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Measured sanctions: legume hosts detect quantitative variation in rhizobium cooperation and punish accordingly

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ABSTRACT

Question: Does severity of punishment vary quantitatively with partner cooperation?

Hypothesis: Sanctions against defecting partners may be crucial for the evolutionary persistence of cooperation. Legume sanctions have been demonstrated when rhizobia either fully defect or fully cooperate, but not when they invest at an intermediate level. We predicted that intermediate rates of cooperation would trigger intermediate sanctions.

Model system: We varied rhizobium cooperation and its importance to the plant by adjusting N_2 concentration, manipulating rhizobia to fix N_2 at about 1%, 17%, 33%, 50%, and 100% of their potential, and/or by adding nitrate.

Results: Fixation and rhizobium fitness were significantly correlated in a regression model suggesting that sanction strength varies with N_2 fixation. Sanction severity was increased by the addition of external nitrate.

Keywords: cheat, cooperation, investment, legume, mutualism, nitrogen fixation, punish, sanction, strategy, symbiosis.

INTRODUCTION

The ubiquitous nature of mutualisms suggests that cooperative behaviour between species is often advantageous to individuals. Yet evolutionary theory begs the question, why expend resources to benefit another species, when resources could be redirected for one's own fitness? The symbioses between legumes and rhizobia (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* species), in which carbohydrates provided by the host legume are exchanged for nitrogen supplied by the rhizobia, are ideal mutualisms to study the dynamics of cooperation. Cooperation has persisted despite lacking the constraints thought to stabilize other mutualisms (see Herre *et al.*, 1999). For instance, rhizobia can survive

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and reproduce in soil in the absence of their host. Horizontal transmission of rhizobia released from nodules (McDermott *et al.*, 1987) to unrelated individual host plants (typically of the same legume species) is common and potentially destabilizing because the reproductive success of rhizobia is not linked to that of their host. Therefore, symbionts might be expected to reallocate resources to their own reproductive fitness (Frank, 1998; Denison, 2000; Simms and Taylor, 2002). Similarly, individual plants are typically infected by more than one strain of rhizobia (Hagen and Hamrick, 1996; Silva *et al.*, 1999). This means that rhizobia that supply their host with nitrogen may indirectly benefit competing strains of rhizobia infecting the same individual plant, creating a classic 'tragedy of the commons' problem (Hardin, 1968), undermining cooperation (Denison, 2000; West *et al.*, 2002a, 2002b). In addition, cooperation is expensive. Legumes invest as much as 20% of net photosynthate production to supporting their rhizobia (Pate, 1986). Similarly, nitrogen fixation is energetically costly to rhizobia, reducing the pool of resources that could potentially be allocated to their own growth and reproduction. If either partner could increase its own fitness by defecting from mutualistic duties, how has this ancient mutualism persisted?

One possible explanation for the evolutionary persistence of the mutualism is that hosts are able to differentially affect the fitness of rhizobium in nodules based on their N_2 fixation rates, perhaps by varying resource allocation (Denison, 2000; Denison and Kiers, 2004a, 2004b). Termed the 'sanctions' hypothesis, we have suggested that these host responses make the reproductive success of the rhizobium strain contingent on the strain exporting nitrate to the host (Denison, 2000; West *et al.*, 2002a, 2002b; Kiers *et al.*, 2003). If the host discriminates among nodules, preferentially supporting those that are fixing more N_2, then cooperation will tend to be favoured. Sanctions are conceptually analogous to 'policing' mechanisms that have been shown to stabilize cooperation within species (Frank, 1995; Ratnieks *et al.*, 2001), 'selective abortion' mechanisms seen in some obligate pollination mutualisms (Pellmyr and Huth, 1994), or 'active retaliation', which Bull and Rice (1991) suggested can be important in maintaining mutualism with either 'partner choice' or 'partner fidelity'.

Differences in nodule growth and/or rhizobia per nodule, associated with differences in rhizobium mutualism, have been seen in soybean (Singleton and Stockinger, 1983; Kiers *et al.*, 2003) and in wild lupine (Simms *et al.*, 2006). Simms *et al.* (2006) called this 'partner choice', consistent with a recent very broad definition (Sachs *et al.*, 2004), but not with a narrower earlier one (Bull and Rice, 1991), which characterized 'repeated or long-term interactions' with one or more partners as 'partner fidelity' instead.

Opportunities for researchers to manipulate mutualistic behaviour directly are rare. However, with rhizobia we are able to modify the cooperative strategy of the symbiont by altering the amount of N_2 in the atmosphere. In previous experiments, we have forced normally mutualistic rhizobia to defect from N_2 fixation by replacing air ($N_2:O_2$, 80:20 v/v) with an argon gas mixture ($Ar:O_2$, 80:20 v/v) containing only a trace amount of N_2 and monitoring the legume host response (Kiers *et al.*, 2003). A reduction in host-mediated O_2 supply to the nodule interior coincided with a 50% reduction in reproductive success of non-cooperative (non-fixing) rhizobia (Kiers *et al.*, 2003). Rhizobia depend on O_2 for aerobic respiration (King and Layzell, 1991), so reduced O_2 influx may result in limited rhizobium reproduction (Kiers *et al.*, 2003), either directly or indirectly.

In past laboratory manipulations, we monitored host response to the most extreme rhizobium strategies: full N_2 fixation or trace N_2 fixation (~1%) (Kiers *et al.*, 2003). By contrast, naturally occurring rhizobium strains often have intermediate levels of cooperation (Burdon *et al.*, 1999; Denton *et al.*, 2000; Thrall *et al.*, 2000). Rhizobium strains show a spectrum of fixation, varying considerably in resources they supply to the host, with net benefits provided by different strains varying as much as ten-fold (Singleton and Stockinger, 1983; Fening and Danso, 2002; Kiers *et al.*, 2002; Provorov and Tikhonovich, 2003). This is similar to other symbioses, such as the mycorrhizal symbiosis (Johnson *et al.*, 1997) and obligate pollination mutualisms (Herre, 1989; Kawakita and Kato, 2004), in which partners often neither fully defect or cooperate, but rather invest at some intermediate level. In such cases, sanctions may also operate at intermediate levels in response to intermediate symbiont investment. The ability to vary sanction strength, like the ability to vary investment (Killingback and Doebeli, 2002; Roberts and Renwick, 2003), may increase the evolutionary stability of cooperation.

Here, we exploit the experimental advantages of the legume–rhizobium system to address the question of variable sanctions, by testing the quantitative dependence of fitness-limiting host sanctions on rhizobium N_2 fixation rates. By altering the composition of gas treatments, we simulated rhizobia with different fixation rates. In a series of three experiments, we determined soybean plant sanction response to rhizobia fixing at three intermediate rates: roughly 17%, 33%, and 50% of potential fixation. Each experiment monitored the reproductive success of rhizobia in a nodule fixing at an intermediate fixation rate, as well as a 'non-fixing' control nodule (fixing only enough N_2 to meet the needs of the rhizobia themselves) and a nodule fixing at 100% fixation (full-fixing nodule). In an additional experiment, we supplied an external source of nitrate to determine how an alternative source of nitrogen affected the intensity of host sanctions. A higher external nitrate concentration means that the cost to the plant of obtaining soil nitrogen decreases, relative to supporting N_2-fixing rhizobia, which we predict would favour sanctions against those rhizobia that might otherwise be tolerated when less soil nitrate is available (West *et al.*, 2002a, 2002b).

MATERIALS AND METHODS

Plant and inoculum growth

Seeds of a semi-dwarf cultivar of soybean (*Glycine max* 'S0066') were grown in growth pouches (Mega International, Minneapolis, MN) as described previously (Kiers *et al.*, 2003). Inoculum of *Bradyrhizobium japonicum* strain USDA 110 ARS (USDA Beltsville, MD) was grown in Modified Arabinose Gluconate (MAG) (Van Berkum, 1990) broth and plants were inoculated 7 days after planting with approximately 1×10^7 *B. japonicum* cells per plant. Plants were grown in a growth chamber (E7/2: Conviron, Winnipeg, Manitoba) under a light intensity of approximately 400 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ supplied with a combination of incandescent and fluorescent lights for a 13-h photoperiod. Temperature was maintained at approximately 22°C. Plants were watered daily with 50 ml of nitrogen-free nutrient solution as described by Blumenthal *et al.* (1997).

$Ar : N_2 : O_2$ treatments

In each experiment, three nodules of similar size on each of six replicate plants were chosen, 14 days after inoculation. Small gaskets made of modelling clay were placed around single nodules, riveted to the pouch with shortened insect pins and fastened with hot melt glue (Fig. 1). This created a gas-tight chamber for each single nodule, without damaging the root system. Gas flowed into each chamber at a rate of 175 $\text{ml} \cdot \text{min}^{-1}$ through 1.6-mm plastic

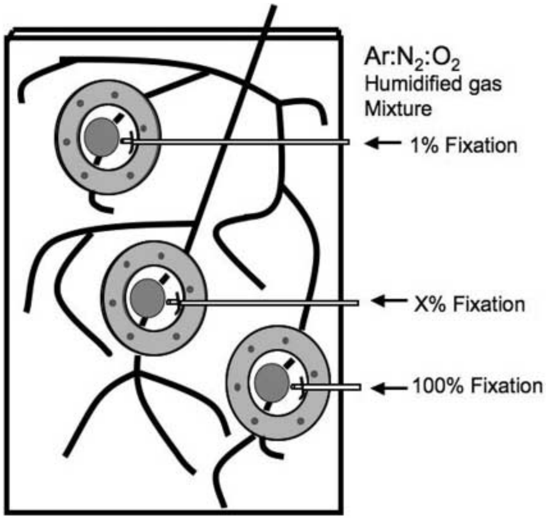

Fig. 1. Gas chamber design for intact soybean plants in pouches. Each of three nodules, surrounded by a clay gasket fastened to the pouch, received (assigned randomly) one of three humidified gas mixtures (Ar : O_2, 1% fixation; Ar : N_2 : O_2, intermediate (X%) fixation; and N_2 : O_2, 100% fixation), all with 20% O_2.

tubing set into the clay, sealed to the pouch with clear adhesive. Using mass flow controllers (Sidetrack 830: Sierra Designs, Monterey, CA), three gas mixtures (Ar : O_2, trace fixation; Ar : N_2 : O_2, intermediate fixation; and N_2 : O_2, full fixation), all with 20% O_2, were delivered through three manifolds, each containing six replicate flow-limiting hypodermic needle capillaries. Humidified gas was supplied to individual nodule chambers. The three nodules on each replicate plant were randomized into three gas treatments. To estimate the external N_2 concentration to achieve the intermediate fixation rates, we assumed an average K_m (N_2) of 0.044 atm (Rasche and Arp, 1989) in the nodule interior, an O_2 flux eight times the N_2 flux (Sinclair and Goudriaan, 1981; Sheehy *et al.*, 1987), and a drop in O_2 concentration from atmospheric outside to near zero in the nodule interior.

Plants in the additional nitrate experiment were grown as described above, either with the addition of 0.25 mmol·l^{-1} $Ca(NO_3)_2$ in the nutrient solution or as no-nitrate controls. Eight plants, each with three single nodules in gas chambers, were randomized to fix N_2 at trace, full, or an intermediate rate of 50%. Plants were further randomized into nitrate or nitrate-free treatments, for a total of four replicates per treatment. Beginning at the initiation of the gas treatments, 50 ml of nitrate or nitrate-free nutrient solution was added to each of the plant pouches and replaced daily.

Nodule harvest

Each set of replicates in each experiment was performed on a different plant. Experiments (each with a different intermediate fixation rate) were sequential with each of the four experiments running for 10 days. Single nodules were weighed, crushed in a tissue homogenizer with 1 ml of H_2O, serially diluted, and plated. Bacterial colonies were grown at 30°C for 10 days and colony-forming units per nodule were calculated from nodule homogenate volume, dilution factor, and the mean of eight plate counts (Kiers *et al.*, 2003). To test the effects of fixation level on rhizobial fitness, data from the three experiments were pooled, natural-log-transformed for homoscedasticity, and a regression analysis was performed using generalized linear modeling, including 'experiment' as a covariable in the PROC-GLM function of SAS (SAS Institute, Cary, NC). Analysis of variance and significant differences among means, using Student-Newman-Keuls tests, were conducted in SAS for the nitrate experiment.

RESULTS

Reproductive success of rhizobia in nodules depended on their ability to fix N_2 (Fig. 2). Level of fixation and rhizobial fitness were significantly and positively correlated ($R^2 = 0.32$, $F_{1,50} = 24.93$, $P < 0.0001$). Rhizobia in nodules fixing at 1, 17, 33, and 50% of potential fixation had a reproductive output that was 37, 40, 61, and 77% that of full-fixing controls, respectively.

The addition of 0.25 $\text{mmol} \cdot \text{l}^{-1}$ $Ca(NO_3)_2$ generally lowered rhizobium reproductive success and nodule weight (Fig. 3). Nodules in the nitrate treatment fixing N_2 at either 50% or 100% potential contained approximately 70% of the number of rhizobia and were 60% the weight of corresponding nodules in the non-nitrate treatment (Fig. 3). Nitrate did not affect nodule weight or reproductive success of non-fixing nodules (Student-Newman-Keuls, $P > 0.05$) (Fig. 3). Within both the nitrate and non-nitrate treatments, rhizobial fitness responded similarly to a reduction in N_2 fixation rate. That is, when N_2 concentration was lowered to limit fixation to a target rate of 50% of potential, reproductive success was decreased to about 85% of potential, with or without nitrate (Fig. 3).

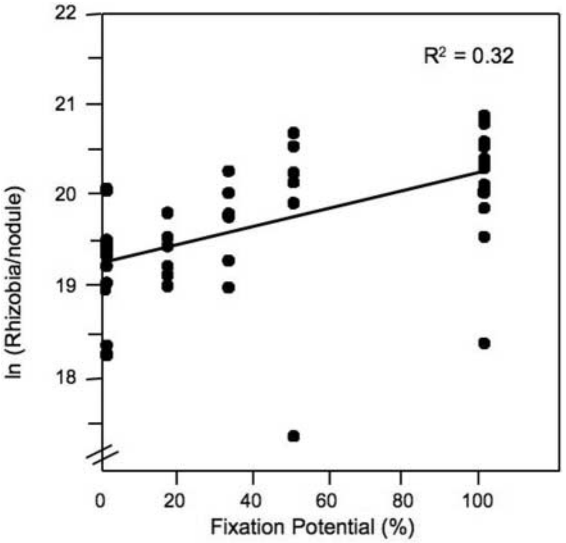

Fig. 2. Natural-log-transformed rhizobial reproductive fitness (as calculated in colony-forming units per nodule) as a function of target fixation rate. Each circle represents an individual nodule. Level of fixation and fitness were significantly correlated in the linear regression model ($R^2 = 0.32$, $F_{1,50} = 24.93$, $P < 0.0001$). Each experiment had six replicate pouches similar to Fig. 1.

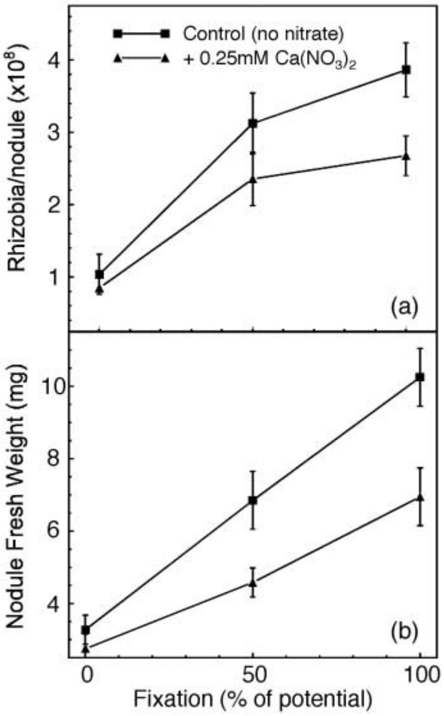

Fig. 3. Effects of reduced N_2 fixation and external nitrate on (a) rhizobia per nodule (as calculated above) and (b) nodule fresh weight. The addition of nitrate reduced rhizobium fitness and nodule weight compared with the non-nitrate controls in nodules fixing at 100% potential and 50% potential (Student-Newman-Keuls, $P < 0.05$, $n = 4$) but not in the non-fixing nodules. In both the nitrate and control treatments, rhizobia fixing at both 100% and 50% outnumbered those fixing at ~1% potential (Student-Newman-Keuls, $P < 0.05$, $n = 4$).

DISCUSSION

Our aim was to determine the effects of variable levels of cooperation (N_2 fixation) on rhizobium reproductive success. The results of this study suggest that rhizobia that fix N_2 at intermediate rates are subject to intermediate sanctions. For example, the fitness of rhizobia fixing at 33% of potential was approximately 1.5 times higher than non-fixing rhizobia and roughly 60% that of fully fixing rhizobia (Fig. 2). Our data provide empirical support for evolutionary models of cooperation that emphasize the importance of variable investments and variable sanctions in the maintenance of mutualisms over evolutionary time (Roberts and Sherratt, 1998; Killingback and Doebeli, 2002; West *et al.*, 2002b; Roberts and Renwick, 2003; Ifti *et al.*, 2004).

The sanction response appears to be a continuous function of N_2 fixation (Fig. 2), although variability among plants does not allow us to rule out, with statistical certainty, the possibility that there is some threshold rate of fixation (probably > 50%) above which plants impose no sanctions. The difference between very mild sanctions and no sanctions may not affect rhizobium evolution if they are outweighed by differences in subsequent survival in the soil, especially if rhizobia that fix less N_2 store more resources per cell. Over most of the range of fixation rates, however, it appears that hosts can distinguish levels of

N_2 fixation in their rhizobial symbionts, and regulate resource allocation to individual nodules accordingly. Individual plants would benefit by sanctioning less-beneficial rhizobia because this conserves scarce photosynthate (West *et al.*, 2002a), even if individual rhizobia never respond to sanctions by fixing more N_2. Because decreased fixation was imposed externally in our experiments, any such plastic responses would have been obscured. The existence of such responses, which would presumably vary among rhizobium genotypes, if they exist at all, remains to be determined.

The severity of the sanction response also varied according to external resource supply. Nitrate decreased the reproductive success and nodule weight of rhizobia in nodules with target N_2 fixation rates of 50% and 100% of potential (Fig. 3). Nitrate exposure has been shown to decrease nodule O_2 permeability (Denison and Harter, 1995) and nodule growth (Fujikake *et al.*, 2003). We previously predicted that increasing nitrogenous fertilizer would favour the evolution of rhizobia with lower N_2 fixation (West *et al.*, 2002a). However, we could not detect a change in the *relative* severity of sanctions with the addition of nitrate. There is a possible source of error in this result, although we consider it unlikely. Suppose the nitrate treatment reduced nodule gas permeability, as has been previously reported with much (40×) higher nitrate concentrations (Denison and Harter, 1995). This would have no effect on the nodule exposed to atmospheric N_2, because N_2 in the nodule interior would still be saturating, even if nodule permeability reduced N_2 influx. But in the nodule with target N_2 fixation of 50%, the actual fixation could be slightly lower in the nitrate treatment than in the no-nitrate control, because lower nodule permeability would cause a greater drop in N_2 concentration across the diffusion barrier that surrounds the nodule interior. A lack of difference between nitrate treatments in the relative severity of sanctions would therefore mean that plants become more, not less, tolerant of lower N_2 fixation when nitrate is available. Although this is unlikely, more experiments are needed to explicitly test whether nitrate can alter the relative abundance of ineffective and effective genotypes in nature.

A major assumption in these and other N_2-limiting experiments is that the growth and reproduction of rhizobia are not directly limited by nitrogen supply. The trace of N_2 in our $Ar:O_2$ (80:20 v/v) reduces fixation to about 1% of normal. The estimated N_2 concentration required to allow complete nitrogen autonomy on rhizobia in the non-fixing treatment is below this 1% figure (Kiers *et al.*, 2003). Furthermore, nodule growth has been shown to be supported by nitrogen imported by the phloem, even when nodules are exporting larger quantities of nitrogen to the xylem (Layzell *et al.*, 1979). There is one report suggesting that replacing N_2 with argon may affect the stomata (McNeill *et al.*, 1993), but in our experiments the shoots were not exposed to argon. We only applied gas treatments to individual nodules, a less severe treatment than exposing the plant or even the entire root, which is a standard method in N_2 fixation research (Atkins *et al.*, 1984; Pate *et al.*, 1984; Denison *et al.*, 1992) with no reported deleterious side-effects.

CONCLUSION

Theory suggests that sanctions could be critical in many cooperative relationships (Frank, 1995; West *et al.*, 2002a, 2002b; Gardner and West, 2004), but there have been few empirical tests of this hypothesis to date. Host punishment has been noted in other mutualistic systems as an important factor in maintaining cooperation, such as in the symbiosis between bobtail squid and the luminescent bacteria, *Vibrio fisheri*, that aid in camouflaging the nocturnal foraging squid via counter-illumination (Sachs *et al.*, 2004). Exploitation in some obligate pollination mutualisms is also apparently limited by host-mediated selective punishment (Pellmyr and Huth, 1994; West and Herre, 1994; Wilson and Addicott, 1998; but see Shapiro and Addicott, 2004; Yu *et al.*, 2004). However, in these systems it is not yet clear if severity of punishment varies quantitatively with the degree of exploitation. A threshold response, as seems more likely for some sanctions such as fruit abscission, could select for only enough mutualism to avoid sanctions. Experimental manipulations of cooperative strategies in these other species may help answer questions about continuous versus threshold responses.

Our data suggest that legume hosts can respond to variable rhizobium performance by imposing sanctions of variable severity. This ensures that rhizobia that reduce their investment in N_2 fixation will pay at least some fitness cost, although this cost could be small in the range from 50 to 100% of potential fixation. Variable sanction severity likely provides individual fitness benefits to legume plants because photosynthate appears to be allocated among nodules in rough proportion to benefits received. This is likely an important selection pressure in maintaining evolutionary stability of the legume–rhizobia symbiosis.

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