

1 **Sexual competition and kin recognition co-shape traits of neighboring dioecious seedlings**

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22 **ABSTRACT**

23 **Plants respond differently to neighbor identity showing plasticity in traits. However, solid**
24 **experiment evidence on the functional traits of dioecious trees shaped by the recognition**
25 **of neighbors with different gender and kinship is scarce. Here we examined the sexual and**
26 **kinship interactions in a dioecious tree species, *Diospyros morrisiana*, by monoculturing**
27 **and pair-culturing seedlings in a transparent gel system. Our results showed that sex-**
28 **specific competition and kin recognition interacted and co-shaped the functional traits of**
29 ***D. morrisiana* seedlings, especially root traits, while intra-sexual and non-kin neighbors**
30 **facilitated the growth of seedlings. This implies kin- and gender-interactions depend on**
31 **different mechanisms, kin selection and niche partitioning respectively, which is critical**
32 **to understand how species coexist and traits are shaped in the nature.**

33

34 **Keywords:** dioecious, *Diospyros morrisiana*, kin recognition, sex competition, intraspecific
35 competition

36 INTRODUCTION

37 How plant species coexist with neighbors determines community structure and understanding
38 these interactions has attracted much research attention (Callaway 2002; Karban 2008; Chen
39 *et al.* 2012; Xu *et al.* 2020). Having limited dispersal ability and a sessile lifestyle, plants
40 often coexist with few neighbors (Inderjit *et al.* 2008; Yang *et al.* 2018), with whom they
41 engage in both above- and below-ground interactions. For example, bean and pea species
42 outcompete neighbors for light by elongating their above-ground stems (Kasperbauer & Hunt
43 1994), and maize roots over-proliferate around nutrient-rich patches to outcompete neighbors
44 (Yu *et al.* 2019). Therefore, plant species are known to mediate this competitive response in
45 the presence of neighbors with different identities, by adjusting their fundamental
46 morphology (Fang *et al.* 2013), physiological traits (Biedrzycki *et al.* 2010), life history traits
47 (Wilczek *et al.* 2009) or resource allocation strategies (Bhatt *et al.* 2011). However, this
48 capacity for neighbor-identity recognition is still poorly understood, particularly with regard
49 to neglect of the individual importance in the community.

50 Although neighbor-identity recognition is known among dioecious species, they are often
51 neglected (Chen *et al.* 2012; Mercer & Eppley 2014; Dong *et al.* 2017). Comprising about 5-
52 6% of all plants, dioecious species have separate male and female individuals, which plays a
53 significant role in maintaining stability in the structure and function of terrestrial ecosystems
54 (Hultine *et al.* 2007; Renner 2014). However, differences in competitive abilities between the
55 sexes remain poorly understood. For example, some studies have demonstrated that females
56 are competitively superior to males in growth and/or survival at high densities (S. Conn &

57 Blum 1981; Lovett Doust *et al.* 1987; Eppley 2006), while others have shown males possess
58 superior resistance and adaptive abilities under environmental stress (Graff *et al.* 2013). If
59 dioecious plants have the capacity of recognizing the gender of conspecific neighbors, they
60 might modulate intensity of inter-sexual and intra-sexual competition. This could play a key
61 role in the maintenance of bias in sex-ratio and/or niche segregation of the sexes.

62 Among dioecious plants, potential competition between the sexes complicates kinship
63 interactions. Mercer & Eppley (Mercer & Eppley 2014) demonstrated that kin and sexual
64 interaction significantly co-affect different traits of *Distichlis spicata*. Competitive traits like
65 root distribution (Dudley & File 2007; Fang *et al.* 2013; Semchenko *et al.* 2014; Li *et al.*
66 2018) and leaf distribution (Murphy & Dudley 2009; Lepik *et al.* 2012) have also been used
67 to identify kin recognition. For example, *Cakile edentula* can discriminate kin through root
68 interactions and accordingly decrease the belowground allocation to favor siblings and
69 *Arabidopsis thaliana* can change the horizontal direction of leaves, reducing leaf masking
70 between related, but not unrelated, neighbors (Crepy & Casal 2015). However, few studies
71 have considered the role of gender and relatedness of neighbors in the process of dioecious
72 species competition and little is known about their interaction (Mercer & Eppley 2014; Dong
73 *et al.* 2017).

74 Early kin selection theory suggested plants might save resources by cooperating with kin
75 and competing with non-kin, leading to fewer competitive traits, higher fitness and
76 contributing to the evolution of kin selection (Hamilton 1964). More recent studies suggest
77 that competition may be greater among more dissimilar genotypes, suggesting species, kin

78 and self/non-self recognition and favoring inclusive fitness (Falik *et al.* 2003; Dudley & File
79 2007; Biedrzycki *et al.* 2010; Mercer 2010; Yang *et al.* 2018). If this pattern holds for
80 dioecious species, then competition among non-kin/inter-sexual combinations should be
81 greater than that among kin/intra-sexual combinations. With increasing intensity of
82 competition, plants will allocate more resources into traits conferring a competitive
83 advantage. This suggests a hypothetical framework, where plant competition is greater with
84 dissimilar genotypes (non-kin and inter-sexual combinations) than with similar genotypes
85 (kin and intra-sexual combinations) (Fig 1a).

86 We tested this hypothesis, setting up kin/non-kin and inter-sexual/intra-sexual treatments
87 together for a dioecious tree species, *Diospyros morrisiana*. Plants were grown in a
88 homogeneous sterile gel medium system, as a novel way to address the kinship and sex
89 interaction without the influence of soil microorganisms. Our aims were to address the
90 following questions: (1) How do dioecious species respond to different sexual neighbors? (2)
91 How do dioecious species respond to kin or non-kin neighbors? (3) Do plants compete greater
92 with dissimilar genotypes (non-kin and inter-sexual combinations in a broad sense) than with
93 similar genotypes (kin and intra-sexual combinations)?

94

95 **MATERIALS AND METHODS**

96 *Plant materials and growth*

97 *Diospyros morrisiana* Hanse (Ebenaceae) is a dioecious subtropical tree growing in eastern
98 Asia, particularly in southern China and Japan (Bawa 1980; Zheng *et al.* 2012). Seeds were

99 collected from two *D. morrisiana* trees: tree A in Heishiding Natural Reserve (23.27° N,
100 111.15° E, Guangdong province, China) and tree B in Jianfengling Natural Reserve (18° N,
101 108° E, Hainan province, China), seeds were marked as seeds A or B, corresponding to the
102 parent tree.

103 Seeds were soaked in sterile water for 30 minutes, surface-sterilized with 15% hydrogen
104 peroxide for 20 minutes. The sterilized seeds were pre-germinated in petri dishes with 0.1L
105 growth medium for 5 days at 30 °C in the dark. After germination, the seedlings were
106 transplanted to a transparent 3D growth cylinder with 20 cm in height and 10 cm in diameter,
107 which was filled with 1.2 L growth medium consisting of half-strength Hoagland solution
108 solidified by 0.2% Phytigel™ (Sigma-Aldrich, German) (Fang *et al.* 2009). The distance
109 between the two neighboring plants was 2 cm (Fang *et al.* 2013). Cylinders were placed in
110 the growth incubators at 26 °C during the day and 21 °C at night in a 12h: 12h (day: night)
111 cycle for seven weeks, while the lower half of the cylinders was covered by aluminum foils
112 to simulate the dark underground environment.

113

114 *Cultivation design*

115 Seedlings from the same mother tree were regarded as kin (Combinations: A-A and B-B),
116 while seedlings from different mother trees were regarded as non-kin (Combinations: A-B).
117 Sexual interactions include inter-sexual (female-male, F-M) and intra-sexual (F-F and M-M).
118 In a single planting, only one seedling was planted in each container as the control group.
119 There were 22 replicates for the single planting treatment (13 females, 9 males), 54 replicates

120 for kin pair treatments, 12 replicates for non-kin pair treatments (total N = 154). Sexual
121 identity could only be identified after harvest by a sex-specific molecular marker method
122 (following section). The valid independent sexual combinations identified post-harvest were
123 29 F-F, 27 F-M and 10 M-M.

124

125 *Sex identification*

126 The sex of dioecious plants is difficult to determine during early developmental stages if
127 plants are not in bloom or bearing fruit (EL-Din Solliman *et al.* 2017). Sex-specific molecular
128 marker identification has been carried out for more than 20 years in the sex determination
129 research in agriculture and horticultural production (Sarkar *et al.* 2017). For *Diospyros* plant
130 species, OGI DNA markers, which control the maleness expression (Akagi *et al.* 2014b), are
131 regarded as an effective method to identify gender in the species. OGI encodes a small RNA
132 that in turn triggers transitive RNAi on a feminizing gene. This OGI marker was used to
133 identify the sexuality of *D. morrisiana* seedlings and to further test the accuracy of OGI DNA
134 markers, we observed 271 *D. morrisiana* mature trees and identified their gender according
135 to flowering or fruiting in Heishiding Natural Reserve in 2017. We collected the leaves of
136 these trees for DNA extraction.

137 The OGI marker primers are OGI-candF1 (5'-CACAGTAGTCATATATTTTTAGC-3')
138 and OGI-spR (5'-CTGGCA CACAAAATATTTTCAACCCT-3') (Akagi *et al.* 2014a). The
139 PCR reaction mixture contained a total volume of 20 μ l including 120 ng template DNA, 600
140 nM forward and reverse primers respectively and 10 μ l 2 \times EasyTaq PCR Super Mix(+dye)

141 (Transgene, Guangzhou, China). The OGI amplification steps consisted of an initial
142 denaturing step at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s,
143 72 °C for 90 s and final 72 °C for 7 min. Results showed that the OGI markers indicate the
144 sex of *D. morrisiana* with 98.6% accuracy.

145

146 *Plant growth traits measurement*

147 Seedling growth was imaged *in situ* every three days after germination using a digital camera
148 (Nikon D600, EFS 60 mm, Japan). Seedlings were harvested seven weeks after germination.
149 Image J software (version 1.49, National Institutes of Health, the USA) and WinRHIZO (Pro
150 2013a, Regent Instrument Inc., Canada) was used to measure a range of morphological traits
151 (Table S1). Shoot and root dry biomass (mg) were measured after drying in an oven at 75 °C
152 for 48 h. Specific root length was calculated as the ratio of root length to root dry biomass.
153 The root/shoot biomass ratio was calculated by dividing root dry biomass by shoot dry
154 biomass.

155

156 *Root spatial distribution measurement*

157 We defined roots present in the resource zone as overlapping and calculated the ratio of
158 overlapping root length to total root length (ROR) (Eq. 1) to evaluate the spatial distribution
159 of roots. L_a is the maximum top root depth, L_b is the maximum root width of two plants, L_b'
160 is the seed distance between two plants (Fig. S1).

$$161 \quad ROR = L_{12} / (L_1 + L_2) \quad (1)$$

162 where L_{12} is the total root length in the overlapping resource zone (region $L_a \times L_b$). L_1
163 and L_2 represent the total root length of each plant in region $L_a \times L_b$ respectively. ROR has
164 values ranging from 0 to 1. When ROR is 0, the root systems are mutually exclusive and no
165 roots are distributed in the resource overlap zone, while when ROR is 1, root systems are
166 completely intertwined.

167

168 *Statistical analyses*

169 Firstly, we analyzed our experiment by using ANOVA to test the effects of sex interaction (F-
170 M, F-F and M-M), kinship (kin and non-kin) and their interactions on the growth traits as
171 well as ROR of *D. morrisiana*. Due to the unbalanced study design, post hoc multiple
172 comparisons of trait parameters among different relatedness and sexual interaction treatments
173 were used and least-squares means were calculated using R package “lsmeans” (Lenth 2016).

174 Secondly, in order to compare the difference of root growth dynamics among planting
175 treatments, we modeled root width and depth of *D. morrisiana* as a function of plant age
176 using the three-parameter logistic model proposed by Paine et al (Paine *et al.* 2012) for each
177 treatment. We also obtained the function-derived absolute growth rate (AGR) and relative
178 growth rate (RGR) from each dynamic model to further visualize the growth dynamic of root.
179 All statistical analyses were performed using R, Version 3.4.2 (R Core Team 2017).

180

181 *Data availability*

182 The data supporting the findings in this study are available from the corresponding author

183 upon reasonable request. Source data are provided with this paper.

184

185 **RESULTS**

186 *Difference of male and female growth in monoculture*

187 In single plantings, males had larger root systems than females (fig2, table S1). When grown
188 alone, after the 7th week, males had significantly larger (P -value = 0.0185) root biomass than
189 females but did not significantly differ from single females in total or shoot biomass (P -
190 value > 0.05; Fig. 2a-c). Total and lateral root length and shoot height of single males was also
191 significantly larger than those of single females (Fig. 2d-h, Table S2).

192

193 *Difference of seedling growth between monoculture and paired culture*

194 Compared with single individuals, male seedlings grown with neighbors had lower root
195 biomass (Fig. 2b), total root length (Fig. 2d), lateral root length (Fig. 2e) and shoot height
196 (Fig. 2f), while females grown with neighbors had larger total biomass (Fig. 2a), total root
197 length (Fig. 2d) and lateral root length (Fig. 2e).

198 During the entire seven weeks, both females and males grown in paired plantings had
199 greater modelled top root depth in intra-sexual (F-F and M-M) than inter-sexual combinations
200 (F-M) (Fig 3a). AGR was a unimodal function of time, reaching a maximum around weeks 3
201 - 5, while RGR had a decreasing sigmoidal response in all combinations (Fig.3b, 3c). Males
202 grown with other males (M_{MM}) had a larger AGR than with other females (M_{FM}) in the first 4
203 weeks (Fig. 3b) and M_{MM} had a larger RGR than M_{FM} in the first 3 weeks (Fig. 3c). F_{FF} had a

204 larger AGR than F_{FM} (Fig. 3b) and the RGR of F_{FF} was slightly higher than F_{FM} (Fig. 3c) over
205 the entire 7 weeks.

206 In all combinations, RGR declined linearly with increasing top root depth (Fig. 3d). RGR
207 in M_{MM} and F_{FF} were always larger than M_{FM} and F_{FM} , respectively. However, root width
208 growth dynamics did not differ for any planting combinations (Fig. S2). Similarly, there were
209 no significant differences in biomass, or other shoot and root traits tested for any planting
210 combinations (Fig. S3).

211

212 ***Kin interactions in females and male***

213 Male seedlings paired with non-kin had significantly larger root/shoot biomass than those
214 grown with kin (Fig. 4a). Female seedlings interacting with non-kin had significantly higher
215 leaf biomass than those grown with kin (Fig. 4b). Moreover, paired non-kin plantings had
216 higher root distribution ratios in the resource overlapping zone (ROR) than those planted with
217 kin, especially in F-F treatments (Fig. 5a). There was no significant difference in the total
218 root length among the six treatments, neither kinship treatments nor sex treatments (Fig. 5b).

219

220 ***Interactions between sex and kin***

221 The total root length in nonkin-FM was significantly larger than kin-FM after 2 weeks (Fig.
222 6a), because the AGR of nonkin-FM was larger than kin-FM in the first 5 weeks (Fig. 6b) and
223 RGR of nonkin-FM was larger than kin-FM in the first 3 weeks (Fig. 6c). Along with the
224 increase of total root length, RGR in nonkin-FM was higher than kin-FM in the whole 7

225 weeks (Fig. 6d).

226 In the 3 kin combinations, there were no significant differences in total root length until
227 the 5th week (Fig. 6a) after this, there were differences in the order kin-FF > kin-MM > kin-
228 FM (Fig. 6a). Absolute growth rate (AGR) and relative growth rate (RGR) of the three kin
229 combinations started to bifurcate during the 3rd and 4th weeks. AGR of kin-FF was larger than
230 that in the other two combinations after the 4th week (Fig. 6b, 6c).

231 In the 3 nonkin combinations, nonkin-FM had largest total root length after the 3rd week
232 (Fig. 6a), which can be directly explained by the largest AGR and RGR of nonkin-FM in the
233 first 4 weeks (Fig. 6b, 6c). The total root length of nonkin-FF exceeded nonkin-MM after
234 week six (Fig. 6a), since the AGR and RGR differed after 4 weeks (Fig. 6b, 6c).

235

236 **DISCUSSION**

237 Our study suggested that seedling growth in *D. morrisiana* was affected by both relatedness
238 and sexual identity in neighboring plants. Kin recognition and sexual competition also led to
239 contrasting fitness consequences in *D. morrisiana* seedlings (Fig. 1b). *D. morrisiana* seedlings
240 grew better with non-kin and intra-sexual neighbors, while competing less with kin and inter-
241 sexual neighbors. This supports our null hypothesis for kin interactions but not that for
242 competition between the sexes. This implies that kin- and gender-interactions depend on
243 different mechanisms, i.e. kin selection theory and niche partitioning theory, respectively, in
244 responding to the identity of neighbors.

245 Kin selection was evident in *D. morrisiana* seedlings, which matched our hypothesis

246 (Fig. 1b). When paired with non-kin neighbors, females and males had significantly higher
247 leaf biomass and root/shoot biomass ratio respectively than those paired with kin neighbors.
248 This does not only support kin recognition in *D. morrisiana* seedlings, but also suggests
249 stronger competition between non-kin than kin individuals. Male seedlings competing with
250 nonkin individuals had significantly higher biomass allocation (root/shoot biomass) than
251 those grown with kin. Female seedlings competing with non-kin individuals had larger leaf
252 biomass than when grown with siblings, allowing females to increase light reception.

253 Kin combinations also had significantly less root length in the resource overlapping zone
254 than non-kin combinations, which suggests kin tend to reduce competition by adjusting their
255 root distribution, especially between female siblings. These results are contrary to Fang *et al.*
256 (Fang *et al.* 2013), who found that rice plants interacting with neighbors of the same
257 genotype had a significantly higher root overlap length compared to those of a different
258 genotype. Reduced overlap in roots in the resource zone might be due to difference in life
259 history traits between rice and *D. morrisiana* used in our study.

260 On the other hand, sexual recognition could be explained by the niche partitioning
261 hypothesis (Fig. 1b), which predicts that relatives overlap more in their niche use and
262 compete more intensely with each other compared with unrelated intraspecies plants (Young
263 1981). This is not consistent with the hypothesis in Fig. 1a. We found that both females and
264 males have greater top root depth in intra-sexual combinations than in inter-sexual
265 combinations, indicating that intra-sexual competition is greater than inter-sexual competition
266 in *D. morrisiana* seedlings. The dioecious grass, *Distichlis spicata*, exhibits a similar trend,

267 with substantially less biomass when grown with inter-sexual than intra-sexual competitors
268 (Mercer 2010). However, biomass and root/shoot biomass ratio in *D. morrisiana* seedlings
269 did not differ between inter-sexual and intra-sexual combinations over the culture period.
270 This suggests an interesting possibility, that plants may adapt to their environment by
271 adjusting their root structures instead of changing root biomass allocation in the seedling
272 stage.

273 Moreover, trait variation between female and male individuals in response to the
274 presence of neighboring plants is considered to be an important factor driving gender-specific
275 growth patterns (Eppley 2006). Females increase their lateral root length when competing
276 with neighbors, indicating asymmetric increase of competitive ability between females and
277 males in sexual competition. The formation of lateral roots presumably improves sink
278 strength, allowing female plants to increase nutrient intake and water acquisition. Such
279 asymmetric sex-specific competition has been found in *Osyris quadripartite*, in which males
280 are more inhibited when competing with neighboring females (Herrera 1988). Similarly,
281 *Distichlis spicata* seedlings, regardless of sex, were six times larger when grown with male
282 than female intraspecies plantings, suggesting females had stronger competitive effects on
283 neighbors than males (Eppley 2006).

284 We conclude that kin selection and niche partitioning mechanisms are together
285 responsible for the sexual competition and kin recognition of the dioecious species seedlings.
286 Future work should look to understand the fitness consequences of kin recognition and
287 gender competition among dioecious species of different ecotypes, which could play

288 important roles in the evolution of coexistence and reproductive strategies in dioecious
289 plants. In addition, we need to know that the mechanism may change because of the absence
290 of microbial communities in the gel cultivation system used in this, and similar, studies. Sex-
291 specific mycorrhizal colonization has been found in several dioecious and gynodioecious
292 plants (Varga 2010, 2013; Varga *et al.* 2017). It has been well studied that arbuscular
293 mycorrhiza could mediate competitive interaction in *Antennaria dioica* (Bais *et al.* 2001;
294 Varga 2015) in which females have higher levels of AM colonization, and benefit more from
295 AM fungi, than males in terms of growth and reproduction. The extent to which microbial
296 factors drive sex-specific effects in the growth of *D. morrisiana* seedlings remains unknown
297 and warrants further studies in the field.

298

299 **Figure Legends**

300 **Figure 1** Theoretical hypothesis for the fitness of females and males in paired plantings, with
301 variation in kinship and for intra- and inter-sexual combinations. (a) where inter-sexual
302 combinations have higher fitness/traits than intra-sexual combinations and non-kins have
303 higher fitness/traits than kins, (b) where intra-sexual combinations have higher fitness/traits
304 than inter-sexual combinations and non-kins have higher fitness/traits than kins. Yellow bars
305 indicate the data ranges of intra-sexual and kin combinations, or inter-sexual and non-kin
306 combinations have larger fitness/traits than the others.

307

308 **Figure 2** Biomass and growth characteristics for female and male individuals in monoculture

309 and pair-culture treatments 7 weeks after germination (a) total biomass, (b) root biomass, (c)
310 shoot biomass, (d) total root length, (e) lateral root length, (f) shoot height. Treatments with
311 the same letter do not differ significantly ($p < 0.05$). Bottom row shows a generalized
312 representative two-dimensional root system under monoculture for (g) female and (h) male
313 *Diospyros morrisiana* plants 7 weeks after germination.

314

315 **Figure 3** Predicted response of *D. morrisiana* from the logistic model grown in monoculture
316 and in combination with different genders. (a) top root depth as a function of time, (b)
317 absolute top root depth growth rate (AGR) as a function of time, (c) relative top root depth
318 growth rate (RGR) as a function of time, and, (d) relative growth rate (RGR) as a function of
319 top root depth. F represents single female, F_{FF} represents intra-sexual (female-female)
320 planting combination (FF), F_{FM} represents inter-sexual (female-male) interaction (FM), M
321 represents single male, M_{MM} represents male in the intra-sexual interactions (MM), M_{FM}
322 represents male in the inter-sexual interactions (FM). Grey shading indicates 95% confidence
323 intervals for the accumulated root growth and growth rates, as derived from population
324 prediction intervals.

325

326 **Figure 4** Biomass allocation of *D. morrisiana* when grown with different kin. (a) Root/Shoot
327 biomass and (b) leaf biomass. Mean+1 SE are given without data transformation. Different
328 letters indicate statistically significant differences ($p < 0.05$) among different treatments.

329

330 **Figure 5** Root growth under different sex and kin combinations (a) ratio of overlapped to
331 total root length (ROR) in the in the resource utilization overlapping zone (b) total root
332 length. Mean +1 SE are given without data transformation ($N = 65$). Different letters indicate
333 significant differences ($p < 0.05$) between treatments: upper-case = kin vs. non-kin; lower-
334 case = sex combinations.

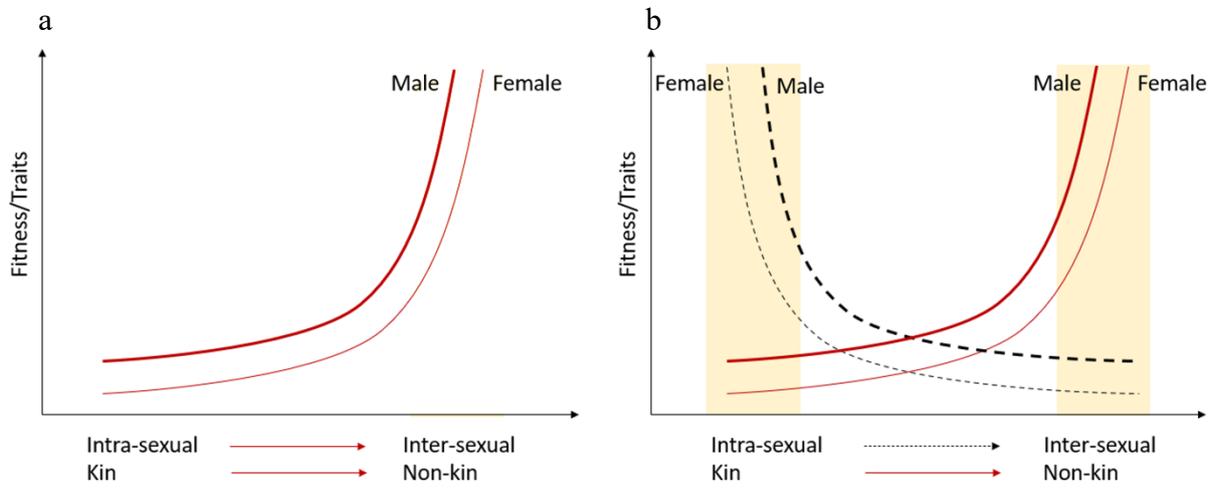
335

336 **Figure 6** Predicted total root length values of *D. morrisiana* from logistic model when
337 interacting with different gender and kin. (a) total root length, (b) absolute growth rate
338 (AGR), (c) relative growth rate (RGR) on the time basis, (d) relative growth rate (RGR) on
339 the total root length basis. Grey shading indicates 95% confidence limits in the estimate for
340 the accumulated growth and the two growth rates, as derived from population prediction
341 intervals.

342

343 Figure 1

344

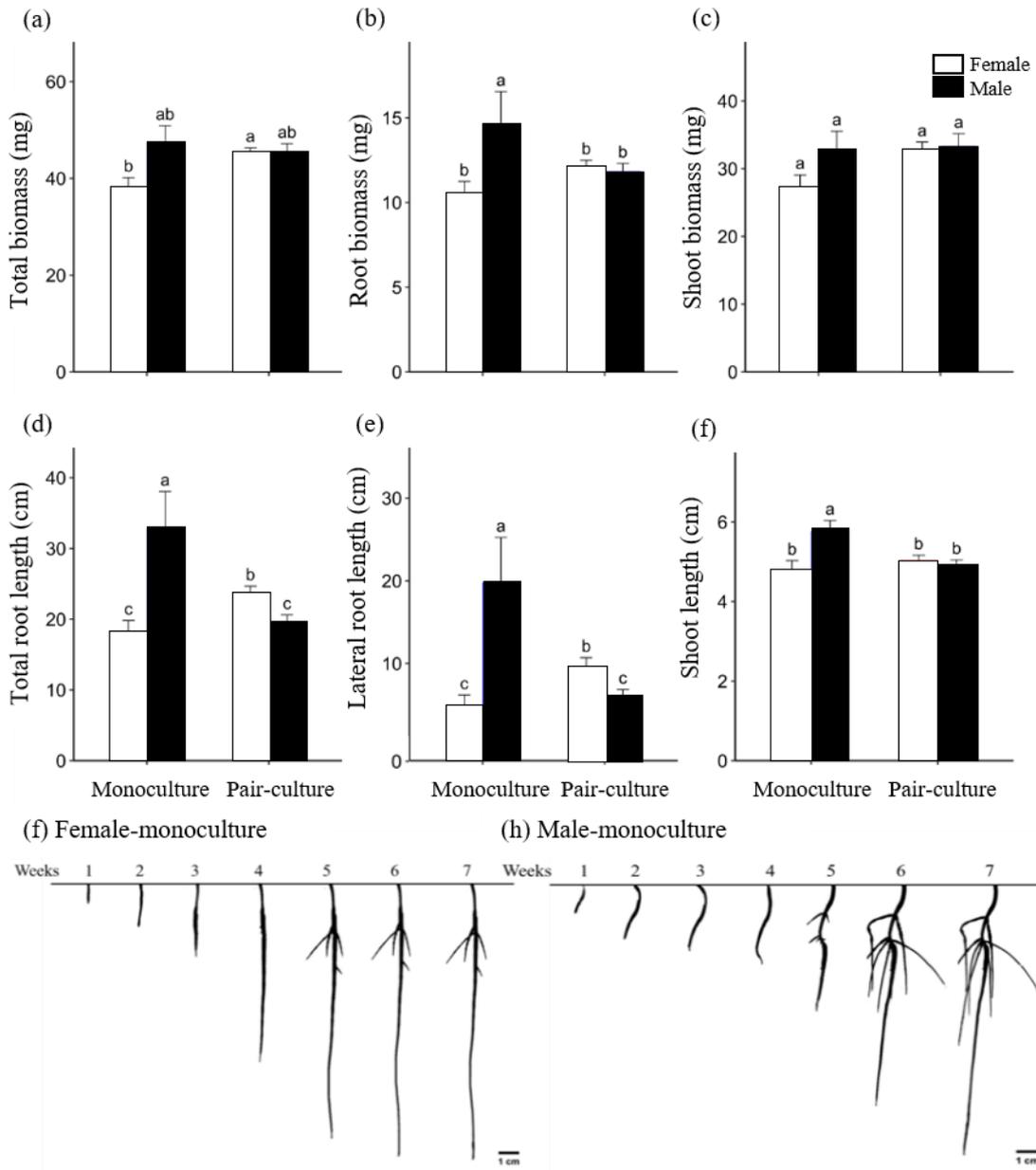


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347 Figure 2

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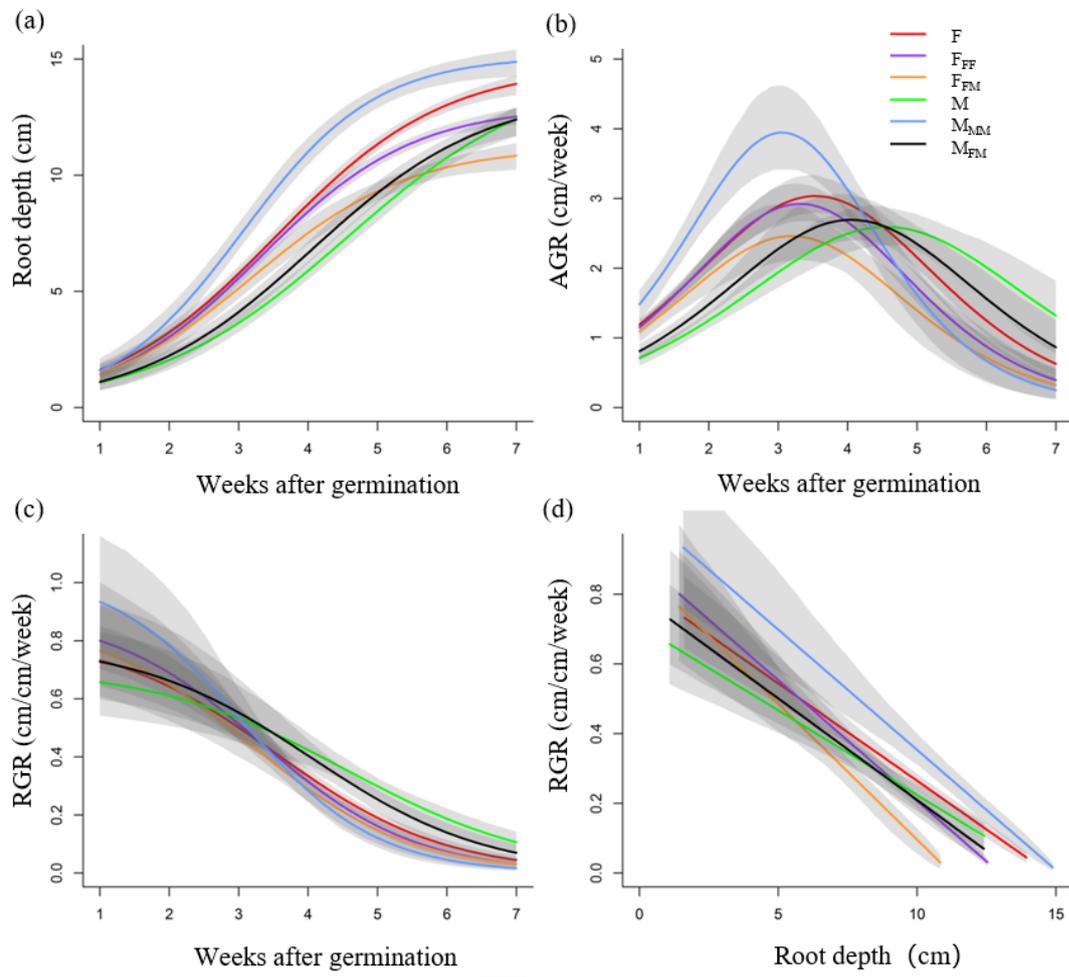
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352 Figure 3

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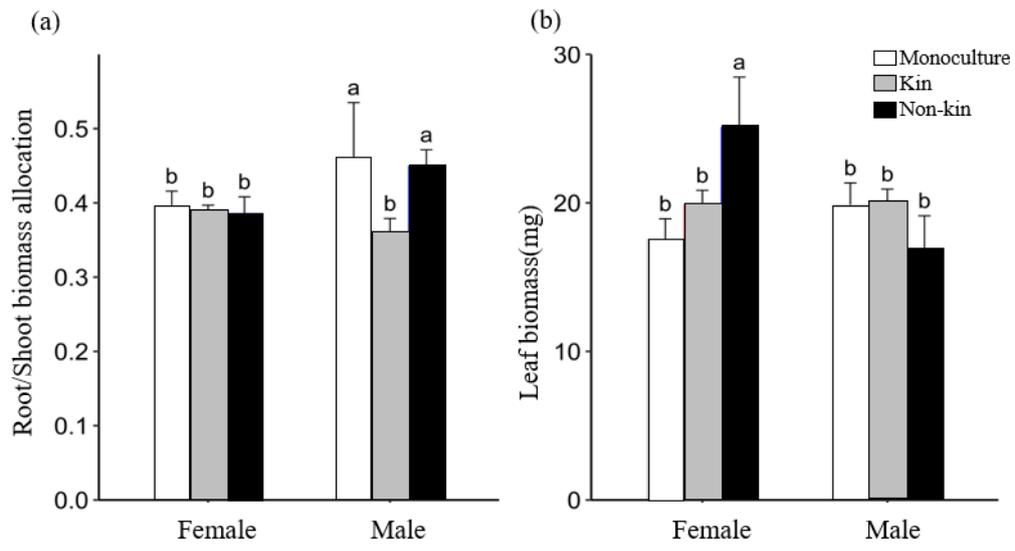
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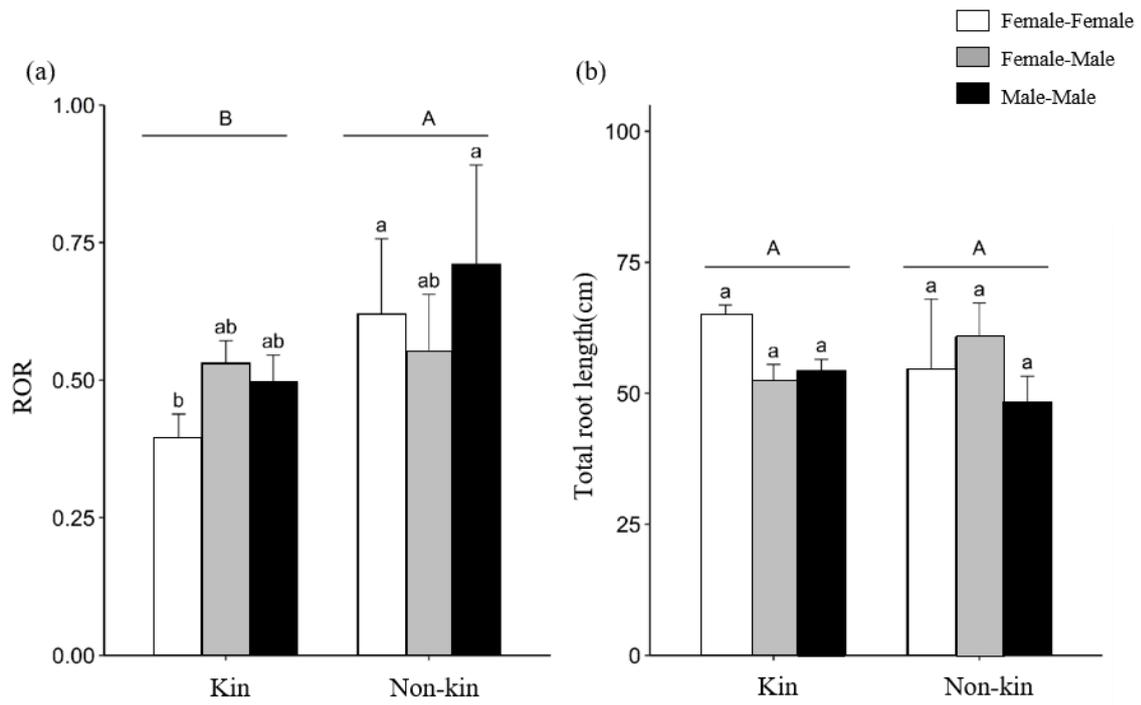
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360 Figure 5

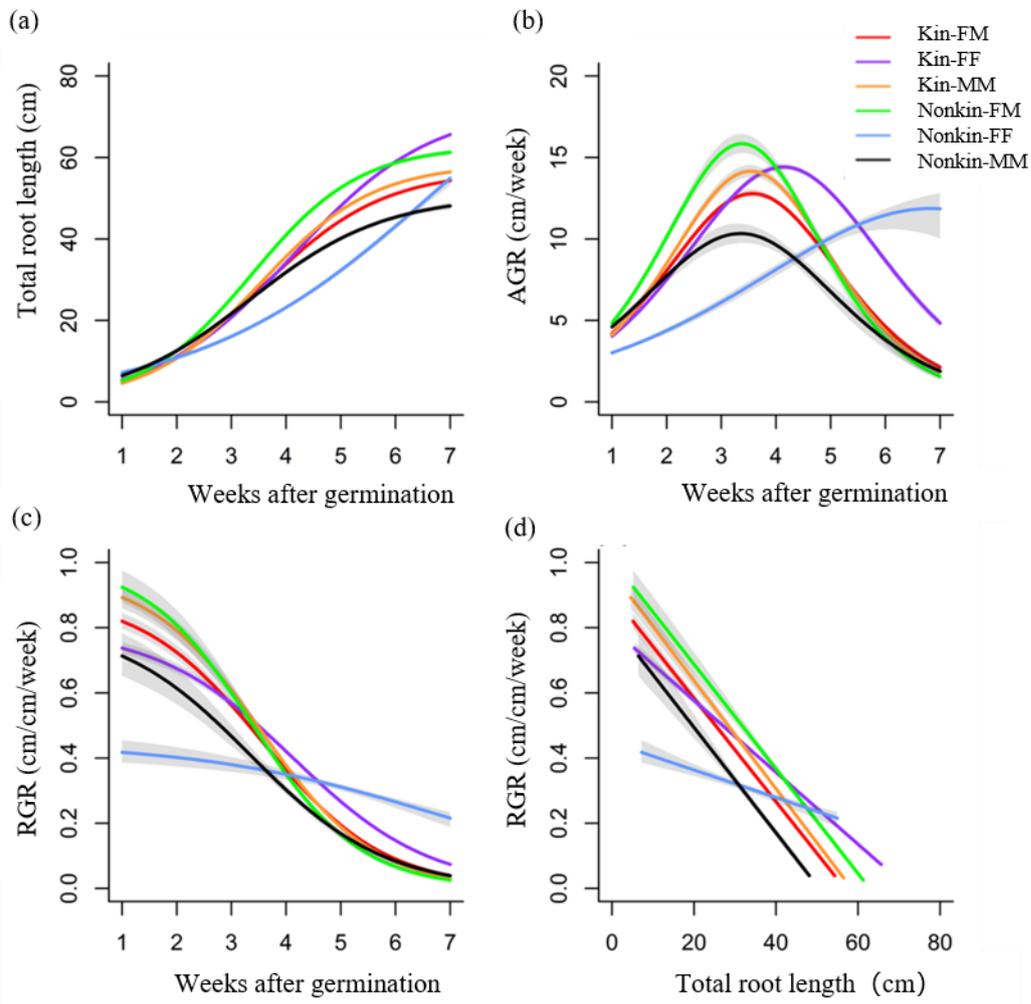


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363 Figure 6

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370

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