

Letter

Single-strain inoculation may create spurious correlations between legume fitness and rhizobial fitness

A recent meta-analysis by Maren Friesen suggests that there is 'widespread fitness alignment' between rhizobia and their legume hosts (Friesen, 2012). The work is motivated by a very important question, namely how is cooperation stabilized between partners in a mutualism? This is a puzzle in the legume–rhizobia partnership because there are potential opportunities for 'cheating' by the rhizobial symbiont. For example, rather than fixing N₂ for the benefit of the host, rhizobial strains can divert more energy to the synthesis of polyhydroxybutyrate (PHB) (Hahn & Studer, 1986; Cevallos *et al.*, 1996), which enhances their own survival and reproduction (Ratcliff *et al.*, 2008). Similarly, rhizobial strains that interfere with plant ethylene signaling increase their own fitness at the expense of the plant (Ratcliff & Denison, 2009). Given this scope for symbiont defection, why hasn't the mutualism collapsed?

Friesen's meta-analysis (Friesen, 2012) uses positive correlations between plant fitness and rhizobial fitness to argue that there is little scope for cheating.

While there may be little scope for cheating where there is only one symbiont genotype per host, this is not likely in the legume–rhizobial mutualism where multiple rhizobial strains, varying in mutualistic benefits, simultaneously interact with a single host. These multiple infections create a potential tragedy of the commons where less mutualistic strains share in the collective benefits, while paying less of the costs (Kiers & Denison, 2008). This decouples the reproductive success of individual strains from the reproductive success of their host.

Why then, the positive correlation identified by Friesen? We would expect a positive correlation if hosts respond differentially to strains varying in their mutualistic benefit, reducing the fitness of less beneficial strains relative to more beneficial strains. Such 'sanctions' have been reported in soybean (Kiers *et al.*, 2003) and lupine (Simms *et al.*, 2006), and recently in alfalfa and pea (Oono *et al.*, 2011). A commentary on Friesen's paper notes that sanctions could explain the reported fitness correlations (De Mita, 2012). However, sanctions may not be universal; moderate amounts of cheating may not trigger host responses (Kiers *et al.*, 2006), and host responses do not always reduce rhizobial fitness (Gubry-Rangin *et al.*, 2010).

Do the positive fitness correlations identified by Friesen's meta-analysis show that there is little inherent scope for cheaters in nature or that sanctions are widespread? Not necessarily either. The main

result presented by Friesen, fitness alignment between legumes and rhizobia, may be a consequence of the methods employed in the analysis. The meta-analysis compared various measures of rhizobial fitness with fitness benefits to their legume hosts (Friesen, 2012). Accurately measuring rhizobial fitness can be difficult (Ratcliff *et al.*, 2012), but there is an even more important issue: nearly all the plant-benefit estimates in the meta-analysis were based on the growth of singly-inoculated plants. This could potentially lead to spurious correlations between plant fitness and rhizobial fitness.

This is most obvious for the reported correlation between plant biomass and nodule biomass. Consider a rhizobial 'cheater' that fixes half as much nitrogen (N), because it diverts resources to its own immediate reproduction or to PHB to support future reproduction. With single-strain inoculation, its host plant will grow only half as large, so it will support less nodule biomass, giving the observed correlation between plant and rhizobial fitness (Fig. 1, left). In the field, however, each plant is infected by several strains. Under these conditions, cheating by only one strain has little effect on overall plant growth or overall nodule biomass. Therefore, resource diversion by the cheater strain would give it high fitness (Fig. 1, right), in contrast to its low fitness with single-strain inoculation. A correlation between plant biomass from single-strain inoculations and the number of nodules per plant raises similar issues. Larger plants can simply support more nodules.

More interesting is the correlation between a strain's competitive success in nodulation and its effect on plant growth, again with single-strain inoculation (Friesen, 2012). This could be evidence for fitness alignment (and compatibility matching) between legumes and rhizobia. But there is an alternative explanation. For an N-starved test seedling with no nodules, even an inefficient nodule is better than none. Rhizobial strains that detect plant roots sooner, exit the persister state more quickly (Ratcliff & Denison, 2010), swim faster, or reproduce faster inside nodules will provide at least some N at a critical stage. With single-strain inoculation, these fast-nodulating strains will result in earlier plant growth (and resulting compound-interest benefits) compared with a slower-nodulating strain, even if the latter provides N at half the carbon cost (Fig. 2). Likewise, faster-nodulating strains will have an advantage in most tests of nodulation competitiveness (Handelsman *et al.*, 1984). So single-strain inoculation emphasizes speed of nodulation, while downplaying the subsequent rate or efficiency of N fixation. This explains the otherwise mysterious correlation between the benefits a strain provides to a (singly-inoculated) plant and its nodulation competitiveness.

Both fast nodulation (early access to N) and N-fixation efficiency (N fixation divided by nitrogenase-linked respiration, for example) enhance plant fitness. Single-strain inoculation emphasizes nodulation speed over fixation efficiency. This would not be a problem if there were a consistent correlation between nodulation speed and fixation efficiency, but they are independent traits. A comparison of

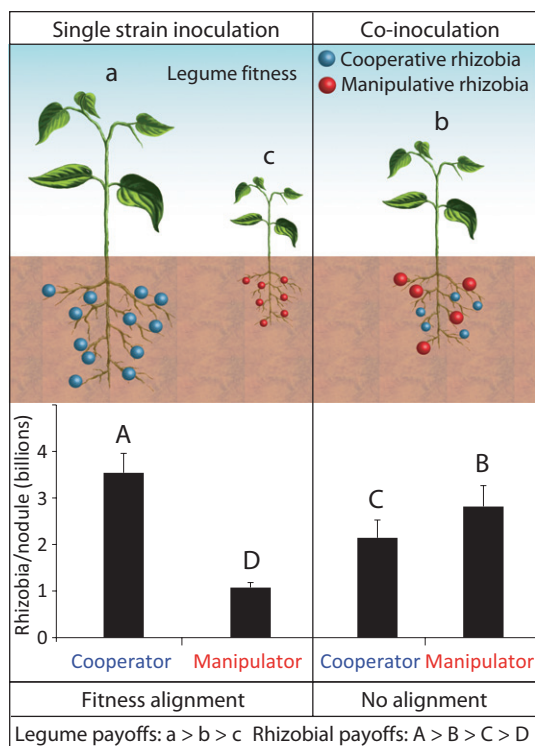


Fig. 1 Evidence for fitness alignment may be biased by single-strain inoculation. Under single-strain inoculation, more cooperative rhizobia directly benefit from greater plant growth, resulting in higher host and symbiont fitness (left). Any rhizobia that provide less host benefit and yet evade sanctions do poorly when alone, but exploit cooperative rhizobia when they coinfect a host (right). Here we illustrate this with a natural example of host manipulation, rhizobitoxine (Rtx) production by *Bradyrhizobium elkanii*. Rtx(+) rhizobia cheat their host, gaining energy-rich polyhydroxybutyrate (PHB) by manipulating host ethylene production, a behavior that reduces legume fitness. We calculated the rhizobial payoff matrix when Rtx(+) rhizobia and an isogenic Rtx(-) mutant either singly infected or coinfecting hosts using data from Ratcliff & Denison (2009). Shown are the number of rhizobia per nodule, corrected for potential reproduction from stored PHB. PHB per cell (pg) was determined by a calibration curve relating forward scatter to PHB per cell ($y = 0.0025x - 0.25$, $r^2 = 0.75$); reproduction from stored PHB was calculated as described in Ratcliff *et al.*, (2012). Bean drawing by Andrea Danti/123rf.com (rights purchased).

three rhizobial strains on 11 host genotypes (Diatloff & Ferguson, 1970) found that strains with faster nodulation resulted in 'an initial growth advantage [but] this was apparently lost by c. 8 wk as the slower nodulating plants began to fix N at a much higher rate'. The strain that consistently provided the greatest benefit at 8 wk took an average of 32 d to nodulate whereas a less beneficial strain took only 25 d. So we cannot assume that faster-nodulating strains are necessarily more efficient.

Friesen also found that, out of 80 mutations reported, none increased rhizobial fitness at the expense of the host. As with the other analyses in this paper, single strain infection limits detection of 'cheater' mutations that would increase rhizobial fitness when plant growth is supported by other, more mutualistic rhizobia.

A better way to compare benefits provided by different rhizobial strains is actually proposed by Friesen (2012). Plants could be

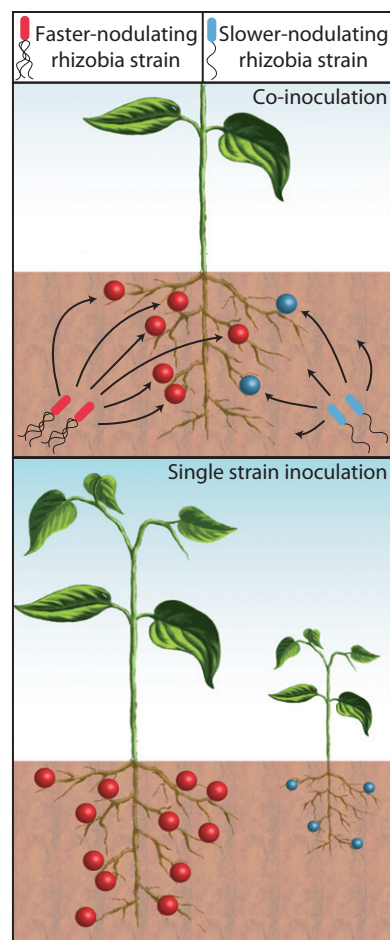


Fig. 2 Faster-nodulating rhizobia form a larger fraction of nodules during coinfection (upper). Faster nodulation may also improve the growth of plants in single-strain infection (lower). This can cause a correlation between nodule occupancy during coinfection and apparent symbiont quality, as assessed by single-strain infection. This correlation may persist even if the faster nodulating strain fixes nitrogen less efficiently.

inoculated with two or more strains, and their relative merits could be assessed by regressing plant growth or seed production on the nodule occupancy by each strain. We have been using this method with various two-strain comparisons, but agree that comparisons among even more strains might be interesting. Nodule occupancy could also be corrected for timing, or nodule biomass could be included. Direct measurements of N-fixation efficiency are also possible (Oono & Denison, 2010).

We applaud Friesen for stimulating interest in rhizobial fitness. Her research shows the need for more empirical work on mixed infections. What we need now is data for a range of rhizobial strains, comparing the fitness benefits they obtain (Ratcliff *et al.*, 2012) and provide in symbiosis in multiply infected plants. It is possible that better methods may lead to the same conclusion drawn by Friesen, but we will not know until these experiments are performed.

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