

COOPERATION IN THE RHIZOSPHERE AND THE “FREE RIDER” PROBLEM

R. FORD DENISON,^{1,7} CAROLINE BLEDSOE,² MICHAEL KAHN,³ FERGAL O’GARA,⁴
ELLEN L. SIMMS,⁵ AND LINDA S. THOMASHOW⁶

¹Agronomy and Range Science, University of California, Davis, California 95616 USA

²Land, Air and Water Resources, University of California, Davis, California 95616 USA

³Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164 USA

⁴Microbiology Department, National University of Ireland, Cork, Ireland

⁵Department of Integrative Biology, University of California, Berkeley, California 94720 USA

⁶USDA-ARS and Department of Plant Pathology, Washington State University, Pullman, Washington 99164 USA

Abstract. Rhizobial bacteria, endomycorrhizal fungi (also known as arbuscular mycorrhizas), and pseudomonad bacteria associated with plant roots can provide substantial benefits to the plants by fixing nitrogen, supplying phosphorus, or controlling root pathogens, respectively. A significant fraction of plant photosynthetic carbon may be used by these associated microorganisms, both to support their beneficial activities and for microbial growth and reproduction. Because many microbial individuals are associated with each individual plant, the individual benefit to a microbe that allocates more resources to its own reproduction (thereby allocating less to fixing N₂, supplying P, or producing antifungal metabolites) would exceed its individual loss from any resulting reduction in collective benefits (mainly plant carbon substrates). An initially rare “free rider” mutant strain might therefore be expected to displace its more cooperative parental strain. Yet, the mycorrhizal and legume–rhizobium mutualisms have persisted (often coexisting with “cheating”) for millions of years. This paper discusses the importance of microbial cooperation (with plants and with other microbes) and possible reasons for its evolutionary persistence in the rhizosphere. In undisturbed soils, spatial structure can favor kin selection, but this may be counterbalanced by the increased likelihood that future competitors will be among the beneficiaries of current cooperation. In loose associations, direct fitness benefits to microorganisms may explain the evolutionary persistence of activities (e.g., production of antifungal compounds) that can benefit plants as a side effect. In closer, more symbiotic, relationships, host sanctions against individuals or clones that fail to perform their symbiotic function may be more important. New molecular methods and other research tools are facilitating research on this topic, and some of these conclusions soon may be revised.

Key words: cheating; kin selection; mutualism; mycorrhizae; pseudomonads; rhizobia; roots; Tragedy of the Commons.

What natural selection cannot do, is to modify the structure of one species, without giving it any advantage, for the good of another species; and though statements to this effect may be found in works of natural history, I cannot find one case which will bear investigation.

—Darwin (1859)

THE PROBLEM

Mutually beneficial interactions between rhizosphere microorganisms and associated plants are ubiquitous and often important to ecosystem function. Symbiotic nitrogen fixation by bacteria in the Rhizobiaceae (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizo-*

bium, or *Azorhizobium* spp.), collectively termed rhizobia, or by *Frankia* spp., in the root nodules of legumes or actinorhizal plants, respectively, is the major N input to many natural and agricultural ecosystems. Mycorrhizal fungi often supply their host plants with mineral nutrients such as P as well as provide other benefits. Some rhizosphere microorganisms are serious root pathogens, but colonization of roots by others, such as *Pseudomonas fluorescens*, can improve plant health by suppressing the pathogens.

Cooperation between microbes and plants is easier to understand, in the context of natural selection, than cooperation among unrelated individuals of the same species. Conspecifics have similar resource requirements and therefore are likely to compete for those resources. Plants compete for light and water with other plants, not with microorganisms. Natural selection may even favor a “beggar my neighbor” strategy (Harper 1977), whereby a plant benefits indirectly by harming neighboring plants (e.g., through preemptive consumption of soil water). In contrast, plants and micro-

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⁷ E-mail: rfdenison@ucdavis.edu

organisms differ in their resource needs and metabolic capabilities. This can favor cooperation between species that have evolved different specialization in resource capture (Schwartz and Hoeksema 1998).

Cooperation between microorganisms and plants may, however, be threatened by intraspecific competition among the microorganisms associated with an individual plant. Rhizobia, for example, compete with other rhizobia infecting the same plant, in the sense that no two rhizobial cells can consume (or hoard) the same plant-supplied organic molecule. Because the activities of a single bacterial cell (rhizobium or pseudomonad) or individual mycorrhizal fungal mycelium can have only a small effect on the health of the plant that benefits them all (mostly by supplying C), competition among microorganisms associated with the same individual plant creates the potential for a Tragedy of the Commons (Hardin 1968).

A number of simple mutations could convert a microorganism genetically disposed to mutualism into a "free rider." Free riders are strains that share in collective benefits, but avoid some individual costs by shirking activities that benefit plants and/or conspecifics. When a free rider strain arises by mutation, will it increase in frequency, will it persist at low frequency, or will it tend to disappear? If fixing N_2 , supplying P, or killing phytopathogenic fungi benefits free riders and "cooperators" equally, then free riders that avoid significant individual costs should quickly displace cooperators.

Some free riders may be almost identical, genetically, to the parental strain. For example, a transposon-induced mutant of *Bradyrhizobium japonicum* retained the ability to nodulate soybean (*Glycine max*), but lost the ability to fix N_2 . When this free-rider strain shared a nodule with its wild-type parent, electron microscopy showed that the free rider accumulated much greater quantities of energy-rich polyhydroxybutyrate (PHB) than the cooperative strain (Hahn and Studer 1986). This presumably reflects trade-offs between the carbon costs of PHB accumulation and of N_2 fixation. Although it apparently has not been demonstrated that hoarding PHB enhances rhizobial survival and reproduction after release back into the soil, PHB content has a dramatic effect on the survival of starved *Azospirillum brasilense* (Tal and Okon 1985). Why, in fact, do rhizobia fix any nitrogen, if costs are borne by an individual strain and benefits are shared with other strains infecting the same host? The spread of microbial freeloaders could (by increasing the cost of symbiosis, relative to costs of direct uptake of N or P from soil) select for plants that exclude microbial symbionts, and could conceivably lead to the local extinction of host plants, but natural selection is blind to future consequences. So, how have these mutualistic symbioses lasted for millions of years?

Current evolutionary theory suggests that cooperation is more likely to persist (i.e., to be evolutionarily

stable) if microbial symbionts are transmitted mainly from parents to offspring, if each host contains only a single genotype of symbiont, and/or if the parties to the mutualism have few other options (Herre et al. 1999). Many important mutualisms do not meet these criteria, however (Wilkinson 2001). Rhizobia, mycorrhizal fungi, and pseudomonads are all transmitted among hosts "horizontally," via the soil, rather than "vertically," via seeds. Similarly, each plant is typically infected by, or associated with, a genetically diverse population of microorganisms. And while many endomycorrhizal fungi are apparently obligate symbionts, that is not the case for all mycorrhizal fungi, or for rhizobia or pseudomonads.

POSSIBLE SOLUTIONS

Spatial structure and kin selection

Herre et al. (1999) also suggested that mutualism is favored by "spatial structure of populations leading to repeated interactions among would-be mutualists," as previously suggested by Hamilton (1964) and others. If so, then soil mixing due to earthworms and other mesofauna or cultivation might threaten the persistence of mutualism. In cultivated soils, the probability that two adjacent nodules on the same plant contain the same bacterial strain may be only slightly greater than random chance (Young et al. 1987), but rhizobial populations of some wild legumes may show greater spatial structure (Souza et al. 1994).

In the absence of disturbance, most microbes of a given species within a given, sufficiently small, volume of rhizosphere soil may be descended from a single individual. If the benefits that result from healthier plants are shared mostly among closely related microbial strains, then kin selection could perhaps favor microbial investment in N_2 fixation (Simms and Bever 1998), P uptake, or plant defense.

On the other hand, greater population viscosity (as might be expected with limited soil mixing) tends also to increase competition among relatives in a way that can decrease the payoff to mutualism (Frank 1998, West et al. 2001, Griffin and West 2002). Empirical data from aboveground mutualistic interactions, consistent with this hypothesis, include the absence of negative correlation between relatedness (among male wasps within a single fig) and intensity of conflict, a result aptly summarized as "vicious fig wasps in viscous populations" (Hardy and Cook 2001).

Consider the analogous belowground situation of a legume infected by two strains of rhizobia. When one strain fixes N_2 and this allows more photosynthesis by the plant, both strains might be rewarded with more resources in the near term. How might indirectly providing more resources to the second strain affect the fitness of the first? If the soil is thoroughly mixed, then the two strains are unlikely to encounter each other again after being released into the soil. In this case,

the fitness effects of allocating bacterial resources to N_2 fixation versus resource storage depend only on short-term costs and benefits, and any benefit to a second, unrelated strain is irrelevant to the fitness of the first. In unmixed soil, however, a rhizobial strain that indirectly provides more resources to another strain aids a future competitor. The situation becomes analogous to that of plants competing for soil resources; a plant that uses more water than it needs may suppress growth of a neighbor that would soon compete with it for N. With many simple dispersal patterns, the decrease in kin selection with increased mixing is exactly balanced by the effects of decreased competition (Taylor 1992). Thus, it is not axiomatic that less mixed soils necessarily favor the evolution of mutualism.

An improved theoretical understanding of the evolution of altruism in viscous populations (Queller 1992, Wilson et al. 2000) highlights the importance of the relative timing of cooperation, dispersal, and competition. If microorganisms that cooperate locally are subsequently more competitive in global competition (due to benefits obtained during their cooperative stage), then this benefit may explain the persistence of cooperation. A full understanding of the effects of spatial structure on mutualism may require analysis at multiple spatial and temporal scales. Trade-offs between microbial fitness in association with plants vs. in life away from plants could also be important.

Horizontal gene transfer is not enough

Horizontal transfer of genes, as opposed to vertical transfer to daughter cells, can have important effects on bacterial genotypes and phenotypes. Uptake of DNA as a nutrient source (Redfield 1993), in combination with DNA repair mechanisms, can sometimes result in incorporation of genes into the bacterial chromosomes. Active transfer of genes among bacteria also occurs, e.g., via conjugation.

Nonsymbiotic rhizobia can acquire the capacity to infect legumes when they receive symbiotic genes from other, sometimes unrelated, strains (Sullivan et al. 1995). This mechanism would not necessarily favor mutualism over parasitism, however. Indeed, horizontal gene transfer has been proposed as a mechanism to maintain virulence in pathogenic bacteria (Smith 2001), when there is an individual cost to producing toxins that provide a collective benefit to the pathogens by increasing access to host resources. It has further been suggested that the rate of horizontal transfer of genes among rhizobia is lower in natural than in agricultural ecosystems (Wernegreen et al. 1997). The apparently higher frequency of horizontal transfer in some settings (e.g., antibiotic resistance in hospitals, or the capacity to nodulate a newly introduced legume host) may be the result of more intense selection (relative to many natural environments) rather than higher rates of horizontal transfer.

Are there other mechanisms that can explain the evolutionary persistence of these mutualisms? We will discuss rhizobia, mycorrhizal fungi, and plant-protecting pseudomonads in turn. These represent a continuum from the most intimate symbiosis (rhizobia typically fix N_2 only inside host cells) through mycorrhizae, which extend from host cells out into the soil, to loose associations between plants and pseudomonads, which may never enter the cells of a healthy host.

Rhizobia

For close-knit symbioses, such as those involving rhizobia or mycorrhizal fungi, plant sanctions or ongoing partner choice may be important (Denison 2000, Simms and Taylor 2002). Strain–host interactions apparently have large effects on symbiotic effectiveness in some (Robinson et al. 2000) but not all (Burdon et al. 1999) cases. It is not known why the relative effectiveness of rhizobia/host combinations varies but the possibility of a large bacteria \times host genetic component complicates the selective landscape considerably in any natural situation where both symbiont populations are heterogeneous.

We use the term “ongoing partner choice” to distinguish it from the partner choice model of Bull and Rice (1991) which “enables one member to differentially reward cooperative vs. uncooperative partners in advance of any possible exploitation.” Legumes must invest resources to support nodule growth and rhizobial reproduction inside the nodule for many days before even the most mutualistic rhizobia fix any N_2 . To varying extents, legumes admit some strains and exclude others. However, because N_2 fixation per se requires the nodule environment, plants are unable reliably to distinguish, at the time of infection, between strains that will or will not eventually fix N_2 (Amarger 1981, Hahn and Studer 1986).

If, however, plants infected by more than one strain of symbiont have a mechanism to monitor each strain’s actual symbiotic performance, they might provide more resources to those that prove to be better mutualists, or sanction strains that prove less mutualistic. This reward or punishment could counter any fitness advantage that “defectors” might obtain (e.g., from hoarding PHB at the expense of N_2 fixation). Plant sanctions or ongoing partner choice could involve decreased resource supply or direct attack, either on less mutualistic nodules or on less mutualistic bacteroids within a nodule (Udvardi and Kahn 1993). Externally, the effects of postinfection sanctions or ongoing partner choice might or might not be apparent, e.g., as premature nodule senescence.

To explain the evolutionary persistence of rhizobial mutualism, sanctions would have to decrease the fitness of less mutualistic rhizobia relative to the better mutualists. In general, rhizobia in legumes whose nodules have determinate growth retain the ability to reproduce after differentiating into nitrogen-fixing bacteroids, so

sanctions against nonfixing bacteroids could explain the evolutionary persistence of symbiotic cooperation in these species. In many legume species with indeterminate nodule growth, bacteroids are terminally differentiated, but these nodules also contain undifferentiated rhizobia that accumulate PHB and are thought to escape into the soil after nodule senescence (Paau et al. 1980). In legume species with indeterminate nodules, sanctions against nonreproductive bacteroids alone would have no evolutionary effect, but whole-nodule sanctions that also affect reproductively viable rhizobia could select for greater mutualism in subsequent rhizobial generations (Denison 2000).

Whereas microbial evolution could rapidly overcome host selectivity based on arbitrary recognition signals, postinfection sanctions based on continuous monitoring of actual performance would drive microbial evolution toward greater mutualism. This would be true whether or not sanctions against a particular nodule depend on the relative performance of other nodules on the same plant (West et al. 2002). Natural selection among plants would only favor such sanctions if individual plants thereby increase benefits (or reduce losses) associated with microbial symbionts.

How severe would host sanctions need to be to counter the rhizobial temptation to defect? Fixing N_2 is energetically expensive, leading to a significant short-term trade-off between N_2 fixation and resource accumulation by rhizobia in a given nodule. A rhizobial mutant unable to accumulate PHB had up to twice the nitrogenase activity and resulted in 50% higher plant dry mass, relative to the wild type (Cevallos et al. 1996), showing that bacteroid behavior can make a substantial difference to host productivity. Rhizobial PHB accumulation is not the same as rhizobial fitness, however. If even good N_2 -fixing bacteroids eventually accumulate PHB as 50% of their dry mass (Wong and Evans 1971), then faster PHB accumulation may enhance rhizobial fitness only when there are risks (e.g., premature nodule senescence) or lost opportunities (e.g., related to the timing of the availability of new hosts) associated with slower PHB accumulation.

The potential fitness benefits of founding a nodule are enormous. One soybean nodule may contain up to 1×10^{10} bacteroids (Bergersen 1982), most of which are reproductively viable (Gresshoff and Rolfe 1978). Some of these bacteroids may be digested by the host, however, before they can escape into the soil (Roth et al. 1987). Rhizobia that lack symbiotic genes altogether presumably never infect plants, but their numbers suggest that they are successful members of soil microbial communities (Segovia et al. 1991, Laguette et al. 1993, Sullivan et al. 1996).

Mycorrhizas

The potential risks and benefits of “cheating” (providing little or no P or other resources to their host plants) may be very different for mycorrhizal fungi

than for rhizobia. Rhizobia can apparently live saprophytically in rhizosphere or soil, whereas growth of many mycorrhizal fungi is entirely dependent on host plants. However, each individual fungus may be attached to several plants, often of more than one species (Simard et al. 1997), so the death of a single host plant will not necessarily kill the fungus.

It is unclear whether the marginal costs of symbiotic cooperation are high enough to give successful mycorrhizal cheaters a major fitness advantage over cooperators. Once a mycorrhizal fungal mycelium has proliferated in the soil, and assuming that it must take up some P for its own use, the additional C cost associated with supplying P to a given host may be small. Nevertheless, even a small individual cost would select against cooperation with the host, if cheaters obtain exactly the same benefits as cooperators sharing the same host.

As in the case of rhizobia, the individual costs or risks associated with cheating might include host responses such as sanctions or rewards. That is, mycorrhizal fungi that provide more P (or benefit the plant in other ways that the plant can detect) might receive more benefits from the host than would less cooperative strains infecting the same host. If these individual benefits were large relative to the individual cost of cooperation, that could explain the evolutionary persistence of mycorrhizal mutualism.

The relevant natural history of this relationship is largely unknown and many of the experiments needed to test these hypotheses will be more difficult with mycorrhizal fungi than with rhizobia. Do hosts enhance mycorrhizal fitness in ways other than by providing photosynthate? Given two mycorrhizal strains with different genetically programmed strategies, how would we measure their relative fitness in the field? Mycorrhizal fungi may increase host fitness in a variety of ways, making it difficult for humans (and perhaps for plant hosts) to calculate overall costs and benefits, even apart from attributing them to a particular strain. In contrast to rhizobia, most of the cells of a mycorrhizal fungus in symbiosis with a plant are in the soil, not inside the host. Greater fungal growth in soil and greater levels of mycorrhizal infection do not always increase P supply to the plant (Sanders and Fitter 1992, Thomson et al. 1994). External variables such as P availability in soil may influence mycorrhizal function by either ecological or evolutionary mechanisms (Johnson 1993).

It is unclear whether host sanctions are even physiologically possible for plants hosting mycorrhizal fungi. Plants may allocate more C to mycorrhizal than to nonmycorrhizal roots (Cairney et al. 1989), but a root segment only 100 μm long often may be infected by two or more different strains (or even species or genera) of a mycorrhizal fungus (Fleming 1985). If a reciprocal exchange of P for C occurs only through each mycorrhizal arbuscule, plants might monitor arbuscule per-

formance (perhaps via sensors or transport systems in the plant membrane that surrounds each arbuscule) and discontinue trade with those that fail to measure up. There is some evidence, however, that mycorrhizal uptake of plant C may also occur through hyphal coils that are spatially separated from the arbuscules (Smith and Smith 1996). If so, then hosts may be unable to distinguish the costs and benefits of strains infecting the same segment of root.

Pseudomonads

Whatever the individual fitness costs and benefits of cooperation with a multiply infected plant, it is clear that collective benefits to rhizobia and mycorrhizal fungi are often tied to host plant health. For pseudomonads, in contrast, the healthiest plants may not provide the greatest collective benefits. Root exudation from dead plants is presumably negligible, but lesions caused by moderate levels of infection by fungal pathogens can increase nutrient availability due to leakage from or lysis of damaged root tissues (Curl and Truelove 1985), and the presence of lesions caused by some pathogens is known to be correlated with increased rhizosphere population sizes of *Pseudomonas* spp. (Mazzola and Cook 1991). Thus, even if the rhizosphere were colonized by a single clone of identical pseudomonads, fitness benefits to the pseudomonads from production of antifungal metabolites might be highest at some intermediate level of plant health. Explanations based on improving plant health are even more problematic, if each individual cell in a genetically diverse microbial community can share in any benefits (e.g., exudates) generated by healthy plants, whether or not that individual cell contributed to plant health through production of antifungal metabolites.

Nevertheless, benefits to plants from pseudomonads producing antifungal metabolites are common (Cook et al. 1995). "Take-all decline" provides one of the most dramatic examples (Rovira et al. 1990). *Gaeumannomyces graminis*, which infects the roots of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), tends to increase when these crops are grown repeatedly (i.e., without rotation to other crops), causing the aptly named "take-all" disease. But continued production of these crops often leads to "take-all decline" due to the development of "suppressive soils" that inhibit growth of *G. graminis* in the rhizosphere. *Trichoderma koningii* and various *Pseudomonas* species have been implicated in take-all decline (Rovira et al. 1990), and phloroglucinols produced by pseudomonads account for the observed suppression of *G. graminis* in some soils (Raaijmakers and Weller 1998, Weller et al. 2002).

There is presumably a metabolic cost to individual *Pseudomonas* cells in producing these antifungal metabolites. Comparisons of growth rates of producing and nonproducing strains, in the absence of plant roots and under conditions that minimize interspecific and

intraspecific competition, could provide some indication of metabolic costs. The evolutionary persistence of antifungal production indicates that inclusive fitness benefits to pseudomonads may outweigh the cost under usual field conditions. It is conceivable that plants could manipulate conditions in the rhizosphere in ways that stimulate production of antifungal compounds by at least some pseudomonad strains. However, if responding to such manipulation had negative effects on pseudomonad growth and reproduction, there would be selection among pseudomonads, whose generation time is shorter than that of plants, for resistance to such manipulation.

Plant effects on the rhizosphere must also influence the course of evolution in microbial populations, but it is unclear whether plants can selectively favor strains most beneficial to themselves. When a single chromosome from a wheat cultivar resistant to root rot was substituted for its homologue in a disease-susceptible cultivar, the resulting cultivar was resistant to root rot and also had a higher percentage of rhizosphere bacteria that produced antifungal metabolites inhibitory to the root-rot fungus, *Cochliobolus sativus*, in vitro (Atkinson et al. 1975). The authors concluded, however, that this association was "entirely coincidental," because treatment of the susceptible line with rhizosphere bacteria from the resistant line did not prevent disease, and because the association between the composition of rhizosphere populations and plant disease resistance was not seen in a second genetic background. One consistent pattern in their data was a positive correlation between disease susceptibility and total numbers of rhizosphere bacteria, especially of bacteria able to break down cellulose and pectin. Whether specific plant genotypes preferentially stimulate rhizosphere populations of bacteria antagonistic to pathogens remains to be determined, but nonselective stimulation of bacterial growth in the rhizosphere may increase, rather than decrease, disease susceptibility. If root exudates attract pathogens, then nonpathogenic bacteria might benefit the plant simply by consuming exudates, thereby preventing them from acting as a beacon.

Even if plants are unable to manipulate pseudomonad numbers or behavior in ways that reliably prevent disease, what about manipulation of pseudomonads by other pseudomonads? Quorum sensing, an interchange of chemical signals that provides microorganisms with information on their local population density, influences production of antimicrobial metabolites, such as phenazines, by some pseudomonads (Bassler 1999). If the benefits of phenazine production depend on simultaneous action by many individual cells, then pseudomonads could benefit, collectively, by producing quorum-sensing signals and by producing phenazines if and only if concentrations of the signal indicate high population density. This would avoid the collective cost of wasteful production of phenazines when the population density is too low to have much effect.

Trade-offs between individual and collective benefits are at the heart of the free rider problem, however, and quorum sensing doesn't automatically solve this problem. Whether production or response to a signal with an individual cost and a collective benefit will be favored by natural selection depends on the genetic relatedness among the communicants (Brookfield 1998) and on the intensity of competition among relatives compared to nonrelatives. There are enough examples of "cheating" in bacteria (Velicer et al. 2000) and even viruses (Turner and Chao 1999) that mindless obedience to such chemical signals cannot be assumed. Mindlessness can be assumed, but not obedience.

We also must consider the role of signals in "eavesdropping" and "deceit" (Stowe et al. 1995). The plant pathogen, *Pythium ultimum*, produces metabolites that downregulate genes that are important for ecological performance of introduced *Pseudomonas* species (Smith et al. 1999). Does production of these chemical signals have a metabolic cost to an individual *Pythium*, while providing them with a collective benefit? If response of pseudomonads to a signal reduces their fitness, *Pseudomonas* strains that ignore these signals should displace those susceptible to them.

At present, we are unable to reject the simplest explanation for production of antifungal metabolites by some *Pseudomonas* strains, namely that single cells incur some individual benefit unrelated to any collective effects, including those collective benefits linked to the health of associated plants. The most likely such benefit is suppression of nearby fungal or bacterial competitors. For strains such as *Pseudomonas fluorescens* 2-79 and *P. aureofaciens* 30-84, the production of phenazine antibiotics contributes to competitiveness on the roots of wheat (Mazzola et al. 1992). In contrast, for *P. fluorescens* F113, the production of the antibiotic 2,4-diacetylphloroglucinol does not appear to be a factor in the competitiveness of the strain on the roots of sugarbeet (Carroll et al. 1995). Metabolites that directly suppress plant-pathogenic fungi could also have some other function, such as serving as a siderophore for the uptake of iron. Siderophores could also suppress pathogenic fungi indirectly, by lowering rhizosphere concentrations of iron below those needed by the fungi (Elad and Baker 1985). In this case, the suppression of plant pathogens could be merely a side effect of competition among bacteria for a limiting resource.

NEW RESEARCH TOOLS

We cannot presently explain the evolutionary persistence of microbial cooperation in the rhizosphere with confidence, even in a single particular case. However, recent advances in both methodology and theory offer the prospect of rapid progress over the next decade.

These tools include isogenic mutants of rhizobia whose altered symbiotic function is linked to alteration of a single gene (Tirichine et al. 2000). Fine-structure

genetic analyses (Raaijmakers and Weller 2001) and genetic modification (Carroll et al. 1995) of *Pseudomonas fluorescens* also are providing insights into its ecological role.

Model systems are also important tools. The widely used model plant *Arabidopsis thaliana* lacks symbiotic interactions such as mycorrhizae and rhizobia. However, model legumes like *Medicago truncatula* (Cook 1999) and *Lotus japonicus* (Handberg and Stougaard 1992) undoubtedly will prove useful.

Recently developed molecular methods include use of the polymerase chain reaction (PCR) to identify rhizobial strains present in soil or in nodules, without the need for DNA purification or culturing (Harrison et al. 1992). Reporter genes (e.g., those using variants of the green fluorescent protein GFP) make it possible to use bacteria as biosensors for root exudates (Bringhurst et al. 2001). Various genomics tools may also prove useful, especially now that the genomes of two rhizobium species have been completely sequenced and sequencing of model legumes is underway (Colebatch et al. 2002).

Molecular biology does not have a monopoly on powerful new tools and methods. Differences in isotopic composition of C from C₃ vs. C₄ plants have been used to identify the source of C for individual mycorrhizal spores (Nakano et al. 1999). Substitution of argon for N₂ can allow experimental control of the timing of rhizobial defection, although conditional mutants might be more useful if mixed nodules are common. Despite its drawbacks (Minchin et al. 1986), acetylene reduction remains useful for minimally invasive measurements of nodule function, as does nodule oximetry (Denison and Layzell 1991), which allows noninvasive measurements of several parameters related to nodule oxygen status.

Useful mutants, new methods, and theoretical advances in evolutionary biology (e.g., theory of viscous populations) are converging in ways that should make cooperation in the rhizosphere much less mysterious within a few years than it is at present.

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