Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*

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The bioprotection performance of *Piriformospora indica* against the root parasite *Fusarium verticillioides* was studied. We found that maize plants first grown with *F. verticillioides* and at day 10 inoculated with *P. indica* showed improvements in biomass, and root length and number as compared with plants grown with *F. verticillioides* alone. To validate our finding that inoculation with *P. indica* suppresses colonization by *F. verticillioides*, we performed PCR analyses using *P. indica* - and *F. verticillioides*-specific primers. Our results showed that inoculation with *P. indica* suppresses further colonization by *F. verticillioides*. We hypothesized that as the colonization by *P. indica* increases, the presence of colonization by *F. verticillioides* decreases. In roots, catalase (CAT), glutathione reductase (GR), glutathione S-transferase (GST) and superoxide dismutase (SOD) activities were found to be higher in *F. verticillioides*-colonized plants than in non-colonized plants. Increased activity of antioxidant enzymes minimizes the chances of oxidative burst (excessive production of reactive oxygen species), and therefore *F. verticillioides* might be protected from the oxidative defence system during colonization. We also observed decreased antioxidant enzyme activities in plants first inoculated with *F. verticillioides* and at day 10 inoculated with *P. indica* as compared with plants inoculated with *F. verticillioides* alone. These decreased antioxidant enzyme activities due to the presence of *P. indica* help the plant to overcome the disease load of *F. verticillioides*. We propose that *P. indica* can be used as a bioprotection agent against the root parasite *F. verticillioides*.

INTRODUCTION

Mycorrhizal associations are among the most widespread, intimate and important symbioses in terrestrial ecosystems. Among these, arbuscular mycorrhiza (AM) represent the most ancient and widespread mycorrhizal symbiosis, and are found in a wide range of land plant species (Harrison, 1997). The successful establishment of this mutualistic association constitutes a strategy to fulfill the nutritional demands of both partners (Kogel et al., 2006). This requires a balance between the defence responses of the host plant and the nutrient demands of the endophyte, resulting in an altered defence-related gene expression, which has been extensively studied during host colonization by obligate biotrophic arbuscular mycorrhizal fungi (AMF). Induction of defence-related genes is most prominent during the early stages of colonization (García-Garrido & Ocampo, 2002), but can also be detected during arbuscule development (Grunwald et al., 2004).

The active resistance of plants to colonization by fungi is often expressed by the hypersensitive reaction (HR) of challenged plant cells, and is characterized by reactive oxygen species (ROS), as well as by induced rapid and localized death of plant tissue at the site of infection (Ingram, 1978; Tenhaken et al., 1995). At the biochemical level, the rapid generation of ROS, such as superoxide (O2•−) and hydrogen peroxide (H2O2), known as the oxidative burst, is the primary defence response against pathogens in the early stages of infection (De Gara et al., 2003). ROS generated upon attack by a pathogen have been proposed to play two different roles: exacerbating the harmful oxidative effect of infection or participating in the defence response by being toxic to the invading pathogen. This contributes to programmed cell death during the HR, driving cell wall reinforcement processes as well as serving as signal molecules for the activation of local and systemic resistance (Álvarez et al., 1998; Corpas et al., 2001; Grant & Loake, 2000; Levine et al., 1994; Mehdy et al., 1996). A
variety of enzyme systems have been proposed to generate ROS in plants. These include a neutrophil-analogous, membrane-bound NADPH oxidase, a lipoxygenase and apoplastic peroxidases (Auh & Murphy, 1995; Bolwell et al., 1995; Croft et al., 1990; Doke, 1983; Doke & Miura, 1995; Levine et al., 1994).

The molecular mechanisms that control the colonization process and AM development are largely unknown. There is evidence showing that differential regulation of the plant defence system could control, at least in part, intraradical fungal growth (Lambais & Mehyd, 1995). Even though AMF can extensively colonize root cortical tissue, the activation of the plant defence responses is limited and transient, and may be restricted to specific cells (Lambais & Mehyd, 1993, 1998; Gianinazzi-Pearson, 1996). The lack of a typical HR in AM suggests that specific mechanisms to attenuate or even suppress plant defence responses may have important roles in controlling AM development. The induction of ROS-scavenging enzymes, such as superoxide dismutase (SOD), peroxidases (POXs) and catalase (CAT), is the most common mechanism for detoxifying ROS synthesized during stress responses (Wojtaszek, 1997; Mittler, 2002). However, the roles of these enzymes in AM are poorly understood. Higher levels of SOD activity are observed in lettuce roots colonized by *Glomus mosseae* or *Glomus deserticola*, compared to non-mycorrhizal controls (Ruiz-Lozano et al., 1996). In tobacco colonized by *G. mosseae*, the transient induction of CAT and ascorbate peroxidase (APX) observed during appressorium formation likely indicates a defence response during the early stages of symbiosis development (Blilou et al., 2000). Induction of CAT has also been observed in nodulated soybean roots colonized by *G. mosseae* (Porcel et al., 2003). The induction of ROS-scavenging enzymes in AM would be an efficient mechanism to attenuate plant defence responses, allowing the AMF to colonize the root cortical tissue. Much information is available regarding the signalling mechanisms that lead to pathogenic interactions between plants and fungi, but little is known about fungus–plant mutualistic symbioses. Recently, Tanaka et al. (2006) have described the role of ROS in regulating the mutualistic interaction between a clavicipitaceous fungal endophyte, *Epichloë festucae*, and its grass host, *Lolium perenne*. They found that plants infected with an *E. festucae* NADPH oxidase (noxA) mutant lose apical dominance, become severely stunted, show precocious senescence, and eventually die. This antagonistic interaction with the host is accompanied by a dramatic increase in endophyte biomass within the plant compared with that in the wild-type. It was proposed that the NoxA isoform plays a specific role in the symbiosis. It was concluded that fungal ROS production is critical in maintaining a mutualistic fungus–plant interaction (Tanaka et al., 2006).

In contrast to AMF, *Piriformospora indica* can be easily cultured axenically, where it asexually forms chlamydospores containing 8–25 nuclei (Verma et al., 1998), *P. indica* shows a broad host spectrum (Varma et al., 1999), including members of the Brassicaceae (such as *Arabidopsis*) which cannot be colonized by AMF. Colonization by *P. indica* is characterized by host cell death and proliferation of the organism in dead cells (Deshmukh et al., 2006; Kogel et al., 2006). This fungus has been found to be involved in promoting the growth of various plants, including cereal crops such as rice, wheat and barley, as well as many Dicotyledoneae (Varma et al., 1999; Peskan-Berghofer et al., 2004). Interaction of the endophytic fungus with *Arabidopsis* roots is accompanied by a considerable requisition of nitrogen from the environment (Peskan-Berghofer et al., 2004). By analysing the interaction of *P. indica* with *Arabidopsis* and tobacco roots it was found that in contrast to mycorrhizal associations, nitrate reduction in the roots is stimulated by *P. indica* (Sherameti et al., 2005). However, recruitment of nitrogen in endophytic interactions differs from that in mycorrhizal interactions, in which the fungus preferentially recruits ammonium rather than nitrate from the soil (Bouckim & Plassard, 2003; Guescini et al., 2003).

In barley, *P. indica* induces resistance to *Fusarium culmorum*, one of the fungal species that causes head blight, as well as systemic resistance to barley powdery mildew *Blumeria graminis* via an unknown mechanism. It has been concluded that this is because of the high antioxidative capacity of plants, which is brought about by fungal colonization (Waller et al., 2005). Additionally, it has been reported that *P. indica* protects barley plants from abiotic stresses such as high salt concentrations (Waller et al., 2005). They found enhanced GR activity in leaves of barley plants colonized with *P. indica*. However, their study did not examine the involvement of other antioxidant enzymes such as CAT, GST and SOD during colonization and establishment of *P. indica*. The present study focuses on the interaction of *P. indica* with maize plants, its role in bioprotection against the root pathogenic fungus *Fusarium verticillioides*, and the activities of various antioxidant enzymes.

**METHODS**

**Plant and fungal culture and growth conditions.** Seeds of maize (*Zea mays* L. Bio 9681) were surface-sterilized for 2 min in ethanol followed by 10 min in a NaClO solution (0.75 % Cl), and finally washed six times with sterile water (Varma et al., 1999). Additionally, seeds were treated with distilled H2O a t6 0 C for 5 min to eliminate naturally occurring *Fusarium* that may have been in or on the corn seed, as described elsewhere (Daniels, 1983). Seeds were germinated on water-agar plates (0.8 % Bacto Agar, Difco) at 25 °C in the dark (Varma et al., 1999). Seedlings were placed in pots (9 cm height by 10 cm diameter) containing expanded clay (2–4 mm diameter). *P. indica* and *F. verticillioides* were cultured on Aspergillus minimal media for 8 days (Hill & Kafer, 2001). Initially, the plants were inoculated with either *P. indica* or *F. verticillioides* by directly mixing the culture in sterile soil and then allowing one set of plants to grow without any fungus for 10 days. For this purpose, plants were grown with 10 g wet fungal mycelium (of either fungus) that was first mixed with 100 ml Hoagland’s solution (Arnon & Hoagland, 1940), and to this 1000 g of sterile soil was added, while in case of control plants, only Hoagland’s solution was added to the same amount of soil and
no mycelium was added. Maize plants were grown in a greenhouse in a controlled environment (30 ± 2 °C, 70 % relative humidity, 16 h photoperiod). They were watered twice and fertilized with Hoagland’s solution once a week. Plants were harvested at different time periods after inoculation with fungi, carefully washed under running tap water, rinsed in deionized autoclaved water and weighed. Root samples were stored in water for 1 h to study colonization; however, for enzyme assays, root tissues were stored in liquid N2.

**Histochemical analysis.** To study colonization, 10 root samples were selected randomly from the maize root. Samples were softened in 10 % KOH solution for 15 min, acidified with 1 M HCl for 10 min, and finally stained with 0.2 % Trypan blue overnight (Dickson *et al*., 1998; Phillips & Hayman, 1970). Samples were destained with 50 % lacto-phenol for 1–2 h prior to observation under a light microscope (Leica type 020-518.500). The distribution of chlamydospores within the root was taken as an index of colonization (Varma *et al*., 1999). The percentage colonization was calculated for the inoculated plants according to a published method (McGonigle *et al*., 1990).

**Role of* P. indica* in bioprotection.** In order to study bioprotection by *P. indica* against the root parasite *F. verticillioides*, all plants were initially grown for 10 days, and subsequently the following sets of plants were used: (1) maize plants grown for 45 days without any fungus (control); (2) maize plants inoculated with *P. indica* alone at day 0 and grown for 45 days; (3) maize plants inoculated with *F. verticillioides* alone at day 0 and grown for 45 days; (4) maize plants first inoculated with *F. verticillioides* at day 0 and at day 10 inoculated with *P. indica* and grown for a total of 45 days; (5) maize plants first inoculated with *P. indica* at day 0 and at day 10 inoculated with *F. verticillioides* and grown for a total of 45 days; (6) maize plants grown simultaneously with both fungi inoculated at day 0 and grown for 45 days. Delayed, alternate and simultaneous inoculation of both fungi were chosen to see the effect of colonization with *P. indica* on bioprotection, i.e. whether there was an effect on the recovery of biomass, and whether there was any change in the number and length of roots, or in the morphological appearance of plants infected with *F. verticillioides*. The growth-promoting effect of the fungus was checked by measuring the dry weight, for which plant materials were treated after harvesting at 100 °C for 72 h in a hot air oven. To demonstrate the antibiotic activity of *P. indica* and *F. verticillioides* to one other, both the fungi were inoculated on the same plate, leaving some distance between the two inocula to allow them to grow without interference from one another. The growth pattern at the hyphal periphery of an individual fungus approaching the hyphal periphery of another was taken as a measure of the antibiotic activity of a particular fungus.

**PCR analyses.** To check the role of *P. indica* in bioprotection against the root pathogen fungus *F. verticillioides*, the following sets of plants were used: (1) maize plants inoculated with *P. indica* alone; (2) plants inoculated with *F. verticillioides* alone; (3) plants inoculated first with *F. verticillioides* and later (at day 10) with *P. indica*. After 5, 15 and 35 days of inoculation, 10 plants were sampled from each set and examined for the presence of *P. indica* and *F. verticillioides* within the root tissues. For PCR comparisons, 0.15 g root tissue of each sample was used to isolate total genomic DNA by the cetyltrimethylammonium bromide (CTAB) method (Bousquet *et al*., 1990). PCR products were carried out in a final volume of 50 µl, containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.01 % (w/v) gelatin, 200 µM dNTPs, 3 µM of each primer, 1 U Taq DNA polymerase and 1 µg genomic DNA as template. Portions of the EF-1-alpha (tef) gene (AJ249912) of *P. indica*, the beta-tubulin (tub2) gene (U27303) of the actin gene (*Jo1238*) of the maize plant were amplified using the following primer pairs: for *P. indica*, PitEFOR (5′-TCGTCCTCTGTCACAAAGATG-3′) and PitERREV (5′-GAGGG-CTCGAGCATGTGT-3′); for *F. verticillioides*, Fvtub-F (5′-GTGCCT-TACCTGACCTGCTCA-3′) and Fvtub-R (5′-GACATCGTAAGTCCT-TCGGG-3′); and for maize, ZmAminF (5′-GTTGCAATGGCCT-GAAATG-3′) and ZmAminR (5′-GACCTGACATCGAGATCT-3′). Reactions were performed in a thermal cycler (Eppendorf) set to the following reaction conditions: denaturation at 94 °C for 5 min, one cycle; for 35 cycles, denaturation at 94 °C for 40 s, annealing at 59 °C, 62 °C and 53 °C for 40 s for *tef*, *tub2* and the actin gene, respectively; and extension at 72 °C for 30 s. Tubes without the DNA template were included in each experiment as a negative control. With a view to using the PCR assay to detect these fungi in maize tissues, primers and total genomic DNA extracted from healthy maize roots were tested to avoid false-positive results by cross-reaction with plant DNA. No samples generated amplified fragments in any experiment for *tef* and *tub2*. Finally, analysis of total genomic DNA extracted from pure cultures of each fungus generated the expected fragments for each of the primer sets tested.

**Antioxidant enzyme activities in maize plants.** In order to study how *P. indica* protects itself from the oxidative defence systems of the plant during colonization, and to determine the impact of colonization with *P. indica* on the antioxidative systems of the plant, antioxidative enzyme activities were checked in the presence and absence of *F. verticillioides*, as well as after delayed inoculation. For this purpose, all experiments and conditions were kept the same as described above. For protein isolation, frozen root and shoot tissues were homogenized at 4 °C in an ice-chilled mortar with liquid N2 in QB buffer (100 mM potassium phosphate buffer, pH 7.8, 1 mM EDTA, 1 % Triton X-100, 15 % glycerol) (Ni *et al*.,1996) (containing no DTT) (for the SOD, CAT and GST assays) with 50 mg polyvinylpyrrolidone (PVP) per gram of tissue (for the GR assay). Crude homogenates were centrifuged at 15 000 g for 15 min at 4 °C, and the supernatant fractions were frozen at −20 °C. Protein content was determined by the Bradford method using BSA as standard (Bradford, 1976).

**SOD assay.** SOD activity was monitored according to a published method (Roth & Gilbert, 1984). One millilitre of reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 100 µM EDTA, 20 µl enzyme extract and 10 mM pyrogallol. The enzyme activity [U (mg protein)⁻¹] was calculated by monitoring the reaction mixture for 120 s (at 60 s intervals) at 420 nm in a spectrophotometer.

**CAT assay.** CAT activity was assayed by measuring the initial rate of H2O2 disappearance using the method of Beers & Sizer (1952). One millilitre of catalase assay reaction mixture contained 0.05 mM sodium phosphate buffer (pH 7.0), 20 µl enzyme extract and 1 mM H2O2. The decrease in H2O2 was followed by a decline in H2O2. A decrease in H2O2 was followed by a decline in H2O2. A decrease in A440 and the activity [U (mg protein)⁻¹] was calculated using a molar absorption coefficient of 40 mM⁻¹ cm⁻¹ for H2O2.

**GST assay.** For the measurement of GST activity, 1 ml reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.5), 20 µl enzyme extract and 2 % 1-chloro-2,4-dinitrobenzene (CDNB). The enzyme activity [U (mg protein)⁻¹] was calculated by monitoring the reaction mixture for 180 s (60 s intervals) at 340 nm in a spectrophotometer, as described elsewhere (Habig *et al*., 1974).

**GR assay.** The activity [U (mg protein)⁻¹] was determined by the oxidation of NADPH at 340 nm with a molar absorption coefficient of 6.2 mM⁻¹ cm⁻¹, as described elsewhere (Nordhoff *et al*., 1993). The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM glutathione (oxidized form, GSSG) and 10 µl enzyme extract (total reaction mixture 1 ml). The reaction was initiated by the addition of NADPH at 25 °C.
Statistical analysis. All graphs were created and statistical calculations performed using Microsoft Excel. The significance of the data obtained was checked with Student’s t test using the program SigmaStat 2.0 (Jandel Corporation).

RESULTS

Interaction of *P. indica* with maize plants and its role in bioprotection against the pathogenic fungus *F. verticillioides*

We observed that *P. indica* colonization in plants is a time-dependent process. We found 20–30% colonization at 10 days, when 1 g fresh culture of *P. indica* was inoculated per pot. We observed a further increase in colonization up to 70% at 20 days (Table 1). Fungal colonization was characterized by intracellular pear-shaped chlamydospores (Fig. 1). We observed distinctive morphological changes in plant roots colonized with *P. indica* as compared with non-colonized (control) plant roots (Fig. 2a). Plants colonized with *F. verticillioides* showed poor root growth as compared with non-colonized plants (Fig. 2a, b, c). We observed an increase in root length in plants colonized with *P. indica* as compared with non-colonized maize plants (*P*<0.05) (Fig. 2b, c). A similar morphological root pattern was also observed in the case of plants first colonized with *F. verticillioides* and at day 10 inoculated with *P. indica* as well as in plants in which simultaneous inoculation with both fungi was carried out (Fig. 2a, b, c).

In the case of dry weight analysis, we observed a significant increase, i.e. 1.8-fold, in 45-day-old maize plants colonized with *P. indica* as compared with non-colonized maize plants (*P*<0.05) (Fig. 3). Plants colonized with *F. verticillioides* showed a 0.36-fold decrease in the dry weight of 45-day-old maize plants (*P*<0.05) as compared with control plants (non-colonized). Inoculation with *P. indica* at day 10 of plants previously colonized with *F. verticillioides* resulted in improved biomass yield, i.e. a 1.8-fold increase in dry weight as compared with plants inoculated with *F. verticillioides* alone (Fig. 3) (*P*<0.05). Maize plants inoculated simultaneously with *P. indica* and *F. verticillioides* at day 0 showed a 1.4-fold increase in dry weight in comparison with controls (plants with no fungus) (Fig. 3). Overall, no significant harmful effects of *F. verticillioides* (on overall plant health, root and shoot systems) were observed in plants previously colonized with *P. indica* and later inoculated with *F. verticillioides* (Figs 2 and 3).

We observed a gradual increase in the band intensity in *P. indica*-inoculated plants during the course of the *tef* gene experiment at 5, 15 and 35 days (Fig. 4a). Similar results were also observed for the *tub2* gene in the case of plants inoculated with *F. verticillioides* alone (Fig. 4b). We also observed an increase in the band intensity for the *tef* gene up to 35 days for plants first inoculated with *F. verticillioides* and later inoculated with *P. indica* (Fig. 4c); however, a gradual decrease was observed in the band intensity of the *tub2* gene in the same samples, and at the end of 35 days a very faint band was observed (Fig. 4d).

Antibiosis assay of *P. indica* and *F. verticillioides*

To check whether the increase in resistance to *F. verticillioides* is mediated by any antibiotics secreted by *P. indica*, an antibiosis assay was performed. We did not observe any inhibition of the growth of the two fungi when grown together, which suggests that *P. indica* does not secrete any antibiotics effective against *F. verticillioides* and vice versa (Fig. 5).

Antioxidant enzyme activities

In the case of roots, it was observed that maize plants colonized with *F. verticillioides* showed a 43-fold increased CAT activity as compared with non-colonized plants, which was statistically significant (*P*<0.05) (Fig. 6a). Under similar conditions, we found increased activities, i.e. 3.2-, 10- and fourfold, for GR, GST and SOD, respectively. Colonization was calculated by the presence of a chlamydospore in selected at random and was divided into 10 pieces. The percentage colonization was 9 ± 6.4% at 5 days, 26 ± 9.1% at 10 days and 70 ± 10.5% at 20 days (Table 1). The significance of the data obtained was checked with Student’s t test using the program SigmaStat 2.0 (Jandel Corporation).

Table 1. Colonization by *P. indica* of maize roots

<table>
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<tr>
<th>Time after inoculation (days)</th>
<th>Percentage colonization*</th>
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<tr>
<td>5</td>
<td>9 ± 6.4</td>
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<tr>
<td>10</td>
<td>26 ± 9.1</td>
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<td>20</td>
<td>70 ± 10.5</td>
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*n* = 10 plants and 100 root pieces. From each plant, one root was selected at random and was divided into 10 pieces. The percentage colonization was calculated by the presence of a chlamydospore in each piece.

Fig. 1. Trypan blue staining of maize plant roots showing intracellular *P. indica* chlamydospores (black arrow) observed at day 10. Bar, 20 μm.

Antioxidant enzyme activities

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respectively; however, only GST activity was found to be significant \((P<0.05)\) \(\text{(Fig. 6b, c, d)}\). In the case of plants colonized with \(P. \text{indica}\), a 23-fold increased activity was found for CAT as compared to non-colonized plants. Under similar conditions, GST and SOD activities were found to be significantly increased by 3.8- and 1.7-fold; however, no significantly increased activity was observed for GR \(\text{(Fig. 6b, c, d)}\). Inoculation with \(P. \text{indica}\) of plants previously colonized with \(F. \text{verticillioides}\) resulted in a sixfold decrease in CAT activity, which was found to be significant \((P<0.05)\) \(\text{(Fig. 6a)}\). However, we did not find a significant difference in CAT activities of plants previously colonized with \(P. \text{indica}\) and later inoculated with \(F. \text{verticillioides}\) and plants colonized with \(P. \text{indica}\) alone. In

**Fig. 2.** (a) Plant shoot and root phenotype demonstrating the bioprotection role of \(P. \text{indica}\) against \(F. \text{verticillioides}\). (C) Maize plants grown for 45 days without any fungus were used as a control; (P) maize plants inoculated with \(P. \text{indica}\) alone at day 0 and grown for 45 days; (F) maize plants inoculated with \(F. \text{verticillioides}\) alone at day 0 and grown for 45 days; (F→P) maize plants first inoculated with \(F. \text{verticillioides}\) at day 0 and at day 10 inoculated with \(P. \text{indica}\) and grown for a total of 45 days; (P→F) maize plants first inoculated with \(P. \text{indica}\) at day 0 and at day 10 inoculated with \(F. \text{verticillioides}\) and grown for a total of 45 days; (P+F) maize plants inoculated simultaneously with both fungi at day 0 and grown for 45 days. Damaged root branches in plants infected with \(F. \text{verticillioides}\) are shown by black arrows, and root branches in plants inoculated with \(P. \text{indica}\) are shown by blue arrows \(\text{(lower panels)}\). (b, c) Change in length of crown roots \(\text{(b)}\) and number of secondary roots \(\text{(c)}\).
In another case, in which simultaneous and alternate inoculation of both fungi was done, plants showed SOD activities almost identical to those of plants colonized with *F. verticillioides* alone (Fig. 6d).

In the case of shoots, plants colonized with *F. verticillioides* did not show a significant increase in CAT activity (Fig. 7a), but GR, GST and SOD activities were found to be increased by 2.5-, nine- and eightfold, respectively, and were found to be significantly increased ($P<0.05$) as compared with non-colonized plants (Fig. 7b, c, d). However, plants colonized with *P. indica* showed a 44-fold increased CAT activity as compared with non-colonized plants, and this was found to be significant ($P<0.05$) (Fig. 7a). Similarly, GR, GST and SOD activities were found to be nine-, 92- and 48-fold increased, respectively (Fig. 7b, c, d). We did not observe any significant increase in CAT and GR activities of plants colonized with both *P. indica* and *F. verticillioides* simultaneously as compared with non-colonized plants (Fig. 7a, b). However, in the case of GST and SOD, we found a significant increase in the activities under the same conditions (Fig. 7c, d). In the case of alternate inoculation (when plants were first inoculated with *Fusarium* and after 10 days with *P. indica*), we found increased activities of CAT, GST and SOD by 23-, nine- and 32-fold, respectively, as compared with control plants, which was significant in all three cases ($P<0.05$) (Fig. 7a, c, d). However, we did not observe any significant change in the GR activity under similar conditions as compared with the control (Fig. 7b). In another condition (when plants first colonized with *P. indica* were inoculated with *Fusarium* after 10 days), we found that CAT, GR, GST and SOD activities were increased significantly as compared with control plants ($P<0.05$), and a maximum 21-fold increased activity was found in the case of SOD (Fig. 7a, b, c, d).

**DISCUSSION**

Almost all terrestrial plants growing in their natural habitats, except limited numbers of plants belonging to the families Amaranthaceae, Chenopodiaceae, Cyperaceae, Juncaceae and Proteaceae, as well as lupins and Cruciferae, are associated with AMF, which are well known for providing a range of benefits to their hosts. Established interactions improve nutrition, resistance to soil-borne...
pathogens, and tolerance to drought and heavy metals (Gosling et al., 2006; Harrier & Watson, 2004). However, the application of fertilizers and biocides, tillage and monocropping decrease the diversity of AMF and reduce potential benefits (Daniell et al., 2001; Oehl et al., 2004; Plenchette et al., 2005). Another difficulty that impedes the application of AMF on a wider scale in the field is that they cannot be cultivated axenically, which complicates the production of inocula.

P. indica holds some promise for practical application because it is simple to propagate in vitro and is accessible to basic physiological and genetic research (Varma et al., 2001). Furthermore, it presumably has a wider host range than most other AM species, and the benefits for the host are comparable with those of AMF, although they result from an interaction involving only one fungus and the host. One remarkable feature of P. indica is its ability to colonize and benefit a variety of unrelated host plants, and this has led to the promotion of this endophyte as a putative biofertilizer and biocontrol agent (Varma et al., 1999; Waller et al., 2005).

In the present study we have studied the role of P. indica in growth-promoting activities, i.e. biomass yield and root integrity of maize plants, as well as its role in bioprotection against the root pathogen fungus F. verticillioides, which is a major parasite of members of the family Gramineae. It causes stalk rot in maize (Christensen & Wilcoxson, 1966), leading to poor stand, reduced root and shoot weight, as well as reduced plant growth and emergence of maize seedlings (Futrell & Kilgore, 1969; Scott & Futrell, 1970). In this study we found that P. indica-colonized plants showed an increase in biomass production as compared with non-colonized plants. This increase in biomass indicates the mycorrhiza-like growth-promoting activity of P. indica. Similar results have also been found by Waller et al. (2005) in the interaction between P. indica and barley plants, which supports our data. Although very little is known of the biochemical mechanisms involved in this association, we hypothesize that the increased plant growth-associated activities in the present study due to P. indica colonization may be attributed to enhanced nutrient uptake (especially of phosphorus and nitrogen), as observed in the case of mycorrhizal associations (Toro et al., 1998; Requena et al., 2001; Sherameti et al., 2005). However, in the present work, we have not studied this, although it warrants investigation.

The colonization with P. indica of roots of maize plants significantly lowers the susceptibility of the latter to F. verticillioides infections as compared with non-colonized plants. The effects of alternate and simultaneous inocu-

Fig. 6. Effect of inoculation of P. indica and F. verticillioides on antioxidant enzyme activities in maize roots. (a) CAT, (b) GR, (c) GST and (d) SOD specific activities compared with those of control plants (C); asterisks show values significantly different from those of the controls (P<0.05). All experimental conditions were the same as described for Fig. 2.
lation with *P. indica* and *F. verticillioides* on the biomass and susceptibility of maize plants were similar, and we found improvements in plant growth, biomass and root integrity. Waller *et al.* (2005) have shown the role of *P. indica* against the leaf parasite *B. graminis*. In the present work we also observed an interesting property of the bioprotective nature of *P. indica* when plants were first inoculated with *F. verticillioides* and at day 10 inoculated with *P. indica*. Our data showed that the presence of the root parasite does not affect plant growth, biomass and root integrity in the presence of *P. indica*. Our data lead us to a conclusion about the role of *P. indica* in biotic tolerance and disease resistance. To validate our results with respect to whether inoculation with *P. indica* suppresses colonization by *F. verticillioides*, we performed PCR analyses using *P. indica*- and *F. verticillioides*-specific primers. Our results showed that inoculation with *P. indica* of plants previously inoculated with *F. verticillioides* suppresses further colonization with *F. verticillioides*, as a very faint band was observed for *F. verticillioides* tub2 at the end of 35 days. On the other hand, an increased band intensity was observed for *P. indica* tef at the end of 35 days. We hypothesize that as colonization by *P. indica* increases, the presence of colonization by *F. verticillioides* decreases. Further plants first inoculated with *F. verticillioides* and at day 10 inoculated with *P. indica* showed an improvement in the total biomass as well as changes to root and shoot morphology. This suggests that *P. indica* helps the plants to resist *F. verticillioides* and hence to recover biomass as well as overall health.

We performed antibiosis assays for the fungi, which revealed that there is no secretion of antibiotics by *P. indica* that inhibit the growth of *F. verticillioides*, suggesting that the biotic tolerance is due to the reprogramming and induction of the resistance of the plant by *P. indica* and is not the result of antibiotic secretion. Similar findings have also been observed by Waller *et al.* (2005) in the case of *P. indica* and its interaction with barley plants, and this supports our findings. However, in a recent study conducted by Serfling *et al.* (2007) to determine the activity of *P. indica* against another pathogenic fungus *B. graminis* under field conditions, it was shown that the symptoms caused by the leaf pathogen did not differ in *P. indica*-colonized plants in comparison with non-colonized plants. However, in another experiment using another pathogenic fungus, *Pseudocercosporella*...
In this work, we observed antioxidant enzyme activity in roots and shoots of maize plants colonized with *P. indica* and *F. verticillioides*. We observed enhanced CAT activity in maize roots colonized with *P. indica*. We observed that plants inoculated with *F. verticillioides* alone showed higher (43-fold) CAT activities than control plants. Goyal *et al.* (1986) observed that water stress results in a 77-fold increase in the activity of CAT in *Rhizobium trifolii*. Induction of CAT activity was associated with higher growth rates of the most infective fungus, suggesting that CAT plays an important role in the regulation of intraradical fungal growth. The scavenging of *H*₂*O*₂ by CAT may be an efficient mechanism to attenuate the elicitation of plant defence responses (Wu *et al.*, 1997), facilitating intraradical fungal growth and differentiation. Significant induction of CAT activity has also been observed in the compatible interaction between *Hordeum vulgare* and *B. graminis Alg-S* (Vanacker *et al.*, 1998). It has been suggested that the induction of NADPH oxidases and the suppression of CAT and APX activities in plants under biotic stress is essential for the induction of programmed cell death (Mittler, 2002). We observed the same pattern of induction of GR, GST and SOD activities in plant roots colonized with *P. indica* and *F. verticillioides* as in the case of CAT activity. In an earlier report, induction of GR activity was observed during the *P. indica*-barley plant interaction, and it was suggested that the higher GR activity maintains an enhanced level of reduced glutathione which is involved in maintaining antioxidant capacity (Waller *et al.*, 2005). In another study, GST was found to be induced during the infection of *Nicotiana benthamiana* with *Colletotrichum orbiculare* (Dean *et al.*, 2005). Those authors further proposed that the most likely role for GSTs in pathogen-infected plants was to suppress necrosis by detoxifying lipid hydroperoxides produced by peroxidation of membranes. We also observed significant induction of SOD activity throughout the experimental period in roots. Our data suggest that induction of SOD in this interaction might be associated with recognition of *P. indica* and activation of the plant defence system. It has been reported that unusually strong induction of antioxidative enzymes during the colonization period results in detoxification of ROS (generated during colonization) and plays a protective role in the interaction between plants and fungi (Alguacil *et al.*, 2003). Increased activity of antioxidant enzymes minimizes the chances of oxidative burst (excessive ROS production), and therefore *P. indica* and *F. verticillioides* might be protected from the oxidative defence system during colonization. Induction of antioxidative enzymes was also observed in shoots of plants colonized with *P. indica* and *F. verticillioides*, but the induction was higher in plants inoculated with *P. indica* than in plants inoculated with *F. verticillioides*. These data suggest the systemic induction of antioxidative defences in the case of colonization by *P. indica*.

The activities of antioxidant enzymes did not vary with alternate and simultaneous inoculation of *P. indica* and *F. verticillioides*. In the present investigation, we found suppressed activities of CAT, GR and GST in roots after alternate and simultaneous inoculation of *P. indica* and *F. verticillioides*; however, induced SOD activity was observed in all three cases of alternate and simultaneous inoculation. Suppression of CAT, GR and GST activities and induction of SOD activity may induce the accumulation of a higher level of *H*₂*O*₂. A higher concentration of *H*₂*O*₂ has also been observed in *P. indica*-colonized wheat plants after infection with *B. graminis f. sp. tritici* (Serfling *et al.*, 2007), which supports our hypothesis of accumulation of *H*₂*O*₂ in maize plants interacting with both *P. indica* and *F. verticillioides*. The action of *H*₂*O*₂ itself and induced defence responses possibly due to elevated *H*₂*O*₂ may reduce infection by and proliferation of *F. verticillioides*, and this may be an explanation of why inoculation of plants with *P. indica* reduces the effects of *F. verticillioides* so that the plant recovers its biomass and normal root proliferation. These observations suggest a slight alteration in the level of antioxidant enzymes, which probably favours the plant–fungal association and increases resistance to the pathogen. Here, we have provided additional information on the mechanism for biotic tolerance and systemic resistance conferred by *P. indica* as well as the antioxidative system, as also proposed by Waller *et al.* (2005).

The colonization of maize plants by *P. indica* leads to increased growth (due to its growth-promoting abilities), systemic resistance to biotic stress and enhanced antioxidant capacity. We show here that *P. indica*-colonized maize plants are more resistant to biotic stress and that the reprogrammed metabolic state, which includes an enhanced antioxidant capacity, does not negatively affect biomass yield. Because *P. indica*, unlike AMF, can easily be propagated on a large scale in axenic culture in the absence of a host plant (Varma *et al.*, 1999), we suggest the consideration of this endophyte as a tool for sustainable agriculture. Exploitation of *P. indica* may not only complement crop-growing strategies but also serve as a model system to study molecular traits that affect disease resistance and grain yields in cereals.

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