Impacts of domestication on the rhizobial mutualism of five legumes across a gradient of nitrogen-fertilisation

Niall Millar[1], Jonah Piovia-Scott[1], Stephanie S. Porter[1*]

[1]School of Biological Sciences, Washington State University, Vancouver WA, USA

*Corresponding author

Contact information

Dr Stephanie Porter: stephanie.porter@wsu.edu

Washington State University Vancouver

14204 NE Salmon Creek Ave

Vancouver, WA 98686

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Abstract

Aims

Domestication and crop breeding has improved plants for human use, but may have unintended consequences for traits that are not selected upon. Disruption in the mycorrhizal mutualism has been observed in crops; possibly due to breeding in fertilised soil. Little is known about whether the legume-rhizobia mutualism can be disrupted by domestication and crop evolution, despite the importance of symbiotic nitrogen fixation. We aimed to identify differences in mutualistic outcomes between five legume cultivars and their wild progenitors in terms of their reactions across varying nitrogen levels.

Methods

With five greenhouse experiments, we characterised symbiosis traits in chickpea, lentil, pea, peanut, and soybean across a gradient of N fertilisation to characterise whether symbiosis traits differ context specifically between wild and domesticated lines.

Results

At lower levels of N addition, wild soybean benefited more from rhizobia than domesticated soybean. Chickpea cultivars abandoned symbiosis at lower N than wild chickpea accessions. Chickpea cultivars reduced per-nodule colony forming units (CFU) in response to N addition more than wild chickpeas, but lentil cultivars reduced CFU

less than lentil accessions. The lentil, pea, and peanut cultivars did not differ from their wild relatives in rhizobial benefit, nor in nodulation response to N addition.

Conclusions

Differences in the regulation of the root nodule symbiosis are evident between domesticated and wild chickpea and soybean, but not lentil, pea, or peanut. This indicates that mutualism disruption - a decrease in the magnitude of the mutually symbiotic interaction - is a possible, but not necessary, consequence of domestication.

Key words: Domestication, evolution, legume, mutualism, nitrogen, rhizobia

Author contributions

Niall Millar (NM) conducted the experiment and wrote the manuscript. Jonah Piovia-Scott assisted with the data analysis. Stephanie Porter (SSP) supervised the practical work and edited the manuscript.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Data availability

The data presented in this manuscript will be archived with Dryad upon acceptance.

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Key words: Domestication, evolution, legume, mutualism, nitrogen, wild relatives, rhizobia

Introduction

Over the course of domestication and crop breeding, artificial selection for traits that ease cultivation has facilitated the expansion of both the human population and those of domesticated plants and animals (Diamond, 2002; Smith, 2016; Zeder, 2017). Crop plants have evolved phenotypes that improve their utility for humans, such as non-shattering seeds, uniform and quick germination, and larger grains or fruits (Abbo *et al.*, 2014). There can, however, be unintended side-effects of domestication and breeding (Kiers *et al.*, 2003; Renaut & Rieseberg, 2015; Kono *et al.*, 2016; Moyers *et al.*, 2018; Sawers *et al.*, 2018). These side-effects include reduced genetic diversity (Smýkal *et al.*, 2018), and accumulation of deleterious mutations, which can reduce biomass, plant height, and starch content (Makino *et al.*, 2018; Valluru *et al.*, 2019). Domestication and breeding can also alter plants' biotic interactions via, for example, reduced chemical defence against herbivores (Chen *et al.*, 2015) and pathogens (Córdova-Campos *et al.*, 2012). However, less research has addressed whether similar side-effects of domestication and breeding have impaired beneficial microbial symbioses (Hetrick *et al.*, 1992, 1993)

Beneficial symbiotic microbes confer substantial benefits to domesticated lineages (Goyal *et al.*, 2021). In agroecosystems, arbuscular mycorrhizal (AM) fungi improve the uptake of phosphorous and the use of water (Smith & Read, 2008) and rhizobia fix 50-70 Tg nitrogen (N) annually at the global scale (Herridge *et al.*, 2008). Given these benefits, it is important to understand how symbiosis with beneficial microbes has evolved as a consequence of crop domestication (Kiers *et al.*, 2007; Martín-Robles *et al.*, 2018; Porter & Sachs, 2020). Symbiosis disruption occurs if there is an evolutionary or ecological decrease in the magnitude of the interaction of the symbiotic partners (Kiers *et al.*, 2003; Porter & Sachs, 2020). Disruption could manifest as reduced benefit from, or reduced investment in, symbiosis.

There is growing evidence of disruption in plant-microbe mutualisms as a consequence of domestication (Hetrick *et al.*, 1993; Ryan *et al.*, 2016; Sawers *et al.*, 2018). For example, the diversity of AM fungal communities associated with wild rice is higher than that of domesticated rice (Parvin *et al.*, 2021). Colonisation by AM fungi is

lower in newer cultivars of sunflower (Turrini *et al.*, 2016) and breadfruit (Xing *et al.*, 2012) than in old cultivars or wild relatives. Post-1950 cultivars of wheat show less dependence on AM fungi than earlier ones (Hetrick *et al.*, 1992, 1993). Cultivars of 14 crops (including cereals and vegetable crops, but not legumes), when fertilised with P, benefit less from mycorrhizae than their wild relatives (Martín-Robles *et al.*, 2018). The phytohormones that were targeted in semi-dwarf grain varieties are regulators of the AM symbiosis, and their manipulation could also have altered symbiotic functioning (Sawers *et al.*, 2018). Furthermore, cultivars that benefit less from symbioses often require higher levels of fertilisation. For example the modern coffee cultivar Caturra supports mycorrhizal fungi with reduced hyphal length compared to other cultivars and has an unusually high demand for fertilisation (Botelho *et al.*, 2011; Lebrón *et al.*, 2012; Abruña *et al.*, 2022).

Similar kinds of disruptions as known from mycorrhizae could also occur in the legume-rhizobium mutualism. Legumes host mutualistic rhizobia in root nodules, wherein the bacteria fix atmospheric N_2 into forms usable by their host, in return for carbohydrates (Venado *et al.*, 2020). While wild plants tend to be N-limited (LeBauer & Treseder, 2008), crop legumes, even if not fertilised directly, are often grown in rotation with crops that are fertilised (Pampana *et al.*, 2018), or in agroecosystems that experience high N deposition (Liu *et al.*, 2006). Under replete soil N conditions, legumes reduce their investment into nodulation and maintenance of the symbiosis (Wendlandt *et al.*, 2019; Friel & Friesen, 2019; Nishida *et al.*, 2021). Artificial selection in plant breeding could select for plants that more readily switch from biological N_2 fixation to direct N acquisition when soil N availability is high (Porter & Sachs, 2020; Liu *et al.*, 2020). If selection like this occurs in high-N environments, then we might expect the plants to be incentivised to begin reducing nodulation at a lower level of N addition than their wild relatives. While such a change in symbiotic response could be beneficial in the short term, it could also contribute to the evolution of less mutualistic rhizobia as a side-effect of N addition (Weese *et al.*, 2015). However, despite the potential for symbiotic disruption under fertile soil conditions (Martín-Robles *et al.*, 2018), few studies exist on the evolution of the legume-rhizobium symbiosis across gradients of N fertilisation (Regus *et al.*, 2017; Oono *et al.*, 2020).

To investigate whether the legume-rhizobial mutualism was disrupted by domestication and breeding, we compared the legume-rhizobium symbiosis in five agriculturally important crops and their wild relatives across a gradient of N fertiliser additions. We first asked, do any of these domesticated legumes benefit less from the rhizobial mutualism than their wild relatives? We hypothesized that, some domesticated crops have evolved a reduced ability to benefit from rhizobia, potentially because of genetic drift, relaxation of natural selection for mutualistic functioning, or adaptation to other aspects of cultivation. Second, we asked, do domesticated legumes cease their mutualism with rhizobia at a lower level of N fertilisation than their wild relatives? We hypothesised that, due to evolution in more fertile conditions, some domesticated crops more readily reduce investment into the rhizobial symbiosis if soil N is abundant.

Materials and Methods

We grew replicate plant lines of five crop/wild relative sets, with or without an inoculation of a rhizobial strain chosen for each set, at a range of N addition levels in the greenhouse. Using generalised linear mixed models (GLMMs), we analysed whether domestication, N fertilisation, inoculation, and their interactions impacted shoot mass, nodule number, and number of viable bacteria per nodule. In particular, we investigated whether domestication has consistently led to reduced rhizobial benefit and/or an increased tendency to halt the mutualism in response to N fertiliser addition across diverse legume crops.

Study systems and experimental designs

We compared cultivars of chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), pea (*Pisum sativum*), peanut (*Arachis hypogaea*), and soybean (*Glycine max*) to accessions of their wild relatives (*Cicer reticulatum, Lens culinaris subsp. orientalis, Pisum sativum subsp. elatius, Arachis duranensis, and Glycine soja,* respectively). These crop/wild relative sets include representatives of three main sub-families of the Fabaceae with domesticated representatives; galegoids (chickpea, lentil, pea), phaseolids (soybean), and dalbergoids (peanut). Table 1 summarises their domestication histories, ploidy, and

criteria for assigning wild progenitors. The seeds of the crop cultivars were sourced from breeders and include two recently released and commonly grown cultivars of each crop legume, except for soybean, for which only one cultivar was used (Table 2). Seeds of wild relatives were provided by the U.S. National Plant Germplasm System and included multiple wild accessions per species (Table 2).

We conducted a greenhouse experiment for each comparison of crop cultivars with wild relative accessions. Each experiment included the following study factors in a complete randomised block design: domestication status (domesticated crop vs wild relative), inoculation with rhizobia (inoculated or uninoculated control), and N fertilisation (one of six-eight ascending levels). Beginning with soybean, we used a design that included 160 pots (each pot containing one plant and constituting an experimental unit) with five replicates of each treatment. For the other four crop/wild relative sets, we scaled up to a design with 360 experimental units with 15 replicates of each treatment. Low germination of wild peanut seeds allowed only for a design with 240 experimental units with four replicates for wild peanut and nine replicates for domesticated peanut. All five experiments were carried out between March and September 2018 in the greenhouse facilities at Washington State University, Vancouver, USA (45°43'57.7"N 122°38'08.4"W).

Plant growth, sampling, and trait measurements

After mechanical scarification, we surface sterilised the seeds with chlorine gas and planted them in SC10R Ray Leach ‘Conetainers’ (Stuewe and Sons Inc., Tangent, Oregon, USA), filled with an autoclaved volumetric 1:1 mixture of quartz sand (grade 0.45) and Sungro Sunshine Mix #1 potting soil (Sun Gro Horticulture, Agawam, MA). Sunshine Mix #1 is made with *Sphagnum* moss, typically low in N content (Aerts *et al.*, 1999), and contains no added N fertiliser. Our intention was to provide an N-limited, but otherwise favourable growth medium. Each pot contained a volume of approximately 164 ml of the 1:1 (v/v) sand-soil mixture. We germinated the seeds in the greenhouse with three 10-minute mist watering cycles each day and 12 hours of supplementary light

per day in addition to natural light. Once enough seeds had germinated, we assigned plants randomly to treatment groups. Each block contained one replicate of each treatment. Due to uneven germination among accessions or cultivars, we distributed wild accessions at random among the treatments.

We applied the first N fertilisation two days before inoculation, to acclimate plants to the soil chemical environment prior to symbiotic association. To compare wild and domesticated legumes at the full range of possible nodulation responses to N, we aimed to expose plants to a range of N spanning zero addition to a level that would cause the abandonment of the rhizobial symbiosis. Basal fertiliser was delivered as a 0.25X Fahraeus solution (Vincent, 1970). To this fertiliser, we added ammonium nitrate (NH_4NO_3) to reach a total of 0, 0.01 g N, 0.1 g N, 0.2 g N, 0.6 g N, or 1.5 g N per 164 ml pot (Table 4) with eight 2 mL applications. For soybean, the first experiment, we used the levels 0, 0.005, 0.01, 0.03, 0.1, 0.2, 0.4, or 0.6 g N, before fewer levels with a broader range were chosen for the other four crop/wild relative sets, to ensure a nodulation-limiting N level would be reached (Table 4). One week after the first fertilisation, we fertilised twice a week, for eight applications in total.

The rhizobial strains we used were provided by the United States Department of Agriculture - Agricultural Research Service (USDA-ARS)(Table 3). Prior to beginning with the experiments, we conducted pilot studies to ensure that the rhizobial strains were beneficial for the wild accessions and cultivars. We inoculated the plants at least two days after the first fertilisation.

We harvested plants after they had received eight bi-weekly fertiliser additions. There were 7-8 weeks between planting and harvest. The shoots were dried at 60^{o}C for 72 hours. We washed the roots and stored them on ice before refrigerating them at 4^{o}C overnight.

Within 24 hours of harvest, we inspected the roots to identify the highest N level at which nodules were consistently present for each crop/wild relative set. We used drop plates (Somasegaran & Hoben, 1994) to compare the number of colony-forming units (CFU) per nodule for freshly harvested nodules at the nodulation-limiting N level to that of nodules at the 0 g N level. To this end, we cut a single nodule from 12

domesticated and 12 wild plants for each crop/wild relative set at each of the two N levels (n=48 nodules per crop/wild relative set). For each plant, we visually estimated and chose the largest single-lobate nodule that was not in close proximity (1-2 mm) to adjacent nodules. Nodules that are closely clustered on an individual root are difficult to remove without being damaged. We surface-sterilised the nodules by vortexing in bleach (7.4% sodium hypochlorite) for 60 seconds, followed by three 60-second rinses in sterilised DI water. We then placed the surface-sterilised nodules in Eppendorf tubes containing 100 µl of liquid Modified Arabinose Gluconate (MAG) media (USDA Agricultural Research Service). Using sterile pestles, we crushed the single nodules in their tubes and stored them on ice. We pipetted 40 µl of each crushed nodule homogenate into 160 µl of MAG media in row A of a 96-well plate, and performed 1:5 serial dilutions from row A-H. For each nodule, we applied one 25 µl drop from rows A, D, E, F, G, & H to each of three replicate MAG agar plates. Each plate contained six drops from six crushed nodules (n=36 drops per plate), for a total of 24 plates per crop/wild relative set. Counting the colonies that formed for appropriate dilutions allowed us to quantify CFU per nodule (provided there was at least one colony). The CFU of a drop was multiplied by its dilution factor (A= 5, D= 625, E= 3125, F= 15625, G= 78125, H= 390625) then by 2.5 (to account for the 40 µl sample of 100 µl homogenate). The mean of these values was taken as the CFU of the original nodule. Drops that grew to saturated lawns, or that yielded no colonies, were not counted. Remaining roots and nodules were frozen at -4^{o}C.

To estimate the degree of benefit gained from symbiosis, we weighed the dried shoots, and to measure investment in the symbiosis, we counted the nodules on thawed roots. We calculated the plants' rhizobial growth response by subtracting the mean shoot mass of uninoculated plants from that of inoculated plants, and dividing by the mean mass of uninoculated plants. Of all the uninoculated plants, 11% formed at least one nodule, likely due to cross-contamination among pots. These included 25 out of 80 uninoculated plants from the soybean experiment, 9 out of 120 uninoculated plants from the peanut experiment, 16 out of 180 uninoculated plants from the chickpea experiment, 12 out of 180 uninoculated plants from the lentil experiment, and 99 out of 180 uninoculated plants from the pea experiment. While varying levels of cross-

contamination occurred, our inference from the experiments should be conservative because all uninoculated plants that formed even a single nodule were removed from the experiment and thus excluded from analyses. All inoculated plants in each crop/wild relative set received the same rhizobial strain (Table 3), so any cross-contamination among inoculated pots must not have severely impacted our findings.

Statistical analyses

To test whether the legume cultivars benefit less from the rhizobial mutualism than their wild relatives, we compared the proportional difference in shoot mass between inoculated and uninoculated plants. We hypothesised that the genetic cost of domestication and/or a relaxation of selection on symbiosis, resulting in a loss of mutualistic benefit, would be reflected by a greater proportional difference in shoot mass between inoculated and uninoculated wild accessions than between inoculated and uninoculated cultivars. We chose to compare proportional differences due to the size difference between the wild and domesticated lines. We analysed shoot weight using general linear mixed models (GLMMs) with Tweedie error distributions and a log link function in glmmTMB (Brooks *et al.*, 2017), in R, version 3.6.0 (R Core Team, 2014). The Tweedie distribution is recommended for continuous data with a large number of zeros (Lecomte *et al.*, 2013; Foster & Bravington, 2013). In five GLMMs, one for each comparison of a crop legume and its wild relatives,, the fixed effects were N level (a continuous variable given by the total N applied with the fertiliser), domestication (crop vs wild), and rhizobial inoculation, the two-way interactions between each pair of these terms, and the three-way interaction among all three. The random effects were experimental block and plant line (i.e., domesticated cultivars or wild accessions). The random effect plant line accounted for the non-independence of the individual plant replicates from the same accession or cultivar, the significance of it indicates differences between the accessions or cultivars within a species. The three-way interaction was the primary test of our hypothesis; the significance of this term indicates both that domesticated and wild plants responded differently to rhizobial inoculation, and that this response changed over the N gradient (Fig. 1, Table 5). The direction and size of the effect shows how the plants reacted to the symbiosis, and how N fertilisation

modulated symbiotic functioning. Wild peanut had low survival, resulting in an uneven distribution of accessions across the experimental treatments. Including plant line as a random effect in the peanut model caused violations of the assumption of equal variance, so we removed plant line to secure a more reliable model.

We hypothesised that, due to breeding in N-fertilised conditions, legume cultivars would have a greater tendency to cease nodulation in response to N addition than their wild relatives. To test this hypothesis, we used GLMMs with Tweedie distributions to model nodulation across the N fertilisation gradient. To account for the fact that domesticated legumes are much larger than their wild relatives (Milla & Matesanz, 2017), we quantified nodulation as the allometric ratio of nodule count per shoot mass. The fixed effects were N level and domestication, and their interaction. The random effects were block and plant line. We ran five versions of this GLMM, one for each comparison between a crop legume and its wild relatives, using data from the inoculated plants only. The N level x domestication interaction allowed us to test whether domestication changes the plants' nodulation response to N. For the analysis of nodule count per unit shoot mass data, we removed three outliers with extreme values. For chickpea, we removed the only plant with nodules at N level 0.6 g N, which alleviated convergence problems for the full model. The inclusion of this outlier did not affect the model outcomes when the random effect of plant line was removed. Plant line did not have a significant effect in the full model. For soybean and pea, we removed the plant with the highest nodule count per unit shoot mass at the N level 0.6, both of which were more than two standard deviations above the mean. Removal of these outliers led to insignificant domestication x N level interactions in soybean and pea. Their removal was appropriate since their inclusion would have made the effect of interaction terms dependent upon a single data point. Additionally, the random effect of plant line was excluded from the model used to analyse nodule count per unit shoot mass data and shoot weight of peanut. This alleviated model convergence problems caused by insufficient replicates in certain accessions. The CFU data was analysed with the same model used for nodulation, with drop plate replicate as an additional random effect. We have excluded statistical results for pea CFU, due to the failure of most plates to grow colonies from the 0.2 g N level. Removing the plant line effect from the CFU model for

peanut caused model convergence problems, which means that we were unable to perform a likelihood ratio test for this effect in peanut.

We used likelihood ratio tests (Lewis *et al.*, 2011) to evaluate the significance of each predictor in all models. To determine the directionality and magnitude of significant effects, particularly of the domestication x N level x rhizobia interaction on shoot mass and of the domestication x N level interaction on nodule number, we used the package 'Effects' (Fox 2003). We compared the slopes and 95% confidence intervals of the factor interactions. Non-overlapping confidence intervals of performance parameters of inoculated and uninoculated soybean were interpreted as evidence that wild soybean benefited more from rhizobial inoculation. To confirm this in an *a posteriori* analysis, we pooled the data across the first five N levels (0, 0.005, 0.01, 0.03, and 0.1 g N) and the highest three (0.2, 0.4, and 0.6 g N). With these pooled datasets, we ran models defining N level as a random effect to check for a significant rhizobia x domestication interaction effect overall.

Results

Mediating effect of domestication on growth response to rhizobial inoculation and N fertilisation

For all legume cultivars and accessions, rhizobial inoculation improved shoot mass, but this benefit decreased with increasing N fertilisation (Fig. 1 a-e). Across the evaluated N levels of 0 – 0.2 g N, rhizobial inoculation increased host plant growth (Inoculation: Chickpea: χ^2: 8.25, $P = 0.004$, lentil: χ^2: 5.5, $P = 0.01$, pea: χ^2: 18.2, $P < 0.001$, peanut: χ^2: 172, $P < 0.001$, soybean, χ^2: 10.4, $P = 0.001$, Fig.1). This is the case despite the lack of a significant rhizobial inoculation effect in the full shoot mass models, except for soybean (Table 5). N fertilisation reduced the benefits of rhizobial inoculation significantly for soybean, (N x Rhiz, χ^2: 12.8, $P < 0.001$, Table 5) and marginally for lentil (N x Rhiz, χ^2: 7.53, $P = 0.08$, Table 5).

For cultivars and wild accessions of chickpea, lentil, pea, and peanut, N fertilisation tended to have a positive effect on shoot mass, up the level of 0.1 – 0.2 g N per plant, above which fertilisation began to have a negative effect on shoot mass (Fig. 1 f-i). The plants were often killed by levels of 1.5 g N per plant, excluding the cultivar and wild accessions of soybean, which were not fertilised at this level. The wild soybean accessions, but not the cultivar, were harmed at 0.6 g N per plant (Fig. 1 j)

Domestication reduced the proportional benefit from rhizobial inoculation and N fertilisation in soybean (Dom x Rhiz x N, χ^2 = 6.15, P = 0.013, Table 5). Across the 0, 0.005, 0.01, 0.03, and 0.1 g N addition levels, the inoculated wild soybean accessions produced 165% more shoot mass than the uninoculated ones, while the soybean cultivar grew 52% larger (Fig. 1e). The shoot mass of inoculated and uninoculated wild soybean accessions differed at or below the 0.1 g N level, but not above (Fig. 2a). Uninoculated plants of the soybean cultivar and the wild soybean accessions did not differ in their response to N fertiliser addition across these lower N levels (post-hoc model excluding inoculated plants, Dom x N, χ^2: 0.2, P =0.6). Across all N levels, the shoot mass of the inoculated and uninoculated soybean cultivar did not differ (Fig. 2b). At low N, the soybean cultivar benefitted less from rhizobial inoculation than the wild soybean accessions (post-hoc model at N levels 0 g - 0.1 g N, Dom x Inoc, χ^2: 19.2, P < 0.001), but at high N, all soybean lines benefited indistinguishably in terms of shoot mass production from rhizobial inoculation (post-hoc model at N levels 0.2 – 0.6 g N, Dom x Inoc, χ^2: 1.33, P = 0.25).

Impact of N fertilisation on nodulation

Overall, fertilisation had a negative effect on nodule numbers per shoot mass in all cultivars and accessions. Domesticated chickpea ceased nodulation at a lower N fertilisation level than wild chickpea (Dom x N, χ^2: 30.6, P<0.001, Table 5, Fig. 3a). Between 0 g N and 0.2 g N addition, chickpea cultivars reduced their nodulation by 99.3%. Across this N addition range wild chickpea accessions reduced their nodulation by only 81% (Fig. 3a). At 0.2 g N, most of the domesticated chickpea plants did not

nodulate, while at this level the wild chickpea plants still had an average of 8 nodules. The response of nodulation to N fertilisation did not differ between cultivars and wild accessions of lentil, pea, peanut, and soybean (Table 5). However, despite the lack of a general wild/domesticated difference in pea, the plant lines differed significantly in their nodulation response to N addition (Plant Line random effect, χ^2: 13.43, $P < 0.001$, Table 5, Fig S1).

In parallel with the reduced nodulation upon N fertilisation, the number of viable rhizobia per nodule, measured as CFU, also generally declined as N fertilisation increased (Fig.3 f-i). CFU in the chickpea cultivars dropped in response to N fertilisation, but in the wild accessions it did not (Dom x N, χ^2: 35.3, P <0.001, Table 5). N fertilisation caused a 94% reduction in CFU in the chickpea cultivars, compared to an increase of 38% in the wild accessions (Fig 3f). In addition to the overall wild/domesticated difference, the individual chickpea plant lines differed significantly in their CFU response to N addition (Plant Line random effect, χ^2: 18.7, $P < 0.001$, Table 5). The wild accessions were more variable in their response to N addition than the cultivars (CFU responses to N addition for each plant line shown in Fig. S2). In contrast, N fertilisation caused a greater reduction in the number of viable bacteria per nodule in the wild lentil accessions than the domesticated lentil cultivars (Dom x N, χ^2: 8.08, P <0.001, Table 5; Fig 3g).

Discussion

Legume crops responded differently to domestication, but symbiotic disruption is uncommon.

We hypothesised that the genetic cost of domestication and/or a relaxation of selection on symbiosis has resulted in a loss of mutualistic benefit for legume cultivars. We expected this to become evident in greater benefits from the mutualism in the wild legume accessions than the cultivars. Growth benefits and the regulation of the rhizobial symbiosis did not differ between the examined wild and domesticated lines of three of five grain legume crops under variable N availability. We examined three aspects of

how domestication may have affected the rhizobial mutualism; the growth benefit that plants receive from rhizobial inoculation, the effect of N addition on nodulation, and bacterial abundance within nodules. Domestication reduced the growth benefit of the mutualism in the soybean cultivar we examined, MN1312CN, and heightened the nodulation response to N addition only in the examined chickpea cultivars. These findings are consistent with a scenario in which the rhizobial mutualism is disrupted in a few domesticated crop legumes compared to their wild relatives, but not the majority of domesticated legumes. All of the examined legumes are among the first plants that have been domesticated, as long ago as 9000 years (Zohary & Hopf, 1973; Tanno & Willcox, 2006; Dillehay *et al.*, 2007; Sonnante *et al.*, 2009; Kim *et al.*, 2012). These were all single domestication events (Husted, 1936; Ellis *et al.*, 1998; Zohary, 1999; Guo *et al.*, 2010) with significant genetic bottlenecks (Abbo *et al.*, 2003; Hyten *et al.*, 2006; Seijo *et al.*, 2007; Alo *et al.*, 2011; Smýkal *et al.*, 2013), and all of these species are diploid, except peanut (De Carvalho Moretzsohn *et al.*, 2004). Despite these commonalities, we observed idiosyncratic responses to domestication.

Domestication does not appear to have disrupted the mutualism across most of the legume crops we examined. In chickpea, lentil, pea, and peanut, our findings of indistinguishable symbiosis traits between wild and domesticated lines is consistent with a scenario in which selection for agronomic traits has neither increased nor decreased the benefit legumes receive from rhizobia. This suggests that the traits that govern the benefits legumes reap from the rhizobial mutualism are not, in general, lost by breeding efforts. Despite a lack of direct selection on the traits that govern the symbiosis, the benefits that legumes receive from rhizobia are, in most cases, maintained, and are not affected by selection on agronomic traits. It has been hypothesised that trade-offs may occur during domestication (Denison, 2015) wherein resources that would have been directed to mutualists like rhizobia and AM fungi have been re-directed to reproductive structures to increase grain yield. (Porter & Sachs, 2020). Such trade-offs do not appear to have occurred during the improvement of most legume crops studied here.

Our results indicate that in most cases, the benefits of the rhizobial mutualism tend to have remained static across the course of crop evolution. This leads us to

question whether there remains potential trait space to be exploited by improving the N efficiency of legume crops through targeted selection (Bennett *et al.*, 2013; Sessitsch & Mitter, 2015; Ryan *et al.*, 2016; Goyal *et al.*, 2021). To investigate this question, it will be important to understand how yield traits and symbiosis traits correlate in modern cultivars. Do the highest-yielding cultivars also show the most efficient regulation of the symbiosis with rhizobia? If so, does this represent the maximum mutualistic potential of legume crops? Even if the ceiling of mutualistic growth benefit has been reached by most crops, our results indicate that certain cultivars may benefit less from rhizobia than their wild relatives, and should have the potential to be improved.

Wild soybean accessions benefited more from rhizobia than a soybean cultivar at low N fertilisation

Under low N availability, the wild soybean accessions benefited more from rhizobial inoculation than the examined soybean cultivar. Rhizobial inoculation increased shoot mass by 165% for the wild soybean accessions but only by 52% for the soybean cultivar, at fertilisation levels of up to 0.1 g N per plant. However, without rhizobia, the wild soybean accessions and the cultivar showed the same positive response to N addition between 0 and 0.1 g N. This suggests that the greater responsiveness of wild soybean to rhizobial inoculation, indeed, must have been related to greater symbiotic functioning, rather than simply a greater responsiveness to nitrogen fertilisation. Although we are unable to state how greater symbiotic functioning is achieved, a variety of mechanisms have been identified (Terpolilli *et al.*, 2012) Our results provide some of the first evidence that rhizobial mutualism benefits could have been reduced by domestication and breeding (Porter & Sachs, 2020). Modern soybean cultivars may be lacking in other symbiotic abilities as well: newer cultivars of soybean may have a reduced ability to sanction against ineffective rhizobia (Kiers *et al.*, 2003, 2007). While the other legume crops we examined also ceased to benefit from rhizobia above 0.1 – 0.2 g N, none of the other legume cultivars differed from their wild relatives in the benefit they received from rhizobia below these levels. The lack of evidence for an evolutionary

disruption of symbiotic benefits in other legume crops than soybean suggests that their domestication and improvement by breeding maintained the beneficial relationship with rhizobia.

Our finding of disruption of the N-fixing rhizobial mutualism in domesticated soybean is congruent with similar symbiotic disruption due to domestication in the mycorrhizal mutualism (Xing *et al.*, 2012; Martín-Robles *et al.*, 2018; Zhang *et al.*, 2019). For example, wild plants benefit from AM fungi both with and without P fertilisation, while domesticated plants benefit only in absence of P fertilisation (Martín-Robles *et al.*, 2018). This suggests that crops have been selected to obtain P directly from soil when fertiliser is applied, instead of using the mycorrhizal symbiosis. Martin-Robles *et al* (2018) report symbiotic disruption related to fertilisation in 14 crops, while we have found only one case of reduced benefit from the rhizobial symbiosis. This could imply that domestication and recent plant breeding could have limited the mycorrhizal mutualism more than the rhizobial mutualism. Plants vary in their ability to sanction against ineffective mycorrhizae (Grman & Kellogg, 2012), and a meta-analysis found 15% of mycorrhizal interactions to be parasitic (Hoeksema *et al.*, 2010). Martin-Robles *et al* (2018) suggest the control of resource trafficking as a potential driver of mycorrhizal disruption. It is possible that disruption of optimal resource allocation and host sanctions are more likely when symbionts are distributed throughout the roots, rather than being confined to nodules, although evidence to the contrary exists (Bever *et al.*, 2009). The benefits of the rhizobial mutualism can be reduced by N fertilisation (Regus *et al.*, 2017; Friel & Friesen, 2019; Nishida *et al.*, 2021), but can be improved by P fertilisation (Singleton *et al.*, 1985; Samago *et al.*, 2018). The benefits of the mycorrhizal mutualism, however, can be reduced by both N and P fertilisation via optimal resource allocation (Treseder, 2004; Egerton-Warburton *et al.*, 2007; Hoeksema *et al.*, 2010; Johnson *et al.*, 2013). This means that if breeding in highly fertilised conditions can disrupt beneficial mutualisms, then this effect could be doubled for mycorrhizae. Therefore disruption of the rhizobial mutualism could be less common than that of the mycorrhizal mutualism. We thus concluded that reduced crop benefit from rhizobia is a possible but not universal consequence of domestication and crop plant breeding.

The reduced rhizobial benefit we observed in domesticated soybean could have multiple explanations. In agricultural settings, selection for traits that are essential for fitness in nature could be relaxed (Lu *et al.*, 2006). Since N fertilisation reduces investment in rhizobia (Glyan'ko *et al.*, 2009; Nishida & Suzaki, 2018a), it could relax selection for maximal rhizobial benefit (Griesmann *et al.*, 2018), and reduce rhizobial propagation (Denison & Kiers, 2011). In our study, the examined wild soybean accessions benefited more from rhizobia than the soybean cultivar at lower levels of fertilisation (where potential N demands must have been only partially covered by fixed N_2 (0, 0.005, 0.01, 0.03, and 0.1 g N per plant, or approximately 0, 3, 5, 16, and 55 kg N ha^{-1}). Soybean is commonly fertilised at 75 kg N ha^{-1} (Gan *et al.*, 2002). This corresponds to the point at which rhizobial benefit declined in our experiment, which means that soybean may be cultivated in conditions where selection for maximal plant symbiotic benefits is relaxed. Responses of legumes to N fertilisation can vary both between and within species. Soybeans are found to benefit from N addition in some locations (Mandic *et al.*, 2015; Bobrecka-Jamro *et al.*, 2018), but not in other locations (De Oliveira *et al.*, 2019; Pannecoucque *et al.*, 2022). In chickpea, pea, field bean (*Vicia faba*), and white lupin (*Lupinus albus*), N addition has been shown not to increase yield, and even reduce it at high N levels (Pampana *et al.*, 2018), which agrees with our findings. If soybeans are more likely to make use of N fertilisation than other legumes, this could indicate a reduced potential to benefit from the rhizobial mutualism..
Alternatively, it is possible that lower benefits from rhizobia in domesticated soybean than in wild soybean in low N conditions indicate that domesticated soybean has evolved to grow successfully without investing as much in the rhizobial symbiosis. Greater fitness in the absence of the symbiosis could give the impression that cultivars benefit less from inoculation than wild soybean accessions, but this is not evidence for mutualism disruption (Sawers *et al.*, 2010; Porter & Sachs, 2020). To detect mutualism disruption as an adaptive response to relaxed selection in crops, future studies should use selection analysis, combined with measurements of N_2-fixation, on a broad range of soybean accessions (McGoey & Stinchcombe, 2009; Campitelli & Stinchcombe, 2013). For such studies, sets of cultivars representing opposite sides of the range of seed size-seed protein tradeoff (Skovbjerg *et al.*, 2020) could be evaluated. *Glycine soja* seeds

can have a greater protein content than those of *Glycine max* (La *et al.*, 2019). Variation in seed protein content may underlie differences in rhizobial benefit. Higher-yielding, lower-protein soybean cultivars may possess less effective rhizobial symbioses, which would imply that crop breeding for oil instead of protein inadvertently selects for disruption of the mutualism.

In contrast to our results, Munoz et al (2016) found that inoculation with efficient rhizobial strains resulted in greater biomass and a greater accumulation of symbiotically fixed N in domesticated than wild soybean. Our study included uninoculated controls of the wild and domesticated soybean that enabled us to compare marginal biomass gains from symbiosis and not only consequence of the differences of the totally fixed N and accumulated biomass.. We used a single domesticated soybean cultivar, and five wild soybean accessions, none of which were included in the set of cultivars and accessions used by Munoz et al (2016). This difference in identity of cultivar/accession choice may explain the contrasting results. Other experiments using different soybean cultivars and carried out at different locations have yielded contrasting results, particularly regarding the growth and yield responses (Mandic *et al.*, 2015; Bobrecka-Jamro *et al.*, 2018; De Oliveira *et al.*, 2019; Pannecoucque *et al.*, 2022). Because we did not include a range of soybean cultivars, further research is needed to establish the general validity of our findings. Experiments with more soybean cultivars and running for longer under field conditions would be helpful in this regard. In the greenhouse, we were unable to replicate the particular climatic and soil conditions that each species is best adapted for. Field experiments carried out in the native ranges of the legumes would allow for a more realistic assessment of their symbiotic potential, particularly considering the disparate geographic ranges of our species. Being grain crops, it will be important to take such experiments beyond the vegetative growth phase. While early dry matter is predictive of yield (Koutroubas *et al.*, 1998), seed yield is the most direct measure of plant fitness.

Future experiments with more plant lines could address another limitation of this study; the low number of replicate lines of domesticated and wild relatives examined for each crop/wild relative set. Examining a more diverse sample of wild vs domesticated

lines for each crop would increase the rigor of the statistical testing of the effect of domestication on symbiosis trait disruption. Due to practical limitations on experimental size, our study was limited in the number of lines we could include. Our findings may, however, motivate future experiments focused on particular crops with a greater sampling of wild and domesticated diversity to achieve greater statistical power to detect domestication effects. In addition to greater diversity within a crop/wild relative set, future experiments could include more distantly related species. In our experiment, the crop/wild relative sets are closely related. Domesticated chickpea has a genetic distance of 0.144 from wild chickpea (Sudupak *et al.*, 2002). Domesticated lentil has a genetic distance between 0.0164 and 0.0182 from wild lentil (Liber *et al.*, 2021). The genomes of domesticated and wild soybean differ by only 0.31% (Kim *et al.*, 2010), and reads of chromosomal pseudomolecules in *domesticated chickpea* and *wild chickpea* show 98.36% median identities (Bertioli *et al.*, 2016). *Pisum sativum subsp. elatius* is considered an adequate wild comparison for *domesticated pea,* but there is some debate on whether particular *elatius* accessions have been cultivated by humans in the past. Including a more distant relative, such as *Pisum fulvum* for pea, could provide a useful outgroup for these comparisons.

A further important consideration for future research will be to describe differing compatibilities of wild and domesticated legumes with different rhizobial strains. Functional mismatches between legumes and rhizobial partners can lead to reduced symbiotic benefits (Terpolilli *et al.*, 2008; Drew & Ballard, 2010; Kazmierczak *et al.*, 2017; Rigg *et al.*, 2021) or reduced investment into the symbiosis (Wendlandt *et al.*, 2019). Although we tested our rhizobial strains to ensure they were beneficial for both the wild and domesticated lines, it is possible that there are better rhizobial partners for each plant line. We may thus have underestimated the potential of wild legumes to benefit from rhizobia because of the use of a generalist strain, isolated from the crop. While it may not be feasible to identify a single “ideal” rhizobial partner for every legume, future experiments comparing wild and domesticated legumes could inoculate with strains isolated from both wild and crop plants. A more diverse selection of rhizobia would increase the chance to reveal the true potential of a legume to benefit from this mutualism, as strains of intermediate quality may be sanctioned (Westhoek *et al.*,

2021). Field-based experiments carried out over a range of latitudes could be used to identify context-specific optimal rhizobial strains (Sprent *et al.*, 2017; Ndungu *et al.*, 2018). Matching legumes to their native rhizobia and soils would provide the most fair test of their symbiotic potential (Sprent *et al.*, 2013; Ramoneda *et al.*, 2021).

Chickpea cultivars reduce nodulation in response to N fertilisation more than wild accessions

A reduction in nodulation is the expected response of legumes to N fertilisation. This response to N fertilisation appears to have been unaltered by domestication and breeding in most of the legume cultivars we tested, except the chickpea cultivars. These chickpea cultivars ceased nodulation at a lower N level than their wild relatives. This stronger response to N fertiliser supports the hypothesis that domestication can lead to earlier downregulation of nodulation in response to N fertilisation, but shows, when compared to the other crops, that this is not a necessary consequence of domestication. Multiple levels of nitrate-mediated symbiosis disruption in domesticated chickpea are evident in our results. Between N additions of 0 – 0.2g, domesticated chickpea reduced nodulation by 99%, while wild chickpea reduced it by only 81%. Furthermore, we also found that post-nodulation effects were strong. At 0.1 g N, the number of viable bacteria per nodule was reduced by 92% in domesticated chickpea, while it was not significantly altered in wild chickpea. It seems that domesticated chickpea plants limited their investment in existing nodules in response to N fertilisation, possibly by diverting photo-assimilates and/or oxygen (Escuredo *et al.*, 1996; Fujikake *et al.*, 2003; Kiers *et al.*, 2003). Our finding in chickpea corroborates previous studies indicating that nodulation in chickpea is sensitive to N-fertilisation, both in isolation (D Cook, pers comm) and compared to other legume crops, such as field beans (Rose *et al.*, 2016; Pampana *et al.*, 2018).

Greater sensitivity of symbiosis to N fertilisation in domesticated chickpea than wild could be adaptive in more fertile agricultural conditions, and thus be selected for. Reducing investments into rhizobial symbionts in response to N fertilisation leaves more C for growth (Nishida & Suzaki, 2018b) and is adaptive in an environment of sufficient N

(Friel & Friesen, 2019). If a greater sensitivity to soil N were adaptive, we might expect to see less benefit from rhizobia at lower N levels for domesticated chickpea, and a greater cost of rhizobia at higher N levels for wild chickpea. However, the greater reduction of nodulation in domesticated chickpea as soil N increased neither improved nor reduced fitness. The same trend was evident in lentil despite the contrasting result between cultivars and wild accessions. Although the wild lentil accessions showed a steeper reduction in per-nodule CFU than the lentil cultivars, this did not confer them a fitness advantage at higher N levels. Adaptation to higher nutrient environments has been reported for domesticated chickpea (Marques *et al.*, 2020). A greater reduction in per-nodule CFU in response to N addition for the wild lentil accessions could be interpreted as evidence of improved control over the symbiosis. However, the chickpea cultivars also showed a greater reduction in nodulation in response to N addition than their wild relatives. This combination of results for chickpea points more directly at a history of strong selection under fertile soil conditions. However, due to the wild lentil accessions' steeper reaction to N addition, we cannot be sure that the same result in the chickpea cultivars implies adaptation to N fertilisation. Future experiments under realistic high and low N conditions in the field will be important to ascertain whether a reduction of investment into rhizobia in response to N fertilisation is adaptive under agricultural conditions in domesticated chickpea.

Conclusion

We found that disruption of the rhizobial symbiosis is a possible, but not necessary consequent effect of crop domestication and breeding. We recorded a variety of responses to domestication; of the five examined legumes, soybean and chickpea showed evidence of disruption to the rhizobial mutualism under domestication, while pea, peanut and lentil did not. Of the lines we examined, domesticated soybean benefitted less from rhizobia than wild soybean at low N levels, and domesticated chickpea reduced its nodulation in response to N fertilisation to a greater extent than wild chickpea. Breeding crops for better relationships with soil microbes is a promising crop improvement strategy (Bennett *et al.*, 2013; Sessitsch & Mitter, 2015; Ryan *et al.*, 2016; Goyal *et al.*, 2021). If the ability of legumes to gain N from rhizobia could be

promoted, it would greatly contribute to the effort of making agriculture more ecologically resilient (Lammerts van Bueren *et al.*, 2018). Introgressing traits from wild ancestors into crops has potential for such crop improvement (Brozynska *et al.*, 2016; Stalker, 2017). Soybean and chickpea in particular could provide fruitful systems with great agronomic relevance for this breeding goal.

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TABLES

Table 1. Timeline of domestication of the studied legume crops. Approximate date of domestication for each crop, its region of origin, the number of independent domestication events, the severity of the known genetic bottleneck(s), and the ploidy of the crop and wild species. Also included is information on the criteria of assigning wild progenitors to each grain legume crop.

	Wild relative	Approx. date of domestication	Region of origin	Number of domestication events	Bottleneck severity	Wild ploidy	Crop ploidy
Soybean, *Glycine max*	*Glycine soja* - (M. Y. Kim et al. 2010; Moon Young Kim et al. 2012; Carter et al. 2004)	6,000-9,000 years ago - (Moon Young Kim et al. 2012)	Northern/central China - (Wang et al. 2016)	Single domestication event - (Guo et al. 2010)	51.1% loss of diversity (Hyten et al. 2006)	Diploid (Kim et al. 2010)	Diploid (Kim et al. 2010)
Peanut, *Arachis hypogaea*	*Arachis duranensis* and *Arachis ipaensis* - (Bertioli et al. 2019; J. G. Seijo et al. 2004). *Arachis monticola* is intermediate ancestor (G. Seijo et al. 2007)	At least ¬8000 years ago (Dillehay et al. 2007; Piperno and Dillehay 2008)	Southern Bolivia - (Milla, Isleib, and Stalker 2005)	Single hybridization event - (Husted 1936). Multiple events possible if always from same diploid parents (J. G. Seijo et al. 2004)	"Large bottleneck" evident due to low variability (G. Seijo et al. 2007)	Diploid (Moretzsohn et al. 2004)	Allotetraploid (Moretzsohn et al. 2004)
Lentil, *Lens culinaris*	*Lens culinaris ssp. Orientalis* (Sonnante, Hammer, and Pignone 2009)	Possible pre-domestication cultivation 13,000 years ago, full domestication 7000-7500 years ago (Sonnante, Hammer, and Pignone 2009)	The Middle East (Zohary 1972)	Single domestication (Zohary 1999)	40% loss of genetic diversity (Alo et al. 2011)	Diploid (Liber et al 2021)	Diploid (Liber et al 2021)
Chickpea, *Cicer arietinum*	*Cicer reticulatum* (Ladizinsky and Adler 1976; Maesen et al. 2009)	9000 years ago (Tanno and Willcox 2006)	The Middle East (Redden and Berger 2009)	Single domestication (Zohary 1999)	4 bottlenecks: Pre-domestication distribution, domestication, change from autumn to spring sowing, and modern breeding. (Abbo, Berger, and Turner 2003)	Diploid (Singh et al. 2008)	Diploid (Singh et al. 2008)
Pea, *Pisum sativum*	*Pisum sativum ssp. elatius* (Kosterin et al. 2010; Smýkal et al. 2017; Jing et al. 2007)	8000-9000 years ago (Zohary and Hopf 1973)	The Middle East (Zohary and Hopf 1973)	Single domestication for *P. sativum* (Ellis et al. 1998), but *P. abyssinicum* is a closely related independent domestication (Jing et al. 2010)	Not well known (Siol et al. 2017), but wild collections have "substantially larger diversity" (Smýkal et al. 2013)	Diploid (Griga & Novak 1990)	Diploid (Griga & Novak 1990)

Table 2. Tested cultivars and wild accessions of grain legumes used in the experiment. Dates of release of cultivars are shown in brackets next to their names. Wild accessions were provided by GRIN's U.S. National Plant Germplasm System.

Fabaceae sub-family	Plant name	Species name	Cultivar/Accession
Hologalegina	Domesticated chickpea	*Cicer arietinum*	Sierra (1992) Sawyer (2007)
	Wild chickpea	*Cicer reticulatum*	PI599050, PI489777, PI599092 CSP, W651045, W651108, W651133, W651141, 2 W651159, 3 W651096, 4 W651045
	Domesticated lentil	*Lens culinaris*	Avondale (2001) Pardina (2009)
	Wild lentil	*Lens culinaris* subsp. *Orientalis*	PI612249, PI615669, PI615670, PI572370, PI572374
	Domesticated pea	*Pisum sativum*	Serge pea Aragorn (2007)
	Wild pea	*Pisum sativum* subsp. *Elatius*	PI344013 PSP, W615008 PSP, PI639974 PSP, PI505059 PSP, PI344538 PSP, PI344010 PSP, PI273209 PSP
Dalbergoids	Domesticated peanut	*Arachis hypogaea*	Jupiter (2000) GA06G (2006)
	Wild peanut	*Arachis duranensis*	PI475884 02 SD, PI219823 05 SD, PI468203 01 SD, PI468372 01 SD, PI497263 01 SD, PI262133 02 SD, PI666083 03 SD,
Phaseolids	Domesticated soybean	*Glycine max*	MN1312CN (2016)
	Wild soybean	*Glycine soja*	PI578346C, PISA74448A, PI342618B, PI458539B, PI522196A

Table 3: Strains of rhizobia applied to crop/wild relative sets. The strains were chosen to be beneficial to both the domesticated and wild legume lines

Legume cultivar/wild relative	Rhizobial partner	Strain ID
Chickpea, *Cicer arietinum*, *C. reticulatum*	*Mesorhizobium ciceri*	USDA 3383
Lentil, *Lens culinaris*, *L. culinaris subsp. orientalis*	*Rhizobium leguminosarum* biovar. *viciae*	USDA 2434
Pea, *Pisum sativum*, *P. sativum subsp. elatius*	*Rhizobium leguminosarum*	USDA 2370
Peanut, *Arachis hypogaea*, *A. duranensis*	*Bradyrhizobium*	USDA 3341
Soybean, *Glycine max*, *G. soja*	*Bradyrhizobium diazoefficiens*	USDA 110

Table 4: Nitrogen fertilization levels in the experiment. Grams of N applied per pot, NH_4NO_3 content of fertiliser in g/L, N molarity of fertiliser, and approximate kg ha^{-1} value of each N level. Kg ha^{-1} values calculated assuming that the surface area of a pot is functionally equal to the same surface area of field soil. Fertiliser was applied in a series of eight 2 ml applications to 164 ml growth compartments filled with a 1:1 (v:v) mixture of soil and sand.

Grams of N applied per 164 ml pot	g/L NH_4NO_3 of fertiliser solution	N molarity of fertiliser solution	Approximate kg ha^{-1} value
0	0	0.00	0
0.005*	0.895	0.02	2.5
0.01	1.79	0.04	5
0.03*	5.37	0.12	15
0.1	17.86	0.45	55
0.2	35.72	0.89	110
0.4*	71.44	1.80	220
0.6	107.15	2.68	330
1.5	267.87	6.69	825

* = N levels applied to soybean only.

Table 5. Generalized mixed-effects models testing the influences of domestication (Dom), N-fertilisation (N), and rhizobial inoculation (Inoc) and the two- and three-way interactions of these factors on plant growth and the root nodule symbiosis of cultivars and wild accessions of grain legumes. The p-values and χ^2 values of likelihood ratio tests are listed. Significant *P*-values are highlighted in bold. Each nodule dilution level was replicated across three identical plates and six dilution levels were evaluated (1:5 dilution to check for non-detectability and the higher dilution levels to get single colonies for enumeration). Rep: replicate plates in the evaluation of the number of viable rhizobial cells per nodule.

	Fixed Effects	Shoot mass		Nodules/Shoot mass		CFU	
		χ2	*p*-value	χ2	*p*-value	χ2	*p*-value
Chickpea	Domestication	14.4	**<0.001**	20.7	**<0.001**	0.36	0.54
	N	277	**<0.001**	258	**<0.001**	9.08	**0.002**
	Inoc	1.41	0.23	-	-	-	-
	Dom x N	17.0	**<0.001**	30.6	**<0.001**	35.3	**<0.001**
	Dom x Inoc	0.86	0.35	-	-	-	-
	N x Inoc	1.68	0.19	-	-	-	-
	Dom x N x Inoc	1.00	0.31	-	-	-	-
	Random Effects						
	Block	0.00	1.00	0	1	6.41	**0.01**
	Plant Line	0.00	1.00	2.39	0.12	18.7	**<0.001**
	Rep	-	-	-	-	0.00	1.00
Lentil	Dom	12.6	**<0.001**	0.81	0.36	0.04	0.82
	N	175	**<0.001**	258	**<0.001**	10.4	**0.001**
	Inoc	0.35	0.54	-	-	-	-
	Dom x N	7.58	**0.005**	0.64	0.42	8.08	**0.004**
	Dom x Inoc	0.42	0.51	-	-	-	-
	N x Inoc	7.53	0.08	-	-	-	-
	Dom x N x Inoc	0.12	0.72	-	-	-	-
	Random Effects						
	Block	0.00	1	0.007	0.93	9.45	**0.002**
	Plant Line	0.00	1	0.07	0.78	1.24	0.26
	Rep	-	-	-	-	0.00	1.00
Pea	Dom	5.08	**0.02**	0.002	0.95		
	N	193	**<0.001**	168	**<0.001**		
	Inoc	1.14	0.28	-	-		
	Dom x N	0.41	0.52	3.31	0.06		
	Dom x Inoc	0.85	0.35	-	-		
	N x Inoc	0.29	0.58	-	-		
	Dom x N x Inoc	0.17	0.67	-	-		
	Random Effects						
	Block	0.15	0.69	0	1		
	Plant Line	0.00	1.00	13.43	**<0.001**		
Peanut	Dom	159	**<0.001**	13.3	**<0.001**	4.7	**0.02**
	N	35.7	**<0.001**	109	**<0.001**	53.1	**<0.001**
	Inoc	0.12	0.72	-	-	-	-
	Dom x N	0.05	0.82	3.02	0.08	3.6	0.057
	Dom x Inoc	0.01	0.91	-	-	-	-
	N x Inoc	0.51	0.47	-	-	-	-
	Dom x N x Inoc	0.62	0.43	-	-	-	-
	Random Effects						
	Block	0.73	0.39	0	1	15.8	**<0.001**
Soybean	Dom	9.63	**<0.001**	0.05	0.81		
	N	29.7	**<0.001**	87.4	**<0.001**		
	Inoc	10.4	**0.001**	-	-		
	Dom x N	0.95	0.33	0.05	0.81		
	Dom x Inoc	0.009	0.92	-	-		
	N x Inoc	12.8	**<0.001**	-	-		
	Dom x N x Inoc	6.15	**0.01**	-	-		
	Random Effects						
	Block	0	1.00	7.19	**0.007**		
	Plant Line	0	1.00	0	1		

1045

Table S1. Generalized mixed-effects models testing the influences of domestication (Dom), N-fertilisation (N), and rhizobial inoculation (Inoc) and the two- and three-way interactions of these factors on shoot mass, with separate models for wild and domesticated legumes. The p-values and χ^2 values of likelihood ratio tests are listed. Significant *P*-values are highlighted in bold.

	Fixed Effects	Shoot mass	
		χ2	*p* -value
Chickpea cultivars	N	148	**<0.001**
	Inoc	0.12	0.72
	N x Inoc	1.68	0.08
	Random Effects		
	Block	0.00	1.00
	Plant Line	0.32	0.57
Lentil cultivars	N	87.6	**<0.001**
	Inoc	0.62	0.42
	N x Inoc	2.59	0.11
	Random Effects		
	Block	0.00	1.00
	Plant Line	0.00	1.00
Pea cultivars	N	119	**<0.001**
	Inoc	0.001	0.97
	N x Inoc	0.03	0.86
	Random Effects		
	Block	1.16	0.28
	Plant Line	0.00	1.00
Peanut cultivars	N	30.9	**<0.001**
	Inoc	0.10	0.75
	N x Inoc	0.80	0.37
	Random Effects		
	Block	0.31	0.58
Soybean cultivar	N	34.3	**<0.001**
	Inoc	14.5	**<0.001**
	N x Inoc	6.06	**0.01**
	Random Effects		
	Block	0	1.00
	Plant Line	0	1.00

	Fixed Effects	Shoot mass	
		χ2	*p* -value
Wild chickpea accesions	N	147	**<0.001**
	Inoc	2.26	0.13
	N x Inoc	0.05	0.82
	Random Effects		
	Block	0.00	1.00
	Plant Line	7.75	0.005
Wild lentil accessions	N	98.3	**<0.001**
	Inoc	0.04	0.84
	N x Inoc	0.31	0.58
	Random Effects		
	Block	0.00	1
	Plant Line	8.91	0.002
Wild pea accessions	N	193	**<0.001**
	Inoc	1.55	0.21
	N x Inoc	0.43	0.51
	Random Effects		
	Block	0.00	1
	Plant Line	0.00	1.00
Wild peanut accessions	N	5.06	**0.02**
	Inoc	0.07	0.79
	N x Inoc	0.43	0.51
	Random Effects		
	Block	0.73	0.39
Wild soybean accessions	N	10.7	**0.001**
	Inoc	3.45	0.060
	N x Inoc	10.1	**<0.001**
	Random Effects		
	Block	0	1.00
	Plant Line	0	1.00

FIGURE LEGENDS

Fig. 1 Growth stimulation by rhizobia decreased with the amount of N applied (a-e). Shoot mass generally increased with N addition up to 0.1 g N per plant. Global mean of shoot mass benefit of rhizobia for domesticated (grey lines) and wild (black lines) plant lines across levels of N addition (**a-e**). P-values shown in a-e are for the

domestication*rhizobial inoculation*N addition interaction (see Table 5). Shoot mass responsiveness is a unit-less ratio calculated as (inoculated shoot mass – uninoculated shoot mass) / (uninoculated shoot mass), with values > 0 indicating benefits from rhizobial inoculation and values <0 indicating growth depression. Means and associated standard errors of shoot mass of domesticated and wild legumes across levels of N fertilisation, with inoculation (solid lines) or without inoculation (dashed lines, **f-j**). See Table S1 for statistical results of shoot weight models run on wild-only and domesticated-only datasets to support differences in **f-j**.

Fig. 2 Domesticated soybean benefitted less from rhizobial inoculation at low N levels than wild soybean Model-based predictions of the reaction of inoculated (solid lines) and uninoculated (dashed lines) wild (a) and domesticated (b) soybean to N fertiliser addition. The predictions and 95% confidence intervals (shaded areas) are based on the mixed-effects model testing the effects of domestication, rhizobia, fertilisation, and the interactions between these factors on soybean shoot mass. The ticks inside the x-axis indicate the evaluated levels of N addition.

Fig. 3 Nodules per unit shoot mass and number of viable rhizobia per nodule generally decreased with addition of N fertiliser. Domesticated chickpea ceased nodulation at a lower N level than the wild chickpea accessions. Colony forming units (CFU) per nodule is a measure of rhizobial symbiont abundance (i.e., viable bacterial cells) per nodule.

Fig. S1 Individual plant lines of domesticated field pea (*Pisum sativum*) and wild *Pisum sativum subsp. elatius* differ in their nodule/shoot mass response to N fertiliser addition. Means and standard errors of nodule/shoot mass of domesticated and wild *Pisum* across levels of N fertilisation. Cultivars shown in grey, wild accessions shown in black.

Fig. S2 Individual plant lines of domesticated chickpea (*Cicer arietinum*) and wild *Cicer reticulatum* differ in their number of viable rhizobia per nodule in response to N fertiliser addition. Means and standard errors of colony forming units (CFU) of domesticated and wild *Cicer* across levels of N fertilisation. CFU per nodule is a measure of rhizobial symbiont abundance (i.e., viable bacterial cells) per nodule. Cultivars shown in grey, wild accessions shown in black.

Figures

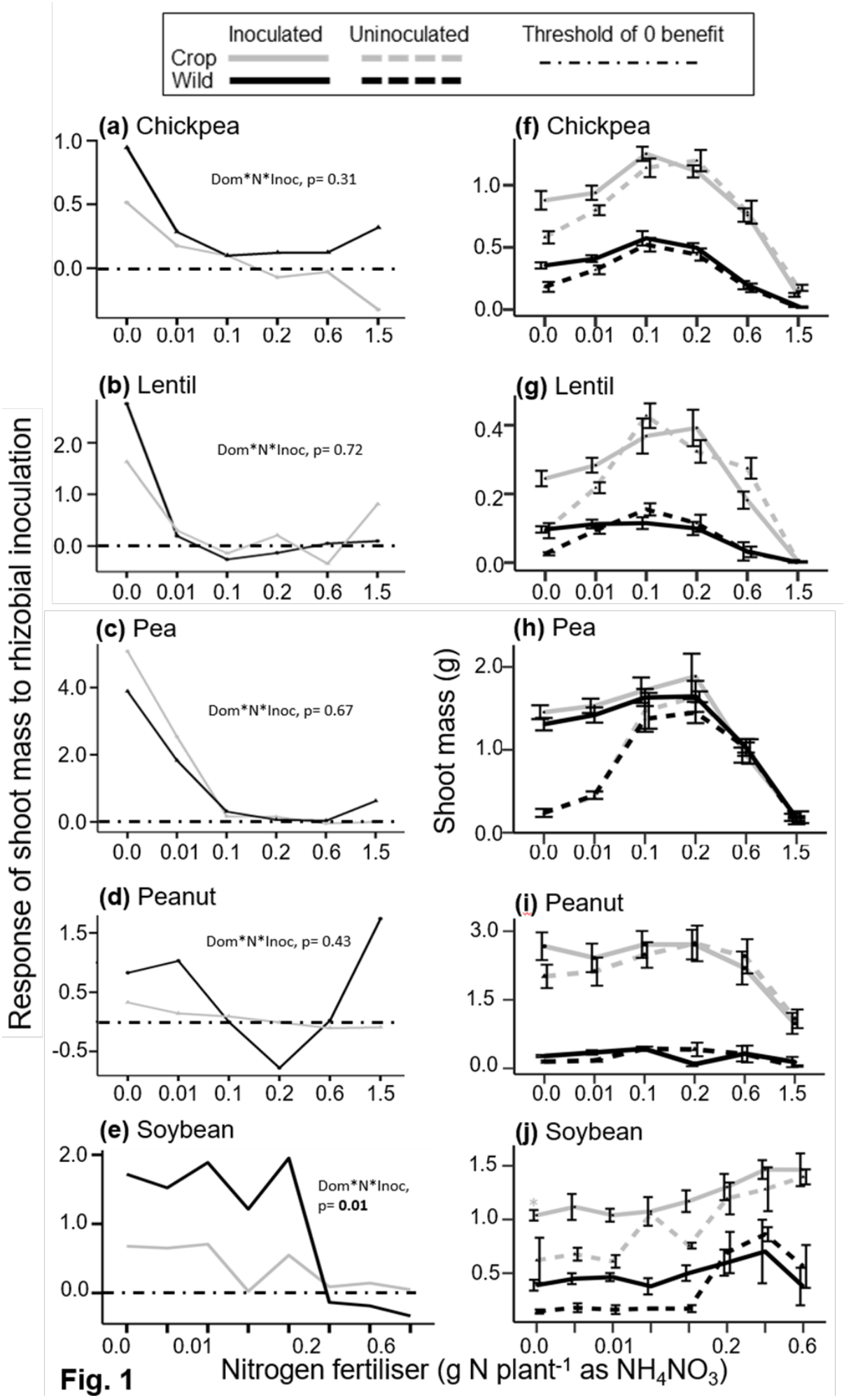
Inoculated
Uninoculated
Threshold of 0 benefit
Crop
Wild
(a) Chickpea
Dom*N*Inoc, p= 0.31
(f) Chickpea
(b) Lentil
Dom*N*Inoc, p= 0.72
(g) Lentil
(c) Pea
Dom*N*Inoc, p= 0.67
(h) Pea
(d) Peanut
Dom*N*Inoc, p= 0.43
(i) Peanut
(e) Soybean
Dom*N*Inoc, p= 0.01
(j) Soybean
Response of shoot mass to rhizobial inoculation
Shoot mass (g)
0.0 0.01 0.1 0.2 0.6 1.5
Nitrogen fertiliser (g N plant-1 as NH4NO3)

Fig. 1

1099

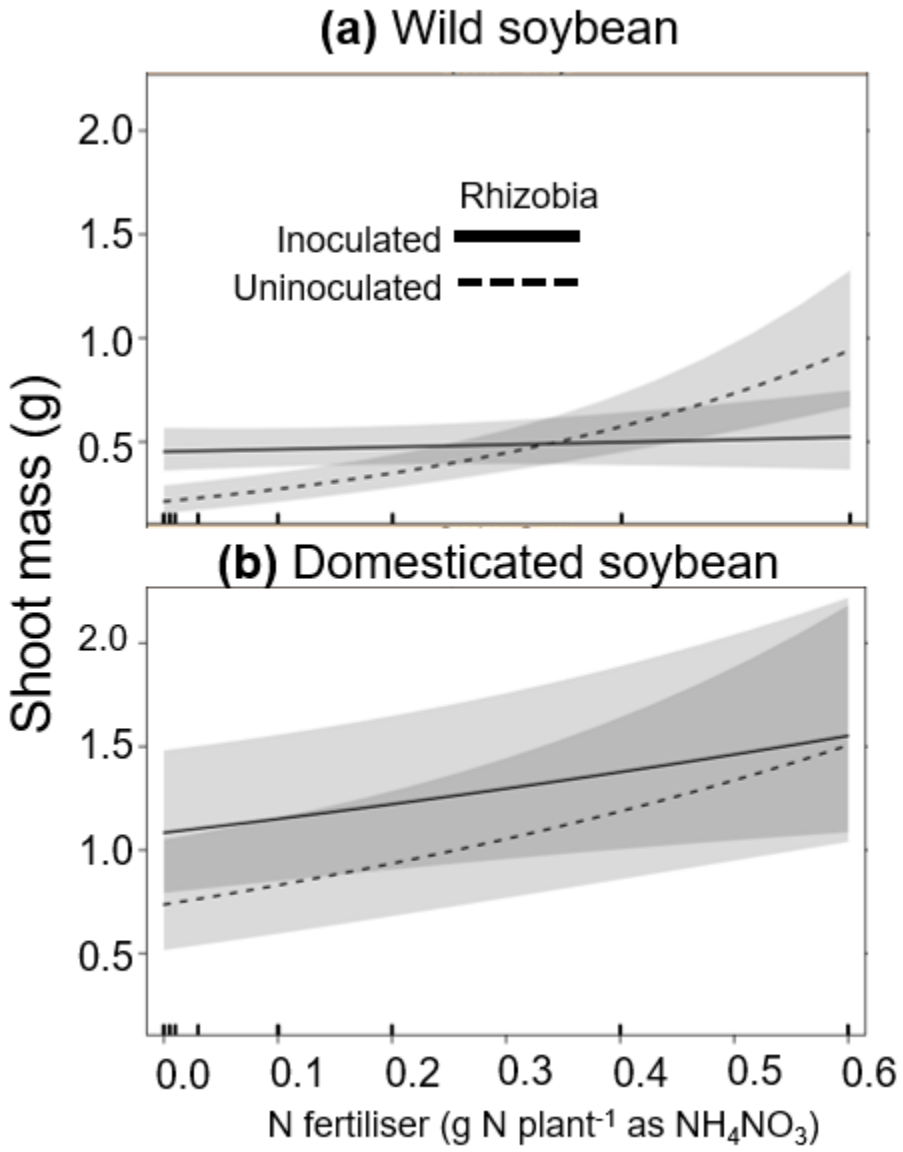
(a) Wild soybean
Rhizobia
Inoculated
Uninoculated
2.0
1.5
1.0
0.5
(b) Domesticated soybean
Shoot mass (g)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
N fertiliser (g N plant-1 as NH4NO3)

Fig. 2

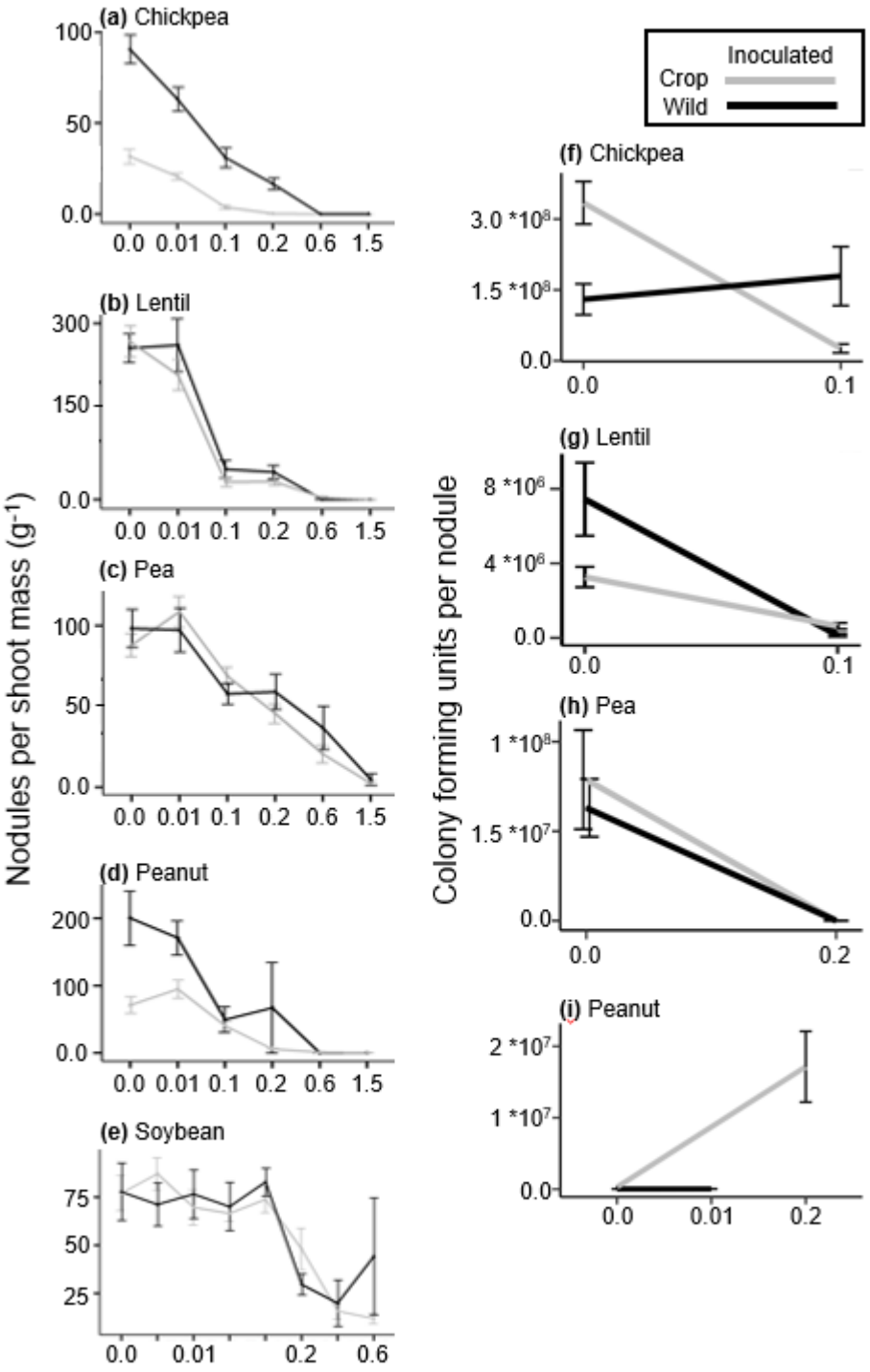
(a) Chickpea
(b) Lentil
(c) Pea
(d) Peanut
(e) Soybean
(f) Chickpea
(g) Lentil
(h) Pea
(i) Peanut
Inoculated
Crop
Wild
Nodules per shoot mass (g-1)
Colony forming units per nodule
Nitrogen fertiliser (g N plant-1 as NH4NO3)

Fig. 3

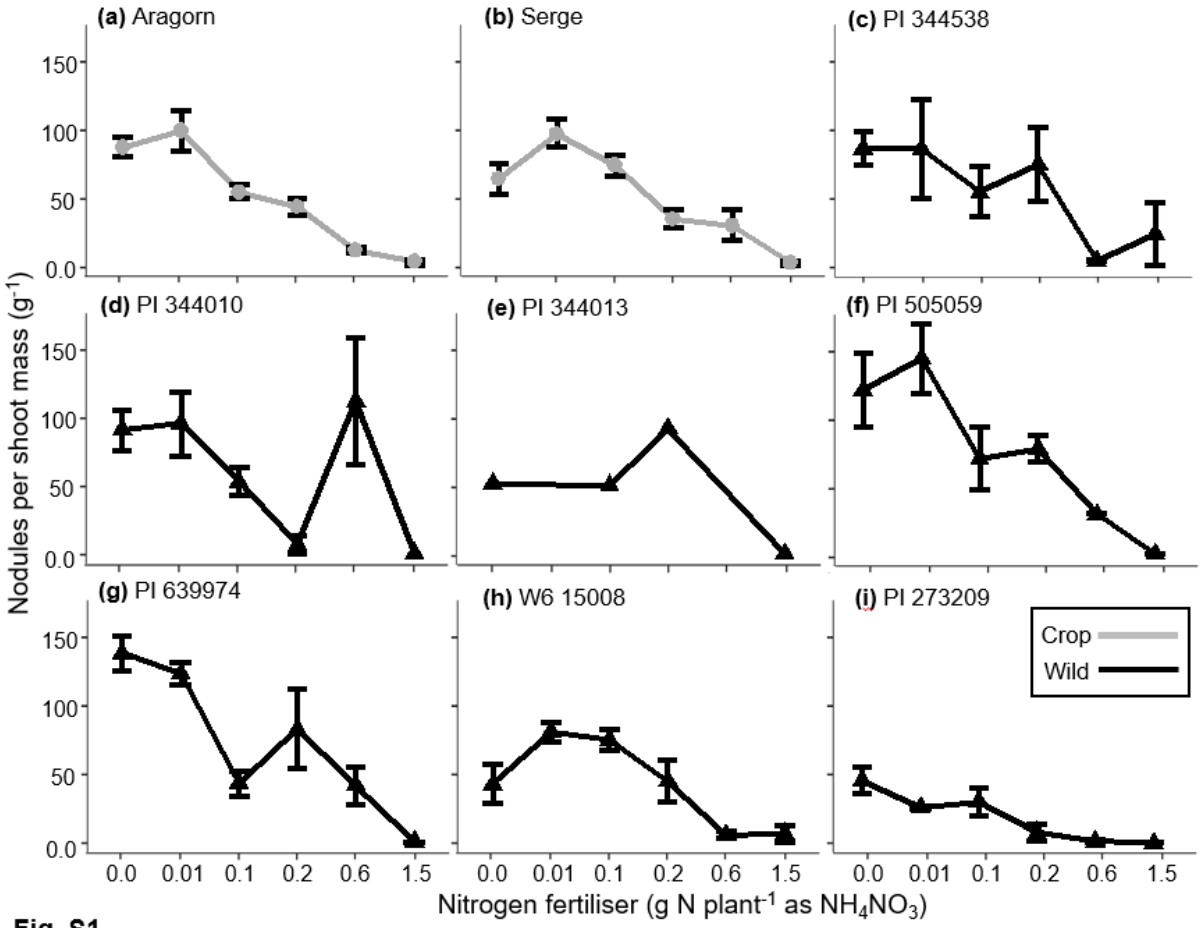
(a) Aragorn
(b) Serge
(c) PI 344538
(d) PI 344010
(e) PI 344013
(f) PI 505059
(g) PI 639974
(h) W6 15008
(i) PI 273209
Crop
Wild
Nodules per shoot mass (g-1)
Nitrogen fertiliser (g N plant-1 as NH4NO3)
0.0
50
100
150
0.0
0.01
0.1
0.2
0.6
1.5

Fig. S1

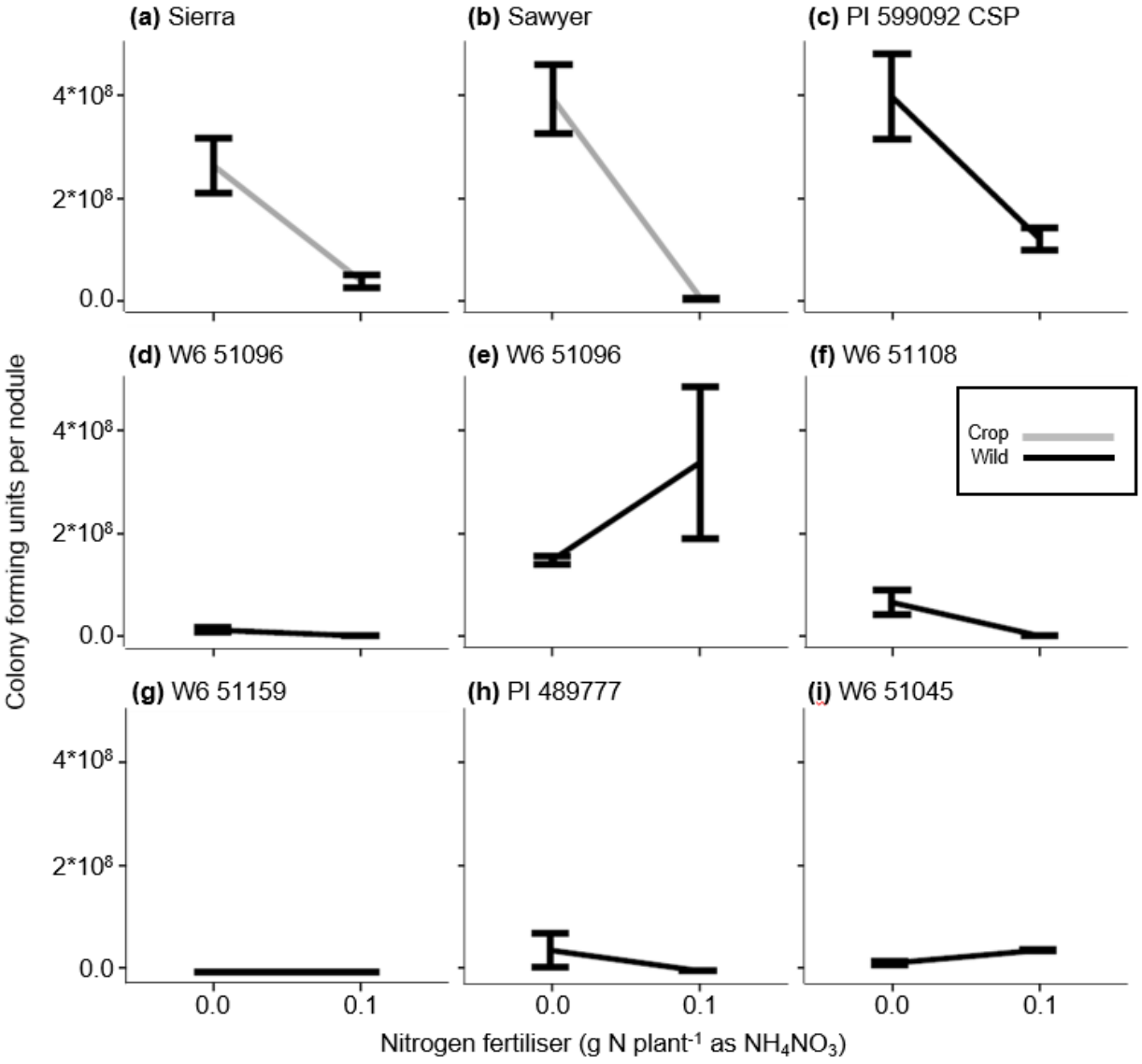
(a) Sierra
(b) Sawyer
(c) PI 599092 CSP
(d) W6 51096
(e) W6 51096
(f) W6 51108
(g) W6 51159
(h) PI 489777
(i) W6 51045
4*10^8
2*10^8
0.0
Colony forming units per nodule
Crop
Wild
0.0
0.1
Nitrogen fertiliser (g N plant-1 as NH4NO3)

Fig. S2