The Mutualistic Fungus *Piriformospora indica* Protects Barley Roots from a Loss of Antioxidant Capacity Caused by the Necrotrophic Pathogen *Fusarium culmorum*

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Fusarium culmorum causes root rot in barley (*Hordeum vulgare*), resulting in severely reduced plant growth and yield. Pretreatment of roots with chlamydospores of the mutualistic root-colonizing basidiomycete *Piriformospora indica* (subdivision Agaricomycotina) prevented necrotization of root tissues and plant growth retardation commonly associated with *Fusarium* root rot. Quantification of *Fusarium* infections with a real-time polymerase chain reaction assay revealed a correlation between root rot symptoms and the relative amount of fungal DNA. *Fusarium*-infected roots showed reduced levels of ascorbate and glutathione (GSH), along with reduced activities of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, GSH reductase, dehydroascorbate reductase, and monodehydroascorbate reductase. Consistent with this, *Fusarium*-infected roots showed elevated levels of lipid hydroperoxides and decreased ratios of reduced to oxidized forms of ascorbate and GSH. In clear contrast, roots treated with *P. indica* prior to inoculation with *F. culmorum* showed levels of ascorbate and GSH that were similar to controls. Likewise, lipid peroxidation and the overall reduction in antioxidant enzyme activities were largely attenuated by *P. indica* in roots challenged by *F. culmorum*. These results suggest that *P. indica* protects roots from necrotrophic pathogens, at least partly, through activating the plant's antioxidant capacity.

Piriformospora indica is a root-colonizing basidiomycete fungus that increases plant growth of a wide range of crop species (Peškan-Berghöfer et al. 2004; Qiang et al. 2012; Weiss et al. 2011). The fungus is known to reproduce asexually by generating thick-walled chlamydospores, and in clear contrast to arbuscular mycorrhiza, can be cultured on synthetic media (Varma et al. 1999; Verma et al. 1998). Plants colonized with *P. indica* exhibit enhanced tolerance against abiotic stress and resistance to microbial pathogens. Several studies have demonstrated that *P. indica* confers salt and drought tolerance to host plants, but the underlying mechanism is not fully elucidated (Baltruschat et al. 2008; Cruz et al. 2010; Franken 2012; Sherameti et al. 2008; Sun et al. 2010; Waller et al. 2005; Zarea et al. 2012).

An important feature of plant responses to environmental stresses is that the balance between production and scavenging of reactive oxygen species (ROS) is shifted towards higher ROS levels (Apel and Hirt 2004). Excess ROS subsequently induce lipid peroxidation of cell membranes and damage to proteins and nucleic acids. Growing evidence suggests that endophytic fungi enhance tolerance of host plants to abiotic stress by altering their antioxidant activity (Hamilton and Bauerle 2012; Rodriguez et al. 2008). Consistent with this, colonization by *P. indica* prevents salt- and drought-induced lipid peroxidation in barley and Chinese cabbage roots, respectively (Baltruschat et al. 2008; Sun et al. 2010). This beneficial effect is associated with significant changes in plant redox metabolism and accumulation of high levels of ascorbate due to increased activities in key antioxidant enzymes, such as dehydroascorbate reductase (DHAR, EC 1.8.5.1) and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) (Baltruschat et al. 2008; Waller et al. 2005). Moreover, upregulation of DHAR and MDHAR in *Arabidopsis thaliana* is essential for a mutualistic association with *P. indica* (Vadassery et al. 2009). In addition to its role in abiotic stress tolerance, *P. indica* confers resistance to a range of microbial pathogens in various crop plants, including barley, lentil, maize, tomato, and wheat (Deshmukh and Kogel 2007; Dolatabadi et al. 2012; Fakhro et al. 2010; Kumar et al. 2009; Serfling et al. 2007; Waller et al. 2005). Most of these studies have been focused on soil-borne diseases such as *Fusarium* root rot of cereals. It is well-established that *F. culmorum* utilizes production of ROS to accelerate cell death and facilitate subsequent infection (Cuzick et al. 2009). Accordingly, an increase in oxidative stress was observed in barley and wheat seedlings affected by *Fusarium* head blight and root rot (Boddu et al. 2006; Desmond et al. 2008; Khoshgofarmanesh et al. 2010).

We show here that *P. indica* counteracts root infections by the necrotrophic pathogen *F. culmorum* and that this beneficial effect is associated with altered antioxidant activity of root cells suited to detoxify pathogen-induced excess ROS.

RESULTS

Quantification of *F. culmorum* in barley roots.

Consistent with earlier studies, three-week-old *P. indica*-colonized barley showed enhanced shoot and root biomass (Fig. 1) (Deshmukh and Kogel 2007; Waller et al. 2005). In contrast, shoot and root biomass was strongly reduced by *Fusarium culmorum* infection within two weeks of inoculation.

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However, when *P. indica*-colonized plants were challenge-inoculated with *F. culmorum*, neither severe root rot symptoms nor growth retardation was observed (Fig. 1).

The ratio of *F. culmorum* DNA to plant DNA was calculated to monitor fungal root infection, by quantitative real-time polymerase chain reaction (qPCR) using primers specific for the fungal *Tri12* gene of the trichothecene pathway and for the translation elongation factor1 α (*EF1 α*) gene from barley. The qPCR analysis confirmed that roots were extensively colonized with *F. culmorum* two weeks after inoculation (Fig. 2). In contrast, preinoculation with *P. indica* resulted in reduced colonization of roots by *F. culmorum*, which is consistent with less root rot-symptom expression and a reduced loss of biomass. No amplification product of the *F. culmorum*-specific *Tri12* gene was observed when template DNA was extracted either from uninoculated or *P. indica*-colonized roots.

***P. indica* protects *Fusarium*-infected roots from a loss of ascorbate and glutathione (GSH).**

We assessed the antioxidant status of barley roots that were colonized either by *P. indica*, *F. culmorum*, or a combination of these fungi. Colonization by *P. indica* resulted in a 2.5-fold increase in ascorbate level and a 70% increase in the ratio of reduced to oxidized ascorbate (dehydroascorbate, DHA) in 3-week-old plants compared with the controls (Fig. 3). In contrast, inoculation of roots with *F. culmorum* caused a 70% reduction in root ascorbate after two weeks, although it did not result in a significant accumulation of DHA. Accordingly, the ratio of ascorbate to DHA decreased by about 70% in *Fusarium*-infected roots. However, when roots were inoculated with *P. indica* one week prior to *F. culmorum*, root ascorbate and DHA levels were similar to that in control plants (Fig. 3).

To extend this analysis, we measured the concentration of reduced GSH in infected and uninfected barley roots. Three weeks after inoculation with *P. indica*, the level of GSH was

slightly, but not significantly, higher in colonized roots as compared with the uncolonized controls (Fig. 4). In contrast, *F. culmorum* infection resulted in about a 40% reduction in the GSH level two weeks after inoculation (Fig. 4). Unlike DHA, the content of oxidized GSH (GSSG) increased significantly (about 2.6-fold) in response to *F. culmorum* infection. Accordingly, the ratio of reduced GSH to GSSG decreased substantially (about fourfold; Fig. 4). As in the case of ascorbate and DHA, depletion of the GSH content and the ratio of GSH to GSSG were prevented by preinoculation with *P. indica*.

Ascorbate-GSH cycle enzymes.

We addressed the question of whether activities of antioxidant enzymes were changed in infected roots and, thus, may contribute to infection-related changes in the redox state of ascorbate and GSH. Elevated cellular ascorbate and GSH levels suggest that enzymes involved in the regeneration of the two antioxidants show increased activities. Consistent with this, *P. indica*-colonized barley roots exhibited an approximately 35% increase in both DHAR and MDHAR activities (Table 1), while ascorbate peroxidase (APX, EC 1.11.1.11) and GSH reductase (GR, EC 1.6.4.2) were only slightly (insignificantly) enhanced. On the contrary, inoculation of plants with *F. culmorum* resulted in a marked reduction in the activities of all the enzymes of the ascorbate-GSH cycle (APX, 60%; GR, 28%; DHAR, 44%; MDHAR, 60%) as compared with controls (Table 1). This reduction of antioxidative enzymes was abolished in *P. indica*-colonized plants in which activities of APX, GR, DHAR, and MDHAR were 3.9-fold, twofold, 1.4-fold, and 1.9-fold higher, respectively, compared with roots infected only with *F. culmorum* (Table 1). When com-

Fig. 1. Shoot and root fresh weight of three-week-old barley plants preinoculated with *Piriformospora indica* and challenged with *Fusarium culmorum*. Two-day-old seedlings were dip-inoculated with 5×10^5 *P. indica* chlamydospores per milliliter or water (mock) before sowing. One-week-old seedlings were transferred to pots containing or not containing inoculum of *F. culmorum*. Control seedlings were mock-inoculated twice at two days and one week. Data are means $\pm$ standard deviation ($n = 4$ plants). The experiment was repeated twice with similar results. Different letters indicate significant differences in shoot and root biomass ($P \leq 0.05$, Tukey test).

Fig. 2. Concentrations of *Fusarium culmorum* DNA in roots of three-week-old barley plants preinoculated with *Piriformospora indica* and challenged with *F. culmorum*. Two-day-old seedlings were dip-inoculated with 5×10^5 chlamydospores per milliliter of *P. indica* or water (mock) before sowing. One-week-old seedlings were transferred to pots containing or not containing inoculum of *F. culmorum*. Control seedlings were mock-inoculated twice at two days and one week. *Fusarium* DNA levels were measured by real-time polymerase chain reaction and were normalized using the plant *EF1 α* assay (Nielsen et al. 2012). Relative biomass of the fungus (means $\pm$ standard deviation) is expressed as the ratio of fungal DNA relative to plant DNA. No amplification product of the *F. culmorum*-specific *Tri12* gene was observed when template DNA was prepared from plants not inoculated with *F. culmorum*. Data are based on three independent experiments run in triplicate. Student's *t*-test indicated significant difference in *F. culmorum* colonization (* $P < 0.05$).

pared with controls, dually inoculated roots showed 60 and 40% greater APX and GR activities, respectively, while DHAR activity was not significantly different and MDHAR activity was 25% less. Together, these data demonstrate that the mutualistic, root-colonizing fungus *P. indica* abolishes detrimental effects on the host plant's antioxidant system caused by the necrotrophic pathogen *F. culmorum*.

***P. indica* protects *Fusarium*-infected roots from loss of superoxide dismutase (SOD) activities.**

Compared with control plants, total activity of SOD (EC 1.15.1.1) was increased by 62% in *P. indica*-colonized three-week-old plants, whereas it was reduced by 56% in roots inoculated with *F. culmorum*. Yet, when *P. indica*-colonized seedlings were challenged with *F. culmorum*, the pathogen-induced reduction in SOD activity was completely abolished (Table 1).

Similarly, activity of catalase (CAT, EC 1.11.1.6) increased significantly in response to *P. indica* (Table 1). We found that *P. indica* elevated the CAT activity by 46% in roots as compared with uncolonized control plants. However, unlike SOD, CAT activity did not change significantly upon inoculation with *F. culmorum* (Table 1).

***P. indica* protects *Fusarium*-infected roots from extensive lipid peroxidation.**

Next, we assessed levels of lipid peroxides (LOOH) in roots of three-week-old plants using the ferrous oxidation xynol orange (FOX) assay (Do et al. 1996). Roots colonized with *P. indica* as well as roots of control plants contained low amounts of LOOH (approximately 70 nmol per gram of fresh weight [FW]; Fig. 5). In contrast, fivefold higher levels of LOOH

were found after inoculation with *F. culmorum*. Notably, pretreatment with *P. indica* at least partially protected roots against lipid peroxidation induced by infection with *F. culmorum* (approximately 160 vs. 330 nmol LOOH per gram of FW; Fig. 5).

DISCUSSION

Abiotic environmental stress and infections by microbial pathogens cause oxidative stress in plants via enhanced generation of ROS (Apel and Hirt 2004). High levels of ROS trigger cellular injury and cell death. To avoid this damage, plants have evolved enzymatic and nonenzymatic antioxidant mechanisms acting in concert to detoxify ROS (Foyer and Noctor 2005). Ascorbate is the major low-molecular weight antioxidant compound playing a central role in the cellular defense against oxidative damage (Conklin et al. 1996; Eltayeb et al. 2007; Zhang et al. 2011). *P. indica*-induced abiotic stress tolerance was shown to be associated with elevated levels of ascorbate and a high ascorbate/DHA ratio, along with increased DHAR and MDHAR enzyme activities in plant roots (Baltruschat et al. 2008; Vadassery et al. 2009; Waller et al. 2005). Moreover, systemic resistance mediated by the root-colonizing endophyte against powdery mildew disease is associated with an increased level of leaf GSH and GR enzyme activity (Waller et al. 2005).

In the present study, we analyzed the size and redox state of total ascorbate and GSH pools in barley roots inoculated with *P. indica* and the necrotrophic fungus *F. culmorum*. The observed decrease in the level of reduced forms of ascorbate and GSH along with the decrease in the ratios of reduced to oxidized forms in *F. culmorum* infected roots (single infection) suggests that the necrotrophic fungus causes detrimental oxid-

Fig. 3. Levels of reduced ascorbate (white bars) and dehydroascorbate (DHA, hatched bars) in roots of three-week-old barley plants preinoculated with *Piriformospora indica* and challenged with *Fusarium culmorum*. Two-day-old seedlings were dip-inoculated with 5×10^5 chlamydospores per milliliter of *P. indica* or water (mock) before sowing. One-week-old seedlings were transferred to pots containing or not containing inoculum of *F. culmorum*. Control seedlings were mock-inoculated twice at two days and one week. Data are means $\pm$ standard deviation ($n = 4$ plants). The experiment was repeated twice with similar results. Different letters indicate significant differences in reduced ascorbate at $P \leq 0.05$ (Tukey test). Levels of DHA did not change significantly at $P \leq 0.05$. FW = fresh weight.

Fig. 4. Levels of reduced glutathione (GSH, white bars) and glutathione disulfide (GSSG, hatched bars) in roots of three-week-old barley plants preinoculated with *Piriformospora indica* and challenged with *Fusarium culmorum*. Two-day-old seedlings were dip-inoculated with 5×10^5 chlamydospores per milliliter of *P. indica* or water (mock) before sowing. One-week-old seedlings were transferred to pots containing or not containing inoculum of *F. culmorum*. Control seedlings were mock-inoculated twice at two days and one week. Data are means $\pm$ standard deviation ($n = 4$ plants). The experiment was repeated twice with similar results. Different letters indicate significant differences in GSH at $P \leq 0.05$ (Tukey test). GSSG level marked with an asterisk is significantly different from that observed in mock-inoculated plants ($P \leq 0.05$). FW = fresh weight.

ative stress. Our data show that *P. indica* could abolish the adverse effect of *Fusarium* infection on ascorbate and GSH in barley roots, as it has previously been demonstrated for salinity-induced stress.

Lipid peroxidation in living organisms subjected to oxidative stress has been widely accepted as an indication of early damage by ROS (Halliwell and Chirico 1993). We observed a fivefold increase in peroxide content of *Fusarium*-infected barley roots. This observation is consistent with previous studies that detected oxidative stress during infection of wheat roots by various *Fusarium* species (Desmond et al. 2008; Khoshgoftarmansh et al. 2010). We found that *P. indica* robustly attenuated the *F. culmorum*-induced accumulation of peroxides.

The present study also confirms that the shift in the redox status to a more oxidizing cellular environment (decreased ascorbate/DHA and GSH/GSSG ratios) in *Fusarium*-infected barley roots is accompanied by a significant reduction in the activities of antioxidative enzymes SOD, APX, GR, DHAR, and MDHAR. Significantly, preinoculation of roots with *P. indica* almost completely abolishes the *Fusarium*-induced decrease in antioxidant capacity. Conflicting results were reported

by Kumar and associates (2009), who found that activities of SOD, CAT, GR, and GST increased markedly in maize roots upon inoculation with *F. verticillioides*, while *P. indica* attenuated the pathogen-induced increase in CAT, GR, and GST activities. The reason for this discrepancy is not yet clear but may be explained by differences in the severity of disease symptoms and in the modulation of the plant's antioxidant system by various mycotoxins with different modes of action produced by *F. culmorum* and *F. verticillioides* in roots of barley and maize, respectively. Maize roots colonized with *F. verticillioides* showed dramatic increases (3.2- to 43-fold over uninoculated control) in enzyme activities depending on the particular antioxidant enzyme (Kumar et al. 2009). This is in sharp contrast to our results, in which *F. culmorum* infection of barley roots resulted in a decrease in SOD, APX, GR, DHAR, and MDHAR activities. We observed the same tendency in tomato roots, in which a decrease in antioxidant capacity in response to *Fusarium oxysporum* f. sp. *lycopersici* was prevented by preinoculation with *P. indica* (unpublished results). In line with our findings, Li and associates (2010) reported that SOD activity and ascorbate content decreased in roots of strawberry plants after inoculation with *F. oxysporum* f. sp. *fragariae*, while preinoculation with the arbuscular mycorrhiza fungus (AMF) *Glomus mosseae* prevented the decline in antioxidants. Similar results were obtained with SOD and ascorbate extracted from stem bases when strawberry plants were inoculated with *Colletotrichum gloeosporioides* causing anthracnose as well as crown and root rot (Li et al. 2010). Furthermore, level of reduced ascorbate and activities of GR, APX, and DHAR decreased in roots of St. John's wort (*Hypericum perforatum*) after inoculation with *C. gloeosporioides* (Richter et al. 2011). In accordance with our finding, the detrimental effect of *C. gloeosporioides* on the antioxidative defense systems in *H. perforatum* roots was completely abolished by AMF (Richter et al. 2011). Taken together, these data suggest that necrotrophic fungi inhibit the antioxidant activity in attacked plant tissues and that root-colonizing mutualistic fungi protect roots from necrotrophic microbes through activation and protection of the plants' antioxidant system.

P. indica does not inhibit the mycelial growth of *F. culmorum* in vitro (Waller et al. 2005), but its effect on the growth of *F. culmorum* in roots had not been quantified. qPCR of the relative abundance of *F. culmorum* and barley DNA was performed in root tissues using specific fungal and plant genomic DNA primers (Nielsen et al. 2012). Reduced relative amounts of *F. culmorum* DNA indicated a significantly lower level of *Fusarium* infection in dually inoculated barley roots as compared with roots with single *F. culmorum* infections. Similar findings were reported for wheat roots inoculated with *P. indica* and *F. graminearum* (Deshmukh et al. 2007), suggesting that *P. indica* does not exert a direct antifungal activity but induces resistance against *Fusarium* infections. However, caution is required in interpreting the qPCR data, because the ratio of *F.*

Fig. 5. Peroxide levels in roots of three-week-old barley plants preinoculated with *Piriformospora indica* and challenged with *Fusarium culmorum*. Two-day-old seedlings were dip-inoculated with 5×10^5 chlamydospores per milliliter of *P. indica* or water (mock) before sowing. One-week-old seedlings were transferred to pots containing or not containing inoculum of *F. culmorum*. Control seedlings were mock-inoculated twice at two days and one week. Lipid peroxidation was measured by the ferrous xylenol orange assay (Do et al. 1996). Hydrogen peroxide was used to construct a standard curve. Data are based on three independent experiments run in triplicate. Different letters indicate significant differences in peroxide levels at $P \leq 0.05$ (Tukey test). FW = fresh weight.

Table 1. Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR) in roots of three-week-old barley plants preinoculated with *Piriformospora indica* and challenged with *Fusarium culmorum*^a

Treatment	SOD (EU/g FW)	CAT ($\mu\text{mol/g FW min}$)	APX ($\mu\text{mol/g FW min}$)	GR (nmol/g FW min)	DHAR (nmol/g FW min)	MDHAR (nmol/g FW min)
Control	576 $\pm$ 77b	70 $\pm$ 8c	4.35 $\pm$ 0.56b	220 $\pm$ 23b	291 $\pm$ 38b	334 $\pm$ 33b
<i>P. indica</i>	933 $\pm$ 115a	102 $\pm$ 12ab	4.93 $\pm$ 0.49b	266 $\pm$ 35ab	396 $\pm$ 35a	445 $\pm$ 29a
<i>F. culmorum</i>	256 $\pm$ 41c	86 $\pm$ 10bc	1.75 $\pm$ 0.31c	159 $\pm$ 25c	164 $\pm$ 19c	133 $\pm$ 19d
<i>P. indica</i> + <i>F. culmorum</i>	836 $\pm$ 99a	118 $\pm$ 9a	6.87 $\pm$ 0.96a	311 $\pm$ 40a	231 $\pm$ 35b	249 $\pm$ 25c

^a Roots of two-day-old seedlings were dip-inoculated with 5×10^5 chlamydospores per milliliter of *P. indica* or water before sowing. One-week-old seedlings were transferred to pots containing or not containing inoculum of *F. culmorum* and cultivated for an additional two weeks before assay. Control seedlings were mock-inoculated twice at two days and one week. Data are means of four independent replicates $\pm$ standard deviation. The experiment was repeated twice with similar results. Different lowercase letters indicate significant differences at $P \leq 0.05$ by Tukey post hoc test. EU = enzyme unit; FW = fresh weight.

culmorum DNA to plant DNA in root samples reflects both fungal abundance and the presence of intact plant cells. *F. culmorum* causes extensive cell death in barley roots, which ultimately results in root rot symptoms. Therefore, the qPCR method might overestimate the abundance of *Fusarium* DNA in necrotized root tissues that contain less-intact plant DNA. Using the qPCR method in greenhouse studies, Strausbaugh and associates (2005) found significant correlations between percent infected root area and *Fusarium* DNA quantities in *F. culmorum*-inoculated wheat and barley roots. However, in plants from field studies, they found no correlation between root-rot severities and amounts of *Fusarium* DNA. Another recent field study showed that development of *Fusarium* crown rot symptoms in wheat often, but not always, correlates with actual *Fusarium* colonization (Hogg et al. 2007). These studies show that qPCR results must be verified by independent methods to detect the fungus in roots. Accordingly, our microscopic analysis confirmed reduced levels of *Fusarium* infection in *P. indica*-preinoculated roots, thereby corroborating our interpretation of qPCR results (not shown).

Consistent with our results, several studies have demonstrated that *P. indica* and other mutualistic fungal endophytes may enable plants to more efficiently scavenge ROS or prevent ROS production under stress conditions (Baltruschat et al. 2008; Rodriguez et al. 2008; Sherameti et al. 2008; Sun et al. 2010; Waller et al. 2005). Our data suggest that antioxidant defense was maintained at a high level in *P. indica*-colonized roots in response to *F. culmorum* infection. It is well-established that necrotrophic pathogens such as *Botrytis cinerea* utilize production of ROS to accelerate cell death and facilitate subsequent infection (Govrin and Levine 2000). A recent study showed that inhibition of the oxidative burst in *Arabidopsis* resulted in resistance to *B. cinerea* infection (Yang et al. 2011). *F. culmorum* infection also triggers a sustained oxidative burst and cell death in the invaded plant tissues (Cuzick et al. 2009). Consistent with this, higher antioxidant capacity was associated with an increase in resistance of transgenic flax (*Linum usitatissimum*) seedlings to *F. culmorum* (Lorenc-Kukuła et al. 2007). Three key enzymes of flavonoid biosynthesis were up-regulated in these flax plants resulting in an increased flavonoid content and high antioxidant capacity.

Based on our results and the aforementioned studies, we propose that the increase in resistance of barley roots to *F. culmorum* is, at least partly, mediated by *P. indica*-induced activation of antioxidant defense. Since higher antioxidant activity diminishes cell death induced by ROS, necrotization of plant tissue is consequently reduced, which is unfavorable to the necrotrophic pathogen. Yet, further studies are required to firmly establish the mechanism of endophyte-mediated resistance against pathogens in plant roots.

MATERIALS AND METHODS

Plant material and fungal inoculation.

Seeds of barley (*Hordeum vulgare* L. cv. Uschi) were surface-sterilized for 10 min in 0.25% sodium hypochlorite, were rinsed thoroughly with water, and were germinated for 2 days at 22°C on sheets of Whatman No. 1 filter paper in petri dishes. Germinated seeds were planted into 200-ml pots (three plants per pot) filled with a 2:1 mixture of expanded clay (Seramis, Masterfoods, Verden, Germany) and Oil-Dri (equivalent to Terra Green, Damolin, Mettmann, Germany), were incubated in a growth chamber at 22°C day and 18°C night cycle, 60% relative humidity, and a photoperiod of 16 h (240 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density), and were fertilized weekly with 0.1% Wuxal top N solution (N/P/K: 12/4/6; Schering, Düsseldorf, Germany).

Agar discs of 0.5 cm diameter covered by mycelium of *P. indica* (DSM 11827; Sharma et al. 2008) were placed in the center of 9-cm petri dishes containing *Aspergillus* minimal medium solidified with 1.5% (wt/vol) agar and were incubated for 6 weeks at 26°C (Peškan-Berghöfer et al. 2004). Then chlamydospores were collected by flooding the surface of the plate with 10 ml of sterile water containing 0.02% (vol/vol) Tween 20, followed by gentle scraping with a spatula. Spore suspension was filtered through two layers of Miracloth (Calbiochem, San Diego, CA, U.S.A.) to remove chunks of mycelium, was centrifuged ($3,000 \times g$, 7 min), and was resuspended in 0.02% Tween 20, and the spore concentration was determined using a haemocytometer. For inoculation with *P. indica*, roots of two-day-old seedlings were immersed in *P. indica* spore suspension ($5 \times 10^5 \text{ ml}^{-1}$) before sowing (Verma et al. 1998). Control plants were treated with water containing 0.02% Tween 20. Root colonization was determined in two-week-old plants by magnified intersections method (McGonigle et al. 1990), after staining root fragments with 0.01% (wt/vol) acid fuchsin in lactoglycerol (Kormanik and McGraw 1982). Nine seedlings (three in each of three pots) were selected at random from each treatment and the whole root system was examined for fungal structures under a Zeiss Axioplan 2 microscope. The rest of the plants were used in further analyses only if all plants chosen for microscopic examination were well-colonized by *P. indica* (colonization was at least 50% among 1-cm-long root segments).

Fusarium culmorum KF 350 was grown on potato dextrose agar plates at 22°C (Jansen et al. 2005). For root inoculation, barley kernels were autoclaved twice for 25 min with a 24-h interval and were then inoculated with conidia of *F. culmorum* and incubated for one week at room temperature before being used as inoculum as described by Waller and associates (2005). One-week-old seedlings were removed from the pots and roots were washed thoroughly with sterile water. Then, seedlings were transplanted to 200-ml pots filled with a 2:1 mixture of expanded clay and Oil-Dri containing or not containing the inoculum (eight to ten infected kernels per pot). Transplanted plants were cultured for an additional two weeks under the same conditions as described above.

Quantification of *F. culmorum* in infected plants.

The ratio of *F. culmorum* DNA to plant DNA was used to monitor the success of *F. culmorum* infection in barley. Roots of three-week-old barley plants were harvested from pot cultures and were washed intensively with sterile water before DNA extraction. DNA was isolated from the whole root system using DNAzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions. Furthermore, pure genomic DNA was isolated from roots of uninoculated plants and from aerial mycelia of *F. culmorum* scraped off the agar to construct calibration curves for a normalized measurement of infection. Extracted DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Primers designed to amplify fragments of either the fungal *Tri12* gene (involved in the trichothecene pathway) from the genomic DNA of *F. culmorum* KF350 (forward, 5'-GCC CAT ATT CGC GAC AAT GT-3' and reverse, 5'-GGC GAA CTG ATG AGT AAC AAA ACC-3') or the plant EF1 α gene from barley genomic DNA (forward, 5'-TCT CTG GGT TTG AGG GTG AC-3' and reverse, 5'-GGC CCT TGT ACC AGT CAA GGT-3') were used (Nicolaisen et al. 2009; Nielsen et al. 2012). Total DNA (100 ng) served as template in each qPCR reaction. Amplifications were performed in a 20- μl volume using 2 $\times$ SYBR FAST master mix (KAPA Biosystems, Woburn, MA, U.S.A.) in a CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA, U.S.A.), according to the follow-

ing program: 3 min at 95°C, 40 cycles of 15 s at 95°C, 10 s at 60°C, 10 s at 72 °C. A melting curve was determined at the end of cycling to verify specificity of amplification. Cycle threshold (Ct) values were calculated automatically by the Bio-Rad CFX Manager software (version 2.1). Individual standard curves were developed by plotting the logarithm of known concentrations of *F. culmorum* DNA and barley DNA (twofold dilution series) against the Ct values. The amount of target DNA for unknown samples was extrapolated from the respective standard curves.

Antioxidant assays.

Roots of three-week-old barley plants were harvested from pot cultures and were washed intensively with sterile water before extraction. The entire excised root system was used for the antioxidant assays. Levels of reduced and oxidized forms of ascorbate and GSH and activities of antioxidant enzymes SOD, CAT, APX, DHAR, MDHAR, and GR were detected spectrophotometrically in root extracts as described (Baltruschat et al. 2008; Harrach et al. 2008).

Peroxide analysis.

Lipid hydroperoxides were extracted and assayed using the FOX assay as described (Do et al. 1996). Roots (0.2 g) were homogenized at 0 to 4°C in 2 ml of methanol containing 0.01% butylated hydroxytoluene (BHT). Following centrifugation (12,000 × g, 10 min, 4°C), the supernatants (0.1 ml) were mixed with 0.7 ml of methanol containing 0.01% BHT. Then, 0.1 ml water containing 2.5 mM FeSO₄, 2.5 mM (NH₄)₂SO₄, and 0.25 M H₂SO₄, as well as 0.1 ml of methanol containing 40 mM BHT and 1.25 mM xylenol orange were added. Samples were incubated at room temperature for 30 min and absorbance at 560 nm was measured. The peroxide content was calculated based on a standard curve created by known concentrations of hydrogen peroxide as described (DeLong et al. 2002). The reactivity of 18:2-derived LOOH with the FOX reagent is nearly identical to H₂O₂ (DeLong et al. 2002).

Statistical analysis.

At least three independent experiments were carried out in each case. Four replicate pots of plants from each treatment were sampled for measurements. Statistical significance was analyzed with Student's *t*-test and analysis of variance followed by the Tukey post hoc test (Statistica 6.1, Statsoft, Tulsa, OK, U.S.A.). Differences were considered to be significant at *P* < 0.05.

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