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# An experimental and modelling exploration of the host-sanction hypothesis in legume–rhizobia mutualism

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ABSTRACT

Despite the importance of mutualism as a key ecological process, its persistence in nature is difficult to explain since the existence of exploitative, "cheating" partners that could erode the interaction is common. By analogy with the proposed policing strategy stabilizing intraspecific cooperation, host sanctions against non-$N_2$ fixing, cheating symbionts have been proposed as a force stabilizing mutualism in legume–*Rhizobium* symbiosis. Following this proposal, penalizations would include decreased nodular rhizobial viability and/or early nodule senescence in nodules occupied by cheating rhizobia. In this work, we analyse the stability of *Rhizobium*–legume symbiosis when non-fixing, cheating strains are present, using an experimental and modelling approach. We used split-root experiments with soybean plants inoculated with two rhizobial strains, a cooperative, normal $N_2$ fixing strain and an isogenic non-fixing, "perfect" cheating mutant derivative that lacks nitrogenase activity but has the same nodulation abilities inoculated to split-root plants. We found no experimental evidence of functioning plant host sanctions to cheater rhizobia based on nodular rhizobia viability and nodule senescence and maturity molecular markers. Based on these experiments, we developed a population dynamic model with and without the inclusion of plant host sanctions. We show that plant populations persist in spite of the presence of cheating rhizobia without the need of incorporating any sanction against the cheater populations in the model, under the realistic assumption that plants can at least get some amount of fixed $N_2$ from the effectively mutualistic rhizobia occupying some nodules. Inclusion of plant sanctions leads to the unrealistic effect of ultimate extinction of cheater strains in soil. Our simulation results are in agreement with increasing experimental evidence and theoretical work showing that mutualisms can persist in presence of cheating partners.



## 1. Introduction

The origin and persistence of mutualism are difficult to explain since the existence of exploitative, "cheating" partners taking benefits but not reciprocating is common (Bronstein, 2001). In the mutualism established between legumes and soil bacteria known as rhizobia, bacteria from soil infect plant's roots and reproduce and differentiate inside root nodules into bacteroids, which are able to fix atmospheric $N_2$ for plant nutrition, receiving carbohydrates in exchange. After nodule senescence, surviving rhizobia are released into the soil where, depending on their viability, they can maintain resident populations (Hirsch, 1996) and reinfect plant's roots in next growing cycle. Plant–rhizobia symbiosis is an example of horizontally transmitted mutualism, since bacteria are acquired from soil and not vertically transmitted from adult plant to seeds.

The occurrence of low $N_2$-fixing and ineffective rhizobia cheating strains in the same plant is common (Singleton and Tavares, 1986; Bronstein, 2001), and accumulation of resources by some non-fixing rhizobia in bacteroid stage has been proposed as cheating advantage at plant's expenses (Denison, 2000). However, this accumulation is a general metabolic consequence of reduced carbon demand from the plant (Lodwig et al., 2003) and not necessarily implies rhizobia further survival advantages (Streeter et al., 1995). Decreased nodular rhizobial viability and/or early nodule senescence have been proposed as plant host sanctions against non-$N_2$ fixing, cheating rhizobia (Denison, 2000; Kiers

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et al., 2003, 2006). A decrease in rhizobial viability was reported when $N_2$-fixing rhizobia were "forced" to cheat soybean plants by replacing normal, $N_2$ containing atmosphere by an Ar:$O_2$ mixture (Kiers et al., 2003, 2006). However, this approach does not really test a sanction from the plant to a true cheating rhizobium sharing the same plant with an effective strain. Besides, exposure to an Ar:$O_2$ atmosphere per se reduces nodule $O_2$ concentration in soybean nodules due to decrease in $O_2$ nodule permeability through a not yet entirely elucidated mechanism (King and Layzell, 1991; Diaz del Castillo and Layzell, 1995; Wei and Layzell, 2006). Therefore we re-examined the plant host-sanctions hypothesis using an experimental method avoiding potentially confounding effects.

We tested the host plant sanction hypothesis using split-root soybean plants of Osumi cultivars, inoculated with two strains of *Bradyrhizobium japonicum*, a highly efficient nitrogen fixing wild-type strain USDA110, and its non-fixing, nifH mutant derivative H1 (Hahn et al., 1984). We tested experimentally the two proposed sanctions, that the plant would reduce viability of cheating rhizobia inside nodules, performing viable rhizobia counts from nodules, and that the plant would cause early senescence of nodules occupied by the cheating strain, by measuring the relative expression of gene markers for nodule senescence and maturity (Alesandrini et al., 2003).

The plant-level experiment we performed allows us to unequivocally test the plant–host-sanction hypothesis. However, the relevant level for studying the long-term behaviour of the system is population level. Since at this level it is not straightforward to perform experiments similar to those we conducted on plants, we studied the long-term dynamics using a population modelling approach. Few modelling attempts on legume–rhizobia mutualism have been made, and the available examples deal with spatial structure of rhizobia and evolution of nitrogen fixation (Bever and Simms, 2000), population genetics of rhizobia (Provorov and Vorobyov, 2000) and the stability of symbiosis mediated by kin selection and plant sanctions against cheating rhizobia (West et al., 2002a, b). Here, based on our experimental approach and results, we analysed the ecological stability of *Rhizobium*–legume symbiosis when "cheating" strains are present, using a population dynamics model with and without the inclusion of plant host sanctions.

## 2. Experimental test of plant sanction hypothesis

### 2.1. Plant split-root experimental setting

Seeds of soybean (*Glycine max*) cultivar Osumi were surface sterilized and germinated. Tip root was removed to generate regrowth of two equally sized half-roots, each placed in a glass tube containing sterilized $N_2$ free liquid Fahraeus nutrient solution (Vincent, 1970). Each tube was inoculated and sealed to prevent cross-contamination, with the appropriate strain of *Bradyrhizobium japonicum*, either the wild type, normally $N_2$ fixing USDA 110 or the Nod+ Fix-, nifH::Tn5 mutant H1 derived from the wild type (Hahn et al., 1984) in the following treatments: half roots of the same plant (USDA110-1/H1-1), or in both roots of the same plant (USDA110-2 or H1-2) (Fig. 1). H1 represents the "perfect" rhizobium cheater since it lacks nitrogenase (the $N_2$ fixing enzyme) activity but shows similar infection and nodule formation levels respect to the wild-type (Hahn et al., 1984). Each tube was carefully filled with nutrient solution as needed, while maintaining the other tube sealed. Plants were placed in a growth chamber with 16 h and 600 $\mu E\,m^{-2}\,s^{-1}$ photosynthetically active radiation at 25 °C, and 8 h darkness at 18 °C. Control uninoculated plants showed no nodulation. Nodule numbers were counted in each half root every three days until nodule production reached

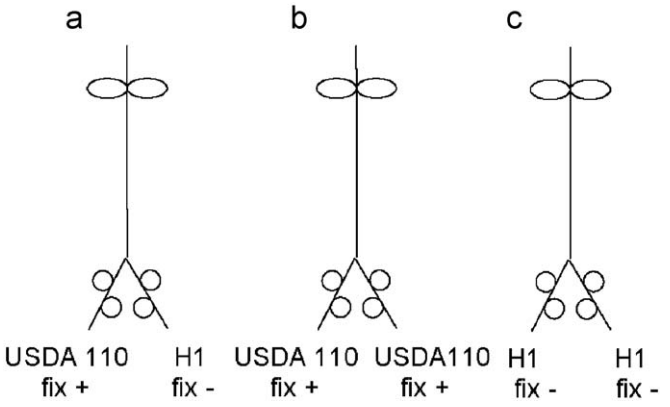


**Fig. 1.** Schematic representation of the split-root plant experiment to test the plant sanction hypothesis. Split roots in each plant were inoculated with *B. japonicum*, either the $N_2$ fixing strain (USDA 110, fix+), or the non-fixing strain (H1, fix–), in three treatments, USDA 110/H1-1 (a), USDA 110-2 (b) or H1-2 (c). At weeks 3, 4 and 5 after inoculation, nodules (represented by circles in roots) were harvested to count viable rhizobia, and to determine expression of senescence and maturity nodule molecular markers.

a plateau. Three, four and five weeks after inoculation nodules of each half root of five plants/treatment were collected. Two nodules per half root were independently weighted and used immediately for rhizobia viable counts. Groups of the remaining nodules were weighted and immediately storaged at −80 °C for further determination of nodule gene marker expression.

### 2.2. Viable rhizobial counts

Two individual nodules from each half-root from five to three plants per treatment for each date were surface sterilized using $Cl_2Hg$ (2.5%), manually crushed, homogenized and resuspended in a buffer containing 0.05 M Tris–HCL and 0.25 mannitol. Appropriate serial dilutions were plated (two replicates per dilution) in yeast extract-mannitol (YEM; Vincent, 1970) supplemented with selective antibiotics depending on the strain (Spc for USDA 110 and Spc+Kan for H1). Plates were incubated at 28 °C for a week or until no further growth was detected, and colony-forming units (c.f.u.) were counted. C.f.u. numbers per nodule and per nodule mass were compared using paired *t*-test analysis on untransformed data (*n* between 10 and 6).

### 2.3. Nodule gene expression

cDNA markers differentially expressed in mature (DD10) and senescent (DD15) soybean nodules (Alesandrini et al., 2003) were used to assess the developmental stage of nodules and to detect any early senescence in the different treatments. DD10 expression increases with nodule development reaching a peak with nodule maturity and then decreases slowly with nodule age, while DD15 expresses only in senescent nodules. Total RNA was extracted and treated with DNAse I (RNeasy Kit, Qiagen) from two nodule groups from each half-root of two plants of each treatment for weeks 3, 4 and 5, previously weighted and frozen (individual nodules did not yield enough RNA). Expression of the nodule markers of senescence DD15 and maturity DD10 was assessed using quantitative real-time PCR (RT-qPCR), with the soybean 18S ribosomal subunit as internal control, using three dilutions and appropriate controls. 20-mer primers were designed with a G/C content of 50–60%, and a Tm of about 60 °C. Length of PCR products ranged between 152 and 180 bp. Primer design software (Primer3) was used to select primer sequences. Secondary structures and dimer formation were checked (Oligo Analyzer

3.0 software). Designed DD15 primers 5′-TGGTTTTCTCCTCCTG-CTGATT-3′ and 5′-GGCAGCATACTCACTTTCACTT-3′, DD10 primers 5′-AGAAGAAGCTGGTGGTATTGGT-3′and 5′-GGAGTTGCTGAGATTG-GATTGA-3′, and 18S primers 5′-TACAACGCGCAAAACCTTACCA-3′and 5′-GTTTCGCTCGTTATAGGACTTG-3′ were purchased from Roche. RT-qPCR was performed with a iCycler iQ real-time PCR detection system from Bio-Rad. Primer efficiencies were between 85% an 100%. RT-qPCR was performed with a iCycler iQ real-time PCR detection system from Bio-Rad, using Reverse Transcriptase SuperScript II and Platinum Taq DNA polymerase (Invitrogen). The cycling program was 1 cycle: 5 min at 94 °C, 30 cycles: 1 min at 94 °C, 1 min at 60 °C and 30 s at 72 °C, and 1 cycle: 10 min at 72 °C. Transcript expression levels of DD15 and DD10 were related to the expression levels of the soybean 18S gene that served as an internal standard. We therefore expressed the standardized transcript expression ct levels as DD15/18S and DD10/18S ratios. Ct ratio values were compared using paired *t*-test analysis ($n = 12$). We checked statistical assumptions for using the *t* test, and they were fairly met in most cases. In a few cases where there was a small departure from normal distribution assumptions we performed non-parametric tests (Mann–Whitney *U*-test), and we found that results were the same as using the *t*-test.

### 2.4. Experimental results

The cheater rhizobial strain showed similar infection and nodule formation levels and temporal patterns respect to the wild-type (Fig. 2). Addressing the first sanction mechanism proposed in the experimental test, results from the rhizobial viability experiments show that the plant is able of tolerating cheating by non-fixing rhizobia when it can get some amount of fixed $N_2$ from at least half of total plant nodules. Obviously, plants with all nodules occupied by cheating rhizobia are not able of maintaining good vegetative conditions and high rhizobia populations as plants partially or exclusively associated with fixing rhizobia (Fig. S1a and b), and ultimately they die due to N starvation about 6 weeks after inoculation (Fig. S1c). Viability of the cheating, non-fixing strain per nodule and per nodule mass was not significantly lower comparing half roots of the same plant separately inoculated with each strain for the two soybean varieties (Fig. 3a and b). Comparing treatments where both half roots of each plant were inoculated with the same strain, cheating rhizobia viability was significantly lower (Fig. 3a and b). In addition, we found no evidence of early nodule senescence in nodules occupied by cheating rhizobia when compared with half roots inoculated with the $N_2$-fixing strain in the same plant (Fig. 4a). Plants with both roots inoculated with the cheating strain showed decreased expression of the senescence marker compared with plants inoculated only with the $N_2$-fixing strain (Fig. 4a). This inversely correlates with the expression of the molecular marker for nodule maturity, showing increased expression in plants with both half roots inoculated with the cheating strain (Fig. 4b).

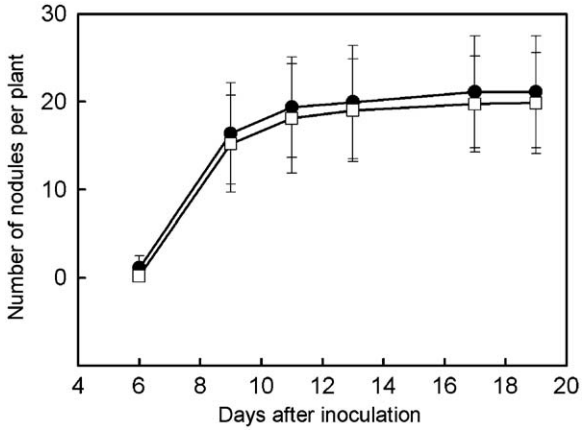


**Fig. 2.** Temporal pattern of nodule production (means ±1s.d.) in the split-root experiment. Nodule numbers were counted in each half root every three days until nodule production reached a plateau, in half roots of the same plant inoculated with the fixing USDA110 strain (circles) or the non-fixing, cheating strain H1 (squares) (treatment USDA110-1/H1-1).

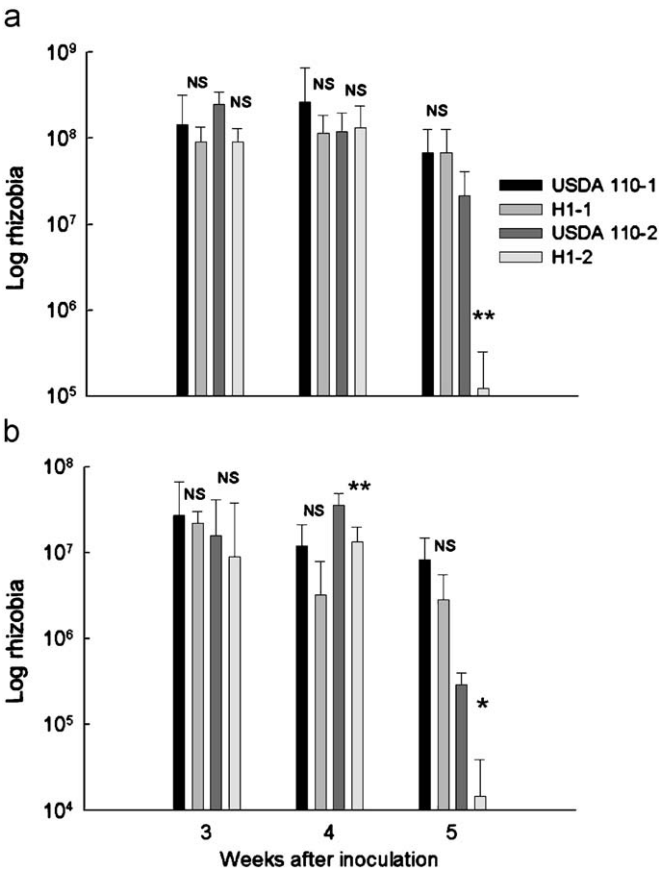


**Fig. 3.** Rhizobia viability per nodule (a) and per nodule mass (b) in the soybean plant split-root experiments. Rhizobia inside nodules infected by the $N_2$-fixing USDA110 strain or the non-fixing, cheating strain H1, either in half roots of the same plant (USDA110-1/H1-1), or in both roots of the same plant (USDA110-2 or H1-2) were counted as colony-forming units (c.f.u.) three, four and five weeks after inoculation. $^{*}P<0.05$, $^{**}P<0.01$ significant differences by paired *t*-tests performed on untransformed data. Bars are means ±1s.d.

## 3. Model development and biological background

The model is built up on the grounds of an experimental approach allowing to directly and unambiguously testing a potential sanction from the plant to a true cheating rhizobium sharing the same plant with an effective strain. To evaluate the effect of the sanctions on the simplest scenario possible, and in agreement with the experimental design, we did not include factors like strain competition and availability of N from soil. We based the model formulation on several biological features of the mutualistic system and the following assumptions, either checked or supported by the experimental test:

- Fixing and non-fixing bacterial strains only differ in their $N_2$ fixing ability, and they have the same ecological abilities (same competition levels in soil and nodule initiation).

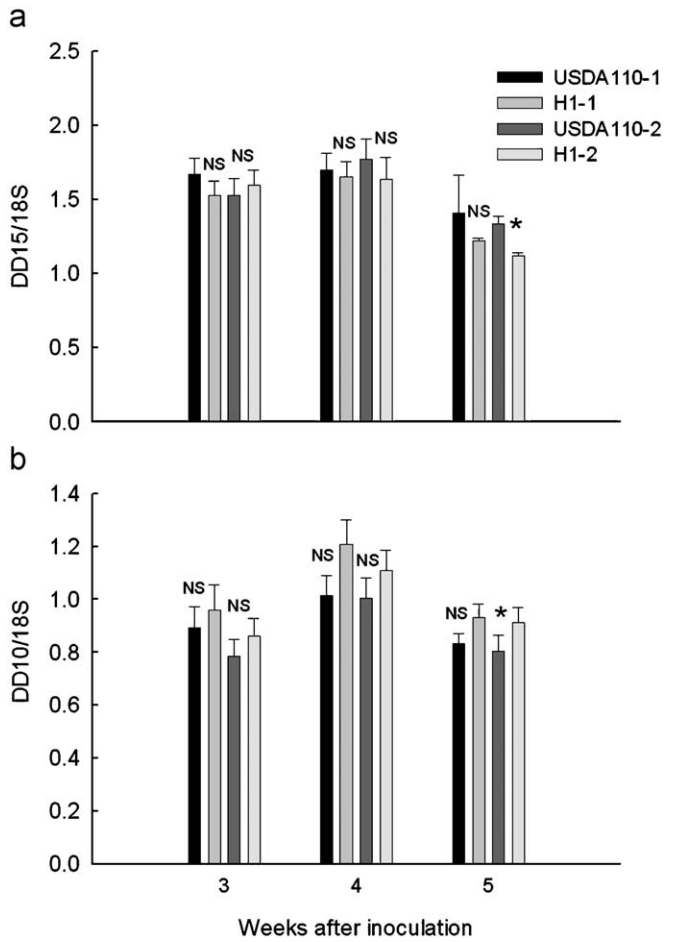


**Fig. 4.** Relative expressions of gene markers DD15 of nodule senescence (a) and DD10 of nodule maturity (b) in nodules from the soybean plant split-root experiments. $^*P<0.05$ significant differences by paired $t$-tests. Bars are means $\pm$1s.d. Treatments as in Fig. 1.

- Nodules are initiated and occupied by a single bacterium of either fixing or non-fixing strain.
- Nodules are occupied to their carrying capacity, are functionally equivalent and metabolically independent of each other.
- At the end of each annual cycle nodules undergo senescence and release surviving bacteria into the soil.
- Fixing and non-fixing nodules can develop and coexist in the same plant.

We discuss next the biological background of the assumptions.

Cheating rhizobia can vary in their $N_2$ fixation ability, from no fixation to low fixation levels compared with highly effective strains. To simplify the system, we deal here with a mutated *Rhizobium* lacking fixation activity but showing similar competitive abilities in soil and infection and nodule formation levels respect to the effective wild-type (Hahn et al., 1984), i.e., the "perfect" *Rhizobium* cheater, which we used in the experimental test. We experimentally checked that nodulation abilities were the same for the two strains (Fig. 2).

The process of encountering between rhizobia and plant is not random, since it involves production of compounds from the plant to attract specific rhizobia into the soil close to roots and competition between rhizobia for root colonization among other factors. However, we can simplify the nodule generation process assuming random probability, since we assume equal ecological abilities and conditions for the mutant and effective strains in soil. A minimum number of compatible rhizobia in the soil is needed to trigger nodule initiation (Amarger and Lobreau, 1982), represented in the plant experiment by the initial amount of bacterial culture inoculated to the plants. We set the time scale to one year, assuming an annual plant and a slow rhizobial turnover in soil. Rhizobial generation times in soil can be very low, affected by environmental conditions like temperature (Wood and Cooper, 1988). The nodule bacteria system is composed by the bacteria growing inside nodules. Each nodule is initiated by a single bacterium that subsequently divides and the derived population fills in the nodule (Gage et al., 1996). Dynamics of bacteria within the nodule is much faster relative to dynamics in the soil free-living state (Gage et al., 1996). After some time, bacterial reproduction in the nodule is constrained, and a nodule carrying capacity for rhizobia is reached. Given that rhizobial population equilibrium inside nodules is reached in a much shorter period than that of bacteria in soil, we assume instantaneous equilibrium and ignore the different stages of nodule development. At the end of the plant's annual growth cycle the nodules undergo senescence and the rhizobia inside them are released into the soil. As previously stated, the number of bacteria coming to the soil from nodules occupied by fixing and non-fixing bacteria can vary if plant sanctions are assumed. In the plant experiment this was tested determining the viability of rhizobia recovered from nodules occupied either by fixing or non-fixing strains.

We modelled the mutualistic plant–rhizobia system described above using three simple logistic mappings. One map represents the plant population and the other two account for the populations of free bacteria living in the soil closely surrounding the root, fixing and non-fixing bacteria. Fig. 5 shows a scheme of the model. We now describe these maps in detail.

We describe the fixing and non-fixing bacterial populations in soil by two coupled logistic maps, modified to take into account the bacteria coming into the soil from the senescent nodules:

$$p_i(t+1) = (p_i(t) + \Delta p_i(t))\left[1 + r_i^s\left(1 - \frac{P_T(t)}{\delta_s}\right)\right] \quad i = +,- \tag{1}$$

$$P_T(t) = p_-(t) + \Delta p_-(t) + p_+(t) + \Delta p_+^N(t) \tag{2}$$

where $p_i$ describes the bacteria population densities in soil, $i = +$, $-$ indicates fixing and non-fixing bacteria, respectively, and $P_T$ is total bacteria population density (i.e., fixing+non-fixing) in soil. The parameter $\delta_s$ stands for the carrying capacity of the soil close to roots in absence of nodulation. An effective carrying capacity greater than $\delta_s$ is set when plants release bacteria at the end of nodulation process. The parameters $r_i^s$ represent the intrinsic reproduction rate of each population in the soil close to roots. Since we are assuming that the only difference between bacterial strains is their nitrogen fixing ability, we will take $r_+^s = r_-^s = r_s$.

The number of the surviving bacteria that returns to the soil ($f_i$) is calculated as $\Delta p_i(t)$. Plants are not able of differentiating fixing

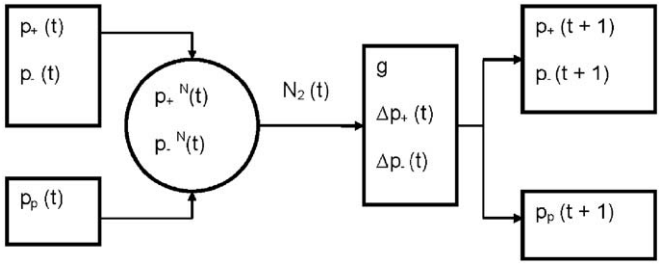


**Fig. 5.** Schematic structure of the model dynamics in a single iteration. Initial values of plant and bacteria populations ($P_p$ for plants; $p_+$ and $p_-$ for bacteria) set the values of bacteria in nodules ($p_+^N$ and $p_-^N$). The bacteria in nodules provide $N_2$ to the plants and the new populations are calculated based on the produced seeds ($g$) and the released bacteria ($\Delta p_+$ and $\Delta p_-$).

from non-fixing bacteria during the root colonization process (Amarger, 1981). Besides, we assume no selection of rhizobia by plants inside nodules, thus we consider $f_i$ the same for both types of bacteria (about $10^{-4}$ of the carrying capacity of a nodule). However, it has been suggested that the plant can recognize the bacterial strains a posteriori on the basis of their fixing ability once they are inside nodules (Denison, 2000; West et al., 2002a). If plants can recognize and sanction the non-fixing rhizobia, the surviving number of non-fixing rhizobia would be lower than the surviving number of the fixing ones (Kiers et al., 2003). To simulate this situation in our model, we allowed the number $f_i$ of surviving bacteria of each type to be different, i.e.

$$\Delta p_i(t) = f_i \frac{\delta_n}{m_s} K_i^N(t) \quad i = +, - \tag{3}$$

where $\delta_n$ is the carrying capacity of each nodule type, $m_s$ is the mass of soil per hectare associated to the crop and $f_+ = f$, $f_- = f(1-\sigma)$. The parameter $\sigma$ represents the sanction intensity the plant applies to the non-fixing bacteria. Its value goes from 0 to 1, where $\sigma = 0$ represents the case without sanction. The number of nodules generated by each type of bacterial strain is $K_i^N(t)$, and it represents a fraction of the total root colonisable sites for nodule initiation $K_s(t)$. According to the hypotheses of this model both rhizobial strains have the same ability to colonize the root and initiate nodules, hence assuming random colonization,

$$K_i^N(t) = \frac{p_i(t)}{p_+(t) + p_-(t)} K_s(t) \Theta(p_i(t) - p_m) \quad i = +, - \tag{4}$$

where $\Theta(x)$ is the step function, i.e., $\Theta(x) = 1$ when $x \geqslant 0$ and $\Theta(x) = 0$ otherwise; the threshold $p_m$ is the minimum bacteria population per g of soil needed to trigger the nodulation process. The maps representing the free bacteria in the soil are coupled to the plant system through the factor $K_s$ (total root colonisable sites for nodule initiation). The more plants there are in the system, the more available colonisable sites there are for nodule initiation. In a first approximation, $K_s$ can be considered proportional to the plant population $P_p(t)$ (number of plant per hectare), i.e.,

$$K_s(t) = nP_p(t) \tag{5}$$

where $n$ is the average number of nodules per plant.

Defining the fraction of fixing bacteria as

$$\alpha(t) = \frac{p_+(t)}{p_+(t) + p_-(t)} \tag{6}$$

and using Eqs. (3)–(6) the maps (1) and (2) can be written as

$$p_+(t+1) = \left(p_+(t) + f_+ \frac{\delta_n}{m_s} \alpha(t) K_s(t) \Theta[p_+(t) - p_m]\right) \times \left[1 + r_s\left(1 - \frac{P_T(t)}{\delta_s}\right)\right] \tag{7}$$

$$p_-(t+1) = \left(p_-(t) + f_- \frac{\delta_n}{m_s} [1 - \alpha(t)] K_s(t) \Theta[p_-(t) - p_m]\right) \times \left[1 + r_s\left(1 - \frac{P_T(t)}{\delta_s}\right)\right] \tag{8}$$

$$P_T(t) = p_-(t) + p_+(t) + \frac{\delta_n}{m_s} \alpha(t) n P_p(t) \times [f_+ \Theta(p_+(t) - p_m) - f_- \Theta(p_-(t) - p_m)] + f_- \frac{\delta_n}{m_s} n P_p(t) \Theta(p_-(t) - p_m) \tag{9}$$

The plant population dynamics can be described by a model of plant spread previously published (Cannas et al., 2003). Briefly, if $\delta_p$ is the carrying capacity of the field where the plants grow, the density population per unit field area is $P_p(t)/\delta_p$. Suppose that such area receives at time $t$+1 $z_s$ seeds from the plant population at time $t$ and that $P_g$ is the probability that a seed germinates and develops into an adult plant. Then, the plant population at time $t$+1 can be assumed proportional to the probability that at least one of the received seeds give rise to an adult plant, i.e.

$$P_p(t+1)/\delta_p = 1 - (1 - P_g)^{z_s}$$

If $g$ is the mean number of seeds produced by a plant in an annual crop, then $z_s = g(t)\, P_p(t)/\delta_p$ and the plant population dynamics is described by

$$P_p(t+1) = \delta_p \left[1 - \exp\left(-g(t) \frac{|\ln(1 - P_g)| P_p(t)}{\delta_p}\right)\right] \tag{10}$$

The number of seeds depends on the amount of available nitrogen for the plants at time $t$. The more nitrogen is available to the plants, the more seeds they produce. We will assume that the amount of nitrogen a plant can obtain depends only on the number of nodules colonized by fixing bacteria; hence, $g$ will be a monotonously increasing function of $K_+^N(t)$. It is also reasonable to assume that there is a maximum number of seeds a plant can produce, denoted as $G$. On the other hand, if there is not enough nitrogen to support the plant seed production, the number of seeds should drop to zero. This means that there is a minimum number of nodules colonized by fixing bacteria required to produce seeds, $K_0$. All the previous assumptions can be modelled by the following expression:

$$g(t) = \begin{cases} G \tanh\left(\dfrac{K_+^N(t) - K_0 P_p(t)}{G P_p(t)}\right) & \text{if } K_+^N(t) - K_0 P_p(t) > 0 \\ 0 & \text{otherwise} \end{cases} \tag{11}$$

Using Eqs. (4)–(6) we arrive to the expression

$$g(t) = \begin{cases} G \tanh\left(\dfrac{\alpha(t) n - K_0}{G}\right) & \text{if } \alpha(t) n > K_0 \text{ and } p_+(t) > p_m \\ 0 & \text{otherwise} \end{cases} \tag{12}$$

It can be noticed that the step function in the mappings for the bacteria Eqs. (7)–(9) acts as a switch, turning on or off the coupling with the plant system. If any bacterial population is below the value of $p_m$ then it does not interact with the plant system and its dynamic is entirely given by its own logistic dynamic in the soil.

### 3.1. Model analysis and results

In this section we compare the behaviour of the model for different values of $\alpha$ and $\sigma = 0$ (without sanction), $\sigma = 0.5$ (moderate sanction) and $\sigma = 1$ (total sanction). In Table 1 we show the values of the parameters that were held constant through the numerical simulations.

#### 3.1.1. No sanction

Under no sanction ($\sigma = 0$), the plants are unable to discriminate among fixing and non-fixing bacteria, and so there is no strain selection. Hence, in our model $f_+ = f_-$, i.e. the number of surviving bacteria that returns to the soil is the same for both type of bacteria. For simplicity, we will consider first the case $p_m = 0$, which describes the limit behaviour when the bacteria populations are larger than $p_m$. When $\sigma = p_m = 0$ the number of fixing bacteria $\alpha$ does not change with time and thus the relative proportion of bacterial populations is determined by its initial value $\alpha(t) = \alpha(0)$. A demonstration is shown in Appendix A.

We found a critical value $\alpha_c$, such that two different dynamical regimes can be distinguished. When $\alpha \leqslant \alpha_c$ the dynamics leads always to the extinction of plants; the smaller the $\alpha$ value, the faster the extinction. This can be understood by looking at Eqs. (10) and (12). If the initial number of fixing bacteria is too

**Table 1**
Parameter values used in the simulations.

| Parameter | Value | Description |
|---|---|---|
| $r_s$ | $10^{-4}$ | Intrinsic rate of growth of bacteria in the soil |
| $\delta_s$ | $10^6\,g^{-1}$ | Soil bacterial carrying capacity (per g of soil) |
| $\delta_n$ | $10^6$ | Nodule's carrying capacity (bacteria per nodule) |
| $f_i$ | $10^{-4}$ of the carrying capacity of a nodule | Surviving bacteria of each type released from nodule |
| $\delta_p$ | $2\times10^5\,ha^{-1}$ | Plants' field carrying capacity |
| $m_s$ | $1.5\times10^5\,g\,ha^{-1}$ | Soil mass per hectare associated to the plant population |
| $n$ | 45 | Typical number of nodules per plant |
| $K_0$ | $0.15\times n$ | Minimum number of fixing nodules per plant needed for seed production |
| $G$ | 55 | Maximum number of viable seeds produced per plant |
| $P_g$ | 0.69 | Probability of a viable seed reaching the adult stage |
| $\sigma$ | 0–1 | Sanction intensity: 0 = no sanction and 1 = maximum sanction |
| $p_m$ | $0–10^3\,g^{-1}$ | Minimum bacteria population per g of soil needed to trigger the nodulation process |

low, very few nodules are created (low fixation levels of $N_2$), the production of seeds is low and therefore the plant population decreases. Since the number of seeds $g$ depends on the bacterial populations only through $\alpha$ and this number remains constant in time (therefore $g$ is also independent of time), the plant population always decreases, even when the fixing bacteria population increases. Once the plants went extinct, the bacterial populations in the soil follow a logistic dynamics until they become stationary. On the other hand, when $\alpha>\alpha_c$ the plant population always reaches a non-zero stationary value. The closer the value of $\alpha$ is to $\alpha_c$ the slower is the convergence to the stationary situation. More details on how the critical value of $\alpha$ can be obtained analytically are given in Appendix B. For the set of parameters values used in this work we have $\alpha_c=0.169$.

When $p_m\neq0$, $\alpha$ changes with time when $p_m>0$ and the overall behaviour depends on the initial values of both types of bacterial populations, instead of depending only on its ratio $\alpha(0)$. The behaviour of the final plant population is more complex now, since it depends on whether $\alpha(t)$ overcomes the critical value $\alpha_c$ (see Appendix B) during the dynamics of the coupled system. However, we found through numerical solutions of the dynamical equations that again both bacterial and plant populations always reach a stationary value for long times. We calculated numerically the dynamics of the system for $p_m=10^3\,g^{-1}$.

If the initial populations of both type of bacteria are below the threshold $p_m$, their dynamics are completely decoupled from the plant system and they develop logistically, while the plants go extinct after the first iteration. If the initial populations of both type of bacteria are above the threshold $p_m$, the dynamics is exactly the same as in the $p_m=0$ case, so again plants survive when $\alpha(0)>\alpha_c$. The main difference with the $p_m=0$ case is that the plant population goes always extinct when $p_+(0)<p_m$, no matter the value of $\alpha(0)$. Fig. 6a shows the typical behaviour of $p_+$, $p_-$ and $P_p$, for $\sigma=0$, $\alpha=0.169$ $\alpha_c$ and $p_m>0$. Simulation started with $\alpha=0.2$, to show that even starting with low population values of fixing rhizobia and comparatively high populations values of non-fixing rhizobia, coexistence between strains is met in the long term. Plants persist at the carrying capacity values since very early simulation steps.

### 3.1.2. Effects of sanction

With moderate sanction ($\sigma=0.5$), i.e., half of the nodules prevented from releasing bacteria into the soil, the plant population survives equally well, but, as expected, a substantial reduction in $p_-$ numbers can be seen and $p_-$ population remains very close to zero. (Fig. 6b). With extreme sanction ($\sigma=1$), the plants halt all the non-fixing bacteria inside the nodules coming into the soil and thus they go extinct very fast (Fig. 6c). The fixing bacterial populations grow faster due to the reinsertion of the bacteria coming from the senescent nodules. It is clear that $\alpha$, the number of fixing bacteria, will increase with time and eventually go to 1. This means that, in the long term when the plant population persists by applying sanctions, only fixing rhizobia will be present in the system.

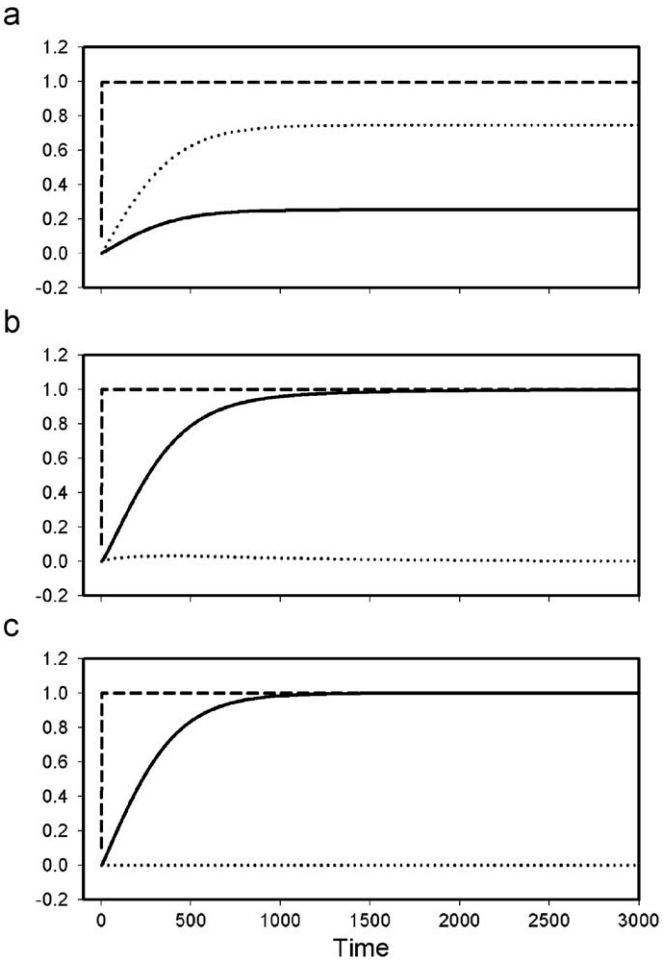


**Fig. 6.** Temporal behaviour of the variables $P_p$ (dashed line), $p_+$ (solid line) and $p_-$ (dotted line) for $\alpha=1.5$ $\alpha_c$, $P_p(0)=0.1$ $\delta_p$, and different values of $\sigma$. (a) $\sigma=0$ (no sanction), (b) $\sigma=0.5$ (moderate sanction), and (c) $\sigma=1$ (total sanction). Plant population is normalized to its carrying capacity and bacterial populations are normalized to their effective carrying capacities. Time is measured in number of iterations. Note that with moderate sanction $p_-$population remains very close to zero.

We calculated numerically the dynamics of the system for $p_m=10^3\,g^{-1}$. When $\alpha<\alpha_c$ and/or $p_+(0)<p_m$ the plant population goes extinct after a few iterations (not shown). When $\alpha>\alpha_c$ and $p_+>p_m$ the population of non-fixing bacteria slowly decreases while the fixing bacteria and plant populations increase until they reach their carrying capacities. The main difference with the case without sanction is that when $p_+>p_m$ the plant population can persist even when the initial proportion of fixing bacteria is smaller than $\alpha_c$, depending on the value of $p_+(0)$. For values of $\alpha$ smaller but close to $\alpha_c$ the plant population can show a non-monotonous behaviour. In this case the plant population decreases in the first iterations but the remaining plants are enough to increase the population of fixing bacteria so that $\alpha(t)$

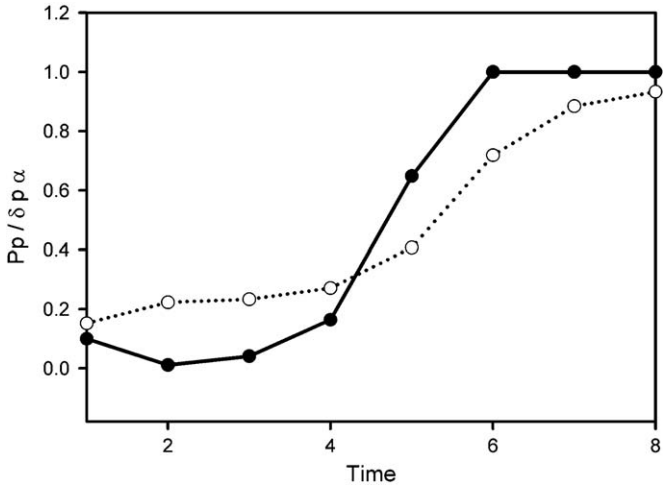


**Fig. 7.** The effect of total sanction on final plant population (fraction of carrying capacity $\delta_p$). $P_p/\delta_p$ (filled circles), $\alpha$ (empty circles). $\alpha_c$ is the dotted horizontal line. When $\alpha$ exceeds the critical value the plant population starts increasing. The initial value of $\alpha$ is 0.9 $\alpha_c$.

exceeds the critical value. This can be observed in more detail in Fig. 7, with total sanction. Hence, the main effect of the sanction is to slightly reduce the required value of $\alpha_c$ for plants to survive. The minimum value of $\alpha$ for which the plants can persist is approximately $\alpha \approx 0.9\ \alpha_c$. However, this effect of $\alpha$ reduction works only in regions of very low values of $p_+$ (up to $1 \times 10^3$ bacteria $g^{-1}$ of soil, Fig. S2).

## 4. Discussion

### 4.1. Hypothesis testing in experimental and modelling contexts

Using a combined experimental and population model approach we showed that coexistence of $N_2$ fixing and non-fixing rhizobia is possible under ecological conditions comparable to some common field conditions. Under a scenario of nitrogen availability restricted to fixing nodules, common in crops and natural vegetation, experimental plants with at least a half-root carrying fixing nodules survive in good conditions. Also, simulated plants are able of maintaining viable populations despite being cheated by non-fixing rhizobia when they can at least get some amount of fixed $N_2$ from the effectively mutualistic rhizobia occupying some nodules. This is a common situation in field (Amarger, 1981; Singleton and Tavares, 1986; Simms et al., 2006). In particular, we showed experimentally that soybean plants do not punish defective, non-fixing rhizobia inside the nodules. From the modelling we showed that plant populations persist in spite of the presence of cheating rhizobia without the need of incorporating any sanction against the cheater populations. Taken together, these results provide evidence against functioning plant host sanctions.

Addressing the first sanction mechanism proposed in the experimental test, results from the rhizobial viability experiments show that the plant is able of tolerating cheating by non-fixing rhizobia when it can get some amount of fixed $N_2$ from at least half of total plant nodules. Plants partially or exclusively associated with fixing rhizobia are able of maintaining good vegetative conditions and high viable rhizobial populations within nodules. Plants with all nodules occupied by cheating rhizobia suffered a severe stress; they were not able of surviving and ultimately died due to N starvation about 6 weeks after inoculation, as expected since rhizobial symbiosis was the only nitrogen source they received. The activity of the legume–*Rhizobium* symbiosis has been found to be extremely sensitive to stress (Zahran and Sprent, 1986; Delgado et al., 1994), and the resulting free radical generation imposes an oxidative stress that provokes bacteroid death (Becana and Klucas, 1992). Thus the decay in rhizobia viability in roots exclusively colonised by non-fixing rhizobia by week 5 is most probably due to a progressive loss of nodule functionality.

Testing the second sanction mechanism, data of expression of nodule maturity and senescence markers provide complementary results to the first mechanism and interesting explanations to the lack of evidence for the sanction hypothesis we found. The finding of no greater senescence in nodules occupied by cheating rhizobia in plants associated with both strains is in agreement with the rhizobial viability results and reinforces the evidence against functioning plant host sanctions. Besides, higher nodule maturation and lower senescence in the extreme case of entirely cheated plants may suggest that cheating rhizobia may be exerting some control over the plant to accelerate nodule development and counteract nodule senescence to get ready early viable populations in face of premature host death by starvation, acting in a true parasitic way (Ferriere et al., 2002). It is known that some rhizobia can overcome the plant controlled nodule initiation (Ma et al., 2002). However, to our knowledge this is the first work providing evidence on a possible control of nodule maturation and senescence by normally nodulating but non-fixing rhizobial strains. This proposed control and possible mechanisms operating behind it deserve to be explored further.

Our results also show that a simple population model can explain the coexistence of fixing and cheating rhizobia strains commonly found in real conditions. Our model predicts a critical fraction of fixing rhizobia in soil ($\alpha_c$), represented by the fixing rhizobia needed to provide a minimum $N_2$ amount for plant population persistence. This is in agreement with works showing that legumes are less likely to colonize new habitats if they fail to find a critical number of suitable $N_2$ fixing rhizobia (Parker, 2001; Parker et al., 2006). Plants with all nodules occupied by cheating rhizobia are not able of maintaining good vegetative conditions and ultimately die due to nitrogen starvation, as showed in the plant experiment. Even under these extreme conditions, a number of non-fixing rhizobia would be released and a viable population of non-fixing, cheater rhizobia would persist in the soil, as we showed in the simulations.

The assumption of no different competitive abilities between strains either in soil or for nodulation was made to set a modelling scenario close to the experimental conditions and to avoid confounding effects. Relaxing the assumption of no ecological differences and assuming for example competitive advantage of cheating strains, the critical fraction ($\alpha_c$) could be even more difficult to meet. This is a common and problematic situation in crops, where a few years after inoculation with highly efficient rhizobia strains nodulation becomes produced by less efficient or even non-fixing strains residing in soil (Amarger, 1981; Singleton and Tavares, 1986; Dowling and Broughton, 1986). Similarly, relaxing the assumption on the restriction of nitrogen source and allowing for plants taking also nitrogen from soil would set conditions even more favourable for cheating rhizobia persistence. Surprisingly, no experimental work assessing the performance of non-fixing and fixing rhizobial strains in soil in legume systems under external nitrogen fertilization is available in the literature. However, we can hypothesize that $\alpha_c$ would become even smaller as part of the required nitrogen could be obtained from soil, and a greater number of non-fixing rhizobia could be thus allowed to compose the total rhizobial soil population.

### 4.2. Inclusion of sanction in the model

We incorporated the plant sanction in the model as a reduction of non-fixing rhizobial survival from nodules to soil, in the same way proposed by authors advocating the need of sanctions for legume–rhizobia mutualism (West et al., 2002a, b). However, in contrast with the modelling approaches followed by these authors, we did neither include any genetic relatedness between rhizobia involving altruism and kin selection nor any hypothesized trade-off involving energetic expenditure on nodules by the plant and nitrogen gain. By simply introducing a minimum number of fixing nodules to guarantee plant survival in absence of other nitrogen source (supported by field information and our own experiments), we showed that sanctions are not needed to explain the legume–rhizobia mutualistic system persistence when cheating rhizobia are present.

When we included plant sanctions in the model, we found that the sanction effect consisted in slightly lowering the value of $\alpha_c$. This effect can be explained as follows. Since the sanction is lowering or halting the return of cheating rhizobia to soil depending on its intensity, it is expected that after few growing cycles mainly fixing rhizobia will be available in the soil for nodulation thus allowing more plants to produce enough viable seeds to reach population equilibrium earlier and with a smaller $\alpha_c$. However, this reduction only operates at unrealistically low values of $p_+$ of $<1\times10^3$ bacteria $g^{-1}$ of soil (Fig. S2), smaller than the number of native rhizobia needed to trigger nodulation ($p_m$) and much smaller than the number needed to support a crop without artificial inoculation, around $5.8\times10^4$ bacteria $g^{-1}$ of soil (Thies et al., 1995). Another predictable result from applying plant sanction is that populations of cheating rhizobia will go extinct from soil with time. As it was previously noted, this is not a realistic situation since cheating strains persist in soil and chronically hamper crop productivity (Amarger, 1981). We conclude that incorporation of plant sanctions to the model is not only unnecessary to explain how plants can persist in presence of cheating rhizobia but furthermore, it leads to unrealistic results.

The two main assumptions behind the sanction hypothesis in mutualisms, that it is costly for the host to be associated with the exploiter, and that mutualism would break unless cheaters are punished, seem not to hold for the majority of mutualistic associations known (Bronstein, 2001). Moreover, for the rhizobia–legume mutualism, costs of being cheated may not be as high as assumed if the host is still able of obtaining benefits from other mutualistic partners, for example in co-infected plants which is a common situation in field (Singleton and Tavares, 1986; Dowling and Broughton, 1986). Punishment evidence in addition to that already obtained under Ar:$O_2$ treatment (Kiers et al., 2003, 2006) is needed to hold the sanction assumption. Another proposed evidence of plant host sanctions, an inverse relationship between nodule size and strain fixation effectiveness in an experiment using *Lupinus arboreus* plants and associated *Bradyrhizobium* spp. was reported (Simms et al., 2006). However, nodule rhizobial population sizes were measured and related to nodule size in different, field collected nodules without recording strain effectiveness (Simms et al., 2006), thus not really testing the main host sanction assumption.

### 4.3. Further potential model extensions

Using a simple population model we were able of explaining the commonly found coexistence of fixing and cheating rhizobial strains in field conditions. However, further extensions providing even more realism could be easily added to our model, for example, co-occupation of the same nodule by strains with different fixation abilities. About 20% of total nodules can be co-occupied by different rhizobial strains in artificial inoculations (Rolfe and Gresshoff, 1980). Effects of co-occupation of nodules by non-fixing rhizobia would be diluted by fixing rhizobia occupying the same nodule, thus not favoring plant sanctions (Denison, 2000).

Another potential model extension is the horizontal transmission of symbiotic plasmids, turning non-nodulating strains into nodulating rhizobia, that is frequent between different strains of rhizobia (Sullivan et al., 1995). This genetic exchange can also be easily added to our model. Effects of symbiotic plasmid transfer would have different effects depending on whether plasmid confers only nodulation abilities or both nodulation and fixation abilities. Transfer of plasmids conferring only nodulation abilities would lead to changes in the frequency of fixing and non-fixing rhizobia both in the soil and inside nodules, and a new equilibrium would be reached depending on the rate of plasmid loss. If transferred plasmids allow for both nodulation and fixation, then it can be expected that fixing rhizobia populations will increase their populations (Provorov and Vorobyov, 2000). Horizontal transfer of symbiotic plasmids could also collaborate in lowering the cheater populations in a similar way as horizontal transfer of virulence factors reduced the effect of cheating from non-producing virulence factor strains in a theoretical model of bacterial pathogenesis (Smith, 2000).

Another model extension to consider is the effect of a point mutation in the symbiotic plasmid changing fixation efficiency. A previous modelling work considering changes in rhizobia fixation efficiency in the context of plant sanctions showed that plants would less likely senesce nodules fixing more N (West et al., 2002b). In our modelling context of no sanctions and non-changing fixation efficiency, we show that the mutualistic system is maintained even in the presence of non-fixing rhizobia. A mutation increasing rhizobia fixation would have an indirect effect increasing plant population densities up to the field carrying capacity. The effect on fixing rhizobia populations would be indirect also, increasing them but only because there would be more plants to nodulate. These extra plants could also be nodulated by non-fixing rhizobia, since we do not assume different competitive abilities for nodulation. So we can expect coexistence of fixing and non-fixing rhizobia, although in different proportions.

Competition between fixing and non-fixing rhizobia for growth in the soil (Hirsch, 1996) and/or nodulation (Amarger, 1981) can also be easily incorporated in our model. Competition in our modelling context would lead to changes in the plant population densities and also in rhizobia frequencies, depending on which strain, fixing or non-fixing, is given the competitive advantage. If fixing rhizobia get the advantage, then we can expect plant populations growing up to the field carrying capacity and non-fixing rhizobia being competitively displaced. On the other hand, if non-fixing rhizobia get the advantage, we can expect the inverse situation. This is a realistic scenario, since it is common to find highly efficient inoculated strains being displaced by less efficient but highly competitive strains in the field (Amarger, 1981).

### 4.4. Theoretical considerations

On more general theoretical grounds, our results support the point of view that cheating does not necessarily menace rhizobia–legume mutualism and that a gradient from mutualism to parasitism can be found in nature. There is increasing empirical evidence that punishment is not always applied to defective mutualistic partners (Ferriere et al., 2002). For example, in a

palm-pollinator mutualistic association, female plants inhibit the development of a weevil pollinator eggs and larvae, benefiting from pollination services but not reciprocating, thus cheating their partner (Dufay and Anstett, 2004). It was expected that the weevils would suspend pollination visits to female plants. However, no evidence of sanctions against female plants was found, and apparently the mutualism persistence is not compromised (Dufay and Anstett, 2004).

In closer mutualistic associations, it is theoretically expected that horizontal transmission will allow symbionts to adopt selfish strategies such as appropriating resources from their current hosts before moving on to new hosts (Bull, 1994). This idea has been tested in a symbiosis comparable to the plant–legume symbiosis, the close algae-jellyfish association, in which the algae benefit their hosts by providing photosynthates in exchange for nitrogen and inorganic nutrients (Muscatine, 1990). Experimental manipulation performed to change the algae natural transmission mode from horizontal to vertical showed higher parasitism in the horizontal mode (Sachs and Wilcox, 2006).

Another system comparable, although with caveats, is the bacteria carrying plasmids. Plasmids transmit vertically but also via horizontal transfer between bacterial cells, and may be lost due to plasmid segregation. Plasmids may confer advantages to bacteria such as antibiotic resistance (Dugatkin et al., 2005). As plasmid bearing is costly to bacteria, cheater bacterial strains lacking the plasmid and living in the vicinity of those carrying them benefit from antibiotic protection provided for plasmids. In these systems, coexistence of cheaters and mutualistic bacteria is common and maintained by frequency-dependent selection (Ellis et al., 2007). It is interesting to think that, in the case of rhizobial symbiotic plasmids the true cheater would be the plasmids carrying genes for nodulation but not for $N_2$ fixation. These plasmids would cheat bacteria carrying effective plasmids, and bacteria carrying ineffective plasmids would cheat the plant in turn. This is an appealing approach to the legume–rhizobia mutualism and we are currently exploring it.

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## Appendix A. Invariance of $\alpha$ for $p_m = 0$ and $\sigma = 0$

In this case we have that $\Theta(p_i - p_m) = 1$ and $f_+ = f_- = f$. Form Eqs. (6)–(8) we have

$$\alpha(t+1) = \frac{p_+(t+1)}{p_+(t+1)+p_-(t+1)} = \frac{p_+(t)+f\frac{\delta_n}{m_s}nP_p(t)\alpha(t)}{p_+(t)+p_-(t)+f\frac{\delta_n}{m_s}nP_p(t)} \tag{A.1}$$

Defining $B(t) = f\delta_n nP_p(t)/m_s$, Eq. (A.1) can be written as

$$p_+(t)[\alpha(t+1)-1] + \alpha(t+1)p_-(t) + B(t)[\alpha(t+1)-\alpha(t)] = 0 \tag{A.2}$$

Noting that,

$$\alpha(t+1) - 1 = -\frac{p_-(t+1)}{p_+(t+1)+p_-(t+1)} \tag{A.3}$$

Eq. (A.2) can be written as

$$\frac{p_+(t+1)p_-(t) - p_+(t)p_-(t+1)}{p_+(t+1)+p_-(t+1)} + B(t)\Delta\alpha(t) = 0 \tag{A.4}$$

where $\Delta\alpha(t) \equiv \alpha(t+1) - \alpha(t)$. We will show that the first term of Eq. (A.4) equals zero:

$$\begin{aligned} &p_+(t+1)p_-(t) - p_+(t)p_-(t+1) \\ &\quad = [p_+(t) + B(t)\alpha(t)]p_-(t) - p_+(t)[p_-(t) + B(t)(1-\alpha(t))] \\ &\quad = B(t)\alpha(t)(p_+(t)+p_-(t)) - B(t)p_+(t) = 0 \end{aligned} \tag{A.5}$$

where we have used again Eqs. (6)–(8). Since $B(t) \neq 0$ we have that $\alpha(t+1) = \alpha(t)$ for all $t$.

## Appendix B. Plants population dynamics: $\alpha_c$

The population dynamics of plants is described by the discrete map Eq. (10), which can be written as

$$x(t+1) = 1 - e^{-\gamma x(t)} \tag{B.1}$$

where $x(t) \equiv P_p(t)/\delta_p$ and $\gamma(t) \equiv g(t)|\ln(1-P_g)|$. Let us assume that $\gamma$ does not depend on time. This case corresponds to $\sigma = p_m = 0$ and the typical shape of the map (B.1) is shown in Fig. B1 for two different values of $\gamma$. Since the map (B.1) is a monotonously increasing function, the long term dynamics will be determined by its fixed points and their stability, i.e., by the solutions of the stationary equation $P_p^*(t+1) = P_p^*(t)$, or equivalently

$$x_* = 1 - e^{-\gamma x_*} \tag{B.2}$$

The trivial fixed point $x^* = 0$, which corresponds to the plants extinction, is always a solution of Eq. (B.2). If $\gamma \leqslant 1$ this is the only solution; if $\gamma > 1$ we see from Fig. B1 that a second, non-trivial solution $x^* = x^*(\gamma)$ appears. The value $x^*(\gamma)$ cannot be obtained analytically and has to be calculated numerically. The stability of a fixed point $x_0$ is given by

$$\left.\frac{\partial}{\partial x}(1-e^{-\gamma x})\right|_{x_0} = \gamma e^{-\gamma x_0} \begin{cases} 1 & \text{unstable} \\ 1 & \text{stable} \end{cases} \tag{B.3}$$

If $\gamma \leqslant 1$, $x_0 = 0$ is stable. If $\gamma > 1$, $x_0 = 0$ is unstable and the new fixed point $x^*$ is stable. Therefore, in the long term the system evolves towards the stationary plant population value $P_p(t) = \delta_p x^*(\gamma)$. From Eq. (12) we see that $\gamma$ is a function of $\alpha$, i.e.

$$\gamma = \begin{cases} |\ln(1-P_g)|G\tanh\left(\dfrac{\alpha n - K_0}{G}\right) & \text{if } \dfrac{K_0}{n} < \alpha \leqslant 1 \\ 0 & \text{if } \alpha < \dfrac{K_0}{n} \end{cases} \tag{B.4}$$

Since $\alpha \leqslant 1$, a necessary condition for the existence of a non-zero stationary plant population is

$$|\ln(1-P_g)|G\tanh\left(\frac{n-K_0}{G}\right) > 1 \tag{B.5}$$

For the range of realistic parameters values of the present system this condition is always satisfied. If Eq. (B.5) holds, a non-zero plant population will be obtained if $\gamma > 1$. Since $\gamma$ is an increasing function of $\alpha$ (see Eq. (B.4)), that means $\alpha > \alpha_c$, where $\gamma(\alpha_c) = 1$, i.e.

$$|\ln(1-P_g)|G\tanh\left(\frac{\alpha_c n - K_0}{G}\right) = 1 \tag{B.6}$$

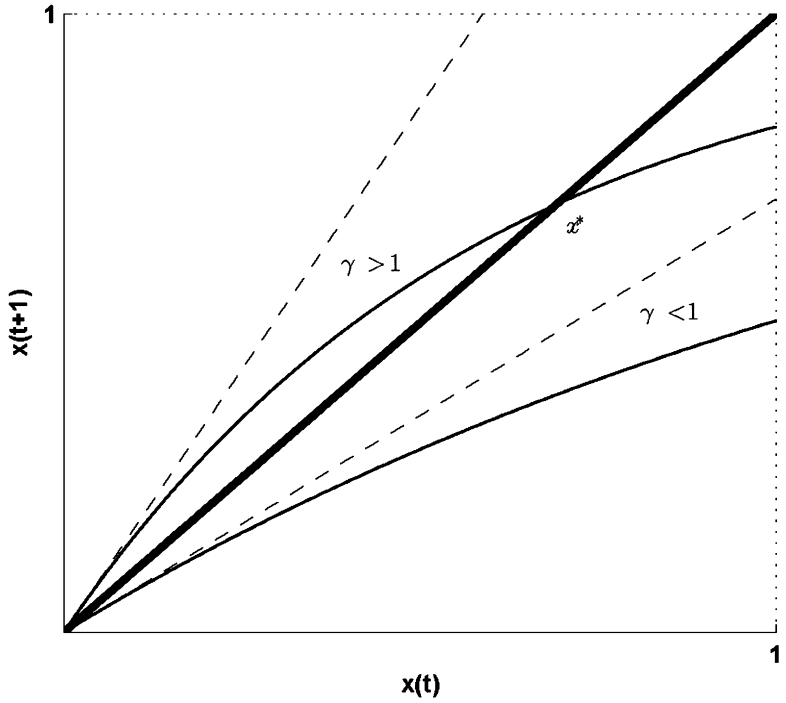


**Fig. B1.** Graphical representation of the map (B.1) for $\gamma>1$ and $\gamma<1$. Dotted lines are tangent to the exponential function $1-\exp(-\gamma x)$ at the origin; the full dark line is the identity function. The exponential and the identity function intersect only when $\gamma>1$, giving rise to a fixed point $x^*\neq 0$.

An approximated solution of this equation can be obtained if

$$0<\alpha_c n - K_0 \ll G \tag{B.7}$$

In this case we can approach $\tanh(x)\cong x$ and from Eq. (B.6) we get

$$\alpha_c = \frac{1}{n}\left(K_0 + \frac{1}{|\ln(1-P_g)|}\right) \tag{B.8}$$

Replacing this value into Eq. (B.7) we obtain the following condition of validity for the expression (B.8):

$$G|\ln(1-P_g)| \gg 1 \tag{B.9}$$

is worth noting that, under the present dynamics, the plants population strictly never reaches its charge capacity $x^*=1$, since that would imply $\gamma\to\propto$ (see Eq. (B.2)). However, for the set of parameter values used in this work it can be shown that $x^*>0.99$ for $\alpha>0.25$, which in practical terms means that the system reaches its charge capacity.

Finally, it is important to stress that, even when $\alpha$ (and therefore $\gamma$) depends on time, the fixed points above analysed are still stationary solutions of the map (B.1). However, their stability (and therefore the way the plant population evolves towards them) may depend in a complex manner on the coupling with the bacteria system. In particular, the instantaneous behaviour of $P_p(t)$ depends on whether $\alpha(t)$ is smaller or larger than $\alpha_c$: if $\alpha(t)>\alpha_c$ the plant population in the next step increases and vice versa. This can lead to a non-monotonous behaviour of the plant population.

## Appendix C. Supplementary material

Supplementary data associated with this article can be found in the online version at 10.1016/j.jtbi.2009.03.033.

## References

Alesandrini, F., Frendo, P., Puppo, A., Hérouart, D., 2003. Isolation of a molecular marker of soybean nodule senescent. Plant Physiol. Biochem. 41, 727–732.

Amarger, N., 1981. Competition for nodule formation between effective and ineffective strains of *Rhizobium meliloti*. Soil Biol. Biochem. 13, 475–480.

Amarger, N., Lobreau, J.P., 1982. Quantitative study of nodulation competitiveness in *Rhizobium* strains. Appl. Environ. Microbiol. 44, 583–588.

Becana, M., Klucas, R.V., 1992. Transition metals in legume root nodules: iron-dependent free radical production increases during nodule senescence. Proc. Natl. Acad. Sci. USA 89, 8958–8962.

Bever, J.D., Simms, E.L., 2000. Evolution of nitrogen fixation in spatially structured populations of *Rhizobium*. Heredity 85, 366–372.

Bronstein, J.L., 2001. The exploitation of mutualisms. Ecol. Lett. 4, 277–287.

Bull, J.J., 1994. Perspective: virulence. Evolution 48, 1423–1437.

Cannas, S.A., Marco, D.E., Paez, S.A., 2003. Modelling biological invasions: species traits, species interactions and habitat heterogeneity. Math. Biosci. 183, 93–110.

Delgado, M.J., Ligero, F., Lluch, C., 1994. Effects of salt stress on growth and nitrogen fixation by pea, faba-bean, common bean and soybean plants. Soil Biol. Biochem. 26, 371–376.

Denison, R.F., 2000. Legume sanctions and the evolution of symbiotic cooperation by Rhizobia. Am. Nat. 156, 567–576.

Diaz del Castillo, L., Layzell, D.B., 1995. Drought stress, permeability to $O_2$, diffusion, and the respiratory kinetics of soybean root nodules. Plant Physiol. 107, 1187–1194.

Dowling, D.N., Broughton, W.J., 1986. Competition for nodulation of legumes. Annu. Rev. Microbiol. 40, 131–157.

Dufay, M., Anstett, M.C., 2004. Cheating is not always punished: killer female plants and pollination by deceit in the dwarf palm *Chamaerops humilis*. J. Evol. Biol. 7, 862–868.

Dugatkin, L.A., Perlin, M., Lucas, J.S., Atlas, R., 2005. Group beneficial traits, frequency-dependent selection and genotypic diversity: an antibiotic resistance paradigm. Proc. R. Soc. London Ser. B Biol. Sci. 272, 79–83.

Ellis, R.J., Lilley, A.K., Lacey, S.J., Murrell, D., Godfray, H.C.J., 2007. Frequency-dependent advantages of plasmid carriage by *Pseudomonas* in homogeneous and spatially structured environments. ISME J. 1, 92–95.

Ferriere, R., Bronstein, J.L., Rinaldi, S., Law, R., Gauduchon, M., 2002. Cheating and the evolutionary stability of mutualisms. Proc. R. Soc. London Ser. B 269, 773–780.

Gage, D.J., Bobo, T., Long, S.R., 1996. Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and Alfalfa (*Medicago sativa*). J. Bacteriol. 178, 7159–7166.

Hahn, M., Meyer, L., Studer, D., Regensburger, B., Hennecke, H., 1984. Insertion and deletion mutations within the *nif* region of *Rhizobium japonicum*. Plant Mol. Biol. 3, 159–168.

Hirsch, P., 1996. Population dynamics of indigenous and genetically modified rhizobia in the field. New Phytol. 133, 159–171.

Kiers, E.T., Rosseau, R.A., West, S.A., Denison, R.F., 2003. Host sanctions and the legume–*Rhizobium* mutualism. Nature 425, 78–81.

Kiers, T.E., Rousseau, R.A., Denison, R.F., 2006. Measured sanctions: legume hosts detect quantitative variation in *Rhizobium* cooperation and punish accordingly. Evol. Ecol. Res. 8, 1077–1086.

King, B.J., Layzell, D.B., 1991. Effect of increases in oxygen concentration during the argon-induced decline in nitrogenase activity in root nodules of soybean. Plant Physiol. 96, 376–381.
Lodwig, E.M., Hosie, A.H.F., Bourde, A., Findlay, K., Allaway, D.A., Karunakaran, R., Downie, J.A., Poole, P.S., 2003. Amino-acid cycling drives nitrogen fixation in the legume–*Rhizobium* symbiosis. Nature 422, 722–726.
Ma, W., Penrose, D.M., Glick, B.R., 2002. Strategies used by rhizobia to lower plant ethylene levels and increase nodulation. Can. J. Microbiol. 48, 947–954.
Muscatine, L., 1990. The role of symbiotic algae in carbon and energy flux in coral reefs. In: Dubinsky, Z. (Ed.), Ecosystems of the World: Coral Reefs. Elsevier, Amsterdam, pp. 75–81.
Parker, M.A., 2001. Mutualism as a constraint on invasion success for legumes and rhizobia. Diversity Distrib. 7, 125–136.
Parker, M.A., Malekand, W., Parker, I.M., 2006. Growth of an invasive legume is symbiont limited in newly occupied habitats. Diversity Distrib. 12, 563–571.
Provorov, N., Vorobyov, N., 2000. Population genetics of rhizobia: construction and analysis of an ''Infection and Release'' model. J. Theor. Biol. 205, 105–119.
Rolfe, B.G., Gresshoff, P.M., 1980. *Rhizobium trifolii* mutant interactions during the establishment of nodulation in white clover. Austr. J. Biol. Sci. 33, 491–504.
Sachs, J.L., Wilcox, T.P., 2006. A shift to parasitism in the jellyfish symbiont *Symbiodinium microadriaticum*. Proc. R. Soc. B 273, 425–429.
Simms, E.L., Taylor, D.L., Povich, J., Shefferson, R.P., Sachs, J.L., Urbina, M., Tausczik, Y., 2006. An empirical test of partner choice mechanisms in a wild legume–*Rhizobium* interaction. Proc. R. Soc. B 273, 77–81.
Singleton, P.W., Tavares, J.W., 1986. Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. Appl. Environ. Microbiol. 51, 1013–1018.
Smith, J., 2000. The social evolution of bacterial pathogenesis. Proc. R. Soc. Lond. B 268, 61–69.
Streeter, J.C., Peters, N.K., Salminen, S.O., Pladys, D., Zhaohua, P., 1995. Fate of nodule-specific polysaccharide produced by *Bradyrhizobium japonicum* bacteroids. Plant Physiol. 107, 857–864.
Sullivan, J.T., Patrick, H.N., Lowther, W.L., Scott, D.B., Ronson, C.W., 1995. Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. Proc. Natl. Acad. Sci. USA 92, 8985–8989.
Thies, J.E., Woomer, P.L., Singleton, P.W., 1995. Enrichment of *Bradyrhizobium* spp. populations in soil due to cropping of the homologous host legume. Soil Biochem. 27, 633–636.
Vincent, J.M., 1970. A Manual for the Practical Study of Root Nodule-Bacteria. Blackwell Scientific Publications, Oxford.
Wei, H., Layzell, D.B., 2006. Adenylate-coupled ion movement. A mechanism for the control of nodule permeability to $O_2$ diffusion. Plant Physiol. 141, 280–287.
West, S.A., Kiers, E.T., Simms, E.L., Denison, R.F., 2002a. Sanctions and mutualism stability: why do rhizobia fix nitrogen? Proc. R. Soc. London Ser. B 269, 685–694.
West, S.A., Kiers, E.T., Pen, I., Denison, R.F., 2002b. Sanctions and mutualism stability: when should less beneficial mutualists be tolerated? J. Evol. Biol. 15, 830–837.
Wood, M., Cooper, J.E., 1988. Acidity, aluminium and multiplication of Rhizobium trifolii: effects of initial inoculum density and growth phase. Soil Biol. Biochem. 20, 83–87.
Zahran, H.H., Sprent, J.I., 1986. Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. Planta 167, 303–309.