THE ROLE OF PATHOGENS IN DETERMINING PLANT RECRUITMENT AND DISTRIBUTION PATTERNS IN A WESTERN AMAZONIAN FLOODPLAIN

by

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ABSTRACT OF THE DISSERTATION

The role of pathogens in determining plant recruitment and distribution patterns in a western Amazonian floodplain

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The main objective of this dissertation was to investigate plant host-pathogen dynamics and evaluate the Janzen and Connell (J-C) hypothesis explaining tropical ecosystem mechanism of diversity maintenance. The first chapter of this dissertation explores the influence of distance from fruiting trees and plant density on fungal disease incidence, insect damage and subsequent mortality of conspecific plants (J-C distance effect). I present novel data about plant pathogens, disease mechanisms, herbivores and host-pathogen interactions for one of the most common plant species of western Amazonia, *Iriartea deltoidea*. I found that insect herbivores are located in the vicinity of fruiting trees causing

high mortality of conspecific seedlings as predicted by the J-C hypothesis. Surprisingly, the J-C distance pattern is not observed for lethal fungal pathogens such as *Diplodia mutila.*

The second chapter evaluates the nature and infection mechanisms of one of the most lethal pathogens found in *I. deltoidea* seedlings: *Diplodia mutila*. This fungus is ubiquitous and a generalist pathogen, causing disease and mortality in several host plants from different families. This characteristic could partially explain why *I. deltoidea* seedlings did not follow a J-C distance pattern. The potential implications of ubiquitous and pathogenic-endophytic fungi effects in tropical ecosystems are discussed. Endophyte-pathogens, hosts, herbivores and environmental conditions interact with each other, determining disease expression or repression.

The third chapter evaluates how environmental conditions, such as light availability, triggers disease expression and potentially defines plant distribution in tropical ecosystems. The ubiquitous and endophytic nature of many fungal pathogens also influences plant recruitment of dispersed propagules.

The fourth chapter examines the fate of dispersed seeds and seedlings in tropical ecosystems. Endophytic fungal pathogens could limit germination of dispersed seeds. Seedling mortality is high when dispersion is spatially and temporally aggregated. However seedling mortality is low when seedlings are randomly dispersed in the forest floor. Seedling mortality of dispersed propagules is produced by the synergistic effect of insect herbivores, fungal pathogens and environmental conditions. I conclude that pathogens in tropical

ecosystems are not just agents of mortality or disease, but also organisms that influence survival and recruitment patterns of plant species.

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I. INTRODUCTION

Conserving tropical biodiversity in the face of multiple environmental changes requires a better understanding of ecosystem processes. One hectare of tropical forest in the Amazon region can host 300 tree species (Valencia et al. 1994) but the processes that allow so many species to coexist remain unclear (Hill and Hill 2001, Terbogh et al. 2002, Wright 2002, Leigh et al. 2004). Several theories emphasize the importance of abiotic factors and stochastic events such as gap dynamics (Hartshorn 1978, Denslow 1987) intermediate disturbance (Connell 1978), spatial heterogeneity of limiting resources (Pacala and Tilman 1994), dispersal limitation (Tilman 1994, Hurtt 1995) and recruitment limitation (Hubbell 1999, Wright 2002). Other theories emphasize the importance of biotic factors and frequency–dependant mechanisms such as the Janzen and Connell hypothesis (Janzen 1970, Connell 1971). The high diversity of animals, fungi, bacteria and organisms living in tropical complex systems are vital for plant dispersal, recruitment and reproduction functions (Terborgh and Andresen 1998, Terbogh et al. 2002, Wright 2002) and could be one of the main drivers maintaining tropical ecosystems. Recent studies indicate that tropical diversity could be maintained by biotic and frequency-dependent mechanisms such as host-enemy dynamics. Data from 7 sites located in tropical ecosystems showed that common species in a local area have higher mortality than locally rare species, increasing diversity of these ecosystems (Wills et al. 1997, Wills 1999, Wills et al. 2006). Mortality of plants is attributed to competition, predators and pathogens. The main objective of this dissertation was to investigate the role of

fungal pathogens in determining plant recruitment and distribution inside tropical undisturbed ecosystems.

The Janzen and Connell hypothesis: Density and Distance effects

Several years ago it was postulated that the presence of a diverse range of pests and pathogens are capable of preventing any one species from dominating the forest (Gillet 1962). Janzen and Connell (Janzen 1970, Connell 1971) emphasized separately the negative effect of pathogens and predators on population recruitment by the adult tree, declining with increasing distance of the juvenile tree from the adult tree or other juvenile trees from now on conspecifics. The Janzen and Connell hypothesis defines two groups of predators the density-response predator and the distance-response predator. The density-response predators rely on the presence of conspecifics while the distance dependant predators are commonly parasites of the adult trees but mortality agents of younger propagules. Janzen emphasizes that “any given predator can belong to both categories”. Most studies in tropical ecology emphasize the importance of density effects (Hubbell et al. 1990, Harms et al. 2000, Hille Ris Lambers et al. 2002, Freckleton and Lewis 2006) and only few of them report distance effects (Augspurger 1984, Augspurger and Kelly 1984, Gilbert et al. 1994).

In the first chapter, the distance and density effects and causes of mortality are studied for one common tree species of southwestern Amazonia. It has been suggested that the isolation of mortality agents of seeds and seedlings in order to study the distance-effect (Gilbert 2005). This study isolated several fungal

pathogens, and provided unique data about their pathogenicity and virulence for the studied plant

Host–specificity of pathogens

The Janzen and Connell hypothesis emphasizes that pathogens and predators have to be host specific:

> "Without host-specificity the offspring would more likely mature close to their parent and regulation of tree density by seed predators would depend on the distance between seed bearing trees of any species serving as foci for these predators. All tree species would be affected by physical conditions favoring certain plant predators, and it is unlikely that these would make any particular tree species extinct or very rare."

The second chapter examines host specificity, pathogenicity and virulence of one pathogen, *Diplodia mutila*, found to be lethal for *Iriartea deltoidea* seedlings. I hypothesized that differences in virulence and pathogenicity of polyphagous fungi could regulate plant populations in natural communities and produce bigger impacts than host specific pathogens.

Plant disease and environmental conditions

Pathogenicity and virulence are relevant concepts in order to understand the nature and implications of plant diseases in tropical ecosystems. These two concepts have been largely ignored so far from tropical ecology literature but see (Gilbert 2005, Gilbert and Webb 2007). Three critical factors or conditions must exist for a plant disease to occur: a susceptible host plant, a source of pathogen, and suitable environmental conditions. The relationship of these factors is called

the ‘disease triangle’. Pathogenicity refers to the ability of an organism to cause disease (i.e, harm the host). This ability represents a genetic component of the pathogen. However, disease and plant mortality are not inevitable outcomes of the host-pathogen interaction; furthermore, pathogens can express a wide range of virulence. Virulence refers to the degree of pathology caused by the pathogenic organism. The extent of virulence is usually correlated with the ability of the pathogen to multiply within the host (Agrios 2005). Additionally, most fungi can be infecting the plant without causing any visible symptom, known as endophytic fungi (Petrini 1986). Endophytes can confer beneficial effects to the plant such as predator defense or physiological advantages for the plant (Arnold et al. 2003, Arnold and Engelbrecht 2007). Endophytic fungi are hyper-abundant and diverse in tropical ecosystems (Lodge et al. 1996, Frohlich and Hyde 1999, Arnold et al. 2000, Arnold 2005, Arnold and Lutzoni 2007). The specific case of *Diplodia mutila* effects explained in the first two chapters shows that endophytic fungi could be beneficial or lethal. This continuum could be highly influenced by environmental conditions. In the third chapter I study *Diplodia mutila* not as a pathogen but as an organism that exists both as a pathogen and as a beneficial endophyte; and how environmental conditions affect the pathogen-endophyte continuum (Schulz and Boyle 2005). I hypothesize that most plants in the tropics follow a similar pattern, where a plant hosts a range of endophytic fungi that act as endophytes or pathogens, depending on environmental conditions and the interaction with other organisms.

Plant diseases affecting dispersed propagules

In the J-C hypothesis all dispersal agents such as frugivorous primates, birds or other mammals contribute to the survival of a given species in a habitat that greatly favors seed predators (Janzen 1970, Connell 1971). This dispersion mechanism allows the seed to “escape” from the area of adult influence and have higher survival probabilities. Animal dispersal is considered one of the most important processes in tropical forest regeneration processes (Janzen 1970, Howe and Smallwood 1982, Link and Di Fiore 2006). Most dispersal processes are aggregated (Russo and Augspurger 2004). If propagules are aggregated and are dispersed far from an adult tree, then density-response predators should produce high mortality. It has been demonstrated that high seed density does not inhibit successful recruitment of the palm *Maximiliana maripa* present in high densities in tapir latrine sites far from fruiting adults experienced significantly lower rates of predation than seeds under adult crowns and in their vicinity (Fragoso et al. 2003). The fourth chapter examines seed and seedling mortality causes of naturally dispersed plants. I hypothesize that endophytic and pathogenic organisms could also be affecting dispersed plants, inducing seed and seedling mortality or protecting the propagule.

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Chapter 1

EFFECTS OF PLANT DISEASES AND INSECT HERBIVORY ON RECRUITMENT OF A COMMON NEOTROPICAL PALM

ABSTRACT

The Janzen and Connell (J-C) hypothesis proposes that plant pathogens and herbivores regulate recruitment of tropical plants near conspecific adults. For 150 days disease incidence, insect damage and mortality rates were monitored on the most common tree species in western Amazonia, *Iriartea deltoidea* (Arecaceae). The causal agents of diseases, insect damage and mortality in *I. deltoidea* seedlings and juveniles were identified and classified into six groups based on their effects on the plant. The proportion of dead seedlings (induced by several different pathogens, insects and random causes) decreased with distance from fruiting adults, consistent with predictions from the J-C hypothesis. Each causal agent of plant damage had a different distance-dependent spatial structure on plant mortality. Stem borers produced high mortality near fruiting trees and the highest mortality rate for young seedlings. Fungal diseases (foliar spots and wilting) and insect herbivory present in most evaluated plants, at all distances, induced initially low mortality rates that increased over time. Logistic regression analysis was used to separate distance and density effects on juvenile mortality and infection rates.

The primary mechanism explaining stem borer attack and mortality of *I. deltoidea* seedlings is distance. Although the study period of 150 days provides only a snapshot of the effects of some diseases and insect herbivores on one plant species, it is suggested that the effects and interactions of several pathogens, endophytes, herbivores and environmental variables are shaping the spatial distribution of *I. deltoidea*.

INTRODUCTION

The Janzen and Connell (J-C) hypothesis predicts high mortality of seeds and juvenile plants near parent trees, a process that facilitates the establishment of heterospecifics (Janzen 1970, Connell 1971). Janzen (1970) proposed three classes of agents that could contribute to distance-dependent recruitment: seed predators, herbivores and pathogens. These agents have been the subjects of scores of investigations (Augspurger 1984, Burkey 1994, Gilbert et al. 1994, Packer and Clay 2000, Norghauer et al. 2006). However, these investigations have provided ambivalent support for the operation of distance effects and no clear consensus has emerged (Hyatt et al. 2003). Several empirical studies suggest that density-dependant mortality could play an important role in tropical species diversity maintenance (Harms et al. 2000). Soil pathogens, such as *Pythium* sp., produced high seedling mortality of susceptible plants at high densities and adequate environmental conditions (Augspurger 1984, Bell et al. 2006). Evidence of distance-dependant mortality was found in temperate forest, where soil pathogens limit plant recruitment in the vicinity of a reproductive tree and allow greater survival of rare but less susceptible tree species (Packer and Clay 2000). However few studies in tropical forest have found distance-dependent plant mortality attributable to pathogens (Augspurger and Kelly 1984, Gilbert et al. 1994, Hood et al. 2004). Some studies suggest a positive relationship between density of conspecifics and seedling recruitment, where the abundance of seeds and seedlings near the fruiting tree masks any distance dependant mortality (Condit et al. 1992, Burkey 1994). Also, in areas of high

adult density, seed shadows of conspecific trees can overlap sufficiently to produce homogenous seed distributions (Bustamante and Simonetti 2000) and it is possible that increased distance from conspecific adults does not enhance seed survival (Hyatt et al. 2003). However, most of these studies focus on few pathogens and/or predators, and most tropical plants potentially host several pathogens or predators (Erwin 1982, Hawksworth 1991). Additionally, three conditions must exist for disease to occur: a susceptible host plant, a pathogen and favorable environmental conditions. The relationship of these factors is called the disease triangle. If only a part of the triangle exists, disease will not occur. Understanding the disease triangle explains why most plants are not susceptible to most pathogens (Agrios 2005). Interactions between herbivores, pathogens and host plants, and their complex interactions with the environment and each other, have been relatively neglected and remain largely unexplored territory.

Fungi and insect diversity estimates in the tropics are high (Erwin 1982, Hawksworth 1991). However, there is limited knowledge about fungal pathogens, disease mechanisms, endophytes and insect herbivores in natural ecosystems. Enhanced appreciation of the role of pathogens and herbivores in forest biology could be obtained if it were possible to isolate disease and mortality agents and to identify them in the field, thereby linking correlations with causation (Gilbert 2005). The identification of plant diseases and their evaluation in natural forest settings still remains a daunting task. The main objective here was to explore the J-C distance effect on survival of the most common tree

species in western Amazonia *Iriartea deltoidea* (Arecaceae), using novel data about plant pathogens, insect herbivores and host-pathogen interactions. This study examined the influence of distance from fruiting trees on fungal disease incidence, insect damage and subsequent mortality and survival rates of conspecific plants.

MATERIALS AND METHODS

The experimental design here attempts to minimize six of the considerations raised by Janzen's original study (Janzen 1970). The predators must be identified; human disturbances/manipulation must be minimized; differential competitive ability of the seedlings (i.e. allelopathy) must be considered; habitat heterogeneity must be accounted for (e.g. often using multiple plot designs); geometric and ecological distance must be compatible and evaluate monoecious species with regular fruiting intervals. Most disease and mortality agents affecting *Iriartea deltoidea* plants were monitored daily. Five fungal pathogens were collected in the field, isolated within the first 4 hours of collection, cultured on Potato Dextrose Agar (PDA), identified based on morphological characterization using three identification sources (Sutton 1980, Ellis and Ellis 1985, Barnett and Hunter 1998) and reinoculated into *Iriartea* seedlings to confirm pathogenicity (Álvarez-Loayza et al. 2008)(Appendix 1). Insect herbivores affecting the plant were collected in the field and identified to genus or family level using two identification sources (Borror et al. 1976, Coto and Saunders 2004). The study sites, described below, are located in an undisturbed natural forest (Terborgh 1983, Gentry 1990), and no artificial manipulations were employed. The study sites had different edaphic conditions, hence, the basic premise is that habitat heterogeneity can be partially averaged out by multiple sampling. The locations were inside lowland tropical forest characterized by flat terrain so that ecological distances may be approximated by geometric distances. *Iriartea deltoidea* is monoecious (Henderson 1990, Sezen et al. 2005). The adults selected for study

fruited in two consecutive years.

Plant: *Iriartea deltoidea*

The palm, *Iriartea deltoidea,* Ruiz & Pavón, (Arecaceae) (Fig. 1), hereafter simply *Iriartea)* is the most common tree in western Amazonia from Ecuador to southeastern Peru (Pitman et al. 2001b). Densities can reach 40 individuals (≥10 cm dbh) per hectare (Pitman et al. 2001a). This monoecious species is widespread and abundant across a wide range of soil types and topographies (Henderson 1990, Clark and Clark 1995, Sezen et al. 2005). Seeds are dispersed by a variety of animals, including spider monkeys, howler monkeys, white-faced monkeys, night monkeys, peccaries, tapirs, several species of rodents (Terborgh 1983), birds, including toucans (Galetti 2000), and bats (Romo 1996). *Iriartea* dispersal distances in a second-growth forest (in Costa Rica) were on the order of 200 m (median), while the maximum seed dispersal distance recorded exceeded 850 m (Sezen et al. 2005). Hence, dispersal distances far exceed the distances between fruiting adults.

Iriartea palms undergo ontogenetic transitions in leaf morphology. Young seedlings produce 3 to 6 round, simple leaves. Subsequent leaves are longer and compound, carrying increasing numbers of pinnae (Terborgh and Davenport 2001). Four age classes, were recognized based on leaf morphology and ease of accessibility for the evaluation of diseases: (1) young seedlings (2) old seedlings (3) juvenile-adults and (4) fruiting trees (Fig. 2). Young seedlings had one or two simple round leaves measuring less than 25 cm. Remnants of the seed were still attached to the newly germinated seedling. Old seedlings had two or more

simple or compound leaves but measured less than 50 cm tall. Old and new seedlings were distinguished by the presence of remnants of the seed and the presence of algae and epifoliar fungi. Algae and epifoliar fungi colonized leaf blades of old seedlings and were absent from new seedlings. Disease symptoms of young and old seedlings were evaluated in all leaves. Juvenile plants had compound leaves and were > 50 cm but <6 m tall. Disease symptoms for juvenile plants were evaluated for only two leaves randomly chosen. Plants taller than 6 m were termed adults. Disease symptoms were visually evaluated only in the lower leaves. Palms designated as fruiting were observed to fruit at least once between 2005 and 2007. Fruiting individuals and adults were too tall (up to 30 m) to be evaluated for disease symptoms. The incidence of plant diseases and mortality agents was tallied only for young and old seedlings.

Study sites

The research was conducted at two field sites in lowland tropical forest in Peru: Cocha Cashu Biological Station (CCBS), Manu National Park (11^{o} 54' S, 71^{o} 22' W, elevation ca. 400 m) and Los Amigos Biological Station (LABS) (12° 34' 07" S, 70° 05' 57" W, elevation ca. 268 m). These sites are 162 km apart and located beside the Rio Manu and Rio Los Amigos, respectively. Mean annual rainfall at CCBS is approximately 2100 mm (10-yr mean), 86% of which occurs during the rainy period (October to May). Mean annual temperature is 24^{o} C, with record extremes of 8^{o} C and 34^{o} C (Terborgh 1983, Gentry 1990) measured

under canopy. The habitat is mature floodplain forest. Mean annual rainfall at LABS is approximate 2850 (6 yr-mean), with a rainy period similar to that at CCBS. Mean annual temperature is 24^{o} C, with record extremes of 8^{o} C and 39° C (Pitman 2007) measured under open canopy. Los Amigos watershed has three different habitats: floodplains, terraced uplands, and hilly uplands (Foster 2001). Ten sample plots were established in mature floodplain forest at CCBS and LABS.

Sampling plots

Ten plots were established in primary floodplain forest, with similar floristic composition and topographic characteristics. Five plots were located at CCBS and five at LABS. Nine plots measured 900 m^2 and one plot located at CCBS measured 2.25 ha. In each plot all *Iriartea* plants were tagged with numbered plastic tags and mapped in an X - Y coordinate system. Height of tallest leaf (cm), number of leaves, color of leaves, diseases on the leaves and stem, and percentage of disease in the affected leaves were recorded for all *Iriartea* plants less than 6 m tall. Presence/absence of diseases was scored, as was insect damage and/or evidence of random mortality agents. Biotic and abiotic factors recorded were: light availability using canopy cover estimates (Welden et al. 1991) and a light meter (Environmental Concepts Plant Light Intensity Meters, LIM2500, USA), proximity to animal latrines or trails, vines surrounding the plant, tree fall gaps and ant nests. Height, light availability, and fruit presence or absence, were recorded for palms taller than 6 m. Plots were monitored three

times after initial establishment. In CCBS, plots were monitored after 7, 45 and 140 days. In LABS, plots were monitored after 5, 52 and 160 days. After 150 days, a few plants could not be found. These were considered to have been lost to random mortality agents.

Iriartea deltoidea pathogens, insect herbivores and random damage

Plant damage and diseases were defined as any response of plant cells and tissues to a pathogenic organism or abiotic and biotic factors that results in adverse changes to the form, function or integrity of the plant (Agrios 2005). Common pathogens and insect pests affecting *Iriartea* were identified in previous research (Álvarez-Loayza et al. 2008) (Alvarez-Loayza unpublished data). For this study, these agents were classified into five groups, hereafter referred to as damage groups, based on the damage they produced on the affected plant. Plants hosting scale insects (Homoptera), crickets, caterpillars and beetles were classified as Group 1 (Fig. 3). Insects were not identified to species, but rather categorized according to the damage they produced (e. g. scale insects, leaf miners, leaf chewers.) Host specificity was not evaluated but all insects identified on seedlings were also found on adult plants. Plants affected by *Diplodia mutila (*hereafter referred as *Diplodia),* a highly pathogenic fungus for *Iriartea* young seedling*s* (Álvarez-Loayza et al. 2008)*,* were classified in Group 2 (Fig. 4). Foliar spots are common diseases in palms and usually do not kill the plant (Elliott et al. 2004). Several fungal pathogens, classified in Group 3, were isolated form *Iriartea* leaf spots: *Colletotrichum gloeosporioides, Pestalotiopsis* sp.,

Microsphaeropsis concentrica, Verticillum sp. and *Fusarium* sp. (Fig. 5). Pathogenicity tests or induced infection by the isolated causal agent, were proved only for *C. gloeosporioides, M. concentrica* and *Pestalotiopsis* sp. Foliar spots can be a symptom of biotic and abiotic stress (Agrios 2005) such as insect damage, water stress and nutrient deficiency (Elliott et al. 2004). Plants were scored as showing foliar spots when insect attack, and unfavorable biotic conditions were not evident even though insect damage and foliar spots may occur simultaneously (García-Guzmán and Dirzo 2001). Plants presenting a characteristic leaf deformation were considered deficient in Boron (Elliott et al. 2004) and classified in Group 4 (Fig. 6). Plants damaged by stem and epicotyl borers, such as caterpillars, beetle larvae or crickets, were assigned to Group 5 (Fig. 7). Epicotyl damage is lethal for the seedling, killing the apical meristem. Random agents producing plant damage such as fallen fronds, peccary rooting, sooty mold fungi (non-pathogenic fungi), monkey feces and urine (Alvarez-Loayza unpublished data), mammal herbivory, and vine collapses were classified as Group 6 (Fig. 8). Most plants were affected by more than one damage group. Plants were assigned to the damage group that was more dominant (percentage of affected leaf surface). Plants with no foliar diseases, insect damage or visible damage were classified as Group 0, hereafter referred to as uninfected plants.

Most fungal pathogens identified in this study are generalists and were found as endophytic fungi in other plant species (Ragazzi et al. 1999, Canon and Simmons 2002, Arnold 2005). *Diplodia mutila* and *Colletotrichum gloeosporioides* were isolated from fruits, seeds, healthy leaflets of adults, and

juveniles of *Iriartea deltoidea, Wendlandiella gracilis, Astrocaryum murumuru* (all Arecaceae), *Oxandra acuminata* (Annonaceae) and *Piper reticulatum* (Piperaceae), indicating that these pathogens are endophytic (Alvarez-Loayza unpublished data) and confirming the polyphagous nature of these pathogens. These pathogens are transmitted by rain, insects, mechanical injuries produced by fallen branches and possibly carried in seeds as endophytes (Alvarez-Loayza unpublished data).

ANALYSIS

In each plot, the minimum distances, (d_{nft}), from all plants in size classes 1, 2 and 3 to the nearest fruiting tree of *Iriartea* were computed using the coordinates of the labeled plant under consideration and the coordinates of the nearest fruiting tree within the plot. The minimum distance between a fruiting adult and plants classified in any of the four age classes described above was deemed as the most ecologically relevant. To investigate the number of diseased plants as a function of distance, seedlings/juveniles were surveyed in concentric 2.5 m annuli centered on a focal fruiting adult, only the first annuli measured 3.25 m in order to account for the extensive root system of the palm (Terborgh and Davenport 2001). The number of plants in each damage group was tallied for each 2.5 m annulus and then divided by the total number of plants located in the selected annulus to yield proportions. One-way ANOVA was used to compare diseases and mortality proportions among plots for each distance group, with Tukey's HSD and t-student tests used to contrast means. All statistical tests were performed with JMP 6.0 (SAS Institute 2005).

Local densities (ρ_{lpd}) were computed by counting the number of *Iriartea* of all sizes within a circle of radius r_d (=50 cm here) of the individual *Iriartea* plant being analyzed. Overall disease incidence- and mortality- rates were separately analyzed with respect to the distance to the nearest fruiting adult (d_{nft}) and local plant density effects (ρ_{lpd}). Because the determination of infectious probabilities for all damage groups requires large sample sizes to account for the wide variation in d_{nft} and ρ_{lpd} , data from all 10 plots were pooled. Both, d_{nft} and ρ_{lpd} ,

are often negatively correlated because higher ρ_{lpd} occurs near fruiting trees. Logistic regression analysis can be used to separate d_{nft} and ρ_{lpd} effects on juvenile mortality and infection rates (Packer and Clay 2000). For the infection rate logistic regression analysis, each *Iriartea* seedling in size class 1, 2 and 3 was assigned a score of 1.0 if infected by one of the 6 agents or zero otherwise. To assess the relative importance of d_{nft} and ρ_{lpd} on infection or mortality rates, the parameters of the logistic regression were computed using only distance to nearest fruiting tree alone, local plant density alone, and their combination (assumed independent). In reality, d_{nft} and ρ_{lpd} are not usually independent but for the purposes of discerning their relative effects on infection or mortality rate, they are treated as independent. Next, the logistic regression was employed with the dependent variable (Y) being the binary state (infected or not) and independent variables being d_{nft} and ρ_{lpd}. Statistical significance is determined based on the coefficients of the logistic regression (*r*). Logistic regression analysis is presented in Appendix 2. All calculations were conducted for r_d = 0.5 m (radius around each plant) and m = 8 (distance groups, by annuli) using Matlab's nonlinear regression routine nlinfit (Mathworks Inc. 2004).

RESULTS

A total of 1068 *Iriartea* plants were mapped in the first census (March 2007). Sixty-three adults produced fruits between December and March 2007, 317 seedlings were considered young at the time of the first census, another 491 were considered old seedlings, from these seedlings 141 had only round leaves and 350 had at least one compound leaf; 197 were considered juveniles-adults (non-fruiting). The remainder, 63 plants, were the number of fruiting adults.

1- Disease incidence and mortality with respect to distance from fruiting trees

Distance dependence for infected plants and recruited plants ceases to exist beyond 11 m with much of the distance decay occurring within the first 10 m, roughly comparable to the diameter of the crown of the fruiting trees (~ 7 m) (Terborgh and Davenport 2001), the zone of influence of fallen fronds (~ 10 m) (Peters 2004) and the mean nearest-neighbor distance between adults in the studied population (8.8 m at CCBS) (Terborgh unpublished data). In the first census, after 5-7 days, plants affected by damage groups 1, 2, 3 and 4 (insects, *D. mutila*, foliar pathogens and random agents) were located near and far from fruiting trees. Most plants affected by damage group 5 (stem borers) were located within the first annulus, 1.25 m, next to the fruiting tree ($P > 0.045$, Fig. 9). After 50 days, similar results were observed. The proportion of plants affected by damage group 5 was significantly higher in the first annulus,

decreasing gradually with distance ($P > 0.0051$, Fig. 9). After 150 days, there was no difference in the incidence of stem borers among distance classes.

Overall mortality appeared to be higher near fruiting trees but no statistical difference was found after 50 days ($P = 0.604$, Fig. 10). However, after 150 days, mortality rates were higher in the first two annuli (within 6.25 m of the fruiting tree) compared to mortality in annuli located farther than 6.25 m ($P = 0.0042$, Fig. 10). The proportion of plants affected by damage groups 1 (insect damage) and 3 (foliar damage) was the highest for all annuli at all times. The proportion of surviving plants after 150 days was somewhat higher far from the fruiting tree but not statistically significant.

To estimate the influence of density and distance on mortality and diseases the analysis was focused for damage group 2 (*Diplodia*) and 5 (stem borers), for censuses 2 and 3 (50 and 150 days). These categories were selected because *Diplodia* shows high virulence in young seedlings and insect borers are devastating to *Iriartea* seedlings. Density-distance effects on mortality were estimated for period 3 (cumulative mortality after 150 days). Both distance (d_{nft}) and density (ρ_{lpd}) are significant predictors of stem borer infections and mortality rates (regression coefficients: $r = 0.73$ and $r = 0.78$ respectively), but d_{nft} were much more significant than ρ_{lpd}. Table 1 summarizes all the logistic regression coefficients and the correlation coefficient (r) between measured and modeled probability of infection (p_i). Figure 11a shows the measured and modeled infection and mortality probabilities as a function of d_{nft} and ρ_{lpd}. For stem borer damage and mortality, the parameters of the logistic regression, β_2 and β_3, are

negative at the 95% confidence interval suggesting statistical significance for both density and distance (Fig. 11b and 11c). Also, the analysis in Table 1 shows that d_{nft} explains 3-6 times more the variability in p_i when compared to ρ_{lpd}. For *Diplodia* infections, the logistic regression explains only 25% of the variance in infection probability, with density effects being statistically insignificant (Fig. 12). The above analysis was repeated for different radii (r_d) around each plant: r_d = 0.2 m and r_d = 1.5 m (not shown here) and the relative importance of d_{nft} over ρ_{lpd} remains qualitatively the same though the overall performance of the logistic regression model degraded (quantified by r).

2- Disease influence over mortality

After 50 days, 9% of the marked plants had died and an additional 16% died after 150 days. Damage group 5 (stem borers) produced 73% of the total plant mortality after 50 days, random agents 22% and *Diplodia* infections contributed 2%. Foliar insects produced the remaining 3% of mortality. After 150 days, stem borers induced 43% of mortality, random damage another 32% and foliar diseases 15%. Boron deficiency produced 4% of mortality, insects 5% and *Diplodia* infections 1% (Fig. 13). Altogether 241 (22%) plants died after 150 days. Most dead plants were young (64%) or old (32%) seedlings.

DISCUSSION

While local density effects cannot be entirely ignored, the primary mechanism explaining stem borer attack and mortality of *Iriartea* seedlings is distance. However, it is important to note that the logistic regression model explains about 50% of the infection probability variance ($r > 0.69$) suggesting that factors other than distance and density are at play, such as abiotic conditions, plant defense mechanisms, pathogen virulence, insect attack rates and spatial variability (Augspurger 1984, Coley and Barone 1996, Sanchez-Hidalgo et al. 1999, Kursar and Coley 2003, Rodriguez et al. 2004). It is important to mention that not all reproductive individuals fruit every year (J. Terborgh unpublished data) therefore there could be other reproductive individuals that did not qualify as fruiting trees in this study. Seedling to adult distances could be overestimated if the actual number of fruiting trees is undercounted. However, our study targeted young seedlings (recently germinated), disease incidence and proximity to conspecific fruiting trees for a period of 150 days.

Mortality rates after 50 and 150 days were higher next to fruiting trees and mostly produced by stem borers. Most young seedlings infested with stem borers were located in the vicinity of the fruiting adults, under closed canopy conditions and few were located far away, possibly near an alternative host of these insects. However, stem borer infection started to decrease after 50-days (Fig. 9) and other damage agents such as foliar diseases started influencing mortality. Young seedlings could be the most susceptible group for stem borers. Older plants may acquire resistance mechanisms. After 150 days, new

seedlings that were not affected by stem borers had more than three leaves and were colonized by a wide variety of foliar pathogens.

The observations recorded in this study began approximately after 3 months of seed deposition for new seedlings and at an arbitrary time for the rest of the palms. Several new seedlings-susceptible individuals- were probably killed before our observations began. Mortality agents for this period of time are unknown but any subsequent mortality observed after monitoring began underestimates the total distance-related post-germination mortality. However the detailed study of pathogens and insect predators was helpful to find a distance-effect produced by stem borers. The study period of 150 days is brief compared to the maturation time of a palm, additional distance-related mortality produced by other mortality agents could continue to occur subsequent to the conclusion of monitoring.

This study does not answer the question as to whether stem borers have a close association with the fruiting tree and whether they are host specific. However the most common stem borer was morphologically identified as a Chrysomelidae: Hispinae larvae (Fig. 7), known to be host specialist (Novotny et al. 1999). Additionally, insect borers have previously been recorded as affecting recruitment of tropical plants (Sullivan 2003). One seed borer, *Coccotrypes* sp. was recorded in 4 of the 10 plots infesting 30% to 100% of the seeds (Alvarez-Loayza unpublished data) and it is a common seed predator for palm species (Janzen 1972). It is possible that the larva of this insect feeds on *Iriartea* seedlings (Sousa et al. 2003). In this study *Coccotrypes* sp. was recorded

feeding in new epicotyls of *Iriartea* (Fig. 7). Host specificity of stem borers and insects damaging seeds of *Iriartea* is unknown. However, *Coccotrypes* sp. extracted from *Iriartea* also infested seeds of *Socratea exhorriza* and *Socratea salarzii* (Alvarez-Loayza unpublished data). Other insect herbivores observed on *Iriartea*, such as scale insects, crickets and Hemiptera were found on other host plants. Additionally, several studies suggest that tropical insects are host-generalists (Novotny et al. 2006). Whether the stem borers of this study were generalists or host specialist, they caused high mortality of young seedlings near fruiting trees, providing evidence of the important role of distance-responsive pathogens and herbivores in regulating plant populations.

Plant diseases and foliar insect damage were not important sources of mortality of *Iriartea* after 50 days. Fungal pathogens such as *Microsphaeropsis concentrica*, *Pestalotiopsis* sp. and *Colletotrichum gloeospoiroides,* are latent and frequently isolated from senescent *Iriartea* leaves (Alvarez-Loayza unpublished data). After 150 days, the proportion of plants killed by fungal diseases increased to 15%. However, plants were considered “killed by foliar pathogens” when the plant was previously recorded as infected with foliar pathogens and found dead after 150 days. Dead seedlings presented distinctive leaf spots indicating the presence of fungal pathogens but it is very likely that these were symptoms of opportunistic pathogens, or endophytic fungi manifesting themselves as saprophytic fungi (feeding from dead plant material). It is strongly suggested that another agent caused seedling mortality (e.g. stem borer, insects, sooty mold, low soil nutrients, etc.).

Boron deficiency affected plants at random locations, inducing 4% mortality after 150 days. These results emphasize the importance of evaluating diseases as pathosystems, where host, pathogen, environment and time are all important (Agrios 2005). Insect herbivores induced mortality after 150 days, most of these insects were crickets or leaf chewers and scale insects, which usually infested leaf blades, interrupting photosynthesis and leading to general plant decay. Random agents of damage such as mammal herbivory, sooty mold fungi and monkey feces killed plants located close and far away from fruiting adults. Sooty mold fungi interrupted photosynthesis of new seedlings growing in spider monkey defecation sites (see Chapter 4). Mechanical damage from falling senescent leaves produced seedling mortality near the fruiting tree as reported previously (Peters 2004). Mammal herbivory (deer) was observed for 2 plants in a period of 33 days at one plot at CCBS (daily observations), it is possible that mammals removed seedlings that were not found after 150 days.

Diplodia was the only pathogen that was closely examined. *Diplodia* was reported to cause seedling mortality in 15 to 30 days, but this fungus, although present, does not reduce fitness of plants larger than 50 cm or plants located in favorable environmental conditions (e.g. shaded understory, well drained soils) (see Chapter 3). Many of the juveniles had typical leaf spots caused by *Diplodia* and were healthy. It seems evident that *Diplodia* infection is not consistent with the J-C hypothesis in spite of evidence that it induces mortality of young seedlings and infects conspecific trees. This random pattern of infection could be produced because this pathogen is present endophytically in several adult

plants and it is transmitted by rain, insects and possibly on or in seeds (see Chapter 3). The implications of endophytism of pathogens are not clear, but some questions may be raised. Is there an advantage for the plant to host a fungus that is a potentially lethal pathogen for its seedlings and an endophyte of adult plants? Could infection by some pathogens enhance fitness of plants? Defensive mutualism has been reported for grasses and endophytic fungi, where a major effect of the fungi is defense of host plants against herbivory (Clay and Holah 1999). The endophytic phase of *Diplodia* and similar fungi colonizing *Iriartea* could be defensive for the plant. Additional studies are needed to evaluate these questions.

The isolation and identification of diseases, insects and other mortality agents of *Iriartea*, and the partitioning of mortality among identified mortality agents distinguishes this study from previous attempts to assess J-C distance effects. The J-C mechanism is only expected to have a strong plant diversity-enhancing role when pathogens show high specificity (Janzen 1970). Current research suggests that plant pathogens and insect herbivores in tropical diverse forests are polyphagous (Lu et al. 2004, Novotny et al. 2006, Gilbert and Webb 2007). Additionally, plant diseases do not occur without favorable environmental conditions or susceptible hosts, and each pathogen has several pathogenic strains that could be highly pathogenic or protect the plant against other virulent strains or pathogens (Agrios 2005). Diseases will also be influenced by plant defense mechanisms, chemical interactions and the presence of endophytic fungi, which are extremely diverse in tropical ecosystems (Okuda et al. 1997,

Sanchez-Hidalgo et al. 1999, Arnold et al. 2003, Arnold 2005, Herre et al. 2007). This study highlights the influence of pathogens and predators on the spatial pattern of seedling mortality independent of host-specificity. Although an observation period of 150 days provides only a snapshot of the effects of some diseases and insect herbivores on one plant species, this study suggests that the interaction of several pathogens, endophytes and herbivores and environmental variables could shape plant spatial patterns.

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Table 1. Equation and parameters of the logistic regression model: $p_i = \frac{1}{1+\exp\left[-\left(\beta_1 + \beta_2 \rho_{lpd,i} + \beta_3 d_{nft,i}\right)\right]}$ determined from nonlinear regression analysis of *Iriartea deltoidea* census plots. The correlation coefficient (*r*) between measured and modeled unknown probabilities of infection (p_i) is also shown. Values in parentheses indicate 95% confidence intervals. For the analysis with independent variables being only $\rho_{lpd,i}$ (density) or $d_{nft,i}$ (distance), β_3 or β_2 (parameters of the logistic regression) are set to zero. The logistic regression model suggests that *D. mutila* infection does not correlate with distance (r = 0.43), mortality inversely correlates with distance (*r* = 0.78), and that stem borer infection inversely correlates with distance (*r* = 0.69).

Model	β_1	β_2	β_3	r
Infection Probability (Month = 3, Mortality)				
Independent variables d_{nft} and ρ_{lpd}	0.64 (-0.13, 1.41)	-0.029 (-0.05, -0.01)	-0.31 (-0.46, -0.17)	**0.78***
Independent variable only d_{nft}				**0.72***
Independent variable only ρ_{lpd}				0.12
Infection Probability (Month = 1, Stem Borers)				
Independent variables d_{nft} and ρ_{lpd}	-1.22 (-1.91, -0.53)	-0.03 (-0.05, -0.01)	-0.20 (-0.32, -0.08)	**0.69***
Independent variable only d_{nft}				**0.60***
Independent variable only ρ_{lpd}				0.16
Infection Probability (Month = 2, Stem Borers)				
Independent variables d_{nft} and ρ_{lpd}	-0.81 (-3.4, 1.76)	0.0 (-0.02, 0.02)	-0.74 (-1.93,0.45)	**0.73***
Independent variable only d_{nft}				**0.73***
Independent variable only ρ_{lpd}				0.04
Infection Probability (Month = 3, *Diplodia mutila*)				
Independent variables d_{nft} and ρ_{lpd}	-1.25 (-2.78, 0.23)	0.0064 (-0.06, 0.07)	-0.0943 (-0.19, 0.02)	0.43
Independent variable only d_{nft}				0.43
Independent variable only ρ_{lpd}				0

*** Significant correlations**

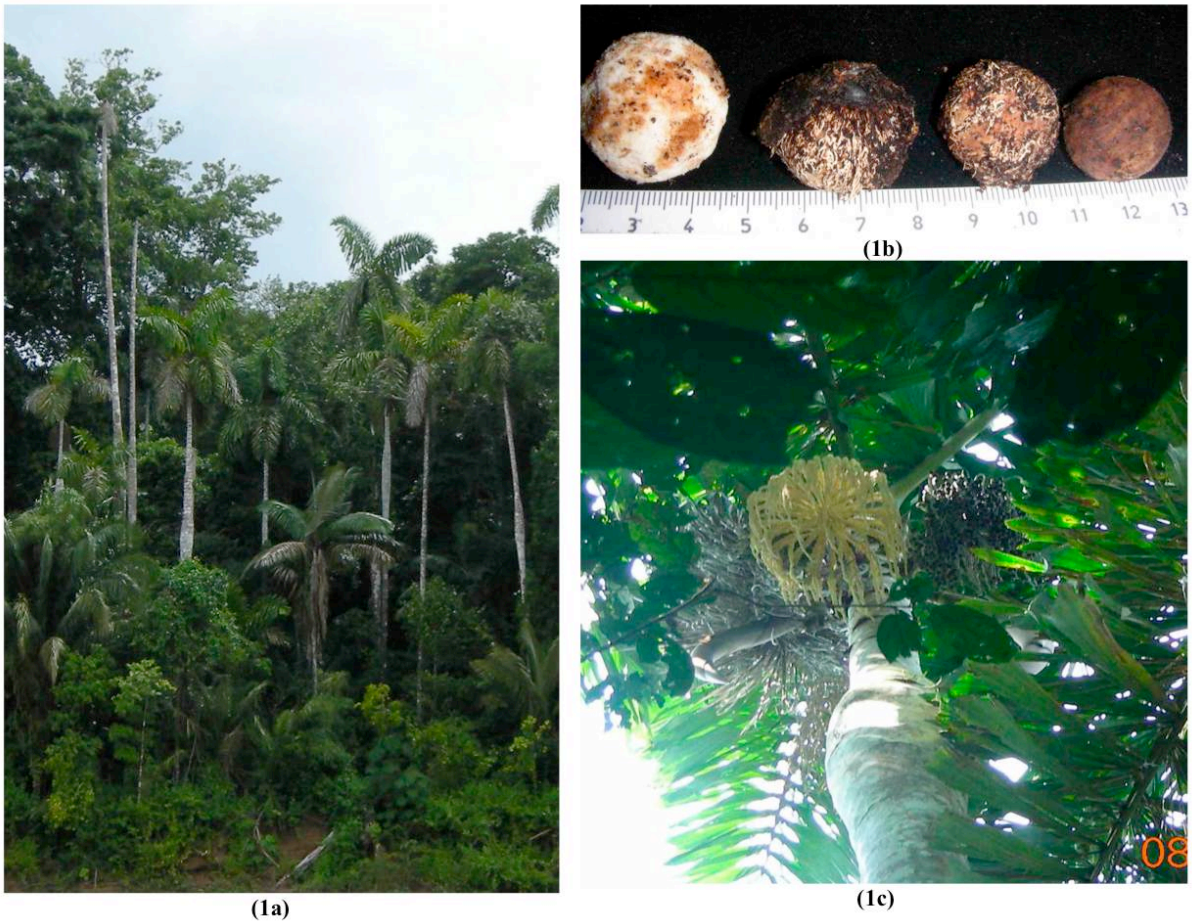

Figure 1. *Iriartea deltoidea,* Ruiz & Pavón, (Arecaceae), (1a) Cluster of adult and juvenile palms (1b) Fruits and seeds (1c) View of the male and female flowers.

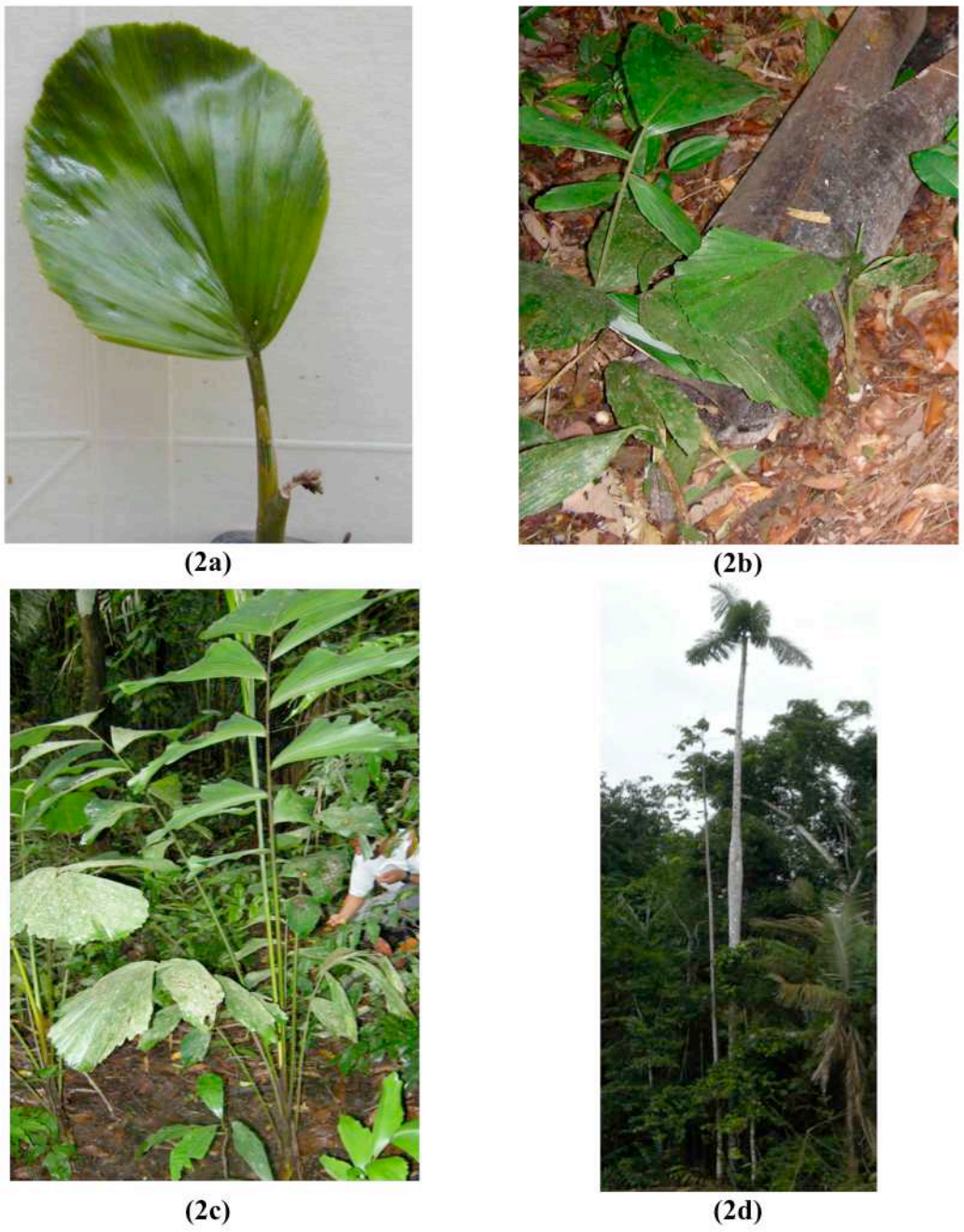

Figure 2. Four age class classification for *Iriartea deltoidea* plants, based on leaf morphology and accessibility to evaluate diseases (2a) young seedlings (2b) old seedlings (2c) juvenile-adults and (2d) fruiting trees.

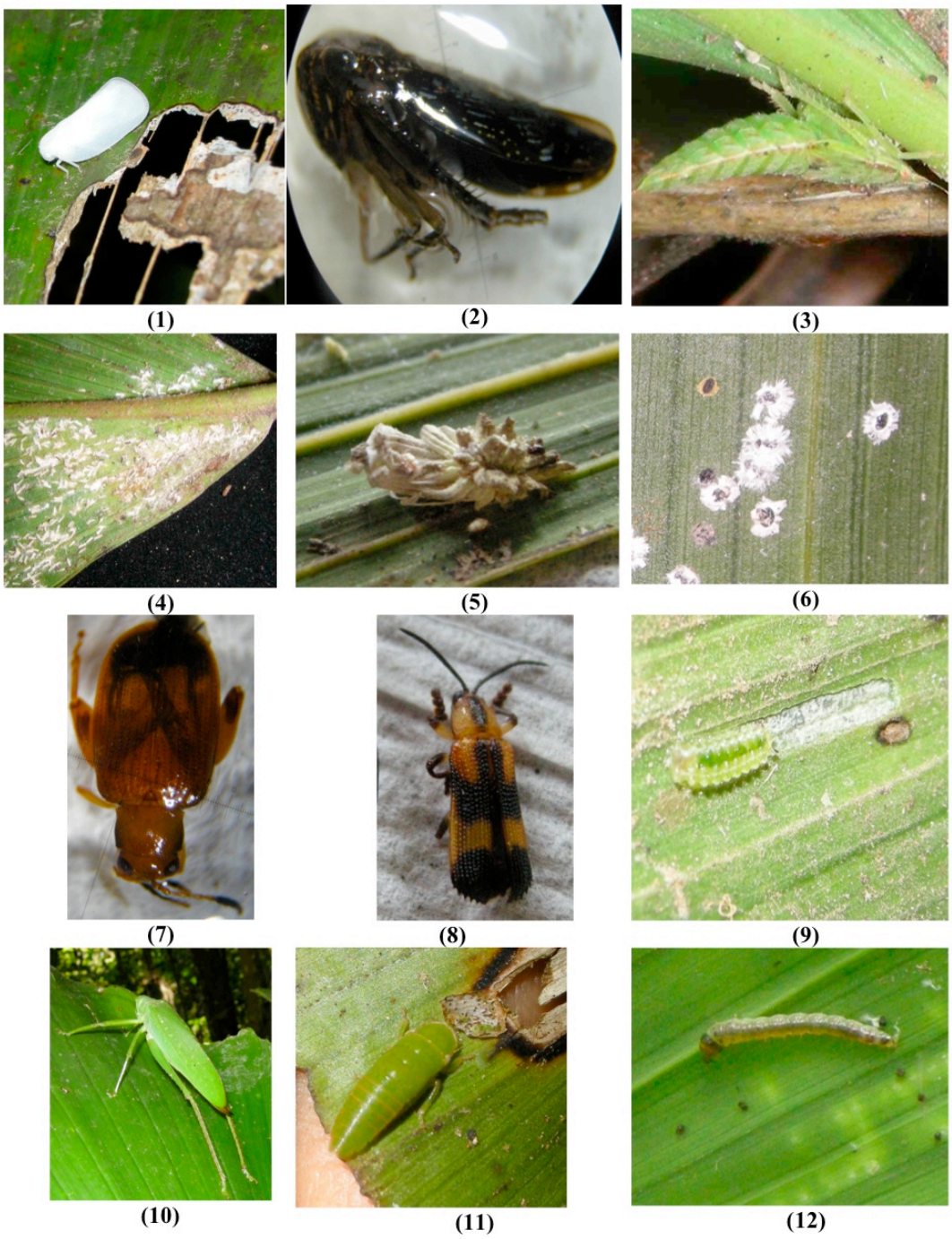

Figure 3. Group 1= insect attack on *Iriartea deltoidea* leaves. **(1)** Homoptera: Flatidae: *Anormenis* sp., **(2)** Homoptera: Cicadellidae, **(3)** Homoptera, **(4)** Homoptera: Diaspididae, **(5)** Homoptera: Diaspididae, **(6)** Homoptera, **(7)** Chrysomelidae: Alticinae, **(8)** Chrysomelidae: Hispinae, **(9)** Chrysomelidae: Cassidiinae: Delocraniini, **(10)** Orthoptera: Tettigoniidae, **(11)** Homoptera, **(12)** Lepidoptera.

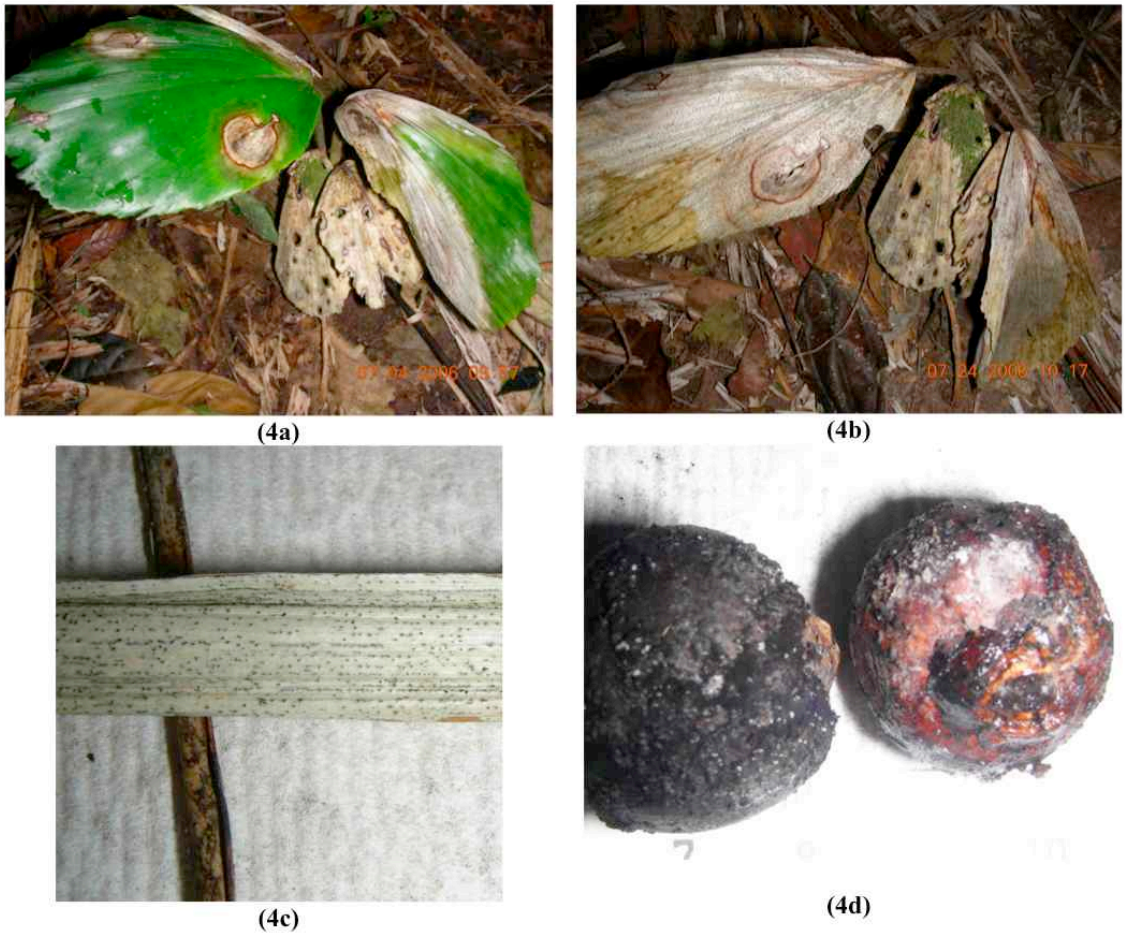

Figure 4. *Diplodia mutila* symptoms on *Iriartea deltoidea* **(4a)** Initial symptoms produced by *Diplodia mutila* in 6-month old *Iriartea deltoidea* seedling **(4b)** the same *Iriartea deltoidea* seedling after 20 days of infection **(4c)** senescent adult leaflet colonized by *Diplodia mutila* **(4d)** fruits infected by *Diplodia mutila*.

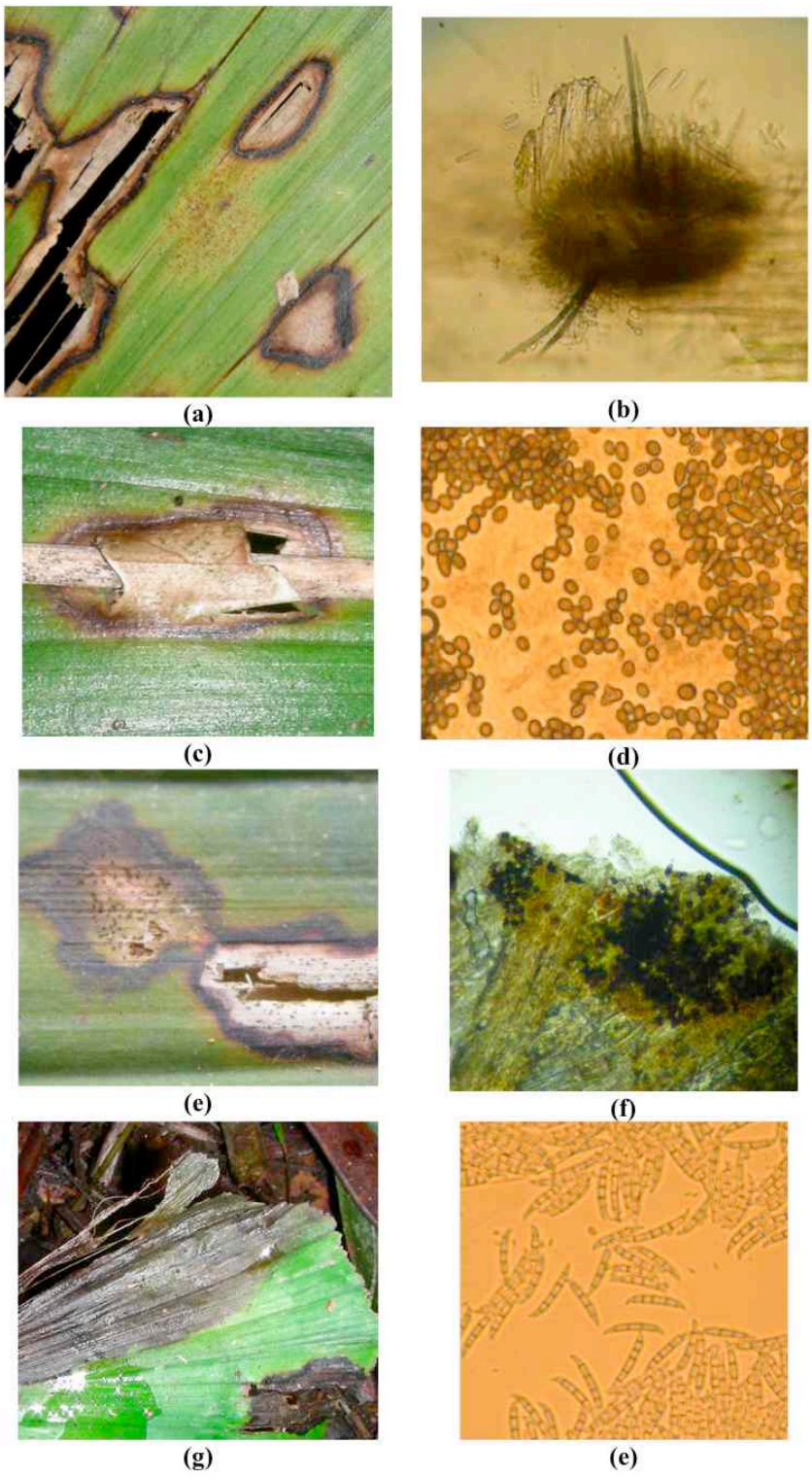

Figure 5. Common foliar pathogens of *Iriartea deltoidea,* (a-b) *Colletotrichum gloesporoides,* (c-d) *Microsphaeropsis concentrica,* (e,f) *Pestalotiopsis* sp., (g-h) *Fusarium* sp.

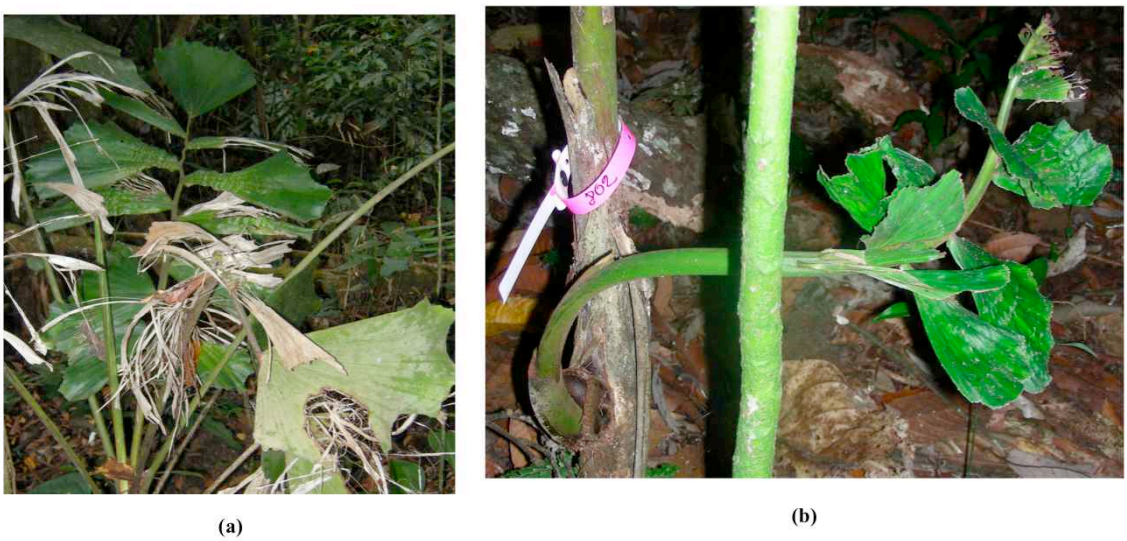

Figure 6. Foliar deformation produced by Boron deficiency in *Iriartea deltoidea* juvenile plants, (a) Leaves of juvenile plant deformed and burned (b) Leaf of juvenile plant growing abnormally damaging the main stem.

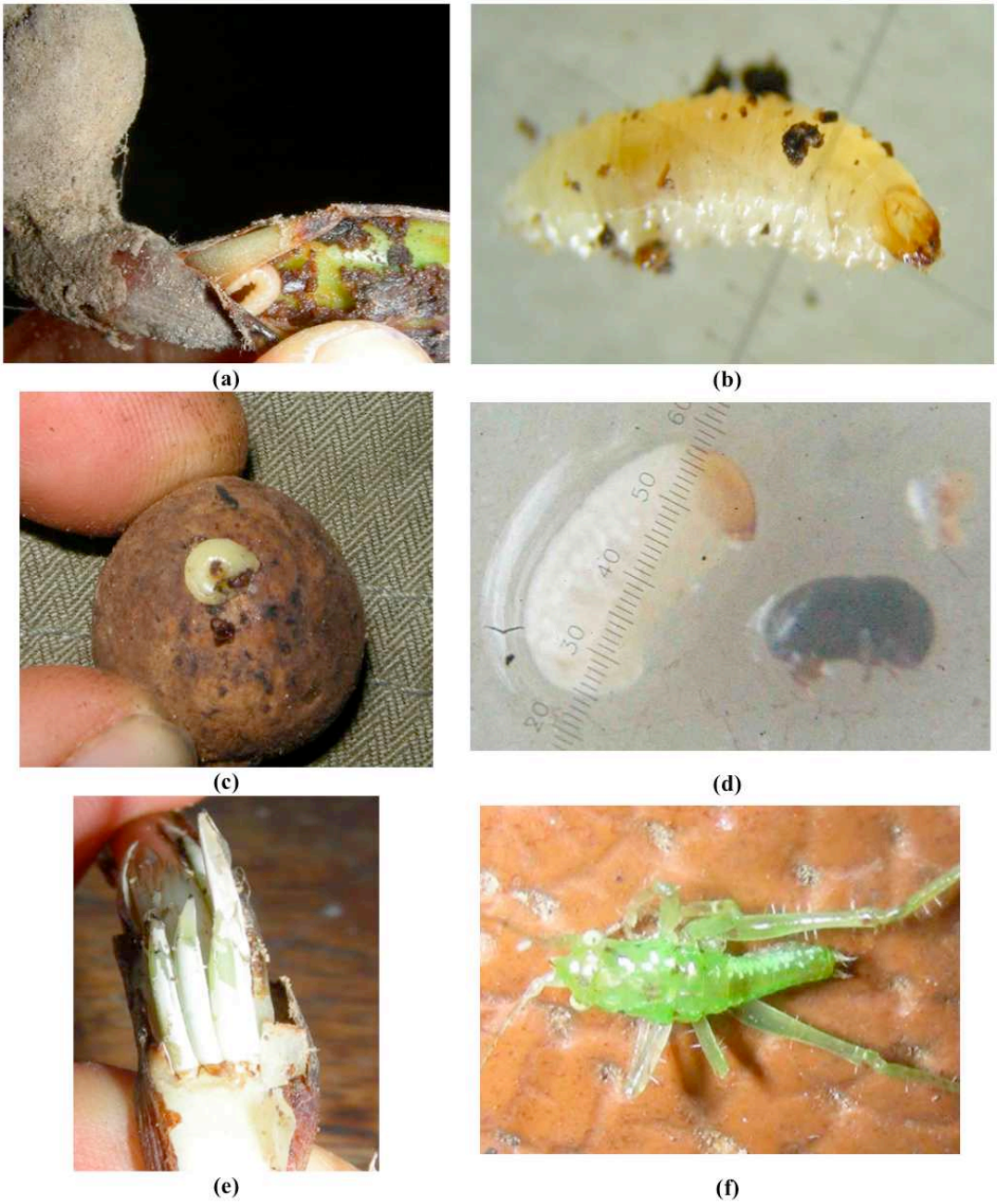

Figure 7. Stem borers attacking *Iriartea deltoidea* young seedlings, (a-b) Most common stem borer found in dead seedlings, identified as Chrysomelidae: Hispinae (c-d) Scolitidae eating young epycotil (e-f) Orthopthera eggs affecting seedlings, causing mortality in most cases.

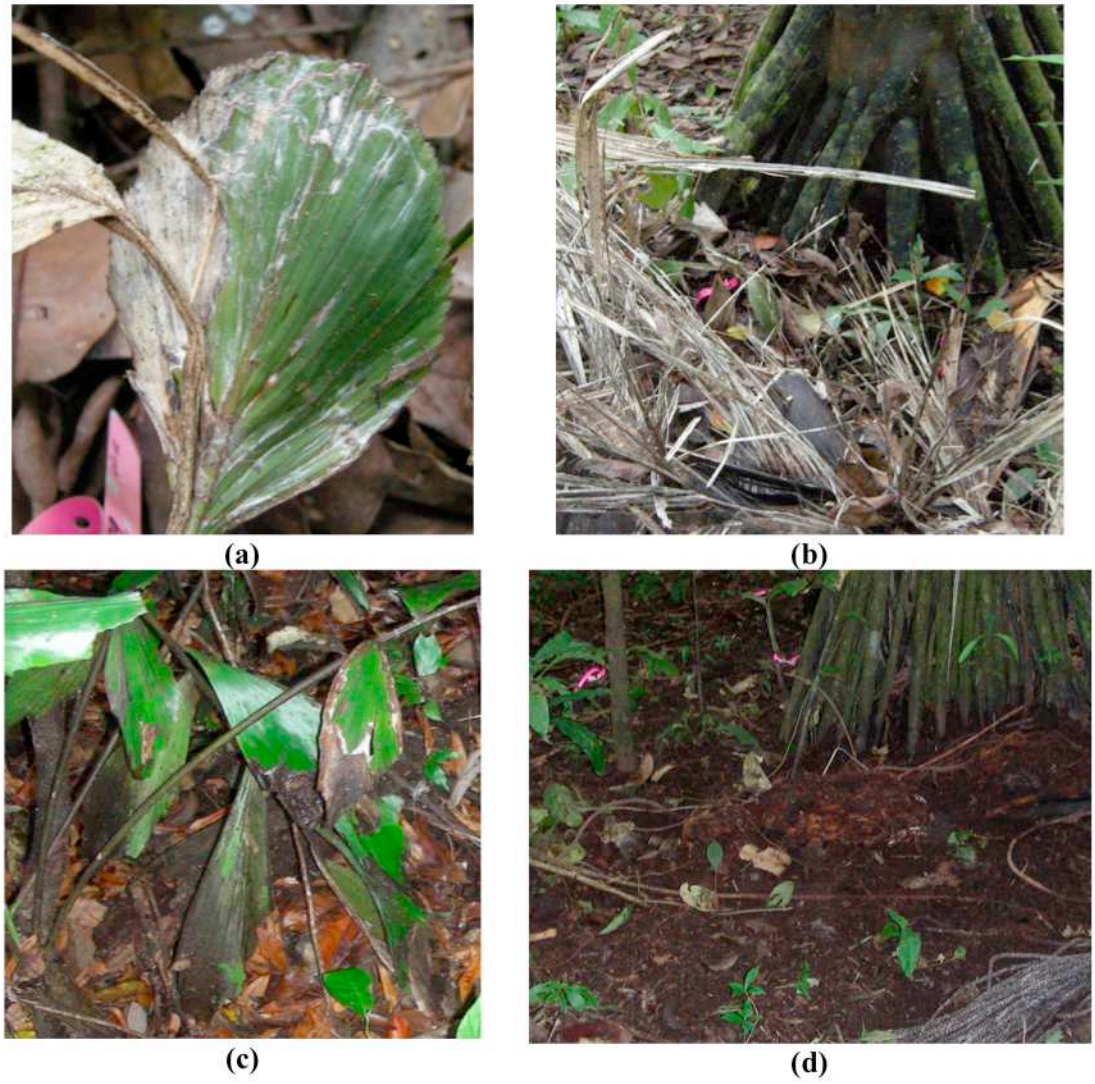

Figure 8. Random mortality agents for *Iriartea deltoidea* plants, (a) White saprophytic fungi infesting a seedling, (b) Fallen branches near adult trees, (c) Sooty mold fungi colonizing plants inside a spider monkey latrine, (d) Plants affected by peccary trampling.

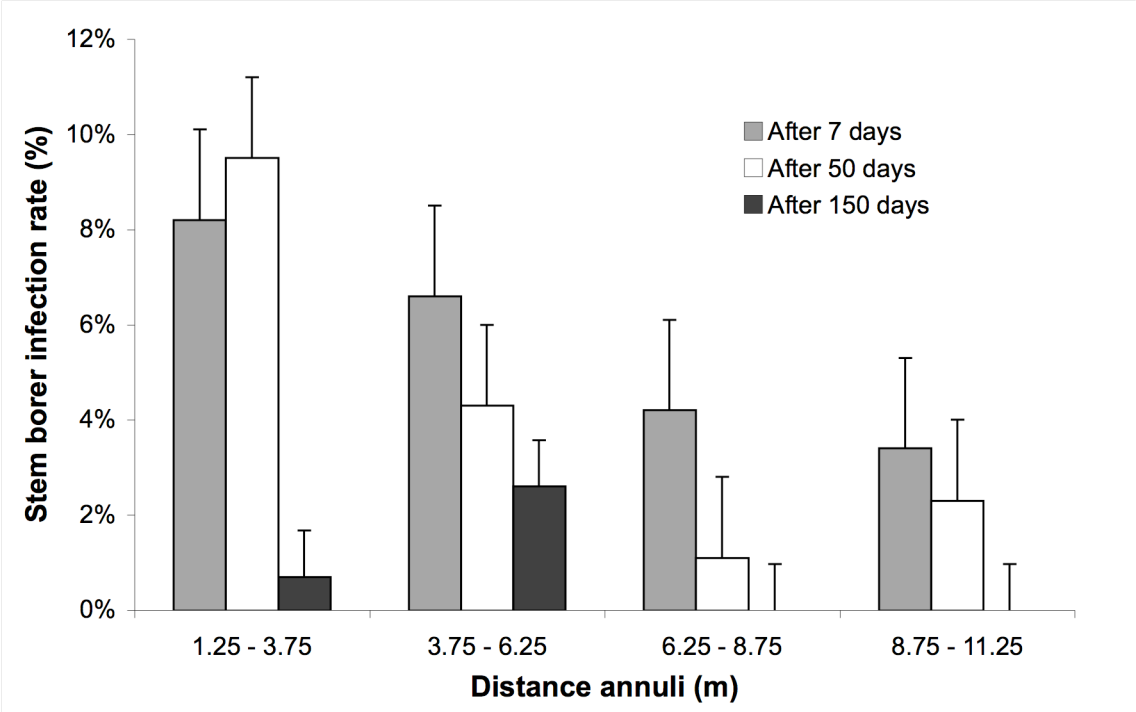

Figure 9. Proportion of plants affected by stem borers in each distance group showing a statistically significant decrease of stem borer incidence with distance from parental tree. The proportion of plants affected by stem borers within 1.25 m was significantly higher in the first census, after 5 days (Tukey-Kramer, n = 10 plots, d.f. = 4, $F = 2.65$, $P > 0.045$) and the second census, after 50 days (Tukey-Kramer, (n = 10 plots, d.f. = 4, $F = 4.28$, $P > 0.0051$). Stem borer attack was lower in the last census.

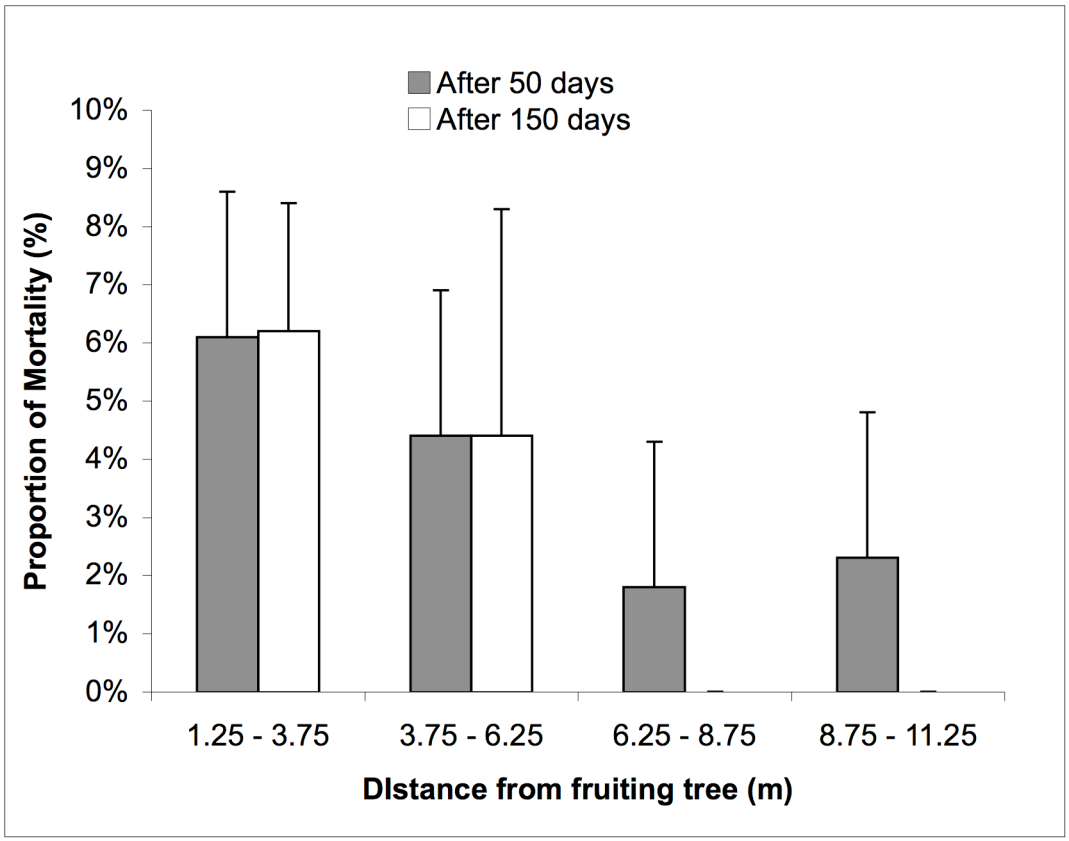

Figure 10. Average overall mortality rates (including all mortality agents) occurring at five concentric annuli around (fruiting) adult trees. There is a decay of overall mortality with distance in the second census (after 50 days), but there was no statistical significance (ANOVA $F_{4, 50}$ = 0. 6344, P = 0.604). Mortality is higher at the first two concentric annuli around, within 6.25 m (ANOVA $\boldsymbol{F}_{4, 50}$ = 2.2377, P = 0.079). Mortality within the first two annuli is significant higher than mortality in the last two annuli (>6.25, < 11.25 m) (t-test $F_{2,50}$ = 9.01, P = 0.0042*).

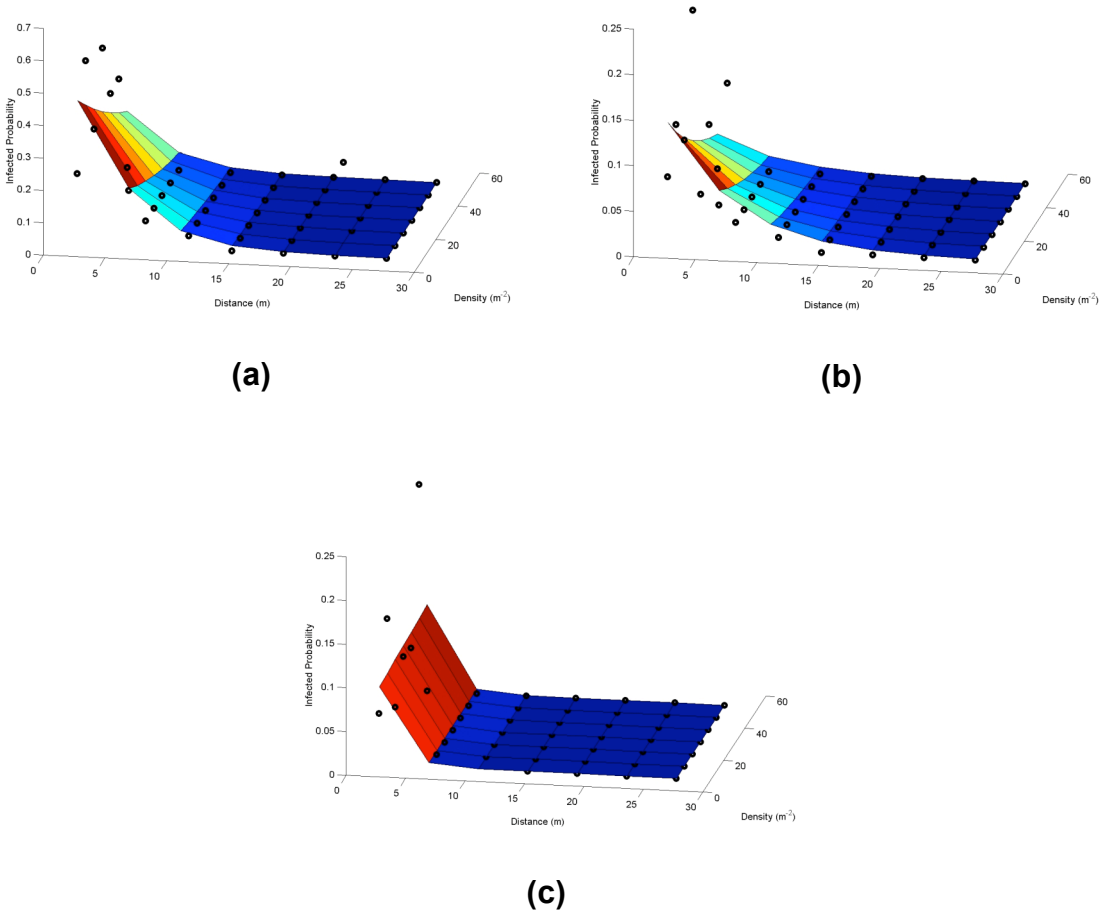

Figure 11. Measured (open circles) and modeled (surface) infection probability as a function of distance to the nearest fruiting tree (d_{nft}) and local plant density (p_{ipd}) for mortality and month 3 (11a) showing that overall mortality correlates with distance from parental plant; stem borers and month 1 (11b) showing that stem borer incidence correlates with distance from parental plant; and stem borers and month 2 (11c). The logistic parameters of the modeled surface along with the correlation coefficient between measured and modeled probabilities are presented in Table 1.

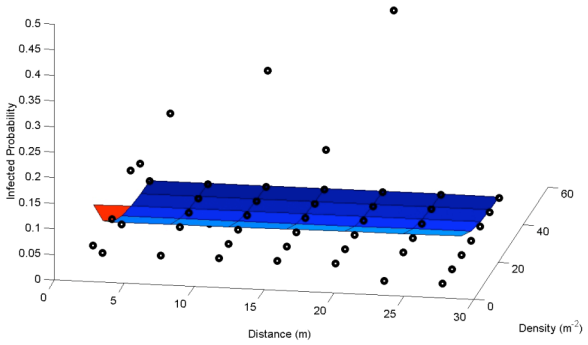

Figure 12. Measured (open circles) and modeled (surface) infection probability as a function of distance to the nearest fruiting tree (d_{nft}) and local plant density (ρ_{lpd}) for disease 2 (*Diplodia mutila*) and month 3 (final census).

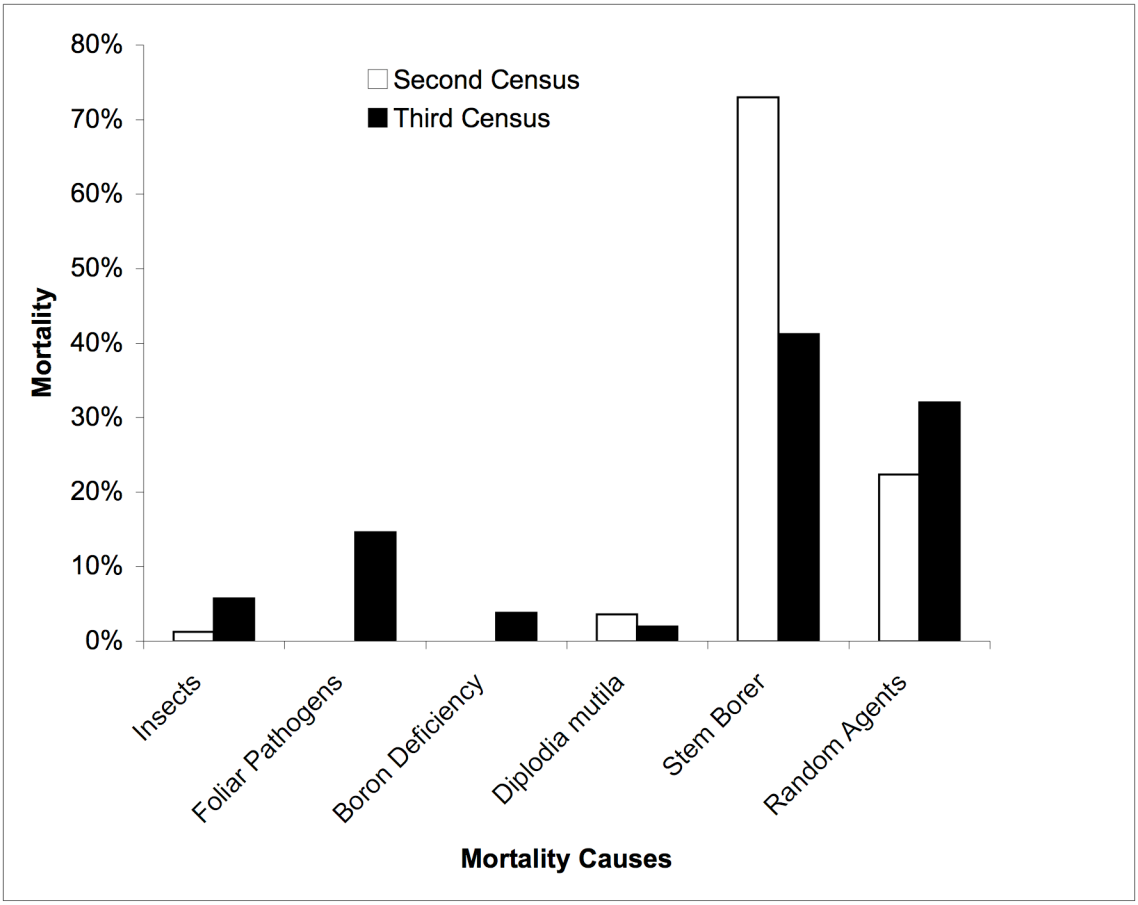

Figure 13. Proportion of *Iriartea deltoidea* seedlings killed by six different damage groups and recorded at Cocha Cashu and Los Amigos Biological Stations after 50 days (second census) and after 150 days (third census).

Chapter 2

HOST RANGE AND PATHOGENICITY OF *DIPLODIA MUTILA*: AN ENDOPHYTE AND PATHOGEN IN TROPICAL FOREST COMMUNITIES

ABSTRACT

There is increasing interest in the role played by plant pathogens and endophytes in maintaining plant diversity in natural plant communities. This study examines host ranges and pathogenicity of the dual endophyte/pathogen *Diplodia mutila* in the natural undisturbed lowland tropical forest plant community of Southwestern Amazonia, Peru through surveys and inoculation experiments. *Diplodia mutila* was isolated from naturally occurring infections on seedlings of the palm *Iriartea deltoidea* in the plant community. The inoculation experiments demonstrated that five palm species (Arecaceae) were highly susceptible to *D. mutila* upon inoculation, 20 plant species had atypical responses and three plant species presented partial or complete defoliation. The pathogen was also isolated from naturally occurring infections of seedlings of *Astrocaryum murumuru* and *Wendlandiella gracilis* (Arecaceae), seeds of *Oxandra acuminata* (Annonaceae) and saplings of *Piper reticulatum* (Piperaceae). *Diplodia mutila* is pathogenic and virulent on a range of dicotyledonous and monocotyledon species. Pathogenicity and virulence vary with plant species, age and environmental conditions. Also, for very young plants of *I. deltoidea*, *D. mutila* is a pathogen resulting in mortality; but for more mature plants the fungus is an asymptomatic endophyte.

INTRODUCTION

Host-specific pathogens, among other biotic agents, have been proposed to limit tree recruitment in tropical forests in a distance-dependent fashion by killing seeds and seedlings in the proximity of reproductive adult trees. Adult trees are assumed to serve as pathogen carriers or reservoirs. Such selective mortality of con-specific propagules enhances tree diversity by suppressing con-specific seedlings arising in the vicinity of the parent tree and allowing other species to establish (Janzen 1970, Connell 1971). Yet, to date, there are few studies of the specificity of particular pathogens over a wide range of potential hosts and plant diseases in natural tropical forest are poorly studied (Zhou & Hyde 2001, Gilbert 2005, Gilbert & Webb 2007). Because of this it is currently difficult to assess impacts in natural plant communities. This research was undertaken to develop data about pathogens that may affect plant recruitment and diversity in natural plant communities.

Host-specialized fungi thus appear to exist in diverse tropical ecosystems, but they may be uncommon and limited to the most common hosts (Gilbert 2005). Host specificity of plant pathogens is well documented by several studies (Zhou & Hyde 2001). In one class of diseases, gene by gene relationships determine disease resistance in the host and virulence of the pathogen (Singh *et al.* 1995, Heath 1996, Cook 1998). However, the gene-by-gene system is characteristic of obligate intracellular pathogens and parasites, but has not been reported in necrotrophic and saprophytic organisms. Other diseases involve the

production of host-specific toxins (Browder & Eversmeyer 1986, Otani 2000). Nevertheless, host-specific toxins have been reported for very few fungi and bacteria compared to the vast number of plant diseases (Newton & Andrivon 1995, Otani 2000). Pathogen specificity of *Pythium* spp. over *Prunus serotina* was reported for temperate forest ecosystems (Packer & Clay 2003) and recently Augspurger and Wilkinson (2007) reported some degree of host specificity for soil pathogens (*Pythium* spp.) on a few tropical plant species. However, most plant diseases located in natural tropical ecosystems are unknown and most importantly, their ecological implications have been poorly studied. If host specific pathogens are important in shaping forest ecosystems and regulating the populations of most tropical plant species, a suitable host needs to be present at sufficiently high densities to ensure that the fungus can colonize new host individuals (Burdon & Chilvers 1982, Lodge *et al.* 1996). However, most tree species in the Amazonian region are rare, occurring at densities of < 1 individual/ha (Pitman *et al.* 2001a). At such low host densities, it will be more difficult for host-specific pathogens to locate new hosts therefore the best strategy for tropical fungi is to be polyphagous (Lu *et al.* 2004). There is recent evidence indicating that polyphagous fungi could be the causal agents for several plant diseases (Gilbert & Webb 2007). Most fungal pathogens in tropical rain forests are polyphagous, but the likelihood that a pathogen can infect two plant species decreases with phylogenetic distance between the plants (Gilbert & Webb 2007); *i.e.* phylogenetic relatedness between plant species could help predict the likely host ranges of plant pathogens.

Pathogenicity and virulence are relevant concepts in order to understand the nature and implications of plant diseases in tropical ecosystems. Three critical factors or conditions must exist for a plant disease to occur: a susceptible host plant, a source of pathogen, and suitable environmental conditions. The relationship of these factors is called the 'disease triangle'. Pathogenicity refers to the ability of an organism to cause disease (i.e, harm the host). This ability represents a genetic component of the pathogen. However, disease and plant mortality are not inevitable outcomes of the host-pathogen interaction; furthermore, pathogens can express a wide range of virulence. Virulence refers to the degree of pathology caused by the pathogenic organism. The extent of virulence is usually correlated with the ability of the pathogen to multiply within the host (Agrios 2005). Differences in virulence and pathogenicity of polyphagous fungi could regulate plant populations in natural communities and produce bigger impacts than host specific pathogens. This study investigates host specificity and virulence of the pathogen *Diplodia mutila,* commonly reported for *Iriartea deltoidea* in undisturbed Amazonian lowland forests (Alvarez-Loayza *et al*. 2008a), by addressing three questions: (1) Is *D. mutila* a host-specific pathogen of *I. deltoidea*? (2) Does virulence of *D. mutila* vary with age of *I. deltoidea* and environmental conditions (i.e. light)? (3) Does virulence of *D. mutila* vary with heterospecific host plants (different plant species)?

MATERIALS AND METHODS

Study Sites

The study was conducted at two localities in the mature floodplain forest area in southeastern Peru: Cocha Cashu Biological Station (CCBS), Manu National Park (11° 54' S, 71° 22' W, elevation ca. 400 m) and Los Amigos Biological Station (LABS) (12° 34' 07" S, 70° 05' 57" W, elevation ca. 268 m). Mean annual rainfall at Cocha Cashu is approximately 2,100 mm (10-yr mean), 86% of which occurs during the rainy period (October to May). Mean annual temperature is 24° C, with recorded extremes of 8° C and 34° C (Gentry 1990, Terborgh 1983). The habitat is mature floodplain forest in the meander plain of a white-water tributary of the Madre de Dios River. Mean annual rainfall at Los Amigos is approximate 2,850 (6-yr mean), with a similar rainy period as at Cocha Cashu. Mean annual temperature is 24° C, with record extremes of 8° C and 39° C (Pitman 2007). Los Amigos watershed has three different habitats: floodplains, terraced uplands, and hilly uplands (Foster 2001).

Plant-Pathogen System

The most common tree in western Amazonia from Ecuador to southeastern Peru is the palm, *Iriartea deltoidea* Ruiz & Pavón (Aracaceae) (Pitman *et al.* 2001a). This monoecious species is widespread and abundant across a wide range of soil types and topographies, recruiting successfully in mature forest conditions (Henderson 1990, Clark & Clark 1995, Svenning 1999, Sezen *et al.* 2005).

The generalist necrotrophic pathogen *Diplodia mutila* Fr. apud Mont. (= *Botryosphaeria stevensii* Shoemaker; Botryosphaeriaceae; Botryosphaeriales; Ascomycota) (Sutton 1980) was reported to cause seedling mortality of *Iriartea deltoidea* young seedlings in a period of 5 to 16 days in undisturbed tropical lowland forest (Alvarez-Loayza *et al.* 2008a.) The diseased leaves presented small circular necrotic spots with black pycnidia- flask shaped asexual structure-producing liquid masses of slowly maturing, non-striate, brown, 1-septate conidia-asexually produced fungal spores (Alvarez-Loayza *et al.* 2008a, Fig. 14, Appendix 1,3).

Host Specificity of *Diplodia mutila*

1. Pathogen Inoculum Preparation

Petri plates containing Potato Dextrose Agar (PDA) (Becton Dickinson & Company) amended with chloramphenicol (100 mgL^{-1}), commonly used to grow fungi, were previously prepared in a laboratory at Rutgers University, wrapped in individual plastic bags and stored inside a transparent plastic container (7.5 L) disinfected with 96% EtOH and 5% NaCl. Tweezers and needles used to manipulate fungi mycelia and spores at CCBS were previously disinfected at Rutgers University. The tools were autoclaved at 121° C for 20 minutes, individually wrapped with sterile absorbent paper and aluminum foil and stored in a disinfected plastic container with 100 g of desiccant (WA Hammond Drierite Company). One thousand toothpicks were autoclaved at Rutgers University in 0.6 L of distilled water. All water was removed before the toothpicks were

separated into groups of approximately 100, sealed into 500-ml Fernbach flasks containing PDA broth, autoclaved at 121°C for 20 min, and allowed to cool at room temperature (Clements *et al.* 2003). All media, toothpicks and tools were carried to CCBS in a waterproof bag.

Pathogen isolation and inoculations were performed at CCBS. A seedling of *I. deltoidea* infected with *D. mutila* was collected using a sterile paper bag and transported to the field laboratory within one hour. Conidial mass from the infected leaf was transferred to a plate of PDA medium using a dissecting microscope. Single-spore isolates were obtained from the conidia by suspending conidia in sterile water and streaking onto PDA medium, after which separated colonies were transferred to a clean plate of PDA with chloramphenicol (Tuite 1969). The procedure was repeated 20 times. Twenty isolates were obtained and kept at ambient temperature (~26^{o} C) and a 12-hour light cycle inside a sterile plastic box (7.5 L) with 50 g of desiccant to control humidity. Conidial production was not observed on PDA, only abundant black mycelium growth. Toothpicks were infested with mycelia by placing them at the growing margins of 5-day old *D. mutila* cultures. The infested toothpicks were incubated at ~26° C for 9 days (Rodrigues da Silva & Juliatti, 2005). *Diplodia mutila* pycnidia and conidia production was recorded on the surface of toothpicks.

2. Inoculation Methods

In December 2006, during the wet season, seedlings of 70 species (36

families, Appendix 4) < 50 cm tall, were inoculated with *D. mutila*, seven replications (minimum) per species. Selection of these plant species was based on their commonness at CCBS (Pitman *et al.* 2001, Alvarez-Loayza, unpublished data, J. Terborgh pers. comm.). Seedling identification was based on morphological characters described in a previous study at CCBS (Alvarez-Loayza unpublished data). Six plant species were not identified to species level, but they were commonly recorded at CCBS (Alvarez-Loayza unpublished data). Four species of palms (*Socratea exorrhiza* (Mart.) H. Wendl., *Oenocarpus bataua* Mart., *Geonoma* sp. 1, and *Geonoma* sp. 2, Appendix 4) were included in the study to investigate whether *D. mutila* shows a phylogenetic signal in host preference. The selected leaf blades were labeled with a unique identification number with permanent marker on the upper surface. Additionally the petiole of the leaf was tagged with a numbered plastic bandette size 4 (National Band & Tag Co). Inoculations were performed in the field by penetrating one leaf blade with one infested toothpick (Young 1943). Most foliar pathogens in tropical forest require wounds for successful penetration (Gilbert & Webb 2007) and the toothpick inoculation method has been previously used with the same pathogen and host plant (Alvarez-Loayza *et al.* 2008a). Control plants were mock-treated with sterile toothpicks. All plants were evaluated for disease presence or absence five times after initial inoculation (after 2, 14, 21, 44 and 150 days). Diameter of necrotic foliar spots caused by *D. mutila* was measured (cm) in affected plants.

3. Field Transects

We established 10 transects (7 m x 1 m) at CCBS and five at LABS to locate leaf infections caused by *D. mutila* in other plant species. Transects were established at 400 m intervals along a selected trail. Two trails were selected at CCBS based on proximity to the field station. The trail at LABS was located in the floodplain and close to the field station. In each transect all saplings less than 1m, and seedlings were measured and identified to species level. Presence/absence of foliar disease symptoms was recorded for each plant. Leaf spots, tip burn, leaf deformation, sooty mold and/or chlorotic leaves were scored as foliar diseases. Leaves showing symptoms similar to the ones produced by *D. mutila* (necrotic circular spots with concentric masses of black pycnidia) were collected in paper bags, labeled and transported to the field laboratory within one hour. Conidia and pycnidia were isolated using the same procedure as above. In two transects at CCBS, we collected seeds of *Oxandra acuminata* Diels (Annonaceae) that presented masses of black pycnidia and conidia corresponding to *D. mutila*. These seeds were under fruiting trees of *O. acuminata*.

4. Isolates and cross inoculations

Cross-inoculations were performed to investigate the possibility that *D. mutila* isolated from other host plants were different strains or pathotypes than that occurring on *I. deltoidea*. Plant species found in the field to be infected with *D. mutila* were: *Astrocaryum murumuru* Mart seedlings and *Wendlandiella*

gracilis Dammer adult palms (Arecaceae), *Oxandra acuminata* seeds (Annonaceae) and saplings of *Piper reticulatum* L. (Piperaceae). *Diplodia mutila* cultures were obtained from conidia produced on each diseased plant. Isolation procedures were the same as the procedure described above. Inoculum was prepared from each of the four isolates using the toothpick technique described above. Using *D. mutila* isolated from each of the host species, three-month old *I. deltoidea* healthy seedlings were inoculated (five replications per isolate), and five seedlings were mock-treated with PDA treated toothpicks. Three two-month old *A. murumuru* seedlings were inoculated with isolates from *W. gracilis*, three with the isolate from *O. acuminata* seeds, three with the isolate from *P. reticulatum,* and three plants were mock-treated with PDA treated toothpicks as above.

Diplodia mutila **virulence on** ***Iriartea deltoidea*** **and five palm species**

Virulence of *D. mutila* was evaluated on *I. deltoidea* seedlings and saplings. Previous field observations suggested that *I. deltoidea* showed ontogenic resistance, defined as loss of susceptibility to a pathogen with maturity (Agrios 2005). Plants of *I. deltoidea* were classified in four age groups based on plant morphology and number of leaves (Rich *et al.* 1995, Terborgh & Davenport 2001). The first group consisted of recently germinated seedlings less than 25 cm with two or less round leaves. The second group included seedlings with more than three round leaves, > 25 cm and juvenile palms less than 1 m tall carrying the first compound leaf. The third group was formed by juvenile plants

more than 1 m and less than 3 m. The fourth group was formed by taller juveniles and adult plants. *Diplodia mutila* was inoculated with two toothpicks on a single leaf of *I. deltoidea* plants belonging to the first three age groups (9 plants per age stage) to evaluate virulence characteristics by age group and ontogenic resistance.

Virulence of *D. mutila* was evaluated in five palms known to be susceptible to *D. mutila* (see results) and two palms that did not develop foliar spots similar to those observed in *I. deltoidea*. Small seedlings (< 40 cm tall) of *Astrocaryum murumuru* Mart., *Oenocarpus bataua, Attalea butyracea* (Mutis ex L.f.) Wess Boer, *Geonoma* sp. 1 and adult plants of *Wendlandiella gracilis* were inoculated (seven plants per species and two toothpicks per leaf) in the field early January 2007-during the wet season- and early July 2007-during the dry season- using the same procedure described above. Each plant was tagged with numbered plastic bandettes size 7. These plants were evaluated every week for a period of 44 days, measuring diameter of foliar necrotic spot and pycnidia formation (number of pycnidia cm^{-2}). Plants inoculated in January 2007 were reevaluated in July 2007.

Diplodia mutila **virulence on** ***Iriartea deltoidea*** **at different light regimes**

Seven seedlings *of I. deltoidea* were inoculated with *D. mutila* at shaded conditions (understory) and seven seedlings were inoculated inside a forest clearing (natural tree fall gap). Necrotic spots were measured once a week for 6 weeks.

ANALYSIS

All analyses were done with JMP statistical software (version 5.1, SAS Institute Inc, Cary, NC). For significant effects, mean comparisons for palm species and *I. deltoidea* age groups were made with Tukey–Kramer HSD and Student's *t*-tests between light environments.

RESULTS

Host Specificity of *Diplodia mutila*

Of the 70 inoculated plant species, five palms species were highly susceptible to *D. mutila* and presented similar symptoms to those observed in *I. deltoidea*. Leaves of *A. murumuru, W. gracilis, Geonoma* sp. 1*, A. butyracea, E. precatoria* Mart. and *O. bataua* showed foliar necrotic spots similar to those seen in *I. deltoidea*. Infected plants of *W. gracilis* and *O. bataua* died after 31 to 72 days, showing necrotic leaves and black pycnidia with liquid masses of conidia. *D. mutila* was re-isolated from infected seedlings. The palms *S. exorrhiza* and *Geonoma* sp. 2 did not show disease symptoms similar to those seen in *I. deltoidea* but presented necrotic scars of 2 to 5 mm width. Sixty-two percent of the plant species did not show any visible reactions (all plants listed in Appendix 4). Thirty-eight percent of plant species had various atypical responses to the inoculation (Table 2). Small necrotic lesions (<5 mm width) around the inoculation point were observed in 20 plant species from different families. Partial defoliation was observed in *Celtis schippii* Standl. (Ulmaceae). Necrosis of inoculated leaves was recorded after a year for *Paullinia obovata* (Ruiz & Pav.) Pers. (Sapindaceae), and *Pourouma cecropiifolia* Mart. (Cecropiaceae). We did not observe pycnidia and conidia production in the dicotyledonous plants mentioned above. A strong defense mechanism was recorded for *Otoba parvifolia* (Markgr.) A.H. Gentry (Myristicaceae), which formed a necrotic lesion (1 cm diameter) around the inoculation point. After several days the plant

eliminated the lesion and the leaf remained healthy. A similar defense mechanism was observed in one seedling of *Virola mollissima* (Poepp. ex A. DC.) Warb. (Myristicaceae). All control seedlings remained healthy.

Diplodia mutila was recorded in 14% of *A. murumuru* evaluated seedlings in five transects at CCBS and two transects at LABS (Fig. 15). The pathogen was also isolated from 25% of the surveyed population of *W. gracilis* adult palms located in three transects at CCBS (Fig. 15) and from 8% of *P. reticulatum* sampled plants (Fig. 16) in one transect also at CCBS. Finally, we recorded 34% of *O. acuminata* seeds infected with *D. mutila* in two transects located at CCBS (Fig. 16, Table 3). The four isolates of *D. mutila* from *O. acuminata*, *W. gracilis*, *P. reticulatum* and *A. murumuru* infected the 20 inoculated *I. deltoidea* seedlings. The inoculated seedlings had the same symptoms and infection period reported for the first isolate of *D. mutila*-obtained from *I. deltoidea*. Similar symptoms and infection periods were observed for the *A. murumuru* inoculated seedlings.

Diplodia mutila **virulence on** ***Iriartea deltoidea,*** **five palm species and the effects of light availability**

The palm *I. deltoidea* presented an ontogenic resistance mechanism. Leaf lesions of young seedlings had an average diametric growth rate of 0.63 ± 0.03 cm day^{-1} and died 15 to 42 days after inoculation in the field. Greenhouse inoculations produced mortality after 5 to 16 days (Alvarez-Loayza *et al.* 2008a). Plants of the second age group (>2 leaves, >25 cm and < 1m size) expressed *D. mutila* symptoms in the inoculated leaf but remained alive after six months. The

inoculated spot increased in diameter (0.16 ± 0.02 cm day^{-1}) but the necrotic lesion was completely eliminated from the leaves. Plants belonging to the third age group (>1m and <3m) developed the characteristic necrotic spot, and the diameter growth rate was also slower (0. 16 ± 0.02 cm day^{-1}) than younger plants (0.63 cm day^{-1}) (Tukey-Kramer, $F_{3,27} = 88.37$, $P = 0.0001$*). After a year the plants in the third group eliminated *D. mutila* necrotic lesion or the complete inoculated leaflet (Fig. 17).

The five palm species susceptible to *D. mutila* showed a virulence range, from high to very low virulence. *Oenocarpus bataua* and *W. gracilis* presented faster diametric necrotic lesion growth rate (0.18 ± 0.024 cm day^{-1}), abundant pycnidia growth (~12 cm^{-2}) and, seedling and adult mortality after 44 days. *Astrocaryum murumuru* developed a small diametric necrotic lesion growth rate (0.1 ± 0.024 cm day^{-1}) and lesions developed few pycnidia (~4 cm^{-2}). The palm *Geonoma* sp. 1 had also a small foliar lesion growth rate (0.06 ± 0.024 cm day^{-1}). The pathogen was less virulent for *A. butyracea* (0.05 ± 0.024 cm day^{-1}). The size of the lesion was relatively small and two of the inoculated plants remained healthy (Tukey-Kramer, $F_{5,34} = 6.308$, $P = 0.0009$*, Fig. 18).

Seedlings of *I. deltoidea* inoculated inside the forest clearing had the highest growth rate of foliar lesions (0.86 ± 0.06 cm day^{-1}) compared to the 0.48 ± 0.05 cm day^{-1} growth rate of foliar lesions of plants inoculated in shaded conditions (Student *t-test* = 4.59, DF = 10, $P > 0.0005$, Fig. 19). Inoculated seedlings in the forest clearing died after ~29 days and seedlings inoculated inside shaded conditions died after ~42 days.

DISCUSSION

Diplodia mutila is pathogenic and virulent on a range of dicotyledonous and monocotyledon species. The four isolates of *D. mutila* obtained from four different plant species produced the same symptoms on *I. deltoidea*, suggesting that these four isolates have similar pathogenic effects and may be part of the same genetic population of the pathogen. *Diplodia mutila* exhibited a preferred host range on palms (Arecaceae). A similar pattern of host recurrent infection was recorded for another ubiquitous and generalist fungus, *Aspergillus flavus*, isolated from seeds of *Unonopsis matthewsii, Oxandra acuminata, Pseudomalmea diclina* (Annonaceae) (Alvarez-Loayza *et al.* 2008b, Appendix 5). The infection produced by *D. mutila* on members of the Arecaceae family and *A. flavus* on members of the Annonaceae family is consistent with the Gilbert and Webb hypothesis that the phylogenetic signal in host range predicts the host ranges of plant pathogens in a local plant community (Gilbert & Webb 2007).

The greater impact and virulence of *D. mutila* on palms indicates some support for the host-specificity component of the Janzen–Connell hypothesis. However, *D. mutila* is distributed worldwide (Sutton 1980), and it was recorded here on members of different families (Piperaceae and Annonaceae). *Aspergillus flavus* was recorded on members of the Myrtaceae and Lecythidaceae families, and it is a generalist pathogen and endophyte (Alvarez-Loayza unpublished data, St. Leger *et al.* 2000, Arrus *et al.* 2005). Based on these results and previous studies (Gilbert & Webb 2007), we suggest that generalist pathogens, with wider host ranges and with the potential to survive on

non-suitable hosts and as endophytic fungi could be more abundant. Generalist pathogens affecting animals and humans have evolutionary advantages over host specialist pathogens such as high levels of genetic diversity and abundant opportunities for cross-species transmission (Woolhouse *et al.* 2001). This mechanism could be operating also for pathogens affecting natural plant communities.

Diplodia mutila was isolated from healthy adult leaves, fallen fronds, fruits and seeds of *I. deltoidea*, indicating that this fungus could be a common endophyte for the palm (Alvarez-Loayza *et al.* 2008b, P. Alvarez-Loayza unpublished data) and could become pathogenic or remain as an endophyte. The endophytic nature of *D. mutila* has also been recorded for other plant species (Ragazzi *et al.* 1999). The role of endophytic fungi needs to be considered when evaluating plant diseases. Several studies indicate that endophytic fungi colonizing tropical plants are very diverse and common (Arnold & Lutzoni 2007). Endophytic fungi colonize their host plants without causing visible symptoms (Petrini 1991). When the endophytes colonize a host and the host tissue is apparently healthy, the relationship between the endophytic fungus and its host may range from latent pathogenesis to mutualistic symbiosis (Arnold *et al.* 2003, Schulz & Boyle 2005, Van Bael *et al.* 2005). It is not clear what are the implications of endophytism of plant pathogens but it is known that endophytic fungi enhance plant resistance to other diseases (Arnold *et al.* 2003) and insect herbivory (Arnold & Lewis 2005). Is there an advantage for the plant to host a fungus that is a potentially lethal pathogen for its seedlings and an

endophyte of adult plants? Defensive mutualism has been reported for grasses and endophytic fungi, where a major effect of the fungi is defense of host plants against herbivory (Clay 1988). *Diplodia mutila* could be acting as a defensive mutualist for *I. deltoidea,* protecting plants from insect and animal herbivores that are lethal for the plant. Additionally, endophytes, by altering competitive hierarchies and diversity within plant communities could alter the relationship between diversity and ecosystems properties (Rudgers & Clay 2005). The abundance of *I. deltoidea* throughout tropical forests could be explained by the presence of endophytic fungi such as *D. mutila* and defensive mutualism interactions.

Diplodia mutila virulence decreased in older *I. deltoidea* plants, indicating that the plant has an ontogenic resistance mechanism. We also found that various plant species outside the Arecaceae family show some disease symptoms in response to *D. mutila* inoculation but their responses vary in severity, ranging from hypersensitivity responses (necrosis of the tissue surrounding lesion) to leaf mortality. *Diplodia mutila* virulence on *I. deltoidea* increases with light availability, confirming that foliar diseases in juvenile palms are more severe when the palms are grown in full sun rather than with some shade (Elliot et al. 2004). Additionally, soil conditions, biotic and abiotic factors, also affect plant susceptibility to *D. mutila*. Seedlings growing in commercial potting soils are susceptible to several diseases and few or none of them survive (Alvarez-Loayza unpublished data, Noblick pers. comm.). We hypothesize that *D. mutila* growing in rich substrates (e.g. PDA, senescent leaves) and favorable conditions (e.g.

young seedlings, susceptible hosts, full illumination, poor soils) should be pathogenic, producing a wide range of toxic metabolites for the plant. *Diplodia mutila* as an endophyte should be "dormant" and since the host plant does not offer favorable conditions there is no o little production of toxic metabolites and/or the plant activates defense mechanisms against the pathogen.

These combined results indicate that plant pathogen effects in undisturbed tropical are extremely complex. In order to evaluate the impacts of plant pathogens and endophytes in tropical ecosystems it is necessary to understand the disease triangle concept and evaluate pathogen virulence, host susceptibility and environmental conditions. Plant diseases will not exist if one of the components is missing. *Iriartea deltoidea* seedlings could be severely affected by *D. mutila* but environmental conditions (i.e. light) and plant defense mechanisms (e.g. ontogenic defense mechanism) can enhance or override pathogen effects. Additionally, plant pathogens and endophytes could be triggering other mechanisms, such as defensive mutualism, enhancing plant recruitment of certain species. Several studies suggest that susceptibility and resistance mechanisms to generalist pathogens, plant chemical defenses and the presence of endophytes are important for plant communities (Okuda *et al.* 1997, Sanchez–Hidalgo *et al.* 1999, Arnold *et al.* 2005, Herre *et al.* 2005, Rudgers & Clay 2005, Herre *et al.* 2007, Murali *et al.* 2007). We suggest that all these mechanisms influenced by environmental variables could explain patterns of commonness and rarity of plant species in the tropics.

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Table 2. Responses of 29 woody plant species and one fern to inoculation by *Diplodia mutila* (n = 7 for all species).

Family	Species	Partial defoliation	Resistance	Necrosis, hypersensitivity	No symptom, only injury	*Diplodia mutila* symptom
Arecaceae	*Astrocaryum murumuru*					100%
Arecaceae	*Attalea butyracea*			15%	29%	56%
Arecaceae	*Euterpe precatoria*			100%		
Arecaceae	*Geonoma* sp. 1				14%	86%
Arecaceae	*Oenocarpus bataua*					100%
Arecaceae	*Socratea exorrhiza*			100%		
Arecaceae	*Wendlandiella gracilis*					100%
Burseraceae	*Protium tenuifolium*			100%		
Cecropiaceae	*Pourouma cecropiifolia*	100%				
Clusiaceae	*Garcinia brasiliensis*			100%		
Cyclanthaceae	*Carludovica palmata*			100%		
Fabaceae	*Mucuna* sp. 1			100%		
Dryoteridaceae	*Tectaria incisa*			43%	57%	
Icacinaceae	*Calatola venezuelana*			86%	14%	
Lauraceae	*Caryodaphnopsis fosteri*			100%		
Lauraceae	*Ocotea oblonga*			100%		
Moraceae	*Ficus maxima*			100%		
Moraceae	*Pseudolmedia laevis*			85%	14%	
Myristicaceae	*Iryanthera olacoides*			100%		
Myristicaceae	*Otoba parvifolia*		100%			
Myristicaceae	*Virola calophylla*			100%		
Myristicaceae	*Virola flexuosa*			100%		
Myristicaceae	*Virola mollissima*	14%			84%	
Myrtaceae	*Eugenia punicifolia*			100%		
Sapindaceae	*Paullinia obovata*	43%		57%		
Sterculiaceae	*Theobroma cacao*			100%		
Ulmaceae	*Celtis schippii*	57%			43%	

Table 3. Plant species infected by *Diplodia mutila* in natural undisturbed tropical forest.

Host Plant	# of individuals	Plant Part Affected	# infected by *Diplodia mutila*	# of Locations	Season
Oxandra acuminata	33	Seed	12	2	Wet
Piper reticulatum	24	Leaf-Sapling	2	1	Dry
Astrocaryum murumuru	103	Leaf-Seedling	14	7	Dry/Wet
Wendlandiella gracilis	21	Leaf-Adult	8	3	Dry/Wet

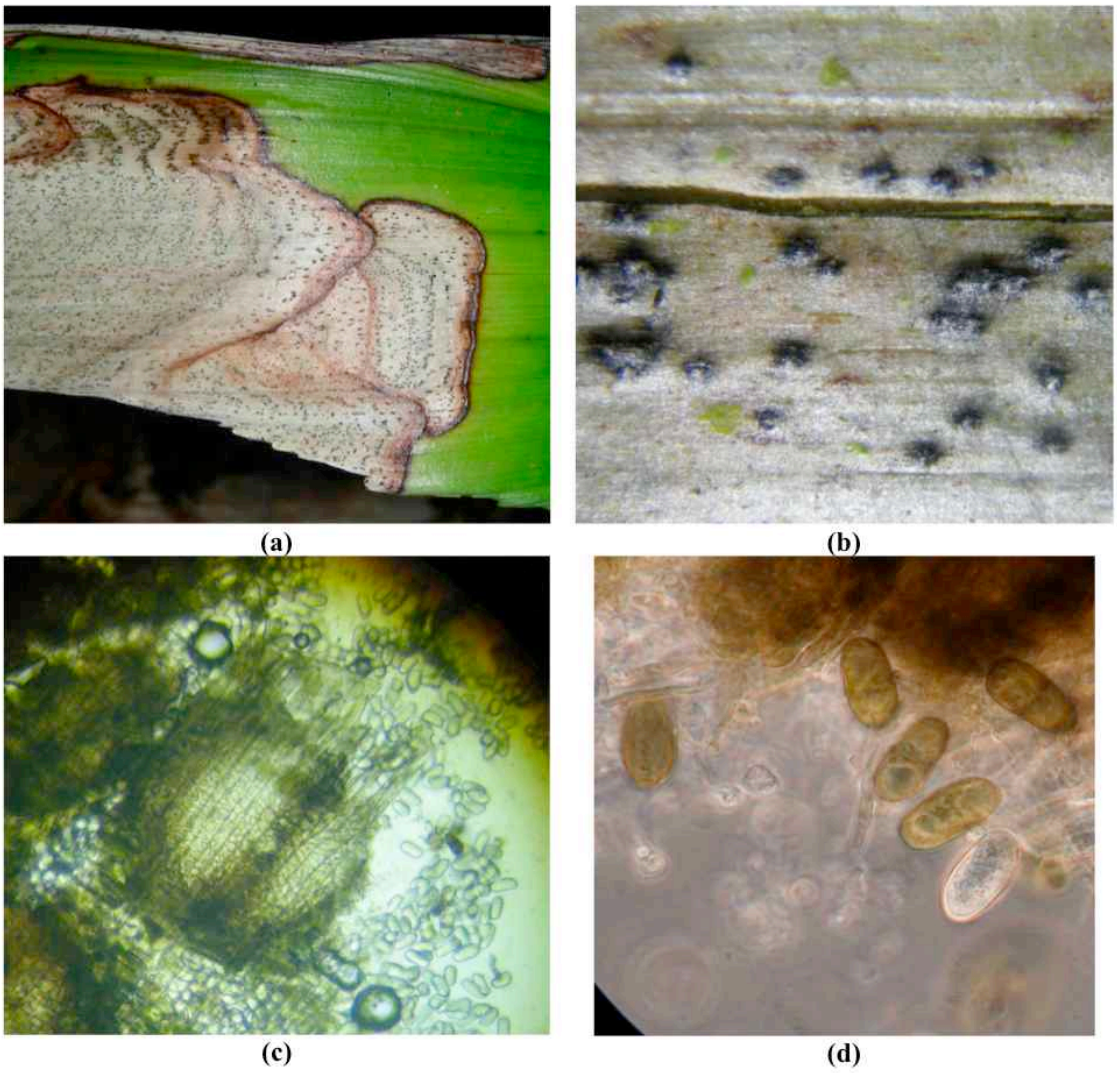

Figure 14. *Diplodia mutila* recorded in seedlings of *Iriartea deltoidea* seedlings, (a) Leaf lesion (b) Pustules observed under a stereoscope (c) Pustules immersed in leaf tissue observed in a compound microscope (d) Mature and immature conidia.

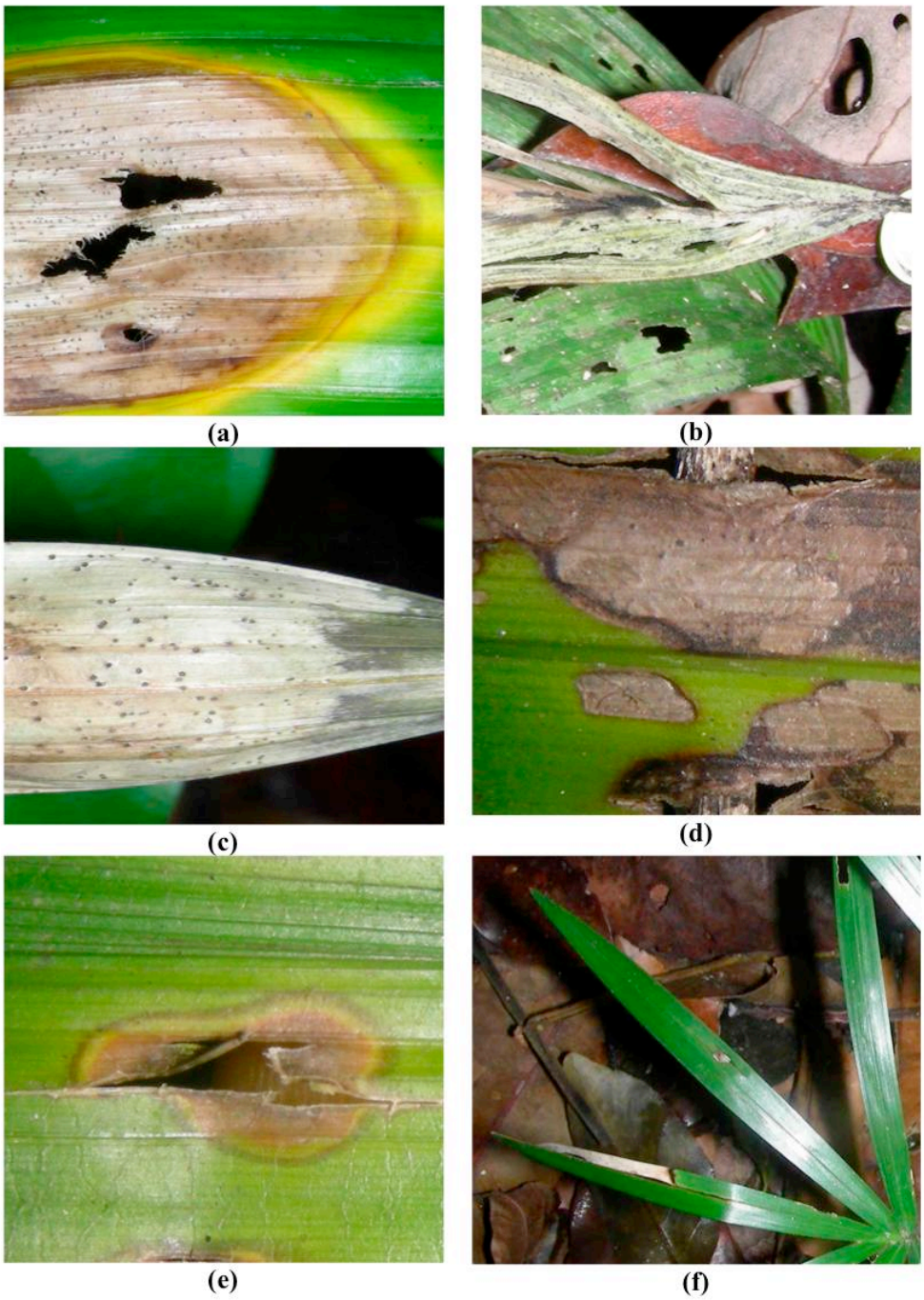

Figure 15. Hosts of *Diplodia mutila* in the palm family (Arecaceae), (a) *Astrocaryum murumuru*, (b) *Wendlandiella gracilis,* (c) *Oenocarpus bataua,* (d) *Geonoma* sp., (e) *Attalea butyraceae,* (f) *Euterpe precatoria.*

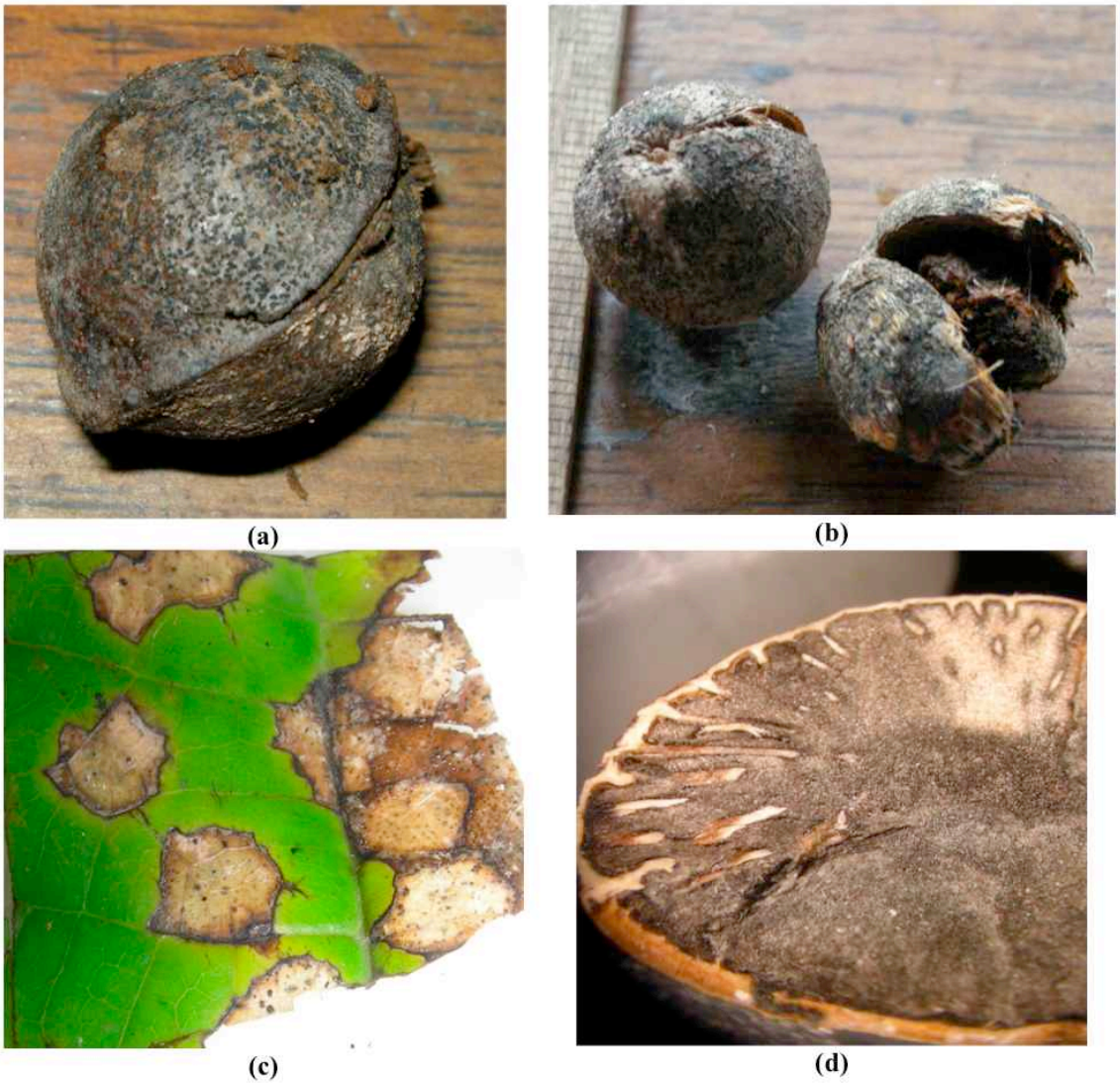

Figure 16. Dicotyledon plants infected with *Diplodia mutila* (a) *Virola calophylla* fruits, (b) *Quararibea wittii* fruits, (c) *Piper* sp. presenting foliar necrosis, with the presence of black pycnidia and production of masses of black conidia (d) *Oxandra acuminata* seeds had black pycnidia over the seed surface and black mycelia in the seed endosperm.

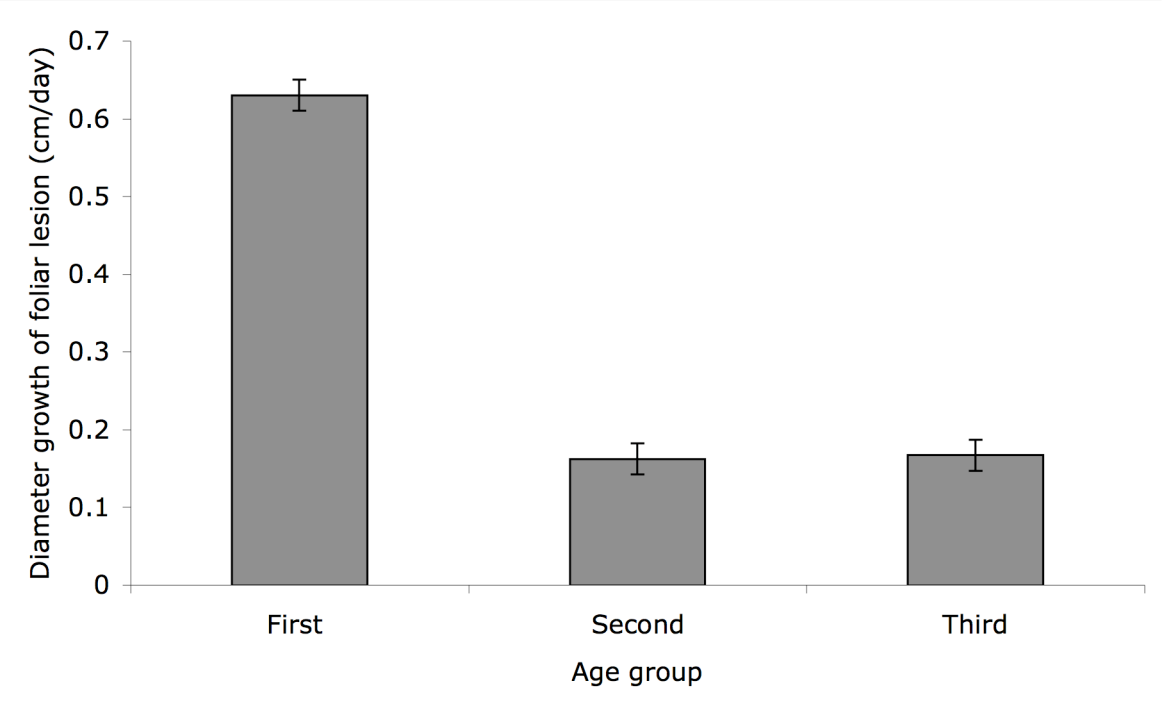

Figure 17. Susceptibility of *Iriartea deltoidea* plants at different age stages to *Diplodia mutila*. Plants of the first age group had the highest diameter growth rate of foliar spots (0.63 ± 0.03 cm/day) and 100% of mortality. Plants of the second age group (>2 leaves, >25 cm and < 1m size) and third age group (>1m and <3m) had lower growth rates of *D. mutila* foliar spots (0.16 ± 0.02 cm/day) (Tukey Kramer, $F_{3,27} = 88.37$, $P = 0.0001$*). After 120 days surviving plants remained healthy, the size of the necrotic spot was constant and in some cases lesions disappeared.

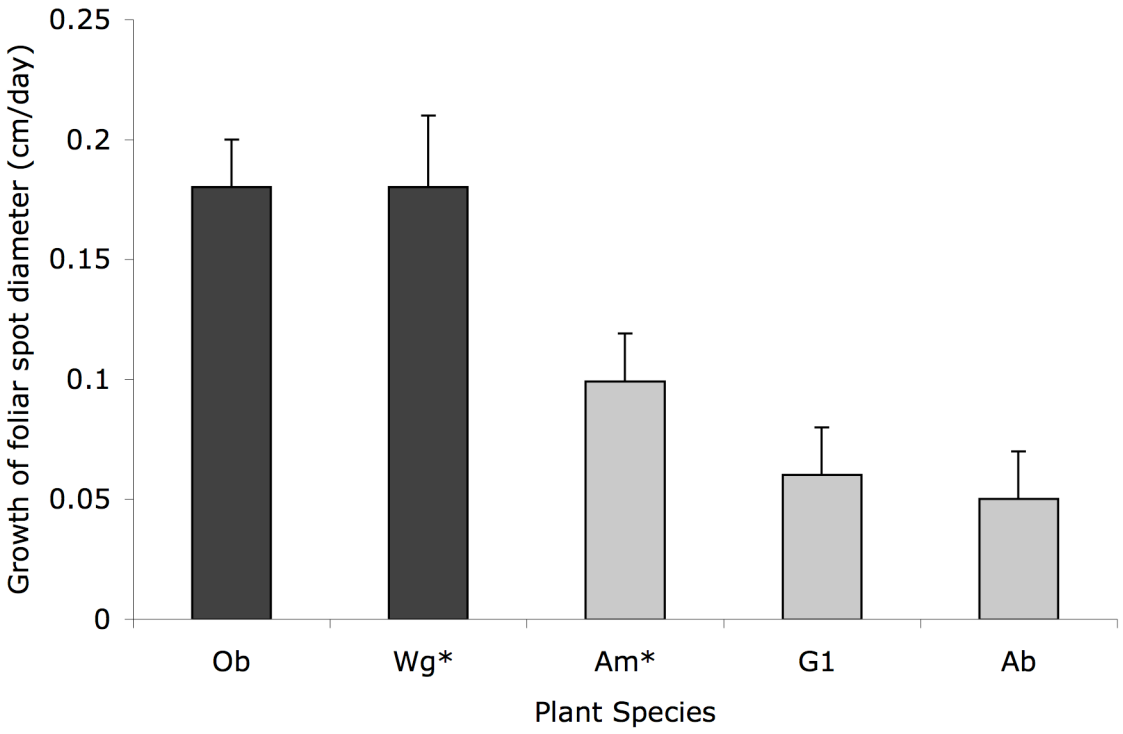

Figure 18. Susceptibility of five plant species to *Diplodia mutila* infection. *Astrocaryum murumuru* (Am), *Geonoma* sp. 1 (G1) and *Attalea butyracea* (Ab) develop necrotic lesions but did not experience mortality. *Oenocarpus bataua* (Ob) and *Wendlandiella gracilis* (Wg) (dark columns) develop necrotic lesions faster and died after ~31 days of inoculation. The size of the lesion was relatively small and two of the inoculated plants remained healthy (Tukey-Kramer, $F_{5,34}$ = 6.308, P = 0.0009*).
* Plant species infected with *D. mutila* in natural conditions.

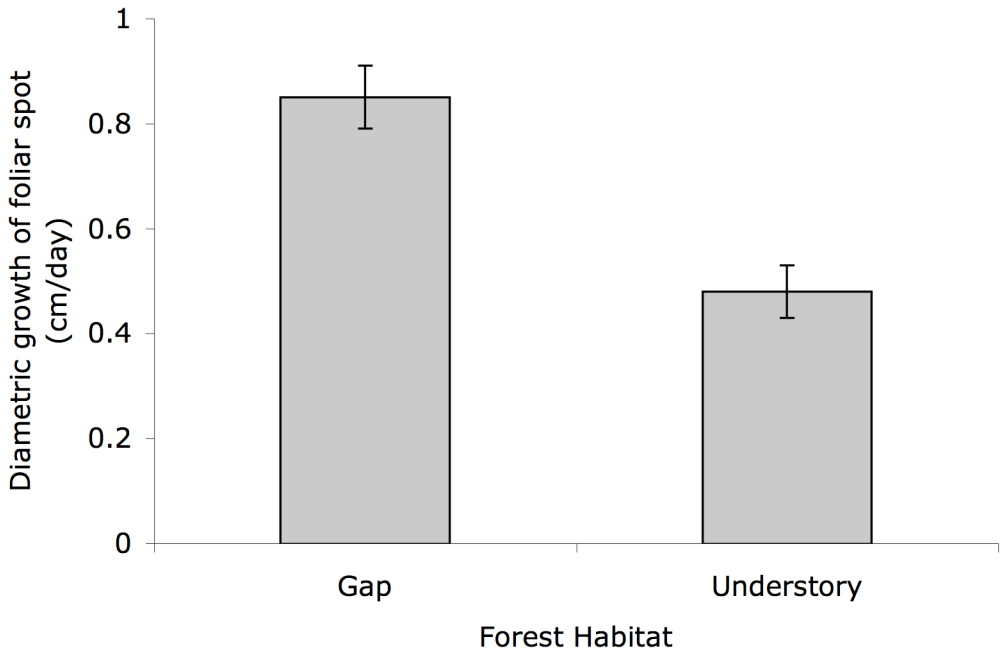

Figure 19. Seedlings of *I. deltoidea* inoculated inside a tree fall gap (forest clearings) had the highest growth rate of foliar lesions (0.86 ± 0.06 cm day^{-1}) compared to the 0.48 ± 0.05 cm day^{-1} growth rate of foliar lesions of plants inoculated in shaded conditions (Student *t-test* = 4.59, DF = 10, *P* > 0.0005).

Chapter 3

LIGHT CONVERTS ENDOSYMBIOTIC FUNGUS TO PATHOGEN, INFLUENCING SEEDLING SURVIVAL AND HOST TREE RECRUITMENT OF TROPICAL PALM IN NATURAL ECOSYSTEMS

ABSTRACT

Tropical plants are colonized by a wide diversity of endophytic fungi. Previous studies have focused on abundance, diversity and have shown that under conditions of stress endophytic fungi become pathogenic. Additionally, other studies show that endophytes alter competitive abilities of host individuals and improve host fitness under abiotic or biotic stress**.** Here we evaluate how a widespread endophytic fungal pathogen infecting a common tropical palm influences plant recruitment and survival in natural ecosystems. The palm, *Iriartea deltoidea,* dominates many wet lowland Neotropical forests. *Diplodia mutila* is a common asymptomatic endophyte in mature plants, however disease and mortality may be expressed in some seedlings, while other seedlings remain disease free. We show that seedlings bearing the endophyte show enhanced resistance to insect herbivory. We investigated the effects of light availability on *D. mutila* disease expression. High light availability triggers pathogenicity and low light favours endosymbiotic development, constraining recruitment of endophyte-infested seedlings to the shaded understory by limiting survival of seedlings in direct light. These results suggest that patterns of plant abundance and the mechanisms maintaining tropical forest biodiversity are the result of a

more complex interplay between abiotic and biotic environments than previously thought. This is the first study that demonstrates that plant endophytes respond to abiotic factors to influence plant distribution in natural ecosystems.

INTRODUCTION

Endophytic fungi that asymptomatically colonize plants (Petrini 1986) are diverse and abundant in tropical ecosystems (Arnold et al. 2000). It has been hypothesized that there are no neutral endophyte-host interactions, but rather involve a balance of antagonism. These organisms can be pathogenic and/or mutualistic (Schulz and Boyle 2005). The variable virulence of the endophyte, the host defence response, and environmental conditions, i.e. the disease triangle (Agrios 2005), could be influencing the endophyte-pathogen continuum inside the host plant. Suboptimal environmental conditions may stress the host defence status, resulting in disease (Kuldau and Yates 2000). Several studies have shown that under conditions of stress, inoculation of endophytes onto plant tissues (Schulz et al. 1998) resulted in disease symptoms (necroses, chlorosis) and (or) growth inhibition of the host with most of the isolates. Additionally, other studies have focused on evaluating how endophytes alter competitive abilities of host individuals and improve host fitness under abiotic or biotic stresses (Arnold et al. 2003, Arnold and Engelbrecht 2007). However, none have looked for or identified environmental factors that alter the behavior of endophytes in natural ecosystems, their relationships to hosts and ecological implications for plant distribution.

Diplodia mutila is a symbiotic endophyte/plant pathogenic fungus infecting the palm *Iriartea deltoidea* (Álvarez-Loayza et al. 2008), which dominates many wet lowland Neotropical forests. The fungus is an asymptomatic endophyte in

mature plants, and disease and mortality are expressed in some seedlings, while others remain disease free. Here we show that seedlings bearing the endophyte show enhanced resistance to insect herbivory. However, high light availability triggers pathogenicity of the fungus, while low light favours endosymbiotic development, constraining recruitment of endophyte-infested seedlings to the shaded understory by limiting survival of seedlings in direct light. These results provide evidence that patterns of plant abundance and the mechanisms maintaining tropical forest biodiversity are the result of a more complex interplay between abiotic and biotic environments than previously thought.

The palm *Iriartea deltoidea* is one of the most dominant tree species in wet lowland and premontane tropical forests of western Amazonia (Pitman et al. 2001, Valencia et al. 2004, Macía and Svenning 2005) and the Chocó- and Central American region (Wattenberg and Breckle 1995, Clark et al. 1999). In contrast to most large palms (Svenning 2001), this species does not depend on large forest gaps for recruitment (Svenning 1999), perhaps related to its peculiar growth strategy (Terborgh and Davenport 2001). However, the inordinate success of *I. deltoidea* in wet New World tropical forests remains an enigma and cannot be explained by morphological attributes such as fruit size or height (Pitman et al. 2001). A partial explanation may be found in the fact that palms have tougher leaves than dicots and thus are less susceptible to insect herbivory (Grubb et al. 2008). In this study we investigate the influence of a common pathogen-endophytic fungus, *Diplodia mutila*, on *I. deltoidea* survival and recruitment. *Diplodia mutila* (Sutton 1980) may be both an asymptomatic

endophyte and a pathogen of *I. deltoidea*, causing mortality in young seedlings after 5 to 16 days of infection and producing foliar spots in adult plants (Álvarez-Loayza et al. 2008) (Fig. 14). In the pathogenic phase, *D. mutila* forms pycnidia, flask-shaped asexual structures that exude masses of uni-cellular to bi-cellular, hyaline to brown conidia (Crous et al. 2006) (Fig. 14). In its endophytic phase the fungus exists only as mycelium within tissues of the host's leaves, stems and seeds (Petrini 1986). *Diplodia mutila* and related species have been reported as endophytes or latent pathogens for several plant species worldwide (Damm et al. 2007, Slippers and Wingfield 2007). This fungus is frequently an asymptomatic endophyte in leaves of healthy juvenile and mature plants, as well as fruits and seeds of *I. deltoidea* (Álvarez-Loayza et al. 2008).

It has been suggested that "a species' abundance at local and large scales may be a simple function of its ability to recruit in close proximity with conspecific adults" (Pitman et al. 2001). Iriartea deltoidea seedlings and juveniles are relatively abundant in proximity to adult trees. Demographic censuses of 518 young I. deltoidea seedlings in 10 plots conducted in 150 days show that distribution of D. mutila, infected seedlings, was not consistent with the Janzen and Connell model of plant infection (Janzen 1970, Connell 1971). The proportion of plants affected by D. mutila was similar near and far from *I. deltoidea* adult plants, ~10% ± 0.05%, $P > 0.3$ (mean ± SE). The proportion of seedlings affected by stem borers within the first 2.5 m was significantly higher near *I. deltoidea* adult plants, 8% ± 0.01% versus 3% ± 0.01%, $P > 0.045$*(mean ± SE). However the proportion of surviving healthy seedlings (no foliar diseases

or insect attack) did not vary significantly with distance from adult palms, ~15% ± 0.05%, $P > 0.9$ (mean ± SE).

MATERIALS AND METHODS

Demographic Censuses

In northeastern Peru (Normand et al. 2006) we arbitrarily placed 102 transects (5 x 500 m, divided in 5 x 5 m subunits) located in mature primary tropical rain forest within 300 km of Iquitos, Peru (excluding transects located in secondary forests, white sand soils, steep topographical conditions and human disturbed forests). Sites in southeastern Peru were located at Cocha Cashu, (CCBS) (Terborgh 1983) and Los Amigos, (LABS) (Pitman 2007). Ten plots were established in May 2007, in primary floodplain forest, with similar floristic composition and topographic characteristics. Five of plots were located at CCBS and five at LABS. Nine plots measured 900 m^2 and one plot at CCBS measured 2.25 ha. In each plot all *I. deltoidea* plants were tagged with numbered plastic tags and mapped in an X - Y coordinate system. The total number of plants located in the 10 plots was 1068: 63 fruiting adults, 518 seedlings and 487 were considered juveniles-adults (non-fruiting) (Appendix 6). We measured height of the tallest photosynthetic leaf (cm) and number of leaves and diameter of foliar spots caused by *D. mutila* (cm) for all seedlings. The amount of disease was calculated by dividing the diameter of the foliar spot by the diameter of the affected leaf and expressed as '% disease'. Disease development over a period of 150 days was calculated by subtracting the % disease in the initial estimate from the final estimate (Fig. 20).

Distribution of seedlings affected by stem borers and *D. mutila*

Plants damaged and killed by epicotyl borers, such as caterpillars, beetle larvae or crickets, were considered as “damaged by stem borers” (Appendix 6). Plants located in the southeastern Peru plots were monitored for presence/absence of *D. mutila* and stem borers, three times after initial establishment (7, 50 and 150 days). In each plot, the minimum distances, from all seedlings to the nearest *I. deltoidea* fruiting plant were computed using the coordinates of the labelled plant under consideration and the coordinates of the nearest fruiting tree within the plot. We surveyed seedlings in 5 concentric 2.5 m annuli centred on a focal fruiting tree. The number of seedlings affected by stem borers and *D. mutila* was tallied for each 2.5 m annulus and then divided by the total number of plants located in the selected annulus to yield proportions. One-way ANOVA was used to compare diseases and mortality proportions among plots for each distance annulus (Tukey's HSD used to contrast means). The proportion of plants affected by stem borers within the first 2.5 m was significantly higher than proportions in the other 4 annuli, in the first census, after ~7 days, 8% ± 0.01%, 6% ± 0.01%, 4% ± 0.01%, 3% ± 0.01%, 3% ± 0.01%, ($F_{4,50}$ = 2.65, P > 0.045*) and the second census, after ~50 days, 10% ± 0.01% , 4% ± 0.01%, 1% ± 0.01%, 2% ± 0.01%, 0.7% ± 0.01% ($F_{4,50}$ = 4.28, P > 0.0051*). Stem borer attack decreased in the last census.

Light availability measurement.

In northeastern Peru light availability was measured using the canopy scope methodology (Brown et al. 2000). In southeastern Peru light availability was estimated above the tallest photosynthetic leaf of each *I. deltoidea* seedling, using the average value of light intensity over the leaf with a light meter (Environmental Concepts Plant Light Intensity Meters, LIM2500, USA). The average value was obtained from three measurements over each plant at 6 am, 12 pm and 5 pm for three consecutive days. The total number of seedlings in the northeastern Peru transects was 660, 94% of seedlings were located at understory conditions (canopy scope <5). The negative correlation between the number of *I. deltoidea* seedlings and canopy openness in the 102 transects (Spearman r = -0.117, $P < 0.05$) was estimated using, n = 280 5-m × 5-m subplots with at least one *I. deltoidea* seedling. Statistical significance was assessed as a one-tailed test and correcting for spatial autocorrelation using Dutilleul's approach for computing the geographically effective degrees of freedom = 227 (Rangel et al. 2006). The total number of seedlings in the southeastern Peru plots was 518 seedlings (less than 25 cm), 91% seedlings were located in dense understory (~55-120 ± 15 μmol m^{-2} s^{-1}).

***Diplodia mutila*–mediated insect protection in *I. deltoidea*.**

On December 2007, ~370 *Coccotrypes* sp. beetles and larvae were extracted from more than 100 fruits and seeds of *I. deltoidea*. In a Petri plate (60 x 15 mm, Fisher Scientific Co. Canada) we placed two 1-cm^2 of PDA (Potato

Dextrose Agar) and two 1-cm^2 of PDA infested with *D. mutila*, covered with squares of non-acidic paper to simulate dark conditions found inside seeds and fruits. PDA was replaced everyday for the duration of the experiment to avoid contamination of non-infested PDA by *D. mutila*. We set up 12 repetitions following this procedure. Six to ten beetles were released in the each Petri plate and monitored daily for 8 to 12 days. Beetles consistently preferred PDA (Appendix 6) and avoided *D. mutila* infested PDA, 4.8 ± 0.14 versus 1.4 ± 0.14, (Repeated Measurement Analysis, Random Effect $F_{4, 456}$ = 160.13, P = 0.0001**). Similar results were also obtained when the experiment was performed using *Coccotrypes* sp. adults and *I. deltoidea* fruits instead of PDA (Appendix 6).

Transplant experiments

We transplanted 30 *I. deltoidea* seedlings from one plot at Cocha Cashu where adults, juveniles, seeds and fruits were colonized by *D. mutila*. Ten seedlings were transplanted to shade conditions, ~55 ± 15 µmol m^{-2} s^{-1}, 10 to a reduced light environment in a greenhouse, ~491 ± 34 µmol m^{-2} s^{-1}, and 10 to full sun exposure, ~1058 ± 23 µmol m^{-2} s^{-1}. All seedlings had 2 leaves and did not have any visible disease symptoms produced by *D. mutila* or any other foliar spot. Light availability was measured three times a day (6 am, 12 pm and 5 pm) for a period of 10 days and all disease symptoms and insect damage were recorded and measured daily. The average daily temperature in the understory and full sun conditions was 23^{o} C ± 3 and 26 ^{o} C ± 5 in the greenhouse.

Laboratory Assays

To assess the effect of light on the fungus, laboratory observations were made on mycelial growth in Water Agar (WA) and Potato Dextrose Agar (PDA). Two photoperiod treatments were employed for five days with six *D. mutila* samples per treatment. The first treatment consisted of 12-hour cycle of darkness and 12-hour cycle of white light (fluorescent, 100 ± 10 μmol m^{-2} s^{-1}), while the second consisted of 21-hour cycle of darkness and 3-hour cycle of light for five days, (6 repetitions per treatment, constant temperature for all treatments).

RESULTS

Diplodia mutila benefits its host plants by enhancing resistance to insect herbivory

Field surveys in the 10 surveyed plots, showed that insect herbivory (stem borers: order Coleoptera) decreased with increasing incidence of *D. mutila* infection ($F_{1, 10} = 18.49$, $P = 0.0026$, $r^2 = 0.69$). Plots with few *D. mutila* infested *I. deltoidea* plants had higher incidence of stem borer mortality, whereas plots with higher incidence of plants colonized by *D. mutila* had lower rates of stem borer-induced mortality. Additional feeding experiments employing *I. deltoidea* fruits and PDA media (Potato Dextrose Agar) colonized by *D. mutila* showed that adults of the beetle *Coccotrypes* sp., and two unidentified species of larvae of the order Coleoptera avoided consumption of fruits and PDA colonized by *D. mutila*. The resistance to insect predators such as stem and seed borers conferred by *D. mutila* may allow *I. deltoidea* to escape the generally high intraspecific density- and distance-dependent mortality and recruit near adult trees (Peters 2003, Queenborough et al. 2007).

High light availability increases plant disease development

This study found that *Diplodia mutila* is beneficial to the plant in understory conditions but strongly reduces the capacity of *I. deltoidea* to recruit in high-light forest gaps. Seedlings of *I. deltoidea* preferentially occur under shady conditions. Extensive sampling at two sites in western Amazonia found out that approximately ~92% of *I. deltoidea* seedlings were found in understory

conditions. Additionally, the foliar necrotic spot symptoms produced by *D. mutila* appeared more frequently in seedlings and juveniles that grew in gaps or diffusely open canopy conditions. Plants with visible symptoms caused by *D. mutila* received significantly higher illumination, 408.3 ± 17.3, than plants with no visible symptoms, 208.2 ± 6.1, $P < 0.0001$, *t* test, n = 808 (mean μmol m^{-2} s^{-1} ± SE). Disease development was faster and more lethal in seedlings with two leaves or less when exposed to higher light conditions ($F_{1,22} = 55.4$, $P = 0.0001$, $r^2 = 0.73$), (Fig. 21). Ontogenic or age-related resistance may be responsible for differences in disease expression between seedlings in different stages of development (Panter and Jones 2002). An additional experiment showed that pathogenicity of *D. mutila* increased with light availability. We inoculated 22 healthy 6-month old *I. deltoidea* seedlings (no foliar spots or insect marks) with *D. mutila*, following inoculation procedures from previous studies (Álvarez-Loayza et al. 2008). Foliar spots produced by *D. mutila* had a higher growth rate and mortality was greater and faster at higher light availability ($F_{1,22} = 93.26$, $P = 0.0001$, $R^2 = 0.816$) (Fig. 22).

Using transplant experiments we demonstrated that increased light availability switched the endosymbiotic phase of the fungus to its pathogenic phase. Diametric growth rate of foliar spots produced by *D. mutila* was higher and faster in full sun conditions, 19.5 ± 2.5 cm/day, than in reduced light, 10.0 ± 2.5 cm/day, and shaded conditions 0.52 ± 2.5 cm/day, analysis of variance (ANOVA), $F_{3,30} = 12.62$, $P = 0.0001$, (mean ± SE) (Fig. 23). *Diplodia mutila*-induced seedling mortality in plants exposed to full sun was 80% after 10 days.

Seedlings under shaded conditions had 10% mortality and seedlings in the greenhouse had 40% mortality.

Laboratory assays showed that fungal growth (measured as diameter of mycelial colonies or as density of mycelium comprising colony) was greater when a 12-hr alternating light-dark cycle was provided than when periods of light were restricted to 3 hours. On Water Agar medium (WA) the average growth rate per day of the colony mycelium for five days was higher under a 12-hour light cycle, 0.52 ± 0.03, than under a 3-hour light cycle, 0.38 ± 0.03, $P > 0.004$, (mean growth rate (cm) per day ± SE). On Potato Dextrose Agar medium (PDA) the average growth rate per day of the colony mycelium was faster and also higher under the longer light period, 1.25 ± 0.01, compared to 1.11 ± 0.11 for the 3-hour photoperiod, $P > 0.018$, (mean growth rate (cm) per day ± SE) and the mycelium was notably denser with more aerial mycelium (Fig. 24). We recorded greater melanization of mycelium in colonies exposed to the longer light period. This was especially evident in colonies grown on PDA. Melanization of mycelium has been linked to enhanced virulence in numerous plant and animal pathogenic fungi (Langfeldera et al. 2003). Colonies grown in PDA under the 12-hour light cycle had significantly faster growth of the central melanized area, 0.71 ± 0.05, than colonies exposed to the 3-hour light treatment, 0.5 ± 0.05, $P > 0.022$, (mean growth rate (cm) per day ± SE), (Fig. 25, Fig. 26). Similar significant results were obtained for colonies growing in WA medium (Fig. 25.)

DISCUSSION

Our field surveys and experiments demonstrate that *D. mutila* has a less destructive effect on *I. deltoidea* seedlings growing under closed canopy conditions than under gap conditions. Pathogenicity of the endophytic phase of this fungus is triggered by increased light availability. Laboratory observations on the pathogen indicate that *D. mutila* mycelial growth and melanin production increase with light exposure. We suggest that higher light intensity could increase both the rate of development of this fungus in plants as well as its virulence. It is apparent that *D. mutila*-colonized *I. deltoidea* seedlings survive better under closed canopy conditions due to the effect of light in triggering the pathogenic phase of the endophyte. When these plants become older seedlings, the pathogen does not seem to affect plant performance even at high light availability and, additionally, may confer other advantages to these plants, i.e., defensive mutualism (Clay and Holah 1999). Endophytes in many plants have been shown to provide hosts with increased herbivore and/or environmental stress resistance (Arnold et al. 2003, Rodriguez et al. 2004, Arnold and Engelbrecht 2007). The case of *D. mutila* demonstrates that the environment can drastically impact how an endosymbiotic fungus affects fitness of its host. Thus, we ask: to what extent are microorganisms really influencing tropical ecosystems? Most ecological research attempting to explain plant distributions has concentrated on: 1) understanding how abiotic factors interact with plants to maintain biodiversity and determine plant abundance in tropical rain forests; or 2) examining how biotic factors such as morphological characters, herbivores, pathogens or seed

dispersers influence these mechanisms and patterns. This case study of *I. deltoidea* shows that host plant characteristics such as age, light-dependent pathogenicity and virulence of an endophyte-pathogen (i.e., *D. mutila)* and endophyte-enhanced defense against insects, are intrinsically connected, influencing patterns of seedling survival and potentially explaining species abundance on larger scales.

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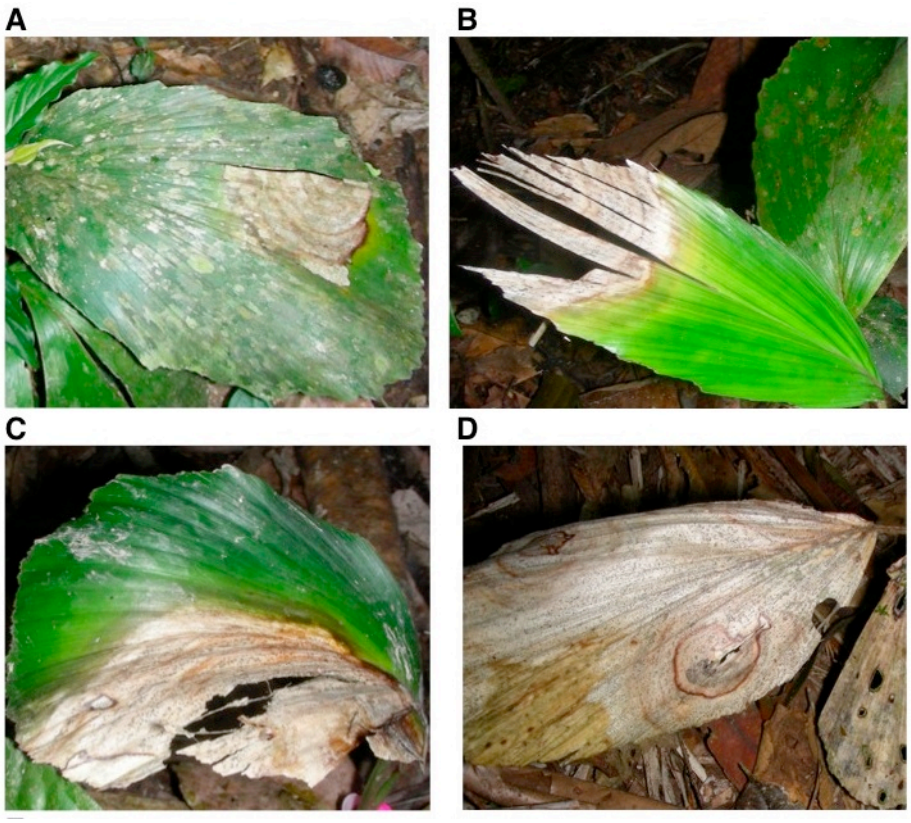

Figure 20. Foliar spots in *Iriartea deltoidea* caused by *Diplodia mutila*, at different infection stages. **(A)** Leaf spot infection for a plant with 2 leaves and one spot covering less than 20% of the leaf **(B)** A plant with two leaves and with a spot covering ~40% of one leaf **(C)** A plant with two leaves and with the two foliar spots covering 50% of both leaves **(D)** Foliar spots covering the entire plant represented 100% of infection. These plants died after 15 to 31 days. **(E)** *Diplodia mutila* pycnidia produced slowly maturing, non-striate, brown, 1-septate conidia measuring 26-28 × 15-20 μm. Liquid conidial darkening and septation was recorded to take place after discharge.

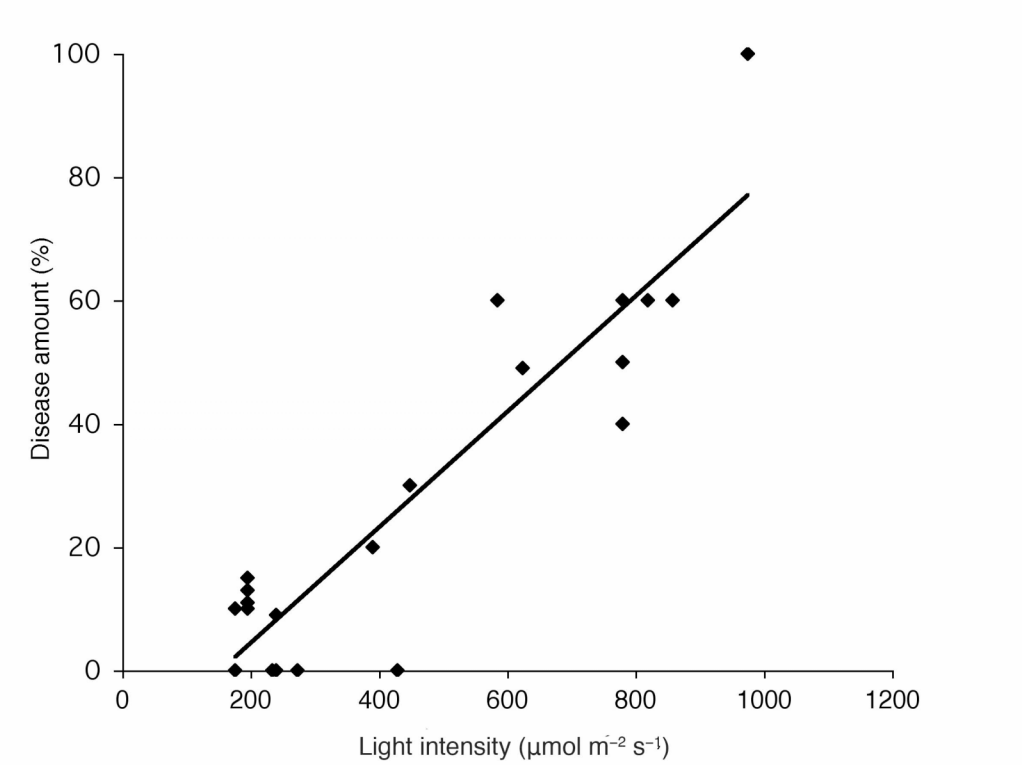

Figure 21. Higher light intensities increased disease development produced by *Diplodia mutila*. For young seedlings with 2 leaves or less there was a significant interaction between amount of infection (% of *D. mutila* foliar spots in *Iriartea deltoidea* leaves) and light level ($F_{1,22}$ = 55.4, P = 0.0001**, r^2 = 0.73)

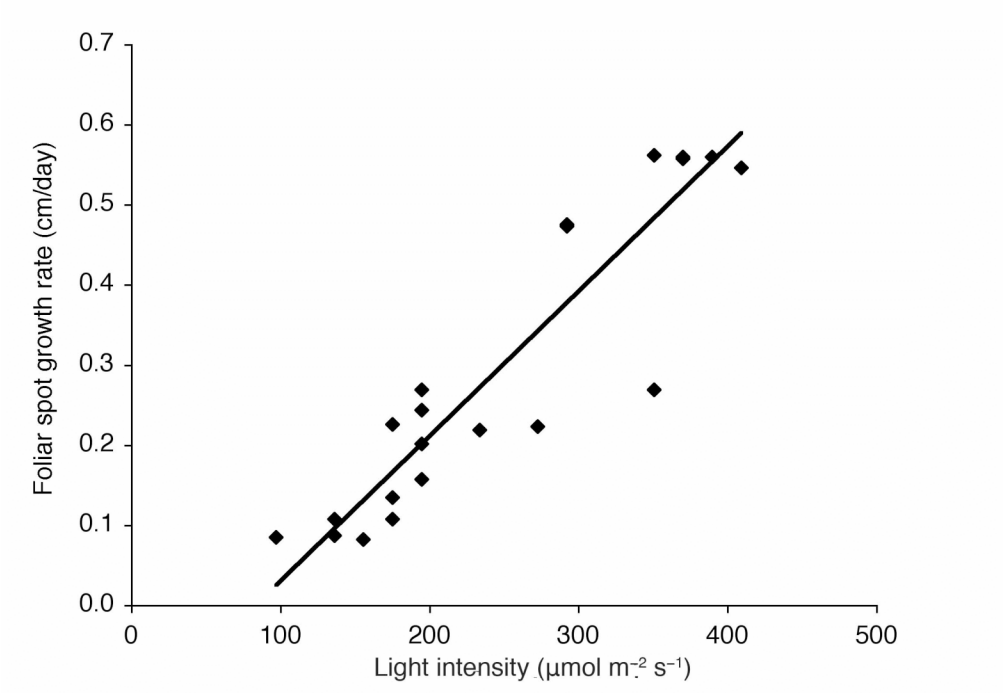

Figure 22. Higher light intensities increased growth of foliar spots produced by *Diplodia mutila*. The diametric growth rate of the foliar spots produced by *D. mutila* was higher at higher light conditions ($F_{1,22}$ = 93.26, P = 0.0001**, r^2 = 0.816).

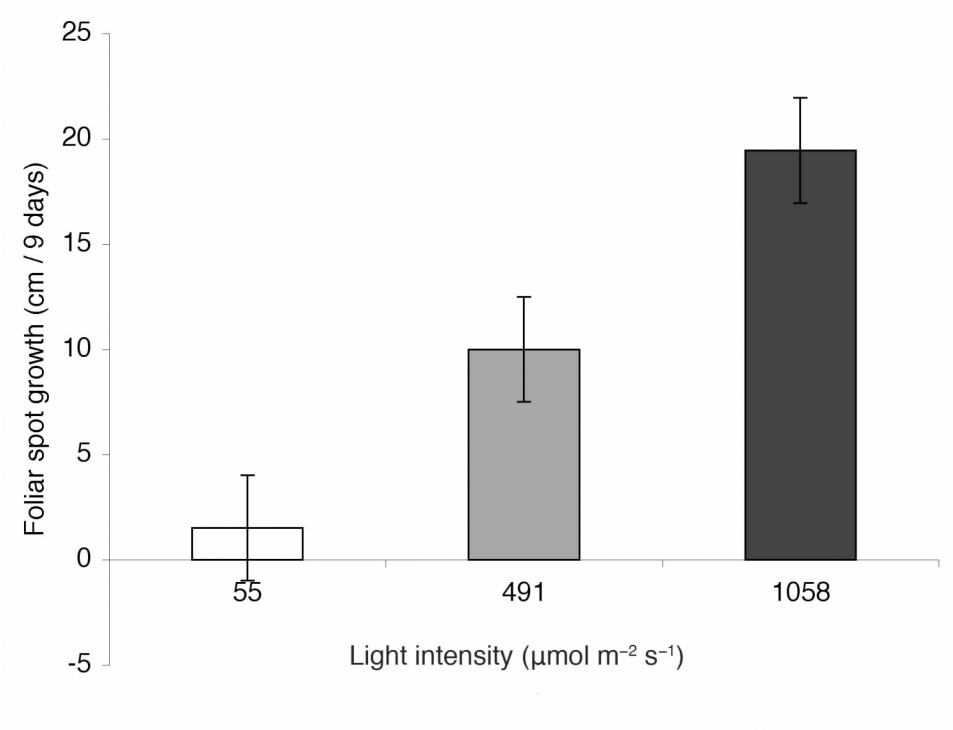

Figure 23. Increased light availability switched the endosymbiotic phase of *D. mutila* to its pathogenic phase. Young seedlings that were colonized with endophytic *Diplodia mutila* showed faster growth rates of diameter of foliar spots (cm) caused by the pathogenic phase of *D. mutila* at higher light intensities (~1058 ± 23 µmol m^{-2} s^{-1}) than seedlings under shaded conditions (~55 ± 15 µmol m^{-2} s^{-1}) (n = 30*, t* test, *P* = 0.0001**). There were also significant differences of foliar spot growth rates among plants growing in the greenhouse (~491 ± 34 µmol m^{-2} s^{-1}) and plants growing under shaded conditions (n = 30*, t* test*, P* = 0.024*). Foliar spot growth rates among plants growing in the greenhouse were lower than plants growing under high light intensities (n = 30*, t* test, *P* = 0.013*), (Tukey Kramer ANOVA, $F_{3,30}$ = 12.62, *P* = 0.0001**).

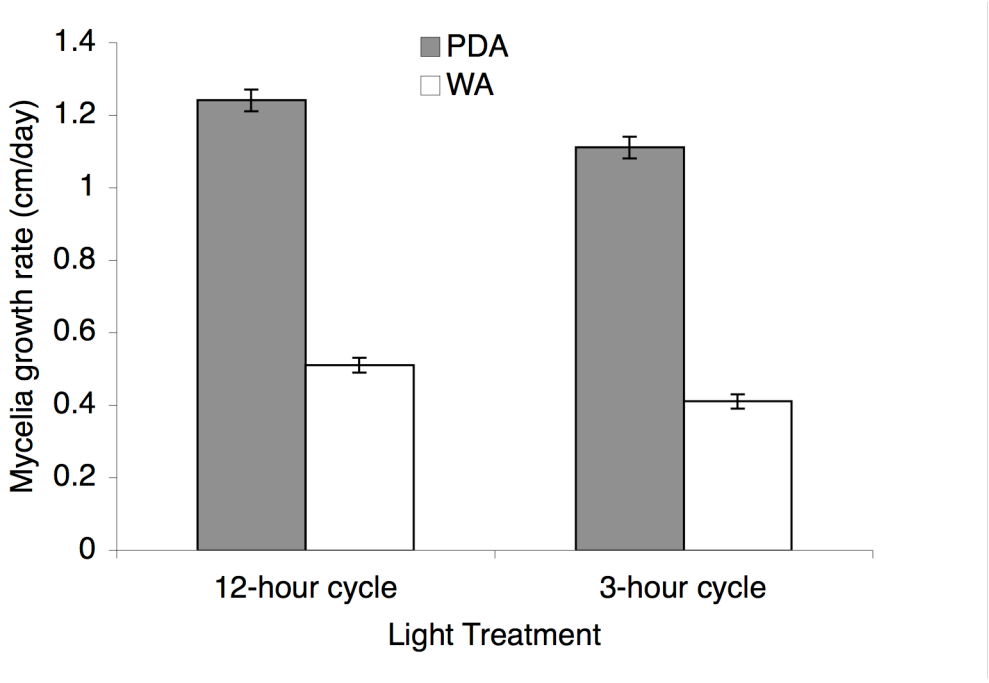

Figure 24. Mycelial radial growth of *Diplodia mutila* on Potato Dextrose Agar (PDA) was faster under a 12-hour cycle than the 3-hour cycle ~1.25 (± 0.03) cm/day vs. 1.11 (± 0.03) cm./day (n = 12, *t* test, *P* > 0.018*). On Water Agar (WA) the average radial growth rate per day of the colony mycelium was ~0.51 cm/day under a 12-hour light cycle; while under a 3-hour light cycle the average growth rate of the colony mycelium was significantly lower, at ~0.41 cm/day after 7 days (n = 12, *t* test, *P* > 0.004*).

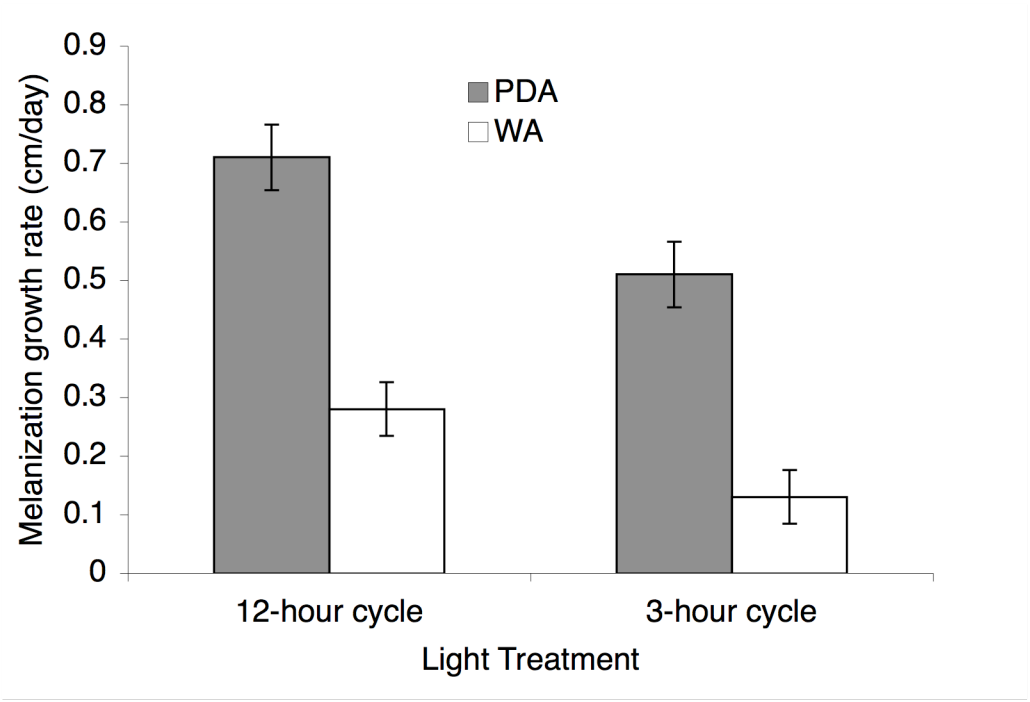

Figure 25. Colonies grown in PDA under the 12-hour light cycle had a more rapid melanization of the central area of the colony (~0.71 cm/day) than colonies exposed to 3-hours of light (~0.5 cm/day) (n =12, *t* test, $P > 0.022$*). Similar results were obtained for colonies growing in WA (n =12, *t* test, $P > 0.0258$*).

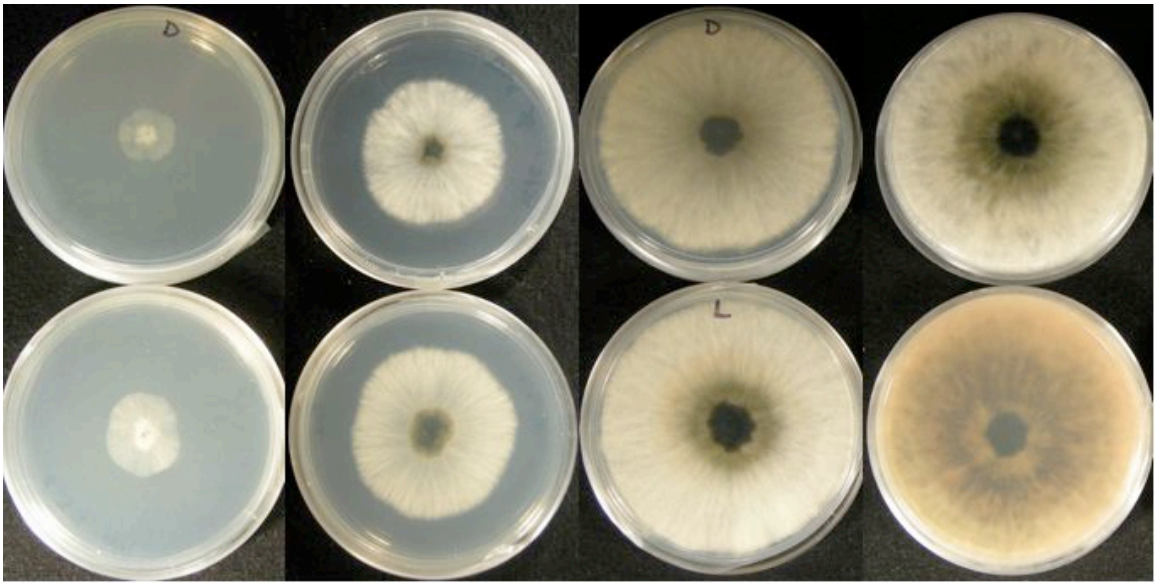

Figure 26. Melanization of colonies of *D. mutila* growing in PDA observed on 4 days (from left to right). Rate of melanization was reduced in the 3-hour cycle treatment (above). Faster melanization was observed in cultures maintained in a 12-hour light cycle (below).

Chapter 4

HIGH MORTALITY OF SEEDS AND SEEDLINGS DISPERSED BY SPIDER MONKEYS

ABSTRACT

Animal seed dispersion is known to increase the probability of plant recruitment, but little is known about the influence of pathogens on dispersed propagules. Here we investigate pathogen-mediated recruitment processes by studying seed mortality and seedling mortality of 114 plant species dispersed under 18 sleeping trees used by spider monkeys (*Ateles belzebuth*) in the lowland tropical moist forest of Manu National Park, Peru. The seed pathogen *Aspergillus flavus* caused 87% seed mortality for two plant species (*Unonopsis matthewsii* and *Oxandra acuminata*). Sites under heavily used sleeping trees showed 89% seedling mortality after one month, 80% of which was influenced by the presence of sooty mold fungi, urine and insect attack. Seed dispersal is generally thought to be an important driver for increased species diversity in tropical ecosystems. Our results suggest that frequency and location of dispersal are crucial to evaluate efficient seed dispersal and plant recruitment. Furthermore, endophytic fungi and ubiquitous oligotrophic pathogenic and non-pathogenic organisms, though often ignored, may offset some of the advantages of dispersal by killing dispersed seeds and seedlings.

INTRODUCTION

Seed dispersal processes are considered important for tropical forest regeneration (Terborgh et al. 2002, Fragoso et al. 2003, Link and Di Fiore 2006). Dispersed seeds are expected to have high survival probability because they escape seed predators, which tend to be concentrated around parent trees (Janzen 1970). Augspurger (1984) reported that the probability of successful seedling recruitment increased with dispersion. Furthermore, Harms et al. (2000) found that seeds of a given species are less likely to become established if the density of that species is high because of pest and pathogen pressure in the tropics, favoring establishment of dispersed seeds. Nevertheless, successful plant recruitment is not only a function of dispersal, but also of how effectively seeds are dispersed, defined by the quantity of seeds dispersed and the quality of dispersal (Schupp 1993). Fruit handling, the effect of passage through the disperser's gut, and dispersal distance are considered important components of dispersal quality (Stevenson 2000, Link and Di Fiore 2006). However, plant recruitment after seed deposition is also a key component of dispersal quality (Chambers and Macmahon 1994, Andresen 2002, Schupp et al. 2002).

Numerous studies provide information about the quantity of seeds ingested by animal dispersers (Jordano and Schupp 2000, Russo 2003), the fate of seeds found in fecal samples (Chapman 1989, Stevenson 2000), and the coupling of behavioral observations of frugivorous animals with the number of seeds they defecate (Chapman 1989, Chapman and Chapman 1996, Andresen 2002). Spider monkeys (*Ateles* spp.) are considered important seed dispersers of

several plant species in Neotropical forests (Stevenson 2000, Terborgh et al. 2002, Russo 2003, Link and Di Fiore 2006).

Comprehensive studies describe seed shadows generated by spider monkeys (Russo and Augspurger 2004, Link and Di Fiore 2006, Russo et al. 2006), and a few have quantified seed survival and seedling recruitment from dispersed seeds by other monkeys (Chapman 1989, Andresen 2002, Russo and Augspurger 2004). However, none of these studies identified the pathogens that damage or kill seeds following dispersal and/or kill recruited seedlings (cf. Augspurger 1984). It is important to consider the role and influence of pathogens after dispersal because they likely play important roles in shaping plant community diversity.

During a previous study of spider monkeys at the Cocha Cashu Biological Station (Cocha Cashu), Russo and Augspurger (2004) found that spider monkeys often slept far from seed sources (~ 48 m) after feeding on *Virola calophylla.* Sites under seed sources and sleeping trees had the lowest per capita seed-to-seedling survival but they had the highest seedling/sapling densities, suggesting that the Janzen and Connell thinning mechanism of clumped plant populations is not strong, originating populations of clumped seedlings and saplings. Nevertheless, causes of mortality were unknown. In a subsequent study at Cocha Cashu, K. N. Gibson observed and monitored annual patterns of seedling recruitment over four years (2003-2006) while conducting a behavioral ecology study of the spider monkeys. An interesting pattern emerged: initially the sites where spider monkeys defecated repeatedly (especially under

sleeping trees) were covered by fecal piles with defecated seeds of many plant species. The seeds established and grew into thick multi-species seedling carpets, then died leaving bare soil (K. N. Gibson unpublished data). Based on these preliminary observations and Howe's study (1989), we hypothesized that dispersed seeds and seedlings may have high mortality due to several ubiquitous oligotrophic pathogenic organisms. The objective of this study was to evaluate this hypothesis by determining mortality causes of dispersed seeds under spider monkey sleeping sites. We focused on seed and seedling pathogens and addressed the following questions: What are the mortality rates of seeds and seedlings germinating under sleeping sites of spider monkeys? What are the primary mortality agents of dispersed seeds and seedlings? Does the frequency of spider monkey visits influence mortality?

MATERIALS AND METHODS

Study Site

The study was conducted at the 10 km^2 Cocha Cashu Biological Station (Cocha Cashu) located in the 18,182 km^2 Manu National Park, Madre de Dios, Peru (11^o 54' S, 71^o 22' W, elevation ca. 400 m). The vegetation is a mixture of mature and successional floodplain lowland undisturbed tropical moist forest. Mean annual rainfall is approximately 2,100 mm (10-yr mean), 86% of which occurs during the wet season (October-May). Mean annual temperature is 24^o C with record extremes of 8^o C and 34^o C (Terborgh 1983, Gentry 1990).

Study animals and sleeping sites

Spider monkeys *(Ateles belzebuth)* are the largest primates in Manu National Park weighing about 8.1 kg (Terborgh 1983, Ohl-Schacherer et al. 2007). Their diet at this study site consists of approximately 146 plant species, composed mainly of fruits (Symington 1987, K. N. Gibson 2008, this study). K. N. Gibson (2008) located 89 spider monkey sleeping trees (trees where the animals were left in the evening and found again the next morning) from 2003 to 2006 during a behavioral ecology study (2,535 observation hrs). The population consists of approximately 102 animals that live in three separate groups in territories of roughly 100-150 ha (K. N. Gibson 2008). Thirty sleeping trees were selected (from the original 89) for this study, 12 of those were excluded because they were located inside flooded areas, peccary (*Tayassu pecari*) wallows, tree-fall gaps or on station trails. During the dry season (June) of 2006 we established 30

plots (ranging from 0.32 to 2 m^2 each) under the 18 study trees, which were all located within an area of approximately 350 ha. These plots were monitored twice a week in the first month and reevaluated after 8 months, during the rainy season. Plots were located beneath the exact place on the branch where the study animals were observed to sleep, defecate, and urinate. The area of the plot was defined by defecation patterns; small plots were established for small localized defecation areas and larger plots consisted of several clumped defecation areas (Fig. 27). Sleeping sites were classified according to how often they were used by spider monkeys and by the relative amount of feces and urine we observed after each night that a tree was slept in. Spider monkeys usually sleep alone or in pairs in different sleeping trees every night, but during multiple nights the same or different animals may sometimes sleep in the same tree and the same spot in that tree (K. N. Gibson unpublished data). Our study animals used sleeping trees more or less often, depending on the home range size and choice of sleeping trees. The variation in the frequency of use of the sleeping sites allowed us to stratify sleeping plots based on use. High Use sites had large amounts (thick multi-layered piles) of feces and urine deposited every day for three or more consecutive days in both dry and wet seasons (Fig. 27). Medium Use sites had traces of feces and urine on one or two days in both seasons. Low Use sites had traces of feces and urine on one day in one season. The thirty sleeping tree plots were grouped as follows: 12 High Use, 6 Medium Use, and 12 Low Use. Additionally, four plots were located between resting and feeding trees of spider monkeys (resting-feeding plots) and established right after we observed

the spider monkeys defecating. Resting-feeding plots had only one defecation event. Light availability was measured using a light intensity meter (Hyrdrofarm LG1 17000). Sleeping trees and existing vegetation in a radius of 5 m around each plot were identified to species level. Seed weight and size, germination type, seedling germination time, and seedling morphology had been determined previously in connection with other research (Pringle et al. 2007, P. Álvarez-Loayza unpublished data).

Mortality rate of seeds and seedlings germinating under sleeping sites

For each season, plots were selected to evaluate seed mortality only if (and just after) we observed the spider monkeys to defecate there. Seed mortality was evaluated inside 18 of the 30 plots in the dry season for one month. In the wet season we evaluated seed mortality inside 25 plots for two weeks. The locations of defecated seeds larger than ~4 mm diameter within each plot were marked at the beginning of the study with plastic toothpicks after the first observed defecation event. All seeds identified as *Ficus* spp., were excluded from the study because most of them are hemiepiphytes and the seedlings germinating in the ground died within very short periods of time (1 to 8 days, P. Alvarez-Loayza unpublished data). Seeds that were removed after the first day of establishment (dung beetle removal) were excluded from the sample size. Seed removal by dung beetles in one day could be extremely high in some plots (~93%) (P. Alvarez-Loayza unpublished data). Only seeds that remained in the plots were considered in the sample size in order to evaluate seed diseases and

mortality causes. Previously established seedlings were identified in all 30 plots and marked with wire and numbered plastic bandettes size 4 (National Band and Tag Co). Seedlings were monitored weekly for 35 days in the dry and wet seasons. Disease symptoms were identified, monitored visually and using photographic sequences. We assumed that observed seedlings emerged from defecated seeds since there were no fruiting trees or vines producing the identified species overhanging a 5 m radius around plots. This assumption is consistent with Howe (1989) and our observation of a highly clumped distribution of several seedling species (~3 species/2 cm^2) most likely germinating from seeds defecated together, and additionally, the seedlings grew in the same locations where we had previously observed fecal piles in the plots.

Mortality agents of dispersed seeds and seedlings

Disease symptoms and mortality causes were recorded weekly for seeds and seedlings in all plots. We grouped mortality agents into five categories: seed and seedling diseases, foliar diseases, epifoliar diseases (Gilbert 2005), insect damage, and animal effect. Organisms that were found attacking seeds and producing damping-off disease (wilting and dead of seedlings) were grouped as seed and seedling diseases. Leaf spots, tip burn, leaf deformation and/or chlorotic leaves were grouped as foliar diseases. Sooty mold fungi (Capnodiaceae) and algal spots were grouped as epifoliar diseases. Sooty mold fungi are non-pathogenic generalists usually living on the droppings of aphids and scale insects (Hemiptera) in plant leaves (Agrios 2005). However, when

sooty mold growth is abundant it interferes with light availability (Anthony et al. 2002), decreasing photosynthetic activity in the plant.

The category Insect Damage was recorded only for seedlings. Caterpillars, leaf miners, stalk borers and cricket herbivory were grouped under this category. Mammalian and insect predation (e.g., rodents, bruchids) was not the focus of this study and thus we did not identify or count the animals that consumed seeds and seedlings inside plots. Mammalian predation was assumed and noted when there was complete removal and/or disappearance of seeds, seedlings, or tags. Insect predation was noted when leaves and stems were chewed and/or damaged by insects.

Identification of pathogenic and non-pathogenic organisms

We collected small portions of diseased tissue (~0.5 mm^2) from the most common and abundant seeds and seedlings in sterile paper bags, labeled with location and host identity, and returned to the field laboratory within one hour. Four pathogenic organisms producing conidia were isolated from plant tissues using the single spore isolation technique (Fox 1993). In the field station laboratory, using a dissecting microscope, conidia from the infected tissue were transferred to a Potato Dextrose Agar (PDA) with chloramphenicol (100 mg/l) medium previously prepared in a laboratory at Rutgers University. Four spores were obtained from the conidia, separated and streaked on the PDA medium and individually transferred to a PDA medium with chloramphenicol. Isolates were kept at an average temperature of 24^{o} C and a 12-hour light cycle inside a sterile box (7.5 L) with 100 gr of desiccant (WA Hammond Drierite Company) to control

humidity. Koch's pathogenicity tests were performed for four pathogens (*Diplodia mutila*, *Aspergillus flavus*, *Microsphaeropsis concentrica* and *Pestalotia* sp.) and their hosts to confirm pathogenicity (Álvarez-Loayza et al. 2008a, Álvarez-Loayza et al. 2008b, P. Álvarez-Loayza unpublished data). Non-pathogenic organisms like sooty mold fungi were isolated using a similar procedure mentioned above, but using spores instead of conidia.

ANALYSIS

In the sleeping tree plots mortality rates and causes of mortality for seeds were quantified for the most abundant species contained in the feces. For seedlings, the importance of pathogens was analyzed by quantifying the proportion of seedlings dying from disease. The relative importance of pathogens and non-pathogenic agents was calculated by dividing the number of disease-related deaths by total observed seedling deaths (Augspurger 1984). Causes of mortality and mortality rates for all seedlings at the end of the dry and wet seasons were compared among animal dispersed seeds under sleeping trees and animal dispersed seeds between resting-feeding trees. To evaluate differences among means we used the Tukey-Kramer HSD-test. Prior to analysis, mortality rates were ArcSin transformed so that data conformed to assumptions of normality. Statistical analyses were performed using JMP 6.0 software (SAS institute).

RESULTS

In the 8-month period of field study, we identified 114 plant species (59 species of seeds and 97 species of seedlings) germinating in the 30 spider monkey plots selected for this study. These numbers are consistent with the number of species identified by Link and Di Fiore (2006) in Ecuador for *Ateles belzebuth*. The total number of evaluated plants was 2,073 seedlings and 1,133 seeds across all sleeping tree and control plots.

Seed Mortality and Causes

During the dry season, we monitored germination and mortality of the most abundant seed species defecated in 18 of the 30 spider monkey plots at the beginning of the census. Initially, 542 seeds were recorded (23 plant species, Appendix 7) defecated in the 18 plots. The four most abundant seed species in the dry season were: *Unonopsis matthewsii, Maytenus magnifolia, Allophylus scrobiculatus,* and *Ziziphus cinnamomum*. We recorded more species in the wet season, when fruit production is higher (Foster 1982, K. N. Gibson et al. unpublished data). During the wet season 591 seeds of 36 plant species (Appendix 7) were defecated in 24 of the 30 sleeping tree plots. The four most abundant species in the wet season were: *Oxandra acuminata*, *Cissus pseudosicyoides*, *Cissus ulmifolia* and *Iriartea deltoidea.* Causes of mortality varied among species (Table 4 and Table 5). *Maytenus magnifolia*, *Allophylus scrobiculatus and Iriartea deltoidea* were susceptible to wilting, caused by *Fusarium oxysporum* and other wilt fungi and bacteria. *Z. cinnamomum* was not

affected by pathogens and had a high germination rate. Mortality rates for *Unonopsis matthewsii* (91%) in the dry season, and *Oxandra acuminata* (83%) in the wet season, was due exclusively to the pathogenic fungus, *A. flavus* (Fig. 27). We did not compare mortality rates and causes among plots or seasons for individual seed and seedling species due to the small sample size. However, similar diseases appeared in both seasons.

Seedling mortality and causes

We analyzed spider monkey sleeping tree plots separately, by season, for the seedling mortality analyses. For the dry season, a Tukey-Kramer test showed significantly higher mortality among sleeping tree plots than resting-feeding tree plots (Fig. 28, $P < 0.05$, $F_{2,34} = 14.32$, $P = 0.0006$). Plots used by monkeys in the dry season had the highest seedling mortality rates (57.6% ± 5.7%) [mean ± SE]. Low mortality rates of seedlings occurred at the resting-feeding plots (2% ± 2%). For the wet season, the Tukey–Kramer test also showed that plots used by monkeys had higher mortality rates of seedlings surviving from the dry season than resting-feeding tree plots (Fig. 28 $P < 0.05$, $F_{2,34} = 11.10$, $P = 0.0027$). Mortality rates of seedlings were lower in the resting-feeding plots (33.5% ± 10%) compared to plots used by monkeys as sleeping sites (74% ± 3.8%). The most important causes of mortality for seedlings growing inside sleeping plots, in both dry and wet season (70%), were sooty mold fungi and burned/wilting leaves (Fig. 27). Sooty mold fungi were identified as *Capnodium* spp., (Reynolds 1971, Hoog and Hermanides-Nijhof 1977). Seedling mortality induced by damping-off diseases was 11% in the dry season

and 4% in the wet season. Insect herbivory was 9% in the dry season and 7% in the wet season. Animal and mechanical damages on seedlings were 2% in the dry season and 15% in the wet season (Fig. 29).

The five most abundant seedling species recorded in the dry season in all plots were: *Allophylus scrobiculatus, Casearia* cf. *decandra, Celtis iguanea, Sloanea obtusifolia* and *Sorocea pileata*, comprising 48% of the total population of seedlings at these sites. All of these species have a short germination time (5 to 25 days), small seeds (< 1 cm length), epigeal germination and small seedlings (< 5 cm) (Table 6). *Allophylus scrobiculatus, C.* cf. *decandra* and *C. iguanea* had the highest mortality rate (92% to 82%) induced mainly by sooty mold fungus. *Sloanea obtusifolia* and *S. pileata* had lower mortality rates (42% to 34%) after 30 days, induced equally by sooty mold and insect attack (Fig. 30). Insect herbivory produced mortality of seedlings inside resting-feeding plots but none of these seedlings presented sooty mold or tip burn symptoms.

Frequency of spider monkey use and mortality

The sleeping sites were stratified based on use: High Use, Medium Use and Low Use. At the end of the dry season (one month after the beginning of the study) we found that High Use plots had 89.5% ± 3.8% of seedling mortality (Fig. 27), Medium Use plots had 57.5% ± 5.3 % of seedling mortality, and Low Use plots had 26% ± 3.8%. At the end of the study, after 8 months, mortality of surviving seedlings was high in High Use plots (85.1% ± 5%) and Medium Use

plots (82.7% ± 7%) and significantly lower (60.9% ± 5%) in Low Use plots (Fig. 31, Tukey-Kramer, $P < 0.05$, $F_{3,28} = 5.93$, $P = 0.0078$).

DISCUSSION

Seed Mortality and Causes

Seed mortality due to pathogens was high for several species. *Aspergillus flavus* was the pathogen causing highest mortality in *Unonopsis matthewsii* and *Oxandra acuminata* (Annonaceae) seeds. *A. flavus* is a facultative saprobe, a well-documented generalist pathogen, and is a common pathogen in the tropics (Arrus et al. 2005, St. Legger et al. 2000). Facultative saprobes necrophytize plant tissues while plants are alive and then change their behavior to degrade plant remains after the plant dies (Agrios 2005). During both the dry and wet season *A. flavus* killed seeds in the same seven plots, suggesting its prevalence in soil at these sites and that favorable conditions for seed infection were ever-present (Janzen 1970, Connell 1971, Howe 1989). Seeds that fell outside of these pathogenic areas were not affected by *A. flavus* and had high germination rates. The non-pathogenic locations (2) were the same for both *O. acuminata* and *U. matthewsii.* There was one *O. acuminata* tree, 2 m away from one sleeping tree plot, but the other 6 plots were not close to any tree in the Annonaceae family, thereby eliminating any confamilial effects over high seed mortality. *Aspergillus flavus* spores are abundant, persistent on plant material and soils, and are easily transmitted, making this fungus a ubiquitous necrotroph pathogen (Klich 2002). There is also some evidence that *A. flavus* may be a seed-transmitted endophyte (Tan and Zou 2001, P. Alvarez-Loayza unpublished data). Additionally, *Fusarium oxysporum* was isolated from four species of seeds. This fungus is ubiquitous in soils and may also be transmitted

endophytically through seeds (Rodrigues and Menezes 2005).

Seedling Mortality and Causes

Overall seedling mortality at spider monkey sleeping sites was high in both the dry and wet seasons, confirming previous empirical observations by K. N. Gibson at Cocha Cashu. Early (<30 day) seedling mortality triggered by sooty mold and urine was found to be high inside High Use and Medium Use spider monkey sleeping tree plots. Sooty mold was not the mortality agent *per se*, but it is likely that the synergistic effects of sooty mold, urine, low photosynthetic rates, and high insect attack slowed plant growth and made plants more vulnerable to damage by insect herbivores (Agrios 2005). Spider monkey urine (or nutrients in the urine) appears to stimulate sooty mold (whose spores are abundant and wind-dispersed). All seedlings that were affected by sooty mold were chlorotic and presented leaf damage. The different mortality rates among the five most common and abundant seedling species could be attributed to seedling life history and morphology. *Allophylus scrobiculatus*, *C. iguanea* and *C.* cf. *decandra* have a fast germination rate (5 to 10 days), small (2 to 3 cm) seedlings and small herbaceous cotyledons and leaves. *Sloanea obtusifolia* and *S. pileata* germinate in 20 to 30 days, are seedlings of medium size (4 to 6 cm), have first leaves of medium size and have a coriaceous texture. Mortality produced by damping off diseases was higher in the dry season presumably because seedlings were relatively young and susceptible to damping-off.

After eight months, mortality rates of surviving seedlings from the dry season were similar for High Use and Medium Use plots (85% and 82% respectively).

The accumulated effect of sooty mold fungi, low photosynthetic rate, and high insect attack on seedlings explained higher mortality in Medium Use plots in the wet season. In the dry season, traces of urine and sooty mold were present in Medium Use plots. Seedlings did not suffer as high mortality in the dry season, but presented disease symptoms (e.g., leaf surface covered by sooty mold, chlorosis, insect attack, tip burn) and died later in the wet season.

Mortality was lower at resting-feeding and Low Use plots (60.9%) compared to High Use and Medium Use plots (~83%). A steady input of resources at defined sites in the forest attracts insect pests and pathogens that chronically kill a large percentage of seeds and saplings at latrine sites but not at random spots that are distant from latrine sites. Seedlings at Low Use and resting-feeding plots still present a clumped distribution, but are not affected by sooty mold, urine effects, and increased insect attack. The seedlings inside Low Use and resting-feeding plots may benefit by escaping from parental trees and from locating pathogen free sites with optimal conditions (Janzen 1970, Connell 1971, Howe 1989). This study confirms that seedlings of scatter-dispersed species rarely survive in dense aggregations under frugivore roosts (Howe 1989) and emphasizes the importance of random events of dispersion in the forest floor, where there is a high probability of escaping seed and seedling pathogens and predators.

Endophytic dissemination in seeds potentially negates benefits from escaping the parental tree (cf. Janzen 1970). *Diplodia mutila* was previously recorded as a mortality agent for *Iriartea deltoidea* fruits, seeds, and seedlings at

Cocha Cashu (Álvarez-Loayza et al. 2007) and *Aspergillus flavus* for *Oxandra acuminata*, and *Unonopsis matthewsii* (Álvarez-Loayza et al. 2008). Mortality for these three plant species could be explained either by the endophytic nature of *D. mutila* (Ragazzi et al. 1999) and *Aspergillus flavus* (Tan and Zou 2001), or their ubiquitous nature. We conclude that successful recruitment and survival depends on (1) the capacity of the seed to find a suitable habitat free of pathogens, (2) the ability to overcome endophytic pathogens (3) scattered dispersion of propagules at random sites in the forest floor and (4) the plant's resistance mechanisms to a wide range of ubiquitous and generalist pathogens.

Broader Impacts

Our data are drawn from a relatively well-protected population of spider monkeys living in a large protected forest national park. Our results are useful as a baseline for comparison and to guide future research on large mammals living under less protected or unnatural conditions. Spider monkeys are among the most vulnerable primates in the world because they reproduce slowly (2-3 year interval between births) and require large tracts of intact forest to feed (Symington 1987). Habitat destruction, fragmentation, and especially hunting have lead to the decline of naturally occurring spider monkey populations over the last few decades (Mittermeier 1987, Peres 1990, de V. Grelle et al. 1999). This study shows that sites where these primates repeatedly defecate are inhospitable at best and mostly deadly for seedlings. While our results are useful for the debate on seed dispersal by animals, it appears that they are critical to our understanding of ecological processes within degraded forests. When the

size of a forest—and in turn, home range of the arboreal primates living there—is reduced, the animals are restricted to sleeping in fewer trees. All sleeping trees then become high use areas that contribute to a decrease in overall seedling recruitment from dispersed seeds. Overall recruitment will be affected on the forest floor by reducing “pathogen-free” sites.

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Table 4. Causes of mortality for the four most abundant seed species contained inside 18 sleeping tree plots of spider monkeys (*Ateles belzebuth*) in the dry season (May-July 2006).

	Unonopsis matthewsii	*Allophylus scrobiculatus*	*Maytenus magnifolia*	*Ziziphus cinnamomum*
Initial number of seeds	164	80	79	43
Dead seeds by wilting	5	10	22	0
Dead seeds by *Aspergillus flavus*	149	0	0	0
Dead seeds by animal	0	11	39	2
Germinated seeds	10	59	18	41
Seedlings killed by insects	10	0	0	10
Seedlings killed by sooty mold + insects	0	59	15	7
Seedlings burned	0	0	0	12
Unknown mortality agent	0	0	0	6
Surviving seedlings after 8 months	0	0	3	6
Surviving seedlings after 12 months	0	0	0	0

Table 5. Causes of mortality for the four most abundant seed species contained inside 24 sleeping tree plots of spider monkeys (*Ateles belzebuth*) in the wet season (December 2006-January 2007).

	Oxandra acuminata	*Cissus pseudocisyiodes*	*Cissus ulmifolia*	*Iriartea deltoidea*
Initial number of seeds	163	67	42	50
Dead seeds by wilting	0	6	14	8
Dead seeds by *Aspergillus flavus*	136	0	0	0
Dead seeds by animal	0	7	0	8
Dead seeds by insects	0	0	0	10
Dead seeds by *Diplodia mutila*	0	0	0	2
Dead seeds – unknown causes	27	1	0	17
Germinated seeds	0	53	28	5
Seedlings killed by sooty mold + insects	0	38	18	0
Seedlings killed by *Diplodia mutila*	0	0	0	2
Seedlings killed by unidentified pathogen	0	15	10	2
Surviving seedlings after 8 months	0	0	0	0
Surviving seedlings after 12 months	0	0	0	1

Table 6. Morphological characteristics of five seedlings species, commonly found in spider monkey sleeping plots at Cocha Cashu Biological Station. The average sizes of seeds and seedlings were calculated using 20 individuals. Mean germination time was estimated using 30 seeds of each plant species.

Plant Species	Seed size (length in cm)	Type of germination	Germination time (days)	Seedling size (cm)	Cotyledon or first leaf texture
Allophylus scrobiculatus	~0.3	Epigeal	~4	~2	Herbaceous
Casearia cf. decandra	~1	Epigeal	~6	~3	Herbaceous
Celtis iguanea	~0.5	Epigeal	~5	~2.5	Herbaceous
Sloanea obtusifolia	~1	Epigeal	~24	~5.5	Coriaceous
Sorocea pileata	~1	Epigeal	~30	~5.5	Coriaceous

Figure 27. Spider monkey feces and effects on seedling recruitment (a) Fecal piles recorded inside High Use sleeping plots of spider monkeys in Cocha Cashu Biological Station. (b) *Oxandra acuminata* seed infected with *Aspergillus flavus*. (c) Sooty mold fungi, *Capnodium* spp., growing on the leaf surface of a seedling of *Sloanea obtusifolia*, which also presented burned spots and wilting. (d) Seedlings inside High Use plots were severely affected by sooty mold, urine and feces impact and insect herbivory.

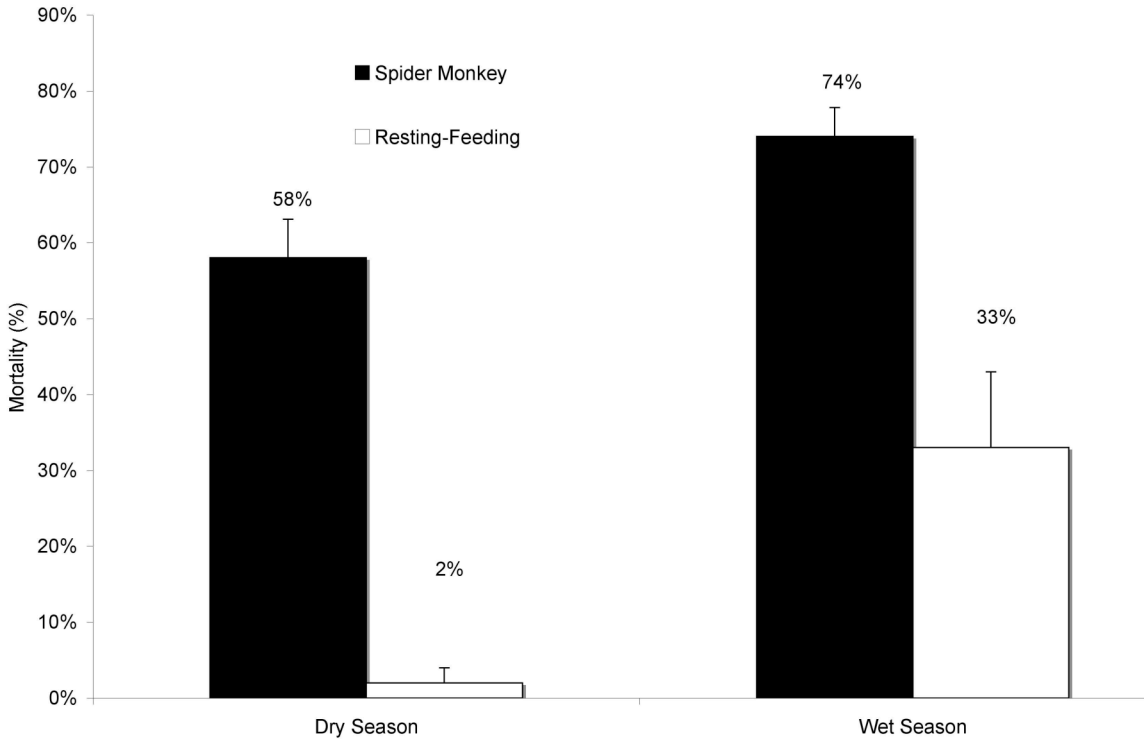

Figure 28. Significant differences in overall seedling mortality rates in 30 spider monkey (*Ateles belzebuth)* plots*,* and 4 resting-feeding tree plots, in the dry and wet seasons. Bars represent the mean percentage (±SE) of mortality rates of seedlings inside these plots. (Tukey-Kramer Test, $P < 0.05$, $F_{2,34} = 11.10$, $P = 0.0027$)

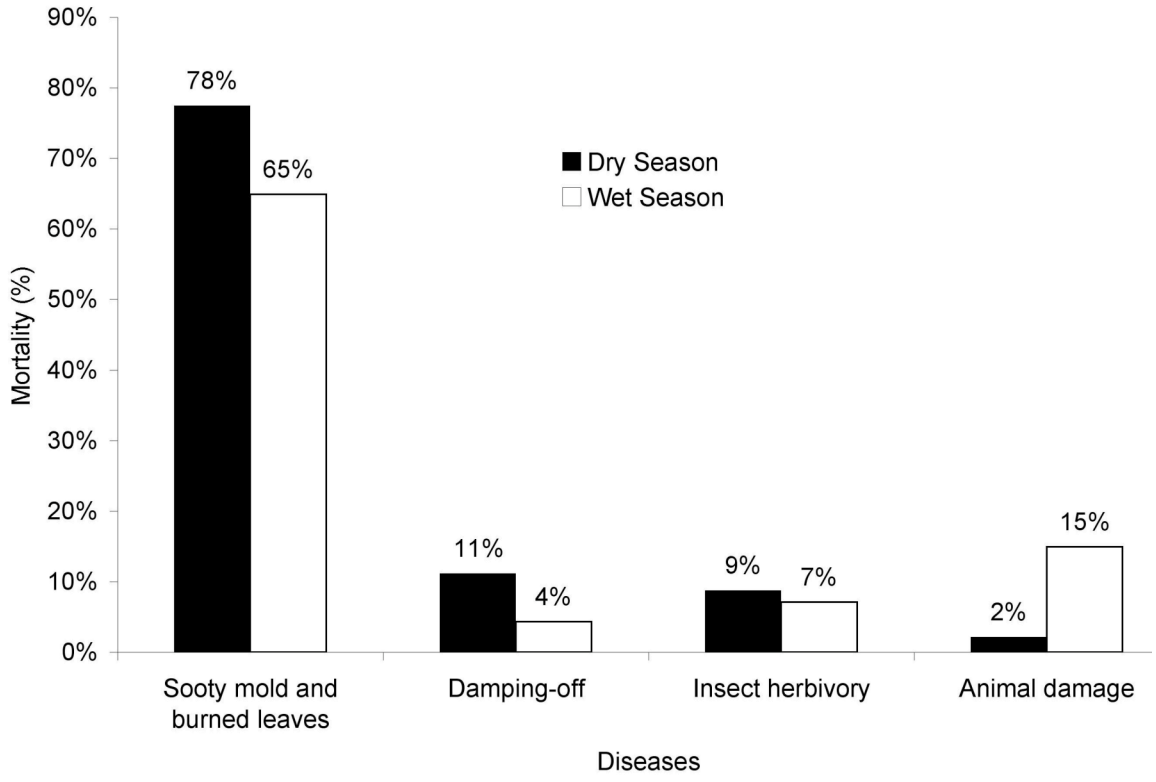

Figure 29. Mortality causes for seedlings growing inside sleeping tree plots. Sooty mold fungi and burned leaves induced high mortality in both seasons. Mortality induced by damping-off diseases, insect herbivory and animal and mechanical damage was lower.

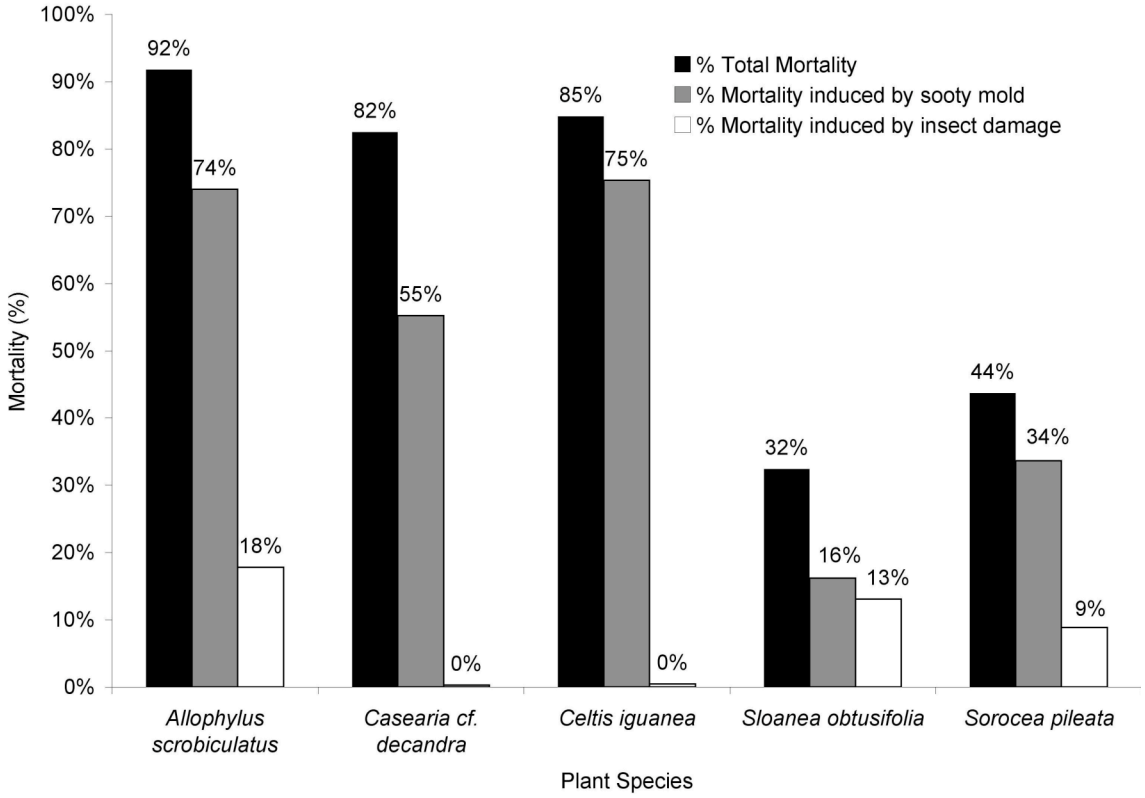

Figure 30. Mortality rates for the most common and abundant seedling species in *A. belzebuth* plots in the dry season. *Allophylus scrobiculatus*, *Casearia* cf. *decandra* and *Celtis iguanea* presented high mortality due to sooty mold, while *Sorocea pileata* and *Sloanea obtusifolia* had lower mortality rates induced by both sooty mold and insect attack.

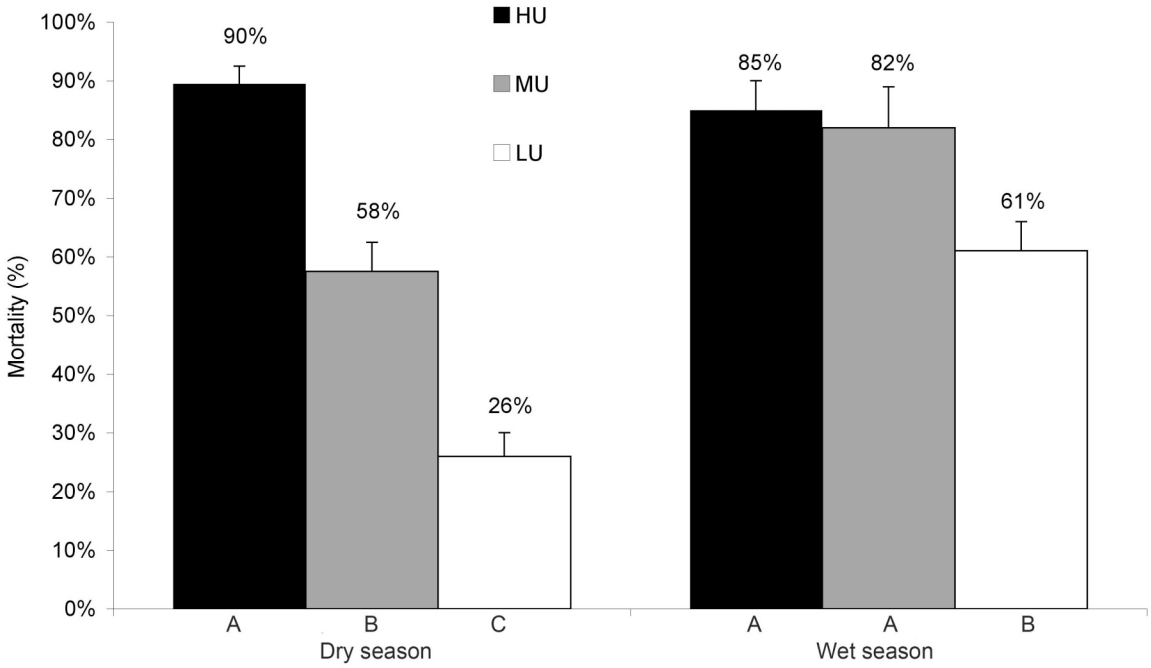

Figure 31. Mortality rates in High Use, Medium Use and Low Use plots in the dry and wet seasons. For the dry season there was a significant difference among plots: HU ≠ MU ≠ LU. For the wet season there was a significant difference on mortality rates of surviving seedlings from the first period: (HU, MU) ≠ (LU). Abbreviations are: HU, High Use, MU, Medium Use, and LU, Low Use. Significant differences in mortality rates among plots by season are represented by different letters (A, B and C). (Tukey-Kramer Test, $P < 0.05$, $F_{3,28} = 5.93$, $P = 0.0078$).

Chapter V

CONCLUSIONS

In the last decades most research on pathogens and endophytes was conducted on agronomic crops and there is little data regarding plant-fungal interactions and host ranges in natural plant populations. Because of this it is currently difficult to assess any impacts in natural plant communities. As far as we are aware this study is unique in examining pathogenicity to many members of a tropical forest plant community using a fungal symbiont that is both asymptomatic endophyte and virulent pathogen. This research project was focused on assessing the impacts of microbial plant symbionts (endophytes and pathogens) on a single tropical forest plant, *Iriartea deltoidea*, distribution of the host plant; and evaluating what roles the abiotic and biotic environments of plants play in defining the effects of the microbial symbionts on plant fitness.

This dissertation strongly focused on one pathogen-endophyte, *Diplodia mutila,* but indicates that generalist pathogens and endophytes may play important roles in natural plant communities. I found that *Diplodia mutila* commonly colonizes healthy plants but also is a virulent pathogen when the plant is young and located in unfavorable environmental conditions. Endophytic fungi could negate benefits of animal dispersal. I report infected plants located far away from any conspecific tree. This pattern of infection could be explained either by vertical (endophyte carried on the seed) or horizontal transmission (spores carried in the rain, insects or wind) and the possibility that *Dipodia mutila*

is a host generalist, therefore it should be infecting other plant species in natural systems. This dissertation found that *Diplodia mutila* was infecting other species in different plant families. This part of the dissertation concludes that differences in virulence, plant susceptibility, and mutualism effects of endophytic pathogens, coupled with environmental conditions (i.e. light) and interactions with predators (i.e. hebivores) will need to be examined for assessment of impacts in natural plant communities.

Previous research has demonstrated that some endosymbionts of plants may be mutualistic, enhancing plant tolerance to biotic and/or abiotic stresses and thus positively affecting plant fitness. It is also clear that mutualism is not absolute, but instead is relative to specific conditions. There is a strong possibility that there is an advantage for the plant to coexist with a potentially lethal pathogen. This fungus could be acting as a protection mechanism for the plant and also as a pathogen. Any association between a microbial symbiont and its plant host is somewhere on the continuum between parasitism and mutualism. I found strong evidence that this fungus protects its host plant from insect herbivores. Infected plants show reduced insect attack and feeding experiments with insects extracted from Iriartea plants showed that insects avoided food material infected with *Diplodia mutila* but do not avoid food material infected with other fungi.

Most ecological research on explaining plant distribution has concentrated on understanding how the abiotic environments interact with plants to determine ultimate distributions of plants; or how biotic factors such as herbivores,

pathogens or mutualists interact with plants to determine plant distribution. The work done for this dissertation shows that neither approach is completely correct and that plant distribution is the result of a complex interplay between the plants, the abiotic environments and the biotic environments. For at least one broadly-distributed tropical palm endophytic fungus (*Diplodia mutila*) we have shown that light triggers the fungus to exhibit its pathogenic phase, impacting host distribution by forcing plants to grow mainly in shaded habitats. The case of *D. mutila* demonstrates that an abiotic factor (level of light) may change expression of the microbial symbiont-plant association and placement along the parasitism-mutualism continuum and drastically impact plant distribution. Endophytes and systemic pathogens are extremely common in tropical plants. It is my hypothesis that the distributions and fitness of many tropical forest plants are affected by endophytic fungi that are responding to abiotic and biotic environmental conditions.

Finally, this dissertation emphasizes the importance of pathogens in natural tropical ecosystems. Several empirical and theoretical studies emphasize the importance of propagule dispersion to increase survival of the plant. This study shows that endophytic fungi such as *Aspergillus flavus* and *Diplodia mutila* negate de benefits of dispersion. *Aspergillus flavus* killed 98% of seeds dispersed by monkeys. Once other species were established we show that the synergistic effects of location of the defecation site, pathogens, insects and environmental factors strongly influences the fate of the seedling. Seedlings do not survive when defecation events are concentrated in one site. These

seedlings are infected with sooty mold fungi *Capnodium* sp., that influences high mortality rates reported in this plots. These combined evidence show that endophytic fungi, pathogens, insect herbivores, generalist and ubiquitous fungi, host plant physiology and environmental conditions strongly influence patterns of plant recruitment in tropical ecosystems.

As a final remark, I want to emphasize the importance of investigating ecological problems combining plant pathology and ecology. It is widely accepted that patterns of plant distribution and abundance in the tropics are strongly influenced by pathogens. I have shown that the interaction of the host plant, pathogen and environment could ultimately define the distribution of one plant species. There are at least 1,500 species in the lowland forest of Manu. I predict that the distribution and abundance of each species could be predicted by the interaction of abiotic and biotic factors on the pathogen-endophyte continuum, where current patterns of abundance are defined by current ecological conditions (i.e. temperature, soil, herbivores) that defines general plant distribution patterns (i.e. preference for wetlands, understory, gaps), but also influences the pathogen-endophyte continuum, strongly affecting mortality and survival of the plants (i.e. defensive mutualism, pathogens, attract herbivores) when located in ideal environmental conditions. This study hopefully contributes to reach the next frontier of tropical ecology, the study of the interaction of abiotic and biotic conditions (i.e. pathogens, microbes, insects, host plants) in tropical ecosystems.

Appendix 1. *First report of Diplodia sp. causing seedling mortality of Iriartea deltoidea palm trees in Peru*

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The palm *Iriartea deltoidea* is one of the most abundant plant species in natural tropical ecosystems (Pitman *et al*., 1999). It is among the species used by native people of the Amazonian region to construct dwellings (Duke & Vasquez, 1994). During the dry season in June 2006, 12 sick seedlings from a sample of 43 seedlings were recorded in one hectare area in lowland tropical forest in Manu National Park, Peru.

The affected leaves presented small circular necrotic spots with black pycnidia producing liquid masses of slowly maturing non-striate brown 1-septate conidia measuring 26-28 x 15-20 μm. One month later, the seedlings were dead and the necrotic leaf spots had grown in size and produced abundant black pycnidia. Symptoms on *I. deltoidea* fruits were similar to those on leaves and collected in June and December 2006 in the same area.

Single conidial isolates from leaves and fruits were grown on Potato Dextrose Agar (PDA) with Chloramphenicol (100 mg/l). Conidial production was induced with near UV light 12-hour light/dark cycle. Based on growth characteristics and morphology of pycnidia and conidia the isolates were identified to genus *Diplodia mutila* Fr. apud Mont. (Sutton, 1980; van Niekerk *et al*., 2004). Fruit and leaf isolates were identical.

Two-month old *I. deltoidea* seedlings grown in a greenhouse were used for pathogenicity tests. Toothpicks were autoclaved and then infested with mycelia by placing them at the growing margins of 5-day old *D. mutila* cultures grown on oatmeal agar. The toothpicks were incubated at 26° C for 4 days (Silva & Juliatti, 2005). Leaves of six plants were inoculated by penetrating leaf blades with infested toothpicks or sterile toothpicks. Toothpicks simulate mechanical damage that *I. deltoidea* seedlings present in natural conditions due to falling fronds and insects. Plants were covered with plastic bags to maintain humidity with a 12-hour light/dark cycle for 16 days. Mean temperature ranged from 22° to 24°C.

Inoculated leaves showed necrotic symptoms identical to those seen in field populations. Infected seedlings died after 5 to 16 days, showing pycnidia with liquid masses of conidia. *D. mutila* was re-isolated from infected seedlings. The control seedlings inoculated with sterile toothpicks remained healthy.

This is the first report of *Diplodia mutila.* causing *Iriartea deltoidea* seedling mortality, although this species has been reported to cause disease on numerous other hosts worldwide (Sutton 1980).

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Appendix 2. *Logistic regression analysis to separate distance effects from local density effects*

In logistic regression, the unknown probabilities p_i of infection are modeled as a function of the independent variables d_{nft} and ρ_{lpd}. The p_i can be computed from Y by clustering the data into $i = 1, 2, ..., m$ classes (labeled as $d_{nft,i}$ and $\rho_{lpd,i}$) and computing p_i from

$$p_i = E\left(\frac{Y_i}{n_i}\middle| d_{nft,i}, \rho_{lpd,i}\right),$$

where E is the expectation operator. Hence, for each distance and density class i, Y can be interpreted as repeated (Bernoulli) trials on infection occurring with probability p_i directly from the data.

The parameters of the logistic regression, $\beta_1, \beta_2, \beta_3$ used to model p_i can be computed from

$$\log\left(\frac{p_i}{1-p_i}\right) = \beta_1 + \beta_2 d_{nft,i} + \beta_2 \rho_{lpd,i},$$

using standard least-squares error minimization or Likelihood estimation. Note that the above equation can be re-arranged to explicitly yield:

$$p_i = \frac{1}{1 + \exp\left[-\left(\beta_1 + \beta_2 d_{nft,i} + \beta_3 \rho_{lpd,i}\right)\right]},$$

and this expression shows how increases in either distance from a fruiting tree or local density impacts the probability of infection. To assess the relative importance of d_{nft} and ρ_{lpd} on infection or mortality rates, the parameters of the logistic regression were computed using only distance to nearest fruiting tree alone, local plant density alone, and their combination (assumed independent).

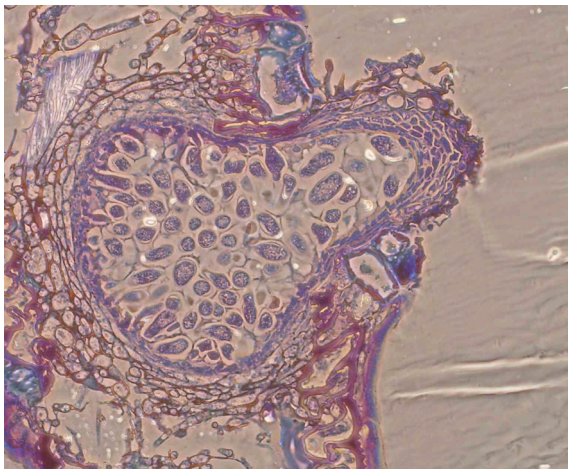
Appendix 3. Description of *Diplodia mutila*

Lesions

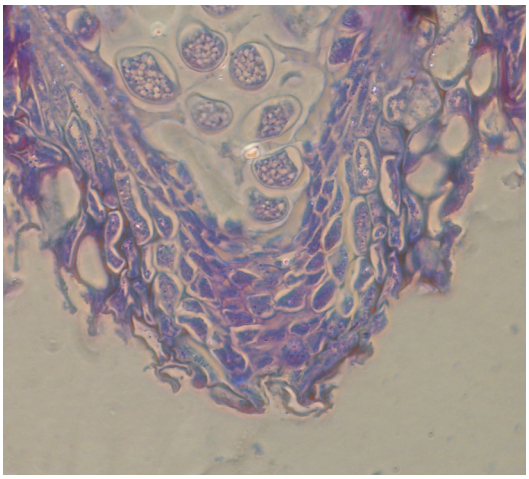
Forming rapidly expanding moist elliptical lesions on *Iriartea deltoidea*, reaching 5 mm after 4 days and 90 mm after 10 days. Pycnidia produced in successive zones in the lesions, with approximately 20 pycnidia per cm^2.

Pycnida Morphology

Pycnidia produced embedded within leaves and exocarp of fruits of *Iriartea deltoidea*. Primordial pycnidia are globose, producing an apical neck meristematic region that ruptures the leaf epidermis as it emerges to the leaf surface. Pycnidial necks curving 90 degrees at the leaf surface. The poroid wall of the pycnidal neck composed of 2-3 parenchymatose layers (measuring 4-10 um, with individual cells measuring 2 x 3 um) with the innermost layer bearing irregular short hemispherical thin-walled papillae (measuring 2-4 um wide x 2 um high).



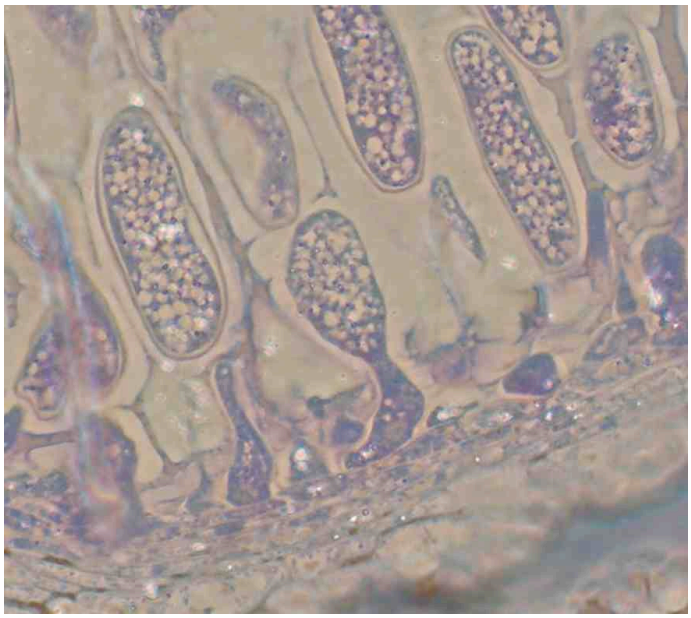
Pycnidia



Ostiole

Pycnidial structure

Individual pycnidia brown subglobose, usually with a somewhat curved neck (measuring 95-100 x 75 - 90 um), usually solitary. The pycnidial wall 25-30 um thick, composed of 6-10 cell layers, with 2-3 thin walled inner layers (measuring 5 um thick) composed of densely staining pseudoparenchymatose ellipsoidal cells (measuring 5-7 length um x 2-3 width um); and 5-7 outer layers (measuring 20-25 um thick) composed of larger ellipsoidal pseudoparenchymatose thick walled cells (measuring 8-20 um length x 2-9 um width) . Outer wall layers showing irregular melanized wall thickenings. Conidia produced on the inner 2-3 layers of the pycnidial wall. Conidiogenous cells holoblastic, irregular in shape, flattened to globose (measuring 4-10 um width by 15-20 um length) with an apical percurrent tubular extension (measuring 7-10 um long by 1-2 um wide), producing several conidia successively.



Conidiogenous cells

Conidia (7-10 um wide x 10-22 um long) produced in masses in the ventor of the pycnidium, cylindrical, at first hyaline, highly granulate internally, with a thick slime layer outer coat; conidia maturing over several days with granules gradually disappearing and forming 1-2 large lipid guttules at opposite ends of the conidium as a central septum develops conidia wall develops a brown melanization. Mature conidia measure 26-28 um length x 15-20 um width and frequently show a truncated base (detachment scar). En masse on the surface of leaves conidia at first cream colored, later becoming brown as conidia mature.

Conidia

Colonies on potato dextrose agar (PDA) (Becton Dickson & Company) reaching 40 mm in diameter after 3 days, cottony, white, with reverse non-pigmented; after 4 days becoming gray to black; chlamydospores forming in aerial mycelium of older cultures, unless induced by prolonged exposure to UV light or culture contamination by bacteria. The teleomorph was not observed on plant tissues or in cultures.

Appendix 4. Plant species inoculated with *Diplodia mutila*

Family	Species	Life form
Acanthaceae	*Justicia appendiculata* Ruiz & Pav	Shrub
Annonaceae	*Duguetia quitarensis* Benth.	Tree
Annonaceae	*Klarobelia candida* Chatrou	Tree
Annonaceae	*Oxandra acuminata* Diels	Tree
Arecaceae	*Astrocaryum murumuru* Mart.	Palm
Arecaceae	*Attalea butyracea* (Mutis ex L.f.) Wess Boer	Palm
Arecaceae	*Euterpe precatoria* Mart.	Palm
Arecaceae	*Geonoma* sp. 1 (morphotype JT6)	Palm
Arecaceae	*Geonoma* sp. 2 (morphotype Tr3)	Palm
Arecaceae	*Oenocarpus bataua* Mart.	Palm
Arecaceae	*Socratea exorrhiza* (Mart.) H. Wendl.	Palm
Arecaceae	*Wendlandiella gracilis* Dammer	Palm
Bombacaceae	*Matisia cordata* Bonpl.	Tree
Bombacaceae	*Quararibea witti* K. Schum. & Ulbr.	Tree
Boraginaceae	*Cordia nodosa* Lam.	Treelet
Burseraceae	*Protium tenuifolium* (Engl.) Engl.	Tree
Cecropiaceae	*Pourouma cecropiifolia* Mart.	Tree
Clusiaceae	*Garcinia brasiliensis* Mart.	Tree
Cyclanthaceae	*Carludovica palmata* Ruiz & Pav.	Tree
Dillenaceae	*Doliocarpus magnificus* Sleumer	Liana
Ebenaceae	*Diospyros pavonii* A. DC.	Tree
Eleocarpaceae	*Sloanea obtusifolia* (Moric.) K. Schum.	Tree
Fabaceae	*Mucuna* sp. 1 (morphotype Tr7)	Tree
Fabaceae	*Inga sp.* "6605" (morphotype Tr72)	Tree
Fabaceae	*Inga edulis* Mart.	Tree
Fabaceae	*Inga sp. 2* (morphotype Alada)	Tree
Fabaceae	*Inga velutina* Willd.	Tree
Fabaceae	*Zygia latifolia* (L.) Fawc. & Rendle	Tree
Flacourtiaceae	*Casearia* cf. *decandra* Jacq.	Shrub
Flacourtiaceae	*Mayna odorata* Aubl.	Shrub
Dryoteridaceae	*Tectaria incisa* Cav.	Fern
Icacinaceae	*Calatola venezuelana* Pittier	Tree
Icacinaceae	*Leretia cordata* Vell.	Liana
Lauraceae	*Caryodaphnopsis fosteri* Van der Werff	Tree
Lauraceae	*Nectandra longifolia* (Ruiz & Pav.) Nees	Tree
Lauraceae	*Ocotea oblonga* (Meisn.) Mez	Tree
Melastomataceae	*Loreya klugii* S.S. Renner	Shrub
Meliaceae	*Guarea kunthiana* A. Juss.	Tree
Meliaceae	*Trichilia elegans* A. Juss.	Tree
Meliaceae	*Trichilia poeppigii* C. DC.	Tree
Moraceae	*Batocarpus amazonicus* (Ducke) Fosberg	Tree
Moraceae	*Brosimum alicastrum* Sw.	Tree

Family	Species	Life form
Moraceae	*Ficus maxima* Mill.	Tree
Moraceae	*Naucleopsis krukovii* (Standl.) C.C. Berg	Tree
Moraceae	*Pseudolmedia laevis* (Ruiz & Pav.) J.F. Macbr.	Tree
Moraceae	*Sorocea pileata* W.C. Burger	Tree
Myristicaceae	*Iryanthera olacoides* (A.C. Sm.) A.C. Sm.	Tree
Myristicaceae	*Otoba parvifolia* (Markgr.) A.H. Gentry	Tree
Myristicaceae	*Virola calophylla* (Spruce) Warb.	Tree
Myristicaceae	*Virola flexuosa* A.C. Sm.	Tree
Myristicaceae	*Virola mollissima* (Poepp. ex A. DC.) Warb.	Tree
Myrtaceae	*Eugenia punicifolia* (Kunth) DC.	Shrub
Olacaceae	*Heisteria acuminata* (Humb. & Bonpl.) Engl.	Tree
Opiliaceae	*Agonandra brasiliensis* Miers	Tree
Piperaceae	*Piper hispidum* Kunth	Shrub
Poaceae	*Guadua sarcocarpa* Londoño & P.M. Peterson	Grass
Rhamnaceae	*Ziziphus cinnamomum* Triana & Planch.	Tree
Rubiaceae	*Faramea multiflora* A. Rich. ex DC.	Shrub
Sapindaceae	*Paullinia obovata* (Ruiz & Pav.) Pers.	Liana
Sapindaceae	*Paullinia* sp. 1 (morphotype winged raquis)	Liana
Sapotaceae	*Pouteria ephedrantha* (A.C. Sm.) T.D. Penn.	Tree
Staphyleaceae	*Huertea glandulosa* Ruiz & Pav.	Tree
Sterculiaceae	*Theobroma cacao* L.	Tree
Theophrastaceae	*Clavija tarapotana* Mez	Shrub
Ulmaceae	*Celtis iguanaea* (Jacq.) Sarg.	Liana
Ulmaceae	*Celtis schippii* Standl.	Tree
Violaceae	*Leonia glycycarpa* Ruiz & Pav.	Tree
Violaceae	*Rinorea viridifolia* Rusby	Shrub
Vitaceae	*Cissus ulmifolia* (Baker) Planch.	Liana

Appendix 5. First Report of *Aspergillus flavus* on *Oxandra acuminata, Malmea diclina* and *Unonopsis matthewsii* seeds in Peru

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The trees *Oxandra acuminata*, *Pseudomalmea diclina* and *Unonopsis matthewsii* (Annonaceae) are sources of wood for houses and other construction for local and native people of the Amazonian region and harvested from natural populations. With the increasing human population and agricultural activities in the Amazonian region forest diversity is impacted. In order to manage the forest communities it is important to understand the dynamics of regeneration of forest plants. Diseases that impact seed and seedling survival are critical in determining ultimate composition of species in the Amazonian forests. During the dry season in June 2006, rotten seeds of *U. matthewsii* (about 90% of 380 seeds) were observed in natural conditions in 7 locations inside an area of 150 ha in lowland tropical forest in Manu National Park, Peru. Infected seeds were open and covered by yellow, dry, powdery, easily liberated conidia. One month later, seeds of *O. acuminata* located in the same locations presented the same infection. In August 2007, *P. diclina* seeds were naturally dispersed (animal dispersion) to these plots and presented similar symptoms. The disease affecting *O. acuminata* was found in two other sampling sites along the river Los Amigos, in Los Amigos research station.

In each occurrence of the disease the pathogen was identified as *Aspergillus flavus* based on morphological characteristics (1,2). Isolation of the pathogen was made on Potato Dextrose Agar (PDA) with chloramphenicol (100 mg/l) incubated at 28°C for 5 to 7 days. Single-spore isolations were made from each plant species and maintained as stock cultures.

For pathogenicity tests, healthy seeds from the three species, obtained from several trees, were previously surface sterilized by dipping in a 0.1% chlorine solution and allowed to dry. To inoculate seeds, a small scalpel was used to make a superficial cut on the seeds, after which conidial suspension (3×10^5 spores/ml distilled water) was pipetted over each wound. Each experiment included 20 inoculated and 20 control seeds; and this was replicated for seeds of each of the three species. Seeds were maintained at ambient temperature in a field station lab and evaluated daily for 10 days. Inoculated seeds of all three species showed symptoms identical to those seen in field populations. Infected seeds died after 2 to 7 days, showing the dry yellow conidia after approximately 1-2 days of decay. All of the control seeds remained healthy. The fungus was re-isolated from infected seeds and demonstrated features consistent with *A. flavus*.

Aspergillus flavus has been reported on numerous host plants worldwide (1,2). However, to our knowledge this is the first report of *A. flavus* causing high seed

mortality of species of Annonaceae in Peru. Our observations suggest that *A. flavus* is one of the important pathogens that impact survival of trees *Oxandra acuminata*, *Pseudomalmea diclina* and *Unonopsis matthewsii* in the natural plant communities where we conducted this study.

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Appendix 6. Supplemental Information for Light converts endosymbiotic fungus to pathogen, influencing seedling survival and host tree recruitment

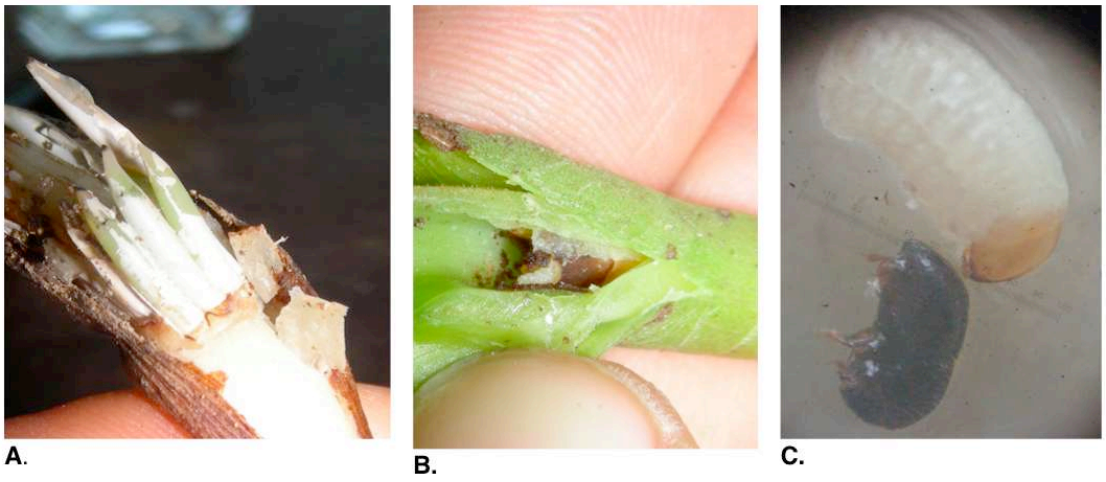
Description of *I. deltoidea* age groups. *Iriartea deltoidea* palms undergo ontogenic transitions in leaf morphology. The first seedling leaves have almost orbicular laminas 5–6 cm in diameter; subsequent leaves are elongate or obovate and longer; leaves longer than ca. 15 cm start producing one pair of pinnae below the elongate end segment and subsequent leaves are still longer an with increasing numbers of pinnae below the large end segment. The juvenile stages of *I. deltoidea* are characterized by pinnate blades in which the blade size and number of pinnae increase as the plant grows, but the pinnae remain flat and the blade two dimensional, even though the pinnae become wider and broadly triangular with increasing age of the plant. An aerial stem is absent in young juveniles, but develops in later stages and increases in both thickness and length throughout the juvenile development. When the stem has reached a few meters in height and the leaves are 1.5-2 m long, aerial stilt-roots start developing at the base of the stem. Eventually the palms reaches mature height with stems 15–20 cm thick and 6–8 meters tall. At this stage the leaves form blades in which the pinnae start dividing into plus/minus linear segments that orient themselves in various angles to the leaf rachis so the blade attains a three-dimensional "bushy" structure. This marks the transition to the subadult stage. The transition to the adults stage is marked by the production of reproductive structures (*S6*). All individuals located in the northeastern Peru transects and southeastern Peru plots were counted, identified to species, and assigned to one of four age classes [seedlings with 1-2 leaves without pinnae; juveniles –all plants larger than seedlings and smaller than adults; subadults – plants as big as adults, but not showing signs of having reproduced; adults – showing signs of having reproduced].

Isolation of endophytes colonizing *I. deltoidea.* Following standard endophyte isolation procedures (*S8*) we isolated *D. mutila* from leaflets of 3 healthy *I. deltoidea* juvenile leaflets. Petri plates containing Malt Extract Agar (MEA) (Becton Dickinson & Company), commonly used to grow endophytes, were previously prepared in a laboratory at Rutgers University, wrapped in individual plastic bags and stored inside a transparent plastic container (7.5 L) disinfected with 96% EtOH and 5% NaCl. Tweezers and needles used to manipulate leaves and fungi at CCBS were previously disinfected at Rutgers University. The tools were autoclaved at 121° C for 20 minutes, individually wrapped with sterile absorbent paper and aluminum foil and stored in a disinfected plastic container with 100 g of desiccant (WA Hammond Drierite Company). At CCBS we selected four *I. deltoidea* juvenile plants, smaller than 2 m and located in 4 trails separated by ~1 km. Two healthy mid leaflets were harvested from one healthy leaf, washed with cold boiled water and processed within 1 h of collection. Healthy leaves were defined as leaves undamaged by

foliar pathogens and/or insects. From each leaflet, we cut 3 adjacent, 2 mm^2 segments from the central part of the leaflet with a sterile scalpel. Leaflet segments were surface sterilized by sequential washes with detergent (Ajax), 70% ethanol (2 min) and 0.5% NaOCl (2 min), and rinsed with sterile water. We obtained the imprint for each side of each leaflet segment in Petri plates containing MEA (*S8*) to control for non-endophytic fungi. Each segment was plated in MEA and evaluated every day. *Diplodia mutila* was isolated from 2 segments (from a total of 36 fragments) from two plants located at different trails. The fungus *Colletotrichum gloeosporioides* was isolated from 6 fragments. Petri plates containing MEA used for the imprint did not have any mycelial growth indicating that fungi cultured from leaf segments were endophytic.

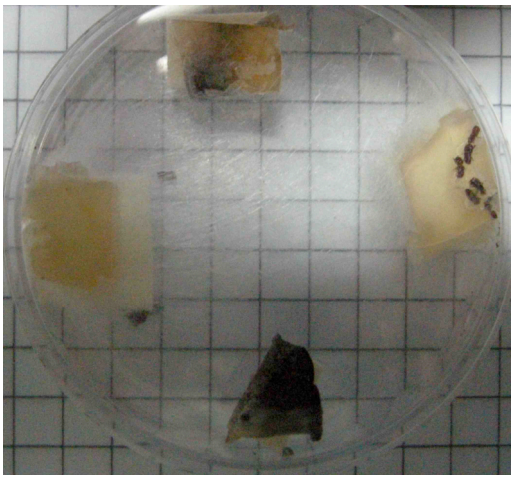
Insect interaction with *Colletotrichum gloeosporioides*

To investigate the possibility that *Coccotrypes* sp. shows avoidance of fungi in general, a similar experiment was set up using PDA colonized by *Colletotrichum gloeosporioides* found in *I. deltoidea* leaves (SI) and 3 beetles per Petri plate. Beetles showed preference for the *C. gloeosporioides* colonized PDA over PDA without the fungus, 1.4 ± 0.08 versus 0.5 ± 0.08, (Repeated Measurement Analysis, Random Effect $F_{4, 224} = 4.61$, $P = 0.0001$**, (mean number of beetles ± SE). These results indicate that *D. mutila* deters insect predation in culture and fruits and C. *gloeosporioides* attracts insect predators.



A. B. C.

Several different insect species were found inside the stems of seedlings of I. deltoidea, including (A) crickets and (B) larvae of unidentified insects for the order Coleoptera. (C) Adult and larvae of *Coccotrypes* sp. were found in *I. deltoidea* fruits and seeds.



Petri plate containing two food substrates: Pure Potato Dextrose Agar (PDA) on the right and left side of the Petri plate and PDA colonized by *Diplodia mutila* at the top and bottom of the Petri plate. *Coccotrypes* sp. preferred pure PDA (right side of the Petri plate) during the 8 to 12-days observational studies.

Appendix 7.

Seed Species: Dry season (18 plots)	Total
Agonandra brasiliensis	4
Allophylus scrobiculatus	80
Calatola microcarpa	5
Celtis iguanea	18
Cissus ulmifolia	20
Connarus sp. 1	1
Cordia ucayalensis	2
Euterpe precatoria	21
Inga edulis	2
Iriartea deltoidea	1
Maripa cf. axilloflora	15
Matayba purgans	12
Matayba RF 13269	11
Maytenus magnifolia	79
Nectandra longifolia	1
Oxandra acuminata	1
Rubiaceae sp. 1	48
Sarcaulis brasiliensis	1
Sloanea obtusifolia	6
Spondias mombin	5
Ocotea sp. red	2
Unonopsis matthewsii	164
Ziziphus cinnamomum	43
Grand Total	542

Seed Species: Wet season (24 plots)	Total
Allophylus glabratus	4
Batocarpus amazonicus	5
Buchenavia grandis	3
Casearia cf. decandra	16
Celtis iguanea	5
Chrysophyllum venezolanum	4
Cissus pseudosicyiodes	67
Cissus ulmifolia	42
Clarisia racemosa	5
Euterpe precatoria	27
Flacourtaceae	4
Guatteria accutisima	9
Inga sp. media	1
Inga sp. grande	2
Iriartea deltoidea	50
Leonia glycicarpa	28
Maripa peruviana	13
Matayba purgans	12
Matisia cordata	18
Matisia rhombifolia	3
Ocotea sp. 1	3
Ocotea sp. 2	3
Otoba parvifolia	8
Oxandra acuminata	163
Oxandra espintana	1
Paullinia sp. 1	22
Pseudolmedia laevis	2
Rheedia acuminata	5
Rheedia brasiliensis	4
Sapium marmieri	22
Socratea exhorriza	6
Sorocea pileata	7
Spondias mombin	4
Strichnos asperula	2
Strichnos erichsonii	18
Theobroma cacao	3
Grand Total	591

Curriculum Vita

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Education

- May, 2009 PhD Degree, Ecology and Evolution, Rutgers The State University of New Jersey, New Brunswick, New Jersey
- 1999 Master Degree, Forestry, West Virginia University, Morgantown, West Virginia
- 1996 Bachelor Degree, School of Agriculture, Universidad Nacional Agraria La Molina, Lima, Peru (class salutatorian).

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