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-N2 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 N2 fixing Rhizobium 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 cheating populations in the model, under the realistic assumption that plants can at least get some amount of fixed N2 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.

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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 N2 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 N2-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-N2 fixing, cheating rhizobia (Denison, 2000; Kiers
A decrease in rhizobial viability was reported when N2-fixing rhizobia were “forced” to cheat soybean plants by replacing normal, N2-containing atmosphere by an Ar:O2 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:O2 atmosphere per se reduces nodule O2 concentration in soybean nodules due to decrease in O2 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., 2003, 2006). However, this approach does not really unequivocally test the plant–host-sanction hypothesis. 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 N2-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 N2 fixing USDA 110 or the nod+ Fix+, nifH1::Tn5 mutant H1 derived from the wild type (Hahn et al., 1984) in the following treatments: half roots of the same plant (USDA110-H1), 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 N2 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 µE m−2 s−1 photosynthetically active radiation at 25 °C, and 8 h darkness at 18 °C. Control uninoculated plants showed no noduleation. Nodule numbers were counted in each half root every three days until nodule production reached 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 stored 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 2% HgCl2 (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 (RNaseasy 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
of fixed N\textsubscript{2} from at least half of total plant nodules. Obviously, cheating by non-fixing rhizobia when it can get some amount viability experiments show that the plant is able of tolerating proposed in the experimental test, results from the rhizobial nodule formation levels and temporal patterns respect to the

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\textsubscript{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\textsubscript{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\textsubscript{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).

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\textsubscript{2} fixing ability, and they have the same ecological abilities (same competition levels in soil and nodule initiation).
experiments. *DD10 of nodule maturity (b) in nodules from the soybean plant split-root
Relative expressions of gene markers DD15 of nodule senescence (a) and
Fig. 4.
A minimum number of compatible rhizobia in the soil is needed
abilities and conditions for the mutant and effective strains in soil.
assuming random probability, since we assume equal ecological
factors. However, we can simplify the nodule generation process
competition between rhizobia for root colonization among other
random, since it involves production of compounds from the plant
the same for the two strains (Fig. 2).
We discuss next the biological background of the assumptions.
Cheating rhizobia can vary in their N₂ 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
either fixing or non-fixing strain.
- Nodules are occupied to their carrying capacity, are function-
ally 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.

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"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 popula-
tion 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 senes-
cence 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:

\[
p_i(t + 1) = (p_i(t) + \Delta p_i(t)) \left[ 1 + r_i^+ \left( 1 - \frac{p_i(t)}{s_i} \right) \right] \quad i = +, -
\]

\[
P_f(t) = p_-(t) + \Delta p_-(t) + p_+(t) + \Delta p_+(t)
\]

where \(p_i\) describes the bacteria population densities in soil, \(i = +, -\) indicates fixing and non-fixing bacteria, respectively, and \(P_f\) is
the total bacteria population density (i.e., fixing+non-fixing) in soil.
The parameter \(s_i\) stands for the carrying capacity of the soil close
to roots in absence of nodulation. An effective carrying capacity
greater than \(s_i\) is set when plants release bacteria at the end of
nodulation process. The parameters \(r_i^+\) 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 \(r_i^+ = r_i^- = r\).

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

<table>
<thead>
<tr>
<th>Nodules</th>
<th>Fixing</th>
<th>Non-fixing</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA110-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USDA110-2</td>
<td></td>
<td></td>
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<tr>
<td>H1-2</td>
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<td></td>
</tr>
</tbody>
</table>

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
±1s.d. Treatments as in Fig. 1.

Fig. 5. Schematic structure of the model dynamics in a single iteration. Initial
values of plant and bacteria populations (\(p_i\) for plants; \(p_+\) and \(p_-\) for bacteria) set
the values of bacteria in nodules (\(p_i^N\)). 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 $f_1$ 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 of surviving bacteria of each type to be different, i.e.

$$\Delta p_i(t) = f_i \frac{\delta_i}{m_i} K_i(t)$$

where $\delta_i$ is the carrying capacity of each nodule type, $m_i$ is the mass of soil per hectare associated to the crop and $f_i = f_1, f_2 = f$ ($1-\sigma$). 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 $K_i(t)$, and it represents a fraction of the total root colonisable sites for nodule initiation $K(t)$. 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,

$$K_i(t) = \frac{P_i(t)}{P_i(t) + P_m(t)} K(t)$$

where $K(t)$ is the total number of plants in the system and its dynamic is entirely given by its own logistic equation and its dynamic is entirely given by its own logistic equation.

$$K_i(t) = n P_i(t)$$

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

Defining the fraction of surviving bacteria as

$$\alpha(t) = \frac{P_1(t)}{P_1(t) + P_m(t)}$$

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

$$P_{i-1}(t+1) = \left( P_i(t) + f \frac{\delta_i}{m_i} \alpha(t) K_i(t) \Theta(P_i(t) - P_m) \right)$$

$$\times \left[ 1 + r_i \left( 1 - \frac{P_i(t)}{\delta_i} \right) \right]$$

(7)

$$P_{i-1}(t+1) = \left( P_m(t) + f \frac{\delta_i}{m_i} [1 - \alpha(t) K_i(t) \Theta(P_i(t) - P_m)] \right)$$

$$\times \left[ 1 + r_i \left( 1 - \frac{P_i(t)}{\delta_i} \right) \right]$$

(8)

$$P_1(t) = P_{i-1}(t) + P_m(t) - \frac{\delta_i}{m_i} \alpha(t) P_1(t)$$

$$\times \left[ f_1 \Theta(P_1(t) - P_m) - f_i \Theta(P_i(t) - P_m) \right]$$

$$+ f \frac{\delta_i}{m_i} n P_1(t) \Theta(P_i(t) - P_m)$$

(9)

The plant population dynamics can be described by a model of plant spread previously published (Cannas et al., 2003). Briefly, if $\delta_i$ is the carrying capacity of the field where the plants grow, the density population per unit field area is $P_i(t)/\delta_i$. Suppose that such area receives at time $t+1$ $z_i$ seeds from the plant population at time $t$ and that $P_0$ 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.

$$P_p(t+1)/P_p(t) = 1 - (1 - P_0)^{z_i}$$

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

$$P_p(t+1) = \delta_p \left[ 1 - \exp \left( -g(t) \frac{\ln(1 - P_0) P_p(t)}{\delta_p} \right) \right]$$

(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 $K_i(t)$. 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, $K_p$. All the previous assumptions can be modelled by the following expression:

$$g(t) = \begin{cases} G \tanh\left( \frac{K_i(t) - K_p P_i(t)}{G P_i(t)} \right) & \text{if } K_i(t) - K_p P_i(t) > 0 \\ 0 & \text{otherwise} \end{cases}$$

(11)

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

$$g(t) = \begin{cases} G \tanh\left( \frac{\alpha(t) n - K_0}{G} \right) & \text{if } \alpha(t) n > K_0 \text{ and } P_m(t) > P_m \\ 0 & \text{otherwise} \end{cases}$$

(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 $f_1 = f_2$, 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 $\alpha < \alpha_c$, 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
low, very few nodules are created (low fixation levels of N₂), the production of seeds is low and therefore the plant population decreases. Since the number of seeds s depends on the bacterial populations only through x 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 population always decreases, even when the fixing bacteria pm extinct after the first iteration. If the initial populations of both type of bacteria are below the threshold pm, 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 pm, the dynamics is exactly the same as in the pm = 0 case, so again plants survive when x(0) > x_c. The main difference with the pm = 0 case is that the plant population goes always extinct when p_o(0) < p_m, no matter the value of x(0). Fig. 6a shows the typical behaviour of p_o, p_p and p_p for σ = 0, α = 0.169 x_c and pm > 0. Simulation started with α = 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 (σ = 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_p numbers can be seen and p_p population remains very close to zero. (Fig. 6b). With extreme sanction (σ = 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, 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. We calculated numerically the dynamics of the system for pm = 10⁴ g⁻¹. When pm ≠ 0, α changes with time when pm > 0 and the overall behaviour depends on the initial values of both types of bacterial populations, instead of depending only on its ratio x(0). The behaviour of the final plant population is more complex now, since it depends on whether α(t) overcomes the critical value x_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 pm = 10³ g⁻¹.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>rₜ</td>
<td>10⁻⁴</td>
<td>Intrinsic rate of growth of bacteria in the soil</td>
</tr>
<tr>
<td>δₜ</td>
<td>10⁵ g⁻¹</td>
<td>Soil bacterial carrying capacity (per g of soil)</td>
</tr>
<tr>
<td>δₚ</td>
<td>10⁵</td>
<td>Nodule’s carrying capacity (bacteria per nodule)</td>
</tr>
<tr>
<td>jₜ</td>
<td>10⁻⁴</td>
<td>Surviving bacteria of each type released from a nodule</td>
</tr>
<tr>
<td>δₚ</td>
<td>2 × 10⁵ ha⁻¹</td>
<td>Plants’ field carrying capacity</td>
</tr>
<tr>
<td>mₚ</td>
<td>1.5 × 10⁸ g ha⁻¹</td>
<td>Soil mass per hectare associated to the plant population</td>
</tr>
<tr>
<td>n</td>
<td>45</td>
<td>Typical number of nodules per plant</td>
</tr>
<tr>
<td>Kₒ</td>
<td>0.15 × n</td>
<td>Minimum number of fixing nodules per plant needed for seed production</td>
</tr>
<tr>
<td>G</td>
<td>55</td>
<td>Maximum number of viable seeds produced per plant</td>
</tr>
<tr>
<td>Pₑ</td>
<td>0.69</td>
<td>Probability of a viable seed reaching the adult stage</td>
</tr>
<tr>
<td>σ</td>
<td>0–1</td>
<td>Sanction intensity: 0 = no sanction and 1 = maximum sanction</td>
</tr>
<tr>
<td>p_m</td>
<td>0–10² g⁻¹</td>
<td>Minimum bacteria population per g of soil needed to trigger the nodulation process</td>
</tr>
</tbody>
</table>

Fig. 6. Temporal behaviour of the variables p_o (dashed line), p_p (solid line) and p_p (dotted line) for α = 1.5, x_c, Pₑ(0) = 0.1 δₚ and different values of σ: (a) σ = 0 (no sanction), (b) σ = 0.5 (moderate sanction), and (c) σ = 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_p population remains very close to zero.
inoculation, as expected since rhizobial symbiosis was the only
ultimately died due to N starvation about 6 weeks after
suffered a severe stress; they were not able of surviving and
nodules. Plants with all nodules occupied by cheating rhizobia
vegetative conditions and high viable rhizobial populations within
associated with fixing rhizobia are able of maintaining good
half of total plant nodules. Plants partially or exclusively
show that the plant is able of tolerating cheating by non-fixing
cheated by non-fixing rhizobia when they can at least get some
availability restricted to fixing nodules, common in crops and
some common field conditions. Under a scenario of nitrogen
rhizobia is possible under ecological conditions comparable to
natural vegetation, experimental plants with at least a half-root
testing in experimental and modelling contexts
Using a combined experimental and population model
approach we showed that coexistence of N₂ 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 N₂ 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 incorporat-
ing any sanction against the cheating 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 N₂ 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–Rhizo-
bium 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 (z_c), represented by the fixing
rhizobia needed to provide a minimum N₂ 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 N₂ 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 (z_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
persistance. 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 z_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.

Fig. 7. The effect of total sanction on final plant population (fraction of carrying
capacity δ_j), P_j/δ_j (filled circles), z (empty circles). z_c is the dotted horizontal line.
When z exceeds the critical value the plant population starts increasing. The initial
value of z is 0.9 z_c.
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 $N_c$. 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 $N_c$. However, this reduction only operates at unrealistically low values of $p$, of $<1 \times 10^5$ 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 $5.8 \times 10^5$ 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 B. japonicum palm-pollinator mutualistic association, female plants inhibit the weevils would suspend pollination visits to female plants. From pollination services but not reciprocating, thus cheating them benefit from antibiotic protection provided for plasmids.

**Appendix A. Invariance of \( x \) for \( p_m = 0 \) and \( \sigma = 0 \)**

In this case we have that \( \Theta(p_1-p_m) = 1 \) and \( f_1 = f_m = f \). Form Eqs. (6)–(8) we have

\[
\alpha(t+1) = \frac{p_+ (t+1)}{p_+ (t+1) + p_- (t+1)}
\]

\[
= \frac{p_+ (t) + \frac{\delta n}{m_0} n P_p (t) \alpha (t)}{p_+ (t) + p_- (t) + \frac{\delta n}{m_0} n P_p (t)}
\]

(A.1)

Defining \( B(t) = f_0 n P_p (t)/m_0 \), Eq. (A.1) can be written as

\[
p_+(t+1) (1+1) + \alpha(t+1) P_- (t) + B(t) [\alpha(t+1) - \alpha(t)] = 0
\]

(A.2)

Noting that,

\[
\alpha(t+1) - 1 = \frac{p_- (t+1)}{p_+ (t+1) + p_- (t+1)}
\]

(A.3)

Eq. (A.2) can be written as

\[
p_+(t+1) P_-(t) - p_-(t) P_-(t+1)
\]

\[
= \frac{p_+ (t) + B(t) [\alpha(t) - \alpha(t)] + B(t) (1 - \alpha(t))}{p_+ (t) + B(t) [\alpha(t) - \alpha(t)] - B(t) \alpha(t) = 0}
\]

(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: \( x_c \)**

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

\[
x(t+1) = 1 - \frac{\alpha^{-\gamma}(t)}{e^\gamma}
\]

(B.1)

where \( x(t) = P_p (t)/\delta_0 \) and \( \gamma(t) = \gamma(t) \ln (1-P_p)/P_p \). Let us assume that \( \gamma \) does not depend on time. This case corresponds to \( \sigma = \sigma_0 = 0 \) 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

\[
x_0 = 1 - \frac{\alpha^{-\gamma}}{e^\gamma}
\]

(B.2)

The trivial fixed point \( x^*=0 \), which corresponds to the plants extinction, is always a solution of Eq. (B.2). If \( \gamma \leq 1 \) 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

\[
\frac{\partial}{\partial x} (1-e^{-\gamma}) x_0 = \gamma e^{-\gamma} x_0
\]

unstable

stable

(B.3)

If \( \gamma \leq 1 \), \( x_0 = 0 \) 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 \( P_p (t) = \delta_0 x^* (\gamma) \). From Eq. (12) we see that \( \gamma \) is a function of \( x_c \), i.e.

\[
\gamma = \begin{cases} \ln (1-P_g)/G \tanh \left( \frac{\alpha_0 - K_0}{G} \right) & \text{if } \frac{K_0}{n} < x < 1 \\ 0 & \text{if } x < \frac{K_0}{n} \end{cases}
\]

(B.4)

Since \( x \leq 1 \), a necessary condition for the existence of a non-zero stationary plant population is

\[
|\ln (1-P_g)/G \tanh \left( \frac{\alpha_0 - K_0}{G} \right) | > 1
\]

(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.

\[
|\ln (1-P_g)/G \tanh \left( \frac{\alpha_0 - K_0}{G} \right) | = 1
\]

(B.6)
An approximated solution of this equation can be obtained if
\[ 0 < x_n - K_0 < G \quad (B.7) \]
In this case we can approach tanh(\(x\)) ≈ \(x\) and from Eq. (B.6) we get
\[ x_c = \frac{1}{n} \left( K_0 + \frac{1}{\ln(1 - P_g)} \right) \quad (B.8) \]
Replacing this value into Eq. (B.7) we obtain the following condition of validity for the expression (B.8):
\[ G | \ln(1 - P_g) | > 1 \quad (B.9) \]
is worth noting that, under the present dynamics, the plants population strictly never reaches its charge capacity \(x^* = 1\), since that would imply \(g \to \infty\) (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 \(x^*\) (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 \(P_g(t)\) depends on whether \(x(t)\) is smaller or larger than \(x_c\); if \(x(t) > x_c\) 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


