

# Legume Sanctions and the Evolution of Symbiotic Cooperation by Rhizobia

R. Ford Denison\*

Agronomy and Range Science, University of California, Davis,  
California 95616-8515

Submitted April 5, 1999; Accepted July 31, 2000

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**ABSTRACT:** The legume-rhizobium symbiosis is an ideal model for studying the factors that limit the evolution of microbial mutualists into parasites. Legumes are unable to consistently recognize parasitic rhizobia that, once established inside plant cells, use plant resources for their own reproduction rather than for  $N_2$  fixation. Evolution of parasitism in rhizobia, driven partly by competition among multiple rhizobial strains infecting the same plant, may be countered by post-infection legume sanctions. Both the biochemical options for rhizobial cheating and the evolutionary effect of legume sanctions depend on differences in rhizobial life history associated with nodule type. In legumes with determinate nodule growth, rhizobia typically retain the ability to reproduce after differentiating into  $N_2$ -fixing bacteroids. Sanctions against individual bacteroids (e.g., acid hydrolases) would therefore select for cooperative rhizobia. In nodules with indeterminate growth, bacteroids generally lose the ability to reproduce, so legume sanctions against bacteroids would have no effect on rhizobial evolution. Whole-nodule sanctions (e.g., decreased nodule  $O_2$  permeability) could be effective, via kin selection of undifferentiated rhizobia that persist in indeterminate nodules and replenish soil populations after nodule senescence. Mixed nodules could reduce the effectiveness of whole-nodule sanctions. The frequency of mixed nodules under field conditions is unknown.

**Keywords:** mutualism, symbiosis, kin selection, plant strategies, nodule  $O_2$  permeability.

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I have found that our knowledge, imperfect though it be, of variation under domestication, afforded the best and safest clue. I may venture to express my conviction of the high value of such studies, although they have been very commonly neglected by naturalists. (Charles Darwin, 1859, p. 27)

Any mutualistic symbiosis raises the question, "Why should

\* E-mail: rfdenison@ucdavis.edu.

a symbiont benefit its host when it would gain immediate advantage by injuring it?" (Maynard Smith 1989, p. 285). The symbiosis between legumes and the rhizobia (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, or *Azorhizobium* spp.) that fix  $N_2$  inside their root nodules is particularly amenable to experimental investigations of this problem. Genetic maps and numerous well-characterized mutants are available for rhizobia, agriculturally important legumes, and "model legume species" (Handberg and Stougaard 1992; Cook 1999). Short generation times and small size make microcosm studies feasible. Symbiotic cooperation ( $N_2$  fixation rate and related variables) can be monitored noninvasively (Denison et al. 1992) and manipulated by substituting argon for  $N_2$ . Given these experimental advantages and the importance of the legume-rhizobium symbiosis in both natural and agricultural ecosystems, it is remarkable how few experiments have explicitly addressed legume or rhizobial strategies that maintain symbiotic cooperation. It is hoped that this article will, first, interest evolutionary biologists in working with this symbiosis and, second, provide a theoretical framework for this system that may lead to more productive hypotheses. This article proposes that rhizobial cooperation is maintained by plant sanctions, whose evolutionary consequences for rhizobia depend on nodule type.

## Cooperation and Conflict with One Rhizobial Strain per Plant

Symbiosis between legumes and rhizobia is often mutually beneficial (Sprent et al. 1987). If a plant is infected by a single strain of mutualistic rhizobia, it can obtain N needed for leaf growth—N that would otherwise require a larger and more expensive root system (Pate 1986)—by supplying the rhizobia with photosynthate. So long as each increment of  $N_2$  fixation leads to greater legume photosynthesis and to more rhizobial access to photosynthate, natural selection among rhizobia should favor symbiotic cooperation, that is, efficient  $N_2$  fixation.

As a legume grows or matures, however, the interests of plant and rhizobia may diverge. Nitrogen invested in

leaves may indirectly increase photosynthate supply to rhizobia (depending on how much self-shading occurs), but N used for seed production is unlikely to do so. Developing seeds compete with rhizobia for photosynthate. Annual plants may mobilize N needed for seedfill from lower leaves rather than continuing to support rhizobia. Perennials may reduce C allocation to N<sub>2</sub> fixation as winter approaches. Even in the ideal case of a plant infected by a single strain of rhizobia, it is unclear whether they should continue fixing N<sub>2</sub> as long as photosynthate remains available, or whether they should defect first, by attacking the nodule and escaping into the soil. The situation is further complicated by conflicts of interest among the multiple rhizobial strains that typically infect each plant and by fundamental differences in the life history of rhizobia infecting different legume species.

### Natural History of the Legume-Rhizobium Symbiosis

Legumes and rhizobia can grow and reproduce independently, but neither usually fixes atmospheric N<sub>2</sub> alone (Sprent et al. 1987). Rhizobia spread among hosts through the soil; they are not transmitted directly from parent to offspring via seed. Symbiosis requires a compatible combination of legume and rhizobial strain, although some legumes (e.g., *Vigna* spp.) and some rhizobia are quite promiscuous. *Rhizobium* sp. strain NGR234, for example, infects over 100 legume genera (Pueppke and Broughton 1999).

The rhizobia are a group of distantly related bacteria, some of which have apparently acquired the ability to infect legumes by gene transfer (Broughton and Perret 1999), sometimes involving plasmids, from other species. Legumes are monophyletic and may be closely related to other plants capable of symbiosis with N<sub>2</sub>-fixing actinomycetes (Soltis et al. 1995).

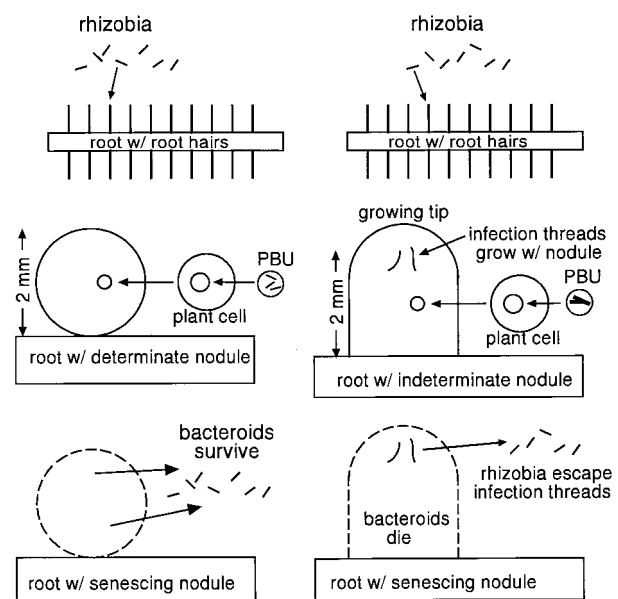
Root exudates from a compatible legume stimulate attachment of symbiotic rhizobia to roots, followed by infection, typically through root hairs. Once inside, rhizobia proliferate inside plant-delimited tubular "infection threads." Division and differentiation of host root cells result in the formation of a nodule, whose structure depends on the legume species (Sutton 1983). Determinate nodules, as found on *Glycine*, *Lotus*, or *Phaseolus* species, usually stop growing within a few weeks, whereas the indeterminate nodules of *Medicago*, *Trifolium*, and *Pisum* species continue growing, giving them an elongated rather than spherical shape.

Rhizobia are released from the infection threads into plant cells, where they are surrounded by a plant-derived peribacteroid membrane. The rhizobia then differentiate into bacteroids, which are typically larger than free-living rhizobia (especially in indeterminate nodules) and may

have irregular shapes. Essentially all N<sub>2</sub> fixation in nodules is carried out by bacteroids. Infection threads persist in indeterminate nodules and continue to invade new plant cells as the nodule elongates, releasing rhizobia that then differentiate into bacteroids in the new cells.

The life spans of individual nodules vary, depending on the legume species, rhizobial strain, and environmental conditions. Indeterminate nodules on woody perennials may last for several years (Sutton 1983). Various researchers have investigated the plant physiology and biochemistry of nodule senescence, but the release of rhizobia from senescing nodules into the soil has received less attention. Sutton's (1983, p. 201) complaint that "modern studies on ... [the] microbiology of nodule senescence ... [are] now overdue" remains largely true today.

The rhizobia released into the soil from determinate nodules have a different life history than those from indeterminate nodules (fig. 1). It is widely, but mistakenly, believed that all N<sub>2</sub>-fixing bacteroids are terminally differentiated and incapable of reproduction (Bergersen 1968;



**Figure 1:** Life cycle of rhizobia from soil to soil via determinate (left panel) or indeterminate (right panel) legume root nodules. Infection via root hairs is typical of both types, but rhizobial differentiation into N<sub>2</sub>-fixing bacteroids differs between the two nodule types. Bacteroids in determinate nodules resemble free-living rhizobia, and there are typically several bacteroids per peribacteroid unit (PBU). Bacteroids in indeterminate nodules are usually larger and morphologically distinct from free-living rhizobia; they are also individually surrounded by a peribacteroid membrane. When determinate nodules senesce, bacteroids survive and replenish soil populations. In indeterminate nodules, terminally differentiated bacteroids die, but undifferentiated rhizobia in the infection threads survive and reproduce in the soil.

Brock 1988; Jimenez and Casadesus 1989). One recent article claimed that “bacteroids lose the ability to reproduce” (Simms and Bever 1998, p. 1713), citing Zhou et al. (1985). Although Zhou et al. did indeed report that bacteroids from clover (*Trifolium repens*) nodules were unable to reproduce, they also found that “reproductive growth of bacteroids from protoplasts and crushed nodules of soybean was regularly observed” (Zhou et al. 1985, p. 473). Similarly, bacteroids in determinate soybean (*Glycine max*) and bean (*Phaseolus*) nodules were found to be viable (Tsien et al. 1977; McDermott et al. 1987), whereas bacteroids from indeterminate alfalfa (*Medicago sativa*) nodules were not (Gresshoff and Rolfe 1978; McDermott et al. 1987; McRae et al. 1989). During nodule senescence, bacteroids of indeterminate pea (*Pisum sativum*) and alfalfa nodules deteriorate, but soybean nodule bacteroids do not (Kijne 1975; Paau and Cowles 1979; Sarath et al. 1986). A comprehensive survey, using detergent sensitivity as a surrogate indicator, concluded that bacteroid reproductive viability is high in rhizobia from *Glycine*, *Lotus*, and *Phaseolus* nodules (all determinate) but low in the indeterminate nodules of *Medicago*, *Trifolium*, and *Pisum* species (Sutton and Paterson 1980).

In general, it appears that only in indeterminate nodules do rhizobia forswear reproduction when they differentiate into bacteroids (Sprent et al. 1987). Even bacteroids from indeterminate nodules may be induced to dedifferentiate under some conditions (Gresshoff et al. 1977), but bacteroids generally deteriorate during the senescence of indeterminate nodules. Soil rhizobial populations are then replenished by undifferentiated rhizobia from the persistent infection threads (Thornton 1930; Vance et al. 1980; Paau et al. 1980). Bacteroids in indeterminate nodules are therefore analogous to the nonreproductive worker ants that defend certain plants (Bronstein 1998). This dependence of bacteroid viability on nodule type, which has profound implications both for rhizobial strategies and for legume counterstrategies, has not received the attention it deserves.

#### Why Do Rhizobia Infect Legumes?

Douglas and Smith (1989) claimed that “no data are available” showing that microbial symbionts benefit from any symbiosis. Similarly, Sprent et al. (1993, p. 71) questioned whether rhizobia benefit from symbiosis “in terms of species continuity.” More appropriately, since natural selection need not benefit a species as a whole, they asked “whether the increase in rhizobial numbers following nodule senescence [and release of rhizobia into soil]... is significantly greater than would occur through microbial multiplication in the rhizosphere.”

The process of infection and nodule formation depends

on a large number of genes in both legumes and rhizobia (Fisher and Long 1992). Mutants that fail to infect legumes presumably arise frequently, but they rarely displace symbiotic rhizobia in soils where legumes are grown. The evolutionary persistence of the symbiosis suggests that rhizobia that infect legumes often leave more progeny than mutants that only colonize the rhizosphere.

A positive contribution of symbiosis to the prevalence of particular rhizobial strains in soil has been demonstrated in various studies. A marked strain reached  $3 \times 10^6$  cells/g in the rhizosphere around decaying nodules, compared to about  $2 \times 10^4$  cells/g in the rhizosphere of young soybean or corn plants (Reyes and Schmidt 1979). Soil counts of another marked strain in plots planted to a nonnodulating soybean for 5 yr were only one-sixth those in plots with a nodulating cultivar (Kuykendall 1989). Application of N fertilizer, which suppresses nodulation, eliminated stimulation by cowpea (*Vigna senensis*) of a symbiotic *Bradyrhizobium* (Thies et al. 1995). Increases in rhizobial populations attributable to symbiosis are not always detectable, and they may be short lived, but this is true of many adaptations in many species.

#### Biochemical Options for Rhizobial Cheating

If there were no opportunity cost to  $N_2$  fixation, natural selection among rhizobia would favor those strains whose bacteroids fix the most  $N_2$ . Frequently, this will increase photosynthesis by the legume host, and the rhizobia will often receive some share of the additional photosynthate. But  $N_2$  fixation is an energy-intensive process; nodules may consume a significant fraction of current photosynthate (Layzell et al. 1981). In the absence of plant sanctions, rhizobia that divert more photosynthate to their own reproduction might leave more descendants than those that fix more  $N_2$ .

Rhizobial growth and reproduction within the nodule (until the space available is filled) may benefit the plant, at least to the extent that the rhizobia differentiate into  $N_2$ -fixing bacteroids. Rhizobia may also hoard plant resources that will increase their reproductive success after nodule senescence. The biochemical options for “cheating” by rhizobia differ between determinate and indeterminate nodules. Because of differences in the reproductive viability of bacteroids, symbiotic cooperation by rhizobia is maintained (or thwarted) by individual selection in nodules with determinate growth and by kin selection in nodules with indeterminate growth. “Hoarding” of plant resources by bacteroids in determinate nodules could increase their chances of survival and reproduction after nodule senescence. Bacteroids in indeterminate nodules, on the other hand, should

redirect plant resources to their reproductive sisters in the infection threads.

The available data support this difference in rhizobial strategy with nodule type. *Bradyrhizobium japonicum* bacteroids in the determinate nodules of soybean (*Glycine max*), for example, accumulate large quantities of energy-rich poly- $\beta$ -hydroxybutyrate (PHB; Kim and Copeland 1996). It has been suggested that the primary role of PHB is “the continuation of  $N_2$  fixation at high rates until the last stages of seed development” (Bergersen et al. 1991, p. 59), but natural selection among rhizobia seems unlikely to favor legume reproduction at the expense of rhizobial reproduction. An earlier study found that PHB “was not sufficient for maintenance of nitrogenase activity under conditions of limited carbohydrate supply” (Wong and Evans 1971, p. 750). In fact, there may be a negative correlation between  $N_2$  fixation rate and PHB accumulation (Kretovich et al. 1977) and an increase in bacteroid PHB in soybean nodules as nitrogenase activity decreases during senescence (Klucas 1974). Rhizobial mutants unable to synthesize PHB actually fix  $N_2$  for longer than their PHB-producing parent (Cevallos et al. 1996). These results are consistent with the hypothesis that hoarding of PHB by bacteroids is at the expense of  $N_2$  fixation. But bacteroids with high PHB reserves may have an advantage (after leaving a senescing nodule) over those with lower reserves, especially if they have to compete with large numbers of other rhizobial strains from other senescing nodules nearby.

In indeterminate nodules, bacteroids of some *Rhizobium meliloti* strains produce “rhizopines,” such as 3-*O*-methylscyllo-inosamine, which are consumed mainly by undifferentiated rhizobia of the same strain (Murphy et al. 1995). Rhizopine supplied by bacteroids could increase the survival and eventual reproduction of undifferentiated rhizobia from infection threads.

#### Mixed Infections and the Need for Legume Sanctions

Given these options for misappropriation of plant resources by rhizobia, natural selection among rhizobia should favor greater  $N_2$  fixation only if the C return from C invested in  $N_2$  fixation (via increased host photosynthesis) is positive. When a plant is infected by a single clone of rhizobia, the rhizobia that fix  $N_2$  may reap much of the benefit, but most plants are infected by more than one strain (Dowling and Broughton 1986). If multiple infections are typical (even assuming, for now, that there is only one strain per nodule), then the “free-rider” scenario of evolutionary game theory (Maynard Smith and Szathmary 1995) predicts an increase in rhizobial parasitism, that is, increasing use of plant resources for rhizobial reproduction rather than  $N_2$  fixation. One should not kill a goose that lays golden eggs, but this assumes “if

you do not kill the golden goose, no one else will either: that is, it assumes that the host is infected by a single clone of symbionts” (Maynard Smith 1989, p. 285). The problem is analogous to the evolution of virulence in multiple parasites infecting a single host (Williams and Nesse 1991; Ebert 1998).

Fortunately, the free-rider scenario appears to be an overly simplistic representation of the legume-rhizobium symbiosis. Schwartz and Hoeksema (1998, p. 1029) suggested that mutualism is favored by natural selection when “the cost of tolerating cheaters is low” (which seems unlikely, given the high metabolic costs associated with nodules) or when “the opportunity to ensure fair trade is high.” Legume sanctions that ensure “fair trade” (by punishing rhizobia that fail to fix  $N_2$ ) may explain the evolutionary persistence of symbiosis, but which legume strategies are likely to be evolutionarily stable?

Some, but not all, earlier hypotheses have implicitly recognized the need for legume sanctions. But none of these hypotheses has adequately addressed the evolutionary implications of either the distinction between determinate and indeterminate nodules or of the possibility that individual nodules may contain more than one rhizobial strain. Udvardi and Kahn (1993) suggested that  $N_2$  fixation may benefit individual bacteroids by maintaining a favorable pH inside the peribacteroid unit (PBU, see fig. 1; Perez-Galdona and Kahn 1994) or by preserving their access to plant resources. Release of ammonia (the first product of  $N_2$  fixation) by bacteroids may counter plant-induced acidification and digestion by acid hydrolases (Brewin 1991). But why differentiate into bacteroids in the first place?

Jimenez and Casadesus (1989) suggested that differentiation into bacteroids is a “reproductive dead end” but that  $N_2$  fixation by bacteroids could benefit related, reproductively viable rhizobia sharing the same nodule by preventing early nodules from senescing early, perhaps at a time of year when rhizobial survival in the soil would be low. Olivieri and Frank (1994) also invoked kin selection, suggesting that  $N_2$  fixation may increase root exudation, thereby benefiting related rhizobia in the rhizosphere. Recent versions of this hypothesis (Provorov 1998; Simms and Bever 1998) suggest that rhizopines produced by bacteroids could be released into the rhizosphere, preferentially benefiting genetically identical rhizosphere populations. It is unlikely, however, that any hypothesis based on nodule exudates can explain symbiotic cooperation by rhizobia. If kin selection is based mainly on benefits to rhizosphere populations, natural selection should favor free-rider mutants that retain the ability to consume rhizopines in the rhizosphere without the costs (infection, nodule formation, fixing enough  $N_2$  to avoid plant sanctions, etc.) associated with rhizopine production. Experiments involving competition among transgenic rhizobia

differing only in the 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 the free-living population of the producing strain” (Gordon et al. 1996, p. 3991). The hypothesis that kin selection among rhizobia mainly involves rhizosphere populations also fails to explain why rhizopine production is not equally common in determinate nodules.

#### Possible Legume Sanctions Against Rhizobial Defectors

Rhizobia apparently have adaptations that allow them to penetrate legume roots without inducing host defenses (Iannetta et al. 1997), but a legume will block new rhizobial infections if N supply from the soil or existing nodules meets demand (Day et al. 1989). Legume control of nodule number apparently involves the plant hormone, ethylene (Penmetza and Cook 1997).

Legumes use a complex system of chemical recognition signals that determine which rhizobial species and/or strains they will admit (Hirsch 1999). Recognizing and excluding parasitic rhizobia at the time of infection would be an ideal strategy for legumes, if this were possible. Recognition signals are not a reliable indicator of symbiotic effectiveness, however. Genetic changes in the extracellular oligosaccharide signals produced by rhizobia near a legume root can cause changes in legume host range (Roche et al. 1991), independent of any differences in eventual  $N_2$  fixation. The elaborate recognition process may facilitate coevolution by limiting the number of rhizobial strains to which a legume must be adapted, but it is an unreliable way to exclude parasitic strains that are closely related to its usual partners. The longer generation times of plants relative to bacteria would put them at a disadvantage in an evolutionary arms race with parasitic rhizobia disguised as cooperative strains. The descendants of a rhizobial strain that fixed  $N_2$  very efficiently last year (many rhizobial generations ago) may retain the recognition signals of the ancestral strain despite a genetic change that greatly increases PHB accumulation at the expense of  $N_2$  fixation. Legumes are unable to recognize newly arisen nonfixing mutants during the infection process (Amarger 1981). Natural selection among legumes should therefore favor postinfection mechanisms that limit losses to rhizobia that prove to be parasitic once admitted.

Possible postinfection sanctions against rhizobial defectors include direct attack on nonfixing bacteroids (e.g., by acid hydrolases), limiting the C supply to nonfixing bacteroids or whole nodules, or limiting nodule  $O_2$  supply (Udvardi and Kahn 1993). The third option is currently supported by the most experimental evidence, but these mechanisms are not mutually exclusive. Visible responses to ineffective rhizobia, such as early nodule senescence

(Rolfe and Gresshoff 1980) or decreased nodule growth (Singleton and Stockinger 1983), could result from any combination of these physiological responses, or perhaps from rhizobia attacking the plant (Thornton 1930). When “autolysis” occurs in senescing nodules, who is digesting whom?

Nodules are the only plant organs, other than leaves, that have the ability to change their gas permeability rapidly and reversibly. It remains to be seen whether the mechanisms of permeability control are similar (Walsh 1995; Minchin 1997; Denison 1998). Nodule  $O_2$  permeability decreases after defoliation, reducing the risk that nitrogenase will be inactivated by increasing nodule interior  $O_2$  concentrations, as a shortage of photosynthate limits respiratory consumption of  $O_2$  (Denison et al. 1992). But nodule permeability often decreases so much that, at least initially,  $O_2$  concentrations actually decrease rather than remaining constant (Denison et al. 1991).

Thornton (1930, p. 118) examined alfalfa and clover nodules undergoing natural or dark-induced senescence and reported that “when no carbohydrate is brought to them, the bacteria [from the infection threads] obtain their energy by attacking the nucleus, cytoplasm, and cell walls of the host tissue.” He suggested that the growth of these rhizobia is normally limited by  $O_2$  supply rather than photosynthate supply. Rhizobia are metabolically capable of consuming various plant cell components, but so long as  $O_2$  is limiting, this additional C source would not increase their growth rate. When the nonstructural carbohydrate supply decreases, however, plant structural components become increasingly tempting. Under this hypothesis, a decrease in nodule  $O_2$  permeability may protect the plant from rampaging rhizobia, in addition to conserving photosynthate and protecting nitrogenase from  $O_2$  inactivation. Although bacteroids may tolerate lower  $O_2$  concentrations than do plant cell mitochondria, the latter are localized near intercellular airspaces, so they may be less sensitive to nodule  $O_2$  supply.

Substitution of argon for  $N_2$  in the atmosphere around the root, thereby preventing  $N_2$  fixation, also causes a decrease in nodule  $O_2$  permeability (Minchin et al. 1983; King and Layzell 1991), which would reduce  $O_2$  supply to rhizobia inside the nodule. This treatment never occurs in nature, but the effects would be similar to infection by parasitic rhizobia that fail to fix  $N_2$ . This nodule permeability response may therefore be adaptive for legumes where parasitic rhizobia are common (Denison 1998). By decreasing the  $O_2$  supply to nodules containing parasitic rhizobia, while maintaining the  $O_2$  supply to nodules with effective rhizobia, a plant might obtain adequate rates of  $N_2$  fixation, while limiting respiration and photosynthate consumption by parasitic rhizobia.

As discussed below, the effectiveness of this strategy

would depend on the variability in rhizobial effectiveness, both among and within nodules, and on whether the  $O_2$  permeability of individual nodules can be regulated independently. Exposing half of a split root system to  $Ar : O_2$  decreased photosynthate partitioning to that half (Singleton and van Kessel 1987), but it is not known whether decreasing  $O_2$  permeability was responsible. Similar experiments with individual nodules have not yet been reported.

### Legume Sanctions, Nodule Type, and the Evolution of Rhizobia

How would different kinds of legume sanctions affect rhizobial evolution? First, assume that different nodules on a given plant may contain different rhizobial strains varying in symbiotic effectiveness (they might still be closely related and serologically indistinguishable) but that no nodule contains more than one strain. In the absence of plant sanctions, competition among rhizobial strains would favor those that allocate more C to reproduction rather than to  $N_2$  fixation.

Does it matter whether plant sanctions against rhizobia that fail to fix  $N_2$  operate at the level of the individual nodule or at the level of the individual bacteroid? Either strategy could benefit the individual plant by reducing wasteful consumption of photosynthate. In determinate nodules, bacteroids are the reproductive units, so either strategy would also tend to decrease the abundance of parasitic rhizobia in the next generation.

In indeterminate nodules, plant sanctions against individual parasitic bacteroids might still benefit the individual plant (some photosynthate would be saved), but sanctions directed solely against the terminally differentiated bacteroids would not prevent reproduction by undifferentiated rhizobia in the same nodule and proliferation of the parasitic strain in the soil. Reducing the C or  $O_2$  supply to an entire nodule that contains only parasitic rhizobia might save more photosynthate than the same sanction against individual bacteroids, for two reasons. First, photosynthate consumption by undifferentiated rhizobia (as well as bacteroids) would be reduced. Second, continued wasteful growth of a nodule that fixes little  $N_2$  might be prevented. In contrast to sanctions against bacteroids alone, whole-nodule sanctions could also limit the future abundance of the parasitic rhizobial strains in the soil, by reducing the reproduction of undifferentiated rhizobia from the infection threads.

### Implications of Mixed Nodules

How would mixed nodules (each containing more than one rhizobial strain) change this picture? Again, it depends on nodule type. Plant sanctions against individual bacteroids

would save photosynthate and, in determinate nodules, would also tend to reduce the frequency of these parasitic strains in soil. Control at the level of the whole nodule would probably be less effective with mixed nodules. If each nodule contains about the same ratio of cooperative to parasitic rhizobia, allocating more photosynthate to some nodules than to others would not increase the  $N_2$  fixation rate per unit photosynthate invested by the host. Similarly, legume sanctions against whole nodules would not alter the comparative advantage of bacteroids that hoard PHB (relative to those that use photosynthate only for  $N_2$  fixation) in determinate nodules. If, however, cooperative rhizobia predominate in some determinate nodules and parasitic strains predominate in others, then host sanctions against those nodules that fix the least  $N_2$  might slow or reverse the evolution of parasitism among rhizobia. An individual plant would also benefit from allocating resources only to the best nodules, assuming that those nodules can fix enough  $N_2$  to meet plant needs. If a plant has only a few large nodules, however, then continuing to support some nodules that contain less mutualistic rhizobia might be a better option than N deficiency.

Neither whole-nodule sanctions nor sanctions against individual bacteroids will necessarily limit the evolution of parasitism among rhizobia in mixed indeterminate nodules. As in determinate nodules, sanctions against whole indeterminate nodules would save little photosynthate, unless there were large differences among nodules in the ratio of cooperative to parasitic rhizobia. (Any evolutionary effect would also depend on differences in the relative abundance of strains in the infection threads.) Plant sanctions against individual bacteroids that fail to fix  $N_2$  in indeterminate nodules would save photosynthate, but because the bacteroids in indeterminate nodules are incapable of reproduction, sanctions against bacteroids would have no direct effect on the evolution of rhizobial parasitism.

Although there may be little incentive for cooperation ( $N_2$  fixation) by bacteroids in multiply infected indeterminate nodules, it might seem that there is also little incentive to "cheat." Even if some of the benefit from  $N_2$  fixation goes to a free rider sharing the nodule, what else can nonreproductive bacteroids do that increases propagation of their genes? As discussed above, they could produce rhizopines, which could then be consumed by genetically identical undifferentiated rhizobia sharing the same nodule.

In singly infected nodules, rhizopine production at the expense of  $N_2$  fixation could be limited by legume sanctions against either bacteroids or whole nodules. In mixed nodules in which the majority of bacteroids in a nodule fix  $N_2$ , however, a significant minority (of another strain) might be able to redirect photosynthate to their undiffer-

entiated sisters via rhizopines without triggering plant sanctions.

### How Common Are Mixed Nodules?

Most of the discussion in the previous section would be academic if mixed nodules were rare. It is, in fact, widely assumed that each nodule contains a single rhizobial strain, but experimental results challenge this hypothesis. When white clover plants were inoculated with a mixture of two rhizobial strains, some nodules contained one strain, some the other, and about 20% contained both (Rolfe and Greshoff 1980). After mixed inoculation of soybean, up to 32% of nodules contained both strains, although the frequency of mixed nodules was less with lower inoculum density (Lindemann et al. 1974). Under some conditions, up to 83% of cowpea nodules formed by a slower-growing rhizobial strain also contained a coinoculated, faster-growing strain (Trinick et al. 1983). Up to 74% of doubly inoculated *Trifolium subterraneum* nodules contained both strains (Demezas and Bottomley 1986). These studies show that both indeterminate and determinate nodules can contain more than one rhizobial strain, but the frequency of mixed nodules under field conditions is unknown (Simms and Bever 1998).

If whole-nodule sanctions are important, natural selection may favor plant mechanisms that limit the number of rhizobial strains to one per nodule. In at least some cases, rhizobia sharing an indeterminate nodule are segregated into separate lobes (Trinick et al. 1989; Sessitsch et al. 1996). It is not clear whether this segregation is controlled by the rhizobia or by the plant—it may involve closely spaced multiple infections—but it might allow targeting of sanctions to one lobe or the other.

### The Tragedy of the Rhizobial Commons

Despite the possibility of conflict, both between legumes and rhizobia and among rhizobial strains, symbiotic cooperation has persisted for millions of years. The prevalence of multiple rhizobial strains per plant should favor the evolution of parasitism, rather than mutualism. It is proposed that this tendency is checked by legume sanctions against rhizobia that fail to fix  $N_2$  once established inside nodule cells.

Indirect conflict among legumes over the nature of those sanctions and their effects on the rhizobial “commons” are also possible. Consider a legume with indeterminate nodules, each containing a different mixture of mutualistic and parasitic rhizobia. Whole-nodule sanctions against those nodules that contain the most parasitic rhizobia could reduce the frequency of parasitic strains, benefiting future generations of legumes growing in the same soil,

including descendants of the plant imposing the sanctions. But if mutualist-dominated nodules were insufficient to meet plant N needs during seedfill, a legume imposing whole-nodule sanctions might leave few descendants to reap this future benefit. Sanctions targeted against individual bacteroids might maximize short-term N return on C investment because little C would be wasted on non-fixing bacteroids, whatever the ratio of mutualistic to parasitic strains in a given nodule. But, in indeterminate nodules, bacteroid sanctions alone would have no effect on future generations of rhizobia in the soil. The overall effects of alternative sanctions on legume-inclusive fitness might depend on the frequency of mixed nodules, seed dispersal distances, etc. Theoretical treatments of parent-offspring conflict (Ellner 1986; Zhang 1998) may be useful in analyzing this problem, but the future beneficiaries of more mutualistic rhizobia may also include unrelated legume competitors. If a perennial legume plant depends mainly on rhizobia released from its own nodules for infection of new roots, that could provide an additional incentive for sanctions that favor cooperative rhizobia.

Bacteroids in determinate nodules show that it is possible to fix  $N_2$  while retaining the capacity to reproduce, so why do bacteroids sacrifice their reproductive viability in nodules with indeterminate growth? By the time the bacteroids cease dividing, the infected cells are packed full of bacteroids. Therefore, any increase in rhizobial numbers depends on new growth of the nodule, which may in turn depend (perhaps indirectly) on  $N_2$  fixation. If more extreme differentiation (e.g., larger cells) results in higher capacity to fix  $N_2$ , as appears to be the case for rhizobia infecting both peanut and cowpea (Sen and Weaver 1984), then kin selection could favor loss of bacteroid viability in favor of continued growth by undifferentiated rhizobia from infection threads. Alternatively, loss of viability might somehow be imposed by the plant host, perhaps through chemical inhibition of cell wall synthesis in rhizobia (Sutton and Paterson 1983). Indeterminate nodules apparently achieve the “germ-soma distinction” (Frank 1996) hypothesized to benefit the host by reducing competition among symbionts. But this separation also prevents the most direct legume sanctions, namely those against non-fixing bacteroids, from having much effect on the spread of parasitic rhizobia.

### Suggestions for Further Research

The legume-rhizobium symbiosis offers numerous opportunities for additional research. Field and microcosm studies are needed on the impact of legume hosts on changes over years in the relative abundance of mutualistic versus parasitic rhizobia. Quantitative estimates of the frequency of mixed nodules in the field should be a high

priority. DNA-based methods (Richardson et al. 1995; Wilson 1995) may be most useful. Lindemann et al. (1974, p. 277) suggested that “most of the doubly infected nodules observed in this study would have shown the presence of only one strain by the usual serological tests.” Photosynthate partitioning and nodule O<sub>2</sub> permeability decrease when an entire root system is exposed to an N<sub>2</sub>-free atmosphere, but the responses of individual nodules need to be determined. Possible mechanisms by which legumes may impose sanctions on individual bacteroids also need to be investigated. *Rhizobium* species with wide host ranges, like *Rhizobium fredii*, which forms determinate nodules on soybean and indeterminate nodules on alfalfa (Hashem et al. 1997), may be particularly useful.

#### Acknowledgments

Thanks to M. Kahn for some leads to the literature; to J. Bronstein, R. Grosberg, D. Phillips, and K. Rice for comments on the manuscript; and to L. Copeland, whose seminar discussing species differences in PHB synthesis stimulated my interest in this topic.

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