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# Host-imposed control mechanisms in legume–rhizobia symbiosis

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Legumes are ecologically and economically important plants that contribute to nutrient cycling and agricultural sustainability, features tied to their intimate symbiosis with nitrogen-fixing rhizobia. Rhizobia vary dramatically in quality, ranging from highly growth-promoting to non-beneficial; therefore, legumes must optimize their symbiosis with rhizobia through host mechanisms that select for beneficial rhizobia and limit losses to non-beneficial strains. In this Perspective, we examine the considerable scientific progress made in decoding host control over rhizobia, empirically examining both molecular and cellular mechanisms and their effects on rhizobia symbiosis and its benefits. We consider pre-infection controls, which require the production and detection of precise molecular signals by the legume to attract and select for compatible rhizobia strains. We also discuss post-infection mechanisms that leverage the nodule-level and cell-level compartmentalization of symbionts to enable host control over rhizobia development and proliferation in planta. These layers of host control each contribute to legume fitness by directing host resources towards a narrowing subset of more-beneficial rhizobia.

Legumes are among the most diverse and ecologically important plant families, colonizing habitats from deserts to rainforests<sup>1</sup>. Their global spread is often linked to their ability to fix atmospheric N<sub>2</sub> with rhizobia bacteria—a trait that requires complex coordination between the host legume and symbiont bacteria<sup>2</sup>. Legumes contribute roughly half of all terrestrial nitrogen fixation<sup>3</sup>, a quarter of agronomic production via crops such as soybean and alfalfa, and a third of human-consumed protein<sup>4</sup>.

To fix N<sub>2</sub> with rhizobia, legumes have evolved organs called nodules, of which they can bear tens to hundreds across their root systems (Box 1). The evolution of nodulation and symbiotic nitrogen fixation probably involved the coordinated expression of multi-step molecular pathways to determine organogenesis and a functional phenotype<sup>5–8</sup>. The legume nodule is a symbiotic organ, whose evolution and function require successful integration between the host and bacteria<sup>9</sup>.

Substantial progress has uncovered molecular mechanisms of nodule organogenesis and nitrogen fixation<sup>8,10–12</sup>.

Rhizobia are defined by their capacity to nodulate legumes and fix nitrogen, and encompass diverse Alphaproteobacteria and Betaproteobacteria<sup>13</sup>. Even closely related rhizobia strains show vast differences in the benefits they provide to legumes, with strains on any given host varying from highly beneficial to non-beneficial. Moreover, nested within lineages of rhizobia that nodulate and benefit legumes are strains that lack symbiosis genes and might be adapted to be saprophytic, commensal or parasitic<sup>14–17</sup>. Unrelated symbionts are predicted to exhibit intense inter-strain competition, lowering host benefit and favouring the evolution of host mechanisms to control infection and minimize symbiont exploitation<sup>18</sup>. Elucidating how hosts detect and respond to the variation in quality of rhizobial microorganisms has only recently become a focus of research, but is important as we seek

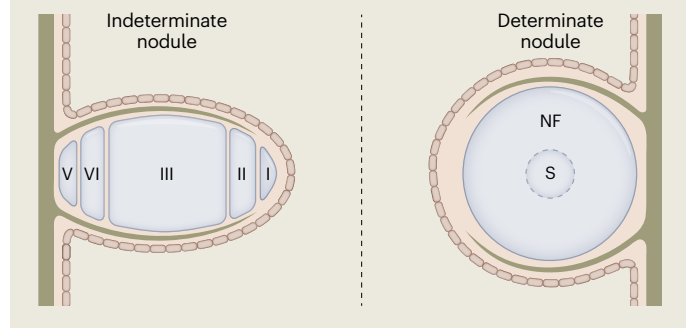
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## BOX 1

## Typical biology of nodulation

Nodules are typically initiated when one or a few rhizobia cells enter root cortical cells via a crack or root hair and are encased by a plant-derived symbiosome membrane, within which they differentiate into bacteroids and can begin fixing nitrogen. Bacteroids typically reside as spatially structured groups within infected plant cells<sup>116</sup> and reach exceptionally high numbers, with 1–50 bacteroids within each symbiosome, and  $10^3$  to  $10^4$  symbiosomes in each infected host cell<sup>106</sup>. Only a subset of rhizobia in soil are compatible with any host, determined by host responsiveness to rhizobia molecular signals, including Nod factors, surface polysaccharides and type III effectors<sup>23,76–79</sup>.

Nodule development varies among legume taxa. Determinate nodules, such as in *Lotus japonicus*, lack a continuous meristem and are spherical, with a core of infected  $N_2$ -fixing cells (NF) surrounded by uninfected host cells. Determinate nodules host homogenous populations of bacteroids, cease growth after their development is complete and allow  $N_2$ -fixing rhizobia to escape back into the soil during nodule senescence, a process initiating from a senescent zone in the nodule center (S). Indeterminate nodules, such as in *M. truncatula*, grow throughout the association between rhizobia and the host, with a spatial gradation of zones, including undifferentiated meristem cells (I), cells being invaded by bacteria and those undergoing symbiotic differentiation into a  $N_2$ -fixing form (II, III), a nitrogen fixation zone (IV) and a senescent zone (V). In some hosts of each nodule type<sup>84</sup>, bacteroids terminally differentiate and cannot escape the nodule<sup>174</sup>, but nonetheless a subset of undifferentiated rhizobia can be released upon nodule senescence<sup>175</sup>. In both nodule types, bacteroids within nodules can fix nitrogen and greatly enhance plant fitness in return for host-derived carbon.



to understand how this mutualism is stabilized by host control and seek to leverage the benefits of rhizobia to sustainably enhance crop nutrition and productivity.

Below we describe mechanisms of infection and host control. Most data come from few, well-studied host taxa. These mechanisms are well established, but should not be viewed as universal, especially as many tropical legumes are understudied<sup>19,20</sup>. We begin by introducing the challenge inherent in all symbioses, which by nature require intimate coordination between individuals of different species with divergent fitness interests. Then we examine two stages of host control, pre-infection and post-infection, which work via different mechanisms to select for beneficial rhizobia and minimize investment in uncooperative strains. Finally, we offer conclusions with some ideas on how the data herein can be used to improve agriculture and sustainability.

## Legume–rhizobium cooperation requires coordination

Legume–rhizobia symbiosis is initiated with a coordinated exchange of molecular signals. Legume roots secrete a cocktail of flavonoids, secondary metabolites that attract rhizobia and initiate nodulation<sup>21,22</sup>. Receptive rhizobia typically respond by secreting lipochitooligosaccharide nodulation factors (Nod factors), which are detected by plant lysine motif receptor-like kinases (LysM)<sup>7</sup>. Nod factors induce root hair curling, which entraps rhizobia cells; trapped rhizobia cells then form an infection thread as an invagination of the plant cell membrane<sup>7,23,24</sup>. Rhizobia reproduce down this tubular structure, either as single clones or multiple strains if these are present in the same nodule<sup>25</sup> (Box 1), are internalized by developing nodule cells and differentiate into intracellular bacteroids that fix  $N_2$  into plant-available forms in exchange for fixed carbon<sup>6,7,16,19,20</sup>.

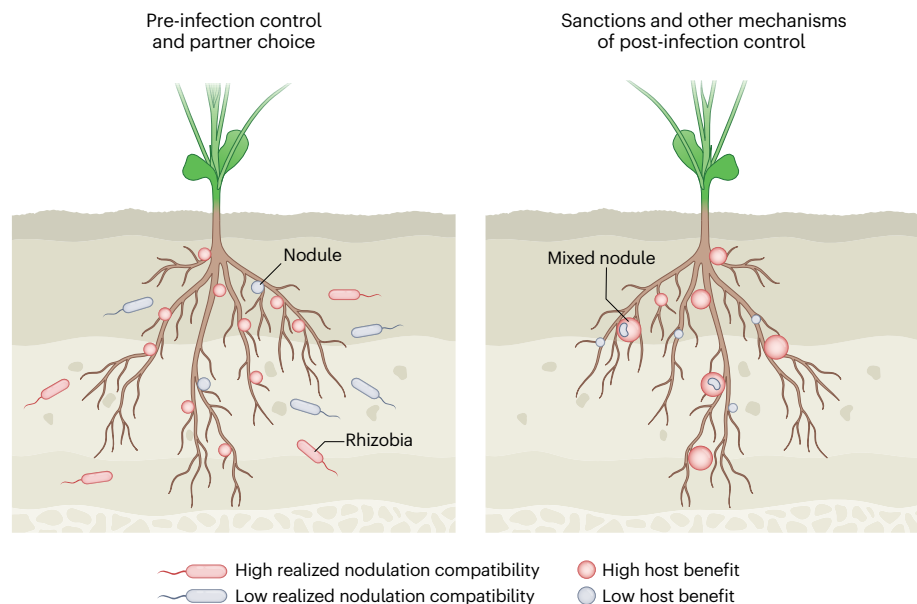
Rhizobia only fix nitrogen inside legume nodules, which provide the microaerobic and energetic requirements of the bacterial nitrogenase. A diffusion barrier made up of cells with few if any intercellular spaces surrounds the nodule interior. This barrier is thought to limit oxygen flux into the nodule interior and maintain microaerobic concentrations, whereas leghaemoglobin-facilitated diffusion of oxygen supports rhizobia respiration<sup>26</sup>. Both partners bear costs for their symbiotic relationship. For example, the host supplies reductant, typically organic acids that are transferred from plant cells to intracellular rhizobia to fuel  $N_2$  fixation, which requires 16 ATP to fix each  $N_2$  molecule. Further,  $N_2$  fixation can compete with rhizobia resource storage and reproduction<sup>27–30</sup>, and requires substantial allocation of a legume's fixed carbon<sup>31</sup>, reducing host fitness if too many nodules are formed<sup>32–34</sup>.

Measuring the net benefits of symbiosis is challenging. Experimentalists investigating legume–rhizobia symbiosis typically inoculate legumes with single rhizobia strains and harvest biomass during vegetative growth. How well these experiments predict a strain's contribution to host fitness under natural conditions, where each plant hosts many rhizobia strains, remains to be determined. For instance, rhizobia strains differ in the speed, duration and N-per-C efficiency of  $N_2$  fixation, and differentially alter plant hormones, traits that can affect plant growth differently in single strain versus mixed-strain settings<sup>35</sup>. Despite methodological uncertainties, these single-strain experiments have revealed that benefits from rhizobia vary among rhizobia genotypes and conditions, including external nitrogen availability, light levels and the host genotype<sup>36–46</sup>. We explore current evidence for how legumes detect and appropriately respond to rhizobia of varying qualities in the following sections (Fig. 1).

## Pre-infection control and partner choice

Pre-infection control mechanisms operate prior to significant resource investment<sup>47–49</sup> (Fig. 2) and can enable hosts to avoid less-beneficial symbionts. Partner choice implies that host preference enhances plant fitness relative to random nodulation, and filtering among strains occurs in advance of nodule organogenesis. Experiments that use clonal and mixed strain inoculations support partner choice, demonstrating that legumes, such as *Acmispon strigosus* and *Medicago truncatula*, can form more nodules with beneficial strains than with non-fixing ones<sup>50</sup> and strain occupancy in nodules following inoculation with a mix of rhizobia strains correlates with the benefit of those rhizobia observed in single inoculation experiments<sup>51</sup>. However, it is difficult to disentangle host effects from those of inter-strain competition among rhizobia, as nodule occupancy patterns are a joint phenotype that depends on both plant and rhizobia genotypes and their interactions<sup>52</sup>.

Several other approaches have established the capacity of legumes to distinguish among rhizobia strains that vary in quality. Split-root experiments eliminate direct interactions between rhizobia strains to show that some hosts can initiate more nodules with a  $N_2$ -fixing strain than a non-fixing one<sup>53</sup>. Labelling rhizobia with fluorescence



**Fig. 1 | Legumes detect and respond to rhizobia of varying benefit via two broad classes of mechanisms.** Left: pre-infection control and partner choice occur if plant production and detection of molecular signals select for beneficial rhizobia (red cells; red nodules show those with beneficial rhizobia) and exclude less-beneficial rhizobia (blue cells; blue nodules show those with less-beneficial rhizobia)<sup>47–49</sup>. Right: sanctions and other post-infection mechanisms are enabled by compartmentalization of symbionts among nodules and infected plant

cells<sup>97,105,106</sup>. Here, hosts control rhizobia development and proliferation in planta, such that nodules and plant cells infected with highly beneficial rhizobia grow rapidly and those infected with less-beneficial rhizobia grow slowly. Both classes of mechanisms can help optimize the benefits of symbiosis by favouring more beneficial rhizobia strains and selecting against those that provide little or no N<sub>2</sub> fixation.

proteins or differentiating them via genotyping of nodule rhizobia reveals that rhizobia strains that result in more plant biomass following single-strain inoculation can also occupy more nodules in mixed inoculation experiments, consistent with plant selection of more beneficial strains<sup>54,55</sup>. Furthermore, stronger partner choice can confer greater benefit. When plants more stringently preferentially nodulate with superior rhizobia, they reap greater rewards<sup>54</sup>. Studies of wild legumes and co-evolved rhizobia reveal additional examples consistent with partner choice (reviewed previously<sup>47</sup>), yet the mechanisms by which plants detect and respond to rhizobia quality in advance of N<sub>2</sub> fixation are still not well-understood.

In experiments that expose legumes to N<sub>2</sub>-fixing rhizobia and isogenic non-fixing mutants, the strains show little difference in nodulation<sup>28,56</sup>. Thus, plants do not appear to detect nitrogen fixation before nodulation. Partner choice probably depends on a reliable linkage between rhizobia genes that encode signals to legumes in advance of N<sub>2</sub> fixation and those that impact benefit to the legume<sup>47</sup>. Genes that encode determinants of compatibility, such as those encoding Nod factors and type III secretion systems, often reside in the same mobile elements as nitrogen-fixation genes<sup>16,57–59</sup>. Host discrimination might maintain linkage among such genes if rhizobia strains with compatible signals that confer high benefit have higher relative fitness than strains that confer lower benefit<sup>39,47,50,60,61</sup>. However, evidence for such genetic linkage is lacking. In fact, loci impacting symbiotic quality reside in genomic regions that are hotspots for gain, loss and recombination, hence any linkage would rapidly break down<sup>16,17,62,63</sup>. This means that elucidating the genetics of partner choice remains challenging. One clear pattern is that hosts bear at least two partner choice mechanisms to preferentially initiate nodules with superior strains, as described below.

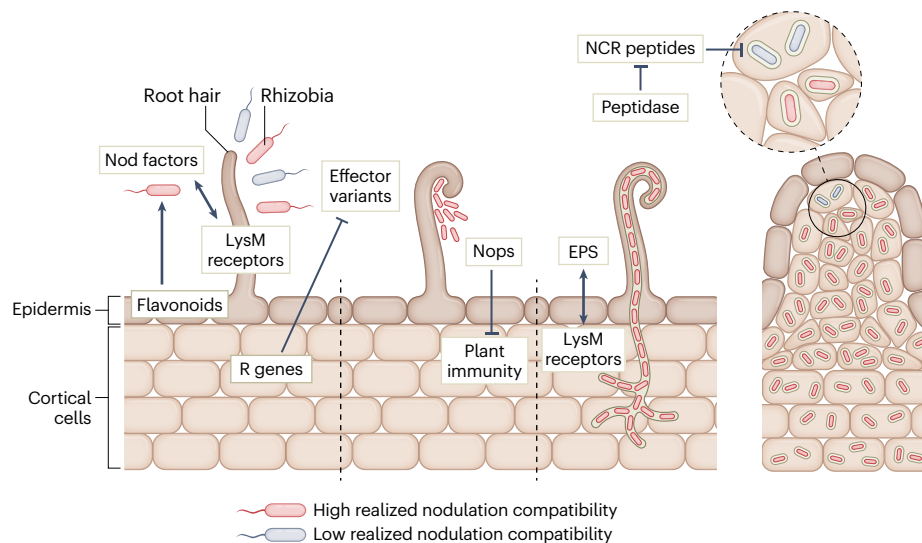
### Fundamental compatibility

A legume genotype's fundamental compatibility defines its ability to establish root nodule infections with specific rhizobia and is determined by the responsiveness of the legume to molecular signals produced by the rhizobia, including Nod factors, surface polysaccharides

and type III effectors (Box 1). Fundamental compatibility contributes to partner choice if compatible strains provide greater benefit than incompatible strains. For example, hosts possessing the *Rj4* allele, an allele common in soybean, are incompatible with many less beneficial, chlorosis-inducing *Bradyrhizobium* strains due to gene-for-gene resistance triggered by the type III effectors these bacterial strains secrete<sup>64–66</sup>. However, near-isogenic lines of soybeans bearing the alternate allele, *rj4*, are compatible with these less-beneficial strains and display reduced fitness relative to *Rj4*-bearing soybeans when inoculated with these strains<sup>64,65</sup>. Thus, the *Rj4* allele modifies soybean's fundamental compatibility in a pattern consistent with partner choice where such uncooperative strains are common.

Variation in legume responsiveness to rhizobia Nod factors is another driver of fundamental compatibility<sup>24,67–69</sup>. Legumes bear LysM receptor-like kinases that determine which Nod factors trigger legume cell calcium oscillations to initiate nodulation<sup>69–72</sup> and can interact with rhizobia surface polysaccharides, which differ in their efficacy for promoting early infection<sup>23,73,74</sup>. Furthermore, legume immune responses can terminate nodule formation. Compatible rhizobia must possess mechanisms to evade legume immune responses<sup>75</sup>; for example, during nodule formation, rhizobia can present surface exopolysaccharides to evade bactericidal actions by the plant immune system, and can secrete effectors<sup>23,76–79</sup>, such as Nodulation outer proteins (Nops), to dampen legume defenses<sup>24,73,80,81</sup>.

Nodule-specific cysteine-rich (NCR) peptides allow some legumes to discriminate amongst rhizobia in a strain-specific manner and help determine fundamental compatibility<sup>24</sup>. By inundating rhizobia with a suite of defensin-like antimicrobial NCR peptides<sup>82,83</sup>, some legumes force rhizobia to differentiate into swollen, non-reproductive, N<sub>2</sub>-fixing bacteroids. Swollen (perhaps non-reproductive) bacteroids are found in legumes with indeterminate nodules and also in some with determinate nodules (Box 1), including those of *Arachis* spp.<sup>84</sup>. In incompatible rhizobia, NCR peptides' antimicrobial properties terminate symbiosis<sup>46</sup>, whereas for compatible rhizobia they are essential to preserve bacteroid viability<sup>85</sup>. Rhizobia can resist host NCR peptides by



**Fig. 2 | Mechanisms of pre-infection control and partner choice.** Pre-infection control by legumes occurs via a multilayer response modulated by back-and-forth molecular communication between plants and rhizobia. The process is initiated by host secretion of species-specific flavonoids<sup>21,22</sup>, a response by compatible rhizobia via production of Nod factors and detection of Nod factors by plant LysM receptors<sup>7</sup>. Compatible rhizobia, for instance, with appropriate Nod factors or type III effector variants, must evade legume immune

responses, via presenting surface exopolysaccharides (EPS) that minimize host bacteriocidal actions or secretion of Nops that dampen legume defences<sup>24,73,80,81</sup>. Hosts can also exhibit gene-for-gene resistance (R genes) against harmful rhizobia that express specific effectors<sup>64–66</sup>. Some legumes further discriminate among rhizobia using antimicrobial NCR peptides, though some rhizobia possess peptidases that can cleave and inactivate host NCR peptides<sup>82,83</sup>.

cleaving them using host-range restriction peptidases<sup>83,86,87</sup>, membrane transport proteins<sup>88</sup> and extracellular polysaccharides<sup>81</sup>. However, none of these mechanisms of strain recognition enable discrimination against mutants that retain the same molecular attributes but fix less nitrogen<sup>28,56</sup>.

Legume taxa vary from specific to generalist in the rhizobia that they nodulate, with a tradeoff between generalism and the benefit gained from symbionts<sup>40</sup>. Symbiotically promiscuous legumes, like the common bean *Phaseolus vulgaris*, allow extremely diverse bacteria to gain access to nodules and can be dominated by strains that are not effective for fixing nitrogen<sup>89</sup>. Loss of specificity could be a by-product of domestication, as the capacity to select beneficial rhizobia can vary markedly with breeding practices and could be tied to the relaxation of plant defenses<sup>90,91</sup>. Work in *Lotus* spp. has uncovered a quantitative trait locus that encodes promiscuity, opening the door to research to better understand its evolution and the mechanistic bases of generalism<sup>92</sup>.

Few molecular mechanisms that restrict fundamental compatibility have been shown to confer an advantage to the host by excluding inferior symbionts. This could be tested using experiments that engineer loss and gain of compatibility function on hosts. While a host may be fundamentally compatible with a broad swath of symbionts, the ability to preferentially initiate nodulation with superior strains amongst diverse compatible options is a distinct trait, which we discuss next.

### Realized nodulation compatibility

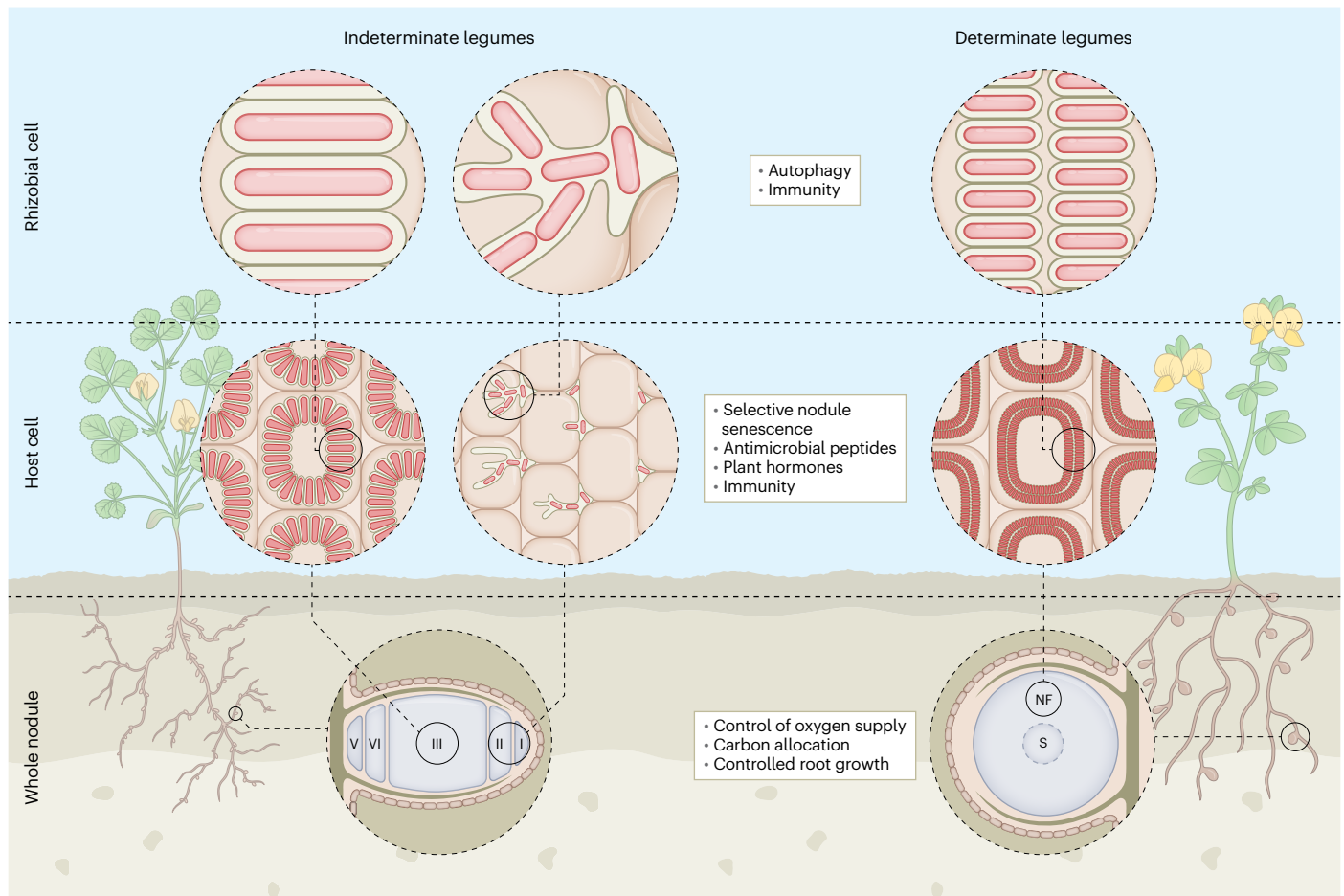
Legumes can preferentially initiate nodules with some fundamentally compatible rhizobia strains over others, which delineates a legume’s realized nodulation. This contributes to partner choice if legumes selectively nodulate with superior compatible strains, as occurs in several legume taxa<sup>50,51</sup>. Surprisingly little is known about how legumes select strains to nodulate among compatible partners<sup>23</sup>. From compatible strains in the rhizosphere, hosts can preferentially nodulate with rhizobia bearing specific early symbiont recognition signaling pathways; for example, a host can preferentially nodulate with strains that express particular Nod factor variants, even if other Nod factor variants are compatible<sup>93</sup>. Other genes that can potentially modulate preferential nodulation include plasmid-encoded transporters,

proteins involved in the biosynthesis of cofactors and proteins related to metabolism<sup>94</sup>. In addition, rhizobia tRNA-derived small RNAs can silence target host genes to promote nodulation success in a specific, localized manner<sup>95</sup>.

Overall, partner choice is susceptible to exploitation because rhizobia can rapidly evolve to be less beneficial to the host yet express the same signals as more beneficial strains. Consistent with this, legumes are typically unable to select superior partners when faced with novel rhizobia or ones in which nitrogen fixation has been knocked out<sup>47,56,96,97</sup>. Even in natural communities, legumes nodulate with non-beneficial strains. Some rhizobia have evolved mixtures of Nod factors that allow them to colonize diverse legumes in which they fix little to no nitrogen<sup>98,99</sup>, whereas others secrete no Nod factors and hitchhike into nodules with strains that do<sup>14,100</sup>. Moreover, successful nodulation can be affected by competitive interactions among rhizobia in the rhizosphere, which are modulated by rhizobia antibiotics<sup>101</sup> and antibiotic resistance traits<sup>102</sup>. To qualify as host-imposed partner choice, these interactions would need to be driven by a host’s ability to generate rhizoplane conditions that favour more beneficial rhizobia<sup>103,104</sup>, which remains unknown. Therefore, it is imperative that legumes detect and respond to rhizobia quality once nitrogen fixation has commenced. We discuss mechanisms involved in these responses in the following section.

### Post-infection control mechanisms

Post-infection control mechanisms enable legumes to preferentially allocate resources to rhizobia in nodules, based on the net benefit provided to the host (Fig. 3). Within post-infection control, hosts may exert sanctions or policing that lead to the accelerated and targeted senescence of nodules or cells within nodules. Alternatively, legumes can impose ‘scaled rewards’ to preferentially allocate resources to higher quality symbionts, which could cause nodules or cells containing more-beneficial rhizobia being larger or better provisioned<sup>97,99,105,106</sup>. Providing more or fewer resources are closely linked<sup>107</sup> as an increase in resources to one nodule is probably linked to a decrease in other nodules on the same plant, and we highlight that hosts might also have the potential to actively harm rhizobia<sup>107,108</sup>.



**Fig. 3 | Post-infection control via sanctions and other mechanisms across the compartmentalized structure of the nodule.** In indeterminate nodules (left, bottom), rhizobia (red) often terminally differentiate into bacteroids in a spatial series (zones II–V) and cannot escape the nodule<sup>174</sup>, but undifferentiated rhizobia (zone I) can be released upon nodule senescence<sup>175</sup>. Zone I is an area of meristematic plant tissue and often has few rhizobia, whereas zone II is where plant cells become infected, and rhizobia undergo differentiation into bacteroids. Within peribacteroid units (shown encased in a dark brown peribacteroid membrane) are N<sub>2</sub>-fixing bacteroids that are often non-reproductive<sup>127</sup> (zone III). These hosts appear to exert a form of whole-nodule sanctions. However, in a nodule infected by more than one strain, it is unknown how sanctions

could target less-effective rhizobia because N<sub>2</sub>-fixing bacteroids in zone III are separated from reproductive clone mates in zone I<sup>105</sup>. For many, but probably not all, determinate nodules<sup>84</sup> (right, bottom), N<sub>2</sub>-fixing bacteroids in the nitrogen fixation zone (NF) can escape back into the soil during nodule senescence by initiating a senescent zone at the nodule core (S). In a nodule infected by more than one strain, sanctions could directly target less-effective rhizobia because N<sub>2</sub>-fixing bacteroids are reproductive<sup>106</sup>. Sanctions probably occur via changes or breakdown of the symbiosome membrane<sup>155</sup>, which releases bacteroids to the hostile plant cytosol<sup>156</sup>. Host immunity and autophagy (degradation of intracellular components, top) are both potential functions that could further mediate host selection against ineffective rhizobia<sup>163,164,167,168</sup>.

### Compartmentalization

Legume physical structures that spatially separate symbionts, namely nodules and the plant cells within them, facilitate host discrimination among rhizobia during symbiosis<sup>109</sup> and reduce direct conflict among symbionts colonizing a single host<sup>110</sup>. Legume control mechanisms should satisfy three criteria: first, they should allow hosts to distinguish among compartments that have more-beneficial or less-beneficial rhizobia; second, they should enable the host to direct resources to more-beneficial rhizobia, ideally even within coinfecting nodules, to increase its fitness return on resource investment; third, they should minimize tissue damage to the host caused by sanctions on rhizobia in infected cells.

Nodules represent compartments for hosts to enact control mechanisms, containing populations of rhizobia that the host can regulate independently. Nodule-level controls would be most effective when nodules contain a single rhizobia strain<sup>111</sup>. If multiple strains share a nodule, this could impair a host's ability to preferentially allocate resources to individual strains. However, cases where ~20% of nodules are co-infected are common in the field<sup>112–114</sup> and the

laboratory<sup>28,51,56,115–120</sup>, with some nodules containing up to six different strains<sup>25</sup>. One solution to this problem is if hosts can enact control at a finer spatial level. Recent work suggests that some legumes can discriminate, at least against completely ineffective rhizobia, even when as much as 50% of the host's nodules are coinfecting with a mixture of effective and ineffective strains<sup>25,50,115,121</sup>. In the next section we discuss possible post-infection control mechanisms.

### Host control over resource supply

Preferential allocation of resources, such as carbon or oxygen, to individual nodules could be a mechanism by which hosts scale rewards to rhizobia performance. Such scaled rewards operate in other symbioses, such as those between plants and arbuscular mycorrhizal fungi<sup>122,123</sup>. Legumes can control nodule permeability to oxygen<sup>26,124,125</sup> and symbiotic rhizobia depend on precise levels of O<sub>2</sub> for aerobic respiration<sup>126</sup>, requiring sufficient O<sub>2</sub> flux for ATP production but low enough levels to prevent damage to nitrogenase<sup>127–129</sup>.

Nodule-level manipulations of rhizobia deflection allow researchers to evaluate legume responses to decreased levels of nitrogen

fixation<sup>130</sup>. For example, when rhizobia are prevented from fixing N<sub>2</sub> using a N<sub>2</sub>-free Ar:O<sub>2</sub> atmosphere, soybean plants decreased the oxygen permeability of nodules and the reproduction of rhizobia decreased. However, these manipulations imperfectly mimic nodules with low N<sub>2</sub> fixation because nitrogenase still consumes resources in the N<sub>2</sub>-free atmosphere, producing hydrogen gas rather than ammonia. Furthermore, it is not clear whether host reduction of O<sub>2</sub> influx is directly responsible for decreased rhizobia reproduction<sup>131</sup>.

Nodules containing more effective N<sub>2</sub>-fixing rhizobia often receive greater carbon allocation by the legume. Regulation of carbon access to the nodule apoplastic space or the infected nodule cells could control rhizobia and nodule growth. Legumes can provide dicarboxylates, primarily succinate and malate, as energy and electron source donors for N<sub>2</sub> fixation by rhizobia. Sucrose is brought to the infected nodule cell via the phloem and metabolized into malate by glycolytic enzymes, specifically phosphoenol pyruvate carboxylase (PEPC) and malate dehydrogenase (MDH). Potentially because of host allocation, nodules containing effective N<sub>2</sub>-fixing rhizobia grow larger and the rhizobia proliferate rapidly, whereas nodules with less-beneficial rhizobia tend to stay smaller and the rhizobia within them divide slowly<sup>96,97,113,115,131–133</sup>.

There are few data on the host control of apoplastic carbon metabolism and its connection with endosymbiotic rhizobia fitness, but some studies suggest that rhizobia can hoard host resources for their own benefit. Rhizobia that store more polyhydroxybutyrate (PHB) during symbiosis can survive longer in the soil between hosts<sup>134</sup>, but a more-beneficial *Rhizobium etli* PHB knockout (PHB<sup>-</sup>) provided bean plants with more nitrogen than an isogenic strain with PHB, presumably because PHB accumulation competes with nitrogen fixation<sup>29</sup>. The PHB<sup>-</sup> strain produced roughly twofold larger nodules than the less-beneficial wild-type strain, consistent with host sanctions against the latter<sup>135</sup>.

Although nodule-level sanctions could help optimize net legume benefits at the level of whole nodules, it is unlikely that ineffective rhizobia in nodules with mixed infections could be targeted by withholding O<sub>2</sub> or carbon<sup>136,137</sup>. It is possible that the peribacteroid membrane could limit carbon influx to particular bacteroids within a nodule<sup>138,139</sup>, although legume control of nodule permeability to gases occurs at the nodule-level and carbon is metabolized via vascular bundles that supply whole nodules. Therefore, control over oxygen flux is unlikely to serve as a mechanism of post-infection control within a co-infected nodule.

### Host mediation of hormones, antimicrobials and amino acids

Phytohormone concentrations are higher in nodules compared with other portions of roots<sup>140,141</sup>, suggesting these molecules might play a role in the regulation of nodule metabolism. There are several sources of evidence for this: together with *CLE* genes, cytokinins are important in the regulation of nodule numbers<sup>142</sup>; plant hormones change in concentration throughout nodule developmental stages<sup>143</sup>; and hormone transport and concentration in nodules is spatially controlled in different cell types<sup>144</sup>. It is conceivable that the spatial control of hormones by hosts could provide a mechanism by which a host could discriminate between beneficial and ineffective rhizobia in mixed nodules, though we lack research that addresses this possibility.

Some legumes secrete antimicrobial peptides that trigger the terminal differentiation of rhizobia into bacteroids, which are incapable of reproduction<sup>145–147</sup> and can show increased N-per-C efficiency<sup>148</sup>. This does not appear to reduce the resource usage and reproduction of less-effective strains as undifferentiated rhizobia clonemates persist in these nodules and are not subject to antimicrobial control<sup>148</sup>. Pea and alfalfa, both of which host non-reproductive bacteroids<sup>133</sup>, exert whole-nodule sanctions that reduce nodule growth. Although sanctions against less-beneficial, non-reproductive bacteroids in mixed nodules could conserve plant resources, they would not selectively harm the corresponding reproductive rhizobia<sup>105</sup>.

### Selective nodule senescence

Nodule senescence is the natural process by which nodules break down<sup>149</sup>. Rhizobia should benefit from a delay in senescence if they can proliferate more within the nodule<sup>150</sup>, whereas legume hosts would benefit from senescing nodules when the costs of harbouring rhizobia outweigh the benefits. Legumes display two types of nodule senescence. Developmental senescence involves nodule maturation and the release of symbionts into the soil, often during fruit set, when legumes shift carbon resources towards seeds and away from nodules. By contrast, induced senescence is triggered if legumes experience stress, such as deficiencies of light<sup>151</sup>, water<sup>152</sup> or the failure of bacterial recognition by the plant<sup>153</sup>. Morphological changes associated with the two types of nodule senescence differ; in the latter, changes progress faster and the contents of the symbiosome are degraded without evidence for legume nutrient remobilization<sup>154</sup>.

Several morphological features of senescence are observed in ineffective nodules. One of the first morphological changes during senescence is breakdown of the symbiosome membrane<sup>155</sup>, which releases bacteroids to the plant cytosol and exposes the rhizobia to the hostile host cell environment<sup>156</sup>. In areas of senescence within indeterminate nodules, proteins involved in the formation of vacuoles accumulate and symbiosomes transform into vacuole-like units<sup>156,157</sup>. Vacuolar fusion machinery might therefore be necessary for controlling symbiosome lysis.

A potentially critical step in nodule senescence is the neutralization of the peribacteroid space that surrounds the symbiosome, an otherwise acidic environment that facilitates import of host resources<sup>158</sup>. For example, non-fixing rhizobia often fail to fully acidify the peribacteroid space, potentially allowing the host to halt symbiosome development<sup>158</sup>.

Senescence is associated with induced expression of cysteine proteases, which mediate senescence<sup>159</sup>. In *Astragalus sinicus*, experimental downregulation of the expression of nodule-specific cysteine protease gene *Asnodf32* delays nodule senescence and extends N<sub>2</sub> fixation<sup>160</sup>. Moreover, the ratio of reactive oxygen species to scavenging enzymes increases during nodule senescence<sup>155,159,161</sup>.

Experimental inoculations with nitrogen fixing and ineffective rhizobia demonstrate that plants can mediate the selective senescence of individual nodules that house ineffective strains. Within *Acmispon* and *Lotus* nodules coinfecting with beneficial and ineffective strains, selective senescence was only associated with individual nodule cells housing ineffective rhizobia<sup>106</sup>. Thus, selective senescence is a promising avenue for investigating host control.

### Immunity

Plants respond to pathogens by producing reactive oxygen species, altering gene expression, remodelling the plant cell wall and producing antimicrobial compounds such as phytoalexins<sup>162</sup>. A common response is rapid, localized programmed cell death that occurs where harmful microorganisms have invaded, known as a hypersensitive response. Many of these responses also occur during rhizobia infection<sup>163,164</sup>; however, the role of immunity in legume sanctions is unresolved. Levels of responsive early gene expression are substantially lower in response to Nod factors than to molecular signals associated with bacterial plant pathogens<sup>165</sup>. The early generation of reactive oxygen species is necessary for rhizobial infection and leads to increases in nitrogen fixation and a delay in senescence<sup>166</sup>. Established nodules are typically not affected by elicitation of host immunity pathways<sup>163</sup>, though the repression of immunity could be reversed as nodules begin to senesce<sup>167</sup>. Lastly, processes similar to a hypersensitive response occur in legume–rhizobia interactions, but in a genotype-specific manner, independent of N<sub>2</sub>-fixing status<sup>168</sup>. The dependence of immune responses on strain identity rather than symbiotic performance means that this process has the same issues as partner choice, in that less-beneficial mutants could retain the identity signals of their more-beneficial ancestors. Moreover, it is unclear how a host immune response could differentially affect strains within the same nodule.

### Summary of post-infection control mechanisms

Like pre-infection mechanisms that fail to exclude some non-fixing rhizobia, post-infection control mechanisms are imperfect. Post-infection control can divert host resources away from less beneficial rhizobia; however, a legume that forms nodules with both a less-beneficial and a more-beneficial strain can have lower fitness than a legume that nodulates exclusively with the more-beneficial strain<sup>55,169</sup>, though this loss of fitness is not ubiquitous<sup>170</sup>. In extreme cases, hosts have no control over parasitic rhizobia: the *Bradyrhizobium elkanii* strain USDA61 produces many nodules on soybean hosts, but fixes little nitrogen<sup>171</sup>, and *B. elkanii* strains that produce the phytotoxin rhizobitoxine induce chlorosis<sup>66</sup>. Less-effective strains also represent an opportunity cost for hosts, but the magnitude of these costs is poorly understood.

Little is known about how legumes sense rhizobia performance. Legumes could assess rhizobia quality using absolute criteria by comparing nodule performance to a fixed threshold, below which plants do not allocate resources to a strain. Alternatively, legumes could use conditional criteria and compare rhizobia performance to that of other nodules on the same plant. Recent research reveals strong support for conditional sanctions: a rhizobium genotype is sanctioned if it is co-inoculated with a strain that confers more benefit to the host, but not if it is co-inoculated with a strain that confers less benefit to the host<sup>54,97</sup>. While the ability to distinguish between cooperative and uncooperative rhizobia can impose no detectable cost on a host legume<sup>169</sup>, it could be that host control is costly in some environments. Although the metabolic costs of sensing nodule performance might be negligible, legume fitness could decrease from shutting down too many nodules or allocating resources to suboptimal nodules. Interestingly, legumes might differ in their ability to sanction, and this could be linked to breeding or agricultural conditions under domestication<sup>90,91,172</sup>, genetic bottlenecks during breeding or long-term fertilization<sup>91,92,173</sup>.

### Conclusion and future perspective

In conclusion, multiple mechanisms of preferential allocation act as a series of sieves, each contributing to legume fitness by directing host resources to a narrowing subset of more beneficial rhizobia<sup>54</sup>. This understanding is critical to future efforts to breed legumes with improved host control. For instance, we must understand how different mechanisms of preferential allocation to rhizobia interact, whether there are trade-offs among host control traits that could constrain their evolution, or if artificial selection could improve multiple host control traits in parallel. If some host control traits sufficiently prevent legume exposure to inferior rhizobia, it is unclear whether this relaxes other mechanisms of preferential allocation. Host control is imperfect, meaning that hosts often reduce, but do not fully eliminate resources conferred to inferior symbionts<sup>96,97</sup>. Low costs and imperfect resource allocation could contribute to the evolution of multiple mechanisms of preferential allocation to rhizobia in legumes, which could provide promising novel targets for crop improvement. Understanding these mechanisms will also be important to engineering microbiomes or designing synthetic symbioses, both of which would require the host's ability to control symbionts that differ in quality. Artificial selection and engineering efforts that fail to consider host control traits risk producing hosts that are overrun by less-beneficial symbionts.

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## Author contributions

S.S.P., S.E.D., R.F.D., E.T.K. and J.L.S. conceived, wrote and edited the paper together.

## Competing interests

The authors declare no competing interests.

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