mini Kit; Qiagen AG, Hilden, Germany). DNA also was isolated from roots of uninoculated plants, and from mycelia harvested by filtration from liquid cultures that were produced as described above to serve as negative and positive controls, respectively, in polymerase chain reaction (PCR) experiments.

We used the annotated sequence (AJ459235) of the β -tubulin gene of *P. indica* to design specific primers and to develop an assay for its detection in host tissues. Primers had the sequences 5'-TTCTGGGAAGTCGTCTCTG-3' and 5'-AGCCAACCATGAGAAGTG-3'. In a total volume of 20 μL , PCR reactions included 0.25 μM each primer, 2.5 mM MgCl_2 , 0.5 mM dNTPs, 1 $\times$ reaction buffer, Taq polymerase at 0.4 U/ μL (all reagents from MBI Fermentas GmbH, St Leon-Roth, Germany), and 100 ng of template DNA. Cycling conditions were 95°C for 180 s; followed by 35 cycles of 95°C for 30 s, 51.5°C for 30 s, and 72°C for 90 s; and a final extension at 72°C for 10 min. Reaction products were analyzed by electrophoresis on a 1% agarose gel. Positive controls included DNA isolated from *P. indica* grown in vitro and produced a band of the anticipated size of 751 bp. Negative controls used either DNA from roots of noninoculated wheat or omitted any template DNA.

Infection assays using wheat pot cultures. Winter wheat (*Triticum aestivum* cv. Kanzler) (Saatzucht Engelen-Büchling e.K., Oberschneiding, Germany), which is highly susceptible to most fungal pathogens, was grown in the greenhouse in pots with a diameter of 13 cm and a height of 11.5 cm. The temperature ranged from 22 to 30°C and the humidity was not controlled. The substratum used was either washed sand (average particle size 220 to 380 μm) (Redsun GmbH & Co. KG, Kevelaer, Germany) or garden soil (Plantaflo GmbH, Vechta, Germany) that was pasteurized by steaming prior to application. Each variant included at least four pots with 4 plants so that a minimum of 16 plants was available. The pots were distributed in a randomized manner and their positions were exchanged once a week.

To determine the germination rate of wheat, 100 ml of mycelial suspensions containing ≈15 g fresh weight each of *P. indica* or *F. culmorum* was produced as described above and poured onto the substratum immediately before the seed were sown. The controls were treated with 100 ml of sterile medium. The results were scored after 7 days of incubation in the greenhouse under the same conditions as above.

For infection assays of wheat plantlets, *P. indica* generally was inoculated 7 days after sowing using the same type of suspension as described above. For dual inoculations, *P. indica* was inoculated 7 days after sowing and then, after an additional 7 days of incubation, challenge inoculation with a pathogen was carried out.

For infection assays of wheat plantlets involving *F. culmorum*, the inoculum was produced as described above. The fresh weights were recorded separately for roots and shoots of plants harvested after 30, 60, 90, and 120 days of growth in the greenhouse under the same conditions as above.

For infection assays of wheat plantlets involving *P. herpotrichoides*, the inoculum was generated as described above. Inoculation was carried out, without inflicting any physical damage to the tissue, by pipetting 800 μL of a spore suspension ($1 \times 10^4 \text{ mL}^{-1}$) to the base of each wheat stem. The inoculated plants were incubated in environmentally controlled growth chambers (Model ILKA 2000/1; Maschinenfabrik Netzschkau, Germany) that were continuously run at 17°C and 76% relative humidity under 16 h of light at 200 $\mu\text{E m}^{-2} \text{ s}^{-1}$ at soil level. At 60 days postinoculation (dpi), fresh weights were recorded separately for roots and shoots. The symptoms were scored by a custom-designed scale that counted the number of brownish tissue layers at the stem base and expressed this as the fraction in percentage of the total layers developed.

For infection assays of wheat plantlets involving *B. graminis* f. sp. *tritici*, the inoculum were dry conidia. Inoculation was performed by shaking infected, heavily sporulating wheat plants above noninfected tester plants. Incubation of the freshly inoculated plants continued in the greenhouse under the same conditions as described above. The numbers of mildew pustules per square centimeter were recorded separately for leaves L2 and L3 at 3, 6, and 9 dpi.

Microscopical techniques. Light microscopy was performed with a Nikon Eclipse 600 epifluorescence microscope (Nikon GmbH, Düsseldorf, Germany). Digital images were taken with a CCD-1300 camera (VDS Vosskühler GmbH, Osnabrück, Germany) and archived and processed with the software package Lucia 4.61 (Nikon GmbH, Düsseldorf, Germany).

To detect hydrogen peroxide production after inoculation with powdery mildew, 1-cm-long segments from the middle of infected leaves (L3) were subjected to staining with 3,3-diaminobenzidine (DAB). For each variant, three-leaf segments were incubated for 8 h in 0.1% DAB (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) with the pH adjusted to 3.8 by HCl. The tissue then was incubated for 16 h in 80% ethanol, 0.15% trichloroacetic acid, and 20% chloroform and stored in 50% glycerol.

To examine the colonization of *Piriformospora indica* in field samples after harvest, 16 root segments per variant were autoclaved in 10% KOH and, afterward, again autoclaved in 0.03% chlorazol black E in a 1:1:1 mixture of lactic acid, glycerol, and water. For each segment, 200 cells were analyzed and the fraction of cells harboring fungal spores was determined.

For microscopical studies of the interaction between *P. indica* and *Pseudocercospora herpotrichoides* occurring in vitro, glass slides were covered with a thin layer of solid complete medium (15). The fungi were inoculated at a distance of 1 cm and allowed to grow for 7 days at 25°C. The slides were incubated for 20 min in Calcofluor White M2R fluorescent brightener 28 (Sigma-Aldrich) (0.01% in distilled water) and washed with 25% glycerol. Three replicates per variant were used to determine the distances between 50 hyphal tips and their respective first branches at the edge of the colony.

Field experiments. Experiments were carried out in the 2002–03 and 2003–04 growing seasons at the field station of the Martin-Luther University Halle-Wittenberg situated at Bad Lauchstädt (federal state of Saxony-Anhalt, Germany, 113 m above sea level; GPS: field of 2002–03, 51°23,61865' North, 11°53,03667' East; field of 2003–04, 51°23,46484' North, 11°52,88095' East). The soil at the field station has a rating index of 94 of 100 possible points (21) and is characterized as clayey silt with loess as the topsoil. For comparison, in the 2003–04 season, we carried out additional field experiments on a field contracted from a local farmer at Salzmünde (federal state of Saxony-Anhalt, Germany, 87 m above sea level; GPS: 51°30,42894' North, 11°48,06916' East). In Salzmünde, the soil is poorer, with a soil rating index of 78, and is characterized as clayey loam, with loess-pararendzina as the topsoil.

Weather data for the Bad Lauchstädt field station had been gathered since 1895. The long-time average temperature is 8.7°C and average yearly precipitation is 483 mm. Corresponding data were not available for the Salzmünde field. Actual weather conditions during the field experiments were as follows: Bad Lauchstädt October 2002 to August 2003, average temperature 9.4°C, accumulated precipitation 460 mm; Bad Lauchstädt October 2003 to August 2004, average temperature 9.0°C, accumulated precipitation 435 mm; Salzmünde October 2003 to August 2004, average temperature 9.0°C, accumulated precipitation 511 mm.

Each field experiment included four replicated blocks. The blocks used a randomized design where each of four variants was represented by a single plot. The plot size was 3 by 12 m. In all experiments, on 15 October, winter wheat cv. Kanzler was sown at a density of 350 grains/m² in rows that were 12 cm apart. This also included a strip of 5 m that completely surrounded the experiment. The four variants differed by the treatments received during the season. One was always an untreated control, another was a fungicide-treated control that received Pronto Plus at 1.5 liter/ha (Bayer AG, Langenfeld, Germany) and Bravo at 1.0 liter/ha (Syngenta GmbH, Maintal, Germany) at stage 60 on the BBCH (Bundesanstalt, Bundessortenamt and Chemical Industry) scale (11), and a third variant received a treatment with a mycelial suspension of *Piriformospora indica* of 3,000 liter/ha in total. A treatment with fungicides containing strobilurines was not included to omit putative yield responses caused by a known side effect. The fourth variant differed between the experiments. Both experiments carried out at Bad Lauchstädt included a *P. indica* treatment using 300 liter/ha in total, whereas the fourth variant from Salzmünde included a treatment with 6,000 liter/ha. Treatments with mycelial suspensions of *P. indica* always were carried out twice, at stages 39 and 55 on the BBCH scale, each time pouring half of the total amount indicated for a given treatment onto the soil using a watering can. None of the variants received any fertilization during the course of the experiment.

We recorded field symptoms caused by several fungal pathogens (i.e., *B. graminis* f. sp. *tritici*, *Septoria tritici*, *S. nodorum*, *Puccinia recondita* f. sp. *tritici*, *F. graminearum*, *F. culmorum*, and *Pseudocercospora herpotrichoides*). Symptoms caused by *P. herpotrichoides* were scored using the same scheme described above for the pot cultures. Scoring of all other disease symptoms relied on published rating schemes (12). Symptoms caused by *S. tritici* and *S. nodorum* were not discriminated. Scoring was performed at the BBCH stages 55, 69, and 85. It should be emphasized that there were no measures taken to increase the natural load of propagules present at the respective plots.

Wheat was harvested on 1 August 2003 and 12 August 2004 at Bad Lauchstädt and on 13 August 2004 at Salzmünde with a small combine harvester designed for field experiments (Massey Fergusson MF 31; AGCO GmbH, Witzenhausen, Germany). First, strips with a width of 1 m were removed at both ends of each block so that the length of the lots used for analysis was reduced

to 10 m. Then, a strip of 1.68 m was harvested in the middle of each plot and used for further analysis. The remaining strips of 0.66 m flanking the former on both sides were discarded. From the entire material harvested from each plot, fresh weights of grains and straw were determined separately. To ascertain the dry weights, two samples of 100 g each were taken from the harvests of each plot and either directly dried for 24 h at 105°C, in the case of straw, or used to determine the water content with a Tecator AP 6060 moisture analyzer (Sinar Technology, Newbury, UK).

Statistical analyses. Analyses were performed with the program JMP (version 4.04; SAS Institute, Cary, NC). We employed the Kruskal-Wallis and *H* tests to assess variation between infection rates of *P. herpotrichoides*. For all other experiments, we applied the Tukey test to determine significant differences between the variants. The confidence limits were at 95%.

RESULTS

***Piriformospora indica* infects and persists in wheat.** We determined the potential of *P. indica* to colonize wheat growing in pot cultures using sand and soil as substrata. Root samples were harvested at regular intervals after inoculation and observed by light microscopy (data not shown). *P. indica* grew from the inoculum toward the root and penetrated the rhizodermis. Within 1 week after inoculation, intracellular hyphae became visible in epidermal cells. *P. indica* formed a dense hyphal network around the root within 2 weeks. Chlamydospores were observed within epidermal and root hair cells after 3 weeks. We did not detect any ingress into the stele or the shoot. On both substrata, differentiation progressed in the same way.

In addition, we also used a sensitive PCR approach to monitor the persistence of *P. indica* in wheat roots. In root DNA from inoculated wheat, *P. indica* was detectable at 7 dpi and remained so over a period of 3 months. DNA isolated from wheat roots that grew in sand always yielded more intense bands than those from soil, suggesting more intense colonization (Fig. 1). Taken together, *P. indica* was able to infect and colonize wheat and to persist in host tissues for a considerable time, similarly to what has been reported for other host plants.

Effects of *P. indica* on root, stem-base and leaf diseases of wheat grown in pot cultures. We determined the influence of *P. indica* and *F. culmorum* on germination of wheat seed. In noninoculated controls, germination rates were 99.3 and 97.9% in garden soil and washed sand, respectively. A significant reduction was observed when *F. culmorum* was inoculated into the soil substratum ($P < 0.001$), resulting in germination rates of 71.4%, which was not found for the sand substratum. When both *P. indica* and *F. culmorum* were inoculated into soil, the germination rate was 90.7%, which was not significantly different from the noninoculated control. Therefore, the detrimental effect of the pathogen was compensated for by the presence of the endophyte. None of the variants from the sand substratum produced

Fig. 1. Polymerase chain reaction (PCR) detection of *Piriformospora indica* in DNA of wheat roots. *P. indica* was inoculated into pot cultures growing either in soil (lanes 1 to 4) or sand (lanes 5 to 8). DNA templates for PCR originate from plants harvested after 7 days (lanes 1 and 5), 14 days (lanes 2 and 6), 30 days (lanes 3 and 7), and 90 days (lanes 4 and 8). Lane 9: root DNA from noninoculated wheat; lane 10: genomic DNA from in vitro cultivated *P. indica*; and lane 11: water control.

significant differences compared with the respective controls (data not shown).

Next, we assessed the potential of *P. indica* to protect wheat plantlets from the root-pathogen *F. culmorum*, the stem-base pathogen *Pseudocercospora herpotrichoides*, and the leaf pathogen *B. graminis* f. sp. *tritici*. In general, plants grown on sand invested more biomass into roots than into shoots (Fig. 2A). The opposite was true for plants grown in soil (Fig. 2B), where the final total biomass harvested was also much higher than in sand. On soil, the endophyte caused only minor changes to the biomass formation of the host in most cases. In contrast, on sand, *Piriformospora indica* had prominent beneficial effects because it roughly doubled the biomass. For this variant, the fresh weights of roots, shoots, and the entire plant were increased significantly at all time points tested when compared with the controls (sand roots, 30, 60, 90, and 120 dpi, $P < 0.0001$; sand shoots, 30 dpi, $P = 0.0008$, and 60, 90, and 120 dpi, $P < 0.0001$; sand total, 30, 60, 90, and 120 dpi, $P < 0.0001$; soil roots, $P = 0.1112$; 60 dpi, $P = 0.015$; 90 dpi, $P = 0.0615$; 120 dpi, $P = 0.0063$; soil shoots, 30 dpi: $P = 0.0014$; 60 dpi, $P = 0.0002$; 90 dpi, $P = 0.0037$; 120 dpi, $P = 0.0583$; soil total, 30 dpi, $P = 0.0343$; 60 dpi, $P = 0.0055$; 90 dpi, $P = 0.1481$; and 120 dpi, $P = 0.3856$). The biomass gains mediated by the endophyte were smaller in the variant where *F. culmorum* was used for challenge inoculation, but still were significant in relation to the controls.

Fig. 2. Effect of *Piriformospora indica* on biomass production of wheat infected with *Fusarium culmorum*. Pot cultures were grown in the greenhouse using **A**, sand or **B**, soil as the substrata. Columns represent the mean ($n = 16$ plants/variant) of total, shoot, and root fresh weights recorded at the indicated day postinoculation (dpi). The upward error bars on top of the columns indicate standard deviation (SD) of the total fresh weight and the downward error bars on the top indicate SD of the shoot fresh weight. The downward error bars within the column represents SD of the root weight. Values that differ significantly from the respective controls (mock inoculation), which were given the significance level 0, are indicated by numbers. Positive and negative numbers represent different levels of significantly increased and decreased fresh weights, respectively. Columns labeled with two numbers belong to both significance levels.

In assays involving *Pseudocercospora herpotrichoides*, in most instances, the pathogen significantly reduced wheat biomass, whereas *Piriformospora indica* significantly increased wheat biomass compared with the respective controls (sand root, $P = 0.0593$; sand shoot, $P = 0.0038$; sand total, $P = 0.0018$; soil root, $P = 0.08$; soil shoot, $P = 0.05$; and soil total, $P = 0.03$) (Fig. 3A).

In variants where *P. indica* was preinoculated, the detrimental effect of the pathogen was not apparent and, in some instances, biomass even increased when compared with the untreated con-

Fig. 3. Effects of *Piriformospora indica* on wheat infected with *Pseudocercospora herpotrichoides*. Plants were grown in sand or soil as the substrata for pot cultures in environmentally controlled growth chambers. Results ($n = 60$ plants/variant) were recorded at 60 days postinoculation. Values that differ significantly from the respective controls (mock inoculation), which were given the significance level 0, are indicated by numbers. Positive and negative numbers represent different levels of significant increases and decreases, respectively. Columns labeled with two numbers belong to both significance levels. **A**, Columns represent the mean of root, shoot, and total fresh weights. The upward error bars on top of the columns indicate standard deviation (SD) of the total fresh weight and the downward error bars on the top indicate SD of the shoot fresh weight. The downward error bars within the column represents SD of the root weight. **B**, Columns show the mean and SD of the percentage of sheath layers at the stem base with brownish symptoms. **C**, Columns show the mean and SD of the average number of sheath layers at the stem base.

trol. Rating of disease symptoms appearing at the stem base indicated that each individual plant treated with *Pseudocercospora herpotrichoides* showed disease symptoms to various degrees. In the absence of *P. indica*, the pathogen produced symptoms in the outer 11% of leaf sheath layers at the stem bases of soil-grown plants and in the outer 13.6% of leaf sheath layers at the stem bases of plants grown on sand (Fig. 3B). Preinoculation with *P. indica* significantly reduced symptoms for both substrata ($P < 0.0001$). Importantly, *P. indica* colonization had an effect on the total number of layers formed (Fig. 3C). Plants that were colonized by the endophyte had significantly more layers than the control but also than the variants where the pathogen was used for challenge inoculation (sand, $P < 0.0001$ and soil, $P = 0.0052$). Plants that were infected by *Pseudocercospora herpotrichoides* only had fewer layers, which was significant for soil.

In assays involving the powdery mildew fungus *B. graminis* f. sp. *tritici*, sand-grown plants generally had more pustules per leaf area than the corresponding plants growing on soil (Fig. 4). For all recordings, the variant that was preinoculated with *P. indica* had fewer mildew pustules than the control. Statistical support for this result was obtained for three of six L2 date-leaf-substrate comparisons and for all six L3 comparisons (sand at 3 dpi: L3, $P < 0.0001$ and L2, $P = 0.0188$; sand at 6 dpi: L3, $P = 0.0295$ and L2, $P = 0.9212$; sand at 9 dpi: L3, $P = 0.1853$ and L2, $P = 0.8952$; soil at 3 dpi: L3, $P < 0.0001$ and L2, $P = 0.4178$; soil at 6 dpi: L3, $P = 0.0041$ and L2, $P = 0.0721$; and soil at 9 dpi: L3, $P = 0.017$ and L2, $P = 0.0445$).

To evaluate putative defense reactions of the host, L3 leaves were harvested between 8 and 72 h postinoculation (hpi) with *B. graminis* and stained with DAB to visualize hydrogen peroxide in situ. We determined the number of DAB-stained epidermal cells per leaf area of plants grown in sand. Preinoculation of the plant with *Piriformospora indica* significantly increased the numbers of stained cells at all recordings in comparison with the controls that only used mildew as the inoculum (8 hpi, $P = 0.0177$; 24 hpi, $P = 0.1113$; 48 hpi, $P = 0.0135$; and 72 hpi, $P = 0.0036$) (Fig. 5). The average sizes of the DAB-stained spots was not significantly different between *P. indica*-treated and control plants (data not shown).

Effects imposed by *P. indica* on wheat grown in the field.

The above experiments established with pot cultures showed that *P. indica* positively affects wheat with respect to biomass and reduction of disease symptoms caused by three fungal pathogens. However, prior to promoting application of *P. indica* in the field, it is essential to assess whether significant effects also are elicited in systems employing conventional agricultural practices. At harvest, we determined grain yields but were unable to record any significant increases mediated by *P. indica* (Fig. 6A). To assess the potential of *P. indica* to produce any effect in the field, we repeated the experiment in the 2003–04 field season at a broader scale. Because the results described above for the pot cultures indicated an effect of the substratum, we set up—in addition to the field in Bad Lauchstädt—a second experiment at a field in Salzmünde that was characterized by poorer soil quality. Furthermore, we increased the amount of mycelial broth of *P. indica* applied as an inoculum at the Salzmünde field. At harvest, we determined straw biomass in addition to grain yield (Fig. 6A). All apparent variation seen for grain yield was again statistically insignificant. However, on the poorer soil of the Salzmünde field, *P. indica* significantly increased straw biomass compared with the untreated control ($P < 0.0001$). Slightly higher straw biomass obtained after a *P. indica* inoculation on the richer soil of the field at Bad Lauchstädt did not prove significant. Fungicide treatment led to significantly higher straw harvests on both soils ($P = 0.0732$).

Symptoms caused by *F. culmorum* and *F. graminearum* were never detected in any of the experiments; therefore, putative

effects exerted by *P. indica* on these pathogens could not be assessed under the prevailing field conditions. All other pathogens occurred in various degrees, depending on the season and the year. None of the symptoms caused by any of the leaf pathogens was reduced significantly by a treatment with *P. indica* when compared with the corresponding controls (data not shown). In contrast, fungicide treatment significantly restricted leaf pathogens in most cases relative to the untreated controls (data not shown). Symptoms caused by *P. herpotrichoides* (i.e., brownish sheath layers at the stem base) did occur in the field and, in most cases, were diminished significantly by *P. indica* (Bad Lauchstädt: 2003, $P = 0.0283$ and 2004, $P = 0.0406$; Salzmünde: 2004, $P = 0.0406$) (Fig. 6B). This was also the case in 2004, when the pathogen was more prevalent than in 2003. The summer of 2003 was exceptionally hot (16,18), a condition that is unfavorable for *P. herpotrichoides* but advantageous for *Piriformospora indica* (Fig. 7A). We determined by microscopy whether and to which extent *P. indica* remained detectable at har-

Fig. 4. Effect of *Piriformospora indica* on pustule formation by *Blumeria graminis* f. sp. *tritici* on wheat. Plants were grown in pot cultures in the greenhouse. Inoculation with *P. indica* was done 7 days prior to inoculation with *B. graminis*. Controls were incubated without receiving an initial *P. indica* inoculum. Columns represent the mean number of pustules and standard deviation ($n = 16$ plants/variant) on L2 and L3 leaves harvested at the indicated days postinoculation (dpi) with powdery mildew conidia; -1 = values that are significantly decreased compared with the respective controls (significance level 0).

Fig. 5. Effect of *Piriformospora indica* on hydrogen peroxide formation by wheat after infection with *Blumeria graminis* f. sp. *tritici*. Plants were grown in pot cultures in the greenhouse. The *P. indica* variants were inoculated 7 days prior to inoculation with *B. graminis*. Controls were not inoculated with *P. indica*. Columns represent the mean and standard deviation ($n = 3$ leaves/variant) of 3,3-diaminobenzidine (DAB)-positive spots counted by microscopy of L3 leaves harvested at the indicated hours postinoculation (hpi) with powdery mildew conidia; 1 = values that differ significantly from the respective controls (significance level 0).

vest and found the endophyte in all 16 plants that were analyzed for each variant that had received a *P. indica* treatment. The extent of fungal colonization did reflect the inoculation level between 600 and 3,000 liters/ha, but not when it exceeded 6,000 liter/ha ($P < 0.0001$) (Fig. 6C).

In vitro interaction of *P. indica* and *Pseudocercospora herpotrichoides*. Because *P. herpotrichoides* was the only pathogen found that was restricted by *Piriformospora indica* under the prevailing field conditions, we examined the interaction between these two fungi in more detail in vitro. First, the fungi were individually grown at temperatures ranging from 5 to 35°C (Fig. 7A). *P. indica* grew much faster than *Pseudocercospora herpotrichoides* at temperatures of 20°C and above. The latter did not grow at 30°C and above, whereas the former continued to grow up to 35°C, where an almost maximal growth rate was observed. At the lower end of the temperature range, *Piriformospora indica* did not grow at 10°C and below. In contrast, *Pseudo-*

cercospora herpotrichoides still grew at 5°C with 46% of the maximal rate. Both fungi grew at the same rate at ≈17°C. Taken together, *Piriformospora indica* has a temperature profile that clearly differs from that of the local pathogen. In another experiment, both fungi were co-inoculated at opposite ends of the agar and their growth was recorded over the same temperature range as described above (Fig. 7A). The growth rates observed for *P. herpotrichoides* were almost unchanged in comparison to the monoculture. However, the growth rates of *Piriformospora indica* were significantly reduced at 20 and 25°C when *Pseudocercospora herpotrichoides* was also present (20°C, $P = 0.0025$ and 25°C, $P = 0.0007$). These temperatures reflected the optimal conditions for the latter. Neither of the fungi overgrew the other. However, at temperatures from 15 to 25°C, we observed, at the contact zone, the formation of an indentation at the growing edge of the mycelium of *Piriformospora indica* (Fig. 7C). At the microscopic level, the

Fig. 6. Effect of *Piriformospora indica* on wheat in the field. Data were recorded at harvest of field experiments conducted at two locations and in two growth periods. Values that differ significantly from the respective controls (significance level 0) are indicated by numbers on top of the columns. Positive and negative numbers represent different levels of significant increases and decreases, respectively. Columns labeled with two numbers belong to both significance levels. **A**, Biomass production. Columns represent the mean and standard deviation (SD) ($n = 4$ plots/variant) of grain and straw dry weights. **B**, Disease symptoms caused by *Pseudocercospora herpotrichoides*. Columns represent the mean and SD ($n = 200$ plants/variant) percentage of sheath layers with brownish symptoms at the stem base. **C**, Extent of root colonization by *Piriformospora indica*. Columns represent the mean and SD ($n = 16$ root segments/variant) of the percentage of root cells stained with chlorazol black E for *P. indica* spores.

Fig. 7. Interaction of *Piriformospora indica* and *Pseudocercospora herpotrichoides* in vitro. **A**, Effect of temperature on fungal growth rates. The fungi were inoculated individually or co-inoculated on opposite sides of a petri dish containing malt extract agar and incubated at the temperatures given at the abscissa. Colonial radii were measured 20 days later. **B**, Branching at hyphal tips of monocultures and co-cultures. Columns represent the mean distance between the hyphal tip at the edge of colonies and the first branch, and standard deviation ($n = 150$ hyphal tips/variant). Values that differ significantly from the respective controls (significance level 0) are indicated by numbers on top of the columns. **C**, Effect of *P. herpotrichoides* on mycelial morphology of *Piriformospora indica* after 24 days of co-culturing at 25°C. **D to K**, Microphotographs from Calcofluor White stained monocultures and co-cultures of **D to G**, *P. indica* and **H to K**, *Pseudocercospora herpotrichoides* showing **D, E, H, and I**, hyphal networks at some distance from the edge of the colony and **F, G, J, and K**, the hyphal tips at the edge of colonies. Bars = 10 μm.

hyphal morphology of *P. indica* was altered when *Pseudocercospora herpotrichoides* grew in close vicinity. The mycelium of *Piriformospora indica*, when grown as a monoculture, formed a regular network (Fig. 7D) with mostly straight hyphae. The hyphal tips appeared straight, with only some branches (Fig. 7F). However, when *P. indica* was co-cultivated with *Pseudocercospora herpotrichoides*, the mycelium of the latter appeared more irregular and exhibited curly hyphae (Fig. 7E). The hyphal tips showed more branches that often remained short (Fig. 7G). In contrast, the morphology of hyphae of *P. herpotrichoides* appeared not to be affected by the presence of *Piriformospora indica* (Fig. 7H and J versus I and K). We measured the distances of the first hyphal branch from the tip and found a significant difference for *P. indica* when the co-culture was compared with the monoculture ($P = 0.0008$) (Fig. 7B). There was no significant difference for *Pseudocercospora herpotrichoides*.

DISCUSSION

Approximately 80% of all terrestrial plants growing in their natural habitats, and also many crop plants, associate with AMF that are well known for providing a range of benefits to their hosts. The technical application of AMF in agriculture has been restricted mainly to organic farming systems. Established interactions can improve nutrition, resistance to soilborne pathogens, and tolerance to drought and heavy metals (6,7). However, the application of fertilizers and biocides, tillage, and monocropping decrease the diversity of AMF and confine potential benefits (3,13,17,19). Other difficulties impeding the application of AMF at a broad scale in the field originate from their obligate biotrophic life style, which complicates the production of inoculum. Furthermore, roots normally are colonized by diverse consortia of AMF, where the contribution of individual species to host vitality is poorly understood and the benefits observed are probably a result of complex ecosystem processes in the mycorrhizosphere (1,4,8,10).

Piriformospora indica holds some promise for practical application because it is simple to propagate in vitro and accessible to basic physiological and genetic research (25). Furthermore, it presumably has an even wider host range than individual AMF species, and the benefits for the host are comparable with that of AMF, although they result from an interaction involving only one fungus and the host. Previously, it had been shown that the basidiomycete *P. indica* exerts several positive effects on colonized host plants when grown in pot cultures (24,26,28). However, it remained uncertain to what extent such results also would be achieved in field experiments that were carried out under regular agricultural conditions. For the work described here, we obtained only a partial confirmation of these earlier results when we treated winter wheat in the field with *P. indica*, which raises doubts as to the broad applicability of *P. indica* for cereal production.

One remarkable feature of *P. indica* is its ability to colonize and benefit a variety of unrelated host plants, which led to the promotion of this endophyte as a putative biofertilizer and biocontrol agent (26,28). Our own pot culture experiments supported this notion because they showed that the interaction of the endophyte with wheat produces a positive effect similar to that reported for other hosts. We were able to prove that *P. indica* colonizes and persists in wheat in a manner comparable with several other plants where this had been tested earlier. The colonization of wheat occurred consistently over the years and conditions tested, even in the field. Additionally, we also observed the reported increase in biomass, but only when a poor sandy substratum was used. The results of the field experiments confirmed this effect of the substratum because significantly enhanced straw production after inoculation with *P. indica* was realized only on the poorer

soils of the Salzmünde plots. However, there were only minor, statistically insignificant increases in grain yields. We cannot rule out the possibility that *P. indica* may significantly improve grain yields if the soil quality used for wheat production is poorer than at the Salzmünde field. In a recent study on the interaction of *P. indica* with barley, Mitscherlich pots were incubated outdoors after the cultures had been established in growth chambers (28). A significant increase in grain yield was observed for one of two cultivars tested. For the moment, it is uncertain whether the poor substrate used in these experiments (i.e., loam/sand, 1:2), the use of another host plant, or the seminatural conditions in the experimental set-up can be correlated with that result. In this context, it should be considered that wheat production in Central Europe is carried out mostly on richer soils. Thus, it is uncertain that the application of *P. indica* under conditions similar to ours may ever lead to a significant increase in grain yields. On the other hand, future research may prove the benefits of this endophyte for subsistence slash-and-burn agricultural systems in the tropics, where nutrient depletion and degradation of soils is rapid and leads to abandonment of fields.

Beyond the effect on biomass, *P. indica* diminished the negative impact of *F. culmorum*, *Pseudocercospora herpotrichoides*, and *Blumeria graminis* f. sp. *tritici* on winter wheat growing in pot cultures. In the field, we observed significantly fewer symptoms of the stem-base pathogen *P. herpotrichoides*. However, there were no significant differences between the controls and the *Piriformospora indica*-treated variants for any of the prevalent leaf pathogens, in any of the years, on any of the fields. Thus, contrasting results were obtained for experiments conducted in the greenhouse and in the field. One might argue that *P. indica* may not have persisted in the host during the duration of the field experiment, but the significant positive effect of *P. indica* on straw production suggests that it must have supported the host actively for a considerable time. In addition, we were able to identify the fungus in all plants examined at the end of the field seasons. Furthermore, *P. indica* treatment caused a significant reduction in disease symptoms for the stem-base pathogen *Pseudocercospora herpotrichoides* in the field, which also suggests that the fungus has successfully established a persisting interaction. Thus, the apparent incapability of *Piriformospora indica* to restrict leaf pathogens in the field must have other reasons. This result was surprising because *P. indica* significantly reduced mildew infections in our greenhouse experiments. In addition, it was reported earlier that preinoculation with *P. indica* induced a systemic response in barley that protected leaves from powdery mildew infections (28). These earlier results have raised hopes that *P. indica* may protect its host even better than AMF that are known to be effective only against many soilborne pathogens but not against leaf pathogens (7). Moreover, heavier disease symptoms often are observed on leaves of plants colonized by AMF. In this respect, under the field conditions tested, *P. indica* did not cause significant beneficial or detrimental effects on the prevalence of leaf pathogens. It remains to be shown whether leaf pathogens will be inhibited significantly in fields where disease pressure is much higher than in this study. It seems that greenhouse experiments can predict only to some degree how far the interaction between the endophyte and the host may affect the impact of pathogens under natural field conditions. This again underlines the necessity of regular field experiments to assess the usefulness of fungal endophytes for crop production.

P. indica reduced symptoms that were caused by *Pseudocercospora herpotrichoides* in the field. To test whether this effect may be linked to the secretion of antifungal proteins or metabolites, we tested sterile-filtered supernatants of liquid cultures from both fungi that grew either alone or as a co-culture. None of the agar-diffusion assays indicated a capability of any fungus to inhibit the other (data not shown). On the other hand, the temperature-dependent growth rates of the two fungi clearly

differ and reflect their geographical origins. *P. herpotrichoides* grows fastest at 20 to 25°C, whereas *Piriformospora indica* does so at 30 to 35°C. When the fungi were confronted directly in vitro on solid media, *Pseudocercospora herpotrichoides* significantly reduced the growth of *Piriformospora indica*, but only at its own optimal temperature range. The pathogen also affected hyphal morphology of *P. indica*. In contrast, there was no obvious effect of the endophyte on the pathogen in the in vitro experiments. These findings may appear contradictory to the field data, where we observed an effect of the endophyte on the disease symptoms caused by the pathogen. It should be noted, however, that there might be no direct contact between the two fungi because *P. indica* colonizes the root, whereas *Pseudocercospora herpotrichoides* infects the stem base. To our knowledge, the question of how likely a direct encounter of these fungi will be in planta remains experimentally untested. Thus, the reduction of the pathogen may be a result, instead, of induced host defense responses related to the phenomena of systemic acquired resistance (28) or priming (2). Our own experiments support this notion because the number of leaf sheath layers at the stem base increased after an inoculation with *Piriformospora indica*, and this seemed to be linked to the reduction of symptoms caused by *Pseudocercospora herpotrichoides*. Induced host defense responses resulting from a preceding inoculation with *Piriformospora indica* also are indicated by increased hydrogen peroxide formation in leaves challenged with powdery mildew. However, under field conditions, this host response may not be effective enough or may not be of sufficient duration to control leaf pathogens.

In conclusion, it is questionable whether *P. indica* may serve as a biofertilizer and a biocontrol agent representing a feasible alternative to current practices in cereal production in Central Europe or comparable regions. *P. indica* is not well suited to grow at lower temperatures and, thus, it might be worthwhile to test whether it may increase grain yields on poor soils receiving no fertilizer, especially in hot climates, where it also may be better able to restrict local pathogens.

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LITERATURE CITED

- Burleigh, S. H., Cavagnaro, T., and Jakobsen, I. 2002. Functional diversity of arbuscular mycorrhizas extends to the expression of plant genes involved in P nutrition. *J. Exp. Bot.* 53:1593-1601.
- Conrath, U., Pieterse, C. M. J., and Mauch-Mani, B. 2002. Priming in plant-pathogen interactions. *Trends Plant Sci.* 7:210-216.
- Daniell, T. J., Husband, R., Fitter, A. H., and Young, J. P. W. 2001. Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. *FEMS Microbiol. Ecol.* 36:203-209.
- Dodd, J. C., Boddington, C. L., Rodriguez, A., Gonzalez-Chavez, C., and Mansur, I. 2000. Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: Form, function and detection. *Plant Soil* 226:131-151.
- Ernst, M., Mendgen, K. W., and Wiersel, S. G. R. 2003. Endophytic fungal mutualists: Seedborne *Stagonospora* spp. enhance reed biomass production in axenic microcosms. *Mol. Plant-Microbe Interact.* 16:580-587.
- Gosling, P., Hodge, A., Goodlass, G., and Bending, G. D. 2006. Arbuscular mycorrhizal fungi and organic farming. *Agric. Ecosyst. Environ.* 113:17-35.
- Harrier, L. A., and Watson, C. A. 2004. The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. *Pest Manage. Sci.* 60:149-157.
- Hart, M. M., and Trevors, J. T. 2005. Microbe management: Application of mycorrhizal fungi in sustainable agriculture. *Front. Ecol. Environ.* 3:533-539.
- Hashiba, T., and Narisawa, K. 2005. The development and endophytic nature of the fungus *Heteroconium chaetospora*. *FEMS Microbiol. Lett.* 252:191-196.
- Helgason, T., Merryweather, J. W., Denison, J., Wilson, P., Young, J. P. W., and Fitter, A. H. 2002. Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. *J. Ecol.* 90:371-384.
- Hess, M., Barralis, G., Bleiholder, H., Buhr, L., Eggers, T., Hack, H., and Stauss, R. 1997. Use of the extended BBCH scale—general for the descriptions of the growth stages of mono- and dicotyledonous weed species. *Weed Res.* 37:433-441.
- James, W. C. 1971. A Manual of Assessment Keys of Plant Diseases. American Phytopathological Society Press, St. Paul, MN.
- Jansa, J., Mozafar, A., Kuhn, G., Anken, T., Ruh, R., Sanders, I. R., and Frossard, E. 2003. Soil tillage affects the community structure of mycorrhizal fungi in maize roots. *Ecol. Appl.* 13:1164-1176.
- Kumari, R., Kishan, H., Bhoon, Y. K., and Varma, A. 2003. Colonization of cruciferous plants by *Piriformospora indica*. *Curr. Sci.* 85:1672-1674.
- Leach, J., Lang, B. R., and Yoder, O. C. 1982. Methods for selection of mutants and in vitro culture of *Cochliobolus heterostrophus*. *J. Gen. Microbiol.* 128:1719-1729.
- Luterbacher, J., Dietrich, D., Xoplaki, E., Grosjean, M., and Wanner, H. 2004. European seasonal and annual temperature variability, trends, and extremes since 1500. *Science* 303:1499-1503.
- Oehl, F., Sieverding, E., Mader, P., Dubois, D., Ineichen, K., Boller, T., and Wiemken, A. 2004. Impact of long-term conventional and organic farming on the diversity of arbuscular mycorrhizal fungi. *Oecologia* 138:574-583.
- Ogi, M., Yamazaki, K., and Tachibana, Y. 2005. The summer northern annular mode and abnormal summer weather in 2003. *Geophys. Res. Lett.* 32:L04706.
- Plenchette, C., Clermont-Dauphin, C., Meynard, J. M., and Fortin, J. A. 2005. Managing arbuscular mycorrhizal fungi in cropping systems. *Can. J. Plant Sci.* 85:31-40.
- Schardl, C. L., Leuchtmann, A., and Spiering, M. J. 2004. Symbioses of grasses with seedborne fungal endophytes. *Annu. Rev. Plant Biol.* 55:315-340.
- Scheffer, F., and Schachtschabel, P. 2002. Lehrbuch der Bodenkunde, 15 ed. Spektrum Akademischer Verlag, Heidelberg, Germany.
- Schüssler, A., Schwarzott, D., and Walker, C. 2001. A new fungal phylum, the Glomeromycota: Phylogeny and evolution. *Mycol. Res.* 105:1413-1421.
- Sherameti, I., Shahollari, B., Venus, Y., Altschmied, L., Varma, A., and Oelmüller, R. 2005. The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. *J. Biol. Chem.* 280:26241-26247.
- Singh, A., Sharma, J., Rexer, K. H., and Varma, A. 2000. Plant productivity determinants beyond minerals, water and light: *Piriformospora indica*—A revolutionary plant growth promoting fungus. *Curr. Sci.* 79:1548-1554.
- Varma, A., Singh, A., Sudha, Sahay, N.S., Sharma, J., Roy, A., Kumari, M., Rana, D., Thakran, S., Deka, D., Bharti, K., Hurek, T., Bleichert, O., Rexer, K. H., Kost, G., Hahn, A., Maier, W., Walter, M., Strack, D., and Kranner, I. 2001. *Piriformospora indica*: An axenically culturable mycorrhiza-like endosymbiotic fungus. In: *The Mycota IX, Fungal Associations*. B. Hock, ed. Springer Verlag, Berlin-Heidelberg.
- Varma, A., Varma, S., Sudha, Sahay, N., Butehorn, B., and Franken, P. 1999. *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. *Appl. Environ. Microbiol.* 65:2741-2744.
- Varma, S., Varma, A., Rexer, K. H., Hassel, A., Kost, G., Sarbhoy, A., Bisen, P., Butehorn, B., and Franken, P. 1998. *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia* 90:896-903.
- Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Huckelhoven, R., Neumann, C., von Wettstein, D., Franken, P., and Kogel, K. H. 2005. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc. Natl. Acad. Sci. USA* 102:13386-13391.