

The Rhizosphere Effect of Plant Kin Recognition: Morphological Responses Combined with Soil Nitrogen Cycling

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Abstract

Aims Kin recognition has been used to explain plant interactions among siblings, but the morphological-based conclusions are various and the mechanism is still fuzzy. Here, we tested the rhizosphere effect of plant kin recognition based on soil nitrogen (N) cycling resulted from root exudates, combined with plant fitness, morphological and physiological performances to examine how plants respond to kin neighbors.

Methods One factorial experimental design of relatedness including either sibling or strangers of *Glycine max* was constructed. After growing about three months, plant morphological traits including plant height, specific leaf area (SLA) and root length as well as plant biomass; physiological traits including root activity, nitrate reductase (NR) activity and contents of chlorophyll; plant N use efficiency of each individuals were measured. Moreover, the production rate of root exudates carbon (C) and N, soil microbial biomass C and N, as well as genes amoA-AOAs, amoA-AOBs, nifH, nirK, nirS and nosZ genes related with soil N were assayed. Finally, the abundances of soil archaea, bacteria and fungi were quantified.

Results Our study showed significant higher plant fitness and physiological growth and N use efficiency in siblings than strangers. The root secreted C rather than secreted N was sensitive to kin identity of *G. max*. Moreover, higher root secreted C quantity of sibling also ignited increasing of soil microbial biomass especially the abundance of Archaea community, and the abundance of amoA-AOAs gene compared to stranger soils. Finally, siblings increased the supply of soil available N and N use efficiency compared to strangers.

Conclusions The rhizosphere changes induced by root exudation resulted in increased fitness and greater resource use efficiency among siblings compared to strangers. These findings suggest that the rhizosphere effect of soil microbial changes and soil N cycling and transformation triggered by the root-exuded C, could be a potential underground feedback mechanism for multiple kin recognition responses.

Introduction

It has been suggested that plants can recognize their kin neighbours and non-self neighbours, and this recognition can have affect direct and inclusive fitness for plants growing with relatives (Hamilton, 1964). Kin recognition is often expressed through decreasing competition by lower investments to fine roots (Gruntman & Novoplansky, 2004; Dudley & File, 2007; Bhatt & Dudley, 2010; Biedrzycki et al., 2010), producing less leaf area (Ninkovic, 2003) or less leaf overlap in kin groups compared to strangers (Crepy & Casal, 2015), and these changes may increase fitness in sibling groups (Tonsor, 1989; Biernaskie, 2011). However, plants may have trade-offs between their traits (Schlichting, 1986; Casper & Jackson, 1997; Cahill & Casper, 2000; Toledo-Aceves & Swaine, 2008), making it difficult to determine if kin recognition in morphological traits has fitness consequences (File et al., 2012; Till-Bottraud & de Villedemereuil, 2016). Some physiological performance traits which can sufficiently reveal plant competitive interactions may also be candidates for kin recognition responses. For example, photosynthesis is likely

to be changed if carbon-use efficiency is affected by neighboring plants (McCormick et al., 2006; Lendenmann et al., 2011). The competition between plants could alter the storage of carbohydrates (Benayas et al., 2003) and thus increase communication by higher roots activity (Yoder, 1999; Callaway & Aschehoug, 2000; Islam et al., 2007; Biedrzycki et al., 2010). Studies have found that plants growing together with their clones decreased their root respiration rate compared to plants growing with non-self neighbours (Meier et al., 2013). However, other physiological responses to the relatedness of neighbours remain less unexplored and need more investigation (Till-Bottraud and de Villedieu, 2016).

In addition to reducing competition, kin recognition could also be revealed through increasing resource use efficiency, especially when the fitness is not available for direct measurements (Cheplick, 1992; James et al., 2005; File et al., 2011, 2012; Meier et al., 2013; Simonsen et al., 2014). Unlike inter-specific plants which access multiple soil resources by consuming different nutrient forms (Mc Kane et al., 2002) or extending roots differently to both horizontal and vertical soil layers (Cahill et al., 2010), kin plants significantly decreased uptake of total nitrogen (N) and NO_3^- -N than strangers to reduce N competition (Zhang et al., 2015), with improved use efficiency of N and other nutritive elements (Maestre et al., 2009; Zhang et al., 2015; Till-Bottraud & de Villedieu, 2016; Li et al., 2017, 2018). Because N is a major nutrient limiting plant growth in most terrestrial ecosystems (LeBauer & Treseder, 2008 Ecology) and sensitive to kin interactions (Zhang et al., 2016; Li et al., 2017, 2018), the amounts and forms of available soil N, their uptake and allocation in plant tissues may play a significant role in kin recognition or competition (Cheplick, 1992; Zhang et al., 2015). Therefore, clarifying how kin recognition affects soil N cycling as well as the related functional genes of soil microbes can provide insights into the mechanisms for plant competition or cooperation with relatives. However, it remains unexplored so far.

Nitrification and denitrification are two critical processes of the soil N cycling, which are mediated by specific functional microbial populations. Nitrification converts ammonia to nitrate via nitrite (Farmaha, 2014; Wrage et al., 2001). The nitrification intensity is thought to be catalyzed primarily by autotrophic ammonia-oxidizing bacteria (AOB) belonging to β - or γ -proteobacteria usually in neutral and alkaline soils (De Boer & Kowalchuk, 2001; Prosser, 1990; Jia & Conrad, 2009; Xia et al., 2011) and by ammonia-oxidizing archaea (AOA) (Konneke et al., 2005; Leininger et al., 2006; Treusch et al., 2005; Venter et al., 2004) especially in acidic soils (Prosser & Nicol, 2012; Yao et al., 2011; Zhang et al., 2012; Gubry-Rangin et al., 2010; Lu et al., 2012). In contrast, denitrification has been considered to be an important pathway for N loss from soil through N_2 and N_2O in terrestrial ecosystems. It can be predicted by the genetic expression of *nirK*, *nirS*, *nosZ* and etc. (Thamdrup and Dalsgaard, 2002; Dalsgaard et al., 2003; Lam et al., 2009). The absorption and use efficiency of available N by plants under N-limited conditions is closely related with the conversion of nitrification and denitrification involved with related functional microbes. Besides, biological N fixation is also another important pathway of N input through fixing atmospheric N_2 to bio-available N, which is achieved by diazotrophs using nitrogenase encoded principally by the genes of *nifH* (encoding the nitrogenase reductase subunit) (Rösch et al., 2002, Warttinen et al., 2008, Tu et al., 2016, Fan et al., 2018). Responses of such functional genes related to soil N cycling to kin interactions

may provide new insights into mechanism-based replenishment to kin response on N use efficiency (NUE).

The rhizosphere microbes can be stimulated by root exudates and thus accelerate soil carbon and nitrogen turnover, affecting the nutrient acquisition of soil microorganisms and plants (Eisenhauer et al, 2012). Numerous studies show that there is a direct relationship between the exudates of plant root system and the soil enzyme activity, because plant root exudates themselves contain enzymes and certainly have impact on the original soil enzyme activity (Dijkstra et al, 2007). Moreover, 20–60% that carbon fixed by plants through photosynthesis is transported to plant roots, and 40%-70% of which is released into rhizosphere as root exudates (Kuzyakov et al, 2000). Therefore, a large amount of carbon and nitrogen accumulate in rhizosphere soil and become the substrate of microbial activities, which affects the growth and structure of microbial community (Singh et al, 2006) as well as the release and circulation of soil nutrients mediated by soil microbes (Dinkerlaker et al., 1989; Fisk et al., 2015). It was found that the increased carbon-nitrogen ratio of plant root exudates could increase the abundance of soil microorganisms, because higher carbon-nitrogen ratio would promote soil microorganisms to secrete more extracellular enzymes to accelerate the decomposition of organic matter and provide carbon and nitrogen for the growth of microorganisms (Zhang et al., 2013). Additionally, root exudates are suggested to be kin signals among plant relatives, but it remains unclear which root exudates participate in kin recognition (Biedrzycki et al., 2010). Therefore, the study of root exudates could not only clarify how the kin recognition signals are passed, but also whether the microbial responses and soil enzyme effect are induced by root exudates response to relatedness.

Kin effects have been reported in some crop species such as *Hordeum vulgare* (Ninkovic, 2003), *Pisum sativum* (Meier et al., 2013), *Lupinus angustifolius* (Milla et al., 2009; 2012), *Oryza sativa* (Fang et al., 2013), perhaps because these crop species are planted widely in the world and are most likely to have relatives growing together and sensitive to N utilization (Hess and De Kroon, 2007; File et al., 2011). In this experiment, we chose *Glycine max* to represent monoculture plants and examine how plant responds to kin neighbors through fitness, morphology, physiology and nutritional absorption performance. We test the following hypotheses: 1) *G. max* plants respond to kin by increasing physiological growth efficiency and fitness; 2) input of plant root exudates (mainly carbon and nitrogen production rate) could be kind of kin signals, and increased of which response amplifies the consequences of growing with kin; and 3) the relatedness of plants in a pot affects functional genes related to the soil N cycling and leads to the differences of NUE between plants growing with kin and strangers. Testing these hypotheses will provide a new perspective on kin interactions and benefit on our understanding of how they evolve (File et al., 2012; Simonsen et al., 2014).

Material And Methods

Experiment Design and Arrangement

Seeds of *Glycine max* (L.) *merr.* were collected from 4 mother plants of one cultivar growing at least 20 meters far away in different plot of the same farm in Gansu Province. Before pollination, we selected some plants with similar size and bagged then with customized transparent waterproof bags to prevent cross-pollination. Those seeds were separated into family groups so that we could construct groups of siblings (seeds from the same mother) and strangers (seeds from different mothers). After germination of seeds in distilled water and growth for three days, two healthy and similarly sized seedlings (to eliminate the size effect) including either siblings (Siblings treatment) or strangers (Strangers treatment) from 4 mother plants were planted randomly as pairs in one pot (10 cm height and 20 cm diameter). The distance between two seedlings was 10 cm and there were 12 replicates of each treatment (experiment 1). At the same time, the same arrangement was repeated for root secretion collection (experiment 2).

Growth Conditions and Management

The soil in planting pots contained 50% sand and 50% humus. We also created six pots without plants as a soil control. Seedlings were then grown in a greenhouse at the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. The temperature of the glasshouse was 28°C in the daytime and 18°C at night time, with a light:dark photoperiod of 16:8 h. The photosynthetic photon fluency rate was $180 \mu\text{M m}^{-2} \text{s}^{-1}$ with relative humidity of approximately 60%. Pots were watered every three days to maintain soil moisture but without fertilization.

Sample Collection

After growing for three months in the greenhouse, plants in experiment 1 were harvested from each plot and 50 g of soil that around to the root system were collected and sieved to through 2mm aperture sieves as rhizosphere soil samples of each individual (45 g were stored at 4°C and 5g were stored at -80°C respectively). Roots were washed for a while and put into 0.5 mM CaCl_2 solution for 3 minutes and then washed by H_2O to removing ^{15}N absorption on the root surface. After measurement of morphological indexes, plant materials were then dried at 65°C for 48 h for biomass, then ground with ball mill (MM2, Retsch, Haan, Germany) for measurements of N contents and $^{15}\text{N}/^{14}\text{N}$.

Root Secretion Collection

At the same time of labelling, plants in experiment 2 were carefully moved out from soil pots and washed gently by sterile water and transplanted in a modified collection device to collect plant root secretion (Pilon et al, 2013). Briefly, a triangular funnel with sterilized glass beads were used to simulate sterile soil pots, and the funnel bottom was fixed with nylon mesh to prevent glass sand from blocking and connected with a vacuum pump to extract root secretion through a plastic hose. The plants in triangular funnels were irrigated with 50 ml carbon-free nutrient solution (0.1 M KH_2PO_4 ; 0.2 M K_2SO_4 ; 0.2 M MgSO_4 ; 0.3 M CaCl_2) and were allowed to grow for 24 h (Yin et al, 2014). The solutions in funnels were pumped out and the funnels were washed twice with the C-free nutrient and pumped out and both were discarded after which we repeated process, and collected all solutions in funnels and dried them under -60°C using a freeze dryer. Then the sediments were diluted to 10 ml with sterile water for analysis C and N of root secretion. After the collection of root exudates, the fresh root and leaves were washed clearly by

distilled water and stored at 4°C for physiological measurements of plant root activity, chlorophyll content, NO₃⁻ content and nitrate reductase (NR) activity both in siblings and strangers.

Soil DNA Extraction and Quantitative PCR

Soil DNA was extracted from 0.25 g samples with the MoBio PowerSoil™ DNA Kit (MO BIO laboratories, CA, US) according to the manufacturers' protocol. Extracted DNA was verified on 1% agarose gel and concentrations were determined photometrically using a Nanodrop ND – 1000 UV – Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Archaeal, bacterial and fungus abundances were quantified by quantitative PCR (qPCR) using the primers of A364aF/A934b, NS1/Fung and 1369F/1492R respectively, while abundances of amoA-AOAs, amoA-AOBs, nifH, nirK, nirS and nosZ genes were using the primers of Arch – amoAF/ Arch – amoAR, amoA – 1F/ amo – 2R, PolF/ PolR, nirKC1F/ nirKC1R, nirSC1F/ nirSC1R and nosZ1F/ nosZ1R respectively. PCR reactions were performed in 25 ml volumes containing 12.5 ml SYBR® Premix Ex Taq™ (TaKaRa Biotechnology, Otsu, Shiga, Japan), 200 nM of each primer and 2 ml of 5 – fold diluted template DNA (1e10 ng). qPCR was performed in an iCycler iQ 5 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) using the specific thermal conditions (Table S1) and standard curves for the qPCR assays generated as described previously (He et al., 2007), and quantification efficiencies varied between 80% – 120% with R² values > 0.99. All qPCR reactions were performed in triplicate.

Measurement of Indicators

(1) Phenotypic Index

plant height, the length of main roots and seed number were measured directly by a ruler and counting. The shoot and root biomass were weighted separately after drying. Two medium sized leaves from each plant were used to measure the average leaf area using a scanner (Epson perfection V700 PHOTO, Long Beach, CA, USA). The specific leaf area (SLA) was calculated by the ratio of leaf area and leaf biomass.

(2) Plant N Use Efficiency

Ground plant materials were weighed 60 mg to measure N accumulation by Elementar Analysensysteme (Vario Max; GmbH, Hanau, Germany). Concentrations of N on the basis of plant dry mass was used to represent NUE of annual plants (Shaver and Melillo, 1984; Birk and Vitousek, 1986). High N concentration indicates low plant NUE.

(3) Physiological Index

Root activity was determined by the triphenyltetrazolium chloride (TTC) reduction method (Islam et al., 2007). Briefly, roots of siblings and strangers were reacted with 0.1 mM phosphate buffer solution (pH 7.0) and 0.4% TTC respectively for 1 h in 37°C. Adding 1 M H₂SO₄ for chromogenic reaction and then read the absorbance of liquid at 485nm. The root activity was expressed by dehydrogenase activity. Chlorophyll content was extracted by acetone at 80%. The absorbances of extract were read at 645 and 663 nm, and the content of chlorophyll was calculated according to the Eq. $20.2A_{645} + 8.02A_{663}$ (Lichtenthaler et al., 1998). As to the NO₃⁻ content, the lateral roots including the root tips were washed clearly by distilled water, weighted 1 g and grinded in 20% acetic acid solution, diluted with the solution to 20 mL. Then we added 0.4 g of the mixed powder (including 100 g of BaSO₄, 2 g of alpha-naphthylamine, 2 g of zinc powder, 4 g of aminobenzene sulfonic acid, 10 g of MnSO₄, 75 g of citric acid) in the solution and shocked it for 3 min and let it stand for 10 min. The reaction mixture was centrifuged for 5 min at 4000×g and determination the absorption of the supernatant at A₅₂₀ nm of the spectrophotometer. Meanwhile, prepare NO₃⁻ standard solutions of concentrations respectively are 10, 20, 30, 40, 60, 80, 100 and 120 mg L⁻¹, and chromogenic reacted with 5% Salicylic acid-sulfuric acid solution. Determination the absorption of the standard solutions at A₅₂₀ nm of the spectrophotometer and gain the standard curves based on the concentrations and absorption. Finally, the concentrations of NO₃⁻ content in plants roots were obtained by matrixing the absorption in the standard curve. Nitrate reductase was measured by vitro method. One gram of plant root was weighed and added to 3 times volume of precooling PBS (pH 7.2), then was ground in an ice bath, and homogenated at 4°C. The homogenate was centrifuged for 5 min at 10000 ×g and quantified 50 µL liquid supernatant, reacted with mixture liquids containing 25 mmol L⁻¹ of PBS, 10 mmol L⁻¹ of KNO₃, 5 mmol L⁻¹ of NaHCO₃, 0.2 mmol L⁻¹ of NaOH and preservation under 25 °C for 30 minutes. Take 250µL of the mixture in a test tube and added 500 µL 1% of sulphonamide reagent and 500 µL of 1% alpha naphthylamine reagent, after 20 min reaction. We read the absorption at A₅₂₀ nm, and calculated the content of NO₂⁻ (per hour per gram fresh weight root, µg g⁻¹) from the reference to the standard curve.

(4) Root Secretion Rate:

The root exudates C and N were measured on a Total Organic Carbon (TOC) analyser (Milti N/C 2100S). The secretion rate of root C and N were calculated by the formula: $v = Vc / mt$. v - the secretion rate (mg g⁻¹ h⁻¹), V- volume of collected root secretion solution in 24 h (L), c- concentration of organic carbon or nitrogen in root exudates (mg L⁻¹), m- dry root biomass (g), t- time of collection, 24 h in this study.

(5) Soil NH₄⁺ and NO₃⁻ Content:

10g fresh soil was extracted with 50 mL 0.05M K₂SO₄ solution (soil-water ratio: 1:5), and oscillated at 200 rpm for 1 h. It was then filtered with qualitative filter paper to obtain the extracted liquid. The NH₄⁺

and NO_3^- content were measured on an auto analyser (AA3, Bran-Luebbe, Germany).

(6) Soil Microbial Biomass C and N

soil microbial C and N were measured by chloroform fumigation extraction (Brookes et al., 1985; Vance et al., 1987). In brief, petri dish containing 10 g fresh soil sample was put into a vacuum dryer, with a beaker containing chloroform without ethanol and some glass balls, and a small beaker containing dilute sodium hydroxide solution. After the lid is closed, the sealing is confirmed and the vacuum pump is used to pump the vacuum to chloroform boiling for 5min, covered the vacuum dryer with a black plastic bag for 24h to sampling. After the fumigation, all the soil was transferred to a 250ml conical flask, and 80ml 0.5 M K_2SO_4 was added, shaken for 1h. At the same time, extraction of 10 g soil without fumigation was performed with the same procedure. Total organic C and N in extracts were measured on a TOC analyzer (Milti N/C 2100S). The microbial biomass was calculated as fumigated soil microbial biomass C/N minus unfumigated soil microbial biomass C/N.

Statistics

Statistical significance was assessed using SPSS 16 (SPSS Inc., Chicago, IL, USA). After the normal distribution for the residuals and the homoscedasticity of the residual variance were tested, ANOVA was used to compare the differences of fitness, biomass, morphology and physiology traits, soil N forms, enzyme activity, gene abundance and alpha diversity between siblings and strangers. A p-value of less than 0.05 was considered to be significant.

Results

Kin Responses for Plant Characteristics

The results of ANOVA showed that siblings allocated more biomass to seeds in focal plants living with siblings than with strangers ($P=0.025$), i.e. increasing the fitness of kin groups. Moreover, there was higher shoot biomass ($P=0.009$) in siblings than strangers (Fig. 1b), but no significant difference for root biomass ($P=0.179$). Plant height ($P=0.011$) and SLA ($P=0.041$), corrected for biomass were greater in siblings than in strangers (Table 1). The root length showed no difference between both groups (Fig. 1a). Siblings showed higher chlorophyll content ($P=0.024$) and nitrate reductase activity ($P=0.019$), but lower root activity ($P=0.062$) compared to strangers (Fig. 2c; Table 1).

Table 1

ANOVA results of fitness, morphology and physiology traits, and nitrogen use differences, root exudate and microbial carbon and nitrogen, and other soil microbial indicators between siblings and strangers. Differences were tested by one-way ANOVA ($P < 0.05$).

Indicators	F-Value	P-Value
Root biomass (g)	1.920	0.179
Shoot biomass (g)	7.917	0.009
Seed biomass (g)	6.340	0.025
Plant height (cm)	5.266	0.011
Root length (cm)	1.102	0.310
SLA (cm ² g ⁻¹)	3.985	0.041
Root NO ₃ ⁻ content (ug g ⁻¹)	1.962	0.480
N reductase activity (ug g ⁻¹ h ⁻¹)	8.102	0.019
Total N accumulation (mg kg ⁻¹)	4.001	0.048
Root activity (ug g ⁻¹ h ⁻¹)	3.966	0.062
Chlorophyll content (mg g ⁻¹)	4.788	0.024
Soil NH ₄ -N (mg kg ⁻¹)	1.123	0.375
Soil NO ₃ -N (mg kg ⁻¹)	10.895	0.008
Root exudate C (mg kg ⁻¹)	33.101	0.006
Root exudate N (mg kg ⁻¹)	2.4808	0.327
Soil microbial C (mg kg ⁻¹)	18.563	0.014
Soil microbial N (mg kg ⁻¹)	6.9691	0.389
AOA (Copies g ⁻¹ dws)	24.152	0.080
AOB (Copies g ⁻¹ dws)	5.156	0.501
Nif (Copies g ⁻¹ dws)	4.895	0.669
nirK (Copies g ⁻¹ dws)	2.520	1.023
nirS (Copies g ⁻¹ dws)	3.152	0.987

Indicators	F-Value	P-Value
NosZ (Copies g ⁻¹ dws)	3.004	0.850
Archaea (Copies g ⁻¹ dws)	28.396	0.004
Bacteria (Copies g ⁻¹ dws)	2.850	0.241
Fungi (Copies g ⁻¹ dws)	4.989	0.520

Although the plant N uptake rate (Fig. 2a; Table 1) and the concentration of NO₃⁻ in plant roots ($P=0.480$) did not differ between siblings and strangers of *G. max* (Fig. 2b; Table 1), higher total N use efficiency (lower N accumulation) ($P=0.048$) of siblings than strangers suggested increased nitrogen use efficiency of siblings.

The root secretion rate of C ($P=0.007$) in root exudates of *G. max* was significantly higher in siblings than strangers. However, the root secretion of N ($P=0.252$) in exudates was not significantly different between siblings and strangers (Fig. 3b; Table 1).

Kin Effects on Soil Characteristics and Soil Microorganisms

Siblings showed significant higher microbial carbon content (MC, $P=0.014$) than stranger rhizosphere soils compare to microbial nitrogen (MN) (Fig. 3a). However, siblings maintained lower soil NO₃⁻ content than strangers ($P=0.008$) compare to NH₄⁺ content of *G. max* (Fig. 3c; Table 1).

The qPCR results of functional genes related to nitrogen cycle showed that significant higher amoA-AOAs abundance in sibling than stranger soils ($P=0.08$; Fig. 4a). However, there showed no significant differences on abundances of genes amoA-AOBs, nirS, nirK and nosZ between sibling and stranger soils (Fig. 4). In terms of the microbial community, sibling soils of *G. max* had higher archaea abundance ($P=0.004$) than strangers compare to the bacteria and fungi abundance (Fig. 5; Table 1).

Discussion

Previous studies have suggested that kin recognition could occur generally in nature, but morphologically based studies have obtained varied conclusions (see to File et al., 2012). We would argue that changes in performance or fitness in theory likely indicate the fitness consequences of changes in adaptive traits, hence changes in potentially adaptive traits (such as root allocation and C exudation) could indicate kin recognition because of which without resource differences suggesting a response to a signal or cue. The current study determined plant kin recognition of *G. max* by not only including the fitness, but also including morphological traits, physiological performances, root exudation, and N uptake as well as microbial biomass C and N, functional genes related to nitrification and denitrification. Integrating these findings, we demonstrated that kin recognition is more nutrient related than previously thought.

Plant fitness (Cheplick, 1992, 2004; File et al., 2012) and morphological responses involved in resource capture (Dudley & File, 2007; Murphy & Dudley, 2009; Bhatt et al., 2010; Biedrzycki et al., 2010; Biernaskie, 2011) in our current study suggested that plants in kin groups show benefit in plant growth efficiency (Lepik et al., 2012). In particular, siblings of *Glycine max* increased their SLA compared to strangers, which was known to reflect the efficient light capture of plants resulting from mutual shading (Ballare et al. 1994; Griffith and Sultan 2005), suggested a cooperative behaviour through increasing the efficiency of light acquisition (Lepik et al. 2012; Crepy and Casal 2015; Li and Xu, 2017). This was also supported by higher chlorophyll content in siblings compare to strangers, which contributes to potential photosynthetic efficiency (Melis, 2009). These changes can also explain better increased size and growth, e. g, height and shoot biomass of siblings living together (Till-Bottraud and de Villedenreuil, 2016).

The lack of significant differences in root length and root biomass allocation suggests that there was no kin response in these belowground traits. However, lower root activity of siblings than strangers suggests lower underground competition for soil nutrients or water (Islam et al., 2007). Higher use efficiency calculated by total N content in siblings than strangers suggested that kin benefit may achieved through increasing the nutrient use efficiency (Lepik et al., 2012) of siblings compared to strangers. Thus, this may suggest that a mutual accommodation was happened among siblings while there was more competition among strangers belowground. This was further supported by higher NR activity in siblings than in strangers, implying higher potential NO_3^- -N transformed to NO_2^- -N and then amino acid for plant growth (Barber, 1984; Baker and Hall, 1988). Thus, the kin recognition responses in N uptake and use efficiency, complementary with the phenotypical performance, may support growing with kin as increasing resource capture efficiency and energy saving of siblings compare to strangers of *G. max*. To summarize, such distinct difference between above- and below-ground parts is ascribed to the ability of legumes such as *G. max* to use atmospheric N_2 through symbiotic rhizobium. As a result, N is not so limited for *G. max* but it requires more C to fuel biological N fixation, thus allocating more C to shoots to enhance photosynthesis. This is supported by similar nitrogenase activity between siblings and strangers (Fig. 4b) as well as higher chlorophyll content in siblings than in strangers (Fig. 2b).

It has been demonstrated in *Arabidopsis* is that plants in kin groups could recognize kin or stranger neighbor through root exudates (Semchenko et al., 2014; Biedrzycki et al. 2010). However, the root exudates are complex, and what substances play key roles on this process was still unknown. We did analyze the main substance of root secreted-C and N and found that root secreted-C rather than secreted-N was sensitive to kin identity of *G. max*, but this is not evidence that root secreted-C provides kin signals. But what is most important is that, plants in kin groups produce more root secreted-C when facing siblings then strangers, which could be a direct available carbon source for rhizosphere microorganisms to activate the growth of rhizosphere microbes and activity (Toberman et al., 2011) and elicit rhizosphere priming effect (Kuzyakov, 2010). This could not only accelerate soil organic matter decomposition (Kuzyakov, 2002; Hopkins et al., 2013; Cheng et al., 2014), but also increase the supply of soil-available N for plants. Thus, root secreted-C could induce rhizosphere microbes changes, and those change may also create feedbacks in the soil nutrient cycle, especially the N mode of occurrence in soil and N absorption

of plants, and thus influence the plant growth or competition. This hypothesized mechanism is supported by higher root exudation of C and N, higher microbial biomass C, higher AOA in siblings than in strangers in our study. Lower soil NO_3^- concentrations in siblings than in strangers are ascribed to higher root uptake, which is supported by higher NR and uptake rates.

Specifically, the N transformation model in sibling plants of *G. max* tended to convert more organic N into nitrate N (the amoA-AOA was higher in sibling than stranger soils) for plant absorption and utilization, and consequently the nitrate N in rhizosphere soil was lower in sibling than stranger soils. Soil N conversion was mainly actuated by soil nitrification process regulated by ammonia oxidizing bacteria and ammonia-oxidizing archaea, and the denitrification process related with denitrifying bacteria, as well as microbial N fixation process. The results exhibited that the abundances of soil archaea and amoA-AOAs genes contributing to soil nitrification were significantly increased in sibling soils compare to stranger soils, while the abundances of genes of NirK, NirS and NosZ contributed to soil denitrification, and gene of nifH contributing to soil N fixation did not significantly differed between siblings and strangers of *G. max*. This could be easily explained because denitrification is generally considered to be intense in an anaerobic environment, but our experimental soil did not create a flooded condition. The result also suggested that compare to soil bacteria and fungi, the significant difference in abundance of soil archaea between sibling and strangers suggested which plays a significant roles on soil nitrification process resulted from plant kin interaction, and plant growing with siblings could have an advantage from soil nitrification process and N retention compared to strangers of *G. max*. Certainly, we should not deny the function of soil bacteria and fungus related with N cycling in plant kin interactions. Therefore, subsequent work should analyze the functional flora and community structure of soil microbes including archaea, bacteria and fungi at the classification level to find the important functional flora involved in nutrient conversion affected by plant kin interactions.

Conclusion

In summary, our results showed the benefits of growing with kin in *G. max* were reflected by increased fitness and resource use efficiency instead of competition among siblings compare to strangers. Moreover, we found evidence that the rhizosphere effects of soil microbial changes and soil N cycling and transformation triggered by the root secreted C, could be a potential underground feedback mechanism of plant kin recognition. We have found that soil archaea community of microorganisms was sensitive both on abundance and function in kin interactions, but we did not identify the functional strains of these communities at the level of a family or genus, which still need further study by high throughput sequencing to reveal more information.

Declarations

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Conflict of Interest:

All of our co-authors have no conflict of interest.

Data Availability Statement:

Research data are not shared.

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Figures

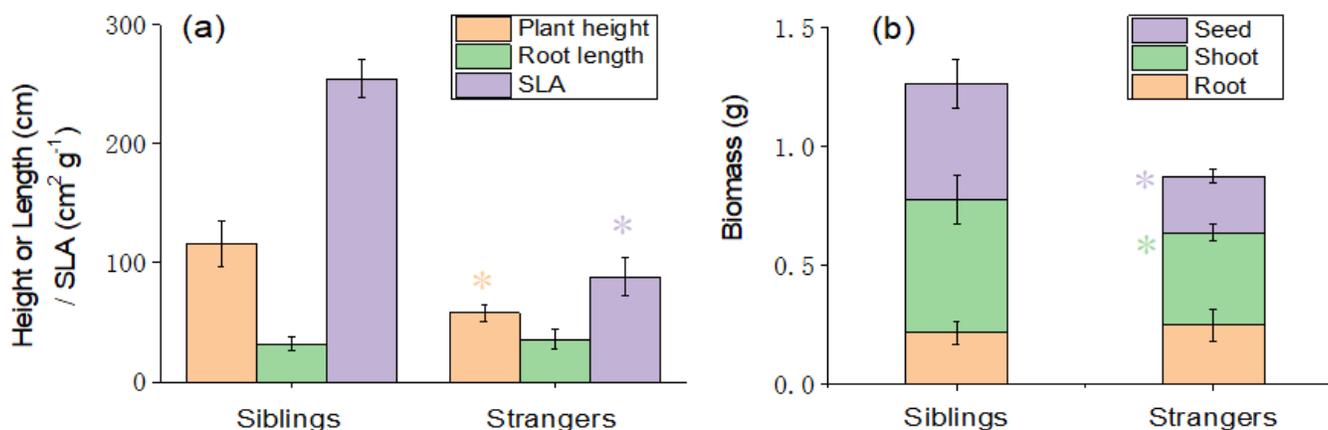


Figure 1

Plant height, root length and SLA (a), the root, shoot and seed biomass (b) in sibling and stranger groups. The means \pm SE of each attribute are presented as bar graphs. The asterisks (*) aside the bars indicate the significant difference between treatments of the corresponding index ($P < 0.05$).

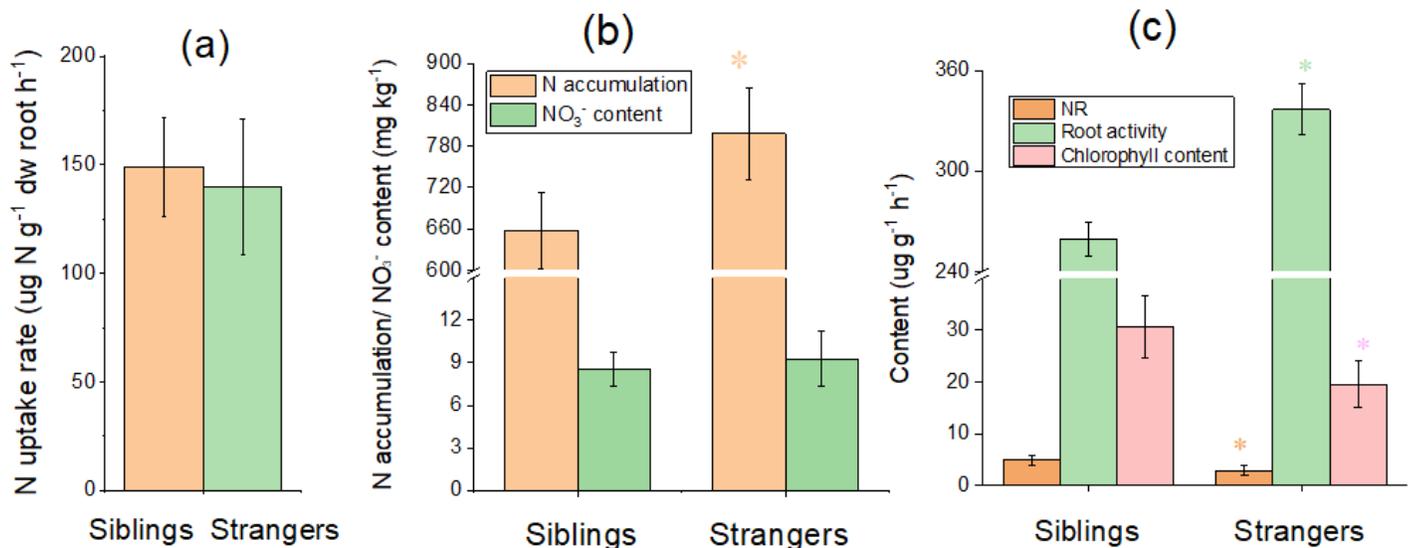


Figure 2

The means \pm SE of attributes of plant N uptake rate (a), plant N accumulation and NO_3^- content (b), Nitrate reductase activity (NR), root activity and Chlorophyll content (c) in sibling and stranger groups are presented as bar graphs. The asterisks (*) aside the bars indicate the significant difference between treatments of the corresponding index ($P < 0.05$).

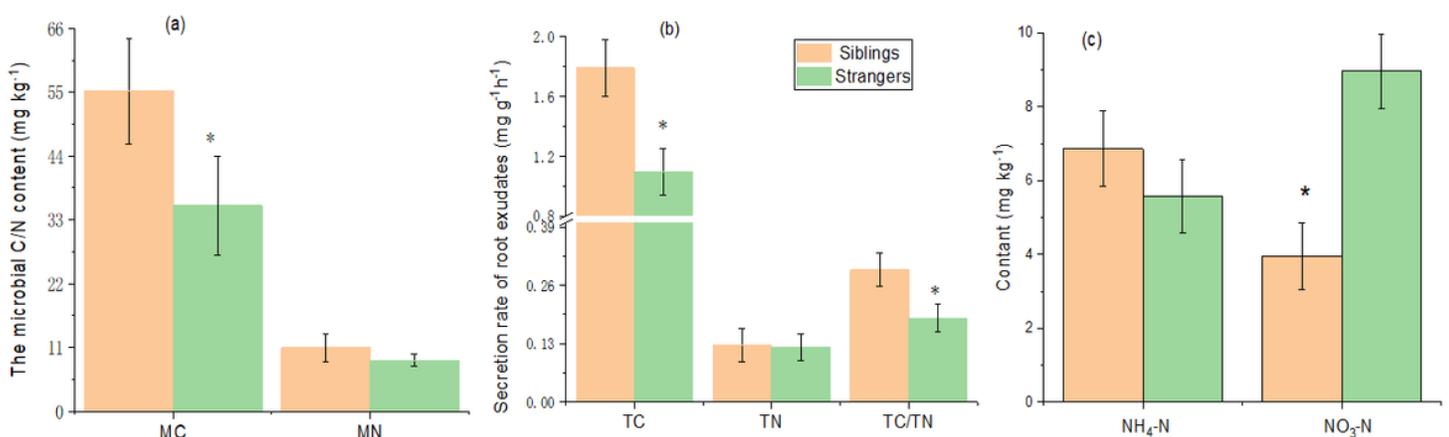


Figure 3

The microbial biomass carbon (MC) and microbial biomass nitrogen (MN) content in rhizosphere soils (a), the TC, TN and TC/TN secretion rate by plant roots (b) and the soil ammonium nitrogen ($\text{NH}_4\text{-N}$) and

nitrate nitrogen (NO₃-N) (c) of siblings and strangers of *G. max*. The asterisks (*) aside the two bars indicate significant differences between siblings and strangers on responding indexes at $P < 0.05$.

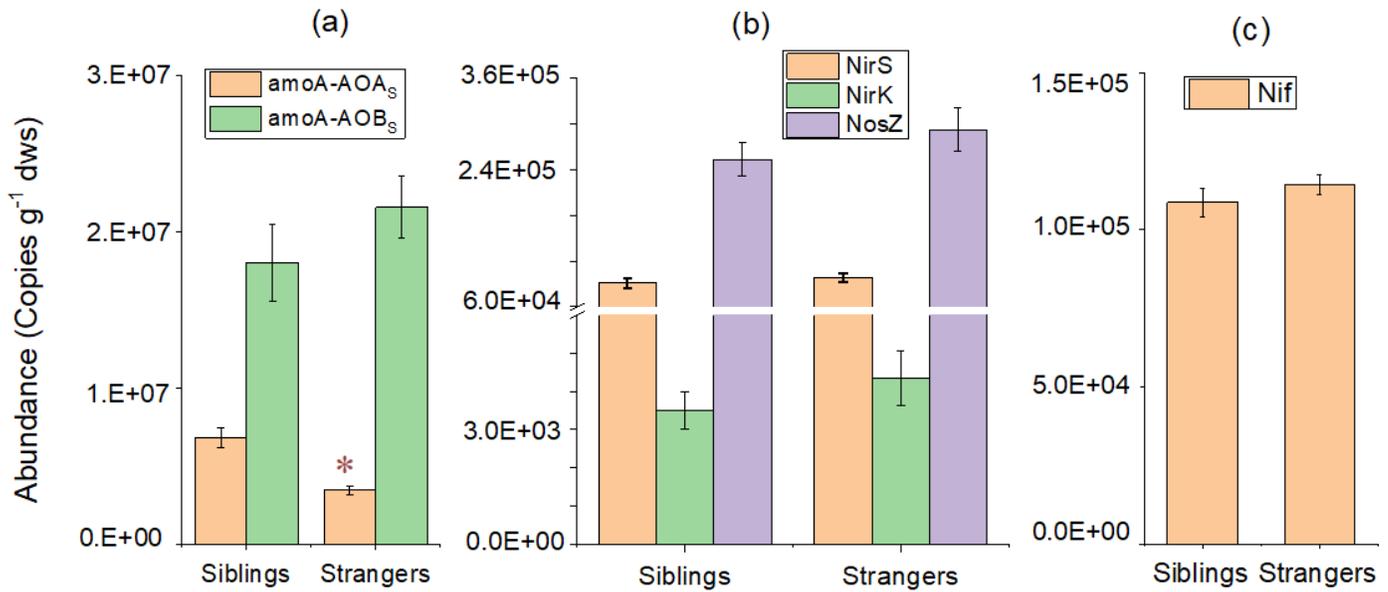


Figure 4

Abundances of genes amoA-AOAs and amoA-AOBs related with nitrification (a), genes of NirS, NirK and NosZ related with denitrification (b), as well as gene of NifH related with microbial nitrogen fixation in rhizosphere soils of siblings and strangers of *G. max*. The asterisks (*) aside the two bars indicate significant differences between the treatments at $P < 0.05$ on responding indexes.

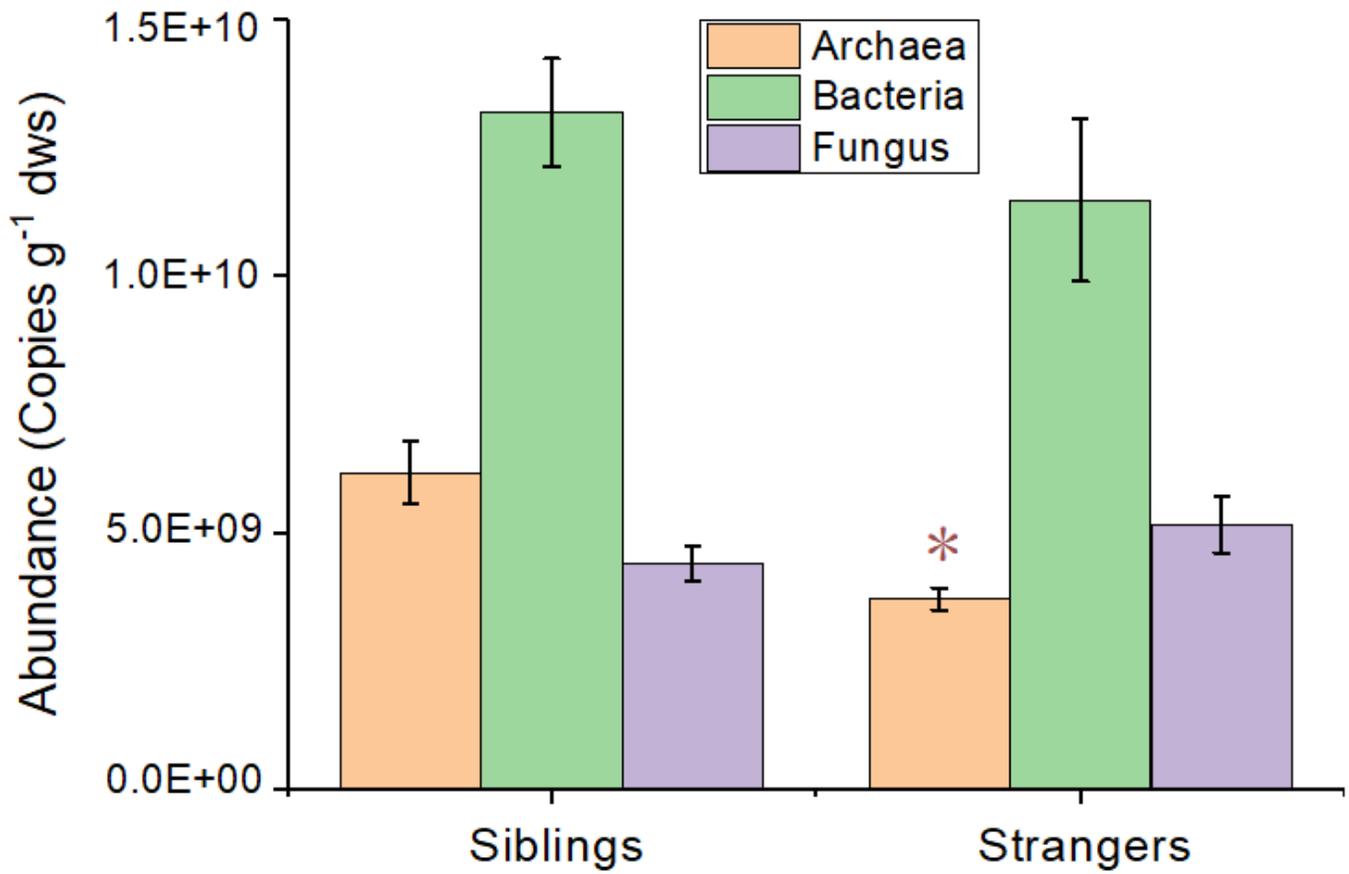


Figure 5

Abundances of soil Archaea, Bacteria and Fungus in rhizosphere soils of siblings and strangers of *G. max*. The asterisks (*) aside the two bars indicate significant differences between the treatments at $P < 0.05$ on responding indexes.

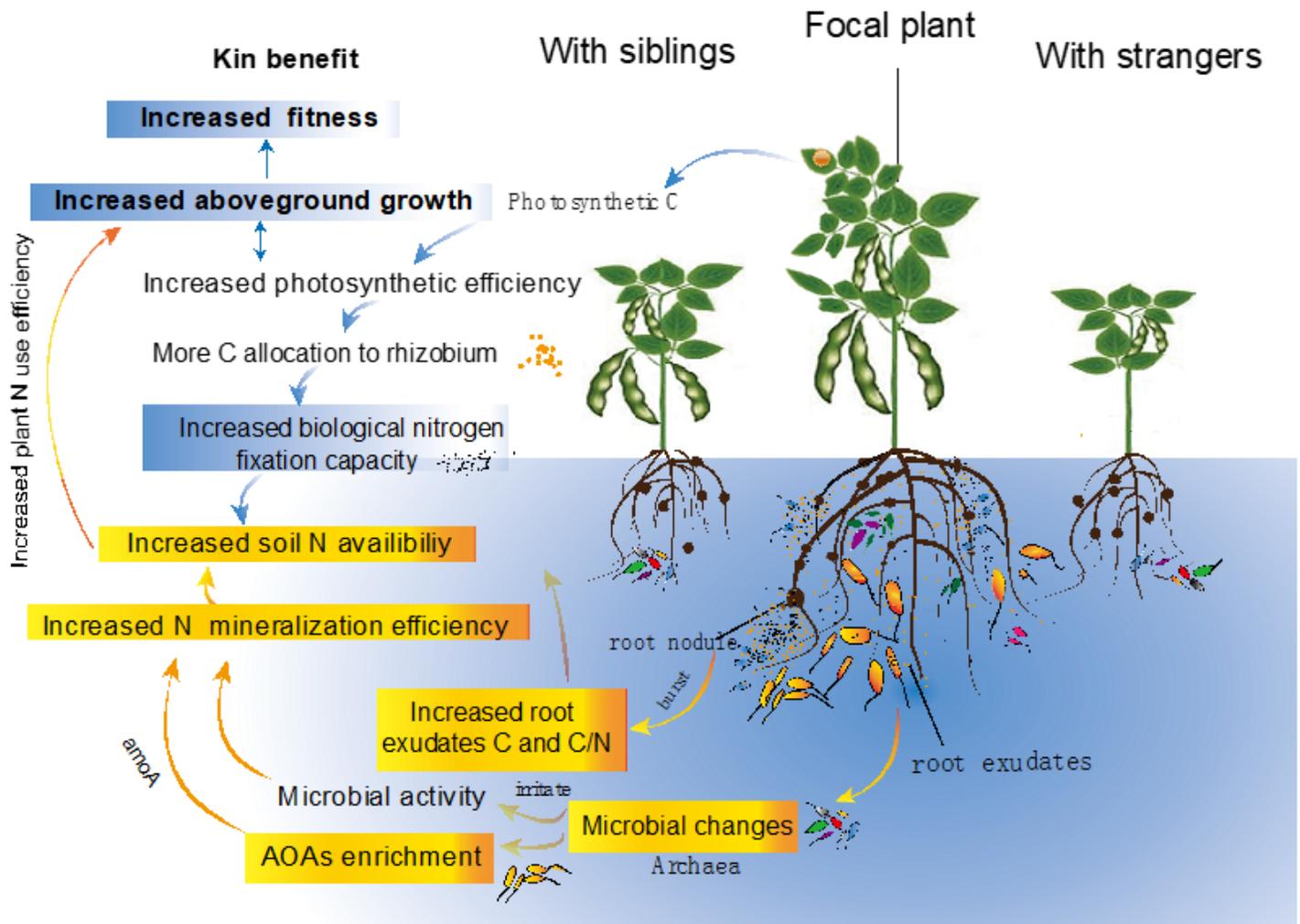


Figure 6

The concept figure: Structure diagram of kin interaction between plant siblings of *G. max* compare to strangers, which was used to explain mechanisms by kin recognition in belowground exudation could result in differences between kin and strangers in N use efficiency, growth, and fitness. In this picture, the focal plant was zoom into two times to see more clearly the above-and below-ground changes compare to the left and right plants. In order to show differences of plant responses to varied neighbours obviously, the focal plant was divided into two parts, the left represents individual performance of focal plant living with siblings, while the right parts represent of which living with strangers.

Supplementary Files

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