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Supplementary information

Temperature sensitivity of woody nitrogen fixation across species and growing temperatures

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Temperature sensitivity of woody nitrogen fixation across species and growing temperatures: Supplementary Information

Plant species, inoculum, and growing conditions

Plants were grown from seeds purchased from online vendors (Sheffield's Seed Company [*Alnus rubra* originating from WA and *Robinia pseudoacacia* originating from Germany; Locke, NY], Rarexoticseeds [*Gliricidia sepium* originating from India; Montreal, QC, CA] and Bountiful Gardens [*Morella cerifera* originating from LA; Willits, CA]) and were inoculated with a nodule slurry and liquid culture from species-specific field-collected nodules. Nodules were collected in FL, OR, FL, and NY for *Morella*, *Alnus*, *Gliricidia* and *Robinia*, respectively. Prior to surface-sterilization, the seed-coat of *Morella* seeds was sanded and cold-treated for 21 days in milled sphagnum moss. Other species did not require pre-treatment prior to surface-sterilization. All seeds were surface-sterilized by submersion in 75% isopropyl alcohol for 10 seconds followed by 3% hydrogen peroxide for 5 minutes. Post surface-sterilization, seeds were rinsed six times with deionized water and submerged in deionized water for 12 hours to imbibe. Seeds were then sown in moist sterilized (autoclaved) growing media (Gran-I-Grit Starter; North Carolina Granite Corporation, NC) in germination trays within each temperature-controlled growth chamber. Upon germination, plants were inoculated with liquid culture and a nodule slurry (see below for details). Once seedlings started fixing N (N fixation estimated by screening seedlings for 5-10 min at 2% C_2H_2; ref. 1) they were transferred to growing media in square 1 L pots.

Upon collection from the field, crushed nodules were used to inoculate a subset of seedlings grown for fresh nodule supply for the experiment. From these seedlings, fresh nodules were collected to prepare inoculum. Nodules were surface-sterilized by vortexing nodules for 60 seconds in a bleach/Contrex lab detergent mix. Post surface-sterilization, nodules were vortexed six times at 20 seconds in deionized water. Nodules were then crushed with a sterile scalpel and cultured on ten yeast mannitol agar plates for each species. Colonies were re-plated from each plate three times before they were placed into yeast mannitol liquid media in culture tubes for one week on a shaker. Liquid media from culture tubes were then combined and plate counts were conducted so that inoculum could be diluted with sterile yeast mannitol liquid media to 5×10^8 cells mL^{-1} for cryopreservation. Inoculum was prepared for cryopreservation by mixing the liquid culture with 30% glycerol and 30% deionized water, placing the mixture in micro-centrifuge tubes, vortexing, and flash-freezing with liquid nitrogen. Following flash-freezing, samples were held in a −30 °C freezer until they were ready to use. The preserved liquid culture was removed from the freezer 2-4 hours prior to inoculating plants to thaw and was diluted to 10^8 cells mL^{-1}. 1 mL, containing 10^8 cells, was then added to each seedling. In addition to inoculating plants with the liquid culture we prepared a nodule slurry for inoculating plants by surface-sterilizing ~5 mL of fresh nodules, crushing the surface-sterilized nodules with a scalpel, diluting the slurry to 15 mL with deionized water and adding 1 mL per seedling. We used this approach to ensure that seedlings had a saturating amount of N-fixing bacteria (liquid culture) and that experimental plants were exposed to a diverse range of bacteria found within nodules in the field (nodule slurry).

Photosynthetic Photon Flux Density (PPFD) during plant growth was approximately 800 µmol m^{-2} s^{-1}, the photoperiod was 14:10 (day:night), relative humidity was 70%, and CO_2 was 400 ppm. Seedlings were grown under low N conditions and were fertilized weekly with 1.5 g N m^{-2} y^{-1} (using NH_4NO_3) and with a modified Hoagland's solution that has all nutrients except N (ref. 2). The addition rate of the modified Hoagland's solution was 0.2 g P m^{-2} y^{-1}, with all other non-N nutrients stoichiometrically balanced to P. Seedlings were grown in trays containing 4 pots each and were watered from the bottom 2-3 times per week so that water was not limiting.

Additional details on N fixation measurements with ARACAS

The incubation chamber was 40.5 L and was made of transparent acrylic. Within the incubation chamber, air was circulated by two explosion-proof muffin fans (MMF24-3B Matticks Industries Inc., Houston, TX). Temperature, controlled by a water bath, was filled with a 1:1 solution of propylene glycol and water (allowing the water bath to cool the solution circulated through the copper coils below 0 °C).

ARACAS can be used to quantify N fixation at any acetylene concentration, including sub-saturating acetylene concentrations (10% is typically considered saturating). Our measurements were made at 2% acetylene to increase safety (>2.5% acetylene is explosive), reduce the potential negative effects of acetylene and ethylene on the plant or bacteria, and optimize the detection threshold for measuring N fixation (3-5 nmol C_2H_4 hr^{-1} with our setup[1]). Acetylene was made in the lab from calcium carbide to improve the precision of measurements because the background ethylene concentration in acetylene tanks is 300-750 times greater than in lab-made acetylene, causing the detection threshold to be 75-600 greater (worse) for tank derived acetylene[1]. For our calculations (equation 1) we used the mean value of k_{eff} (0.0045 [0.0010, 0.0105] hr^{-1} [mean and 95% CI] for our chamber setup[1]) and the mean value of K_{m} for each species and growing temperature because the uncertainty in estimated k_{eff} and K_{m} were assumed to be primarily caused by variation across incubations (for k_{eff}) and individual plants (for K_{m}). For the purposes of this manuscript, we measured how N fixation changed for an individual plant (and for an individual incubation) as a function of temperature or as a function of time at a constant temperature, so the variation in k_{eff} and K_{m} across incubations and plants was not relevant to our calculations.

K_{m} was estimated in independent plants (Supplementary Tables 4, 5) by measuring ethylene production at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 10% acetylene in an ascending order (but with an additional, and final, measurement at 2% acetylene to determine if the rate of ethylene production significantly changed during the course of measurement; this only affected measurements at 40 °C, as discussed below). Ethylene production data were fit with a Michaelis-Menten curve at the species level to estimate V_{max} and K_{m}, with V_{max} as a free parameter that varies for individual plants. Here, V_{max} differs from $V_{\mathrm{max}}(t)$ in equation 1 because it was determined from the set of incubations spanning a range of acetylene concentrations and represents an average rate for an individual plant over stable environmental conditions. K_{m} was estimated for each species (n=4 species) grown at each temperature regime (n=3 growing temperatures) and at measurement temperatures of 20 to 40 °C at 5 °C intervals (n=5-7 measurement temperatures). In most cases (all species except for *Morella*) there was a significant decline in ethylene production at 40 °C during the measurement of ethylene production as a function of acetylene. As a result, K_{m} estimated at 40 °C was only used for *Morella* grown at the warm growing temperature. K_{m} was estimated in two separate ways: as a linear function of the measurement temperature (with a separate intercept for each growing temperature) and as a fixed parameter that is independent of the growing and measurement temperatures. Where K_{m} varied with temperature, the mean of the growing temperature-specific intercepts was used for all growing temperatures with a species. The 95% CI for the intercept was determined via parametric bootstrapping[3] a normal distribution. The N fixation temperature response curves in the main text do not allow for a temperature effect on K_{m}. N fixation temperature response curves where K_{m} can vary with temperature are presented in Supplementary Tables 11, 12 and Supplementary Figs. 15-17. Where K_{m} varied with temperature, optima were at higher temperatures and rates at warm temperatures were greater than in the version without a temperature-dependent K_{m}, so the results in the main text err on the side of underestimating temperature optima. The same models (i.e., modified beta with linear acclimation of all parameters for *Morella*, *Alnus*, and *Robinia* and modified beta with linear acclimation of T_{min} and T_{opt} for *Gliricidia*) were used for the N fixation temperature responses where K_{m} can vary with temperature. Because N fixation was often low below 20 °C and declined rapidly at high temperatures, K_{m} was difficult to estimate beyond our temperature range (20-35 or 20-40 °C). Where

the linear function of K_m with respect to the measurement temperature was used, we extrapolated K_m values to temperatures below 20 °C and above 35 or 40 °C.

$\frac{dE(t)}{dt}$ was corrected for background rates of ethylene production in the absence of acetylene using the following steps. First, $\frac{dE(t)}{dt}$ at 0% acetylene (from the same incubations used to determine K_m; but also including the 0% acetylene incubations at 40 °C) was linearly regressed as a function of incubation temperature to test for a temperature effect on the rate of background ethylene production. This test involved two linear regressions. One with a common slope across species and a separate intercept for each species. The other with a common slope across growing temperatures and a separate intercept for each growing temperature. In both cases the slope was not significantly different from 0, so we assumed that temperature had no effect on background rates of ethylene production. Second, we calculated the background rate of ethylene production prior to injecting acetylene for each individual on which we measured a temperature response of N fixation and each individual that was measured at a constant temperature through time. This background rate was subtracted from the rate of ethylene production following the injection of acetylene ($\frac{dE(t)}{dt}_{\text{used}} = \frac{dE(t)}{dt}_{\text{measured}} - \frac{dE(t)}{dt}_{\text{produced without acetylene}}$).

Prior to analysis, data were visually inspected and artifacts (anomalously low or high ethylene points) were removed (i.e., 2.0 % of N fixation temperature response data). Artifacts were more common under conditions of high humidity and could have occurred due to disruptions in the flow rate through tubing connecting the incubation chamber to the Picarro ethylene analyser (i.e., as a result of condensation in the tubing).

Conversion factor (i.e., N:C_2H_4) calculations

As noted in the Methods, calculations of the conversion factor used to convert nitrogenase activity to fixed N follow ref. 4. First, the isotopic content (in atom % ^{15}N) of $^{15}N_2$ in the atmosphere of the syringe during the incubations was calculated according to:

$$\%^{15}N_{\text{atm,inc}} = \left(\%^{15}N_{\text{gas}} \times f_r\right) + \left(\%^{15}N_{\text{env}} \times (1 - f_r)\right), \quad (S1)$$

where $\%^{15}N_{\text{gas}}$ is the percent of ^{15}N atoms in the N_2 added to the syringe, f_r is the fraction of the syringe's headspace replaced with the ^{15}N enriched gas mixture ($^{15}N_2$ and O_2), and $\%^{15}N_{\text{env}}$ is the percent of ^{15}N atoms in the environment. For our incubations $\%^{15}N_{\text{gas}} = 98\%$, $f_r = 0.2$, and $\%^{15}N_{\text{env}} = 0.3663\%$, so $\%^{15}N_{\text{atm,inc}} = 19.9\%$. Next, the % N in the nodules that was derived from N fixation during the $^{15}N_2$ incubation was calculated as:

$$\%N_{\text{fix,inc}} = \frac{\%^{15}N_{\text{samp}} - \%^{15}N_{\text{env}}}{\%^{15}N_{\text{atm,inc}} - \%^{15}N_{\text{env}}} \times 100, \quad (S2)$$

where $\%^{15}N_{\text{samp}}$ is the % of N atoms that were ^{15}N in the nodules following the $^{15}N_2$ incubation. N fixation rates (in units of g N g^{-1} dry nodule min^{-1}) were then calculated as:

$$SNF = \frac{N_{\text{samp}} \times \%N_{\text{fix,inc}}}{M \times I}, \quad (S3)$$

where N_{samp} was the mass of N (in grams) of the nodules for each sample, M was the total nodule mass of each sample (also in grams), and I was the incubation time (i.e., 30 minutes). Following unit conversion to mol N g^{-1} dry nodule hr^{-1}, we calculated the ratio of fixed N to $V_{\max}(t)$ (units of mol C_2H_4 g^{-1} dry nodule hr^{-1}) as mol N:mol C_2H_4. These data are presented at the level of each species (Supplementary Table 13).

To test for an effect of temperature on the conversion factor, data were linearly regressed as a function of incubation temperature with a common slope across species and a separate intercept for each species. This was done for both linear and log-transformed conversion factor data. In both cases, the slope of the regression was not significant, so we used a conversion factor that was constant across temperature. Outliers below 1 and above 7 (4±3, of which 26% of data were outside of this range; 4 is the theoretical conversion factor) were removed prior to analysis.

Additional details on modeling the possible change in N fixation due to prolonged exposure to high temperature

We fit a rising and falling function, using a piecewise regression approach, to the N fixation measurements made at constant temperatures for 6-hour intervals. This was done separately for each species at each growing temperature. This differs from our approach with collecting the temperature response curve data, where, as noted in the Methods, we kept conditions constant for 15 minutes at 10 °C prior to starting the temperature ramp. N fixation rates were typically very low at 10 °C, and even sometimes below the detection threshold, thus preventing us from using the approach described here for modeling the N fixation temperature response curves. The rising function is a monomolecular function[5] and accounts for the initial increase in N fixation rates (e.g., the role of diffusion through nodules) upon the injection of acetylene and the falling function is a modified negative sigmoid function that allows N fixation to equilibrate above zero:

$$y = \begin{cases} a(1 - e^{-qt}) \; if \; t < t_{\mathrm{break}} \\ a\left(\dfrac{1-d}{1+be^{ct}} + d\right) \; if \; t \geq t_{\mathrm{break}} \end{cases}. \qquad (S4)$$

In equation S4 the variables are y, the N fixation rate (mol N_2 hr^{-1} $plant^{-1}$ or mol C_2H_4 hr^{-1} $plant^{-1}$; these units are related by a constant [Supplementary Table 13]), and t, time (hr). The parameters are a (mol N_2 hr^{-1} $plant^{-1}$ or mol C_2H_4 hr^{-1} $plant^{-1}$), which is the saturated (maximum) N fixation rate for the rising function and the starting (maximum) N fixation rate for the falling function; q (hr^{-1}), which controls the rate of increase in the rising function; b (unitless) and c (hr^{-1}), which control the shape and rate over which N fixation declines over time; and d (unitless), the proportion of the maximum rate at which N fixation equilibrates. The breakpoint, t_{break} (hr), was fit separately for each individual measurement of N fixation through time and at a constant temperature. The median value of t_{break} was 0.34 hr. In cases where model convergence was not achieved, the breakpoint was visually determined.

To account for how the shape of the N fixation temperature response curve was affected by time and temperature, we focused on the falling function and how b and d changed as a function of the difference between the predicted optimum temperature (T_{opt}) from the species- and growing temperature-specific N fixation temperature response curves and the measurement temperature (T_{meas}; $\tau = T_{\mathrm{opt}} - T_{\mathrm{meas}}$). Redefining temperature in this way allows acclimation to affect how quickly N fixation rates drop at a specific absolute temperature for a given species and growing temperature. τ was also a better predictor than T_{meas} for how N fixation changed as a function of time. After redefining temperature, b and d were modelled with all species and growing temperatures pooled as:

$$b(\tau) = e^{\alpha_{\mathrm{b}} e^{\beta_{\mathrm{b}} \tau}} - 1, \qquad (S5)$$

$$d(\tau) = \frac{1}{(1 + \alpha_{\mathrm{d}} e^{\beta_{\mathrm{d}} \tau})}. \qquad (S6)$$

Equation S5 allows b, which cannot be negative, to increase nonlinearly with τ. It can also handle cases where $b = 0$ (which occurred when there was no decline in the rate during the measurement interval). This function fit the data better than a simple exponential function. Equation S6 has a negative sigmoidal shape and is bounded between 0 and 1, as it represents the long-term equilibrium value of the N fixation rate (e.g., if the measurement interval was infinite). All parameters (i.e., α_{b}, β_{b}, α_{d}, and β_{d}) are

positive in equation S5 and S6. We removed data from three individuals for calculating $b(\tau)$, $d(\tau)$, and c due to b values that were between 5 and 7 (which we considered outliers; all other values were between 0 and 0.92). We then plugged $b(\tau)$ and $d(\tau)$ and the median estimate of c into the falling function in equation S4 and normalized to 1 at $t = 0$ (which cancels a) to show how N fixation drops as a function of time and temperature:

$$y_{normalized} = \left(\frac{1 + b(\tau)}{1 + b(\tau)d(\tau)}\right)\left(\frac{1 - d(\tau)}{1 + b(\tau)e^{ct}} + d(\tau)\right). \quad (S7)$$

Equation S7 (which is a falling function; figure 4a) was then used together with equation 5 to simulate the N fixation temperature response curves for different hypothetical rates of heating. First, we determined how much N fixation declined due to our heating rate of *c*. 6 °C hr^{-1}. With this, we simulated what each temperature response datapoint would be had it not been affected by the time spent at all temperatures below that point. We then refit the N fixation temperature response function (equation 5 with the possibility of linear acclimation of parameters to the growing temperature) to these simulated data. Finally, equation S7 was reapplied to simulate N fixation temperature response curves for different hypothetical rates of heating. Parameter values for equations S5-S7 are available in Supplementary Table 14.

Information about the source of data for the Houlton et al. (2008) temperature response function

The Houlton *et al.* (2008) temperature response function (ref. 6; plotted in Fig. 2 and Supplementary Fig. 16) is a normal function fit to data from ref. 7–12. Of these six studies, one measured the temperature response of symbiotic N fixation[10], while the other five measured temperature responses of asymbiotic N fixation. The symbiotic N fixation study was for an herbaceous plant species (*Trifolium vesiculosum*), where the integrated amount of N that was fixed for plants grown at different temperatures was measured (thus the temperature response confounds acclimation with the instantaneous temperature response). We then searched for the latitudinal origin of the N-fixing organisms from the six studies[7–12]. In some, latitude was listed[8,11]. In other cases, we determined latitude from listed location names[7,9,10,12]. For ref. 9, N-fixing organisms were collected from two locations in New South Wales, Australia: Gunnedah (31.0 °S) and Cowra (33.8 °S). We used the mean of the two locations (32.4 °S) as the latitude for ref. 9. For ref. 10, two strains of *Rhizobium* were utilized, one from Research Seeds Urbana, St. Louis, MO (38.6 °N) and one from Lipha Tech, Inc., Milwaukee, WI (43.1 °N). We used the mean (40.9 °N) of the two locations as the latitude for ref. 10. For ref. 12, N-fixing organisms were collected from multiple sites across Finland, which spans *c*. 60-70 °N. We used the 65 °N as the latitude for ref. 12.

Supplementary Table 1 | Parameters for the temperature response of N fixation with acclimation to growing temperature. Parameters are for equations 5-6, the modified beta function with linear acclimation of all parameters to the growing temperature. These values and equations, along with mean growing temperatures as inputs, form the curves and parameter values seen in Figs. 1-3. See Supplementary Tables 6, 8 for ΔAIC values used for model selection. *Morella* and *Gliricidia* were binned for the tropical function and *Alnus* and *Robinia* were binned for the temperate function.

	T_{min} (95% CI)		T_{opt} (95% CI)		T_{max} (95% CI)	
Plant Species	Slope [°C °C^{-1}]	Intercept [°C]	Slope [°C °C^{-1}]	Intercept [°C]	Slope [°C °C^{-1}]	Intercept [°C]
Morella cerifera	1.158	−19.40	0.783	14.56	0.184	40.89
(tropical, actinorhizal)	(1.137, 1.178)	(−19.87, −18.92)	(0.780, 0.786)	(14.48, 14.63)	(0.179, 0.189)	(40.78, 41.00)
Alnus rubra	0.512	−20.60	0.066	31.20	0.030	41.85
(temperate, actinorhizal)	(0.452, 0.543)	(−21.43, −19.02)	(0.064, 0.067)	(31.17, 31.25)	(0.028, 0.032)	(41.80, 41.91)
Gliricidia sepium	0.865	−13.94	0.416	23.89	*	45.48
(tropical, rhizobial)	(0.825, 0.905)	(−14.88, −12.99)	(0.411, 0.421)	(23.78, 24.00)		(45.46, 45.51)
Robinia pseudoacacia	0.936	−15.84	0.024	31.44	0.052	44.70
(temperate, rhizobial)	(0.917, 0.958)	(−16.42, −15.32)	(0.021, 0.028)	(31.35, 31.53)	(0.047, 0.056)	(44.61, 44.80)
Tropical	0.932	−14.87	0.574	19.72	*	45.35
	(0.913, 0.953)	(−15.37, −14.36)	(0.571, 0.577)	(19.65, 19.79)		(45.33, 45.37)
Temperate	0.697	−14.93	0.047	31.24	0.009	43.82
	(0.676, 0.716)	(−15.48, −14.43)	(0.045, 0.049)	(31.19, 31.28)	(0.006, 0.011)	(43.76, 43.88)

*Displaying parameters of a model with acclimation of T_{min} and T_{opt}, but not T_{max}

Supplementary Table 2 | Parameters for the temperature response of photosynthesis. Parameters are for equations 5-6, the modified beta function with linear acclimation of T_{opt} to the growing temperature but no acclimation of T_{min} or T_{max}. These values and equations, along with mean growing temperatures as inputs, form the curves and parameter values seen in Figs. 2-3 and Supplementary Figs. 1-4 and 16-17. See Supplementary Tables 7 and 9 for ΔAIC_c values used for model selection.

	T_{min} (95% CI)	T_{opt} (95% CI)		T_{max} (95% CI)
Plant Species	[°C]	Slope [°C °C^{-1}]	Intercept [°C]	[°C]
A_{275}				
Morella cerifera	3.11	0.238	22.83	41.67
(tropical, actinorhizal)	(−1.37, 7.09)	(0.079, 0.385)	(18.81, 26.80)	(41.05, 42.23)
Alnus rubra	1.12	0.339	19.10	42.27
(temperate, actinorhizal)	(−2.63, 4.92)	(0.230, 0.454)	(16.47, 21.68)	(41.81, 42.77)
Gliricidia sepium	8.14	0.637	12.90	42.06
(tropical, rhizobial)	(7.02, 9.34)	(0.483, 0.789)	(9.26, 16.62)	(41.53, 42.53)
Robinia pseudoacacia	5.51	0.194	22.88	41.23
(temperate, rhizobial)	(3.10, 7.89)	(0.066, 0.327)	(19.52, 26.20)	(40.85, 41.63)
A_{sat}				
Morella cerifera	−0.50	0.163	25.99	42.30
(tropical, actinorhizal)	(−6.87, 5.93)	(0.026, 0.310)	(22.23, 29.55)	(41.62, 42.94)
Alnus rubra	−2.32	0.417	18.45	42.98
(temperate, actinorhizal)	(−8.45, 3.71)	(0.312, 0.522)	(15.95, 21.07)	(42.37, 43.53)
Gliricidia sepium	10.12	0.553	14.26	42.66
(tropical, rhizobial)	(10.08, 10.16)	(0.381, 0.730)	(9.92, 18.44)	(42.02, 43.28)
Robinia pseudoacacia	3.94	0.160	24.62	42.33
(temperate, rhizobial)	(0.54, 7.40)	(0.022, 0.302)	(21.15, 28.14)	(41.74, 42.91)

Supplementary Table 3 | Parameters for the temperature response of N fixation without acclimation to growing temperature. Parameters are for equation 5, the modified beta function. Species-level functions and the tropical and temperate functions are from Supplementary Table 1, with growing temperature set to 23.5 °C for *Morella, Gliricidia*, and the tropical function and 18.5 °C for *Alnus, Robinia*, and the temperate function. These growing temperature differences are meant to account for the tropical species typically experiencing warmer growing conditions than temperate species. The overall function was fit with all species and growing temperatures binned and without the possibility of acclimation, in contrast to the parameters shown in Supplementary Table 1.

	T_{min} (95% CI)	T_{opt} (95% CI)	T_{max} (95% CI)
Plant Species	[°C]	[°C]	[°C]
Morella cerifera	7.81	32.94	45.21
(tropical, actinorhizal)	(7.67, 7.94)	(32.92, 32.97)	(45.19, 45.23)
Alnus rubra	−11.12	32.42	42.40
(temperate, actinorhizal)	(−11.55, −10.68)	(32.40, 32.44)	(42.39, 42.42)
Gliricidia sepium	6.40	33.66	45.48
(tropical, rhizobial)	(6.21, 6.58)	(33.63, 33.68)	(45.46, 45.51)
Robinia pseudoacacia	1.48	31.89	45.66
(temperate, rhizobial)	(1.32, 1.63)	(31.87, 31.92)	(45.63, 45.68)
Tropical	7.04	33.22	45.35
	(6.93, 7.17)	(33.20, 33.23)	(45.33, 45.37)
Temperate	−2.04	32.10	43.98
	(−2.23, −1.85)	(32.08, 32.12)	(43.96, 44.00)
Overall	1.41	32.71	44.83
	(1.24, 1.51)	(32.70, 32.73)	(44.81, 44.84)

Supplementary Table 4 | The Michaelis-Menten half-saturation constant (K_m) for measuring nitrogenase activity with acetylene. These K_m estimates (in units of % acetylene) were used to calculate the values shown in the figures in the main text and do not allow for an effect of measurement temperature nor growing temperature.

	K_m (95% CI)
Plant Species	[%]
Morella cerifera	2.13 (1.91, 2.37)
Alnus rubra	1.65 (1.46, 1.90)
Gliricidia sepium	2.77 (2.60, 2.96)
Robinia pseudoacacia	2.39 (2.23, 2.53)

Supplementary Table 5 | The Michaelis-Menten half-saturation constant (K_m) for measuring nitrogenase activity with acetylene that includes the possibility of a linear effect with measurement temperature. K_m estimates (in units of % acetylene) were significantly correlated with measurement temperature in all species except *Alnus rubra*. K_m for *Alnus rubra* represents pooled data from all measurement and growing temperatures. See Supplementary Tables 11, 12 and Supplementary Figs. 15-17 for N fixation temperature response curves using these K_m values.

	Intercept (95% CI)	Slope (95% CI)
Plant Species	[%]	[% °C^{-1}]
Morella cerifera	0.06 (−0.36, 0.47)	0.071 (0.048, 0.097)
Alnus rubra	1.65 (1.46, 1.90)	NS
Gliricidia sepium	1.29 (0.71, 1.88)	0.038 (0.012, 0.078)
Robinia pseudoacacia	1.26 (0.86, 1.64)	0.046 (0.030, 0.072)

Supplementary Table 6 | ΔAIC values for temperature response functions of N fixation. The modified beta function (equation 5) was the best function for modeling the temperature response of N fixation. Functions were fit at the treatment level (species x growing temperature) with one negative log-likelihood function for all species and growing temperatures.

Response Variable	Model	ΔAIC
N-fixation (n = 661225)	**Modified beta**	**0**
	Peaked Arrhenius	67201
	Quadratic	315073
	Normal	135651

Supplementary Table 7 | ΔAIC_c values for temperature response functions of photosynthesis. The modified beta function (equation 5) was the best function for modeling the temperature response of both A_{275} and A_{sat}. Functions were fit at the treatment level (species x growing temperature) with one negative log-likelihood function for all species and growing temperatures.

Response Variable	Model	ΔAIC_c
A_{275} (n = 292)	**Modified beta**	**0**
	Peaked Arrhenius	94.0
	Quadratic	212.7
	Normal	27.8
A_{sat} (n = 300)	**Modified beta**	**0**
	Peaked Arrhenius	54.8
	Quadratic	211.6
	Normal	19.8

Supplementary Table 8 | ΔAIC values for the possibility of linear acclimation of the modified beta function parameters to the growing temperature used to model N fixation. For each species, the best model had all parameters acclimating to temperature.

Plant Species	Model	ΔAIC
Morella cerifera (tropical; actinorhizal) n = 152712	$\boldsymbol{T_{min}, T_{opt}, T_{max} = f(T_{growth})}$	**0**
	$T_{min}, T_{opt} = f(T_{growth})$	5113
	$T_{min}, T_{max} = f(T_{growth})$	100607
	$T_{opt}, T_{max} = f(T_{growth})$	8976
	$T_{min} = f(T_{growth})$	109344
	$T_{opt} = f(T_{growth})$	11859
	$T_{max} = f(T_{growth})$	156340
	$T_{min}, T_{opt}, T_{max} \neq f(T_{growth})$	156357
Alnus rubra (temperate; actinorhizal) n = 175589	$\boldsymbol{T_{min}, T_{opt}, T_{max} = f(T_{growth})}$	**0**
	$T_{min}, T_{opt} = f(T_{growth})$	586
	$T_{min}, T_{max} = f(T_{growth})$	2979
	$T_{opt}, T_{max} = f(T_{growth})$	483
	$T_{min} = f(T_{growth})$	3945
	$T_{opt} = f(T_{growth})$	755
	$T_{max} = f(T_{growth})$	9350
	$T_{min}, T_{opt}, T_{max} \neq f(T_{growth})$	9432
Gliricidia sepium (tropical; rhizobial) n = 164694	$\boldsymbol{T_{min}, T_{opt}, T_{max} = f(T_{growth})}$	**0**
	$T_{min}, T_{opt} = f(T_{growth})$*	7679
	$T_{min}, T_{max} = f(T_{growth})$	23042
	$T_{opt}, T_{max} = f(T_{growth})$	206
	$T_{min} = f(T_{growth})$	28060
	$T_{opt} = f(T_{growth})$	9466
	$T_{max} = f(T_{growth})$	35787
	$T_{min}, T_{opt}, T_{max} \neq f(T_{growth})$	54540
Robinia pseudoacacia (temperate; rhizobial) n = 168230	$\boldsymbol{T_{min}, T_{opt}, T_{max} = f(T_{growth})}$	**0**
	$T_{min}, T_{opt} = f(T_{growth})$	306
	$T_{min}, T_{max} = f(T_{growth})$	112
	$T_{opt}, T_{max} = f(T_{growth})$	7722
	$T_{min} = f(T_{growth})$	463
	$T_{opt} = f(T_{growth})$	7858
	$T_{max} = f(T_{growth})$	13850
	$T_{min}, T_{opt}, T_{max} \neq f(T_{growth})$	14389

*Used this model because slope was negative when $T_{max} = f(T_{growth})$; we consider this negative slope a statistical artifact rather than a biologically meaningful result

Supplementary Table 9 | ΔAIC_c values for the possibility of linear acclimation of the modified beta function parameters to the growing temperature used to model photosynthesis. Functions that failed to converge have ΔAIC_c values listed as NA.

Response Variable	Model	ΔAIC_c
	$T_{min}, T_{opt}, T_{max} = f(T_{growth})$	NA
	$T_{min}, T_{opt} = f(T_{growth})$	NA
	$T_{min}, T_{max} = f(T_{growth})$	NA
A_{275}	$\mathbf{T_{opt}, T_{max} = f(T_{growth})}$	**0**
n = 292	$T_{min} = f(T_{growth})$	NA
	$T_{opt} = f(T_{growth})$*	0.6
	$T_{max} = f(T_{growth})$	68.7
	$T_{min}, T_{opt}, T_{max} \neq f(T_{growth})$	87.0
	$T_{min}, T_{opt}, T_{max} = f(T_{growth})$	NA
	$T_{min}, T_{opt} = f(T_{growth})$	12.4
	$T_{min}, T_{max} = f(T_{growth})$	98.0
A_{sat}	$\mathbf{T_{opt}, T_{max} = f(T_{growth})}$	**0**
n = 300	$T_{min} = f(T_{growth})$	158.7
	$T_{opt} = f(T_{growth})$*	5.3
	$T_{max} = f(T_{growth})$	72.4
	$T_{min}, T_{opt}, T_{max} \neq f(T_{growth})$	98.7

*Used this model because slope for T_{max} was sometimes negative for the $T_{opt}, T_{max} = f(T_{growth})$ model; we consider this negative slope a statistical artifact rather than a biologically meaningful result.

Supplementary Table 10 | ΔAIC_c values for the possibility of linear acclimation of the normal function parameters (equation 2) to the growing temperature used to model leaf respiration. For each species, the best model had both parameters acclimating to temperature.

Plant Species	Model	ΔAIC_c
Morella cerifera (tropical; actinorhizal) n = 277	$\boldsymbol{T_{opt}, s = f(T_{growth})}$	**0**
	$T_{opt} = f(T_{growth})$	37.8
	$s = f(T_{growth})$	34.6
	$T_{opt}, s \neq f(T_{growth})$	49.9
Alnus rubra (temperate; actinorhizal) n = 279	$\boldsymbol{T_{opt}, s = f(T_{growth})}$	**0**
	$T_{opt} = f(T_{growth})$	126.2
	$s = f(T_{growth})$	24.6
	$T_{opt}, s \neq f(T_{growth})$	23.4
Gliricidia sepium (tropical; rhizobial) n = 248	$\boldsymbol{T_{opt}, s = f(T_{growth})}$	**0**
	$T_{opt} = f(T_{growth})$	172.4
	$s = f(T_{growth})$	7.9
	$T_{opt}, s \neq f(T_{growth})$	22.6
Robinia pseudoacacia (temperate; rhizobial) n = 248	$\boldsymbol{T_{opt}, s = f(T_{growth})}$	**0**
	$T_{opt} = f(T_{growth})$	271.1
	$s = f(T_{growth})$	3.1
	$T_{opt}, s \neq f(T_{growth})$	1.9

Supplementary Table 11 | Parameters for the temperature response of N fixation with acclimation to growing temperature where K_m can vary with temperature. Parameters are for equations 5-6, the modified beta function with linear acclimation of all parameters to the growing temperature. Here, the N fixation rates that were fit with equations 5-6 were derived using the K_m functions in Supplementary Table 5. This contrasts with the parameters in Supplementary Table 1, which were generated using K_m values from Supplementary Table 4. These values and equations, along with mean growing temperatures as inputs, form the curves and parameter values seen in Supplementary Figs. 15-17. As in Supplementary Table 1, *Morella* and *Gliricidia* were binned for the tropical function and *Alnus* and *Robinia* were binned for the temperate function.

	T_{min} (95% CI)		T_{opt} (95% CI)		T_{max} (95% CI)	
Plant Species	Slope [°C °C^{-1}]	Intercept [°C]	Slope [°C °C^{-1}]	Intercept [°C]	Slope [°C °C^{-1}]	Intercept [°C]
Morella cerifera	1.154	−22.26	0.633	19.74	0.153	41.51
(tropical, actinorhizal)	(1.126, 1.176)	(−22.94, −21.57)	(0.630, 0.636)	(19.68, 19.81)	(0.149, 0.157)	(41.41, 41.60)
Alnus rubra	0.512	−20.60	0.066	31.20	0.030	41.85
(temperate, actinorhizal)	(0.452, 0.543)	(−21.43, −19.02)	(0.064, 0.067)	(31.17, 31.25)	(0.028, 0.032)	(41.80, 41.91)
Gliricidia sepium	1.067	−17.66	0.322	26.65	*	45.83
(tropical, rhizobial)	(1.037, 1.104)	(−18.56, −16.68)	(0.319, 0.327)	(26.55, 26.75)		(45.80, 45.85)
Robinia pseudoacacia	0.947	−16.35	**	33.01	0.064	44.53
(temperate, rhizobial)	(0.928, 0.966)	(−16.87, −15.83)		(32.99, 33.03)	(0.060, 0.068)	(44.44, 44.63)
Tropical	0.943	−15.54	0.468	23.24	*	45.44
	(0.921, 0.965)	(−16.07, −15.00)	(0.466, 0.471)	(23.18, 23.31)		(45.42, 45.46)
Temperate	0.676	−14.32	0.027	32.12	*	44.24
	(0.657, 0.696)	(−14.88, −13.76)	(0.025, 0.029)	(32.07, 32.17)		(44.23, 44.25)

*Displaying parameters of a model with acclimation of T_{min} and T_{opt}, but not T_{max}

**Displaying parameters of a model with acclimation of T_{min} and T_{max}, but not T_{opt} because the slope for T_{opt} did not differ significantly from "0" in the model with acclimation of all parameters

Supplementary Table 12 | Parameters for the temperature response of N fixation without acclimation to growing temperature where K_m can vary with temperature. Just as Supplementary Table 11 is the analog where K_m can vary with temperature for Supplementary Table 1, this is the analog where K_m can vary with temperature for Supplementary Table 3.

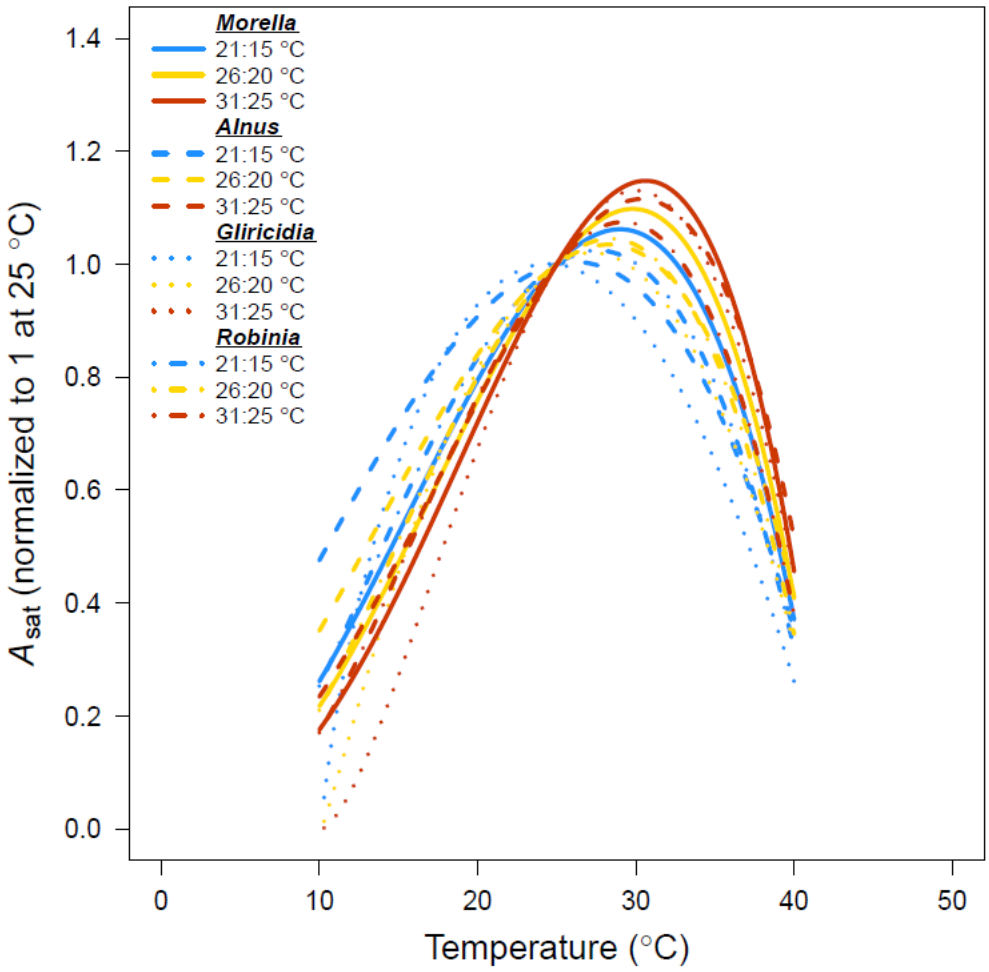
	T_{min} (95% CI)	T_{opt} (95% CI)	T_{max} (95% CI)
Plant Species	[°C]	[°C]	[°C]
Morella cerifera	4.86	34.62	45.11
(tropical, actinorhizal)	(4.65 5.04)	(34.60, 34.63)	(45.09, 45.12)
Alnus rubra	−11.12	32.42	42.40
(temperate, actinorhizal)	(−11.55, −10.68)	(32.40, 32.44)	(42.39, 42.42)
Gliricidia sepium	7.41	34.21	45.83
(tropical, rhizobial)	(7.22, 7.59)	(34.19, 34.24)	(45.80, 45.85)
Robinia pseudoacacia	1.17	33.01	45.72
(temperate, rhizobial)	(1.00, 1.34)	(32.99, 33.03)	(45.70, 45.74)
Tropical	6.63	34.25	45.44
	(6.49, 6.76)	(34.23, 34.26)	(45.42, 45.46)
Temperate	−1.82	32.62	44.24
	(−2.03, −1.61)	(32.60, 32.64)	(44.23, 44.25)
Overall	1.90	33.44	45.05
	(1.80, 2.05)	(33.42, 33.45)	(45.04, 45.06)

Supplementary Table 13 | Conversion factors for paired ARACAS and $^{15}N_2$ incubations on severed nodules. The sample size (n) for each species is the sample size after outliers were removed.

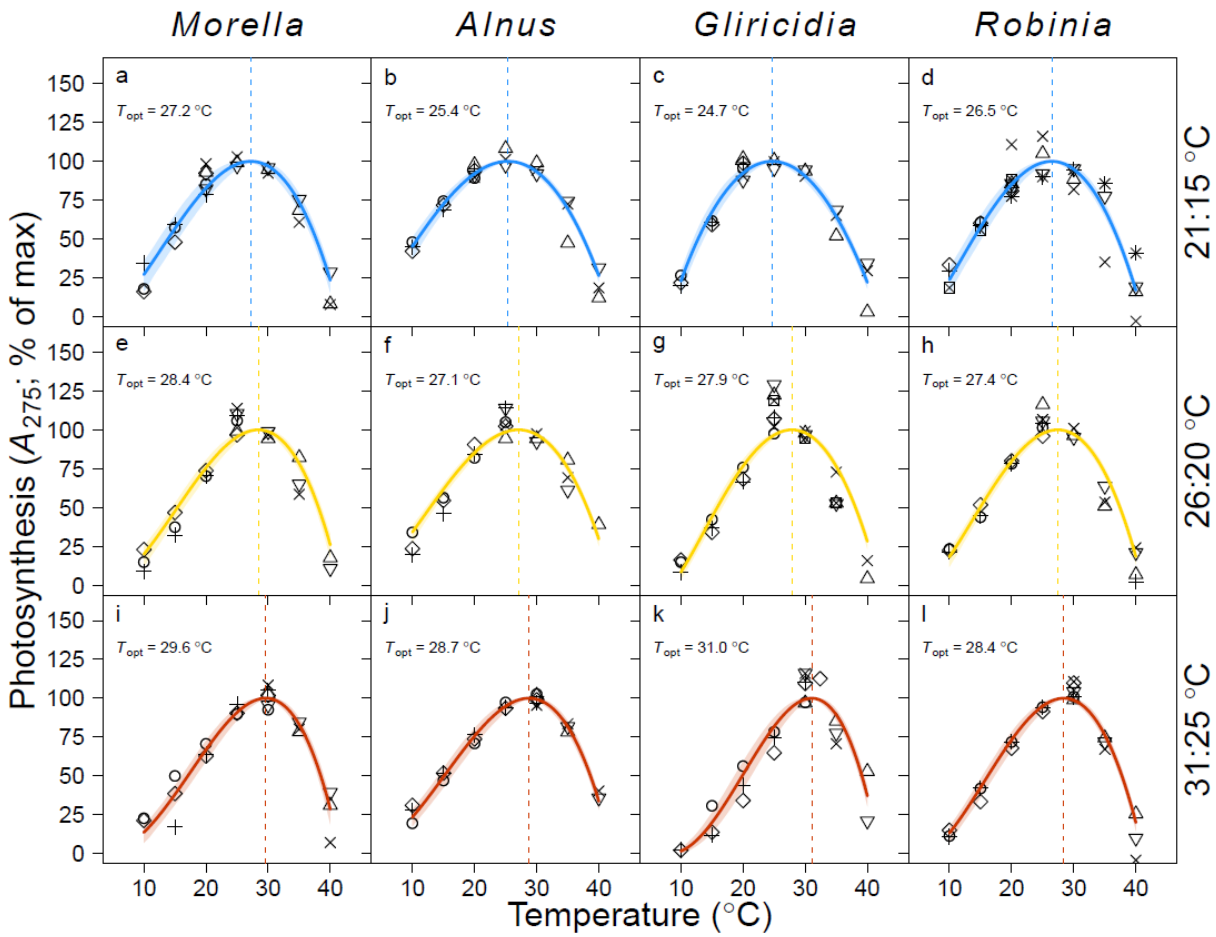
	Conversion Factor (mol C_2H_4 mol N_2^{-1})	
Plant Species	Mean	SE
Morella cerifera (n=17)	4.98	0.39
Alnus rubra (n=14)	3.15	0.40
Gliricidia sepium (n=13)	3.84	0.47
Robinia pseudoacacia (n=15)	4.27	0.33

Supplementary Table 14 | Parameters, units, and values used in equations S5-S7.

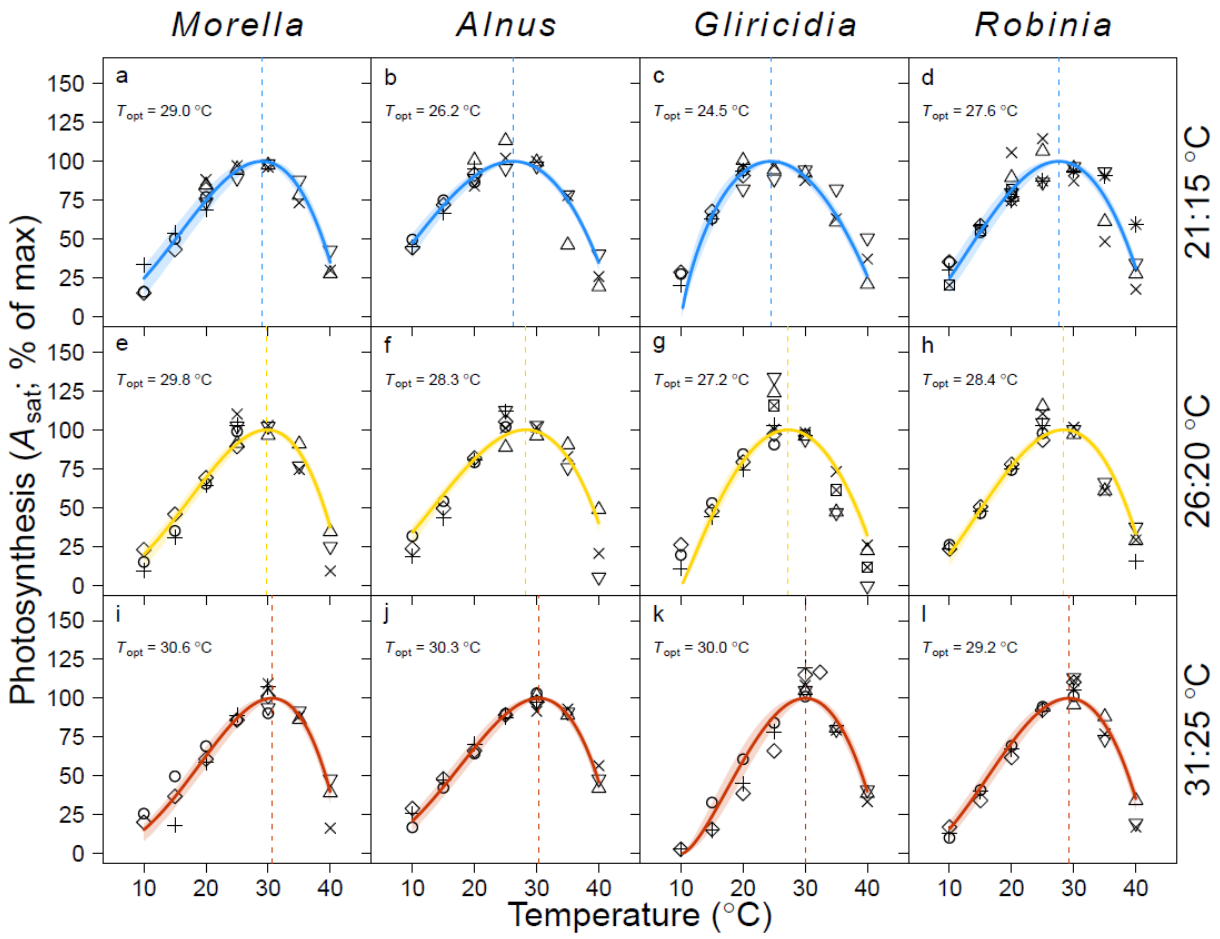
Parameter	Units	Value	95% CI
α_b	unitless	0.030	(0.009, 0.068)
β_b	$°C^{-1}$	0.292	(0.188, 0.444)
c	hr^{-1}	0.957	(0.479, 7.534)
α_d	unitless	0.253	(0.167, 0.374)
β_d	$°C^{-1}$	0.655	(0.398, 0.987)



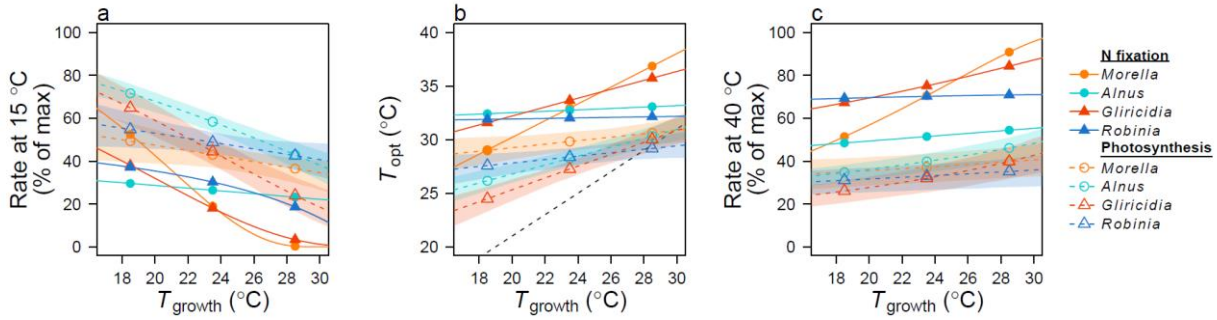
Supplementary Figure 1 | Photosynthesis at an atmospheric CO_2 of 400 ppm acclimates to growing temperature. Maximum likelihood-estimated temperature response functions represent species-level fits with linear acclimation of the optimum temperature to the growing temperature. This differs from Fig. 2b in that A_{sat} is displayed here instead of A_{275}.



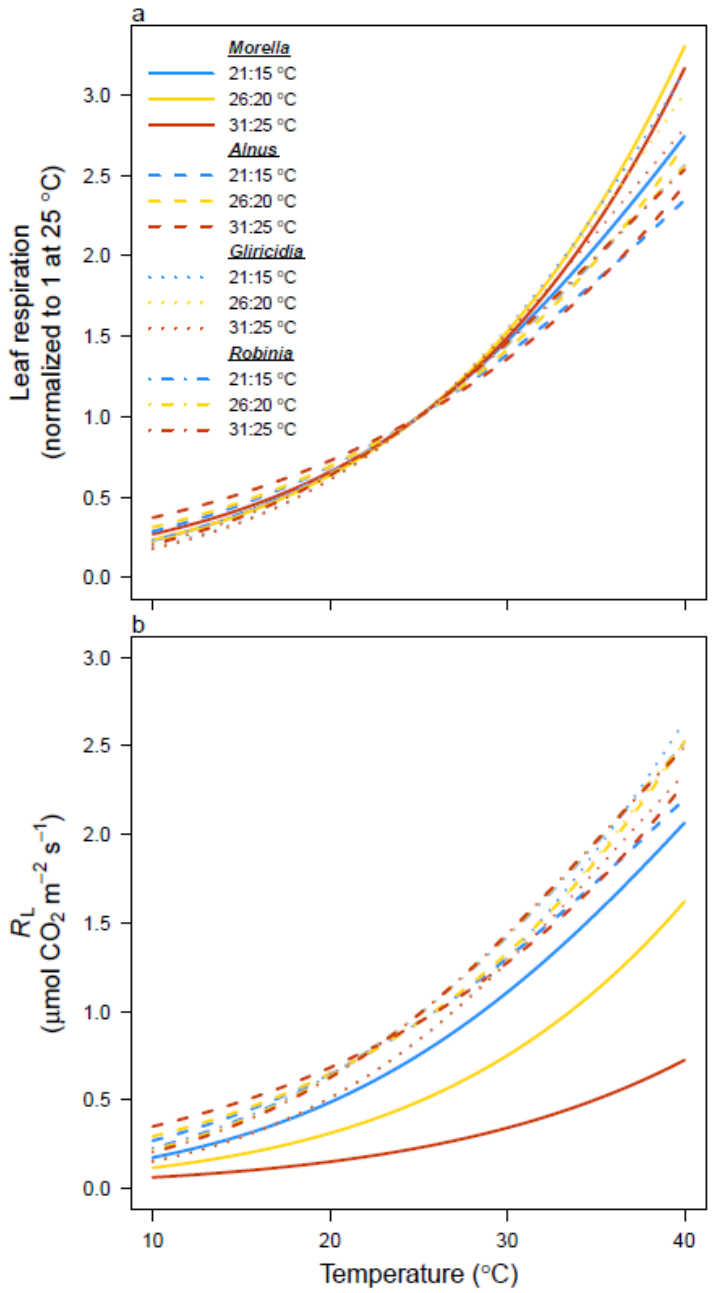
Supplementary Figure 2 | Data and temperature response curves of photosynthesis at a C_i of 275 ppm CO_2 in four tree species (columns) grown under a range of growing temperatures (rows). Photosynthesis data collected on individual leaves (points; different symbols within panel represent different leaves) are shown with maximum likelihood-estimated temperature response functions (curves) for cool (**a**-**d**; blue), intermediate (**e**-**h**; gold), and warm (**i**-**l**; red) growing conditions. Individual leaves were from three individual plants per treatment, where one leaf per plant was used for ascending measurements and a separate leaf was used for descending measurements (see Methods). In these temperature response functions the optimum temperature (T_{opt}, which is listed and visualized with a vertical dashed line in each panel) linearly acclimates to the growing temperature (T_{growth} = day:night temperature in legend). Consequently, the curves shown in each column are fit to all the data in the column along with their respective growing temperatures (colors vary with growing temperature). Shading represents 95% CI. Six individual leaves (one ascending and one descending from the daytime growing temperature per individual plant) were measured for each treatment (species x growing temperature), apart from *Gliricidia* at 26:20 °C (n=7) and *Robinia* at 21:15 °C (n=8). These are the same curves shown in Fig. 2b, shown here with the underlying data.



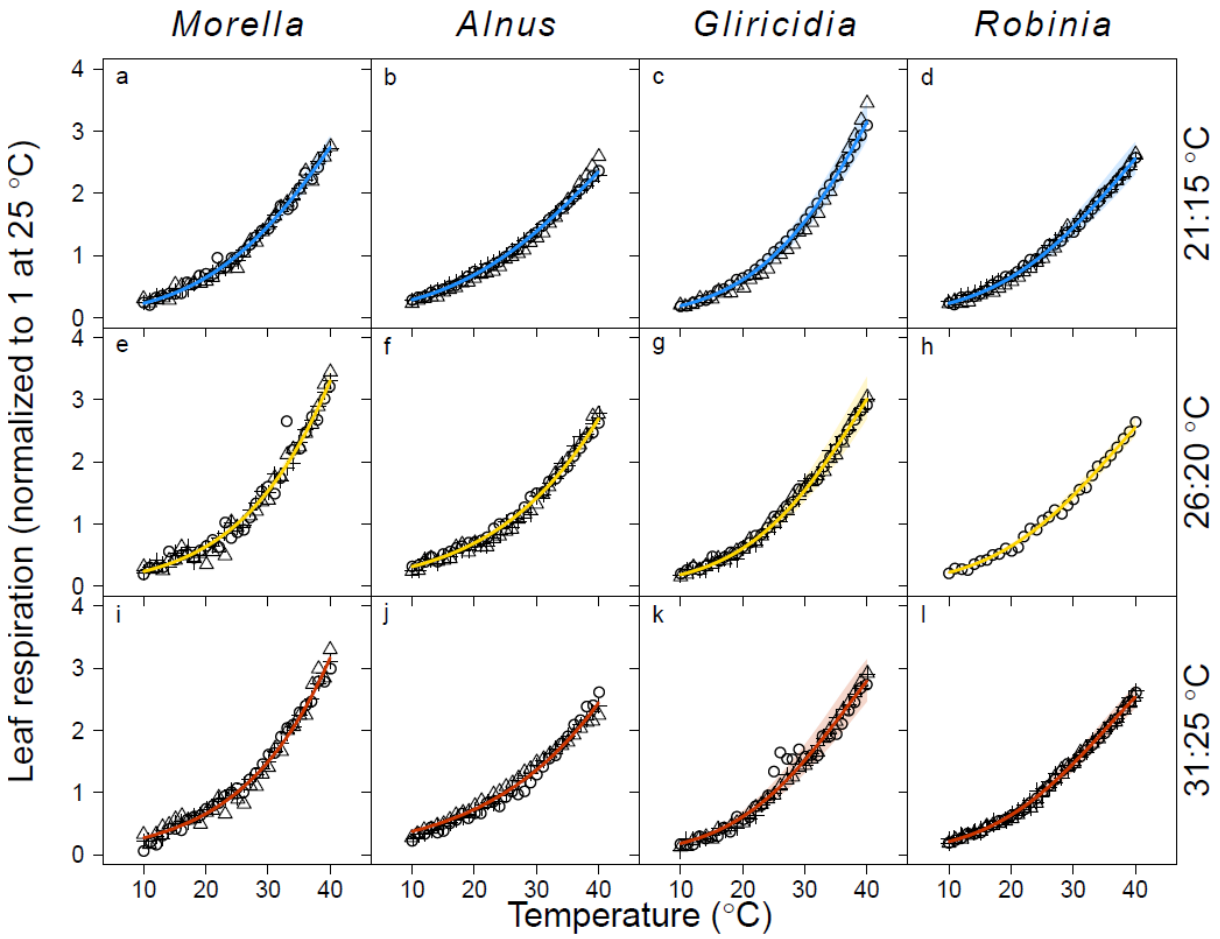
Supplementary Figure 3 | Data and temperature response curves of photosynthesis at an atmospheric CO_2 of 400 ppm CO_2 in four tree species (columns) grown under a range of growing temperatures (rows). This differs from Supplementary Fig. 2 in that A_{sat} is displayed here instead of A_{275}. These are the same maximum likelihood-estimated curves shown in Supplementary Fig. 1, shown here with the underlying data. Shading represents 95% CI.



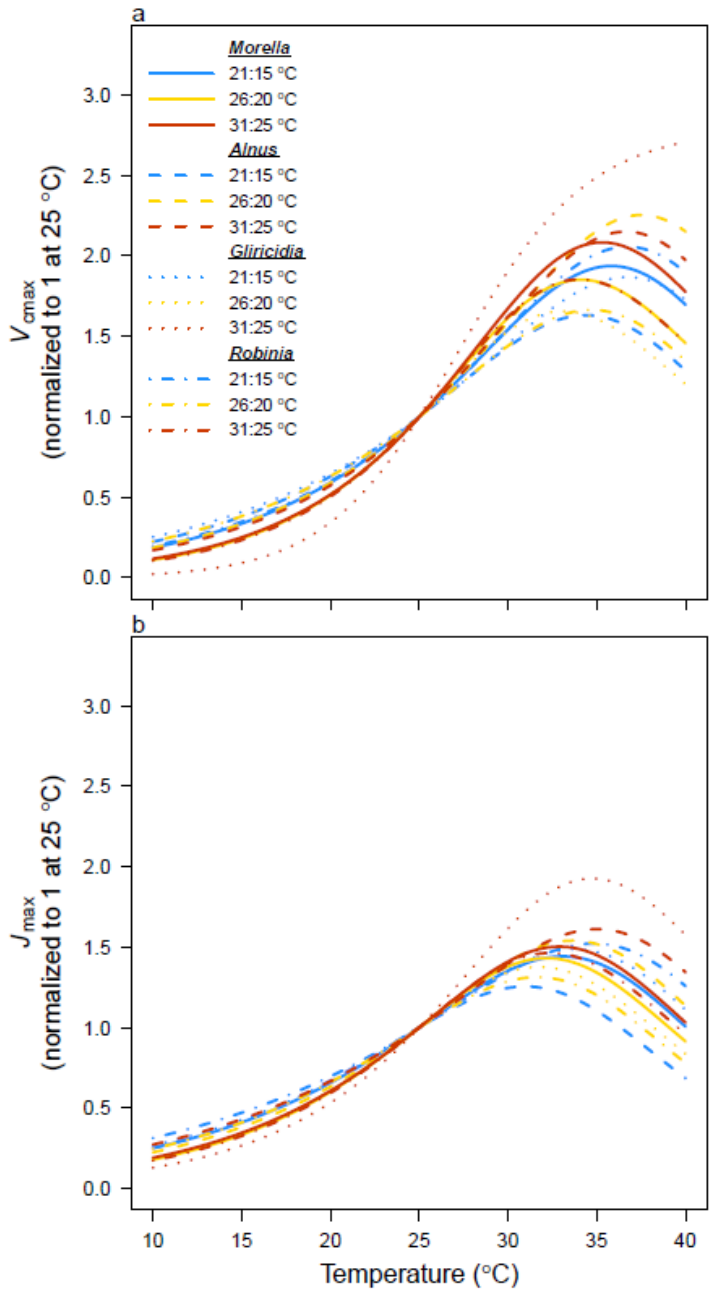
Supplementary Figure 4 | Thermal acclimation of N fixation and photosynthesis at an atmospheric CO_2 of 400 ppm. Details as in Fig. 3, except that A_{sat} is plotted instead of A_{275}. Shading represents 95% CI.



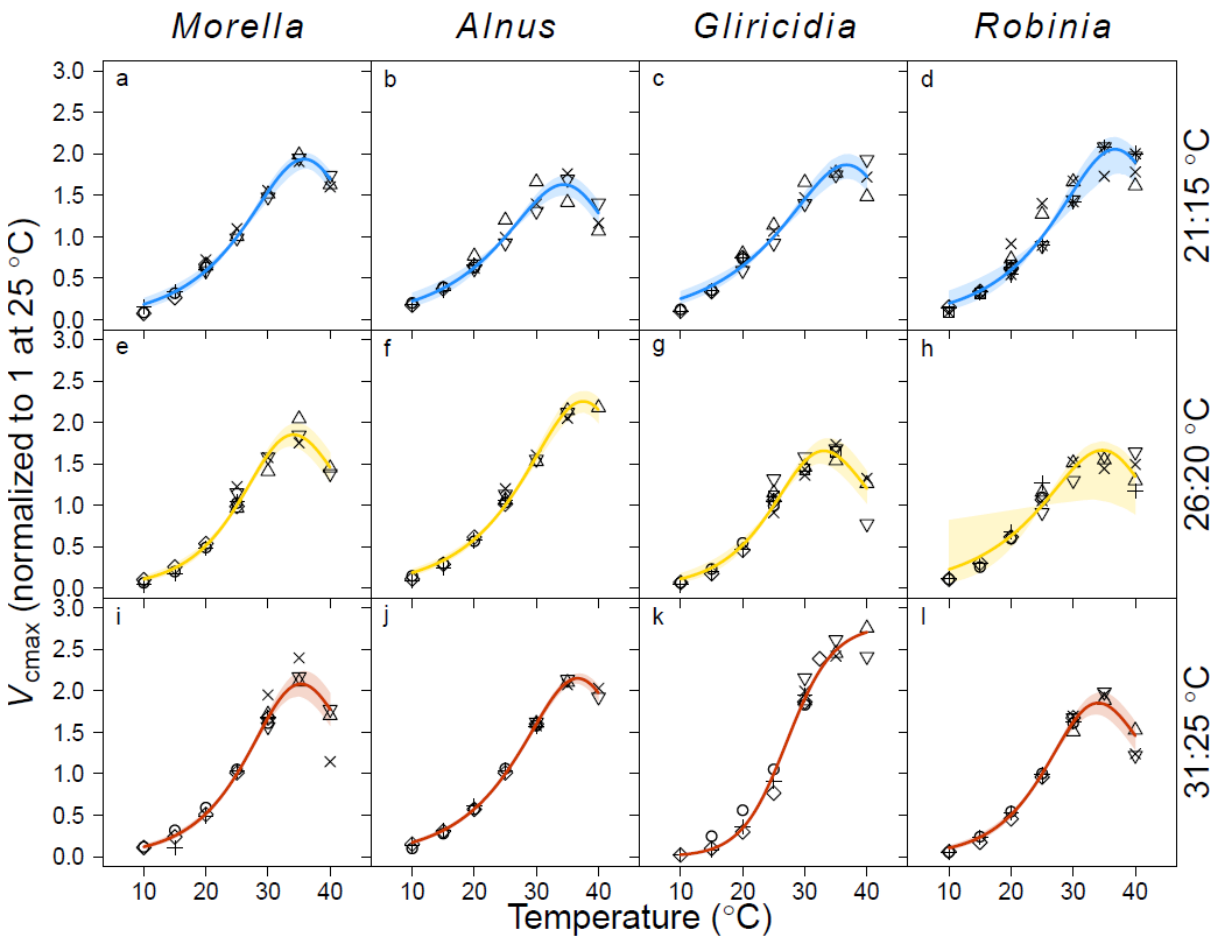
Supplementary Figure 5 | Leaf respiration temperature response (a) and its effect on leaf respiration in the light (R_L) (b) across species and growing temperatures. Maximum likelihood-estimated temperature response functions represent species-level fits with linear acclimation of the optimum temperature and the standard deviation of the normal function (equation 2) to the growing temperature. R_L significantly decreased with the growing temperature in *Morella* (**b**) but not in other species.



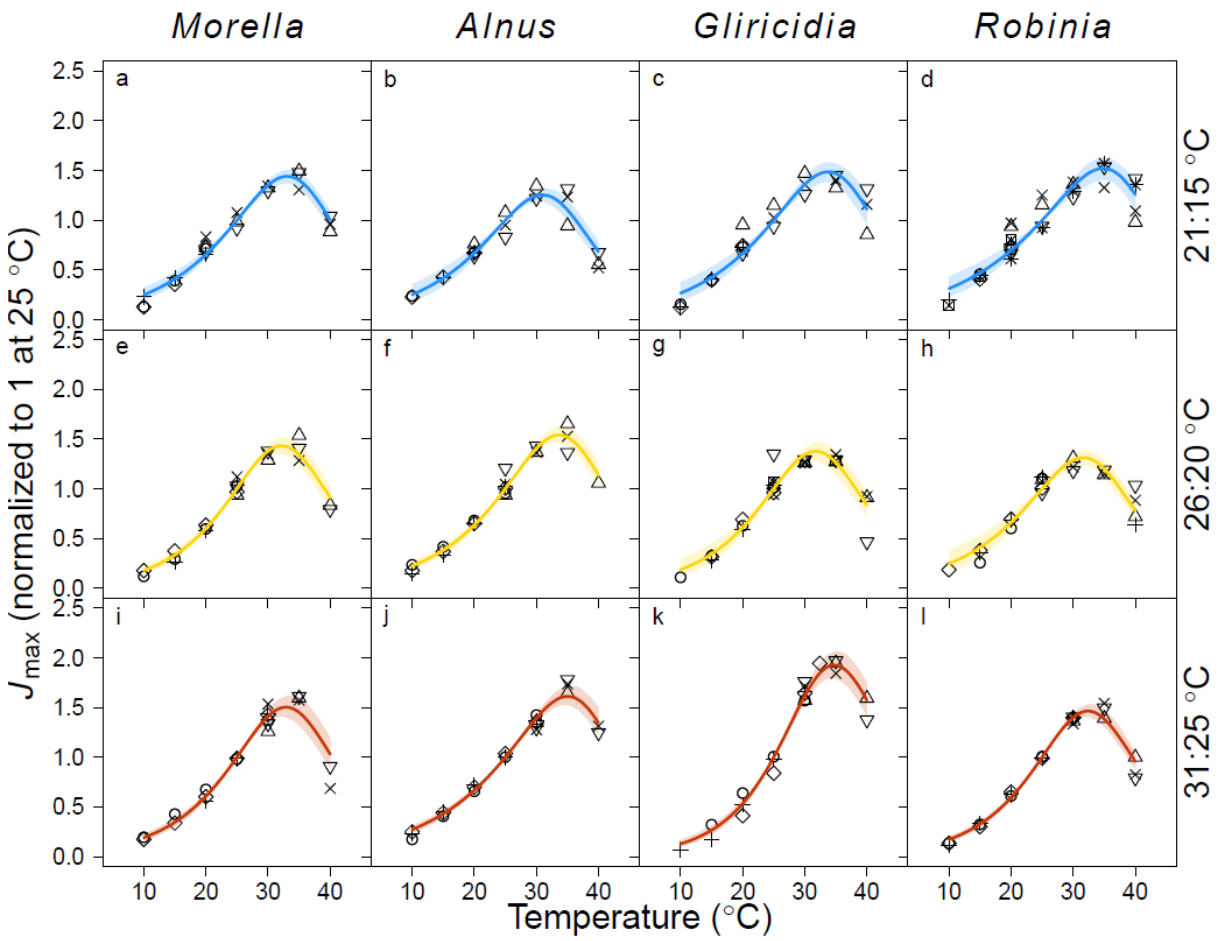
Supplementary Figure 6 | Data and temperature response curves of leaf respiration in four tree species (columns) grown under a range of growing temperatures (rows). Leaf respiration data collected on individual leaves from separate individuals (points; different symbols within panel represent different leaves) are shown with maximum likelihood-estimated temperature response functions (curves) for cool (**a**-**d**; blue), intermediate (**e**-**h**; gold), and warm (**i**-**l**; red) growing conditions. Maximum likelihood fits are at the species level, with linear acclimation of the optimum temperature and the standard deviation of the normal function (equation 2) to the growing temperature. R_D estimates are normalized to 1 at 25 °C. Other details as in Supplementary Figs. 2-3. Three individual leaves (all from separate plants) were measured for each treatment (species x growing temperature), apart from *Gliricidia* at 21:15 °C (n=2) and *Robinia* at 26:20 °C (n=1) and 31:25 °C (n=4). However, because model fits were at the species level, the total number of leaves (equivalently, the number of plants) used for each function was 9, 9, 8, and 8 for *Morella*, *Alnus*, *Gliricidia*, and *Robinia*, respectively.



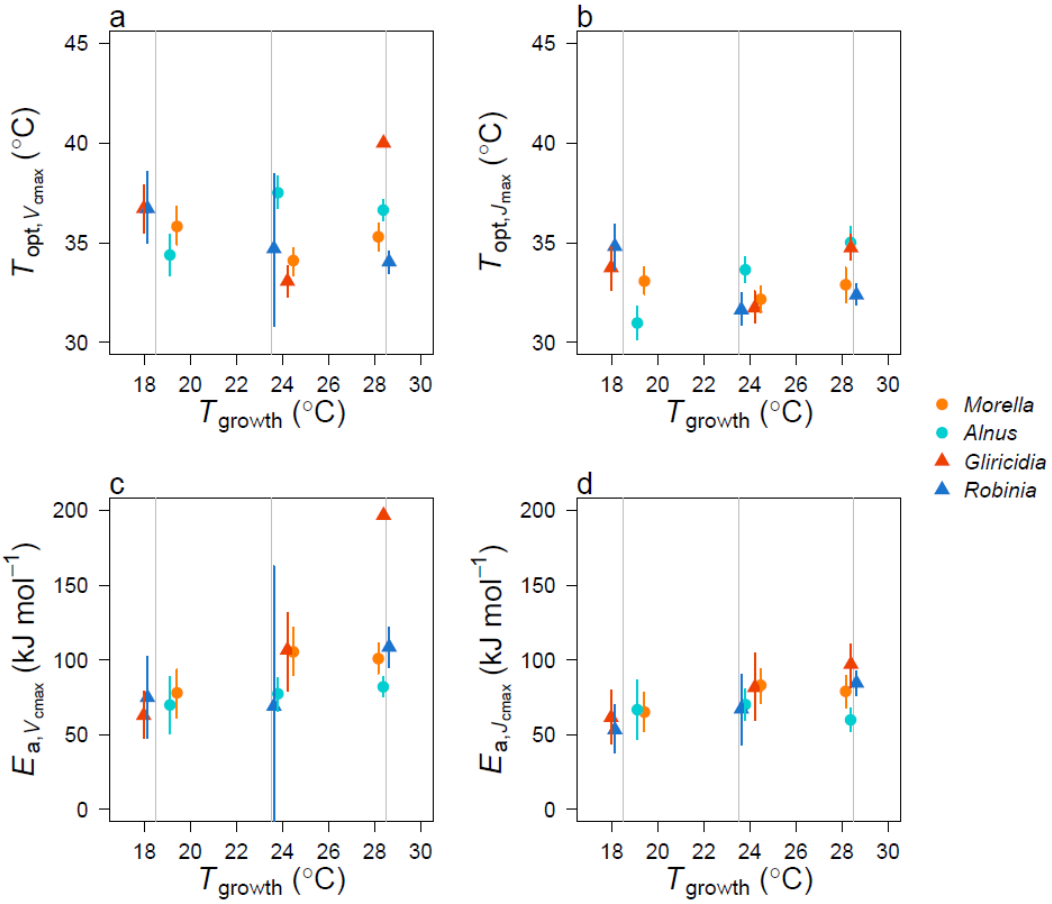
Supplementary Figure 7 | Temperature responses of V_{cmax} and J_{max} as affected by species and growing temperature. V_{cmax} and J_{max} are modelled at the treatment level with a peaked-Arrhenius function (equation 4).



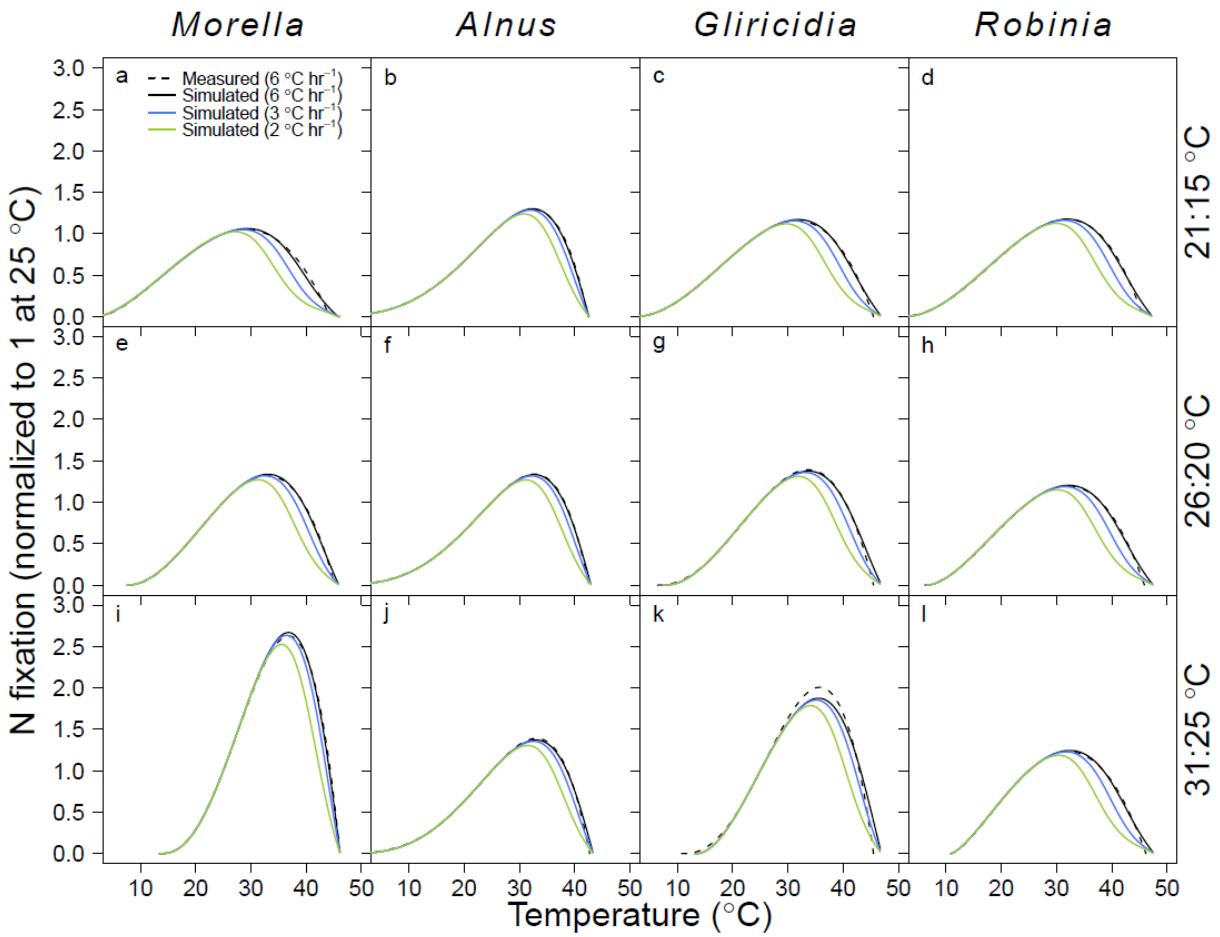
Supplementary Figure 8 | Data and temperature response curves of V_{cmax} in four tree species (columns) grown under a range of growing temperatures (rows). The number of sample leaves (equivalently, the number of sample plants) is the same as for photosynthesis, as data are derived from the same set of measurements. The curves shown represent treatment-level maximum likelihood-estimated temperature response functions. *Gliricidia* at 31:25 °C does not have an estimated 95% CI because our negative log-likelihood function did not converge sufficiently, likely due to the unclear peak at higher temperatures. All other details as in Supplementary Fig. 6.



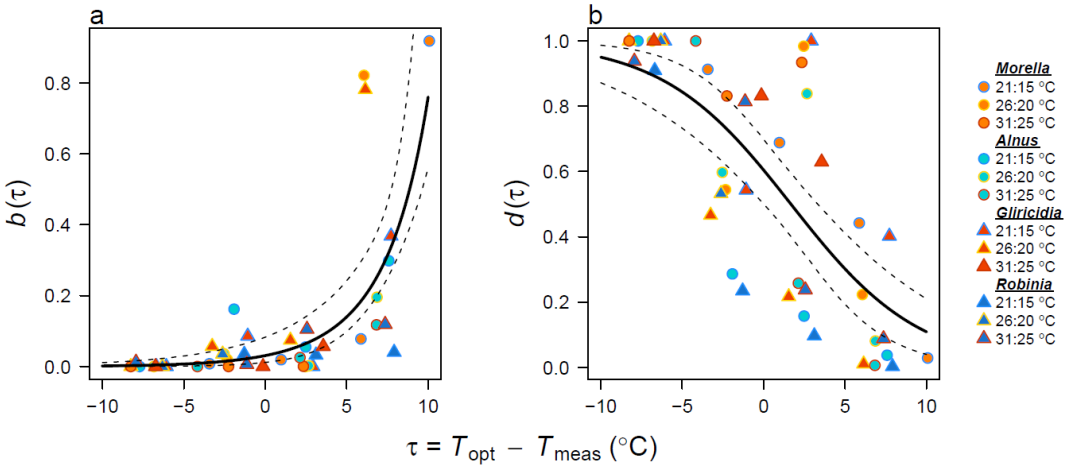
Supplementary Figure 9 | Data and temperature response curves of J_{max} in four tree species (columns) grown under a range of growing temperatures (rows). Details as in Supplementary Fig. 8. Shading represents 95% CI.



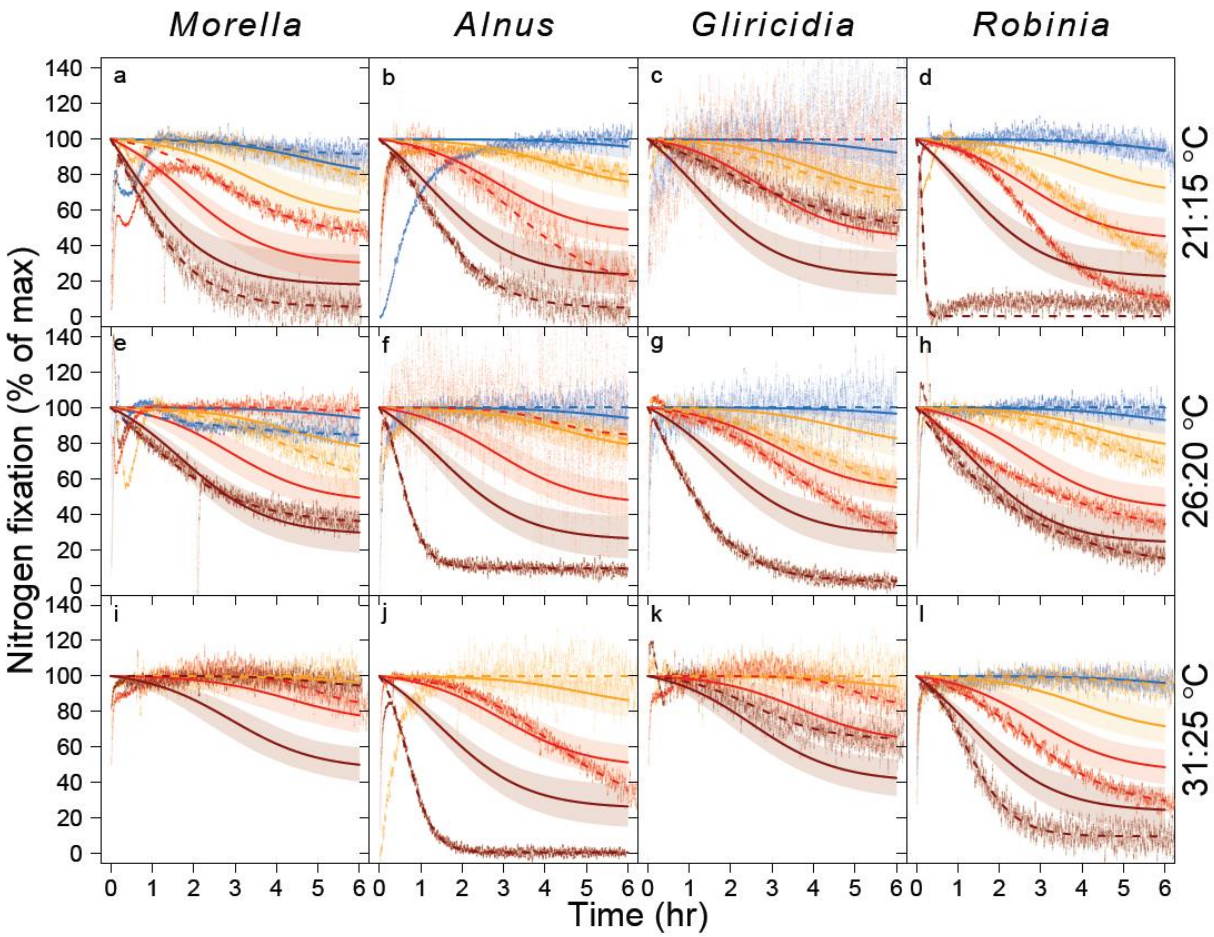
Supplementary Figure 10 | Parameter estimates for optimal temperature (T_{opt}) and H_a, which controls the exponential rise in rates below T_{opt}. Maximum likelihood-estimated parameters are for each species and growing temperature for the photosynthetic parameters V_{cmax} (**a**, **c**) and J_{max} (**b**, **d**). A small amount of horizontal random noise was added to increase visibility; each cluster represents the same growing temperature (the gray vertical lines). Bars show 95% CIs. See caption in Supplementary Fig. 8 for explanation of missing 95% CI for V_{cmax} of *Gliricidia* at 31:25 °C.



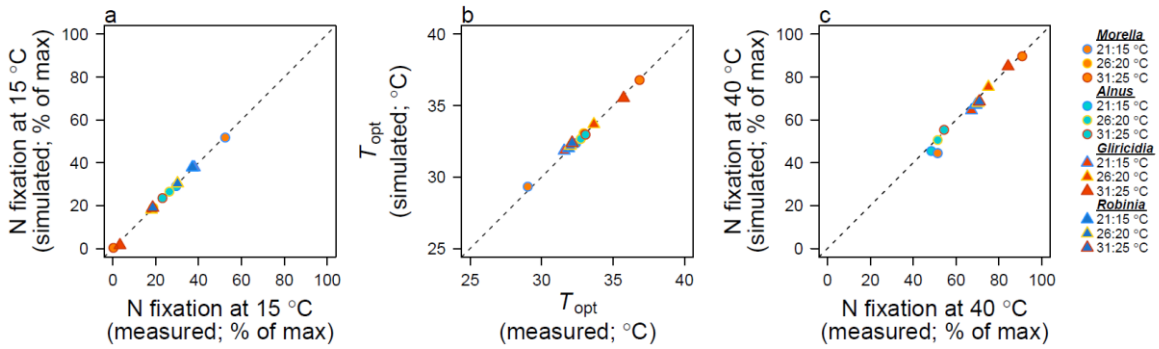
Supplementary Figure 11 | Temperature response curves of N fixation depend on the rate of heating. Measured curves represent fits of equation S7 to N fixation data collected at a rate of 6 °C hr^{-1} while simulated curves show how the rate of heating changes the expected temperature response curve.



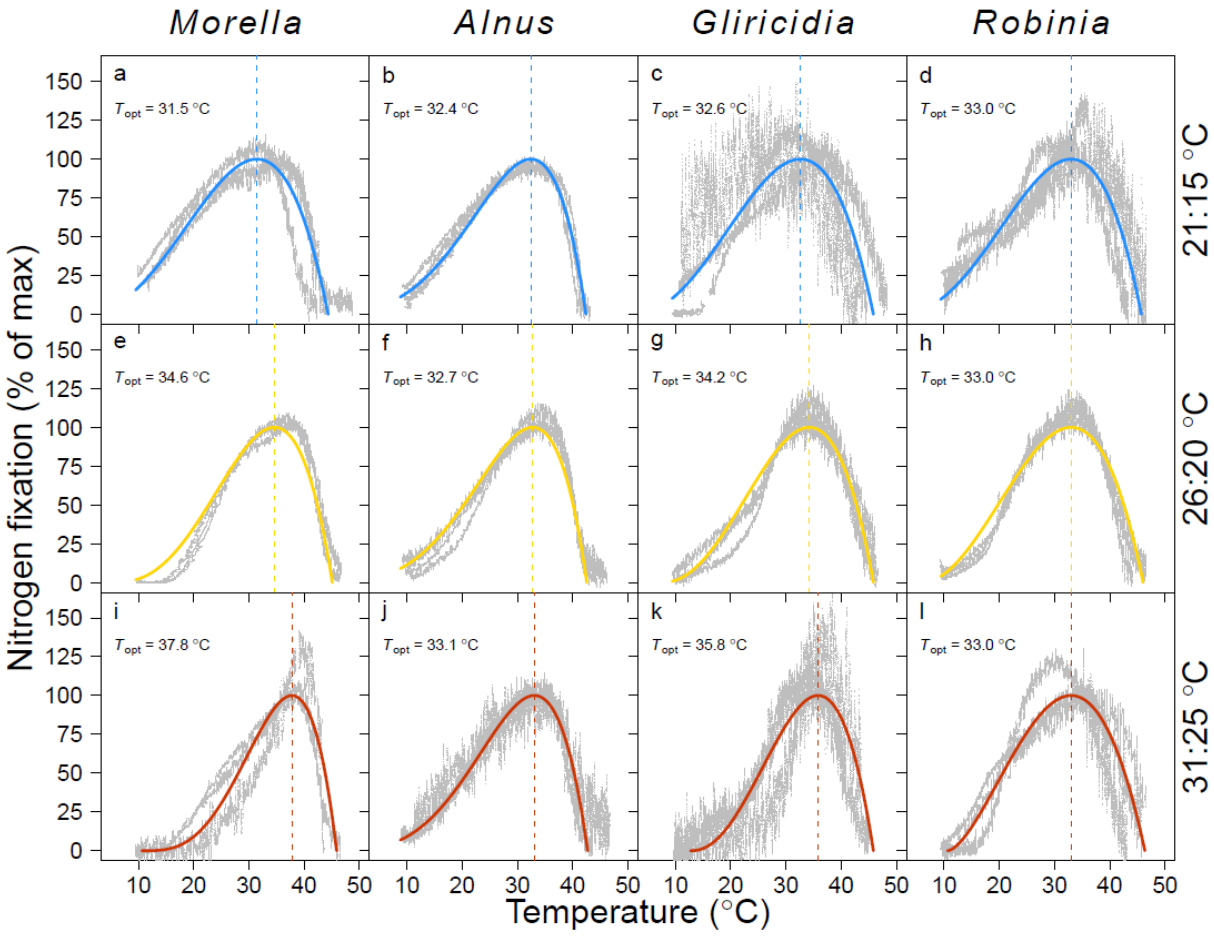
Supplementary Figure 12 | The temperature response of parameters *b* and *d* from equations S4, S7. The solid curves represent maximum likelihood-estimates and the dashed curves represent 95% CIs for equations S5 (**a**) and S6 (**b**). Temperature is defined as tau (τ), the difference between the optimum temperature and the measurement temperature for each species at each growing temperature. Model fits are across all species and growing temperatures.



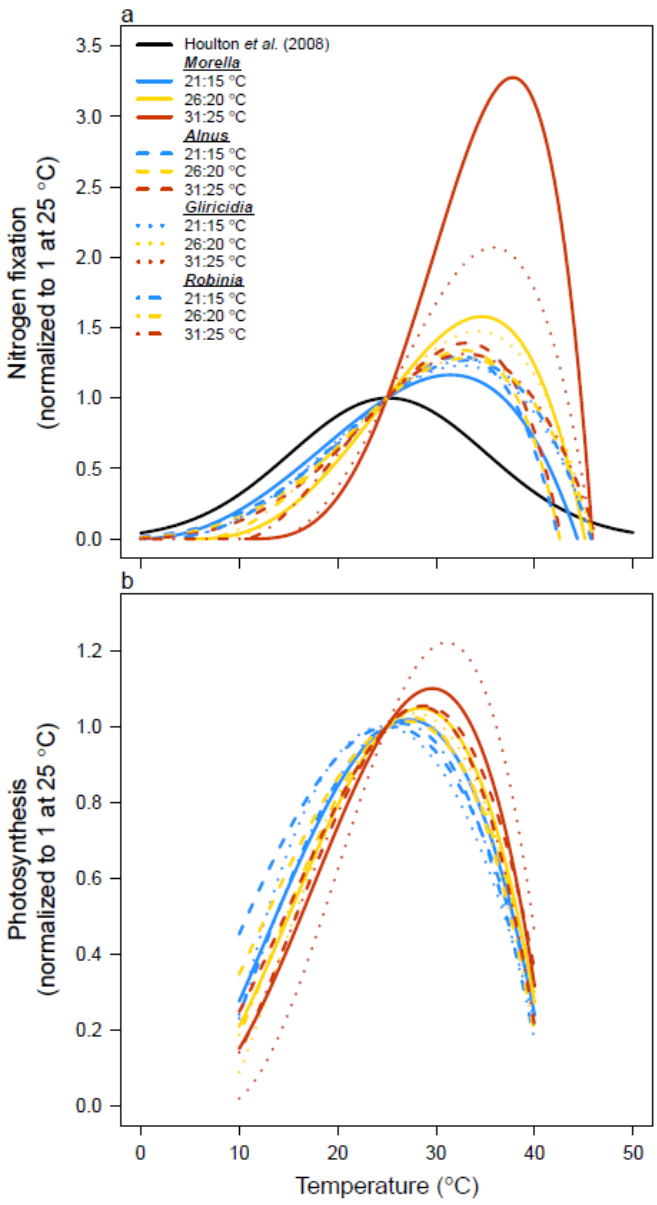
Supplementary Figure 13 | Data, individual-run model fits, and overall model predictions for how N fixation at constant temperatures declines over time. Data (small points) were fit with piecewise nonlinear functions (equation S4). Colors represent measurement temperatures: 25 °C in blue, 30 °C in orange, 35 °C in red, and 40 °C in brown. Fits of the function that accounts for declining N fixation after initial equilibration (equation S4; dashed curves [and the dotted curve for the 35 °C measurement of *Gliricidia* at the growing temperature of 21:15 °C]) were used to develop an overall model (equation S7; solid curves) for how temperature and length of exposure determined the temperature response of N fixation. Shading represents 95% CI of the overall model simulated at different measurement temperatures for a given species and growing temperature. For 31:25 °C, data at 25 °C were only collected for *Robinia*.



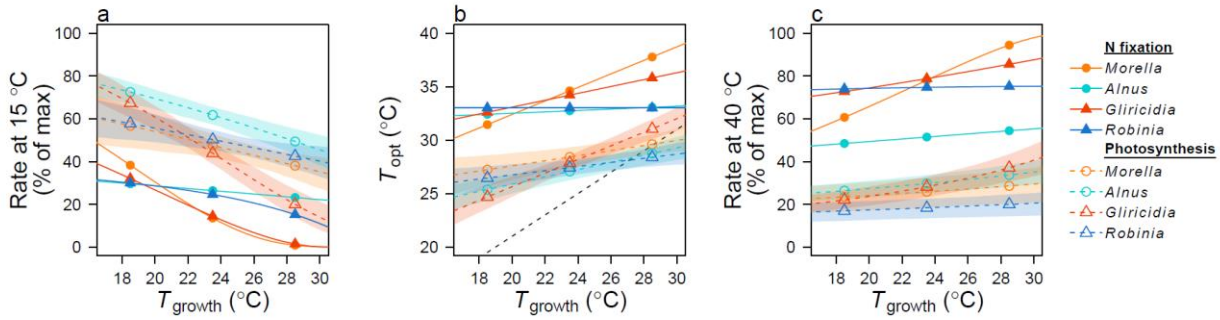
Supplementary Figure 14 | Comparisons of the relative N fixation rate at 15 °C (a), the optimum temperature for N fixation, and the relative N fixation rate at 40 °C (c) for measured vs. simulated temperature response curves at a heating rate of 6 °C hr^{-1}.



Supplementary Figure 15 | Data and temperature response curves of N fixation in four tree species grown under a range of growing temperatures, where K_m can vary with temperature. Details as in Fig. 1.



Supplementary Figure 16 | Temperature response curves of N fixation where K_m can vary with temperature, as compared to the Houlton *et al*. 2008 function and photosynthesis at a C_i of 275 ppm CO_2. Details as in Fig. 2 except for the scale of the y-axis in **a**.



Supplementary Figure 17 | Thermal acclimation of optimal temperatures and rates at low and high temperatures for N fixation where K_m can vary with temperature. Details as in Fig. 3. Shading represents 95% CI.

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