Human selection and the relaxation of legume defences against ineffective rhizobia

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Enforcement mechanisms are thought to be important in maintaining mutualistic cooperation between species. A clear example of an enforcement mechanism is how legumes impose sanctions on rhizobial symbionts that fail to provide sufficient fixed N₂. However, with domestication and breeding in high-soil-N environments, humans may have altered these natural legume defences and reduced the agricultural benefits of the symbiosis. Using six genotypes of soya beans, representing 60 years of breeding, we show that, as a group, older cultivars were better able to maintain fitness than newer cultivars (seed production) when infected with a mixture of effective and ineffective rhizobial strains. Additionally, we found small differences among cultivars in the ratio of effective : ineffective rhizobia released from their nodules, an indicator of future rhizobial strain fitness. When infected by symbionts varying in quality, legume defences against poor-quality partners have apparently worsened under decades of artificial selection.

Keywords: cheat; nitrogen fixation; mutualism; partner choice; punish; sanction

1. INTRODUCTION

Cooperation can be undermined by ‘cheaters’, which ‘cooperate less... but are potentially able to gain the benefit of others cooperating’ (West *et al.* 2007). Many mutualisms, including those between ants and plants, plants and pollinators, and cleaner and client fish are plagued by low-quality mutualists (Bronstein 2001; Sachs *et al.* 2004). In the legume–rhizobia mutualism model, clear differences in partner quality have been found, with different rhizobium isolates from the same soil giving up to 10-fold differences in host–plant growth (Burdon *et al.* 1999). Rhizobial strains of sub-optimal effectiveness have been isolated from fields worldwide and are often cited as a factor contributing to poor agronomic yields (Erdman 1950; Labandera & Vincent 1975; Hafeez *et al.* 2001; Denton *et al.* 2000; Fening & Danso 2002).

Although microbial symbionts generally benefit from healthier hosts, the interests of the host and symbiont are not perfectly aligned. For rhizobia, trade-offs exist between fixing nitrogen for the benefit of the plant versus hoarding resources like polyhydroxybutyrate (Hahn & Studer 1986; Cevallos *et al.* 1996; Denison & Kiers 2004a,b; Akçay & Roughgarden 2007), which can enhance future survival or reproduction (Cai *et al.* 2000).

Theory predicts that diversion of resources away from N₂ fixation to a rhizobium’s own reproductive fitness will be favoured by natural selection, especially when the strain is competing against other strains infecting the same host,

even if there is a shared cost due to a less healthy host (Frank 1998; Denison 2000; Crespi 2001; West *et al.* 2002a,b). Multiple rhizobium strains per plant (Hagen & Hamrick 1996; Silva *et al.* 1999) can create a ‘Tragedy of the commons’ problem (Hardin 1968), potentially destabilizing the mutualism over evolutionary time.

However, recent experiments suggest that legume ‘host sanctions’ (Denison 2000) against less-effective rhizobial strains may stabilize the mutualism by allowing legumes to direct more resources to those nodules containing the best strain (Kiers *et al.* 2003, 2006). Legumes that preferentially allocate resources to nodule rhizobia on the basis of their actual symbiotic performance are likely to promote the evolution of higher levels of N₂ fixation while directly increasing their own fitness (West *et al.* 2002a,b). Sanctions can result in nodule growth differences, which have been documented in soya bean (Singleton & Stockinger 1983; Kiers *et al.* 2003, 2006) and recently in wild lupines (Simms *et al.* 2006). These sanctions tend to increase the N return on a plant’s C investment.

Studies suggest that a host–plant’s interaction with rhizobia is a heritable trait that may be modified in plant breeding programmes (Herridge & Danso 1995; Devine & Kuykendall 1996; Herridge *et al.* 2001). Therefore, sanction ability (strength and sensitivity) may vary among legume genotypes, depending on the natural or artificial selection pressures to which their ancestors were exposed. This difference in sanction ability may be most pronounced between our modern crops and their ‘less-improved’ ancestors owing to breeding practices. Breeding under high fertilizer regimes became common in the 1940s (Gizlice *et al.* 1993), although a few programmes have bred legume cultivars in low-soil-N environments (Döbereiner 1997).

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Table 1. Name, year released, maturity group, control seed yields ($\pm$ s.e.) with no rhizobial inocula and pedigree of six short-season soya beans (*Glycine max* (L.) Merr.)

cultivar	year released	maturity group	control yields (g m^{-2})	effective-strain yields (g m^{-2})	pedigree
<i>type: older</i>					
Kabott ^a	1937	00	116 (± 35)	208 (± 48)	From Ninguta Manchuria collection
Pagoda	1939	00	148 (± 48)	210 (± 47)	Mandarin/Manitoba Brown
Flambeau	1948	00	180 (± 41)	203 (± 41)	selection from early Russian variety
<i>type: newer</i>					
Maple Glen	1987	00	219 (± 54)	294 (± 60)	BD22115-13/Premier
AC Harmony	1992	00	218 (± 52)	286 (± 50)	Maple Presto/Williams/2/Weber
AC Rodeo ^b	2001	00	203 (± 56)	309 (± 61)	Maple Arrow/PI 361088B/3*Maple Glen/3/AC Harmony

^a Adapted from table 1 (Voldeng *et al.* 1997).

^b Personal communications (E. Cober, Agriculture & Agri-food Canada).

It has been proposed that breeding for yield under high fertilizer regimes could relax sanctions or other loss-limiting traits (Kiers *et al.* 2002). Alternatively, increased fertilizer regimes may favour genotypes that are less tolerant of marginally effective strains (stricter sanctions) because the plant only needs a few (top-performing) nodules to make up the small deficit in soil N. We therefore ask whether there have been any trends in the sanctioning response over decades. This is an important question for two reasons; we may be: (i) selecting for cultivars that fail to maximize net benefits derived from symbiosis and (ii) inadvertently encouraging the spread of less-effective symbionts.

Since, under natural conditions, legumes are infected by multiple symbionts that vary in quality (Souza *et al.* 1994; Hagen & Hamrick 1996; Silva *et al.* 1999), we address the question of whether older cultivars are better able to maximize their fitness with a mixture of effective and ineffective rhizobial strains than their modern counterparts. No plant can force a rhizobium lacking nitrogenase to fix N_2 , but do cultivars differ in their ability to allocate resources selectively among nodules on the basis of N_2 fixation?

An important secondary question is how multiple symbionts per plant interact in influencing plant growth. Heath & Tiffin (2007) recently reported that a mixture of rhizobium strains was worse for *Medicago truncatula* growth than growth with the worst single strain in a high-N environment. On the other hand, Maherali & Klironomos (2007) found that mixtures of phylogenetically distant mycorrhizal fungi enhanced *Plantago* growth more than any single strain alone.

In a 2-year field experiment, we compared six soya bean cultivars, representing over 60 years of breeding, for their ability to benefit from effective rhizobia in the presence of an ineffective strain. An additional laboratory study examined differences in rhizobium reproduction in nodules of the two oldest and two newest cultivars to see whether genotypes that best tolerated rhizobium mixtures (perhaps by imposing sanctions on non-fixing nodules) might also selectively enrich the soil with the better strain. Our ultimate aim is to determine how soya bean genotypes differ in their response to a mixture of ineffective and effective rhizobia, including effects on the rhizobium populations and the dispersion of legumes for subsequent soya bean crops.

2. MATERIAL AND METHODS

(a) Field experiment

Seeds of six cultivars (table 1) of *Glycine max* maturity group 00 (Eastern Cereal and Oilseed Research Centre, Agriculture & Agri-food Canada) were grown in a split-plot design at the Maine Agricultural Experiment Station, Highmoor Farm (latitude $44^{\circ}17'43.31''$ N, longitude, $69^{\circ}59'09.83''$ W), Monmouth, Maine. The cultivars were chosen from the list of short-season cultivars either selected, bred or with parents from a breeding programme in Ottawa, Canada. They were selected based on their similar growth habitats, traceable pedigree and ability to represent distinct periods of breeding (Morrison *et al.* 1999; E. Cober 2002, personal communication). In the first year, cultivars were grown on Dixfield fine sandy loam, coarse loamy, mixed frigid typic Haplothord (Moran & Schupp 2003) with a pH of 6.2, 4.3% organic matter and $4.25 \mu\text{g g}^{-1}$ KCl-extractable nitrate at time of planting. The experiment was repeated for the second year under the same soil conditions in an adjacent plot. Air temperature in the summer growing season of both years was between 16 and 20°C . Precipitation from June–September was 28.5 cm in 2002 and 33.2 cm in 2003. Plots were rainfed with no additional irrigation. Fields had no history of soya bean cultivation.

Prior to planting, inoculum of *Bradyrhizobium japonicum* USDA 110 ARS (USDA Beltsville, MD), an effective strain resistant to streptomycin and rifampicin (Kuykendall & Weber 1978) was grown in modified arabinose gluconate (MAG; van Berkum 1990). Ineffective strain USDA 110 LI (USDA Beltsville, MD), a derivative of USDA 110 lacking symbiotic N_2 -fixation ability (Kuykendall & Elkan 1976) was grown in yeast extract-mannitol broth (YEM). Both strains were grown for 8 days in a rotary shaker until late exponential growth phase ($A_{420}=0.5\text{--}1.5$) and samples were plated to determine cell densities. One hundred millilitres of the cultures were injected into 250 g of irradiated sterile peat to grow for four weeks.

Each plot, inoculated with one of an effective, ineffective, 1 : 1 mix of effective : ineffective or the rhizobium-free control treatment, contained six randomized sub-plots, one for each cultivar type. Seeds were inoculated with 1 g of bacterial inoculum (5×10^4 cells/seed, either 110 ARS, 110 LI or a 1 : 1 mixture) or 1 g of sterile peat moss (control). Sub-plots measured 2×2 m with 250 seeds per plot grown in five rows spaced 0.4 m apart, with a 0.5 m path between sub-plots. Each plot was surrounded by a buffer strip of uninoculated

soya bean as a barrier against rhizobial contamination. Each of four treatment plots, containing each of six cultivars, was replicated three times for a total of 72 sub-plots. The same plot system was used in the adjacent field in the second year.

No nodules were found in biweekly examination of the root systems from buffer strip soya beans, consistent with the absence of a previously established *B. japonicum* rhizobium population and a lack of cross-contamination between plots. Control plots remained nodule-free in both years. Each year, root systems from five plants from the inner three rows of each sub-plot were harvested at the end of July and all nodules were collected and pooled by sub-plot. A sub-sample of 5–10 nodules from each sub-plot was preserved in 50% glycerol and kept on ice. Within 24 hours, these nodules were weighed and surface sterilized in 3% (V/V) hypochlorite (50% commercial bleach) for 3 min. After sterilization, nodules were rinsed five times in sterile water. Nodules (pooled by plant) were crushed, plated either on YMA (ineffective treatment), MAG plates containing 400 µg ml⁻¹ streptomycin and 40 µg ml⁻¹ rifampicin (effective treatment) or both YMA and MAG plates (mixed treatment) and counted as rhizobia per nodule as previously described (Kiers *et al.* 2003).

Chlorophyll meter readings (Minolta, SPAD-502), used as a proxy for midseason leaf N content, were made at the beginning of podfill (R3 development stage; Fehr *et al.* 1971) from 10 or more locations per sub-plot on the second or third soya bean leaflet from the growing point of randomly selected plants. Plants were harvested by hand the second week of September. Only the inner 1 m² was harvested to avoid edge effects. Dried pods were removed from plants and run through a drum roller upright Pullman plot thresher to separate seeds from pods. Seeds were weighed and sub-samples were ground to a fine powder for nitrogen analysis using a Geo 20/20 IRMS (PDZ Europa Ltd., Crewe, UK) at the UC Davis Stable Isotope Facility.

'Yield difference' was calculated for each cultivar by subtracting their uninoculated control plot yield from their treatment yields (e.g. effective, ineffective, mixed). This value is proportional to the cultivar's absolute N from fixation because approximately 90% of N accumulates in the seeds, regardless of soya bean ancestry (Cregan & Yaklich 1986). However, a given percentage of benefit from inoculation will give a larger absolute yield difference with modern cultivars because there is an estimated 0.5% per year yield improvement due to plant breeding and selection (Voldeng *et al.* 1997; Morrison *et al.* 1999, 2000). We therefore calculated a 'yield difference ratio' for the mixed treatment (mixed-control yield)/(effective-control yield). This measures a cultivar's symbiotic response to a mixed inoculum compared with the ideal response with an effective N₂-fixing strain.

For the analysis, we fitted a linear mixed-effects model using the linear mixed-effects function (lme) in R software (R Core Team 2007). Treatment, type and the treatment × type interaction were designated as fixed effects and cultivar was treated as a random effect nested within type. This random effect structure was determined using likelihood ratio tests to compare models with simpler (random intercepts for cultivars) and more complex (including cultivar interactions with fixed effects) random effects structures (i.e. comparisons with more complex models, all $p > 0.05$). A likelihood ratio test of models with and without a random effect for 'year' was non-significant and therefore data from the 2 years were pooled. Absolute seed yield, yield difference (treatment-control), seed N, leaf chlorophyll and

rhizobia per nodule were used as response variables. Yield difference ratio for the mixed treatment (calculated as described above) was analysed using the lme function with cultivar as a random effect. Standard model checking plots were used to check that there was no obvious inequality of their variance residuals and that they were approximately normally distributed.

(b) Laboratory experiment

To investigate possible differences in rhizobium strain selectivity among cultivars, the two oldest cultivars (Kabott and Pagoda) and the two most recently released cultivars (AC Harmony and AC Rodeo) were grown in an additional laboratory experiment. Seeds of the four cultivars were surface sterilized in 0.5% (v/v) hypochlorite for 5 min, rinsed with sterile water six times and soaked overnight. Seed coats were removed and seeds were planted in growth pouches (Mega International, Minneapolis, MN). Six replicates of each cultivar were maintained in a growth chamber (E7/2: Conviron, Winnipeg, Manitoba) with a 13 hours photoperiod (400 µmol m⁻² s⁻¹) at 22°C.

Inoculum of *B. japonicum* strain USDA 110 ARS or 110 LI was grown, as above, in either MAG or YEM, respectively. Cells were rinsed free of media. Plants were completely randomized into four treatments 5 days after planting and inoculated with 1 × 10⁷ cells per plant of either 110 ARS (effective), 110 LI (ineffective), 110 ARS:110 LI (1 : 1 mixture) or no *B. japonicum* (control). Nitrogen-free nutrient solution (Blumenthal *et al.* 1997) was replaced daily. Plants were grown for five weeks.

Root systems were harvested and nodules were counted and weighed. All nodules were sterilized in 3% (V/V) hypochlorite (50% commercial bleach) for 3 min and rinsed in a continuous flow of sterile water for 8 min. Nodules were crushed, plated and colony forming units (cfu) counted as described (Kiers *et al.* 2003). Nodules from mixed treatments were surface sterilized and plated on YMA plates. Colonies were then replicated on MAG plates containing antibiotics, as above, to determine the proportion of effective to ineffective bacteria. Plant tops were oven-dried for 48 hours at 60°C, weighed, and stem and leaves were homogenized by ball milling before N analysis as described above. The laboratory data were analysed as a CRD with the four cultivars as a fixed factor and three types of rhizobial inoculation (or the control) as the treatment. Analysis of variance was performed with the response variables nodule number, nodule weight, plant dry weight, N per plant and proportion of effective : ineffective rhizobia in nodules using the GLM procedure (SAS Institute), and mean separation was calculated using Tukey's HSD ($p = 0.05$). Levene's test confirmed the homogeneity of variances.

3. RESULTS

(a) Field experiment

Inoculation with effective N₂-fixing bacteria led to significantly higher seed yield, leaf chlorophyll and percentage of seed N when compared with the ineffective inoculum ($p < 0.05$; table 2). Averaging across cultivars, the mixed treatment of 1 : 1 (effective : ineffective) was not statistically different from the effective treatment in yield and percentage of seed N ($p > 0.05$) but did result in lower chlorophyll and rhizobia per nodule (table 2). Results for the control treatment were similar to the ineffective

Table 2. Mean treatment effects ($\pm$ s.e.) on soya bean cultivars inoculated with three different inocula and an uninoculated control, averaged over six cultivars and two growing seasons.

measurement	treatment			
	effective	mixed	ineffective	control
seed yield (g m^{-2})	252 (± 20.9) ^a	233 (± 19.7) a	174 (± 18.0) b	181 (± 19.5) b
chlorophyll (SPAD)	36.6 (± 0.5) ^a	29.6 (± 0.7) b	21.6 (± 1.1) c	26.1 (± 0.8) d
seed N (%)	6.24 (± 0.07) a	6.16 (± 0.09) a	5.01 (± 0.07) b	4.99 (± 0.05) b
rhizobia/nodule ($\times 10^8$)	2.7 (± 0.3) a	1.8 (± 0.1) b	2.0 (± 0.3)ab	—

^a Means within rows followed by the same letter are not significantly different (Tukey HSD, $p < 0.05$).

Figure 1. Yield differences of six cultivars inoculated with either (a) effective inoculum or (b) 1 : 1 mixture of effective and ineffective inoculum (mixed inoculum). To compare the inoculum effects while standardizing for base yield differences, values were calculated as difference (increase or decrease) from replicated control plots in which cultivars were grown with no inoculum. Means within a graph followed by the same letter are not statistically different (Tukey HSD, $p < 0.05$).

treatment, except in chlorophyll measurements, in which ineffective-rhizobium plots were significantly lower than even the control (table 2).

Analyses of yield difference, roughly proportional to the cultivar's absolute N from fixation, showed a significant effect of inoculation treatment ($F_{2,98} = 25.64$, $p < 0.001$, figure 1) and a strong treatment $\times$ type interaction ($F_{2,98} = 9.98$, $p = 0.0001$). Within the mixed treatment (figure 1b), comparisons among individual cultivars found that the oldest cultivar, Kabott, showed the greatest positive yield increase. For each of three older cultivars, inoculation with a mixture of rhizobia gave yield differences that were not significantly different from those obtained with effective rhizobia ($p > 0.05$). By contrast,

Figure 2. (a) Yield difference ratios of cultivars under a 1 : 1 mixture of effective : ineffective rhizobia (mixed inoculum). Ratios were calculated for each cultivars as yield difference (see figure 1) in mixed treatment over yield difference for effective treatment: (mixed yield-control)/(effective yield-control). Dotted line at 1.0 indicates a ratio in which symbiotic benefits are maximized under mixed treatment conditions. (b) Absolute field yields of cultivars under a 1 : 1 mixture of effective : ineffective rhizobium treatment (mixed inoculum). Legend same as figure 1.

the three newer cultivars showed significantly less yield benefit from the mixed treatment relative to the effective treatment ($p < 0.05$, figure 1).

The yield difference ratio measures the ability of cultivars to achieve their full symbiotic potential when exposed to mixed rhizobial populations. Comparisons between type (old versus new cultivars) found that older cultivars show a significantly higher yield difference ratio, relative to newer cultivars ($F_{1,4} = 7.45$, $p = 0.05$; figure 2a). Ratios for newer cultivars were all less than 0.5, meaning that these cultivars received less than half as much benefit from the mixed rhizobia as from the

Table 3. Average nodule number per root system, fresh weight per nodule and plant dry weight ($\pm$ s.e.) of pouch-grown cultivars (two older types and two newer types) inoculated with either effective or ineffective rhizobia.

year released	nodule number (per root)		weight per nodule (mg)		plant dry weight (g)	
	effective	ineffective	effective	ineffective	effective	ineffective
1937 Kabott	52.7 ($\pm$ 9.2)a ^a	4.7 ($\pm$ 0.6)a	9.00 ($\pm$ 3.6)a	23.4 ($\pm$ 8.2)a	0.517 ($\pm$ 0.09)a	0.361 ($\pm$ 0.03)ab
1939 Pagoda	74.0 ($\pm$ 8.2)a	6.5 ($\pm$ 0.6)a	5.0 ($\pm$ 0.3)a	16.4 ($\pm$ 0.5)a	0.589 ($\pm$ 0.02)ab	0.226 ($\pm$ 0.02)a
1992 AC Harmony	68.7 ($\pm$ 8.4)a	14.0 ($\pm$ 1.7)a	4.3 ($\pm$ 0.2)a	18.2 ($\pm$ 4.2)a	0.643 ($\pm$ 0.09)ab	0.327 ($\pm$ 0.04)ab
2001 AC Rodeo	49.0 ($\pm$ 4.4)a	16.5 ($\pm$ 2.6)a	5.2 ($\pm$ 0.3)a	12.4 ($\pm$ 2.1)a	0.690 ($\pm$ 0.07)b	0.399 ($\pm$ 0.06)b

^a Within cultivars (rows), effective and ineffective treatments resulted in significantly different means for all three measured parameters (Tukey HSD, $p < 0.05$). Within treatments (columns) means followed by the same letter are not significantly different (Tukey HSD, $p > 0.05$).

effective rhizobia. Ratios for older cultivars appeared to exceed 1.0, but their 95% confidence intervals all included 1.0.

Under the mixed-inoculum treatment, there were no longer significant differences in absolute seed yield among cultivars (figure 2b). That means that the yield advantage of the new cultivars in the effective inoculum treatment (table 1) due to selection and breeding (Morrison *et al.* 1999, 2000) vanished when exposed to a mixed-inoculum treatment. Leaf chlorophyll estimates at podfill stage differed little among cultivars exposed to a mixed-inoculum treatment (data not shown). Measurements of seed N were highest in the old cultivar Flambeau in the mixed treatment and lowest in the new cultivar AC Harmony, but differences were small and insignificant among other cultivars (data not shown).

(b) Laboratory experiment

The two oldest and two newest cultivars were grown in an additional laboratory experiment to measure nodulation differences more accurately. Final nodule number per plant and plant dry weight were significantly less when inoculated with the ineffective strain than when inoculated with the effective strain ($p < 0.05$) for all cultivars (table 3). Nodule number did not differ among cultivars within treatments (table 3). Weight per nodule was higher, partly compensating for nodule number, as has been noted previously in soya bean (Ikeda 1999), when cultivars were inoculated with the ineffective strain than with the effective strain ($p < 0.05$, table 3). Within all three inoculum treatments, cultivars did not differ significantly from each other in weight per nodule ($p > 0.05$).

In the mixed treatment, the older cultivar Pagoda had a significantly larger proportion of the effective strain in its nodules (by plate counts) than new cultivar, AC Harmony (figure 3a). No other significant differences were found for proportion of effective strains among cultivars. Similarly, in the mixed treatment, g of N per plant was apparently lowest in AC Harmony, but differences were not statistically significant ($p > 0.05$) among the cultivars (figure 3b).

4. DISCUSSION

From the perspective of the legume host, the mixed rhizobium treatment is the closest approximation to both natural and agronomic conditions (Souza *et al.* 1994;

Figure 3. (a) Proportion of effective rhizobia from nodules of four cultivars of pouch-grown plants inoculated with a 1 : 1 mixture of effective : ineffective rhizobia. Nodules were harvested and pooled from each root system, sterilized and crushed to determine the proportion of effective to ineffective rhizobia per plant and (b) total nitrogen (N) per plant at harvest in mixed-inoculum treatment. Means within a graph followed by the same letter are not statistically significant (Tukey HSD $p < 0.05$). Legend key is the same as figure 1.

Hagen & Hamrick 1996; Silva *et al.* 1999; Denton *et al.* 2002; Fening & Danso 2002). The ability of cultivars to maximize benefits from a mixed rhizobial population, relative to an effective rhizobial population, was calculated as the yield difference ratio (i.e. yield difference for mixed/effective rhizobial treatments). Here we found a clear performance advantage of older cultivars (figure 2a).

Mixed rhizobial populations are ubiquitous. Are they a problem for legume hosts? Singleton & Stockinger (1983) found that plants inoculated with mixtures of effective and ineffective rhizobia were able to compensate for low-quality partners by allocating resources preferentially to

nodules containing the best strains, so that plants where only 75% nodules were effective still had 95% of the N accumulation of plants with 100% effective nodules. This is in agreement with our findings here for older soya bean genotypes. By contrast, Heath & Tiffin (2007) found that *Medicago truncatula* plants had lower fitness when inoculated with a 1 : 1 mixture of strains than when inoculated with the worse single strain alone, at least under high-N conditions. They suggested possible strain–strain antagonism in reducing plant performance. Our results for newer soya bean genotypes were between these two cases, in that the fitness benefits with mixed inoculum were better than with the ineffective strain, but worse than the effective strain.

Our experimental system differed from that of Heath & Tiffin (2007) in many ways, including: (i) larger differences in effectiveness among our strains (effective versus completely non-fixing), (ii) field versus greenhouse conditions, and (iii) legumes with determinate versus indeterminate nodules. The last difference may be of particular importance because the viability of rhizobia from some plants with indeterminate nodules (e.g. in *Medicago*) is apparently limited to those rhizobia not yet fixing N₂. Our results and those of Singleton & Stockinger (1983) were from soya beans, in whose determinate nodules the rhizobia that fix N₂ remain viable (Denison 2000). It is not yet understood how sanctions operate in multiply infected indeterminate nodules (Denison & Kiers 2004a), or how strains might interact within such nodules (Knapp *et al.* 1990). If their results reflect antagonism among strains within mixed nodules, this effect might be swamped when there are larger differences in mutualism (and sanctions) between strains.

In older cultivars, yield difference ratios apparently (but not significantly) greater than 1.0 (figure 2a) are surprising because they suggest that older cultivars were able to gain even more benefit from the mixed population of rhizobia than from the effective strain alone. Mycorrhizal fungi microcosm experiments (van der Heijden *et al.* 1998; Maherali & Klironomos 2007) have shown complementary benefits from mixtures of fungal strains, perhaps due to their ability to access different soil resources. However, similar complementarity between effective and ineffective rhizobium strains would not be expected.

The newer cultivars had a fitness advantage, as is expected with increased adaptation through progress in selection and breeding, when only effective rhizobia were present (table 1). This yield advantage of new cultivars (a trend estimated at 0.5% per year yield increase, Morrison *et al.* (1999)) disappeared when we simulated a more realistic mixture of rhizobia, combining effective and ineffective strains (figure 2b). Given that mixed rhizobium soils are the norm, these results raise the question of whether older (and generally lower yielding) cultivars, with their ability to reduce yield losses under mixed-inoculation conditions, may be preferable genotypes in soils where ineffective rhizobia are common, or at least useful in breeding for those conditions.

This ability of certain plant genotypes to perform better under mixed conditions may be due to strain selectivity of better rhizobium strains into nodules or post-infection sanctions, in which plants detect and senesce (or at least reduce resource supply to) nodules containing

less-effective symbionts (Denison 2000; Kiers *et al.* 2003). The resulting differences among strains in nodulation success, and in rhizobia per nodule, are also key to future rhizobial strain fitness (Moawad *et al.* 1984; Brockwell *et al.* 1987). When cultivars were inoculated with only an ineffective strain in our laboratory experiment, we found no significant difference among genotypes. All cultivars had a similarly low absolute number of nodules when inoculated with this particular ineffective strain (table 3). With mixed inoculation, however, we found a small difference in apparent strain selectivity among cultivars, with higher proportions of effective rhizobia in the nodules of the older cultivar Pagoda (figure 3a).

The effective strain occupied up to 10 times as many nodules as the ineffective strain, with single-strain inoculation (table 3), yet the effective strain occupied no more than 60% of nodules with mixed inoculation (figure 3). It seems unlikely that this discrepancy can be explained by differences in survival in the rhizosphere. Instead, host plants must respond differently to the ineffective strain when the effective strain was present rather than absent. This could reflect either host selectivity during the infection process or early nodule abortion based on failure to fix N₂ (an example of sanctions). Some previous work has shown that ineffective rhizobia can be just as competitive for nodulation (Kuykendall & Elkan 1976; Amarger 1981; Hahn & Studer 1986). On the other hand, soya beans exclude some (but not all) of rhizobia with a particularly harmful, chlorosis-inducing phenotype (Devine & Kuykendall 1996) and there is evidence of selectivity of specific *B. japonicum* strains by soya bean (Caldwell & Vest 1968; Ferrey *et al.* 1994) and *Medicago truncatula* (Heath & Tiffin 2007) genotypes. The extent to which recognition systems can effectively exclude ineffective rhizobia under typically mixed field conditions deserves further attention (Savka *et al.* 2002).

5. CONCLUSIONS

Under conditions of multiple infection, enforcement mechanisms against poor-quality partners are particularly important in stabilizing cooperation. Through decades of artificial selection, have these enforcement mechanisms in legumes been compromised? Results from our field and laboratory experiments suggest that older cultivars may be better able to limit losses to mixed N₂-fixing bacteria populations that vary in quality. This may not be the only case of human interference in symbiotic stability. In the absence of positive selection by breeders for improved symbiosis, less-mutualistic symbiotic relationships may have similarly arisen in the arbuscular mycorrhizal symbiosis (Manske 1990; Johnson & Pfleger 1992). As in the legume symbiosis, host plants are typically infected by multiple mycorrhizal symbionts (Vandenkoornhuyse *et al.* 2002; Scheublin *et al.* 2004).

Interactions among symbionts infecting the same host plant deserve additional attention, given the contrast between our results, especially for older cultivars, and those of Heath & Tiffin (2007). Antagonistic interactions between rhizobium strains within the same nodule are plausible, perhaps involving bacteriocins (Goel *et al.* 1999), but only if different strains occupy the same nodules. Because nodules containing more than one rhizobial strain would also undermine nodule-level

sanctions (Denison 2000), they should be a priority for future research.

We wish to thank Andy Gardner, Jason Hoeksema and Stuart West for comments that greatly improved the manuscript; Malcolm Morrison and Elroy Cober for soya bean germplasm and advice on experimental design; Andy Hector, Laura Lewis, Chris Hartley and Larry Tuber for help with the statistical analysis; Peter Graham for novel laboratory techniques; Highmoor Farm for field advice; Tim Griffin for local seeds and Raivo Vihman, Matt Spurlock, Amy and Jeff Burchstead, Dana Pratt, Johanna Horn and the JED collective for gruelling field work.

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