Chapter II

MAINTAINING COOPERATION IN
THE LEGUME-RHIZOBIA SYMBIOSIS:
IDENTIFYING SELECTION PRESSURES AND MECHANISMS

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1. INTRODUCTION

Mutualistic symbioses include a wide range of interactions among a diverse set of organisms. The symbiosis between legumes and rhizobia is a classic mutualistic relationship. In return for carbohydrates provided by the host legume, the rhizobia supply nitrogen to the legume. Although reciprocally beneficial, the stability of this cooperative relationship poses a dilemma to evolutionary theory. Why do symbioses persist over millennia, if additional resources could be gained by either partner through the exploitation of the mutualism? N_2 fixation is clearly beneficial to the host plant because it supplies nitrogen needed for growth and photosynthesis. But N_2 fixation is energetically costly to the bacteria, and hence reduces the resources that could be allocated to their growth and reproduction. Why then do rhizobia fix N_2 to benefit the host plant when expending those resources on reproduction could increase the fitness of the rhizobia themselves? We have suggested (Denison, 2000; West *et al.* 2002 a,b) and then shown experimentally (Kiers *et al.*, 2003) that legumes penalize rhizobia that fail to fix N_2. Such sanctions selectively favor the most beneficial rhizobia, and hence can stabilize the mutualistic interaction (Denison, 2000; West *et al.*, 2002a,b).

2. EXPLAINING COOPERATION: THE PROBLEM

Basic alignment of interests between host and symbiont will tend to select, in an evolutionary sense, for continued cooperation. Current theory suggests that the stability of cooperation is promoted by "passage of symbiont from parent to offspring (vertical transmission), genotypic uniformity of symbionts within individual hosts, spatial structure of populations leading to repeated interactions between would-be mutualists and restricted options outside the relationship for both partners " (Herre *et al.*, 1999). However, symbioses between legumes and rhizobia follow none of the restrictions above, with the possible exception of spatial structuring. Rhizobia are saprophytes and can reproduce outside the host. Rhizobia are transmitted horizontally through the soil, not passed directly from parent to offspring, and plants are typically infected by more than one strain of rhizobia (Lindemann *et al.*, 1974; Dowling and Broughton, 1986; Vlassak and Vanderleyden, 1997). This last point implies that rhizobia that supply their host with nitrogen may indirectly benefit competing strains of rhizobia infecting the same individual plant.

To be more specific, if an individual plant hosts a single lineage of rhizobial symbiont, then the shared fate of host and symbiont may favor cooperation (Herre, 1993; Frank, 1994a, 1998; Crespi, 2001). But as the number of strains per plant increases, evolutionary theory predicts a rise in symbiont parasitism (Axelrod and Hamilton, 1981; Frank 1994 a,b; Leigh and Rowell, 1995; Maynard Smith and Szathmary, 1995). This is the classic tragedy of the commons problem from human economics (Hardin, 1968). Although unwavering cooperation among lineages infecting a given plant would increase their collective access to resources, each symbiont lineage is selected to allocate resources mainly to its own growth and reproduction, to the detriment of its host and fitness of other lineages. Consequently, 'free-rider' rhizobia, those that cheat by extracting carbohydrates from the host while fixing little to no N_2, are predicted to spread at the expense of efficient N_2-fixing strains (Denison, 2000; West *et al.*, 2002a).

Data from the field have confirmed the existence of less effective rhizobia, as rhizobial strains have been shown to vary considerably in the benefits they provide to the host (Thrall *et al.*, 2000; Denton *et al.*, 2000). Strains fixing little to no N_2 are widespread in both natural and agricultural soils (Singleton and Stockinger, 1983; Burdon *et al.*, 1999). N_2 fixation is an energy intensive process (Gutschick, 1981). Clear trade-offs exist between allocation of carbohydrate to respiration in support of N_2 fixation versus hoarding of carbohydrate by rhizobia to support their own growth and reproduction (Denison, 2000). This is demonstrated in the work of Hahn and Studer (1986), who showed that *Bradyrhizobium japonicum* mutants that do not fix N_2 accumulate higher amounts of the energy-rich poly-β-hydroxybutyrate (PHB) than their N_2-fixing parent strains, when both strains share the same nodule. Cevallos *et al.* (1996) found that rhizobial mutants that were unable to synthesize PHB would continue to fix N_2 for longer periods than the PHB producing parents. These data suggest a negative correlation between PHB accumulation and N_2 fixation rate (Kretovich *et al.*, 1977) as well as a possible individual benefit for rhizobia that cheat

their plant hosts by failing to invest in N_2 fixation (Denison, 2000).

Cooperation may still be maintained in the presence of cheating symbionts if costs associated with tolerating cheaters are negligible (Schwartz and Hoeksema, 1998). However, investment in nodule production and maintenance is high for legumes, consuming as much as 20% of net photosynthate production (Pate, 1986), suggesting that supporting ineffective rhizobia is a substantial drain on the host. Investment in nodules can pay off for plants if the increase in net photosynthesis supported by N_2 fixation (Bethlenfalvay *et al.*, 1978) exceeds the photosynthate cost, but there is no guarantee that all rhizobia admitted will provide a net benefit.

The tendency of hosts to associate with better cooperators may increase the evolutionary stability of certain mutualisms (Ferriere *et al.*, 2001). If plants could distinguish between marginally effective and highly effective rhizobial strains at time of infection, there would be little host carbohydrate loss to cheating strains. But because nitrogen fixation does not begin until several days after infection, it appears pre-infection recognition and exclusion of cheaters would have to be based solely on signaling mechanisms. Despite elaborate recognition signals (Hirsh, 1999), legumes do not seem to exclude parasitic rhizobia that are closely related to their usual symbiotic partners (Amarger, 1981; Hahn and Studer 1986). The short generation time of rhizobia compared to plants and the ability of rhizobia to modify their own extracellular signals (Roche *et al.*, 1991), perhaps mimicking cooperative strains, favors the parasitic symbiont in breaking possible signaling codes.

Given that there are high energy costs of mutualism, that the hosts lack an ability to recognize parasitic rhizobia at time of infection, and that genetically diverse symbiont populations on the host create a tragedy of the commons, we would expect less effective rhizobia to proliferate in soil populations. Indeed, a breakdown in cooperation seems inevitable over only a few generations. How, then do we explain the stability of the legume-rhizobia symbiosis over tens of millions of years?

3. EXPLAINING COOPERATION: THE HYPOTHESES

3.1. Hypothesis 1: Shared Interest

A classical explanation for cooperation between different species is based on the concept of 'shared interest' (Frank, 1998). It might seem that rhizobia have a shared interest in increasing the overall plant growth and photosynthesis of their host by providing the host with nitrogen (Bethlenfalvay *et al.*, 1978), because this would presumably increase overall levels of resources available to the rhizobia. In this hypothesis, high levels of N_2 fixation are favored because cooperating with the host results in increased resource supply to all of the rhizobia infecting a given plant, giving their descendants a competitive advantage against other rhizobia. However, the conditions for cooperation to be maintained under a shared interest model are quite restrictive. West *et al.* (2002a) present a series of models which suggest natural selection will maintain high levels N_2 fixation in rhizobia if (i) there is high relatedness (*r*) among rhizobial strains sharing a host and (ii) the relative importance

of N_2 fixation to overall plant photosynthesis (source of carbohydrates for rhizobia) is high (*s is low if soil N is scarce, for example*) (Figure 1). As these parameters change to low relatedness of rhizobial strains and low relative importance of N_2 fixation to plant resources, low levels of N_2 fixation are predicted (West *et al.*, 2002a).
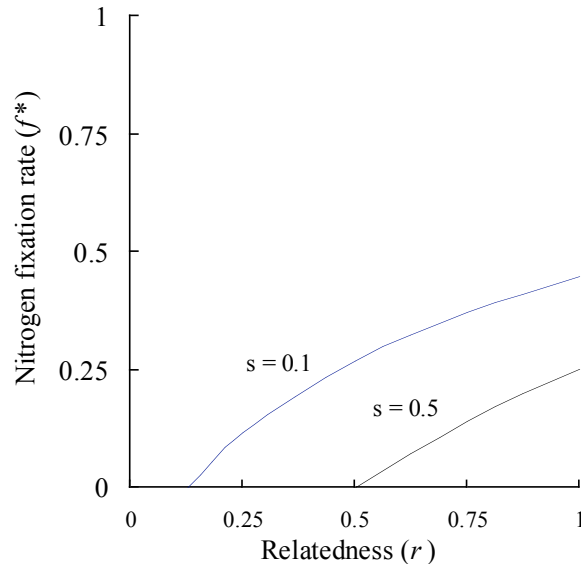

Figure 1: Shared Interest Model. The unbeatable N_2-fixation rate (f*) plotted against the relatedness between the rhizobial strains in a plant (r). Different lines represent values of (s), the relative importance of N_2 fixation by rhizobia to plant resources and growth. Low rates of fixation are predicted when there is low relatedness of the rhizobia on a root system and when N_2 fixation has a smaller effect (high s) on plant resources. Reproduced from West et al.*(2002a) with permission of the Royal Society.*

Most plants are infected by more than one strain of rhizobia (Dowling and Broughton, 1986). These high numbers of rhizobial strains infecting each plant host create conditions that select for low levels of N_2 fixation. Data from electrophoretic markers suggest an average of approximately 10 rhizobial strains per plant, with a range of 4-18 (Hagen and Hamrick, 1996 *a,b*; Souza *et al.*, 1997). This selection for a breakdown in cooperation in a multiply infected host is analogous to the evolution of virulence in multiple parasites infecting a single host (Williams and Nesse, 1991; Herre, 1993; Ebert, 1998; Frank, 1998). Each symbiont is selected to increase its own growth at the expense of costly cooperative efforts like N_2 fixation (Axelrod and Hamilton, 1981; Leigh and Rowell, 1995; Maynard Smith and Szathmary, 1995). The shared interest hypothesis fails to explain the maintenance of cooperation as multiple symbiont strains on the host create a tragedy of the commons in which individual interests are selected above collective interests.

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3.2. Hypothesis 2: Kin Selection in the Rhizosphere

The hypothesis that the evolutionary persistence of N_2 fixation depends on the channeling of plant resources to related kin in the soil rhizosphere has been suggested in several papers (Olivieri and Frank, 1994; Simms and Bever, 1998; Bever and Simms, 2000; Crespi, 2001). The rhizosphere hypothesis relies on a kin-selected benefit of resources supplied by altruistic kin (Jimenez and Casadesus, 1989) inside the nodule. It assumes that the nodule rhizobia that fix N_2 are terminally differentiated and at a reproductive dead end (but see below), and can best perpetuate their genes by shuttling resources to reproductively viable (undifferentiated) kin in the rhizosphere (Simms and Bever, 1998). It maintains that N_2 fixation provides greater root exudations and that these resources can be used by genetically-related rhizobia in the soil. A more specific version of the rhizosphere hypothesis is known as the 'rhizopine concept', which assumes that inositol-like compounds called 'rhizopines' are excreted by N_2-fixing nodules and then metabolized mainly by specific rhizobial kin in the soil (Olivieri and Frank, 1994; Murphy *et al.*, 1995; Simms and Bever, 1998).

The rhizopine hypothesis is supported by the following observations: (i) the expression of genes for the synthesis of rhizopines is controlled by genes linked to N_2-fixing activity; (ii) in *Sinorhizobium meliloti* and *Rhizobium leguminosarum*, Sym plasmids encode for rhizopine metabolism, and (iii) nodulation competitiveness is improved by rhizopine-catabolizing ability (Provorov, 1998). However, competition experiments using transgenic rhizobia differing in their ability to produce or consume rhizopines "were not consistent with the hypothesis that the sole role of rhizopines is to act as proprietary growth substances for free-living populations of the producing strain" (Gordon *et al.*, 1996).

Fierce competition among soil bacteria (including unrelated saprophytes and both nonmutualistic and mutualistic rhizobia) for resources in the rhizosphere undercut the effectiveness of resource shuttling by nodule rhizobia to their soil kin (West *et al.*, 2002*a*). Open access to plant exudates could increase the relative fitness of rhizosphere mutualists and nonmutualists alike (Denison *et al.*, 2003). The rhizosphere hypothesis acknowledges this vulnerability to invasion by unrelated soil bacteria but counters the problem by suggesting that rhizopines can only be metabolized by a few rhizobial strains, and that spatial structuring of soil (poor mixing to limit dispersal of rhizobia) will prevent invasion by unrelated strains. It is suggested that rhizopine specificity restricts resource consumption to related mutualists while spatial structuring increases relatedness between nodule and soil rhizobia resulting in the direct transfer of resources between nodule and soil kin (Simms and Bever, 1998; Bever and Simms, 2000).

Nonetheless, the rhizosphere hypothesis is unlikely to explain cooperative stability for several reasons:

(i) The rhizosphere hypothesis assumes that rhizobia in the nodule leave no direct descendants. Although nodules differ in their ability to leave direct descendants,

species with determinate nodules (*Glycine*, *Lotus*, and *Phaseolus*) tend to have bacteroids (the differentiated N_2-fixing rhizobial form) that do retain their reproductive ability (Gresshoff and Rolfe, 1978; Zhou *et al.*, 1985; McDermott *et al.*, 1987). This implies that selection can act directly on the N_2-fixing form of rhizobia (the bacteroids) and need not rely on a rhizosphere kin-selected benefit (Denison, 2000). Even in indeterminate nodules (*Pisum*, *Trifolium* and *Medicago*), the undifferentiated rhizobia found in the infection threads are thought to be reproductively viable (Sprent *et al.*, 1987) and recolonize the soil after nodule senescence (Vance *et al.*, 1980; Timmers *et al.*, 2000). Natural selection would therefore favor bacteroids that preferentially shuttle resources to these genetically identical kin within the nodule, which may contain more rhizobia than 10 kg of soil (West *et al.*, 2002*a*), rather than to a mixed population of rhizobia and other species in the rhizosphere. West *et al.* (2002*a*) suggested that accumulation of the energy-rich PHB molecule (potentially useful for reproduction and soil survival of rhizobia) could be useful to researchers in identifying the rhizobial form that survives senescence of the nodule. In determinate nodules, differentiated bacteroids hoard PHB, whereas in indeterminate nodules it is the undifferentiated rhizobia (and not bacteroids) that accumulate PHB, at least in some species (Wong and Evans, 1971; Vance *et al.*, 1980). Importantly, in both nodule types, some reproductively viable rhizobia are thought to survive nodule senescence, although natural selection will act on different forms (differentiated rhizobia in determinate nodules and undifferentiated rhizobia in indeterminate nodules). We therefore reject the assumption that all nodule rhizobia are reproductively dead and that soil kin are the only form that can benefit from N_2 fixation. If there are possible legume-rhizobia combinations that do not result in the reproduction of any direct descendants from rhizobia inside nodules, then kin-selected rhizosphere benefits may prove to be an important selective mechanism in those cases.

(ii) The rhizosphere hypothesis suggests that spatial structuring (population viscosity) of rhizobial soil populations is key to the stability of the legume-rhizobia mutualism (Bever and Simms, 2000). Although spatial structuring of populations may increase kin-selected benefits by reducing mixing with nonmutualistic strains, increased spatial structuring also increases local competition among clonally identical rhizobia, which could reduce any kin-selected benefits for greater N_2 fixation (Queller, 1992; Frank, 1998; West *et al.*, 2002*a*). Indeed, under the simplest scenario, the benefits of spatial structuring to kin selection are often exactly balanced by changes in scale of competition (Taylor, 1992; Queller, 1994; Frank, 1998; West *et al.*, 2001).

(iii) The rhizosphere hypothesis proposes that specific exudates from the host plant selectively benefit related kin in the rhizosphere. Specificity of rhizopines might prevent some soil bacteria from being able to catabolize rhizopines, but mutants closely related to the parental strain could easily arise. For instance, a rhizobial mutant that lost the genes for infecting legumes but retained the ability to metabolize rhizopines would outcompete and displace its mutualistic parent strain, assuming that the benefits of symbiosis to rhizobia depend on rhizopine exudation to the rhizosphere (Denison, 2000). Even if specific rhizosphere resources were available, benefits to rhizosphere rhizobia are likely trivial when compared to resource benefits gained by rhizobia *inside* the nodule (Denison, 2000; West *et al*., 2002*a*). Carbon is shuttled to bacteroids and fuels fixation as well as the creation of new bacteroids (Lodwig and

Poole, 2003). Access to carbon resources is presumably greatest for rhizobia inside the nodule, rather than in the rhizosphere. Any resources exuded from the root will be the object of fierce competition with saprophytic and nonmutualist bacteria (Denison, 2000; West *et al.*, 2002a). These bacteria have been shown to outnumber rhizobia by two to three orders of magnitude (Hirsch, 1996) and are likely better competitors for most resources than rhizobia -- rhizopines may be an exception -- as their entire life cycle is spent in the soil. In contrast, hoarding of PHB by bacteroids in determinate nodules or transfer of rhizopines to reproductive clonemates inside indeterminate nodules can specifically support nodule populations of rhizobia. Clearly, fitness benefits to rhizobia in nodules outweigh benefits to rhizobia in the soil, but basic research is still needed to be certain whether increases in rhizobial soil populations following nodule senescence (Brockwell *et al.*, 1987; Bushby, 1993) are the result of shuttling resources to rhizosphere populations or the release of viable rhizobia from the nodule.

3.3. Hypothesis 3: Amino-Acid Cycling and Bacteroid Environment

Cycling of amino acids has been proposed as a mechanism to directly link a bacteroid's own N_2 fixation with the supply of resource received, not just the overall C or N status of the host plant (Lodwig *et al.*, 2003). The coupled exchange is proposed to involve the transfer of amino acids to the nodule bacteroids, which permits the shut down of ammonium assimilation. The bacteroids cycle the amino acids and return them to the host, for asparagine biosynthesis in the case of pea (*Pisum sativum*) (Lodwig *et al.*, 2003). This type of dependence would act as a selective pressure to maintain cooperative stability in which neither partner dominates the symbiosis (Sprent, 2003). A more generalized model that coupled bacteroid carbon metabolism to plant nitrogen metabolism has been proposed by Kahn *et al*. (1985).

We agree with Sprent (2003) that 'checks and balances' are the key to maintaining cooperation between legume plants and their symbiotic N_2-fixing rhizobia. However, in order for exchange-control systems to have any evolutionary effect, they must actually reduce the survival and reproduction of those rhizobial strains failing to provide the plant host with nitrogen (Kiers *et al*., 2003). In nodules on pea, the host plant studied by Lodwig *et al.* (2003), withholding resources to the differentiated N_2-fixing bacteroids would not impose effective selection pressure because pea bacteroids are unable to reproduce anyway (Gresshoff and Rolfe, 1978; Zhou *et al.*, 1985; McDermott *et al.*, 1987). Upon senescence of pea nodules, therefore, the bacteroids die and only the undifferentiated rhizobia, which never fixed N_2 in symbiosis, escape into the soil from dying pea nodules (see above). Consequently, bacteroid-directed resource control does not provide a generalized evolutionary explanation for the maintenance of the legume-rhizobial mutualism in pea, or other species with indeterminate nodules (Kiers *et al.*, 2003). Even if bacteroids were reproductive in this species, the exchange control system would need to affect their survival and reproduction, not just their ability to export fixed N. Lodwig *et al.* (2003) did not track the reproductive success of the rhizobia in the nodules and therefore did not establish

whether failure to cycle amino acids actually reduced the reproductive fitness of bacteroids.

Plant-mediated bacteroid control could be important for rhizobia whose bacteroids do have direct descendants (Denison, 2000). Udvardi and Kahn (1993) suggested that individual bacteroids could benefit from N_2 fixation because fixation maintains an appropriate pH inside the peribacteroid unit that envelops the bacteroids and also ensures access to plant resources by coupling plant N metabolism to bacteroid C metabolism. Control of bacteroid oxygen supply (Layzell and Hunt., 1990), coupled with the export of fixed nitrogen, may also act to limit the spread of cheating rhizobia (Udvardi and Kahn, 1993; Denison, 2000). Brewin (1991) proposed that to oppose plant-induced digestion at low pH, bacteroids release ammonia. These mechanisms of bacteroid-directed exchange control could be important, but the suggested physiological links between the failure to fix N_2 and reductions in rhizobial fitness have not been demonstrated.

3.4. Hypothesis 4: Post-Infection Sanctions

One idea that has recently attracted much attention is that cooperation between species can be enforced if individuals are able to actively reward cooperation or punish less cooperative behavior. This idea has been termed 'sanctions' (Denison, 2000) and is conceptually analogous to 'policing' mechanisms that have been shown to stabilize cooperation within species (Frank, 1995; Ratnieks *et al.*, 2001). Plant sanctions can be defined as the preferential supply of resources (or alternatively the severing of resources) to nodules based on the amount of N_2 supplied by the nodules (Denison, 2000; Simms and Taylor, 2002; West *et al.*, 2002*a,b*; Kiers *et al.*, 2003).

The sanctions hypothesis proposes the host makes the reproductive success of rhizobial strains contingent on their ability to export N. This would provide a selection pressure to maintain rhizobial fixation even under conditions where fixation rates are otherwise predicted to be low (*i.e.*, when the tragedy of the commons occurs, with high genetic diversity and low relatedness (*r*) of symbionts). This idea has been shown to be theoretically robust in models and not dependent upon whether plants assess the level of N_2 fixation absolutely, or relative to the other rhizobia infecting the plant (West *et al.*, 2002*a,b*). In contrast to the low rates of fixation demonstrated in a shared interest model (Figure 1), high fixation rates are predicted when plants preferentially supply resources to nodules fixing more N_2 (Figure 2). Importantly, the predicted fixation rate shows very little sensitivity to (*r*), the relatedness of rhizobial strains in a plant. The high within-host diversity of symbionts found would therefore have little influence on the fixation rate of the strains (West *et al.*, 2002*a*). In the presence of nodule sanctions, rhizobia that fix little N_2 are denied access to resources, even if (perhaps *especially* if) other nodules on the same host plant are supplying plenty of N. This essentially eliminates the tragedy of the commons on the level of the root system. Kin selection remains an important selection component, but on the level of the individual nodule, not on the level of the root or surrounding rhizosphere (West *et al.*, 2002*a*).

If the evolutionary persistence of symbiotic N_2 fixation by rhizobia is the result of selection pressures by the host, are there similar selection pressures on the hosts to

favor the evolution of these sanctions? In clear-cut cases of mutualistic partners that defect (*e.g.*, yucca moths, Pellmyr and Huth, 1994), imposition of sanctions (preferential abortion of flowers) creates an obvious selective advantage. But when partners may provide some intermediate level of benefit, sanctions may actually inflict a cost on the host. For natural selection among plants to favor the evolution of sanctions, they must provide a fitness benefit to individual plants. By simultaneously considering the optimal fraction of nodules senesced and the initial investment in nodules, West *et al.* (2002*b*) concluded that sanctions are favored when the advantage of increasing the efficiency of the symbiont partner is greater than the expenditure of reducing the *total* advantage of the partners (*i.e.*, reducing the overall amount of N_2 fixation). This result, coupled with the finding that increasing the severity of sanctions selects for more mutualistic behavior of symbionts (West *et al.*, 2002*a*) suggests a co-evolutionary mechanism for continued stability of cooperation.
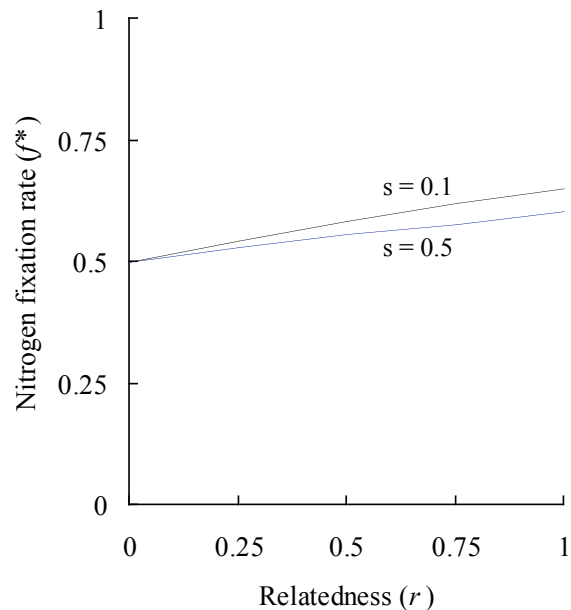

Figure 2: Fixed-threshold plant sanctions. The unbeatable N_2-fixation rate (f*) plotted against the relatedness between the rhizobial strains in a plant (r). Different lines represent values of (s), the relative importance of N_2 fixation by rhizobia to plant resources and growth. High rates of N_2-fixation are predicted regardless of relatedness (r) of the rhizobia and effect on plant resources (s) because of selective partitioning of resources. Reproduced from West et al. *(2002a) with permission of the Royal Society.*

The models of West *et al.* (2002*a,b*) described above assume nodule-level sanctions. That is, a host is assumed to have the ability to identify individual nodules that are evading their N_2-fixing duties. Kiers *et al.* (2003) tested this assumption and

the general sanctions hypothesis by mimicking cheating strains and monitoring host response. In a series of experiments at three spatial scales (whole plant, half root systems and individual nodules), fixing strains were forced to cheat when air was replaced with an N_2-free atmosphere. By replacing air (N_2:O_2, 80:20 v/v) with argon (Ar:O_2, 80:20 v/v), and a trace of N_2, N_2 fixation was reduce to about 1% of normal, low enough to elicit a potential plant response while ensuring no direct N limitation to rhizobia. Nodules were allowed to develop under standard atmospheric conditions and then cheating was imposed on target nodules by manipulating the atmosphere to prevent further N_2 fixation. The benefits of this method include precise control of when and where rhizobia fix N_2, and the absence of possible confounding differences between strains, such as basic metabolic differences that might be associated with non-fixing strains.

The results demonstrated a significant fitness cost to those rhizobia failing to fix N_2 (Kiers *et al.*, 2003). As predicted by the sanctions hypothesis, N_2 fixing rhizobia consistently grew to larger numbers than non-fixing rhizobia. At all three spatial scales tested (plant, half root, or single nodule level), plants detected cheating by rhizobia and responded in ways that reduced their fitness. Two-fold differences in population size were found between rhizobia released into the microcosm rhizosphere after only one plant generation – *i.e.*, a 50% fitness cost for not fixing N_2. Survival of these N_2-fixing rhizobia over five months in sterile sand was also significantly higher than survival of non-fixing rhizobia, possibility indicating differences in stored resources. Differences in resource allocation between fixing and non-fixing treatments were observed when host plants were exposed to both cheating and N_2-fixing rhizobia on a single root system (split-root and single nodule experiments). When given the option of selective partitioning between fixing and non-fixing nodules, the host consistently supplied more resources (as shown by higher nodule fresh weight and higher root dry weights) to the N_2-fixing treatment.

The host plant is essentially faced with two options to successfully sanction cheating rhizobia; either reduce resources required for rhizobial growth or attack the rhizobia directly. A direct attack (*e.g..*, using acid hydrolysis) on non-fixing nodules could be effective in reducing losses to non-fixing rhizobia and (as a side-effect) in preventing cheating populations from escaping into the soil. Reducing carbohydrate supply to the nodules could limit the spread of cheating bacteria, but it has been reported that when nodules are starved of carbohydrates the rhizobia begin to attack host cell organelles and cell walls to obtain their energy (Thornton, 1930). If hosts could limit respiration of the rhizobia by decreasing internal nodule O_2 concentration, this might restrict growth of non-fixing nodule rhizobia (Udvardi and Kahn, 1993; Denison, 2000) and also decrease the likelihood of a rhizobial attack on the plant. Changes in nodule O_2 permeability had previously been documented in response to cessation of N_2 fixation on a whole-root system basis (Sheehy *et al.*, 1983) and to various stresses (Denison *et al.*, 1983; Hartwig *et al.*, 1987).

Preventing individual nodules from fixing N_2 elicited a direct plant response (Kiers *et al.*, 2003). Within 48 hours, nodule permeability to O_2 and internal nodule O_2 concentration were significantly lower in non-fixing nodules than in N_2-fixing nodules (Figure 3). This has been suggested as the primary mechanism for the sanctioning of cheating rhizobia (Kiers *et al.*, 2003). As indicated by a lack of significant differences

in O_2-saturated respiration, photosynthate supply did not seem to limit rhizobial respiration more than in the control. However, decreasing nodule O_2 concentration need not be the sole mechanism for sanctioning control. Legumes may use a suite of control mechanisms that limit the spread of cheating bacteria. It is suspected that the response of lowering O_2 concentration is specific to rhizobial defection, and not simply lack of N, because nodule O_2 permeability also decreases when soil N supply increases (Denison and Harter, 1995).
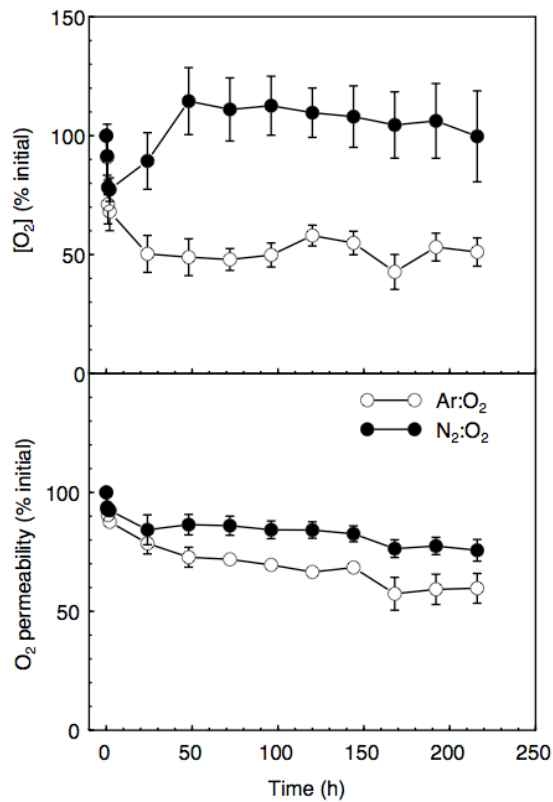

Figure 3: O_2 relations in single nodules where rhizobia were allowed to fix (N_2:O_2) or prevented from fixing (Ar:O_2). Within 48 hours, non-fixing nodules had significantly lower interior O_2 concentration under 20% O_2 as calculated from leghaemoglobin oxygenation. Data are presented as % of initial concentration to standardize for any initial differences.

It appears that, on average, rhizobia that infect legumes and fix N_2 must leave more descendants (higher inclusive fitness) than those that do not. Fixation of N_2 by rhizobia appears to be maintained through domination by one partner, enforcing good behavior by the other, perhaps through manipulation of resources such as O_2. The penalty for noncompliance (sanctions) acts to reduce the fitness of the cheating partner and create a strong selection pressure for continued cooperation.

4. CHEATING AND MIXED NODULES

We have presented evidence in agreement with other symbiotic systems (West and Herre, 1994) that one partner (the legume) dominates the relationship via asymmetrical control of the mutualistic rhizobial partner by reducing O_2 supply to nodules that fail to fix N_2.

If this is a strong selection pressure, why then do cheaters exist at all? One possible explanation is that multiple strains of rhizobia can infect single nodules, and that sanctions occur at the level of the nodule (or more generally that there is genetic diversity of symbionts at the level at which sanctions are applied). Nodule-level sanctions will be most effective if there is only one strain of rhizobia per nodule (Denison, 2000) but some single nodules do contain more than one (Rolfe and Gresshoff, 1980; Trinick *et al.*, 1983; Demezas and Bottomley, 1986). When rhizobial strains share a nodule, there is a degree of protection for the defecting strain. Cheating strains have been shown to accumulate PHB at the expense of the fixing strain when sharing a single nodule (Hahn and Studer, 1986).

This can therefore create a tragedy of the commons at a different level, undercutting the evolutionary effects of nodule-level sanctions (Denison, 2000) and making sanctions at the nodule level only partially effective. Though the frequency of mixed nodules under field conditions is largely unknown (Simms and Bever, 1998), the persistence of the cheating polymorphism may well be a result of strains sharing a nodule. Plant sanctions could be aimed at individual bacteroids within a nodule (Udvardi and Kahn, 1993; Denison, 2000) and in determinate nodules, such as soybeans, this could effectively reduce the occurrence of more parasitic strains. However, in indeterminate nodules, sanctions against bacteroids would be ineffective at limiting the evolution of parasitism because selection acts only on the reproductively viable fraction of the nodule, the undifferentiated rhizobia in the infection threads. If the abundance of cheaters in a particular indeterminate nodule is high, the nodule could be tagged for sanctions, but in mixed nodules this could affect both fixing and non-fixing strains. There is a great need for empirical work to determine the frequency of mixed nodules in the field, and further work on the mechanism by which sanctions occur. Comparing occurrences of mixed rhizobial genotypes in determinate and indeterminate nodules could clarify the importance of different selection pressures, such as kin selection in indeterminate nodules.

5. FUTURE DIRECTIONS

COOPERATION IN THE LEGUME-RHIZOBIA SYMBIOSIS

Sanctions could potentially be very important in stabilizing mutualisms between species because they can select for cooperation under a wide range of conditions, especially in cases where cooperation is hard to explain by other theories. Model predictions and empirical data support the sanctions hypothesis, though several simplifying assumptions have been made. For instance, static models were used to identify plant and rhizobial strategies favored by natural selection (West *et al.*, 2002 *a,b*). If different stages of plant growth were incorporated into dynamic models, we could predict growth-dependent N_2 fixation rates and associated sanction severity. Because N_2-fixing strategies for rhizobia may differ over the lifetime of a plant, increasingly sophisticated models could examine rhizobial strategies for strains infecting perennial and annual legumes. The static models assumed that less mutualistic rhizobia fixed less N_2 from the start (West *et al*., 2002*b*). The more complex tactic of fixing N_2 initially and then defecting could be advantageous to rhizobia attempting to avoid sanctions. More detailed models will be useful in exploring the selective advantages of specific rhizobial and plant strategies but are unlikely to annul the basic conclusions presented above.

Proper testing of these model predictions will require both field and microcosm studies. Estimates of the frequency of mixed nodules will be important in establishing how selection pressures may differ in determinate and indeterminate nodules. Field data monitoring changes in relative abundance of less mutualistic rhizobia in response to agricultural practices (Kiers *et al.*, 2002) and changes in vegetation, could be useful predictors in the evolution and subsequent management of symbiont communities.

An implication of this work is that shifts in symbiotic functioning could also result from agronomic breeding practices. Studies suggest that a host plant's ability to form effective symbioses is a heritable trait that may be selected for or against in plant breeding programs (Maske, 1989; Hetrick *et al.*, 1993). It is conceivable that breeding, under high fertilizer regimes, could also have modified the ability of legumes to enforce cooperation of symbionts. Presumably, dependence on biologically fixed nitrogen would be greater in the wild ancestors of agronomic crops with natural selection favoring legumes able to detect and sanction ineffective rhizobia. Studies are currently underway to evaluate how 70 years of soybean breeding have modified the sanctioning response. This is an important question because we may be selecting for cultivars that fail to maximize the amount of nutrients derived from symbiosis, and perhaps also inadvertently encouraging the spread of less effective symbionts.

Microcosm studies have the potential to yield interesting results as both host and symbiont can be grown and manipulated in a laboratory setting. Future studies could consider the complexity of 'conditional outcomes' on host and symbiont strategies. This complexity includes the influences of changes in resource availability, increases and decreases of genetic diversity of symbiont populations, and variable fixation strategies. Altering the timing and composition of gas treatments to simulate rhizobia with different fixation patterns will be useful to determine how closely plants can track changes in rhizobial fixation.

Experiments could aim to determine if plants evaluate rhizobia on a 'relative' basis to other strains infecting the plant or if plants employ a 'fixed' sanction strategy on

rhizobial symbionts based on a predetermined fixation minimum (West *et al.*, 2002a). Further data are needed to determine the level at which sanctions are applied in different species (nodule or individual bacteroid) and to determine whether there are differences in sanctioning strategy between indeterminate and determinate nodules. These questions need to be part of a larger query to investigate the fate and resource hoarding of rhizobia from senescing indeterminate and determinate nodules. Monitoring the accumulation and/or transfer of energy rich compounds might be useful in future studies to link resource dynamics with rhizobial fitness success.

6. CONCLUSION

Controlling the reproductive fate of a symbiont can impose a strong selective pressure for cooperation. Host sanctions or other indirect methods of resource control may be central in favoring cooperation in a diversity of mutualistic symbiosis (West and Herre, 1994; Pellmyr and Huth, 1994; Yu, 2001; Heinrich and Boyd, 2001; Bshary and Grutter, 2002; Gardner and West, 2004). The dominating partner, in this case apparently the legume, imposes a penalty on the symbiont for noncompliance. Both organisms still benefit from the mutualistic relationship but the control is asymmetrical. Given the importance of the legume-rhizobia symbiosis to natural and agricultural ecosystems, identifying selection pressures which maintain this cooperation should become a priority. Research dedicated to discovering the factors that stabilize the legume-rhizobia symbiosis may help us manipulate selection pressures to maximize the benefits derived from the symbiosis. Understanding the evolution of cooperation in the legume-rhizobia symbiosis may also provide a theoretical framework to evaluate host control in other mutualistic systems.

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FIGURE LEGENDS

Figure 1: Shared Interest Model. The unbeatable N_2-fixation rate (f*) plotted against the relatedness between the rhizobia strains in a plant (r). Different lines represent values of (s), the relative importance of N_2 fixation by rhizobia to plant resources and growth. Low rates of fixation are predicted when there is low relatedness of rhizobia on a root system and when N_2 fixation has a smaller effect (high s) on plant resources. Reproduced with permission of the Royal Society.

Figure 2: Fixed-plant sanctions. The unbeatable N_2-fixation rate (f*) plotted against the relatedness between the rhizobia strains in a plant (r). Different lines represent values of (s), the relative importance of N_2 fixation by rhizobia to plant resources and growth. High rates of N_2-fixation are predicted regardless of relatedness (r) of rhizobia and effect on plant resources (s) because of selective partitioning of resources. Reproduced with permission of the Royal Society.

Figure 3: O_2 relations in single nodules where rhizobia were allowed to fix ($N_2:O_2$) or prevented from fixing ($Ar:O_2$). Within 48 hours, non-fixing nodules had significantly lower interior O_2 concentration under 20% O_2 as calculated from leghaemoglobin oxygenation. Data are presented as % of initial concentration to standardize for any initial differences. Reproduced with permission of Nature Publishing Group.