Sanctions, Cooperation, and the Stability of Plant-Rhizosphere Mutualisms

E. Toby Kiers[1] and R. Ford Denison[2]

[1]Faculteit der Aard – en Levenswetenschappen, De Boelelaan 1085, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands; email: ekiers@falw.vu.nl

[2]Department of Ecology, Evolution, & Behavior, University of Minnesota, St. Paul, Minnesota 55108; email: denis036@umn.edu

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Abstract

There are both costs and benefits for host plants that associate with microbes in the rhizosphere. Typically, an individual plant associates with multiple microbial genotypes varying in mutualistic benefit. This creates a potential tragedy of the commons where less-mutualistic strains potentially share in the collective benefits, while paying less of the costs. Therefore, maintaining cooperation over the course of evolution requires specific mechanisms that reduce the fitness benefits from "cheating." Sanctions that discriminate among partners based on actual symbiotic performance are a key mechanism in rhizobia and may exist in many rhizosphere mutualisms, including rhizobia, mycorrhizal fungi, root endophytes, and perhaps free-living rhizosphere microbes. Where they exist, sanctions may take different forms depending on the system. Despite sanctions, less-effective symbionts still persist. We suggest this is because of mixed infection at spatial scales that limit the effects of sanctions, variation among plants in the strength of sanctions, and conflicting selection regimes.

INTRODUCTION

Potential and Actual Benefits of Plant-Rhizosphere Mutualisms

Cooperation: behavior whose evolutionary stability depends on actual benefits to another

Cheater: individuals not cooperating (or cooperating less), but potentially able to gain benefits from cooperation by others

Plants receive substantial benefits from rhizosphere organisms, including rhizobia, mycorrhizae, and root endophytes. Nitrogen fixation (N_2 fixation), increased phosphorus (P) supply, and pathogen protection by rhizosphere microorganisms can provide hosts with measurable fitness advantages. A substantial portion of host photosynthetic carbon (C) goes to support the activities, growth, and reproduction of associated microbes, with 15–20% going to growth and respiration of rhizobium-containing nodules (Minchin & Pate 1973), 4–20% to mycorrhizal fungi (Johnson et al. 1997), and about 15% exuded or otherwise lost to the rhizosphere (Morgan et al. 2005), where it supports a variety of microbes. Cooperation between host plants and microbes should benefit both partners, given that organisms differ in their resource needs and metabolic capabilities (Hoeksema & Schwartz 2003). Mutual benefit does not guarantee that cooperation is evolutionarily stable, however.

Most plants interact with several genotypes or strains of microbe, or even multiple species in the case of mycorrhizal fungi. Such multiple infections can create a potential tragedy of the commons (Hardin 1968). The tragedy is that cooperative partners that supply their hosts with resources indirectly aid competing strains (including noncooperative ones) colonizing the same individual (Denison et al. 2003a). Within-host competition can therefore destabilize mutualisms (West et al. 2002a,b). This is why models based on a single clone of symbiont per host are irrelevant to most rhizosphere mutualisms, which involve multiple strains per plant. The evolutionary consequences of hosting multiple partners is a recurring theme in rhizosphere mutualisms (Denison 2000, Denison et al. 2003a, Kiers et al. 2002, Sachs et al. 2004). This review discusses the tragedy of the commons in plant-rhizosphere mutualisms and its various solutions. First, we discuss the general problems associated with costly microbial-plant mutualisms and then examine three classes of rhizosphere microbes: N_2-fixing rhizobia, arbuscular mycorrhizal fungi, and less-studied mutualisms with endophytic or free-living rhizosphere microbes.

The Rhizosphere as a Tragic Commons

Microbes may benefit from associating with healthier plants, but what if benefits are shared with "cheaters" that contribute less to keeping plants healthy? We endorse a recent definition of cheaters as "individuals who do not cooperate (or cooperate less than their fair share), but are potentially able to gain the benefit of others cooperating" (West et al. 2007). For cooperation between microbes and plants, however, the common currency needed for an uncontroversial definition of fair share might be elusive. This problem can be simplified if we instead think of cooperation among microbes, investing in the health of a shared plant host upon which they all depend. Then, a fair share becomes an equal share, and microbial cheaters can be identified by how their performance compares with other strains. A cheating rhizobium genotype, for example, is one that diverts more resources to its own reproduction, and away from N_2 fixation that by increasing host plant photosynthesis (Bethlenfalvay et al. 1978) can benefit fellow rhizobia of any strain on the same host plant.

For cooperation among microbes of the same species, we can apply the well-developed, but widely misunderstood, theory of kin selection (Griffin & West 2002, West et al. 2008). The theory is that alleles that are individually costly but collectively beneficial disappear unless they preferentially benefit others, usually kin, that share the same alleles. Hamilton (1964) showed that an altruism allele with a fitness cost c to an actor A will only spread if fitness benefits b to group B, weighted by their relatedness r to A, are large enough. That is, if $br > c$.

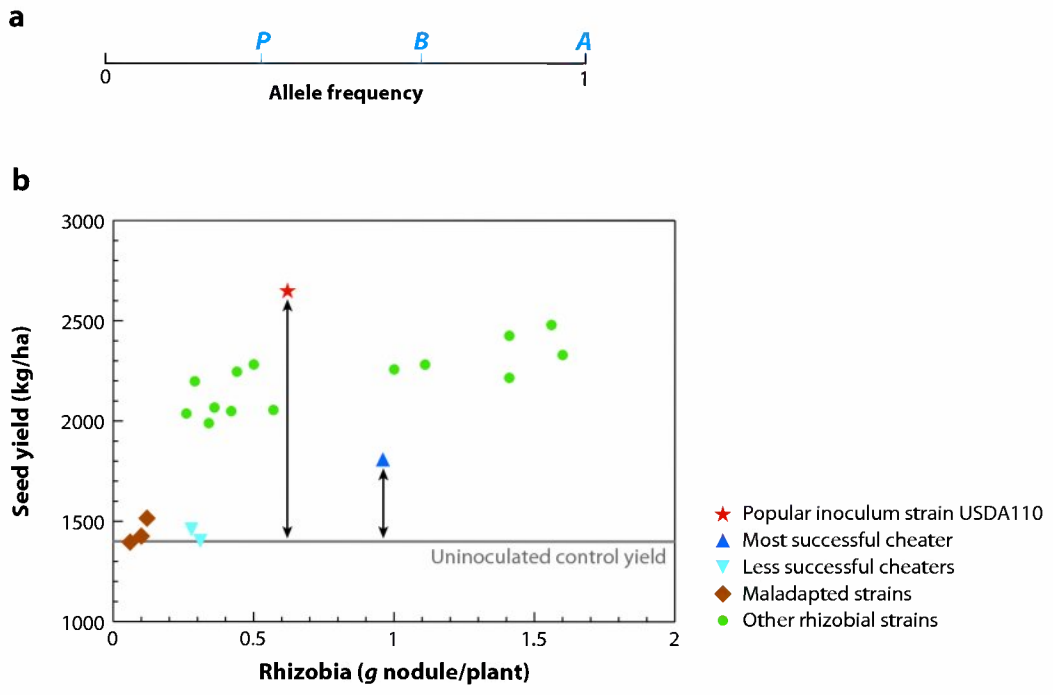

Figure 1

(*a*) Graphical explanation of Hamilton's *r*. *A* is an individual with the altruism allele. *B* is the population of beneficiaries; some or all may have the altruism allele. *P* is mean allele frequency in the overall population, which includes both *A* and *B*. Hamilton's *r* is the distance from *P* to *B*, as a fraction of the distance from *P* to *A* (from Grafen 1985). (*b*) Benefits of symbiosis to soybeans (seed yield) and rhizobia (assumed proportional to *g* nodule/plant) for 21 *Bradyrhizobium japonicum* strains. Horizontal line shows yield of uninoculated control plants. Double-headed arrows show benefits from the popular inoculum strain USDA110 (*red star*) and the most successful cheater (*dark blue triangle*). Two other cheaters that obtained less benefit from symbiosis (*light blue upside-down triangles*) and three apparently maladapted strains that gained almost no benefit (*brown diamonds*) are also shown. Data from Abel & Erdman (1964); graphical approach suggested by Ezra Lyon.

The *r* in Hamilton's rule is not necessarily the relatedness calculated from genealogical trees (Griffin & West 2002). Two microbial cells may have the same allele owing to clonal descent from a common ancestor, in which case they will also be identical at most other loci; but they could also have acquired the same allele by horizontal gene transfer, while being unrelated at most loci. Especially in such cases, we have found Grafen's (1985) graphical explanation of Hamilton's *r* (**Figure 1*a***) particularly useful. Consider the case where individual *A* has the altruism allele (fixing more N_2, say), so the frequency of that allele is 1 for *A*, as plotted. (In diploid species, there could be heterozygotes with an altruism allele frequency of 0.5.) *B* is the average frequency of the allele among beneficiaries of *A*'s altruism, while *P* is its average frequency in the overall population. Grafen (1985) defined Hamilton's *r* as how far *B* is toward *A* from *P*. In the diagram, *B* is halfway from *P* to *A*, so *r* is 0.5. With *B* halfway between *P* and *A*, reproduction by a random beneficiary will increase the frequency of the allele in the overall population, but only half as much as if *A* reproduced. Therefore, to maintain the altruism allele, any fitness loss by *A* must be balanced by a twofold fitness increase for *B*, exactly consistent with Hamilton's equation.

Hamilton's *r*: coefficient in Hamilton's rule; $c < br$, where c is the fitness cost to the actor and b is the benefits to kin

But what if the benefits of *A*'s altruism are shared with the population *P*, with which *A* and *B* compete for resources? Reproduction by a random member of *P* will not change the allele's

frequency, on average. If B were identical to P, then B would be 0% of the way from P to A, Hamilton's r would be zero, and the individual cost of altruism would lead to its disappearance. Limited dispersal of microbes in soil tends to make B more similar to A, because many beneficiaries of A's altruism are clonemates (genetically identical, owing to clonal reproduction) of A. But limited dispersal also moves P closer to B. The net effect is that limited dispersal (e.g., close proximity on the same plant root) does not automatically favor altruism (Griffin & West 2002).

Sanctions: selection imposed by a partner that rewards cooperative behavior and punishes less cooperative behavior

This means that, in order for a microbial allele that benefits plants to persist, microbes with the allele must benefit relative to those with alternate alleles. Furthermore, the benefit, multiplied by Hamilton's r, must exceed the cost. In general, limited dispersal is not sufficient to bring this about. The tragedy of the commons therefore appears to apply to the rhizosphere.

Let us illustrate with the example of rhizobia fixing N_2 for their host plants. By supplying its host with nitrogen (N), an individual rhizobium enhances host photosynthesis, thereby potentially increasing the rhizobium's own access to photosynthate. But when several rhizobial strains infect the same individual plant, a rhizobium cell that fixes N benefits not only its own kin, but also other rhizobium strains that compete for host resources and future nodulation opportunities (Denison 2000).

The question can be framed as follows: Are benefits to other rhizobia, weighted by Hamilton's r, less than the cost to a rhizobium cell of fixing N_2? Assume that a plant is host to 10 equally abundant rhizobium strains. Then Hamilton's r for an allele that increases N_2 fixation and is found in only one strain would be about 0.1, if the frequency of that allele in the overall population is near zero. But r could be as low as zero if the allele frequency in the overall population is also 0.1; if $B = P$, then B is 0% of the way toward A from P. In other words, if the rhizobia infecting a given plant have the same allele frequency as the overall population, then any activity that benefits all rhizobia on that plant equally (N_2 fixation increasing photosynthesis, say) will not change the allele frequency in the population.

West et al. (2002b) modeled the evolutionarily stable strategy for rhizobium investment in N_2 fixation as a function of Hamilton's r for rhizobia within a plant. When it was assumed that increased host-plant photosynthesis (owing to rhizobium N_2 fixation) benefits all rhizobia on the plant equally, the model predicted decreasing investment in N_2 fixation as r decreased. With typical numbers of strains per plant (range 4–18; Hagen & Hamrick 1996, Silva et al. 1999), the predicted N_2 fixation fell to zero.

Similar arguments apply even more strongly for mycorrhizal fungi and for rhizosphere microbes near the root surface. Selection for cooperation imposed by differences in the survival of groups, such as the microbes associated with an individual plant, is undermined by migration between groups (Kerr et al. 2006, Levin & Kilmer 1974). Rhizobia inside a nodule cannot leave until the nodule decays, whereas mycorrhizal fungi and root-surface microbes can associate with a new host at any time. The conclusion from these simulations (West et al. 2002b) is therefore broadly applicable to rhizosphere mutualisms: Collective benefits to microbes cannot explain the evolutionary persistence of activities that are metabolically costly to individual microbial cells. Some combination of two explanations is required. (*a*) An activity that may happen to benefit the host may not be as costly to microbes as it seems, because it generates benefits to individual microbes that do not depend on whether the plant also benefits, or (*b*) the relevant spatial scale for Hamilton's r may be smaller than the whole plant, so that r is correspondingly higher. In this review, we discuss how the relative importance of these mechanisms differs for rhizobium, mycorrhizal, and other microbial mutualisms. Host sanctions imposed on individual nodules are a combination of these two mechanisms, as discussed below.

SANCTIONS AND THE LEGUME-RHIZOBIA MUTUALISM

The Challenge of Less-Beneficial Rhizobia

Although rhizobia (bacteria classified as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, etc.) are textbook examples of cooperative symbionts, in reality rhizobia cover the entire range from mutualists to parasites, varying dramatically in the N_2-fixing benefits provided to their hosts (Ballard et al. 2002; Denton et al. 2000; Heath & Tiffin 2007; Thrall et al. 2000, 2007). Strains of rhizobia can vary as much as tenfold in net host benefits, even when derived from a single location (Burdon et al. 1999).

Some rhizobium strains provide little benefit to host plants because they form few nodules under the conditions tested or because they reproduce poorly in nodules. Free-living rhizobia that are unable to nodulate legumes are common in some soils (Laguerre et al. 1993, Denison & Kiers 2004a, Segovia et al. 1991, Sullivan et al. 1996). Such strains have little effect on plants, positive or negative, and are not the focus of this section. Here, we focus on comparing mutualists with cheater strains that are highly competitive for nodulation and abundant inside nodules, but provide little or no benefit to plants.

Although data on net benefits to host plants have rarely been expressed on a per rhizobium cell basis, rhizobium numbers can be approximately proportional to nodule weight (Kiers et al. 2003), which is more commonly measured. **Figure 1*b*** shows the variation in benefit provided to a soybean host cultivar "Lee" from 21 different *Bradyrhizobium japonicum* strains as a function of nodule weight per plant (data from Abel & Erdman 1964). Three strains (*diamonds*) gave little plant benefit (yield similar to uninoculated controls) but also little rhizobium benefit (low nodule weight per plant). These strains may be incompatible with this particular host rather than cheaters. Two of three cheaters (triangles) also provided negligible plant benefit. Because their nodule weights were similar to several strains that were much more beneficial to the plant, the plant benefit these cheaters provided per rhizobium cell must have been much less.

The most successful cheater provided less than half as much yield benefit as inoculum strain USDA110 (*star*; compare lengths of two *double-headed arrows*). But this strain had a higher fitness than USDA110 (i.e., more nodule weight per plant). Although single-strain inoculation experiments like this are a common way of comparing strains, most plants in the field host multiple strains. A 50:50 mixture of USDA110 and this particular cheater would presumably give plant growth intermediate to that with the two strains separately. In that case, the fitness of USDA110 would be reduced even more, while the cheater's fitness would increase. This illustrates one reason why highly beneficial inoculum strains tend not to persist over years: The N_2 that cooperative strains fix can benefit cheaters hosted by the same plant. Note that defining a strain as a cheater is based on how its performance compares with other strains. It may help to think of the concept of cheating as cheating other rhizobia, not whether the strain provides a net benefit to the host in single-strain inoculation. In **Figure 1*b***, the cheater just discussed provided a small net benefit to the host relative to no rhizobia, whereas the two other cheaters provided none. Similarly, we would classify rhizobia that form unusually large nodules on pea plants, presumably enhancing their own fitness, but support only half the plant growth of strains that form smaller nodules (Laguerre et al. 2007) as cheaters. In most soils, total rhizobium populations vastly exceed nodulation opportunities; the disappearance of cheaters would therefore benefit plants, because they compete with better strains for nodulation.

We hypothesize that the spectrum in effectiveness of rhizobial strains, once inside nodules, can best be understood in the context of rhizobium strategies to maximize benefit from symbiosis

while limiting costs. Respiration in support of N_2 fixation for the benefit of the plant is expensive for rhizobia (Gutschick 1981), consuming C compounds that they could otherwise have used to support their own reproduction (Denison 2000, Kretovich et al. 1977). If some strains can limit costs by "free-riding" on other strains, they will be favored by selection and spread (Denison et al. 2003a). However, cheating is far from universal. Strains that fix more N_2 must somehow benefit in ways that cheater strains on the same plant do not. We explore possible mechanisms below.

Partner Choice: Choosing Partners Based on Recognition Signals

One potential mechanism to stabilize cooperation would be to simply chose the best partner from the start. In theory, plants could identify cheaters, based on preinfection signals, and prevent them from infecting roots. But, despite elaborate signaling between plants and rhizobia (Jones et al. 2007), legumes do not seem to exclude parasitic rhizobia that are closely related to their usual symbiotic partners (Hahn & Studer 1986, Kuykendall & Elkan 1976). For instance, Amarger (1981) reported that in five out of six cases, competitive ability did not differ between effective strains and their ineffective mutants. In contrast to these results, Champion et al. (1992) found that soybeans inoculated with a 1:1 mixture of effective:ineffective strains resulted in more nodules formed by the effective strain than would be found by chance. This is consistent with the observation that soybeans exclude some (but not all) rhizobia with a particularly harmful, chlorosis-inducing phenotype (Devine & Kuykendall 1996), and that other legumes limit infection by specific ineffective strains (Kiers et al. 2007, Materon & Zibilske 2001).

One problem with relying on signals is that they are not always honest (Edwards & Yu 2007). The short generation time of rhizobia compared to plants means that selection could favor the evolution of rhizobial strains with modified signals that mimic those of cooperative strains, regardless of their true benefits. The extent to which recognition systems can effectively exclude ineffective rhizobia deserves further attention, but this mechanism seems unlikely to solve the problem of cheating. There will always be strong selection for cheaters that mimic the signals of their cooperative competitors in order to gain access to a plant host. Honest signaling between cooperating species is rarely absolute (Szamado 2000).

Sanctions: Rewards or Punishments Based on Actual Symbiotic Performance

Actual benefits, unlike signals, are a better measure of mutualism. If host plants are able to monitor benefits from rhizobia and discriminate against nodules that are fixing less N_2 (Udvardi & Kahn 1993), then cooperation can be favored. We have termed this the sanctions hypothesis (Denison 2000). Modeling has shown that major investment in N_2 fixation becomes an evolutionarily stable strategy for rhizobia when sanctions are imposed against individual nodules that fix less N_2 (West et al. 2002b). This is because, although Hamilton's r for rhizobia at the scale of the whole plant may be <0.1, as discussed above, r could be 1.0 for a single clone of rhizobia within a single nodule. By fixing more N_2, a rhizobium cell could help protect millions of its clonemates from nodule-level sanctions.

The sanctions hypothesis was recently tested using soybean. Rhizobia that are normally mutualistic were forced to defect from N_2 fixation by replacing air with an Ar:O_2 mixture that contained only a trace amount of N_2 (Kiers et al. 2003). A decrease in host-mediated O_2 supply to the nodule interior coincided with a 50% reduction in rhizobium reproduction in the nonfixing nodule. Rhizobia and plant cells depend on O_2 for aerobic respiration. A decrease in O_2 influx was therefore hypothesized to limit rhizobium reproduction (Kiers et al. 2003), either directly or indirectly (e.g., via an O_2 effect on C supply to rhizobia from the plant cells in which they are found).

Intermediate Punishment

Like other symbioses, such as obligate pollination mutualisms (Kawakita & Kato 2004) and mycorrhizal interactions (Jones & Smith 2004), partners in the legume-rhizobia symbiosis often neither fully defect nor cooperate, but rather invest at some intermediate level (**Figure 1*b***). The problem is, if sanctions are imposed only on the most extreme cheaters, then intermediate levels of cheating will still evolve (Killingback & Doebeli 2002, Roberts & Renwick 2003). Do intermediate sanctions exist?

Controlling N_2 concentration around individual nodules allows us to manipulate the rate of N_2 fixation so that plant response to various levels of cooperation can be determined. When three intermediate rates of N_2 fixation were compared, rhizobium cells per nodule (after 10 days) increased linearly with N_2 fixation, although nodules fixing at 50% of potential had about 75% as many rhizobia as fixing controls (Kiers et al. 2006). These results show that soybeans also respond to intermediate levels of cheating by rhizobia. Is it not known, however, if plants can punish other mediocre microbial mutualists (e.g., mycorrhizae, endophytes) similarly.

Partner Choice, Sanctions, or the Same Thing?

One of the most visible consequences of legume sanctions can be seen in nodule growth. Decreased nodule growth in response to less-beneficial rhizobia has been found in soybean (Champion et al. 1992; Kiers et al. 2003, 2006; Singleton & Stockinger 1983), and most recently in wild lupine (Simms et al. 2006). Simms et al. (2006) invoke partner choice to describe their findings, yet is choice, *sensu stricto*, operating? Their wild lupines did not exclude less cooperative rhizobial strains in greenhouse trials. Therefore, effective partner choice, originally defined as a one-time interaction with choice occurring "in advance of any possible exploitation" (Bull & Rice 1991) did not occur. However, nodules occupied by these less-beneficial strains were significantly smaller, so they presumably contained fewer rhizobia. This is consistent with our definition of sanctions and Bull & Rice's (1991) definition of partner fidelity, i.e., a continuing interaction where those who "at any time fail to co-operate with their partner can be penalized."

Are partner choice and sanctions the same thing? We see sanctions as biological analogs of "policing" mechanisms that have been shown to stabilize cooperation within species (Ratnieks et al. 2001), "selective abortion" mechanisms seen in some obligate pollination mutualisms (Pellmyr & Huth 1994), and processes involving "active retaliation" in partner fidelity, as defined above (Bull & Rice 1991). These are all ways of defining mechanisms that support good partners while punishing exploiters. In contrast, partner choice as redefined by Sachs et al. (2004) covers "several forms, ranging from establishing cooperation with only one of several potential partners to altering the duration of cooperation with a partner according to its actions, to actually reducing the fitness of selfish partners." This new definition is so broad that it covers a whole range of qualitatively different interactions among partners. We would argue that the first of these possibilities (establishing cooperation with only one of several potential partners) is partner choice, but we would use sanction to describe the last two forms, as explained below.

There are a few important differences between partner choice and sanctions. The first is the difference between signals and actual behavior. Mate choice based on arbitrary signals (Castellano & Cermelli 2006) and legumes that admit only certain rhizobia based on chemical signals (Kinkema et al. 2006) are good examples of true partner choice. In contrast, sanctions alter the exchange of benefits or duration of an interaction based on actual behavior in a relationship.

The second difference lies in relative versus absolute standards. Partner choice implies a comparison between two or more partners. In contrast, punishment or sanctions do not necessarily

depend on what any other partner or potential partner is doing. King & Layzell (1991) found that nodule-interior O_2 concentration decreased in a soybean host when the whole root system was in an N_2-free atmosphere. This is analogous to a plant root system infected by only cheating rhizobia. If, as we hypothesize, it is O_2 supply that limits rhizobium reproduction during sanctions (Kiers et al. 2003), this is sanctions without choice. It may turn out that comparisons are sometimes involved, because plants have been shaped by selection in response to variation between strains, but apparently comparisons are not essential.

Finally, it is essential to stress that sanctions, as we have defined them, do not imply an expectation of changed behavior, in contrast to sanctions in human interactions. It is unknown whether rhizobia can change their behavior in response to sanctions, but sanctions will certainly change the evolution of rhizobium populations.

GIVEN SANCTIONS, WHY DO LESS-EFFECTIVE RHIZOBIA STILL EXIST?

Given that sanctions exist, how do less-beneficial rhizobia persist at all? Although legume sanctions are less severe than the flower abortion seen in some yuccas (Pellmyr & Huth 1994), rhizobia that fail to fix N_2 produce only half as many descendants inside nodules (Kiers et al. 2003). Therefore nonfixing, parasitic strains should be completely displaced by N_2-fixing strains within a few generations (Denison & Kiers 2004a). Yet this has not happened.

The cheating polymorphism may persist for some combination of the following reasons: (*a*) Rhizobia in mixed nodules may escape sanctions; (*b*) conflicting selection regimes may limit adaptation to particular hosts; (*c*) some legumes may impose only weak sanctions, perhaps due to human modification; and (*d*) rhizobia may biochemically manipulate the host.

Mixed Nodules Reduce the Evolutionary Effects of Host Sanctions

If each nodule only contains one strain of rhizobia, then sanctions imposed at the level of individual nodules will reduce the relative fitness of strains that fix less N_2. Cheating rhizobia could find a haven in mixed nodules, however. If a soybean nodule with equal numbers of fixing and nonfixing rhizobia (so 50% of potential N_2 fixation) imposes sanctions that limit rhizobium reproduction to 75% of potential (Kiers et al. 2006), the two strains may not be affected equally. The nonfixing strain could redirect resources from N_2 fixation into current reproduction. Or it could hoard C as polyhydroxybutyrate (PHB), which is more abundant in nonfixing rhizobia (Hahn & Studer 1986). PHB can increase rhizobium survival and even reproduction under C-limited conditions (Ratcliff et al. 2008).

If sanctions cause cheating rhizobia to have lower fitness in single-strain nodules but higher fitness than their co-occupants in mixed nodules, then the balance between fixing and nonfixing strains would depend on the field frequency of mixed nodules. Mixed nodules are common when plants are inoculated with high numbers of rhizobia in the laboratory (Demezas & Bottomley 1986, Gage 2002, Rolfe & Gresshoff 1980). Data under field conditions are limited, but Moawad & Schmidt (1987) found 12–32% of soybean nodules were mixed.

Conflicting Selection Regimes

Some less-mutualistic strains may not be consistent cheaters at all, but represent mismatched host-symbiont partnerships. This could perhaps explain half of the less-mutualistic strains in **Figure 1*b***.

This hypothesis is discussed extensively in the mycorrhizal section below, but potentially applies to the legume-rhizobia symbiosis as well.

Studies using native versus foreign soils as rhizobial inoculum show that selection imposed by one host genotype may favor rhizobia that are less beneficial to another host genotype. In such experiments, host plants tend to grow best when inoculated with their own soils, indicating local coadaptation (Parker et al. 2006). In a study of clover populations in Norway, plants from the north gave higher yields when nodulated by rhizobia from the north than from the south (Svenning et al. 1991). In a similar study, substituting a native for a nonnative rhizobial strain resulted in a 39% increase in seed biomass per plant. In eight populations this effect ranged from –13% to 182% (Parker 1995). Related results were interpreted by Heath & Tiffin (2007) to suggest that plant benefit depended upon the rhizobium strain–plant population combination, implying there is no "universally most beneficial partner."

However, local adaptation is not universal. Howieson (2001) reported that rhizobium strains from different Mediterranean islands often give better host plant growth than rhizobia from the same island. Thrall et al. (2007) found that while one species of *Acacia* grew significantly better with rhizobial populations from its own soils, another species grew equally well with rhizobia from other soils. Even more important, the rank order of effectiveness for rhizobial populations was similar for the two host species, suggesting that ineffective rhizobial populations were consistently poor, regardless of host plant. Although selection imposed by alternate hosts may play a role in maintaining the polymorphism for effectiveness, the consistently poor performance of some rhizobial strains cannot be explained solely by this mechanism.

Strict Sanctions May Not be Universal

We expect sanctions to be widespread, but tests in additional species are needed. Nodule responses to N_2-free atmospheres are apparently similar in other legumes (Minchin et al. 1983), although effects on rhizobium fitness were not measured. The best evidence for host sanctions, based on actual counts of rhizobia/nodule, comes from our experiments with soybean (Kiers et al. 2003, 2006). There is also indirect evidence for sanctions in lupine, based on differences in nodule size (Simms et al. 2006). In addition, Lodwig et al. (2003) showed that bacteroids in pea nodules may depend on the host for amino acids, but starving bacteroids would have no direct evolutionary effect because bacteroids in pea nodules are nonreproductive (Kijne 1975).

Symbiotic N_2 fixation is thought to have evolved some 60 Mya (Doyle 1998). Presumably since that time, natural selection has favored legumes able to defend themselves against cheating rhizobia. However, strong sanctions may not be universal. In particular, with the advent of modern breeding and increased fertilizer use, humans may have inadvertently altered natural legume defenses (Kiers et al. 2002). Using genotypes of soybeans representing 60 years of breeding, we showed that recently released cultivars are more adversely affected by a mixture of fixing and nonfixing rhizobia than are older cultivars. However, it is not yet clear whether this was due to sanctions or partner choice (Kiers et al. 2007). Such examples illustrate how human-mediated change may facilitate the persistence of cheating genotypes.

Other Possible Mechanisms

Can any of these mechanisms explain the successful cheater in **Figure 1*b***? This strain may have benefited from finding a haven in mixed nodules in the past, or its adaptation to a particular host may have been reduced by past selection imposed by other hosts. But its apparently high fitness with single-strain inoculation is not explained by either of these mechanisms. Maybe soybean cultivar

"Lee" imposes only weak sanctions, or maybe this rhizobium strain has some other mechanism to escape sanctions. Some rhizobia are known to increase nodulation success by interfering with plant ethylene signaling (Ma et al. 2002); perhaps some rhizobia also interfere with plant signals that trigger sanctions. More research on these types of mechanisms are needed.

SANCTIONS AND THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS

Cheating in Mycorrhizae

In considering cooperation between arbuscular mycorrhizal fungi (AMF) and plants, definitions of cheating based on fair share (West et al. 2007) are as problematic as defining fair share for plant and bacterial partners in legume-rhizobium cooperation. If soil P fertility is high, perhaps a strain that allocates nothing to its own reproduction and does everything possible to benefit the host would still be a net drain on the host, but can we still call it a cheater? As with rhizobia, the problem is simplified if we think instead of cooperation among mycorrhizal fungal strains in keeping their shared host healthy. Then cheaters can be identified by how their performance compares with other strains. However, the question of net benefits to the host is nonetheless important, as discussed below.

One complication is that, unlike N_2-fixing symbioses, benefits of AMF are diverse (Newsham et al. 1995) and context dependent (Jones & Smith 2004, Li et al. 2008). Benefits can include peripheral functions such as pathogen protection (Herre et al. 2007) and drought avoidance (Ruiz-Lozano 2003). There is no one single attribute (such as N_2 fixation) to compare measurements of host benefit, and it is impossible to employ an experimental design to explore all the diverse conditions under which the relationship is potentially beneficial (Jones & Smith 2004, Fitter 2006).

However, here we assume that the repeated failure of certain AMF strains to provide a measurable benefit (Burleigh et al. 2002; Graham & Abbott 2000; Koch et al. 2006; Smith et al. 2003, 2004; Violi et al. 2007) is not solely a question of context, but could also represent a successful evolutionary strategy (Kiers & van der Heijden 2006). We now know that AMF strains differ dramatically in metabolic strategies such as amount of C extracted from their hosts (Jakobsen et al. 1992), amount of lipids allocated to storage (van Aarle & Olsson 2003), the transfer of P to their host plants (Boddington & Dodd 1999), C transport capacities across symbiotic interfaces (Dickson et al. 1999), and colonization and hyphal-length allocation (Smith et al. 2000, Hart & Reader 2005). Fungal allocation strategies can determine benefits conferred to the host. For instance, extraradical hyphae and arbuscules are positively correlated with benefits provided to host plants (Johnson 1993), whereas vesicles tend to indicate storage benefiting the fungus. Similarly, gaining access to new hosts (and carbohydrate resources) requires increased investment by AMF in large runner hyphae (rather than absorptive hyphae) (Hart & Reader 2005), which essentially drain the host plant while promoting the fungus (Graham & Abbott 2000). We conclude that the effectiveness of AMF is not always context dependent and that symbiotic performance may have a strong genetic basis (see Munkvold et al. 2004).

Do Networks Undermine Sanctions in the Mycorrhizal Symbiosis?

If, as suggested above, mycorrhizal strains vary dramatically in their mutualistic functioning, it is reasonable to suggest that plants employ mechanisms to maximize the most beneficial mycorrhizal interactions. If hosts waste fewer resources on supporting less-mutualistic symbionts, this could create a strong selection pressure against suboptimal mycorrhizal strains (Kiers & van der Heijden 2006). But can sanctions effectively operate in the mycorrhizal symbiosis?

Unlike the legume-rhizobia symbiosis, in the mycorrhizal symbiosis both host and symbiont simultaneously interact with several partners, making it impossible for the host plant to fully enslave the symbiont (Frean & Abraham 2004). Fungal strains can associate with a number of host plants through common mycorrhizal networks (see Selosse et al. 2006 for review). This has consequences for the effectiveness of host-imposed punishment. A fungus denied resources—or even attacked—by one host plant may survive and flourish with resources from another individual plant.

How then would plant-mediated sanctions operate under these conditions? Can sanctions evolve under conditions where the severity of punishment is potentially buffered by interactions with another host? One problem is that punishment is potentially costly to the host. Linking sanctions to a direct fitness benefit for the host is even more problematic for the plant because of these complex mycorrhizal networks. For host-imposed sanctions to evolve, there must be an individual fitness benefit of the sanction to the host (West et al. 2002a). Altruistic punishment, common in humans (Fehr & Rockenbach 2004), is not expected in plants.

Unfortunately, at this stage there are more questions than answers, and new modeling approaches to understanding these questions are emerging (Landis & Fraser 2008). There is strong evidence for favored partnerships between plant and fungal symbionts, but we do not know how beneficial partnerships are promoted or maintained. Coexisting plant species in a certain space harbor distinct AMF communities (Vandenkoornhuyse et al. 2003). In some instances, host-fungal combinations have specific functional advantages, such as protection against herbivory (Bennett & Bever 2007), increased P uptake (Helgason et al. 2002), and uptake of different P sources (Reynolds et al. 2006). However, mycorrhizal communities hosted by plants can also exhibit maladaptive associations, with particular fungal strains proliferating in number, even though these strains may be less beneficial than others to their host plant (Bever 2002).

Potential Sanctioning Mechanisms

Diverse communities of unrelated AMF intermingle on a small spatial scale (Alkan et al. 2006), making mechanisms to select and reward intermixed beneficial fungi difficult to envision from an anatomical and physiological perspective (Smith & Smith 1996). However, despite this biological complexity, there are several approaches host plants may employ to discriminate and punish interspersed fungal partners.

At the cellular level, plants could control fungal cheaters using various compounds (e.g., flavonoids, phytoalexins, H_2O_2) (Vierheilig 2004) to inhibit fungal colonization (Tawaraya et al. 1998). Plants may actively digest hyphae (Imhof 1999) or accumulate compounds that are inhibitory to hyphal growth (Guenoune et al. 2001). However, to determine which strains to inhibit and which to promote the host plant requires some mechanism to evaluate different strains. We have previously suggested that host plants link the release of resources (e.g., carbohydrate supply) to the amount of nutrients (e.g., P, N) transferred from fungal symbionts (Kiers & van der Heijden 2006). Reciprocally, the degree to which fungal strains expend resources for the benefit of the host plant could depend on the resources gained from the host in the interaction. This type of exchange can be best understood in the context of enforced cooperation (West et al. 2007).

Interestingly, experimental evidence for fungi imposing sanctions on plants is strong. Bücking & Shachar-Hill (2005) utilized root organ cultures to show that a host plant's carbohydrate supply can trigger the release of phosphate into the mycorrhizal interface (Bücking & Shachar-Hill 2005, Solaiman & Saito 2001). When there is less carbohydrate supply to the fungus, the cytoplasmic concentration of inorganic phosphate in the hyphae is decreased, more phosphate is converted into polyphosphates (a pool not accessible for the host plant), and the P concentration in the interface is reduced. But by this mechanism, it appears that the fungal partner is punishing stingy host

plants, rather than the host plant punishing the fungal strain. It is currently not known whether plants detect locally enhanced phosphate concentrations in the root and increase C allocation at a spatial scale fine enough to selectively benefit more mutualistic fungi.

Molecular Evidence for Sanctions

Despite the multiple benefits that AMF may provide, at least some symbiotic P transfer may occur in all AMF symbioses (Javot et al. 2007, Smith et al. 2003), so P transfer may be key to sanctions. Recent studies are focusing on mycorrhiza-specific plant P-transporters which are required for P transfer and the symbiosis in general (Javot et al. 2007, Maeda et al. 2006). Experimentally disabling the P-transporter MtPT4 in *Medicago truncatula* led to premature arbuscle death, and a sixfold decrease in extraradical hyphal length (Javot et al. 2007). This suggests that (*a*) active P transport is required to sustain the AMF symbiosis and (*b*) one of the partners may be inducing premature death of the arbuscles before further resources are invested (Javot et al. 2007).

Is death of the arbuscule a fungal response (e.g., because of reduced C transfer) or a sanction imposed by the host plant? The usual defenses of host plants are not typically triggered by AMF (Harrison 2005), but accelerated cell death under host control has been proposed under certain conditions (Genre & Bonfante 2002). In *Lotus japonicus*, disruption of the plant's mycorrhiza-inducible P transporter resulted in fewer arbuscules, more idioblasts (distinct types of cells that can contain phenolic compounds), and more necrotic symbiotic root nodules, compared to controls (Maeda et al. 2006). The researchers suggested that the increase in idioblast cells might reflect a defense response, extending to both AMF and rhizobia, with the symbiosis further suppressed through changes of phytohormone levels (e.g., jasomate). Increased idioblast production, correlated with this reduced P transfer, essentially terminated all symbiotic interactions, including those with mutualistic rhizobia. Now, it is crucial to determine the scale and accuracy of this type of plant response. Knocking out P transport in the fungal partner, rather than the plant, would better determine how host plants respond to nonbeneficial symbionts.

AMF can also affect plant P supply indirectly. Colonization of *Tagetes patula* by *Glomus etunicatum* induced expression and secretion of a plant-derived acid phosphatase, which acted to further liberate P in the rhizosphere (Ezawa et al. 2005). It is not clear how this induced expression could help the fungal symbiont preferentially gain more C from the host. Furthermore, this mechanism would liberate P in the rhizosphere where it would presumably be available to other plants competing with the host plant. Such examples of collective benefit in the rhizosphere are the newest challenges to explain in an evolutionary framework, and the focus of the following section.

RHIZOSPHERE AND ENDOPHYTIC MUTUALISM

Rhizosphere microbes may cooperate less than endophytes. Endophytic microbes inside plant roots and rhizosphere microbes near plant roots can benefit plants in various ways, including through an improved nutrient supply, protection against pathogens or high temperature, and production of phytohormones that may benefit the plant (Barea et al. 2005). The question is whether any of these qualify as cooperation. Maintaining microbial cooperation linked to benefiting host plants requires specific mechanisms, such as sanctions, that are not needed for the microbial activities that benefit plants only as a side effect. Microbial activities will evolve to track plant needs only where such mechanisms are operating.

Cooperation with host plants seems more likely to be evolutionarily stable for endophytes than for microbes in the rhizosphere. Restricted access to the root interior may limit the number of clones of a given endophyte species per plant, leading to a higher value for Hamilton's r than

in the rhizosphere. Whether Hamilton's r for endophytes is high enough (especially, perhaps, in seedlings) to maintain cooperation simply through the shared benefits of a healthier host (i.e., without sanctions) remains to be seen. Hosts may also affect the fitness of microbes within their root tissues to a greater degree than those on the root surface, so sanctions are more plausible within root tissue, mechanistically. We know of no actual evidence for sanctions on endophytic microbes, but if there is substantial population structure among endophytes within roots, something as simple as differential C allocation to (or even differential survival of) different root segments might work.

Each subsection below discusses a specific mechanism by which rhizosphere or endophytic microbes might benefit plants, beginning with a brief assessment of its likely importance in the field. Our main concern is whether beneficial activities can be explained by direct benefits to the microbes themselves, or whether cooperation is operating such that selection depends on benefiting plants.

Nutritional Mutualisms

Some bacteria fix N_2 in the soil or in association with plant roots. Bacteria may also solubilize phosphate or iron, making them more available to plants. Natural selection may favor N_2 fixation and phosphate solubilization to meet the N and P needs of rhizosphere microbes themselves, but natural selection will not favor fixing N_2 beyond the needs of the microbes, nor will it favor mechanisms to transfer these resources to plants, if there is no associated benefit for the particular microbes responsible. For root endophytes, undiscovered sanction-like mechanisms could perhaps target benefits to microbes that fix more N_2 and share it with plants.

A recent field study (Sahin et al. 2004) inoculated barley plants with three *Bacillus* strains; one was shown to solubilize phosphate and the others apparently were N_2 fixers. Together, these three strains increased barley yield from 2.70 (unfertilized control) to 2.95 t ha^{-1}. This difference was significant, but small relative to benefits from N and P fertilizer, which increased yield to 3.38 t ha^{-1}. It is not certain that even this small yield increase was due to phosphate solubilization or N_2 fixation, however, as inoculum strains were "selected for their antifungal and antibacterial properties in the previous studies" (Sahin et al. 2004). Endophytic N_2 fixers associated with sugar cane roots have been credited with significant N contributions, ranging from 60 kgN ha^{-1} $year^{-1}$ in field studies (Boddey et al. 1995) to 150 kgN ha^{-1} $year^{-1}$ in container studies. There are various concerns with container studies (e.g., edge effects), including some noted by the original researchers (Urquiaga et al. 1992); more field data are needed.

These N contributions may exceed what we would expect from direct selection for meeting the N needs of microbes themselves. If we hypothesize that N_2 fixation by endophytic bacteria is a genuine example of cooperation, with selection dependent on benefiting plants, then research on the mechanisms that maintain this cooperative relationship would be worthwhile. Do we find spatial structuring of N_2-fixing bacterial populations inside roots and spatial patterns of plant resource allocation to root segments correlated with N_2 fixation, such that genotypes that fix more N_2 are preferentially supported? If so, then endophytic N_2 fixers may be subject to a cruder version of the sanctions that maintain N_2 fixation in rhizobia.

An interesting study of N_2 fixation in *Gluconacetobacter diazotrophicus* (Sevilla et al. 2001) seems more consistent with direct microbe benefit for N_2 fixation than with sanctions. A nonfixing mutant had only slightly lower fitness (68% as many cells per gram of plant) than the N_2-fixing wildtype, even with single-strain inoculation (Hamilton's $r = 1$). Furthermore, Sevilla et al. (2001) calculated that the amount of N fixed may have been similar to the amount of N in the bacterial cells themselves. Individual selection could favor some N_2 fixation, but only something equivalent to sanctions would select for sharing fixed N with the plant.

Antimicrobial Mutualisms

Many rhizosphere species protect plants from root pathogens (Cook et al. 1995). We have suggested previously (Denison et al. 2003a) that plant protection could be a by-product of individual selection for production of antibiotics that microbial cells use against competitors or predators. The healthiest plants may not release the most exudates into the rhizosphere (Curl & Truelove 1985), so antibiotic-producing pseudomonads may be more abundant near root lesions (Mazzola & Cook 1991). Furthermore, any benefits associated with protecting plants would be shared with microbes that did not contribute to that protection. Data showing a role for phenazine antibiotics in microbial competitiveness (Mazzola et al. 1992) are consistent with our hypothesis that antibiotic production does not depend on whether plants benefit. This conclusion is emphasized by more recent results, including bacteriocin-mediated antagonism among *G. diazotrophicus* strains (Munoz-Rojas et al. 2005) and a role for antibiotics in defense against predation by protozoa and competition with other bacteria (Jousset et al. 2006, 2008).

Protection against High Soil Temperature

A fungal endophyte has been shown to enhance plant tolerance to high soil temperature (Redman et al. 2002), apparently using information coded by a virus (Marquez et al. 2007). The physiological mechanism is unknown, but what evolutionary mechanisms could maintain the beneficial fungal/viral activity? Three hypotheses seem worth pursuing, two of which would qualify as fungus/virus cooperation with the plant: (*a*) If individual plants are typically infected by a single fungus/virus genotype, then strains that keep their host alive will have higher fitness. (*b*) Sophisticated plant sanctions based on this trait seem unlikely, but greater survival of root segments occupied mainly by more-beneficial strains might work. (*c*) Individual or kin selection, not dependent on plant benefit, could be involved. For example, if fungal cells excrete some chemical that protects their own membranes or that of nearby kin from high temperature, that same chemical might protect the plant, as a side-effect.

Information as a Commodity: Honest Signaling or Manipulation?

Many microbes produce molecules that influence plant growth. Influencing plants is not the same as benefiting plants, however. For microbial signals to consistently benefit plants, three conditions would have to be met: (*a*) the microbes need information not otherwise available to the plant, (*b*) selection among microbes must favor honest signaling, rather than manipulation of plants, and (*c*) the plant must integrate microbial signals with other information in a way that increases its fitness. For example, soil microbes near a root might detect a nearby N source and signal the presence of that resource, stimulating root growth in their direction. But if microbes benefit from root growth, selection would favor the production of false signals. Even if the microbial signal was "correct," plants that respond might neglect other, more urgent needs. We therefore consider it unlikely that microbial signaling benefits plants consistently. The microbial mimicking of plant hormones is more likely manipulation than cooperation. There is no signal-based "love parade beneath our feet" (Somers et al. 2004).

What evidence is needed to disprove (Kinraide & Denison 2003) our hypothesis that the evolution of microbial phytohormone production is not dependent on benefits to plants? (*a*) Effects on plant growth would need to be directly linked to microbial signals, rather than other microbial activities discussed above; (*b*) field data, not pot studies, are needed to see effects on plant allocation in its evolutionary context; (*c*) data would need to show increases in plant reproduction (fitness), not just root growth or total biomass. An increase in growth, at the expense of reproduction, is a fairly common result of manipulation by parasites (Yu & Pierce 1998).

These criteria are met by few, if any, published studies. A comparison between the auxin-producing wildtype and a nonauxin mutant of *Azospirillum brasilense* provided convincing evidence that auxin production by this strain can increase root hair production in wheat, at the expense of decreased root length (Dobbelaere et al. 1999), although it does not exclude the possibility that any benefits observed in the field could involve other mechanisms, such as N_2 fixation (Vanderleyden & Steenhoudt 2006). Increased root hair production could benefit bacteria nearby, but is trading root hairs for root length beneficial to the plant? In field experiments with wheat in Belgium, inoculation with *Azospirillum* increased shoots per plant and root weight approximately 30% each (Dobbelaere et al. 2001). This result represents a reverse of trends in wheat breeding responsible for much of the increase in yield potential over decades, namely fewer shoots per plant (but more plants per square meter) and more allocation to grain at the expense of leaves, stem, and root (Denison et al. 2003b). These changes in shoots per plant and root allocation "did not result in higher yields," a result attributed to wet weather. Results of farmer trials in Mexico were reportedly more promising. In Israel, inoculation increased early growth of five wheat cultivars, but final yields were lower in two cultivars and higher in only one (Kapulnik et al. 1987). So benefits from *Azospirillum* are inconsistent and perhaps context-dependent, as we would expect if the bacteria were manipulating plants for their own benefit.

Rhizosphere and Endophyte Mutualism: Expanding Sanctions?

Although some microbes associated with plant roots are pathogenic, many others are beneficial. This said, we see no clear evidence of cooperation, (e.g., selection linked to actual plant benefits). Cooperation between endophytic microbes and plants is plausible, if microbial populations in roots are sufficiently structured and if plant benefits to microbes selectively favor those that are more beneficial. Any mechanism that selectively favors some fraction of the microbes associated with a plant's roots, based on the benefits they provide the plant, would be functionally equivalent to sanctions, from the viewpoint of the microbes. One could argue that the term sanctions has additional implications, such as some minimum level of physiological complexity or evidence that the plant response is itself maintained by natural selection. A delay in this discussion is needed until additional candidate sanctions have been discovered.

FUTURE DIRECTIONS

Given multiple microbial genotypes per individual plant, sanctions seem essential to maintain microbial cooperation. But do plants actually have mechanisms that can selectively benefit more-mutualistic mycorrhizal fungi or endophytic bacteria, given realistic levels of spatial population structure for microbes within a plant? Do seedlings, perhaps, have few enough microbial genotypes per individual plant that microbial fitness is more strongly linked to plant health? Laboratory and field data are needed.

Models that assume one symbiont genotype per individual plant are highly misleading; more realistic models could be useful. For rhizobia, incorporating the effects of mixed nodules would be useful, as would greater attention to plasticity. If rhizobia can detect a second strain in a nodule, what should they do? For mycorrhizae, does it matter that a fungus is connected to several different hosts simultaneously, rather than in successive generations? Given rapid microbial evolution, what hypotheses related to signaling are plausible enough to merit experimental tests? The most promising approach to understanding cooperation in the rhizosphere will involve physiological studies designed with insights from evolutionary theory.

SUMMARY POINTS

1. In order for a microbial allele that benefits plants to persist, microbes with the allele must benefit relative to potential competitors on the same host that have alternative alleles.
2. With several competing microbe genotypes per individual plant host, collective benefits from associating with a healthier host cannot explain the evolutionary persistence of cooperation.
3. Signals can be counterfeited and therefore are not reliable for assessing the true nature of partner quality.
4. The problem of hosting multiple partners can be solved if host plants are able to discriminate among symbionts, preferentially supporting cooperators or punishing cheaters.
5. Depending on the system, sanctions can take different forms. A rhizobium cell is sanctioned inside a single nodule, whereas any sanctions against a mycorrhizal fungus (connected to several plants) may be buffered by interactions with other plant partners. Sanctions are difficult to impose in loosely associated rhizosphere mutualisms.
6. For free-living rhizosphere microbes, individual selection could favor benefits (nutritional, protection, etc.) for the microbe itself, but only something equivalent to sanctions would select for sharing these benefits with the plant host.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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