

Sanctions and mutualism stability: why do rhizobia fix nitrogen?

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Why do rhizobia expend resources on fixing N₂ for the benefit of their host plant, when they could use those resources for their own reproduction? We present a series of theoretical models which counter the hypotheses that N₂ fixation is favoured because it (i) increases the exudation of useful resources to related rhizobia in the nearby soil, or (ii) increases plant growth and therefore the resources available for rhizobia growth. Instead, we suggest that appreciable levels of N₂ fixation are only favoured when plants preferentially supply more resources to (or are less likely to senesce) nodules that are fixing more N₂ (termed plant sanctions). The implications for different agricultural practices and mutualism stability in general are discussed.

Keywords: coevolution; kin selection; mycorrhizae; parasite; symbiosis; virulence

1. INTRODUCTION

Mutualisms are reciprocally beneficial relationships (interactions) between organisms. Despite their widespread occurrence, the existence of mutualisms poses a problem for evolutionary theory (Leigh & Rowell 1995; Herre *et al.* 1999). Why should an organism perform a behaviour (usually with some short-term cost) that provides a benefit for an individual of a different species (Maynard Smith 1989)? Here we are concerned with the mutualism between legumes and the rhizobia (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, or *Azorhizobium* spp.) that fix atmospheric N₂ inside their root nodules. Specifically, why do rhizobia expend resources on fixing N₂ for the benefit of their host plant (indirectly benefiting rhizobial competitors that share the same plant), when they could use those resources for their own reproduction?

Several recent papers have suggested that the evolutionary persistence of N₂ fixation might depend on kin selection towards rhizobia in the soil nearest the root (termed rhizosphere (Olivieri & Frank 1994; Simms & Bever 1998; Bever & Simms 2000; Crespi 2001)). It has been hypothesized that N₂ fixation leads to a greater exudation of root resources that can then be used by rhizobia in the rhizosphere. This would provide a kin-selected benefit when limited dispersal leads to increased relatedness between N₂ fixing rhizobia in the nodule and in the rhizosphere, resulting in the channelling of resources to reproductively viable kin in the soil. However, this hypothesis is unlikely to be correct because: (i) it assumes that rhizobia inside nodules leave no direct descendants in the soil, which is generally not the case (Denison 2000); (ii) the kin-selected benefits of altruism towards related rhizobia in the rhizosphere are likely to be largely negated by competition

between related rhizobia (West *et al.* 2001); (iii) the fitness benefits towards rhizobia in the rhizosphere are likely to be negligible compared with those towards rhizobia in the root nodules. We elaborate on these points in § 3.

These points suggest that we must ask what level of N₂ fixation, if any, can be favoured when considering the fitness of rhizobia inside the root nodules of a plant. We first examine if N₂ fixation can be favoured because it increases plant growth and therefore the resources available for rhizobia growth (Jimenez & Casadesus 1989). This hypothesis relies on kin selection between rhizobia in the different nodules of a plant, and we find that it can only favour N₂ fixation under extremely restrictive conditions that are not likely to apply (§ 4). We then examine if N₂ fixing can be favoured when plants preferentially supply more resources to (or are less likely to senesce) nodules that are fixing more N₂ (termed plant sanctions; § 5 (Denison 2000)). This hypothesis relies on kin selection between the rhizobia in the same nodule, and is able to favour N₂ fixation under an extremely wide range of conditions.

2. NATURAL HISTORY OF THE LEGUME–RHIZOBIUM MUTUALISM

The relevant aspects of the legume–rhizobia mutualism are described in detail by Denison (2000). Briefly, rhizobia are soil bacteria which can sometimes survive in soil for years without their legume hosts, and non-symbiotic rhizobia may be common. When a suitable host is available, a few of the rhizobia surrounding a legume root will infect it, triggering the formation of root nodules, in which they multiply. In 'effective' symbioses, many of the rhizobia eventually differentiate into the bacteroid form that is capable of fixing N₂. Nodules can take several forms, although the best characterized are: (i) those with determinate growth which are approximately spherical when mature, with bacteroids throughout; and (ii) those with indeterminate growth which become elongated, with

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undifferentiated rhizobia mainly in persistent infection threads near the tip, and bacteroids nearer the root.

3. WHY KIN SELECTION TOWARDS RHIZOBIA IN THE RHIZOSPHERE IS UNLIKELY TO BE IMPORTANT

It has been suggested that the main driving force for N_2 fixation is that it increases the root exudation of materials that can support rhizobial metabolism, and that this provides a kin-selected benefit to related rhizobia in the rhizosphere (Olivieri & Frank 1994; Simms & Bever 1998; Bever & Simms 2000; Crespi 2001). In this section we argue that this mechanism is unlikely to be generally important for several reasons.

First, this hypothesis is often based on the assumption that the rhizobia in a root nodule are at a reproductive dead end. However, this is not usually the case (Denison 2000). In species with determinate nodules, the N_2 -fixing bacteroids themselves typically retain the ability to reproduce. Bacteroids extracted from inside soyabean cells (excluding the possibility of contamination by undifferentiated extracellular rhizobia) have been observed to divide by video microscopy (Zhou *et al.* 1985) and grown in culture (Gresshoff & Rolfe 1978). Bacteroids in senescing nodules have little decrease in respiration rate or in protein content (Sarath *et al.* 1986). In species with indeterminate nodules, it is the undifferentiated rhizobia within the infection threads of root nodules, rather than bacteroids, that typically survive senescence and recolonize the soil (Thornton 1930; Vance *et al.* 1980; Timmers *et al.* 2000). We suggest that hoarding of the energy-rich polymer, polyhydroxybutyrate (PHB), is a useful tag for the rhizobial form that typically survives nodule senescence. Bacteroids in some determinate nodules may accumulate 50% PHB by weight (Wong & Evans 1971), but in at least some indeterminate nodules it is the undifferentiated cells that accumulate PHB. Nonetheless, whichever form within the root is able to reproduce, it is the reproductively viable rhizobia escaping from senescing nodules that are thought to explain the increase in soil populations of rhizobia (both in absolute numbers and relative to other bacteria) after nodule senescence (Brockwell *et al.* 1987; Bushby 1993).

Second, any kin-selected benefits towards rhizobia in the rhizosphere would probably be reduced by competition between relatives. Kin selection towards rhizobia in the rhizosphere relies on the fact that those rhizobia are related to the rhizobia in the root nodules, and that this relatedness arises through limited dispersal (population viscosity or spatial structuring; Bever & Simms 2000). However, this same limited dispersal will lead to increased competition between relatives which opposes kin selection (Queller 1994; Frank 1998; West *et al.* 2001). The extent to which competition between relatives opposes kin selection has been shown to depend upon the form of dispersal (Queller 1992; Kelly 1994; Mitteldorf & Wilson 2000). However, a wide number of models have suggested that for simple dispersal patterns, these opposing forces of kin selection and competition exactly cancel (e.g. Taylor 1992*a,b*; Wilson *et al.* 1992), in which case there would be negligible kin selection towards rhizobia in the rhizosphere.

Third, rhizobia inside nodules vastly outnumber conspecifics in the soil. For example, a single soyabean nodule can contain 2.6×10^5 bacteroid-containing cells, each containing 3.8×10^4 bacteroids, for a total of almost 10^{10} bacteroids per nodule (Bergersen 1982). There may be 10^5 or fewer rhizobia per g in bulk soil, even after growth of a compatible host (Kucey & Hynes 1989; Thies *et al.* 1995), and rhizosphere counts rarely exceed 10^6 per g, except immediately after release of rhizobia from senescing nodules (Herridge *et al.* 1984; Moawad *et al.* 1984). Therefore, one soyabean nodule may contain as many rhizobia as 10–100 kg of soil. If there are 4×10^5 soyabean plants per hectare, each with 100 nodules containing 10^{10} bacteroids, and if the 2×10^6 kg of soil in the surface layer of that hectare contains 10^5 rhizobia per g, then 99.95% of reproductively viable rhizobia are those in the nodules. Following nodule senescence, rhizobia densities in the soil are increased substantially, and this is thought to be due to the extremely numerous reproductively viable rhizobia escaping from senescing nodules (Reyes & Schmidt 1979; Brockwell *et al.* 1987; Bushby 1993). This increase in rhizobia density is sufficient even to alter significantly the species composition in the soil—for example, *Bradyrhizobium japonicum* constituted less than 1% of total bacteria in the rhizosphere of *Glycine max* during the ‘period of active nodulation’, but up to 20% of rhizosphere bacteria after rhizobia were released from senescing nodules (Moawad *et al.* 1984). Although there is then a fairly rapid decrease in rhizobia densities, rhizobia populations, even several years later, exceed those that are observed following a non-host (Triplett *et al.* 1993), even if the non-host is also a legume (Kucey & Hynes 1989).

Fourth, rhizobia inside a root nodule are likely to have greater access to plant resources than those in the bulk soil. Legumes depend on bacteroids to fix N_2 and (in indeterminate nodules) on undifferentiated rhizobia as a source of new bacteroids as the nodule grows, so they supply them with carbon, typically as malate (Day *et al.* 1995), and other resources. The rhizobia within a nodule have the greatest access to these resources (as clearly shown by PHB accumulation), even highly specific growth substrates such as the ‘rhizopines’ (complex energy rich molecules produced by 14% of strains of *Sinorhizobium meliloti* bacteroids, and which are mainly consumed by undifferentiated bacteria of those strains (Murphy *et al.* 1995)). Furthermore, there will be strong competition for any resources which do exude from the root. Numerous saprophytic bacteria, including non-symbiotic rhizobia, exist in the soil and rhizosphere (Segovia *et al.* 1991; Laguerre *et al.* 1993). These bacteria, which outnumber rhizobia in the soil by two or three orders of magnitude (Hirsch 1996), spend their entire life cycle in the soil, and so it is probable that they will be more efficient at utilizing and competing for root exudates in the soil (due to being specialized on this lifestyle). These saprophytes will reduce the benefit that related rhizobia in the soil are able to derive from root exudates. In some cases the saprophytes (including non-symbiotic rhizobia) could have many of the metabolic capabilities of the symbiotic strains, even the ability to compete for highly specific growth substrates such as the ‘rhizopines’.

Our above points are generalizations based upon current biological knowledge of the mutualism between rhizo-

bia and legumes. They suggest that kin selection towards rhizobia in the rhizosphere is likely to be substantially less important than selection within plants. Although it is possible to envisage a scenario in which this might not be the case, this would require extremely severe assumptions for which there is no evidence. In addition, as our conclusions are based upon a generalized rhizobium, we would like to stress also that there may be some interesting, as yet undiscovered, exceptions. We cannot exclude the possibility that there are some legume–rhizobium combinations in which rhizobia inside a nodule have no direct descendants. For example, are there legumes, analogous to non-photosynthetic orchids and monotropes which parasitize mycorrhizae that usually experience a more mutualistic relationship with plants (Taylor & Bruns 1997), that are able to parasitize their rhizobial partners by killing most of the bacteroids and vegetative bacteria in the nodule? A hypothetical legume species that digested so many of the rhizobia inside its nodules that rhizobia founding nodules leave fewer descendants than those that do not, would select against rhizobial genes for infecting roots. But if the rhizobium-eating species were sufficiently rare, and mimicked the recognition signals of a more common and more rewarding host, it might nonetheless succeed in such deceit.

4. MODEL I: CAN NITROGEN FIXATION BE FAVOURED IN THE ABSENCE OF PLANT SANCTIONS?

In this section we examine whether N_2 fixation by rhizobia can be favoured because it increases plant growth and therefore the resources available for rhizobial growth. This represents the simplest possible scenario, assuming that plants neither preferentially supply more resources, nor are less likely to senesce nodules that are fixing more N_2 (i.e. there are no plant sanctions), and ignores any benefits to rhizobia in the rhizosphere.

We consider a plant that is infected by strains of rhizobia whose average relatedness is given by r (e.g. if n equally abundant strains infect a plant then $r = 1/n$). When we consider the influence of relatedness on N_2 -fixation rates we are not hypothesizing that rhizobia alter their symbiotic behaviour in response to the relatedness between infecting strains within a plant. Rather, we are asking how natural selection would alter rhizobial behaviour over a number of generations of selection, for scenarios with different relatedness.

We consider the fitness of a focal rhizobium strain whose bacteroids have a N_2 -fixation rate of f . The average rate of N_2 fixation by the bacteroids of all the rhizobial strains in the plant containing our focal strain is assumed to be z (i.e. the average of all the rhizobial strains, including the focal strain whose fitness we are considering). The N_2 -fixation rate (f or z) can vary between zero and unity, and represents the proportion of the rhizobial energy budget that is allocated to N_2 fixation, relative to the storage of energy resources. Our aim is to determine the unbeatable value of f , the N_2 -fixation rate that cannot be outcompeted by any other strategy.

We assume that the fitness of rhizobia in a nodule is directly proportional to the amount of resources that they can store (e.g. as PHB) before the nodule senesces. We

assume that the rate of N_2 fixation influences the amount of resource storage by rhizobia (and therefore their fitness) in two ways:

- (i) Under N-limited conditions, increased levels of N_2 fixation will be good for the plant (e.g. by increasing photosynthesis), and so will increase the amount of resources (e.g. photosynthate) circulating in the plant that can be utilized by the rhizobia. For example, Bethlenfalvai *et al.* (1978) found that rhizobial strains with higher N_2 -fixation rates increased net photosynthesis rates of the common pea (*Pisum sativum*). This leads to a positive effect on rhizobial fitness of increased N_2 fixation. We allow for this by assuming that the resources available to rhizobia are given by P , which is a function of z and s (we use z , the mean rate of N_2 fixation by the bacteroids in the plant, as we are assuming initially that all rhizobial strains infecting a plant have equal access to plant resources). The parameter s determines the relative importance of N_2 fixation by rhizobia to plant resources and growth— s can vary between zero and infinity, with lower values meaning that N_2 fixation by rhizobia is more important to plant resources and growth (and therefore rhizobial fitness). This definition of s incorporates a number of biologically important factors into a single parameter, and we discuss it further below. Most of our predictions are obtained without specifying a relationship for P , and when one is required for illustrative purposes (e.g. figures) we assume that $P = s + z$.
- (ii) The higher the N_2 -fixation rate of a strain, the less it is able to put into storing resources for future reproduction. Denison (2000) discussed the evidence for trade-offs between N_2 fixation and the accumulation of resources such as PHB. A clear example comes from the work of Hahn & Studer (1986) who showed that the rate of PHB accumulation was considerably higher in a *B. japonicum* mutant that did not fix N_2 than in the N_2 -fixing parent strain. We allow for this by assuming a negative effect on rhizobial fitness due to the cost of N_2 fixation: fitness is proportional to $(1 - f)$.

The parameter s incorporates two main factors: (i) how important is nitrogen acquisition to the plant growth—photosynthesis rate (and therefore to the photosynthate supply to rhizobia), and (ii) how important is N_2 fixation by rhizobia relative to direct nitrogen acquisition from the soil by the plant. Higher values of s suggest that N_2 fixation by rhizobia is less important to the plant photosynthesis rate, because nitrogen is less limiting to the plant, or the plant can obtain greater amounts of nitrogen directly from the soil. One useful limiting case with which to consider this is a scenario when the photosynthesis rate depends entirely upon current nitrogen acquisition, in which case s would represent the ratio of nitrogen that the plant obtains from the soil to the maximum rate at which rhizobia in the plant could fix N_2 (i.e. $s \rightarrow \infty$ would mean that the plant is obtaining all of its nitrogen directly from the soil; $s = 1$ would mean that if rhizobia were fixing at the maximum rate possible ($f = 1$) they would provide one

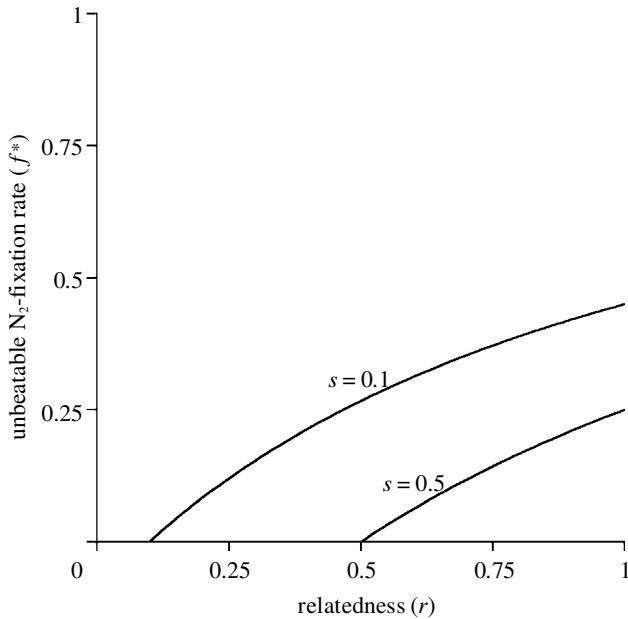


Figure 1. No plant sanctions. The unbeatable N_2 -fixation rate (f^*) plotted against the relatedness between the rhizobia strains in a plant (r). Different lines represent different values of s , the relative importance of N_2 fixation by rhizobia to plant resources and growth (lower values of s signify that N_2 fixation by rhizobia has a larger effect on plant growth and therefore resource availability). Competition is assumed to be global.

half of the plant's nitrogen, and $s = 0$ would mean that rhizobia supply all of the plant's nitrogen).

The overall fitness of a rhizobial strain (W) is given by the circulating resources multiplied by the proportion of resources that they store:

$$W = P(1 - f). \quad (4.1)$$

This equation illustrates the trade-off that is fundamental to this model. Increasing the N_2 -fixation rate is good (beneficial) because it leads to more resources that can be utilized (through the contribution of f to z), but bad (costly) because it decreases the rate at which a given strain accumulates those resources to support its own growth and reproduction. A crucial aspect of this trade-off is that the benefit is shared with the rhizobia infecting all the nodules in a plant, and so its importance will depend upon the relatedness between the strains of rhizobia infecting the plant (lower relatedness means weaker kin selection), whereas the cost is directly paid by individual strains. In Appendix A we show that equation (4.1) leads to three predictions:

- (i) The unbeatable rate of N_2 fixation (f^*) is positively correlated with the relatedness of rhizobial strains in a plant (r) (figure 1). As relatedness (r) increases the benefits (increased resources) of fixing N_2 in any particular nodule will be shared with closer relatives, increasing the kin-selected benefits of N_2 fixing.
- (ii) The unbeatable rate of N_2 fixation (f^*) declines as the importance to the plant of N_2 fixed by rhizobia decreases (i.e. as s increases; figure 1), for example, when plants are able to obtain a greater amount of

nitrogen directly from the soil. This occurs because N_2 fixation by rhizobia has a smaller effect on the overall level of circulating resources, and so decreases the benefit of N_2 fixation.

- (iii) Below a certain relatedness (r), N_2 fixation is not favoured. For example, in the special case where $P = s + z$, it can be shown that N_2 fixation is not favoured when $r < s$. Estimates of the relatedness (r) can be obtained from data on the number of different rhizobial strains infecting individual plants—electrophoretic markers suggest an average of approximately 10 (range 4–18 (Hagen & Hamrick 1996a,b; Souza *et al.* 1997; Silva *et al.* 1999)). Assuming these strains are equally abundant would give $r = 0.1$, suggesting that N_2 fixation will only be favoured (and at very low levels) when it has a very large effect on plant resources (i.e. $s < 0.1$). Further, the limitations of these and other markers means they are likely to underestimate the actual number of strains infecting each plant.

Overall, the results of this model suggest that the conditions under which N_2 fixation is favoured by natural selection are restrictive, requiring high r and low s . Furthermore, even when some N_2 fixation is favoured, it is predicted that rhizobia will use only a small fraction of their resources for N_2 fixation (i.e. low f^* ; figure 1). In addition, the model described above assumed implicitly that after leaving root nodules, competition among rhizobia (for soil resources or for access to new hosts) is global, that is, that the rhizobia from a single plant are not likely to have to compete with each other. This may not be the case, as limited dispersal (spatial structure) means that rhizobia that emerged from the same plant might be more likely to be competitors in the future (termed local competition). In Appendix B we show that as competition becomes more local, even lower levels of N_2 fixation are favoured (figure 2).

More generally, the results of this model agree with previous work on the evolution of mutualisms. A number of previous models have shown that increased genetic diversity of symbionts (lower r) favours less mutualistic and more parasitic behaviour (e.g. Axelrod & Hamilton 1981; Frank 1994a,b; Leigh & Rowell 1995; Maynard Smith & Szathmary 1995). In addition, a model which predicted mutualism to evolve with ease assumed that only one symbiont interacted with each host per generation (i.e. $r = 1$ (Doebeli & Knowlton 1998)).

5. MODEL II: CAN NITROGEN FIXATION BE FAVOURED BY PLANT SANCTIONS?

In this section we examine how plant sanctions influence the unbeatable N_2 -fixation rate. By plant sanctions we mean that the plant preferentially gives more resources to (or is less likely to senesce) nodules which are fixing more N_2 . In all cases we assume that there is only one rhizobium strain per nodule (leading to the more restrictive definition of f and z as the average rate of N_2 fixation in the nodules containing the focal lineage whose fitness we are considering (f), and the average rate of N_2 fixation in all the nodules of the plant (z), an assumption that we

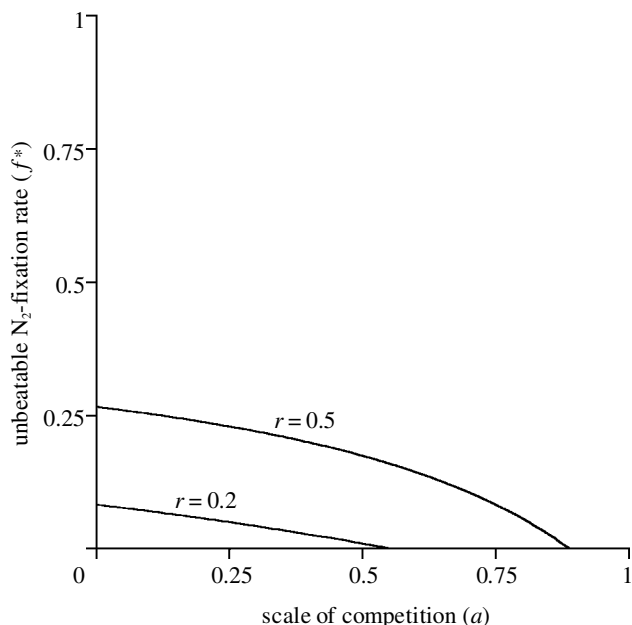


Figure 2. No plant sanctions. The unbeatable N_2 -fixation rate (f^*) plotted against the scale of competition between rhizobia (a). Competition varies from global ($a = 0$), among all members of the population, to local ($a = 1$), when rhizobia emerging from a nodule only compete with rhizobia emerging from the same plant. Different lines represent different relatedness between the rhizobia strains infecting a plant (r).

shall return to in § 6). We distinguish two different mechanisms with which plant sanctions could occur.

First, we consider a ‘fixed’ rule, where sanctions occur at a very local scale in response to the absolute level of N_2 fixation in a nodule, irrespective of the N_2 -fixation rate at other nodules. This possibility would require a relatively simple control mechanism. For example, plants could reduce the O_2 supply to a nodule dependent upon the N_2 -fixation rate (Udvardi & Kahn 1993; Denison 2000). Second, we consider a ‘relative’ rule, where sanctions are applied to a nodule in response to the N_2 -fixation rate at that nodule relative to the N_2 -fixation rate at other nodules. This possibility would require a more complicated control mechanism that allowed the relative N_2 -fixation rate at different nodules to be assessed. We allow for plant sanctions by extending the fitness equation to

$$W = P(1 - f)G, \quad (5.1)$$

where G is the fraction of resources supplied to a particular nodule, and a function of f and z .

(a) Model IIa: fixed-rule plant sanctions

In order to consider fixed-rule plant sanctions we assume that the resources supplied to a nodule (G) increase with the rate of N_2 fixation in that nodule (f), and do not depend upon the rate of N_2 fixation elsewhere (z). The simplest possible equation for this relationship is $G = f$, and whilst this is used for the figures, we show in Appendix A that our qualitative predictions hold more generally.

In Appendix A we show that, as in the no sanctions model (model I), the fixed rule sanctions model predicts that the unbeatable rate of N_2 fixation (f^*) should: (i)

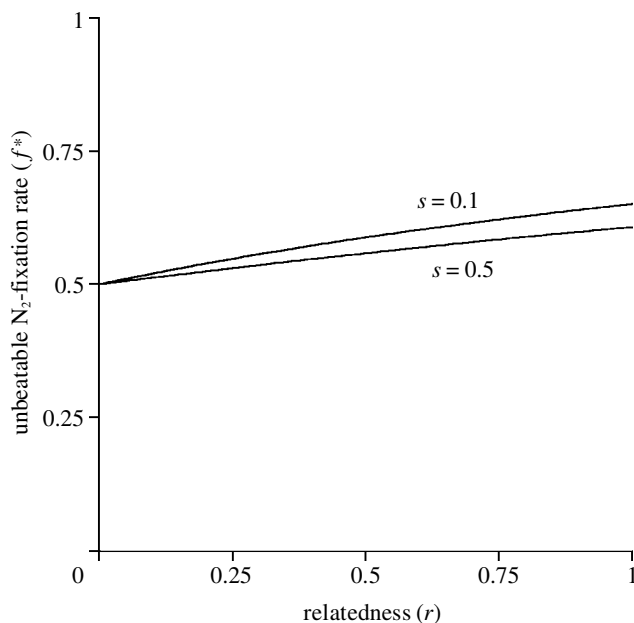


Figure 3. Fixed-rule plant sanctions. The unbeatable N_2 -fixation rate (f^*) plotted against the relatedness between the rhizobia strains in a plant (r). See legend to figure 1.

increase with the relatedness among rhizobial strains in the plant (higher r), and (ii) decrease as N_2 fixation by rhizobia becomes less important to the plant (higher s ; for example, if more N_2 can be obtained directly from the soil) (figure 3). However, with fixed sanctions: (i) the unbeatable N_2 -fixation rate (f^*) is considerably higher for a given set of parameter values (indeed the lowest value of f^* predicted by this model, is higher than the maximum value predicted by the no sanctions model—compare figures 1 and 3); (ii) variation in relatedness (r) and the importance of N_2 fixation by rhizobia to the plant (s) make relatively less difference to the unbeatable N_2 -fixation rate (f^*); (iii) the unbeatable N_2 -fixation rate (f^*) asymptotes at positive levels—for example, in the special case when $P = s + z$ and $G = f$, f^* it is never predicted to be below 0.5 (as $r \rightarrow 0$, $f^* \rightarrow 0.5$, irrespective of s) (figure 3).

The predictions of this model differ from those of the no sanctions model (model I) because plant sanctions impose an additional fitness benefit of N_2 fixation to the bacteria within a nodule—the more a strain fixes N_2 in a nodule, the greater share of resources the plant provides to that nodule. This benefit relies on kin selection at the level of the nodule, and acts even when kin selection towards other nodules in the plant is non-existent (i.e. $r \rightarrow 0$), explaining why high levels of N_2 fixation can still be favoured ($f^* = 0.5$ when $r = 0$). Furthermore, this additional fitness benefit of N_2 fixation to bacteria in the nodule can be by far the most important factor driving N_2 -fixation rates, as shown by the fact that the unbeatable N_2 -fixation rate changes relatively little between $r = 1$ (maximum possible kin-selected benefit within a plant) and $r = 0$ (minimum possible kin-selected benefit within a plant) (figure 3).

Overall, this model shows that plant sanctions can favour high levels of N_2 fixation. Importantly, the predicted unbeatable N_2 -fixation rate (f^*) shows little sensitivity to the relatedness between rhizobial strains in a plant (r) or

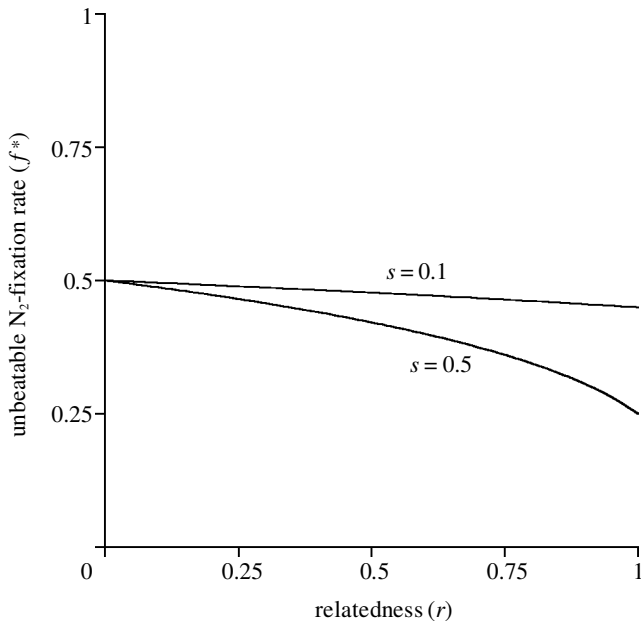


Figure 4. Relative-rule plant sanctions. The unbeatable N_2 -fixation rate (f^*) plotted against the relatedness between the rhizobia strains in a plant (r). See legend to figure 1.

the relative importance to plant growth and resource availability of N_2 fixed by rhizobia (s). Furthermore, this model still favours N_2 fixation when rhizobia that emerge from the same plant are more likely to be competitors in the future (local competition). In Appendix A we show that as competition becomes more local, lower levels of N_2 fixation are favoured, but that this asymptotes at a minimum N_2 -fixation rate (e.g. $f^* = 0.5$ for the special case considered above).

(b) Model IIb: relative-rule plant sanctions

We assume that the amount of resources supplied by a plant to a particular nodule (or the likelihood that the nodule is not senesced) is proportional to the rate at which N_2 is being fixed in that nodule, relative to the average for all nodules (i.e. $G = f/z$). Consequently the amount of resources that a nodule obtains depends not only upon the N_2 -fixation rate in that nodule, but also the N_2 -fixation rate in other nodules. This form of $G(f, z)$ provides a direct fitness benefit of nitrogen fixation (f), and also a negative effect on other rhizobial strains in the plant ($1/z$; the higher the N_2 -fixation rate of a strain, the less resources will be given to other strains in the plant).

In Appendix A we show that, as in the no sanctions model (I) and fixed sanctions model (IIa), this relative rule sanctions model predicts that the rate of N_2 fixation (f^*) should decrease as the plant can obtain more nitrogen directly (higher s) (figure 4). In addition, as with fixed sanctions (model IIa), this relative sanctions model is able to predict much higher N_2 -fixation rates (f^*) than the no sanctions model (I). However, in contrast to the other models, relative sanctions can lead to the unbeatable N_2 -fixation rate (f^*) decreasing with higher relatedness between rhizobial strains infecting each plant (higher r). In the extreme, the N_2 -fixation rate (f^*) asymptotes at positive levels—for example, in the special case when $P = s + z$, f^* is never predicted to be above 0.5 (as $r \rightarrow 0$, $f^* \rightarrow 0.5$; figure 4).

The negative relationship between fixation rate and the relatedness of rhizobial strains infecting each plant arises because a relative rule means that increasing the N_2 -fixation rate in a nodule decreases the proportion of resources that are given by the plant to other nodules. Consequently, a higher rate of N_2 fixation has a negative effect on the fitness of the other nodules, which decreases the inclusive fitness of a strain, more as they occupy a greater fraction of the nodules in a plant (i.e. higher r). This negative effect on the fitness of other nodules has a greater influence than the positive effect of making the plant produce more resources overall, because the positive effect is diluted by the relative importance to the plant of N_2 fixing, the parameter s (i.e. increasing the value of z decreases the ratio $(s+z)/z$ as long as $s > 0$; as $s \rightarrow 0$, this ratio tends to unity, and changes in r do not influence the unbeatable N_2 -fixation rate).

Overall, this model shows that relative-rule plant sanctions can favour high levels of N_2 fixation. However, the level of N_2 fixation is lower than fixed-rule plant sanctions, except when high numbers of rhizobial strains infect each plant (in which case both models predict that $f = 0.5$). In Appendix B we show that when rhizobia that emerge from the same plant are more likely to be competitors (more local competition) the relative rule model suggests that higher levels of N_2 fixation are favoured, asymptoting at a maximum N_2 -fixation rate of 0.5. Consequently, if high numbers of rhizobial strains infect each plant, or competition is relatively local, then the fixed and relative rule sanction models predict the same level of N_2 fixation.

6. DISCUSSION

(a) Why fix nitrogen?

We suggest that N_2 fixation is unlikely to be favoured merely because it increases plant growth and therefore the resources available for rhizobial growth (in the root nodule or rhizosphere). Instead our results support the suggestion of Denison (2000; see also Simms & Taylor 2002) that plant sanctions are required for appreciable levels of N_2 fixation to be favoured. Plant sanctions are defined as plants preferentially supplying more resources to (or being less likely to senesce) nodules that are fixing more N_2 . Plant sanctions offer a fitness benefit to N_2 fixation in any particular nodule, to the reproductively viable bacteroids in determinate nodules, and to the genetically identical (clonal) undifferentiated bacteria in the infection threads of indeterminate nodules. It is important to realize that kin selection is still an important component of sanction models, only at the level of the nodule (i.e. the level at which we have assumed sanctions occur), and not at the level of the plant or surrounding soil. Although the predicted N_2 -fixation rate can differ between different types of sanctions (fixed or relative rules), this difference is negligible for the conditions that are likely to dominate in the field (high numbers of rhizobial strains per plant and local competition between rhizobia in the soil).

Our models predict how N_2 -fixation rates will evolve in response to different agricultural practices. As well as suggesting how to 'get the best' out of rhizobia, this means that agricultural systems provide excellent opportunities for testing our predictions. Addition of inorganic or

organic nitrogenous fertilizers would increase the amount of nitrogen that plants can obtain directly (higher s), and so favour the evolution of rhizobia with lower levels of N_2 fixation (less so when plant sanctions occur, although this may be a result of our assumption that the severity of plant sanctions is independent of s). Although few studies have documented the effects of long-term nitrogenous fertilizer use on the symbiotic stability of rhizobia, we expect a similar pattern in mycorrhizae, and there is support for this prediction from the literature. Arbuscular mycorrhizal communities show a decrease in symbiotic performance under cumulative phosphorus fertilizer regimes (Johnson 1993; Thingstrup *et al.* 1998; Kahiluoto *et al.* 2000). Mechanical disturbance of the soil by cultivation (tilling) will mix up rhizobial strains; that has two consequences, the net effects of which are difficult to predict. First, tilling could increase the number of strains that infect each plant (higher n), favouring lower (no sanctions or fixed rule sanctions) or higher (relative rule sanctions) levels of N_2 fixation. Second, tilling could make competition between rhizobia in the soil more global, decreasing competition between related rhizobia, favouring higher (no sanctions or fixed rule sanctions) or lower (relative rule sanctions) levels of N_2 fixation. Although few studies have specifically addressed this prediction, Ferreira *et al.* (2000) found increased symbiotic performance of rhizobia isolated from no-till plots in soyabean-wheat and soyabean-wheat-maize rotations.

(b) Future directions

Our models suggest several important lines of empirical research. In particular: (i) How many rhizobial strains infect individual plants? (ii) What are the forms of the relationships between N_2 fixation and total plant resources or resource hoarding by rhizobia in nodules? (iii) How often do multiple rhizobial strains infect single nodules under field conditions (Lindemann *et al.* 1974; Johnston & Beringer 1976) and do plants have ways of minimizing the chances of this occurring? (iv) Do plant sanctions exist (Denison 2000)? (v) If plant sanctions exist, are they based on relative or absolute rules, do they depend upon resource availability (p), and at what level do they occur (nodule or bacteroid—sanctions at the bacteroid level in determinate nodules would enable N_2 fixation to be favoured in the absence of kin selection at even the nodule level)? (vi) How does the 'effectiveness' (genetic predisposition to fix N_2 under defined symbiotic conditions) of rhizobia correlate with the agricultural practices experienced by a population which have therefore shaped their recent evolutionary history? To date, questions (ii)–(vi) have been almost completely neglected.

We have made several simplifying assumptions with our models, and there are a number of ways in which they could be expanded. In particular, we have assumed a static model, without different stages of plant growth. Although this simplification provides an approximation, more subtle and state dependent N_2 -fixation rates could be predicted by dynamic models (Mangel & Clark 1988). For example, higher levels of N_2 fixation may be favoured early in the growth of an annual plant, when each increment of nitrogen leads to an increase in leaf growth and therefore in the photosynthetic rate. N_2 fixed later may instead be used for seed production, which does not increase the circulating

level of resources available for nodule bacteria. The situation could become even more complicated in a perennial legume. However, in addition to providing an approximation of more complex systems, it should be noted that our model might apply directly to a system with prolonged and continuous vegetative growth, such as a grazed legume pasture or a juvenile leguminous tree. In our sanctions model we assumed also that each nodule is infected by a single rhizobial strain. If multiple strains could infect a nodule then this might favour lower levels of N_2 fixation if plant sanctions are applied only on a whole-nodule basis (determinate and indeterminate nodules), but not if they are applied only at a bacteroid level (determinate nodules). In addition, multiple strains in a nodule could offer some interesting opportunities for rhizobia that exploited the N_2 fixation of others. None the less, we believe that our qualitative conclusions are unlikely to be overturned by more detailed models, and that empirical work is required to explore the most useful ways in which to extend the theory.

(c) Mutualism stability

To conclude, our results emphasize a general point about the stability of mutualisms. Current theory suggests that mutualisms are best viewed as reciprocal exploitations that none the less provide net benefits to each partner (Herre *et al.* 1999). However, this does not imply a balanced exploitation. Indeed, West & Herre (1994) have suggested that a major factor which could help stabilize mutualisms is the dominance of one partner that enforces 'good behaviour' on the other (especially by the partner that provides resources and has a direct self interest in the reproduction of both partners (see also Murray 1985; Bull & Rice 1991; Leigh & Rowell 1995; Noe & Hammerstein 1995; Schwartz & Hoeksema 1998; Wilkinson & Sherratt 2001)). For example, in the fig-fig wasp pollinator mutualisms, the pollinator wasps have no interest in the short term reproductive interests of the host plants, whereas the plants require the pollinator offspring to disperse their pollen (and so 'good behaviour' by figs does not have to be enforced to the same extent that it must be in their wasps). In this case the fig appears to be the controlling partner in many aspects of the mutualism (Herre 1989), such as having a proportion of 'unbeatable' seeds in which the wasps cannot develop (West & Herre 1994). Similarly, in the yucca-yucca moth system, the yuccas preferentially abort fruit which contain an excessive number of pollinators, and in which all or a high proportion of their seeds would be eaten (Pellmyr & Huth 1994; see also Murray 1985). Here, in the case of the legume-rhizobia mutualism, we suggest that the major factor enforcing good behaviour (N_2 fixation) in rhizobia is the threat of sanctions from their plant host. Our model explicitly shows the importance of sanctions, and although developed for rhizobia, the underlying model could be applied easily to other mutualisms, such as mycorrhizae.

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APPENDIX A

General model

All our results are derived using the 'direct fitness' formulation of inclusive fitness (Taylor & Frank 1996; Frank 1998). The fitness of the focal lineage (W) is given by

$$W = P(1 - f)G, \quad (\text{A } 1)$$

where f is the N_2 -fixing rate of the focal lineage and z is the mean N_2 -fixing rate of rhizobia in that plant. P is a function of s and z and represents the amount of resources circulating in the plant that can be (depending upon sanctions) available to the rhizobia— P is assumed to be an increasing function of the resources supplied by the rhizobia to the plant ($dP/dz > 0$ and $d^2P/dz^2 \leq 0$) as well as the nitrogen obtained directly from the soil (s). G is a function of f (fixed and relative rule sanctions) and z (relative rule sanctions), and measures the relative amount of resources that are provided to a nodule depending upon the rate at which N_2 is fixed (i.e. it determines if there are plant sanctions, and if so what form they take). Various physiological parameters, such as the efficiency of photosynthesis and N_2 fixation, could be explicitly entered into the fitness equation but they would cancel out when solving for the unbeatable N_2 -fixation rate, and so would not alter our predictions. Following Taylor & Frank (1996), the unbeatable N_2 -fixing rate (f^*) is found by solving $dW/dx|_{f=z=f^*} = 0$, with the derivative of W being obtained using the chain rule, and the phenotypic derivatives replaced with the corresponding relatedness coefficient (r).

For no plant sanctions (model I; $G = 1$) the equilibrium condition is given by

$$r(1 - f^*)\frac{\partial P}{\partial z} - P = 0. \quad (\text{A } 2)$$

The influence of r on f^* can be found with implicit differentiation. Writing $H(f^*, r, s)$ for the left-hand side of equation (A 2), we obtain

$$\frac{df^*}{dr} = -\frac{\partial H/\partial r}{\partial H/\partial f^*}. \quad (\text{A } 3)$$

Equation (A 3) gives N/D , where $N = (1 - f^*)P'$, and $D = P'(1 + r)P'(1 - f^*)r$, both of which are positive. Consequently, f^* is positively correlated with r . Similarly it can be shown that f^* is negatively correlated with s . In the special case where $P = s + z$, we obtain $f^* = (r - s)/(1 + r)$.

For the case of fixed rule sanctions (model IIa), G is a positive function of f ($dG/df > 0$ and $d^2G/df^2 \leq 0$) and does not depend upon z . In this case the equilibrium condition is given by $P(1 - f^*)G' - PG + rS'(1 - f^*)G = 0$, and it can be shown with implicit differentiation that f^* is positively correlated with r and negatively correlated with s . In the special case where $P = s + z$, we obtain $f^* = 1 - 2s + r + \sqrt{(4s + 4s^2 + (1 + r)^2)/2(2 + r)}$.

For the case of relative rule sanctions (model IIb), G is a positive function of f and a negative function of z . In this

case the equilibrium condition is given by $P((1 - f) \times G' - G) + r(1 - f)(P'G + PG') = 0$. In this case the shape of the relationship between f^* and r is not independent of G , even if we assume G is simply a function of f/z . The reason for this is that it will depend upon the relative effect of z in P and G . For example, if $P = s + z$, and $G = f/z$, then the latter effect will be greater (see § 5b for further discussion) and f^* will be negatively correlated with r . Specifically

$$f^* = \frac{1}{4}(1 + s(r - 2) + \sqrt{1 + s(4 - 6r) + s^2(r - 2)^2}).$$

APPENDIX B

Scale of competition

The above models assume implicitly that competition (density-dependent population regulation) occurs on a global scale. To allow for more local competition we add in the parameter a , following Frank (1998, p. 114; see also fig. 3 of West *et al.* (2001)). The parameter a measures the spatial scale of density-dependent competition. An increase in the reproductive success of rhizobia in the same plant by a proportion x increases local competition by a factor ax . An increase in the average reproductive success of rhizobia in the population by a proportion y increases global competition by a factor $(1 - a)y$. The parameter a can vary from zero to 1.0: when $a = 0$ competition is global between rhizobia from all plants in the population, and we obtain the results given above; when $a = 1$ competition is local, and rhizobia lineages only compete with rhizobia from the same plant. When the scale of competition is taken into account, the fitness of the focal lineage (W) is given by

$$W(f, z, \bar{z}) = \frac{P(s, z)(1 - f)G(f, z)}{aP(s, z)(1 - z)G(z, z) + (1 - a)P(s, \bar{z})(1 - \bar{z})G(f, \bar{z})}, \quad (\text{A } 4)$$

where \bar{z} is the mean N_2 -fixation rate in the population. The denominator is the intensity of competition, given by a times the average reproduction of rhizobial lineages in the plant, plus $(1 - a)$ times the average reproduction of rhizobial lineages in the population. The unbeatable N_2 -fixation rate (f^*) is found as above, following Taylor & Frank (1996).

For the case with no plant sanctions (model I), or fixed-rule plant sanctions (model IIa), it can be shown by implicit differentiation that f^* decreases with r . The specific solutions for the case in which $P = s + z$ are

$$\begin{aligned} f^* &= \frac{r - s - ar(1 - s)}{r + 1 - 2ar}, \\ f^* &= \frac{1 - 2s + r - 2ar(1 - s)}{2(2 + r) - 6ar} \\ &\quad + \frac{\sqrt{4s + 4s^2 + (1 + r)^2 - 4ar(2s^2 + 2s^2 + 1 + r) + 4a^2r^2(s^2 + s + 1)}}{2(2 + r) - 6ar}, \\ f^* &= \frac{1}{4(ar - 1)}(1 - ar - s(2 - ar - r)) \\ &\quad + \frac{\sqrt{8s(1 - r)(1 - ar) + (1 - ar - s(2 - r - ar))^2}}{4(ar - 1)}, \end{aligned} \quad (\text{A } 5)$$

for no plant sanctions (model I; $G = 1$), fixed rule sanctions (model IIa, with $G = f$), and relative rule sanctions (model IIb, with $G = f/z$), respectively. Note that: (i) when

$a = 0$, the solutions in equation (A 5) simplify to those given in Appendix A which did not allow for spatial scale; (ii) when $a = 1$, the solutions in equation (A 5) simplify to the same values as when $r = 0$ in the solutions in Appendix A (one way of thinking about local competition is that it reduces relatedness, where relatedness is measured at the scale at which competition occurs (Queller 1994)); (iii) our model allows the relatedness (r) to vary independently of the spatial scale of competition (a) following Frank (1998)—an alternative would have been to assume a specific pattern of dispersal and allow them to covary, with r depending upon a (see Taylor 1992a,b).

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