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

Diana E. Marco[1, 6], Juan P. Carbajal[2], Sergio Cannas[3,6], Rebeca Pérez-Arnedo[4], Ángeles Hidalgo-Perea[5], José Olivares[4], José E. Ruiz-Sainz[5] and Juan Sanjuán[4]

1Laboratorio de Ecología Matemática, Area de Producción Orgánica, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Ciudad Universitaria, CC 509, 5000 Córdoba, Argentina

2 Artificial Intelligence Laboratory, Dept. of Informatics, University of Zurich Andreasstr. 15, CH-8050 Zurich, Switzerland.

3 Instituto de Física de la Facultad de Matemática, Astronomía y Física (IFFMAMAF-CONICET), Universidad Nacional de Córdoba,Ciudad Universitaria, 5000 Córdoba, Argentina.

4 Dept. Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC. Prof. Albareda 1, 18008 Granada, Spain.

5 Dept. Microbiología, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes, 41012 Sevilla, Spain.

6 Members of the National Research Council (CONICET), Argentina.

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 analyze the stability of Rhizobium-legume symbiosis when "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 merely reduces the time needed for reaching plant population equilibrium and 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 or even improve in presence of cheating partners.

Keywords: mutualism, cheating, legume-rhizobia symbiosis, host sanctions, experimentally-based modelling.

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 reproduce and differentiate inside root nodules into bacteroids 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, can maintain resident populations (Hirsch, 1996). 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, 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 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 et al., 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). 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). 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 (Alessandrini et al., 2003). We show that soybean plants do not punish defective, non-fixing rhizobia inside the nodules.

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., 2002 a,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. 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 merely reduces the time needed for reaching plant population equilibrium and leads to the unrealistic effect of ultimate extinction of cheater strains in soil.

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). 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 µEm-2 s-1 photosynthetically active radiation at 25 EC, and 8 h darkness at 18 EC. Control uninoculated plants showed no nodulation. 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 storaged at -80 EC 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, homogeneized and resuspended in a buffer containing 0.05M Tris-HCL and 0.25 manitol. 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 EC for a week or until no further growth was detected, and colony-forming units (c.f.u.) were counted. c.f.u. numbers were compared using paired t-test analysis on untransformed data (n between 10 to 6).

2.3. Nodule gene expression

cDNA markers differentially expressed in mature (DD10) and senescent (DD15) soybean nodules (Alessandrini 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 DD1022 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 EC. Length of PCR products ranged between 152-180 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′-TGGTTTTCTCCTCCTGCTGATT-3′ and 5-GGCAGCATACTCACTTTCACTT-3′, DD10 primers 5′-AGAAGAAGCTGGTGGTATTGGT-3′and 5′-GGAGTTGCTGAGATTGGATTGA-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 EC, 30 cycles: 1 min at 94

EC, 1 min at 60 EC and 30 s at 72 EC, and 1 cycle: 10 min at 72 EC. 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).

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, 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 mass was not significantly lower comparing half roots of the same plant separately inoculated with each strain for the two soybean varieties (Fig. 3). Comparing treatments where both half roots of each plant were inoculated with the same strain, cheating rhizobia viability was significantly lower (Fig. 3). 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). In an apparently puzzling way, 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). However, this 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, and in agreement with the experimental design, we avoided factors like strain competition.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 (competition in soil and nodule initiation).

* 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 rhizosphere 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 rhizosphere 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 rhizosphere (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:

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

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

where p_i describe the bacteria population densities in soil, $i \in [+, -]$ indicates fixing and non-fixing bacteria respectively and P_T is total bacteria population in soil. The parameter δ_s stands for the carrying capacity of the rhizosphere.

The parameters $r_i^{\,s}$ represents the intrinsic reproduction rate of each population in the rhizosphere. 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 rhizosphere is represented by $\Delta p_i^{\,N}(t)$. If no host sanction is assumed, i.e., plants are not able of differentiating fixing

from non-fixing bacteria during the root colonization process (Amarger, 1981), we consider this number the same for both types of bacteria (about $f = 10^{-4}$ 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.

$$\Delta p_i^N(t) = f_i \frac{p_i^N(t)}{m_s} = f_i \frac{\delta_n}{m_s} K_i^N(t) \qquad (3)$$

where δ_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 σ represents the sanction intensity the plant applies to the non-fixing bacteria. It 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. 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,

$$K_i^N(t) = \begin{cases} \dfrac{p_i(t)}{p_+(t) + p_-(t)} & \text{If } p_i(t) \geq p_m \\ 0 & \text{otherwise} \end{cases}$$

where the threshold p_m is the minimum bacteria population per g of soil needed to trigger the nodulation process. Defining the number of fixing bacteria as

$$\alpha(t) = \frac{p_+(t)}{p_+(t) + p_-(t)} \qquad (5)$$

the maps (1) can be written as

$$p_+(t+1) = \left(p_+(t) + f_+\delta_n \alpha(t) K_s\, \Theta\left[p_+(t) - p_m\right]\right)\left[1 + r_s\left(1 - \frac{P_T(t)}{\delta_s}\right)\right] \qquad (6)$$

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

where the step function $\Theta(x) = 1$ when $x \geq 1$ and $\Theta(x) = 0$ otherwise.

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.,

$$K_s(t) = nPp(t) \qquad (8)$$

where n is the average number of nodules per plant.

The plant population dynamics can be described by a model of plant spread previously published (Cannas et al., 2003). Briefly, if δ_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 ns 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., $p_p(t+1) / \delta_p = 1 - (1 - P_g)^{n_s}$. If g is the number of seeds produced by a plant in a annual crop, then $n_s = g\, p_p(t) / \delta_p$ and

The plant population dynamics is described by

$$p_p(t+1) = \delta_p \left[1 - e^{-g \frac{\left|\ln(1-P_g)\right| P_p(t)}{\delta_p}} \right] \qquad (9)$$

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

$$g(t) = G \tanh\left(\frac{K_+^{\,N}(t) - K_0 p_p(t)}{G\, p_p(t)} \right) \qquad (10)$$

Using Eqs.(4)-(8) we arrive to the expression

$$g(t) = \begin{cases} G \tanh\left(\dfrac{\alpha(t) n \Theta\left(p_+(t) - p_m\right) K_0}{G} \right) & \text{If} \quad \alpha(t) n > K_0 \quad \text{and} \quad p_+(t) > p_m \\ 0 & \text{otherwise} \end{cases} \qquad (11)$$

Finally, using Eqs.(2),(3),(4),(5) and (8), the total bacteria population at time t can be written as

$$P_T(t) = p_-(t) + p_+(t) + \frac{\delta_n \alpha(t) n\, p_p(t)}{m_s}\left[f_+ \Theta\left(p(t) - p_m\right) - f_- \Theta\left(p_-(t) - p_m\right)\right] + f_- \frac{\delta_n n\, p_p(t)}{m_s} \Theta\left(p_-(t) - p_m\right) \qquad (12)$$

It can be noticed that the step function in the mappings for the bacteria 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 dynamic in the rhizosphere.

3.1. Model analysis and results

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

Under no sanction (σ = 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 α does not change with time and thus the relative proportion of bacterial populations is determined by its initial value α (t) = α (0). A demonstration is shown in Appendix A.

We found a critical value α_c, such that two different dynamical regimes can be distinguished. When $\alpha \leq \alpha_c$ the dynamics leads always to the extinction of plants; the smaller the α value, the faster the extinction. This can be understood by looking at Eqs. (9) and (11). If the initial number of fixing bacteria is too 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 bacteria populations

only through α 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 extinguished, the bacterial populations in the rhizosphere 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. Again, the closer the value of α to α_c the slower the convergence to the stationary situation. More details on how the critical value of α 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 $p_m \neq 0$, α 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 α (0). The behaviour of the final plant population is more complex now, since it depends on whether α (t) overcomes the critical value α_c (see Appendix B) during the dynamics of the coupled system. However, we found that again both bacterial and plant populations always reach a stationary value for long times.

If the initial populations of both type of bacteria are below the threshold p_m, their dynamics is completely decoupled from the plant system and they develop logistically, while the plants go extinguished 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 extinguished when $p_+(0) < p_m$, no matter the value of α (0). Fig. 6 a shows the typical behaviour of p_+, p_- and p_p, for $\sigma = 0$, $\alpha = 1.5\ \alpha_c$ and $p_m > 0$.

With intermediate 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 (Fig. 6b).

With extreme sanction ($\sigma = 1$), the plants halt all the non-fixing bacteria inside the nodules coming into the soil. In this situation the only way the non-fixing bacteria may persist in the system is due to their reproduction in the soil (Fig. 6c). The fixing bacterial populations grow faster due to the reinsertion of the bacteria coming from the senescent nodules. It is clear that α, 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 $p_m = 10^3\ g^{-1}$.

When $\alpha < \alpha_c$ and/or $p_+(0) < p_m$ the plant population is extinguished after a few iterations (not shown), as in the case with no sanction. 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 number of fixing bacteria is smaller than α_c, depending of the value of $p_+(0)$. For values of α smaller but close to α_c the plant population can show a non-monotonous behaviour. In this case the number of seeds and the plant population decrease in the first iterations but the remaining plants are enough to increase the population of fixing bacteria so that $\alpha(t)$ exceeds the critical value. This can be observed in more detail in Fig. 7. Hence, the main effect of the sanction is to reduce the required initial value of α for the plants to survive. The minimum value of α for which this effect

can be observed is approximately $\alpha \approx 0.9\ \alpha_c$. However, this reduction operates at unrealistically low values of α.

Another effect of the presence of sanction is to reduce slightly the time needed for populations to reach a stationary state, as shown also in Fig. 6c.

4. Discussion

Using a combined experimental and population model approach we showed that ecological persistence of legume- rhizobia mutualism under field comparable conditions is not compromised by the presence of non-fixing, cheating rhizobia in the symbiotic system. Under a restrictive scenario, that the only source of nitrogen is from symbiosis, experimental plants survive in good conditions and 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, which is a common situation in field (Amarger, 1981; Singleton and Tavares, 1986; Simms et al., 2006). Taken together, the experimental results and the simulation outcomes 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 rhizobial populations. Plants with all nodules occupied by cheating rhizobia are not able of surviving and

ultimately they die due to N starvation about 6 weeks after inoculation, as expected since rhizobial symbiosis was the only nitrogen source. 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 are 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 further tested.

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 number (α_c) of total soil rhizobia population size represented by fixing rhizobia needed to provide a minimum N_2 amount for plant population persistence. In our knowledge, this critical number has not been experimentally determined yet. 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, but still 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 ensures a source of cheating strains for nodulation in the next plant population cycles. Relaxing the assumption of no ecological differences and assuming for example competitive advantage of cheating strains, the critical number (α) 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 favorable 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 the critical α 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.

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., 2002 a,b). However, in contrast with the modelling approaches followed by these authors, we did not include any genetic relatedness between rhizobia involving 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 we included plant sanctions in the model, we found that results did not change significantly. The main effects consisted in reducing the time needed for plants to reach population equilibrium and to lower the critical α. Since the sanction is lowering or halting the return of cheating rhizobia to soil, 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 α. In any case, a minimum bacteria population is needed to trigger nodulation. Another expected result from applying plant sanction is that populations of cheating rhizobia will go extinguished from soil with time. As previously noted, this is not a realistic situation since persistent cheating strains may chronically hamper crop productivity (Amarger, 1981).

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 complications 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). Another potential complication 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. However, none

of these further complications is expected to pave the way for the plant sanction hypothesis.

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 a field experiment using Lupinus arboreus plants and associated Bradyrhizobium spp. was reported (Simms et al., 2006). However, nodule rhizobial population sizes were measured and related only to nodule size and not to strain efficiency in independently field collected nodules (Simms et al., 2006), thus not really testing the main host sanction assumption.

On more general theoretical grounds, our results support the point of view that cheating does not necessarily menace rhizobia-legume mutualism. 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). Coexistence of cheaters and true mutualistic partners is also theoretically possible (Ferriere et al., 2002; Foster and Kokko, 2006).

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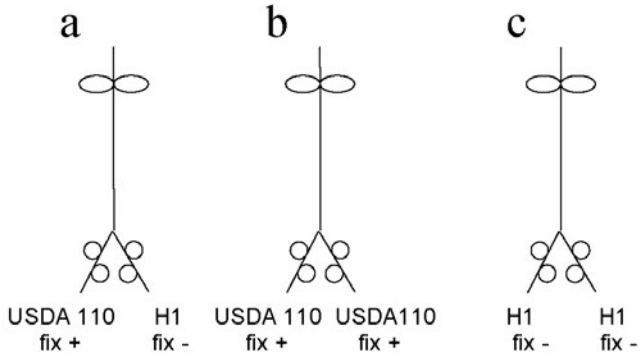

Figure 1. Schematic representation of the split-root plant experiment to test the plant sanction hypothesis. Split roots in each plant were inoculated

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.

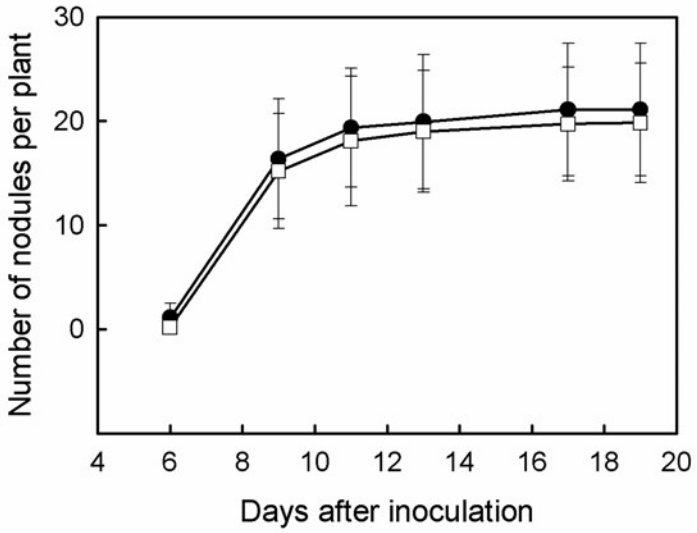

Figure 2. Temporal pattern of nodule production (means " 1 s.d.) in the split-root experiment. Nodule numbers were counted in each half root every three days until nodule production reached a plateau, in half roots of the same plant inoculated with the fixing USDA110 strain (circles) or the non-fixing,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

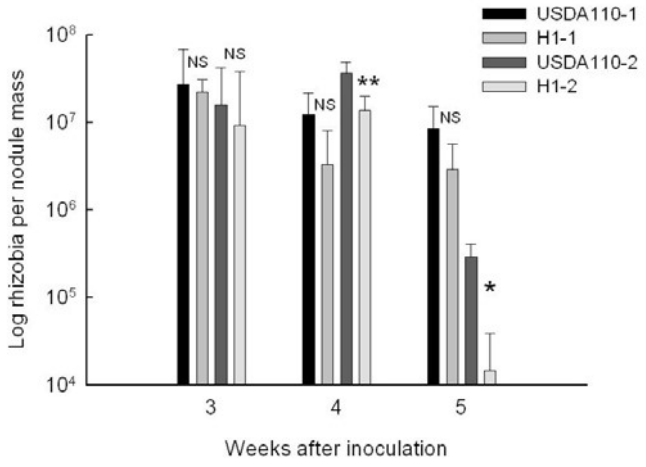

Figure 3. Rhizobia viability per nodule mass in the soybean plant split-root experiments. Rhizobia inside nodules infected by the N_2-fixing USDA110 strain

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 " 1 s.d.

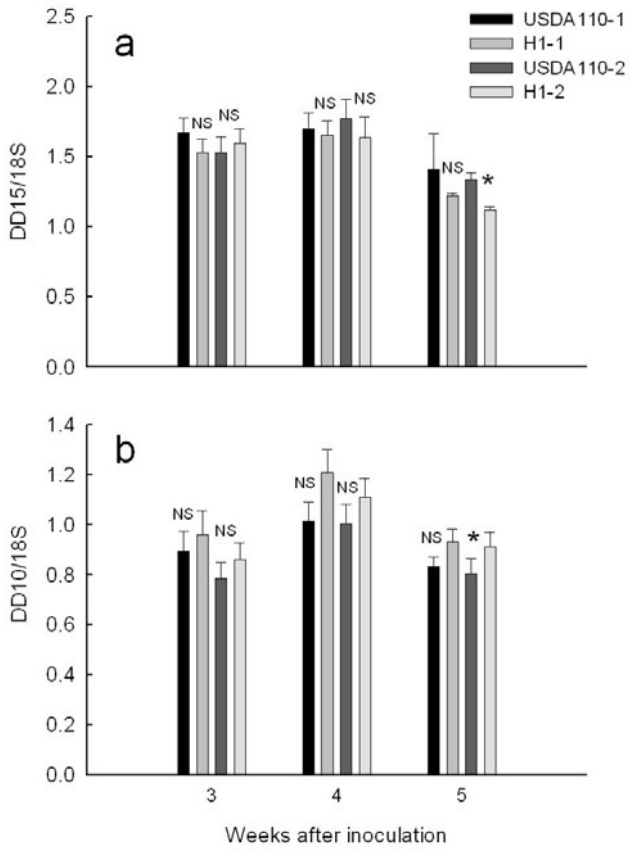

Figure 4. Relative expressions of gene markers DD15 of nodule senescence (a) and DD10 of nodule maturity (b) in nodules from the

soybean plant split-root experiments. *P < 0.05 significant differences by paired t-tests. Bars are means " 1 s.d. Treatments as in Fig. 1.

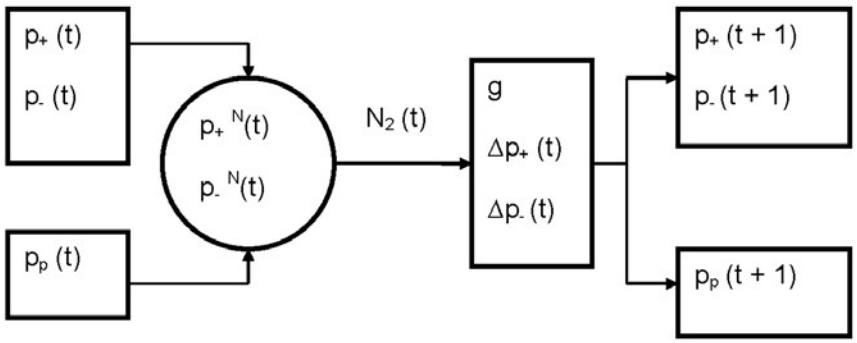

Figure 5. Schematic structure of the model dynamics in a single iteration. Initial values of plant and bacteria populations (p_p for plants; p_+ and p_- for bacteria) set the values of bacteria in nodules (p^N_+ and p^N_-); K_s represents the number of nodules available for colonisation and KN the number of colonised nodules. 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 (Δp_+ and Δp_-).

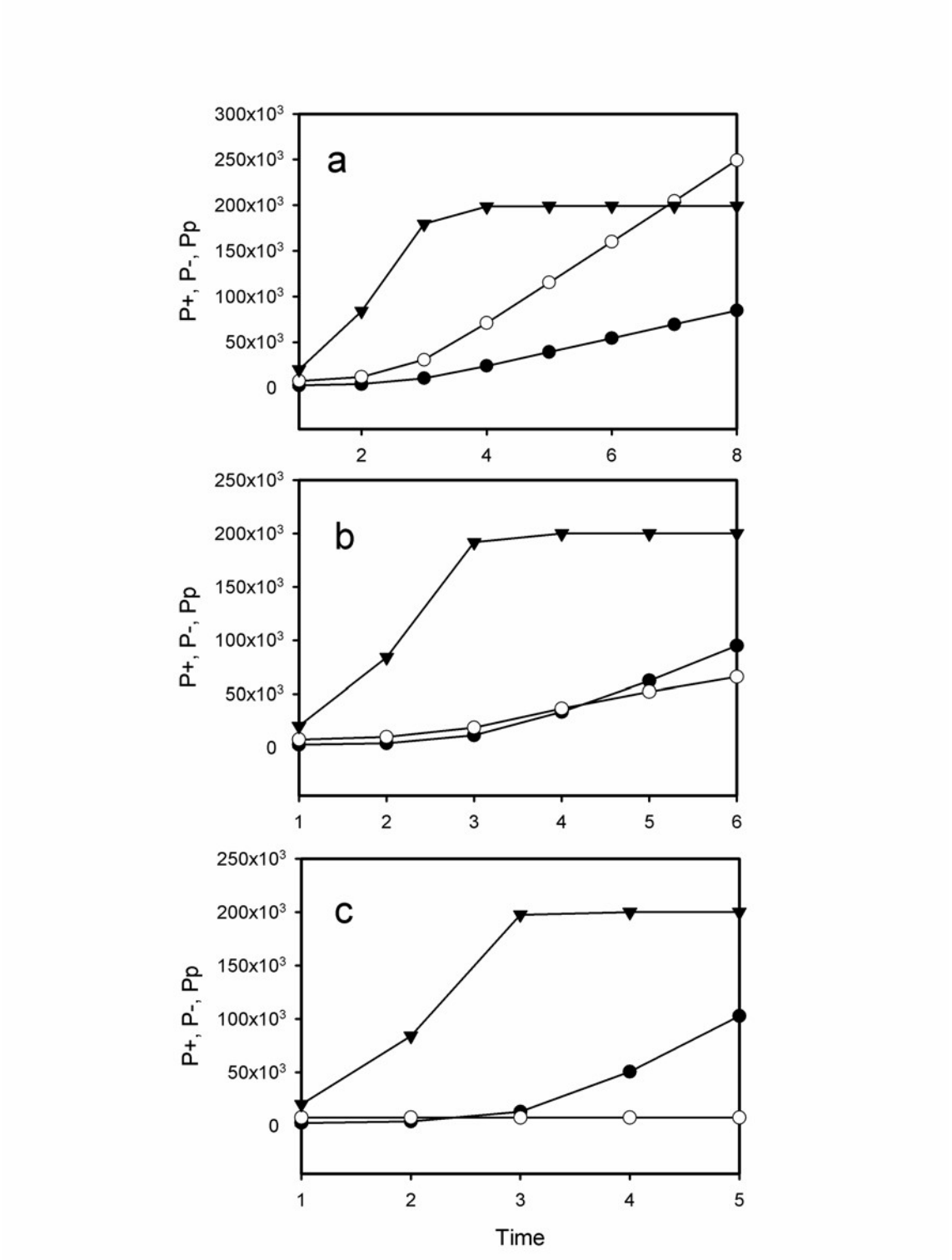

Figure 6. Temporal behaviour of the variables p_p (triangles), p_+ (filled circles) and p_- (empty circles) for $\alpha = 1.5\ \alpha_c$, $p_p(0) = 0.1\ \delta_p$, and different

values of σ. (a) σ = 0 (no sanction), (b) σ = 0.5 (moderate sanction), and (c) σ = 1 (total sanction). Time is measured in number of iterations.

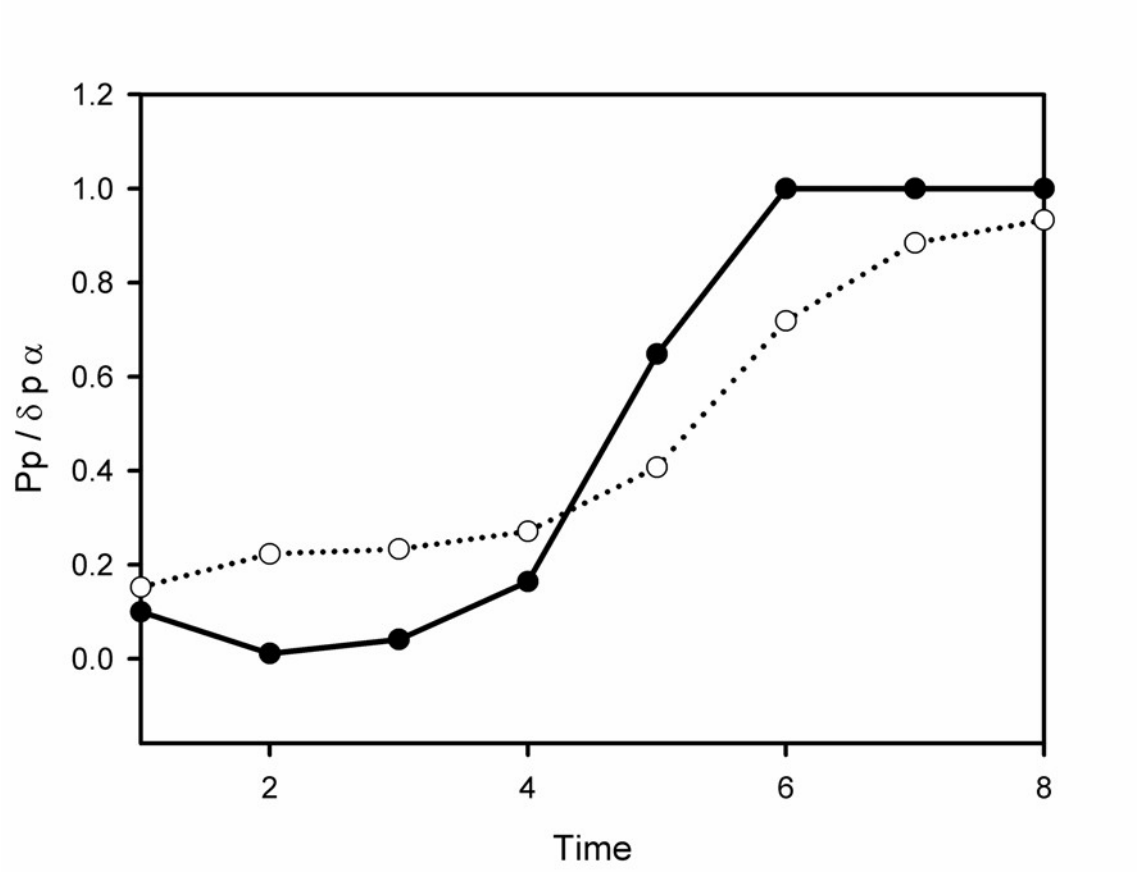

Figure 7. The effect of sanction on final plant population (fraction of carrying capacity δ_p). p_p/δ_p (filled circles), α (empty circles). α_c is the dotted horizontal line. When α exceeds the critical value the plant population starts increasing. The initial value of α is 0.9 αc.

Parameter	Value	Description
r_s	$10^{-4}\ g^{-1}$	Intrinsic rate of growth of bacteria in the rhizosphere (per g of soil)
δ_s	$10^{6}\ g^{-1}$	Rhizosphere´s carrying capacity (per g of soil)
δ_n	10^{6}	Nodule´s carrying capacity
δ_p	$2 \times 10^{5}\ Ha^{-1}$	Plants´ field carrying capacity
m_s	$1.5 \times 10^{5}\ g \cdot Ha^{-1}$	Soil mass per hectare associated to 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
σ	0-1	Sanction intensity 0 = No sanction, 1 = maximum sanction
p_m	$0 - 10^3\ g^{-1}$	Minimum bacteria population per g of soil needed to trigger the nodulation process

Table 1. Parameter values used in the simulations.

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

In the following appendix it is shown that the magnitude α is a constant for these particular values of the parameters p_m and σ.

The definition of α given in 6 applied for t + 1 produces

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

$$= \frac{p_+(t) + f\delta_n n p_p(t)\alpha(t)}{p_+(t) + p_-(t) + f\delta_n n p_p(t)}$$

the second term of the equality comes from (16) and (17) where the second factor was canceled. Should be noted that for $p_m = 0$ and σ = 0 corresponds Θ ($p_i - p_m$) = 1 and $f_+ = f_- = f$.

Replacing $f \delta_n n p_p(t) = B(t)$ and equating to zero produces,

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

$$p_+(t)[\alpha(t+1) - 1] + \alpha(t+1)p_-(t) + B(t)[\alpha(t+1) - \alpha(t)] = 0 \tag{A.3}$$

Noting that,

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

It can be written,

$$-\frac{p_+(t)p_-(t+1)}{p_+(t+1) + p_-(t+1)} + \frac{p_+(t+1)p_-(t)}{p_+(t+1) + p_-(t-1)} + B(t)\Delta\alpha(t) = 0 \tag{A.5}$$

$$\frac{p_+(t+1)p_-(t) - p_+(t)p_-(t+1)}{p_+(t+1) + p_-(t+1)} + B(t)\Delta\alpha(t) = 0 \tag{A.6}$$

Analising the first term in A.6 it is noted that

$p_+(t+1)p_-(t) - p_+(t)p_-(t+1) =$

$= [p_+(t) + B(t)\alpha(t)]\, p_-(t) - p_+(t)[p_-(t) + B(t)(1 - \alpha(t))] =$

$= p_+(t)p_-(t) - p_+(t)p_-(t) + B(t)\alpha(t)(p_+(t) + p_-(t)) - B(t)p_+(t) =$

$$= B(t)\frac{p_+(t)}{p_+(t)+p_-(t)}\left[p_+(t)\ p_-(t)\right] - B(t)\,p_+(t) =$$

$= B(t)\,p_+(t) - B(t)p_+(t) = 0$ (A.7)

$\Rightarrow p_+(t+1)p_-(t) - p_+(t)p_-(t+1) = 0\ \forall\ t$ (A.8)

The first term is zero hence, according to A.6, the second term is also zero.

This means that while $B(t) \neq 0$ then $\Delta\alpha(t) = 0$.

Appendix B. Obtention of α_c whitout sanction

In this appendix a formula for the value of α_c when $\sigma = 0$ is derived. A general analysis of fixed points of the map representing the plants in the model is presented.

The equation for the fixed points of the system is,

$p_P(t+1) = p_P(t)$

In the particular case of the map representing the plants,

$$p_p(t+1) = \delta p\left(1 - e^{-\frac{p(t)g}{k}}\right)$$

From what has been presented so far, it is known that g is a constant in this case and depends only on the parameters and the initial value of α, i.e. the initial proportion of fixing bacteria. Applying a change of variables it is written as,

$$x(t) = \frac{p(t)}{\delta_p}$$

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

where

$$\gamma = \frac{\delta_p g}{k} \qquad \text{(B.1)}$$

Hence the equation for the fixed point is written as follows,

$$x^* = 1 - e^{-\gamma x^*} \qquad \text{(B.2)}$$

$x^* = 0$ is always a solution. From the figure B.1 it can be observed that there is another solution if and only if $\gamma > 1$. Noting that,

$$g = \begin{cases} G \tanh\left(\frac{\alpha n - k_0}{G}\right) & \text{If} \quad \frac{K_0}{n} < \alpha \leq 1 \\ 0 & \text{If} \quad \alpha \leq \frac{K_0}{n} \end{cases}$$

and replacing in B.1 it is obtained that,

$$\frac{\delta_p G}{k} \tanh\left(\frac{\alpha n - K_0}{G}\right) > 1$$

$$k < \delta_p G \tanh\left(\frac{\alpha n - K_0}{G}\right)$$

One extreme situation is when $\alpha = 1$, this means that all the bacteria in the rizhosphere can fix nitrogen. In that case the plant population should survive and for that it must be provided that,

$$k < \delta_p G \tanh\left(\frac{n - K_0}{G}\right) \qquad \text{(B.3)}$$

All the values of k under this condition allow to find the value of α for which the bifurcation occurs, i.e. $\alpha = \alpha_c$ so that $\gamma = 1$. It This can been expressed as follows,

$$k < \delta_p G \tanh\left(\frac{\alpha_c n - K_0}{G}\right)$$

If only are considered the values of α_c such that,

$$\alpha_c n - K_0 \ll G \wedge \alpha_c n - K_0 > 0 \qquad \text{(B.4)}$$

the hyperbolic tangent could be expressed in first order,

$$k < \delta_p G\left(\frac{\alpha_c n - K_0}{G}\right) \qquad \text{(B.5)}$$

The value of α_c is the value of α such that the argument of the hyperbolic tangent is zero $\alpha = \frac{K_0}{n}$ incremented by the ratio of the normalization parameter k and the factor $n\delta_p$ which corresponds to the maximun value of K_s (total colonizable sites).

Replacing this value in the hyperbolic tangent it is obtained,

$$\tanh\left(\frac{\left(\frac{k}{n\delta_p} + \frac{K_0}{n}\right) n - K_0}{G}\right) = \tanh\left(\frac{k}{\delta_p G}\right)$$

and using the condition (B.4) it follows that,

$$k \ll \delta_p G \qquad \text{(B.6)}$$

Stability of the fixed points

The stability of the fixed point is given by the derivative of the map evaluated in the fixed point. For the values $\alpha > \alpha_c$ there are two possible solutions (two fixed points), $x^* = 0$ and $x^* = P$. The value P can not be obtained analytically but if the fixed point $x^* = 0$ is unstable then the other fixed point $x^* = P$ is an attractor.

The stability of a fixed point is given by,

$$\frac{\partial}{\partial x}\left(1 - e^{-\gamma x}\right) \Big|_{x^* = \gamma e^{-\gamma x}} \begin{cases} > 1 & \text{Unstable} \\ < 1 & \text{Stable} \end{cases}$$

If $\gamma < 1$ the only solution is $x^* = 0$ and is and attractor. If $\gamma > 1$, $x^* = 0$ is unstable and $x^* = P$ is stable. Therefore, after the bifurcation the system evolves towards the nonzero fixed point.

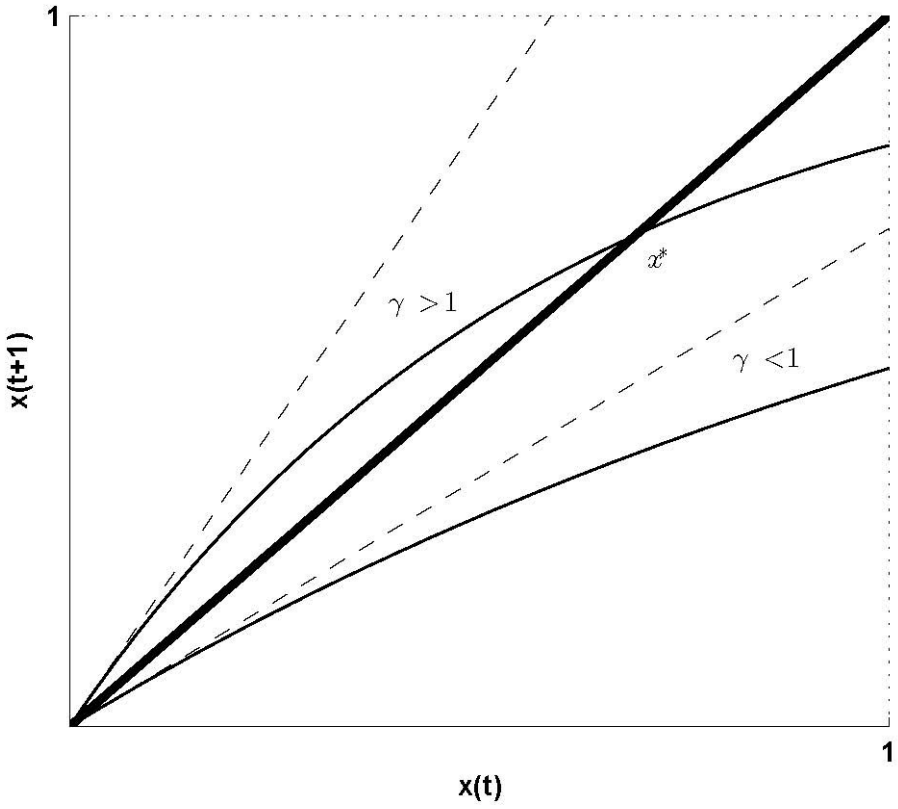

Figure B.1. Graphical representation of the fixed point equation. The dashed line is tanget to the exponential at the origin, the dark line is the identity function. The exponential and the identity function intersect only when $\gamma \geq 1$, hence there is a bifurcation when the tangent line at the origin equals the diagonal, $\gamma = 1$.

Supplementary material available for this article:

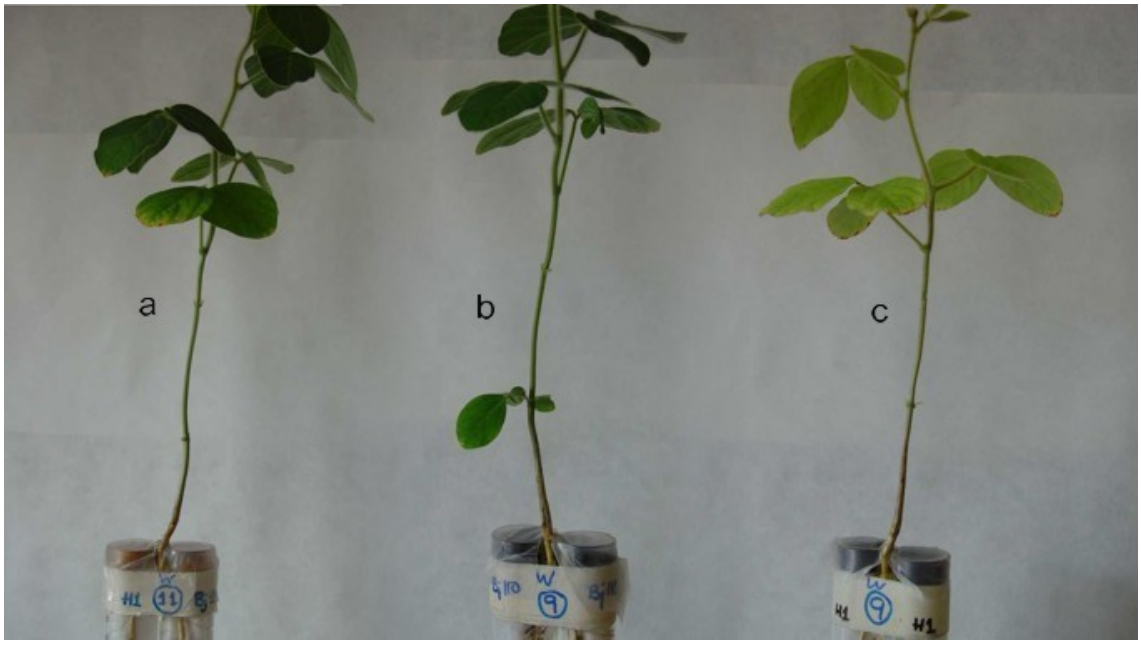

Figure S1. Split-root soybean plants inoculated with the N_2-fixing USDA110 strain or the non-fixing, cheating strain H1, either in half roots of the same plant (USDA110-1/H1-1, a), or in both roots of the same plant (USDA110-2, b, or H1-2, c). After 6 weeks of inoculation, plants A, B showed no evidence of stress, but plant C, with both roots inoculated with the non-fixing strain H1, showed extreme N starvation.