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Analysis of a negative plant–soil feedback in a subtropical monsoon forest

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Summary

1. The Janzen–Connell hypothesis provides explanations for species coexistence and predicts that recruitment of tree juveniles is reduced by host-specific enemies, particularly soil biota. Previous studies, however, have not fully addressed the aspect of host specificity. Using a legume tree (*Ormosia glaberrima*) in a subtropical monsoon forest as a model, we experimentally investigated the mechanisms underlying a Janzen–Connell effect.

2. A negative plant–soil feedback was identified by a field census and confirmed by planting surface-sterilized seeds at different densities around focal trees. Fungicides were applied to test whether the effects were caused by fungi. In a growth-room inoculation experiment, effects of collected soil samples on seedling survival were examined and compared to soil samples treated with fungicides. Sequencing of the internal transcribed spacer region (ITS) sequence of the 28S ribosomal RNA gene was used for pathogen identification. The fungus causing seedling mortality was isolated and characterized by ITS rDNA sequencing and inoculation experiments.

3. A Janzen–Connell effect was identified at a field site, in which *O. glaberrima* was a locally common species. *In situ* treatments with fungicides and a corresponding growth-room simulation experiment showed that seedling survival depended on the distance to focal adult trees and that a fungal pathogen attacked seeds and seedlings. No negative plant–soil feedback was observed at another field site with a single *O. glaberrima* tree, indicating a locally rare species advantage.

4. The disease-inducing fungus was identified as *Fusarium oxysporum*. Inoculation experiments showed that the isolated fungus was pathogenic on *O. glaberrima* seedlings, but non-pathogenic on seedlings from other tree species co-occurring with *O. glaberrima*. Moreover, susceptibility of *O. glaberrima* depended on seed provenance (likely genotype).

5. *Synthesis*. We demonstrate that an observed negative plant–soil feedback on a locally common legume tree is caused by a host-specific pathogen. Our data fully support the criteria of spatially unequal pathogen distribution and host specificity proposed in the Janzen–Connell model. Taken the interaction between *O. glaberrima* and *F. oxysporum* as a paradigm, we suggest that host-specific pathogens, locally accumulated around parent trees, are important determinants of tree community structure.

Key-words: 28S ribosomal RNA gene, distance-dependent seedling mortality, *Fusarium oxysporum*, host specificity, Janzen–Connell hypothesis, plant–soil (below-ground) interactions, recruitment of juveniles, soil pathogen, subtropical forest

Introduction

Although various hypotheses have been proposed by theoretical ecologists, exact mechanisms underlying species coexistence still remain unclear (Hubbell 2001; Wright 2002; Volkov *et al.* 2005). The Janzen–Connell hypothesis, independently proposed by Janzen and Connell (Janzen 1970; Connell 1971), has received certain support from empirical data, particularly with respect to the impact of soil pathogens on plant communities in natural ecosystems (Augspurger 1983; Harms *et al.* 2000; Packer & Clay 2000; Bever 2003; Reinhart *et al.* 2005; Petermann *et al.* 2008; van der Heijden, Bardgett & van Straalen 2008; Reinhart & Clay 2009; Mangan *et al.* 2010; Swamy *et al.* 2011). The Janzen–Connell hypothesis predicts that juveniles closely located to adult trees at high density will suffer increased mortality and that these effects are due to propagation or attraction of natural enemies (herbivores and pathogens).

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Hence, tree species with high competitiveness are prevented from being predominant, particularly in tropical and subtropical forests (Janzen 1970; Connell 1971; Clark & Clark 1984).

An important element of the Janzen–Connell hypothesis is that enemies inducing such negative plant–soil feedback responses are unequally distributed in forests and that their impact decreases with increasing distance to host trees. Similarly, increasing density of neighbouring adult trees of the same species (i.e. conspecifics) is predicted to promote attack by these enemies. Due to these distance- and density-dependent effects, locally rare tree species in a tree community should display an increased recruitment of juveniles (Janzen 1970; Connell 1971; Webb & Peart 1999; Volkov *et al.* 2005). Accordingly, recent studies reported that seedlings of rare species display higher survival rates than those of abundant species in forest communities (e.g. Queenborough *et al.* 2007; Chen *et al.* 2010).

Host specificity is a crucial assumption of the Janzen–Connell hypothesis, and it was originally proposed that a given species attacks a specific host plant (Janzen 1970; Connell 1971). More recently, studies have shown that enemies or pathogens attack several evolutionary-related hosts with similar morphological and chemical traits (Farrell 2001; Novotny *et al.* 2002; Gilbert & Webb 2007; Liu *et al.* 2012). Phylogenetic and spatial analysis of tree community structures can provide clues to the host range of corresponding enemies (e.g. Webb *et al.* 2002; Gilbert & Webb 2007). However, only inoculation experiments with an isolated pathogen can provide a clear answer to the question whether a given pathogen is host specific (Konno, Iwamoto & Seiwa 2011).

Microbes accumulated in the soil around parent trees are prominent candidates for inducing a negative plant–soil feedback. Microbial pathogens possess relatively low dispersal ability in the soil (Augspurger & Wilkinson 2007), and their transmission rates likely depend on the density of host plants (Burdon & Chilvers 1982). According to the Janzen–Connell hypothesis, a single adult tree (locally rare species) located at a sufficient distance from aggregated conspecific adult trees (locally common species) should display a locally rare species advantage by 'escaping' from pathogens (Hubbell & Foster 1986). So far, a number of papers provided information on distance- and density-dependent effects of soil pathogens in tropical and temperate ecosystems (Harms *et al.* 2000; Packer & Clay 2000; Bell, Freckleton & Lewis 2006; Petermann *et al.* 2008; Mangan *et al.* 2010; Swamy *et al.* 2011). Effects of specific pathogenic microbes on juveniles of parent trees were often indirectly demonstrated, however (Freckleton & Lewis 2006). For example, experiments with fungicides in the field (Bell, Freckleton & Lewis 2006; Bagchi *et al.* 2010) or under experimental conditions (Packer & Clay 2000; Liu *et al.* 2012) provided evidence that certain soil fungi represent the most important group of soil pathogens. A number of papers reported identification and isolation of pathogenic fungi causing damping-off disease of tree juveniles, but their host range was not tested in inoculation experiments (Mills & Bever 1998; Packer & Clay 2000; Augspurger & Wilkinson 2007; Reinhart & Clay 2009). A recent cross-inoculation study on the multihost fungus *Colletotrichum anthrisci* showed that specific isolates differed in virulence depending on the host plant, and it was suggested that such differences are sufficient to cause Janzen–Connell effects (Konno, Iwamoto & Seiwa 2011).

Here, we examined a Janzen–Connell effect around adult *Ormosia glaberrima* trees in a 1-ha square of a subtropical monsoon forest. The negative plant–soil feedback was not observed around a single tree at another field site, suggesting a locally rare species advantage. We addressed the questions whether the negative plant–soil feedback is caused by a pathogenic fungus and whether effects are stronger when the density of *O. glaberrima* seeds is experimentally increased around parent trees. We then identified and isolated the disease-causing fungus and characterized its host specificity in an inoculation experiment with *O. glaberrima* and heterospecific tree species. The results fulfilled the criterion of host specificity proposed in the Janzen–Connell model.

Materials and methods

STUDY SITES AND STUDY SPECIES

Heishiding Nature Reserve (Guangdong Province, China; 111°49′09″–111°55′0″ E, 23°25′15″–23°30′02″ N, 150–927 m above sea level) covers *c.* 4200 ha of 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.* 2000).

Ormosia glaberrima Wu, a species belonging to the Fabaceae (Papilionoideae, Sophoreae) family, is one of the most common species in the lowland part of the Heishiding Nature Reserve (Luo 2009). Adult trees produce large amounts of seeds in typical mast years at an interval of 3–4 years (W. N. Ye, pers. comm.). *Ormosia glaberrima* was chosen as model species in this study, as seeds could be stored, rapidly germinated after surface sterilization, lacked seed-born pathogens and were obviously not eaten by seed herbivores (see Table S1 in Supporting Information).

A study site was chosen in October 2008, and a 1-ha plot was established in March 2009 (named site 1). Positions of *O. glaberrima* saplings and trees with a diameter at breast height (DBH) ≥ 1 cm were mapped (Fig. S1), their DBH was measured and the different growth stages categorized according to He, Legendre & LaFrankie (1997). Species coexisting with *O. glaberrima* trees at this study sites are listed in the Supporting Information (Table S2).

At site 1, five adult *O. glaberrima* trees were chosen and 1 × 10 m sample belts were established to investigate seed and seedling density (four belts per focal tree). Sample belts satisfied the criterion that other *O. glaberrima* trees had a minimal distance of 20 m to any point in the belt. In November 2008, when seed fall was over, the number of fallen seeds was determined for each sample belt. At the same time, large amounts of seeds were collected from site 1 for the later experiments. Moreover, *O. glaberrima* seeds were also collected from another 1-ha plot (named site 2), which contained a single adult *O. glaberrima* tree. Site 2 was *c.* 1.2 km away from site 1, and no *O. glaberrima* trees were found between these two sites (Table S2, Fig. S1).

First emergence of *O. glaberrima* seedlings was observed in early April 2009, and germination of most seeds occurred in the middle of April, when newly emerging seedlings were counted and tagged with

a plastic band in each established sample belt. During the following months, all tagged surviving seedlings were censused and newly occurring seedlings were tagged monthly.

MANIPULATIVE FIELD EXPERIMENT

A fungal exclusion experiment was conducted at different distances from five focal trees at study site 1. Two arcs (of sectors with a central angle of 30°) were established for each focal tree, and test plots (1.2 × 2 m) were set up at 0, 5, 10, 15 and 20 m on both arcs. These arcs satisfied the criteria that no other conspecific adult trees occurred within 20 m to any test plot. All plots were divided into five subplots (1.2 × 0.4 m). Two subplots on one side were treated with fungicide and two subplots on the other side were sprayed with the same amount of water as a control. The middle subplot was left untreated as a buffer zone to limit a potential drift of fungicide to the control subplots. Subplots (except the buffer zone) were divided into three test squares (0.4 × 0.4 m). *Ormosia glaberrima* seeds (surface sterilized with concentrated sulphuric acid) were planted into the central area (0.2 × 0.2 m) of each square at different densities (4, 9 and 16 seeds per square, respectively).

Fungicides were applied every 2 weeks according to the manufacturers' recommendations: 0.25 g m^{-2} of granular Ridomil Gold 25 G (Syngenta Ltd, Basel, Switzerland) and 0.5 g m^{-2} of Carbendazim + Quintozene 40% WP (Meibang Pharmaceutical Corporation, Xi'an, China; dissolved in water; 500 mL m^{-2}). Granular Ridomil Gold 25 G is a systemic fungicide, which effectively inhibits oomycetes (see e.g. Bell, Freckleton & Lewis 2006; Reinhart & Clay 2009), and Carbendazim + Quintozene 40% WP is a systemic, broad-spectrum fungicide against various fungi, such as *Fusarium* and *Rhizoctonia*. Control squares received the same volume of water without fungicides. After planting of seeds, most of the seedlings emerged within 2 weeks. The whole field experiment lasted 40 weeks, and seedling survival was determined every 2 weeks during the first 10 weeks and every 5 weeks until the end of the experiment.

SIMULATION UNDER GROWTH-ROOM CONDITIONS

At site 1, samples from the upper soil (*c*. 10 cm in depth; *c*. 10 kg) next to each established test plot (see above) were collected, resulting in two soil samples per chosen distance (0, 5, 10, 15 and 20 m) for each *O. glaberrima* tree. Soil samples were transported to the laboratory in Guangzhou and then sieved (mesh diameter: 0.2 cm) to eliminate seeds. Every soil sample was divided into three parts: one part was left untreated (intact soil with microbes), the second part was treated with fungicides as described above, and the third part was sterilized by gamma-radiation (Huada Radiation Corporation, Guangzhou, China). Each test unit consisted of a 250-mL soil sample in a sterilized 300-mL plastic vessel (upper jar) linked with cotton wicks to a similar vessel (lower jar), which was filled with 250 mL of sterilized water (Fig. S3). Surface-sterilized *O. glaberrima* seeds (from site 1 or site 2) were transferred into the jars at two different densities (low density: one seed per jar; high density: four seeds per jar). The soil samples were then covered with a 1-cm layer of sterilized expanded clay (*c*. 1 mm in diameter) to prevent contamination by ambient microbes. In total, 600 jar units (five focal trees × five different distances × three different soil treatments × two seed densities × two seed provenances × two replicates) were prepared. Jars were kept in an air-conditioned growth-room at 24 ± 2 °C, 80–95% relative humidity and low-light conditions (photosynthetically active radiation ≈ 25 μE s^{-1} m^{-2}; 12 h day^{-1}) to simulate under canopy conditions. Positions of jars in the growth-room were randomly changed every week. The experiment lasted for 40 weeks and seedling survival was determined every 2 weeks. At site 2, samples from the upper soil were collected as described above, and 120 jar units were prepared (one focal tree × five different distances × three different soil treatments × two seed densities × two seed provenances × two replicates).

PATHOGEN IDENTIFICATION

DNA was directly extracted from rotten *O. glaberrima* seeds (collected around the five focal trees at site 1) according to a described procedure (Gallery, Dalling & Arnold 2007) using a DNA purification kit for fungal DNA (Omega Bio-Technology incorporation, Norcross, GA, USA). Polymerase chain reactions (PCRs) were achieved on an Eastwin EDC-810 thermocycler (Eastwin Lifescience Incorporation, Beijing, China) with primers ITS1 and ITS4 as previously described (Arnold & Lutzoni 2007). These primers are specific primers for amplification of the ribosomal internal transcribed spacer region (ITS rDNA) of the 28S ribosomal RNA gene (Martin & Rygiewicz 2005). Reaction mixtures were analysed on ethidium bromide-stained agarose gels. Amplicons were purified from agarose gels with a DNA gel extraction kit (Omega Bio-Tek Incorporation) and sequenced by Invitrogen (Invitrogen Incorporation, Guangzhou, China) using the primers ITS1 and ITS4. Obtained sequences were compared with nucleotide data bases using the BLASTN algorithm (Altschul *et al.* 1997) at the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/).

PATHOGEN ISOLATION

Fungi from infected *O. glaberrima* plants (rotten seeds and seedlings) were isolated according to a standard isolation method (Yu 1979). Two kinds of agar plates were chosen: 2% (w/v) potato dextrose agar (PDA) and 2% (w/v) corn meal agar (CMA) (both obtained from Qingdao Hope Bio-Technology Corporation, Qingdao, China). Agar plates were supplemented with 100 mg L^{-1} kanamycin to inhibit the growth of bacteria. Sealed plates were incubated at 24 °C in the dark for 2 weeks, and appearing filamentous fungi were purified on fresh plates to obtain fungal isolates derived from single spores or hyphal tips (Leach & Clapham 1992). Purified isolates were cultivated in liquid Sabouraud dextrose medium (Qingdao Hope Bio-Technology Corporation). Total genomic DNA isolation, PCRs (PCR products in the range of 400–600 bp), and analysis of obtained sequences were performed as described above. Sequences were submitted to the GenBank data base (accession numbers JN002164 to JN002181).

PATHOGENICITY TESTS

Fungal isolates were divided into two groups according to their ITS sequences (Table S3). Group 1 contained fungal isolates which could eventually be pathogenic, and group 2 represented isolates which were predicted to be non-pathogenic. Each group (as a mixture) was used for a first inoculation test with *O. glaberrima* seeds or seedlings (seeds from site 1). Surface-sterilized *O. glaberrima* seeds were transferred into pasteurized 300-mL plastic jar units (250 mL gamma-radiated soil collected from test site 1 in the upper jar and 250 mL distilled water in the lower jar; Fig. S3) and then inoculated with 10 mL conidial suspension (*c*. 1.5 × 10^9 conidia per mL). Inoculation at seedling stage was performed with 2-week-old seedlings according to a previously described method (Lichtenzveig *et al.* 2006). The soil was then covered with a 1-cm layer of sterilized expanded clay, and the upper jar was covered by an inverted plastic vessel to ensure moist

conditions (Augspurger & Wilkinson 2007). Each inoculation was performed with 16 replicates (one seed or seedling per jar unit). Inoculated plants and non-inoculated controls were placed randomly in the growth-room and kept under growth conditions indicated above. As plants inoculated by group 1 (but not by group 2) showed disease symptoms, all isolates of group 1 were individually tested in a similar second inoculation experiment, which revealed that *Fusarium oxysporum* (isolate F06) was pathogenic. The fungus was successfully re-isolated from rotten seeds and dying seedlings to fulfil Koch's postulates.

To examine host specificity, *F. oxysporum* was inoculated on *O. glaberrima* (seeds from either site 1 or site 2) as well as with co-existing tree species, namely *Schefflera octophylla* (Lour.) Harms, *Cryptocarya concinna* Hance and *Castanopsis fabri* Hance. Inoculation with 2-week-old seedlings was performed as described above. Each inoculation was performed with 16 replicates (one seedling per jar unit). Seedling survival was determined 60 days post-inoculation.

STATISTICAL ANALYSIS

The manipulative field experiment and the growth-room simulation experiment were statistically analysed by a generalized linear mixed model (GLMM):

$$Y_{ijkr} \sim \text{binomial}(p_{ijkr})$$

$$\pi_{ijkr} \sim \text{logit}(p_{ijkr}) = \frac{p_{ijkr}}{1-p_{ijkr}} = \alpha + \beta_0[\text{fixed effects}] + [1|\text{random effect}]$$

where Y_{ijkr} is the binary response variable (dead or alive seedlings) for a given *O. glaberrima* seedling in test quadrats/jars *i*, which has been exposed to a fungicide/gamma-radiation treatment *j* (no treatments in controls); *k* is the planting density of seeds into blots/jars, *r* is one of the five focal trees, and p_{ijkr} is the predicted seedling survival at the end of the experiment. Distance (test quadrats/jars), fungicide/gamma-radiation, seed density and two-way interactions (e.g. distance × fungicide) were treated as fixed effects and focal trees as random effect. In all procedures, the Laplace approximation method was used. Likelihood ratio statistics were applied to analyse variation among focal trees (random effect). Odds ratios (seedling survival of plants exposed to fungicide treatment vs. control treatment without fungicide) were chosen to express the effect of fungicide treatment on survival of seedlings at different distances from a focal tree. Odds ratios > 1 (95% confidence interval does not overlap 1) indicate positive effects of fungicide on seedling survival, and odds ratios not significantly different from 1 indicate no effect. Furthermore, odds ratios were calculated to illustrate that effects of fungicide treatment and soil sterilization on seedling survival were similar. The null hypothesis that seedling survival was independent of fungal inoculation was tested using Pearson's chi-square test. All analyses were performed using the statistical programming language R, version 2.12.0 (R Development Core Team 2010).

Results

IDENTIFICATION OF A NEGATIVE PLANT–SOIL FEEDBACK

At site 1, there were 26 mature trees (DBH ≥ 9 cm), 14 premature trees (4 cm ≤ DBH < 9 cm), 31 juveniles (DBH < 4 cm; height ≥ 50 cm) and five dead mature trees of *O. glaberrima* (Fig. S1). Around five focal adult trees, seeds were censused after the seedfall in winter 2008. Although several seedlings were met in April 2009, no surviving seedlings were found in October 2010, suggesting that a soil pathogen inhibited recruitment of *O. glaberrima* juveniles (Fig. 1b). At site 2, however, seedling density gradually increased over time, indicating no negative soil–plant feedback (Fig. S2).

FIELD EXPERIMENT

A fungicide treatment experiment at site 1 was performed in which seeds at different densities were planted around five focal adult *O. glaberrima* trees. Fungicide application had a strong positive effect on seedling survival (Table 1; Fig. 2a,b). Seedling survival depended on the distance from the focal tree. Values were significantly reduced at low distance, for example, close to parent trees (Table 1). Accordingly, the benefit of the fungicide treatment decreased with increasing distance (Table 1; Fig. 2a,b). Compared to seeds from site 1, seeds collected from the single tree at site 2 appeared to be less susceptible to the fungal pathogen. Effects of increasing planting density on seedling survival were not observed (Table 1).

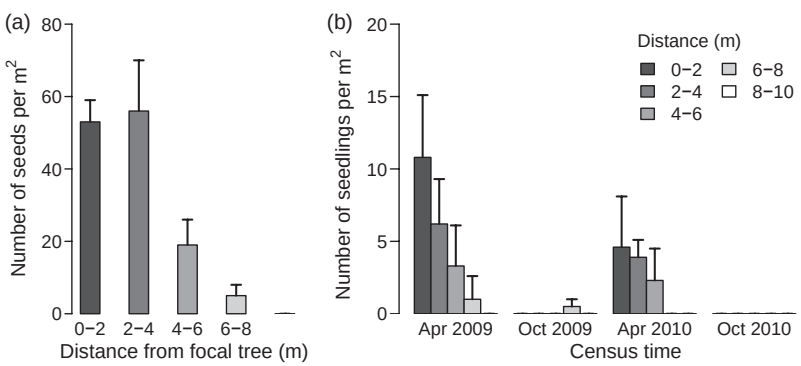

Fig. 1. Seed density (a) and seedling density (b) at indicated distance from five focal *Ormosia glaberrima* trees at site 1. Seed density was determined after seed fall in November 2008. No seed fall was observed during the following 2 years. Seedling number of October 2009 was defined as the sum of surviving seedlings tagged in April 2009 and non-tagged seedlings, which emerged between April and October. Using the same method, the number of seedlings was determined in April 2010 and October 2010. Data indicate means ± SD. The census was carried out every month (for simplicity, only four time points are shown).

Table 1. Results of a GLMM examining the effects of distance (0, 5, 10, 15 and 20 m from focal tree), fungicide treatment, planting density (4, 9 and 20 seeds per test square), seed provenance (seeds from site 1 and from a single tree at site 2, respectively) and two-way interactions (e.g. density × fungicide) on seeding survival (number of emerged and survived seedlings) of *Ormosia glaberrima* in the manipulative field experiment

Fixed effects	Estimate (SE)	*Z*-value	$P(>\|z\|)$
Distance	0.019 (0.008)	2.384	**0.017**
Fungicide	−2.355 (0.268)	−8.774	**< 2e−16**
Density	−0.008 (0.013)	−0.642	0.521
Seed provenance	0.199 (0.082)	2.416	**0.016**
Distance × fungicide	0.144 (0.012)	11.570	**< 2e−16**
Density × fungicide	0.001 (0.018)	0.079	0.937
Random effects	d.f.	χ^2	$P(>\|Chi\|)$
Focal adult tree	1	1.723	0.189

Significant results are shown in boldface type.

Seedling emergence data of the experiment for the three used planting densities are shown in the Supporting Information (Table S4). The interaction planting density × fungicide did also not affect seedling survival (Table 1). Variations among focal trees were not significant, suggesting accumulation of a fungal pathogen around each of the five focal trees (Table 1). In control test squares (without fungicide treatment) at a 0-m distance from focal trees, strong effects of a fungal pathogen on seedling survival were observed for the first 8 weeks of the experiment. During the following weeks, no seedlings died. However, dead seedlings were observed during the last weeks of the experiment, presumably due to the beginning of the dry season (Fig. S4a).

GROWTH-ROOM EXPERIMENT

A growth-room experiment with soil collected around focal trees at different distances showed similar results (Table 2; Fig. 2c,d). Survival of seedlings planted into jars differed for the soil samples, for example, survival decreased at a close distance to focal parent trees (Table 2). A sterilization (gamma-radiation) or fungicide treatment of soil samples resulted in significantly increased seedling survival (Table 2; Fig. 3c,d). The benefit of sterilization decreased with increasing distance from parent trees. Seedling survival was not significantly different between the sterilization and fungicide treatments (Fig. 2c,d), suggesting that a disease-inducing fungus caused the negative plant–soil feedback. The seed provenance was also found to affect seedling survival (Table 2). The use of seeds collected from site 1 (Fig. 2c) resulted in higher odds ratio values as compared to seeds from site 2 (Fig. 2d). Neither the seed density (number of planted seeds per jar) nor the interaction seed density × sterilization had an effect on seedling survival

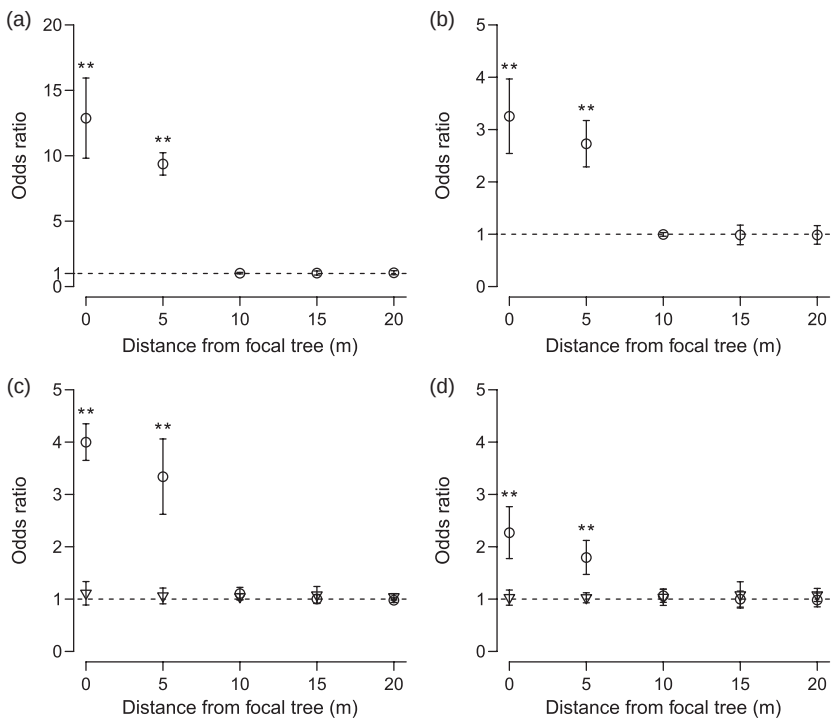

Fig. 2. Effects of fungicide and gamma-radiation treatments (odds ratios of survival of seedlings) as a function of the distance from focal *Ormosia glaberrima* trees in the manipulative field experiment (a: seeds from site 1, b: seeds from site 2) and in the growth-room experiment (c: seeds from site 1, d: seeds from site 2). Each focal tree is treated as a replication. No significant differences for seedling survival among focal trees were detected (see Table 1). Odds ratios of seedling survival (fungicide/no fungicide) are indicated as circles and odds ratios of seedling survival (gamma-radiation/fungicide) as triangles. 95% confidence intervals are indicated by bars. Odds ratios significantly different from 1 (95% confidence interval does not overlap 1) are marked with asterisks (**).

Table 2. Results of a GLMM examining the effects of distance (soil collected at 0, 5, 10, 15 and 20 m from focal tree), sterilization treatment (gamma-radiation of soil), planting density (1 and 4 seeds per jar), seed provenance (seeds from site 1 and from a single tree at site 2, respectively) and two-way interactions (e.g. density × sterilization) on seeding survival (number of emerged and survived seedlings) of *Ormosia glaberrima* in the growth-room simulation experiment

Fixed effects	Estimate (SE)	*Z*-value	$P(>\|z\|)$
Distance	0.171 (0.017)	9.794	**< 2e−16**
Sterilization	2.605 (0.592)	4.397	**1.10e−05**
Density	−0.092 (0.091)	−1.018	0.309
Seed provenance	0.363 (0.175)	2.076	**0.038**
Distance × sterilization	0.128 (0.151)	−5.940	**2.85e−09**
Density × sterilization	0.001 (0.018)	0.849	0.396
Random effects	d.f.	χ^2	$P(>\|\text{Chi}\|)$
Focal tree	1	1.509	0.219

Significant results are shown in boldface type.

(Table 2). Seedling emergence data for the two used planting densities are shown in the Supporting Information (Table S5). Strongest pathogen effects on seedling survival were found for non-treated soil samples (collected at 0-m distance from trees) during the first 8 weeks of the experiment. Seedling survival remained constant at a low level during the following weeks, indicating that the first weeks after germination were most critical for survival of seedlings (Fig. S4b).

A similar fungal exclusion experiment in the growth-room was also performed with soil samples from site 2. The results did not provide any indications for the presence of a fungal pathogen (Fig. S5).

IDENTIFICATION AND ISOLATION OF THE PATHOGEN

For identification of the pathogen, rotten *O. glaberrima* seeds around the five focal trees at site 1 were collected and DNA was isolated. PCRs were performed with primers ITS1 and ITS4 to amplify the fungal ribosomal internal transcribed spacer region (ITS rDNA). Out of 50 independent samples, an amplicon of expected size was obtained for nine PCRs. The corresponding agarose gel stained by ethidium bromide is shown (Fig. 3). The amplicons were sequenced and nine identical sequences were obtained (GenBank accession No JN002169). A comparison with nucleotide data bases using the BLASTN algorithm revealed that the obtained sequences were most similar to ITS rDNA sequences of the *F. oxysporum* Schlecht group. Thus, based on ITS rDNA sequencing, *O. glaberrima* seeds around all five focal parent trees were infected by the fungus *F. oxysporum* and no other fungus could be detected by the used PCR approach.

To isolate the identified pathogen, fungi were isolated from rotten seeds or dying seedlings collected around the five focal trees. In total, 18 morphologically different isolates (numbered F01–F18) could be distinguished (Table 3). ITS rDNA sequencing for 10 isolates of F06 indicated that *F. oxysporum* was isolated. Sequences of the *F. oxysporum* isolates were identical to the sequences obtained by direct PCRs with rotten seeds (GenBank accession No JN002169).

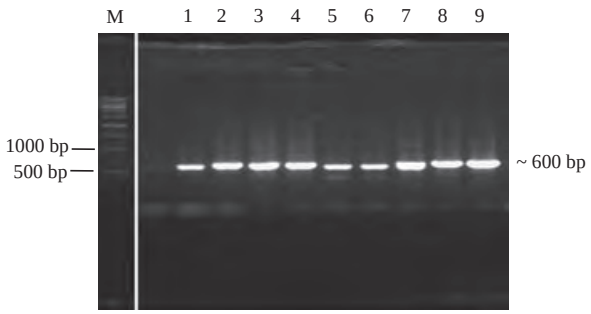

Fig. 3. Analysis of PCRs performed with template DNA extracted from rotten *Ormosia glaberrima* seeds around focal trees at site 1. Nine PCRs with ITS1 and ITS4 primers resulted in amplicons of expected size (*c.* 600 bp), which were eluted from the shown agarose gel and then sequenced (identical ITS rDNA sequences for all nine samples; GenBank accession No JN002169). Abbreviations: M, molecular weight markers; lanes 1 and 2, seeds from tree 1; lane 3, seeds from tree 2; lanes 4 and 5, seeds from tree 3; lanes 6 and 7, seeds from tree 4; lanes 8 and 9, seeds from tree 5.

A series of inoculation experiments with *O. glaberrima* demonstrated that *F. oxysporum* (F06) could efficiently decrease seedling survival of *O. glaberrima*, whereas the other isolated fungi did not (Table S3).

TEST OF HOST SPECIFICITY

To test host specificity, *F. oxysporum* (F06) was inoculated on *O. glaberrima* seedlings, which derived from seeds collected either from site 1 or site 2. Seeds from *S. octophylla*, *C. concinna* and *C. fabri* trees were included into the inoculation experiment. These three species were found to be typical representatives of tree communities in the Heishiding Nature Reserve (see also Table S2). Other tree species could not be tested, as seed availability was limited and efforts failed to initiate germination of collected seeds. The fungus only induced disease symptoms on *O. glaberrima*, whereas the other tested species were resistant. Similar to the previous experiments, the seed provenance had an impact on seedling mortality ($\chi^2 = 34.2$, d.f. = 1, $P = 5e{-}09$; Fig. 4).

Discussion

A field experiment with fungicides at site 1 and a corresponding growth-room simulation experiment with collected soil samples clearly indicated a negative plant–soil feedback caused by a fungal pathogen. The experiments showed that the disease-inducing fungus was mainly located close to the focal parent trees. The fungus, identified as *F. oxysporum*, was pathogenic on *O. glaberrima*, but non-pathogenic on other tree species.

According to the Janzen–Connell hypothesis, host-specific natural enemies prevent dominant species from being predominant, thereby providing space for locally rare species (Janzen 1970; Connell 1971; Clark & Clark 1984). Many trees display

Table 3. Isolation and ITS rDNA sequences of fungi associated with seeds or seedlings of *Ormosia glaberrima*

Isolate	Origin	Isolation medium	Number of isolates	Number of sequences	GenBank accession No.	Most related sequence (species name and accession No.)
F01	Dying seedling	PDA	6	3	JN002164	*Pestalotiopsis sp.* (FJ947050.1)
F02	Rotten seed	PDA	3	3	JN002165	*Pestalotiopsis sp.* (GU723442.1)
F03	Dying seedling, rotted seed	PDA	7	4	JN002166	*Pestalotiopsis clavispora* (HM999902.1)
F04	Rotten seed	PDA	8	4	JN002167	*Pestalotiopsis sydowiana* (HQ248207.1)
F05	Dying seedling	PDA	2	2	JN002168	*Botryosphaeria parva* (DQ499154.1)
F06	Dying seedling, rotten seed	PDA	41	10	JN002169	*Fusarium oxysporum* (AY684919.1)
F07	Dying seedling	CMA	4	4	JN002170	*Alternaria compacta* (EU128529.1)
F08	Rotten seed	PDA	3	1	JN002171	*Paraconiothyrium hawaiiense* (HM751092.1)
F09	Rotten seed	CMA	5	2	JN002172	*Bionectria ochroleuca* (GU929189.1)
F10	Dying seedling	CMA	3	1	JN002173	*Annulohypoxylon atroroseum* (EF488415.1)
F11	Dying seedling	PDA	11	4	JN002174	*Trichoderma erinaceum* (EU280106.1)
F12	Rotted seed	PDA/CMA	9	3	JN002175	*Penicillium pinophilum* (HQ589151.1)
F13	Rotted seed	PDA	6	3	JN002176	*Penicillium janthinellum* (AY373921.1)
F14	Dying seedling	PDA	5	2	JN002177	*Penicillium janthinellum* (AB293968.1)
F15	Dying seedling	PDA	21	4	JN002178	*Penicillium* sp. (FJ379828.1)
F16	Dying seedling	PDA	8	3	JN002179	*Hypocrea koningii* (GU176484.1)
F17	Rotted seed	CMA	2	1	JN002180	*Peziza ostracoderma* (FJ537076.1)
F18	Sick seedling	PDA/CMA	16	4	JN002181	*Hypocreales sp.* (GQ923983.1)

Five dying seedlings and five rotten seeds were collected from each focal tree (50 samples in total). Fungi were isolated on agar plates containing the indicated medium (PDA and CMA). Fungal isolates were distinguished based on morphological characteristics (numbered from F01 to F18). ITS rDNA was amplified by PCR using DNA from isolates as a template. Obtained sequences were submitted to GenBank and compared with nucleotide databases using the BLASTN algorithm.

low seed dispersal (Condit *et al*. 2000), for example, seed and seedling densities rapidly decrease with increasing distance from the parent tree as shown for *O. glaberrima* (Fig. 1). Negative effects of seedling density on seedling survival have been reported in previous studies testing the Janzen–Connell hypothesis (Packer & Clay 2000; Bell, Freckleton & Lewis 2006; Li *et al*. 2009; Bagchi *et al*. 2010). In the performed field experiment, however, such a relationship was not found. The factors seedling density and distance from parent tree were decoupled in our experiment. The results showed that the experimentally manipulated seed density *in situ* had no direct effect on seedling survival under the tested conditions (Table 1). Similar data were also obtained from the growth-room simulation experiment (Table 2). Nevertheless, it is possible that high seedling density promotes propagation of *F. oxysporum* over time (perhaps years), thereby forming a distance-dependent negative plant–soil feedback around each parent tree. Alternatively, *F. oxysporum* could accumulate around parent trees if the fungus infected roots of the parent tree itself. Several decades are needed for forest trees to develop from juveniles to mature trees. Such a long peroid likely facilitates pathogen accumulation as compared to annual species (Packer & Clay 2004).

Our PCRs with DNA from rotten seeds followed by ITS rDNA sequencing showed that *F. oxysporum* was present around all five focal trees at site 1, whereas no indications for the presence of pathogenic soil fungi were obtained for site 2 (Figs S2 and S5). It is tempting to speculate that the single tree at site 2 'escaped' from the *F. oxysporum* population of site 1 and this is reminiscent to invasive species lacking natural enemies, a phenomenon explained by the enemy release hypothesis (Keane & Crawley 2002; Mitchell & Power 2003). Such a locally rare species advantage will likely be reduced over time (Hawkes 2007). We suggest that the probability of pathogen attack will increase with increasing age and density of adult trees. As a consequence, a natural 'tree rotation' (analogous to crop rotation in agriculture) will occur under natural conditions in forests, thereby promoting coexistence of many species in different successional stages.

Although attack by soil-borne pathogens has been frequently invoked to explain seedling mortality of juveniles around parent plants, few studies have isolated and identified corresponding fungi (Klironomos *et al*. 2000; Packer & Clay 2000). Oomycetes have received most attention in studies testing the Janzen–Connell hypothesis (Augspurger 1984; Mills & Bever 1998; Bell, Freckleton & Lewis 2006; Reinhart & Clay 2009). Effects of other fungi may also be important, however (Bagchi *et al*. 2010; Konno, Iwamoto & Seiwa 2011; Maron *et al*. 2011). In this study, we have identified *F. oxysporum* as disease-inducing fungus of *O. glaberrima* seedlings. *Fusarium oxysporum* is a genetically heterogeneous polytypic morphospecies, which includes pathogenic as well as non-pathogenic strains. Various pathogenic strains of *F. oxysporum* cause wilt disease of various agronomically important crop plants, including legumes. Pathogenic *F. oxysporum* variants usually possess a narrow host range and isolates infecting identical hosts have been grouped into the taxonomic hierarchy 'forma specialis' (Gordon & Martyn 1997). Although most pathogenic *F. oxysporum* strains have been studied in agricultural context, wilt disease induced by *F. oxysporum* has been also

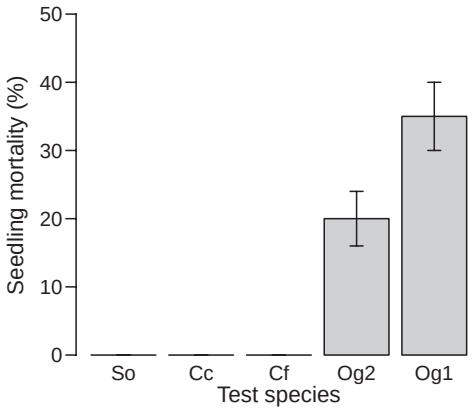

Fig. 4. Effect of isolated *Fusarium oxysporum* on survival of seedlings of *Ormosia glaberrima* and tree species coexisting with *O. glaberrima*. Seedlings (16 seedlings; 1 plant per jar) were individually inoculated with a conidial suspension. Plants were incubated under growth-room conditions and mortality of seedlings was determined at the end of the experiment (60 days post-inoculation). Non-inoculated control plants showed 100% survival. Data indicate means ± SD. Abbreviations: So, *Schefflera octophylla*; Cc, *Cryptocarya concinna*; Cf, *Castanopsis fabri*; Og1, *O. glaberrima* (seeds from site 1); Og2, *O. glaberrima* (seeds from site 2).

reported for legume trees such as *Acacia koa* (Gardner 1980) and *Albizia julibrissin* (Stipes & Phipps 1975).

Fusarium oxysporum not only negatively affected survival of *O. glaberrima* seedlings, but also seed germination (pre-emergence stage). Therefore, effects of soil pathogens on *O. glaberrima* in our experiments were expressed as seedling/seed ratio (number of survived seedlings divided by planted seeds). Although restraining effects of soil pathogens on young seedlings have been found to be crucial (Harms *et al.* 2000; Bell, Freckleton & Lewis 2006; Bagchi *et al.* 2010), effects of pathogens during the pre-emergence stage should not be neglected (Hille Ris Lambers, Clark & Beckage 2002; Bell, Freckleton & Lewis 2006). Gardner (1980) reported that *A. koa* seeds were infected by *F. oxysporum* forma specialis *koae* and that the fungus was seed-borne. In our study, however, surface-sterilized *O. glaberrima* seeds contained no detectable fungi, indicating that the pathogen was soil borne.

Host specificity is an explicit assumption of the Janzen–Connell hypothesis (Janzen 1970; Connell 1971; Packer & Clay 2000; Augspurger & Wilkinson 2007). Although this assumption has been challenged by findings of certain studies (Novotny *et al.* 2002; Gilbert & Webb 2007), our results indicate that the characterized *F. oxysporum* isolate was pathogenic on *O. glaberrima*, but not on other tested tree species. Interestingly, seed provenance (*O. glaberrima* seeds collected from site 1 vs. site 2) influenced pathogen-induced seedling mortality in the field experiment (Table 1) as well as in the growth-room experiment (Table 2; Fig. 4). Trees at site 1 could be genetically different from the single tree at site 2 or their seed quality could be reduced (although their average seed weight was not different; 100-seed weight is 23.1 ± 0.27 g for seeds from site 1, and 100-seed weight is 22.7 ± 0.42 g for seeds from site 2; Student's t-test, $t = 1.705$, d.f. = 6.868, $P = 0.133$). A previous study showed that focal *Prunus serotina* trees differed with respect to pathogen effects on recruitment of juveniles (Packer & Clay 2003). In our study, however, no significant differences for seedling survival among the five focal trees at site 1 were detected. Based on these findings, we suggest that the trees at site 1 may be genetically closely related, but different from the single tree at site 2. Future work is required to genetically analyse seeds from individual *O. glaberrima* trees and to test whether genetic variations are crucial for seedling survival in the interaction with *F. oxysporum*. It is worth noting that host specificity in plant–pathogen interactions can depend on a single mutation in the genome of either the pathogen (virulence and avirulence genes) or the host plant (resistance genes), resulting in 'gene-for-gene' interactions (Thompson & Burdon 1992; Gandon *et al.* 1996; Thrall & Burdon 1997). Thus, the seed provenance of host plants should not be neglected when testing the Janzen–Connell hypothesis in future studies.

Conclusion and outlook

This study provides empirical data that a negative plant–soil feedback was caused by a specific soil-borne fungus, which accumulated in a distance-dependent pattern around parent trees. At study site 1, *F. oxysporum* apparently keeps self-replacement of *O. glaberrima* in check and therefore fulfils an essential prerequisite of the Janzen–Connell hypothesis. Taken the identified interaction as a paradigm, we suggest that many other host-specific pathogens contribute to the maintenance of tree community structure. Identification and analysis of pathogens from other dominant tree species and determination of their host preference will provide the basis for future simulation experiments with whole plant communities. Test plot-based pathogen exclusion and inoculation experiments in the field should be combined with growth-room simulations. Particular attention should be paid to factors modulating pathogenicity and disease such as humidity, light conditions, host genotype, seed quality, inoculum density and pathogen-suppressing microbes. Experiments are also required to localize tree pathogens *in situ* by PCR-based techniques to obtain more information on their temporal-spatial distribution in forests.

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Supporting Information

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

Table S1. Reasons for selection of *O. glaberrima* as focal tree species.

Table S2. Tree communities at the two *O. glaberrima* sites (1 ha plot).

Table S3. Pathogenicity of isolated fungi on seeds and seedlings of *O. glaberrima*.

Table S4. Seedling emergence (expressed in percentages) in the fungal exclusion experiment at site 1.

Table S5. Seedling emergence (expressed in percentages) in the growth-room simulation experiment.

Figure S1. *O. glaberrima* trees situated at site 1 and site 2 in the Heishiding Nature Reserve.

Figure S2. Seed density and seedling density at indicated distance from the single adult *O. glaberrima* tree at site 2.

Figure S3. Example of a plastic jar unit used in this study.

Figure S4. Time course of seedling survival (seedling/seed ratio) of *O. glaberrima* in the field and greenhouse experiments.

Figure S5. Seedling survival (seedling/seed ratio) of *O. glaberrima* in the growth-room simulation experiment with soil samples from site 2.

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Supporting information

Table S1 Reasons for selection of *O. glaberrima* as model species in this study

	Characteristics
1.	*O. glaberrima* is a characteristic tree species of the Heishiding Nature Reserve.
2.	Seeds could be collected in sufficient amounts.
3.	Stored seeds could germinate at a high rate.
4.	Seeds could be successfully surface-sterilized.
5.	No seed-borne microbes were detected in surface-sterilized seeds.
6.	Non-germinated seeds with their impermeable and hard seed coat were not attacked by microbes in a pre-experiment.
7.	No indications for any seed herbivores were obtained in a pilot experiment, suggesting that the cardinal-red color of *O. glaberrima* seeds has an aposematic function.

Table S2 Tree communities at the two sites (1 ha-plot)

Species	Family	Individual number	
		site 1	site 2
Ixonanthes chinensis	Ixonanthaceae	175	16
Cryptocarya concinna	Lauraceae	126	80
Neolitsea phanerophlebia	Lauraceae	106	1
Xanthophyllum hainanense	Polygalaceae	70	49
Reevesia thyrsoidea	Sterculiaceae	67	12
Symplocos adenophylla	Symplocaceae	59	16
Itea chinensis	Escalloniaceae	53	0
Quercus chungii	Fagaceae	51	0
Machilus breviflora	Lauraceae	51	128
Altingia chinensis	Hamamelidaceae	45	0
Sinosideroxylon pedunculatum	Sapotaceae	44	0
Ormosia glaberrima	Fabaceae	43	1
Rapanea neriifolia	Myrsinaceae	39	0
Syzygium hancei	Myrtaceae	38	0
Schefflera octophylla	Araliaceae	33	13
Ardisia quinquegona	Myrsinaceae	37	32
Artocarpus styracifolius	Moraceae	30	37
Castanopsis fabri	Fagaceae	23	1
Lithocarpus litseifolius	Fagaceae	23	2
Engelhardia fenzelii	Juglandaceae	19	43
Ilex memecylifolia	Aquifoliaceae	28	12
Schima superba	Theaceae	19	14
Lindera chunii	Lauraceae	18	7
Pithecellobium lucidum	Fabaceae	17	28
Cinnamomum porrectum	Lauraceae	12	4
Illicium henryi	Illiciaceae	12	0
Engelhardia roxburghiana	Juglandaceae	11	23
Pinus massoniana	Pinaceae	11	23

Lithocarpus haipinii	Fagaceae	9	5
Litsea acutivena	Lauraceae	9	3
Randia canthioides	Rubiaceae	9	5
Ternstroemia gymnanthera	Theaceae	9	14
Camellia caudata	Theaceae	8	13
Diospyros morrisiana	Ebenaceae	7	27
Machilus chinensis	Lauraceae	7	27
Canavium album	Burevaceae	6	9
Lithocarpus lohangwu	Fagaceae	6	0
Litsea rotundifolia	Lauraceae	6	0
Meliosma fordii	Sabiaceae	6	0
Carallia diplopetala	Rhizophoraceae	5	12
Cinnamomum austrosinense	Lauraceae	5	13
Ilex pubilimba	Aquifoliaceae	5	1
Machilus velutina	Lauraceae	5	98
Symplocos anomala	Symplocaceae	5	9
Acmena acuminatissima	Myrtaceae	4	5
Daphnipyllum oldhamih	Daphniphyllaceae	4	1
Castanopsis carlesii	Fagaceae	3	15
Castanopsis kawakamii	Fagaceae	3	15
Cercis sp.	Fabaceae	3	2
Garcinia multiflora	Guttiferae	3	15
Litsea elongata	Lauraceae	3	2
Sapium discolor	Euphorbiaceae	3	14
Styrax confusa	Styracaceae	3	7
Toxicodendron succedaneum	Anacardiaceae	3	15
Camellia sinenis	Theaceae	2	3
Antidesme micropyllum	Euphorbiaceae	1	15
Choerospondias axillaris	Anacardiaceae	1	5
Elaeocarpus sylvestris	Elaeocarpaceae	1	30
Garcinia oligantha	Guttiferae	1	4
Ilex dasyphylla	Aquifoliaceae	1	8
Symplocos lancifolia	Symplocaceae	1	9
Itea chinensis	Saxifragaceae	0	126
Lithocarpus hancei	Fagaceae	0	29
Altingia chinensis	Hamamelidaceae	0	21
Evodia lepta	Rutaceae	0	13

Ardisia elegans	Myrsinaceae	0	12
Carallia brachiata	Rhizophoraceae	0	11
Syzygium rehderianum	Myrtaceae	0	11
Craibiodendron scleranthum	Ericaceae	0	7
Ormosia pachycarpa	Fabaceae	0	5
Saurauia tristyla	Actinidiaceae	0	5
Sinosideroxylon wightianum	Sapotaceae	0	5
Vitex quinata	Verbenaceae	0	4
Acer tutcheri	Aceraceae	0	3
Helicia cochinchinensis	Proteaceae	0	3
Lithocarpus corneus	Fagaceae	0	3
Pterospermum heterophyllum	Sterculiaceae	0	3
Symplocos lancifolia	Symplocaceae	0	2
Castanopsis nigrescens	Fagaceae	0	1
Cinnamomum pauciflorum	Lauraceae	0	1
Elaeocarpus deciplens	Elaeocarpaceae	0	1
Ficus superba	Moraceae	0	1
Ilex fragilis	Aquifoliaceae	0	1
Michelia foveolata	Magnoliaceae	0	1
Zanthoxylum dissitum	*Rutaceae*	0	1
Unkown species	----	61	81

Species used for testing host specificity of *F. oxysporum* are shown in boldface type.

Table S3 Pathogenicity of isolated fungi on *O. glaberrima* seeds and seedlings

Group	Isolate	Disease symptoms
1	Mixture F01-F07	+/+
	F01	-/-
	F02	-/-
	F03	-/-
	F04	-/-
	F05	-/-
	F06	+/+
	F07	-/-
2	Mixture F08-F18	-/-

Isolates were divided into two groups according to literature indicated below (Stipes & Phipps 1975; Gardner 1980; McQuilken & Hopkins 2001; Recorbet *et al.* 2003; Jeewon, Liew & Hyde 2004; Slippers *et al.* 2004; Wei & Xu 2004; Garibaldi, Bertetti & Gullino 2008). Group 1 contained potential pathogens and isolates of group 2 were predicted to be non-pathogenic isolates. Seeds or seedlings were inoculated with conidial suspensions and kept under growth-room conditions. Pathogenicity tests were first performed with indicated mixtures and then individually with each isolate of group 1. Abbreviations: +/+, significant disease symptoms during the stages of pre-germination (poor seed germination, fungal colonization of seed coat) and post-germination (reduced growth, leaf chlorosis, seedling death), respectively; -/- no significant disease symptoms.

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Table S4 Seedling emergence (expressed in percentages) in the fungal exclusion experiment at site 1 two weeks after planting seeds into test squares. Seeds collected from site 1 or site 2 were planted at high (H), medium (M) and low (L) density (16, 9 and 4 seeds per test square, respectively). Data indicate means ± SD

Seed prove-nance	**Treatment**	**Distance from focal tree (m)**														
		0			**5**			**10**			**15**			**20**		
		H (%)	M (%)	L (%)	H (%)	M (%)	L (%)	H (%)	M (%)	L (%)	H (%)	M (%)	L (%)	H (%)	M (%)	L (%)
site 1	control	41.7±7.4	45.7±9.9	45.0±11.2	47.5±9.4	48.9±9.7	40.0±13.7	75.0±8.8	64.5±14.5	70.0±20.1	71.2±12.9	68.9±9.3	75.0±17.7	74.1±4.0	71.4±12.7	80.0±11.2
	fungicide	68.8±12.5	68.9±14.5	65.0±13.7	66.3±9.4	68.9±14.5	70.0±11.2	70.0±13.0	68.9±9.3	75.0±17.7	76.3±12.0	71.4±12.7	75.0±25.0	74.5±5.8	71.1±12.7	80.0±20.1
site 2	control	50.0±9.8	51.1±12.7	50.0±17.7	46.3±3.4	53.3±9.3	55.0±11.2	71.3±9.4	68.9±12.2	75.0±17.7	73.8±12.0	71.4±12.7	75.0±25.0	71.3±13.0	71.1±12.7	80.0±20.1
	fungicide	66.3±9.5	62.3±9.9	75.0±17.7	67.5±9.3	68.9±14.5	75.0±17.7	67.5±9.3	71.4±12.7	75.0±17.7	72.5±13.0	71.4±12.7	75.0±17.7	71.3±13.0	66.7±13.6	80.0±11.1

Table S5 Seedling emergence (expressed in percentages) in the growth-room simulation experiment two weeks after planting seeds into jars. Seeds collected from site 1 or site 2 were planted at high (H) and low (L) density (4 and 1 seeds per jar, respectively). Data indicate means ± SD

Seed prove-nance	**Treatment**	**Distance from focal tree (m)**									
		0		**5**		**10**		**15**		**20**	
		H (%)	L (%)	H (%)	L (%)	H (%)	L (%)	H (%)	L (%)	H (%)	L (%)
site 1	control	55.0±14.3	50.0±35.4	50.0	55.0±14.3	77.5±10.5	80.0±27.4	85.0±10.5	90.0±22.4	85.0±10.5	90.0±22.4
	fungicide	80.5±14.3	90.0±22.4	90.0±10.5	80.0±14.3	85.0±10.6	90.0±22.4	87.5±8.8	90.0±22.4	90.0±10.5	80.0±14.3
	gamma-radiation	90.0±10.4	90.0±22.4	90.0±16.2	90.0±22.4	95.0±6.8	80.0±27.3	87.5±8.8	100.0	90.0±10.4	90.0±22.4
site 2	control	60.0±10.5	50.0	50.0±22.4	60.0±27.4	90.0±10.5	80.0±27.4	85.0±10.5	90.0±22.4	85.0±10.5	90.0±22.4
	fungicide	87.5±8.8	90.0±22.4	95.0±6.8	80.0±27.4	87.5±8.8	90.0±22.4	85.0±13.7	90.0±22.4	82.5±11.2	100.0
	gamma-radiation	90.0±13.7	90.0±22.4	80.0±6.8	100.0	92.5±6.8	80.0±27.4	85.0±10.5	100.0	90.0±5.6	90.0±22.4

Fig. S1 *O. glaberrima* trees situated at site 1 and site 2 in the Heishiding Nature Reserve. Insert figures: Distribution of *O. glaberrima* at the two sites. Individuals were categorized into mature trees with DBH ≥ 9 cm (○), dead trees (Δ), premature trees with DBH 4 ≤ DBH < 9 cm (●), and juveniles at a height ≥ 50 cm and DBH < 4 cm (●).

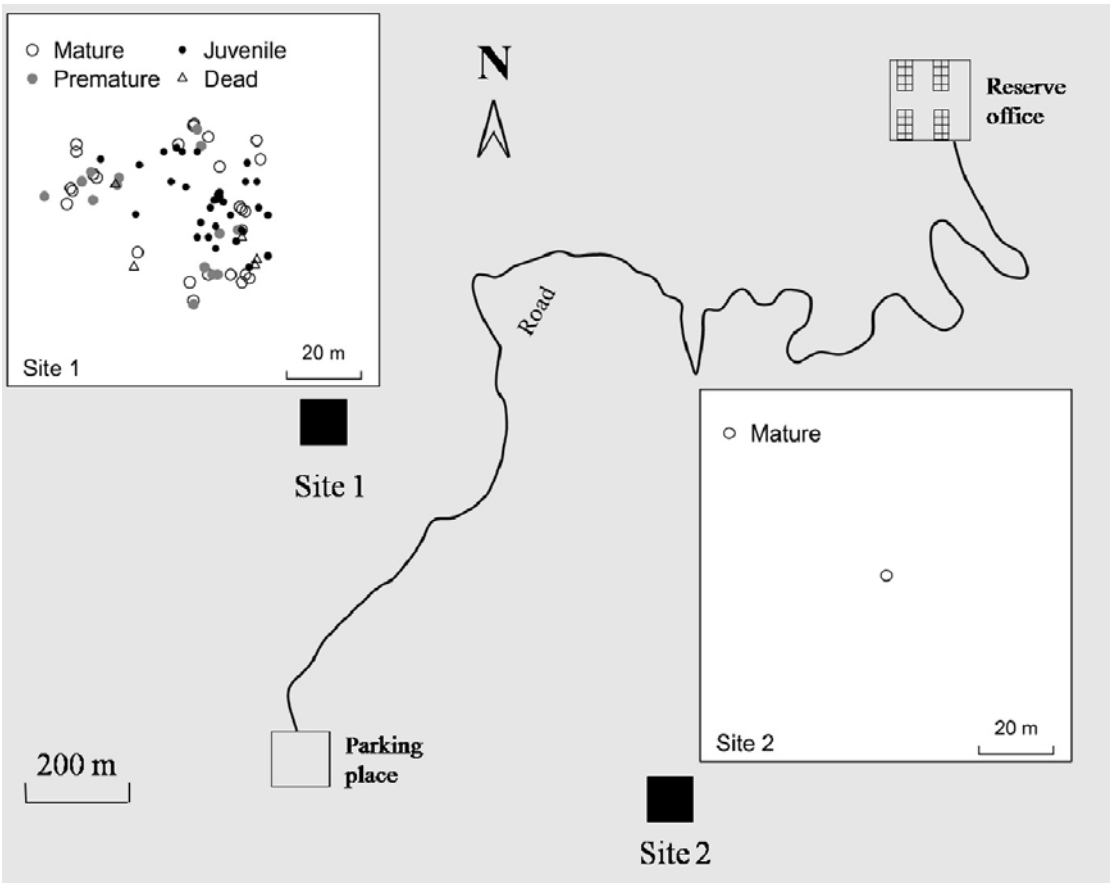

Fig. S2 Seed density (a) and seedling density (b) at indicated distance from the single mature tree at site 2. Seed density was determined after seed fall in November 2008. No seed fall was observed during the following two years. Seedling number of October 2009 was defined as the sum of surviving seedlings tagged in April 2009 and non-tagged seedlings, which emerged between April and October. Using the same method, the number of seedlings was determined in April 2010 and October 2010. Data indicate means ± SD.

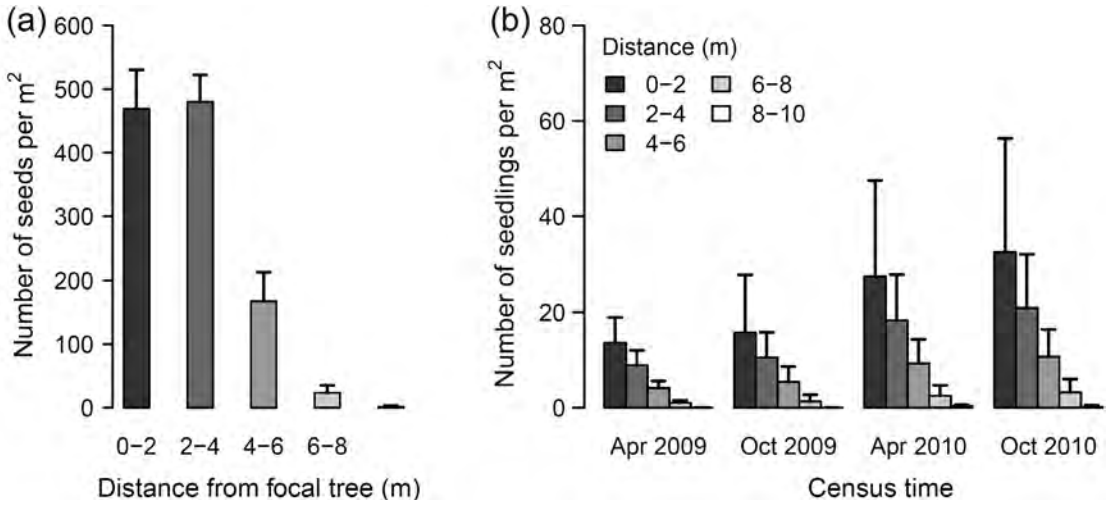

Fig. S3 Example of a plastic jar unit used in this study. The upper vessel was connected with the lower vessel by three cotton wicks. After sterilization of the jar unit, the upper vessel was filled with 250 ml of soil covered with a layer of sterilized expanded clay. The lower vessel was filled with sterilized distilled water (bar = 1 cm).

Fig. S4 Time course of seedling survival (seedling/seed ratio) of *O. glaberrima* (seeds only from site 1) during the manipulative field experiment at site 1 (a) and the corresponding growth-room simulation experiment (b). Data of the field experiment indicate means ± SD for test squares located at 0 m distance from focal trees. The seedling survival at the time of planting corresponds to the germination percentage (90 ± 7.07%). Data of the simulation experiment indicate means ± SD for soil collected from the same locations.

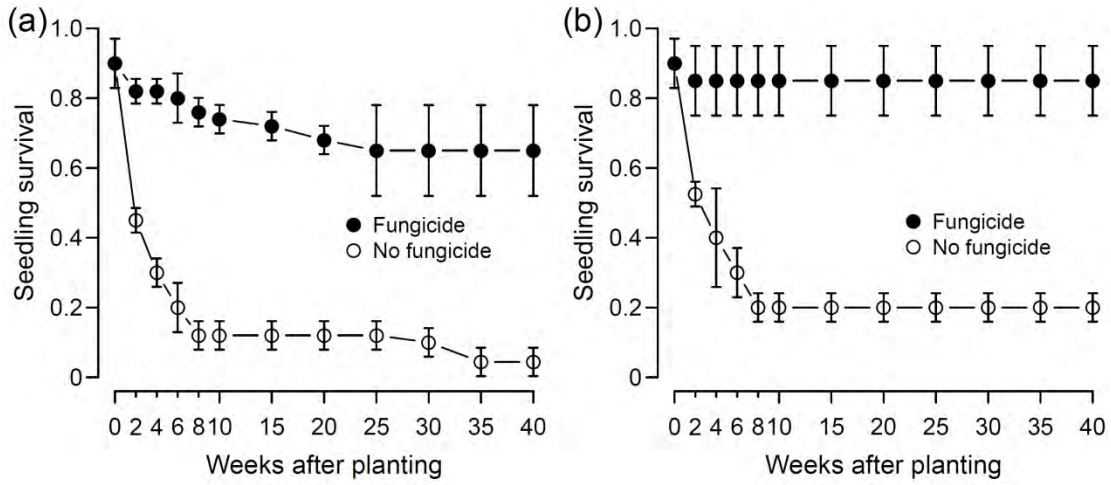

Fig. S5 Seedling survival (seedling/seed ratio) of *O. glaberrima* in the growth-room simulation experiment with soil samples from site 2. Soil samples around the single adult *O. glaberrima* tree were collected at indicated distance. Soil samples were treated with fungicides, sterilized by gamma-radiation, or left untreated. Surface-sterilized seeds of different provenances were planted into jars and kept under growth-room conditions for 40 weeks. Data indicate means ± SD at the end of the experiment (40 weeks after planting). Abbreviations: C, without sterilization; F, fungicide treatment; G, gamma-radiation treatment; Site 1, seeds collected from site 1; Site 2, seeds collected from site 2.