Medicago truncatula adjusts root proliferation, nodule formation, and partner choice in response to local N heterogeneity

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Plant and Soil

Medicago truncatula adjusts root proliferation, nodule formation, and partner choice in response to local N heterogeneity --Manuscript Draft--

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Abstract:	<p>ABSTRACT</p> <p>Aims Increases in anthropogenic nitrogen (N) deposition are expected to lead to a higher mean and lower variance of N in the soil, which may disproportionately affect leguminous plants that acquire N both symbiotically via rhizobia and abiotically from soil. Here, we investigate the effects of changing N-heterogeneity at a fine spatial scale on the legume-rhizobium symbiosis.</p> <p>Methods We used a split-root system to introduce local soil N-heterogeneity, while maintaining the same plant-level amount of N. We treated root halves of five inbred lines of Medicago truncatula (HapMap lines 267, 270, 276, 279, and 313) with 50%-50%, 20%-80%, 10%-90%, or 2%-98% N and inoculated them with two Ensifer meliloti strains that differ in N-fixation ability.</p> <p>Results Although shoot biomass was unaffected by N-heterogeneity, plant lines adjusted their relative investments in nodulation versus root proliferation to optimize N-acquisition; high-N root halves invested more into root proliferation, while low-N root halves invested more into nodule formation. We detected hidden plasticity in partner choice: although all plants formed more nodules with the more effective N-fixing strain, roots experiencing greater N-limitation increased preference for effective N-fixing rhizobia.</p>	

	<p>Furthermore, there was significant variation among lines in root proliferation and nodulation responses to N-heterogeneity.</p> <p>Conclusion</p> <p>In response to N-heterogeneity, <i>M. truncatula</i> exhibited phenotypic plasticity in root proliferation, nodulation, and partner choice at the half-root level. We suggest that plasticity in response to rhizosphere conditions makes legumes potentially resilient to changes in N-heterogeneity, particularly in a time of anthropogenic N deposition.</p>
Response to Reviewers:	<p>Response to Reviewer:</p> <p>>>The title, "Medicago truncatula adjusts root proliferation, nodule formation, and partner choice to cope with local N heterogeneity" still suggests belowground plasticity is a means of enhancing performance or fitness for <i>M. truncatula</i> across heterogeneous environments, which is not supported by their data. I think a title something like, "Medicago truncatula adjusts root proliferation, nodule formation, and partner choice to in response to local N heterogeneity" would be a more accurate description of the findings.</p> <p>We agree that rewording the title would be appropriate given our last set of changes, and have updated it to "Medicago truncatula adjusts root proliferation, nodule formation, and partner choice in response to local N heterogeneity" (Line 1).</p> <p>>>"Medicago" and "Medicago truncatula" are non consistently italicized in the text.</p> <p>Thank you for bringing this to our attention. In this revision we cleaned up italicization in text (Line 378), as well as some minor formatting issues in the references (Line 499, 539, 544).</p> <p>Sincerely,</p> <p>Xuechun Zhang et al.</p>

***Medicago truncatula* adjusts root proliferation, nodule formation, and partner choice in response to local N heterogeneity**

Running title: Response of legumes to nitrogen heterogeneity

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ABSTRACT

Aims

Increases in anthropogenic nitrogen (N) deposition are expected to lead to an overall higher mean and lower variance of N in the soil, which may disproportionately affect leguminous plants that acquire N both symbiotically via rhizobia and abiotically from soil. Here, we investigate the effects of changing N-heterogeneity at a fine spatial scale on the legume-rhizobium symbiosis.

Methods

We used a split-root system to introduce local soil N-heterogeneity, while maintaining the same overall plant-level amount of N. We treated root halves of five inbred lines of *Medicago truncatula* (HapMap lines 267, 270, 276, 279, and 313) with 50%-50%, 20%-80%, 10%-90%, or 2%-98% N and inoculated them with two *Ensifer meliloti* strains that differ in N-fixation ability.

Results

Although shoot biomass was unaffected by N-heterogeneity, plant lines adjusted their relative investments in nodulation versus root proliferation to optimize N-acquisition; high-N root halves tended to invest more into root proliferation, while low-N root halves invested more into nodule formation. We detected hidden plasticity in partner choice: although all plants formed more nodules with the more effective N-fixing strain, roots experiencing greater N-limitation increased preference for effective N-fixing rhizobia. Furthermore, there was significant variation among plant lines in root proliferation and nodulation responses to N-heterogeneity.

Conclusion

In response to N-heterogeneity, *M. truncatula* exhibited phenotypic plasticity in root proliferation, nodulation, and partner choice at the local half-root level. We suggest that plasticity in response to

37 rhizosphere conditions makes legumes potentially resilient to changes in N-heterogeneity, particularly in
38 a time of anthropogenic N deposition.

39

40 **Keywords:** nitrogen deposition, legume-rhizobium symbiosis, *Medicago truncatula*, *Ensifer meliloti*,
41 nitrogen acquisition, root foraging, nodulation, partner choice, mutualism

INTRODUCTION

Bioaccessible forms of nitrogen (N) such as nitrate or ammonia often limit terrestrial plant growth (Vitousek et al. 1997). Anthropogenic N deposition via runoff or atmospheric N emissions increases overall soil N availability and significantly impacts plant communities by reducing species diversity (Bobbink et al. 2010; Moreau et al. 2015). This species loss is often non-random, as N-efficient species are subjected to greater interspecific competition from species ordinarily limited by N (Walter et al. 2017; Gilliam et al. 2016). Moreover, this deposition can also result in the loss of N-heterogeneity, coined the N-homogeneity hypothesis (Gilliam 2006), and is likely to continue as a result of future anthropogenic activities (Lamarque et al. 2013; Gilliam et al. 2019). Research in support of the N-homogeneity hypothesis suggests that the spatial heterogeneity of N and other resources affects plant community composition at a large scale, but the sensitivity of plant communities is modulated by the spatial grain of heterogeneous patches (Hutchings et al. 2003). In addition to impacting plant communities, changes in soil N have been shown to affect soil microbial communities, with increased N reducing plant carbon allocation to root microbes (Carara et al. 2018). Of particular concern is how a shift in soil N-heterogeneity may impact plant species that rely on microbial partners for nutrients (Bobbink et al. 2010; Gilliam et al. 2019), yet we know relatively little about the effects of altered N-heterogeneity on symbiotic N-fixing microbes and their host plant species.

As an additional means to acquire N, legumes such as *Medicago truncatula* have evolved a symbiosis with N-fixing bacteria called rhizobia that are capable of converting atmospheric nitrogen (N₂) into plant-usable forms. In return, rhizobia reside within specialized nodules on plant roots and receive a carbon source from their host. The legume-rhizobium symbiosis has been described as one of the most ecologically and economically important N-fixing symbiotic associations, particularly for its relevance to agricultural production (Wagner 2011) and for understanding mutualism evolution (Heath and Tiffin 2007). However, this symbiosis exhibits a great degree of plasticity in response to a variety of biotic and abiotic factors including variation in the N-fixing ability of different rhizobium strains (Parker 1995; Thrall et al. 2000; Heath and Tiffin, 2007) and the availability and distribution of nutrients within the soil,

as often demonstrated by split-root experiments (Ruffel et al. 2008; Jeudy et al. 2010; Laguerre et al. 2012; reviewed in Larraínzar et al. 2014). The legume-rhizobium symbiosis is particularly sensitive to shifts in soil N availability, and although several previous studies have investigated how changes in mean soil N affect the symbiosis, we know relatively little about the effects of altered N-heterogeneity on this important interaction.

Legumes are capable of optimizing N acquisition under changing N conditions in several ways. First, legume roots can “forage” for N in N-rich patches in the soil, such that roots proliferate more in high- than low-N patches (Robinson et al. 1999; Forde and Lorenzo 2001; Ruffel et al. 2008; Ruffel et al. 2011; Batstone et al. 2017). Second, roots downregulate new nodule formation in high-N environments (Streeter and Wong 1988; reviewed in Carroll and Mathews 1990; Bollman and Vessey 2006; Kassaw et al. 2015), suggesting that additional nodulation is costly when a plant has sufficient N. Nodulation is not only regulated systemically but also locally, whereby N-supplied root sections form fewer nodules compared to N-limited sections (Blumenthal et al. 2006; Jeudy et al. 2010; Lin et al. 2018). These results suggest that not only can legumes optimize N acquisition under changing mean N conditions, but also under heterogeneous soil N conditions. Finally, when faced with rhizobia that vary in their quality as N-fixing symbionts, legumes can optimize N uptake by preferentially associating with or allocating resources to more effective rhizobium strains, traits often termed “partner choice” or legume “sanctions,” respectively (Bull and Rice 1991; Kiers et al. 2003; Heath and Tiffin 2009; Laguerre et al. 2012; reviewed in Frederickson 2013). For example, *Medicago truncatula* forms ~63% more nodules with an effective rhizobium strain (*Ensifer meliloti* 1022) than with an ineffective strain (*Ensifer meliloti* 1021) (Batstone et al. 2017). Whether legumes can simultaneously adjust root proliferation, nodulation, and partner choice in response to N-heterogeneity remains to be tested.

The addition of soil N is thought to impact the legume-rhizobium symbiosis in several ways. Within the lifespan of a plant, soil N addition increases plant fitness and decreases overall nodulation (Thomas et al. 2000; Salvagiotti et al. 2008; Zhang et al. 2011), but does not appear to affect the ability of plants to “choose” effective N-fixing rhizobia; the ratio of effective to ineffective N-fixing rhizobia did

not significantly differ between plants growing under N-limited versus N-rich conditions (Regus et al. 2014; Grillo et al. 2016). On the other hand, the long-term addition of N fertilizer to field plots over 22 years resulted in the evolution of ineffective N-fixing rhizobia (Weese et al. 2015), either because legumes evolved to rely less on rhizobia as a N source, thus relaxing partner choice (Kiers et al. 2007), or because selection favored the evolution of less beneficial rhizobia that can still nodulate plants but provide less N in return (Klinger et al. 2016). These previous studies examined the effects of changing mean soil N levels; to our knowledge, no study has examined the effects of changing soil N heterogeneity on nodulation and partner choice. While mean soil N is predicted to increase over the long term (Bobbink et al. 2010), soil N heterogeneity is likely to fluctuate much more rapidly, and could have important, yet unaccounted for, consequences on the legume-rhizobium symbiosis (Galloway et al. 2004, Gilliam 2006).

We conducted a split-root experiment using five lines of the model legume *Medicago truncatula* and two strains of rhizobia, one ineffective and one effective at fixing N (*E. meliloti* 1021 and *E. meliloti* 1022, respectively). We chose the five lines based on the results of a previous study (Batstone et al. 2017) that demonstrated that *M. truncatula* genotypes vary in traits such as partner choice; the five *M. truncatula* lines studied here span the full range of trait values. By holding total soil N available to each plant constant while changing the proportion of soil N available to each half-root, we tested whether increasing N-heterogeneity affects several key symbiosis traits, specifically plant performance, root growth, nodulation, and partner choice. This topic is of particular relevance, given the ongoing changes to the global N landscape from atmospheric N deposition and other anthropogenic N inputs that could reduce N heterogeneity and potentially, plant diversity (Gilliam 2006). We predicted that plants should be able to compensate for differences in N-heterogeneity between treatments, with no difference in plant performance, by optimizing abiotic and symbiotic N-uptake. Specifically, we predicted that with increasing N-heterogeneity, root halves experiencing greater N should invest relatively less into nodulation with the effective N-fixing strain, consistent with the reduced dependency on rhizobia in high-N environments found in other experiments (Thomas et al. 2000; Salvagiotti et al. 2008; Zhang et al.

2011). Our study is the first to use a split-root technique to investigate how legumes optimize both symbiotic and abiotic N-uptake under varying degrees of N-heterogeneity.

MATERIALS AND METHODS

Study system

Medicago truncatula, barrel medic, is an annual legume native to the Mediterranean region and commonly used in legume-rhizobium experiments. We inoculated *M. truncatula* with two strains of the rhizobium *Ensifer meliloti* (Em1021 and Em1022) that differ in their effectiveness as N-fixers. Inoculation with Em1022 alone results in almost 40% greater plant biomass than inoculation with Em1021 (Batstone et al. 2017), and Em1022 fixes around three-times as much nitrogen on *M. truncatula* A17 compared to Em1021 (Terpolilli et al. 2008). We received Em1021 isolates from Turlough Finan at McMaster University, Canada, and Em1022 isolates from Jason Terpolilli at Murdoch University, Australia. We prepared 480 *M. truncatula* seeds from five inbred lines (HapMap (HM) lines 267, 270, 276, 279, and 313) obtained from the *Medicago* HapMap Project (<http://www.medicagohapmap.org/hapmap/germplasm>). The five lines we used were originally collected from Algeria (HM lines 267 and 276), Portugal (HM line 270), Morocco (HM line 279), and an introduced population in Canada (HM line 313), and then selfed to generate seed stocks provided to us by the HapMap Project. We specifically chose these lines because they were included in a previous split-root experiment (Batstone et al. 2017) that, upon reanalysis (see results), found significant variation in each line's ability to both forage for N in the soil when rhizobia were absent, and to preferentially associate with the higher-quality strain when N-fertilizer was absent.

Plant growth setup

Seed preparation was staggered over 12 days. Each day, an equal number of seeds from each of the five lines was processed (i.e., 8 replicates per line per day), giving us a total of 40 plants per day. Following the split-root protocol of Kassaw and Frugoli (2012) and Batstone et al. (2017), we scarified and surface sterilized the seeds, and after a week of growth over Fahräeus agar, we cut the shoot-root junction to

induce the growth of separate root halves. We then planted successful split-root halves into a system of paired Cone-tainers™ (Stuewe & Sons, Tangent, OR, USA) attached side by side. Each half-root grew in its own isolated soil environment, but they shared aboveground plant parts, allowing us to compare both the local and systemic responses to changing N heterogeneity. We used sterilized washed river sand (New Canadian Lumber, Toronto, Canada) as a substrate for root growth, and plugged the bottom of each Cone-tainer™ with clean polyester cotton to prevent sand from draining (Supp. Info. Methods S1 and S2). We harvested a total of 338 plants that successfully generated split-roots and survived until harvest, averaging 17 replicates per line per treatment. Of these, we scored nodule occupancy on 128 plants, an average of six replicates per line per treatment. We cultured bacteria from a total of 1,743 nodules, but analyzed data from only the 1,095 that were unambiguously scored as either Em1021 or Em1022, excluding 52 mixed nodules (4.52% of all nodules scored).

N-heterogeneity treatments

We randomly assigned plants to N-heterogeneity treatments that varied in the degree of soil N available to each half-root. In other words, each half-root received a different N concentration, even though the total N available to both halves was the same across all treatments (3.13 mM, as in Batstone et al. 2017). We used four different N-heterogeneity treatments, corresponding to 50% and 50% of the total N distributed between the two root halves for the control, then 80% and 20%, 90% and 10%, and 98% and 2% respectively (exact recipes in Supplementary Information). Each root half in the 50:50 treatment was randomly designated as a ‘high-N’ or ‘low-N’ root half for statistical analyses, but they received an identical amount of fertilizer. To isolate the effects of N only, we kept calcium and potassium constant among fertilizers to ensure that these other macronutrients would not affect root responses (Moreau et al. 2008). Autoclaved fertilizers were poured into sterilized 50 mL Falcon Tubes (Corning Life Sciences) that were then securely attached to the bottom of each Cone-tainer™, and a sterilized wick (Blueline polypropylene rope, 3/8-in diameter) was used to span from three-quarters of the way into the Cone-tainer™ to the very bottom of the Falcon tube below. Thus, each plant received two Falcon tubes filled with the same (control) or different fertilizers corresponding to each treatment. Fertilizer was consistently

wicked up into the soil from the attached Falcon tube, and we added additional fertilizer of the same treatment whenever it ran low to ensure a constant supply (Supp. Info. Methods S3). In our experiment, each half-root received a mixed inoculum of both *E. meliloti* 1021 and *E. meliloti* 1022 (see details below). For comparison, we also re-analyzed data from Batstone et al. (2017) to assess how the same five plant lines (267, 270, 276, 279, and 313) proliferated their root systems in response to 80% versus 20% N in the absence of rhizobia, and how they formed nodules with an effective (Em1022) or ineffective (Em1021) N-fixing strain when growing in a split-root system in the absence of additional N fertilizer (Fig. S1).

Rhizobia inoculation

Rhizobia strains were individually streaked onto solid Tryptone Yeast (TY) medium, and a single individual colony was chosen to be inoculated into liquid TY medium for three days in a shaking incubator set to 29 °C and 200 rpm. We recorded the OD₆₀₀ of each strain to make sure growth was consistent across days (OD₆₀₀ ~ 0.1), and then created a 2:1 mix of Em1021:Em1022 strains, providing each plant with 1 mL of inoculum in total. We used a higher proportion of Em1021 because Em1022 is better at forming nodules with *M. truncatula* (Terpolilli et al. 2008), and will often be the only strain present in nodules if Em1021:Em1022 inoculation ratios are too low (Batstone et al. 2017) (Supp. Info. Methods S4). Plants then grew in a growth chamber set to 23 °C/18 °C day/night temperature, 70% humidity, and 300 µmol m⁻² s⁻¹ photosynthetically active radiation (PAR) for a total of 31 days post-inoculation; plants were approximately two months old at this point, after including the time required for generating split-roots. At the end of this period we harvested all plants, and dissected up to five nodules per root half to later measure strain occupancy (Supp. Info. Methods S5). Previously, total nodule biomass was found to account for less than 10% of total root biomass, indicating that excising a maximum of five nodules would represent only a small portion of root biomass (Jeudy et al. 2010, supplementary information). On average, we removed 0.43 more nodules from low-side roots compared to high-side roots. There was an average of 2.68 and 2.10 nodules remaining on the low-N and high-N root halves respectively, constituting a minimal effect on root biomass comparisons. Finally, we separated

the shoot from the remaining two root sections, dried them in an oven set to 60 °C for three days, and measured the dried biomass of each of the parts.

Nodule occupancy

Dissected nodules were thoroughly dried in vials filled with silica gel and cotton on top, and stored at 4 °C to prevent degradation until they were processed. After imbibing overnight in distilled water, we surface-sterilized nodules using ethanol (15 s), bleach (15 s), and five changes of distilled water to remove bleach, and then crushed each individual nodule and streaked its contents onto a solid TY media plate. After three days of growth in an incubator set to 29 °C, we identified the rhizobium strain contained inside each nodule based on colony morphology; Em1021 forms small, isolated opaque colonies, while Em1022 forms large, mucoid colonies. In addition to plates that clearly showed either Em1021 or Em1022, we noted plates with insufficient growth or both strains (i.e., mixed nodules) (Supp. Info. Methods S6).

Statistical analyses

To investigate the effects of N-heterogeneity on several key symbiosis-related traits at both the whole-plant and local half-root levels, we used a generalized linear mixed model (GLMM) framework executed in R using the lme4 package (Bates et al. 2015). For whole-plant traits (shoot biomass, total nodule number, root biomass, and partner choice), we included the main and interactive fixed effects of N-heterogeneity treatment (four levels, ordered by increasing heterogeneity) and plant line (five levels), as well as the random effects of batch (i.e., day) and tray (nested within batch). Partner choice in this case was calculated by dividing the number of nodules a plant formed with the effective N-fixing strain by the total number of nodules formed with both strains. For half-root responses (root biomass, nodule number), we included the fixed main and interactive effects of N-heterogeneity treatment, plant line, root-half (i.e., high-N or low-N, nested within treatment), and either nodule number or root biomass as a covariate, respectively, as well as the random effects of batch, tray (nested within batch), and plant ID (nested within tray). To examine partner choice at the half-root level, we used the same model as for nodule number, except that we additionally included the main and interactive effects of strain (two levels, either

Em1022 or Em1021). For models with nodule number as a response, we additionally included an observation-level term as a random effect to account for overdispersion (Elston et al. 2001; Harrison 2014). We determined the best-fit probability distribution of each model by choosing the one that gave the lowest AIC value. The analysis of deviance used Wald Chi-squared tests (CAR package in R; Fox and Weisberg 2011) with type III sums of squares to make sure results did not depend on the order in which model terms were tested. We checked model fit by plotting scaled residuals versus fitted values. Finally, we used the lsmeans package in R (v.2.25; Lenth et al. 2015) to conduct post-hoc analyses based on least squares means. Specifically, we compared root halves (i.e., low- versus high-N) in each treatment over plant lines, as well as for each treatment within plant lines using Dunnett (1995)'s contrasting method.

RESULTS

Whole-plant results

Shoot biomass was similar across N-heterogeneity treatments ($p=0.612$), but differed significantly among plant lines ($p<0.001$; Fig. S2). Plant line 270 in particular did not germinate well and we discovered during harvest that many plants established only one successful root half. Overall, plants formed many more nodules with the effective N-fixing strain than with the ineffective strain, with an average Em1022-occupancy rate of 76.5% ($\pm 1.60\%$ SE), but there was no effect of N-heterogeneity treatment on partner choice at the whole-plant level nor a significant difference among lines (see Fig. S2d). We also found a non-significant ($p > 0.1$) main effect of N-heterogeneity on total root biomass and nodule number. Similar to our shoot biomass results, plant lines significantly differed ($p < 0.001$) in terms of total root biomass and nodule number ($p<0.001$), but not N-heterogeneity treatment at the whole-plant level (Fig. S2, Table S1).

Half-root results

In contrast to whole-plant traits, we observed many differences among N-heterogeneity treatments at the half-root level. We found a significant interaction between N-heterogeneity treatment and root half for root biomass ($p < 0.05$, Table S2); independent of plant line, as N-heterogeneity increased, high-N root

halves grew larger than low-N root halves, although the difference between root halves was non-significant in the 10:90 treatment (Fig. 1 “All lines” panel). Moreover, the significant three-way interaction among N-heterogeneity treatment, root half, and plant line suggests that plant genotypes differed in their ability to respond to N-heterogeneity via root proliferation ($p < 0.01$, Table S2); plant line 279 clearly showed the expected response of increasing root biomass as N became increasingly available on the high-N side, while plant line 270 did not show any trend with increasing N-heterogeneity (Fig. 1).

Similarly, root halves tended to form more nodules as they became more N-limited, compared to when they were locally supplied with greater N concentrations (Fig. 2 “All lines” panel). Again, we found a significant three-way interaction among line, N-heterogeneity treatment, and root half ($p < 0.01$, Table S2), suggesting that plant lines varied in their ability to regulate nodulation in response to N-heterogeneity; plant line 313 showed an increasing, although saturating, response to N-heterogeneity on the low-N side, while other lines, such as 270 and 279, did not show much difference between root halves with increasing N-heterogeneity (Fig. 2). We also found a significant three-way interaction among root biomass, N-heterogeneity treatment, and root half for number of nodules ($p < 0.01$, Table S2); with increasing N-heterogeneity, there were correspondingly greater differences between low-N and high-N root halves in the number of nodules formed per unit of root biomass (Fig. 3). Specifically, high-N root halves tended to form fewer nodules per unit root biomass as local N availability increased (Fig. 3). In addition, the magnitude of this difference depended on rhizobium strain (i.e., four-way interaction among root biomass $\times$ treatment $\times$ root half $\times$ strain, $p < 0.001$, Table S3), and was mostly driven by the effective N-fixing strain Em1022 (Fig. 4). Finally, we found a significant three-way interaction among N-heterogeneity treatment, root half, and rhizobium strain ($p < 0.05$, Table S3), indicating that root halves formed significantly more nodules with the effective N-fixing strain Em1022 when they were more N-limited, while they formed similar numbers of nodules with the ineffective strain Em1021 across N-heterogeneity treatments (Fig. 4). In other words, low-N root halves exerted stronger partner choice when N was more limiting.

Re-analyzing data from Batstone et al. (2017), we found that in the absence of rhizobia, high-N root halves also had higher root biomass than low-N root halves (Fig. S1 B, Table S4). Specifically, there was a significant effect of treatment (20% versus 80% N) on root biomass ($p < 0.001$), and a significant interaction effect between treatment and plant line on root biomass ($p < 0.05$), suggesting that plant lines differ in their ability to forage for N. When rhizobia were present and N-fertilizer absent, roots inoculated with the effective N-fixer Em1022 formed more nodules overall ($p < 0.001$), and a significant interaction between treatment and plant line on nodule number was evident ($p < 0.001$), suggesting that plant lines differed in their ability to preferentially associate with the effective N-fixer Em1022 (Fig. S1 D). Our analysis revealed lines 313 and 267 to be the worst root foragers, but the most selective in their associations, forming most of their nodules with the more effective N-fixing strain (Fig. S1 D). In contrast, line 279 was best at root foraging, but was indiscriminate, forming similar nodule numbers with both strains (Fig. S1 B, D). Lines 276 and 270 were moderate root foragers, but formed similar or more nodules with the ineffective N-fixing strain, respectively (Fig. S1 B, D).

DISCUSSION

Plant responses to N-heterogeneity

Overall, we found that legumes can maintain performance even under extreme N-heterogeneity by finely balancing local root proliferation and nodulation in a strain-specific manner. Despite increasing N-heterogeneity, aboveground plant biomass remained constant as half-roots adjusted their investment in growth versus nodule formation. High-N root halves foraged for abiotic N by increasing root proliferation (Fig. 1), while low-N root halves foraged for symbiotic N by making more nodules (Fig. 2). While our findings of root foraging and nodulation regulation in response to N fertilizer are not new (Streeter 1985, Carroll and Mathews 1990, Salvagiotti et al. 2008), our experiment is the first to demonstrate that as N-heterogeneity increases, plants form more nodules with a more effective N-fixer (Em1022) on N-starved root halves (Fig. 4), showing that partner choice can respond to local N conditions. Our results are likely conservative, as splitting the roots of plants had some adverse effects on growth and development that could dampen the magnitude of differences observed among N-heterogeneity treatments (Batstone et al.

2017). Our split-root study revealed that despite differences in overall vigor among lines (some lines outperformed others, and lines displayed varying magnitude of responses to N-heterogeneity), all plant lines were somewhat able to modulate local root responses, suggesting that *M. truncatula* could be resilient to changing N regime.

Our study is consistent with numerous previous findings (e.g. Streeter 1985; also reviewed in Streeter and Wong 1988) that show N-limited roots upregulate nodulation relative to N-supplied roots; this pattern is demonstrated in every line except 270 (Fig. 2). We also show that this upregulation tends to increase with increasing N-heterogeneity, but saturates in the most extreme N-heterogeneity treatment (Fig. 2), perhaps because roots on the N-limited side are comparatively smaller (Fig. 1), and thus, space constraints put an upper limit on nodulation. By regressing nodule number against root biomass, we found that the nodules formed per unit root biomass depended on both N-heterogeneity treatment and rhizobium strain (Figs. 3 and 4, Table S3); as N-heterogeneity increased, roots experiencing greater N-limitation made more nodules per unit root biomass with the effective N-fixing strain Em1022, but the number of ineffective Em1021 nodules remained relatively constant across treatments (Fig. 4, Table S3). Because we inoculated half-roots with both rhizobium strains in all N-heterogeneity treatments, we cannot determine how each strain may have altered root responses to N-heterogeneity independently. However, our results clearly show that *M. truncatula* optimizes N-acquisition by adjusting root growth and nodulation with the effective N-fixing strain when N is limiting. The upregulation of nodulation on N-limiting roots further supports that N plays a role in modulating nodule formation and warrants further investigation into pathways that affect the number and distribution of nodules on the root system.

The novelty of our study is that we uncovered hidden plasticity in partner choice at the half-root level; root halves experiencing greater N-limitation not only exhibited greater nodulation relative to root biomass, but this was driven by increased partner choice (Figs. 3 and 4, Table S3). At the whole-plant level, our results reaffirm that *M. truncatula* exhibits significant partner choice when presented with both effective and ineffective rhizobium strains (e.g., Heath and Tiffin 2009; Gubry-Rangin et al. 2010; Batstone et al. 2017). But, plants did not exhibit plasticity in partner choice in response to N-

heterogeneity at the whole-plant level (Table S1), reaffirming previous studies that found consistent levels of partner choice when plants were exposed to different mean amounts of N in the soil (Laguerre et al. 2012; Regus et al. 2014; Grillo et al. 2016). However, by examining the local root-level responses to changing N conditions, we indeed find that plants can modulate the strength of partner choice by forming more nodules with a more effective N-fixer when N is limiting (Fig. 4). Furthermore, our results suggest that abiotic N-heterogeneity may change the spatial distribution of rhizobia in the rhizosphere; given that high-N half-roots nodulated significantly less overall, we expect rhizobia (and especially beneficial rhizobia) to reach higher densities in low-N patches. Future studies would benefit from assessing the fine-scale spatial distribution of rhizobia in the soil as it relates to N-availability.

Genetic variation in plant responses to N-heterogeneity

Most previous experiments examining *M. truncatula*'s ability to optimize N-uptake focused on a single plant line or used an additional mutant line (e.g., Ruffel et al. 2008; Jeudy et al. 2010; Laguerre et al. 2012). The five plant lines we used were previously found to vary in their ability to forage for N in the soil and preferentially associate with an effective N-fixer when these traits were measured individually (Batstone et al. 2017; Fig. S1). Our experiment uncovered significant genetic variation in root foraging and nodulation when both N fertilizer and rhizobia were present (Table S2). For example, line 270 exhibited almost no difference in root biomass or nodulation even at the most extreme N-heterogeneity treatment (Figs. 1, 2), indicating that some lines are either unable to cope with N heterogeneity effectively or are using different mechanisms to cope with N-heterogeneity. In contrast, line 279 demonstrated effective root foraging consistent with previous studies (e.g., Hodge et al. 1999; Robinson et al. 1999; Batstone et al. 2017), whereby N-supplied roots grew increasingly larger as N-heterogeneity increased (Fig. 1). Our study uncovered significant variation among *M. truncatula* lines in their ability to plastically respond to N-heterogeneity, and significant differences in fitness (shoot biomass) among lines, but no significant line x treatment effect on plant fitness; e.g., the least plastic and least fit line (270) performed equally poorly in all N-heterogeneity treatments. We might have expected line 270 to outperform more plastic lines in the 50:50 treatment, when other plant lines could not capitalize on their ability to adjust N

acquisition in response to N-heterogeneity, but the costs of phenotypic plasticity are notoriously difficult to measure (Auld et al. 2010) and measuring selection on plasticity in *M. truncatula* would likely require phenotyping many more than five plant lines across multiple N-heterogeneity environments.

Comparing results from the same five lines between experiments (i.e., present study versus Batstone et al. 2017) revealed several similarities, but also obvious differences. First, when we re-analyzed published data from Batstone et al. (2017) on root foraging in the absence of rhizobia, we similarly found that line 279 was best at root foraging while the other plant lines only marginally increased root growth under high-N conditions (Fig. S1 B), suggesting that line 279's superior root foraging ability is not altered significantly by the presence of rhizobia. In contrast, plant line 313 showed much greater root proliferation into 80%-N when rhizobia were present (Fig. 1) than absent (Fig. S1 B), supporting other studies that find rhizobia can alter the root foraging capabilities of the plant (e.g., Goh et al. 2016). The best root forager (i.e., 279) and choosiest plant line (i.e., 313) from Batstone et al. (2017) were also the top two performing lines in our present study (Fig. S2A), suggesting that effectively coping with N-heterogeneity may be achieved through alternative strategies, such as specializing in abiotic or symbiotic N-acquisition. In our present study, we found that plant lines differed in their plastic responses to increasing heterogeneity, supporting a theory of alternative strategies for coping with N-heterogeneity (Fig. 1, Fig. 2, Table S2). A major difference between the two experiments, however, is that in contrast to Batstone et al. (2017), we did not find significant genetic variation in partner choice (Table S3); all lines tended to upregulate nodulation with the more effective N-fixer when roots became increasingly N-limited (Fig. 4). This difference is likely due to the fact that in Batstone et al. (2017), strains were inoculated separately onto root halves, whereas in our study, both strains were inoculated onto both root halves. Eliminating spatial separation between strains permits antagonistic strain-strain interactions, and could modify the plant's ability to discriminate between strains, both of which are expected to affect partner choice as evident in the present study.

Under environmental shifts in nutrient availability, fixed strategies are at risk of being outcompeted by more flexible genotypes, leading to local and community shifts in genotypic

composition. In contrast, legumes such as *M. truncatula* could be more effective at coping with shifting N heterogeneity by fine-tuning both abiotic and symbiotic N-acquisition traits including root foraging, nodulation, and partner choice. The genetic variation we observed in local root responses to N, combined with the accessions and genomic resources available through the *Medicago* Hapmap project, make *M. truncatula* a good candidate species for understanding the mechanisms that legumes use to cope with changing N conditions. Future studies could utilize many different *M. truncatula* genotypes to address whether N-acquisition traits are under selection, and how N-heterogeneity may alter selection for these traits.

CONCLUSIONS

Given the dramatic rise of agricultural inputs to the global N cycle, understanding the traits and mechanisms that organisms use to better cope with increasing N-heterogeneity is pressing. Here we improved our understanding of how *M. truncatula* acquires N symbiotically and abiotically under homogeneous versus heterogeneous N conditions, and our results emphasize the substantial phenotypic plasticity of root investment in both proliferation and strain-specific nodulation. We suggest that this plasticity makes legumes potentially resilient to changes in N-heterogeneity, as evidenced by the similar performance of plants across treatments. More research into the legume-rhizobium mutualism will give us greater insight into the future of mutualistic systems as N heterogeneity continues to decrease from anthropogenic activities.

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406

407 **Conflict of Interest**

408 The authors submit that they have no conflicts of interest to declare at this time.

409

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546
547

FIGURE LEGENDS

Figure 1: Root foraging depends on N-heterogeneity treatment and plant line. Dry root biomass (least squares means $\pm$ 1 SE) of low-N (open symbols) and high-N (filled symbols) root halves in each N-heterogeneity treatment (50:50, 20:80, 10:90, 2:98) for each plant line (from top-left: 270, 276, 267, 313, and 279) and across all plant lines (bottom-right, “All lines”). Symbols above means represent significant (* = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.001) and marginally significant (■ = p-values between 0.05-0.1) contrasts between root halves at each treatment level (*sensu* Dunnett 1995). See Table S2 for complete GLMM results.

Figure 2: Nodulation is affected by N-heterogeneity treatment and plant line. Nodule number (least squares means $\pm$ 1 SE) of low-N (open symbols) and high-N (filled symbols) root halves in each N-heterogeneity treatment (50:50, 20:80, 10:90, 2:98) for each plant line (from top-left: 270, 276, 267, 313, and 279) and across all plant lines (bottom-right, “all lines”). Symbols above means represent significant (* = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.001) and marginally significant (■ = p-value between 0.05-0.1) contrasts between root halves at each treatment level. See Table S2 for complete GLMM results.

Figure 3: Nodule number per unit root biomass depends on N-heterogeneity treatment. Relationships between nodule number and root biomass for low-N (open symbols) and high-N (filled symbols) root halves in each N-heterogeneity treatment (from left: 50:50, 20:80, 10:90, 2:98). Data from all plant lines and both rhizobium strains were pooled for each treatment. Grey shading represents standard error. Solid and dashed lines show linear regression lines for high-N and low-N root halves, respectively. P-values based on model regression coefficients indicate a significant difference in slope when comparing between root halves at each N-heterogeneity treatment except the control (50:50). See Table S3 for complete GLMM results.

Figure 4: Plasticity in partner choice as roots become more N-limited. The number of Em1021 (left) and Em1022 (right) nodules (least squares $\pm$ 1 SE) on low-N (open symbols) and high-N (filled symbols) root halves in each N-heterogeneity treatment (50:50, 20:80, 10:90, 2:98). Data from all plant lines were pooled for each treatment. Symbols above means represent significant (* = p-value <0.05; ** = p-value < 0.01) contrasts between root halves. See Table S3 for complete GLMM results.

SUPPORTING INFORMATION

Figure S1. Shoot and root responses to fertilizer (A, B) or rhizobia (C, D) in a previous split-root experiment (Batstone et al. 2017). Dry shoot biomass (A) and dry root-half biomass (B) responses (mean $\pm$ 1SE) to N treatments of 20% and 80% of total available N, for low-N and high-N root halves, respectively. Plants in A and B were not inoculated with rhizobia, and remained rhizobium-free throughout the experiment. In contrast, C and D show dry shoot biomass (C) and nodule number (D) responses (mean $\pm$ 1SE) to rhizobia that differ in their ability to fix N, with Em1021 being ineffective and Em1022 being effective. Plants in C and D were given an N-free fertilizer throughout the experiment. See Table S4 for complete GLMM results.

Figure S2. Whole-plant responses (mean $\pm$ 1SE) with all treatments combined for each plant line. Nodule count and dry root biomass results are pooled from both low-N and high-N root halves. Numbers next to points show sample size for each line. See Table S1 for complete GLMM results.

Figure S3. Photo of Experimental Setup, displaying *M. truncatula* in a split-root setup with each root half in a separate Cone-tainer™ (Stuewe & Sons, Tangent, OR, USA) attached side by side.

Table S1. Analysis of deviance (ANODEV) tables for whole-plant responses, with the main effects of plant line, N-heterogeneity treatment, and their interaction. Random effects included batch and tray. Significant p-values (< 0.05) are highlighted in bold.

Table S2. ANODEV tables for half-root responses, including dry root biomass and nodule number, with main effects of plant line, N-heterogeneity treatment, root half nested within treatment, a covariate (either nodule number or dry root biomass, respectively), and all interactions. Random effects included batch, tray, and plant. Significant p-values (< 0.05) are highlighted in bold. Marginally significant p-values (0.05-0.1) are italicized.

607

608 **Table S3.** ANODEV table for partner choice, with main effects of plant line, N-heterogeneity treatment,
609 root half nested within treatment, dry root biomass as a covariate, strain, and all interactions. Random
610 effects included batch, tray, plant. Significant p-values (< 0.05) are highlighted in bold. Marginally
611 significant p-values (0.05-0.1) are italicized.

612

613 **Table S4.** ANODEV table based on data from a previous half-root experiment (Batstone et al. 2017) that
614 included dry shoot and root biomass and nodule number as responses, with the main effects of plant line,
615 treatment (20% or 80% -N; inoculated with Em1021 or Em1022) and their interaction. Random effects
616 included tray and plant (root biomass model only). Significant p-values (< 0.05) are highlighted in bold.

617

618 **Methods S1-6.** Details of split-root generation, making up fertilizers, rhizobia inoculation, harvesting
619 plants, and determining nodule occupancy.

620

621

Figure 1

Figure 2

Figure 3

Figure 4

Em1021

Click here to access/download;line figure;Zhang Wang et al. Figure 4.pdf

Em1022

Figure A1

Shoot biomass (mg)

B

Root biomass (mg)

C

D

Figure S2

1 **SUPPORTING INFORMATION – Tables**

2 **Table S1.** Whole-plant models. Accompanies Fig. S1.

3

	Shoot biomass (g) ¹			Root biomass (g) ²		
	χ^2	df	P-value	χ^2	df	P-value
(Intercept)	344.342	1	<0.001	196.934	1	<0.001
treatment	1.814	3	0.612	1.162	3	0.762
line	239.307	4	<0.001	243.535	4	<0.001
treat×line	4.964	12	0.959	8.612	12	0.736
	Total nodules (no.) ³			Partner choice ⁴		
(Intercept)	178.380	1	<0.001	107.720	1	<0.001
treatment	1.161	3	0.762	1.073	3	0.784
line	197.579	4	<0.001	3.035	4	0.552
treat×line	12.912	12	0.375	16.030	11	0.140

4 ¹ Gamma distribution with square-root link

5 ² Gamma distribution with square-root link

6 ³ Poisson distribution with square-root link, observation-level effect included as random effect

7 ⁴ Linear mixed model (i.e., Gaussian distribution with identity link)

8 **Table S2.** Local half-root level models. Accompanies Figs. 1 and 2.
9

	Root biomass (g) ¹			Nodules (no.) ²		
	χ^2	df	P-value	χ^2	df	P-value
(Intercept)	102.467	1	<0.001	85.699	1	<0.001
Covariate	211.267	1	<0.001	221.065	1	<0.001
line	43.842	4	<0.001	48.812	4	<0.001
treatment	6.449	3	0.092	2.676	3	0.444
treatment×half	10.697	4	0.030	7.253	4	0.123
covariate×treatment	8.350	3	0.039	1.236	3	0.745
line×treatment	10.512	12	0.571	13.675	12	0.322
covariate×treatment×half	5.537	4	0.237	27.731	4	<0.001
line×treatment×half	35.789	16	0.003	32.442	16	0.009

10 ¹ Gamma distribution with square-root link

11 ² Poisson distribution with square-root link, observation-level effect included as random effect

12 **Table S3.** Local half-root level model accounting for strain. Accompanies Figs. 3 and 4.
13

Nodules (no.)			
	χ^2	df	P-value
(Intercept)	386.936	1	<0.001
root	137.207	1	<0.001
line	47.193	4	<0.001
treatment	4.915	3	0.178
strain	113.838	1	<0.001
treatment×half	0.961	4	0.916
root×treatment	8.671	3	0.034
line×treatment	12.297	12	0.422
root×strain	0.852	1	0.356
line×strain	6.175	4	0.186
<i>treatment×strain</i>	<i>7.243</i>	<i>3</i>	<i>0.065</i>
root×treatment×half	15.809	4	0.003
line×treatment×half	10.259	16	0.853
treatment×half×strain	10.618	4	0.031
root×treatment×strain	21.824	3	<0.001
line×treatment×strain	25.915	12	0.011
root×treatment×half×strain	10.669	4	0.031
line×treatment×half×strain	17.765	16	0.338

14 ¹ Poisson distribution with square-root link, observation-level effect included as random effect

Table S4. Local half-root models re-analyzed from a previous split-root experiment (Batstone et al., 2017). Accompanies Fig. S2.

	Shoot biomass (g) ¹			Root biomass (g) ¹		
	χ^2	df	P-value	χ^2	df	P-value
(Intercept)	166.040	1	<0.001	266.793	1	<0.001
line	6.452	4	0.168	7.346	4	0.119
treatment	NA	NA	NA	18.446	1	<0.001
line×treatment	NA	NA	NA	11.516	4	0.021
	Shoot biomass (g) ¹			Nodules (no.) ²		
	χ^2	df	P-value	χ^2	df	P-value
(Intercept)	1854.000	1	<0.001	182.990	1	<0.001
line	44.850	4	<0.001	40.781	4	<0.001
treatment	NA	NA	NA	13.334	1	<0.001
line×treatment	NA	NA	NA	66.958	4	<0.001

¹ Gamma distribution with square-root link

² Poisson distribution with square-root link

Top half: Plants (N = 41, average ~ 8 replicates per line) of the same five lines as used in the present experiment (270, 276, 313, 267, 279) grew under rhizobia-free conditions, and split-roots were treated either with 20% or 80% N, the exact same as the 20:80 treatment used in the present experiment. Bottom half: Plants (N = 27, average ~ 5 replicates per line) of the same five lines grew under nitrogen-free conditions, and split-roots were inoculated either with the less or more effective N-fixers Em1021 and Em1022, respectively. For all models, we used generalized mixed model (GLMM) framework in R. Models analyzing shoot biomass as the response included the fixed effect of line and the random effect of tray, while GLMMs for root biomass and nodule number included the fixed effects of line, treatment (20% or 80%; Em1021 or Em1022), and their interaction, as well as the random effects of tray and plant. GLMMs were conducted using the lme4 package in R (Bates et al., 2015), and model assumptions were checked by visual inspection of the residuals versus fitted values. ANOVAs were based on type III sums of squares so that term order did not affect results.

SUPPORTING INFORMATION - METHODS

Methods S1. Seed sterilization and preparation

1. Each seed was scarified by giving it a small cut opposite the embryo with a razor.
2. We submerged the seed in 95% ethanol for 30 seconds, then washed it in bleach for 5 minutes, rinsed it under distilled water for 5 minutes, and finally soaked it in distilled water for 20 minutes.
 - Sterilization following the methods detailed in Gorton et al. (2012)
3. After sterilization, we stored the seeds in the dark at 4 °C for two days. Afterwards, we removed the seedlings and continued to keep them in the dark for one day at room temperature.
 - Vernalization following the methods detailed in Kassaw and Frugoli (2012)
4. Using Fahræus agar overlaid with autoclaved filter paper, we placed the seedlings vertically, five per plate, covered with another layer of filter paper. These were left alone for five days.
5. Afterwards, we cut the seedling with an alcohol- and flame-sterilized razor at the junction between the root and the shoot, and transferred the shoot to a new agar plate with autoclaved filter paper.
6. We grew the plants for another five days, and then removed the top filter paper layer. At this point, a split-root should begin to emerge.

Methods S2. Planting

Planting occurred six days after exposing the roots, when we transferred plants that had successfully generated equal split roots into a system with a pair of Cone-tainers™ attached side by side. The Cone-tainers™ each had an autoclaved polypropylene rope wick (Blueline, Canada) embedded in autoclaved sand (New Canadian Lumber & Building Supplies, Toronto, ON, Canada) that spanned the length of the Cone-tainers™ to draw water up. Cotton at the base of the system prevented sand from washing out.

1. First, we saturated the sand with water before planting each plant by hand following the methods detailed in Barker et al. (2006).
 2. We attached falcon tubes with liquid fertilizer of the relevant treatment to the bottom of the each Cone-tainer™.
 3. The plants were added to the soil, placing each root half into one of the two Cone-tainers™. The roots were then covered with soil.
 4. We kept the plants in a tray covered by plastic bags to maintain humidity and reduce contamination, and placed the trays in a growth chamber programmed for a 16-hour photoperiod, a constant temperature of 25°C, and 70% humidity.
 5. After one week of growth to allow the roots to grow and acclimate, we inoculated every Cone-tainer™ with a mixed rhizobial culture of Em1021 and Em1022 (more on this below).
 6. After two weeks of growth, we removed the plastic bags covering the trays.
 7. After three weeks of growth, we replaced all the fertilizer tubes with a new supply.
- Plants were watered daily, using just enough distilled water to keep the surface of the sand moist.

For plants that were ineffective at absorbing fertilizer from below due to issues with the wick setup, we applied the fertilizer from the surface for both of the root halves, even if only one half had an issue. This was necessary to minimize differences in environmental conditions between root halves. The plants grew for 51 days in total.

Methods S3. Fertilizer

We used four different treatments, corresponding to 50% and 50% of the total N distributed between the half-roots for the control, then 80% and 20%, 90% and 10%, and 98% and 2% respectively. The total amount of N received by each plant was held constant at 3.13 mM N. The macronutrients in the fertilizer were KNO_3 , $\text{Ca}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, K_2SO_4 , K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaCl (exact concentrations given in chart below), and Fe-EDTA (made up according to Barker et al. [2006], Appendix 2.1 for Fahræus medium). The micronutrients (all with final concentration of 1 ug/l) were MnSO_4 , ZnSO_4 , CuSO_4 , H_3BO_3 , and NaMoO_4 . Based on table 2 of Moreau et al. (2008), we decreased the concentration of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ as the concentration of $\text{Ca}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ increased to account for calcium, and we decreased the concentration of K_2SO_4 as the concentration of KNO_3 increased to account for potassium.

N-heterogeneity treatments		Macronutrients list (mM)						
Proportion N	Concentration N (mM)	KNO_3	$\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$	$\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	K_2SO_4	K_2HPO_4	$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	NaCl
2%	0.063	0.016	0.047	4.953	1.384	2.400	2.000	0.200
98%	3.0625	0.766	2.297	2.703	0.634	2.400	2.000	0.200
10%	0.313	0.078	0.234	4.766	1.322	2.400	2.000	0.200
90%	2.8125	0.703	2.109	2.891	0.697	2.400	2.000	0.200
20%	0.625	0.156	0.469	4.531	1.244	2.400	2.000	0.200
80%	2.5	0.625	1.875	3.125	0.775	2.400	2.000	0.200
50%	1.563	0.391	1.172	3.828	1.009	2.400	2.000	0.200

Methods S4. Rhizobia inoculation

1. We streaked rhizobia onto tryptone-yeast (TY) media plates and incubated them at 28°C for 3 days.
2. To create a 2:1 ratio of Em1021:Em1022 strains, we looped bacteria from pure cultures that grew on the plates into a Falcon tube with 50mL of liquid TY media for Em1021, and a Falcon tube with 25 mL of liquid TY media for Em1022. We stored both tubes along with a blank TY media tube in a shaking incubator at 28°C for 3 days.
3. Afterwards, we measured the optical density (OD600) of each strain's culture compared against a blank TY media using a photometer. The average readings across the groups were 0.256 A for 1021 ($\pm$ SD 0.048), and 0.358 A for 1022 ($\pm$ SD 0.043).
4. Immediately after obtaining the readings, we mixed the inoculum together in a 2:1 ratio (Em1021:Em1022) and inoculated each root half with 1 mL of the combined inoculum using a multi-pipettor.

Methods S5. Harvesting

We separated the shoot from the root halves at the beginning of harvesting, and took note of the following information from each plant:

1. The identification information (i.e. plant number, batch number, genotype, treatment)
2. The total number of trifoliate leaves
3. The total number of nodules on each root half
4. The dry weight of the aboveground portion of the plant, and each root half.

We dissected viable nodules from each root half, up to a maximum of 5 per root half. We placed the dissected nodules in an Eppendorf tube with dry silica and cotton balls and labelled them.

These nodules were then kept refrigerated at 4 °C. We placed the remaining plant parts separately into individual coin envelopes and dried them at 60°C for 2 days before taking dry weight measurements.

Methods S6. Nodule imbibing and sterilization

1. The preserved and dehydrated nodules were transferred from one silica-containing tube to a new 1.5 mL tube filled with distilled water for rehydration by using featherweight forceps.
2. The imbibed nodules were placed into the fridge for two to eight hours until fully rehydrated prior to subsequent sterilization. The low temperature environment is necessary for imbibing process to prevent nodule degradation.
3. After two to eight hours, we used glass Pasteur pipettes for liquid transferring during sterilization.
4. Nodules were immersed in 95%-100% ethanol (EtOH) for ten seconds, being inverted continually throughout this time so each nodule would be immersed. The EtOH was then discarded using the same pipette.
5. A second pipette was used to add 6% hypochlorite, inverting the tube for another ten seconds. The hypochlorite was then discarded using the same pipette.
6. A third pipette was used to remove the hypochlorite completely, by filling and emptying the tube with distilled water five times.
7. After sterilization, distilled water was added to the tube for subsequent plating.

141 Note: It was important to use three pipettes during each step of sterilization and also plate
142 nodules right after sterilization because even though most of the hypochlorite was removed, trace
143 amounts can degrade nodules if left for more than a few hours.

144 8. To begin plating, each surface-sterilized nodule was placed on the lid of a sterile petri
145 dish and crushed using alcohol- and flame-sterilized forceps.

146 9. A sterile cotton swab was used to absorb the contents of the crushed nodule and evenly
147 streak the surface of the agar medium containing a mixture of tryptone, yeast, and salt
148 ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$).

149 10. All plates were then placed in an incubator set to 29°C for the next 72 hours for bacterial
150 replication.

Supporting Information References

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