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1 **The role of herbivorous insects and pathogens in the regeneration**
2 **dynamics of *Guazuma ulmifolia* in Panama**

3

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39

40 **Abstract** A significant proportion of mortality of rainforest trees occurs during the early
41 life stages (seeds and seedlings), but mortality agents are often elusive. Our study
42 investigated the role of herbivorous insects and pathogens in the early regeneration
43 dynamics of *Guazuma ulmifolia* (Malvaceae), an important tree species in agroforestry
44 in Central America. We reared pre-dispersal insect seed predators from *G. ulmifolia*
45 seeds in Panama. We also carried out an experiment, controlling insects and pathogens
46 using insecticide and/or fungicide treatments, as well as seed density, and compared
47 survivorship of *G. ulmifolia* seeds and seedlings among treatments and relative to
48 untreated control plots. We observed (1) high pre-dispersal attack (92%) of the fruits of
49 *G. ulmifolia*, mostly by anobiine and bruchine beetles; (2) negligible post-dispersal
50 attack of isolated seeds by insects and pathogens; (3) slow growth and high mortality (>
51 95%) of seedlings after 14 weeks; (4) low insect damage on seedlings; and (5) a strong
52 positive correlation between seedling mortality and rainfall. We conclude that for *G.*
53 *ulmifolia* at our study site the pre-dispersal seed stage is by far the most sensitive stage
54 to insects and that their influence on seedling mortality appears to be slight as compared
55 to that of inclement weather. Thus, the regeneration of this important tree species may
56 depend on effective primary dispersal of seeds by vertebrates (before most of the seed
57 crop is lost to insects), conditioned by suitable conditions in which the seedlings can
58 grow.

59

60 **Keywords** Anobiidae; fungal pathogen; negative density-dependence;
61 rainforest; seed predation

62

63 **Introduction**

64 In tropical rainforests a significant proportion of tree mortality occurs during the
65 early life stages of the plant (seeds and seedlings), and patterns of mortality at

66 these early stages of life may be critical in influencing the diversity of older
67 individuals (Massad 2013; Green et al. 2014). There is growing evidence that
68 interactions between plants and host-specific insect herbivores and pathogens
69 could be a central mechanism for explaining plant diversity (Leigh et al. 2004). A
70 mechanism proposed to facilitate the coexistence of tree species in tropical forests
71 is negative density-dependence or the so-called Janzen-Connell effect (Janzen
72 1970; Connell 1971; Bell et al. 2006; Comita et al. 2010; Bagchi et al. 2014).
73 Seeds are most likely to disperse to sites near their parent trees, but seeds and
74 seedlings are also more likely to survive away from parents where they are less
75 likely to be attacked by host-specific insects and pathogens, which may disperse
76 contagiously from parent trees. Negative density-dependence is the result of the
77 proliferation of species-specific herbivores and pathogens among hosts in areas of
78 high conspecific plant densities, and the negative correlation between enemy
79 propagation and the distance from parent trees to their nearby offspring (Janzen
80 1970; Connell 1971; Bell et al. 2006; Comita et al. 2010; Bagchi et al. 2014).
81
82 Insects, more than herbivorous mammals, are responsible for most herbivory on
83 seedlings in tropical rainforests (Cruz and Dirzo 1987). In addition, many insects, but
84 very few mammals, preferentially attack seeds or plants near conspecific adults
85 (Hammond and Brown 1998). While there have been many studies assessing insect
86 herbivory (foliar damage) and resulting seedling mortality (review in Massad 2013),
87 community studies targeting the insects feeding on rainforest seedlings are relatively
88 rare (Basset 1999; Alvarez-Loayza and Terborgh 2011). Ecologists often infer the
89 impact of insect herbivores on plants by measuring the apparent or rate of leaf damage
90 (Coley 1983; review in Lowman 1984), because of the relatively low density of
91 herbivores in tropical rainforests (Novotny and Basset 2000). However, such studies are

92 known to underestimate the magnitude and impact of herbivory significantly, because
93 herbivores may eat whole leaves, or disproportionately damage buds and young or
94 expanding leaves (Lowman 1984). This issue may be critical during the seed-to-
95 seedling transition (germinating seed or very young seedling). Although damage by sap-
96 sucking insects on seeds or seedlings may be difficult to quantify, it may nevertheless
97 lower plant fitness significantly via direct action or the spreading of diseases (Nault and
98 Ammar 1989).

99

100 Seedling establishment and survival in tropical rainforests may also be influenced by
101 pathogens, especially fungal pathogens (Augspurger 1984; Gilbert et al. 1994; Mangan
102 et al. 2010; Bagchi et al. 2014). For example, Augspurger (1984) reported that on Barro
103 Colorado Island in Panama, dependent on the tree species, 2-74% of seedlings may be
104 dead from disease two months after germination. Disease incidence is often host-density
105 dependent and may have the potential to regulate host spatial distribution (Augspurger
106 1984; Gilbert et al. 1994). Further, interactions between herbivory and fungal pathogens
107 may in some cases be significant and affected by anthropogenic disturbance (Benítez-
108 Malvido et al. 1999).

109 One way to assess the effect of insect herbivores or fungal pathogens on rainforest seeds
110 or seedlings is to exclude them with (a) insecticide and/or fungicide or (b) mesh
111 enclosures in treatment plots and compare survivorship of seeds or seedlings between
112 treatment plots and control plots (Swamy and Terborgh 2010; Bagchi *et al.* 2014).

113 Bagchi *et al.* (2014) used method (a) to study the role of insects and fungal pathogens in
114 causing mortality to seeds and young seedlings in a rainforest in Belize. They found that
115 spraying insecticides on seeds and germinating seedlings increased overall seedling
116 survival dramatically (by a factor of 2.7). The benefits of insecticide application varied
117 among plant species, so that excluding insects changed the structure of the seedling

118 community considerably. These initial results suggest that insects and pathogens have
119 an important role in driving plant dynamics and diversity.

120

121 Several studies have used insecticide and fungicide applications to assess the role of
122 insects and pathogens in maintaining plant diversity and causing conspecific negative
123 density-dependent plant survival as predicted by the Janzen-Connell hypothesis (e.g.
124 Bell *et al.* 2006; Bagchi *et al.* 2014; Gripenberg *et al.* 2014; Fricke *et al.* 2015).

125 However, to our knowledge, none of the insecticide studies have followed the fate of the
126 seeds and seedlings in close enough detail to allow any conclusions to be drawn
127 regarding which stage in the early life of plants was most likely to suffer from enemy
128 mediated mortality, and how insecticide treatment would impact insect herbivores
129 specific to these stages. Moreover, since most studies assessing the role of enemies on
130 plant recruitment have applied insecticides and fungicides separately, little is known
131 about the potential interactive effects of insect and pathogen attacks (e.g. Bagchi *et al.*
132 2014; Gripenberg *et al.* 2014).

133

134 The main objectives of this study were to investigate the impacts of insect herbivores
135 and fungal pathogens on the early regeneration of a tree species of great importance in
136 agroforestry in Central America and Mexico, *Guazuma ulmifolia* Lam. (Batis *et al.*
137 1999). *Guazuma ulmifolia* is one of the most common trees in agricultural areas in
138 Central America, where it grows in fields, primary and secondary forests (Orwa *et al.*
139 2009). Elucidating the major causes of mortality of this species at the early stages can
140 help drafting recommendations to foster the regeneration of this species and forest
141 reestablishment during the process of rehabilitation of degraded soils (Griscom *et al.*
142 2009).

143

144 Our protocol involved assessing both the damage on seeds caused by insect predators
145 and the potential joint effects of insect herbivores and fungal pathogens on seed
146 germination and the survival of young seedlings. We then evaluated (1) which stage
147 (seed, seed-to-seedling transition, seedling) is particularly vulnerable to insect
148 herbivores and pathogens; (2) whether the negative impact of insect herbivores at the
149 seedling stage is higher than that of fungal pathogens; and (3) whether seed or seedling
150 density affects the action of insect herbivores and fungal pathogens, as predicted by the
151 Janzen-Connell hypothesis (Alvarez-Loayza and Terborgh 2011).. Finally, with these
152 data in hand, we briefly discuss their implication for the regeneration of *G. ulmifolia* in
153 agroforestry landscapes of Central America.

154

155 **Material and methods**

156 ***Study sites***

157 Insect seed predators were reared from *Guazuma ulmifolia* fruits collected on Barro
158 Colorado Island (BCI) and at a nearby site, El Charco (ELC). As the use of insecticide
159 is not allowed on BCI, our experiment assessing the role of insects and pathogens at the
160 seed-to-seedling transition stage was conducted at ELC. Barro Colorado island is a
161 biological reserve (9.15°N, 79.85°W, elevation 120-160 m) of 1,500 ha covered by
162 lowland rainforest and created by the opening of the Panama Canal in 1914. A
163 permanent ForestGEO vegetation plot (Anderson-Teixeira et al. 2014) of 50 ha is
164 located in the centre of the island. The annual average rainfall is 2551 mm and annual
165 average daily maximum air temperature is 26.3 °C. Details about the island and the
166 rainforest plot are reported in Anderson-Teixeira et al. (2014). ELC (9°502.58"N,
167 79°39048.24"W, elevation 100 m) is ca 20 km from BCI and is part of the 19,545 ha
168 Soberania National Park. There is a one ha ForestGEO plot at ELC and salient
169 differences between the vegetation on BCI and that of ELC and other ForestGEO plots

170 in the area are discussed in Pyke et al. (2001). ELC is a secondary rainforest while BCI
171 includes both mature secondary forest and old-growth forest. Annual average rainfall is
172 also lower (2050 mm) at ELC than at BCI (Pyke et al. 2001).

173

174 ***Study plant***

175 *Guazuma ulmifolia* (Lam. 1789, Malvaceae, "Guácimo" in Panama) was selected for
176 this study because of the relatively high availability of seeds at the study sites. It is also
177 an important tree species in agroforestry, being used for firewood, medical treatment,
178 the production of living fences and rehabilitation of degraded soils by fostering forest
179 regeneration (Batis et al. 1999; Griscom et al. 2009). Other advantages of selecting this
180 species in the context of our study included long-lived seeds (+18 months, Sautu et al.
181 2006) and rather quick germination (10-143 days after sowing, Roman et al. 2012;
182 average 52.5 days: Sautu et al. 2006).

183

184 Of the seven species in the *Guazuma* genus, *G. ulmifolia* is the only one to occur in
185 Panama (Robyns and Cautrecasas 1964). The tree is 4-25 m tall, with a broad and
186 irregular crown and grey bark. It grows at low or medium elevation, in lowland dry or
187 wet forests, from Mexico to Paraguay, most commonly in areas where annual rainfall is
188 700-2500 mm (average 1,956 mm), and in a variety of soils (Francis 1991; CONAFOR
189 2018). In Panama, it is commonly observed on the Pacific side, where it flowers and
190 produces fruits from January to July. The species is partially deciduous during the dry
191 season (Roman et al. 2012). *Guazuma ulmifolia* is a pioneer species and usually
192 constitutes only a small percentage of basal area in secondary forests (Francis 1991). On
193 BCI, the 50 ha plot of old growth forest includes 64 individuals of this species
194 (ForestGEO 2018). At ELC *G. ulmifolia* is not present in the small one ha plot (and was

195 also not recorded within or near our experimental blocks) but is relatively frequent
196 nearby (i.e., within 500 m).

197

198 Individual *Guazuma* trees can produce up to 5,000-10,000 fruits per crop. The capsule
199 of the fruit is elliptical or hemispherical, measuring between 18-25mm long, and 14-
200 22mm wide. When mature, it is black (Plate S1), contains a hydrophilic mucilage, and
201 includes 60-68 small seeds averaging 2 mm length and 0.0042 g dry weight (Robyns
202 and Cautrecasas 1964; Francis 1991; Roman et al. 2012). Fruits usually mature from
203 September to April and may be observed for long periods on trees (Batis et al. 1999).
204 Fruits are eaten by peccaries, tapirs, deer, agoutis and squirrels, as well as domestic
205 livestock; bats are efficient seed dispersers (Janzen 1983; Roman et al. 2012). In
206 Mexico and Costa Rica 12-42 % of seeds have been reported to be destroyed by several
207 species of bruchine beetles (Johnson and Kingsolver 1971; Janzen 1975, 1983).

208

209 ***Insect rearing from seeds***

210 For rearing insects we collected 320 fruits freshly fallen (without apparent
211 decomposition) from *G. ulmifolia* on BCI. They were obtained between 2011-2013 from
212 22 trees (average 14.5 fruits collected per tree). To assess pre-dispersal attack (i.e.,
213 while seeds are still on trees) of *G. ulmifolia* fruits, in 2016 we collected 100 fruits
214 directly from the crown of 5 trees near the 1ha plot in ELC (20 fruits collected per tree).
215 In both cases fruits were stored in individual plastic pots, lined with tissue paper and
216 covered with 1 mm mesh netting for ventilation and to avoid subsequent
217 colonization/contamination of fruits. Rearing pots were checked twice weekly, and any
218 emerging insects were collected, preserved, mounted and then identified with the
219 assistance of taxonomists (see Acknowledgements) and/or with molecular techniques
220 (Ratnasingham and Hebert 2013). Fruits were stored for 3 months and then dissected to

221 check for the presence of developing larvae. We obtained DNA Cytochrome c oxidase
222 subunit I (COI, ‘DNA barcode’) sequences from legs of representative insect specimens
223 and we used Barcode Index Numbers (BINs) derived from insect sequences to delineate
224 species (Ratnasingham and Hebert 2013). Molecular data were uploaded in the Barcode
225 of Life project “BCISP” (<http://www.barcodinglife.org/>). Insect vouchers were
226 deposited at the Fairchild Museum and Smithsonian Tropical Research Institute in
227 Panama.

228

229 ***Monitoring of seeds and seedlings***

230 Ten experimental blocks (B01 to B10) were set up near the permanent plot at ELC
231 (coordinates in Table S1; the distance between blocks varied from 16 to 69 m). Each
232 block (5.5m x 2.5m) consisted of 8 quadrats of 1 m², in which 4 treatments were applied
233 to two seed densities (10 and 25 seeds per m², see below). Control quadrats (coded C10
234 and C25) were sprayed with water, insecticide quadrats (I10 and I25) were sprayed with
235 insecticide, fungicide quadrats (F10 and F25) were sprayed with fungicide, and
236 combined treatment quadrats (I+F10 and I+F25) were sprayed with insecticide and
237 fungicide. Quadrats (one replicate of each treatment combination per block) were
238 located at random within each block (Table S1). Litterfall traps on BCI (Wright et al.
239 2004) were used to estimate natural *G. ulmifolia* seed densities. In 2013, average seed
240 density of *G. ulmifolia* at ELC was 1 seed m⁻² y⁻¹ (n=50 traps, L. Markesteijn and O.
241 Lewis unpubl. data). On BCI, for the period 1987-2011, seed density for this species
242 was 6.1 seeds m⁻² y⁻¹ (n=200 traps, Wright et al. 2004; S.J. Wright unpubl. data). The
243 density of seeds buried in the soil was much lower, < 0.6 seeds m⁻² (Dalling et al. 1997).
244 Hence for our experiment we considered a high natural seed density of 10 seeds m⁻² y⁻¹
245 and a very high seed density of 25 seeds m⁻² y⁻¹.

246

247 We used the insecticide ENGEO 247 SC (Syngenta SA, Basel, Switzerland) with active
248 ingredients thiamethoxam (a neonicotinoid) 141 g L⁻¹ and lambda-cyhalothrin (a
249 pyrethroid) 106 g L⁻¹. According to the manufacturer, thiamethoxam has root-, leaf- and
250 stem-systemic activity, while Lambda-cyhalothrin has a strong contact action. We used
251 0.0025ml m⁻² in 50 ml sprayed on each quadrat every week, as recommended by the
252 manufacturer and used in previous studies (e.g. Bagchi et al. 2014). ENGEO is a broad-
253 spectrum insecticide, commonly used against herbivorous insects in crops. At the
254 volume used here, ENGEO has very few ecotoxic and health risks. Similarly, we used
255 the fungicide AMISTAR TOP 325 SC (Syngenta SA, Basel, Switzerland), with active
256 ingredients difenoconazole 125 g L⁻¹ and azoxystrobin 250 g L⁻¹. AMISTAR was
257 sprayed at levels of 0.1ml m⁻² in 1000 ml on each quadrat every week, as used by
258 Bagchi et al. (2014) and at slightly higher level than recommended by the manufacturer
259 (0.05 ml m⁻²). Difenoconazole is a broad-spectrum fungicide, while azoxystrobin is a
260 systemic fungicide commonly used in agriculture.

261

262 In total 1,400 fresh seeds of *G. ulmifolia*, collected from different trees near ELC, were
263 sown into the experimental blocks in May 2016, at the onset of the rainy season. Prior
264 to sowing, capsules were opened and seeds were checked for insect damage. Only
265 apparently intact seeds were sown. To mimic near natural conditions, seeds were not
266 treated to increase germination rates (Sautu et al. 2006). Blocks were first cleared of
267 litter and seeds, and seeds of *G. ulmifolia* were then sown and the litter carefully
268 replaced. Within each quadrat seeds were sown individually in 25 areas of 20 x 20 cm
269 delineated by a sowing frame. In quadrats with a seed density of 10 seeds m⁻² y⁻¹, seeds
270 were positioned in 10 randomly selected 20 x 20 cm areas, whereas at a seed density of
271 25 seeds m⁻² y⁻¹, each seed occupied one of the 25 areas. Each seed was coded and
272 marked with a plastic coffee stirrer for the purpose of monitoring. After sowing, each

273 quadrat was sprayed weekly with a hand mister with 50 ml of water (control) or with 50
274 ml of the corresponding treatment for a total duration of 14 weeks (to August 2016).
275 After this date surviving seedlings were measured and monitored at a lower frequency
276 until May 2017, one year after the start of the experiment. Every week, the status of
277 each seed or seedling was scored individually as Seed (no germination), Seedling
278 (germination occurred, seedling growing, no insect damage), Damaged (seedling
279 growing with damage) and Dead (seedling germinated but subsequently died). We
280 initially planned to score seedlings in the Damaged category further using 10 different
281 subcategories (including chewing, mining, galling, skeletonizing, etc.), but only three of
282 these categories proved to be useful for *G. ulmifolia* seedlings: (a) leaf chewing (on side
283 of leaves; possible agents Lepidoptera and Orthoptera); (b) shrivelled and/or necrotic
284 areas (possible agent: fungi); and (c) unknown reason (i.e., damage could not be
285 attributed with certainty). Seedlings of species other than *G. ulmifolia* were removed
286 from quadrats throughout the monitoring period. At the end of the experiment, we
287 measured the height of surviving seedlings.

288

289 ***Statistical analyses***

290 We first ensured that there was no or little spatial pseudoreplication in our data by
291 running simple Mantel tests (1,000 randomizations) between the dependent variables
292 (see below) and the coordinates of the blocks. There was no obvious spatial
293 autocorrelation for any of the response variables: germinated seeds: Mantel's $t = 0.092$,
294 $p = 0.303$; dead seedlings: $t = 0.117$, $p = 0.248$; damaged seedlings: $t = -0.079$, $p =$
295 0.654 . We used general linear models (GLM) to test the effect of treatment (Control,
296 Fungicide, Insecticide and Insecticide + Fungicide), seedling density (10 or 25 seeds)
297 and the interaction between these two fixed effects on the three dependent variables, at
298 the end of the 14 monitoring weeks: number and percentage of germinated seeds,

299 seedling mortality (number and percentage of dead seedlings) and number and
300 percentage of seedlings damaged. All statistical analyses were conducted using R v
301 3.3.3 (R Development Core Team 2011) in the RStudio (Racine 2012) integrated
302 development environment. The glm function was used with dependent variables
303 expressed as percentages to compare the two seed densities. Significance of each fixed
304 effect in the GLMs was assessed using the Anova function of the package 'car' in R
305 (Fox and Weisberg 2017) and is provided in the Results section together with the
306 likelihood-ratio chi-square ($LR\chi^2$) and the corresponding degrees of freedom. We
307 empirically modelled seedling survival over time with a rational model ($y = (a + b * x) /$
308 $(1 + c * x + d * x^2)$; where y is the number of seedlings growing and x is the time in
309 weeks), which in almost all cases tested had the highest coefficient of determination
310 (R^2) and the lowest Akaike information criterion corrected (AICC) of all models tested
311 with CurveExpert Professional (Hyams 2011). We tested significant differences
312 between fitted models using Kolmogorov-Smirnov two samples tests. We used
313 available rainfall data from BCI
314 (http://biogeodb.stri.si.edu/physical_monitoring/research/barrocolorado) to check for
315 possible correlation with the mortality of seedlings at ELC. Means \pm s.e. are reported in
316 text and figures.

317

318 **Results**

319 ***Insects reared from fruits***

320 The insects reared from the fruits of *G. ulmifolia* are summarized in Table 1, notes on
321 their ecology are presented in Appendix S1, and some species are illustrated in Plate S1.
322 They included 15 species, mostly Coleoptera. Six beetle species (four Bruchinae,
323 *Amblycerus* spp. and two Anobiinae, *Lasioderma* spp.) can be considered as seed
324 predators, are likely to be host-specific to *G. ulmifolia* and represented 69% of

325 individuals reared. Further, one lepidopteran seed predator was also reared and
326 considered to be generalist (Appendix S1). The percentage of Anobiinae to the total
327 number of insects reared was higher at ELC (90%) than at BCI (38%). Faunal
328 differences between ELC and BCI may partly be due to pre- and post-dispersal fruit
329 attack as the percentage of fungal-feeders, scavengers or sapromycetophagous insects
330 reared was higher at BCI (26.3%) than at ELC (5.4%). At ELC, out of the 100 fruits
331 obtained from trees and reared (i.e., pre-dispersal stage), 92% of fruits were attacked by
332 Anobiinae.

333

334 *Seed germination*

335 After 14 weeks of monitoring, 36.1% of the 1,400 sown seeds had germinated (Table
336 S2). Peak germination occurred during the onset of the rainy season, in the third week
337 of monitoring (24.1%). The average percentage of germinated seeds per block ranged
338 from 30.8% (quadrats I25) to 42.0% (quadrats F10) but varied little between treatments
339 and seed densities (Fig. 1; Table S2). This was confirmed by the GLM, which indicated
340 no significant effect of combined insecticide and fungicide treatment on germination
341 ($LR\chi^2 = 0.17$, $df = 3$, $p = 0.98$), seed density ($LR\chi^2 = 1.57$, $df = 1$, $p = 0.20$), nor their
342 interaction ($LR\chi^2 = 1.30$, $df = 3$, $p = 0.72$).

343

344 *Survival, growth and mortality of seedlings*

345 Overall, seedling mortality was very high, and in total 95.7% of germinated seeds were
346 dead by Week 14 (Table S3). Average seedling mortality (percentage of dead seedlings
347 to total germinated seeds) per block varied little and ranged from 88.6% in quadrats F25
348 to 97.8% in quadrats I10 (Fig. 2). GLM models confirmed that (a) treatments had no
349 effect on the survival of seedlings ($LR\chi^2 = 5.24$, $df = 3$, $p = 0.15$); (b) seed densities had
350 no effect on seedling survival $LR\chi^2 = 0.0048$, $df = 1$, $p = 0.94$); and (c) the interaction

351 between the two variables had no impact on seedling survival ($LR\chi^2 = 1.30$, $df = 3$, $p =$
352 0.72). Seedling survival over time could be fitted to rational models (Fig. 3; range of R^2
353 0.899 to 0.968). At densities of 10 seeds per m^2 , seedling survival was similar in control
354 and I10 quadrats. However, it was slightly better in F10 quadrats than in control
355 quadrats and this difference was significant (Kolmogorov-Smirnov two samples test
356 between the two fitted models, max. diff. = 0.267 , $p = 0.002$). Patterns of survival of
357 seedlings were similar when sown at densities of 10 seeds per m^2 or 25 seeds per m^2 . At
358 densities of 25 seeds per m^2 , seedling survival was higher in F25 quadrats than in
359 control quadrats (max. diff. = 0.327 , $p < 0.0001$), and also slightly higher in I+F25
360 quadrats than in control quadrats (max. diff. = 0.228 , $p = 0.011$). However, these
361 differences were small (Fig. 3).

362

363 The highest seedling mortality was in weeks 4 and 5 (weeks of the 27 May and 3
364 June 2016), with a total of 77 and 86 dead seedlings, respectively. During week 4,
365 only one week after the highest rate of seed germination (Fig. 3), 25.7% of
366 seedlings growing the preceding week died. Weekly seedling mortality was not
367 correlated with the amount of rainfall every monitoring week ($r = -0.08$, $p > 0.80$;
368 excluding weeks 1 and 2 during which few seeds germinated and no seedlings
369 died). However, the cumulative mortality of seedlings during weeks 3 to 14 was
370 strongly and positively correlated with the cumulative amount of rainfall during
371 the same period ($r = 0.93$, $p < 0.0001$).

372

373 After 14 weeks of monitoring, only 24 seedlings were alive, including 9 damaged
374 seedlings, spread only over 4 blocks. The rational models estimated that after one year
375 of monitoring, a total of 9 seedlings for the whole experiment would still be alive. This
376 was not the case: the last two seedlings died on 5 May 2017, one year after the

377 beginning of the experiment, in block 07, quadrat I+F25. The growth and biomass of
378 surviving seedlings in week 14 were also rather slow and low. They measured on
379 average 30.0 ± 1.91 mm and the two last seedlings to die were 125 and 140 mm tall.
380 Since the sample size of surviving seedlings in week 14 was low, we performed a
381 simple ANOVA that indicated that neither treatment ($F_{3,1} = 0.896$, $p = 0.48$) nor seed
382 density ($F_{3,1} = 0.713$, $p = 0.42$) had any effect on seedling height.

383

384 ***Damage to seedlings***

385 Overall, 100 out of 506 seedlings that germinated were damaged (19.8%, Table S4). In
386 80% of cases, damage was identified as originating from leaf-chewing insects, 19% of
387 cases involved fungal necrosis, whereas the final 1% could not be attributed with
388 certainty. The mean percentage of damaged seedlings relative to total germinated seeds
389 per block varied from 15.5% (F10) to 25.2% (C10), with no clear positive effect of
390 insecticide (Fig. 4). GLM models indicated that neither the combined insecticide and
391 fungicide treatment ($LR\chi^2 = 0.60$, $df = 3$, $p = 0.89$), nor seed density treatments (density
392 ($LR\chi^2 = 0.27$, $df = 1$, $p = 0.60$), nor their interaction ($LR\chi^2 = 0.26$, $df = 3$, $p = 0.98$) had
393 a significant effect on the number of damaged seedlings. The relatively low percentage
394 of seedlings damaged, as compared to the mortality of apparently undamaged seedlings
395 (80.2%) suggests that insects were not responsible for most seedling mortality. This was
396 confirmed by the rarity with which insects were observed damaging seedlings during
397 day-time fieldwork.

398

399 **Discussion**

400 *Which stage is particularly sensitive to insect herbivores?*

401 Regarding this question, we observed that: (a) pre-dispersal attack of the fruits of
402 *Guazuma ulmifolia* is very high (up to 92% of fruits attacked) and the result of the

403 action of several species of insect seed predators, mainly anobiine and bruchine beetles
404 (*Lasioderma* spp. and *Amblycerus* spp.). (b) In Panama, and contrary to what was
405 observed in Costa Rica (Janzen, 1975), anobiine beetles were responsible for most of
406 the fruit damage, not bruchine beetles. (c) Post-dispersal attack of isolated *G. ulmifolia*
407 seeds appears to be rare, as rates of seed germination (overall 36%) were not affected
408 significantly by either insecticide or fungicide treatments, nor by seed density. (d) Once
409 seeds germinated, seedling mortality was high (> 95% after 14 weeks of monitoring)
410 but was not reduced by our insecticide or fungicide treatments and was not affected by
411 seed density.

412

413 Because of very high pre-dispersal attack of *G. ulmifolia* fruits at ELC, the number of
414 viable seeds that can germinate on the ground is probably considerably reduced. Janzen
415 (1975) indicates that in Costa Rica in almost all *G. ulmifolia* fruits attacked by
416 *Amblycerus cistelinus*, all seeds in the fruit are destroyed by the time the larva matures.
417 The same author reports that anobiine beetles drill through large number of seeds but
418 that they rarely kill > 50% of the seeds in a fruit and infest usually < 5% of fruits in the
419 field. Although we have no precise rate of seed attack per se, it presumably remains
420 high because of the high infestation rate of fruits and should be a strong limiting factor
421 in the early regeneration of *G. ulmifolia* at ELC. The distance to other reproductive
422 conspecific trees, as well as the dispersal distance of insect seed predators, could also
423 explain different rates of fruit attack per tree (Janzen 1975), but this was not quantified
424 in this study. Fruits stay on trees for a long time; they are dormant and immature for 5-6
425 months and then mature over 3 months (Janzen 1982). *Amblycerus* spp. attack fruits
426 when maturing (Janzen, 1975), but long periods of fruit exposure on trees may be
427 favourable to other seed predators, such as anobiine beetles.

428

429 In contrast, we believe that post-dispersal attack of *G. ulmifolia* seeds by insects is
430 infrequent. *Amblycerus* spp. do not oviposit on fallen fruits even if there are many on
431 the ground (Janzen 1975). Our insecticide treatment did not affect the rate of
432 germination of isolated seeds, which anyway are small and hence unlikely to be
433 attacked by insects (Moles *et al.* 2003). Although *G. ulmifolia* seeds lack polyphenols as
434 defensive compounds, they invest in protective tissue, as almost 70% of the seed dry
435 weight is made up by a hard seed coat (Gripenberg *et al.* 2018; S. Gripenberg, pers.
436 obs.). This could further impede attack of isolated seeds. Furthermore, the lack of
437 improved seed germination or seedling survival in our insecticide and fungicide
438 treatments (see below) suggests that the pre-dispersal seed stage is by far the stage most
439 sensitive to insect enemies.

440

441 *Is the negative impact of insect herbivores at the seedling stage higher than that*
442 *of fungal pathogens? Agents of seedling mortality*

443 In relation to this question we further observed that: (a) seedling survival over time was
444 weakly and positively influenced by the fungicide treatment. (b) There was a strong
445 positive correlation between weekly cumulative seedling mortality and cumulative
446 rainfall. (c) About 20% of seeds that germinated were damaged, mostly by leaf-chewing
447 insects, but neither the treatments nor seed density affected the number of damaged
448 seedlings. (d) Insect herbivores were not observed feeding on seedlings during day-
449 time. (e) Seedling growth was slow (3cm tall after 14 weeks), and all 506 seedlings that
450 germinated out of 1,400 seeds sown were dead after one year.

451

452 Leaves of *G. ulmifolia* on mature trees are readily attacked by a variety of insect
453 herbivores (Janzen 1983), but reports of damage on conspecific seedlings are, to the
454 best of our knowledge, not available. Insects feeding on *G. ulmifolia* seedlings may be

455 active during night-time, as suggested by the low but nevertheless standing leaf damage
456 suffered by seedlings (16 % of germinated seeds). It is also unlikely that seedling
457 mortality could be explained by the near presence conspecific mature trees and
458 contagion of insect herbivores, as *G. ulmifolia* is not very common at ELC. These
459 arguments, along with the lack of insecticide treatments on the survival of seedlings or
460 their damage, suggest that insect herbivores (leaf-chewing and sap-sucking insects)
461 infrequently attacked *G. ulmifolia* seedlings at ELC, and are not primarily responsible
462 for seedling mortality. This could be explained by the low foliar biomass represented by
463 these seedlings (Basset 1999).

464

465 Apart from a weak positive effect of fungicide treatment on seedling demography
466 (survival in time; as reported in Bagchi et al. 2014), our data do not provide evidence
467 for fungal pathogens being important in the early regeneration of *G. ulmifolia*. When
468 seedlings were damaged, and the source of damage could be unequivocally scored,
469 more seedlings were apparently damaged by insects rather than by fungi. However, it
470 may be very difficult to score visually seedlings damaged by fungi. Furthermore, our
471 fungicide treatment may have been more effective against endophytic pathogens than
472 soil pathogens, which may attack both seeds and seedlings (Mangan et al., 2010). The
473 lack of fungicide effects may also be explained differently. Strobel et al. (2007) reported
474 on a strain of *Muscodor albus* isolated in leaves of *G. ulmifolia* in Ecuador and able to
475 colonize conspecific seedlings. This endophytic fungus produces a series of volatile
476 organic compounds with antibiotic activity that collectively act to kill other fungi or
477 bacteria, while there are no external symptoms on its host. It may be argued that this
478 fungus lives in its host in a symbiotic condition, providing protection from pathogens
479 while surviving and growing on plant nutrients (Strobel et al. 2007). If *G. ulmifolia*

480 produces its own fungicides, then the lack of strong effect of our fungicide treatment is
481 not surprising.
482
483 July 2016 should have been a period of active growth for *G. ulmifolia* seedlings that
484 germinated in May 2016. July 2016 was the second rainiest July (monthly sum
485 486.8mm) on BCI since records began in 1925, only surpassed by July 1956 (496.6mm;
486 http://biogeodb.stri.si.edu/physical_monitoring/research/barrocolorado). In these
487 conditions high rainfall may have caused soil anoxia and some rot in seeds and
488 seedlings, as suggested by the strong correlation between cumulative rainfall and
489 seedling mortality during the experiment. Further, *G. ulmifolia* seedlings have a
490 relatively low tolerance to flooding (Lopez and Kursar 2003), and heavy rain may also
491 damage stems and foliage or uproot the seedlings. Thus, we strongly suspect that the
492 main agent of seedling mortality during the experiment was unfavourable rainfall
493 conditions, but we stress that the patterns recorded may not apply under all conditions.

494

495 *Does seed or seedling density affects the action of enemies? Relations with the*
496 *Janzen-Connell hypothesis*

497 For the densities considered in this study, post-dispersal seed and seedling density did
498 not greatly affect the negative action of insect herbivores and fungal pathogens. The
499 lack of difference in seedling survival between control quadrats sown at two different
500 seed densities also suggest that intra-specific competition of seedlings is not responsible
501 for their high mortality (Alvarez-Loayza and Terborgh 2011), at least for the densities
502 considered in this study, which were high as compared to natural seed densities for this
503 species in Panama.

504

505 Since post-dispersal attack of *G. ulmifolia* seeds and seedlings by insects at ELC was
506 infrequent, this suggests that Janzen-Connell effects due to insect herbivores
507 specifically, if they exist, may be rather weak for this tree species. Comita et al. (2010)
508 examined the strength of negative density-dependence for 180 plant species on nearby
509 BCI, investigating seedling survival as function of conspecific seedling abundance
510 (Table S2 in Comita et al. 2010), they found that the strength of conspecific neighbor
511 effects were rather weak for *G. ulmifolia*, which ranked 158th out of 180 species (i.e. in
512 the lower quartile of species). Nonetheless their data indicate negative density-
513 dependence for *G. ulmifolia*, raising the question of which biotic agent may be
514 responsible.

515

516 Since *Amblycerus* spp. only oviposit in fruits on trees (Janzen 1975), they are probably
517 not involved directly in this negative density-dependence. Perhaps a more plausible
518 explanation is that rapid ingestion of *G. ulmifolia* fruits by dispersal agents (mammals,
519 birds and bats; Janzen 1982) results in small clusters of seeds, germinating close
520 together corresponding to a single defecation (Howe 1989; Lawson et al. 2012). Seeds
521 and seedlings would be unlikely to be attacked by insects, as suggested by our
522 experiment, and these clusters are potentially very isolated from other conspecifics
523 (Lawson et al. 2012).

524

525 *Possible biases in our study*

526 First, as indicated by further monitoring of blocks after 14 weeks, seeds were unlikely
527 to germinate after the end of our experiment. This suggests that the length of the study
528 and the germination rate of seeds were appropriate for our experiment (and see
529 Gripenberg et al. 2014). Yet, the overall growth of seedlings was slow: after nearly 4
530 months of monitoring, most seedlings were dead and survivors were < 5cm tall,

531 whereas in greenhouse conditions after the same time period, seedlings are usually 30-
532 40 cm tall (Francis 1991). Seedling biomass, not measured in the present study, was
533 presumably low with, on average, only 3-5 leaves amounting to 4 cm² for each survivor
534 at the end of the experiment. Further, seedlings survived only a short time at ELC:
535 27.5% of germinated seedlings survived only one week. In these conditions, selecting a
536 different tree species for our experiment, with rapid growth and high foliar biomass,
537 would have perhaps helped to quantify better seedling mortality due to insect
538 herbivores.

539

540 Second, the overall germination rate of *G. ulmifolia* seeds (36%) was higher at ELC
541 than that reported in the literature for Panama (natural conditions, 20%: Sautu et al.
542 2006). Many seeds germinated quickly, within three weeks of being sown. This
543 suggests that light limitation at ELC was not a major factor compromising seedling
544 performance of *G. ulmifolia* at the early stages of the life cycle of this species, which
545 was the focus of our study. Indeed, this pioneer species can grow in a variety of habitats
546 (Francis 1991), but we acknowledge that (1) our experimental conditions might have
547 been different from open habitats where *G. ulmifolia* also grows and (2) that the longer-
548 term survival of the seedlings and saplings of this species might be compromised by
549 limited light. Whether light-demanding pioneers are ultimately able to grow to maturity
550 in a given location will depend on changes to the light environment *subsequent* to
551 establishment. In a longer-term perspective, light gaps will form in the forest
552 subsequent to seedling establishment, providing initially shaded seedlings with the
553 conditions necessary to grow towards maturity. Thus, it is relevant to look at the early
554 stages of seedling growth under relatively shaded conditions because the outcome of
555 this phase in plant demography sets the template for future establishment.

556 Third, an unknown proportion of seeds was also probably lost in our experiment before
557 germination. We observed damage due to vertebrates crossing the blocks (i.e.,
558 excavated soil, seed labels chewed), most likely caused by agoutis, *Dasyprocta*
559 *punctata*, and coatis, *Nasua narica*. We also suspect that ants, particularly *Atta* spp.,
560 removed some of the seeds used in the experiment. In Colombia, several ant species,
561 such as *Atta cephalotes*, are known to remove preferentially in open pastures small tree
562 seeds including *G. ulmifolia* (Escobar-Ramírez et al. 2012). On BCI, a high proportion
563 of *G. ulmifolia* seeds are removed secondarily, presumably by ants (Ruzi et al., 2017).
564 However, our insecticide treatment at ELC should have impacted negatively such seed
565 removal by ants. Heavy rainfall may have also washed some of the small seeds out of
566 the experimental blocks. The very small size of the seeds made it impossible to search
567 for individual seeds at the end of the experiment and assess their fate. Selecting tree
568 species with large seeds would thus be useful for future experiments. We believe that
569 pre-germination loss to vertebrates is probably infrequent because of the low biomass
570 represented by isolated seeds, but we cannot quantify loss of seeds due to removal by
571 ants or by rainfall.

572

573 Our factorial design insured that all treatment groups were exposed to the same
574 experimental conditions. All experimental blocks were thus likely to be affected in the
575 same way by additional environmental factors (such as light conditions, seed removal
576 by vertebrates and rainfall) which were not controlled in our study.

577

578 **Conclusion**

579 Our study generated new information on the role of natural enemies in the survival of
580 *Guazuma ulmifolia* at the early stages of the life cycle. We conclude that the pre-
581 dispersal seed stage is by far the stage most vulnerable to insect enemies, that the main

582 agent of seedling mortality during the experiment was probably inclement rainfall, and
583 that Janzen-Connell effects that may be specifically due to insect herbivores are
584 probably rather weak for this tree species. The regeneration of *G. ulmifolia* in the forest
585 of ELC appears to be difficult but not to be the outcome of the action of insect
586 herbivores after seeds are dispersed. This illustrates that each tree species may respond
587 differently to pathogens and herbivores (Gripenberg et al. 2014), and that further work
588 should aim at tracking the possible existence of suites of species that share a common
589 response, perhaps partly dictated by conserved morphological and chemical traits (Paine
590 et al. 2012).

591

592 What are the implications of our findings for the regeneration of this important tree
593 species in agroforestry? Without addressing what may be suitable conditions for
594 seedling growth, the regeneration of this species may further depend on effective
595 primary dispersal of seeds by vertebrates, before most of the seed crop is lost to insects.
596 In this situation, local conservation of dispersal agents may be crucial to enhance escape
597 of seed predation by insects. For example, artificial roosters for frugivorous bats, which
598 disperse seeds of *G. ulmifolia* (Roman et al. 2012), may greatly help the regeneration of
599 this species and the reestablishment of vegetation in disturbed areas (Silveira et al.
600 2011).

601

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617

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761 in tropical forests? A 17-year record from Panama. *Ecology* 85: 484–489.

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763 **Supplementary material**

764 Table S1-S4, Appendix S1, Plate S1.

765 **Table 1.** Summary of insects reared (total number of individuals per species) from fruits
 766 of *G. ulmifolia* at BCI (320 fruits) and ELC (100 fruits). Barcode Index Number (BIN)
 767 indicated when available and is linked to available insect pictures. Host specificity was
 768 inferred from a larger study on insect seed predators on BCI (Gripenberg et al. 2018).
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Taxa	BIN	BCI	ELC	Notes
COLEOPTERA				
Chrysomelidae - Bruchinae				
<i>Amblycerus cistelinus</i> (Gyllenhal, 1833)	BOLD:ACG0463	72	0	Seed predator, host specific on <i>G. ulmifolia</i>
<i>Amblycerus</i> sp. bru39SG	BOLD:ACJ3956	39	3	Seed predator, host specific on <i>G. ulmifolia</i>
<i>Amblycerus</i> sp. bru30SG	BOLD:ABW8381	15	0	Seed predator, host specific on <i>G. ulmifolia</i>
<i>Mimosestes</i> sp. 1YB	-	0	1	Seed predator
Ptinidae – Anobiinae				
<i>Lasioderma</i> sp. ano2SG	BOLD:ACF0770	242	291	Seed predator, host specific on <i>G. ulmifolia</i>
<i>Lasioderma</i> sp. ano4SG	-	1	0	Seed predator
Bostrichidae				
<i>Lyctus</i> sp.	-	0	2	Wood borer
Silvanidae				
<i>Ahasverus advena</i> (Waltl, 1832)	-	1	0	Fungal-feeder, cosmopolitan pest of stored products
<i>Cathartus</i> sp. 1SG	-	115	0	Probably fungal-feeder
Unidentified ELC sp. 1	-	0	1	Probably fungal-feeder
Unidentified ELC sp. 2	-	0	1	Probably fungal-feeder
Cucujidae				
Unidentified - kuj1SG	-	63	0	Ecology unknown
Nitidulidae				
<i>Stelidota</i> sp. 5SG	-	2	0	Sapromycetophagous?, generalist, reared from several hosts
Unidentified larva	BOLD:ACL7065	29	0	Sapromycetophagous?, may be larvae of <i>Stelidota</i> sp. 5SG
<i>Stelidota</i> sp. 6SG	-	0	1	Sapromycetophagous?, generalist, reared from several hosts
Curculionidae – Scolytinae				
<i>Xyleborus</i> sp. 1YB	-	0	1	Wood borer
Corylophidae				
Unidentified Corylophidae	-	0	11	Fungal-feeder
LEPIDOPTERA				
Autostichidae				
<i>Deoclona xanthoselene</i> (Walsingham, 1911)	BOLD:ACF0463	51	0	Seed predator, generalist, reared from several hosts
Blastobasidae				
Blas lep37SG	BOLD:ABV2151	2	1	Scavenger, generalist, reared from several hosts
Blas lep31SG	BOLD:ABV2158	1	2	Scavenger, generalist, reared from several hosts
HYMENOPTERA				
Braconidae				
Unidentified Braconidae	-	0	1	Parasitoid
Pteromalidae				
Unidentified Pteromalidae	-	1	7	Parasitoid
TOTAL		634	323	

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772 **LEGENDS OF FIGURES**

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774 **Fig. 1.** Average percentage of germinated seeds per block, detailed by treatment (C =
775 control, I = insecticide, F = fungicide, I+F = insecticide and fungicide) and seed
776 densities (light bars: 10 seeds m⁻² y⁻¹; dark bars: 25 seeds m⁻² y⁻¹).

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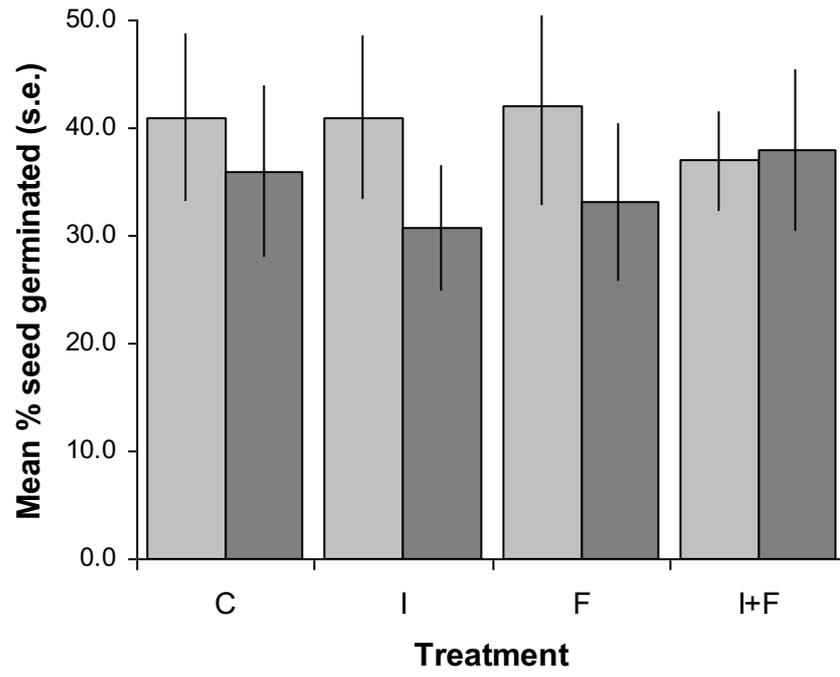
778 **Fig. 2.** Average percentage of dead seedlings per block, detailed by treatment (C, I, F,
779 I+F, coded as in Fig. 1) and seed densities (light bars: 10 seeds m⁻² y⁻¹; dark bars: 25
780 seeds m⁻² y⁻¹).

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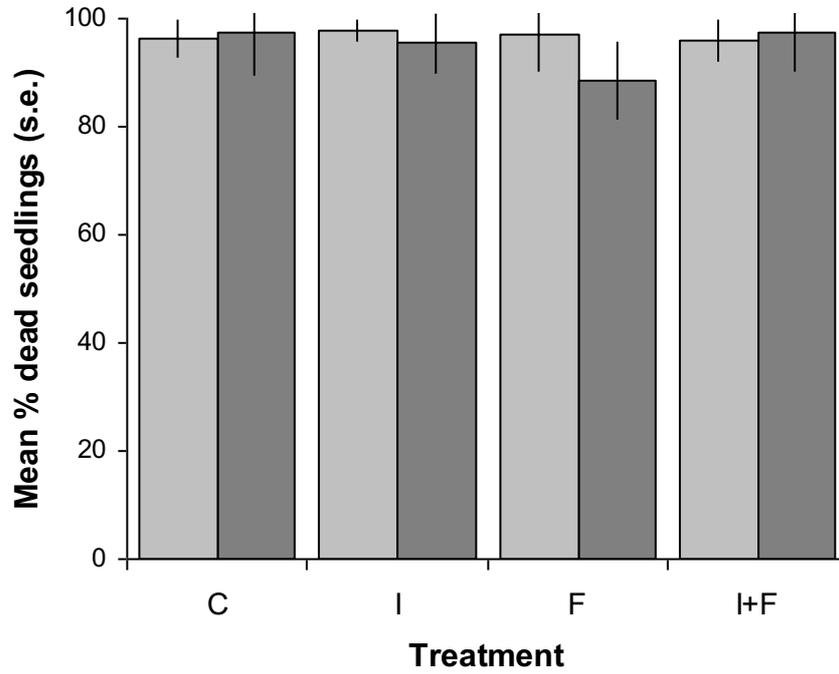
782 **Fig. 3.** Number of seedlings growing plotted against time for each treatment (coded as
783 in Fig. 1 and plotted with different colors) and seed densities of (a) 10 seeds per m² and
784 (b) 25 seeds per m². Observations indicated by symbols, fitted rational models indicated
785 by continuous lines (control identified by thick blue line).

786 **Fig. 4.** Average percentage of damaged seedlings per block, detailed by treatment (C, I,
787 F, I+F, coded as in Fig. 1) and seed densities (light bars: 10 seeds m⁻² y⁻¹; dark bars: 25
788 seeds m⁻² y⁻¹).

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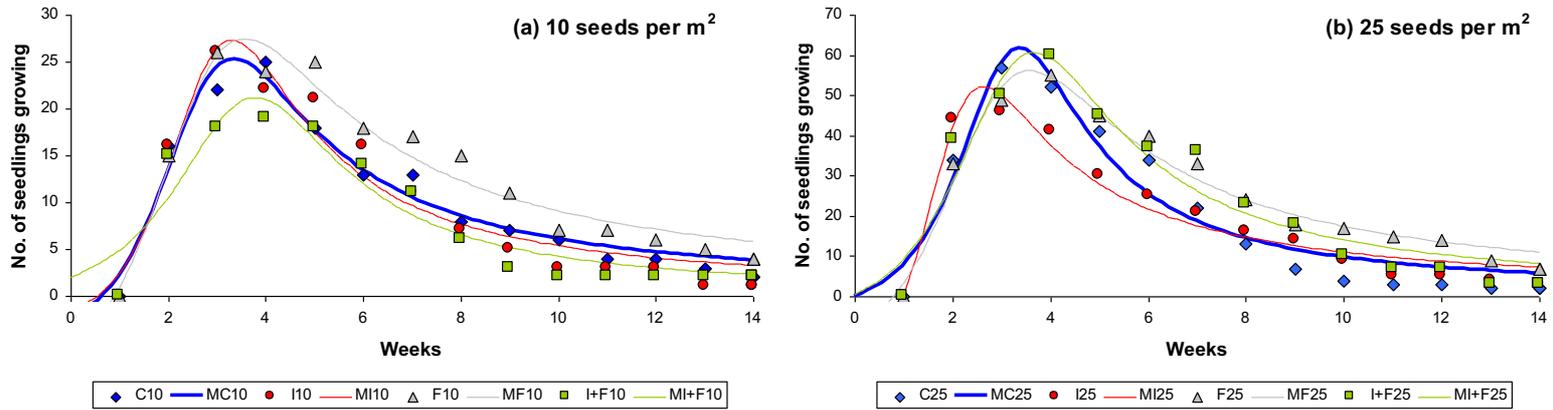


791 **Fig. 1.** Average percentage of germinated seeds per block, detailed by treatment (C =
 792 control, I = insecticide, F = fungicide, I+F = insecticide and fungicide) and seed
 793 densities (light bars: 10 seeds m⁻² y⁻¹; dark bars: 25 seeds m⁻² y⁻¹).



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795 **Fig. 2.** Average percentage of dead seedlings per block, detailed by treatment (C, I, F,
 796 I+F, coded as in Fig. 1) and seed densities (light bars: 10 seeds m⁻² y⁻¹; dark bars: 25



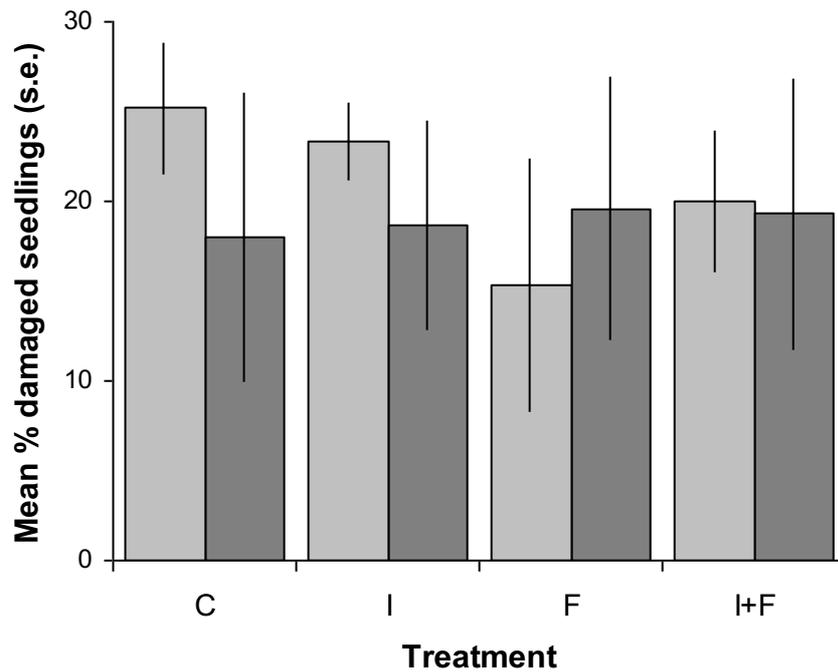
797 seeds m⁻² y⁻¹).

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Fig. 3. Number of seedlings growing plotted against time for each treatment (coded as in Fig. 1 and plotted with different colors) and seed densities of (a) 10 seeds per m² and (b) 25 seeds per m². Observations indicated by symbols, fitted rational models indicated by continuous lines (control identified by thick blue line).



811 **Fig. 4.** Average percentage of damaged seedlings per block, detailed by treatment (C, I,
 812 F, I+F, coded as in Fig. 1) and seed densities (light bars: 10 seeds m⁻² y⁻¹; dark bars: 25
 813 seeds m⁻² y⁻¹).

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