

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

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Type of article: Letters

*Running title:* Sexual competition and kin recognition of dioecious plants

Word count: Abstract 121; Main text 5544

Number of references: 46

Number of figures and tables: 6 figures in the main text; 2 tables and 3 figures in the supporting

information

## ABSTRACT

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

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

## INTRODUCTION

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

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

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

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

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

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

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

## **MATERIALS AND METHODS**

### *Plant materials and growth*

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

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

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

#### *Cultivation design*

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

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

### *Sex identification*

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

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

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

#### *Plant growth traits measurement*

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

#### *Root spatial distribution measurement*

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

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



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

### *Statistical analyses*

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

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

### *Data availability*

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

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

## RESULTS

### *Difference of male and female growth in monoculture*

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

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

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

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

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

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

#### ***Kin interactions in females and male***

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

#### ***Interactions between sex and kin***

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

weeks (Fig. 6d).

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

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

## DISCUSSION

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

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

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

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

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

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

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

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

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

## Figure Legends

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

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

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

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

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



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

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

Figure 1

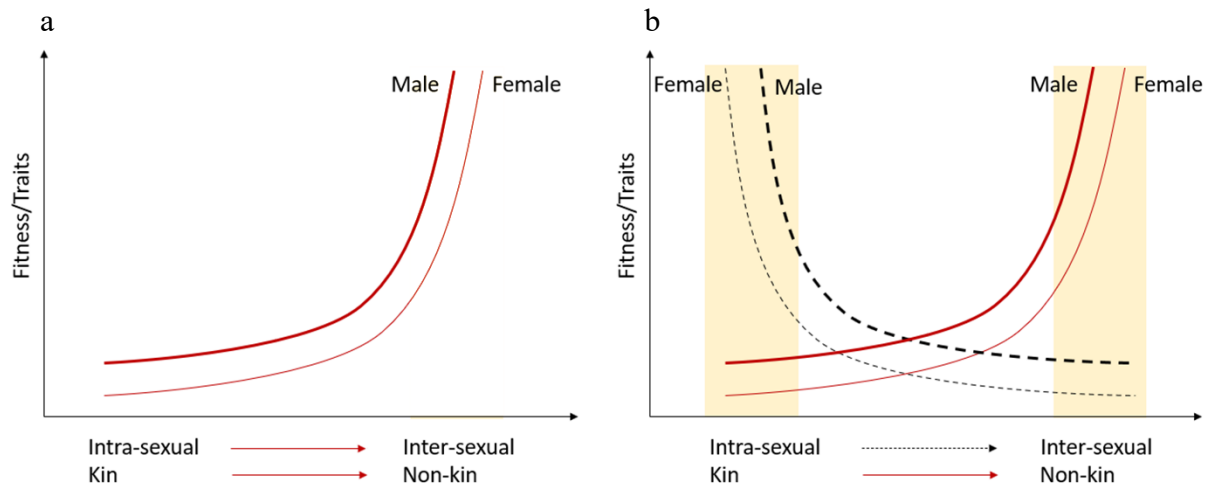


Figure 2

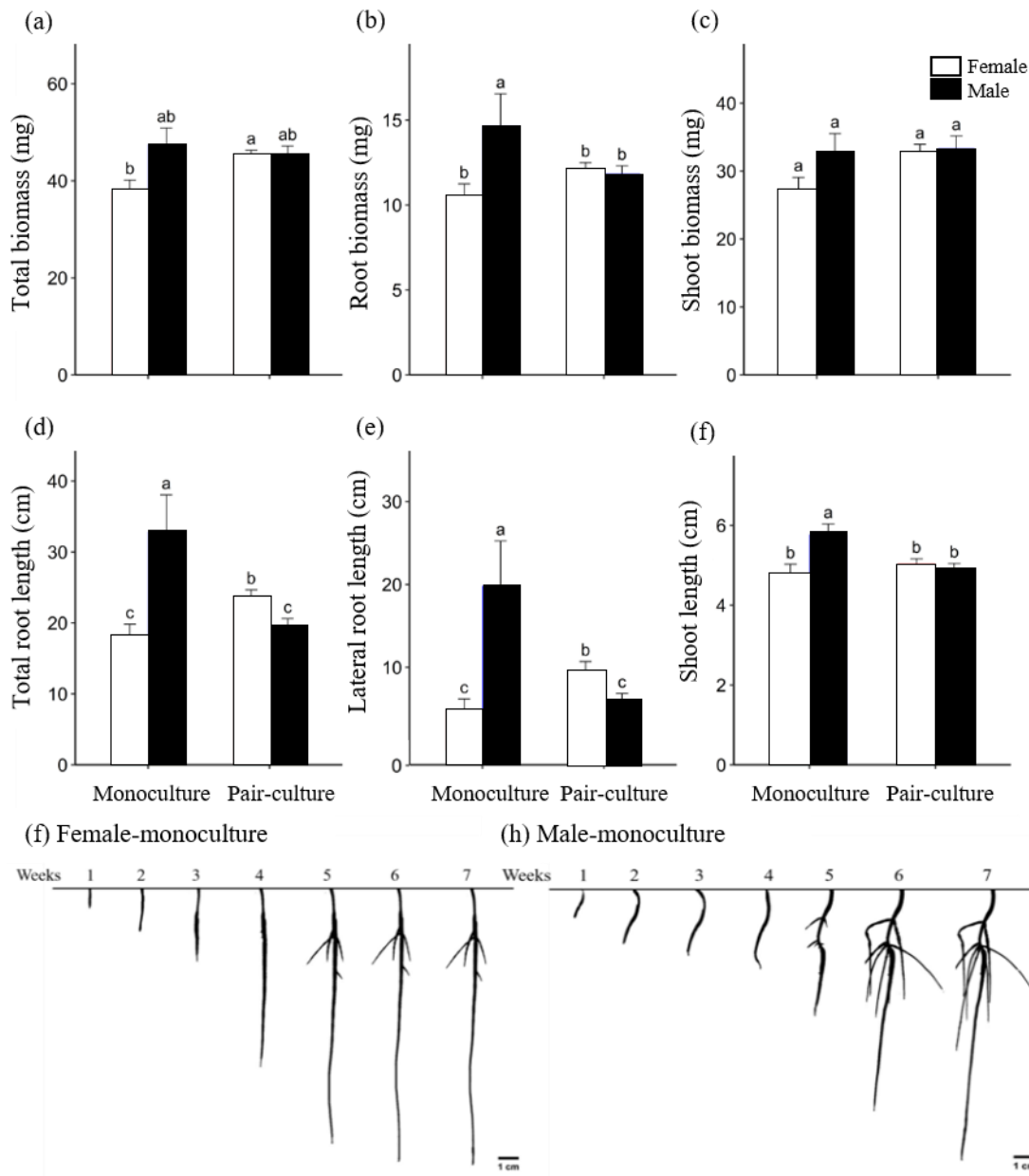
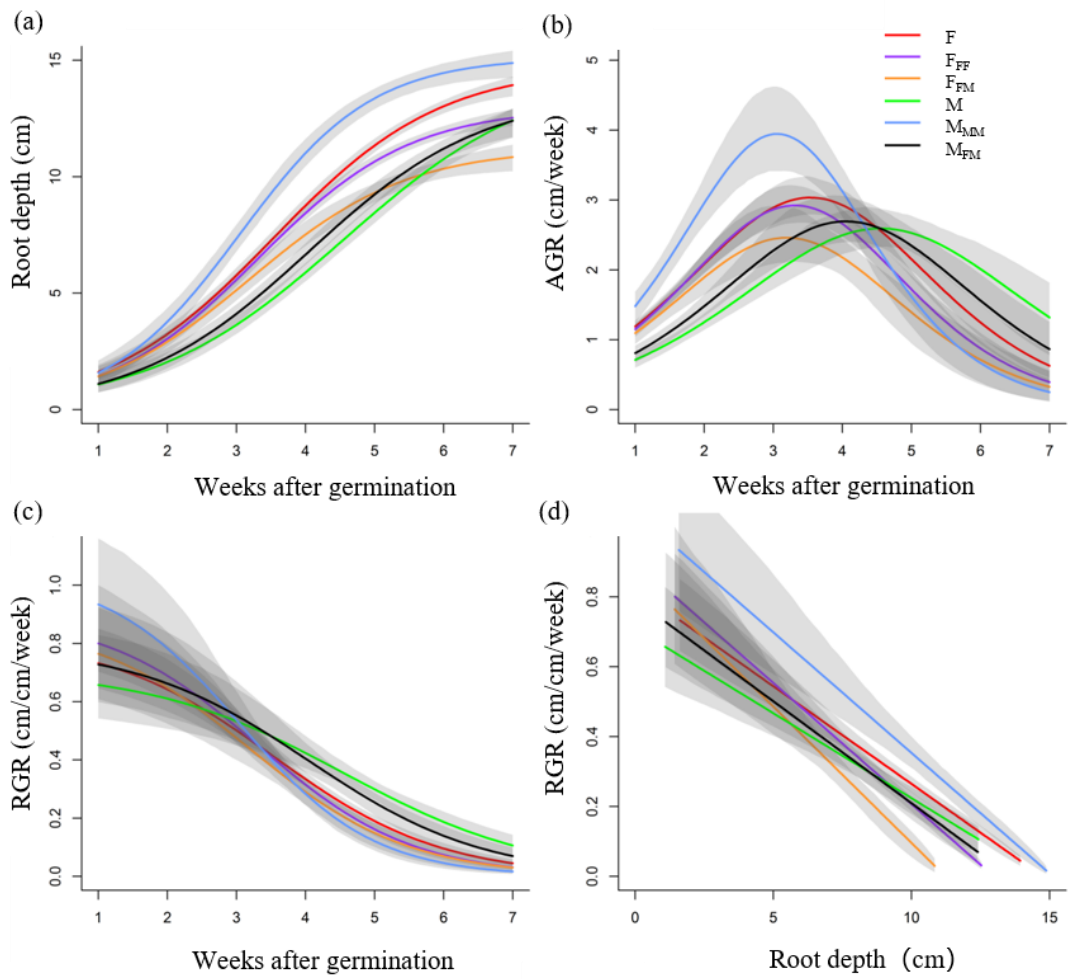
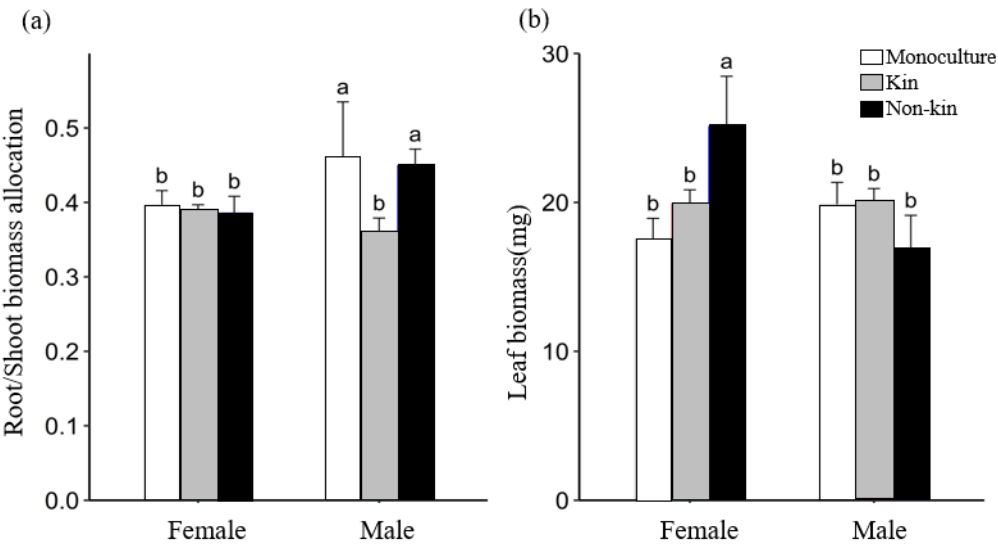


Figure 3



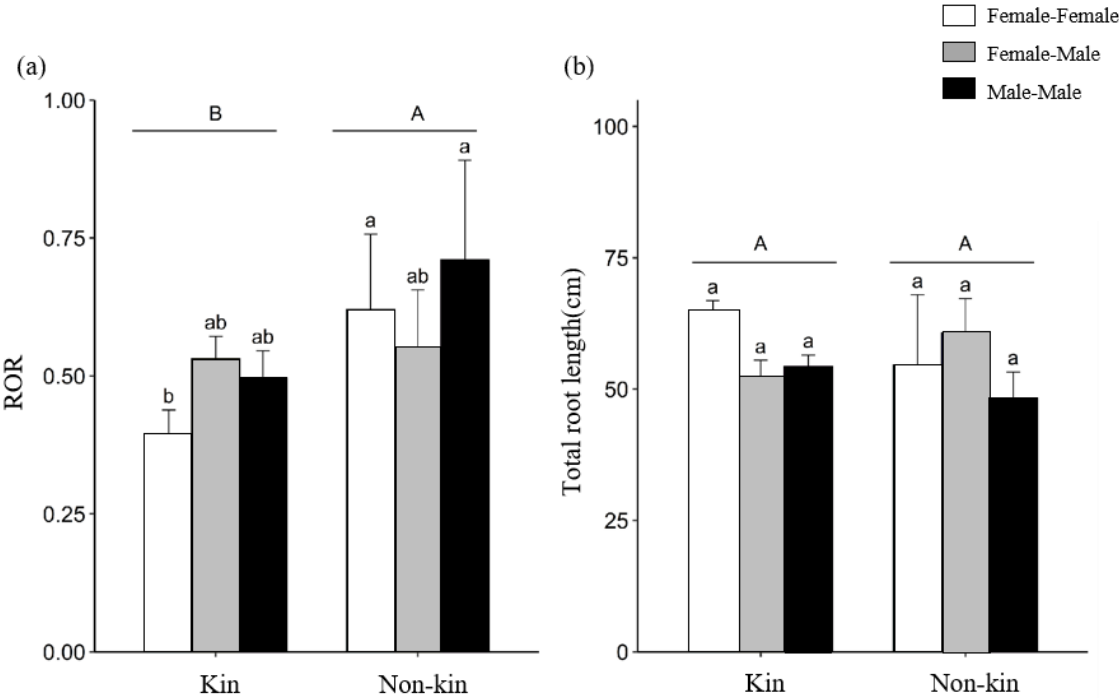
357 Figure 4

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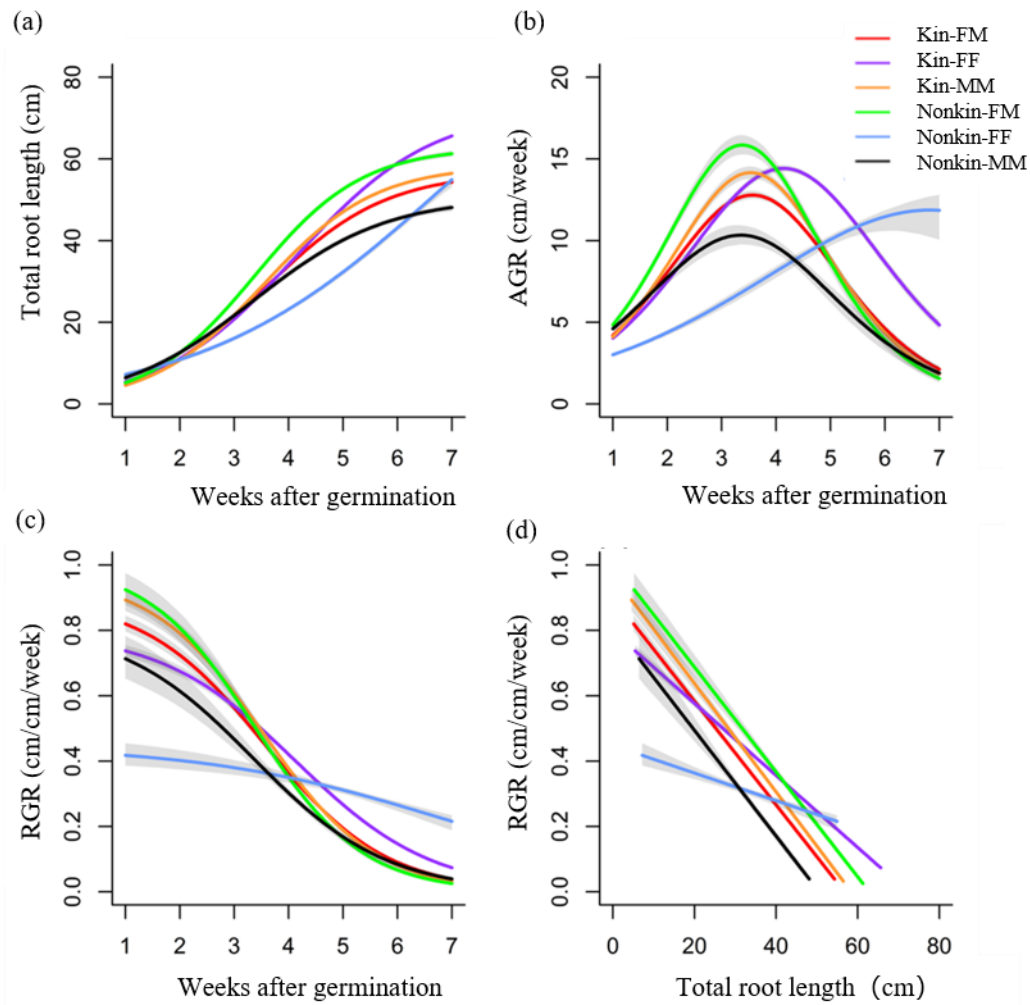
360 Figure 5



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Figure 6



## ACKNOWLEDGMENTS

This work was supported by National Non-profit Institute Research Grant of CAF (CAFYBB2017QC003) and NSFC (31370441, 31670628).

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