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Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates

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The legume–rhizobia symbiosis is a classical mutualism where fixed carbon and nitrogen are exchanged between the species. Nonetheless, the plant carbon that fuels nitrogen (N_2) fixation could be diverted to rhizobial reproduction by 'cheaters'—rhizobial strains that fix less N_2 but potentially gain the benefit of fixation by other rhizobia. Host sanctions can decrease the relative fitness of less-beneficial reproductive bacteroids and prevent cheaters from breaking down the mutualism. However, in certain legume species, only undifferentiated rhizobia reproduce, while only terminally differentiated rhizobial bacteroids fix nitrogen. Sanctions were, therefore, tested in two legume species that host non-reproductive bacteroids. We demonstrate that even legume species that host non-reproductive bacteroids, specifically pea and alfalfa, can severely sanction undifferentiated rhizobia when bacteroids within the same nodule fail to fix N_2. Hence, host sanctions by a diverse set of legumes play a role in maintaining N_2 fixation.

Keywords: pea; alfalfa; sanction; mutualism; bacteroid differentiation; rhizobia

1. INTRODUCTION

Mutualisms—cooperative relationships between different species—are ubiquitous and often ancient [1]. 'Cheaters', which have been defined as individuals or genotypes that cooperate less while benefiting from the cooperation of others [2,3], have been reported in many mutualisms [4]. One of the major questions in evolutionary biology is why ancient mutualisms have not been broken down by such cheaters [1,5–7]. For example, in the legume–rhizobia symbiosis, where fixed carbon and nitrogen are exchanged, a cheating rhizobial strain might divert resources from nitrogen (N_2) fixation, which benefits its legume host, to its own reproduction instead. Legume hosts are typically infected by several strains, creating a potential tragedy of the commons in which cheaters would displace mutualists in the absence of mechanisms that increase the fitness of the latter [8,9]. Two such mechanisms are 'host sanctions', which reduce the fitness of cheaters (or defective partners) [10–12], and 'partner choice', where each partner could identify and reject forming relationships with cheaters (or defectives) [11,13–15]. In the legume–rhizobia symbiosis, both types of mechanism of stabilization have been demonstrated [10,11,14–16], but it is still debated whether these mechanisms are universal (i.e. found in all legume host species) [14,17].

In particular, sanctions might be expected to vary among legume host species due to a dichotomy in host effects on rhizobial life history [17]. In nodules of species like soybean (*Glycine max*) and lupine (*Lupinus arboreus*) [10,16], rhizobial bacteroids (the differentiated, N_2-fixing form) retain the ability to reproduce. But inside nodules of some other legume host species, like pea (*Pisum sativum*) and alfalfa (*Medicago sativa*), rhizobial bacteroids are swollen and terminally differentiated. Some of their clonemates remain undifferentiated within these nodules, however, acting as a germline for additional N_2-fixing bacteroids, as well as for soil populations that may nodulate the next generation of plants. Like sterile workers of eusocial insect colonies, or bacterial cells that self-destruct to release chemicals benefiting surrounding bacteria, the inherited behaviour of non-reproductive bacteroids (e.g. N_2 fixation) has evolved because it preferentially benefits others carrying the same alleles [18]. However, in contrast to sterile workers and self-destructing bacteria, the loss of reproductive viability of bacteroids is not an inherited behaviour. Instead, it is imposed by the legume host, apparently via certain peptides [19] that evolved repeatedly among the legumes [20].

There are two reasons why host sanctions might be less common in legumes hosting non-reproductive bacteroids. First, although reducing plant resource allocation to individual bacteroids that fail to fix N_2 would benefit hosts by conserving resources [21], this would not necessarily constitute sanctions if they fail to also affect the undifferentiated rhizobia from which future generations are descended [17]. Sanctions against the whole nodule could, on the other hand, affect both bacteroids and undifferentiated reproductives. The inclusive fitness of

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non-reproductive bacteroids—the survival and reproduction of the clonal undifferentiated rhizobia that carry the same genes as the bacteroids—would then be lowered. Second, cheaters (i.e. rhizobia that actually benefit from fixing less N_2) may be less common when bacteroids are non-reproductive. Reproductive bacteroids pay an opportunity cost when fixing N_2, because the carbon received from plants could otherwise be used for bacteroid reproduction or stored for future reproduction when in the soil. However, it is unclear whether non-reproductive bacteroids can increase their inclusive fitness by fixing less N_2 [17] (i.e. whether there is any opportunity cost for fixation). A few rhizobial strains that associate with hosts imposing terminal differentiation of bacteroids are known to synthesize rhizopines, nutritional inositol compounds [22] that non-reproductive bacteroids synthesize by diverting carbon use from N_2 fixation and that undifferentiated rhizobia can catabolize. Without a mechanism such as rhizopines that allows non-reproductive bacteroids to increase their inclusive fitness at the expense of N_2 fixation, the interests of rhizobia and host plants would be aligned [17]. This could lead to fewer cheating rhizobia and a weakened selection for mechanisms of host sanctions among legumes hosting non-reproductive bacteroids.

Nonetheless, legume hosts might benefit from reducing resource allocation to nodules containing less-beneficial rhizobia (perhaps defective mutants, rather than cheaters that actually benefit from fixing less N_2), which might function as sanctions as a side-effect. However, host species that induce terminal differentiation of bacteroids have so far not been shown to sanction less-beneficial rhizobia, based on a lack of significant difference in nodule size among strains differing in benefits to the host [15], or no difference in the number of viable (undifferentiated) rhizobia per nodule between a fixing and a non-fixing strain [14].

Decreased reproduction (in nodules) of less-beneficial rhizobia is a necessary but not sufficient condition to demonstrate performance-based host sanctions. Genotypically distinct strains (even isogenic ones) can have pleiotropic differences that may interfere with nodulation [23] or bacteroid development [24]. A strain that reproduces poorly in nodules (prior to differentiation into bacteroids) would fix less N_2 per nodule, like a cheater, but cause and effect would be reversed from host sanctions. Such a strain would fix less N_2 because it reproduced poorly in the nodule, rather than *vice versa*. Ideally, therefore, we should compare ongoing rhizobial reproduction in nodules that start with similar numbers of bacteroids, but differ in N_2 fixation. Kiers *et al.* [10] did this, simulating cheaters in soybean, by reducing atmospheric N_2 around nodules to near zero. In a split-root experiment, they found about 50 per cent fewer rhizobia per non-fixing nodule (in $Ar:O_2$) compared with fixing nodules (in air) containing the same strain.

In this study, we applied the split-root $Ar:O_2$ method of Kiers *et al.* [10] to two species that host non-reproductive bacteroids. We measured rhizobial fitness (reproductive rhizobia per nodule) directly with plate counting and also compared the amount of energy-rich polyhydroxybutyrate (PHB) in the reproductive clonemates of fixing and non-fixing bacteroids, a resource that could be crucial for rhizobial fitness [25].

2. MATERIAL AND METHODS

(a) *Plant growth conditions and rhizobial inoculum*

Seeds of peas (*P. sativum* cv. 'Green Arrow'; Henry Field's Seed & Nursery Co., Aurora, IN, USA) and alfalfa (*M. sativa* 'ARC'; Keith Henjum, Department of Agronomy and Plant Genetics, University of Minnesota, Saint Paul, MN, USA) were surface-sterilized with 0.09 per cent hydrogen peroxide for 3 min, rinsed with sterile deionized water and planted in split plastic pouches containing nitrogen-free Fahraeus nutrient media [26]. *Rhizobium leguminosarum* A34 and *Sinorhizobium meliloti* MP6 were grown in tryptone yeast media with antibiotics (500 $\mu g\,ml^{-1}$ streptomycin for A34 and 400 $\mu g\,ml^{-1}$ streptomycin and 6 $\mu g\,ml^{-1}$ tetracycline for MP6).

Between 4 and 7 days after germination, the main seedling roots were cut 1–2 cm below the cotyledons to allow even formation of lateral roots between the two halves of the split pouches. After development of split roots, plants were inoculated with 1 ml (approx. 10^9 cells) of stationary-phase rhizobial inoculum on each half of their roots. Peas were inoculated with *R. leguminosarum* A34 and alfalfa with *S. meliloti* MP6.

(b) *Nitrogen-free gas treatment*

Split-root plants inoculated in pouches were transferred into flat glass chambers with silicone dividers between root halves [10]. A mass-flow control system mixed pure argon (Ar) and oxygen (O_2) gases, approximately in the ratio of 79 : 21. This N_2-free $Ar:O_2$ mixture was introduced into one half of each chamber (randomly chosen) while pure air was introduced into the other half at a slightly lower flow rate, to prevent any leakage of N_2 into the N_2-free side. Gases were introduced near the bottom of each side (approx. 75 ml in volume) and flowed out around the plant stem. Three or more nodules of comparable sizes were identified from each side before treatments were assigned. For peas, one experiment ($n = 5$) compared younger nodules with initial nodule lengths between 1.0 and 2.3 mm, and a second ($n = 8$) compared older nodules with initial nodule lengths between 2.0 and 3.9 mm. Initial alfalfa nodule lengths ranged from 1.0 to 2.0 mm in a set of nine plants. Nodules were harvested after 10 days of gas treatments. Flow rates on the $Ar:O_2$ side were approximately 60 $ml\,min^{-1}$ for peas to conserve gas, but increased to 90 $ml\,min^{-1}$ for alfalfa after a preliminary experiment suggested incursion of atmospheric N_2 at the lower flow rate. These rates are still less than the 130 $ml\,min^{-1}$ used previously with soybeans by Kiers *et al.* [10].

(c) *Rhizobia fitness (reproductives per nodule and polyhydroxybutyrate per reproductive) assessment*

Nodules were harvested and rinsed with sterile deionized water three times before being crushed in ascorbic acid buffer [27]. There was no surface sterilization performed on the nodules since this had previously led to some rhizobial death, as evidenced by low colony counts relative to flow cytometric counts. *Sinorhizobium meliloti* MP6 and *R. leguminosarum* A34 could be accurately counted on media containing strain-specific antibiotics with serial dilution, as evidenced by corresponding flow counts.

Nodule extracts (both bacteroids and undifferentiated rhizobia) were stained with Nile red and analysed for mean PHB (pg) per undifferentiated rhizobial cell in the flow cytometer. Smaller undifferentiated rhizobia could be

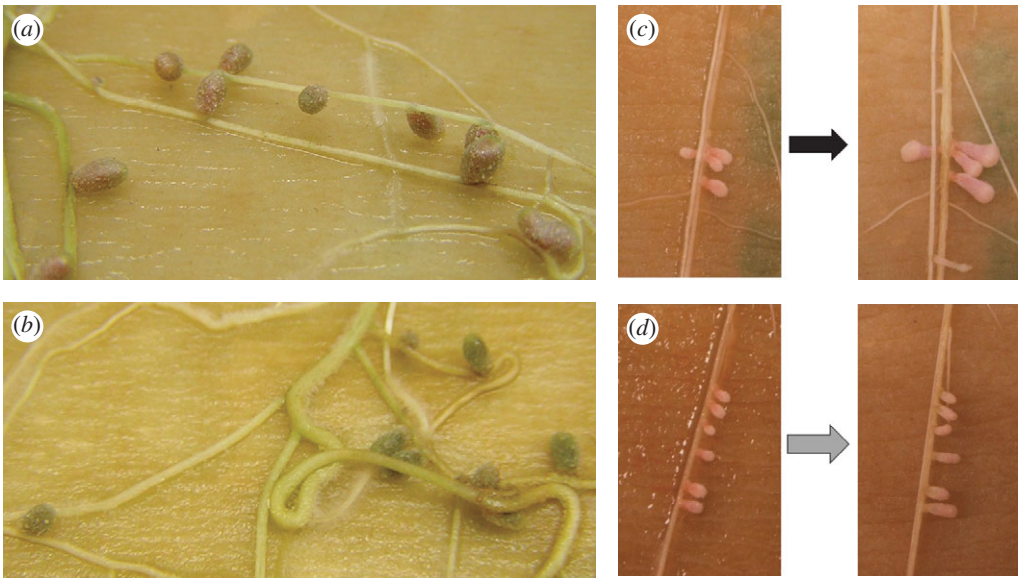

Figure 1. Split-root experiments of peas and alfalfa: half of nodulated roots were exposed to a $Ar:O_2$ (79 : 21) mixture for 10 days while the other half received purified air. (*a*) N_2-fixing pea nodules after the treatment were pinkish-red while (*b*) non-fixing nodules on $Ar:O_2$ side were green and senescing. (*c*) Alfalfa nodules before and after air treatment. (*d*) Alfalfa nodules before and after $Ar:O_2$ treatment, from the same host plant as (*c*).

distinguished from larger swollen bacteroids because they have lower forward and side scatter with the flow cytometer [20].

(d) *Statistics*

Number of undifferentiated rhizobia per nodule, PHB (pg) per undifferentiated rhizobial cell and nodule fresh weights were compared between $Ar:O_2$ and control treatments using a two-tailed paired *t*-test. Regressions of nodule weight to viable rhizobia per nodule were compared between treatments using Student's *t* in R. Regressions were tested for significant positive slopes using an *F*-test in R.

3. RESULTS

(a) *Sanctions in peas*

After 10 days in N_2-free atmosphere ($Ar:O_2$), pea nodules were visibly senescing compared with control treatment nodules (figure 1*a*,*b*). Plate counting revealed young non-fixing nodules contained only 25 per cent as many undifferentiated rhizobial cells as fixing nodules. In the second experiment with older nodules, non-fixing nodules contained only 10 per cent as many rhizobia (figure 2*a*). Apparent differences in nodule weight were not statistically significant in either younger nodules ($p = 0.07$) or older ones ($p = 0.43$; figure 2*c*). There was a significant positive relationship between the number of reproductively viable rhizobia and nodule weight among fixing nodules ($r^2 = 0.56$, 6.6×10^8 cells $g^{-1} + 5.3 \times 10^5$ cells, $p < 0.0001$), and a much weaker positive relationship for the non-fixing nodules ($r^2 = 0.04$, 7.1×10^7 cells $g^{-1} + 2.5 \times 10^5$ cells, $p < 0.05$). The slopes were significantly different between the two treatments (figure 3; d.f. = 187, $t = 2.18$, $p < 0.05$).

There was no significant difference in PHB per cell between treatments for younger nodules at the end of the experiment, but in older nodules PHB per undifferentiated rhizobial cell was 70 per cent greater in the N_2-fixing control than in nodules in the N_2-free $Ar:O_2$ treatment (figure 2*b*).

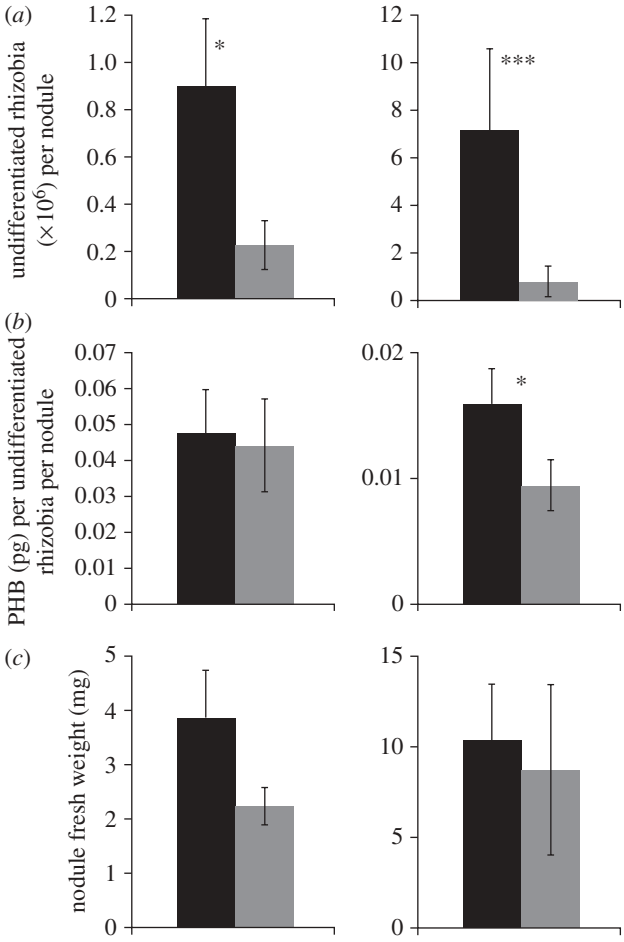

Figure 2. Comparison of (*a*) reproductive rhizobia per nodule, (*b*) PHB per cell in small reproductive rhizobia and (*c*) nodule fresh weight after 10 day argon ($Ar:O_2$, grey bars) versus control ($N_2:O_2$, black bars) treatments, in young (left charts) versus old (right charts) pea nodules. Error bars indicate 1 s.d. Paired two-tailed equal variance *t*-tests were performed. $*p < 0.05$, $***p < 0.001$.

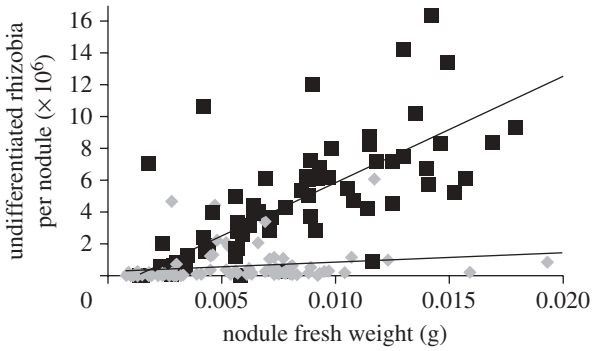

Figure 3. Regressions of nodule fresh weight to number of colony-forming rhizobia per nodule of the two treatments ($N_2 : O_2$, black squares versus $Ar : O_2$, grey diamonds) for peas (d.f. 187, $t = 2.18$, $p < 0.05$).

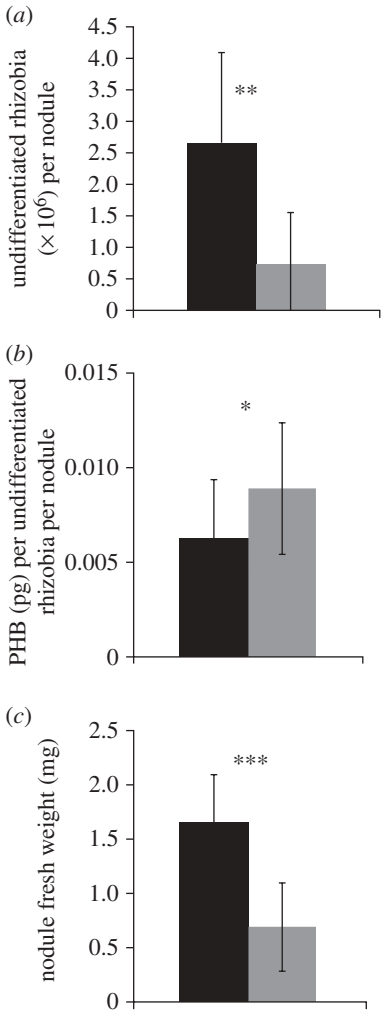

Figure 4. Comparison of (*a*) reproductive rhizobia per nodule, (*b*) PHB per reproductive (non-swollen) rhizobial cell and (*c*) nodule fresh weights after 10 days of N_2-free air ($Ar : O_2$) treatment (grey bars) on young alfalfa nodules and controls ($N_2 : O_2$, black bars). Error bars indicate 1 s.d. Paired two-tailed equal variance *t*-tests were performed. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(b) *Sanctions in alfalfa*

As in pea, alfalfa nodules in N_2-free air contained fewer viable rhizobia per nodule (27% of controls; figure 4*a*). They had lower nodule fresh weights than control nodules (figure 4*a*,*c*) after 10 days. Nodule weights were weakly correlated with number of viable rhizobia per nodule ($r^2 = 0.17$ for $Ar : O_2$ and $r^2 = 0.38$ for control; not shown) and had significantly positive slopes for both treatments (1.0×10^9 cells (g of nodule)$^{-1}$ control, $p < 0.0001$, and 9.3×10^8 cells (g of nodule)$^{-1}$ for $Ar : O_2$, $p < 0.0001$). Unlike pea nodules, these regressions of viable rhizobia to nodule weights were not different between treatments for alfalfa (d.f. = 134, $t = 0.34$, $p = 0.73$). PHB per undifferentiated rhizobial cell per nodule was significantly greater in the nodules in N_2-free $Ar : O_2$ than in N_2-fixing nodules in air (figure 4*b*), contrary to results from peas.

4. DISCUSSION

Rhizobia in nodules of some legume species, including peas and alfalfa, apparently lose the ability to reproduce when they terminally differentiate into N_2-fixing bacteroids. A fraction of the rhizobia (clonally identical to these bacteroids) within the same nodule remains undifferentiated and reproductive. Previous work suggested that responses of these hosts to less-beneficial rhizobia do not reduce rhizobial fitness (i.e. they do not impose sanctions) [14,15]. This was in contrast to results for soybean or lupines [10,16], whose nodules only contain reproductive bacteroids, motivating us to test host sanctions in legume species with non-reproductive bacteroids using gas treatments (eliminating differences other than N_2 fixation among strains) and direct measures of rhizobial fitness (plate counting and PHB by flow cytometry). Our results provide clear evidence that even host species with non-reproductive bacteroids can impose sanctions, at least when rhizobia fix almost no N_2. Hence, mutualism by non-reproductive bacteroids can be facilitated by kin selection, via the exposure of their undifferentiated clonemates to host-plant sanctions.

The apparent lack of sanctions in *Medicago truncatula* in Heath & Tiffin's [15] study, based on no consistent relationship between the benefits that strains provided (counts of leaves or fruits, with single-strain inoculation) and strain fitness (estimated from weight per nodule), may be due to small differences in N_2 fixation among their strains. Soybean rhizobia with small differences in N_2 fixation do not necessarily trigger sanctions either [28]. Furthermore, with only small differences in mutualism among strains, determining which strain is most mutualistic becomes challenging. A strain that makes fewer nodules may give less benefit per plant during single-strain inoculation, but sanctions presumably depend on benefits (or benefit : cost ratio) per nodule. Hence, measuring strain efficiency, rather than host-plant growth during single-strain inoculation, may deserve more attention in the future, as differences among strains in the carbon cost of supporting rhizobia may be as important as their relative nitrogen contribution [29].

An apparent lack of sanctions in *M. truncatula* was also found in Gubry-Rangin *et al.*'s [14] experiment where plants were co-inoculated with a fixing and non-fixing strain. Nodules containing the fixing strain were significantly larger—more than twice the weight of nodules with the non-fixing strain—but this difference was not reflected in differences in plate counts of viable rhizobia per nodule. The nodule size difference suggests that the plants shut off resources to under-performing nodules,

but apparently this did not limit rhizobial reproduction. This outcome may be specific to their rhizobial strains. Positive correlations between nodule weight and number of viable rhizobia per nodule are commonly reported, including indeterminate nodules of *M. truncatula* [30] and *L. arboreus* [16]. However, some studies have found instances where there were more rhizobia per nodule mass in nodules that fixed less N_2 [31] or greater nodule mass for rhizobial strains that fixed relatively little N_2 [32]. In our study, both nodule weights and number of viable rhizobia per nodule were usually significantly greater for fixing than for non-fixing nodules (figures 2*a*,*c* and 3*a*,*c*).

In older pea nodules, however, nodule weights were not significantly different (figures 2*c* and 3), yet rhizobial numbers were lower in non-fixing nodules. This is essentially the opposite of what Gubry-Rangin *et al.* [14] found with *M. truncatula*. These older pea nodule results suggest that, although nodules had stopped growing, either the undifferentiated rhizobia continued to reproduce more in the fixing nodules or they died in the non-fixing nodules. It is unlikely that rhizobia inside a nodule would starve to death during a 10 day treatment, but perhaps some plant factors acted as an antibiotic within the nodule, allowing hosts easier access to amino acids etc. in the rhizobia. Our results highlight the importance of actual counts for the estimation of rhizobial fitness instead of relying on nodule size as a proxy, especially if nodule weights only marginally change at later stages but rhizobial numbers change significantly. Even counts may not fully reflect rhizobial fitness of different rhizobial phenotypes if PHB per rhizobial cell could significantly contribute to rhizobial fitness [25]. In both peas and alfalfa, however, we detected less than 0.1 pg of PHB per cell, which may not be enough to fuel much reproduction [25]. Therefore, we doubt that the differences we detected in PHB would overturn the fitness differences we measured in terms of cell numbers.

While it is now clear that host sanctions are possible even in legume species that impose terminal differentiation of bacteroids, it is not known whether sanctions play a role equal to or greater than pre-infection partner choice in maintaining the mutualism. Nodulation competition between effective parent strains and ineffective mutants appears to depend on the particular mutation, and results will range from effective strains forming more nodules (suggesting pre-infection partner choice [11,14]) to ineffective mutants forming an equal or greater number of nodules ([33] and references therein). Pre-infection partner choice seems a theoretically unlikely mechanism for consistently stabilizing rhizobial mutualism, given the advantage of the rhizobia's shorter generation time in any evolutionary arms race with hosts, but it does appear to be effective against a subset of less-beneficial rhizobia [11,14,15].

By inducing terminal differentiation of bacteroids, plants may reduce bacteroid cheating options, but it appears that peas and alfalfa can, nonetheless, sanction their rhizobia even more severely than soybeans, in terms of relative viable cell numbers per nodule. Hence, cheating or defective non-reproductive bacteroids may be common enough in the soil to maintain selection for negative host responses for which the evolutionary effects on rhizobia meet our definition of host sanctions. However, the results of Heath & Tiffin [15] and Kiers *et al.* [28] suggest that intermediate levels of fixation may not trigger sanctions. If fairly minor diversion of resources from N_2 fixation to rhizobial reproduction can significantly increase rhizobial fitness, with minimal risk of triggering sanctions, then most rhizobial strains in the field are likely to be mediocre.

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