



Medicago truncatula supernodulation mutants *sun4* and *lss* show enhanced seed yield and seed nitrogen allocation from nitrogen fixation under low nitrogen availability

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Abstract

Background and Aims Symbiotic nitrogen fixation in legumes has an energetic cost, which the host controls through inhibition of nodulation in response to externally available nitrogen. Autoregulation of nodulation (AON) mutants supernodulate, even in the presence of sufficient nitrogen. Here, we tested whether three AON mutants of *Medicago truncatula*, *sun4*, *rdn1-1* and *lss*, display fitness costs due to supernodulation, and to what extent this is dependent on the supply of nitrogen.

Methods We grew wild type (WT) and AON mutants under controlled conditions with a supply of either 0.5 or 2 mM of ¹⁵N-labelled nitrate until seed maturation, and determined biomass, seed yield, tissue N content, and N derived from nitrogen fixation. Plants were either inoculated with *Sinorhizobium meliloti* or left uninoculated.

Results The *sun4* and *lss*, but not *rdn1-1* mutant showed significantly higher seed yield than the WT

plants in the low (0.5 mM) nitrate treatment, but only if they were inoculated with rhizobia. The mutants allocated significantly higher proportion of N derived from nitrogen fixation to seeds. At the higher (2 mM) nitrate concentration, mutants and WT showed similar biomass and N derived from nitrogen fixation.

Conclusions We conclude that, despite the higher potential energetic cost of supernodulation, nodulated *sun4* and *lss* AON mutants can have a fitness advantage (higher yield and biomass) under low N due to higher nitrogen fixation over the whole growth period, and that they allocate more fixed N and more biomass into their seeds than WT plants, at least under controlled conditions.

Keywords Autoregulation · Nitrogen fixation · Nodulation · Resource allocation

Introduction

The formation of nitrogen-fixing nodules provides legumes with a source of fixed nitrogen following conversion of dinitrogen gas into ammonia by symbiotic bacteria called rhizobia. Rhizobia are soil bacteria that infect legume roots and induce the formation of root nodules, in which rhizobia are housed intracellularly in symbiosomes. In exchange for fixed N, the plant provides its symbionts with fixed carbon in the form of organic acids (Udvardi and Poole 2013). Because of this cost, the symbiosis is inhibited in the

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presence of sufficient concentrations of fixed N, such as nitrate or ammonium, in the soil, when the cost of N uptake from nitrate or ammonium are estimated to be less than that of nodulation (Andrews et al. 2009; Voisin et al. 2003). Conversely, CO₂ enrichment can partially overcome the inhibition of nodulation by nitrate (Butterly et al. 2016). Nodule numbers are also adjusted internally through autoregulation of nodulation (AON), i.e., a minimum number of nodules on the root system systemically represses further nodules until the C/N ratio is sufficiently increased through further plant growth (Ferguson et al. 2019).

Both AON and N regulation of nodulation are controlled by peptides of the CLE [CLAVATA3 (CLV3)/endosperm-surrounding region] family that are synthesised in the root after perception of either external N or following the perception of Nod factors (Mortier et al. 2010; Okamoto et al. 2009; Reid et al. 2011a, b). In *M. truncatula*, nitrate induces the expression of CLE35 which inhibits nodulation (Lebedeva et al. 2020; Mens et al. 2021; Moreau et al. 2021). *Medicago* CLE peptides CLE12 and 13 are induced in response to Nod factor perception and travel to the shoot, most likely in the xylem (Hastwell et al. 2015; Mortier et al. 2010). In *M. truncatula*, CLE12 requires tri-arabinylation, which is most likely catalysed by RDN (ROOT DETERMINED NODULATION) (Imin et al. 2018; Kassaw et al. 2017; Schnabel et al. 2011). The peptides are perceived by a leucine-rich repeat receptor-like kinase in the shoot (Okamoto et al. 2013), which is encoded by *SUNN* (*SUPER NUMERIC NODULES*) in *M. truncatula* (Schnabel et al. 2005). Similar to the regulation of meristem activity by CLV1, the *SUNN* receptor kinase interacts with other proteins that mediate CLE peptide signaling, including BAM2 (*BARELY ANY MERISTEM 2*; Thomas and Frugoli 2024), CLV2 and CORYNE (Crook et al. 2016).

In *M. truncatula*, an additional locus named *LSS* (*LIKE SUNN SUPERNODULATION*; Schnabel et al. 2010) has been identified that controls the expression of *SUNN*. Like *sun* mutants, the *lss* mutant supernodulates. Activation of the AON receptor-like kinase in the shoot leads to mobilisation of a miRNA signal (miR2111) to the root that inhibits nodulation (Tsikou et al. 2018; Zhang et al. 2021), partly through altering the expression of the Nod factor receptor NFP (Nod Factor Perception) and two *TML* (*TOO MUCH LOVE*) genes that negatively regulate nodulation

(Gautrat et al. 2019; Takahara et al. 2013). However, in *M. truncatula*, *TML* expression in the roots of the *sun4* mutant was found to be similar to WT, suggesting a somewhat different role of *TML* compared to *Lotus* (Schnabel et al. 2023).

AON mutants have multiple phenotypes, but the most characteristic is supernodulation, in which the initial formation of nodules does not result in inhibition of subsequent nodulation events. In addition, AON mutants of different legume species are typically tolerant to the inhibition of nodulation by nitrate (e.g., Day et al. 1986, 1989; Goh et al. 2019; Jeudy et al. 2010; Sagan et al. 1995; Schnabel et al. 2005; Wopereis et al. 2000) and this was how AON mutants were first identified in soybean (Carroll et al. 1985). The nitrate tolerance of AON mutants can be explained by the fact that perception of the nitrate-induced CLE peptides, such as CLE35 in *M. truncatula*, requires the action of the AON receptor-like kinase, *MtSUNN* or *LjHAR1* (Mens et al. 2021; Okamoto et al. 2009; Reid et al. 2011a). The *sun4*, *lss* and *rdn1* mutants still show some sensitivity of nodule number control to nitrate, but less than the WT (Goh et al. 2019; Jin et al. 2012; Schnabel et al. 2010). The control of nodule numbers through systemic and local signals is complex and involves at least three pathways in *M. truncatula* (Chaulagain and Frugoli 2021), including nitrate signalling through the COMPACT ROOT ARCHITECTURE 2 (*CRA2*) receptor that interacts with the negative regulation of nodulation by *SUNN* (e.g., Gautrat et al. 2019, 2020; Ivanovici et al. 2023; Laffont et al. 2019, 2020; Luo et al. 2021; Moreau et al. 2021).

Mutants defective in AON are also typically smaller than WT plants (e.g., Buzas and Gresshoff 2007; Jin et al. 2012; Novák 2010; Novák et al. 2011; Schnabel et al. 2005; Voisin et al. 2013; Wopereis et al. 2000). Interestingly, the reduced growth of AON phenotype is also observed when the plants are not nodulated, although the growth reduction is more pronounced in nodulated plants, likely because of the energy demand of excessive numbers of nodules (e.g., Goh et al. 2019; Voisin et al. 2013). This explanation is partially supported by studies that increased atmospheric CO₂ concentration in order to test whether increased C supply would rescue the growth defect of AON mutants. Elevated CO₂ supply caused a relatively greater increase in shoot biomass in the *rdn1-1* mutant of *M. truncatula* compared to WT plants

after six weeks (Qiao et al. 2021), and a relatively and totally greater increase in shoot biomass in the *rdn1-1* and *sun4* mutant of *M. truncatula* compared to WT after nine weeks (Zhang et al. 2023). This suggests at least a partial carbon limitation of the AON mutants. Expression of *SUNN* and *RDN1* in *M. truncatula* is restricted to cells in the vasculature surrounding the phloem, suggesting that the gene products have a role in phloem loading (Schnabel et al. 2011, 2012). This could potentially alter allocation of phloem-transported substrates that limit plant growth.

However, the growth and yield responses of different legume AON mutants depend on the species and mutant investigated, the growth conditions and the length of the experiment. For example, glasshouse experiments covering the first ~6 weeks of vegetative growth consistently show reduced shoot and root length and biomass of AON mutants, while nodule number and/or nodule biomass per plant and tissue nitrogen concentrations are typically significantly increased (e.g., Day et al. 1989; Goh et al. 2019; Hansen et al. 1992; Jin et al. 2012; Qiao et al. 2021; Voisin et al. 2013). Field trials with AON mutants of soybean at various sites show some yield reduction in the mutants compared to WT plants (e.g., Song et al. 1995), but these are not as extreme as biomass reductions seen in AON mutants grown over shorter times. Higher nitrogenase activity per plant (Qiao et al. 2021) and tissue N contents (Goh et al. 2019) have also been observed in inoculated *sun4*, *lss* and *rdn1* mutants compared to WT plants, while nitrogenase activity and fixed N per nodule were lower in AON mutants compared to wild type plants (Qiao et al. 2021). This begs the question whether increased provision of N through nitrogen fixation in the AON mutants would, over time, compensate for the increased cost of supernodulation, and to what extent this depends on the provision of external N sources to the plant, especially in view of increasing N fertiliser prices and the high environmental costs of N fertiliser use (Gu et al. 2023).

Here, we tested whether a long term, controlled experiment with defined amounts of N availability would elucidate the effect of long-term differences in nodule number and nitrogen fixation of Medicago AON mutants on N allocation and yield. We investigated the differences in seed and biomass yield, N content and % N derived from N fixation in the *M. truncatula* *sun4*, *rdn1-1* and *lss* mutants compared

to WT plants grown under very low compared to low but sufficient N availability in a controlled glasshouse experiment. We hypothesised that the AON mutants would show increases in yield and N content under N limitation because of their ability to fix more nitrogen over a full life cycle.

Materials and methods

Seed germination and plant growth conditions

We used the *M. truncatula* *rdn1-1* (Schnabel et al. 2011) and *lss* (Schnabel et al. 2010) mutants derived from cultivar Jemalong A17, as well as the *sun4* mutant, derived from the related Jemalong J5 cultivar (Schnabel et al. 2005). Jemalong A17 was used as the WT because no obvious phenotypic differences have been observed between cultivars Jemalong A17 and J5 (Schnabel et al. 2005). We note that the *rdn1-1* mutant contains a 103 kb deletion that includes *RDN1* as well as 17 other annotated genes, but no significant differences in nodule number or root length were observed between *rdn1-1* and other *rdn1* mutants (Schnabel et al. 2011).

Seeds were scarified using sandpaper and surface sterilized in 6.25% (w/v) sodium hypochlorite for 10 min. Seeds were then washed with sterile water five times and imbibed in sterile water for 3 h. Seeds were transferred onto Fåhræus medium (Fåhræus 1957), vernalized at 4 °C for 2 days and germinated at 25 °C in the dark overnight. Uniform seedlings were transferred into pots (170 mm (W) × 170 mm (L) × 240 mm (H), 7 L) containing fine perlite (Australian Perlite Pty Ltd., Australia). Fine perlite was pre-washed with tap water and pre-soaked with nutrient solution (nitrogen-free Fåhræus medium) overnight prior to seedling transfer. Small black gravel stones (~0.5 cm diameter) were placed onto each pot to prevent surface algae growth. Plants were grown in a glasshouse with natural lighting and temperature conditions set at 25 °C Day/20 °C night. Each pot was watered with 500 mL of Fåhræus medium twice a week for the first month and after that, three times a week.

Rhizobial growth condition and inoculation

Sinorhizobium meliloti strain WSM 1022 (Terpolilli et al. 2008) was cultured on Bergersen's modified

medium (BMM) agar (Rolfe et al. 1980). For plant inoculation, a liquid culture was diluted with BMM to an optical density (at 600 nm) of 0.1. Each pot was inoculated with 1 mL of bacterial culture 3 days after transferring germinated seedlings to the pots.

Glasshouse experimental design

The glasshouse experiment was designed with three factors: genotype (4), inoculation (2) and two levels of nitrate with five replicates for each, a total of 80 plants. The inoculated and uninoculated plants were grown in separate but adjacent rooms with the same orientation in the same glasshouse to avoid rhizobium contamination in the non-inoculated plants. The plants in each glasshouse room were divided in five blocks of eight plants. Each block contained each individual genotype-nitrate concentration. Each individual genotype-nitrate concentration was randomly placed in the block growth area, areas were matched between rooms. The plants were grown from autumn to spring (March to September) in Canberra, Australia, until the full growing cycle of each individual plant was completed, the plants stopped producing seeds and the leaves were dry. At six weeks after germination, we collected one fully developed leaf per plant to determine total N and $\delta^{15}\text{N}$ at an early stage. Nodule numbers were counted after 6 weeks in a separate set of plants to the main experiment because nodule numbers could not be reliably counted at the end of the main experiment due to the extensive root systems formed by that stage. The experimental measurements are summarised in Supplement Fig. 1.

Isotopic ^{15}N nitrate treatments

Potassium nitrate (KNO_3) was used as the source of nitrate added into Fåhræus medium. Two different concentrations were used, 0.5 and 2 mM. While the 2 mM concentration was still relatively low compared to other studies, we previously determined that a concentration of 2 mM KNO_3 was sufficient to significantly reduce nodule density in the same genotypes of *M. truncatula* compared to lower N concentrations, while shoot % N was almost as high as after growth with 10 mM nitrate (Goh et al. 2016). KNO_3 (Sigma Aldrich, St Louis, MO, USA) was enriched with ^{15}N at 1% atom excess. Potassium chloride was used to balance the ionic strength

of the nutrient solution (Schortemeyer et al. 1999). Plants were watered two or three times a week with liquid Fåhræus medium containing specific nitrate concentrations according to the experimental design. Nitrate treatments started two days after rhizobial inoculation. Each pot received the same amount of liquid Fåhræus medium.

Plant phenotyping

To avoid entanglement between plants and assist with plant handling, *M. truncatula* plants were loosely bound-up on a wooden stake using paper tape during the plant growth period. Ripe seed pods were collected from each plant for the duration of the experiment. After the plants were fully senesced and the shoot had dried out, each plant was harvested. Roots of plants were examined for nodules, and originally un-inoculated plants with nodules present on their root system (due to contaminating bacteria in the glasshouse) were excluded from the analysis. Shoots and seed pods were dried in an oven for a minimum of 3 days at 65 °C and weighed. Seeds were extracted from a known quantity of seed pods and weighed. The fraction of seeds per seed pod was used to calculate the total seed yield per plant. We used final plant biomass and seed yield as our proxies for fitness.

Total nitrogen content

Dried shoot material and seeds were ground using a TissueLyser LT (Qiagen, Australia). For the shoot measurements, the whole shoot (leaves and stems, but not pods or seeds) was ground up into a fine powder and a subsample of that was used. For the seed measurements, at least 100 seeds of each plant were ground up and mixed (unless the whole plant made less than 100 seeds, in which case all of the seeds were used), and a subsample was measured. For each plant sample, 2.0–2.5 mg of dry and finely ground material was weighed into a tin foil capsule and the weight recorded. Content of total N and C, as well as $\delta^{15}\text{N}$, was determined from the ground tissues using an Isoprime isotope-ratio mass spectrometer (Elementar). The references have been calibrated for total %N using White Wings flour as the reference. $\delta^{15}\text{N}$ was measured versus air.

Measurements of nitrogen derived from the atmosphere

The nitrogen derived from the atmosphere (Nd_{fa}) and the amount of N derived from nitrogen fixation (%N fixed) were calculated using the ¹⁵N dilution method as described previously (Goh et al. 2016).

Nd_{fa} was determined by this equation: Nd_{fa} = 1 - (atom excess of N₂-fixing plant / atom excess of reference plant). Atom excess was calculated by subtracting 0.3663%, which is the atmospheric natural abundance atom of %¹⁵N, from the atom% obtained from mass spectrometer output. The reference plant used in this calculation was the mean of the uninoculated plants grown at the same condition as the inoculated plants. %N fixed was calculated as %N fixed = Nd_{fa} × %N content of N₂-fixing plants. Total (shoot or seed) N fixed was calculated as %N fixed × dry biomass (shoot or seed).

Statistical analysis

We initially ran a three-way Analysis of Variance to assess effects of nitrogen, inoculation and genotype using R Studio (Version 1.3.1093; R Core Team 2023, Table 1). The effect of the nitrogen and inoculation treatments were so strong they obscured our

capacity to detect differences among genotypes, and so we subsequently tested for differences amongst the genotypes using one-way analysis of variance (ANOVA) within each of the four experimental nitrogen by inoculation treatments (low nitrate not inoculated, high nitrate not inoculated, low nitrate inoculated, high nitrate inoculated). Post hoc analysis using the Tukey's multiple comparison analysis method was used to test the differences among genotypes. These analyses were done using GraphPad Prism version 5.0 (GraphPad Software, Inc., USA), using the mean and standard error of mean (SEM). Linear regressions in Figs. 2 and 3 were calculated in R and visualised using the ggplot function (Wickham 2016).

Results

Shoot biomass and nitrogen content

To test if excessive nodule formation on *M. truncatula* roots result in a fitness penalty for the AON mutants, and whether this is dependent on N supply, we grew *M. truncatula* WT and AON mutants *rdn1-1*, *sun4* and *lss* under the following environmental conditions, 0.5 mM or 2 mM nitrate without rhizobia, and then also at 0.5 mM nitrate with rhizobia or 2 mM nitrate

Table 1 Three-way ANOVA results for all phenotypes and factors. Significant p-values are indicated with: $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***

Response	Nitrate	Genotype	Rhizobia	Nitrate*Genotype	Nitrate*Rhizobia	Genotype*Rhizobia	Nitrate*Genotype*Rhizobia
Shoot dry weight	<2.2e-16 ***	0.0005407 ***	<2.2e-16 ***	0.3011660	0.0203793 *	0.0310386 *	0.9364420
Total N Shoot	7.842-04 ***	0.1162558	<2.2e-16 ***	0.4940293	0.5336943	0.3834692	0.6246150
%N Shoot	7.092e-08 ***	0.0584410	<2.2e-16 ***	0.5919535	0.0004482 ***	0.1451092	0.2886648
Δ ¹⁵ N Shoot	<2.2e-16 ***	0.0606785	<2.2e-16 ***	0.0002655 ***	<2.2e-16 ***	0.0025916 **	0.2389547
Seed dry weight	<2.2e-16 ***	0.4450898	<2.2e-16 ***	0.0001009 ***	0.5416201	0.1895830	0.0770989
Total N Seed	1.473e-15 ***	0.05786	<2.2e-16 ***	8.439e-05 ***	0.03263 *	0.18229	0.05833
%N Seed	0.23066	0.02533 *	<2.2e-16 ***	0.77456	2.924e-06 ***	0.15999	0.76786
Δ ¹⁵ N Seed	<2.2e-16 ***	6.539e-10 ***	<2.2e-16 ***	0.03364 *	1.177e-13 ***	0.33307	0.62951
Seed pod weight	<2.2e-16 ***	0.2350895	<2.2e-16 ***	0.0007899 ***	0.1767002	0.2058650	0.1659738
Seed fraction	0.5506	0.5123	3.851e-15 ***	0.4413	0.3827	0.9525	0.8725
Total N fixed Shoot	0.1210	0.1892		0.2750			
%N fixed Shoot	1.852e-08 ***	0.02428 *		0.24158			
%NDFA Shoot	<2.2e-16 ***	1.261e-07 ***		0.000714 ***			
Total N fixed Seed	5.046e-07 ***	0.0001029 ***		0.0044970 **			
%N fixed Seed	<2.2e-16 ***	0.000161 ***		0.893986			
%NDFA Seed	<2.2e-16 ***	0.01e-08 ***		0.09704			
%N Leaf	2.037e-12 ***	0.1133	<2.2e-16 ***	0.3495			
Δ ¹⁵ N Leaf	<2e-16 ***	0.1208	<2e-16 ***	0.3782			

with rhizobia. To ascertain the nodulation phenotypes of the mutants, nodules were counted after 6 weeks in a separate set of plants (Table 2), showing significantly higher numbers in the *sun4* and *lss* mutants compared to the WT at both nitrate concentrations. The *rdn1-1* mutant showed intermediate nodule numbers, that were significantly higher than WT nodules only at 2 mM nitrate.

After being grown to maturity, shoot dry weight (leaves and stems without pods) increased significantly in response to both increasing nitrate and inoculation with rhizobia in both the WT and AON mutants (Table 1). Shoot dry weight for plants grown without rhizobia in the 0.5 mM nitrate treatment was almost negligible and increased ~20–25 fold with inoculation of rhizobia, while rhizobia increased shoot biomass only ~2–threefold with the 2 mM nitrate treatment (Table 2).

With the 0.5 mM nitrate treatment without rhizobia, shoot dry weight was significantly ($p < 0.05$, Table 2) reduced in the *sun4* and *lss* mutants compared to the WT plants, whereas the shoot dry weight in the *rdn* mutant did not change significantly compared to WT plants under the same condition. In the other growth conditions, no significant differences between WT and mutants could be detected. Shoot biomass was generally the lowest in the *lss* mutant, with a significant reduction in biomass compared to the *sun4* mutant under 2 mM nitrate without rhizobia, and a significant reduction under 0.5 mM nitrate compared to the *rdn1-1* mutant in the presence of rhizobia, but no significant differences to WT plants.

The nitrogen fraction (%N) of the shoots was determined in all four treatments, to investigate if the supernodulation phenotype in the mutants changed the N content in the shoot. Inoculation with rhizobia significantly ($p < 0.05$, Table 1) increased the %N in both the WT and AON mutants, at both nitrate levels. However, the *sun4* and *lss* mutants showed a significantly higher %N in the shoot compared to the WT in the 2 mM nitrate plus rhizobia treatment ($p < 0.05$, Table 2).

The percentage of nitrogen derived from the atmosphere (%Ndfa) was significantly altered by nitrate and inoculation (Table 1) and showed a significant increase in all three supernodulating mutants compared to the WT in the 0.5 mM nitrate treatment (Table 2), whereas in the 2 mM nitrate treatment only the *sun4* and *lss* mutants showed a significantly

higher %Ndfa in the shoots compared to the WT. While %Ndfa reached >90% under 0.5 mM nitrate, the values reached only ~65–70% under 2 mM nitrate. Although a significant higher %Ndfa could be observed in both nitrate treatments in *sun4* and *lss* mutants compared to the WT, this did not result in higher total shoot fixed nitrogen content at either nitrate level (Table 2). The total amount of N fixed in the shoot was not significantly affected by nitrate or genotype (Tables 1 and 2).

Seed biomass and nitrogen content

Seed pods of the plants were collected throughout the full growth cycle of the plants. As with the shoot growth, the uninoculated plants grown in the 0.5 mM nitrate conditions struggled throughout the growth cycle; only one WT and one *rdn1-1* mutant produced any seed pods, therefore the uninoculated 0.5 mM nitrate condition was omitted from the seed yield statistical analysis.

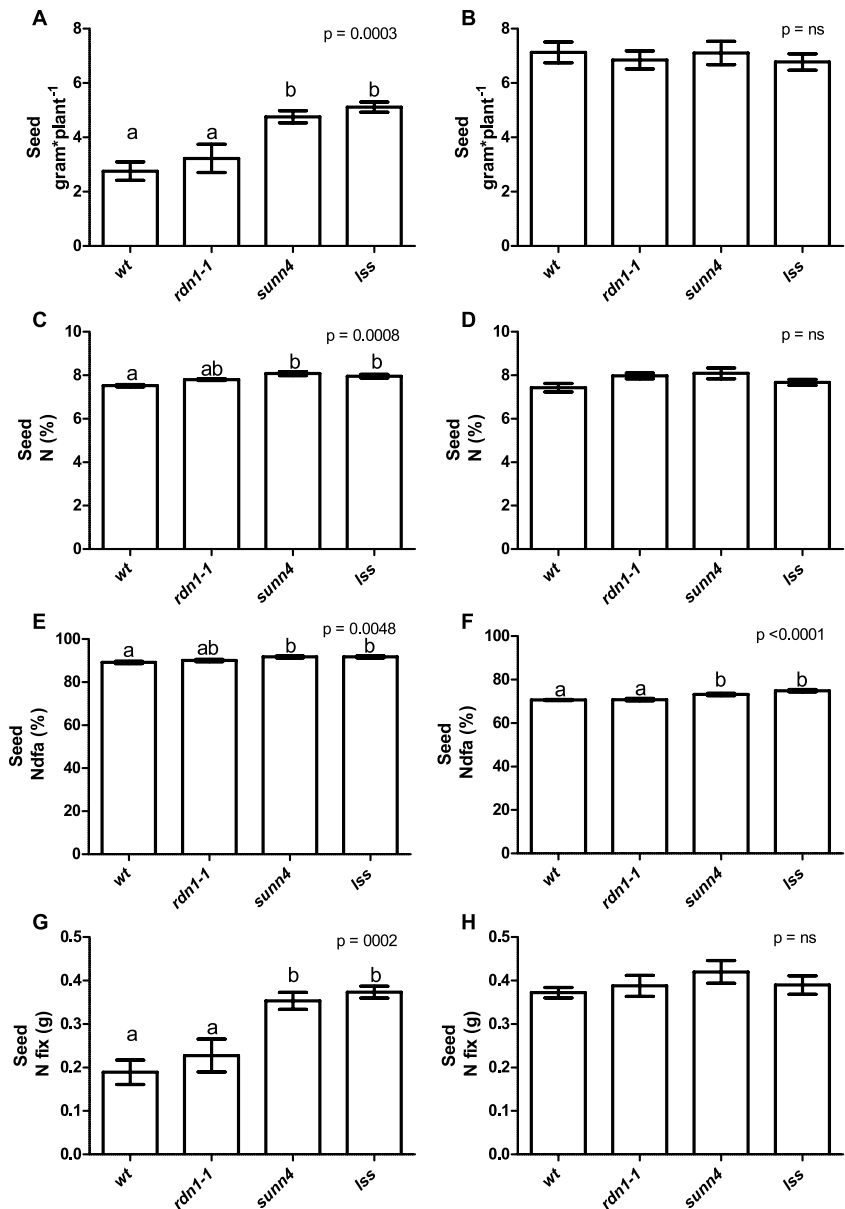
Inoculation significantly increased the seed pod yield in both nitrate conditions in the WT and AON mutants (Supplement Table 1). All three AON mutants grown in low nitrate conditions in the presence of rhizobia showed a significant increase in seed pod yield compared to the WT plants. The increase in seed pod yield was especially apparent in the *sun4* and *lss* mutant with an increase of an average 1.6–1.7 fold, respectively. No significant changes in seed pod yield could be detected between genotypes at the 2 mM nitrate concentration.

Seeds were extracted from the seed pods to investigate if there was a difference in seed yield between the wildtype and mutants in all growing conditions. No significant differences in the fraction of seeds formed per seed pod could be detected between wildtype and mutants in any of the tested conditions (Supplement Table 1). Inoculation with rhizobia significantly increased seed yield, with a >20-fold increase at 0.5 mM nitrate and a 3–fourfold increase at 2 mM nitrate. Seed yield also increased significantly with the 2 mM nitrate application (Fig. 1). In addition, total seed yield per plant in the *sun4* and *lss* mutants was significantly higher compared to the WT in the inoculated low nitrate condition. No significant genotypic differences were found at the higher nitrate treatment.

Table 2 Plant phenotypes under different nitrate and inoculation treatments. The table shows means and standard errors. Shoot phenotypes were measured at maturity after 6 months. Leaf and nodule phenotypes were measured after 6 weeks as indicated. Data were analysed by one-way ANOVA with Tukey post hoc test using a p value threshold of 0.05 within each of the four treatment inoculation conditions. Pairwise differences are indicated by letters. Any means not sharing the same letter were significantly different at $p < 0.05$. No pairwise comparisons were made when the ANOVA was not significant (-). Sample sizes are indicated at the top of the table. All sample sizes were initially five, but contaminated uninoculated plants were omitted from the analysis

	0.5 mM nitrate, uninoculated					2 mM nitrate, uninoculated					0.5 mM nitrate, inoculated					2 mM nitrate, inoculated				
	Wt (n=3)	<i>rdn1-1</i> (n=4)	<i>sun4</i> (n=4)	<i>lss</i> (n=4)	Wt (n=5)	<i>rdn1-1</i> (n=4)	<i>sun4</i> (n=4)	<i>lss</i> (n=4)	Wt (n=5)	<i>rdn1-1</i> (n=5)	<i>sun4</i> (n=5)	<i>lss</i> (n=5)	Wt (n=5)	<i>rdn1-1</i> (n=5)	<i>sun4</i> (n=5)	<i>lss</i> (n=5)				
Shoot dry-weight (g)	Mean	1.20	1.09	0.65	0.72	12.74	11.16	13.89	10.93	27.08	30.39	25.85	25.39	36.25	34.87	30.89	37.54			
	SE	0.09	0.05	0.08	0.15	0.81	0.31	0.24	0.97	1.13	0.96	0.66	1.70	2.31	2.35	1.41	1.75			
Shoot N (%)	Mean	a	a	b	b	ab	ab	a	b	ab	a	ab	b	-	-	-	-			
	SE	1.47	1.52	1.52	1.57	0.93	0.86	0.93	0.98	2.16	2.13	2.35	2.23	1.63	2.26	2.31	2.04			
Shoot Ndfa (%)	SE	0.06	0.07	0.14	0.09	0.04	0.02	0.05	0.07	0.13	0.02	0.13	0.22	0.10	0.09	0.15	0.14			
		-	-	-	-	-	-	-	-	-	-	-	-	a	a	b	b			
Shoot Ndfa (%)	Mean	NA	NA	NA	NA	NA	NA	NA	NA	90.42	92.44	92.20	91.97	64.36	70.65	71.11	67.08			
	SE	NA	NA	NA	NA	NA	NA	NA	NA	0.35	0.24	0.35	0.35	1.23	0.43	0.81	0.60			
Shoot N fix (g)		-	-	-	-	-	-	-	-	a	b	b	b	a	s	b	b			
	Mean	NA	NA	NA	NA	NA	NA	NA	NA	0.52	0.63	0.51	0.55	0.41	0.52	0.56	0.51			
Shoot N fix (g)	SE	NA	NA	NA	NA	NA	NA	NA	NA	0.02	0.07	0.01	0.04	0.05	0.05	0.05	0.05			
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Leaf N (%)	Mean	NA	NA	NA	NA	NA	NA	NA	NA	4.20	4.63	4.82	4.92	36.25	34.87	30.89	37.54			
	SE	NA	NA	NA	NA	NA	NA	NA	NA	0.04	0.34	0.14	0.17	2.31	2.35	1.41	1.75			
Leaf N (%)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	Mean	NA	NA	NA	NA	NA	NA	NA	NA	126.40	89.47	123.30	89.43	1.63	2.26	2.31	2.04			
Leaf $\delta^{15}N$ (‰), 6 weeks	SE	NA	NA	NA	NA	NA	NA	NA	NA	18.29	14.48	23.06	19.68	0.10	0.09	0.15	0.14			
		-	-	-	-	-	-	-	-	-	-	-	-	a	a	b	b			
Nodule No., 6 weeks	Mean	NA	NA	NA	NA	NA	NA	NA	NA	14.67	42.17	177.60	135.80	64.36	70.65	71.11	67.08			
	SE	NA	NA	NA	NA	NA	NA	NA	NA	1.09	6.07	19.87	23.09	1.23	0.43	0.81	0.60			
		-	-	-	-	-	-	-	-	a	a	c	c	a	b	c	c			

Fig. 1 Seed phenotypes of WT and AON mutants in response to different N treatments. **A, C, E, G:** 0.5 mM N with inoculation at maturity. **B, D, F, H:** 2 mM N with inoculation. Figure shows means and standard error (SEM). Data were analysed by one-way ANOVA with Tukey post hoc test using a p value threshold of 0.05. The p value for each ANOVA is shown in the respective panels. Pairwise differences are indicated by letters. Any means not sharing the same letter were significantly different at $p < 0.05$. No pairwise comparisons were made when the ANOVA was not significant (ns). $n = 5$



There was a statistically significant (Table 1) increase in seed %N after rhizobium inoculation, increasing from ~4.5 to >7.5% at low N and from ~5.7 to >7% at higher N, whereas nitrate treatment did not alter seed %N (Table 1, Fig. 1). Within the low nitrate plus rhizobia treatment, the seeds of *sunn4* and *lss* mutants had slightly but significantly higher %N compared to seeds of the WT plants. The higher %N in seed of the *sunn4* and *lss* mutant plants in combination with the higher seed yield in the *sunn4* and *lss* mutants resulted in a significantly

higher total nitrogen yield associated with seeds in those mutants compared to the wildtype in the low nitrate plus rhizobia conditions. Seed %N and seed total N content were not affected by genotype in the higher nitrate treatments (Fig. 1; Supplement Table 1).

Calculations of the percentage seed nitrogen derived from the atmosphere (%NDFA) confirmed a significant increase in the *sunn4* and *lss* mutant seeds grown at both nitrate treatments (Fig. 1; Supplement Table 1). Similar to the values found for

the shoot, %Nd_{fa} of seeds reached ~90% at 0.5 mM nitrate and ~70% at 2 mM nitrate. Calculations using the %Nd_{fa} in seeds and the total seed N similarly showed that a significant larger part of the total N in the seeds was derived from the symbiotic nitrogen fixation in the *sun4* and *lss* mutants when grown at low nitrate and in the *sun4* mutant when grown at higher nitrate compared to the WT grown under the same conditions.

The total fixed nitrogen deposited in seeds was also significantly increased in *sun4* and *lss* mutants compared to WT plants at 0.5 mM nitrate, with an almost twofold difference. No significant increase in total fixed N in the seed of *rdn1-1* plants was found (Fig. 1). Total fixed nitrogen in the seeds at 2 mM nitrate did not differ between genotypes. In contrast to fixed N in the shoot, the fixed N in the seed continued to increase at 2 mM compared to 0.5 mM nitrate in WT and *rdn1-1* plants, but not in the *sun4* and *lss* mutants (Fig. 1; Supplement Table 1).

The increase in %NDFA in seeds was associated with an increase in yield across all four genotypes ($R^2=0.75$) in the 0.5 mM but not the 2 mM nitrate ($R^2=0.0008$) treatment (Fig. 2). This relationship was largely driven by the high %NDFA and high yield in the *sun4* and *lss* mutants at the low nitrate treatment. These results suggest that the AON mutants *sun4*

and *lss*, and to a lesser extent *rdn1-1*, which showed an intermediate phenotype, can convert the higher amounts of fixed nitrogen into higher seed yield.

The percentage of N in seeds could be predicted by the percentage of N and the $\delta^{15}\text{N}$ (indicating nitrogen fixation capacity) in the leaves of the same plants at six weeks post germination (Fig. 3). In the non-fixing (uninoculated low N) controls, %NDFA could not be calculated at 6 weeks because not enough material was available as the plants were very small.

While leaf N% was already increased in inoculated *sun4* and *lss* plants compared to WT at six weeks, this difference was only statistically significant at 2 mM nitrate (Table 2). $\delta^{15}\text{N}$ was also already decreased in the mutants at that stage, indicating active nitrogen fixation (Table 2), but again, this was only significant at 2 mM nitrate, possibly because the additional nitrate accelerated plant growth at the early stages after germination.

Discussion

Autoregulation of nodulation is a mechanism that controls nodule numbers in response to nitrogen demand and carbon supply. Previous studies examining the growth of AON mutants in different legume

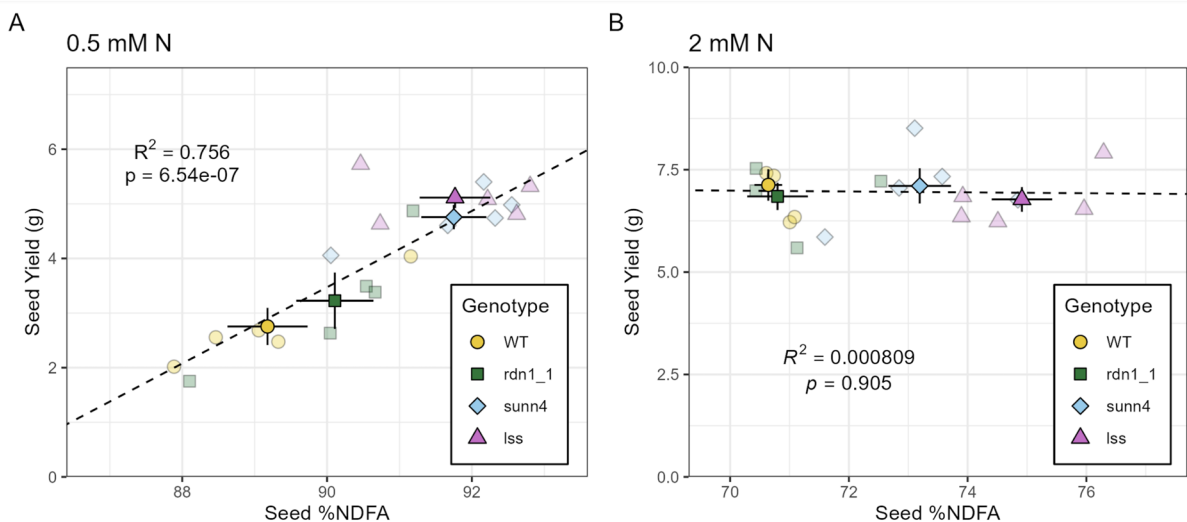


Fig. 2 Correlation between %NDFA in seeds and final seed yield in nodulated WT and AON mutants in different N treatments at maturity. **A**) Plants grown in 0.5 mM nitrate; **B**)

Plants grown in 2 mM nitrate. Each solid data point shows the average and SEM of five replicates (replicates shown in faded colours). Regressions were calculated based on all replicates

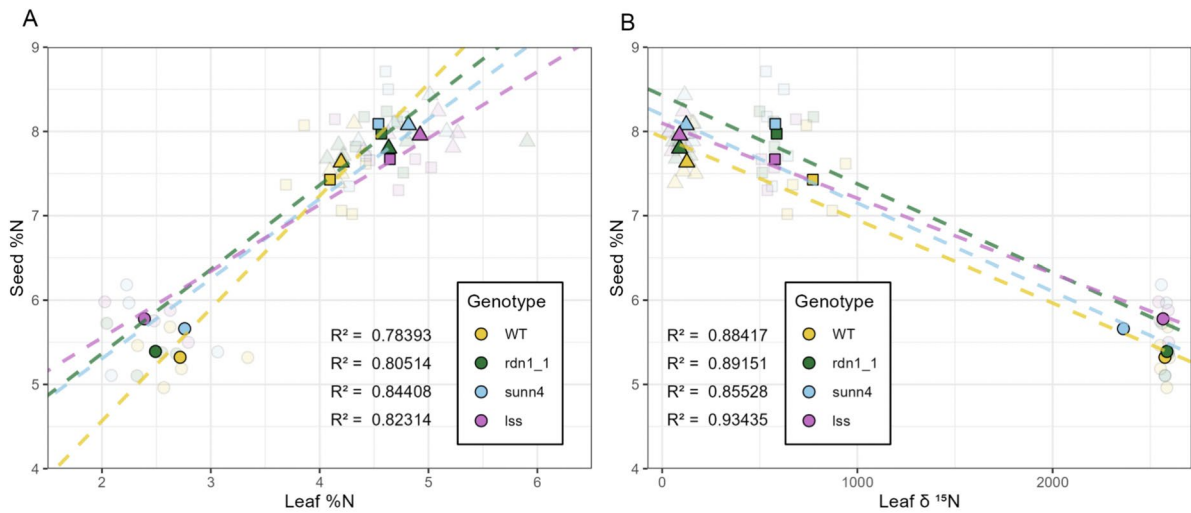


Fig. 3 Correlation between nitrogen status in leaves at 6 weeks after germination and the seed N concentration at the end of the plants' growth period. **a** shows %N, **b** shows $\delta^{15}N$ correlated with seed %N. Correlations are shown separately for each genotype using all replicates. Each solid data point is the

average of five replicates, with faded symbols showing individual replicates. All regressions were statistically significant for all genotypes with $p < 0.0001$. Uninoculated plants at 0.5 mM nitrate were not included because leaves were too small at six weeks

species have consistently found them to be smaller than WT plants, although the mechanisms behind this are not understood. Smaller size and biomass of AON mutants has generally been observed under nodulated and non-nodulated conditions, but typically the growth defect is more extreme under nodulated conditions, suggested to be a result of the high carbon demand of nodules, both for their establishment and for the ATP cost of nitrogen fixation (e.g., Schnabel et al. 2005; Goh et al. 2019; Hansen et al. 1989; Voisin et al. 2003). In general, studies have found no significant limitation of nitrogen fixation by photosynthate supply, but rather by nodule C metabolism (Vance and Heichel 1991). However, most of the previous studies measured plant size or biomass at early growth stages. In contrast, our study found that when grown to maturity over six months in a glasshouse, none of the AON mutants showed a reduction in biomass or yield when nodulated. Surprisingly, under lower N (0.5 mM nitrate), the AON mutants *lss* and *sunn4* had higher seed yield and higher seed N concentrations than WT plants. Yield and N concentrations in the AON mutants at the higher N concentration (2 mM nitrate) was similar to WT, suggesting that these AON mutants have a fitness advantage when N supply is so low that the additional numbers

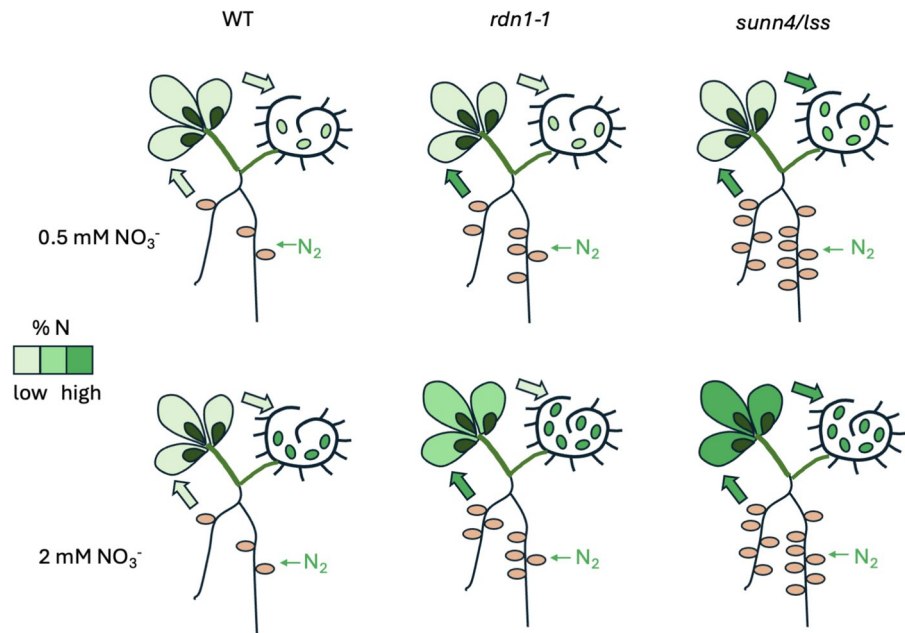
of nodules and the increased nitrogen fixation provide an advantage and furthermore, that the cost of nodulation does not result in reduction in growth or seed production at these N levels. The results are summarised in a model in Fig. 4. While the mechanism of the increased seed yield and N concentration in the AON mutants is not known, it could involve changes in N partitioning between nodules, roots, leaves and seeds, investment of N in different photosynthetic proteins, or hormonal feedback regulation between shoot and root.

Our results bring up three questions that are examined further below: 1) Why was no shoot biomass penalty observed in the AON mutants in this study? 2) What caused the increased seed yield and seed N concentration in the *sunn4* and *lss* mutants? 3) Why were different seed yield phenotypes observed in the *sunn4* and *lss* mutants compared to the *rdn1-1* mutant?

1) Why was no shoot biomass penalty observed in the AON mutants in this study?

Plant size and biomass can be limited by both C and N. While nodulation and nitrogen fixation are C sinks, they provide additional nitrogen that can be invested into photosynthesis for additional

Fig. 4 Model showing the relative differences in shoot and seed biomass and % N in the different genotypes at the two applied N levels in inoculated plants. Shoot/seed biomass are indicated by the size of the leaf/number of seeds inside the pod. %N in the tissue is indicated by the intensity of the green colour. The arrows indicate the presumptive relative amounts of fixed N delivered to leaves and subsequently to seeds



carbon gain. Carbon fixation can be limited by Rubisco, and it has been estimated that around 20% of leaf N (40% of the leaf soluble protein) is invested into Rubisco (Evans and Clark 2019). However, in nodulated plants, there is a time lag between nitrogen fixation and additional N investment into carbon fixation and subsequent biomass allocation into leaves and then seeds. Therefore, to account for the cumulative effect of nitrogen fixation, we grew plants to maturity in our study. While many previous studies found reduced size and lower shoot and root biomass of AON mutants at the seedling stage or before seed set, we found that by maturity, AON mutants generally had no biomass penalty compared to the WT. One exception was that in non-nodulated plants, there was a biomass reduction in the *sunn4* and *lss* mutants in the low N (0.5 mM KNO_3) treatment. This suggests that the biomass reduction of AON mutants can be overcome by additional N provision, either through nitrate addition or through nitrogen fixation.

This advantage is likely to be cumulative over several months, and this might explain why at earlier stages of growth, e.g., six weeks, this advantage is not realised. In a previous, similarly designed experiment, at six weeks, the *M. truncatula sunn4*, *rdn1-1* and *lss* mutants show

reduced biomass in pot experiments, but this depended on the nitrogen supply and the nodulation status (Goh et al. 2019). At 0 or 0.1 mM of nitrate supply, the biomass of AON mutants was only slightly reduced compared WT in uninoculated plants, while this difference was larger in nodulated plants. However, at 2 or 10 mM nitrate supply, AON mutants showed a much larger biomass reduction over the WT. In addition, in that study, *lss* and *sunn4* mutants also showed much higher nodule density than WT or *rdn1-1* plants, at both low and high N levels (Goh et al. 2019). We found similarly high nodule numbers in the *lss* and *sunn4* mutants in this study at six weeks, and intermediate nodule numbers in the *rdn1-1* mutant. In the future, it would be interesting to test whether a difference in the onset of nitrogen fixation in the AON mutants could explain the differences at early plant growth stages.

In our study, all inoculated AON mutants showed higher total amounts of % N derived from nitrogen fixation in shoots under the low N treatment compared to the WT, and this was also the case in the *sunn4* and *lss* mutants at the higher N treatment (Table 2). Shoot N concentrations were similar in mutants and WT plants, except for the inoculated plants at the 2 mM nitrate treatment, in which the *sunn4* and *lss* mutants showed a

higher leaf N concentration than WT and *rdn1-1* had an intermediate phenotype. In a prior study at six weeks of growth, *M. truncatula* AON mutants have higher shoot N concentrations than WT at low N concentrations, while this difference disappears at 2 or 10 mM nitrate (Goh et al. 2019). Likewise, that study shows that root N concentrations of AON mutants are significantly higher than in WT plants at low and higher N supply at six weeks of growth (Goh et al. 2019). In the present study we also saw higher leaf %N and lower $\delta^{15}\text{N}$ at six weeks in inoculated AON mutants, although this was only significant at 2 mM nitrate. We suggest that this additional fixed N in the AON mutants might not be translated into higher biomass at early growth stages, but over time, it can compensate for the biomass penalty in the AON mutants. Thus, N-limited WT plants are likely limited in carbon fixation over time, while AON mutants might sustain carbon fixation due to higher nitrogen fixation, with subsequent higher N availability for Rubisco and other parts of the photosynthetic machinery. This is supported by measurements of active Rubisco content of leaves, which is significantly increased in inoculated plants of the *sun4* mutant compared to WT plants at ambient (and elevated) CO_2 concentrations (Zhang et al. 2023). In the soybean AON mutant *nts382*, extending the vegetative period through grafting with a tropical line with prolonged juvenile growth increased seed yield compared to the ungrafted line, confirming that prolonged assimilation can overcome the initial biomass penalty in AON mutants (Sebunruang and Lantican 1998). Overall, the nitrogen fixation measurements in our current study show that supernodulation can be an advantage at very low N and in the presence of rhizobia over long time periods, most likely because of the additional nitrogen fixation they realise over time that likely has a net benefit in photosynthesis and subsequent yield. AON likely exists though because in natural environments, available soil N concentrations are typically higher than those used here. However, AON depends not only on total available N but the C:N ratio. When legumes evolved around 80 million years ago, atmospheric CO_2 concentrations were much higher than at present, and an AON mechanism may not have existed at

that time. As CO_2 concentrations fell over time, AON would have become more important (Mathesius 2022).

- 2) What caused the increased seed yield in the *sun4* and *lss* mutants?

It was interesting to observe that at the 0.5 mM N treatment, the *sun4* and *lss* mutants showed an increased seed yield as well as a higher percentage of N derived from nitrogen fixation invested in the seeds, but not shoots. This suggests that additional nitrogen fixation in these mutants can be specifically invested in the seeds (Fig. 2). That this was not observed at 2 mM nitrate indicates that there was sufficient external N available to provide N for carbon fixation and C allocation to seeds. A glasshouse study with a root-determined hypernodulating mutant of *Lotus japonicus*, *Ljrdh1*, also showed a higher pod + seed yield of the mutant compared to the WT plants, and this was also accompanied by higher nitrogen fixation in the mutant. However, this was not measured at a whole plant level, and seed N was not determined (Yokota et al. 2009). In our work, the correlations between seed %N and seed yield showed a general positive trend, but also indicated that in inoculated plants, addition of 2 mM compared to 0.5 mM nitrate was able to increase yield without increasing seed %N, likely because of increased shoot biomass at 2 mM nitrate. The fact that seed %N was predicted by leaf %N and leaf $\delta^{15}\text{N}$ at six weeks after germination shows that early measurements of nitrogen fixation could be used for breeding legumes with higher seed nitrogen content.

At earlier growth stages, nodule numbers and nitrogen fixation capacity were found to be a good predictor of seed yield in bean (Fernández-Luqueño et al. 2012), and the length of the nitrogen fixation period was also positively correlated with yield (Fernández-Luqueño et al. 2008). Similarly, crosses of soybean cultivars with AON mutants resulted in the generation of lines with higher nodule numbers, increased vegetative growth period and significantly higher yields than the WT parent, especially under low N in the field (Takahashi et al. 2003). However, other studies in WT accessions of soybean found that increasing biological nitrogen fixation can result in a yield penalty or a reduction in harvest

index, i.e., reduced allocation to seeds compared to shoot (Tamagno et al. 2018). In chickpea, no clear association between nitrogen fixation and yield was found in a number of common accessions grown in the field (Sadras et al. 2016). However, a meta-analysis of different grain legumes found a generally positive correlation between nitrogen fixation and yield (Salon et al. 2001).

Field studies of soybean AON mutants showed a similar or reduced seed yield of AON mutants compared to WT plants at maturity (Maloney and Oplinger 1997; Pracht et al. 1994; Song et al. 1995). Soil concentrations of N were not measured in those studies, but were likely higher than the 2 mM solution provided here. Thus, the benefit of additional nitrogen fixation potential of the AON mutants was likely not realised at the higher N concentrations. This could be because the energetic cost of nitrogen fixation is higher than the cost of taking up nitrate or other N sources from the soil. For example, field experiments with white lupin suggested an estimated carbon loss of around 25% in nitrogen-fixing compared to nitrate fed plants, likely through higher respiration in the nitrogen fixing plants, while biomass was only slightly reduced and tissue N was increased in nitrogen-fixing compared to nitrate-fed plants (Pate et al. 1979).

A recent study in soybean found that intermediate nodule numbers in a *rhizobially induced cle1a/2a (ric1a/2a)* mutant, i.e., defective in the peptides that trigger AON, resulted in higher N and C fixation, and subsequent seed yield and protein content in (N-fertilised) field-grown plants, compared to WT and the *nark* mutant (Zhong et al. 2024). Interestingly, the intermediate nodule numbers we found in the *Medicago rdn1-1* mutant compared to *sun4/lss* mutants did not result in higher yield or N content, pointing to gene or species-specific differences between *Medicago* and soybean.

Presumably, AON mutants of different legume species differ in their nitrogen fixation ability, sensitivity to external N, as well as C and N allocation to leaves and seeds. For example, legumes species differ in the mechanisms used for compensating for increased carbon demand from nodules, e.g., through increases in photosynthesis

or by restricting root growth, and they also differ in the ability to fix nitrogen after the vegetative period (Schulze et al. 1994). Another reason for the observed differences between *Medicago* and soybean AON mutants might be the severity of the supernodulation phenotypes of individual mutants. In addition, cultivated soybean shoot development is determinate (Tian et al. 2010) and seeds mature in a more synchronised way than in *Medicago truncatula*, in which seed mature continuously over several weeks on indeterminately developing shoots (Bucciarelli et al. 2006). Thus, it is also possible that the differences in phenology between soybean and *Medicago* contributed to the different findings on yield in the soybean compared to *Medicago* AON mutants.

Further differences could arise through comparison of glasshouse with field studies. In the field, yield can be affected by many variables that could limit nitrogen fixation, in particular water availability and P nutrition, which were not limiting in our experiments, but could limit nitrogen fixation in the field (e.g., Rogers et al. 2006; Sulieman et al. 2013). Being pot-grown, plants in this study may have also been limited in root growth compared to field studied (Passioura 2006). In addition, higher leaf N concentrations could make plants more susceptible to herbivores in the field (Simonsen and Stinchcombe 2014). One or more of these factors might explain why prior field trials indicate that seed yield of soybean AON mutants is not significantly different to, or is lower than WT plants (Maloney and Oplinger 1997; Pracht et al. 1994; Song et al. 1995; Wu and Harper 1991). However, it is also possible that the advantage is only expressed at low to moderate N levels and as such is difficult to detect. From an evolutionary perspective one would posit that if such mutants had an overall fitness advantage, then nodulation levels of WT lines would generally be under positive selection. Nonetheless, even if they are not advantageous on average, they could have potential application in specific resource conditions.

- 3) Why were different seed yield phenotypes observed in the *sun4* and *lss* mutants compared to the *rdn1-1* mutant?

One interesting finding in our study was the different phenotypes of the *sun4* and *lss* com-

pared to the *rdn1-1* mutant, with the latter not displaying the increased seed yield, seed N content or %Ndfa in seeds, although seed pod yield was also significantly increased at low nitrate after inoculation (Supplementary Table 1). The *rdn1-1* mutant also showed a less severe supernodulation phenotype compared to *sun4* and *lss* mutants after six weeks of growth in the glasshouse (similar to Goh et al. 2019). Qiao et al. (2021) show that at ambient CO₂, the *rdn1-1* mutant has slightly higher fixed N per plant compared to *sun4* plants, but both mutants have similar nitrogenase activity per plant and per nodule at 6 weeks of age. In this study, shoot %Ndfa was similarly increased in *rdn1-1* compared to the *sun4* and *lss* mutants. Overall, these results indicate that the *rdn1-1* mutant is not defective in nitrogen fixation or leaf N accumulation, but that partitioning of the shoot N and C to seeds may be affected, at least under the conditions used here.

These findings might be a result of the *rdn1-1* mutation itself or be indirectly affected by other genes or loci that are deleted in this mutant (Schnabel et al. 2011), and thus other *rdn* mutants will have to be analysed to test if there is a specific role of RDN in seed C and N allocation. A transcriptome analysis of gene expression changes in shoots and roots of *sun4* and the *rdn1-2* mutant with WT plants showed largely overlapping gene expression changes between *sun4* and *rdn1-2* tissues, but some unique gene expression changes in each mutant (Schnabel et al. 2023).

Conclusions

Overall, our results show that nodulated AON mutants of *M. truncatula* do not have a growth penalty when grown to maturity under very low N supply, at least under controlled conditions. Rather, it appears that the investment of the additional N derived from nitrogen fixation into carbon fixation over time subsequently translated into higher seed yield with higher seed N concentrations. The influence of inoculation at very low N was substantial, with biomass and yield increases >20 fold. With the increasing price of fertilisers and the enormous cost of nitrogen pollution, agricultural practices that minimise N inputs are becoming more important (Gu et al. 2023). Our

study suggests that the *M. truncatula* AON mutants *sun4* and *lss* have the potential to be utilised under very low nitrogen inputs. Whether this advantage can be realised in the field will have to be tested in the future.

Future long-term studies could focus on the mechanisms by which AON mutants of a range of species control C and N allocation over time from seed germination to maturity across a continuous gradient of N availability and in concert with other resource limitations. Such experiments may be key to explaining the significantly smaller biomass observed at early growth stages, but potential for higher yields at maturity, at least at low to moderate N levels, and may consequently have potential application for reduced N production systems.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to declare.

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