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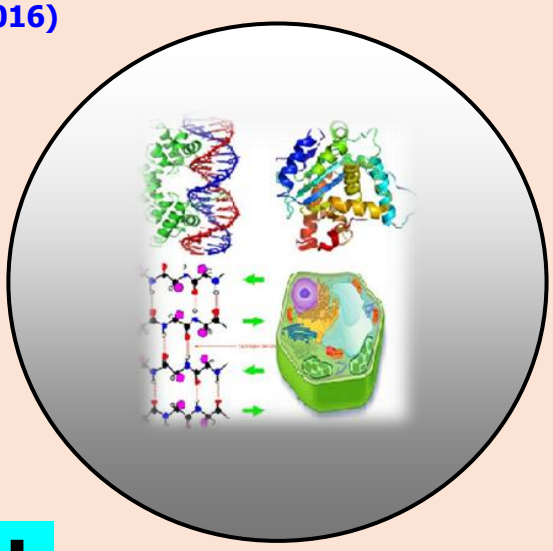

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RESEARCH PAPER

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Role of Rhizobium Strains in Symbiotic Nitrogen Fixation

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ABSTRACT

Nitrogen is a critical limiting element for plant growth and production. It is a major component of chlorophyll, the most important pigment needed for photosynthesis, as well as amino acids, the key building blocks of proteins.

Resource mutualisms such as the symbiosis between legumes and nitrogen-fixing rhizobia are context dependent and are sensitive to various aspects of the environment, including nitrogen (N) addition. Mutualist hosts such as legumes are also thought to use mechanisms such as partner choice to discriminate among potential symbionts that vary in partner quality (fitness benefits conferred to hosts) and thus impose selection on rhizobium populations. Biological nitrogen fixation (BNF), discovered by Beijerinck in 1901, carried out by a specialized group of prokaryotes. These organisms utilize the enzyme nitrogenase to catalyze the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). Plants can readily assimilate NH_3 to produce the aforementioned nitrogenous biomolecules. These prokaryotes include aquatic organisms, such as cyanobacteria, free-living soil bacteria, such as Azotobacter, bacteria that form associative relationships with plants, such as Azospirillum, and most importantly, bacteria, such as Rhizobium and Bradyrhizobium, that form symbioses with legumes and other plants.

Key words: Mutualism, Rhizobium Sp, Ecosystem and Nitrogen Fixation.

INTRODUCTION

The growth of all organisms depends on the availability of mineral nutrients, and none is more important than nitrogen, which is required in large amounts as an essential component of proteins, nucleic acids and other cellular constituents. There is an abundant supply of nitrogen in the earth's atmosphere - nearly 79% in the form of N_2 gas. However, N_2 is unavailable for use by most organisms because there is a triple bond between the two nitrogen atoms, making the molecule almost inert. In order for nitrogen to be used for growth it must be "fixed" (combined) in the form of ammonium (NH_4) or nitrate (NO_3) ions. The weathering of rocks releases these ions so slowly that it has a neglible effect on the availability of fixed nitrogen. So, nitrogen is often the limiting factor for growth and biomass production in all environments where there is suitable climate and availability of water to support life Apple by 1984.

Microorganisms have a central role in almost all aspects of nitrogen availability and thus for life support on earth some bacteria can convert N_2 into ammonia by the process termed nitrogen fixation; these bacteria are either free-living or form symbiotic associations with plants or other organisms (e.g. termites, protozoa) other bacteria bring about transformations of ammonia to nitrate, and of nitrate to N_2 or other nitrogen gases many bacteria and fungi degrade organic matter, releasing fixed nitrogen for reuse by other organisms. All these processes contribute to the nitrogen cycle. Mutualistic interactions are fundamental components of every ecosystem on Earth, span all domains of life, and contribute immensely to ecosystem function. In resource mutualisms, partners trade in limiting resources such that both species enjoy higher fitness. Higher-quality mutualists provide more resources and thus increase the fitness of their partners over those of lower-quality mutualists; therefore, partner quality can be viewed as a quantitative trait of one mutualist, measured as the effects it has on its partner's fitness, and composed of genetic and environmental effects .Partner quality is quite variable in nature, but theoretical models of mutualism cannot easily explain how such variation is maintained in the face of selection. One explanation for the maintenance of mutualism variation is context-dependency; if selection acting on mutualist populations shifts depending on the environment, then variable environments (biotic, abiotic) can maintain genetic variation in partner quality. Resource mutualisms might be particularly context dependent, as the availability and thus the value of traded commodities varies over spatial and temporal scales. Thus, understanding whether known agents of selection in mutualisms vary with the environment is part of a general understanding of how resource mutualisms (co)evolve. Here, we tested for environmental context dependence in plant rhizobial mutualisms by examining plasticity in host associations in response to external variation in nitrogen (Benson and Silvester, 1993).

Table 1. Show Nitrogen fixation important function.

Type of fixation	**N_2 fixed (10^{12} g per year, or 10^6 metric tons per year)**
Non-biological	
Industrial	about 50
Combustion	about 20
Lightning	about 10
Total	**about 80**
Biological	
Agricultural land	about 90
Forest and non-agricultural land	about 50
Sea	about 35
Total	**about 175**
Data from various sources, compiled by DF Bezdicek & AC Kennedy, in *Microorganisms in Action* (eds. JM Lynch & JE Hobbie). Blackwell Scientific Publications 1998.	

Plants in the legume family (Fabaceae) exchange photosynthate for plant-available forms of N generated by N-fixing bacteria (collectively termed rhizobia) that are housed in root nodules. The legume–rhizobium symbiosis is ecologically and economically significant, as it is the main source of non anthropogenically fixed N in terrestrial systems (Vitousek et al., 1997; Cleveland et al., 1999). The Fabaceae is also one of the plant families (Doyle, 1998; Werner et al., 2014) and the second most important crop family behind the grasses (Graham and Vance, 2003). Since there is no vertical transmission of symbionts, plant hosts must acquire rhizobial partners anew with each generation, and these prospective plant hosts are faced with a diverse range of potential rhizobium species and genotypes .The rhizobium with which a plant associates is potentially very important, since rhizobia can vary considerably in terms of the fitness benefits they confer to the plant host. Current research suggests that legumes possess mechanisms that mitigate the potential impacts of less-beneficial rhizobia on host fitness (or conversely, reward better mutualist partners) and thus potentially act as agents of selection on rhizobium partner quality.

There is evidence that plants can allocate fewer resources to nodules that generate less fixed N, resulting in smaller nodules containing fewer rhizobium offspring Many legumes also seem to be able to discriminate among rhizobia and preferentially form nodules with strains that confer greater fitness benefits ("partner choice). It is important to note, however, that there is likely not a single universally most beneficial strain of rhizobia; instead, the degree of fitness benefits provided by a rhizobial symbiont depends on the genotype of the host plant (G × G interactions) and the environmental context (G × E). Furthermore, the ability to discriminate among rhizobium partners of varying quality appears to be genetically variable in natural populations of legumes. The dependence of rhizobium partner quality and host benefits on the environment suggests that host-mediated selection on rhizobium populations and resulting evolutionary effects on rhizobium partner quality will also be context dependent Napoli, 1975. Recent human activity has elevated soil nitrogen (N) availability in both natural and agricultural systems through direct fertilization and atmospheric deposition. If soil N is readily available, it presumably becomes less costly for a plant to simply acquire N from the environment rather than invest resources into symbiosis. Legumes typically decrease nodulation in response to N addition suggesting that symbiosis is costly to maintain. For these reasons, increases in soil N are expected to disrupt the legume–rhizobium mutualism by weakening the positive fitness feedbacks between partners that maintain mutualism. Indeed, recent evidence indicates that N fertilization leads to changes in partner quality in rhizobium populations both in the short term (one season; and long term (22 yr). though the exact way in which N addition altered selection on rhizobium populations remains unresolved (Fattah, 2008). Together, this previous knowledge of the legume–rhizobium relationship given rise to the prediction that legumes should invest less in discriminating mechanisms when N is readily available. Regus and colleagues (2014) recently studied stabilizing mechanisms under N addition in *Lotus strigosus* and found no evidence that partner choice or sanctions changed in response to high N. We build on this work by studying these mechanisms in a different legume–rhizobium system, incorporating different N conditions and host genetic variation. Using a manipulative greenhouse experiment with three genotypes of the model legume *Medicago truncatula* and a mixed inoculum of three genotypes of its primary symbiont *Ensifermeliloti* across three N levels, we asked: (1) Do the frequencies of rhizobium strain occupancies (of host nodules) depend on the N level? (2) Do the sizes of host nodules inhabited by particular rhizobium strains vary across N levels? (3) How does context dependency in partner choice, or plant responses to mutualist quality, affect the maintenance and evolution of these traits?

MATERIAL AND METHODS

Used a factorial experiment, consisting of three plant maternal families, three N levels, and five replicates (3 × 3 × 5 = 45 pots total) grown in sterile Magenta box leonard jars (Heath et al., 2010) in the University of Toronto plant growth facilities in December of 2007. All plants received the same inoculum of an equal mixture of three strains (48e, 35c, 30a: *Chat c*, *Sals b*, and *Naut a,* respectively, from Soil was autoclave-sterilized and wet with ∼30 mL nutrient solution before inoculation with $\sim 10^8$ total rhizobium cells in 1 mL diluted culture broth. The soil surface was covered with a layer of sterile sand, and pots were bottom-watered with sterile nutrient solution throughout the experiment. We kept Magenta pots filled with 1/4 strength Fahreus solution with intermediate and high N treatments supplemented with 0.1 mM or 1.0 mM KNO_3, respectively. N levels used were those chosen by to replicate the range of reported N levels near fertilized agricultural fields while not eliminating nodulation by *Medicago.* Plants were harvested after 6 weeks. We clipped the aboveground tissue for drying and measuring plant aboveground biomass and counted total nodules on each plant. For each of 10 nodules from each plant, we assigned a size score (1–4; 1 = very small and white; 2 = small but pink; 3 = medium/average (∼2 mm); 4 = large), then isolated and genotyped the rhizobium strain inhabiting the nodule using previously described procedures to assess nodule occupancy Briefly, nodules were surface-sterilized, crushed, and plated before DNA extraction and genotyping using a post-PCR restriction digestion assay designed specifically to differentiate these three *E. meliloti* strains. Of 441 genotyped nodules, 11 nodules were obviously of mixed occupancy even after culturing and were excluded from further analysis. We used fixed-effects ANOVA implemented in the program JMP (version 12, SAS Institute, Cary, North Carolina, USA) to test for the effects of plant family, N level, and their interaction on the two main response variables (nodule occupancy and nodule size) and nodule number and plant biomass. For nodule occupancy, we analyzed the frequency of each strain separately in three separate ANOVA models; then, we created a "nodule occupancy" variable by assigning each plant a 1, 2, or 3 based on which strain was dominant in its nodules (1 = strain 30a, 2 = strain 35c, or 3 = strain 48e).

Similarly, for nodule size, we first analyzed all plant nodules together, then separately for each occupying strain. Results in the separate and combined analyses were similar for both occupancy and size; therefore, we present the latter analysis in the paper for simplicity for per-strain results). Least-squares means (lsmeans) for each plant family from the per-strain analyses for traits X and Y were regressed against the partner quality means (from a previous single-strain inoculation using the same families, strains, and N levels; Heath et al., 2010) to assess partner choice and plant allocation responses to partner quality in this experiment. Partner choice would be indicated by a positive relationship between strain partner quality in single strain inoculation and that strain's occupancy in mixed inoculation; sanctioning would be indicated by a positive relationship between strain partner quality in single inoculation and that strain's nodule size in mixed inoculation (Vadakattu and Paterson, 2006).

RESULTS

As expected, plant biomass increased with elevated N levels and varied among plant families (Table 1). Nodule number ranged from 3 to 64 per plant and varied among plant families, but did not respond significantly to N level or the plant family × N interaction (Table 1). Nevertheless, nodule number and biomass were positively correlated (R^2 = 0.1743, P = 0.0048; Table 1; Appendix S1, see Supplemental Data with the online version of this article), suggesting that even the highest N treatment (1 mM) was not enough to saturate the growth benefits of association with rhizobia.

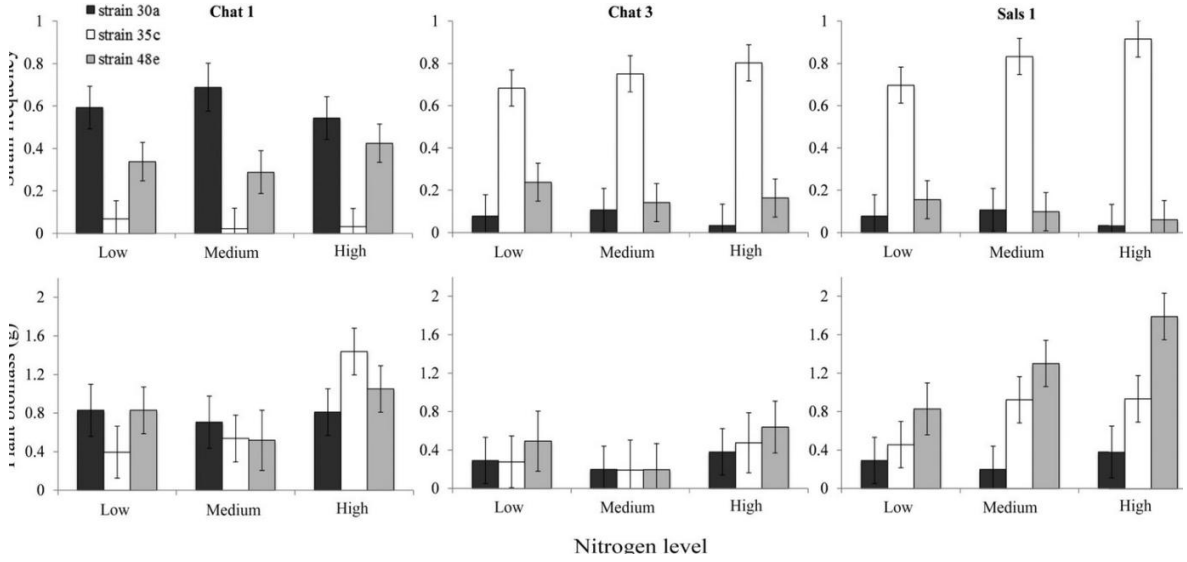

Fig 1. Show Nitrogen level and relation to rhizobium strains.

Nodule occupancy varied significantly among plant families, but was not altered by N addition or the plant family by N interaction). Plant family Chat 1 associated most often with strains 30a and 48e, with mean occupancy of N treatments ranging from 54.3 to 59.3% and 33.8 to 42.5% respectively, and rarely associated with strain 35c (mean occupancy of N treatments ranging from 1.2 to 6.9%; Fig. 1). Conversely, plant families Chat 3 and Sals 1 predominantly associated with strain 35c, with 68.4–80.3% and 71.1–91.5% of nodules being occupied with 35c among N treatments, respectively. Nodule size also varied significantly among plant families, but did not respond to N level or the interaction Overall, Chat 1 had larger nodules than the other plant families There was no evidence that nodule occupancy or size among the three rhizobium strains became more similar with increasing N level; in other words, plants did not become less choosy as N increased (Fig. 1).

Mechanism of biological nitrogen fixation

Biological nitrogen fixation can be represented by the following equation, in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen ions).

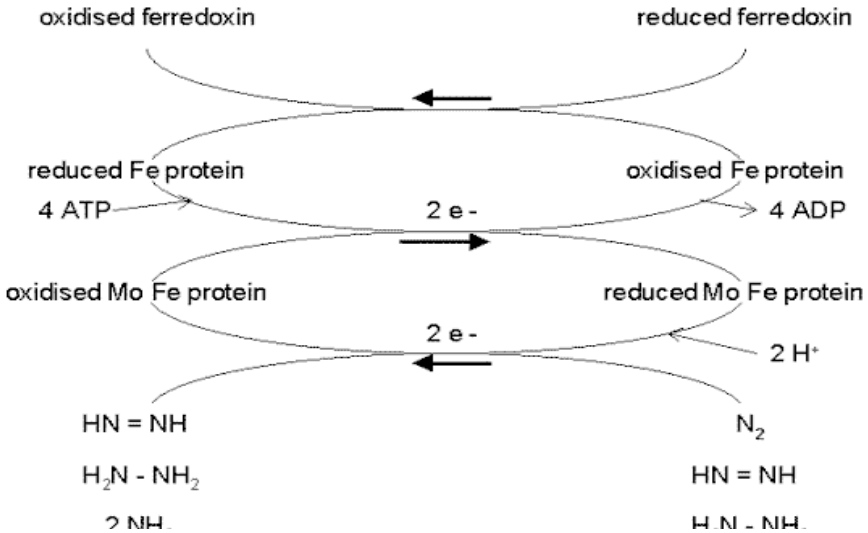

Fig 2. Show N_2 fixation program and Fe anf Mo.

Fig 3. Part of a clover root system bearing naturally occurring nodules of *Rhizobium*. Each nodule is about 2-3 mm long.

Fig 4. Clover root nodules at higher magnification, showing two partly crushed nodules (arrowheads) with pink-colored contents.

$N_2 + 8H+ + 8e^- + 16$ ATP = $2NH_3 + H_2 + 16$ADP + 16 Pi

This reaction is performed exclusively by prokaryotes (the bacteria and related organisms), using an enzyme complex termed nitrogenase. This enzyme consists of two proteins - an iron protein and a molybdenum-iron protein, as shown below (Herridge et al., 2008). The reactions occur while N_2 is bound to the nitrogenase enzyme complex. The Fe protein is first reduced by electrons donated by ferredoxin. Then the reduced Fe protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to H_2N-NH_2, and this in turn is reduced to $2NH_3$. Depending on the type of microorganism, the reduced ferredoxin which supplies electrons for this process is generated by photosynthesis, respiration or fermentation. There is a remarkable degree of functional conservation between the nitrogenase proteins of all nitrogen-fixing bacteria. The Fe protein and the Mo-Fe protein have been isolated from many of these bacteria, and nitrogen fixation can be shown to occur in cell-free systems in a laboratory when the Fe protein of one species is mixed with the Mo-Fe protein of another bacterium, even if the species are very distantly related (Hubbell and Kidder, 2009).

Symbiotic nitrogen fixation

1. Legume symbioses

The most familiar examples of nitrogen-fixing symbioses are the root nodules of legumes (peas, beans, clover, etc.). In these leguminous associations the bacteria usually are *Rhizobium* species, but the root nodules of soybeans, chickpea and some other legumes are formed by small-celled rhizobia termed *Bradyrhizobium*. Nodules on some tropical leguminous plants are formed by yet other genera. In all cases the bacteria "invade" the plant and cause the formation of a nodule by inducing localised proliferation of the plant host cells. Yet the bacteria always remain separated from the host cytoplasm by being enclosed in a membrane - a necessary feature in symbioses (see the image below).

Cyanobacterial associations

The photosynthetic cyanobacteria often live as free-living organisms in pioneer habitats such as desert soils (see cyanobacteria) or as symbionts with lichens in other pioneer habitats. They also form symbiotic associations with other organisms such as the water fern *Azolla*, and cycads. The association with *Azolla*, where cyanobacteria (*Anabaena azollae*) are harboured in the leaves, has sometimes been shown to be important for nitrogen inputs in rice paddies, especially if the fern is allowed to grow and then ploughed into the soil to release nitrogen before the rice crop is sown. A symbiotic association of cyanobacteria with cycads is shown below. The first image shows a pot-grown plant. The second image shows a close-up of the soil surface in this pot. Short, club-shaped, branching roots have grown into the aerial environment. These aerial roots contain a nitrogen-fixing cyanobacterial symbiont.

Fig 5. Nodules on some tropical leguminous plants.

This limitation may not apply to the bacteria that live in root nodules or other intimate symbiotic associations with plants. It has been estimated that nitrogen fixation in the nodules of clover roots or other leguminous plants may consume as much as 20% of the total photosynthate. In addition to these intimate and specialized symbiotic associations, there are several free-living nitrogen-fixing bacteria that grow in close association with plants. For example, *Azospirillum* species have been shown to fix nitrogen when growing in the root zone (rhizosphere) or tropical grasses, and even of maize plants in field conditions. Similarly, *Azotobacter* species can fix nitrogen in the rhizosphere of several plants. In both cases the bacteria grow at the expense of sugars and other nutrients that leak from the roots. However, these bacteria can make only a small contribution to the nitrogen nutrition of the plant, because nitrogen-fixation is an energy-expensive process, and large amounts of organic nutrients are not continuously available to microbes in the rhizosphere Kamst, et al. 1998.

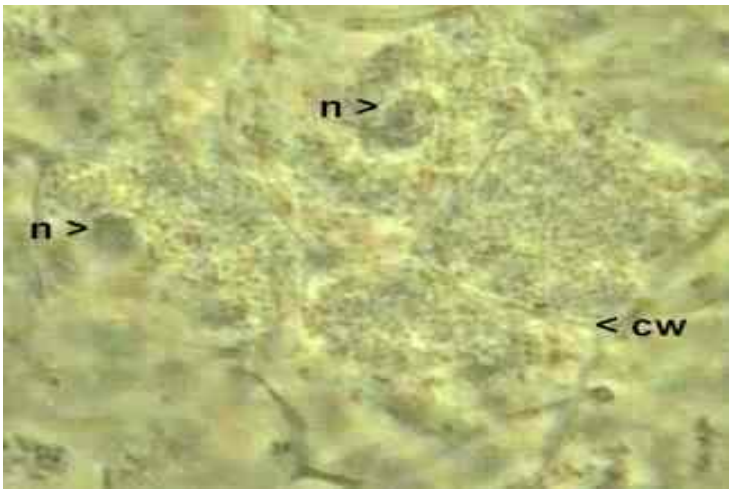

Fig 6. Showing four root cells.

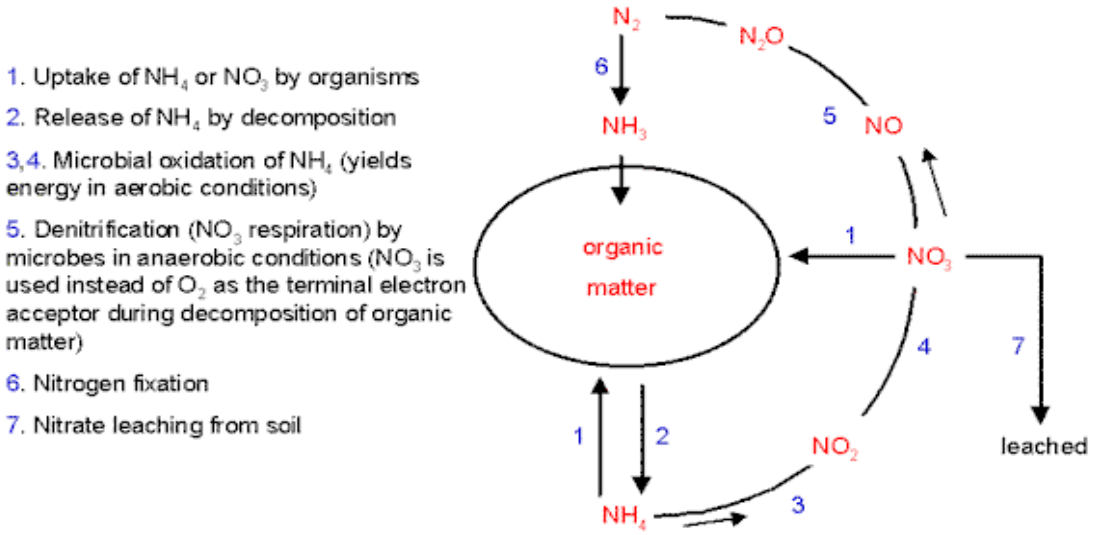

Fig 7. Stages in cycle of fixation and nitrate leaching from soil.

Part of a crushed root nodule of a pea plant, showing four root cells containing colonies of *Rhizobium*. The nuclei (n) of two root cells are shown; cw indicates the cell wall that separates two plant cells. Although it cannot be seen clearly in this image, the bacteria occur in clusters which are enclosed in membranes, separating them from the cytoplasm of the plant cells (Kamst et al., 1998). In nodules where nitrogen-fixation is occurring, the plant tissues contain the oxygen-scavenging molecule, leghaemoglobin (serving the same function as the oxygen-carrying haemoglobin in blood).

The function of this molecule in nodules is to reduce the amount of free oxygen, and thereby to protect the nitrogen-fixing enzyme nitrogenase, which is irreversibly inactivated by oxygen.

The nitrogen cycle

The diagram below shows an overview of the nitrogen cycle in soil or aquatic environments. At any one time a large proportion of the total fixed nitrogen will be locked up in the biomass or in the dead remains of organisms (shown collectively as "organic matter"). So, the only nitrogen available to support new growth will be that which is supplied by nitrogen fixation from the atmosphere (pathway 6 in the diagram) or by the release of ammonium or simple organic nitrogen compounds through the decomposition of organic matter (pathway 2).

CONCLUSIONS

Despite the lack of context dependence, plant genotypes respond very differently to mixed populations of rhizobia, suggesting that these traits are genetically variable and thus could evolve in response to longer-term increases in N.

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