Explaining coexistence of nitrogen fixing and non-fixing rhizobia in legume-rhizobia mutualism using mathematical modeling

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ABSTRACT

In the mutualism established between legumes and soil bacteria known as rhizobia, bacteria from soil infect plants roots and reproduce inside root nodules where they fix atmospheric N_2 for plant nutrition, receiving carbohydrates in exchange. Host-plant sanctions against non N_2 fixing, cheating bacterial symbionts have been proposed to act in the legume-*Rhizobium* symbiosis, to preserve the mutualistic relationship. Sanctions include decreased rhizobial survival in nodules occupied by cheating rhizobia. Previously, a simple population model experimentally based showed that the coexistence of fixing and cheating rhizobia strains commonly found in field conditions is possible, and that the inclusion of sanctions leads to the extinction of cheating strains in soil. Here, we extend the previous model to include other factors that could complicate the sanction scenario, like horizontal transmission of symbiotic plasmids, turning non-nodulating strains into nodulating rhizobia, and competition between fixing and cheating strains for nodulation. In agreement with previous results, we show that plant populations persist even in the presence of cheating rhizobia without incorporating any sanction against the cheater populations in the model, under the realistic assumption that plants can at least get some amount of fixed N_2 from the effectively mutualistic rhizobia occupying some nodules. Inclusion of plant sanctions leads to the unrealistic extinction of cheater strains in soil. Our results agree with increasing experimental evidence and theoretical work showing that mutualisms can persist in presence of cheating partners.

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1. Introduction

In the mutualism established between legumes and soil bacteria known as rhizobia, bacteria from soil infect plants roots and reproduce inside root nodules, where they fix atmospheric nitrogen 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 [1] and reinfect plants roots in the next growing cycle. This naturally occurring mutualism has been since long used in agriculture to add rhizobia to the crops as inoculants, to replace or at least reduce the use of nitrogen fertilizers, mainly as nitrates, which causes many environmental and human health problems. Field added nitrates leach to water contributing to eutrophication and the blooming of algae that diminishes oxygen water content,

thus causing fish death. Nitrate contamination of drinking water is of great concern, since it can cause methemoglobinemia, especially in children [2]. Also, a nitrate metabolite, nitrosamine, is carcinogenic [3].

In the *Rhizobium*-legume mutualism, partner benefits are clear: plant receives nitrogen from *Rhizobium* bacteria inside the nodules and bacteria receive carbon compounds from the plant. However, strains of nodulating rhizobia that do not fix (or fix low) nitrogen are common in the soil and even coexisting in the same plant [4]. Nodulation by ineffective rhizobia is an example of "cheating" (receiving benefits but not reciprocating) by a partner mutualist [5]. This presents the problem of how can cooperation be maintained if partners pursuit only self-benefit? In the legume-rhizobia symbiosis, decreased nodular rhizobial viability and/or early nodule senescence have been proposed as plant host sanctions against non fixing, cheating rhizobia [6–8]. However, in a previous work combining experiments and mathematical modeling no evidence of plant host sanctions was found [9]. In that first approach, the ecological stability of *Rhizobium*-legume symbiosis, when "cheat-

ing" strains were present, was analyzed using a population dynamics model with and without the inclusion of plant host sanctions. Here, we extend the previous model [9] to include other factors that confer more realistic conditions, and could complicate the sanction scenario, like horizontal transmission of symbiotic (*sym*) plasmids, turning non-nodulating strains into nodulating rhizobia [10], and competition between fixing and cheating strains for nodulation [11]. Transfer of plasmids conferring nodulation abilities would lead to changes in the frequency of fixing and non-fixing rhizobia population both in the soil and coming from nodules. Competition in our modeling context would lead to changes in the rhizobial population densities, depending on which strain, fixing or non-fixing, is given the competitive advantage.

The inclusion of these two factors will allow for a new and deeper understanding of the Rhizobium-legume symbiotic persistence in the rhizobial cheating and host-sanction context.

2. Model development and biological background

The model is based on 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 [12]. Details of the biological framework of the model are given in [9]. Briefly, the model formulation is based on several biological features of the mutualistic system and the following assumptions, either checked or supported by experimental tests:

- *Sym* plasmid is the only factor that confers nodulation ability and fixing capacity (fix + or fix −). It can be transferred from plasmid-carrying bacteria (fix + and fix −) to recipient bacteria without plasmid (plasmid-free bacteria) in the soil [10]. A single bacterium can carry only one *sym* plasmid (fix + or fix −) at a time.
- Fixing and non-fixing bacterial strains differ in their N_2 fixing ability and competition levels for nodule initiation [11,12]
- Nodules are initiated and occupied by a single bacterium of either fixing or non-fixing strain [13].
- Nodules are occupied to their carrying capacity, and are functionally equivalent and metabolically independent of each other [6].
- Fixing and non-fixing nodules can develop and coexist in the same plant [12].
- There is a minimum number of fixing nodules per plant needed to develop and produce seeds [12].
- At the end of each annual cycle nodules undergo senescence and release surviving bacteria into the soil [1].
- The number of bacteria coming to the soil from nodules occupied by fixing and non-fixing bacteria can vary if plant sanctions are assumed [6].
- The time scale is one year, assuming an annual plant and a slow rhizobial turnover in soil [1].

We modeled the mutualistic plant-rhizobia system described above using logistic mappings. Equations represent the plant population, the populations of free bacteria living in the soil closely surrounding the root, fixing and non-fixing bacteria, and plasmid-free bacteria, and bacteria inside nodules. Fig. 1 shows a scheme of the model. Details of the biological framework of the model are given in [9]. Briefly, we describe the fixing and non-fixing bacteria and recipient bacteria without plasmid populations in soil by two coupled logistic maps, modified to take into account the bacteria coming into the soil from the senescent nodules (fix +, fix −), and
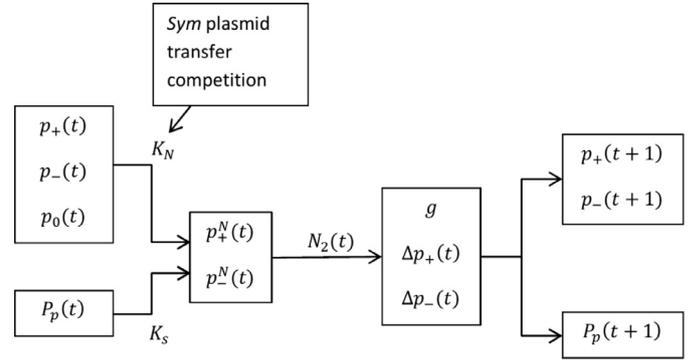

Fig. 1. Schematic structure of the model dynamics in a single iteration. Initial values of plant and bacteria populations (P_p for plants; p_+, p_- and p_0 for bacteria in soil) set the number of nodules formed after *sym* plasmid transfer and competition between fix + and fix − rhizobia, and so the values of bacteria in nodules (p_+^N and p_-^N) to be released in soil after nodule senescence. 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_-). The total number of nodules generated by bacterial strains K_N and K_s denotes the total root colonisable sites for nodule initiation.

already in the soil (plasmid-free bacteria):

$$p_i(t+1) = (p_i(t) + \Delta p_i(t))\left(1 + r_i^s\left(1 - \frac{P_T(t)}{\delta_s}\right)\right)$$
$$+ \beta p_0(t) p_i(t) - \tau p_i(t), \quad i = +, -, \tag{1}$$

$$p_0(t+1) = p_0(t)\left(1 + r_0^s\left(1 - \frac{P_T(t)}{\delta_s}\right)\right)$$
$$- (p_0(t)\beta - \tau)(p_+(t) + p_-(t)), \tag{2}$$

$$P_T(t) = p_+(t) + \Delta p_+(t) + p_-(t) + \Delta p_-(t) + p_0(t), \tag{3}$$

where p_i describes the bacteria population densities in soil, $i = +, -, 0$ indicates fixing and non-fixing bacteria, and plasmid-free bacteria respectively, and P_T is the total bacteria population density (i.e., fixing plus non-fixing plus plasmid-free bacteria) in soil. The parameter δ_s stands for the carrying capacity of the soil close to roots in absence of nodulation. An effective carrying capacity greater than δ_s is set when plants release bacteria at the end of nodulation process. Parameter β stands for the probability of horizontal *sym* plasmid transfer from fix + or fix − bacteria to plasmid-free bacteria and its value was set at 10^{-6} events per recipient cell [14]. Constant τ is the probability of *sym* plasmid loss and its value was set at 10^{-3} events per *sym* plasmid carrying cell [15].

The parameter r_i^s represents the intrinsic reproduction rate of each population in the soil close to roots, assumed to be equal $r_+^s = r_-^s = r_0^s = r^s$. The total number of the surviving bacteria that returns to the soil at time t is calculated as $\Delta p_i(t)$. Dynamics of p_0 occurs exclusively in the soil, and is affected by *sym* plasmid transfer (β) and plasmid loss (τ). Plants are not able to differentiate fixing from non-fixing bacteria during the root colonization process [11]. If we assume no selection of rhizobia by plants inside nodules, the number f_i of surviving bacteria of each type released from a nodule will be the same for fixing and non fixing bacteria (about 10^{-4} of the carrying capacity of a nodule). If we consider that 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 [6,7,16]. In this last case, we allowed the number f_i of surviving bacteria of each type to be different, i.e.

$$\Delta p_i(t) = \frac{f_i \delta_n}{m_s} K_i^N(t), \quad i = +, -, \tag{4}$$

Table 1
Model parameters.

Parameter	Value	Description
r_i^s	10^{-1}	Intrinsic rate of growth of bacteria in the soil ($i = +, -, 0$) [1].
δ_s	$10^6 g^{-1}$	Soil bacterial carrying capacity (per g of soil) [1].
β	10^{-6}	Probability of *sym* plasmid transfer (event per recipient cell) [14].
τ	10^{-3}	Probability of *sym* plasmid loss (event per *sym* plasmid carrying cell) [15].
C	0.8–1.8	Competition coefficient [18].
w	0.4	Competition coefficient [17].
φ	$0 - 1$	Fraction of nodulation sites effectively forming nodules [12].
δ_n	10^6	Nodule's carrying capacity (bacteria per nodule) [12].
f	10^{-4}	Surviving bacteria released from nodule [12].
δ_p	$2 \times 10^5 Ha^{-1}$	Plants' field carrying capacity [9].
m_s	$1.5 \times 10^5\, g\, Ha^{-1}$	Soil mass per hectare associated to the plant population [9].
n	45	Typical number of nodules per plant [12].
K_0	$0.15 \times n$	Minimum number of fixing nodules per plant needed for seed production [12].
G	55	Maximum number of viable seeds produced per plant [9].
Π_g	0.69	Probability of a viable seed reaching the adult stage [9].
σ	$0 - 1$	Sanction intensity 0 = No sanction, 1 = maximum sanction.
p_m	$0 - 10^2 g^{-1}$	Minimum bacteria population per g of soil needed to trigger the nodulation process [12].

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 that plant applies to the non-fixing bacteria. Its value goes from 0 to 1, where $\sigma = 0$ represents the case without sanction.

The number of nodules generated by each type of bacterial strain is $K_i^N(t)$, and it represents a fraction of the total root colonisable sites for nodule initiation $\varphi K_s(t)$. We allow for both rhizobial strains to vary in their ability to colonize the root and initiate nodules.

If $p_+(t) + p_-(t) \geq p_m$ then K_+^N and K_-^N are defined as the solution of the system:

$$\frac{K_+^N}{K_-^N} = C\left(\frac{p_+}{p_-}\right)^w, \tag{5}$$

$$K_+^N + K_-^N = \varphi K_s, \tag{6}$$

where φ is the fraction of nodulation sites effectively forming nodules. On the other hand, if $p_+(t) + p_-(t) < p_m$ then $K_+^N = K_-^N = 0$. Here $K_s(t) = nP_p(t)$ is the number of sites available for nodulation, where n is the average number of nodules per plant considered proportional to the plant population $P_p(t)$ (number of plants per hectare), the threshold p_m is the minimum bacteria population per gram of soil needed to trigger the nodulation process, C and w are the competition coefficient and exponent respectively in Amarger's equation [17]. When $C = 1$ and $w = 1$ there is no competition between fix + and fix − strains for nodule initiation, and so the nodulation is assumed to be at random. If $C > 1$ then strain fix + is more competitive. For simulations, C values were taken between 0.8 and 1.8 [18], and w was fixed at 0.4 [17]. 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 plant population is defined by

$$P_p(t + 1) = \delta_p\left[1 - \exp\left(-g(t)\frac{|\log(1 - \Pi_g)|P_p(t)}{\delta_p}\right)\right]. \tag{7}$$

Briefly, δ_p is the plant carrying capacity of the field, Π_g is the probability that a seed germinates and develops into an adult plant, and g is the mean number of seeds produced by a plant in an annual crop, that is assumed to depend on the number of nodules colonized by fixing bacteria and the plant population itself, i.e. $g(t) = h(K_+^N(t), P_p(t))$ for a given function of two variables h.

The number of seeds depends on the amount of available nitrogen for 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, h will be a monotonously increasing function of K_+^N. 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 K_0 colonized by fixing bacteria required to produce seeds. Notice that plants that not reach K_0 do not complete their development and ultimately die from nitrogen starvation [12].

All the previous assumptions can be modeled by the following expression

$$h(K_+^N, P_p) = \begin{cases} G \tanh\left(\dfrac{K_+^N - K_0 P_p}{G P_p}\right), & \text{if } K_+^N - K_0 P_p > 0, \\ 0, & \text{otherwise.} \end{cases} \tag{8}$$

If the sum of bacterial populations $p_+ + p_-$ is below the value of p_m then it does not interact with the plant system and the plant dynamic is entirely given by its own logistic dynamic in the soil.

3. Model analysis and results

We compare the behavior of the model looking at bacteria and plant population dynamics in soil, for different values of C (the competition coefficient) and $\sigma = 0$ (without sanction), $\sigma = 0.5$ (moderate sanction) and $\sigma = 1$ (total sanction). Sensitivity analysis showed that changing the value of w (slope of the competition equation) does not significantly influence population dynamics of strains and plants. In Table 1 we show the values of the parameters that were used through the numerical simulations. Initial conditions were: $p_0 = 200$, $p_+ = 400$, $p_- = 400$ and $P_p = 20,000$.

Assuming no sanction ($\sigma = 0$), plants are unable to discriminate among fix + and fix − rhizobia, 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 strains. In all simulations we consider the case in which $p_+(0) + p_-(0) \geq p_m$, i.e. there is enough rhizobia in soil to elicit nodulation. In all the considered cases without sanction, the population of free-plasmid bacteria p_0 remains in the system even with very low values, reaching an equilibrium in which the plasmid transfer rate and the plasmid loss rate are balanced.

The populations of plasmid bearing strains p_+ and p_- show a fast growth during the first initial cycles, to reach equilibrium at about 500 years, with relative values of p_+ and p_- depending on the competition coefficient C. Notice that increasing values of C
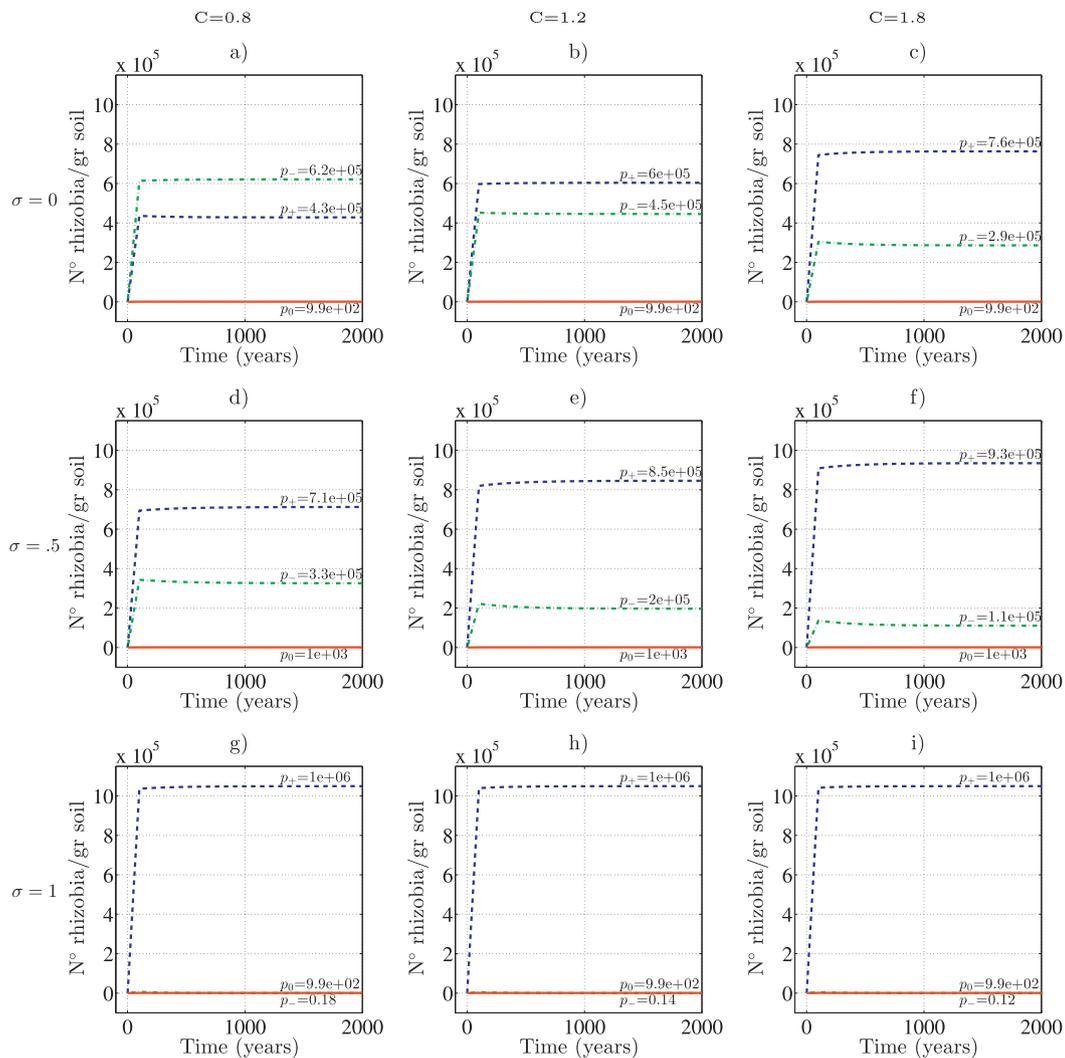
Fig. 2. Simulation results showing dynamics of strains populations in soil, fix+ (p_+), fix− (p_-) and plasmid-free rhizobia (p_0) under different sanction (σ) and competitive ability (C) scenarios. First row corresponds to no sanction ($\sigma = 0$), second row corresponds to moderate sanction ($\sigma = 0.5$) and third row corresponds to total sanction ($\sigma = 1$). First column corresponds to $C = 0.8$, second column corresponds to $C = 1.2$ and third column corresponds to $C = 1.8$.

show increasing relative population of p_+ with respect to p_-, although both populations coexist in time anyways. Indeed, we can see that rising the competitive ability of the fix + strain ($C = 0.8$–1.8), has the effect of increasing its population even more in at least one order of magnitude (see Fig. 2a, b and c). Plant population stabilizes at 2×10^5 plants/hectare within a few cycles, and it is not influenced by changes in strains' competitive abilities.

Notice that the fact that plant population converges to the value $\delta_p = 2 \times 10^5$ can be proved analytically, by showing that δ_p is a fixed point of Eq. (7); in other words, that if the carrying capacity δ_p is reached at time t then the population of plants stabilizes at this value for later times. Indeed, let us suppose that there exists a time t_* such that $P_p(t_*) = \delta_p$ (i.e. the carrying capacity is reached at t_*). Then, since right hand side of Eq. (7) is increasing we have $P_p(t_* + 1) \geq P_p(t_*) = \delta_p$. But we also have that $P_p(t) \leq \delta_p$ for all t. Consequently, $\delta_p \leq P_p(t_* + 1) \leq \delta_p$, which let us conclude that $P_p(t_* + 1) = \delta_p$. Analysis of steady states of bacterial populations is quite more challenging and it is discussed in the Supplementary material.

Allowing for moderate sanction ($\sigma = 0.5$), i.e. half of the nodules prevented from releasing bacteria into the soil, the overall behavior of the bacteria populations is qualitatively similar to the case without sanction. The advantage of p_+ over p_- is in-

creased even with the lower C value. A stable coexistence between the strains is reached at times similar to those without sanction (see Fig. 2d, e and f). Plant population stabilizes at 2×10^5 plants/hectare within a few cycles, and again, it is not influenced by changes in strains' competitive abilities.

Considering total sanction ($\sigma = 1$), plants do not allow the non-fixing bacteria inside the nodules to come into the soil. While the fix – rhizobia go extinct (less than one bacterium per gram of soil) very early, the fixing bacterial populations grow to higher levels due to the reinsertion of the bacteria coming from the senescent nodules. Varying competition ability for the fixing strains does not exert any effect in a scenario of total sanction (see Fig. 2g, h and i). This means that, in the long term when the plant population apply extreme sanctions, only fixing rhizobia will be present in the system. As in the previous cases, plant population stabilizes at 2×10^5 plants/hectare within a few cycles, and it is not influenced by changes in strains' competitive abilities.

Fig. 3 shows the ratio of fix + and fix – populations varying the strains' competitive abilities under different sanction scenarios. From here we can observe that the large time behaviors of the two populations are very similar with no sanction or moderate sanction while the influence of a greater competitive ability of the fix + strain is reflected in a slight ratio increase (notice that
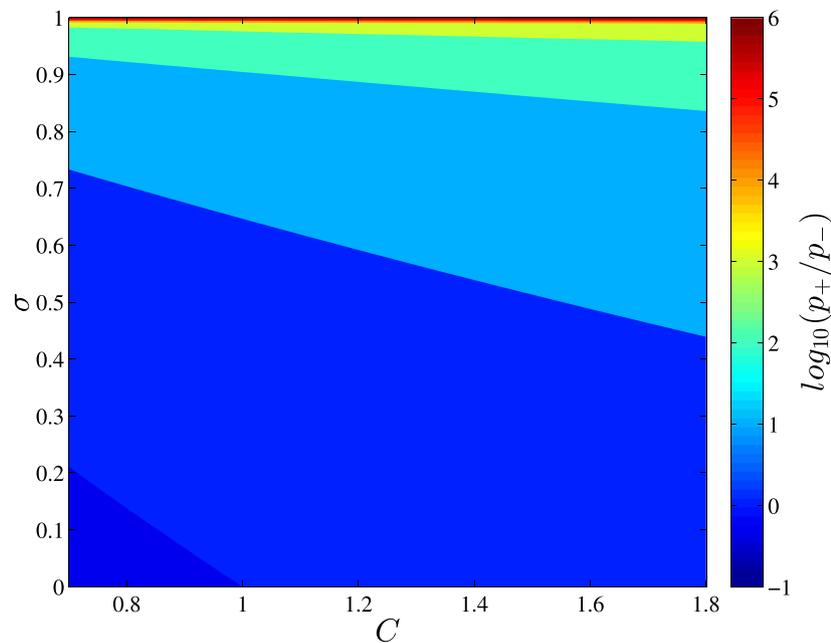
Fig. 3. Simulation results showing the ratio of strains populations in soil, fix$+$($p+$), fix$-$($p-$) under different sanction scenarios and competition coefficients, for large time. Horizontal axis represents competition coefficient (C) values from 0.8 to 1.8, vertical axis corresponds to sanction values (σ) in the interval from 0 to 1 while the intensity of the colors stands for the ratio of strain populations at final time, represented as the $\log_{10}(p_+/p_-)$.

color stripes become wider as C increases). However, the application of total sanction renders a higher ratio increase favoring the fix$+$strain and showing the extinction of the fix$-$strain.

Sensitivity analysis performed to analyze the influence of horizontal transfer of the *sym* plasmid, performing simulations without *sym* transfer under the same conditions of sanction and competition, showed that the overall system behavior is maintained. However, there is a small quantitative effect, with the population of p_0 increasing (since there is no flux from p_0 to p_+ and p_-), and the populations of p_+ and p_- decreasing (because there is no influx from p_0). The effect is small since the rate of *sym* plasmid transfer is low, according to the used experimental values (see Fig. S1 Supplementary material).

4. Discussion and conclusions

Our results are in agreement with previous results of the model [9], showing that a simple population model including strain competition can explain the coexistence of fixing and non-fixing, cheating rhizobia strains, as it commonly occurs in field conditions. Also as previously found, inclusion of plant sanctions is not required to explain strain coexistence. Plant populations are able to maintain a stable equilibrium provided they get fixing rhizobia needed to provide a minimum N_2 amount, despite being cheated by non-fixing rhizobia.

The assumption of no different competitive abilities between strains for nodulation made in the previous version of the model was relaxed and instead, fixing and non-fixing were allowed to compete for nodulation sites. We explored a range of competition coefficient values, changing competitive advantage from fix$-$to fix$+$strains. This is an interesting scenario for agricultural practices, since a common and problematic situation in crops occurs, when in spite of field inoculation with highly efficient rhizobia strains, after a few years nodulation becomes produced by more competitive but less efficient or even non-fixing strains residing in soil [4,11,19]. Our results showed that even slight improvements in nodulation competitiveness of the fixing strains used in inoculants could result in significant raises in fixing strains populations

in soil with the resulting reduction in the use of nitrogen fertilizers. The inclusion of horizontal transfer of the symbiotic plasmid, conferring nodulation ability to soil rhizobia, is quite challenging since finding experimental values is difficult. We based our parameter value for β on the experimental results reported by Kinkle and Schmidt [14], who recorded horizontal transfer of a symbiotic plasmid between *Sinhorizobium fredii* and *Rhizobium legumi-nosarum* strains nodulating pea, under different conditions (sterile and non sterile soil, humidity, temperature and inoculum amount).

We found that horizontal transfer of the *sym* plasmid exerts a quantitative effect on rhizobia in the soil, although this effect is small since the rate of *sym* plasmid transfer is low, according to the used experimental values [14]. Transfer of *sym* plasmid does not so far represent an important factor in the context of the mutualistic system dynamics explored in this work, where pre-existence in the soil of nodulating rhizobia is assumed (like in agricultural systems artificially inoculated). However, since transmission of *sym* plasmid confers the ability of nodulating and eventually fixing nitrogen, it may represent an important factor in fields harboring non-nodulating rhizobia that could eventually became nodulating (and fixing) through *sym* plasmid transfer from inoculated or invading nodulating strains [10].

In this modeling context, plants receive nitrogen only from rhizobia fixation, in consequence, plants that not reach a minimum amount of nitrogen from fixing nodules do not complete their development and ultimately die from nitrogen starvation (supported by experimental results, [12]). So, the inclusion in the model of sanctions from plants to non-fixing rhizobia [6], as well as in the previous version of the model, was made in the context of plants reaching a minimum number of fixing nodules. What it can be concluded from this scenario is that, provided the plants reach a minimum number of fixing nodules and applying moderate sanctions, the cheating and the fixing strains coexist in the system and the plant population survives and stabilizes over time. Coexistence is also obtained with no sanctions, so we conclude that considering the host sanction hypothesis is not necessary to explain coexistence of cheating and fixing strains. Furthermore, stronger sanctions as proposed by Denison [6], lead to the disappearance of

cheating strains from the system. However, field evidence shows that cheating strains persist in soil and chronically hamper crop yields [11].

From a theoretical point of view, our results support the idea that cheating does not necessarily endanger rhizobia-legume mutualism and that a gradient from mutualism to parasitism can be found in nature [5]. There is an increasing wealth of theoretical and empirical evidence that cheating is widespread and punishment is rarely applied to defective mutualistic partners, and that other factors like herbivory can influence mutualisms [20,21]. Furthermore, costs of cheating in rhizobia-legume mutualism may not be as high as assumed if the host is still able of obtaining benefits from other mutualistic partners, as in co-infected plants that are common in field [4,19]. This situation will be incorporated in the model in further versions, allowing for co-occupation of the same nodule by strains with different fixation abilities. About 20% of total nodules are usually co-occupied at field by different rhizobial strains in artificial inoculations [22]. We expect the effects of co-occupation of nodules by non-fixing rhizobia will be diluted by fixing rhizobia occupying the same nodule, thus not favoring plant sanctions.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at 10.1016/j.mbs.2017.07.001.

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