The influence of seed source, habitat, and fungi on *Cecropia* seed survival in two neotropical forests

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INTRODUCTION

Seed dispersal confers two fundamental advantages to plants: an escape from sources of mortality that are concentrated around parents, and an increased probability of colonizing suitable habitats or microhabitats (Howe and Smallwood, 1982). For pioneer species in old-growth forest, dispersal is especially important for recruitment since pioneers primarily germinate and establish in tree fall gaps. Since gaps are ephemeral and their locations are largely unpredictable (e.g., Hubbell and Foster, 1986), recruitment success by pioneers increases with fecundity and with the range and uniformity of seed dispersal (Murray, 1988; Dalling and John, 2006). Life history trade-offs, however, can weaken selection for traits that maximize dispersal. For example, small seeds can be produced in greater numbers but have lower probabilities of establishment (Smith and Fretwell, 1974; Dalling and Hubbell, 2002). Furthermore, small-seeded species may be limited to recruiting in specialized microsites, such as those that lack leaf litter or maintain favorable soil moisture conditions (Metcalfe and Grubb, 1997; Engelbrecht *et al.,* 2006)

For pioneer species with limited dispersal in space, recruitment success can be increased by maintaining soil seed banks that disperse seeds over time. Seeds of some species can persist in a viable state in the soil for up to several decades (Dalling *et al.,* 1997; Murray and Garcia, 2002). These seeds germinate in response to cues that reflect favorable conditions for seedling establishment, such as high red:far red ratios of light irradiance (Vázquez-Yanes and Smith, 1982; Vázquez-Yanes *et al*., 1990), elevated soil moisture and nitrate concentrations (Daws *et al.,* 2002), and fluctuating soil temperatures (Vázquez-Yanes, 1974; Vázquez-Yanes and Orozco-Segovia, 1982). Seed persistence

may be a critical trait for some pioneers with very low fecundity or with establishment requirements that are rare, such as unusually large and infrequent disturbances. For tropical pioneers with broader establishment requirements, seed banks can increase recruitment success by buffering against inter-annual variation in seed production and unpredictable germination opportunities (e.g., Cheke *et al.*, 1979; Hall and Swaine, 1980; Putz and Appanah, 1987).

Demographic effects of fungi in seed banks

If recruitment of pioneers is largely dependent upon even transient seed banks, then understanding the spatial distribution of seed mortality in the soil may be important for predicting seedling establishment. When seeds land on the soil surface, rodents, ants, and beetles may be important seed predators (e.g., Levey and Byrne, 1993; Kaspari, 1996; Fornara and Dalling, 2005). Fungi account for some mortality on the soil surface (Jones, 1994) but once seeds are incorporated into soil, infection by fungi and other micro-organisms may become the dominant source of seed mortality. Declines in seed viability in soil have often been attributed to infection by pathogenic fungi (Dalling *et al.*, 1998b; Baskin and Baskin, 1998; Alvarez-Buylla and Martinez-Ramos, 1990).

Within *Cecropia*, seed mortality from fungal infection appears to vary among species. For example, Murray and Garcia (2002) found that *Cecropia polyphlebia* seeds are largely resistant to fungal attack in lower montane rain forest soils, while Dalling *et al*. (1998b) found that up to 50% of *Cecropia insignis* mortality in the soil could be attributed to fungal pathogens in a moist semi-deciduous tropical forest. However, the

degree to which different seed characteristics or environmental conditions influence these patterns remains unknown.

Seeds from the same mother plant may also differ in their susceptibility to fungi. For example, variation in nutritional status during seed development or damage to the seed coat during dispersal can influence intrinsic susceptibility to infection (Wulff, 1986; Fenner and Thompson, 2005, and references therein; see Levey *et al.,* Chapter 2 this volume). Seeds also may harbor asymptomatic infections by endophytic fungi (fungi that colonize and live within living plant tissues without causing disease), which accumulate via contagious spread (*i.e.*, horizontal transmission; Arnold, 2002) or by vertical transmission from maternal plants (e.g., Bose, 1947). There is some evidence that endophytes infect *Cecropia* seeds prior to dispersal (Gallery *et al.,* 2006), although the frequency of these infections is still being determined. Fungal colonization at the predispersal stage may affect post-dispersal seed survival in the soil as endophytic colonization has been shown to confer increased resistance to subsequent infection by pathogens (Arnold *et al.,* 2003).

Mortality from fungal infection appears to be especially important for small-seeded species with thin fruit or seed walls (Crist and Friese, 1993; Fenner and Thompson, 2005). In temperate and sub-tropical grassland communities, fungicide treatments increased the survival of buried seeds by 10-30% (Lonsdale 1993; Leishman *et al.*, 2000; Blaney and Kotanen, 2002). Fungicide treatments also increased survival of *Betula papyrifera* (white birch) seeds in the forest understory in Ontario, Canada; 8% of seeds germinated in the control (water) treatment compared to 32% in the fungicide treatment (O'Hanlon-Manners and Kotanen, 2004). Survival of *Miconia argentea* and

Cecropia insignis seeds buried for six months in mature forest on Barro Colorado Island (BCI), Panama, increased from 5-10% for untreated seeds to 50% for seeds treated with a broad-spectrum fungicide (Captan; Dalling *et al.*, 1998b). Similar increases in survival were found in a greenhouse experiment at La Selva, Costa Rica, when *Cecropia insignis* seeds potted in forest soil were treated with the fungicide Benomyl (R. Gallery, 2002 unpublished results). However, these studies did not address the spatial distribution of fungi in soils or identify the fungal species involved in seed mortality.

More recent experiments have begun to address the diversity and spatial structure of seed-infecting fungal communities in tropical forests. Gallery *et al.* (2006) examined seed survival and fungal infection of seeds of four *Cecropia* species (*C. insignis, C. longipes, C. obtusifolia* and *C. peltata*) buried in common gardens below crowns of *C. insignis* trees on BCI. Seed survival varied among burial sites and *Cecropia* species, with the lowest survival found in *C. insignis*. The question remains whether these patterns reflect variation in fungus-induced seed mortality.

Results from fungal culturing and direct PCR of dead seeds show that soil-incubated *Cecropia* seeds are infected with a highly diverse assemblage of fungi (primarily Ascomycota) comprised of a few common and many rare species (Gallery *et al.*, 2006). Randomization tests coupled with Jaccard's index of similarity were used to determine whether fungal community composition was more strongly influenced by burial sites (distinct tree crowns) or by the species identity of the buried seeds. Although the most common genotypes of fungi were cultivated from all sites and from all *Cecropia* species, seeds shared more fungal species when buried beneath the same crown than

when below different crowns. Furthermore, the communities of fungi isolated from seeds were more similar within a *Cecropia* species than among species.

While providing a first resolution of the spatial and host-associated patterns of fungal infection in this tropical pioneer genus, these results fail to resolve the importance of host-generalist versus host-specific fungi in determining seed mortality patterns. If the common, host-generalist fungi are predominantly responsible for seed mortality, then opportunities for escape from pathogens via seed dispersal may be limited. On the other hand, if locally distributed fungi and/or fungi with strong host preferences contribute significantly to seed mortality, then the probability of a seed surviving fungal attack will depend greatly on where the seed lands, thereby contributing to spatial variation in recruitment success.

In this chapter, we describe two experiments that examine spatial variation in survival of *Cecropia* seeds in the seed bank. The objective of this study was to assess the relative importance of the intrinsic traits of *Cecropia* species, including provenances and maternal seed sources, the heterogeneity in seed-infecting fungal communities (Gallery *et al.*, 2006), and certain environmental conditions on the survival of seeds in soil. First, we use reciprocal transplant experiments between two provenances of co-occurring *Cecropia* species to broadly test the relative effects of environment and seed source on seed survival. We then examine local variation in survivorship within the dispersal range of individual *Cecropia* trees and ask whether dispersal away from habitat conditions associated with *Cecropia* crowns (*i.e.*, high conspecific seed bank density and litter cover) increases seed survival in the soil. We also test whether seeds experience lower survival below their parent crown compared to below conspecific crowns. Finally, we

present new data highlighting fungi occurring in soil-incubated *C. insignis* seeds at the La Selva Biological Station, Costa Rica, and compare these results to a larger dataset from Barro Colorado Island, Panama (Gallery *et al.,* 2006, A.E. Arnold, 2006 unpublished results). We use these data to discuss the potential for local and regional-scale differences in fungal communities to influence *Cecropia* seed survival.

METHODS

Study sites and species

We examined the survival of *Cecropia* seeds in soils at two neotropical sites, Barro Colorado Island (BCI), Panama (9˚ 9' N, 79˚ 51' W), and La Selva Biological Station, Costa Rica (10˚ 26' N, 83˚ 59' W). These two sites are separated by 475 km and experience distinctly different patterns of rainfall and seasonality. The semi-deciduous forest on BCI experiences an intense, four-month dry season from January to May, which accounts for less than 10% of its annual precipitation (annual mean = 2600 mm; Windsor, 1990). In contrast, La Selva's aseasonal, wet forest receives approximately 4000 mm of rain annually (Sanford *et al.,* 1994) with no less than 100 mm of rain in any month. Despite these differences, old growth forests at these sites share many species of vascular plants (Gentry, 1990).

The study species, *Cecropia insignis* Liebm. and *C. obtusifolia* Bertol. (Urticaceae; Sytsma *et al.,* 2002) are dioecious pioneer trees common in lowland moist and wet forests in Central America (Holdridge *et al.,* 1971; Croat, 1978). *C. insignis* is

one of the most common pioneer tree species at both study sites and one of the most abundant species in the seed bank (Putz, 1983; Young *et al.*, 1987; Dalling *et al.*, 1995, 1997; Dupuy and Chazdon, 1998). *C. obtusifolia* is more abundant in younger forests at BCI and La Selva (Croat, 1978; Hammell, 1986), nonetheless, its seeds are still regularly found in the seed bank of mature forest (Young *et al.*, 1987; Dalling *et al.*, 1995; 1997).

At both sites, *Cecropia obtusifolia* fruits nearly continuously, with peak fruiting from February-August, while *C. insignis* fruits from April-July with peak fruit production in early-June (Croat 1978; Milton 1991). Thus the peak of seed production for BCI and La Selva overlaps with the period of the highest frequency of gap formation, which generally correlates with the wet season on BCI (May-December) and/or exceptionally wet periods in both forests (Hartshorn, 1978; Brokaw, 1982; Garwood, 1983). Although *Cecropia* seeds are widely dispersed by bats, birds and monkeys, below-crown seed rain is also very high (e.g., 64,000 *C. insignis* seeds/m^2/yr below fruiting individuals on BCI; Dalling *et al.*, 1998b). Seeds of both species are small (mean fresh mass = 0.6 mg; Dalling *et al.*, 1997; Gallery *et al.*, 2006). Like most common small-seeded species that do not fruit year-round, seeds of *C. insignis* and *C. obtusifolia* tend to be seasonally abundant in the seed bank, with most seeds surviving less than a year (Alvarez-Buylla and Martinez-Ramos, 1990; Dalling *et al*., 1997; 1998a).

General protocol

We conducted two experiments to investigate potential sources of variation in spatial heterogeneity of seed survival in the seed bank. In each we used a common protocol

based on a reciprocal burial design, whereby seeds from different maternal sources were buried under conspecific crowns. Seeds were collected from seed traps or directly from the crowns of *C. insignis* and *C. obtusifolia* individuals (maternal sources) at both BCI and La Selva. Immediately after collection, seeds were removed from infructescences and rinsed in a 10% Clorox® bleach (0.5% sodium hypochlorite) solution for two minutes to remove surface contaminants. Seeds were then surface-dried under sterile conditions in a darkroom and sorted by maternal source into lots of 30, each of which was mixed with 10 g of sterilized forest soil (autoclaved at 115°C for two hours) and enclosed in a nylon mesh bag (0.5-mm mesh size). Four seed bags from each maternal source were buried in soil at a depth of 3 cm in one 3 m x 3 m plot below each focal crown. After five months of incubation (July-December, which encompasses the greatest seasonal fluctuation in seed bank densities of *Cecropia*; Dalling *et al.,* 1998b), bags were recovered and germination trials were conducted for eight weeks to determine the percentage of seeds surviving. Bags contents (seeds and soil) were transferred into Petri dishes lined with sterile filter paper, watered with filtered water, sealed with Parafilm, and placed in ambient air growth-houses (mean max. = 30.8ºC ± 1.0ºC, mean min. = 23.9ºC ± 0.8ºC) with 30% full sunlight and high red:far red irradiance to induce germination (see Dalling *et al.,* 1998b). Seed survival was measured as the proportion of 30 seeds that germinated (radicle and cotyledon emergence) adjusted for the initial viability, which was determined at the onset of the experiment with a sub sample of 100 seeds from each maternal source.

Experiment 1: Is seed source or environment a better predictor of seed survival?

Previous results showed differences in seed survival among four sympatric *Cecropia* species when buried in common gardens below *C. insignis* crowns on BCI (Gallery *et al.*, 2006). *Cecropia insignis* seeds experienced the lowest survival, with foreign seeds (from La Selva) suffering lower survival than local seeds (from BCI). Seed survival also differed significantly among crowns. These differences may result from intrinsic seed characteristics that vary among *Cecropia* species, from small-scale spatial differences and/or host-affinities of fungal communities capable of infecting *Cecropia* seeds, and from small-scale variation in environmental conditions (e.g., soil temperature and moisture, litter cover) that influence seed-fungal interactions in soil.

For this experiment, we established common gardens by burying seed bags concurrently at both BCI and La Selva. We used seeds from six individuals (maternal sources) of *C. insignis* from BCI and five from La Selva, and four *C. obtusifolia* from both BCI and La Selva. We used an incomplete reciprocal design where seeds were buried (1) below their maternal crown and two other randomly chosen conspecifics in their 'home' site (e.g., BCI seeds at BCI), or (2) three randomly chosen conspecifics in the 'away' site (e.g., BCI seeds at La Selva) (N = 11 for *C. insignis*, N = 8 for *C. obtusifolia*). Using the general methods described above, seed bags were field-incubated from July-December 2001.

This experiment allowed us to examine two co-occurring *Cecropia* species with similar seed traits (e.g., seed mass, seed longevity in soil, susceptibility to infection by particular fungi; Gallery *et al.*, 2006) and determine the relative importance of seed source and environmental conditions on seed survival. Transplanting seeds over a large geographic scale (475 km between BCI and La Selva), but still within the species' natural

range tested whether seed source (local or novel provenance) affected seed survival. In turn, transplants below different crowns within sites tested whether seeds suffer lower survival below their own parent's crown. This result may be expected if the fungal communities in a below-crown area are locally adapted to the genotypes of seed they most commonly encounter (see Gilbert, 2005); however, evidence for such local adaptation in natural systems is lacking (but see Parker, 1985). Alternatively, similar survival of different seed sources buried below the same crown and/or site-specific patterns of seed survival would suggest that extrinsic factors play a large role in determining where, and, for how long a seed will survive.

Experiment 2: How does local dispersal affect seed fate?

Limited seed dispersal implies that the highest densities of seeds and their specialist natural enemies should be found below crowns of reproductive adults (Janzen, 1970; Connell, 1971). For *Cecropia,* seed densities in the soil decline logarithmically with increasing distance from fruiting crowns (Alvarez-Buylla and Martinez-Ramos, 1990; Dalling *et al.*, 1998a). Annually replenished seed banks below reproductive crowns may create a positive feedback with fungal inoculum resulting in overall high inoculum densities in high seed bank areas (e.g., Bever, 1994). Therefore, seed mortality due to fungal infection may be highest in the seed-dense areas below reproductive crowns.

Seed survival may also depend on other environmental conditions associated with tree crowns. For example, litter cover may influence seed survival indirectly by changing soil moisture and nutrient conditions that promote fungal activity and infection potential

(Agrios, 1997; Agarwal and Sinclair, 1987), and/or change community composition of fungi and micro-invertebrates in soil and litter (Facelli and Pickett, 1991). Results from large-scale litter manipulation plots in Panama reveal higher saprophytic fungal biomass (hyphal length) in the top 2 cm of soil under litter-addition plots than under litter-removal plots (A. Vincent, Panama, 2006, personal communication). If similar fungi infect both *Cecropia* leaf and seed tissue (A.E. Arnold, 2006 unpublished results) then the inoculum provided by *Cecropia* leaf litter may overwhelm any potentially density-dependent effects that arise from seed to seed transmission of fungi in the soil.

We addressed this issue by conducting a second experiment in July 2003 in which seed bags were buried (1) below female *C. insignis* crowns, (2) in plots at least 50 m from *C. insignis* trees, and (3) below male *C. insignis* crowns. This approach allowed us to test for the effect of high versus low *C. insignis* litter and potential pathogens associated with adult trees on seed survival (e.g., Gilbert, 1995; Packer and Clay, 2000) independent of the effects of high versus low seed bank density.

We buried *C. insignis* seeds from six BCI and six La Selva trees (N = 12) in 3 m x 3 m plots (1) below six fruiting (female) *C. insignis* crowns, (2) at six locations 50 m away from each of those fruiting individuals, and (3) below six flowering (male) *C. insignis* crowns at least 50 m from the nearest fruiting *C. insignis*. All seed sources were buried in all plots. To address the temporal aspect of seed survival, a sub-sample of seed bags (432; from the six BCI seed sources x 4 bags/source x 18 plots) was removed after 1 month and the remaining bags (864; from the six BCI and six La Selva sources) were left to incubate for five months.

Natural *C. insignis* seed bank densities were measured the first week of August, which corresponds with the end of the fruiting season and peak *C. insignis* seed bank densities (Dalling *et al*., 1998b). Densities were measured by coring 235-cm^3 (10 cm diameter x 3 cm depth) of soil at nine randomly chosen locations within the plots below each of the *C. insignis* crowns. Soil cores were spread thinly (0.5 cm) over sand flats (3 cm deep) in an ambient-air growing house and watered regularly. Seedlings were tagged upon emergence and grown until they could be positively identified; no seedlings died before they could be identified (protocol follows Dalling *et al.,* 1997).

Leaf litter cover was measured at ten randomly chosen locations within each plot. To correspond with seed bank measurements, litter cover was measured the last week of July using a point intercept method (Elzinga *et al.*, 2001). A 0.5-m long galvanized steel rod (2 mm diameter) was pushed through the litter and the number of times a leaf touched the rod was recorded.

A reduced version of the experiment was conducted concurrently at La Selva (N = 12). Duplicate seed lots to the BCI experiment were used, but plots were established only (1) below six fruiting female *C. insignis* crowns and (2) 50 m away from those individuals. No seed bank density or litter thickness measurements were taken.

Data analyses

The percent of viable seeds that germinated after five months of soil incubation was analyzed using factorial analysis of variance (ANOVA) with type III sums of squares (proc mixed, SAS Institute v. 9.1, Cary, NC, USA). Prior to analysis, germination data

were logit-transformed to approximate normality. Below-crown plots were nested within site and maternal sources of seeds were nested within provenance. Maternal source was treated as a random effect and was used to test provenance. Because the four bags per maternal source incubated at a given below-crown plot could not be considered independent (Gallery *et al.,* 2006), mean germination of the four bags was used in each model.

RESULTS AND DISCUSSION

Experiment 1: Environment is a better predictor of seed survivorship than seed source

Seed survival at BCI and La Selva were significantly different for both *C. insignis* and *C. obtusifolia. Cecropia insignis* seeds experienced higher survival when buried at BCI than at La Selva ($F_{(1, 17)} = 24.41$, $P < 0.001$, Fig. 23.1a) while the opposite pattern was true for *C. obtusifolia* ($F_{(1, 7)} = 19.53$, $P = 0.0031$, Fig. 23.1b). BCI and La Selva experience distinctly different seasonality and total amount of rainfall, but the effects, if any, that these factors have on *Cecropia* seed banks remain equivocal.

Within each site, seed survival differed among crowns for both *Cecropia* species. Seed survival under particular crowns ranged from 6.8% (±6.6%) to 61.9% (±8.0%) for *C. insignis* ($F_{(9, 17)} = 1.92$, $P = 0.11$), and from 9.9% (±6.3%) to 79.3% (±8.3%) for *C.* obtusifolia ($F_{(6, 7)} = 4.00$, $P = 0.046$). If survival was determined only by species-level traits then we would expect similar survival within a species regardless of where seeds

are buried. Instead, these results suggest that seed survival is also a consequence of the location to which seeds are dispersed and incorporated into the seed bank (see also Fenner and Thompson, 2005). Therefore, differences in habitat conditions and/or variation in biotic conditions (including fungal communities) contribute greatly to variation in seed survival in seed banks.

The effect of provenance was significant for *C. insignis* ($F_{(1, 17)} = 5.29$, $P = 0.035$), with La Selva seed sources experiencing slightly higher survival than BCI seed sources at both sites. Provenance was not significant for *C. obtusifolia* ($F_{(1, 7)} = 0.39$, $P = 0.55$). The relatively similar survival between BCI and La Selva seed sources for both species at both BCI and La Selva suggests that rare or 'foreign' *Cecropia* seed sources are unlikely to have a substantial survival advantage (Fig. 23.1a and b). However, it remained unclear whether source effects are important at smaller (local) scales. To explore this, we assessed survival for seeds buried beneath their mothers vs. other conspecific crowns. We found that seeds buried beneath their mothers did not suffer lower survival than seeds buried under other conspecific female crowns (results similar at BCI and La Selva for both *C. insignis* [t = 0.93, df = 1, 17, $P = 0.36$; linear contrasts within ANOVA] and *C. obtusifolia* [t = 0.10, df = 1, 7, $P = 0.92$]). Thus, maternal sources of seed on a small or local scale did not appear to be important in shaping seed survival.

Previous results showed that *C. insignis* and *C. obtusifolia* do not differ in the incidence of fungal infection when buried in common gardens (Gallery *et al.,* 2006). Given that these species experience similar infection rates, the particular fungi infecting seeds may account for the different survival patterns within forest communities. Our

current research examining the local- and geographic-scale differences in *Cecropia* seed-infecting fungal communities will enable us to determine whether different fungi are responsible for the different seed survival patterns in these two sites.

Experiment 2: Dispersal away from C. insignis crowns increases seed survival

On BCI, seed survival was high after 1 month of burial and did not differ for *C. insignis* seeds buried beneath female crowns (80.1% ± 3.4%), below male crowns (78.4% ± 4.0%), or 50 m away from female crowns (78.5% ± 4.1%). After five months of burial, however, survival was lower for seeds buried below female or male *C. insignis* vs. 50 m away (Fig 23.2a; t = 5.25, df = 2, 16, $P < 0.001$; linear contrasts within ANOVA). At La Selva, survival was also lower for seeds buried for five months below female *C. insignis* versus 50 m away (Fig 23.2b; t = 4.23, df = 1, 20, $P < 0.001$). This pattern is consistent with results from a similar seed-incubation experiment in a tropical forest in Los Tuxtlas, Mexico: *C. obtusifolia* seed survival in sites 10-28 m from the nearest fruiting *C. obtusifolia* was higher than that of seeds incubated below crowns (Alvarez-Buylla and Martinez-Ramos**,** 1990). Seed survival did not differ significantly below female and male *C. insignis* on BCI (Fig 23.2a; t = 1.03, df = 2, 16, $P = 0.32$). Together, these results suggest that regardless of parental/nonparental status of seed sources, conditions below the crowns of *Cecropia* are generally unfavorable for conspecific seed survival.

Similar to the results from Experiment 1, the effect of provenance was not significant for *C. insignis* ($F_{(1, 10)} = 0.16$, $P = 0.70$). Focusing on seed survival below female *C. insignis*, seeds did not experience lower survival below their maternal crowns compared to below other conspecifics ($F_{(1, 87)} = 2.03$, $P = 0.16$). These results suggest

that seeds dispersed to areas below conspecific crowns are at an equal disadvantage to seeds falling below their maternal crown. They therefore fail to provide evidence for feedback mechanisms that select against common host genotypes either at single-individual or single-population scales.

Experiment 2: Effects of seed density and litter inputs on seed survival

Seed bank densities varied below crowns, but, on average, were 2.5 times higher below females (446 ± 55 seeds/m^2) than below males (170 ± 59 seeds/m^2; two sample t-test, t = 1.44, df =1, 10, $P < 0.05$). Of the 54 cores taken below male crowns, 44% yielded viable *C. insignis* seeds. In contrast, 76% of the 54 cores taken below female crowns contained viable *C. insignis* seeds. Seed survival below female (high seed bank density) and male (low seed bank density) *C. insignis* on BCI was not significantly different (Fig 23.2a), suggesting that seed survival is not primarily driven by factors that are sensitive to local seed density (see also Dalling *et al.,* 1998b).

At BCI, we found that the litter layer was patchier and overall litter thickness was higher below *Cecropia* crowns relative to 50 m away from crowns. Percent cover under *Cecropia* crowns ranged from 85% (male) to 90% (female), relative to 72% at sites 50 m away. Furthermore, 16% of the ground sampled in the 50-m plots was covered with a layer of three or more leaves, compared to 41% of plots below female crowns and 33% of plots below male crowns. The litter composition below male and female *C. insignis* trees was comprised primarily of *C. insignis* leaves, whereas no *C. insignis* leaves were encountered in any plots 50 m away (B. Wolfe and R.E. Gallery, 2003 unpublished results). In some cases, litter thickness reached up to nine *Cecropia* leaves below male

and female crowns. Mean litter cover below male and female *C. insignis* crowns was similar (1.8 leaves ± 0.19 below females; 2.2 leaves ± 0.21 below males; t = 0.66, df = 2, 15, $P = 0.51$) and greater than litter cover in plots 50 m from *Cecropia* crowns (1.0 leaves ± 0.20; t = 1.8, df = 2, 15, $P < 0.05$, linear contrasts within ANOVA), suggesting a relationship between the presence of *Cecropia* litter and survival of *Cecropia* seeds. Similarities in litter composition below male and female *Cecropia* trees may therefore play a role in explaining the similar below-crown seed survival rates.

Leaf litter could directly influence seed survival below *C. insignis* individuals if fungi that are present in *Cecropia* leaves contribute to the inoculum that infects seeds. In tropical forests, pathogen transmission from parent to offspring through deposits of aerial spores or leaves has been suggested with seedlings (Gilbert, 1995; Mancini *et al*., 2001), but not with seeds. Direct transmission is not necessary given that, in the absence of a suitable host or under unfavorable conditions, many fungi are capable of long-term persistence in soil (e.g. *Fusarium, Rhizoctonia*) either as saprophytes or as mycelia and sclerotia (masses of mycelium) in dead parent material, or as resting or other types of spores (Bruehl, 1987; Agrios, 1997).

Genotype comparisons with an existing database of endophyte sequence data indicate that several fungal taxa isolated from *C. insignis* seeds show high sequence affinity (99%) at a fast-evolving locus (internal transcribed spacer region) to isolates obtained as endophytes from living leaf tissue and as saprophytes from leaf litter (A.E. Arnold, 2006 unpublished results). Studies of leaf-litter and wood-decay fungi in Panama (e.g., Cornejo *et al.* 1994; Ferrer and Gilbert, 2003) suggest that saprophytes are non-randomly distributed with regard to host species, and thus may demonstrate differential

host affinity. Given that the litter composition below male and female *C. insignis* trees was comprised primarily of *C. insignis* leaves, the observed spatial structure of fungal communities below individual *Cecropia* crowns (Gallery *et al.,* 2006) could be largely influenced by leaf litter fungi.

LINKING *CECROPIA* SEED SURVIVAL TO FUNGAL COMMUNITY COMPOSITION

Cecropia seed-infecting fungi

To fully understand the ecological importance of seed-infecting fungi for recruitment of pioneer species, it is critical to assess the abundance, species richness, and species composition of endophytes, saprophytes, and pathogens associated with seeds. Our previous studies of canopy-collected and soil-incubated seeds of four sympatric *Cecropia* species on BCI indicated that taxonomically diverse communities of fungi infect *Cecropia* seeds and have potentially important impacts on seed persistence in the soil seed bank (Gallery *et al.,* 2006). At the time, we lacked comparable data to determine whether fungal communities are similar at La Selva.

Here we examined representative seed-infecting fungi from surface-sterilized *C. insignis* seeds incubated in soil below six *C. insignis* crowns for one month at La Selva, Costa Rica, in 2003. After surface-sterilization, seeds were plated on 2% malt extract agar (MEA) in sterile Petri dishes. MEA is a general medium that has been shown previously to encourage growth by diverse microfungi (e.g., Fröhlich and Hyde**,** 1999),

though it likely underestimates the number of fungi present. From 250 haphazardly chosen seeds, 32 distinct fungal morphotypes emerged in culture, with seven morphotypes accounting for 72% of the total isolates. This pattern of relative abundance is consistent with seed-infecting fungi from other tropical forests (Gallery *et al.*, 2006) and temperate ecosystems (Schafer and Kotanen, 2004) and with that of other diverse groups of tropical fungi, for which a few species are very common, but the vast majority of species are rare (e.g., Fröhlich and Hyde, 1999; Arnold *et al.*, 2000; Gilbert *et al.*, 2002).

Preliminary molecular analyses focused on 43 representative isolates that comprised rare and common morphotypes. Methods for DNA extraction, PCR, sequencing, and sequence assembly followed Arnold *et al.* (2006). We extracted total genomic DNA from living cultures on MEA plates using a modified phenol:chloroform method. We used diluted DNA (1:10 dilution of extraction yields) in PCR to amplify the ITS (internal transcribed spacer) and 5.8s regions of the nuclear ribosomal repeats using primers ITS1F and ITS4 or LR3 (White *et al.* 1990; see also http://www.lutzonilab.net), which are effective for diverse Ascomycota, Basidiomycota, and Zygomycota. PCR products were cleaned and sequenced for both forward and reverse reads, and all contigs were verified manually before submission to BLAST searches of the NCBI GenBank database for provisional identification at higher taxonomic levels.

The identified morphotypes include representatives of diverse orders of Ascomycota, including Eurotiales, Chaetothyriales, Pleosporales, Sordariales, Hypocreales, Diaporthales, and Phyllachorales (Table 23.1). These orders are frequently represented among endophytic fungi inhabiting leaves of trees at our study sites (Arnold

and Lutzoni, 2006) and also contain numerous pathogens and saprophytic species. Several orders containing putatively saprophytic Basidiomycota also were recovered. High affinity matches based on BLAST searches of GenBank, coupled with phylogenetic analysis of isolate sequences among top matches (Arnold, 2002; Arnold and Lutzoni, 2006), indicate that diverse species of Ascomycota, including *Rhinocladiella, Botryosphaeria, Nectria, Fusarium, Chaetomium, Alternaria,* and *Colletotrichum* occur within seeds of *C. insignis* (Table 23.1). Several genotypes were associated preferentially with viable or inviable seeds, providing a basis for future experimental analyses (Table 23.1).

These data are largely congruent with findings from a larger sampling of 220 fungi from *Cecropia* seeds at BCI (Gallery *et al*., 2006). In that study, species of *Nectria, Fusarium* and *Chaetomium* were especially common, with *Phomopsis, Botryosphaeria,* and other taxa isolated at lower frequencies. Genotype comparisons show that despite the recovery of some genera at both La Selva and BCI, only five genotypes recovered at La Selva were also represented at BCI (comparisons based on 99% ITS sequence similarity). Shared genotypes included the most common *Fusarium* genotype at BCI and a genotype that does not match named sequences in GenBank, but which corresponds in phylogenetic analyses to *Rhinocladiella.* However, 64% of genotypes recovered from BCI and La Selva have been found only once, and thus could not be compared across sites. Among the genotypes found more than once, 85% were specific to only BCI or La Selva. Together, these observations indicate that distinct fungal communities capable of infecting *Cecropia* seeds occur at each site. Large differences in seed survival rates

observed between sites (Experiment 1) may therefore indicate that the frequency with which seeds encounter pathogenic fungi also varies among these forests.

CONCLUSIONS

Together the experiments in this chapter demonstrate that environmental conditions in the seed bank have potentially greater influence on *Cecropia* seed survival than maternal sources or intrinsic seed characteristics. Contrary to the expectation based on local adaptation of fungal communities to their hosts, we found that seeds did not experience lower survival in their local site (BCI vs. La Selva), and that survival was not lower for seeds buried below their maternal crowns as opposed to other conspecific crowns. Many abiotic factors likely influence *Cecropia* seed survival in soil and need to be examined in greater detail. For example, in temperate and tropical systems, significant sources of seed mortality have been attributed to the direct effects of fluctuations in soil moisture (Schafer and Kotanen, 2003) and temperature (Lonsdale, 1993), and to the indirect effects of these factors on microbial activity.

Cecropia seeds dispersed away from fruiting crowns and incorporated into the seed bank have higher survival than seeds that are incorporated into soils below conspecific crowns (fruiting or not). We examined two conditions associated with *Cecropia* below-crown sites and found that *Cecropia* litter cover may have a greater effect on seed survival than high *Cecropia* seed bank density. Litter cover can create moist microhabitats and increase saprophytic fungal biomass in general, which may negatively affect seed survival in soil. Our data also show that many fungi associated with live leaf

tissue and litter infect *Cecropia* seeds, suggesting litter could even provide a direct inoculum source of fungi capable of infecting seeds.

DIRECTIONS FOR FUTURE RESEARCH

The specificity of fungal-seed interactions and the ecological factors influencing the activity and diversity of these fungi are only beginning to be examined. Fungi recovered from soil-incubated *Cecropia* seeds likely include seed pathogens and parasites, saprophytes, and endophyte species that may be harmless or beneficial to a particular host. While most common fungal genotypes isolated appear to be widespread generalists, Gallery *et al.* (2006) found some evidence that other genotypes are either very patchily distributed or demonstrate affinities for particular hosts. In a study using similar experimental protocols, Schafer and Kotanen (2004) found evidence for both generalist and specialist seed pathogens of four co-occurring grasses in temperate meadows. Further, they found grass seeds differed in their susceptibility to a range of fungi they commonly encounter in soil. These species-specific interactions among seeds and fungi suggest fungi have the potential to differentially limit recruitment of susceptible hosts. However, to determine the community-wide effects of fungi on a particular host, it will be necessary to identify the dominant seed pathogens and determine their spatial distribution in soils.

We have identified putative *C. insignis* seed pathogens by considering seed viability associated with particular fungal isolates (Table 23.1; Gallery *et al.,* 2006). The next step is to determine whether these isolates are responsible for killing their host

seeds. Traditional approaches in plant pathology, which focus on satisfying Koch's postulates (Agrios, 1997), argue for the importance of re-inoculation studies to determine causality. By infecting freshly collected, asymptomatic seeds with our focal isolates, assessing seed survival, and re-isolating the inoculants from live or dead seeds, we hope to identify at least some of the fungal pathogens of *Cecropia* seeds in Panama and Costa Rica. These results will provide the framework for developing future hypotheses aimed at testing species-specific interactions among fungi and seeds in tropical soils.

Acknowledgements

We thank the organizers of the Fourth International Symposium-Workshop on Frugivory and Seed Dispersal for the opportunity to contribute to this volume: Andrew Dennis, Ronda Green, Eugene Schupp, and David Westcott. This research was supported by NSF DEB-0343953 and DEB-0516564 to JWD and AEA with additional support from an NSF Postdoctoral Fellowship in Microbial Biology to AEA (DEB-0200413), a Mellon STRI-OTS Comparative Research Grant to JWD and REG, and the UIUC Undergraduate Mentoring in Environmental Biology Program (UMEB) to BTW. We thank the Smithsonian Tropical Research Institute and the Organization for Tropical Studies for providing facilities, logistical support, and permission to conduct this research at Barro Colorado Island and La Selva Biological Station. We thank Deborah and David Clark for use of their *Cecropia* TREES database at La Selva. The TREES project is funded by NSF's LTREB program (DEB- 0129038) and TEAM project of Conservation International with support from the Gordon and Betty Moore Foundation. We also thank

François Lutzoni for logistical support at Duke University and Evelyn Sanchez, Olman Paniagua and Gilbreth Hurtado for field assistance.

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Table 23.1. Identities of 43 representative *Cecropia* seed-infecting fungi based on high affinity matches from BLAST searches (in GenBank), coupled with phylogenetic analysis of isolate sequences among top matches. Fungi were isolated from *Cecropia insignis* seeds following soil incubation in the forest understory of the La Selva Biological Station, Costa Rica. Data indicate number of isolates sharing that genotype, and the number of times a genotype was recovered from germinated (viable) or un-germinated (inviable) seeds. Taxa that are of uncertain placement at the ordinal or family levels are listed as I.S. (incertae sedis). Cases in which top BLAST matches were to unidentified sequences are marked N/I (not identified). Taxon names are given for N/I sequences if placement was confirmed by phylogenetic analysis. Ordinal names marked with asterisks are Basidiomycota; all others are Ascomycota. ITS sequences are available on request from the authors.

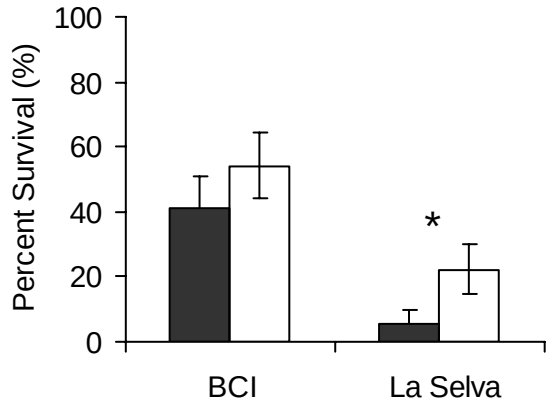
Order	**Family**	**Genus**	**Isolates**	**Seed status**	
				Viable	**Inviable**
Chaetothyriales	Herpotrichiellaceae	*Rhinocladiella*	7	1	6
Aphyllophorales*	Corticiaceae	*Athelia*	4	-	4
Diaporthales	Valsaceae	*Phomopsis*	3	-	3
N/I, I.S. Ascomycota	N/I, Botryosphaeriaceae	*N/I, Botryosphaeria*	3	-	3
Diaporthales	Valsaceae	*Diaporthe*	2	-	2
Diaporthales	Valsaceae	*N/I, Diaporthe/Phomopsis*	2	-	2
Eurotiales	Trichocomaceae	*Penicillium*	2	-	2
Hypocreales	Mitosporic hypocreales	*Fusarium*	2	1	1
N/I, Pleosporales	N/I, Pleosporaceae	*N/I, Alternaria*	2	1	1
Phyllachorales	Phyllachoraceae	*Colletotrichum*	2	2	-
Pleosporales	Pleosporaceae	*Curvularia*	2	-	2
Agaricales*	Pleurotaceae	*Pleurotus*	1	1	-
Aphyllophorales*	Polyporaceae	*Ceriporiopsis*	1	-	1
Eurotiales	Trichocomaceae	*Emericella*	1	1	-
Hypocreales	Clavicipitaceae	N/I, Unknown	1	1	-
Hypocreales	Nectriaceae	*Nectria*	1	1	-
I.S. Ascomycota	Botryosphaeriaceae	*Botryosphaeria*	1	-	1
I.S. Ascomycota	Dothioraceae	*Aureobasidium*	1	-	1
I.S. Ascomycota	Mycosphaerellaceae	*Cladosporium*	1	-	1
I.S. Ascomycota	Botryosphaeriaceae	*N/I, Botryosphaeria*	1	1	-
Saccharomycetales	Saccharomycetaceae	*Debaryomyces*	1	-	1
Sordariales	Chaetomiaceae	*Chaetomium*	1	-	1
Tremellales*	I.S., Tremellales	*Cryptococcus*	1	-	1

Figure 23.1. Percent seed survival of (a) *C. insignis* and (b) *C. obtusifolia* seeds in plots below crowns of fruiting (female) conspecific trees at BCI and La Selva. Bars show differences in seed survival between provenances; dark bars represent the mean (± 1 SE) percent germination of BCI seed sources and white bars represent the mean (± 1 SE) percent germination of La Selva seed sources. (a) *C. insignis*: La Selva seed sources (white bars) experienced higher survival than BCI seed sources (dark bars) at both sites (* $P < 0.05$). Survival was higher at BCI than at La Selva regardless of seed source. (b) *C. obtusifolia:* La Selva (white bars) and BCI (dark bars) seed sources experienced similar survival at both sites. Survival was higher at La Selva than at BCI regardless of seed source.

Figure 23.2. Percent seed survival of *C. insignis* seeds after five months of incubation in plots (1) below six crowns of fruiting (female) *C. insignis* trees, (2) six plots 50 m from those fruiting individuals, and (3) below six crowns of flowering (male) *C. insignis* trees at least 50 m from the nearest fruiting *C. insignis* at BCI (a). Seeds were not buried below male *C. insignis* trees at La Selva (b). Bars show differences in seed survival between provenances, which were not significant in any burial plot in either site. Dark bars represent the mean (± 1 SE) percent germination of six BCI seed sources; white bars represent the mean (± 1 SE) percent germination of six La Selva seed sources. Different letters show significant ($P < 0.05$) differences in survival among burial plots at BCI and La Selva.

1 Fig 23.1:

(a) *C. insignis*



(b) *C. obtusifolia*

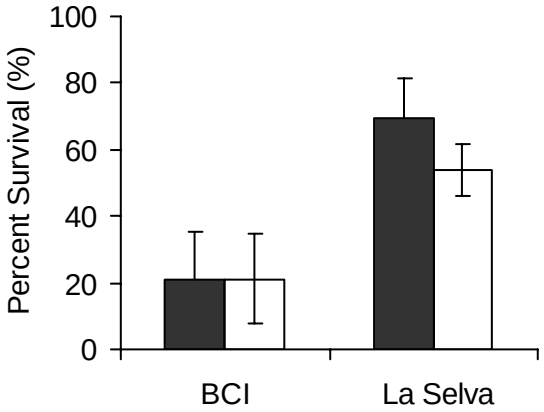
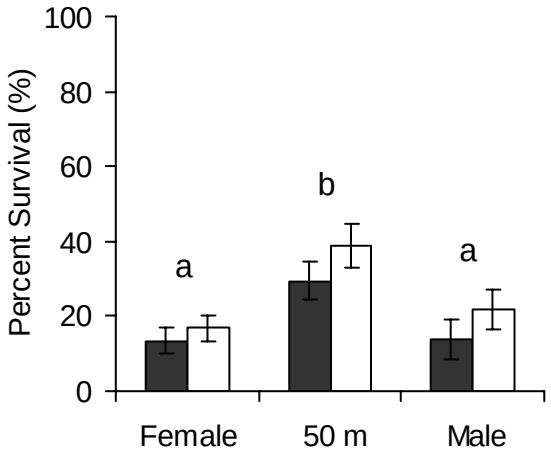


Fig. 23.2

(a) BCI



(b) La Selva