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Host sanctions and the legume–rhizobium mutualism

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Explaining mutualistic cooperation between species remains one of the greatest problems for evolutionary biology^{1–4}. Why do symbionts provide costly services to a host, indirectly benefiting competitors sharing the same individual host? Host monitoring of symbiont performance and the imposition of sanctions on ‘cheats’ could stabilize mutualism^{5,6}. Here we show that soybeans

penalize rhizobia that fail to fix N₂ inside their root nodules. We prevented a normally mutualistic rhizobium strain from co-operating (fixing N₂) by replacing air with an N₂-free atmosphere (Ar:O₂). A series of experiments at three spatial scales (whole plants, half root systems and individual nodules) demonstrated that forcing non-cooperation (analogous to cheating) decreased the reproductive success of rhizobia by about 50%. Non-invasive monitoring implicated decreased O₂ supply as a possible mechanism for sanctions against cheating rhizobia. More generally, such sanctions by one or both partners may be important in stabilizing a wide range of mutualistic symbioses.

Mutually beneficial symbiotic relationships between species are ubiquitous, but their evolutionary persistence is puzzling in many cases^{1–3}. If each individual plant or animal host is infected by a single symbiont lineage, then the host and symbiont have a shared interest that may favour cooperation. This is especially so if the symbiont is transmitted vertically, from parent to offspring^{2,7}. However, many mutualisms involve multiple symbiont genotypes per individual host and horizontal transmission of symbionts among unrelated host individuals^{1,2,7}. In this case, each symbiont lineage is selected to increase its own growth and fitness selfishly, at the expense of its host and the other lineages^{2,7}. This is the classic Tragedy of the Commons problem, common to economic and social theory². The tragedy is that while the symbionts as a group could obtain more resources from their host with prudent cooperation, this is not evolutionarily stable because each symbiont lineage gains by selfishly pursuing its own short-term interests.

One possible solution is selection imposed by hosts rewarding

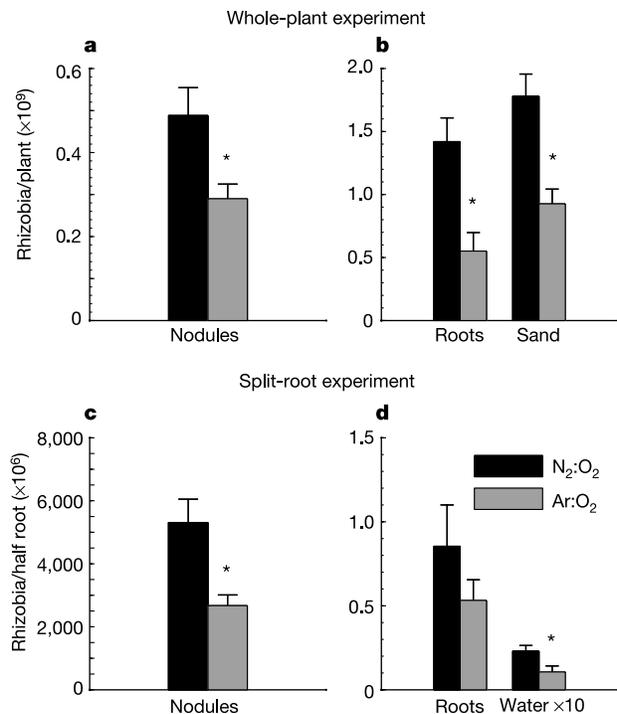


Figure 1 Rhizobia fixing N₂ grew to larger numbers in whole-plant and split-root experiments. Rhizobia allowed (N₂:O₂) or prevented (Ar:O₂) from fixing N₂ by experimental manipulation of atmosphere at the whole-plant (a, b) or split-root (c, d) level were counted (antibiotic media), from nodules (a, c), on the root surface (b, d) and in the surrounding sand (b) or water (d). Counts from water (d) were multiplied by ten for scaling. *Significant differences by ANOVA or paired *t*-test: a, *P* < 0.005, *N* = 11 pairs; b, root fraction, *P* < 0.01, and sand fraction, *P* < 0.01, *N* = 11; c, *P* < 0.001, *N* = 12 plants; d, root fraction, *P* = 0.24, and water fraction, *P* < 0.01, *N* = 12.

cooperation or punishing less cooperative behaviour^{3-6,8,9}. This 'sanctions' hypothesis is an evolutionary analogue of the 'policing' that can stabilize cooperation within species¹⁰, such as within social insect colonies¹¹. Although sanctions could be important in stabilizing symbiotic mutualisms between species⁸, the difficulty of manipulating most mutualisms experimentally has precluded previous experimental tests of the sanctions hypothesis, or indeed of alternative hypotheses.

Here, we test the sanctions hypothesis with the legume-rhizobium mutualism. Rhizobia are bacteria that fix N₂ within the root nodules of their host legume plants. N₂ fixation is clearly beneficial to the host plant, because it supplies nitrogen needed for growth and photosynthesis. But N₂ fixation (at rates that greatly exceed the nitrogen needs of rhizobia) is energetically costly to the bacteria, and hence reduces the resources that could be allocated to their own growth and reproduction^{5,6}. A single legume plant is typically infected by several different bacterial lineages⁵, creating a potential tragedy of the commons. Consequently, if plants treat fixing and non-fixing nodules similarly (that is, no sanctions), natural selection will favour rhizobia that invest very little in N₂ fixation^{5,6}. Rhizobia vary greatly in the benefits they provide to legumes. Strains that fix little or no N₂ after they form root nodules on legumes are common in some soils^{12,13}. Given the cost of N₂ fixation, why haven't these cheats completely displaced cooperators? Some mutualisms may be stabilized by the tendency of individuals to associate selectively with better cooperators¹⁴, but legumes cannot consistently recognize and exclude non-fixing rhizobia from infecting their roots^{15,16}.

The legume-rhizobium system offers exceptional opportunities to test the sanctions hypothesis. We can force rhizobia to cheat by replacing air (N₂:O₂, 80:20 v/v) with a gas mixture (Ar:O₂, 80:20 v/v) containing only traces of N₂ (about 0.03% v/v). We estimate that this treatment reduces N₂ fixation to about 1% of normal, based on a K_m (half-saturation N₂ concentration) of about 3%¹⁷. This method allows precise control of when and where rhizobia fix N₂, without possible confounding effects associated with non-fixing strains. We used this method with soybean (*Glycine max*) and its symbiont *Bradyrhizobium japonicum*. These rhizobia are often mutualistic, but 'ineffective strains', which take plant resources but fix little or no N₂, are widespread^{13,15,16}. We forced rhizobial cheating in: (1) whole plants; (2) one-half of the root system; or (3) individual nodules. In each case, we imposed cheating by exposing target nodules to a nearly N₂-free atmosphere and exposed control nodules

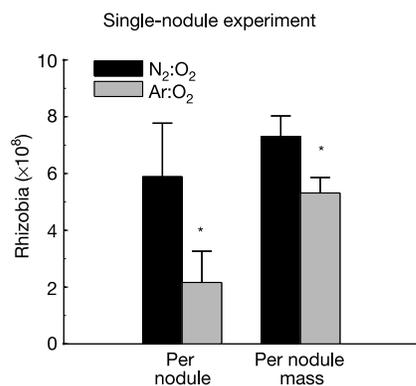


Figure 2 Rhizobia fixing N₂ grew to larger numbers in the single-nodule experiment. Rhizobia allowed to fix (N₂:O₂) or prevented from fixing (Ar:O₂) N₂ were counted (antibiotic media) on a per-nodule and per-nodule-mass basis after 10 d of treatment. *Significant differences by paired *t*-test with *N* = 6 experiments: per nodule, *P* < 0.05; per nodule mass, *P* < 0.01.

to air. In the absence of sanctions, we would expect rhizobia fixing little N₂ to direct more resources to their own growth and reproduction. In contrast, if host plants detect the near-cessation of N₂ fixation and apply effective sanctions, then we would predict greater growth and reproduction in the rhizobia allowed to fix N₂ normally.

As predicted by the sanctions hypothesis, forcing rhizobia to cheat by preventing N₂ fixation led to a significant decrease in their fitness. N₂-fixing rhizobia consistently grew to larger numbers than non-fixing rhizobia in nodules, whether cheating was forced at the plant (Fig. 1a), half-root (Fig. 1c), or nodule level (Fig. 2). In addition, there was a twofold difference (after one plant generation) in release of rhizobia into surrounding sand (Fig. 1b) or nutrient solution (Fig. 1d). Furthermore, rhizobia that had fixed N₂ in nodules had greater survival in sand over five months than rhizobia from the non-fixing treatment (paired *t*-test, *P* < 0.01, *N* = 12).

The decrease in fitness of the non-fixing rhizobia was associated with a decrease in resource allocation to non-fixing nodules by host plants, as indicated by nodule mass. In experiments where rhizobia were forced to cheat at the half-root or individual-nodule level, each host plant had both fixing and non-fixing nodules, allowing selective partitioning of resources by the host plant. Consistent with the sanctions hypothesis, final nodule fresh weight was higher

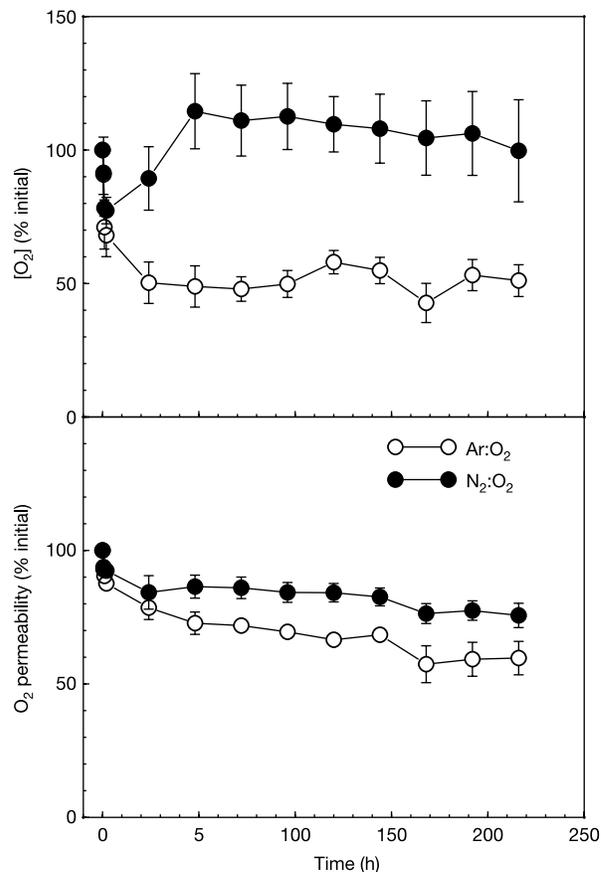


Figure 3 O₂ relations in single nodules where rhizobia were allowed to fix (N₂:O₂) or prevented from fixing (Ar:O₂) N₂. Within 48 h, non-fixing nodules had significantly lower nodule interior O₂ concentration under 20% O₂, as calculated from leghaemoglobin oxygenation (paired *t*-test, *P* < 0.001, *N* = 6), and significantly lower O₂ permeability (paired *t*-test, *P* < 0.05, *N* = 6), relative to controls. Data are presented as % of initial concentration to standardize for any initial differences. A correction for increasing nodule size in controls would have further increased permeability differences between the treatments.

in N₂-fixing nodules, both in the split-root experiment (paired *t*-test, *P* < 0.001, *N* = 12) and in the single-nodule experiment (paired *t*-test, *P* < 0.05, *N* = 6). In addition, root dry weights were higher on the N₂-fixing side in the split-root experiment (paired *t*-test, *P* < 0.05, *N* = 12). These results demonstrate how differences in resource allocation^{13,18} at the nodule level are linked to differences in rhizobial fitness.

What is the mechanism by which these sanctions are carried out? Host plants could impose sanctions on non-fixing nodules by attacking rhizobia directly or by decreasing the supply of any resource required for growth^{5,19}. It appears that a decrease in O₂ supply may be the primary mechanism. Nodule interior O₂ concentration and nodule O₂ permeability were both lower in non-fixing nodules within 48 h of the initiation of the experiment (Fig. 3). This decrease in nodule interior O₂ concentration, previously seen in whole-plant experiments²⁰, is the opposite of what would have happened if photosynthate supply had decreased enough to limit respiration in the nodule interior. A lack of significant differences between treatments in O₂-saturated respiration rate (paired *t*-test, *P* = 0.47, *N* = 6) also indicated that photosynthate supply did not limit respiration more in non-fixing nodules. Nodule O₂ permeability responds to various conditions that affect nitrogen supply and demand^{21,22}, but responses to soil nitrogen are in the opposite direction (that is, greater O₂ permeability when less nitrogen is available)²³ from the response we found to differences in N₂ fixation. Our results therefore appear to be a specific response to rhizobial defection.

A key assumption in these experiments is that nitrogen supply is unlikely to limit the growth or reproduction of rhizobia in non-fixing nodules directly. Much of the nitrogen needed for nodule growth is imported from the phloem, even in nodules that are exporting much larger quantities of nitrogen to the xylem²⁴. Even under the conservative assumption that plants force complete

nitrogen autonomy on rhizobia in non-fixing nodules, we still estimate that even 1% of the N₂ fixation rate in air would provide enough nitrogen to prevent any direct limitation on rhizobial growth. Specifically, if we assume an N₂ fixation rate in air of 2.6 mg N per g of dry weight of nodule per h (0.276 mmol H₂ and 3 mol H₂ per mol N₂)²⁰ and 2.5 mg bacteroid N per g dry weight of nodule (15 mg bacteroid protein and 6 mg protein per mg N)²⁵, the bacteroids (the differentiated, N₂-fixing form of rhizobia) in nodules exposed to air would fix enough nitrogen to double in less than an hour. Even at only 1% of the N₂-fixation rate in air, bacteroids in the Ar:O₂ treatment would fix enough nitrogen to have quadrupled their numbers during the 240 h duration of our single-nodule experiments.

The nodules in our experiment contained only one strain of rhizobia. Mixed nodules can occur, but there is little information on their frequency under field conditions⁵. The potential tragedy of the commons that results from multiple strains per host^{1,3} could also apply to mixed individual nodules. Mixed nodules might reduce the evolutionary effects of nodule-level sanctions if cheats sharing a nodule with mutualists are somewhat protected from nodule-level sanctions⁵. The sanctions reported here are less severe than the flower abortion seen in some yuccas⁹. If rhizobial cheats accumulate more resources than mutualists in the same nodule, as seen by electron microscopy¹⁶, this could perhaps explain the persistence of cheats, despite the fitness cost of cheating in single-strain nodules.

Sanctions directed at specific bacteroids within nodules could be effective in mixed nodules, but only in species in which bacteroids retain the ability to reproduce. Ironically, the most recent evidence for sanctions against bacteroids comes from pea nodules²⁶. In contrast to soybean nodules, bacteroids in pea nodules leave no descendants^{5,27,28}, so denying them resources would have no direct effect on the evolutionary maintenance of cooperation. Only undifferentiated rhizobia, which never fixed N₂, escape into the soil after pea nodules senesce (Fig. 4). Whole-nodule sanctions, such as cutting off O₂ supply, could affect the survival and reproduction of all rhizobia in the nodule interior. This would impose selection on whichever form is reproductive, and therefore central to the evolution of a given species⁵.

Our results support the hypothesis that legumes select for more cooperative rhizobia by imposing sanctions on the basis of the amount of N₂ that rhizobia fix once established inside nodules. The hypothesis that host sanctions could lead to the evolutionary stabilization of the legume–rhizobium mutualism has been shown previously to be theoretically robust^{6,8}. More generally, sanctions are one way in which the host can control the resource environment of their symbiont, and hence impose a selective environment that favours cooperative behaviour. Mechanisms that can do this, such as sanctions and other more indirect methods^{1,3}, could be important in stabilizing a wide range of mutualistic symbioses. This is because they can favour cooperation when cooperation is otherwise hardest to explain: when there are many symbiont strains per host and there is horizontal symbiont transmission among unrelated host individuals^{1,2,7}. □

Methods

We used an Ar:O₂ atmosphere with only traces of N₂ (about 0.03%, by mass spectrometry) to mimic rhizobial cheats that suddenly stop fixing N₂. In future experiments, we could alter the timing and composition of gas treatments to simulate rhizobia with different fixation patterns (for example, fixing N₂ at 25% of potential).

Whole-plant experiment

Seeds of a dwarf cultivar of soybean (*Glycine max*; cv. T243, Strain PI 548224, USDA Soybean Germplasm Collection) were sterilized, germinated and planted into autoclaved 700 ml chambers made from stacked Magenta GA-7 culture boxes filled with quartz sand. An air-driven pump recirculated sterile N-free nutrient solution in each chamber. *Bradyrhizobium japonicum* strain USDA 110 ARS was injected into the sand at the base of each seedling, 7 d after planting. Plants were grown with photosynthetically active radiation of 600 μE m⁻² s⁻¹ and 14 h photoperiod. Replicate plants were grouped into four blocks based on acetylene reduction estimates of initial nitrogenase activity and

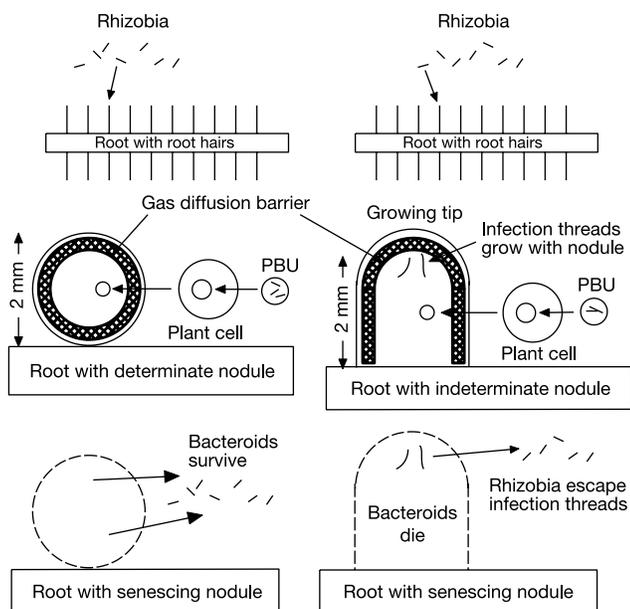


Figure 4 Nodule structure and the life history of rhizobia. After rhizobia differentiate into N₂-fixing bacteroids, they lose the ability to reproduce in nodules with indeterminate growth (for example, pea), but not in nodules with determinate growth (for example, soybean)⁵. The gas permeability of the O₂ diffusion barrier is under the control of the plant in both types of nodule^{21,22}. The peribacteroid unit (PBU) consists of one or more bacteroids surrounded by a plant membrane, which could perhaps allow sanctions at the bacteroid level.

randomly assigned to N₂-fixing and non-fixing treatments. Either N₂:O₂ or Ar:O₂ was delivered through perforated plastic tubing 1 cm above the base, at 100 ml min⁻¹. Three months after planting, nodules were removed from roots. Roots were cut, vortexed, and sonicated in a FS20 'watch-bath' type sonicator in 0.01% Tween 20. The extractant was diluted 10⁶-fold and spread on MAG antibiotic-containing plates. Intact nodules were removed from roots, counted, weighed and crushed in a tissue homogenizer, diluted and plated. Sand from each box was homogenized for 30 min in a sterile flask containing sterile 0.01% Tween 20, on a flask rotator. A liquid subsample was removed from the sand mixture 3 cm below the water line, diluted by 10⁴ and plated. Colonies grew for 10 d at 32 °C and colony-forming units (c.f.u., mean of eight plates) were recorded.

Split-root experiment

Seeds of *G. max* semidwarf variety 'S0066' were sterilized, germinated, and inoculated with approximately 10⁷ cells per seedling. Twelve plants, each with two similar root halves (resulting from regrowth after root-tip removal), were transplanted to hydroponic chambers, with similar nodule numbers on each half of a chamber divided by a silicone gel seal. Chamber halves were randomized into two treatments, either N₂:O₂ or Ar:O₂ (80:20, v/v) at 130 ml min⁻¹, 5 d after transplanting. H₂ production was measured to confirm disruption of N₂ fixation²⁹ by Ar:O₂. Five weeks after transplanting, roots, nodules and rhizobia in nutrient solution were processed as described above for the 12 replicates, each a paired comparison. For survival assays, nodule homogenate was diluted and added at an estimated 10⁵ rhizobia per g sterile sand. Twenty weeks later, rhizobial populations were determined by plate counts.

Single-nodule experiment

Six independent replicate experiments used *G. max* 'S0066' grown in plastic growth pouches and inoculated as above. Fifteen days later, two nodules of equal size were selected per plant. Fixing and non-fixing treatments were randomized. Chambers of 2 cm diameter were positioned around intact nodules, with 250 ml min⁻¹ of humidified N₂:O₂ or Ar:O₂ flowing through each chamber. Fractional oxygenation of leghaemoglobin under air, nodule O₂ permeability, and O₂-saturated respiration rate were measured daily as previously described^{23,30}. Briefly, nodules were exposed successively to 20, 0, 70 and 0% O₂ while fractional oxygenation of the nodule protein leghaemoglobin was measured by non-invasive spectrophotometry. O₂ permeability was calculated from the rate of increase in oxygenation after switching to 70% O₂, after correcting for respiration, which was calculated from the rate of oxygenation decrease as interior O₂ fell from O₂-saturated to O₂-limited concentrations after switching to 0% O₂. After 10 d, nodules were weighed, crushed, and assayed for c.f.u. per nodule and per g of nodule. Analyses of variance and Tukey's studentized range test for whole-root, and paired *t*-tests for split-root and single nodule experiments were conducted using SAS software (SAS Institute).

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Functional genetic analysis of mouse chromosome 11

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Now that the mouse and human genome sequences are complete, biologists need systematic approaches to determine the function of each gene^{1,2}. A powerful way to discover gene function is to determine the consequence of mutations in living organisms. Large-scale production of mouse mutations with the point mutagen N-ethyl-N-nitrosourea (ENU) is a key strategy for analysing the human genome because mouse mutants will reveal functions unique to mammals, and many may model human diseases³. To examine genes conserved between human and mouse, we performed a recessive ENU mutagenesis screen that uses a balancer chromosome, inversion chromosome 11 (refs 4, 5). Initially identified in the fruitfly, balancer chromosomes are valuable genetic tools that allow the easy isolation of mutations on selected chromosomes⁶. Here we show the isolation of 230 new recessive mouse mutations, 88 of which are on chromosome 11. This genetic strategy efficiently generates and maps mutations on a single chromosome, even as mutations throughout the genome are discovered. The mutations reveal new defects in haematopoiesis, craniofacial and cardiovascular development, and fertility.