

New Phytologist Supporting Information Methods S1 & S2

Article title: Legumes versus rhizobia: a model for ongoing conflict in symbiosis

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Methods S1: Methods and references used for Figure 2

Overview of the acetylene reduction assay

The acetylene reduction assay (ARA) was popularized by Hardy *et al.* (1968) and has become a mainstay of research into nitrogen fixation rates in root- and stem-nodulating plants. Briefly, the assay harnesses the natural ability of nitrogenase to reduce a variety of substrates, which includes protons (to H₂), dinitrogen (to ammonia), and acetylene (to ethylene). In atmospheres of up to 10% acetylene, nitrogenase preferentially reduces acetylene over other available substrates, and nitrogen fixation rates can be approximated from ARA-determined nitrogenase activity by dividing the latter by some empirically-determined constant (usually 3-4). Nitrogenase activity is not equal to nitrogen fixation rate because, under normal physiological conditions, some nitrogenase activity is used to produce H₂. Parsons *et al.* (1992) assumed an nitrogenase activity-to-nitrogen fixation ratio of 4:1 and calculated the ARA value that would be required to sustain the known growth rate of a *Sesbania rostrata* seedling of known nitrogen content; they found their ARA prediction (293 $\mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ h}^{-1}$) closely matched their empirical ARA measurements (270-280 $\mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ h}^{-1}$), supporting the utility of this assay for approximating nitrogen fixation rates.

The acetylene reduction assay has been critiqued because common assay conditions increase the resistance of the nodular oxygen diffusion barrier, which artificially reduces nitrogenase activity (Minchin *et al.*, 1983, 1986). Problematic assay conditions include disrupting the tissues being sampled (i.e., removing shoots from roots or removing nodules from roots) and incubating tissues in acetylene for longer than 10 minutes, although this “acetylene-induced decline” in nitrogenase activity depends on plant species, inoculum, and assay conditions (Vessey, 1994).

Literature search

We extracted measurements of nitrogenase activity from published acetylene reduction assays that used either intact plants, nodulated roots or stems, or excised nodules. We used Web of Science search terms ‘nitrogenase’ + ‘nodule’ and examined publications from January 2013 to August 2017. To increase coverage of specific taxa, we also searched tribal or generic names in conjunction with the search term “acetylene” and examined publications dating back to 1987.

Extracting acetylene reduction assay (ARA) data from publications

We used one ARA value per plant species (or subspecies/cultivar) per publication to account for data from the same experiment likely exhibiting more similarity than data from different experiments. In studies that published multiple ARA values for the same species (or subspecies/cultivars), as when ARA was measured in different treatments or over a growing season, we used the highest reported ARA value for the species (or subspecies/cultivar) or the value of the control treatment when stressors/manipulations had been applied. When data had to be extracted from a figure, we manually measured the height of bars/points and standardized to experimental units using the scale bar. Of 199 references uncovered during the literature search, we retained 215 data points from 106 references. References were excluded if we found that ARA was performed on bacterial cultures (instead of nodules) or if we could not standardize the reported ARA units to common units (see below).

Standardizing ARA data to common units

ARA data were standardized to units of $\mu\text{mol ethylene produced g}^{-1} \text{ nodule dry weight (DW) h}^{-1}$ to be presented in Figure 2 as specific nitrogenase activity (SNA). When ARA data were originally

reported per nodule fresh weight (FW), we adjusted to DW by multiplying by 0.25 (assuming nodule DW was 25% nodule FW; we found nodule DW varied from 10-35% of nodule FW based on studies where nodule DW and FW were presented together). When ARA data were reported per plant or per nodule, we adjusted to per g nodule DW using nodule mass data reported in the same publication.

Assigning plant genera to clades

Plant genera were assigned to one of four clades of root-nodulating plants according to the phylogenies in Doyle (2011) and Lewis (2005):

Non-legumes: includes *Parasponia* and actinorhizal taxa (Rosales, Cucurbitales, and Fagales)

Early-diverging legumes: includes MCC clade, dalbergioids (s.l.), and genistoids (s.l.)

Warm-season legumes: includes milletiods (s.l.) and Tribe Indigoferae

Cool-season legumes: includes robiniods (s.l.) and the IRLC legumes

Assigning nodule trait values to plant genera

We assigned each plant genus one of two values for each of four nodule traits, as follows:

Infection method	R = root hair infection, intracellular infection	C = crack entry, epidermal infection, intercellular infection
Nodule development	D = determinate growth	I = indeterminate growth
Symbiont sequestration	F = fixation threads, persistent infection threads, N-fixing hyphae (for actinorhizal plants)	S = symbiosomes
Symbiont differentiation	N = nonterminal, reversible, nonswollen bacteroids	T = terminal, irreversible, swollen/elongated bacteroids, bacteroids with reduced viability

We searched the literature for evidence of which trait values occurred in each genus in our dataset. Publications used to support trait value assignments are found in Table S1B. In many cases we could not find publications supporting specific genera, and so we inferred trait values from related taxa (also see Table S1B). Occasionally a genus showed evidence for both trait values; these were indicated as ‘mixed’ (M).

Testing effects of tissue treatment on nitrogenase activity

For all analyses, specific nitrogenase activity values were log-transformed to improve normality. All statistics were performed in JMP Pro 13 (SAS Institute Inc., Cary, NC, USA).

To address the concern that nitrogenase activity declines after disrupting assayed tissue, we categorized each data point with regard to tissue treatment prior to ARA measurement: ‘nodule’ (n = 80) included nodules excised from roots with or without a portion of the subtending root attached, ‘roots or stems’ (n = 79) included nodulated roots (or nodulating stems for stem-nodulating species) detached from the rest of the plant, and ‘intact plants’ (n = 36) included whole plants measured in or out of their growth medium. SNA varied significantly

by tissue treatment ($F_{2,193} = 3.3992$, $P = 0.0354$), with intact plants tending to have greater nitrogenase activity than roots or stems, which tended to have greater nitrogenase activity than nodules. Pairwise differences between tissue treatments were not significant, but we confirm the general trend that less-disrupted tissues exhibit greater levels of nitrogenase activity.

Testing effects of acetylene incubation time on nitrogenase activity

To address the concern that nitrogenase activity declines after exposing tissues to acetylene for more than 10 minutes, we assembled data for the amount of time plant tissues were incubated in acetylene prior to measuring ethylene production. In several cases, ethylene was measured at several time points after acetylene was added and the reported SNA values were not tied to specific time points. When multiple incubation times were available for a data point, we chose the shortest incubation time. The 180 SNA data points for which we could collect acetylene incubation time values ranged from 0.75 minutes to 1140 minutes. There was no significant effect of acetylene incubation time on SNA ($P = 0.2285$), but when the six data points with incubation times greater than 240 minutes were excluded, there was a significant negative relationship ($R^2 = 0.052$, $P = 0.0014$). We next binned each data point into acetylene incubation times of 0.75-10 minutes ($n = 24$), 11-30 minutes ($n = 68$), 31-60 minutes ($n = 62$), and 61-1440 minutes ($n = 26$) and found that SNA varied significantly among incubation time bins ($F_{3,177} = 9.1479$, $P < 0.0001$). Incubation times of 0-10 minutes had significantly greater SNA than any other time bin, which did not differ from each other. Thus, we confirm the trend that nitrogenase activity decreases after more than 10 minutes of exposure to acetylene.

Testing effects of plant clade and nodule traits on nitrogenase activity (Methods S1 Table 1)

To account for variable numbers of data points within each plant genus (ranging from 1-30; see Table S1B), we calculated genus-level SNA means and used this smaller dataset to examine SNA variation among plant clades and nodule traits using ANOVA and independent-samples t -tests, respectively.

Using the entire dataset ('all data,' 58 genus means from 215 data points), we did not detect significant variation in SNA among clades or between alternative trait values for any of the four nodule traits we tabulated (infection method, nodule development, symbiont sequestration, or symbiont differentiation). Next, we re-calculated genus means using just data points collected under more optimal ARA conditions (i.e., from intact plants, or under acetylene incubation times of up to 10, 30, or 60 minutes). We repeated our analyses using these filtered datasets and still failed to find differences in SNA among clades or nodule types, suggesting that plant evolution has not significantly shaped nitrogenase activity. However, these results could also be due to the loss of statistical power from the smaller sample sizes in these filtered datasets. We are thus cautious in interpreting these negative results too strongly and support future research on this important question.

Testing effects of nodule traits on nitrogenase activity within each plant clade (Methods S1 Table 2)

For each nodule trait, we examined clades in which each trait value occurred in at least three genera (excluding genera marked with 'M' or 'U') and tested for effects of alternative trait values on SNA using an independent-samples t -test. When filtered datasets were used (0.75-10 minute or 0.75-60 minute acetylene incubations), we found no significant effects of nodule trait values on nitrogenase activity within any clade. When the entire dataset was used, we found that non-legumes with root hair infection had significantly greater nitrogenase activity than nonlegumes with crack infection (Methods S1 Table 2).

Methods S1 Table 1. Effects of plant clade and nodule traits on nitrogenase activity using different data subsets. For each embedded table, “n” refers to the number of plant genera in each level of each tested factor.

	Intact plants ONLY 20 genera (from 36 data points)	C₂H₂ incubation times 0.75-10 min ONLY 13 genera (from 24 data points)	C₂H₂ incubation times 0.75-30 min ONLY 34 genera (from 92 data points)	C₂H₂ incubation times 0.75-60 min ONLY 53 genera (from 154 data points)	All Data 58 genera (from 215 data points)	
Plant Clade	$F_{3,17} = 0.5009, P = 0.6869$					
	$F_{3,10} = 0.3810, P = 0.3810$					
	$F_{3,31} = 0.2650, P = 0.8500$					
	$F_{3,50} = 0.6251, P = 0.6022$					
	$F_{3,55} = 1.5341, P = 0.2162$					
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
Non-leg	7	1.46	4	2.10	9	1.33
Early leg	4	1.56	2	3.29	7	1.23
Warm leg	6	1.21	3	1.73	11	1.14
Cool leg	3	0.23	4	1.91	7	1.43
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
Non-leg	10	0.93	12	0.82	10	0.86
Early leg	16	1.38	17	1.30	16	1.38
Warm leg	18	1.02	19	0.83	18	1.02
Cool leg	9	0.86	10	0.46	9	0.86
Infection method	$t = 1.03, P = 0.3205$					
	$t = 0.41, P = 0.6893$					
	$t = 1.70, P = 0.1005$					
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
R	12	1.52	9	2.38	22	1.47
C	5	0.66	2	2.09	9	1.03
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
R	33	1.31	34	1.10	33	1.31
C	16	0.85	18	0.78	16	0.85
Nodule development	$t = -0.01, P = 0.9892$					
	$t = 2.13, P = 0.0594$					
	$t = 0.37, P = 0.7116$					
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
I	13	1.15	7	2.61	21	1.31
D	6	1.16	5	1.48	12	1.22
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
I	34	1.16	39	0.93	34	1.16
D	18	0.94	18	0.83	18	0.94
Symbiont compartmentalization	$t = -0.47, P = 0.6477$					
	$t = 0.09, P = 0.9331$					
	$t = -0.26, P = 0.7958$					
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
F	7	1.46	4	2.10	9	1.33
S	12	1.10	9	2.16	24	1.26
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
F	11	0.84	13	0.75	11	0.84
S	40	1.19	43	0.98	40	1.19
Symbiont Differentiation	$t = -1.08, P = 0.2957$					
	$t = 0.48, P = 0.6395$					
	$t = 1.16, P = 0.2551$					
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
T	11	0.89	7	2.27	16	1.41
N	9	1.62	6	1.99	17	1.13
	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)	n	Mean log ₁₀ (SNA)
T	24	0.92	27	0.70	24	0.92
N	27	1.26	29	1.10	27	1.26

R = root hair infection, C = crack infection, I = indeterminate nodule development, D = determinate nodule development, F = fixation threads, S = symbiosomes, T = terminal symbiont differentiation, N = nonterminal symbiont differentiation

Methods S1 Table 2. Comparison of nitrogenase activity between alternative nodule trait values for each main clade of N-fixing plants studied here, using all available data. For each embedded table, “n” refers to the number of plant genera with the indicated trait value. When there were no genera having a particular trait value, we filled the “mean” field with “NA” (not applicable). When a particular trait value was only found in one genus, preventing a *t*-test from being performed, we also filled the *t*-test field with NA.

All Data				
Infection method	Clade	n	Mean $\log_{10}(\text{SNA})$	<i>t</i> -test
	Non-leg	R = 5	1.45	<i>t</i> = 2.95
		C = 7	0.38	<i>P</i> = 0.0145
	Early leg	R = 8	1.90	<i>t</i> = 1.48
		C = 7	1.05	<i>P</i> = 0.1627
	Warm leg	R = 14	0.89	<i>t</i> = -0.16
		C = 4	0.99	<i>P</i> = 0.8773
	Cool leg	R = 7	0.37	NA
		C = 0	NA	
	Nodule development	Clade	n	Mean $\log_{10}(\text{SNA})$
Non-leg		I = 12	0.82	NA
		D = 0	NA	
Early leg		I = 14	1.41	<i>t</i> = 0.79
		D = 3	0.79	<i>P</i> = 0.4404
Warm leg		I = 5	0.64	<i>t</i> = -0.43
		D = 14	0.89	<i>P</i> = 0.6695
Cool leg		I = 8	0.41	NA
		D = 1	0.09	
Symbiont compartmentalization		Clade	n	Mean $\log_{10}(\text{SNA})$
	Non-leg	F = 12	0.82	NA
		S = 0	NA	
	Early leg	F = 1	-0.08	NA
		S = 14	1.56	
	Warm leg	F = 0	NA	NA
		S = 19	0.83	
	Cool leg	F = 0	NA	NA
		S = 10	0.46	
	Symbiont differentiation	Clade	n	Mean $\log_{10}(\text{SNA})$
Non-leg		T = 11	0.90	NA
		N = 1	0.04	
Early leg		T = 4	0.76	<i>t</i> = -1.19
		N = 11	1.61	<i>P</i> = 0.2571
Warm leg		T = 5	0.87	<i>t</i> = 0.11
		N = 14	0.81	<i>P</i> = 0.9160
Cool leg		T = 7	0.25	<i>t</i> = -1.27
		N = 3	0.93	<i>P</i> = 0.2408

R = root hair infection, C = crack infection, I = indeterminate nodule development, D = determinate nodule development, F = fixation threads, S = symbiosomes, T = terminal symbiont differentiation, N = nonterminal symbiont differentiation

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Methods S2: Methods and references used for Figure 3

Literature search

Host investment data was measured from published studies containing light or electron micrographs. We used Web of Science to find articles published through August 1st, 2017 which resulted in a total of 85 articles and 577 data points. We limited our search to the years 2013-2017 and searched for the topic 'nodul*'. The search was then refined by '*rhizob*', 'legum*', 'lupin*', 'parasponia', 'aeschynomene*', 'frankia', and 'alder' individually. We were unable to find usable images for parasponia and thus expanded our search by one year to 2012.

Extracting data from literature

Light and electron micrograph figures were analyzed in ImageJ using the published scale bars. Images were excluded if they did not provide scale bars, their resolution was too low, or if the infection was not beneficial due to host, symbiont or environment effects. The proportion of the nodule infected was calculated using light microscopy images containing whole nodule sections. We measured stained plant cells as the infected area and used our best judgement to only measure cells in the nitrogen fixation zone. This value was then divided by the entire nodule section area to get the proportion of the nodule that is infected. Average area of an infected plant cell was calculated as the total area of intact and whole infected cells divided by the total number of whole cells in the light microscopy image. Bacteroid density and bacteroids per symbiosome were calculated using transmission electron microscopy images. For bacteroid density we counted the total number of bacteroids in an image and divided by the area of that image. For bacteroids per symbiosome we counted the total number of bacteroids in the image and divided by the total number of symbiosomes in the image.

Categorizing data

Plant genera were assigned to one of four clades of root-nodulating plants according to the phylogenies in Doyle (2011) and Lewis (2005):

Non-legumes: includes *Parasponia* and actinorhizal taxa (Rosales, Cucurbitales, and Fagales)

Early-diverging legumes: includes MCC clade, dalbergioids (s.l.), and genistoids (s.l.)

Warm-season legumes: includes milletiods (s.l.) and Tribe Indigofereae

Cool-season legumes: includes robiniods (s.l.) and the IRLC legumes

We searched the source publications for evidence of terminal bacteroid differentiation (TBD) versus non-TBD for each host-symbiont combination which generated usable data (Table S2). When bacteroids were swollen, elongated, or had reduced viability after escaping a nodule we considered this as TBD. If we were unable to find evidence of TBD in the original paper, we used Web of Science to search for evidence of TBD for the species of interest. Most data was categorized according to Oono et al. (2010), and in some instances we were unable to find evidence of TBD or non-TBD. These data points were left as unknown and excluded from TBD versus non-TBD analyses.

Data analysis

For each measurement taken we calculated the mean values for each genus. These genus-level mean values were then used to compare all clades using analysis of variance (ANOVA) with a post-hoc Tukey HSD test when applicable. In order to compare the effects of TBD versus non-

TBD we used a Student's t-test on all data in a given measurement. All statistics were performed in JMP Pro 13 (SAS Institute Inc., Cary, NC, USA).

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