

# Distance-dependent effects of pathogenic fungi on seedlings of a legume tree: impaired nodule formation and identification of antagonistic rhizosphere bacteria

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## Summary

**1.** The Janzen-Connell (JC) hypothesis proposes that diversity of tree communities is promoted by species-specific enemies that suppress seedling recruitment. Pathogenic soil-borne fungi are often responsible for JC effects. However, previous ecological studies have not placed JC effects in the context of beneficial soil bacteria. Using the JC effect surrounding a subtropical legume tree (*Ormosia glaberrima*) as a model, we characterized tripartite interactions between seedlings, soil fungi and plant-associated bacteria.

**2.** Survival of seedlings grown in soil inocula collected at close distances to focal adult trees was reduced. Half of the experimental units were treated with fungicides to confirm the presence of pathogenic fungi. In a parallel experiment, nitrogen-fixing rhizobia induced fewer nodules on *O. glaberrima* seedlings when soil inocula were collected closer to focal adult trees. An inoculation experiment with rhizobial isolates promoted nodulation of seedlings grown in soil taken at close distances to adult trees, suggesting that rhizobial densities are suboptimal at these locations.

**3.** *Fusarium oxysporum*, a pathogen of *O. glaberrima* seedlings, produced exudates that inhibited growth of most bacteria isolated from nodules. Accordingly, *F. oxysporum* negatively affected rhizosphere colonization of rhizobia in a co-inoculation experiment. Contrariwise, certain *Burkholderia* isolates (from nodules and the rhizosphere) were identified that possess the ability to inhibit growth of *F. oxysporum*. The majority of the antagonistic bacteria were isolated from locations with fungal pathogens, suggesting competition between fungi and rhizosphere bacteria.

**4.** Exclusion of pathogenic fungi by application of fungicides promoted nodulation of *O. glaberrima* seedlings under growth-room and field conditions.

**5. Synthesis.** Our findings indicate that soil-borne pathogens surrounding adult trees are microbial keystone species that locally influence interactions between seedlings and plant-associated bacteria.

**Key-words:** antifungal compounds, *Burkholderia*, *Fusarium*, Janzen-Connell hypothesis, nodulation, plant–soil (below-ground) interactions, rhizobia, symbiosis

## Introduction

Plant-microbe interactions can strongly affect ecosystem function and plant diversity (Wardle *et al.* 2004; van der Heijden, Bardgett & van Straalen 2008; Bardgett & van der Putten 2014). Increasing empirical and experimental data provide evidence that soil-derived pathogens negatively affect seedling recruitment in forests. The Janzen-Connell (JC) hypothesis (Janzen 1970; Connell 1971) proposes that high diversity of

tree communities is related to species-specific pathogens that eliminate the offspring in the neighbourhood of seed-producing adult trees. Soil-borne fungi appear to be the main agents causing JC effects and corresponding fungi have been identified in a number of studies (e.g. Packer & Clay 2000; Thompson *et al.* 2010; Liu *et al.* 2012a). In the subtropical Heishiding Nature Reserve (Guangdong, China), JC effects were found in the neighbourhood of various trees (Liu *et al.* 2012a,b, 2015a; Xu *et al.* 2014; Liang *et al.* 2015). Seedling recruitment of the legume tree *Ormosia glaberrima* was impaired by fungi such as *Fusarium oxysporum* at close distance to focal adult trees (Liu *et al.* 2012a).

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Beneficial microbes promote plant growth and therefore possess the potential to be used as inoculum strains in agriculture (Antoun *et al.* 1998; Avis *et al.* 2008; Hayat *et al.* 2010). They often colonize the rhizosphere and synthesize particular compounds for host plants (e.g. phytohormone producing bacteria). Some of them facilitate the uptake of certain nutrients from the soil (e.g.  $\text{Fe}^{3+}$  uptake via siderophores) while others protect plants from diseases (e.g. antagonistic rhizosphere bacteria producing toxins against pathogens) (Haas & Defago 2005; Hayat *et al.* 2010; Pieterse *et al.* 2014). Various beneficial microbes establish a mutualistic symbiosis with their host plants. In interactions between plants and mycorrhizal fungi, the roots receive from the fungus nutrients, particularly phosphorus (van der Heijden, Bardgett & van Straalen 2008). In nodules of legumes (Fabaceae family), symbiotic bacteria, commonly referred as rhizobia, provide the host plant with fixed nitrogen. Young legume roots are infected by rhizobia (usually via root hairs) and form nodules, a new root organ containing plenty of infected cells. The rhizobia within nodules differentiate into bacteroids and use their nitrogenase enzyme complex for fixation of nitrogen (reduction of atmospheric dinitrogen to ammonia) to the benefit of the host plant (Perret, Staehelin & Broughton 2000). Increasing evidence is provided that effects of beneficial microbes on plant productivity influence the composition of plant communities, thereby affecting plant diversity (e.g. Van der Heijden *et al.* 1998, 2006; Klironomos 2000; Spehn *et al.* 2002; Öpik *et al.* 2006; Wagg *et al.* 2011).

Although distance-dependent effects of fungal soil pathogens on seedling survival have been frequently observed by researchers testing the JC hypothesis (e.g. Packer & Clay 2000; Xu *et al.* 2014), plant–bacteria associations were not examined in these studies. Here, we aimed to investigate whether JC effects caused by fungal pathogens affect nodule formation of *O. glaberrima*. We hypothesized that low seedling survival is driven by increased fungal attack closer to adult trees and that surviving seedlings produce less nodules at these locations. We also expected that pathogenic fungi competitively coexist with rhizobia in the host rhizosphere. Furthermore, we wondered whether there are antagonistic bacteria that can suppress growth of fungal pathogens. An overview of the performed experiments is shown in Fig. 1.

## Materials and methods

### STUDY SITES AND PLANT SPECIES

Heishiding Nature Reserve (Guangdong Province, China; about 4200 ha in size) is a subtropical evergreen broad-leaved monsoon forest. Annual precipitation is 1744 mm in average, with a humid season from April to September and a dry season from October to March (Yu *et al.* 1999). *Ormosia glaberrima* Wu, a species belonging to the Fabaceae (Papilionoideae, Sophoreae) family, was chosen as study species. This species is a common legume tree in the lowland part of the Heishiding Nature Reserve. Soil samples were taken at different distances to focal trees located at different sites of the reserve (Fig. S1 in Supporting Information). The collected soil was used to

set up experiments with *O. glaberrima* seedlings under growth-room conditions. In a first test with soil from 12 focal trees located at six sites, we aimed to determine whether (i) seedling survival is reduced in soil samples closer to the focal tree, (ii) whether this effect was driven by fungal pathogens (by adding fungicides to half of the experimental units), and (iii) whether there are antagonistic bacteria in the rhizosphere of surviving seedlings. In a parallel experiment with the same soil samples, we aimed to determine whether nodulation of seedlings is lower when seedlings are grown in soil collected closer to the focal trees. Soil samples taken at different distances to five adult trees at site 1 (Fig. S1; Liu *et al.* 2012a) were used for further experiments.

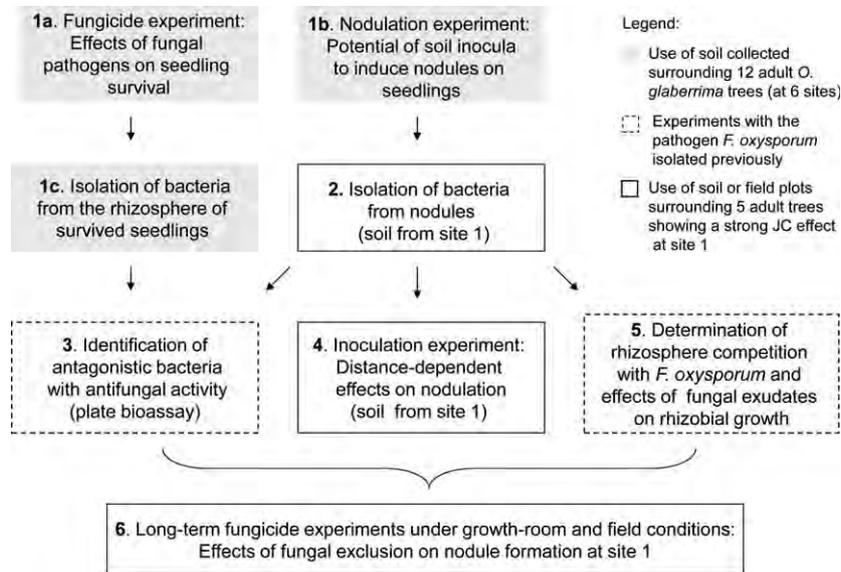
### FUNGI EXCLUSION AND NODULATION TESTS IN THE GROWTH-ROOM

Soil material was collected at different distances (0, 5, 10, 15 and 20 m) to 12 adult *O. glaberrima* trees (located at six sites). The soil samples were taken in autumn and used for growth-room experiments in Guangzhou. Sterilized 300-mL plastic jar units linked with a cotton wick were used for plant growth as described (Liu *et al.* 2012a). Briefly, the upper jar was filled with 250 mL of the collected soil material and the lower jar contained 250 mL of sterilized nitrogen-free B&D nutrient solution. Surface-sterilized *O. glaberrima* seeds were left for germination under sterile conditions for 2 weeks. Seedlings were then placed into the prepared plastic jar units (one seedling per jar unit). Effects of soil-borne fungal pathogens on seedling survival were examined by application of fungicides to half of the plants. A sterile fungicide solution (20 mL per jar unit) was applied every 14 days for 8 weeks. The solution contained  $0.25 \text{ g L}^{-1}$  granular Ridomil Gold 25 G (Syngenta Ltd, Basel, Switzerland) and  $0.5 \text{ g L}^{-1}$  Carbendazim + Quintozene 40% WP (Meibang Pharmaceutical Corporation, Xi'an, China). Jar units of the control group were treated with the same volume of autoclaved water.

In a parallel growth room experiment, aliquots of the soil collected from the 12 adult trees were used to examine whether they contain nodule-inducing rhizobia and whether there are distance-dependent variations in nodule formation. The same growth system was used except that the upper jar units contained 250 mL of collected soil and autoclaved vermiculite in the ratio 1:1 (without fungicides). Examples of nodules formed on *O. glaberrima* roots are shown in Fig. S2.

All jars were kept at constant temperature ( $24 \pm 2 \text{ }^\circ\text{C}$ ) but different humidity and light conditions were used in the two tests. Plants of the fungi exclusion experiment were kept at 80–95% relative humidity and low-light conditions (photosynthetically active radiation  $\approx 25 \mu\text{E s}^{-1} \text{ m}^{-2}$ ; 12 h per day), which are favourable for fungal infection. The nodulation experiment was performed at 40–55% relative humidity and high-light conditions (photosynthetically active radiation  $\approx 850 \mu\text{E s}^{-1} \text{ m}^{-2}$ , 12 h per day), which are ideal for nodule formation. Under high-light/low-humidity conditions, plant biomass accumulation of 25-weeks-old seedlings was about threefold higher than under low-light/high-humidity conditions (soils without fungicides; Fig. S3).

In total, 1320 jar units [(12 adult trees  $\times$  5 distances  $\times$  11 replicates  $\times$  2 (control and treatment)] for the fungi exclusion experiment and 660 jar units (12 adult trees  $\times$  5 distances  $\times$  11 replicates) for the nodulation experiment were prepared. Positions of jars in the growth-room were randomly changed every week. Both experiments lasted 25 weeks. Seedling survival was examined for the fungi exclusion experiment. For the nodulation experiment, number of formed nodules and total plant dry weight were determined.



**Fig. 1.** Overview of experiments performed in this study. An initial experiment with soil collected at different distances to 12 *O. glaberrima* trees at six sites (see Fig. S1 in Supporting Information) was conducted to determine JC effects (fungi exclusion experiment; 1a), to investigate distance-dependent nodule formation (1b), and to isolate rhizosphere bacteria from surviving seedlings (1c). In another experiment (2), nodule bacteria were isolated from formed nodules using soil inocula from focal trees showing a strong JC effect (five trees at site 1). Bacteria isolated from the rhizosphere (1c) or nodules (2) were then screened for their potential to inhibit growth of the fungus *F. oxysporum*, a pathogen isolated previously from *O. glaberrima* (3). An inoculation experiment with isolated rhizobia and different soils from the five trees at site 1 was conducted to test whether rhizobial density is suboptimal for nodulation when soil inocula derive from locations close to adult trees (4). Effects of *F. oxysporum* on rhizobia were further investigated in a rhizosphere competition experiment and by analysing the impact of fungal exudates on bacterial growth in a plate assay (5). Finally, long-term experiments with fungicides were performed under growth-room and field conditions to investigate whether nodulation of *O. glaberrima* seedlings is promoted in the absence of pathogenic fungi (6).

Furthermore, soil samples were collected from different locations (five distances) surrounding five adult trees at site 1 in two series (in successive periods; totally 975 seedlings) to isolate rhizobia from nodules of *O. glaberrima* seedlings and to perform an inoculation experiment (see below).

Finally, soil samples were collected from the 0-m and 5-m distances to five adult trees (at site 1) and used for a long-term fungi exclusion experiment in which nodule formation was examined after 60 weeks. Seedlings grown in totally 260 jar units [5 adult trees  $\times$  2 distances  $\times$  13 replicates  $\times$  2 (fungicide treatment and control)] were kept under low-light/high-humidity conditions. The fungicide treatment was performed like in the previous fungi exclusion experiment. At the time of harvest, seedling survival was counted. Nodule parameters (number of nodules; nodule fresh weight) and plant dry weight were determined for surviving seedlings.

#### ISOLATION OF RHIZOBIA FROM NODULES

Bacteria were isolated from nodules of seedlings that were grown in soil taken at different distances to five focal trees located at site 1. Isolation and identification of nodule bacteria was performed according to Somasegaran & Hoben (1994). Colonies on TY (Beringer 1974) plates supplemented with 20  $\mu\text{g mL}^{-1}$  cycloheximide were purified by transferring bacteria on fresh plates. To obtain genomic DNA (Chen & Kuo 1993), liquid cultures of isolates (1 isolate per nodule) were kept at 27 °C on a rotary shaker (220 rpm) until  $\text{OD}_{600} \approx 0.5$  was reached. The DNA was then used for PCR amplification of the 16S ribosomal RNA gene with degenerate primers (Weisburg *et al.* 1991). Synthesis of the primers and sequencing were carried out by Invitrogen (Guangzhou, China). Phylogenetic analysis was performed with CLUSTALX 2.1 (Conway Institute, University College Dublin, Dublin, Ireland) and

PHYLIP 3.695 (University of Washington, Seattle, WA, USA) software packages by using the neighbour-joining method with Kimura's two-parameter model (Kimura 1980).

#### ISOLATION OF ANTAGONISTIC BACTERIA FROM THE RHIZOSPHERE OF *O. GLABERRIMA* SEEDLINGS

Antagonistic bacteria secreting antifungal compounds were isolated from the rhizosphere of surviving *O. glaberrima* seedlings grown in soil collected around 12 adult trees at six sites (using control soil without fungicide treatment; see above). When available, five seedlings (grown in five jars) per location (0, 5, 10, 15 and 20 m distances to focal trees) were used. In total, 14 100 rhizosphere isolates were tested for potential antifungal activity. At the time of harvest (25 weeks after planting seedlings into the jars), *O. glaberrima* root tips (1 cm) were removed and roots, soaked in 1 mL of Ringer's solution (Klement, Rudolph & Sands 1990), were vigorously shaken on a vortex (model BE3100, Qilin Beier Instrument Manufacturing Co., Haimen, Jiangsu, China) for 15 min at 2500 rpm. Isolated rhizosphere bacteria grown on threefold diluted TY plates containing 20  $\mu\text{g mL}^{-1}$  cycloheximide (60 colonies per seedling) were randomly selected to test their ability to inhibit hyphal growth of *F. oxysporum* (isolate F06; Liu *et al.* 2012a). A given bacterial isolate was first placed at two sites of a potato dextrose agar (PDA, Qingdao Hope Bio-Technology Corporation, Qingdao, China) plate. The PDA plates were incubated at 27 °C for 48 h in the dark and mycelium (6 mm in diameter) from the periphery of a 7-day-old culture of *F. oxysporum* on PDA agar was then placed in the centre of the plate. Plates containing mycelium without bacteria were included into the experiment. Plates were incubated at 27 °C until the mycelium reached the bacterial colonies (ca. 10 days). In the same way, bacteria isolated from

*O. glaberrima* nodules (Table S1) were examined for potential antifungal activity.

#### INOCULATION OF *O. GLABERRIMA* SEEDLINGS WITH RHIZOBIA

An inoculation experiment with *O. glaberrima* seedlings and rhizobia was performed with different soil samples to increase rhizobial density in the rhizosphere. Soil was collected at different distances (0, 5, 10, 15 and 20 m) to five adult *O. glaberrima* trees at site 1. Growth conditions for nodule formation (high-light/low-humidity) were used as described above. Inoculation was performed 10 days after planting of *O. glaberrima* seedlings into the jar units. Two *Burkholderia* strains (SY26, SY74) and three *Rhizobium* strains (SY197, SY6 and SY273), available at the time of the experiment (Table S1), were used as a mixed inoculum. Strains grown in liquid TY medium were mixed equally ( $OD_{600} \approx 1.2$ ). After centrifugation, bacterial pellets were re-suspended in 10 mM  $MgSO_4$  and adjusted to  $OD_{600} \approx 0.2$ . Each seedling was inoculated with 3 mL of bacterial suspension containing a mixture of these five strains or mock-inoculated with 3 mL of 10 mM  $MgSO_4$ . In total, 650 jar units were prepared: 325 jar units (5 adult trees  $\times$  5 distances  $\times$  13 replicates) were inoculated and 325 served as controls. Plants were harvested 25 weeks post inoculation and the number of nodules and plant dry weight were determined.

#### DETERMINATION OF RHIZOSPHERE COMPETITION BETWEEN RHIZOBIA AND *F. OXYSPORUM*

To analyse the effect of pathogenic fungi on rhizobia–host interactions, a rhizosphere competition experiment was performed with a rhizobial population and the pathogen *F. oxysporum*. *O. glaberrima* seeds were left to germinate for 15 days on 0.5% (w/v) water agar plates. Seedlings (root length of ca. 3 cm) were soaked for 20 min in a suspension containing the 15 rhizobial strains isolated from *O. glaberrima* nodules. The rhizobial population consisted of the 15 rhizobial strains listed in Table S1. These bacteria were trapped from soil at site 1 from where *F. oxysporum* was isolated previously (isolate F06; Liu *et al.* 2012a). Bacteria were grown in liquid TY medium ( $OD_{600} \approx 0.4$ ) and then mixed (1:1) with 1% (w/v) methylcellulose (Sigma Chemical Co., St. Louis, MO, USA). The coated seedlings were air-dried for 30 min under sterile conditions. Seedlings coated with rhizobia were then planted into jar units containing 250 mL of sterilized vermiculite and expanded clay (ratio 1:1) and  $10^8$  *F. oxysporum* spores. Control plants were grown in the substrate without spores. Low-light/high-humidity conditions (see above) were used in the growth-room. Seven and 14 days after planting, *O. glaberrima* root tips (1 cm) were removed and roots soaked in 1 mL of Ringer's solution were shaken on a vortex (model BE3100, Qilin Beier Instrument Manufacturing Co.) at 2500 rpm for 15 min. Population sizes of rhizobia in the rhizosphere (colony forming units) were determined by dilution plating on solid TY medium supplemented with 20  $\mu$ g mL<sup>-1</sup> cycloheximide.

#### INHIBITION EFFECTS OF *F. OXYSPORUM* EXUDATES ON RHIZOBIA

To analyse antibacterial activity of *F. oxysporum*, effects of fungal exudates on rhizobial growth were tested on agar plates according to Hoffman, Garrison & Dohlman (2002). *F. oxysporum* was shaken (180 rpm at 27 °C) in Sabouraud medium (Qingdao Hope Bio-Technology Corporation) for 10 days. Culture supernatants (100 mL) were collected by centrifugation at 12 857 g. The material was dried in a

speed-vac evaporator and re-suspended in 20  $\mu$ L H<sub>2</sub>O. Paper filter disks, each impregnated with 11  $\mu$ L of the solution, were then placed on 2% (w/v) TY agar plates, on which test rhizobia were spread before. Concentrated Sabouraud medium without *F. oxysporum* was used as a control. Plates were sealed with parafilm, incubated at 27 °C for 3 days and then photographed. The 15 rhizobial strains shown in Table S1 were used for the bioassay. Halo-like inhibition zones of bacterial growth surrounding the paper filter disks indicated presence of antibacterial compounds in exudates of *F. oxysporum*.

#### FIELD EXPERIMENT

A fungal exclusion experiment with *O. glaberrima* seedlings planted into fungicide-treated plots at site 1 of the Heishiding Nature Reserve has been initiated as reported (Liu *et al.* 2012a). Briefly, seedlings were planted at the distance of 0, 5, 10 15 and 20 m to 5 adult *O. glaberrima* trees and plots were separated into two halves. One half was treated with fungicide (Ridomil Gold 25 G; Carbendazim + Quintozene 40% WP) while the other half served as a control. Fungicides were applied during the first 38 weeks. Seedlings were kept in the field for another 56 weeks without any treatment. The number of nodules per plant and the biomass of surviving seedlings were determined at the end of the experiment.

#### STATISTICAL ANALYSIS

Fungi exclusion by treatment with fungicides and the parallel nodulation experiments with soil collected from 12 adult trees at six sites were statistically analysed by a generalized linear mixed model (GLMM) as follows: Distance to focal adult trees (location where soil was collected surrounding adult trees) and fungicide treatment were considered as fixed factors and the 12 focal trees as random effect in the fungal exclusion experiment. In the nodulation experiment, the distance to adult trees was considered as a fixed factor and the 12 focal trees as random effect. Binomial distribution and logarithm-link function for the fungal exclusion experiment and Poisson distribution and log-link function for the nodulation experiment were used to test the effect of distance to adult trees on seedling survival and nodule formation, respectively. Pearson's chi-square tests were employed to analyse the effects of fungicide treatment on seedling survival for each distance in the initial experiment with soil from 12 adult trees at six sites and for a long-term experiment with soil from five adult trees at site 1. Where indicated (growth-room and field experiments), effects of rhizobial inoculation and fungicides on nodulation and biomass of *O. glaberrima* seedlings (as compared to mock-treated controls) were analysed by independent *t* tests. Likewise, *t* tests were employed for analysis of effects of *F. oxysporum* inoculation on the density of rhizobia (colony forming units) in the rhizosphere of *O. glaberrima* seedlings. Data (nodule number, nodule fresh weight, plant dry weight and colony forming units) were log transformed to fit normal distribution if necessary. All statistical analyses were performed using the statistical programming language R software, version 2.12.0 (R Development Core Team 2010).

## Results

#### SOIL-BORNE EFFECTS ON SEEDLING SURVIVAL AND NODULE FORMATION

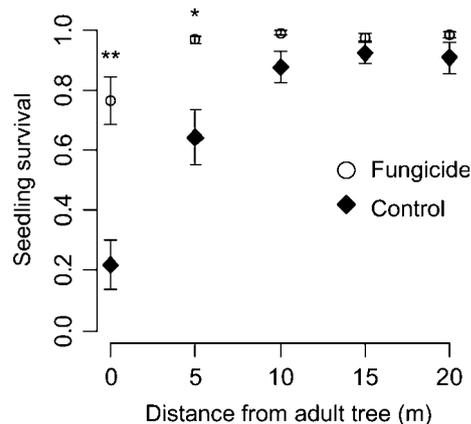
A treatment with fungicides promoted survival of *O. glaberrima* seedlings grown in soils from different locations surrounding adult trees (Fig. 2 and Table 1). Effects of the

fungicide treatment considerably varied with respect to the 12 focal adult trees around which the soil samples were collected. Seedling survival was also distance-dependent. Survival of seedlings grown in soil collected close to adult trees (0 and 5 m distance) was increased by application of the fungicides, indicating a JC effect caused by fungal pathogens such as *F. oxysporum* (Liu *et al.* 2012a).

The results of a parallel experiment showed that the soil inocula collected at different distances to the 12 adult trees differently influenced nodule formation (Table 2). Significantly fewer nodules were formed when seedlings were grown in soil inocula collected at close distances (0–5 m;  $n = 24$ ) in comparison to inocula taken at greater distance (10–20 m;  $n = 36$ ) ( $t$  test;  $t = -2.5$ ,  $P = 0.0122$ ; Fig. S4). Likewise, the use of soil inocula collected in the neighbourhood of adult trees (0–5 m) resulted in reduced plant biomass as compared to inocula taken farther away (10–20 m) ( $t$  test;  $t = -4.2$ ,  $P < 0.0001$ ; Fig. S4). Taken the data of these two experiments together, they indicate that effects of soil-borne pathogenic fungi and nodule formation are both distance-dependent.

#### ISOLATION OF RHIZOBIA AND ANTAGONISTIC BACTERIA

Bacteria were isolated from nodules that were harvested from *O. glaberrima* seedlings grown in soil collected from trees showing an obvious JC effect (five trees at site 1 of the reserve). In total, 337 nodule associated bacteria were retrieved and 220 isolates could be classified as rhizobia based on 16S rRNA gene sequence analysis. Accordingly, the rhizobia could be grouped into 15 distinct strains (with different 16S rRNA sequences) that belong to the genera *Rhi-*



**Fig. 2.** Effect of fungicide application on survival of *O. glaberrima* seedlings grown in different soils under controlled growth-room conditions. Soil samples were collected at indicated distances to 12 adult trees at six sites. The fungal exclusion experiment with 11 seedlings per location and treatment was conducted at low-light/high-humidity conditions and lasted 25 weeks. Data indicate means  $\pm$  SE (locations treated as replicates;  $n = 12$ ). Significant differences of seedling survival in response to the fungicide treatment at a given distance are marked with asterisks (Pearson's chi-square test;  $P < 0.01$ , \*\*,  $P < 0.05$ , \*).

**Table 1.** Results of a GLMM examining the effects of fungicide treatment and distance (soil collected at 0, 5, 10, 15 and 20 m from 12 focal adult trees) on seedling survival of *O. glaberrima* in a growth-room experiment (low-light/high-humidity conditions)

Fixed effects	Estimate coefficients (SE)	Z-value	$P$ ( $> z $ )
Distance	0.2354 (0.0380)	6.201	$<2e-16^*$
Fungicide	-2.4132 (0.2759)	-8.747	$<2e-16^*$
Random effect	$\chi^2$		$P$ ( $>$ Chisq)
Adult trees	18.996		1.31e-05*

\*Significant result.

**Table 2.** Results of a GLMM examining the effects of distance (soil collected at 0, 5, 10, 15 and 20 m from 12 focal adult trees) on nodulation (nodule number) of *O. glaberrima* seedlings in a growth-room experiment (high-light/low-humidity conditions)

Fixed effects	Estimate coefficients (SE)	Z-value	$P$ ( $> z $ )
Distance	0.0087 (0.0020)	4.424	9.68e-06*
Random effect	$\chi^2$		$P$ ( $>$ Chisq)
Adult trees	393.41		$<2e-16^*$

\*Significant result.

*zobium* ( $\alpha$ -rhizobia), *Mesorhizobium* ( $\alpha$ -rhizobia) and *Burkholderia* ( $\beta$ -rhizobia) (Table S1). A corresponding phylogenetic tree is shown in Fig. S5. *Rhizobium* bacteria were frequently isolated from nodules. *Mesorhizobium* isolates were less often obtained and all isolates derived from nodules of seedlings grown in soil collected at a middle-distance (5–15 m). Remarkably, *Burkholderia* isolates were often trapped when seedlings were grown in soil collected in the close neighbourhood (0–5 m distance) of adult trees (Table S1).

As seedlings could establish symbiosis with rhizobia, we wondered whether antagonistic bacteria secreting antifungal compounds are also associated with *O. glaberrima*. Seedlings grown in different soils from 12 adult trees (see above) were used for isolation of rhizosphere bacteria. Each isolate was then individually tested for antifungal activity on agar plates using *F. oxysporum* (isolate F06), a pathogenic fungus previously isolated from *O. glaberrima* seedlings (Liu *et al.* 2012a). An overview of the obtained results is shown in the Tables S2 and S3. Most antagonistic rhizosphere bacteria (18 of totally 23 isolates) were trapped from seedlings grown in soil from locations containing pathogenic fungi as deduced from the corresponding fungal exclusion experiment with fungicides (Fig. 2). These findings provide clues for direct fungus–bacteria interactions in the rhizosphere of *O. glaberrima*. Based on 16S rRNA sequence analysis, 22 antagonistic bacteria were classified as *Burkholderia* and one as *Pseudomonas* (Table S3). Fig. S6 shows the results of a corresponding phylogenetic analysis for the *Burkholderia* 16S rRNA sequences obtained in this study (strains isolated from the rhizosphere and from nodules).

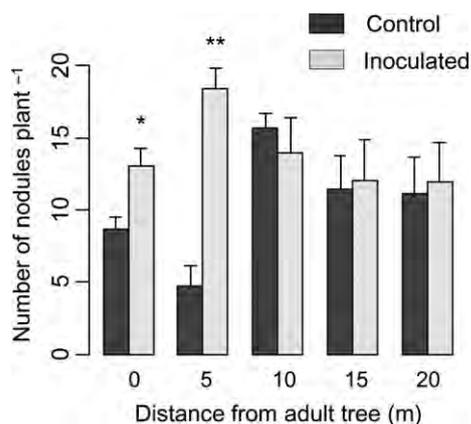
The rhizobial strains that were isolated from *O. glaberrima* nodules were also subjected to the plant bioassay with *F. oxysporum*. Interestingly, *Burkholderia* strain SY28 efficiently inhibited growth of this fungus. Fungal growth inhibition caused by SY28 was also observed when various other fungi were tested. However, three fungi appear to be resistant to the antifungal compounds secreted by SY28 (Fig. S7).

#### DISTANCE DEPENDENT EFFECTS OF RHIZOBIAL INOCULATION

The results of the initial nodulation experiment indicated suboptimal nodulation in close neighbourhood of focal trees. We therefore performed an inoculation experiment to test whether the population density of rhizobia is a limiting factor for nodule formation. Soil samples were collected at different distances to five adult trees located at site 1. Bacterial suspensions of five rhizobial strains (see Table S1) were mixed and used as an artificial inoculum. The number of formed nodules was compared between inoculated and non-inoculated seedlings. As shown in Fig. 3, increased nodulation in response to rhizobial inoculation was found for the close distances (soil samples collected at 0 and 5 m from adult trees), whereas no significant inoculation effects were seen for soil samples taken more far away (10–20 m). These data indicate that rhizobial densities are suboptimal at locations close to adult trees and suggest a potential competition between rhizobia and pathogenic fungi in this zone.

#### COMPETITION BETWEEN RHIZOBIA AND *F. OXYSPORUM* IN THE RHIZOSPHERE OF *O. GLABERRIMA*

To analyse competition between rhizobia and pathogenic fungi experimentally, we co-inoculated *O. glaberrima* seedlings with



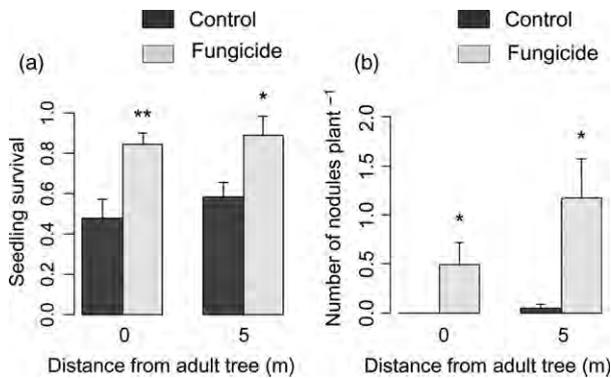
**Fig. 3.** Effects of rhizobial inoculation on nodulation of *O. glaberrima* seedlings grown in soil collected at different distances to five adult trees at site 1. The growth-room experiment with a mixed inoculum (five strains) and soil from totally 25 locations (13 seedlings per location) was conducted at high-light/low-humidity conditions and lasted 25 weeks. Data indicate means  $\pm$  SE (locations treated as replicates;  $n = 5$ ). Significant inoculation effects are marked with asterisks ( $t$  tests;  $P < 0.01$ , \*\*;  $P < 0.05$ , \*).

15 rhizobial strains (Table S1) and the pathogen *F. oxysporum* previously isolated from *O. glaberrima*. When analysed 14 days post inoculation, the rhizobial population in the rhizosphere was significantly lower in the presence of the fungus ( $t$  test,  $n = 6$ ;  $t = 4.2$ ,  $P = 0.0424$ ; Fig. S8). These data indicate that *F. oxysporum* negatively influenced rhizobial colonization of the rhizosphere. In accordance with these findings, a bioassay with rhizobia and *F. oxysporum* crude extract showed that fungal exudates inhibited growth of most tested strains. However, two *Burkholderia* strains (SY164 and SY28) as well as a *Mesorhizobium* strain (SY247) showed normal growth, indicating a resistance mechanism against one or several antibacterial compounds produced by *F. oxysporum* (Fig. S9).

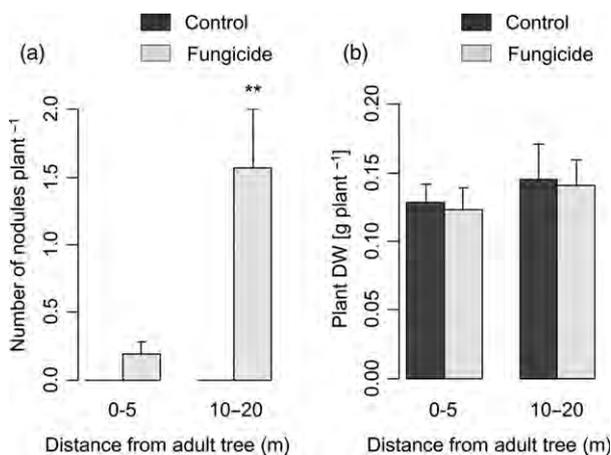
#### FUNGAL EXCLUSION PROMOTES NODULATION OF *O. GLABERRIMA* SEEDLINGS

Taken the data from the performed experiments together, they suggest that nodulation of *O. glaberrima* seedlings in the close neighbourhood of adult trees is negatively influenced by pathogenic fungi such as *F. oxysporum*. To test this hypothesis, a fungi exclusion experiment with soil inocula from five adult trees at site 1 was conducted. Soil samples (0 and 5-m distances) were chosen for the long-term growth-room experiment. As expected, seedling survival was significantly increased by the fungicide treatment, confirming the presence of pathogenic fungi in these soils (Pearson's chi-square test,  $n = 5$ ;  $\chi^2 = 6.7$ ,  $P = 0.0097$  for the 0-m distance;  $\chi^2 = 4.2$ ,  $P = 0.0412$  for the 5-m distance). Nodules on surviving seedlings were not (0-m distance) or only occasionally (5-m distance) observed on control seedlings grown in soil without fungicide. In response to the fungicide treatment, nodulation of seedlings occurred for soil from both distances and the number of nodules increased 27-fold for the 5-m distance ( $t$  test,  $n = 5$ ;  $t = 2.8$ ,  $P = 0.0067$ ; Fig. 4). Similar data were also observed with respect to the nodule biomass per seedling as shown in Fig. S10. The fungicide treatment slightly reduced the total plant biomass in this experiment (for the 0-m distance significantly;  $t$  test,  $n = 5$ ;  $t = 2.3$ ,  $P = 0.0298$ ). These findings indicate that exclusion of fungi strongly promoted nodulation under growth-room conditions.

Finally, we investigated whether exclusion of fungi also influences nodulation of *O. glaberrima* seedlings in the field. Nodule formation was examined for seedlings in plots surrounding adult trees at site 1. Halves of these plots were repeatedly treated either with fungicides or water alone. At the time of harvest, seedlings survived in plots surrounding three trees. Remarkably, nodules were only formed on roots of seedlings that were grown in soil treated with fungicides. Moreover, the number of nodules per seedling at the 10–20 m distance (8 plots;  $n = 8$ ) was significantly higher than that at the 0–5 m distance (9 plots;  $n = 9$ ) ( $t$  test;  $t = -3.3$ ,  $P = 0.0055$ , Fig. 5a). The fungicide application resulted in reduced plant biomass accumulation but differences were not significant (Fig. 5b). Hence, exclusion of fungi in the soil enabled rhizobia to induce nodules on *O. glaberrima* seedlings under field conditions at site 1.



**Fig. 4.** Fungal exclusion promotes nodulation on *O. glaberrima* roots in a long-term growth-room experiment. Soil samples were collected in the neighbourhood of five adult trees and treated either with fungicide or water as a control. The experiment was conducted at low-light/high-humidity conditions and lasted 60 weeks. At the time of harvest, seedling survival (a) and the number of nodules of surviving plants (b) were determined. Data indicate means  $\pm$  SE (locations treated as replicates;  $n = 5$ ). Significant effects of fungicide treatment at indicated distances are marked with asterisks (Pearson's chi-square tests for (a) and  $t$  tests for (b);  $n = 5$ ;  $P < 0.01$ , \*\*;  $P < 0.05$ , \*).



**Fig. 5.** Fungicide application enables nodulation of *O. glaberrima* seedlings in a field experiment. Seedlings were grown in plots surrounding adult trees at site 1. Plots were treated with fungicide or not during the first 38 weeks. At the time of harvest (94 weeks post planting), the number of nodules (a) and the biomass (b) of surviving seedlings were determined. Data indicate means  $\pm$  SE (plots treated as replicates). Asterisks indicate a significant effect of fungicide treatment on nodulation at the 10–20 m distance ( $t$  test;  $P < 0.01$ , \*\*).

## Discussion

JC effects of soil pathogens, also known as negative soil feedbacks, provide explanations for species co-existence in forests with high species diversity (Packer & Clay 2000; Petermann *et al.* 2008; Bagchi *et al.* 2010; Fricke, Tewksbury & Rogers 2014; Xu *et al.* 2014; Spear, Coley & Kursar 2015). According to the JC hypothesis, seedling recruitment depends on the distance of seedlings to their parent trees. Low movement of pathogens, particularly soil fungi, is a prerequisite for variations in seedling survival surrounding adult trees (Chen *et al.* 2010; Comita *et al.* 2010; Spiegel & Nathan 2012; Souza,

Franco & Callaway 2013; Fricke, Tewksbury & Rogers 2014). In most cases, JC effects of pathogenic fungi have been indirectly quantified by fungicide experiments in which seedling survival was examined. Using this approach, we determined the strength of a JC effect on *O. glaberrima* seedlings for different soil inocula taken at different distances to focal adult trees. Strong JC effects we found for soil collected at a close neighbourhood (0–5 m distance) of adult trees (Fig. 2), suggesting that pathogenic fungi accumulate surrounding adult trees in a ring-shaped way. Our work confirms that soil inocula from five trees at site 1 of the Heishiding Reserve contain pathogenic fungi as reported previously (Liu *et al.* 2012a). Soil from a single tree located at another site of the reserve (tree 8 at study site 2; Fig. S1) also showed a JC effect, whereas no (or very little) distance-dependent effects of pathogenic fungi on this tree were reported previously (Liu *et al.* 2012a) and in a more recent paper (Liu *et al.* 2015b). The latter article claimed that single adult trees at low density sites show significantly lower JC effects as compared to trees at abundant sites. However, these conclusions (drawn from data of three single trees) are inconsistent with the present data on tree 8. The strength of a given JC effect is certainly influenced by environmental variations such as humidity and light conditions (Fig. S3). Future long-term experiments are required to study the occurrence and strength of JC effects over space and time.

Some *O. glaberrima* seedlings were not killed by the fungal pathogens in our tests, even when low-light/high-humidity conditions were chosen (Fig. 2). Accordingly, surviving seedlings grown in soil collected at close distance to adult trees could be characterized with respect to their associations with rhizobia and antagonistic rhizosphere bacteria. Under high-light/low-humidity conditions, pathogen effects were largely suppressed and seedlings were well-nodulated (Figs 3 and S4). These findings highlight the ability of *O. glaberrima* seedlings to survive in different environments and provide explanations for resistance to fungal infection when microclimatic conditions change, e.g. due to a fallen tree. Similar modulating effects of light and humidity on development of fungal diseases (Augsburger 1984; McCarthy-Neumann & Ibáñez 2013) and nodulation of legumes (Ta & Faris 1988; Bonomi *et al.* 2012) have been reported in other studies.

The findings of our paper suggest that pathogenic fungi surrounding adult trees such as *F. oxysporum* are microbial keystone species that have a great impact on other plant-associated microbes (Berry & Widder 2014). Removal of these fungi by fungicide application strongly promoted nodule formation in growth-room and field experiments (Figs 4 and 5). Moreover, our findings point to the possibility that the presence of these fungi is a driving force for establishment of bacterial populations with antagonistic properties. Most of these bacteria (18 of 23 isolates; Table S2) originated from locations for which a fungicide effect on seedling survival was found. In other words, rhizosphere bacteria producing antifungal compounds may have a selective advantage at locations where pathogenic soil fungi are abundant. These findings provide first clues on a possible relationship between

antagonistic bacteria and pathogenic fungi causing a JC effect on *O. glaberrima* seedlings. Future inoculation experiments are required to examine the ability of the isolated antagonistic bacteria to attenuate JC effects. We suggest that seedlings associated with antagonistic bacteria are more resistant to fungal infection (Avis *et al.* 2008; Pieterse *et al.* 2014).

The antagonistic bacteria, with the exception of a *Pseudomonas* isolate, were classified as bacteria belonging to the genus *Burkholderia* (Table S3). Antifungal compounds produced by *Burkholderia* strains include glycopeptides (xylocandins, cepacidines; Bisacchi *et al.* 1987; Lee *et al.* 1994), cyclic lipopeptides (so-called burkholdines; Tawfik *et al.* 2010), pyrrolnitrin, phenazine (Cartwright, Chilton & Benson 1995), 4-quinolinones (Moon *et al.* 1996), 2-hydroxy-methyl-chroman-4-one (Kang *et al.* 2004) and various volatiles (Groenhagen *et al.* 2013; Tenorio-Salgado *et al.* 2013). Moreover, certain *Burkholderia* strains establish symbiosis with fungi of the genus *Rhizopus* and produce rhizoxin, a phytotoxin acting on  $\beta$ -tubulin (Partida-Martinez & Hertweck 2005). *Burkholderia* bacteria show very different lifestyles: free-living environmental strains, pathogenic bacteria (including opportunistic human pathogens) and symbiotic bacteria (including nitrogen fixers) have been identified (Winsor *et al.* 2008). Based on 16S rRNA sequence analysis, most *Burkholderia* strains isolated from *O. glaberrima* nodules are related to nodule-inducing *Burkholderia* species (group A of *Burkholderia* species; Estrada-de los Santos *et al.* 2013). Certain isolated strains however, namely SY27, SY28 and SY164 (Fig. S6) may belong to the *B. cepacia* complex (group B of *Burkholderia* species) and perhaps represent rather nodule-associated bacteria than nodule-inducing nitrogen fixers. It is worth mentioning in this context that strain SY28 produces compounds that efficiently impair fungal growth (Fig. S7).

A direct interaction between fungal pathogens and nodule bacteria is further supported by the finding that the characterized *F. oxysporum* isolate secretes antibacterial compounds. Growth of most rhizobia was inhibited under our test conditions (Fig. S9). Production of antimicrobial compounds, including the mycotoxin fusaric acid, has been described for various *F. oxysporum* strains. Such compounds may have a negative impact on co-existing bacteria (Notz *et al.* 2002; de Boer *et al.* 2005; Folman *et al.* 2008).

Plant-dependent effects of fungal pathogens on nodulation of *O. glaberrima* seedlings likely also exist. Fungal pathogens can impair establishment of symbiosis by weakening host plants and activation of plant defense reactions (Van Loon 2007; Evangelisti, Rey & Schornack 2014) may impair nodule formation. The trade-off between production of defense compounds and nodule formation appears to be actively controlled by the host plant. For example, *Lotus japonicus* plants treated with flg22 (flagellin peptide elicitor) showed increased defense gene expression, whereas nodule formation was considerably reduced (Lopez-Gomez *et al.* 2012). Furthermore, competitiveness of fungal pathogens such as *F. oxysporum* and rhizobia in the rhizosphere (Fig. S8) may depend on root exudates and their altered composition in response to pathogen infection (Bais *et al.* 2006; Raaijmakers *et al.* 2009). It is

worth mentioning that mutualistic arbuscular mycorrhizal fungi can reduce diseases and thus counteract JC effects (Avis *et al.* 2008; Liang *et al.* 2015). However, no mycorrhizal fungi were observed in roots of *O. glaberrima* seedlings in our experiments (L. Liu, Z.-P. Xie & C. Staehelin, unpublished observations).

Rhizobial inoculation resulted in increased nodule formation of seedlings grown in soil taken at close distance to focal adult trees showing a JC effect (Fig. 3). These findings indicate that the density of rhizobia was likely a limiting factor for nodule formation. Limited availability of rhizobia can impair the performance of legumes such as invasive *Acacia* species (Wandrag *et al.* 2013). Likewise, studies on soybean and other agricultural legumes showed that an increasing number of rhizobia in the host rhizosphere can result in a linear increase in nodulation (Elegba & Rennie 1984; Patrick & Lowther 1995; Deaker, Roughley & Kennedy 2004). However, inoculation effects can be negatively influenced by indigenous rhizobial populations with high nodulation competitiveness (Singleton & Tavares 1986; Thies, Singleton & Bohlool 1991). Like for *O. glaberrima* (Table S1), phylogenetically diverse bacteria have been isolated from *Acacia* nodules. Inoculation experiments with two Australian *Acacia* species indicated that plant growth varied depending on the rhizobial inoculum and that co-inoculation with several strains often reduced plant growth as compared to inoculation with single strains (Barrett *et al.* 2015). Future inoculation experiments are required to examine the benefits of specific rhizobial strains on *O. glaberrima* growth and to test at which life stage nitrogen-fixation in nodules translates into a fitness advantage. Taking into consideration that nodule formation of seedlings can be reduced by fungal pathogens at close distance to adult trees, we expect a similar effect on tree juveniles. In other words, young trees may have a better nitrogen supply when they are localized far from parent trees.

## Conclusion and outlook

The findings of this study indicate that pathogenic fungi such as *F. oxysporum* are microbial keystone species that influence other plant-associated soil microbes. Our data indicate that soil fungi accumulate at a close distance to adult *O. glaberrima* trees and negatively affect nodulation of seedlings. Removal of fungal pathogens by fungicides resulted in stimulated nodule formation, indicating a relationship between fungal pathogens and nitrogen fixing bacteria. Moreover, our findings provide clues that the presence of pathogenic soil fungi around adult trees promotes accumulation of antagonistic *Burkholderia* strains in the rhizosphere of seedlings. Hence, pathogenic fungi surrounding focal adult trees locally affect the composition of microbial communities. The resulting spatial heterogeneity of microbes (Bissett *et al.* 2010; Hanson *et al.* 2012) likely has major implications on microhabitat and fitness of seedlings and tree juveniles. Future studies are required to examine the dynamic relationship between fungi causing JC effects and beneficial bacteria under experimental growth-room conditions and in their natural

environment. Antagonistic bacteria with the potential to inhibit growth of fungal pathogens are expected to attenuate JC effects. Special attention should be paid to light and humidity conditions, which may have crucial effects on tripartite plant-fungus-bacteria interactions.

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## Data accessibility

Nucleotide sequences have been deposited at the GenBank data base: Accession numbers KP687347, KP687348, KP687349, KP687350, KP687354, KP687358, KP687359, KP687360, KP687366, KP687368, KP687369, KP687370, KP687371, KP687377, KP687379 and KP687398 to KP687410.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Strains isolated from nodules of *O. glaberrima* seedlings grown in soil collected around five adult trees at site 1 of the Heishiding Nature Reserve.

**Table S2.** Frequency and origin of antagonistic bacteria isolated from the rhizosphere of *O. glaberrima* seedlings.

**Table S3.** List of antagonistic bacteria isolated from the rhizosphere of *O. glaberrima* seedlings.

**Figure S1.** Location of study sites in the Heishiding Nature Reserve.

**Figure S2.** Examples for nodules formed on *O. glaberrima* seedlings.

**Figure S3.** Plant biomass of 25-weeks-old *O. glaberrima* seedlings grown under different growth conditions.

**Figure S4.** Nodulation of *O. glaberrima* seedlings grown in soil collected at different distances to 12 focal adult trees.

**Figure S5.** Phylogenetic analysis of isolated rhizobial strains (SY strains) based on 16S rRNA sequences.

**Figure S6.** Phylogenetic analysis of *Burkholderia* strains isolated in this study based on 16S rRNA sequences.

**Figure S7.** Antagonistic activity of SY28 (*Burkholderia* sp.) against various fungi.

**Figure S8.** Density of rhizobia on roots of *O. glaberrima* grown in a sterilized substrate supplemented with and without *F. oxysporum* spores.

**Figure S9.** Effects of *F. oxysporum* exudates on rhizobial growth.

**Figure S10.** Nodule biomass and plant dry weight of *O. glaberrima* seedlings in a long-term fungi exclusion experiment.

## SUPPORTING INFORMATION

Lan Liu, Shixiao Yu, Zhi-Ping Xie and Christian Staehelin

Distance-dependent effects of pathogenic fungi on seedlings of a legume tree: impaired nodule formation and identification of antagonistic rhizosphere bacteria.

This file contains Tables S1-S3 and Figures S1 to S10:

**Table S1.** Strains isolated from nodules of *O. glaberrima* seedlings grown in soil collected around 5 adult trees at site 1 of the Heishiding Nature Reserve.

**Table S2.** Frequency and origin of antagonistic bacteria isolated from the rhizosphere of *O. glaberrima* seedlings.

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**Table S1.** Strains isolated from nodules of *O. glaberrima* seedlings grown in soil collected around 5 adult trees at site 1 of the Heishiding Nature Reserve.

Strain *	GenBank accession No.	Most related 16S rRNA sequence (species name and accession No.)	Number of isolates	Distance from adult tree (m)
SY6	KP687379	<i>Rhizobium tropici</i> CAF440 (FJ405381.1)	94	13 isolates at 0 m, 10 at 5 m, 21 at 10 m, 32 at 15 m, and 18 at 20 m
SY25	KP687371	<i>Burkholderia</i> sp. SBH-7 (AB366329.1)	12	2 isolates at 0 m, 4 at 5 m, 3 at 10 m, 1 at 15 m, and 2 at 20 m
SY26	KP687370	<i>Burkholderia</i> sp. ASS3 (AB303631.2)	15	2 isolates at 0 m, 10 at 5 m, and 3 at 15 m
SY27	KP687369	<i>Burkholderia cepacia</i> CNR22 (AB114607.1)	3	2 isolates at 0 m and 1 at 5 m
SY28	KP687368	<i>Burkholderia</i> sp. B35 (JX010979.1)	7	1 isolate at 0 m, 1 at 5 m, 3 at 10 m, and 2 at 15 m
SY74	KP687366	<i>Burkholderia</i> sp. SBH-16 (AB366338.1)	10	5 isolates at 0 m, 2 at 5 m, and 3 at 20 m
SY80	KP687348	<i>Mesorhizobium plurifarum</i> JN192 (KF150431.1)	5	5 isolates at 15 m
SY124	KP687360	<i>Rhizobium tropici</i> P4-6 (HM852122.1)	2	1 isolate at 0 m and 1 at 20 m
SY134	KP687359	<i>Rhizobium</i> sp. Leb-15 (AF511494.1)	9	8 isolates at 10 m and 1 at 20 m
SY135	KP687358	<i>Rhizobium</i> sp. lebi-5 (AY490119.1)	5	2 isolates at 10 m, 2 at 15 m, and 1 at 20 m
SY164	KP687354	<i>Burkholderia</i> sp. A40 (KF479551.1)	3	3 isolates at 20 m
SY171	KP687349	<i>Mesorhizobium</i> sp. CCANP64 (HF931057.1)	4	3 isolates at 10 m and 1 at 15 m
SY197	KP687377	<i>Rhizobium</i> sp. 2394 (JX174271.1)	32	5 isolates at 0 m, 5 at 5 m, 14 at 10 m, 1 at 15 m, and 7 at 20 m
SY247	KP687347	<i>Mesorhizobium</i> sp. D16 (JF913978.1)	13	2 isolates at 5 m, 1 at 10 m, and 10 at 15 m
SY273	KP687350	<i>Rhizobium tropici</i> CIAT899 (NR102511.1)	6	6 isolates at 20 m

\* Based on the 16S rRNA gene sequence analysis, 220 isolates were classified as rhizobia. Isolates with identical sequences were considered to belong to the same strain. Strains SY26, SY74, SY197, SY6 and SY273 were used for the inoculation experiment performed in this study.

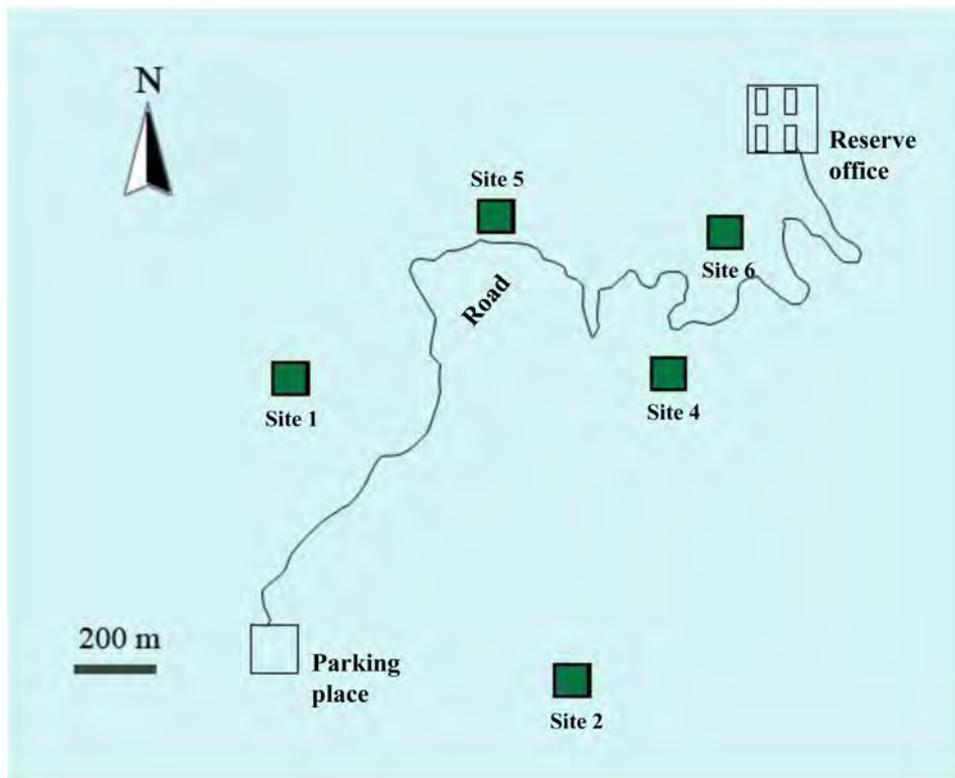
**Table S2.** Frequency and origin of antagonistic bacteria isolated from the rhizosphere of *O. glaberrima* seedlings.

Characteristics	Value	%	Comments
Total number of bacterial isolates	14100		60 isolates per surviving seedling
Number and frequency (%) of antagonistic isolates among the 14100 tested isolates	23	0.16	16S RNA sequences of 22 isolates related to those of <i>Burkholderia</i> sp. and one sequence related to a <i>Pseudomonas aeruginosa</i> strain (see Table S3)
Number of adult trees (among the 12 trees) from which soil inocula with antagonistic bacteria were collected	5	41.7	Soil inocula collected from 12 trees at 5 different distances (60 locations)
Number of identified locations containing antagonistic bacteria (among the 60 locations)	10	16.7	
Number of locations with antagonistic bacteria among the 31 locations with effects of pathogenic fungi on <i>O. glaberrima</i> seedlings	7	22.6	Presence of pathogenic fungi at 31 locations as deduced from the effect of fungicides on seedling survival (Fig. 2).
Number of locations with antagonistic bacteria among the 29 locations lacking effects of pathogenic fungi on <i>O. glaberrima</i> seedlings	3	10.3	Absence of pathogenic fungi at 29 locations as deduced from the lacking effect of fungicides on seedling survival (Fig. 2).
Number of antagonistic isolates (among the 23 antagonistic isolates) from locations containing pathogenic fungi	18	78.3	Antagonistic isolates obtained for 7 locations surrounding 3 trees (trees 4, 5 and 7)
Number of antagonistic isolates (among the 23 antagonistic isolates) from locations lacking effects of pathogenic fungi	5	21.7	Antagonistic isolates obtained for 3 locations surrounding 2 trees (trees 10 and 11)

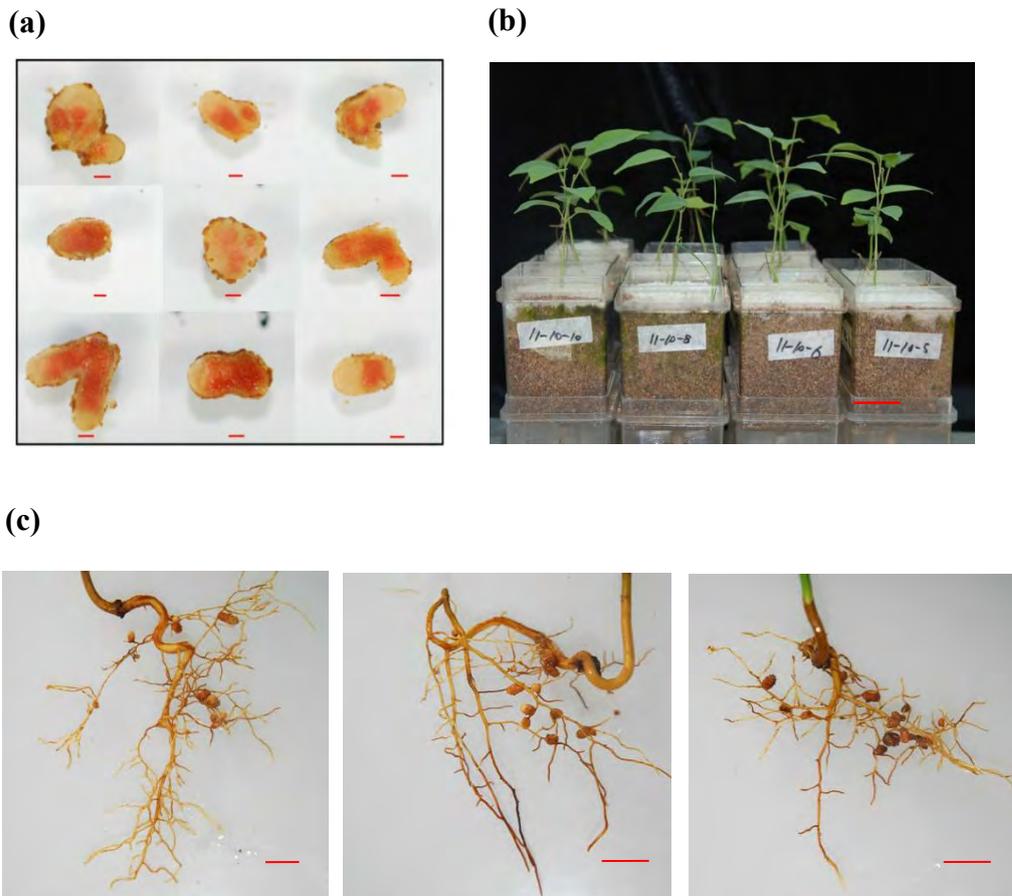
**Table S3.** List of antagonistic bacteria isolated from the rhizosphere of *O. glaberrima* seedlings.

Isolate	GenBank accession No (16S rRNA) *	Distance from adult tree (m)	Tree No	Most related 16S rRNA sequence (species name and accession No.)
A1	KP687398	5	7	<i>Burkholderia</i> sp. B35 (JX010979.1)
A2	KP687398	15	5	
A8	KP687398	0	4	
A9	KP687398	20	4	
A10	KP687398	20	11	
A12	KP687398	10	7	
A17	KP687398	10	10	
A20	KP687398	10	10	
A13	KP687399	10	10	
A27	KP687400	15	5	
A3	KP687401	10	5	<i>Burkholderia cepacia</i> ATCC 21809 (AY741338.1)
A6	KP687404	15	10	
A7	KP687404	5	5	
A11	KP687402	5	5	
A14	KP687403	5	5	
A18	KP687404	5	5	
A19	KP687404	5	5	
A4	KP687405	5	5	
A22	KP687407	5	5	<i>Burkholderia</i> sp. (JX010990.1)
A16	KP687406	5	5	
A5	KP687408	5	5	<i>Burkholderia</i> sp. (JQ518344.1)
A21	KP687409	5	5	<i>Burkholderia</i> sp. (KF479562.1)
A15	KP687410	10	7	<i>Pseudomonas aeruginosa</i> (JQ659980.1)

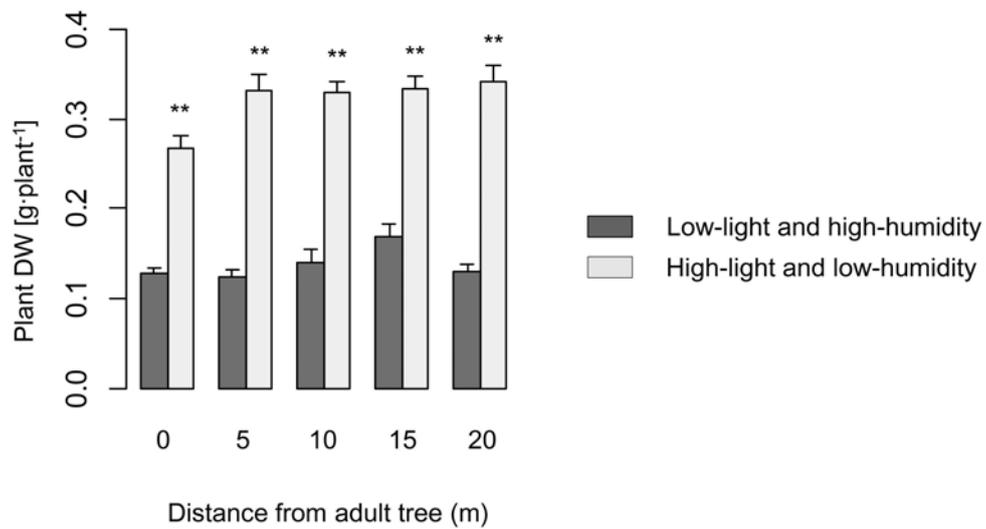
\* Isolates with different 16S rRNA sequences possess different accession numbers.



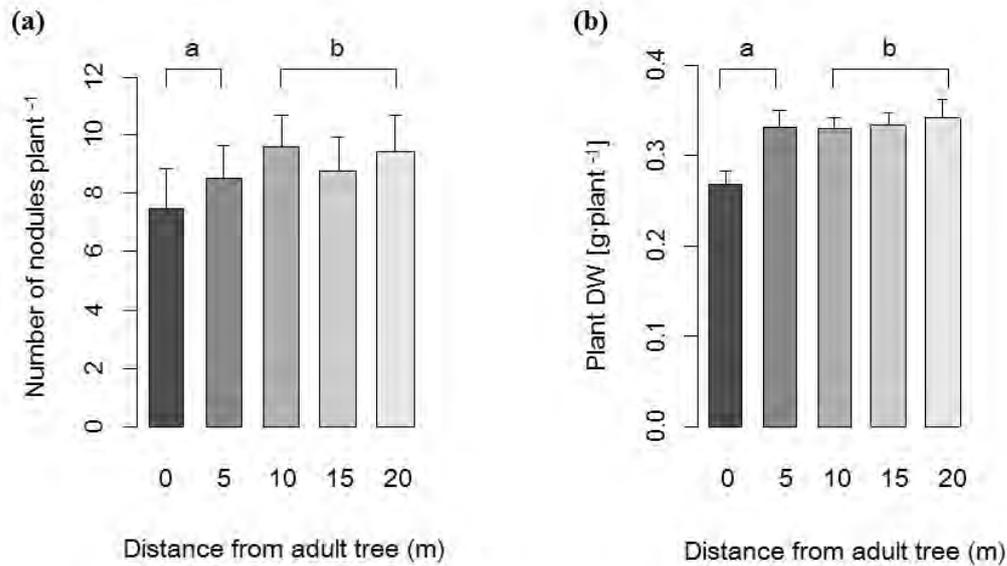
**Figure S1.** Location of study sites in the Heishiding Nature Reserve. Tree 7 at site 3 is outside the map. Initial tests were performed with soil from all 6 sites (trees 1, 2 and 3 at site 1; tree 8 at site 2; tree 7 at site 3; trees 4, 5 and 6 at site 4; tree 9 at site 5; trees 10, 11 and 12 at site 6). Further experiments were performed with soil from site 1 (trees 1, 2, 3, 13 and 14).



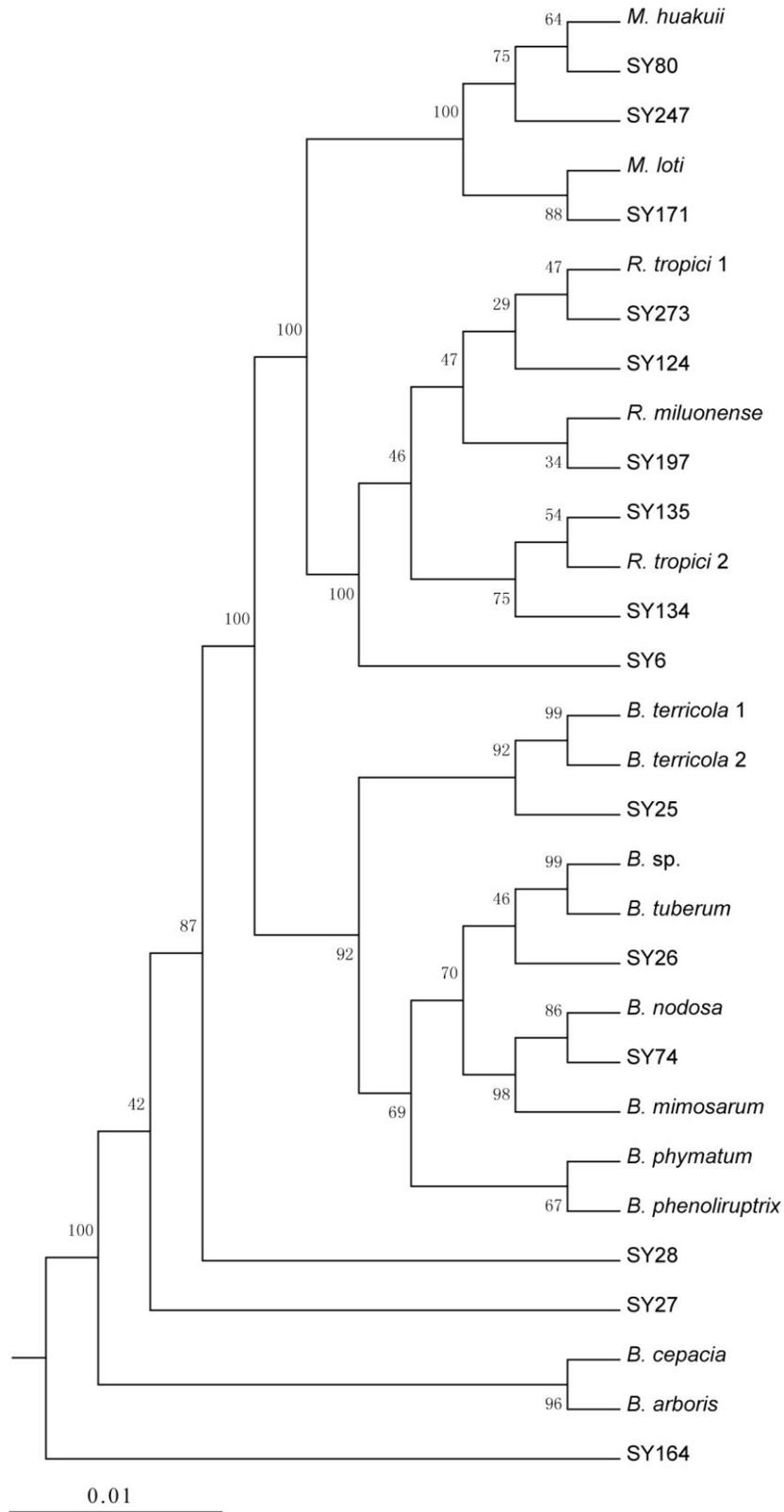
**Figure S2.** Examples for nodules formed on *O. glaberrima* seedlings. Seedlings were grown in soil collected around adult trees at site 1 of the Heishiding Nature Reserve. (a) Example of effective pink nodules cut into two parts (bar = 0.1 cm). (b) Aerial part of nodulated seedlings (bar = 5 cm). (c) Nodulated roots of *O. glaberrima* seedlings (bar = 1 cm).



**Figure S3.** Plant biomass of 25-weeks-old *O. glaberrima* seedlings grown under different growth conditions. Seedlings were grown in different soils collected at indicated distances to 12 focal adult trees at 6 different sites. High-humidity/low-light conditions were chosen to examine effects of pathogenic fungi (fungi exclusion experiment; see Fig. 2 and Table 1), whereas low-humidity/high-light conditions were used in a parallel nodulation experiment (see Fig. S4). Data indicate means  $\pm$  SE (locations treated as replicates;  $n = 12$ ). Plant biomass (dry weight, DW) at the time of harvest was significantly increased for seedlings under low-humidity/high-light conditions ( $t$  tests,  $n = 12$ ;  $P < 0.01$ , \*\*).

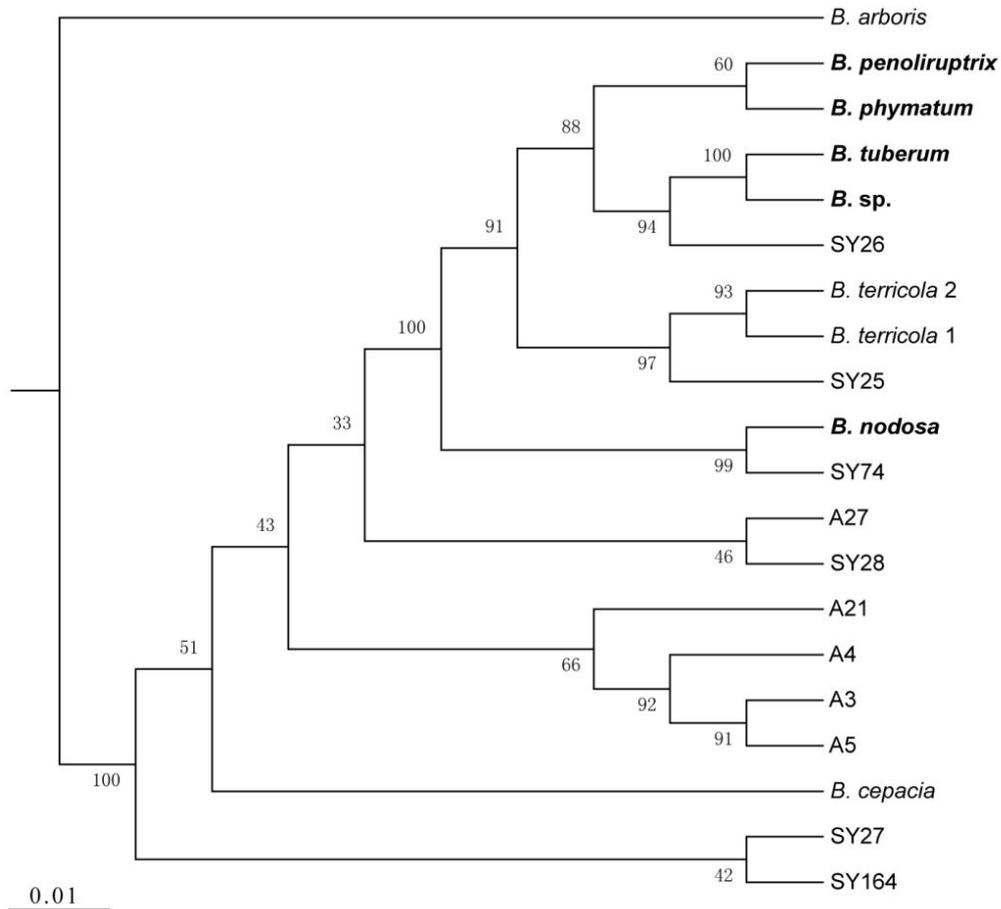


**Figure S4.** Nodulation of *O. glaberrima* seedlings grown in soil collected at different distances to 12 focal adult trees at 6 different sites. Plants were kept in a growth-room under high-light/low-humidity conditions favorable for nodule formation. (a) Number of nodules per plant formed on seedlings. (b) Plant biomass of seedling (DW stands for dry weight). Data indicate means  $\pm$  SE (locations treated as replicates;  $n=12$ ). Nodule formation and plant biomass of seedlings grown in soil inocula collected at close distance (a, 0-5 m) are significantly different from those taken at greater distance (b, 10-20 m) ( $t$  tests,  $P < 0.05$ ).

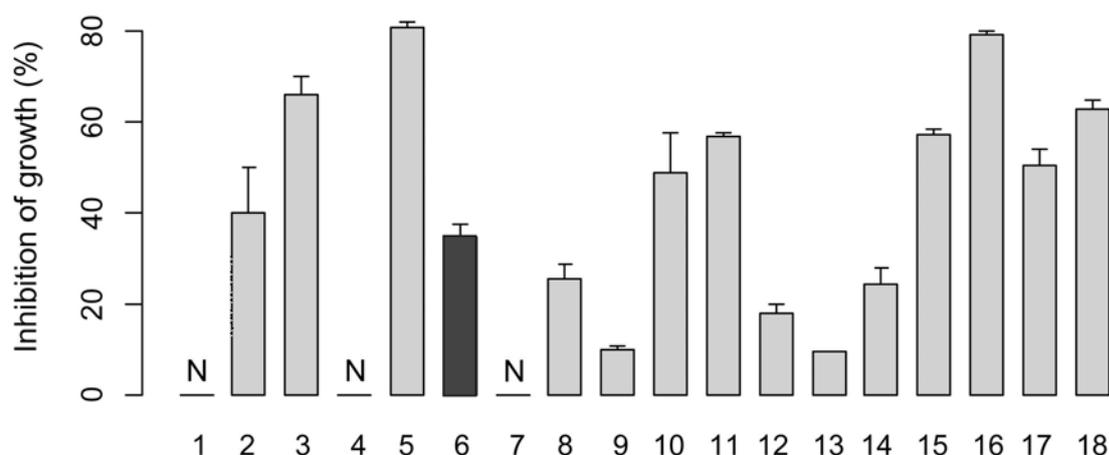


**Figure S5.** Phylogenetic analysis of isolated rhizobial strains (SY strains) based on 16S rRNA sequences. Bootstrap values calculated from 1,000 replicates are indicated at branching points. The scale bar represents  $\approx 1\%$  of nucleotide changes between close

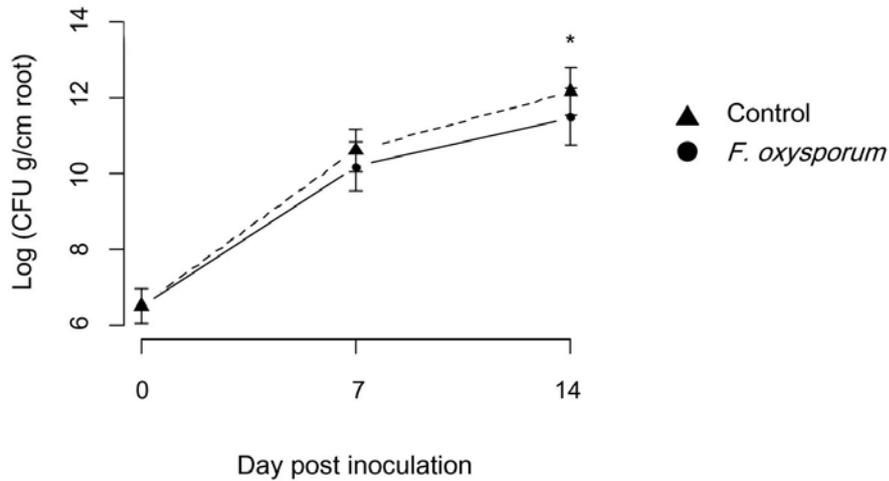
relatives. Following reference strains were used: *R. tropici* 1 (*Rhizobium tropici* USDA 9039, X67233), *R. tropici* 2 (*Rhizobium tropici* USDA 9030<sup>T</sup>, X67234), *M. loti* (*Mesorhizobium loti* LMG 6125<sup>T</sup>, X67229), *M. huakuii* (*Mesorhizobium huakuii* USDA 4779, D12797.1), *R. miluonense* (*Rhizobium miluonense* CPN9, KC907875.1), *B. terricola* 1 (*Burkholderia terricola* BRUESC290, KF031499), *B. terricola* 2 (*Burkholderia terricola* strain BRUESC219, KF031500.1), *B. tuberum* (*Burkholderia tuberum* strain STM678, NR\_027554.1), *B. nodosa* (*Burkholderia nodosa*, AY533861.1), *B. mimosarum* (*Burkholderia mimosarum*, HE864350.1), *B. phymatum* (*Burkholderia phymatum* STM815, NR\_027555.1), *B. phenoliruptrix* (*Burkholderia phenoliruptrix* BR3459a, NR\_102849.1), *B. cepacia* (*Burkholderia cepacia* ORS 3307, EF054874.1), *B. arboris* (*Burkholderia arboris* 12c, JF792427.1), and *B. sp.* (*Burkholderia sp.* CCGE1002, CP002014.1.)



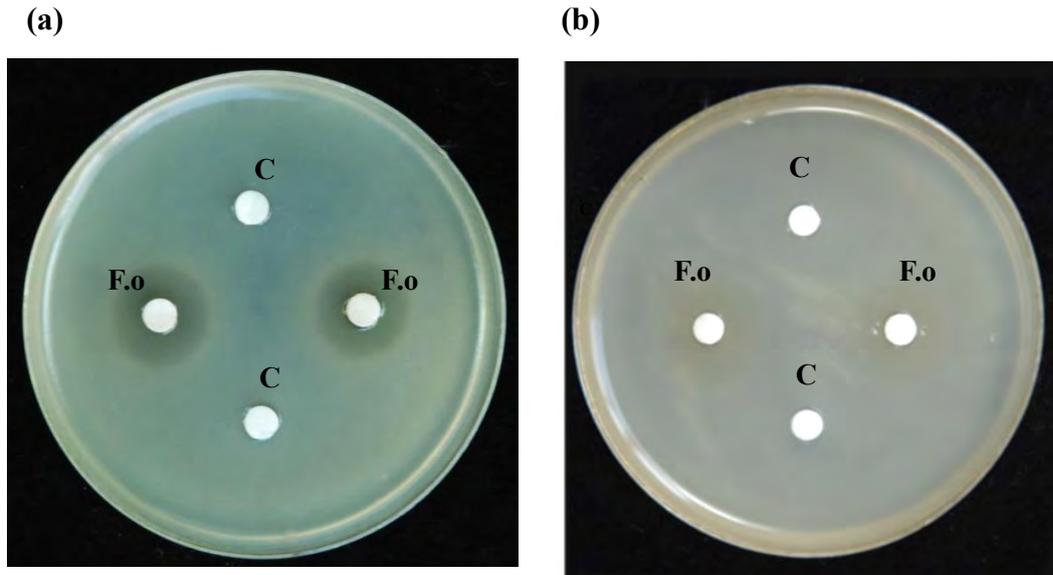
**Figure S6.** Phylogenetic analysis of *Burkholderia* strains isolated in this study based on 16S rRNA sequences. Bootstrap values calculated from 1,000 replicates are indicated at branching points. The scale bar represents  $\approx 1\%$  of nucleotide changes between close relatives. Following strains were used: *B. terricola 1* (*Burkholderia terricola* BRUESC290, KF031499), *B. terricola 2* (*Burkholderia terricola* strain BRUESC219, KF031500.1), *B. tuberum* (*Burkholderia tuberum* strain STM678, NR\_027554.1), *B. nodosa* (*Burkholderia nodosa*, AY533861.1), *B. phymatum* (*Burkholderia phymatum* STM815, NR\_027555.1), *B. phenoliruptrix* (*Burkholderia phenoliruptrix* BR3459a, NR\_102849.1), *B. cepacia* (*Burkholderia cepacia* ORS 3307, EF054874.1), *B. arboris* (*Burkholderia arboris* 12c, JF792427.1), *B. sp.* (*Burkholderia* sp. CCGE1002, CP002014.1.) A3-5, A21 and A27 are antagonistic *Burkholderia* isolated from the *O. glaberrima* rhizosphere. SY25 to SY28, SY74 and SY164 are *Burkholderia* strains isolated from nodules of *O. glaberrima* (for strain information, see Tables S1 and S3). *Burkholderia* reference strains reported to induce nodules on legumes are indicated in boldface type.



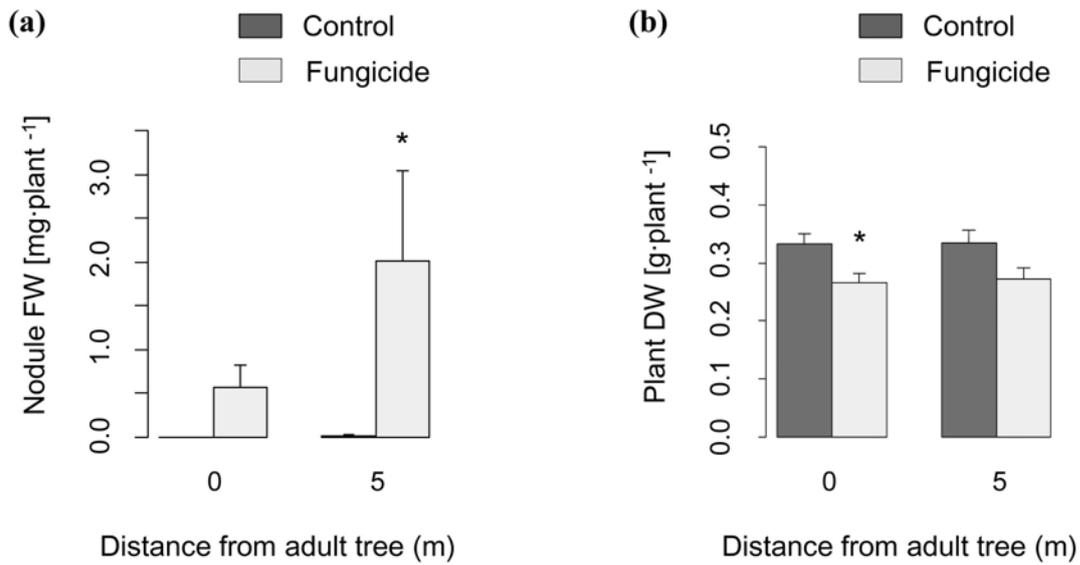
**Figure S7.** Antagonistic activity of SY28 (*Burkholderia* sp.) against various fungi. SY28 bacteria and test fungi were co-cultivated on potato dextrose agar plates. Inhibition percentages were calculated from  $[(R_1 - R_2)/R_1] \times 100$  where  $R_1$  is the farthest radial distance from the fungal mycelium to the SY28 colony and  $R_2$  is the distance on a line between the inoculation positions of the test fungus and SY28 (Whipps 1987, New Phytol., 107, 127-142). Data indicate means  $\pm$  SE. N indicates that no inhibition effect was detected. Following fungi were used (accession number of 16S rRNA sequences are shown in parentheses): 1: *Pestalotiopsis* sp. (FJ947050.1), 2: *Pestalotiopsis* sp. (GU723442.1), 3: *Pestalotiopsis clavispora* (HM999902.1), 4: *Pestalotiopsis sydowiana* (HQ248207.1), 5: *Botryosphaeria parva* (DQ499154.1), 6: ***Fusarium oxysporum*** (AY684919.1), 7: *Alternaria compacta* (EU128529.1), 8: *Paraconiothyrium hawaiiense* (HM7510092.1), 9: *Bionectria ochroleuca* (GU929189.1), 10: *Annulohypoxyloa trorseum* (EF488415.1), 11: *Trichoderma erinaceum* (EU280106.1), 12: *Penicillium pinophilum* (HQ589151.1), 13: *Penicillium janthinellum* (AY373921.1), 14: *Penicillium pinophilum* (AB293968.1), 15: *Penicillium* sp. (FJ379828.1), 16: *Hypocrea koningii* (GU176484.1), 17: *Peziza ostracoderma* (FJ537076.1), 18: *Hypocreales* sp. (GQ923983.1). *Fusarium oxysporum* (isolate F06; Liu *et al.* 2012, J. Ecol. 100, 1019-1028) is shown in boldface type and was also used in the rhizosphere competition experiment (Fig. S8).



**Figure S8.** Density of rhizobia on roots of *O. glaberrima* grown in a sterilized substrate supplemented with and without *F. oxysporum* spores. Seedlings were harvested 7 and 14 days post inoculation (pi). The rhizosphere population density of the inoculated rhizobial population was determined by the dilution plating method (CFU stands for colony forming units). Data indicate means  $\pm$  SE (seedlings treated as replicates;  $n = 6$ ). The significant difference between *F. oxysporum* inoculated seedlings and control seedlings at 14 days p.i. is marked by an asterisk ( $t$  test,  $n = 6$ ;  $t = 4.2$ ,  $P = 0.0424$ ).



**Figure S9.** Effects of *F. oxysporum* exudates on rhizobial growth. Paper filter disks impregnated with material from concentrated supernatants of *F. oxysporum* cultures (“F.o.”) or with concentrated Sabouraud medium as a control (“C”) were placed on on TY agar plates containing a given rhizobial strain. The photos show the results for strain (a) SY124 and (b) SY247 (after 3 days incubation at 27°C). Clear halos around the F.o. filter disks were observed for all tested rhizobial strains (see Table S1) except for *Mesorhizobium* strain SY247 and two *Burkholderia* strains (SY164 and SY28).



**Figure S10.** Nodule biomass and plant dry weight of *O. glaberrima* seedlings in a long-term fungi exclusion experiment. Soil samples were collected from locations in the close neighborhood (0-m and 5-m distances) of 5 adult trees at site 1. One half was treated with fungicides and the other half with water (control). The growth room experiment was conducted at high humidity/low light conditions and lasted 60 weeks. At the time of harvest, nodule biomass (a) and total plant dry (b) were determined. Corresponding data for seedling survival and nodule number per plant are shown in Fig. 4. Data indicate means  $\pm$ SE (locations treated as replicates; n=5). Significant effects of fungicides at a given distance are marked with asterisks (*t* tests, n=5;  $p < 0.05$ , \*).