

1 The mutualistic fungus *Piriformospora indica* protects barley roots from a loss of antioxidant  
2 capacity caused by the necrotrophic pathogen *Fusarium culmorum*

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15

16 **ABSTRACT**

17 *Fusarium culmorum* causes root rot in barley (*Hordeum vulgare*), resulting in severely  
18 reduced plant growth and yield. Pretreatment of roots with chlamydospores of the mutualistic  
19 root-colonizing basidiomycete *Piriformospora indica* (Agaricomycotina) prevented  
20 necrotization of root tissues and plant growth retardation commonly associated with *Fusarium*  
21 root rot. Quantification of *Fusarium* infections with a real-time PCR assay revealed a  
22 correlation between root rot symptoms and the relative amount of fungal DNA. *Fusarium*-  
23 infected roots showed reduced levels of ascorbate and glutathione (GSH), along with reduced  
24 activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase  
25 (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and

1 monodehydroascorbate reductase (MDHAR). Consistent with this, Fusarium-infected roots  
2 showed elevated levels of lipid hydroperoxides and decreased ratios of reduced to oxidized  
3 forms of ascorbate and glutathione. In clear contrast, roots treated with *P. indica* prior to  
4 inoculation with *F. culmorum* showed levels of ascorbate and GSH that were similar to  
5 controls. Likewise, lipid peroxidation and the overall reduction in antioxidant enzyme  
6 activities were largely attenuated by *P. indica* in roots challenged by *F. culmorum*. These  
7 results suggest that *P. indica* protects roots from necrotrophic pathogens at least partly,  
8 through activating the plant's antioxidant capacity.

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10

## 11 INTRODUCTION

12

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

23 An important feature of plant responses to environmental stresses is that the balance  
24 between production and scavenging of reactive oxygen species (ROS) is shifted towards  
25 higher ROS levels (Apel and Hirt 2004). Excess ROS subsequently induces lipid peroxidation

1 of cell membranes and damage to proteins and nucleic acids. Growing evidence suggests that  
2 endophytic fungi enhance tolerance of host plants to abiotic stress by altering their antioxidant  
3 activity (Hamilton and Bauerle 2012; Rodriguez et al. 2008). Consistent with this,  
4 colonization by *P. indica* prevents salt- and drought-induced lipid peroxidation in barley and  
5 Chinese cabbage roots, respectively (Baltruschat et al. 2008; Sun et al. 2010). This beneficial  
6 effect is associated with significant changes in plant redox metabolism and accumulation of  
7 high levels of ascorbate due to increased activities in key antioxidant enzymes, such as  
8 dehydroascorbate reductase (DHAR, EC 1.8.5.1) and monodehydroascorbate reductase  
9 (MDHAR, EC 1.6.5.4) (Baltruschat et al. 2008; Waller et al. 2005). Moreover, up-regulation  
10 of DHAR and MDHAR in *Arabidopsis thaliana* is essential for a mutualistic association with  
11 *P. indica* (Vadassery et al. 2009). In addition to its role in abiotic stress tolerance, *P. indica*  
12 confers resistance to a range of microbial pathogens in various crop plants, including barley,  
13 lentil, maize, tomato, and wheat (Deshmukh and Kogel 2007; Dolatabadi et al. 2012; Fakhro  
14 et al. 2010; Kumar et al. 2009; Serfling et al. 2007; Waller et al. 2005). Most of these studies  
15 have been focused on soil-borne diseases such as Fusarium root rot of cereals. It is well  
16 established that *F. culmorum* utilizes production of ROS to accelerate cell death and facilitate  
17 subsequent infection (Cuzick et al. 2009). Accordingly, an increase in oxidative stress was  
18 observed in barley and wheat seedlings affected by Fusarium head blight and root rot (Boddu  
19 et al. 2006; Desmond et al. 2008; Khoshgoftarmanesh et al. 2010).

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

23

## 24 **RESULTS**

### 25 **Quantification of *F. culmorum* in barley roots**

1 Consistent with earlier studies, three-week-old *P. indica*-colonized barley showed enhanced  
2 shoot and root biomass (Fig. 1; see also Deshmukh and Kogel 2007; Waller et al. 2005). In  
3 contrast, shoot and root biomass was strongly reduced by *Fusarium culmorum* infection  
4 within 2 weeks of inoculation. However, when *P. indica*-colonized plants were challenge-  
5 inoculated with *F. culmorum*, neither severe root rot symptoms nor growth retardation was  
6 observed (Fig. 1).

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

16

### 17 ***P. indica* protects Fusarium-infected roots from a loss of ascorbate and glutathione**

18 We assessed the antioxidant status of barley roots that were colonized either by *P. indica*, *F.*  
19 *culmorum*, or a combination of these fungi. Colonization by *P. indica* resulted in a 2.5-fold  
20 increase in ascorbate level and a 70% increase in the ratio of reduced to oxidized ascorbate  
21 (dehydroascorbate, DHA) in 3-week-old plants compared with the controls (Fig. 3). In  
22 contrast, inoculation of roots with *F. culmorum* caused a 70% reduction in root ascorbate after  
23 2 weeks, although it did not result in a significant accumulation of DHA. Accordingly, the  
24 ratio of ascorbate to DHA decreased by about 70% in *Fusarium*-infected roots. However,

1 when roots were inoculated with *P. indica* one week prior to *F. culmorum*, root ascorbate and  
2 DHA levels were similar to that in control plants (Fig. 3).

3 To extend this analysis, we measured the concentration of reduced glutathione (GSH)  
4 in infected and uninfected barley roots. Three weeks after inoculation with *P. indica*, GSH  
5 was slightly, but not significantly, higher in colonized roots as compared to the uncolonized  
6 controls (Fig. 4). In contrast, *F. culmorum* infection resulted in about 40% reduction in the  
7 GSH level 2 weeks after inoculation (Fig. 4). Unlike DHA, the content of oxidized  
8 glutathione (GSSG) increased significantly (about 2.6-fold) in response to *F. culmorum*  
9 infection. Accordingly, the ratio of reduced to oxidized glutathione decreased substantially  
10 (about 4-fold; Fig. 4). As in the case of ascorbate and DHA, depletion of the GSH content and  
11 the ratio of GSH to GSSG were prevented by preinoculation with *P. indica*.

12

### 13 **Ascorbate-glutathione cycle enzymes**

14 We addressed the question of whether activities of antioxidant enzymes were changed in  
15 infected roots and thus may contribute to infection-related changes in the redox state of  
16 ascorbate and glutathione. Elevated cellular ascorbate and GSH levels suggest that enzymes  
17 involved in the regeneration of the two antioxidants show increased activities. Consistent with  
18 this, *P. indica*-colonized barley roots exhibited approximately 35% increase in both DHAR  
19 and MDHAR activities (Table 1) while ascorbate peroxidase (APX, EC 1.11.1.11) and  
20 glutathione reductase (GR, EC 1.6.4.2) were only slightly (insignificantly) enhanced. On the  
21 contrary, inoculation of plants with *F. culmorum* resulted in a marked reduction in the  
22 activities of all the enzymes of the ascorbate-glutathione cycle (APX, 60%; GR, 28%; DHAR,  
23 44%; MDHAR; 60%) as compared with controls (Table 1). This reduction of antioxidative  
24 enzymes was abolished in *P. indica* preinoculated plants where activities of APX, GR,  
25 DHAR, and MDHAR were 3.9-fold, 2-fold, 1.4-fold, and 1.9-fold higher, respectively,

1 compared with roots infected only with *F. culmorum* (Table 1). When compared to controls,  
2 dually inoculated roots showed 60% and 40% higher APX and GR activities, respectively,  
3 while DHAR activity was not significantly different and MDHAR activity was 25% lower.  
4 Together, these data demonstrate that the mutualistic, root-colonizing fungus *P.indica*  
5 abolishes detrimental effects on the host plant's antioxidant system caused by the  
6 necrotrophic pathogen *F. culmorum*.

7

#### 8 ***P. indica* protects Fusarium-infected roots from loss of superoxide dismutase activities**

9 Compared to control plants, total activity of superoxide dismutase (SOD, EC 1.15.1.1) was  
10 increased by 62% in *P. indica*-colonized 3-week-old plants, whereas it was reduced by 56%  
11 in roots inoculated with *F. culmorum*. Yet, when *P. indica*-colonized seedlings were  
12 challenged with *F. culmorum*, the pathogen-induced reduction in SOD activity was  
13 completely abolished (Table 1).

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

18

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

20 Next, we assessed levels of lipid peroxides (LOOH) in roots of 3-week-old plants using the  
21 ferrous oxidation xylenol orange (FOX) assay (Do et al. 1996). Roots colonized with *P.*  
22 *indica*, as well as roots of control plants, contained low amounts of LOOHs (approx. 70 nmol  
23 g<sup>-1</sup> FW; Fig. 5). In contrast, 5-fold higher amounts of LOOHs were found after inoculation  
24 with *F. culmorum*. Notably, pretreatment with *P. indica* at least partially protected roots

1 against lipid peroxidation induced by infection with *F. culmorum* (approx. 160 vs. 330 nmol  
2 g<sup>-1</sup> FW (Fig. 5).

3

#### 4 **DISCUSSION**

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

17 In the present study, we analyzed the size and redox state of total ascorbate and  
18 glutathione pools in barley roots inoculated with *P. indica* and the necrotrophic fungus *F.*  
19 *culmorum*. The observed decrease in the level of reduced forms of ascorbate and glutathione  
20 along with the decrease in the ratios of reduced to oxidized forms in *F. culmorum* infected  
21 roots (single infection) suggests that the necrotrophic fungus causes detrimental oxidative  
22 stress. Our data show that *P. indica* could abolish the adverse effect of *Fusarium* infection on  
23 ascorbate and glutathione in barley roots, as it has previously been demonstrated for salinity-  
24 induced stress.

1 Lipid peroxidation in living organisms subjected to oxidative stress has been widely  
2 accepted as an indication of early damage by ROS (Halliwell and Chirico 1993). We observed  
3 a 5-fold increase in peroxide content of *Fusarium*-infected barley roots. This observation is  
4 consistent with previous studies that detected oxidative stress during infection of wheat roots  
5 by various *Fusarium* species (Desmond et al. 2008; Khoshgoftarmanesh et al. 2010). We  
6 found that *P. indica* robustly attenuated the *F. culmorum*-induced accumulation of peroxides.

7 The present study also confirms that the shift in the redox status to a more oxidizing  
8 cellular environment (decreased ascorbate/DHA and GSH/GSSG ratios) in *Fusarium*-infected  
9 barley roots is accompanied by a significant reduction in the activities of antioxidative  
10 enzymes SOD, APX, GR, DHAR, and MDHAR. Significantly, preinoculation of roots with  
11 *P. indica* almost completely abolishes the *Fusarium*-induced decrease in antioxidant capacity.  
12 Conflicting results were reported by Kumar et al. (2009), who found that activities of SOD,  
13 CAT, GR, and GST increased markedly in maize roots upon inoculation with *F.*  
14 *verticillioides*, while *P. indica* attenuated the pathogen-induced increase in CAT, GR, and  
15 GST activities. The reason for this discrepancy is not yet clear but may be explained by  
16 differences in the severity of disease symptoms, and in the modulation of the plant's  
17 antioxidant system by various mycotoxins with different modes of action produced by *F.*  
18 *culmorum* and *F. verticillioides* in roots of barley and maize, respectively. Maize roots  
19 colonized with *F. verticillioides* showed dramatic increases (3.2- to 43-fold over uninoculated  
20 control) in enzyme activities depending on the particular antioxidant enzyme (Kumar et al.  
21 2009). This is in sharp contrast to our results, in which *F. culmorum* infection of barley roots  
22 resulted in a decrease in SOD, APX, GR, DHAR, and MDHAR activities. We observed the  
23 same tendency in tomato roots, where a decrease in antioxidant capacity in response to  
24 *Fusarium oxysporum* f. sp. *lycopersici* was prevented by preinoculation with *P. indica*  
25 (unpublished results of the authors). In line with our findings, Li et al. (2010) reported that



1 SOD activity and ascorbate content decreased in roots of strawberry plants after inoculation  
2 with *F. oxysporum* f. sp. *fragariae*, while preinoculation with the arbuscular mycorrhiza  
3 fungus (AMF) *Glomus mosseae* prevented the decline in antioxidants. Similar results were  
4 obtained with SOD and ascorbate extracted from stem bases, when strawberry plants were  
5 inoculated with *Colletotrichum gloeosporioides* causing anthracnose as well as crown and  
6 root rot (Li et al. 2010). Furthermore, level of reduced ascorbate and activities of GR, APX,  
7 and DHAR decreased in roots of St. John's wort (*Hypericum perforatum*) after inoculation  
8 with *C. gloeosporioides* (Richter et al. 2011). In accordance with our finding, the detrimental  
9 effect of *C. gloeosporioides* on the antioxidative defense systems in *H. perforatum* roots was  
10 completely abolished by AMF (Richter et al. 2011). Taken together, these data suggest that  
11 necrotrophic fungi inhibit the antioxidant activity in attacked plant tissues, and that root-  
12 colonizing mutualistic fungi protect roots from necrotrophic microbes through activation /  
13 protection of the plants' antioxidant system.

14 *P. indica* does not inhibit the mycelial growth of *F. culmorum* *in vitro* (Waller et al. 2005),  
15 but its effect on the growth of *F. culmorum* in roots had not been quantified. Real-time PCR  
16 quantification of the relative abundance of *F. culmorum* and barley DNA was performed in  
17 root tissues using specific fungal and plant genomic DNA primers (Nielsen et al. 2012).  
18 Reduced relative amounts of *F. culmorum* DNA indicated a significantly lower level of  
19 *Fusarium* infection in dually inoculated barley roots as compared to roots with single *F.*  
20 *culmorum* infections. Similar findings were reported for wheat roots inoculated with *P. indica*  
21 and *F. graminearum* (Deshmukh et al. 2007), suggesting that *P. indica* does not exert a direct  
22 antifungal activity but induces resistance against *Fusarium* infections. However, caution is  
23 required in interpreting the qPCR data because the ratio of *F. culmorum* DNA to plant DNA  
24 in root samples reflects both fungal abundance and presence of intact plant cells. *F. culmorum*  
25 causes extensive cell death in barley roots which ultimately results in root rot symptoms.

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

12 Consistent with our results, several studies have demonstrated that *P. indica* and other  
13 mutualistic fungal endophytes may enable plants to more efficiently scavenge ROS or prevent  
14 ROS production under stress conditions (Baltruschat et al. 2008; Rodriguez et al. 2008  
15 Sherameti et al. 2008; Sun et al. 2010; Waller et al. 2005). Our data suggest that antioxidant  
16 defense was maintained at a high level in *P. indica*-colonized roots in response to *F.*  
17 *culmorum* infection. It is well established that necrotrophic pathogens such as *Botrytis cinerea*  
18 utilize production of ROS to accelerate cell death and facilitate subsequent infection (Govrin  
19 and Levine 2000). A recent study showed that inhibition of the oxidative burst in *Arabidopsis*  
20 resulted in resistance to *B. cinerea* infection (Yang et al. 2011). *F. culmorum* infection also  
21 triggers a sustained oxidative burst and cell death in the invaded plant tissues (Cuzick et al.  
22 2009). Consistent with this, higher antioxidant capacity was associated with an increase in  
23 resistance of transgenic flax (*Linum usitatissimum*) seedlings to *F. culmorum* (Lorenc-Kukuła  
24 et al. 2007). Three key enzymes of flavonoid biosynthesis were upregulated in these flax  
25 plants resulting in an increased flavonoid content and high antioxidant capacity.

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

7

## 8 **MATERIALS AND METHODS**

9

### 10 **Plant material and fungal inoculation**

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

19           Agar discs of 0.5 cm diameter covered by mycelium of *P. indica* (DSM 11827;  
20 Sharma et al. 2008) were placed in the centre of 9-cm petri dishes containing *Aspergillus*  
21 minimal medium solidified with 1.5% (wt/v) agar and incubated for 6 weeks at 26°C (Peškan-  
22 Berghöfer et al. 2004). Then chlamydospores were collected by flooding the surface of the  
23 plate with 10 ml of sterile water containing 0.02% (v/v) Tween 20 followed by gentle  
24 scraping with a spatula. Spore suspension was filtered through two layers of Miracloth  
25 (Calbiochem) to remove chunks of mycelium, centrifuged (3000 g, 7 min), resuspended in

1 0.02% Tween 20, and the spore concentration was determined using a haemocytometer. For  
2 inoculation with *P. indica*, roots of 2-day-old seedlings were immersed in *P. indica* spore  
3 suspension ( $5 \times 10^5 \text{ ml}^{-1}$ ) before sowing (Verma et al. 1998). Control plants were treated with  
4 water containing 0.02% Tween 20. Root colonization was determined in 2-week-old plants by  
5 magnified intersections method (McGonigle et al. 1990) after staining root fragments with  
6 0.01% (w/v) acid fuchsin in lactoglycerol (Kormanik and McGraw 1982). Nine seedlings  
7 (three in each of three pots) were selected at random from each treatment and the whole root  
8 system was examined for fungal structures under a Zeiss Axioplan 2 microscope. The rest of  
9 the plants were used in further analyses only if all plants chosen for microscopic examination  
10 were well-colonized by *P. indica* (colonization was at least 50% among 1-cm-long root  
11 segments).

12 *Fusarium culmorum* strain KF 350 was grown on potato dextrose agar plates at 22°C  
13 (Jansen et al. 2005). For root inoculation, barley kernels were autoclaved twice for 25 min  
14 with a 24-h interval, then inoculated with conidia of *F. culmorum*, and incubated for one week  
15 at room temperature before being used as inoculum as described by Waller et al. (2005). One-  
16 week-old seedlings were removed from the pots and roots were washed thoroughly with  
17 sterile water. Then seedlings were transplanted to 200 ml pots filled with a 2:1 mixture of  
18 expanded clay and Oil-Dri containing or not containing the inoculum (8-10 infected kernels  
19 per pot). Transplanted plants were cultured for additional 2 weeks under the same conditions  
20 as described above.

21

## 22 **Quantification of *F. culmorum* in infected plants**

23 The ratio of *F. culmorum* DNA to plant DNA was used to monitor the success of *F.*  
24 *culmorum* infection in barley. Roots of 3-week-old barley plants were harvested from pot  
25 cultures and washed intensively with sterile water before DNA extraction. DNA was isolated

1 from the whole root system using DNAzol reagent (Invitrogen) following the manufacturer's  
2 instructions. Furthermore, pure genomic DNA was isolated from roots of uninoculated plants  
3 and from aerial mycelia of *F. culmorum* scraped off the agar to construct calibration curves  
4 for a normalized measurement of infection. Extracted DNA was quantified using a NanoDrop  
5 1000 spectrophotometer (Thermo Fisher Scientific). Primers designed to amplify fragments  
6 either of the fungal *Tri12* gene (involved in the trichothecene pathway) from the genomic  
7 DNA of *F. culmorum* KF350 (forward, 5'- GCC CAT ATT CGC GAC AAT GT-3' and  
8 reverse, 5'- GGC GAA CTG ATG AGT AAC AAA ACC-3'), or the plant *EF1α* gene from  
9 barley genomic DNA (forward, 5'- TCT CTG GGT TTG AGG GTG AC-3' and reverse, 5'-  
10 GGC CCT TGT ACC AGT CAA GGT-3') were used (Nicolaisen et al. 2009; Nielsen et al.  
11 2012). Hundred ng of total DNA served as template in each qPCR reaction. Amplifications  
12 were performed in 20 µl volume using 2× SYBR FAST Master Mix (KAPA Biosystems) in a  
13 CFX96 Real-Time System (Bio-Rad Laboratories) according to the following program: three  
14 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  
15 determined at the end of cycling to verify specificity of amplification. Cycle threshold (Ct)  
16 values were calculated automatically by the Bio-Rad CFX Manager Software (version 2.1).  
17 Individual standard curves were developed by plotting the logarithm of known concentrations  
18 of *F. culmorum* DNA and barley DNA (twofold dilution series) against the Ct values. The  
19 amount of target DNA for unknown samples was extrapolated from the respective standard  
20 curves. To normalize gene quantification between different samples, the amount of fungal  
21 *Tri12* was divided by the amount of plant *EF1α* quantified in infected roots.

22

### 23 **Antioxidant assays**

24 Roots of 3-week-old barley plants were harvested from pot cultures and washed intensively  
25 with sterile water before extraction. The entire excised root system was used for the

1 antioxidant assays. Levels of reduced and oxidized forms of ascorbate and glutathione, and  
2 activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate  
3 peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase  
4 (MDAR), and glutathione reductase (GR) were detected spectrophotometrically in root  
5 extracts as described (Baltruschat et al. 2008; Harrach et al. 2008)

6

### 7 **Peroxide analysis**

8 Lipid hydroperoxides were extracted and assayed using the ferrous oxidation/xylanol orange  
9 (FOX) assay as described (Do et al. 1996). Roots (0.2 g) were homogenized at 0-4°C in 2 ml  
10 methanol containing 0.01% butylated hydroxytoluene (BHT). Following centrifugation  
11 (12,000 g, 10 min, 4°C), the supernatants (0.1 ml) were mixed with 0.7 ml of methanol  
12 containing 0.01% BHT. Then 0.1 ml water containing 2.5 mM FeSO<sub>4</sub>, 2.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>,  
13 and 0.25 M H<sub>2</sub>SO<sub>4</sub>, as well as 0.1 ml methanol containing 40 mM BHT and 1.25 mM xylanol  
14 orange were added. Samples were incubated at room temperature for 30 min, and absorbance  
15 at 560 nm was measured. The peroxide content was calculated based on a standard curve  
16 created by known concentrations of hydrogen peroxide as described (DeLong et al. 2002).  
17 The reactivity of 18:2-derived LOOHs with the FOX reagent is nearly identical to H<sub>2</sub>O<sub>2</sub>  
18 (DeLong et al. 2002).

19

### 20 **Statistical analysis**

21 At least three independent experiments were carried out in each case. Four replicate pots of  
22 plants from each treatment were sampled for measurements. Statistical significance was  
23 analyzed with Students *t*-test and ANOVA followed by Tukey post hoc test (Statistica 6.1,  
24 Statsoft). Differences were considered to be significant at  $P < 0.05$ .

25

1

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6

7 **Author Contributions**

8 K.H.K, H.B., and B.B. designed research; B.D.H., H.B., and J.F. performed research; J.F.  
9 analyzed data; and B.B., J.F., and K.H.K. wrote the paper.

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## TABLES

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 3-week-old barley plants preinoculated with *Piriformospora indica* and challenged with *Fusarium culmorum*. Roots of 2-day-old seedlings were dip-inoculated with  $5 \times 10^5$  chlamydo spores ml<sup>-1</sup> 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 additional 2 weeks before assay. Control seedlings were mock-inoculated twice at 2 days and 1 week.

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

Data are means of 4 independent replicates  $\pm$  SD. 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



1 **FIGURE CAPTIONS**

2

3 Fig. 1. Shoot and root fresh weight of 3-week-old barley plants preinoculated with  
4 *Piriformospora indica* and challenged with *Fusarium culmorum*. Two-day-old seedlings were  
5 dip-inoculated with  $5 \times 10^5$  *P. indica* chlamydospores  $\text{ml}^{-1}$  or water (mock) before sowing.  
6 One-week-old seedlings were transferred to pots containing or not containing inoculum of *F.*  
7 *culmorum*. Control seedlings were mock-inoculated twice at 2 days and 1 week. Data are  
8 means  $\pm$  SD (n= 4 plants). The experiment was repeated twice with similar results. Different  
9 letters indicate significant differences in shoot and root biomass ( $P \leq 0.05$ , Tukey test).

10

11 Fig. 2. Concentrations of *Fusarium culmorum* DNA in roots of 3-week-old barley plants  
12 preinoculated with *P. indica* and challenged with *F. culmorum*. Two-day-old seedlings were  
13 dip-inoculated with  $5 \times 10^5$  chlamydospores  $\text{ml}^{-1}$  of *P. indica* or water (mock) before sowing.  
14 One-week-old seedlings were transferred to pots containing or not containing inoculum of *F.*  
15 *culmorum*. Control seedlings were mock-inoculated twice at 2 days and 1 week. *Fusarium*  
16 DNA levels were measured by real-time PCR and normalized using the plant EF1 $\alpha$  assay  
17 (Nielsen et al. 2012). Relative biomass of the fungus (means  $\pm$  SD) is expressed as the ratio of  
18 fungal DNA relative to plant DNA. No amplification product of the *F. culmorum*-specific  
19 *Tri12* gene was observed when template DNA was prepared from plants not inoculated with  
20 *F. culmorum*. Data are based on three independent experiments run in triplicate. Students *t*-  
21 test indicated significant difference in *F. culmorum* colonization (\*  $P < 0.05$ ).

22

23 Fig. 3. Levels of reduced ascorbate (white bars) and dehydroascorbate (DHA, hatched bars) in  
24 roots of 3-week-old barley plants preinoculated with *P. indica* and challenged with *F.*  
25 *culmorum*. Two-day-old seedlings were dip-inoculated with  $5 \times 10^5$  chlamydospores  $\text{ml}^{-1}$  of *P.*

1 *indica* or water (mock) before sowing. One-week-old seedlings were transferred to pots  
 2 containing or not containing inoculum of *F. culmorum*. Control seedlings were mock-  
 3 inoculated twice at 2 days and 1 week. Data are means  $\pm$  SD (n= 4 plants). The experiment  
 4 was repeated twice with similar results. Different letters indicate significant differences in  
 5 reduced ascorbate at  $P \leq 0.05$  (Tukey test). Levels of DHA did not change significantly at  
 6  $P \leq 0.05$ . FW, fresh weight.

7

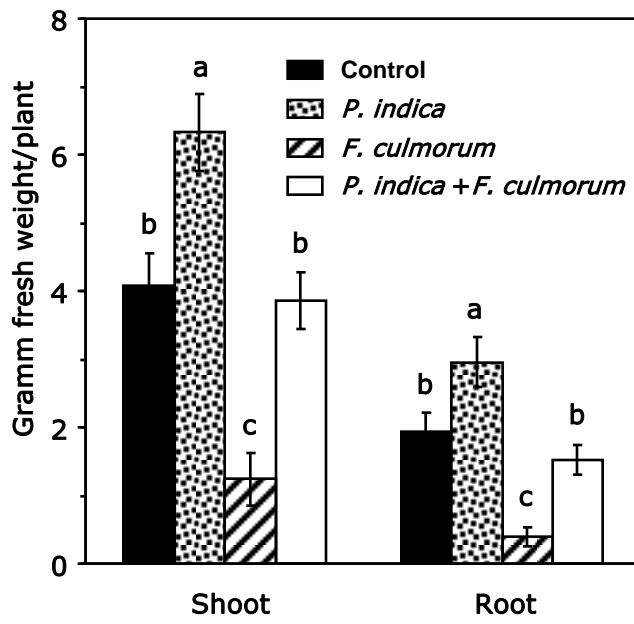
8 Fig. 4. Levels of glutathione (GSH, white bars) and glutathione disulfide (GSSG, hatched  
 9 bars) in roots of 3-week-old barley plants preinoculated with *P. indica* and challenged with *F.*  
 10 *culmorum*. Two-day-old seedlings were dip-inoculated with  $5 \times 10^5$  chlamydozoospores  $\text{ml}^{-1}$  of *P.*  
 11 *indica* or water (mock) before sowing. One-week-old seedlings were transferred to pots  
 12 containing or not containing inoculum of *F. culmorum*. Control seedlings were mock-  
 13 inoculated twice at 2 days and 1 week. Data are means  $\pm$  SD (n= 4 plants). The experiment  
 14 was repeated twice with similar results. Different letters indicate significant differences in  
 15 reduced ascorbate at  $P \leq 0.05$  (Tukey test). GSSG level marked with an asterisk is significantly  
 16 different from that observed in mock-inoculated plants ( $P \leq 0.05$ ). FW, fresh weight.

17

18 Fig. 5. Peroxide levels in roots of 3-week-old barley plants preinoculated with *P. indica* and  
 19 challenged with *F. culmorum*. Two-day-old seedlings were dip-inoculated with  $5 \times 10^5$   
 20 chlamydozoospores  $\text{ml}^{-1}$  of *P. indica* or water (mock) before sowing. One-week-old seedlings  
 21 were transferred to pots containing or not containing inoculum of *F. culmorum*. Control  
 22 seedlings were mock-inoculated twice at 2 days and 1 week. Lipid peroxidation was measured  
 23 by the ferrous xylene orange assay (Do et al. 1996). Hydrogen peroxide was used to  
 24 construct a standard curve. Data are based on three independent experiments run in triplicate.

- 1 Different letters indicate significant differences in peroxide levels at  $P \leq 0.05$  (Tukey test). FW,
- 2 fresh weight.

Fig. 1. Harrach, B.D., MPMI



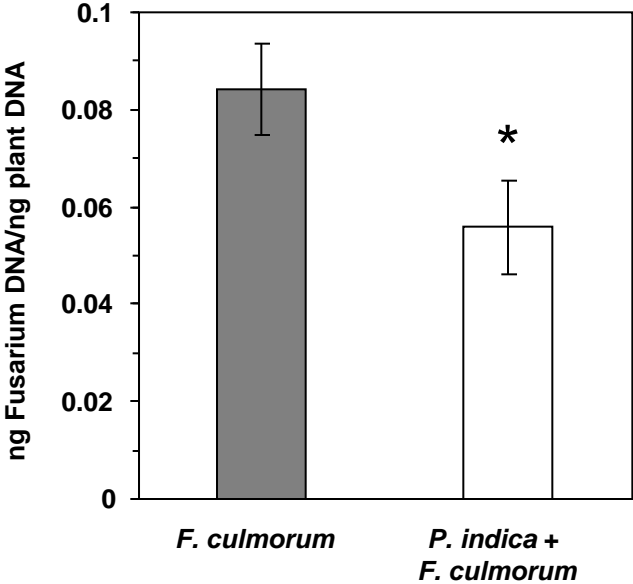


Fig. 3. Harrach, B.D., MPMI

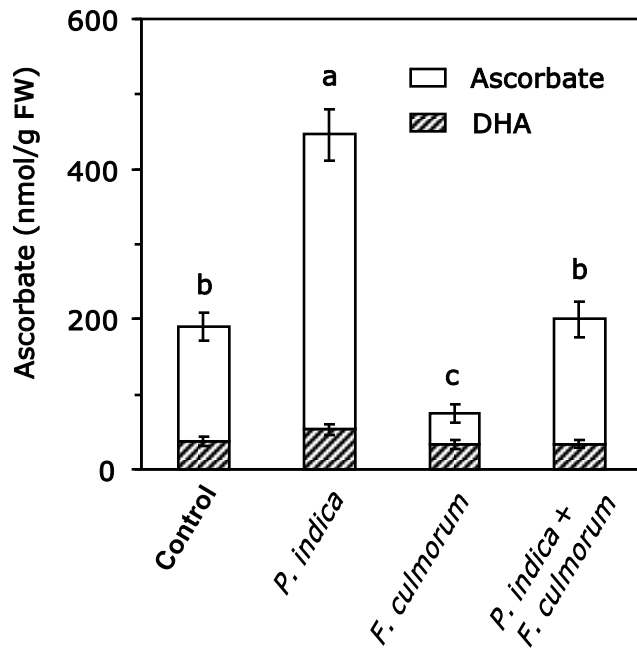


Fig. 4. Harrach, B.D., MPMI

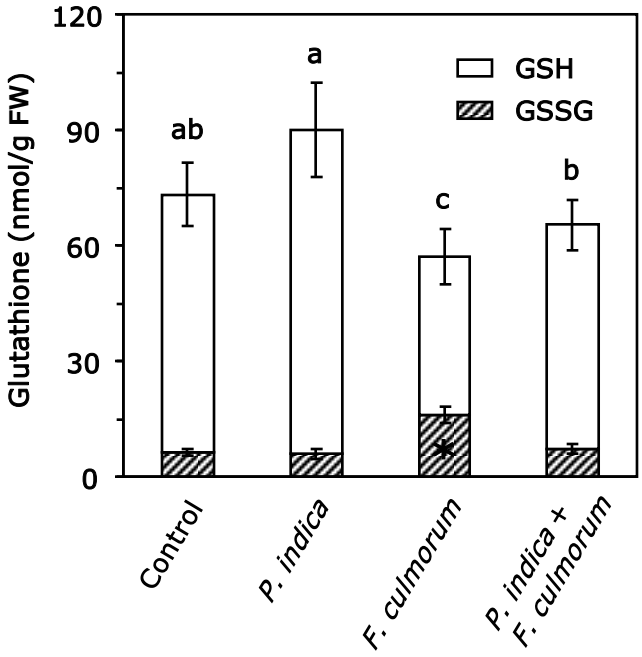


Fig. 5. Harrach, B.D., MPMI

