



Intraspecific interactions in the annual legume *Medicago minima* are shaped by both genetic variation for competitive ability and reduced competition among kin

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Abstract

Documenting if plants exhibit kin competition avoidance in intraspecific plant interactions is relevant both to improve crop growth, and to understand diversity and composition in natural plant communities. However, a number of confounding mechanisms complicates detecting kin competition avoidance from experiments comparing plants growing with kin and non-kin neighbors. We conducted complementary greenhouse experiments using genotypes from four populations of the annual *Medicago minima*, which in a previous study showed higher survival when interacting with kin relative to non-kin. We show that genotypes vary in kin competition avoidance, and in competitive ability, but find no indication of complementary resource use. Importantly, from our first experiment of root growth behavior, we know that some genotypes exhibit kin competition avoidance. Yet, the variation in competitive ability we find in our second experiment, where plants grow in mini communities together with either kin or unrelated genotypes, can alone explain the variation we observe in growth and biomass among communities. In our case, the genotypes with highest competitive ability were also those that showed kin competition avoidance. This confounding effect obscured the disentangling of mechanisms underlying difference in growth between kin and non-kin interactions. When silencing root exudates by adding activated carbon to a subset of our genotype combinations, we found increased size asymmetry of plants grown together, and mostly in kin communities. This suggests that plants recognize the identity of neighbors via root exudates, and compete less with neighbors recognized as kin. To detect kin competition avoidance we suggest designing experiments that pair unrelated genotypes with similar competitive abilities. Such design, combined with silencing root exudates would be powerful to detect whether plants show kin competition avoidance or not.

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Introduction

In nature, plants frequently interact with conspecifics. Because most plant populations show genetic structure, interactions between conspecifics often occur between

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genetically closely related individuals i.e. kin (Hamrick & Godt, 1996). It is generally expected that conspecifics have very similar resource requirements and thus that they compete strongly with each other. If genetically related conspecifics (i.e. kin) have more similar resource requirements than unrelated conspecifics, competition between kin should be even stronger (MacArthur & Levins, 1967; Young, 1981). However, when some studies reported reduced root competition in kin interaction experiments (e.g. Bhatt, Khandelwal & Dudley, 2010; Biedrzycki, Jilany, Dudley & Bais, 2010; Dudley & File, 2007), it spurred further interest in understanding if avoidance of competition among kin may frequently operate in plants (e.g. Callaway, 2007; Dudley, 2015; Ehlers & Bilde, 2019; Milla, Forero, Escudero & Iriondo, 2009). Kin competition avoidance has implications for our understanding of species co-existence (Ehlers, David, Damgaard & Lenormand, 2016) and is of interest in crop breeding (Kiers & Dennison, 2014; Weiner, Du, Zhang, Qin & Min-Li, 2017).

Establishing whether plants exhibit kin competition avoidance solely based on competition experiments comparing kin and non-kin interactions is not straightforward, because plants are highly plastic in their response to the environment (Novoplansky, 2009). When plant roots encounter roots from a neighbor plant it usually triggers increased root proliferation. This can in turn incur a “cost of competition” because increased root proliferation can mean diminished aboveground biomass (Gersani, Brown, O'Brien, Maina & Abramsky, 2001; O'Brien, Gersani & Brown, 2005; Semchenko, Saar & Lepik, 2014). Thus, if plants reduce root competition towards kin neighbors, plants may grow better with kin due to a reduced cost of competition. However, the mere observation that either a focal plant, or all plants grown together, grow better in kin relative to non-kin competition experiments is not proof of kin competition avoidance, as several confounding effects may also explain this result (Fig. 1).

First, genetic variation for competitive ability, such as variation in growth rate, and ability to suppress neighbor plants will influence the growth of plants regardless of whether kin competition avoidance occurs or not (e.g. Biernaski, 2011; Cahill, Kembel & Gustafson, 2005; Masclaux et al., 2010). One may expect competitively superior genotypes to grow larger in non-kin communities where they interact with competitively inferior neighbors, compared to kin-communities, where plants with similar competitive ability surround them. For competitively inferior genotypes, plants may grow larger in kin-communities, compared to when they are surrounded by non-kin genotypes that are competitively superior. Therefore, if there is genetic variation in competitive ability in the set of genotypes tested, differences in size asymmetry between kin and non-kin communities can be expected also in the absence of kin competition avoidance. Indeed, a higher variance in biomass among plants growing in non-kin groups relative to plants growing in kin groups is a common observation

(Donohue, 2003; Simonsen, Chow & Stinchcombe, 2014; Tonsor, 1989). Importantly, the larger variance in growth rate and size among plants in non-kin groups, can result in lower mean performance (biomass, or seed set) of the plants growing in non-kin groups because of diminishing return of investments in resource uptake or growth (Ehlers & Bilde, 2019; Masclaux et al., 2010; Simonsen et al., 2014). Higher mean performance of plants in kin communities can thus be higher simply due to more similar competitive abilities and hence is not proof of kin competition avoidance.

Second, if the genotypes tested happen to have complementary niche traits (e.g. differ in rooting depth) that allow plants to exploit resources without interfering with each other's niche, we may observe higher performance in non-kin communities. When two species compete in the same environment, character displacement may allow the species to co-exist by exploiting different niches (Pfennig, Rice & Martin, 2006). With a similar reasoning, different conspecific genotypes that vary in niche traits may grow better if genotypes compete less as they occupy slightly different niches. Resource partitioning and kin competition avoidance are, however, not mutually exclusive (File, Murphy & Dudley, 2012). Higher performance of plants grown in non-kin communities is therefore not proof that there is no kin competition avoidance.

Third, from kin selection theory, we may expect plants to behave altruistically towards their kin, i.e. that plants “pay a cost” as they sacrifice some of their own fitness in order to increase that of related neighbor plants. In this case, a focal plant that avoids competing with kin may grow smaller with kin neighbors compared to non-kin neighbors. However, as pointed out by Dudley (2015), kin competition avoidance does not need to be an altruistic trait in order to be favored by kin selection. Reduced competition towards kin does not necessarily result in a cost for the plant. Reduced root growth could instead reduce cost of competition that otherwise arises due to a diminishing return in aboveground biomass, by neighbors triggering an increased root proliferation in a focal plant (Gersani et al., 2001). Reduced cost of competition among kin could be a non-costly cooperative rather than a costly altruistic trait (Dudley, 2015).

The above illustrates that, without prior information on whether the plants exhibit kin competition avoidance, inferring what mechanisms are underlying competition outcomes in kin and non-kin competition experiments is very challenging.

Here, to gain insight into the mechanism underlying positive kin interactions, we used a set of genotypes, which in a previous study (Ehlers et al., 2016) showed a higher survival when grown together with a kin relative to non-kin. We therefore hypothesized that these genotypes may have a plastic growth response modulated by neighbor relatedness, and may exhibit kin competition avoidance. To further investigate the possible underlying mechanisms, we set up three different experiments. One experiment was designed to examine the root growth behavior of seedlings grown in

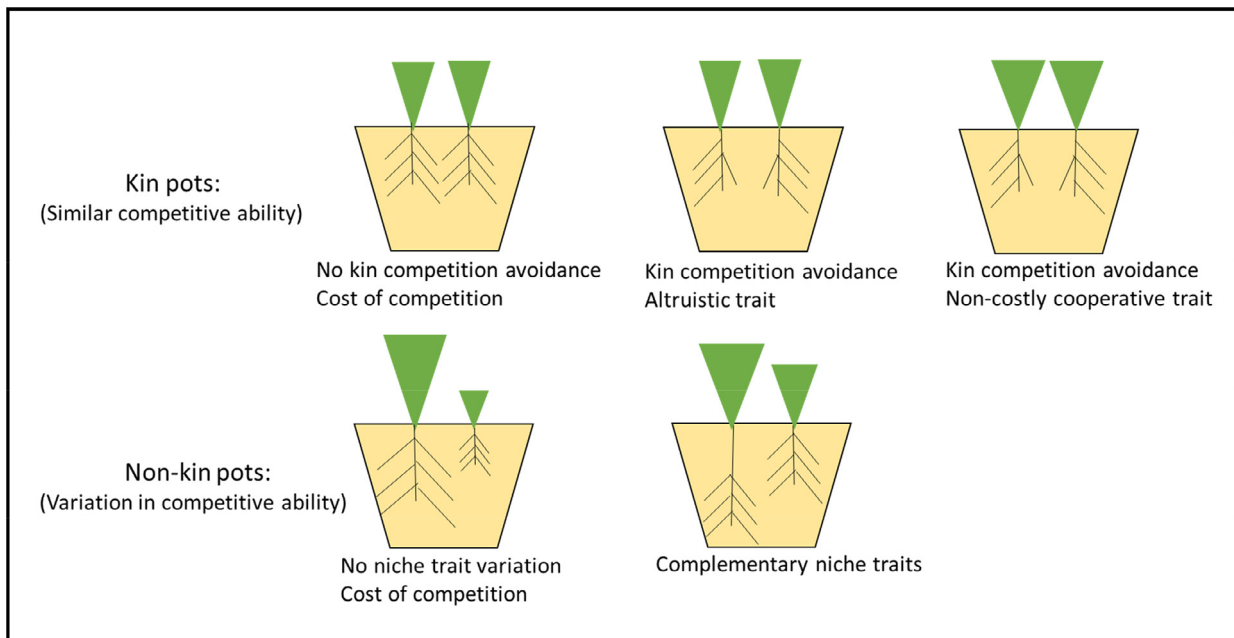


Fig. 1. Conceptual figure of mechanisms that can affect differences in plant growth between kin and non-kin pots. With presence of genetic variation in competitive ability, kin plants are competitively more similar than non-kin plants. Therefore, the variance in biomass among-plants within a pot is expected to be lowest in kin pots irrespective of whether they show kin competition avoidance or not. Plants that show kin competition avoidance (here shown as reduced root growth towards kin) are not expected to experience a cost of competition (e.g. a cost when an increased investment in roots results in decreased aboveground biomass). Kin competition avoidance can be an altruistic trait where a plant sacrifices some of its own fitness to increase that of the kin neighbor, or it can be a non-costly cooperative trait. Some non-kin plant pairs may show variation in niche traits that allow them to exploit different niches (here shown as variation in rooting depth).

pairwise competition, using a setting that allowed monitoring root growth over time towards a neighbor that was either a kin or a non-kin. A second experiment was set up to mimic different genetic neighborhoods a given genotype may experience under natural conditions. Rather than growing plants in pairwise competition, we used mini-communities consisting of four plants in a pot, with one central plant surrounded by three neighbor plants that were either kin, or different combinations of non-kin. This experiment ran from seedlings to adult plants, and we determined leaf growth rate, aboveground shoot biomass of all plants, and root biomass of the focal (central) plants. Lastly, we performed a third experiment, where we replicated a subset of the genotype combinations used in the second experiment, and added activated carbon to the soil. Activated carbon has the potential to silence root exudates and, provided these exudates act as neighbor recognition cues (Callaway, Ridenour, Laboski, Weir & Vivanco, 2005; Lau et al., 2008; Semchenko et al., 2014), prevent plants from recognizing their kin.

We used our first experiment to determine if genotypes showed a reduced root competition towards their kin. We infer that plants exhibit kin competition avoidance, if they show less root growth toward a kin neighbor relative to a non-kin neighbor.

In our second experiment, we hypothesize:

- 1) If there is genotypic variation in competitive ability, plants in kin pots should grow at a similar rate, and attain a more similar size compared to plants in non-kin pots (i.e. the variance in biomass among plants within pots should be smaller in kin treatments).
- 2) Focal genotypes that are competitively superior should grow larger in non-kin pots where they meet competitively inferior genotypes, and vice-versa for competitively inferior genotypes.
- 3) If genotypes exhibit kin competition avoidance, focal plants should have a lower root biomass in kin pots relative to non-kin pots. If this behavior is not an altruistic trait, focal plants that reduce their root growth when growing with kin should not pay a cost in terms of reduced shoot biomass.
- 4) If genotypes differ in niche traits and exploit resources differently, the total biomass of all plants in a pot should be larger in pots containing unrelated genotypes.

In our third experiment, we hypothesize:

- 5) If root exudates act as a cue to recognize a kin, and if plants avoid root competition with kin, silencing these signals (with activated carbon) should result in increased

competition among plants in kin communities but not among plants in non-kin communities.

Materials and methods

Study species

Medicago minima L. is an annual legume widespread around the Mediterranean basin. This species is autogamous and produces spiny dehiscent fruits typically containing 5–8 seeds each. Fruits mostly disperse close to the maternal individual, as they remain on the withered mother plant until the end of the life cycle, but they can also be passively dispersed by grazers attached to their fur, and possibly by ants (Wolff & Debussche, 1999). The population structure of *M. minima* has not been studied, but we assume that such structure exists. The reason is that closely related annual *Medicago truncatula* has a very similar biology and exhibits a population genetic structure; densely populated kin patches alternate with more heterogeneous patches composed of several genotypes (Bonnin, Ronfort, Wozniak & Olivieri, 2001; Siol, Bonnin, Olivieri, Prossperi & Ronfort, 2007). Due to the strong similarity in life-history traits between the two species (same habitat, annual, selfing, similar seed dispersal), we expect *M. minima* to exhibit a similar population genetic structure.

The four genotypes of *M. minima* originated from four different sites (one genotype per site) in the garrigue vegetation in the valley of St. Martin de Londres, Southern France. The distance between sampling sites varied from 500 m to 2 km, thus genotypes originated from similar climatic condition. Due to the autogamous mating system, seeds from different maternal plants within a site may still be from the same genotype. Therefore, only one genotype from each of the four sampling sites was used. Seeds from one maternal plant per site were grown in the greenhouse for one generation to remove maternal effects. Seedlings emerging from fruits from the same maternal plant are full sibs, and hereon we refer to them as kin.

Experimental set up

In all experiments, plants were grown in a mix of sand, greenhouse soil and vermiculite in proportions 1:1:1. To ensure germination, we scarified seeds with sand paper and placed them on wet filter paper for 48 h before sowing them onto seed trays placed in a greenhouse. Greenhouse temperature was set to a minimum temperature of 15 °C. After germination, seedlings were transplanted into pots. As seeds germinated over a couple of days there was a small variation in seedling size both within and among genotypes, and care was taken to initially match similarly sized seedlings for the same pot.

Experiment 1: We set up a pairwise comparison experiment in which each of the four target genotypes was grown with either a kin or a non-kin neighbor. Due to limitation in seed availability from some genotypes, we were not able to set up a full factorial experiment. We combined each focal genotype with two different non-kin genotypes, thus resulting in one kin treatment and two non-kin treatments for each focal genotype (Table 1). Each of these genotype combinations was replicated three times.

Pairs of plants were sowed at a distance of 2.5 cm in transparent plastic containers (30 cm × 25 cm × 3 cm) that allowed observing and measuring the direction of root growth. We kept the plastic containers tilted at a 50° angle, thus forcing roots to grow against the downward-facing surface. To avoid exposure of roots to light, we kept the downward-facing side of each container covered with a black plastic slate that was removed when recording root growth. Growth and direction of roots were monitored twice a week. A transparent millimeter plastic foil glued over the container's surface was used to mark root growth of each plant over time. Seedlings of *Medicago minima* have one primary root growing vertically, and a number of lateral roots developing subsequently from the primary root. Secondary roots can develop in any direction, but due to the experimental set up in which containers with reduced width were kept inclined, we forced lateral roots to grow in two dimensions, either toward or away from neighbors (Appendix A). This allowed easy categorization of roots growing either away or toward the neighbor plants.

After 6 weeks, when the roots of several plants reached the bottom of the containers the experiment was ended. We harvested each individual, and roots were carefully separated from soil, washed, oven dried at 70 °C degrees for 72 h and biomass estimated using a high-precision scale (Mettler Toledo AX504, $d = 0.1$ mg).

After completion of the experiment, the millimeter paper foils on which root development was recorded, were scanned and processed using the imaging software Image-J (Lobet, Pagès & Draye, 2011) to estimate number of lateral roots, and root length.

Experiment 2: Four seedlings of *M. minima* were planted in 20 cm diameter pots (volume 4 L). One focal genotype was

Table 1. Combinations of kin (K) and non-kin (NK) treatments used for both experiment 1 and experiment 2. Additional kin and non-kin treatments where activated carbon was added are marked with a “c”. The top row indicates the focal genotypes, while the column indicates the identity of the neighbor or surrounding genotypes.

	Focal genotype	SM1	SM2	SM3	SM4
Neighbours	SM1	K + c	NK + c		NK
	SM2	NK	K	NK	
	SM3	NK		K	NK
	SM4		NK	NK + c	K + c

located at the center of the pot and surrounded by three equidistant individuals. Focal genotypes were exposed to three different treatments based on surrounding plants' identity: 1) *kin*: the surrounding individuals belonged to the same genotype as the focal; 2) *non-kin*: the three surrounding individuals belonged to the same genotype, but were different from the focal one; 3) *mix*: the surrounding individuals belonged to three different genotypes different from each other and from the focal one. The three treatments were meant to represent different scenarios of relatedness among conspecifics that may occur in nature. A genotype is either growing in a kin patch of its own genotype (*kin*), in a kin patch of another genotype (*non-kin*), or in a patch of mixed genotypes.

Genotype combinations used for non-kin treatments were the same as those in experiment 1 (Table 1). Each combination treatment was replicated four times for each genotype (N pots = 64, N plants = 256).

Diameter and number of leaves on focal and neighbor plants was recorded weekly for three weeks to estimate early growth rate. Afterwards, due to technical difficulties of separating plants growing together, we restricted our measures to only record diameter of focal plants. After 16 weeks, aboveground biomass of all plants (focal and surrounding) was harvested individually. Roots of the focal plant was separated from the roots of the three surrounding plants, and washed with tap water. We note that separating adult focal plant roots from surrounding plants roots was difficult and somewhat error-prone, as roots were often highly intermingled. All harvested plant material was dried at 70 °C degrees for 72 h and weighed using a high-precision scale (Mettler Toledo AX504, $d = 0.1$ mg). Our plants did not produce flowers before the experiments ended. Hence, we were not able to obtain data on fruit set.

Experiment 3: We set up a third experiment consisting of a subset of the kin and non-kin treatments used in experiment 2 (three replicates for each) and added 20 ml activated carbon/l of soil. We did not have enough seedlings available to make a complete replicate of all the interactions from experiment 2, and we had no a priori knowledge of genotype specific behaviors, so genotypes assigned to either a kin or a non-kin experiment were chosen at random. Activated carbon can dampen the effects of root exudates, which is known in some plants to mediate neighbor identity recognition (Chen, Doring & Anten, 2012; Semchenko et al., 2014). The aboveground biomass of all plants was harvested after first harvesting the plants from our second experiment. Hence, plants in our third experiment grew one week longer in the mini-communities compared to experiment 2. Plants were dried and had their biomass obtained similar to the experiments above.

Statistical analyses

Experiment 1: For each focal plant, we estimated root behavior of focal plants by calculating the difference

between the number of lateral roots growing away from and growing towards the neighbor, standardized by the plant's total number of roots (calculated as: $(\text{roots}_{\text{away}} - \text{roots}_{\text{toward}}) / (\text{roots}_{\text{away}} + \text{roots}_{\text{toward}})$). The same calculation was applied to root length measured in cm. We applied linear models to test the effect of focal genotype, treatment (*kin* vs. *non-kin*) and their interaction on root behavior (creating separate models for number of roots and roots length).

Subsequently, we tested the effects of focal genotype identity and treatment (*kin* vs. *non-kin*) on belowground biomass (using generalized linear models with gamma distribution and identity link function) and on the roots-to-total biomass ratio (using linear models). Identity of neighbor genotypes was not specified in the model due to the limited number of replicates available.

Experiment 2: We calculated early radial growth and early leaf growth as the difference in respectively maximum diameter and number of leaves between week 2 and week 1. We applied linear regression models to test how early leaf growth of focal and surrounding individuals varied across treatments. We applied linear models to test for the effects of focal genotype, treatment (*kin*, *non-kin* and *mix*) and their interaction on early radial growth and final biomass of focal plants. We also tested how the identity of focal and surrounding genotypes affected the biomass production of focal plants from kin and non-kin communities using linear models.

Based on the biomass of focal plants, we calculated a relative interaction index (RII) modified from Armas, Ordiales and Pugnaire (2004). RII was calculated for each focal genotype separately. We first calculated the mean biomass attained by each focal genotype in kin treatments, and then used it as a baseline for comparing the biomass attained by the same focal genotype in each pot of non-kin and mix treatments. RII was calculated as follows for comparing kin and non-kin treatments $K_{\text{mean}} - \text{NK} / K_{\text{mean}} + \text{NK}$ and for comparing kin and mix treatments $K_{\text{mean}} - \text{Mix} / K_{\text{mean}} + \text{Mix}$. The index is symmetric around zero and ranges between +1 and -1. In our case, positive values of RII indicated that higher biomass was attained in kin compared to non-kin or mix treatments, and vice versa for negative values of RII.

To test how total pot biomass and how among-plants-within-pot variance (a measure of asymmetric size competition) in biomass changed across treatments, we used linear models. We also used linear models to test how within-pot variance in biomass and total pot biomass changed across treatments (*kin* vs. *non-kin*) in response to activated carbon (Experiment 3). For this analysis, within-pot variance was log-transformed.

All statistical analyses were conducted using the statistical software R version 3.5.3 (R Core Team, 2019). We carried out power analysis to estimate the power of our statistical tests, using the package *pwr* (Campely, 2018) and the function *pwr.f2.test*. Post-hoc tests were carried out using pairwise comparisons in the package *emmeans* (Lenth, 2019).

Results

Do plant genotypes avoid root competition with their kin?

In experiment 1, we found that root growth (both number and length of roots) away vs. toward a neighbor varied across focal genotypes and treatments (Table 2, Fig. 2A). Two genotypes (SM2 and SM3) grew more roots away from their neighbor when this was a kin compared to non-kin, a response consistent with avoiding root competition with kin. In contrast, one genotype (SM1) grew more roots toward kin neighbors compared to non-kin, and no differential root growth in response to neighbor identity was detected for the fourth genotype (SM4). These results were consistent for both the number of lateral roots (Table 2, Fig. 2A) and for the length of roots (Table 2, Fig. 2B). Power analysis indicated that the power of the test for number of roots was 0.83 and for root length 0.87.

We did not find any effect of treatment (kin vs. non-kin) on the root-to-total biomass ratio ($df = 1, 31, F = 0.14, p = 0.70$) of the six-weeks-old seedlings. Identity of focal genotype had an effect only on belowground biomass ($df = 3,31, F = 4.79, p = 0.007$), where seedlings of the focal genotype SM1 had obtained the lowest root biomass.

Does growth of genotypes differ between kin and different non-kin interaction treatments?

From our mini communities (experiment 2) a linear regression for growth of focal and surrounding plants leaves (Fig. 3A-C) revealed different slopes across treatments. There was a significant positive relationship between leaf growth of focal and surrounding plants in the kin treatments ($df = 1,31; F = 8.94, p = 0.009$), but not in mix ($df = 1,14; F = 0.19, p = 0.64$) and non-kin ($df = 1,30; F = 0.15, p = 0.69$) treatments. Thus, the leaf growth rate was more

Table 2. Results of linear models conducted on the number and the total length of roots growing away vs. toward a neighbor, in response to identity of focal genotype, treatment (kin or non-kin) and their interaction (Experiment 1).

No. roots	df	F	P
Focal genotype	3,28	4.42	0.012
Treatment	1,28	13.25	0.001
FG*T	3,28	7.68	0.001
Length of roots	df	F	P
Focal genotype	3,28	3.4	0.031
Treatment	1,28	14.6	0.001
FG*T	3,28	8.37	<0.001

similar among plants growing in the same pot, when they grew with kin.

A more similar growth among plants in the kin communities was also confirmed by analysis of among-plant within-pot variance in adult biomass. Although total pot biomass (Fig. 4A) was similar across treatments ($df = 2,61; F = 0.38, p = 0.68$), the within-pot variance in biomass (Fig. 4B) varied substantially across treatments ($df = 2,61; F = 3.59, p = 0.03, power = 0.622$). Variance in the aboveground adult biomass among plants within pots was three times higher in mix than in kin treatments, showing that mix treatments had the largest size asymmetry among plants grown together.

Genetic variation for competitive ability?

Genotypes differed significantly in growth rate estimated as early radial growth (Table 3). Genotype SM1 had the slowest and genotype SM3 the fastest growth rate, however no significant effect of treatment was found (Table 3).

We found no effects of genotype identity ($df = 3,58; F = 2.31, p = 0.08$) or treatment ($df = 2,58; F = 0.17, p = 0.83$) on aboveground biomass of adult focal plants. However, biomass of focal plants varied with genotype identity of surrounding plants. When analyzing for the effect of surrounding genotype identity on biomass of focal plants in kin and non-kin treatments, we found a significant effect of identity of surrounding genotype ($df = 3,41; F = 2.93, p = 0.04$). Focal plants surrounded by genotype SM1 attained the highest biomass and focal plant surrounded by genotype SM3 the lowest (Fig. 5). Together with the higher size asymmetry among unrelated plants growing together in pots with unrelated neighbors (Fig. 4B), and the difference in growth rate (Table 3) these results strongly indicate presence of genetic variation for competitive ability among genotypes. Genotype SM1 had the slowest growth rate and the least ability to suppress growth of neighbor plants, and SM3 with the fastest growth rate suppressed growth of neighbor plants the most.

The relative interaction index (RII) differed among adult genotypes ($df = 3,43; F = 4.72; p = 0.006$) but not between non-kin and mix treatments ($df = 1,43, F = 0.49; p = 0.48$). Here positive RII values indicate that a focal genotype produces a higher biomass in kin communities, and vice versa for negative values. SM2 and SM3 had the smallest RII values, and SM1 and SM4 the highest (Fig. 6). Thus when growing with kin, focal genotypes SM2 and SM3 attained the lowest aboveground biomass whereas SM1 and SM4 attained their highest.

Root biomass of focal adult plants differed among genotypes ($df = 3,52; F = 10.27; p < 0.001$), but showed no significant effect of treatment ($df = 2,52, F = 0.056; p = 0.94$) or interaction between genotype and treatment ($df = 6, 52; F = 0.64, p = 0.7$). Despite the lack of interaction effect, we note that SM2 and SM3 tended to have lower root biomass

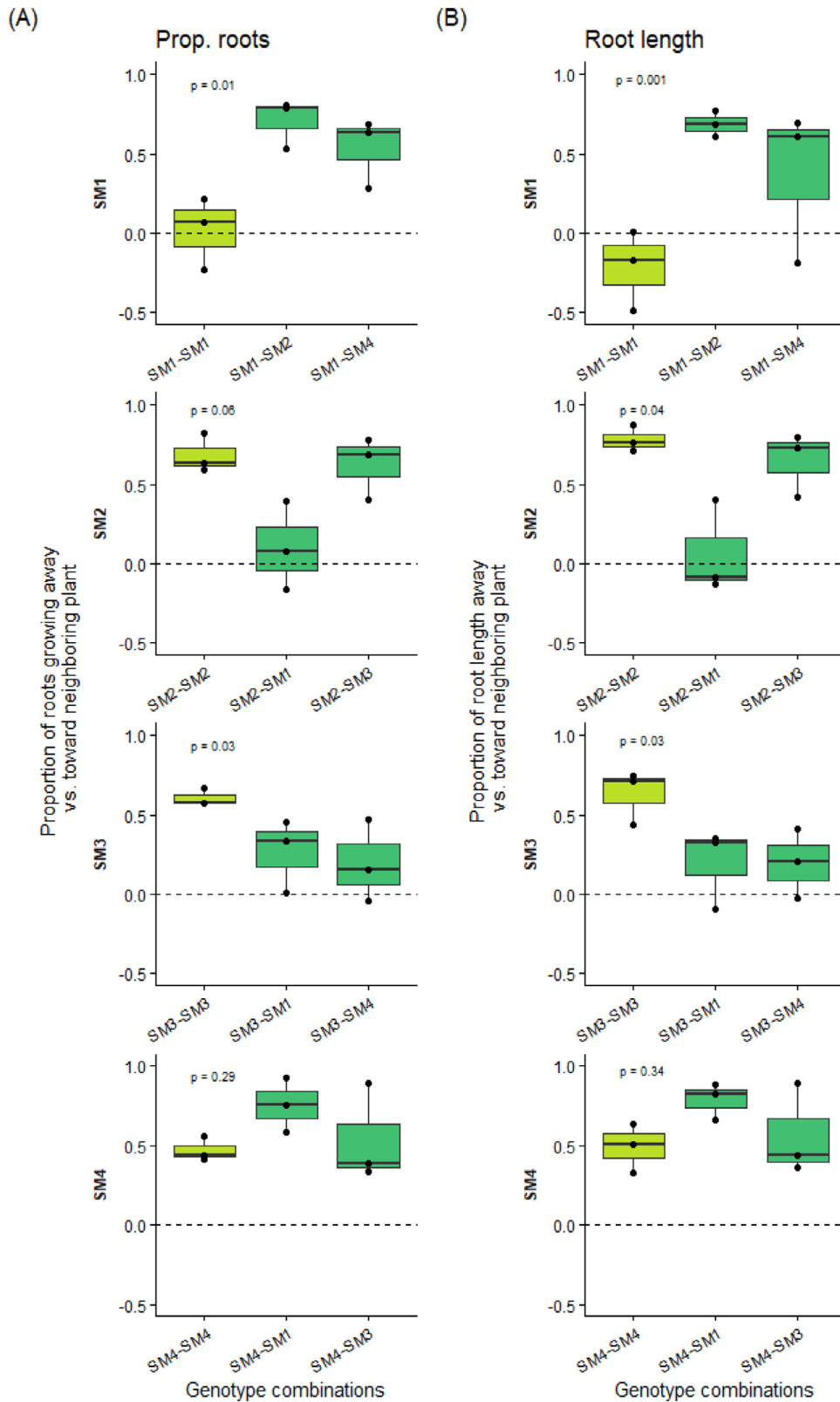


Fig. 2. Boxplots of (A) proportion of roots growing away vs. toward the neighboring plant, (B) proportion of root length growing away vs. toward the neighboring plant, (experiment 1). Each dot represents one individual replicate. Positive values indicate that a higher number of roots and higher root growth (length measured in cm) were recorded away vs. toward the neighbor. Panels represent focal genotypes (SM1, SM2, SM3, SM4) separately, and on each panel we report the genotype combinations for kin (in light green) and non-kin (dark green) treatments. *P*-values report results of Tukey HSD posthoc tests between kin and non-kin treatments, where non-kin treatment is the combined effect across the different non-kin genotypes.

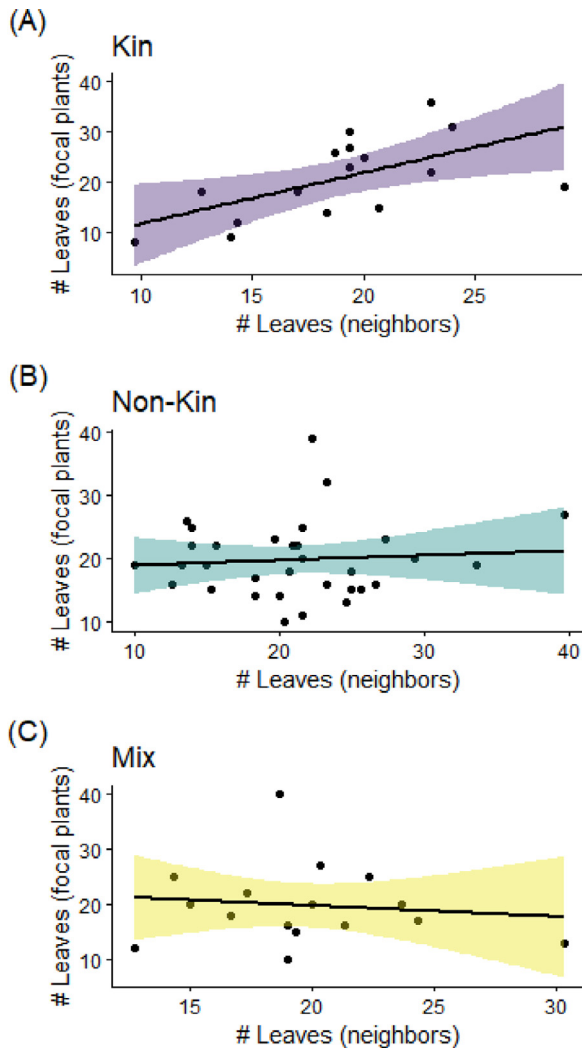


Fig. 3. Scatterplots representing the relationship between the numbers of leaves produced by focal plants as a function of the mean number of leaves produced by neighbor plants within each pot for (A) kin, (B) non-kin and (C) mix treatments. In each graph, single dots represent pots (experiment 2).

in kin communities compared to mixed communities, where SM1 and SM4 showed the opposite trend (Fig. 7).

Do root exudates act as recognition cue for neighbor identity?

The addition of activated carbon dramatically altered growth of plants. When soil was treated with activated carbon, the among-plant within-pot variance (power analysis = 0.74) in biomass increased 2.7 times for kin treatments and 1.04 times in non-kin treatments (Table 4, Fig. 4D). The total biomass in pots treated with activated carbon was 19% higher in kin treatments and 2% higher in non-kin treatments (power analysis = 0.69) (Table 4, Fig. 4C). The addition of activated carbon increased both the growth, and the size

asymmetry of plants grown together and especially in kin communities.

Discussion

Kin competition avoidance in plants is of high interest to fundamental understanding of plant interactions, and for improving crop growth by reducing competition among plants (Kiers & Dennison, 2014; Weiner et al., 2017). So far, evidence for reduced kin competition in cultivated species is scarce and, altruistic or cooperative properties might have been lost in modern crop varieties bred for high individual yield (Montazeaud et al., 2020). This suggests that it may be more likely to find plastic growth responses to neighbor identity in natural plant populations. Below, we discuss our results in the light of different possible mechanisms, and the complications of designing studies that can differentiate among these.

Our first experiment tested whether plants of *Medicago minima* altered their root growth toward their neighbor depending on neighbor relatedness. We found that the response to neighbor relatedness was genotype-specific. In general, all genotypes grew more roots away-from than toward their neighbors, which can be attributed to our experimental design, where two plants were grown in close proximity to each other leaving most space for root growth in the direction away from the neighbor (Appendix A). Two of the focal genotypes had a response to neighbors consistent with avoiding kin root competition. Genotypes SM2 and SM3 grew proportionally fewer roots toward their kin neighbors. In contrast, SM1 grew more roots toward its kin neighbors relative to non-kin. This genotype had also the slowest growth rate, and obtained the lowest biomass. When growing with a kin of similar slow growth rate, it may simply have resulted in more space to proliferate roots toward kin compared to more fast-growing non-kin neighbors. As the root behavior experiments was ended after six weeks, we do not know if SM1 may have shown reduced root competition at a later time, once its roots had grown in closer proximity to neighbor kin roots.

As hypothesized, for experiment 2, we did find that plants growing with kin had more equal growth rates, and obtained a more similar size compared to plants growing with unrelated genotypes. This result is consistent with kin communities being composed of competitively equivalent individuals, as also reported in previous studies (Donohue, 2003; Simonsen et al., 2014; Tonsor, 1989). Although only using four genotypes, we found that these varied in growth rate and in ability to suppress neighbors. SM2 and SM3 had the fastest growth rate, and suppressed neighbor plants the most. These genotypes were therefore likely competitively superior to SM1 and SM4. Focal individuals of the two competitively superior genotypes also had the lowest values of our RII estimates compared to the two less competitive (“inferior”) genotypes. This is consistent with

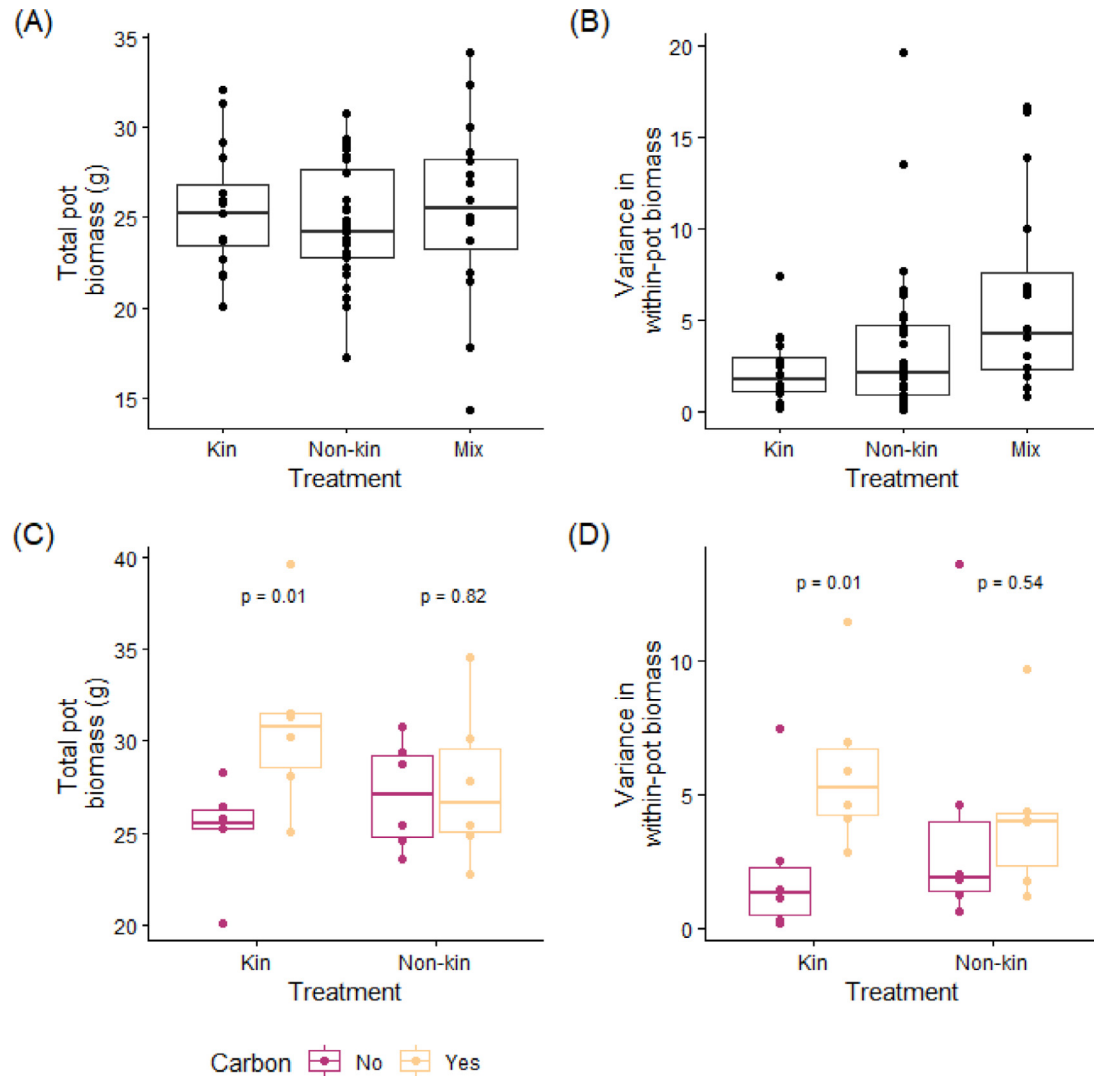


Fig. 4. (A, B) Boxplots representing total biomass and variance in within-pot biomass for untreated soil; (C, D) Boxplots comparing total pot biomass and variance in within-pot biomass for kin and non-kin treatments in untreated soil vs. soil with activated carbon (experiment 3). Dots indicate single pots.

our hypothesis, that competitively superior genotypes should grow largest in non-kin pots where they interact with competitively inferior plants, and vice versa for competitively inferior genotypes. Genotypes SM2 and SM3 did tend to have lower root biomass in kin communities compared to when they were growing in mixed communities, consistent with reduced root competition kin pots, and increased root

growth when interacting with many different genotypes. If reduced root growth towards kin is an altruistic trait, we expect this to result in a lower shoot biomass compared to a situation where the plant does not avoid root competition. We found no difference in shoot biomass of focal plants across treatments; this suggests that kin competition avoidance was not costly to the focal plant. However, the fact that the two competitively superior genotypes were also the only ones that showed reduced kin competition (experiment 1), complicates the interpretation of the growth patterns observed in experiment 2. The differences in growth and biomass from experiment 2 can be explained solely by variation in competitive ability, even though our first experiment showed that at least two of our four genotypes did show kin competition avoidance. Lower root biomass with kin compared to non-kin could, in our case, be the result of similarly high competitive ability among kin rather than kin competition avoidance.

Table 3. Results of linear models for early radial growth of focal individuals in response to focal genotype and treatment (Experiment 2), power analysis = 0.86.

Radial growth	df	F	P
Focal genotype	3,52	3.03	0.037
Treatment	2,52	2.32	0.108
FG*T	6,52	0.95	0.46

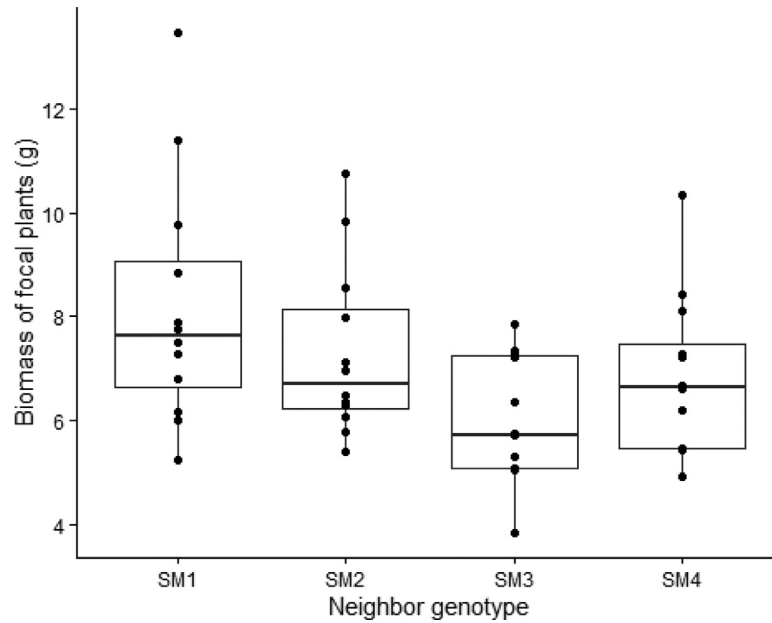


Fig. 5. Boxplot representing biomass of focal plants averaged across genotypes in response to the identity of the surrounding genotype using the treatments kin and non-kin where the identity of surrounding plants are the same (experiment 2). Dots represent single focal plants.

We found no difference in total pot biomass among treatments, and hence no indication that our genotypes varied in niche traits giving rise to resource partitioning. Intraspecific variation in functional vegetative traits that can relate to resource partitioning, occurs across environmental gradients, whereas genotypes from the same climatic regions have comparably similar vegetative traits (Kuppler et al., 2020). When studying kin competition avoidance, it is usually preferred to use genotypes that have a history of co-occurrence, originating from the same populations or from different

populations within the same study region (as in our study). However, at this spatial scale, we expect modest environmental gradients making it less likely that genotypes differ in vegetative traits that give rise to niche complementarity.

In our third experiment, we found that activated carbon increased overall biomass of plants. This may be a consequence of the carbon added, but we also note that these pots were harvested one week later than the pots grown without activated carbon. More importantly, we found that although activated carbon increased overall growth, it also increased

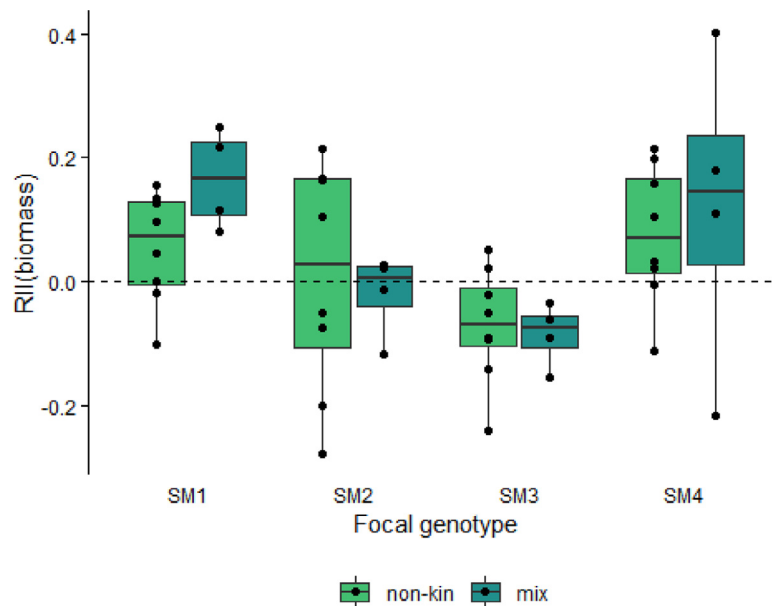


Fig. 6. The relative interaction index (RII) for each genotype. Positive values indicate that a focal plant obtained a higher biomass in kin communities compared to either non-kin or mix, and vice-versa for negative values.

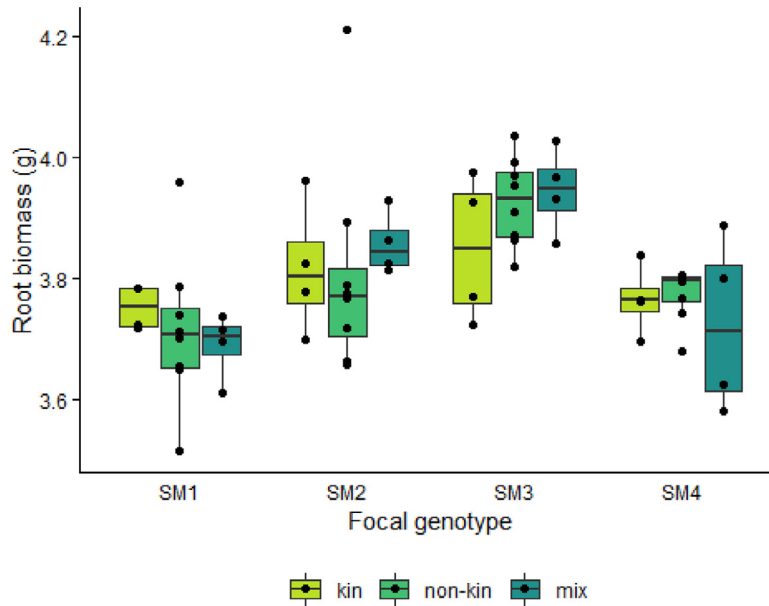


Fig. 7. Boxplot of the root biomass of focal plants growing in pots of either kin, non-kin, or mix treatments.

size asymmetry among plants growing in the same pot. This increase was much higher in kin communities, suggesting an increased competition among the kin plants when prevented from recognizing these as kin. However, this experiment was done on a subset of the treatment combinations used in our second experiment. The kin pots with activated carbon consisted – by chance – of genotypes SM1 and SM4, which in our first experiment did not show reduced kin competition. Yet, when silencing the root exudates, these genotypes showed an increased competition towards their kin neighbors, suggesting that these genotypes – the slowest growing ones – may exhibit kin competition avoidance occurring at a later stage than the duration of our first experiment.

In conclusion, our three complementary experiments on genotypes that were a priori expected to show positive kin interactions, illustrate the challenge of understanding the mechanisms underlying the different performance in kin vs.

non-kin conditions. Interpreting the outcome is complicated by the variation in competitive ability, and that such variation was confounded with variation in kin competition avoidance among genotypes. It is noteworthy that the genotypes with the highest competitive ability were also the genotypes showing the strongest kin avoidance for root competition, suggesting that kin competition avoidance may evolve jointly with competitive ability. Genotypes that are both highly competitive against non-kin and able to reduce their competition toward kin can become highly successful. However, this needs further studies using a larger number of genotypes than the present study. We suggest that experiments ideally should consist of genotypes chosen based on prior knowledge of their competitive ability. This would allow to control for competitive similarity (by using different genotypes of similar competitive ability) when comparing the outcomes of interaction between kin versus non-kin interactions. Furthermore, such a design, combined with treatments that prevent plants from recognizing neighbor identity could be particularly powerful to disentangle reduced kin competition from competitively equivalent but unrelated neighbors.

Table 4. Linear models on the within pot variance in biomass among plants and total pot biomass in response to treatment (kin vs. non-kin), application of activated carbon, and their interaction.

Within-pot variance	df	F	P
Treatment	1,20	0.124	0.728
Carbon	1,20	5.95	0.024
Treatment*Carbon	1,20	2.49	0.130

Pot total biomass	df	F	P
Treatment	1,20	0.22	0.674
Carbon	1,20	4.15	0.056
Treatment*Carbon	1,20	2.84	0.103

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.baae.2021.03.003.

References

- Armas, C., Ordoles, R., & Pugnaire, F. I. (2004). Measuring plant interactions: A new comparative index. *Ecology*, *85*, 2682–2686.
- Bhatt, M. V., Khandelwal, A., & Dudley, S. A. (2010). Kin recognition, not competitive interactions, predicts root allocation in young *Cakile edentula* seedling pairs. *New Phytologist*, *189*(4), 1135–1142. doi:10.1111/j.1469-8137.2010.03548.x.
- Biedrzycki, M. L., Jilany, T. A., Dudley, S. A., & Bais, H. P. (2010). Root exudates mediate kin recognition in plants. *Communicative & Integrative Biology*, *3*(1), 28–35.
- Biernaskie, J. M. (2011). Evidence for competition and cooperation among climbing plants. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1714), 1989–1996. doi:10.1098/rspb.2010.1771.
- Bonnin, I., Ronfort, J., Wozniak, F., & Olivieri, I. (2001). Spatial effects and rare outcrossing events in *Medicago truncatula* (Fabaceae). *Molecular Ecology*, *10*(6), 1371–1383.
- Cahill, F. J. J., Kembel, S. W., & Gustafson, D. J. (2005). Differential genetic influences on competitive effect and response in *Arabidopsis thaliana*. *Journal of Ecology*, *93*(5), 958–967.
- Callaway, R. M., Ridenour, W. M., Laboski, T., Weir, T., & Vivanco, J. M. (2005). Natural selection for resistance to the allelopathic effects of invasive plants. *Journal of Ecology*, *93*(3), 576–583.
- Champely, S. (2018). Package ‘pwr’. Retrieved from <http://cran.r-project.org/package=pwr>
- Chen, B. J., Doring, H. J., & Anten, N. P. (2012). Detect thy neighbor: Identity recognition at the root level in plants. *Plant Science*, *195*, 157–167.
- Core Team., R. (2019). *R: A language and environment for statistical computing* Vienna, Austria: R Foundation for Statistical Computing. Retrieved from <https://www.R-project.org/>.
- Donohue, K. (2003). The influence of neighbor relatedness on multilevel selection in the Great Lakes sea rocket. *The American Naturalist*, *162*(1), 77–92. doi:10.1086/375299.
- Dudley, S. A. (2015). Plant cooperation.. *AoB Plants*, *7* Plv113.
- Dudley, S. A., & File, A. L. (2007). Kin recognition in an annual plant. *Biology Letters*, *3*(4), 435–438.
- Ehlers, B. K., & Bilde, T. (2019). Inclusive fitness, asymmetric competition and kin selection in plants. *Oikos (Copenhagen, Denmark)*, *128*(6), 765–774. doi:10.1111/oik.06390.
- Ehlers, B. K., David, P., Damgaard, C. F., & Lenormand, T. (2016). Competitor relatedness, indirect soil effects and plant coexistence. *Journal of Ecology*, *104*(4), 1126–1135. doi:10.1111/1365-2745.12568.
- File, A. L., Murphy, G. P., & Dudley, S. A. (2012). Fitness consequences of plants growing with siblings: Reconciling kin selection, niche partitioning and competitive ability. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1727), 209–218.
- Gersani, M., Brown, J. S., O'Brien, E. E., Maina, G. M., & Abramsky, Z. (2001). Tragedy of the commons as a result of root competition. *Journal of Ecology*, *89*, 660–669.
- Hamrick, J. L., & Godt, M. J. W. (1996). Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, *351*, 1291–1298.
- Kiers, E. T., & Denison, R. F. (2014). Inclusive fitness in agriculture. *Philosophical transactions of the Royal Society B*, *369*, 20130367.
- Kuppler, J., Albert, C. H., Ames, G. M., Armbruster, W. S., Boenish, G., Boucher, F. C., et al. (2020). lobar gradients in intraspecific variation in vegetative and floral traits are partially associated with climate and species richness. *Global Ecology and Biogeography*, *29*, 992–1007.
- Lau, J. A., Puliafico, K. P., Kopshever, J. A., Steltzer, H., Jarvis, E. P., Schwarzländer, M., & Hufbauer, R. A. (2008). Inference of allelopathy is complicated by effects of activated carbon on plant growth. *New Phytologist*, *178*(2), 412–423. doi:10.1111/j.1469-8137.2007.02360.x.
- Lenth, R. (2019). *Emmeans: Estimated marginal means, aka least-squares means*. Retrieved from <https://CRAN.R-project.org/package=emmeans>.
- Lobet, G., Pagès, L., & Draye, X. (2011). A novel image-analysis toolbox enabling quantitative analysis of root system architecture. *Plant Physiology*, *157*(1), 29–39.
- MacArthur, R., & Levins, R. (1967). The limiting similarity, convergence, and divergence of coexisting species. *The American Naturalist*, *101*(921), 377–385.
- Masclaux, F., Hammond, R. L., Meunier, J., Gouhier-Darimont, C., Keller, L., & Reymond, P. (2010). Competitive ability not kinship affects growth of *Arabidopsis thaliana* accessions. *New Phytologist*, *185*(1), 322–331. doi:10.1111/j.1469-8137.2009.03057.x.
- Milla, R., Forero, D. M., Escudero, A., & Iriondo, J. M. (2009). Growing with siblings: A common ground for cooperation or for fiercer competition among plants? *Proceedings of the Royal Society B: Biological Sciences*, *276*(1667), 2531–2540. doi:10.1098/rspb.2009.0369.
- Montazeaud, G., Rousset, F., Fort, F., Violle, C., Fréville, H., & Gandon, S. (2020). Farming plant cooperation in crops. *Proceedings of the Royal Society B*, *287*, 20191290.
- Novoplansky, A. (2009). Picking battles wisely: Plant behaviour under competition. *Plant Cell and Environment*, *32*, 726–741.
- O'Brien, E. E., Gersani, M., & Brown, J. S. (2005). Root proliferation and seed yield in response to spatial heterogeneity of below-ground competition. *New Phytologist*, *168*, 401–412.
- Pfennig, D. W., Rice, A. M., & Martin, R. A. (2006). Ecological opportunity and phenotypic plasticity interact to promote character displacement and species coexistence. *Ecology*, *87*, 769–779.
- Semchenko, M., Saar, S., & Lepik, A. (2014). Plant root exudates mediate neighbor recognition and trigger complex behavioural changes. *New Phytologist*, *204*(3), 631–637. doi:10.1111/nph.12930.
- Simonsen, A. K., Chow, T., & Stinchcombe, J. R. (2014). Reduced plant competition among kin can be explained by Jensen's

- inequality. *Ecology and Evolution*, 4(23), 4454–4466. doi:10.1002/ece3.1312.
- Siol, M., Bonnin, I., Olivieri, I., Prosperi, J. M., & Ronfort, J. (2007). Effective population size associated with self-fertilization: Lessons from temporal changes in allele frequencies in the selfing annual *Medicago truncatula*. *Journal of Evolutionary Biology*, 20(6), 2349–2360. doi:10.1111/j.1420-9101.2007.01409.x.
- Tonsor, S. J. (1989). Relatedness and Intraspecific competition in *Plantago lanceolata*. *American Naturalist*, 134, 897–906.
- Weiner, J., Du, Y.-L., Zhang, C., Qin, X.-L., & Min-Li, F. (2017). Evolutionary agroecology: Individual fitness and population yield in wheat (*Triticum aestivum*). *Ecology*, 98, 2261–2266.
- Wolff, A., & Debussche, M. (1999). Ants as seed dispersers in a mediterranean old-field succession. *Oikos (Copenhagen, Denmark)*, 84, 443–453.
- Young, J. (1981). Sib competition can favor sex in two ways. *Journal of Theoretical Biology*, 88(4), 755–756.

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