Research paper

Increased dependence on nitrogen-fixation of a native legume in competition with an invasive plant

Meixu Han [a,1], Haiyang Zhang [b,1], Mingchao Liu [c], Jinqi Tang [d], Xiaocheng Guo [c], Weizheng Ren [a], Yong Zhao [a], Qingpei Yang [a], Binglin Guo [a], Qinwen Han [a], Yulong Feng [c], Zhipei Feng [a], Honghui Wu [e,f], Xitian Yang [a], Deliang Kong [a,*]

[a] College of Forestry, Henan Agricultural University, Zhengzhou 450002, China
[b] College of Life Science, Hebei University, Baoding 071002, China
[c] Liaoning Key Laboratory for Biological Invasions and Global Changes, College of Bioscience and Biotechnology, Shenyang Agricultural University, Shenyang, Liaoning 110866, China
[d] College of Horticulture, China Agricultural University, Beijing 100193, China
[e] State Key Laboratory of Efficient Utilization of Arid and Semi-arid Arable Land in Northern China, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[f] Key Laboratory of Arable Land Quality Monitoring and Evaluation, Ministry of Agriculture and Rural Affairs, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China

ARTICLE INFO

ABSTRACT

Suppression of roots and/or their symbiotic microorganisms, such as mycorrhizal fungi and rhizobia, is an effective way for alien plants to outcompete native plants. However, little is known about how invasive and native plants interact with the *quantity* and *activity* of nutrient-acquisition agents. Here a pot experiment was conducted with monoculture and mixed plantings of an invasive plant, *Xanthium strumarium*, and a common native legume, *Glycine max*. We measured traits related to root and nodule quantity and activity and mycorrhizal colonization. Compared to the monoculture, fine root *quantity* (biomass, surface area) and *activity* (root nitrogen (N) concentration, acid phosphatase activity) of *G. max* decreased in mixed plantings; nodule *quantity* (biomass) decreased by 45%, while nodule *activity* in N-fixing via rhizobium increased by 106%; mycorrhizal colonization was unaffected. Contribution of N fixation to leaf N content in *G. max* increased in the mixed plantings, and this increase was attributed to a decrease in the rhizosphere soil N of *G. max* in the mixed plantings. Increased root *quantity* and *activity*, along with a higher mycorrhizal association was observed in *X. strumarium* in the mixed compared to monoculture. Together, the invasive plant did not directly scavenge N from nodule-fixed N, but rather depleted the rhizosphere soil N of the legume, thereby stimulating the *activity* of N-fixation and increasing the dependence of the native legume on this N source. The *quantity−activity* framework holds promise for future studies on how native legumes respond to alien plant invasions.

1. Introduction

Invasive plants have greatly reduced global biodiversity and sustainability (Bajwa et al., 2019; Hudgins et al., 2023). This is particularly true in agroecosystems with monoculture planting and excessive fertilization, which incur a high risk of alien plant invasion (Chen et al., 2013). Mounting evidence has shown that invasive plants can gain a competitive advantage over native plants by suppressing nutrient acquisition of native plants from symbiotic microbes such as mycorrhizal fungi and/or rhizobia (Grove et al., 2017; Birnbaum et al., 2018; Liu et al., 2022). Additionally, invasive plant roots have been observed to exhibit increased mycorrhizal associations, further contributing to their invasiveness (Tian et al., 2021; Sun et al., 2022; Yu et al., 2022).

Legume plants produce abundant nitrogen (N) through symbiotic nitrogen fixation (SNF) in their nodules (Zhong et al., 2023). Studies on plant intercropping have found that SNF-derived N can be utilized by mycorrhizal fungi associated with gramineous plants. These fungi can colonize legume nodules and transfer N to gramineous plants (He et al., 2003; Ingraffia et al., 2019). On the other hand, SNF-derived N can be released as root exudates from legume roots or during the decomposition of nodules after their death. This N is then taken up by the roots of gramineous plants (Thilakarathna et al., 2016; Zhang et al., 2020). Therefore, it is reasonable to speculate that invasive plants may also benefit from the SNF-derived N of native legumes through these mechanisms, although SNF processes can be inhibited by invasive plants. However, few studies have explored its potential contribution to the invasiveness of alien plants. Legume plants associate with both rhizobia and mycorrhizal fungi, and both associations are energy costly (Wang et al., 2021; Li et al., 2022). However, little is known about how these two symbiotic associations respond to alien plant invasion. For example, it is unclear whether both associations in native legumes are reduced or whether one symbiotic association is reduced and the other is facilitated by competition with invasive plants. Clarifying this issue would provide valuable insights for optimizing symbiotic associations (such as rhizobia, mycorrhizal fungi, or both) of crops in response to alien plant invasion.

The ability of roots to acquire nutrients depends on both the *quantity* and *activity* of the roots involved in the acquisition process (Zhang et al., 2023, 2024). Root *quantity* refers to the total surface area of the roots involved in foraging for soil nutrients. Root *activity* is often represented by nutrient content, such as N, or by enzymes activity (e.g., root acid phosphatase, Apase) in the roots, as these factors are closely related to nutrient uptake by roots (Bardgett et al., 2014; Han and Zhu 2021; Han et al., 2022). Recent studies have demonstrated that there can be a decoupling of the *quantity* and *activity* of roots during nutrient acquisition (Bergmann et al., 2020; Zhang et al., 2024). Therefore, to gain a comprehensive understanding of how native and invasive plants compete for soil nutrients via their roots, it is necessary to consider both the *quantity* and *activity* of roots. Furthermore, the N-fixing ability of legumes depends on the *quantity* (number or biomass of nodules) and *activity* (N-fixing rate by nodules) of nodules. Increased N-fixation *activity* has frequently been observed with fewer nodules in legumes, given the high energy demands of the N-fixation process in the nodules (McCulloch and Porder 2020; Ke et al., 2022). Therefore, both the *quantity* and *activity* of the nodules should be considered when assessing the amount of SNF-derived N. However, to date, few studies have explored the responses of both roots and N-fixation nodules in native legumes to invasive plants within the framework of *quantity−activity* for nutrient acquisition. It remains unclear how the *quantity* and *activity* of roots vary in coordination or independently of the nodules.

In this study, we aim to test two hypotheses by conducting monoculture and mixed planting experiments with a noxious invasive plant, *Xanthium strumarium*, and an important native legume crop in China, *Glycine max*. The hypotheses are as follows: (1) *X. strumarium* can compete with *G. max* for nodule-fixed N by increasing the *quantity* and *activity* of its roots, as well as enhancing its mycorrhizal association. (2) when in competition with *X. strumarium*, both the *quantity* of the roots and nodules in *G. max* will decrease, whereas the *activities* of these agents for nutrient acquisition will increase, aiming to alleviate growth suppression of the legume caused by the invasive plants.

2. Materials and methods

2.1. Plant materials and experimental design

The study was conducted at the research station at Shenyang Agricultural University (123°33′E, 41°48′N) where the elevation is 59 m. Mean annual temperature is 8.1 °C, and mean annual precipitation is 722 mm. Here, *Xanthium strumarium* was selected as the invasive plant and *Glycine max* as the native plant. *X. strumarium* is an annual herb, native to North America, and has become a harmful invasive plant in northeast China (Iqbal et al., 2020, 2021; Chen et al., 2022). This invasive plant becomes a significant threat to agriculture field crops, particularly *G. max*, which is widely planted in northeast China (Iqbal et al., 2020, 2021).

In mid-October 2017, we collected seeds from mature individual plants of *X. strumarium* near Qipanshan, Shenyang. Seeds of *G. max* were bought from Dafeng Seed Industry Development Co., Ltd, Shenyang China. In late April 2018, both seeds of the invasive and native plants were disinfected with 0.5% potassium permanganate solution, washed with distilled water, and then sown in pots (25 cm × 16 cm × 14 cm) filled with 4 kg soil. The soils samples were collected from a forest stand near Dongling Park, Shenyang. Briefly, the top soil was sampled, sieved, air-dried and subsequently mixed with sterilized river sands (see Chen et al., 2022 for details). Initial soil physical and chemical characters were as follows: soil pH, 7.34; organic matter content, 44 g kg^{-1}; total N content, 0.22 g kg^{-1}; total phosphorus content, 0.06 g kg^{-1}; total potassium content, 1.48 g kg^{-1}; available N content, 125.67 mg kg^{-1}; available phosphorus content, 3.10 mg kg^{-1}; available potassium content, 55 mg kg^{-1}.

In mid-May, seedlings were thinned to one individual plant per pot for the monoculture plantings, and one *G. max* individual and one *X. strumarium* individual per pot for the mixture. Only one plant individual was set in the monoculture plantings, a little different from studies with two individuals in this planting usually aiming to account for intraspecific competition (McNickle 2020). However, we aimed to explore how the invasive plants compete with the native legume plant. That is, we focus mainly on the interspecific competition of the soil resource between the invasive and the native plants rather than the intraspecific competition within each species. Therefore, the set of one individual in the monoculture plantings can fulfill such purpose on interspecific competition, as has been adopted in previous studies (McNickle 2020; Chen et al., 2022). Five replicates were established for each planting types. In each pot, root physiological traits (acid phosphatase), mycorrhizal colonization rate and *activity* of biological N-fixation were measured. However, due to the destructive sampling nature of these measurements, the root systems could not be kept intact to obtain data on root biomass. Therefore, an additional five replicates were set up for both monoculture and mixed plantings to measure plant biomass and leaf morphological traits.

2.2. Plant sampling and trait measurements

Plants were harvested in late July 2018. Shoot biomass was measured after the samples were dried at 60 °C for 48 h. Plant roots were separated into coarse (diameter > 2 mm) and fine (diameter < 2 mm) roots, and their biomass was measured separately.

Total leaf area of an individual plant was measured by Li-3000C (Li-COR, Lincoln, NE, USA), and then the leaf biomass was measured. Randomly selected sub-samples of fine roots were scanned, and the images were used to calculate fine root surface using WinRhizo Pro (2016 software, Regent Instruments, Canada). The scanned roots were then dried and weighed. Total surface area

of fine roots of an individual plant was calculated according to the portion of the fine root sub-sample to total fine root biomass of the individual plant.

Root acid phosphatase activity (Apase) was determined following Png et al. (2017), using *para*-nitrophenyl phosphate (*p*NPP) as the substrate (Png et al., 2017; Kavka et al., 2021). Briefly, fresh root samples were ground, shaken and centrifuged. The supernatant was then terminated with sodium hydroxide, and the concentration of para-nitrophenol (*p*NP) was calculated by UV−VIS spectrophotometer (UV−Vis Spectrophotometer, Shimadzu, Japan). Root acid phosphatase activity (Apase) was calculated as: Apase = *p*NP/(reaction time × root weight) (see Method S1 for details).

Mycorrhizal colonization rate was measured following previous studies (Trouvelot et al., 1986; Chen et al., 2022; Bi et al., 2023). Briefly, 50 root segments (~1 cm in length) were randomly selected including terminal two root branch orders usually with the highest mycorrhizal colonization rate in the root system. The root segments were consecutively soaked into KOH solution, acetic acid solution, and then dyed in acetic acid ink observation of mycorrhizal colonization using a microscope (Nikon MODEL ECLIPSE Ni-U, Japan). Mycorrhizal colonization rate was divided into five categories, each with a weight of 0.95, 0.70, 0.30, 0.05 and 0.01, respectively. Mycorrhizal colonization rate was then calculated as follows:

$$\text{MYC}(\%) = \frac{0.95 \times n_5 + 0.70 \times n_4 + 0.30 \times n_3 + 0.05 \times n_2 + 0.01 \times n_1}{50} \times 100\%$$

$n_1 - n_5$ is the number of root segments examined in each above mycorrhizal colonization category.

The *quantity* aspect of the N-fixing strategy for the soybean was assessed by the nodule number and nodule biomass per plant as well as the biomass of a single nodule. The *activity* aspect of the N-fixing strategy was indicated by the nitrogenase iron protein (*nifH*) gene copies in the nodule where higher *nifH* gene copies indicate higher N-fixation rate of the nodule (Yang et al., 2020, 2023; Libourel et al., 2023). The *nifH* gene copies were determined by the following steps: 1) Nodule DNA extraction; 2) Quantitative real-time PCR assay; 3) Quantify the *nifH* gene present in the samples (see Method S2 for details).

We also collected bulk soil and rhizosphere soil in each pot to measure soil total C and N contents as well as δ^{15}N content. Briefly, the pots were destroyed to expose plant roots. The soil easily shaken off the roots was treated as bulk soil and the soil adhering to roots and not easily shaken off was carefully collected as rhizosphere soil. The soil samples were then ground using GT200 to determine soil C and N content using Elementary analytical instrument (Elementary analytical instrument, Germany). Soil δ^{15}N content was measured using SerCon Integra 2 Integrated EA-IRMS isotope mass spectrometer (SeCron, Cheshire Crewe, UK). The C, N and δ^{15}N contents for leaves and roots were measured in a similar procedure. The contribution of total leaf N by the N-fixation (%Ndfa) was calculated following previous studies (Balboa and Ciampitti 2020; Cox et al., 2022):

$$\%\text{Ndfa} = \frac{\delta^{15}N_{(X.\ strumarium)} - \delta^{15}N_{(G.\ max)}}{\delta^{15}N_{(X.\ strumarium)} - B} \times 100$$

X. strumarium was used as the reference plant. Leaf δ^{15}N of the reference plant ($\delta^{15}N_{(X.\ strumarium)}$) is calculated as the average leaf δ^{15}N of this plant across the monoculture and mixed plantings (see Fig. 2). $\delta^{15}N_{(G.\ max)}$ is the leaf δ^{15}N in each pot with *G. max*.

B is the leaf δ^{15}N value of legume plants depending solely on atmospheric N for their N source. We used B value was −2.7‰ across legume species (Balboa and Ciampitti 2020). B values for soybean from all previous glasshouse studies ranged from −2.7‰ to −1.98‰ (Balboa and Ciampitti 2020) with an average of −1.97‰. In our study, the lowest leaf δ^{15}N of the soybean was −2.67‰, and therefore we used the lowest B value of previous studies (i.e., −2.7‰) to calculate %Ndfa in our study. The absolute amount of N in *G. max* leaves contributed by N-fixation of nodules (i.e., Ndfa amount) was calculated as:

$$\text{Ndfa amount} = \frac{\%\text{Ndfa} \times \text{leaf N content}}{100}$$

In addition, we used leaf δ^{15}N of *X. strumarium* in monoculture plantings as leaf δ^{15}N of reference plants (see Fig. S8). The results of %Ndfa in *G. max* in the monoculture and mixed plantings using this new leaf δ^{15}N of the reference plant (Fig. S8) were very similar to that based on the above reference plants. This suggests that the selection of reference plant leaf δ^{15}N did not alter the result of leaf % Ndfa in the native legume plant. Therefore, we only presented the result based on the average leaf δ^{15}N of the reference plant across the monoculture and mixed plantings.

2.3. Data analysis

The difference in leaf and root traits of the native or invasive plant between monoculture and mixture was analyzed using independent sample *t* test. Linear regressions were employed to explore trait relationships among roots, leaves, nodules and soil nutrients. Significant level was set at 0.05, and $0.05 < p < 0.1$ was considered as marginal significance. All the analyses were performed in R 4.2.0 (R core team).

3. Results

3.1. Responses of above- and belowground plant biomass to competition

Comparing with *Glycine max* in monoculture plantings, biomass of *G. max* in the mixture decreased by 41% for total ($p < 0.01$), 43% for shoot ($p < 0.01$), 36% for root compartment ($p < 0.01$), respectively (Fig. S1A−C). In contrast, biomass of *Xanthium strumarium* showed no significant difference between monocultures and mixtures (all *p* values > 0.05) (Fig. S1A−C).

3.2. Responses of leaf and root traits to competition

Compared with monoculture, values of *G. max* in the mixture decreased by 39% for leaf biomass ($p < 0.01$) (Fig. S2A), 38% for total leaf area ($p < 0.01$) (Fig. S2B), and 42% for total leaf N content ($p < 0.01$) (Fig. S2D). No difference was observed for leaf N concentration ($p = 0.62$) between monoculture and mixture (Fig. S2C). By contrast, only leaf biomass (−16%, $p = 0.02$) but not leaf area, leaf N concentration and total leaf N content was significantly reduced when growing together with *G. max* compared with values from monoculture (Fig. S2B−D).

Compared with monoculture, fine root biomass of *G. max* in the mixtures decreased by 36% ($p < 0.01$), total fine root surface area decreased by 45% ($p < 0.01$), fine root nitrogen (RN) concentration decreased by 17% ($p = 0.02$), and acid phosphatase activity decreased by 33% ($p < 0.01$) (Fig. 1A−D). By contrast, *X. strumarium* in mixture increased fine root N concentration by 40% ($p = 0.016$) while showing no significant differences for fine
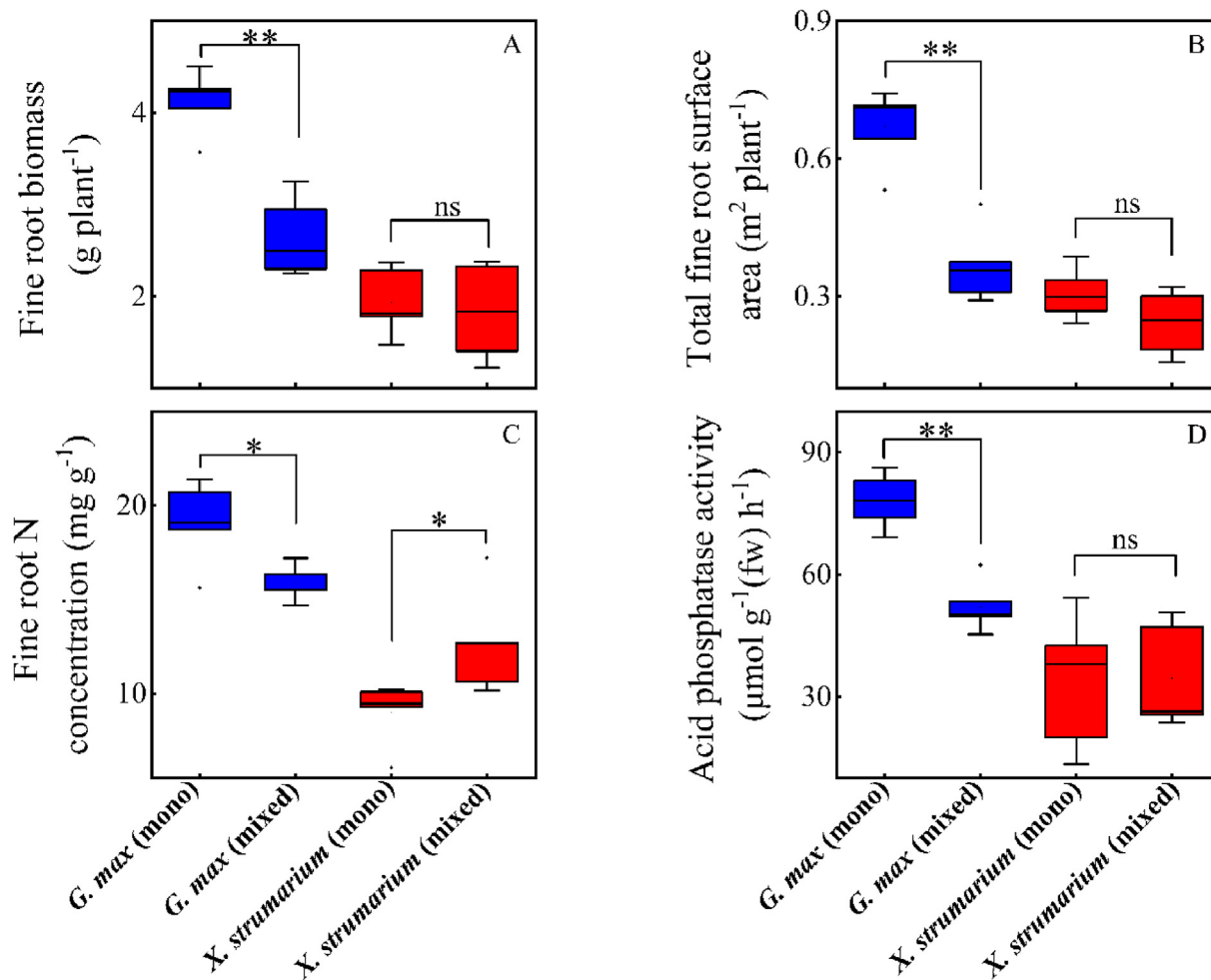
Fig. 1. Fine root traits of *Glycine max* and *Xanthium strumarium* in monoculture (mono) and mixture (mixed). (A) Fine root biomass, (B) Total fine root surface area, (C) Fine root N concentration, (D) Acid phosphatase activity. **, $p < 0.01$; *, $p < 0.05$; ns, non-significant.

root biomass, total fine root surface area per plant and acid phosphatase activity compared with *X. strumarium* in the monoculture (Fig. 1).

3.3. Responses of N-fixing and mycorrhizal strategy to competition

Compared with monoculture plantings, nodule number per plant of *Glycine max* in mixed plantings decreased by 49% ($p < 0.01$), nodules biomass per plant decreased by 45% ($p < 0.01$), single nodule biomass remained unchanged ($p = 0.94$), while *nifH* gene copies increased by 106% ($p < 0.01$) (Fig. 2A–C). Compared with *G. max* in monoculture plantings, proportion of total leaf N derived from atmospheric N-fixation (%Ndfa) increased in mixed plantings of *G. max* ($p = 0.002$, Fig. 2D), while absolute amount of Ndfa remained the same ($p = 0.41$, Fig. 2E). In addition, *nifH* gene copies in nodules of the native legume were positively correlated with %Ndfa ($R^2 = 0.51$, $p = 0.02$, Fig. 2F).

The isotope measurements indicated that leaf δ^{15}N of *G. max* decreased in the mixed plantings compared to the monoculture, whereas leaf δ^{15}N of *Xanthium strumarium* increased in the mixed plantings compared to the monoculture (Fig. S3). In addition, mixed plantings, compared with monoculture, had no effect on mycorrhizal colonization rate of *G. max* ($p = 0.86$), whereas significantly increased mycorrhizal colonization rate of *X. strumarium* by 42% ($p < 0.001$) (Fig. 3).

3.4. Response of rhizosphere soil N of Glycine max to competition

Compared with monoculture plantings, rhizosphere soil N of *G. max* in mixed plantings decreased by 7% ($p = 0.038$) (Fig. 4A). Such effect of mixed plantings on rhizosphere soil N were not significant for *X. strumarium* ($p = 0.64$) (Fig. S4). In addition, rhizosphere soil N of *G. max* was positively correlated with nodule biomass (Fig. 4B) and negatively correlated with *nifH* gene copies (Fig. 4C).

4. Discussion

4.1. Nodule-fixed N from the native legume may not be utilized by the invasive plant

Typically, legume plants produce abundant N resources from the N fixation of their nodules, which can be utilized by the roots and/or mycorrhizal fungi of plants coexisting with legume plants (Zhang et al., 2020). If this facilitation effect is present in the *Glycine max-Xanthium strumarium* system, we would expect to observe depleted leaf δ^{15}N values of *X. strumarium* given that the nodule-fixed N is depleted in δ^{15}N. Surprisingly, our results showed that leaf δ^{15}N of *X. strumarium* increased rather than decreased in mixed plantings compared to monoculture plantings (Fig. S3). This suggests that *X. strumarium* may not directly utilize biologically fixed N from *G. max*, contradicting hypothesis 1.
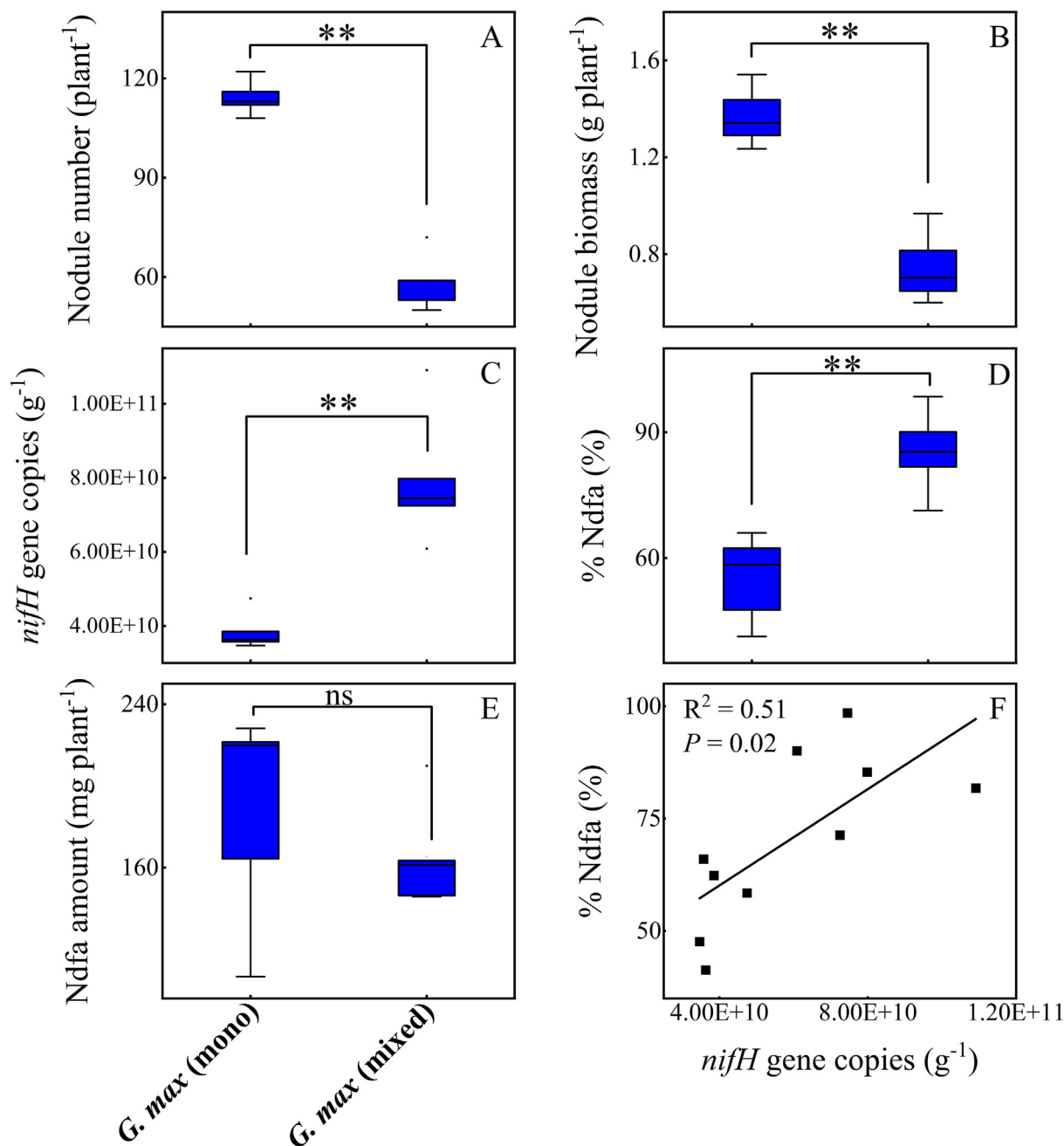
Fig. 2. N-fixing related traits of *Glycine max* in monoculture (mono) and mixture (mixed). (A) Nodule number per plant, (B) Nodule biomass per plant, (C) *nifH* gene copy number per unit nodule weight, (D) proportion of total leaf N contributed by N-fixation of nodules (%Ndfa), (E) absolute leaf N content per plant contributed by N-fixation of nodules (Ndfa amount). (F) Relationships between *nifH* gene copies and %Ndfa. %Ndfa: proportion of total leaf N contributed by N-fixation of nodules. **, $p < 0.01$; ns, non-significant.

We speculate that the lack of utilization of nodule-fixed N by the invasive plant could be attributed to several factors. First, the nodules facing with *X. strumarium* may exhibit greater resistance, potentially through the production of defensive chemicals, against infection by the mycorrhizal fungi of the invasive plant (Weston and Mathesius 2013; Semchenko et al., 2014; Kong et al., 2018). Second, reduced root *activity* owing to interspecific competition could lead to a significant decrease in root exudates from the legume (Fig. 1C) (Sun et al., 2020), thereby reducing the availability of nodule-fixed N. Given that N fixation is an energy-demanding process, legume roots in competition with invasive plant may reduce exudates containing nodule-fixed N. Third, nodule numbers were greatly reduced in the mixed planting (Fig. 2A), which could limit the colonization of these nodules by the mycorrhizal fungi of the invasive plants. Although we currently lack direct evidence for these mechanisms, investigating them would greatly enhance our understanding of how legumes respond and adapt to the invasion of alien plants. Considering the typically strong competitive ability of invasive plants for soil resources, it is possible that the conservation of the nodule-fixed N against exploitation by *X. strumarium* could be applicable to other legumes as well. However, this speculation warrants further investigation.
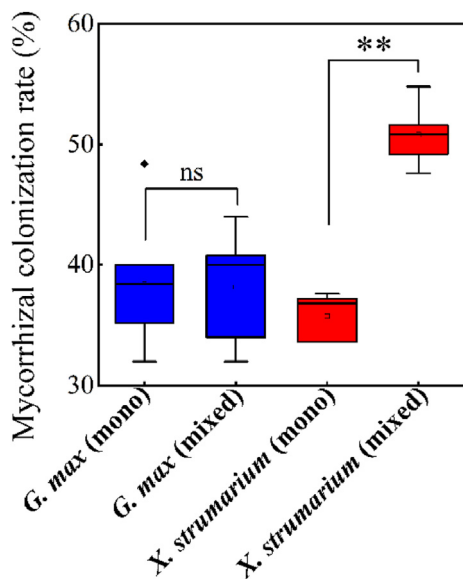
Fig. 3. Mycorrhizal colonization rate of *Glycine max* and *Xanthium strumarium* in monoculture (mono) and mixture (mixed). **, $p < 0.01$; ns, non-significant.

4.2. Belowground interactions between the native legume and the invasive plant

While *X. strumarium* may not have access to the nodule-fixed N, this invasive plant can still suppress *G. max* by outcompeting the native plant for soil resources (see Method S3 in the supporting information). Contrary to our hypothesis 2, in the presence of competition from *X. strumarium*, both the *quantity* and *activity* of the native legume's roots were reduced (Fig. 1). This could be due to the greatly enhanced belowground competitive ability of the invasive plant, including higher root *quantity* and *activity* and higher mycorrhizal colonization rate of this plant in mixed plantings compared to monoculture plantings (Figs. 1 and 3). Therefore, intensive competition from the invasive plant could reduce soil resources to a low level (Fig. 4), thereby suppressing the compensatory increase in the root *activity* of the native legume.

In contrast to legume roots, our results showed a decrease in nodule *quantity* and an increase in nodule *activity* in mixed plantings compared to monoculture plantings. This response is due to

the intense competition for soil resources by the invasive plant *X. strumarium* (Fig. 4). For example, the reduction in rhizosphere soil N content of the native legume caused by *X. strumarium* (Fig. S5A) can limit plant growth, and hence reduce carbon allocation to nodule production (Fig. S6). Meanwhile, the lower rhizosphere soil N content of the legume can downregulate genes that inhibit N-fixing and/or upregulate genes that stimulate N-fixing, as reported in previous studies, leading to higher nodule *activity* (Salvagiotti et al., 2008; Li et al., 2009, 2022; Lin et al., 2018).

Considering the responses of nutrient acquisition strategies by legume roots, nodules, and mycorrhizal fungi (which remain unchanged, see Fig. 3) to the invasive plant *X. strumarium*, we anticipated an increased contribution by the nodule-fixed N to the legume N acquisition. This expectation was supported by the isotopic evidence of a higher percentage of %Ndfa for *G. max* in mixed plantings than in monoculture plantings (Fig. 2D), as well as a positive relationship between %Ndfa and nodule *activity* (Fig. 2F). Together, our results demonstrate that the invasive plant increase nodule *activity* in the native legume through strong competition for soil nutrients, thereby enhancing the dependence of the native legume on nodule-fixed N (Fig. 2D).

4.3. Competition of soil resources by mycorrhizal fungi of the invasive plant

Consistent with our expectation, the invasive plant, *X. strumarium*, had higher mycorrhizal association in the mixture plantings than that in the monoculture plantings. If the increased mycorrhizal association in the invasive plant is used to acquire the rhizosphere soil N of the native legume, we would expect a negative relationship between the two variables. However, our result revealed a positive relationship between mycorrhizal association of *X. strumarium* and the rhizosphere soil N content of *G. max* (Fig. S5B). We also noted a higher root P acquisition ability in the mixed compared to monoculture plantings, as indicated by an increased proportion of total root Apase accounted for by *X. strumarium* in the mixed plantings (Fig. S7). This indirectly suggests that growth of *X. strumarium* in the mixed planting may be limited by P availability. Therefore, the increased mycorrhizal association of the invasive plant is likely used to acquire P rather than N in the rhizosphere soil of the native legume. Taken together, our findings suggest that roots and mycorrhizal fungi of *X. strumarium* may synergistically work to promote P acquisition in competition with the native legume.
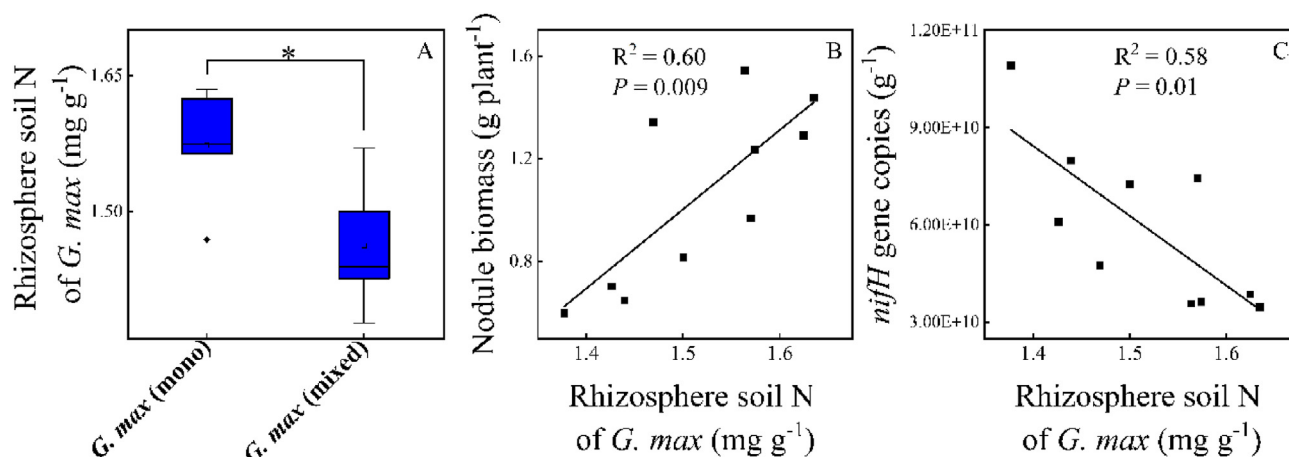

Fig. 4. Rhizosphere soil N of *Glycine max* in different plantation (A) and its relationship with nodule biomass (B) and *nifH* gene copies (C). mono: monoculture, mixed: mixture. *, $p < 0.05$.
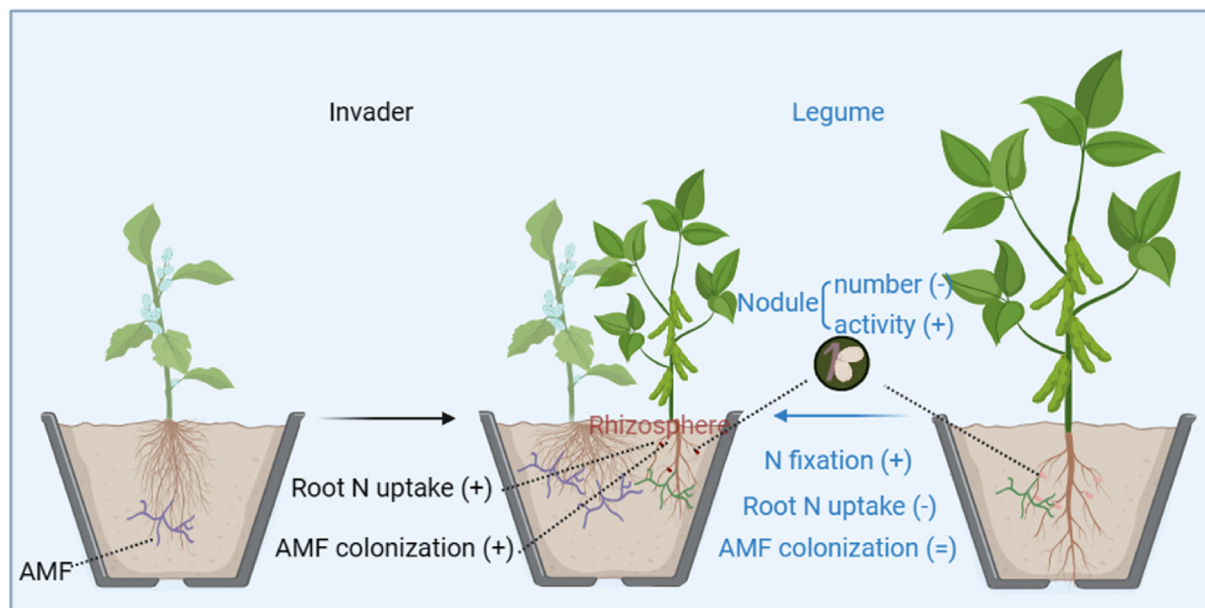
Fig. 5. Conceptual model for belowground resource competition between the invader (*Xanthium strumarium*) and the native legume (*Glycine max*). When in mixed plantings, the invader, *X. strumarium*, increases nutrient acquisition through its higher root nitrogen (N) uptake from the rhizosphere soil of the native legume. Meanwhile the invasive plant shifts to be with higher arbuscular mycorrhizal colonization (AMF) in the mixed plantings likely facilitating phosphorus acquisition from rhizosphere soil of the native legume. Depletion of rhizosphere soil N of the native legume by the invasive plant reduces native root N uptake and nodule *quantity* while greatly stimulates nodule *activity* for N-fixation, consequently resulting a higher contribution of N-fixing to the native plant N acquisition. '+', '−' and '=' indicate increase, decrease and no change of belowground nutrient acquisition in the mixed relative to the monoculture plantings, respectively.

Interestingly, the mycorrhizal association with *G. max* roots was unaffected in the mixed plantings (Fig. 3). Previous evidence has shown that *G. max*, in competition with the invasive plant, increases its dependence on biologically fixed N, which could entail an increased need for P for stoichiometric homeostasis of the N:P ratio (Zhong et al., 2023). As aforementioned, increased mycorrhizal association of *X. strumarium* in the mixture plantings can enhance its competition for soil P. Meanwhile, root Apase was reduced in the mixed planting. Therefore, keeping the mycorrhizal association of *G. max* unchanged in mixed plantings could be an effective way to partially meet the increased P demand of the native legume in mixed plantings.

5. Conclusion

Although the native legume plant can produce plenty of N through N-fixing by nodules, the invasive plant, *Xanthium strumarium*, does not appear to scavenge this fixed N from nodules. Instead, the invasive plant, with higher root *activity* and *quantity*, suppressed N acquisition by the roots of the native legume, while increasing its dependence on legume N-fixation by stimulating nodule *activity* in N-fixing (Fig. 5). Meanwhile, increased mycorrhizal association of *X. strumarium* seems to facilitate exploitation of rhizosphere soil P from the native legume. Therefore, the *activity−quantity* framework shows promise for enhancing our understanding of how alien plants successfully invade native legumes.

This study had certain limitations that should be addressed in future studies. First, it is important to note that we only examined one invasive plant species and one native legume species, which may restrict the generalizability of our findings. However, our study highlights the significant roles of invasive plant roots and mycorrhizal fungi in resource competition, specifically for N and P, which are common traits of invasive plants. Additionally, the mechanism underlying the increased dependence of the native legume on nodule-fixed N is commonly observed in legume plants, i.e.,

stimulating nodule *activity* through the depletion of rhizosphere soil N in legumes by an invasive competitor. Therefore, despite the limitations mentioned above, our conclusions based on a few species may have broader applicability to other invasive plants and native legumes. Second, future studies could consider incorporating native weedy species, such as gramineous or herbaceous species, as a comparison to invasive plants to elucidate the differences between invasive plants and weedy species in driving belowground interspecific competition. Third, although our study focused on examining belowground resource competition as a contributing factor to invasive success, it is crucial to consider the role of allelochemicals released by invasive plants in suppressing the growth of native plants. Therefore, future studies should consider both the allelopathy and resource competition of invasive plants relative to native legumes. Finally, future studies should concentrate on the composition of mycorrhizal fungi and the rhizobium community, which are important for nutrient acquisition from the soil or atmosphere.

Data availability statement

Data are available in Dryad Digital Repository, a publicly available database, or acquirable on request of the corresponding author.

CRediT authorship contribution statement

Meixu Han: Writing − review & editing, Writing − original draft. **Haiyang Zhang:** Writing − review & editing, Formal analysis. **Mingchao Liu:** Supervision, Resources. **Jinqi Tang:** Investigation, Data curation. **Xiaocheng Guo:** Investigation. **Weizheng Ren:** Validation. **Yong Zhao:** Funding acquisition. **Qingpei Yang:** Software. **Binglin Guo:** Software. **Qinwen Han:** Data curation. **Yulong Feng:** Supervision, Project administration. **Zhipei Feng:**

Methodology. **Honghui Wu:** Writing − review & editing. **Xitian Yang:** Funding acquisition. **Deliang Kong:** Writing − review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2024.04.003.

References

Bajwa, A.A., Farooq, M., Nawaz, A., et al., 2019. Impact of invasive plant species on the livelihoods of farming households: evidence from *Parthenium hysterophorus* invasion in rural Punjab, Pakistan. Biol. Invasions 21, 3285–3304. https://doi.org/10.1007/s10530-019-02047-0.

Balboa, G.R., Ciampitti, I.A., 2020. Estimating biological nitrogen fixation in field-grown soybeans: impact of B value. Plant Soil 446, 195–210. https://doi.org/10.1007/s11104-019-04317-1.

Bardgett, R.D., Mommer, L., De Vries, F.T., 2014. Going underground: root traits as drivers of ecosystem processes. Trends Ecol. Evol. 29, 692–699. https://doi.org/10.1016/j.tree.2014.10.006.

Bergmann, J., Weigelt, A., van der Plas, F., et al., 2020. The fungal collaboration gradient dominates the root economics space in plants. Sci. Adv. 6. https://doi.org/10.1126/sciadv.aba3756.

Bi, B., Yin, Q., Hao, Z., 2023. Root phosphatase activity is a competitive trait affiliated with the conservation gradient in root economic space. For. Ecosyst. 10, 100111. https://doi.org/10.1016/j.fecs.2023.100111.

Birnbaum, C., Morald, T.K., Tibbett, M., et al., 2018. Effect of plant root symbionts on performance of native woody species in competition with an invasive grass in multispecies microcosms. Ecol. Evol. 8, 8652–8664. https://doi.org/10.1002/ece3.4397.

Chen, G.Q., He, Y.H., Qiang, S., 2013. Increasing seriousness of plant invasions in croplands of eastern China in relation to changing farming practices: a case study. PLoS One 8, e74136. https://doi.org/10.1371/journal.pone.0074136.

Chen, J., Zhang, H.Y., Liu, M.C., et al., 2022. Plant invasions facilitated by suppression of root nutrient acquisition rather than by disruption of mycorrhizal association in the native plant. Plant Divers. 44, 499–504. https://doi.org/10.1016/j.pld.2021.12.004.

Cox, A., Boots-Haupt, L., Brasier, K., et al., 2022. Using δ^{15}N to screen for nitrogen fixation: reference plant position and species. Agron. J. 114, 1842–1850. https://doi.org/10.1002/agj2.21032.

Grove, S., Haubensak, K.A., Gehring, C., et al., 2017. Mycorrhizae, invasions, and the temporal dynamics of mutualism disruption. J. Ecol. 105, 1496–1508. https://doi.org/10.1111/1365-2745.12853.

Han, M., Chen, Y., Li, R., et al., 2022. Root phosphatase activity aligns with the collaboration gradient of the root economics space. New Phytol. 234, 837–849. https://doi.org/10.1111/nph.17906.

Han, M., Zhu, B., 2021. Linking root respiration to chemistry and morphology across species. Global Change Biol. 27, 190–201. https://doi.org/10.1111/gcb.15391.

He, X., Critchley, C.N.R., Bledsoe, C.S., 2003. Nitrogen transfer within and between plants through common mycorrhizal networks (CMNs). Crit. Rev. Plant Sci. 22, 531–567.

Hudgins, E.J., Cuthbert, R.N., Haubrock, P.J., et al., 2023. Unevenly distributed biological invasion costs among origin and recipient regions. Nat. Sustain. https://doi.org/10.1038/s41893-023-01124-6.

Ingraffia, R., Amato, G., Frenda, A., et al., 2019. Impacts of arbuscular mycorrhizal fungi on nutrient uptake, N$_2$ fixation, N transfer, and growth in a wheat/faba bean intercropping system. PLoS One 14, e0213672. https://doi.org/10.1371/journal.pone.0213672.

Iqbal, M.F., Feng, Y.L., Feng, W.W., et al., 2021. Ecological impacts of the invasive plant *Xanthium strumarium* and the impacts of three aboveground herbivores on the invader. Ecol. Indicat. 131, 108140. https://doi.org/10.1016/j.ecolind.2021.108140.

Iqbal, M.F., Liu, M.C., Iram, A., et al., 2020. Effects of the invasive plant *Xanthium strumarium* on diversity of native plant species: a competitive analysis approach in North and Northeast China. PLoS One 15, e0228476. https://doi.org/10.1371/journal.pone.0228476.

Kavka, M., Korn, K., Hazarika, M., et al., 2021. Potato root and leaf phosphatase activity in response to P deprivation. J. Plant Nutr. Soil Sci. 184, 668–677. https://doi.org/10.1002/jpln.202100112.

Ke, X., Xiao, H., Peng, Y., et al., 2022. Phosphoenolpyruvate reallocation links nitrogen fixation rates to root nodule energy state. Science 378, 971–977. https://doi.org/10.1126/science.abq8591.

Kong, C.H., Zhang, S.Z., Li, Y.H., et al., 2018. Plant neighbor detection and allelochemical response are driven by root-secreted signaling chemicals. Nat. Commun. 9, 3867. https://doi.org/10.1038/s41467-018-06429-1.

Li, Q., Zhang, H., Song, Y., et al., 2022. Alanine synthesized by alanine dehydrogenase enables ammonium-tolerant nitrogen fixation in *Paenibacillus sabinae* T27. Proc. Natl. Acad. Sci. U.S.A. 119, e2215855119. https://doi.org/10.1073/pnas.2215855119.

Li, Y.Y., Yu, C.B., Cheng, X., et al., 2009. Intercropping alleviates the inhibitory effect of N fertilization on nodulation and symbiotic N$_2$ fixation of faba bean. Plant Soil 323, 295–308. https://doi.org/10.1007/s11104-009-9938-8.

Libourel, C., Keller, J., Brichet, L., et al., 2023. Comparative phylotranscriptomics reveals ancestral and derived root nodule symbiosis programmes. Nat. Plants 9, 1067–1080. https://doi.org/10.1038/s41477-023-01441-w.

Lin, J.S., Li, X., Luo, Z., et al., 2018. NIN interacts with NLPs to mediate nitrate inhibition of nodulation in *Medicago truncatula*. Nat. Plants 4, 942–952. https://doi.org/10.1038/s41477-018-0261-3.

Liu, Y., Huang, W., Yang, Q., et al., 2022. Research advances of plant invasion ecology over past 10 years. Biodivers. Sci. 30, 23438. https://doi.org/10.17520/biods.2022438.

McCulloch, L., Porder, S., 2020. Lower nodule biomass with increased nitrogenase efficiency in *Robinia pseudoacacia* seedlings when grown under low soil phosphorus conditions. SN Appl. Sci. 2. https://doi.org/10.1007/s42452-020-03518-z.

McNickle, G.G., 2020. Interpreting plant root responses to nutrients, neighbours and pot volume depends on researchers' assumptions. Funct. Ecol. 34, 2199–2209. https://doi.org/10.1111/1365-2435.13517.

Png, G.K., Turner, B.L., Albornoz, F.E., et al., 2017. Greater root phosphatase activity in nitrogen-fixing rhizobial but not actinorhizal plants with declining phosphorus availability. J. Ecol. 105, 1246–1255. https://doi.org/10.1111/1365-2745.12758.

Salvagiotti, F., Cassman, K.G., Specht, J.E., et al., 2008. Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. Field Crops Res. 108, 1–13. https://doi.org/10.1016/j.fcr.2008.03.001.

Semchenko, M., Saar, S., Lepik, A., 2014. Plant root exudates mediate neighbour recognition and trigger complex behavioural changes. New Phytol. 204, 631–637. https://doi.org/10.1111/nph.12930.

Sun, D., Yang, X., Wang, Y., et al., 2022. Stronger mutualistic interactions with arbuscular mycorrhizal fungi help Asteraceae invaders outcompete the phylogenetically related natives. New Phytol. 236, 1487–1496. https://doi.org/10.1111/nph.18435.

Sun, L., Ataka, M., Han, M., et al., 2020. Root exudation as a major competitive fine-root functional trait of 18 coexisting species in a subtropical forest. New Phytol. 229. https://doi.org/10.1111/nph.16865.

Thilakarathna, M.S., McElroy, M.S., Chapagain, T., et al., 2016. Belowground nitrogen transfer from legumes to non-legumes under managed herbaceous cropping systems. A review. Agron. Sustain. Dev. 36, 58. https://doi.org/10.1007/s13593-016-0396-4.

Tian, B., Pei, Y., Huang, W., et al., 2021. Increasing flavonoid concentrations in root exudates enhance associations between arbuscular mycorrhizal fungi and an invasive plant. ISME J. 15, 1919–1930. https://doi.org/10.1038/s41396-021-00894-1.

Trouvelot, A., Kough, J.L., Gianinazzi-Pearson, V., 1986. Physiological and genetical aspects of mycorrhizae: proceedings of the 1st european symposium on mycorrhizae. In: Gianinazzi, S., Gianinazzi-Pearson, V. (Eds.), European Symposium on Mycorrhizae. INRA Press, Paris, pp. 217–221.

Wang, T., Guo, J., Peng, Y., et al., 2021. Light-induced mobile factors from shoots regulate rhizobium-triggered soybean root nodulation. Science 374, 65–71. https://doi.org/10.1126/science.abh2890.

Weston, L.A., Mathesius, U., 2013. Flavonoids: their structure, biosynthesis and role in the rhizosphere, including allelopathy. J. Chem. Ecol. 39, 283–297. https://doi.org/10.1007/s10886-013-0248-5.

Yang, J., Xiang, N., Liu, Y., et al., 2023. Organelle-dependent polyprotein designs enable stoichiometric expression of nitrogen fixation components targeted to

mitochondria. Proc. Natl. Acad. Sci. U.S.A. 120, e2305142120. https://doi.org/10.1073/pnas.2305142120.

Yang, Y., Liu, L., Singh, R.P., et al., 2020. Nodule and root zone microbiota of salt-tolerant wild soybean in coastal sand and saline-alkali soil. Front. Microbiol. 11, 2178. https://doi.org/10.3389/fmicb.2020.523142.

Yu, H., He, Y., Zhang, W., et al., 2022. Greater chemical signaling in root exudates enhances soil mutualistic associations in invasive plants compared to natives. New Phytol. 236, 1140–1153. https://doi.org/10.1111/nph.18289.

Zhang, H., Wang, X., Gao, Y., et al., 2020. Short-term N transfer from alfalfa to maize is dependent more on arbuscular mycorrhizal fungi than root exudates in N deficient soil. Plant Soil 446, 23–41. https://doi.org/10.1007/s11104-019-04333-1.

Zhang, Y., Cao, J.J., Yang, Q.P., et al., 2023. The worldwide allometric relationship in anatomical structures for plant roots. Plant Divers. 46, 621–629. https://doi.org/10.1016/j.pld.2023.05.002.

Zhang, Y., Cao, J.J., Lu, M.Z., et al., 2024. The origin of bi-dimensionality in plant root traits. Trends Ecol. Evol. 39, 78–88. https://doi.org/10.1016/j.tree.2023.09.002.

Zhong, Y., Tian, J., Li, X., et al., 2023. Cooperative interactions between nitrogen fixation and phosphorus nutrition in legumes. New Phytol. 237, 734–745. https://doi.org/10.1111/nph.18593.