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Phylogenetic ecology applied to enrichment planting of tropical native tree species

Daniella Schweizer^{a,*}, Gregory S. Gilbert^{a,b}, Karen D. Holl^a^a Environmental Studies Department, University of California, Santa Cruz, CA 95064, USA^b Smithsonian Tropical Research Institute, Balboa, Ancón, Panama

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ABSTRACT

Enrichment planting within established plantations or secondary forests is a common strategy to enhance forest recovery, given that later successional forest species tend to have low dispersal and limited recruitment into these sites. It is difficult, however, to predict how species of seedlings will perform when planted under different overstory species. The field of phylogenetic ecology offers tools to help guide the selection of seedlings, drawing on the evolutionary conservatism of important functional traits. We evaluated the survival, growth, foliar disease, and herbivory of various native tropical tree seedlings at different evolutionary distances from monospecific stands of trees beneath which they were planted. We expected that seedlings planted under conspecific overstory trees would have low survival and growth and high percent foliar damage (as predicted by the Janzen–Connell Hypothesis), and that seedling performance would improve steadily with phylogenetic distance between seedling and overstory species. We found that seedlings planted under conspecific canopies had lower survivorship, reduced growth, and greater foliar damage than seedlings planted under canopies of different tree species. An overall increase in seedling performance with greater phylogenetic distance between seedling and overstory species was dominated by the contrast in performance between conspecific pairs and seedlings planted beneath extra-familial overstory species; but lack of available congeneric pairing limited inference about interactions among close relatives. Most pathogenic fungi isolated from enrichment-planted seedlings also caused disease when inoculated on the overstory tree species where the seedlings had been planted; this is consistent with overstory trees being an important reservoir of pathogens that affect seedlings. We conclude that enrichment planting with species more distantly related to those that dominate the canopy should enhance seedling's performance. Closer analysis at the congeneric level is warranted because of expected strong biotic interactions at close phylogenetic distances.

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1. Introduction

Restoring tropical forests on abandoned agricultural lands often requires human intervention. Lack of seed dispersal, competition from exotic grasses, and stressful biotic conditions combine to slow or prevent natural succession to pre-deforestation communities (Holl, 1999; Aide et al., 2000; Cole et al., 2010). One promising restoration strategy is to establish early successional species tolerant of open conditions, followed by enrichment planting of desired woody species under an established canopy (Lamb, 1998, 2011; Paquette et al., 2006). This approach can accelerate succession, increase biodiversity, and enhance carbon sequestration (Ashton et al., 1998; Schulze, 2008; Keefe et al., 2009; Paquette et al., 2009). However, choosing combinations of overstory and enrichment species that are likely to be successful is largely a process of trial and error.

Empirical studies that inform choices of enrichment species have mainly focused on the effect of light (i.e. how canopy species vary in the depth of shade they create and how seedlings vary in their shade tolerance) and on competition among species (Ashton et al., 1998; Menalled et al., 1998; Pena-Claros et al., 2002; Schuler and Robison, 2010). Fewer studies have looked at other interactions that affect species performance. Some studies have found high pest incidence on seedlings planted under monospecific stands of common forestry species in *Eucalyptus*, *Pinus*, and *Dipterocarpaceae* (Kirton and Cheng, 2007; Lombardero et al., 2008; Zhou et al., 2008; Chungu et al., 2010). The predictive power of how particular seedling species will perform under a given overstory species is limited by the small number of species that have been evaluated. Here we combine a large experimental test of the performance of enrichment-planted tree species under established overstory trees with the analytical tools of phylogenetic ecology to evaluate whether phylogenetic relationships provide a predictive framework for choosing species for successful enrichment planting.

* Corresponding author. Address: Environmental Studies Department, University of California, 1156 High Street, Santa Cruz, CA 95064, USA. Tel.: +1 831 334 7802.

E-mail address: daniellaschweizer@gmail.com (D. Schweizer).

Ecologically important traits are often phylogenetically conserved (e.g., drought tolerance is conserved within the cacti) (Farrell, 2001; Blomberg et al., 2003; Chazdon et al., 2003). Conservation of ecologically important traits can mean that closely related species are able to occupy similar habitats (Kozak and Wiens, 2006; Donoghue, 2008; Wiens et al., 2010), but also that they are more likely to compete strongly for resources (Elton, 1946; Dayan and Simberloff, 2005; Wilson and Stubbs, 2012) and share pests and pathogens (Novotny et al., 2002a, 2002b; Gilbert and Webb, 2007). Indeed, biotic interactions of all types are phylogenetically conserved across the entire tree of life (Gomez et al., 2010), and evolutionary distance between plant species can be useful as an index of the likelihood that species share important ecological traits. For instance, phylogenetic distance is a good and easily determined predictor of whether a pathogen or pest of one plant species will cause disease on another (Gilbert and Webb, 2007; Ness et al., 2011; Gilbert et al., 2012). This phylogenetic signal in host range of pests and pathogens may provide an integrative index of the likely performance of seedlings growing under established canopies of different tree species.

We know from numerous empirical tests of the Janzen–Connell Hypothesis (Janzen, 1970; Connell, 1971) that tropical tree seedlings and saplings growing closer to conspecific adults often perform worse and suffer greater attack from natural enemies than do seedlings growing under a canopy of heterospecific trees (e.g. Clark and Clark, 1984; Hubbell et al., 1990; Gilbert et al., 1994; Metz et al., 2010; Bagchi et al., 2011; Paine et al., 2012). One recent experimental test of the Janzen–Connell Hypothesis found that seedlings survive significantly less in soil from below conspecific trees than in heterospecific soil due to the presence of soil pathogens (Liu et al., 2012). What is less clear is whether there is a continuous increase in performance with phylogenetic distance of heterospecifics; or instead, are all heterospecific canopies broadly equivalent for increased seedling performance regardless of their phylogenetic distance to the seedlings?

We tested whether phylogenetic distance between overstory trees and seedlings planted beneath them is a useful predictor of seedling growth and survival, as well as of damage to leaves by pests and pathogens shared with the overstory trees. In addition, we conducted a cross-inoculation experiment to test whether the likelihood of developing foliar disease symptoms decreased continuously with phylogenetic distance between seedling and overstory species. We expected that seedlings closely related to the overstory tree species would have lower performance and higher foliar damage than those more distantly related. This study aims to evaluate whether this phylogenetic approach could provide a useful tool for restoration practitioners to select which species may be planted together most successfully when species-specific empirical data are lacking. Evolutionary relationships may reduce the need for extensive multi-species field-testing and guide more efficient empirical testing. The incorporation of phylogenetic diversity in ecosystems restoration may promote ecosystem stability through increased trait and functional diversity (Forest et al., 2007; Cadotte et al., 2012; Tan et al., 2012; Verdu et al., 2012).

2. Materials and methods

2.1. Study sites

We conducted this study in “The Native Species Reforestation Project” (PRORENA), located in Soberania National Park in the Panama Canal Watershed, Republic of Panama. PRORENA was established by the Center for Tropical Forest Science at the Smithsonian Tropical Research Institute (STRI) and by the Yale Tropical Resources Institute to assess the forestry potential of na-

tive tree species and promote their use in forestry and reforestation (Wishnie et al., 2007).

Soberania National Park has a strongly seasonal climate with a mean annual rainfall of 2226 mm and 4.1 dry months annually between December and May (dry months have < 100 mm rainfall). Soberania National Park overlies tropical ultisols that are predominantly clay or silty clay (Park et al., 2010). Most of the park is covered by secondary tropical rain forest. The PRORENA study site was deforested prior to the 1960s and then farmed for several decades until it was incorporated into the park in the 1980s. Prior to the PRORENA project, the plot was fallow for at least 10 years and was invaded by dense stands of the exotic grass *Saccharum spontaneum* L. (Wishnie et al., 2007).

2.2. Enrichment planting design and methods

Overstory tree species at PRORENA were planted in 2003 in 9-m × 12-m monospecific plots with each overstory tree species planted in three randomly-selected, replicate plots (Fig. A1). Trees were planted at an initial density of 20 trees per plot. Understory vegetation was kept clear with machetes and string trimmers for 2 years following planting, and the planted trees were sprayed with insecticide. After 2 years, the plots were thinned to leave ten trees spaced at 6 m, and understory clearing and pesticide treatment stopped (Wishnie et al., 2007). Of the 24 native tree species originally planted, we chose 11 overstory species that had good growth and that produced a closed canopy at least during the rainy season (i.e., some species are dry-season deciduous) (Table 1).

We planted a total of 2512 seedlings from 20 species and 14 families beneath the canopies of the 11 chosen overstory species (Fig. A1). We chose seedling species that would cover the widest breadth possible of phylogenetic distances (time of independent evolution) from the overstory species from those available as nursery seedlings, which resulted in planting between four and six different seedling species beneath each overstory tree species (Table 2 and Appendix A2). Some seedling species, such as *Copaifera aromatica*, *Swietenia macrophylla* and *Tabebuia rosea*, were planted more than other species since they represented a similar evolutionary distance beneath most overstory species. In this experimental design we replicated the phylogenetic distances not the seedlings species identity. The number of initially planted seedlings beneath each overstory species varied from 12 to 17 per seedling species. We included the number of initially planted individuals as a variable in the growth and survival models to test its effect.

The phylogenetic distances were calculated using the Phylomatic tool in Phylocom, version 4.1 (Webb et al., 2008). We used the most recent, maximally resolved Angiosperm tree (R20080417.new) as backbone to our phylogenetic tree, which is based on the APG3 classification and we dated it using Wikstrom ages (Wikstrom et al., 2001; Stevens, 2008). The phylogenetic distance between the seedling and the overstory species ranged from 0 to 233.1 My of independent evolution. Time of independent evolution is twice the time to most recent common ancestor. For the species in this study, 0 My corresponds to conspecifics, 57.2–87.8 My to confamilials, 90.5 My corresponds to the single conordinal pair in the study (*T. rosea* beneath *Tectona grandis*), and 91+ My to extraordinals. Congeneric species were not available for this study.

The design of this study is such that the independent variable of interest is the phylogenetic distance between the seedlings and the tree species beneath which they were planted, rather than the species identity of the seedlings or of the overstory species. Due to this experimental design only three species – *Colubrina glandulosa*, *Pachira quinata*, and *Terminalia amazonia* – were planted under canopies of both conspecifics and heterospecifics (Table 2). These

Table 1

Percent canopy openness of the various overstorey species. Shown is the mean of three measurements in each of the three replicate plots per species \pm sd. Foliar periodicity was determined from the ratio of dry:wet season canopy openness, where: evergreen ≤ 1.5 ; semideciduous 1.51–3; deciduous >3.0 . Wet season measurements were taken in August 2009 and dry season measurements in March 2010.

Overstorey species	Canopy cover%		Foliar periodicity
	Wet season	Dry season	
<i>Acacia mangium</i> Willd.	12.7 \pm 1.6	19.2 \pm 0.2	Evergreen
<i>Colubrina glandulosa</i> Perkins	18.0 \pm 5.2	33.3 \pm 0.2	Semi-deciduous
<i>Dyphisa robinoides</i> (Mill.) M. Sousa	13.4 \pm 1.8	18.1 \pm 3.9	Evergreen
<i>Gliricidia sepium</i> Kunth ex Steud.	11.4 \pm 3.6	16.7 \pm 5.8	Evergreen
<i>Inga punctata</i> Willd.	11.5 \pm 0.6	20.7 \pm 3.6	Semi-deciduous
<i>Luehea seemannii</i> Triana & Planch.	18.0 \pm 9.5	18.8 \pm 0.1	Evergreen
<i>Ochroma pyramidale</i> (Cav. ex Lam.) Urb.	18.7 \pm 4.0	30.8 \pm 5.2	Semi-deciduous
<i>Pachira quinata</i> (Jacq.) W.S. Alverson	12.8 \pm 3.2	38.2 \pm 5.3	Deciduous
<i>Spondias mombin</i> L.	24.6 \pm 2.3	32.3 \pm 3.5	Evergreen
<i>Tectona grandis</i> L. f.	6.8 \pm 0.9	44.7 \pm 4.4	Deciduous
<i>Terminalia amazonia</i> (J.F. Gmel.)	12.4 \pm 3.5	20.3 \pm 0.7	Semi-deciduous

Table 2

Seedling species used in this study, and their survival for 23 months under conspecific or heterospecific canopy species. SI: shade intolerant, IST: intermediate shade tolerant, ST: shade tolerant.

Species	Family	Light tolerance	# Planted	% Survival under	
				Con.	Hetero.
<i>Acacia mangium</i> Willd	Fabaceae	SI ^a	50	4.0	–
<i>Anacardium excelsum</i> (Bertero & Balb. ex Kunth) Skeels	Anacardiaceae	IST ^b	42	–	61.9
<i>Chrysophyllum cainito</i> L.	Sapotaceae	ST ^a	42	–	64.3
<i>Colubrina glandulosa</i> Perkins	Rhamnaceae	SI ^a	150	26.0	73.3
<i>Copaifera aromatica</i> Dwyer	Fabaceae	ST ^c	469	–	75.9
<i>Cordia alliodora</i> (Ruiz & Pav.) Oken	Boraginaceae	SI ^{d,e}	42	–	54.8
<i>Dyphisa robinoides</i> (Mill.) M. Sousa	Fabaceae	SI ^a	17	100.0	–
<i>Gliricidia sepium</i> Kunth ex Steud.	Fabaceae	SI ^a	51	7.9	–
<i>Inga punctata</i> Willd.	Fabaceae	SI ^a	43	60.3	–
<i>Luehea seemannii</i> Triana and Planch	Tiliaceae	SI ^d	50	32.0	–
<i>Manilkara zapota</i> (L.) van Royen	Sapotaceae	ST ^e	41	–	82.6
<i>Ochroma pyramidale</i> (Cav. ex Lam.) Urb.	Malvaceae	SI ^d	19	0.0	–
<i>Pachira quinata</i> (Jacq.) W.S. Alverson	Bombacaceae	IST ^c	82	45.2	67.5
<i>Sapium glandulosum</i> L. Morong	Euphorbiaceae	IST ^f	92	–	11.8
<i>Spondias mombin</i> L.	Anacardiaceae	SI ^d	40	4.8	–
<i>Sterculia apetala</i> (Jacq.) Karst.	Sterculiaceae	SI ^d	78	–	56.4
<i>Swietenia macrophylla</i> King	Meliaceae	ST ^g	506	–	87.7
<i>Tabebuia rosea</i> (Bertol.) DC.	Bignoniaceae	IST ^d	516	–	68.8
<i>Tectona grandis</i> L. f.	Verbenaceae	SI ^a	43	66.7	–
<i>Terminalia amazonia</i> (J.F. Gmel.) Exell.	Combretaceae	IST ^{e,h}	139	60.0	88.1

^a World Agroforestry Centre, n.d.

^b Plath et al. 2011.

^c Wishnie et al. 2007.

^d Condit et al. 1996.

^e Piotto 2007.

^f The Country Day School, n.d.

^g Ramos and Delamo 1992.

^h Redondo-Brenes and Montagnini 2006.

three species provide a means to evaluate effects of phylogenetic distance to the overstorey species with a standard reference performance (growth under conspecifics vs. growth under heterospecifics).

Most seedlings came from the PRORENA nurseries and were at least 6 months old at the time of outplanting. For some species, supplemental seedlings were collected from a nearby forest a month before planting (five species; seedlings > 1 year old) or bought from commercial nurseries (three species). Post hoc testing showed no significant differences in survivorship among seedling sources for all but two species: *Sapium glandulosum* seedlings collected from the forest had lower survival than those grown in the nursery, and *S. macrophylla* seedlings from the PRORENA nursery grew better than those from other nurseries (data not shown). Given the minimal effect of source for most species, and randomization of seedlings from different sources across the experiment, seedling source was not further included in analytical models of seedling performance.

The average size of seedlings at outplanting ranged from 9 to 21 cm for most species. Three species were much larger: *S. macrophylla*, *Gliricidia sepium*, and *Dyphisa robinoides* had average heights of 30.6, 48.5, and 87.9 cm, respectively. We included initial height in the models to test its effect. From June to August 2008, between 14 (for overstorey trees with six enrichment-planted species) and 17 individuals (for overstorey trees with four species) of each species were planted in each of the plots for a total of 68–84 seedlings per plot. Seedlings were randomly planted along four planting lines in each plot, at 0.5-m separation between seedlings within a line, and 1-m separation between the lines and the edge of plot.

Seedlings were planted into 3 years of understory regrowth. The understories of the different overstorey tree species varied substantially, most notably in density of the invasive grass *S. spontaneum*. This grass is highly shade intolerant and was less dense or absent under tree species that had closed canopy. It was much more common in plots where overstorey species produced less shade (*Ochroma pyramidale*, *Spondias mombin*, *Luehea seemannii*, *C. glandulosa*).

Where *S. spontaneum* was too dense to allow planting, it was cut back with machetes along the planting line, but it re-grew within 2 months. We did not control *S. spontaneum* regrowth after planting, nor did we clear any other understory vegetation.

2.3. Performance and foliar damage data collection

We recorded survival and height for each seedling at five census times over 2 years: September 2008 (1 month after planting), January 2009 (5 months after planting, dry season), August 2009 (12 months after planting, wet season), March 2010 (19 months after planting, dry season), and August 2010 (23 months after planting, wet season). Leaf area and stem basal width were measured during censuses at 19 and 23 months. Height was measured from the base of the plant to its apical bud. In all censuses except the first, we counted the total number of leaves, the number of leaves with disease symptoms, and the number of leaves with herbivory. This provided an estimate of the average percent of leaves damaged by herbivory or disease.

2.4. Biophysical variables

Because seedlings performance is strongly affected by biophysical variables that vary among plantations of different overstory species, we measured canopy openness, percent ground cover, and soil macronutrients (NO_3 , NH_4^+ , and P) as potential covariates that could affect seedling performance.

Canopy openness is a proxy for irradiance, temperature and relative humidity in the understory (Gilbert et al., 2007). For canopy openness, we took three hemispherical canopy photos per plot during the wet and dry season using a fish-eye lens mounted on a leveled digital camera (Nikon Coolpix, model 995). Pictures were taken at dusk and always facing north. We took pictures with the camera placed on a tripod 1 m above the ground. The program Gap Light Analyzer v.2 (Frazer et al., 1999) was used to calculate the percent canopy openness from the photos; estimates were averaged for each of the three photos taken at a given camera height. We estimated cover of broad-leaved species, ferns, grass, bare ground, and litter (to the nearest percent) in 1-m² quadrats placed every 3 m along a 15-m diagonal transect on each plot.

We collected 5-cm diameter \times 10-cm deep soil cores at three randomly located points in each 9 \times 12-m plot and combined the samples for analysis. Soil samples were kept cool (4 °C) until extraction of the nutrients, which was done within 2–5 h. We extracted P using 25 ml of Mehlich-III extracting solution on 2.5 g of soil. For NO_3 and NH_4^+ extractions, we used 20 ml of potassium chloride (KCl) extracting solution on 2 g of soil (detailed protocols can be found at: <https://ctfs.arnarb.harvard.edu/webatlas/datasets/bci/soilmaps/BCIsoil.html>; see John et al., 2007 for a published version of these methods). Subsequent estimation of NO_3 and NH_4^+ was conducted using Lachat Quickchem method 12-107-04-1-B (NO_3) and 10-107-06-1-K (NH_4^+). KCl solution was employed as the carrier and to make combined standards between 0.5 and 20 mg N L⁻¹ for NH_4^+ and NO_3 . P was determined via ICP spectrometry.

2.5. Cross inoculation

We isolated pathogenic necrotrophic foliar fungi from the planted seedlings and inoculated them on the overstory tree species to assess the phylogenetic signal of shared pathogens. Between July and August 2010, we collected one diseased leaf from every surviving seedling in the field and processed a diseased leaf to isolate pathogenic foliar fungi following the methodology of Gilbert and Webb (2007). The fungal mycelia were allowed to grow for 5–7 days, time after which, a piece of mycelium-filled agar was

placed on a new Petri dish filled with 2% malt extract agar (MEA) and allowed to grow as a pure culture of the fungus. When multiple fungal morphotypes were apparent, each one was transferred to a separate dish. Less than 2% of the cultures could not be purified to single strains and were discarded. Each strain was preserved by placing actively growing mycelia plugs in a 2-ml cryovial and filling the vial with sterile water.

One week after fungal strain purification, strains were selected to conduct pathogenicity tests on seedlings of the enrichment-planted species and of the overstory species. Ten individual seedlings from each of the enrichment and overstory species were prepared by PRORENA nursery staff and placed in a greenhouse under partial shade with overhead netting. We included 659 fungal strains isolated from the 20 enrichment-planted seedlings in the PRORENA plots. Each strain was inoculated onto one leaf of each of two seedlings: (1) the seedling species from which it was isolated (i.e., Koch's proof of pathogenicity testing) and (2) the species of overstory tree beneath which the original seedling had been growing.

Inoculations and subsequent scoring of plant susceptibility followed the methodology employed by Gilbert and Webb (2007). One week after the inoculations, the leaves were harvested, and the disease symptoms (e.g., necrosis) recorded. If the inoculated leaf developed disease symptoms, that plant species was recorded as "susceptible" to that particular strain. If the control treatment (fungus-free mock inoculation) developed disease symptoms, that strain was excluded from the analyses because it indicated potential contamination; this occurred in <1% of the inoculations. A sample of 241 strains shown to be pathogenic on the original host species through the greenhouse tests were then inoculated using the same inoculation methods on the overstory tree species in the field.

We identified a subset of the isolated foliar fungi (173 of 659 strains) by extracting and amplifying nuclear ribosomal DNA (rDNA) from the mycelia of pure cultures using the *Extract-N-Amp Plant* PCR Kit from Sigma–Aldrich Laboratories (www.sigmaaldrich.com/). We selected fungal cultures to represent the range of morphotypes, which allowed us to characterize the diversity of foliar fungi isolated from the seedlings.

We amplified the ITS region using primers ITS 1 (TCCGTAGGT-GAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) (Gardes and Bruns, 1993; Larena et al., 1999). All PCRs contained 4.4 μ l NDI Water, 10 μ l Extract-N-Amp mix, 0.8 μ l of each of the primers (Forward and Reverse), and 4 μ l of DNA template for a 20- μ l reaction volume. The PCR protocol used was an initial 94 °C for 3 min, followed by 34 cycles of 94 °C for 30 s (denaturing phase), 54 °C for 30 s (annealing phase) and 72 °C for 1 min (extension phase) with one final extension of 72 °C for 10 min (final extension). All PCR sequencing was conducted at the UC Berkeley DNA Sequencing Facilities (<http://mcb.berkeley.edu/barker/dnaseq/services>). The sequenced fungi were further identified to the genus level through BLAST searches (<http://blast.ncbi.nlm.nih.gov/>).

2.6. Data analysis

2.6.1. Seedling performance using conspecific–heterospecific comparisons

Since most studies on seedling–overstory interactions have used a simple conspecific–heterospecific framework, we employed *t*-test to compare proportion surviving, growth rate (cm month⁻¹), and foliar damage (Area under Damage Progress Curve) for seedlings planted under conspecific overstory species vs. those planted under heterospecific overstory species, regardless of phylogenetic distance. Area under the Damage Progress Curve (AUDPC) integrates percent foliar damage over time using the mid-point or trapezoidal integration method of Campbell and Madden (1990):

$$\text{AUSPC} = \sum_i^{n-1} \frac{(y_i + y_{i+1})}{2} (t_{i+1} - t_i) \quad (1)$$

where n is the number of assessments, y_i is the percent damage at time i . We standardized AUDPC by dividing it over the total time of the study (23 months) so that it ranged from 0% to 100% foliar damage (Campbell and Madden, 1990).

2.6.2. Effect of phylogenetic distance on seedling performance and foliar damage

We estimated time to death, measured as the number of days from outplanting to the first census at which a seedling was recorded as dead. Such survival data are right censored when a seedling outlived the 23-month study period. The expected time of survival, and the impact of various independent variables (and their interactions) on survival were estimated from these data using Cox proportional hazards regression (Cox, 1972), which provides an integrated measure of differences in survival. Akaike Information Criterion was used to select the best-fit model of the survival data. The initial form of the Cox regression of the hazard function h is shown in the following equation:

$$\begin{aligned} \log h_i(t) = & \alpha(t) + \beta_1(\text{phylogenetic distance})_i \\ & + \beta_2(\text{canopy openness})_i \\ & + \beta_3(\text{ground cover (e.g., litter)})_i \\ & + \beta_4(\text{soil nitrogen})_i + \beta_5(\text{soil phosphorus})_i \\ & + \beta_6(\text{height at planting}) \\ & + \beta_7(\text{number of individuals planted}). \end{aligned} \quad (2)$$

As a second metric, we calculated the proportion of planted seedlings that survived the full 23 months of the study and used linear regression to fit survival as a function of phylogenetic distance between the planted seedling and the overstory species.

Seedling height, basal area, and foliar area were all strongly correlated ($R^2 > 0.73$ and $P < 0.0001$ for all pairwise correlations; data not shown); therefore, we used only seedling height for analysis of plant growth. Growth was calculated as monthly height change between censuses (e.g., $\text{height}_{\text{census2}} - \text{height}_{\text{census1}} / (t_{\text{census2}} - t_{\text{census1}})$) and averaged for all seedlings of a species within each plot. Seedling species that died and resprouted had a negative height change.

Seedling growth was analyzed employing a repeated measures design with time in months as the repeated measure, replicate plots nested within overstory species as a random effect, and phylogenetic distance, the different biophysical variables (canopy openness, ground cover, soil N and P), and initial height as covariates. Up to three way interactions were explored. Between-effects variables with a $P < 0.05$ for the intercept (i.e., interaction between the covariates and the categorical variable) were kept in the final model. We tried various regression approaches to fit to inter-census growth as a function of phylogenetic distance to explore the effect of this single variable, and a linear model best fit the data.

Foliar damage was estimated as the number of leaves showing disease symptoms and/or herbivory divided by the total number of leaves. Percent foliar damage was integrated over time by calculating the Area under the Damage Progress Curve (AUDPC) to evaluate whether the cumulative damage over time was explained by phylogenetic distance to the overstory species. Individuals that died were considered completely damaged. This assumption may inflate the importance of pests and pathogens to seedling survival, but provides an integrated estimate of the effect of phylogenetic distance on seedling health. AUDPC results were fitted to the independent variables using various regression approaches, with phylogenetic distance and the biophysical variables as explanatory variables. Linear regression was the best fit for the data and is re-

ported. The Akaike information criterion was used for model selection.

2.7. Cross inoculations

We used a χ^2 test to evaluate whether there was an association between pathogenicity of fungal isolates on the seedling species of origin and pathogenicity on the associated overstory tree species. We tested our hypothesis of a continuous decline in disease susceptibility with phylogenetic distance between overstory tree species and understory seedling species using logistic regression models with the $\log_{10}(\text{phylogenetic distance} + 1)$ as the independent variable, and the diseased/healthy status of individuals as the response variable. We fit these models to both the overstory species inoculated in the greenhouse and the overstory trees inoculated in the field.

All statistical analysis were conducted using the programs JMP 9.0.0 (SAS-Institute, 2010) and R 2.10.1 (R-Development-Core-Team, 2009) and $P < 0.05$ is considered significant throughout.

3. Results

3.1. Seedling survival

Of the 2512 seedlings planted, 1650 (65.7%) survived to the last census (23 months) (Table 2). Seedlings planted under heterospecific overstories had significantly greater survival (mean% = 66.1 ± 20.4) than did seedlings planted under conspecific canopies (mean% = 37.0 ± 32.4 , $t_{21} = 2.6$, $P = 0.0166$); the three species planted under both conspecific and heterospecific canopies followed the same pattern (Fig. 1A). Seedling survival increased with phylogenetic distance from the overstory species (Fig. 2A and Appendix A3) and was greater when planted under overstory species with more open canopies (i.e., were deciduous) during the dry season (Cox proportional hazards model, increased relative risk of mortality for phylogenetic distance = 0.9943 (95% CI = 0.9936–0.9950), relative risk increase for canopy openness in the dry season = 0.9941 (95% CI = 0.9882–1.0000), $\chi^2 = 241.3$, $df = 2$, $P < 0.0001$, $R^2 = 0.08$). The phylogenetic signal in percent survival was not significant when the analysis was restricted to family-level distances and greater (Cox proportional hazards model without conspecifics: relative risk increase of mortality for phylogenetic distance = 0.9952 (95% CI = 0.9890–1.0011), $\chi^2 = 3.2$, $df = 1$, $P = 0.0765$, $R^2 = 0.01$).

Shade intolerant seedling species had a significantly higher risk of death (Shade intolerant: relative risk increase = 3.46 (95% CI 2.94–4.06), $\chi^2 = 277.7$, $df = 2$, $P < 0.0001$, $R^2 = 0.09$). However, mortality decreased significantly with phylogenetic distance when shade intolerants were removed from the analysis (Cox proportional hazards model: relative risk increase = 1.0030 (95% CI 0.9962–0.9984), $\chi^2 = 30.4$, $df = 1$, $P < 0.0001$, $R^2 = 0.01$). Biophysical variables, number planted and initial height did not explain a significant amount of variation in seedling survival.

3.2. Seedling growth

Growth rates for the whole 23-months census period were higher when seedlings were growing beneath heterospecifics (Fig. 1B). The effect of treatment on differences in growth rates increased with time (repeated measures ANOVA showed significant time \times treatment interaction: Time: $F_{2,4,341.4} = 8.7$, $P \leq 0.0001$; Time \times Relationship to Overstory (cons or hetero): $F_{2,4,341.4} = 11.2$, $P < 0.0001$, with G-G correction for violation of sphericity). When phylogenetic distance was analyzed as a continuous variable, growth increased as a function of time and phylogenetic distance

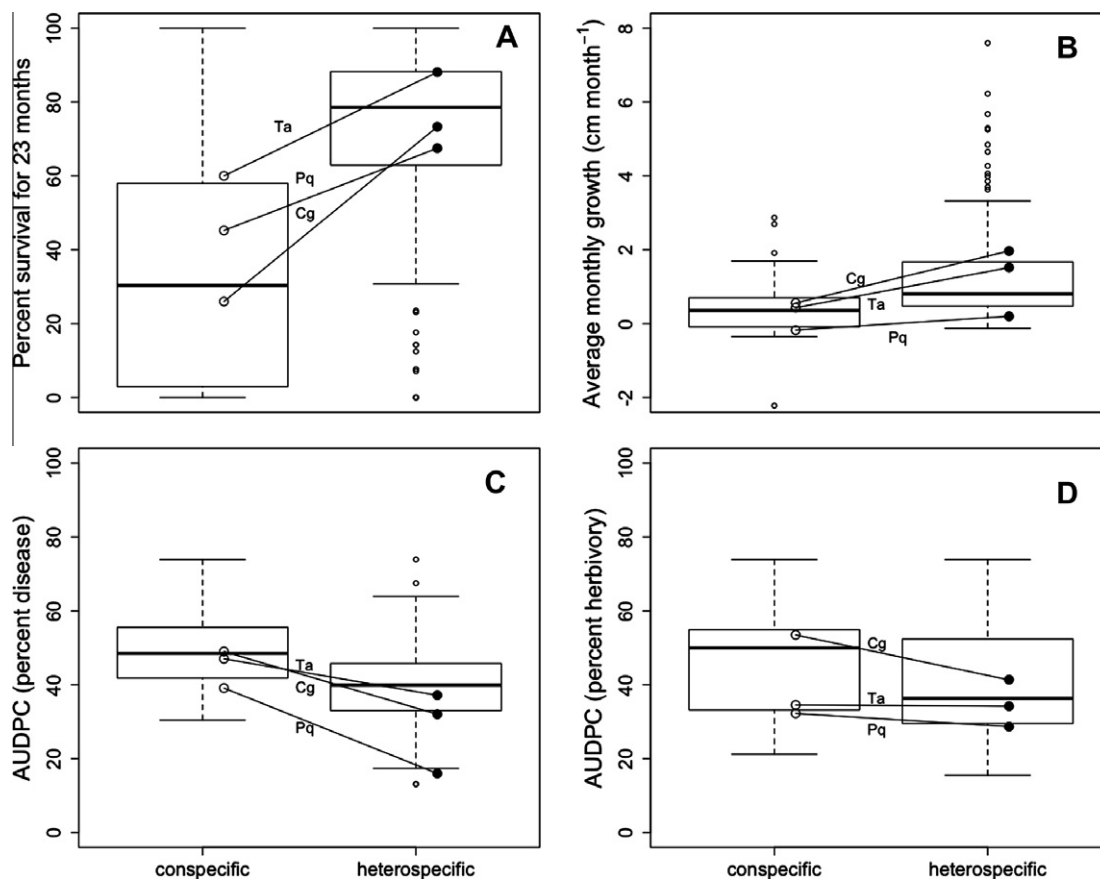


Fig. 1. Seedling performance under conspecific and heterospecific overstories, 23 months after planting. Each overstory tree species had a conspecific planted beneath for a total of 11 conspecific pairings. In addition, a variety of heterospecifics ($n = 4\text{--}6$ seedling species per overstory species), from confamilials to very far relatives, were planted for a total of 46 heterospecific pairings. (A) Percent survival ($t_{21} = 2.6, P = 0.0166$), and (B) monthly growth rate ($t_{34,6} = -3.3, P = 0.0024$) were lower, (C) foliar disease (Area Under the Damage Progress Curve (AUDPC)) (AUDPC_{disease}: $t_{46,3} = 4.7, P = 0.0002$), and (D) foliar herbivory (AUDPC_{herbivory}: $t_{45,3} = 2.4, P = 0.0234$) were greater under conspecific canopies. The boxplots show the median, 1st and 3rd quartiles, minimum and maximum values. The dots represent the mean of the three replicate overstory species plots for the three seedling species planted both under conspecifics and under heterospecifics (survival: $t_4 = 2.8, P = 0.0484$, growth: $t_{21} = -2.5, P = 0.0189$, AUDPC_{disease}: $t_{17,8} = 3.8, P = 0.0014$, AUDPC_{herbivory}: $t_{14,8} = 0.9, P = 0.3965$) (Cg = *Colubrina glandulosa*, Pq = *Pachira quinata*, Ta = *Terminalia amazonia*).

between the seedling and overstory species (repeated measures ANOVA: Time \times Phylogenetic Distance: $F_{2,3,330,6} = 4.9, P = 0.0065$, Appendix A3). However, there was no significant effect of phylogenetic distance when the analysis was restricted to family-level distances and greater (Fig. 2B).

Even though soil macronutrients (NH_4^+ , NH_3 , and P) did not explain a significant amount of variation in the overall growth models, seedlings grew significantly more under legumes (mean = $1.9 \text{ cm month}^{-1} \pm 3.2$) than under non-legumes (Mean = $0.94 \text{ cm month}^{-1} \pm 2.6, t_{445,3} = 3.6, P = 0.0004$).

3.3. Foliar damage

Foliar disease and herbivory were greater on seedlings growing under conspecific than under heterospecific overstories (Fig. 1C and D), when analyzed using the Area Under the Disease Progress Curve (AUDPC) as a temporally integrated measure of disease and herbivory prevalence. Similar effects of the overstory were found when the analysis was restricted to the three species planted under both conspecific and heterospecific canopies (Fig. 1C and D).

Foliar disease (as AUDPC) declined with phylogenetic distance between the seedling and overstory species (Appendix A3). This effect disappeared when the analysis included only seedling – overstory pairs at family-level or more (Fig. 2C). Foliar herbivory did not show a significant phylogenetic signal (Fig. 2D). This resulted mainly because seedlings of *T. rosea*, which is a distant relative to

most overstory species (233.1 My phylogenetic distance from 92% of the overstory species) employed in this study, always experienced high herbivory (>65% on average). When this species was excluded from the analysis, however, herbivory declined significantly with phylogenetic distance (AUDPC = $45.1 - 0.0545 \times$ phylogenetic distance, $F_{1,133} = 19.2, P < 0.0001$).

3.4. Cross inoculations with fungal pathogens

We conducted greenhouse inoculations with 659 strains of foliar fungi isolated from diseased individuals of the 20 species of enrichment-planted seedlings. Each of the 659 strains was inoculated on a seedling of the host of origin and on a seedling of the overstory species beneath which that seedling was growing. We further inoculated the 431 fungal strains proven to be pathogenic on the host of origin (i.e., positive Koch's proof of pathogenicity) on the associated overstory species in the field. Several inoculated leaves were lost through external factors, leaving 639 tests of pathogenicity on original host species, 622 on associated overstory species in the greenhouse and 241 in the field (Table 3A).

We sequenced 173 strains, which were selected to represent the diversity of pathogenic fungal morphotypes when grown in pure culture. BLAST searches showed that most of the isolates were from common genera of plant pathogenic fungi *Diaporthe*, *Fusarium*, *Glomerella*, *Nigrospora*, and *Pestalotiopsis*, plus occasional collections of *Botryosphaeria*, *Calonectria*, *Cordyceps*, *Corynespora*,

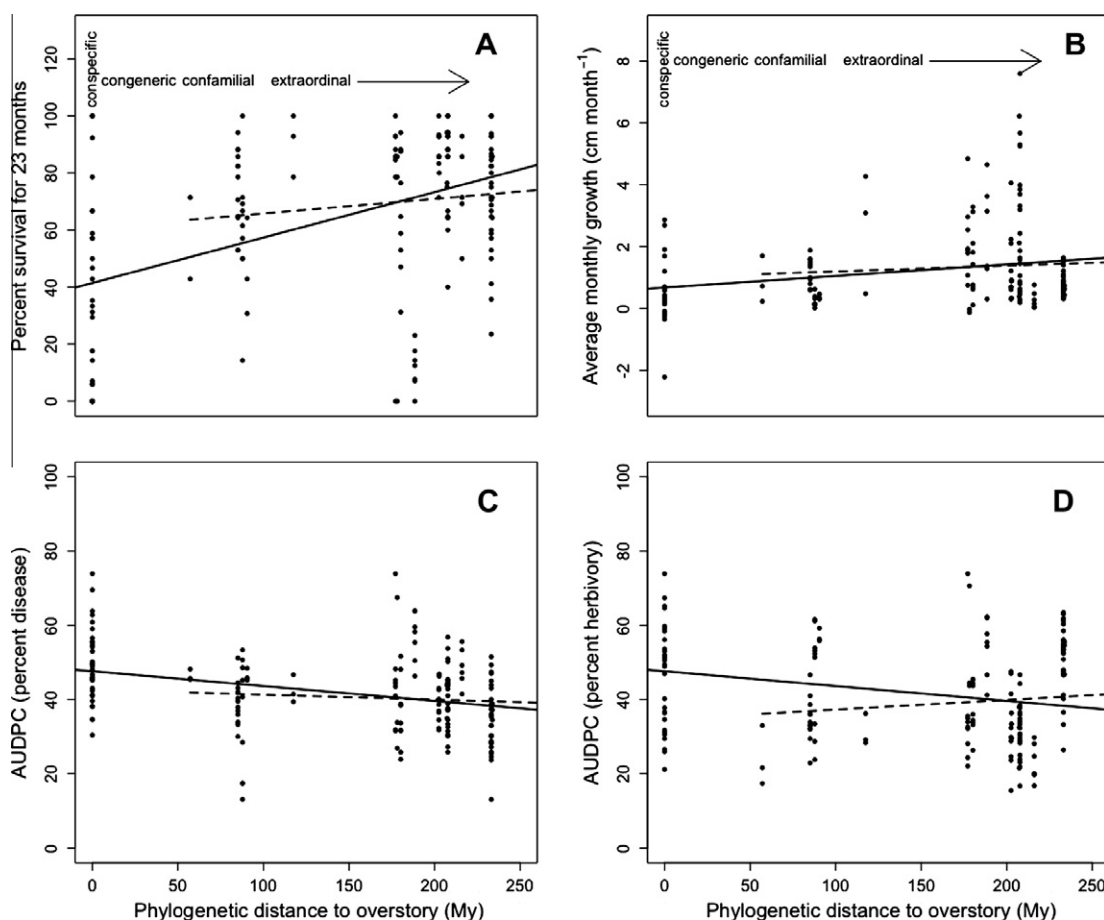


Fig. 2. Phylogenetic signal, with linear fit, in (A) percent seedling survival, where each point is the percent seedlings surviving 23 months, for a particular seedling-overstory species combination (percent survival = $41.5 + 0.159 \times$ phylogenetic distance, $F_{1,166} = 43.6$, $P < 0.0001$, $R^2 = 0.21$; no conspecifics: percent survival = $60.2 + 0.0645 \times$ phylogenetic distance, $F_{1,134} = 2.9$, $P = 0.0987$, $R^2 = 0.02$), (B) monthly growth rate (mean growth rate (cm month^{-1}) = $0.68 + 0.0037 \times$ phylogenetic distance, $F_{1,151} = 7.4$, $P = 0.0074$, $R^2 = 0.05$; no conspecifics: monthly growth rate = $0.99 + 0.0022 \times$ phylogenetic distance, $F_{1,129} = 0.9$, $P = 0.3514$, $R^2 = 0.01$), and (C) foliar disease (AUDPC_{disease} = $47.7 - 0.04002 \times$ phylogenetic distance, $F_{1,166} = 18.8$, $P < 0.0001$, $R^2 = 0.10$; no conspecifics: AUDPC_{disease} = $42.7 - 0.0152 \times$ phylogenetic distance, $F_{1,134} = 0.9$, $P = 0.3547$, $R^2 = 0.01$), (D) foliar herbivory (Area Under the Disease Progress Curve; AUDPC_{herbivory} = $43.2 - 0.0143 \times$ phylogenetic distance, $F_{1,166} = 1.4$, $P = 0.2369$, $R^2 = 0.01$; no conspecifics: AUDPC_{herbivory} = $34.5 + 0.0298 \times$ phylogenetic distance, $F_{1,134} = 2.03$, $P = 0.1565$, $R^2 = 0.01$). Phylogenetic distance is given as estimated million years of independent evolution between the seedling and overstory tree species. Solid line is the regression including all data; dashed line is the regression excluding conspecific data from the analysis.

Table 3

(A) Results of pathogenicity testing on original host species and overstory species, both in the greenhouse and the field. (B) For the 601 strains for which data were available on both original host and overstory species in the greenhouse inoculations, fungi were more likely to be pathogenic on both hosts or neither than expected ($\chi^2 = 101.8$, $df = 1$, $P < 0.0001$).

A. Inoculation results				
Target	# Pathogenic	# Asymptomatic	Total	Percent
Host species	431	208	639	67.4
Overstory spp. in greenhouse	375	247	622	60.3
Overstory spp. in field	179	62	241	74.3
B. Number of fungal strains that caused disease when inoculated onto				
Original host sp.	Overstory sp.		Total	
	Yes	No		
Yes	298	64	362	
No	102	137	239	
Total	400	201	601	

Daldinia, *Monacrosporium*, *Penicillium*, *Saccharomyces*, *Trametes*, *Trichoderma*, and a number of undetermined endophytes.

Inoculations produced symptoms on over 60% of the individuals regardless of whether it was the host of origin or its associated overstory species, both in the greenhouse and in the field (Table 3A). On the greenhouse cross-inoculations, for the 601

strains for which data were available on both original host and overstory species, fungi were much more likely than expected to be pathogenic on both the seedling and overstory species, or to be pathogenic on neither (Table 3B). Of those fungi pathogenic on the original host species, 82% were also pathogenic on the overstory tree species. Phylogenetic distance between source seedling

and overstory species was not predictive of the probability that a pathogen of the seedling species would also cause disease on the associated overstory species in the greenhouse ($\text{logit}(\text{proportion diseased}) = 0.71 - 0.064 \times \log_{10}(\text{phylogenetic distance} + 1)$, $P = 0.1888$, $n = 622$) or in the field ($\text{logit}(\text{proportion diseased}) = 1.3 - 0.056 \times \log_{10}(\text{phylogenetic distance} + 1)$, $P = 0.5246$, $n = 241$).

4. Discussion

Our results indicate that tree seedlings survive poorly, grow less, and suffer more foliar damage when planted under conspecific overstories. Lower performance of enrichment-planted seedlings beneath conspecific canopies is what would be expected from Janzen–Connell type mechanisms that commonly affect seedlings in tropical forests. Strong and pervasive negative effects on performance of seedlings close to a parent tree or when conspecific densities are high are often the result of higher host-specific shared pests and pathogens near a conspecific adult tree (e.g., Augspurger and Kelly, 1984; Gilbert et al., 1994; Metz et al., 2010; Liu et al., 2012; Paine et al., 2012).

The classical approach to testing for Janzen–Connell effects has been to compare impacts on seedlings growing near conspecific vs. heterospecific adults (e.g., Augspurger, 1984; Augspurger and Kelly, 1984; Clark and Clark, 1984; Hubbell et al., 1990; Gilbert et al., 1994; Wills et al., 1997; Harms et al., 2000). This binary approach is the simplest phylogenetic comparison, treating all heterospecifics as equivalent and assuming that natural enemies are mostly host-species specialists. We know, however, that the host ranges of most enemies include a number of host species that are phylogenetically clustered (Novotny and Basset, 2005; Weiblen et al., 2006; Gilbert and Webb, 2007; Maherali and Klironomos, 2007; Pearse and Hipp, 2009; Gilbert et al., 2012; Wilson et al., 2012). Liu et al. (2012) experimentally tested that seedlings survive poorly when planted on the soil of conspecifics due to the presence of shared soil pathogens but that this negative effect decreases gradually beyond conspecifics.

Similarly, local plant community assembly processes are often shaped by species interactions that have a clear phylogenetic signal (e.g., Webb et al., 2006; Dyer et al., 2010; Gibson et al., 2010; Gonzalez et al., 2009; Mangan et al., 2010; Metz et al., 2010). In our experiment, a binary conspecific–heterospecific comparison captured much of the effect of overstory species on seedling performance, rather than there being a strong continuous relationship between phylogenetic distance on seedling performance. Because seedling availability prevented including congeneric (short phylogenetic distance) comparisons in our experiment, we were unable to assess the effects among close relatives, which is where the strongest conservatism of interactions has been shown (e.g., Becerra and Venable, 1999; Weiblen et al., 2006; Gilbert and Webb, 2007; Burns and Strauss, 2012). Closely examining the performance of close relatives (e.g., congeners) would be useful in clarifying the breadth of phylogenetic effects on seedling performance and the utility of phylogenetic approaches in selecting species for planting.

In our cross inoculation study, the strong majority (82%) of those fungi that were pathogenic on their original host seedling species were also pathogenic on the overstory species (100% of conspecific tests and 71% of heterospecific inoculations). In addition, 74% of those pathogen were able to cause disease on overstory trees inoculated in the field despite recognized differences in susceptibility of canopy vs. understory leaves to infection (Gilbert, 1995; Gilbert et al., 2007). Although we did not conduct widespread host-range testing in this study, such polyphagy is expected among pathogens (Barrett et al., 2009). Extensive testing of foliar

pathogen host range in a nearby forest, using the same methods as employed here, showed that fungi were able to cause disease on a median of 28% of their neighboring host species (Gilbert and Webb, 2007). Although pathogens are likely to spread among neighboring plants in the understory, this high rate of susceptibility of heterospecific overstory plants to pathogens isolated from seedlings suggests that overstory tree species are likely to serve as important reservoirs of pathogens for the seedlings. This is to be expected as the overstory trees have a large foliar area positioned above the seedlings from which rain and gravity can carry fungal spores.

Our results are consistent with the overstory foliage being an important source of inoculum for seedling diseases, but we also found a significantly higher proportion of diseased seedlings when planted under conspecific overstories, which agrees with the results of previous studies (Gilbert and Webb, 2007; Gilbert et al., 2012; Liu et al., 2012). There is a stronger probability of disease sharing between conspecifics and congeners, with a sharp decline in disease sharing beyond close relatives. This stronger likelihood of sharing hosts among close relatives with a long tail that extends to very far relatives has also been reported for tropical leaf chewing insects, challenging previous assumptions of high host specificity in the tropics (Novotny et al., 2002b). In the current study this phylogenetic signal was weak beyond a conspecific–heterospecific effect. The large source of spores from the canopy above could create a swamping effect that overcomes any true underlying phylogenetic signal in host sharing and facilitates acquisition of new hosts (Parker and Gilbert, 2004). In addition, rapid evolution of fungi that permits local adaptation to common hosts is facilitated by the faster reproduction cycle of the fungi compared to the plant host (Gandon et al., 1996; Burdon and Thrall, 2000; Capelle and Neema, 2005; Springer, 2007; Konno et al., 2011).

By choosing overstory species that formed a closed canopy, we indirectly chose a set of overstory species that were mostly light demanding (Table 2). However, phylogenetic distance, even though weaker, was still a significant predictor of survival even after the light-demanding species were removed from the data set. There is no reason to expect that the effects of a conspecific overstory were limited to poor performance of light-demanding seedlings in a shady environment.

Our experiment was designed to test whether phylogenetic relationships between seedling and overstory plants could be used to predict the likely performance of enrichment-planted seedlings in an established understory; avoiding the need for species-specific empirical data. However, idiosyncratic traits among species led to great variation in the data. For example, seedlings planted under legumes grew more than under non-legumes, regardless of their phylogenetic distance from the overstory species. Foliar herbivory was significantly affected by the identity of the seedling; *T. rosea* suffered uniformly heavy herbivory regardless of the overstory species. Such idiosyncrasies can obscure a broadly significant phylogenetic signal in field experiments and reduce the predictive capacity for selecting success of enrichment-planted species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foreco.2013.02.012>.

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