Light converts endosymbiotic fungus to pathogen, influencing seedling survival and host tree recruitment

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Endophytic fungi that asymptomatically colonize plants [1] are diverse and abundant in tropical ecosystems [2]. These organisms can be weakly pathogenic [3] and/or mutualistic, frequently enabling plants to adapt to extreme environments, alter competitive abilities of host individuals and improve host fitness under abiotic or biotic stresses [4,5,6]. *Diplodia mutila* is a symbiotic endophyte/plant pathogenic fungus infecting the palm *Iriartea deltoidea* [7], 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 favors 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 [8-10] and the Chocó- and Central American region [11,12]. In contrast to most large palms [13], this species does not depend on large forest gaps for recruitment [14], perhaps related to its peculiar growth strategy [15]. 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 [10]. A partial explanation may be found in the fact that palms have tougher leaves than dicots and thus are less susceptible to insect herbivory [16]. In this study we investigate the influence of a common pathogen-endophytic fungus, *Diplodia mutila*, on *I. deltoidea* survival and recruitment. *Diplodia mutila* [17] 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 [7] (Fig. 1A-D). 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 [18] (Fig. 1E). In its endophytic phase the fungus exists only as mycelium within tissues of the host's leaves, stems and seeds [1]. *Diplodia mutila* and related species have been reported as endophytes or latent pathogens for several plant species worldwide [19,20]. This fungus is frequently an asymptomatic endophyte in leaves of healthy juvenile and mature plants, as well as fruits and seeds of *I. deltoidea* [7].

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" [10]. *Iriartea deltoidea* seedlings and juveniles are relatively abundant in proximity to adult trees. Demographic censuses of 518 *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 [21,22]. 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).

We found evidence that *D. mutila* benefits its host plants by enhancing resistance to herbivory by some insects. 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 [23,24].

78 This study found that *Diplodia mutila* is beneficial to the plant in understory
79 conditions but strongly reduces the capacity of *I. deltoidea* to recruit in high-light forest
80 gaps. Seedlings of *I. deltoidea* preferentially occur under shady conditions. Extensive
81 sampling at two sites in western Amazonia found out that approximately ~92% of *I.*
82 *deltoidea* seedlings were found in understory conditions. Additionally, the foliar
83 necrotic spot symptoms produced by *D. mutila* appeared more frequently in seedlings
84 and juveniles that grew in gaps or diffusely open canopy conditions. Plants with visible
85 symptoms caused by *D. mutila* received significantly higher illumination, 408.3 ± 17.3,
86 than plants with no visible symptoms, 208.2 ± 6.1, $P < 0.0001$, *t* test, n = 808 (mean
87 μmol m^{-2} s^{-1} ± SE). Disease development was faster and more lethal in seedlings with
88 two leaves or less when exposed to higher light conditions ($F_{1,22} = 55.4$, $P = 0.0001$,
89 $r^2 = 0.73$), (Fig. 2A). Ontogenic or age-related resistance may be responsible for
90 differences in disease expression between seedlings in different stages of development
91 [25]. An additional experiment showed that pathogenicity of *D. mutila* increased with
92 light availability. We inoculated 22 healthy 6-month old *I. deltoidea* seedlings (no
93 foliar spots or insect marks) with *D. mutila*, following inoculation procedures from
94 previous studies [7]. Foliar spots produced by *D. mutila* had a higher growth rate and
95 mortality was greater and faster at higher light availability ($F_{1,22} = 93.26$, $P = 0.0001$,
96 $R^2 = 0.816$) (Fig. 2B).

97 Using transplant experiments we demonstrated that increased light availability
98 switched the endosymbiotic phase of the fungus to its pathogenic phase. Diametric
99 growth rate of foliar spots produced by *D. mutila* was higher and faster in full sun
100 conditions, 19.5 ± 2.5 cm/day, than in reduced light, 10.0 ± 2.5 cm/day, and shaded
101 conditions 0.52 ± 2.5 cm/day, analysis of variance (ANOVA), $F_{3,30} = 12.62$, $P =$
102 0.0001, (mean ± SE) (Fig 3). *Diplodia mutila*-induced seedling mortality in plants
103 exposed to full sun was 80% after 10 days. Seedlings under shaded conditions had 10%
104 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. 4A). 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 [26]. 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. 4B, Fig. 4D). Similar significant results were obtained for colonies growing in WA medium (Fig. 4B.)

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 [27]. Endophytes in many plants have been shown to provide hosts with increased herbivore and/or environmental stress resistance [4-6]. 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.

Methods

Demographic Censuses

In northeastern Peru 28 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) [29] and Los Amigos, (LABS) [30]. 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.*

158 *deltoidea* plants were tagged with numbered plastic tags and mapped in an X - Y
159 coordinate system. The total number of plants located in the 10 plots was 1068: 63
160 fruiting adults, 518 seedlings and 487 were considered juveniles-adults (non-fruiting)
161 (SI). We measured height of the tallest photosynthetic leaf (cm) and number of leaves
162 and diameter of foliar spots caused by *D. mutila* (cm) for all seedlings. The amount of
163 disease was calculated by dividing the diameter of the foliar spot by the diameter of the
164 affected leaf and expressed as '% disease'. Disease development over a period of 150
165 days was calculated by subtracting the % disease in the initial estimate from the final
166 estimate.

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

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

184 0.01%, 2% ± 0.01%, 0.7% ± 0.01% ($F_{4,50}$ = 4.28, P > 0.0051*). Stem borer attack
185 decreased in the last census.

186 **Light availability measurement.**

187 In northeastern Peru light availability was measured using the canopy scope
188 methodology [31]. In southeastern Peru light availability was estimated above the tallest
189 photosynthetic leaf of each *I. deltoidea* seedling, using the average value of light
190 intensity over the leaf with a light meter (Environmental Concepts Plant Light Intensity
191 Meters, LIM2500, USA). The average value was obtained from three measurements
192 over each plant at 6 am, 12 pm and 5 pm for three consecutive days. The total number
193 of seedlings in the northeastern Peru transects was 660, 94% of seedlings were located
194 at understory conditions (canopy scope <5). The negative correlation between the
195 number of *I. deltoidea* seedlings and canopy openness in the 102 transects (Spearman *r*
196 = -0.117, $P < 0.05$) was estimated using, n = 280 5-m × 5-m subplots with at least one *I.*
197 *deltoidea* seedling. Statistical significance was assessed as a one-tailed test and
198 correcting for spatial autocorrelation using Dutilleul's approach for computing the
199 geographically effective degrees of freedom = 227 [32]. The total number of seedlings in
200 the southeastern Peru plots was 518 seedlings (less than 25 cm), 91% seedlings were
201 located in dense understory (~55-120 ± 15 μmol m^{-2} s^{-1}).

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

203 On December 2007, ~370 *Coccotrypes* sp. beetles and larvae were extracted from
204 more than 100 fruits and seeds of *I. deltoidea*. In a Petri plate (60 x 15 mm, Fisher
205 Scientific Co. Canada) we placed two 1-cm^2 of PDA (Potato Dextrose Agar) and two 1-
206 cm^2 of PDA infested with *D. mutila*, covered with squares of non-acidic paper to
207 simulate dark conditions found inside seeds and fruits. PDA was replaced everyday for
208 the duration of the experiment to avoid contamination of non-infested PDA by *D.*
209 *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 (SI) 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 (SI).

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° C ± 3 and 26 ° 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 was 24° C).

Supplementary Information accompanies the paper on **www.nature.com/nature**.'

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Figure 1 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 2 Higher light intensities increased disease development produced by *Diplodia mutila*. **(A)** For young seedlings with 2 leaves or less there was a

259 significant interaction between amount of infection (% of *D. mutila* foliar spots in
260 *Iriartea deltoidea* leaves) and light level ($F_{1,22}$ = 55.4, P = 0.0001**, r^2 = 0.73).
261 **(B)** The diametric growth rate of the foliar spots produced by *D. mutila* was
262 higher at higher light conditions ($F_{1,22}$ = 93.26, P = 0.0001**, r^2 = 0.816).

263

264 **Figure 3** Increased light availability switched the endosymbiotic phase of *D.*
265 *mutila* to its pathogenic phase. Young seedlings that were colonized with
266 endophytic *Diplodia mutila* showed faster growth rates of diameter of foliar
267 spots (cm) caused by the pathogenic phase of *D. mutila* at higher light
268 intensities (~1058 ± 23 µmol m^{-2} s^{-1}) than seedlings under shaded conditions
269 (~55 ± 15 µmol m^{-2} s^{-1}) (n = 30, *t* test, P = 0.0001**). There were also
270 significant differences of foliar spot growth rates among plants growing in the
271 greenhouse (~491 ± 34 µmol m^{-2} s^{-1}) and plants growing under shaded
272 conditions (n = 30, *t* test, P = 0.024*). Foliar spot growth rates among plants
273 growing in the greenhouse were lower than plants growing under high light
274 intensities (n = 30, *t* test, P = 0.013*), (Tukey Kramer ANOVA, $F_{3,30}$ = 12.62, P
275 = 0.0001**).
276

277 **Figure 4 (A)** Mycelial radial growth of *Diplodia mutila* on Potato Dextrose Agar
278 (PDA) was faster under a 12-hour cycle than the 3-hour cycle ~1.25 (± 0.03)
279 cm/day vs. 1.11 (± 0.03) cm./day (n = 12, *t* test, P > 0.018*). On Water Agar
280 (WA) the average radial growth rate per day of the colony mycelium was ~0.51
281 cm/day under a 12-hour light cycle; while under a 3-hour light cycle the average
282 growth rate of the colony mycelium was significantly lower, at ~0.41 cm/day
283 after 7 days (n = 12, *t* test, P > 0.004***). (B)** Colonies grown in PDA under the
284 12-hour light cycle had a more rapid melanization of the central area of the

285 colony (~0.71 cm/day) than colonies exposed to 3-hours of light (~0.5 cm/day)
286 (n =12, *t* test, *P* > 0.022*). Similar results were obtained for colonies growing in
287 WA (n =12, *t* test, *P* > 0.0258*). **(C)** Melanization of colonies of *D. mutila*
288 growing in PDA observed on 4 days (from left to right). Rate of melanization
289 was reduced in the 3-hour cycle treatment (above). Faster melanization was
290 observed in cultures maintained in a 12-hour light cycle (below).

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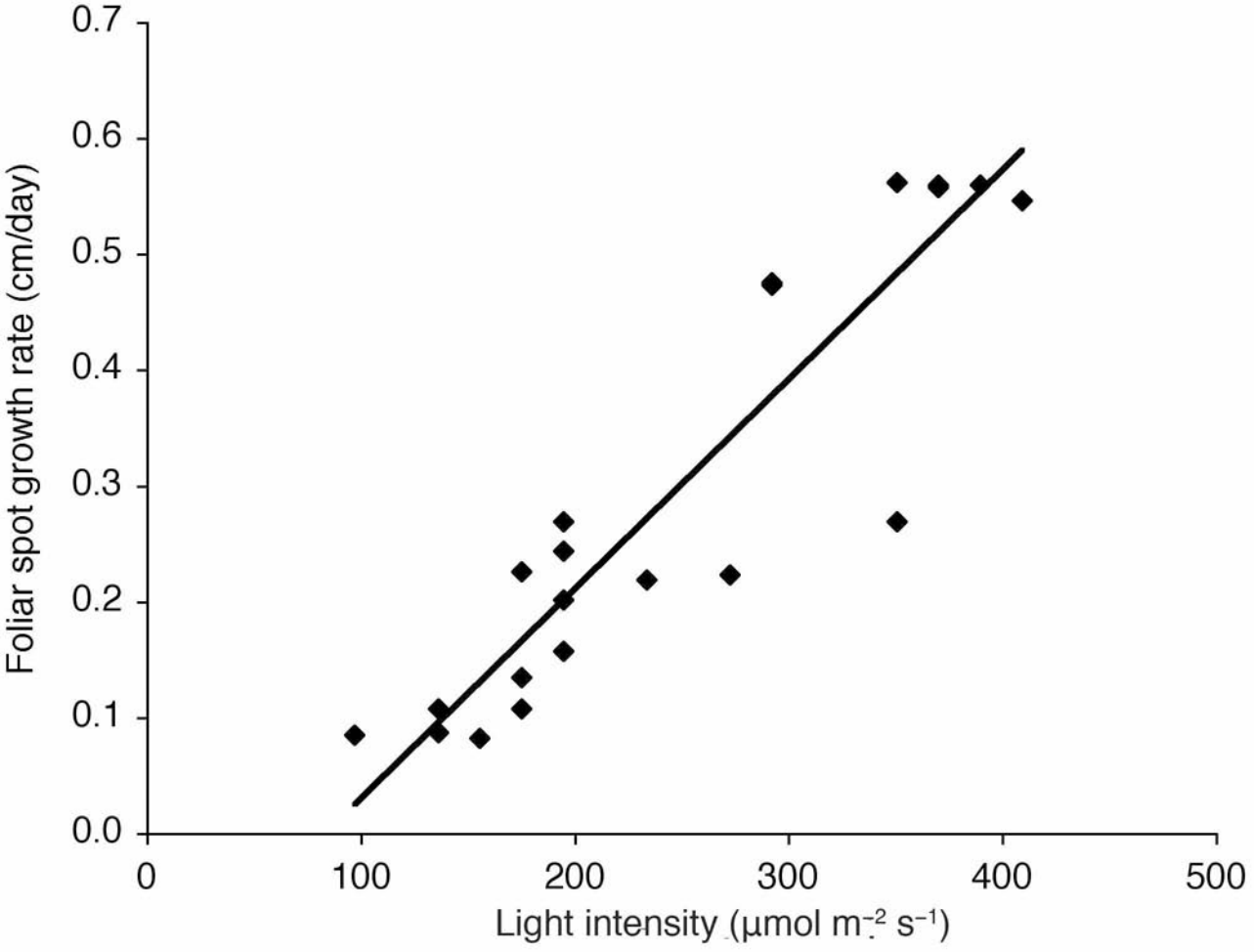
A


B


C


D


E
10 µm


0.7
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0.5
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0.3
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Foliar spot growth rate (cm/day)
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400
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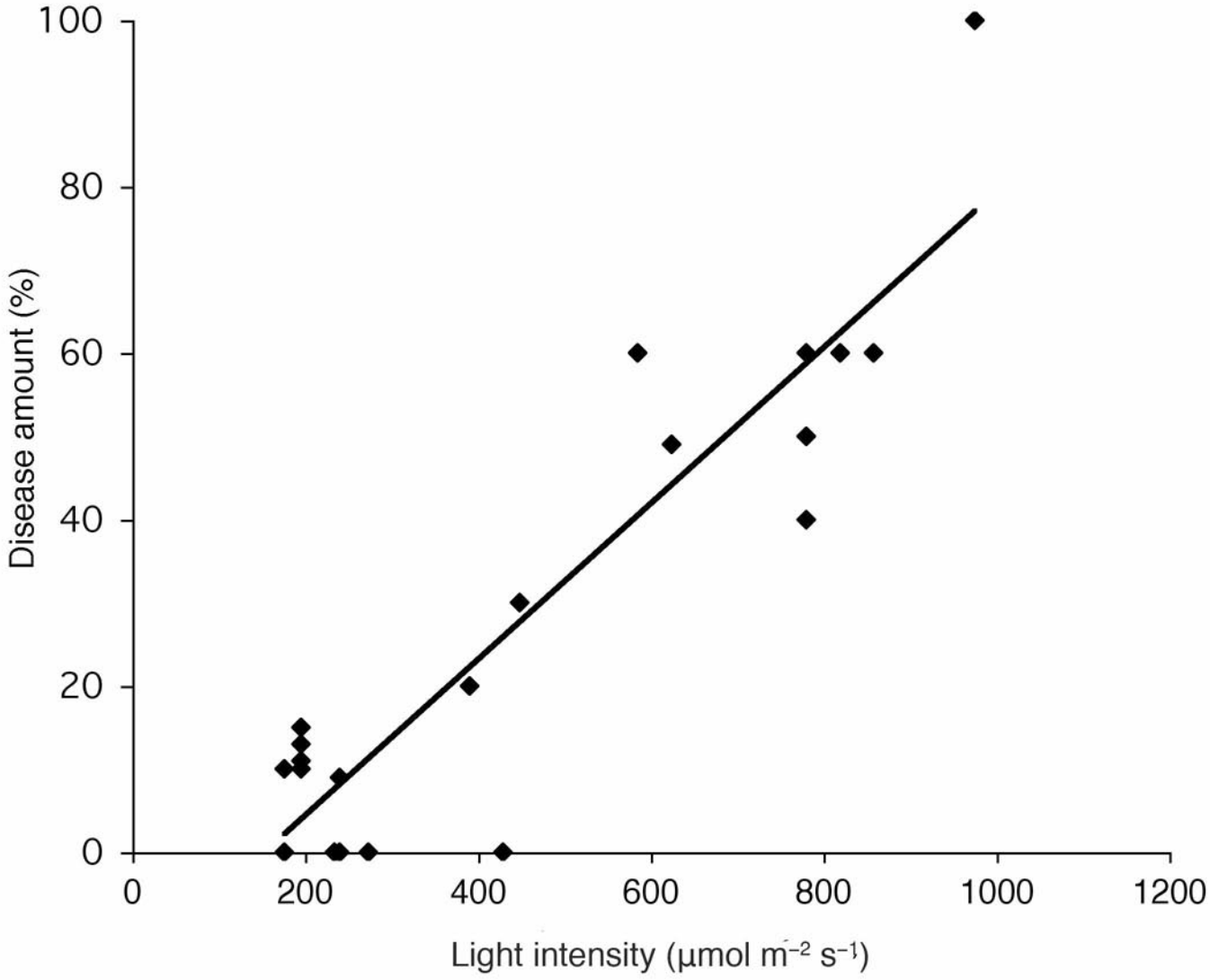
Light intensity (µmol m-2 s-1)


100
80
60
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20
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Disease amount (%)
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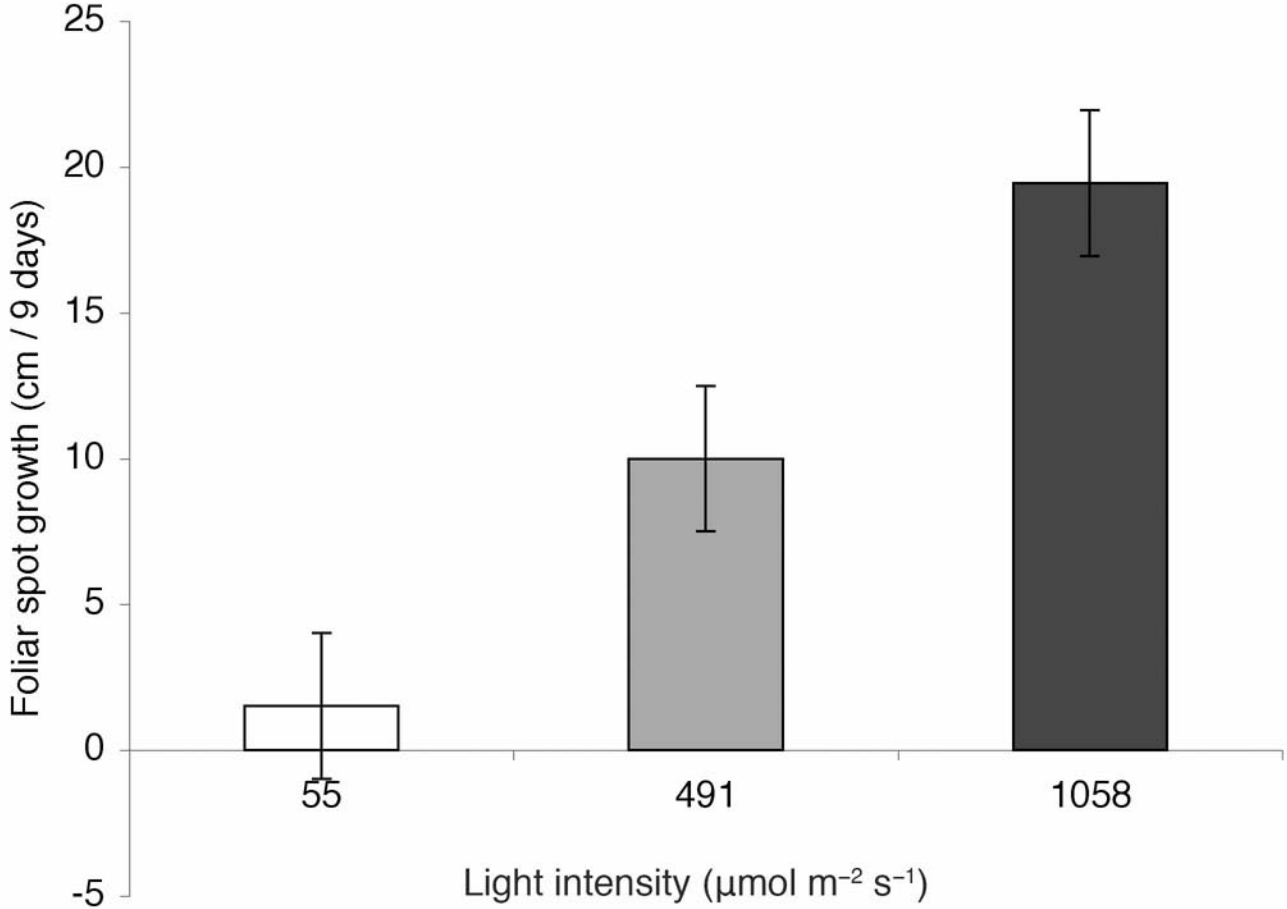
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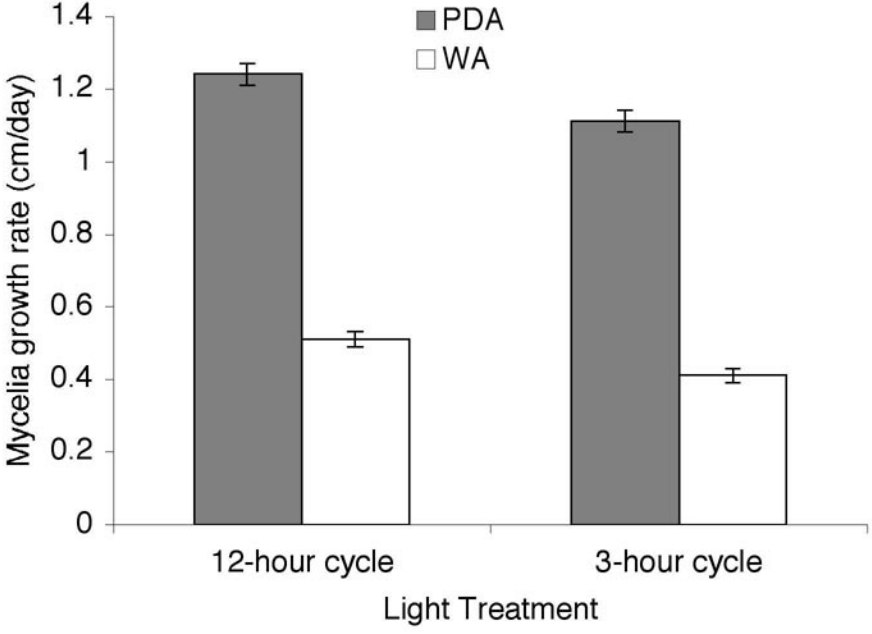
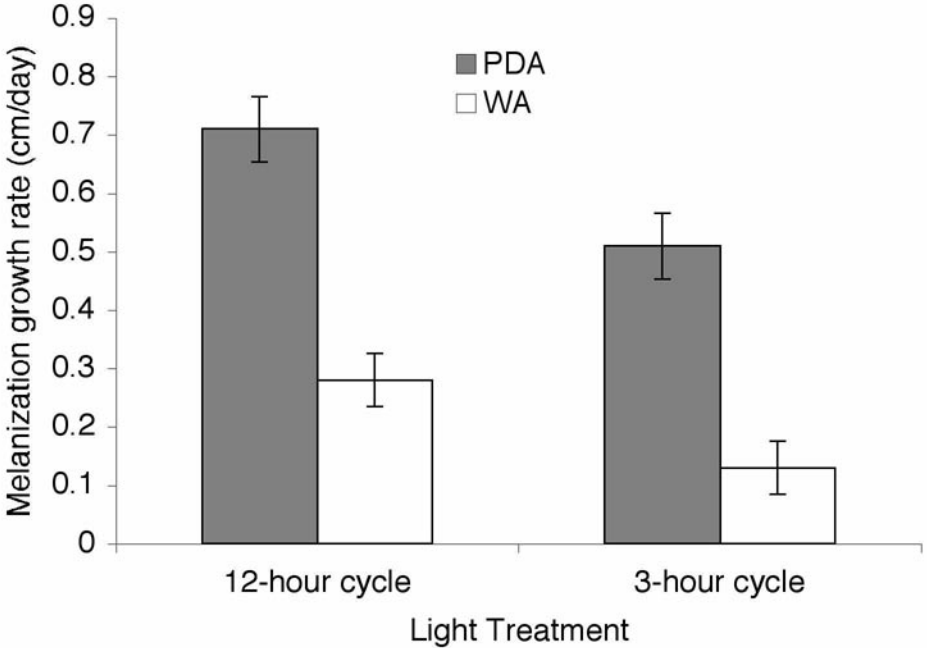
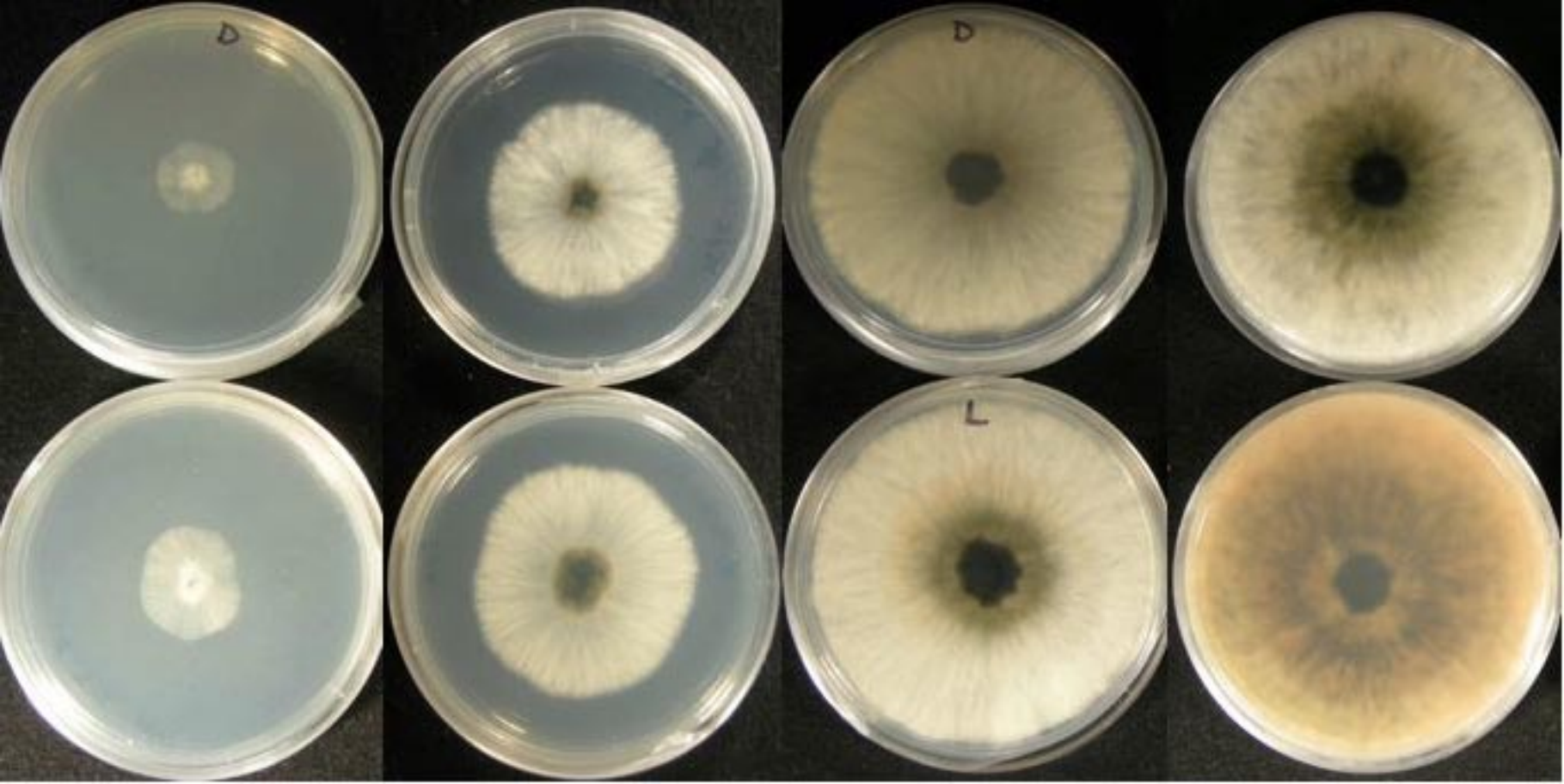
10
5
0
-5
Foliar spot growth (cm / 9 days)
55
491
1058
Light intensity (µmol m⁻² s⁻¹)


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mycelia growth rate (cm/day)
PDA
WA
12-hour cycle
3-hour cycle
Light Treatment


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Melanization growth rate (cm/day)
PDA
WA
12-hour cycle
3-hour cycle
Light Treatment


D
D
L