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Opinion

Pseudomonas in the spotlight: emerging roles in the nodule microbiome

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While rhizobia have long been recognised as the primary colonisers of legume nodules, microbiome studies have revealed the presence of other bacteria in these organs. This opinion delves into the factors shaping the nodule microbiome and explores the potential roles of non-rhizobial endophytes, focusing particularly on *Pseudomonas* as prominent players. We explore the mechanisms by which *Pseudomonas* colonise nodules, their interactions with rhizobia, and their remarkable potential to promote plant growth and protect against pathogens. Furthermore, we discuss the promising prospects of using *Pseudomonas* as inoculants alongside rhizobia to enhance crop growth and promote sustainable agricultural practices.

Highlights

The nodule microbiome is dominated by symbiotic rhizobia, but other bacteria, in particular *Pseudomonas*, are also frequent members.

Nodule-associated *Pseudomonas* can promote plant health by alleviating abiotic stress, stimulating plant growth, and protecting against pathogens.

Pseudomonas exhibit synergistic interactions with nitrogen-fixing rhizobia and antagonism with parasitic rhizobia.

Pseudomonas use various strategies to colonise nodules.

Rhizobiales bacteria dominate the nodule microbiome but they are not alone

Legumes are important crops and have been recognised as pillars of sustainable agriculture due to their low reliance on synthetic nitrogen fertilisers [1]. They establish endosymbiotic relationships with nitrogen-fixing **rhizobia** (see Glossary). Rhizobia reside inside cells of specialised root organs called **nodules** [2], which are infected via tubular structures called infection threads [3]. Legumes create a unique environment inside nodules, characterised by high bacterial density, low free oxygen concentrations, and an abundant supply of photosynthates and metals such as iron and molybdenum [4–6]. For decades, the inside of nodules was believed to be exclusively colonised by rhizobia, with other microbes isolated from nodules dismissed as mere 'contaminants' [7–9]. In recent years, with the advancement of **microbiome** studies, it has become evident that nodule **microbiota** is not restricted to rhizobia and includes various microbial taxa, which are now recognised as potential modulators of legume health [10].

The composition of the nodule microbiome is strongly influenced by the soil microbiota [11], with soil properties, such as pH, playing crucial roles [12–15]. The plant genotype also shapes the nodule microbiome. The host genotype determines the compatibility with the rhizobial **symbiont** [16] and influences the composition of non-rhizobia **nodule endophytes**. For example, in a study involving different *Glycine max* **cultivars**, the cumulative **relative abundances** of non-rhizobia bacteria ranged from 1% to 34% in the most extreme cultivars, with Pseudomonadaceae nearly undetected in some cultivars but comprising up to 19.8% in others [17]. It has also been shown that nodule endophytes are present throughout a nodule's life cycle, albeit at different relative abundances [18]. This raises questions about how these bacteria influence the symbiosis and plant productivity.

The composition of nodule endophytes often varies across studies, even among plants of the same species. For instance, while nodules of some *Vigna* species may host up to seven different genera [19], other studies have reported as many as 25 [14]. This variability limits our ability to precisely define core nodule endophytes for a given species and calls into question the standards applied in different studies (Box 1). Nonetheless, certain trends can be identified, as some endophytes are more frequently associated with nodules than others. These bacteria include

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Box 1. Methodological considerations in nodule microbiome research

Methodological variations, including surface sterilisation, DNA extraction, primer selection, and taxonomic classification, pose challenges to comparing findings across nodule microbiome studies. Effective nodule surface sterilisation is crucial due to lower microbial diversity inside nodules compared with the rhizosphere and **rhizoplane**, aiming to distinguish between epiphytic and endophytic bacteria. Common chemical sterilisation methods involve NaOCl, EtOH, or $HgCl_2$ at various concentrations and durations, followed by washing in sterile water [53,63,80,90,91]. Some studies employ physical disruption techniques like sonication, either exclusively [86] or in combination with NaOCl treatment [61,92]. However, the optimal incubation length and sterilant concentration remain unclear, and their suitability for different plant species and nodule types is uncertain. The risk of contamination from **epiphytes** largely depends on the robustness of the sterilisation method. Therefore, thorough sterilisation is essential to minimise the overestimation of nodule endophytes.

DNA extraction and PCR amplification methods can introduce greater biases than sequencing and classification, compromising quantitative microbiome analyses [93]. Primer selection is crucial in taxonomical identification, with the 16S rRNA gene being predominantly used. However, different hypervariable regions may not capture consistent genetic variation across genera, requiring different primer combinations for amplification. Moreover, primers with varying affinity to different bacterial taxa may lead to biased results [94]. Binding energy differences due to GC content and template concentration can also introduce biases when selecting primers [95,96].

Stringency of sequencing reads clustering is another factor. For example, **operational taxonomic units (OTUs)** clustered at 97% similarity capture less diversity than **amplicon sequence variants (ASVs)** that cluster reads 100% identical [97]. Some reference databases cannot assign taxonomy to the same level as others when particular primers are used, exacerbating taxonomical assignment issues due to a large number of unique reads obtained.

A final challenge in studying nodule microbial communities is determining their spatial distribution and distinguishing whether bacteria are true nodule endophytes or mere epiphytes. Sterilisation methods generally remove bacteria but not their DNA, making it essential to spatially localise microbes [98]. In some cases, bacteria cannot be isolated or genetically modified, impairing fluorescent protein tagging. A powerful alternative is multiplex fluorescence *in situ* hybridisation (FISH) techniques such as combinatorial labelling and spectral imaging (CLASI)-FISH, which allows the spatial visualisation of up to hundreds of distinct microorganisms [99,100]. Only a few studies in the field have truly verified that nodule microbiota comprise genuine nodule endophytes.

Arthrobacter, *Bacillus*, *Chitinophaga*, *Chryseobacterium*, *Enterobacter*, *Flavobacterium*, *Niastella*, *Novosphingobium*, *Pseudomonas*, *Sphingobacterium*, *Sphingomonas*, and *Streptomyces* (Figure 1). It is noteworthy that *Pseudomonas* and *Bacillus* stand out as the most frequently identified in nodules using both culture-dependent [20] and culture-independent methods (Figure 1).

A meta-analysis of culture-dependent studies revealed that *Pseudomonas* inhabit nodules of taxonomically diverse hosts [20]. For instance, *Pseudomonas* strains have been isolated from nodules of various wild legumes (e.g., *Scorpiurus*, *Sophora*, *Vachellia*) as well as cultivated hosts (e.g., *Cicer*, *Glycine*, *Phaseolus*) under different experimental conditions [20]. They are, in general, fast growing and able to thrive in a broad range of media, making them easy to isolate [21]. Moreover, approximately 73% of all microbiome studies reviewed in this opinion find *Pseudomonas* inside nodules, making them over twice as prevalent as the next most frequent non-rhizobial nodule endophytes (Figure 1). *Pseudomonas* have been detected in nodules of *Arachis*, *Cicer*, *Glycine*, *Lotus*, *Medicago*, and *Vigna* species (Figure 1). The only legumes that showed no *Pseudomonas* colonisation on sequencing of the nodule microbiome were *Acacia*, *Phaseolus*, and *Prosopis*. However, *Pseudomonas* have been isolated from *Acacia* [22,23] and *Phaseolus* [24] nodules, suggesting that their absence in those microbiome studies may be due to factors other than incompatibility with the host plant. Whether host factors known to influence *Pseudomonas* plant colonisation, such as primary metabolism [25], secondary metabolites [26], and immunity [27], also play a role in nodule colonisation or whether additional factors are involved remains to be elucidated.

In this opinion, we present evidence for the potential of nodule-endophytic *Pseudomonas* bacteria to enhance legume health and propose them as models for the study of tripartite interactions among host plants, rhizobia, and nodule endophytes. We delve into the mechanisms by which *Pseudomonas* are likely to colonise nodules, their interactions with rhizobia, and their ability to

Glossary

Amplicon sequence variant (ASV): unit of taxonomic analysis that comprises a unique DNA sequence obtained from high-throughput sequencing of marker genes and that differs from other sequences by unique polymorphisms.

Cultivar: plant variant that is cultivated and has been produced by selective breeding.

Epiphyte: microorganism residing on the surface of plant organs, typically having beneficial effects on the plant.

Induced systemic resistance (ISR): a defence mechanism activated by beneficial microorganisms, enhancing pathogen resistance usually through the jasmonate or ethylene signalling pathways at a whole-plant level.

Microbiome: a community of microorganisms living in a specific habitat, along with their interactions and effects on their environment.

Microbiota: microorganisms living in a specific environment.

Nodule: specialised root organ where symbiotic rhizobia are hosted intracellularly.

Nodule endophyte: non-rhizobial bacteria residing within nodule tissues without causing harm, and often benefiting the host.

Operational taxonomic unit (OTU): taxonomic unit in which organisms with closely related sequences are grouped together based on a similarity threshold (typically 97% similarity).

Pathogen: a microorganism causing plant diseases and disrupting normal plant functions ultimately leading to reduced growth, yield, and sometimes plant death.

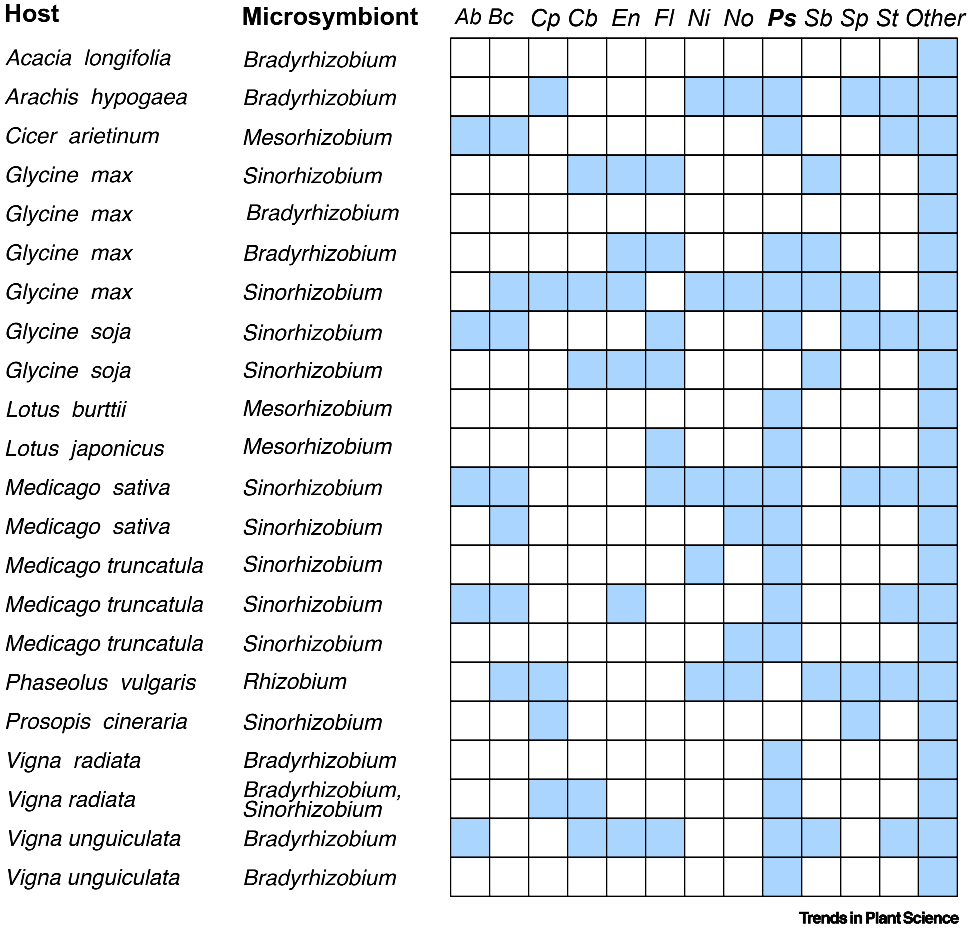
Relative abundance: the percentage of an organism belonging to a particular taxon relative to the total number of organisms in a sample.

Rhizobia: paraphyletic group of bacteria capable of forming nitrogen-fixing endosymbiosis with legume plants.

Rhizoplane: the region directly adjacent to the root surface where microbes attach.

Rhizosphere: the area surrounding plant roots, influenced by the chemical, biological and physical activity of the root.

Siderophore: small, high-affinity iron-chelating molecules secreted by microbes.



Host	Microsymbiont	*Ab*	*Bc*	*Cp*	*Cb*	*En*	*Fl*	*Ni*	*No*	***Ps***	*Sb*	*Sp*	*St*	*Other*
Acacia longifolia	*Bradyrhizobium*	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦
Arachis hypogaea	*Bradyrhizobium*	⬜	⬜	🟦	⬜	⬜	⬜	🟦	🟦	🟦	⬜	🟦	🟦	🟦
Cicer arietinum	*Mesorhizobium*	🟦	🟦	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	⬜	🟦	🟦
Glycine max	*Sinorhizobium*	⬜	⬜	⬜	🟦	🟦	🟦	⬜	⬜	⬜	🟦	⬜	⬜	🟦
Glycine max	*Bradyrhizobium*	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦
Glycine max	*Bradyrhizobium*	⬜	⬜	⬜	⬜	🟦	🟦	⬜	⬜	🟦	🟦	⬜	⬜	🟦
Glycine max	*Sinorhizobium*	⬜	🟦	🟦	🟦	🟦	⬜	🟦	🟦	🟦	🟦	🟦	⬜	🟦
Glycine soja	*Sinorhizobium*	🟦	🟦	⬜	⬜	⬜	🟦	⬜	⬜	🟦	⬜	🟦	🟦	🟦
Glycine soja	*Sinorhizobium*	⬜	⬜	⬜	🟦	🟦	🟦	⬜	⬜	⬜	🟦	⬜	⬜	🟦
Lotus burttii	*Mesorhizobium*	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	⬜	⬜	🟦
Lotus japonicus	*Mesorhizobium*	⬜	⬜	⬜	⬜	⬜	🟦	⬜	⬜	🟦	⬜	⬜	⬜	🟦
Medicago sativa	*Sinorhizobium*	🟦	🟦	⬜	⬜	⬜	🟦	🟦	🟦	🟦	⬜	🟦	🟦	🟦
Medicago sativa	*Sinorhizobium*	⬜	🟦	⬜	⬜	⬜	⬜	⬜	🟦	🟦	⬜	⬜	⬜	🟦
Medicago truncatula	*Sinorhizobium*	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	🟦	⬜	⬜	⬜	🟦
Medicago truncatula	*Sinorhizobium*	🟦	🟦	⬜	⬜	🟦	⬜	⬜	⬜	🟦	⬜	⬜	🟦	🟦
Medicago truncatula	*Sinorhizobium*	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦	🟦	⬜	⬜	⬜	🟦
Phaseolus vulgaris	*Rhizobium*	⬜	🟦	🟦	⬜	⬜	⬜	🟦	🟦	⬜	🟦	🟦	🟦	🟦
Prosopis cineraria	*Sinorhizobium*	⬜	⬜	🟦	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	🟦
Vigna radiata	*Bradyrhizobium*	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	⬜	⬜	🟦
Vigna radiata	*Bradyrhizobium, Sinorhizobium*	⬜	⬜	🟦	🟦	⬜	⬜	⬜	⬜	🟦	⬜	⬜	⬜	🟦
Vigna unguiculata	*Bradyrhizobium*	🟦	⬜	⬜	🟦	🟦	🟦	⬜	⬜	🟦	🟦	⬜	🟦	🟦
Vigna unguiculata	*Bradyrhizobium*	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	⬜	⬜	🟦

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Figure 1. Taxonomic composition of the microbiome in legume nodules. Presence (blue boxes) or absence (white boxes) of the 12 most frequently detected genera in microbiome studies of surface-sterilised legume nodules. Each row corresponds to a different study. Data were extracted from references [11,12,14,18,61,63,78–91]. Abbreviations: *Ab*, *Arthrobacter*; *Bc*, *Bacillus*; *Cp*, *Chitinophaga*; *Cb*, *Chryseobacterium*; *En*, *Enterobacter*; *Fl*, *Flavobacterium*; *Ni*, *Niastella*; *No*, *Novosphingobium*; *Ps*, *Pseudomonas*; *Sb*, *Sphingobacterium*; *Sp*, *Sphingomonas*; *St*, *Streptomyces*. 'Other' indicates other taxa. *Pseudomonas* are highlighted in bold.

promote plant growth and protect against **pathogens**. We discuss the intriguing finding that they can selectively antagonise cheater rhizobia while not affecting nitrogen-fixing symbionts. We highlight limitations of current approaches used to study nodule microbiota (Box 1) and raise key open questions aimed at guiding the future direction of this emerging field.

Symbiont: an organism that lives in close association with another organism, often but not always providing mutual benefits to both partners.

Can nodule-isolated *Pseudomonas* promote the health of legumes?

A broad spectrum of *Pseudomonas* isolates exhibit plant-growth promoting traits, including the alleviation of abiotic stress, phytohormone production, mineral solubilisation, and pathogen protection (Figure 2, Key figure and Table S1 in the supplemental information online). These traits are complementary to symbiotic nitrogen fixation and thus nodule-isolated *Pseudomonas* hold potential as bioinoculants for legumes.

Alleviation of abiotic stress

Abiotic stress can drastically reduce legume yield. Some nodule endophytes diminish plant stress caused by salinity and heavy metals (Table S1). For example, *Pseudomonas* sp. Zy-2-1 from

Key figure

Potential roles of nodule *Pseudomonas* during root nodule symbiosis

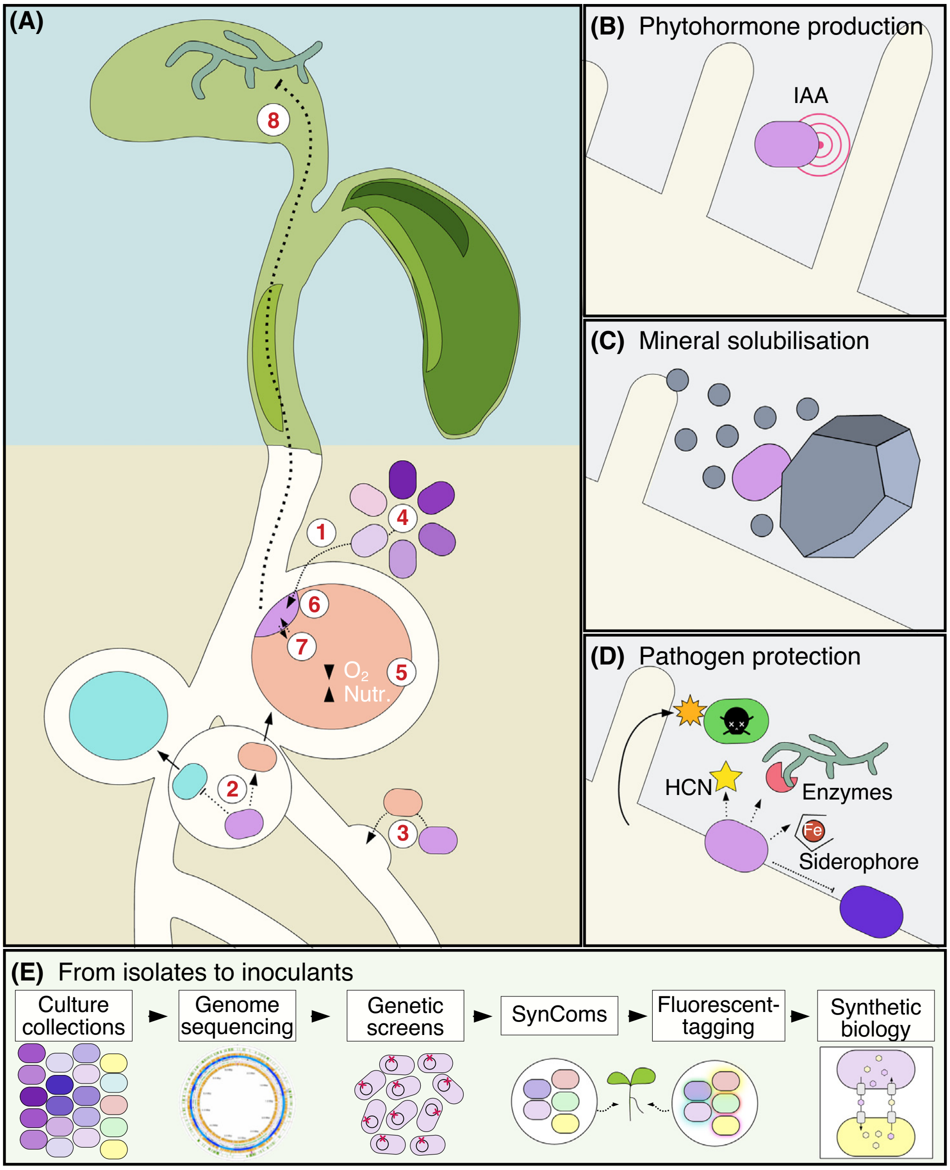

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Figure 2. (A) Scheme illustrating open questions about the interactions of *Pseudomonas* (purple), beneficial rhizobia (peach), and cheater rhizobia (cyan) with each other and with the host plant. Outstanding questions are numbered as follows. (1) Do legumes selectively recruit *Pseudomonas* from the rhizosphere? (2) How do *Pseudomonas* differentially mediate nodulation? (3) How do *Pseudomonas* colonise nodules? (4) What is the genetic makeup of nodule-endophytic *Pseudomonas*? (5) How do *Pseudomonas* adapt to the nodule environment? (6) What is the host response to nodule-endophytic *Pseudomonas*? (7) Is there metabolic exchange between *Pseudomonas* and the host? (8) Do nodule-endophytic *Pseudomonas* induce distal pathogen protection? (B–D) Plant health beneficial activities by *Pseudomonas* include auxin production (B), nutrient acquisition (C), and pathogen protection (D). (E) Proposed steps to gain mechanistic understanding of the function of nodule-colonising *Pseudomonas*. Abbreviations: IAA, indole-3-acetic acid; Nutr., nutrients.

Sphaerophyssa salsula nodules alleviated growth reduction in *Medicago lupulina* under moderate Cu^{2+} stress [28]. Similarly, *Pseudomonas* sp. N4 and N8 from *Medicago* nodules tolerated heavy metals *in vitro* and significantly increased heavy metal accumulation in *Medicago* roots [29]. All these strains were found to exhibit aminocyclopropane-1-carboxylate (ACC) deaminase activity [28,29], a common mechanism to mitigate the stress response.

Phytohormone production

Many *Pseudomonas* isolated from both soil and nodules produce indole-3-acetic acid (IAA) [28,30] (Figure 2B), an auxin phytohormone that regulates cell elongation, tissue differentiation, and organ formation among other processes [31]. Auxins control root development [32], and thereby nutrient and water uptake. Exogeneous IAA application promotes the induction of adventitious and lateral roots [33]. IAA-producing *Pseudomonas* sp. GR12-2 promoted root growth in canola seedlings and adventitious roots on mung bean cuttings, unlike the IAA-deficient *ipdc* mutant [34]. Nodule-isolated *Pseudomonas* often produce IAA *in vitro* (Table S1). Multiple studies have shown that strains producing IAA can promote root growth on co-inoculation with rhizobia, as well as correlating with an increase in nodule number, plant weight, and the nitrogen content of shoots (Table S1). However, no report has demonstrated that nodule-isolated *Pseudomonas* can produce IAA *in planta*, nor that IAA production is indeed causing the observed phenotypes in legumes.

Phosphate solubilisation

Phosphorous is crucial for plant growth and is especially limiting for nitrogen fixation [35]. Phosphorous in the soil forms insoluble complexes with calcium, iron, and aluminium, limiting its accessibility to plants [36]. Phosphate-solubilising *Pseudomonas* release organic acids that lower pH, making phosphate more accessible [37–39] (Figure 2C). However not all phosphate-solubilising bacteria can readily solubilise all phosphate complexes. Berza and colleagues found that while many nodule isolates could solubilise tricalcium phosphate, this was not the case for aluminium phosphate and iron phosphate [40]. Phosphate-solubilising *Pseudomonas* have been shown to increase growth in *Arachis*, *Glycine*, and *Pisum* among others (Table S1). However, molecular evidence is lacking on whether solubilisation of phosphate by these strains results in increased plant phosphorus uptake, or that this drives the observed growth benefits.

Can nodule-isolated *Pseudomonas* protect against pathogens?

Plant-associated *Pseudomonas* can protect against phytopathogens through competition for resources, synthesis of antimicrobial compounds, and induction of systemic resistance in hosts [41] (Figure 2D). However, only a few studies have explored the protective potential of nodule-associated *Pseudomonas*, and the precise mechanisms controlling these effects *in planta* remain unknown.

Siderophore production

Pseudomonas can outcompete pathogens for essential nutrients such as iron, phosphate, sulphur, and nitrogen [42]. *Pseudomonas* species acquire iron through **siderophores**, which they use to compete with pathogens [43]. Nodule-isolated *Pseudomonas* also produce siderophores, although few studies show the role of siderophores in pathogen suppression. For instance, *Pseudomonas* sp. DD201, isolated from *G. max*, has been shown to produce siderophores and to inhibit the Oomycetes pathogen *Phytophthora sojae* 01 [44]. Similarly, Noreen and colleagues reported that fluorescent *Pseudomonas* strains isolated from *Leucaena leucocephala* nodules produced siderophores and suppressed fungal pathogens, including *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani*, and *Fusarium oxysporum* [45].

Antimicrobial activity

Pseudomonas can kill other microbes through the production of antimicrobial metabolites [46], proteolytic enzymes [47], and bacteriocins [48]. For instance, phenazines produced by *Pseudomonas* sp. LV inhibited mycelial growth of *Botrytis cinerea*, the causal agent of grey mould disease [49]. Proteases secreted by *Pseudomonas protegens* CHA0 (formerly *Pseudomonas fluorescens* CHA0) inhibited egg hatching and killed the root-knot nematode *Meloidogyne incognita* [50]. Tailocins, phage tail-like bacteriocins, supressed competitors in metapopulations of the leaf pathogen *Pseudomonas viridiflava* [51]. Some of these activities are also present in nodule-isolated *Pseudomonas* (Table S1). Strains with phenazine-producing genes, isolated from nodules of *Lotus corniculatus* and *Medicago sativa*, had antifungal activities [52]. Additionally, *Pseudomonas* strains isolated from *G. max* nodules have shown both bactericidal and fungicidal activities *in vitro* and *in planta* [53]. However*,* more research is required to understand the regulation of these antimicrobial mechanisms in the **rhizosphere** of legumes and within nodules.

Induced systemic resistance

Pseudomonas species, particularly fluorescent strains, trigger **induced systemic resistance (ISR)** in host plants. ISR is triggered by various molecules, including cell envelope components, iron-regulated metabolites, and antibiotics [54]. For example, *P. protegens* CHA0 produces orfamide, a cyclic lipopeptide that triggers ISR against *Cochliobolus miyabeanus* and reduces leaf lesions in rice [55]. Combinations of strains can enhance protective efficiency, as shown by combining *Pseudomonas capeferrum* WCS358, which produces the siderophore pseudobactin, and *Pseudomonas* sp. RE8, capable of triggering ISR but lacking pseudobactin production, against *F. oxysporum* [56]. Finally, the nodule-endophytic *Pseudomonas* sp. Sneb1990 stimulated local and systemic immunity, which limited *M. incognita* infection and induced salicylic acid and jasmonic acid marker genes in tomato [57]. However, to our knowledge, there are currently no reports of ISR triggered in legumes by nodule-isolated strains, presenting an unexplored research avenue.

How *Pseudomonas* impact nodulation?

Co-inoculation with *Pseudomonas* can increase nodulation (Table S1). For instance, co-inoculation of *Rhizobium* sp. SV20, *Rhizobium leguminosarum* bv. *viciae* SV15, and *Enterobacter clocae* SV27 with *Pseudomonas* sp. SV23 increases nodule numbers in *Vicia faba* [58]. However, it is important to note that *Pseudomonas* can also have the opposite effect, and lead to a reduction in nodule numbers. For instance, co-inoculating *Bradyrhizobium japonicum* A1017 with *Pseudomonas brassicacearum* WCS365 (formerly *P. fluorescens* WCS365) has been reported to decrease the nodule numbers of *G. max* [59]. Similarly, co-inoculation of *Phaseolus vulgaris* cv. Daisy with *Rhizobium phaseoli* RCR3662 and *Pseudomonas* sp. strains 1 and 2 resulted in a decrease in nodule numbers [60]. Interestingly, some nodule-isolated *Pseudomonas*, like strain Lb2C2, decreased ineffective nodulation of *Lotus japonicus* Gifu by *Rhizobium* sp. BW8-2 but not effective nodulation with a beneficial *Mesorhizobium* strain [61]. Whether the promotion or suppression of the nodulation is mediated directly through microbe–microbe interaction or through *Pseudomonas*-mediated stimulation of host responses remains to be investigated. Understanding of the underlying mechanisms could lead to the use of *Pseudomonas* not only to stimulate nodulation but also to control it.

How do *Pseudomonas* colonise nodules?

In terms of host colonisation, nodule-associated *Pseudomonas* can be classified into two categories: (i) those capable of nodulating legumes independent of rhizobia; and (ii) those that colonise nodules alongside rhizobia. Although rare, some *Pseudomonas* isolates have been described to nodulate on their own. For instance, *Pseudomonas* sp. Ch10048 was found to nodulate *Robinia pseudoacacia* without rhizobia [62]. This isolate harbours a *nodA* gene similar to that of *Mesorhizobium loti* Ch90 [62]. Similarly, *Pseudomonas* sp. GLU4 had a *nodC* gene and induced white, non-fixing nodules on *M. sativa*, while lacking *nodA* and *nifH* genes [63]. *Pseudomonas* sp. D5, isolated from *Acacia confusa* nodules, was shown not only to nodulate *A. confusa*, *Acacia crassicarpa*, and *Acacia mangium* under axenic conditions but also to harbour *nodA* and *nifH* genes with similarity to *Bradyrhizobium* sp. and even to exhibit nitrogenase activity [23]. The mechanisms by which these *Pseudomonas* infect nodules have not been elucidated. However, it is tempting to speculate that they may have acquired the *nod* genes by horizontal gene transfer, thereby gaining the capability to induce nodule formation and potentially even infection threads. Experimental evolution assays have shown that a pathogenic *Ralstonia solanacearum* strain, when transformed with a symbiotic plasmid from *Cupriavidus taiwanensis*, can induce nodulation, the formation of infection threads, and even nodule cell infection after gaining mutations in pathogenicity genes and rewiring of regulatory circuits [64].

Most commonly, however, nodule-associated *Pseudomonas* lack the ability to induce nodule formation on their own and depend on symbiotic rhizobia to initiate the process [65]. It remains unclear whether these *Pseudomonas* strains rely on rhizobia for infection or can independently colonise nodules through alternative mechanisms. One possibility is crack-entry infection, where bacteria enter through natural openings in the root [65], as *Pseudomonas* have been observed to accumulate within the nodule apoplast [66]. Another possibility is that *Pseudomonas* hitchhike on infection threads induced by symbiotic rhizobia. Non-symbiotic rhizobia can co-colonise infection threads induced by *Mesorhizobium* in a Nod factor- and exopolysaccharide-dependant manner, which results in the co-infection of nodule cells [67]. Additionally, *Pseudomonas* have been found to colonise nodules intracellularly [61]. For example, *Pseudomonas* sp. PLb11B was unable to nodulate *Lotus burttii* on its own, or colonise ineffective nodules formed by *Rhizobium* sp. BW8-2, but colonised nodules alongside *Mesorhizobium* sp. Qb1E3-1 intracellularly [61]. This correlated with the mode of infection of the rhizobia, as *Rhizobium* and *Mesorhizobium* strains have been described to colonise *L. burttii*, a promiscuous host [68], using different modes of infection [69]. In the future, it will be interesting to investigate whether the mode of nodule entry of the rhizobial symbiont is the controlling factor that dictates *Pseudomonas* nodule colonisation and whether specific interactions between *Pseudomonas* and different rhizobia enable or exclude the hitchhiking of *Pseudomonas* in rhizobia-induced infection threads.

What do *Pseudomonas* do and how do they survive inside nodules?

Many of the beneficial traits of *Pseudomonas* described in previous sections could be performed epiphytically or in the rhizosphere, but what role do *Pseudomonas* play inside nodules? Are they merely opportunists taking advantage of the nutrient-rich nodule environment or could they, along with other nodule endophytes, be providing services to the legume host? Some *Pseudomonas* have the ability to fix nitrogen. For instance, *Pseudomonas stutzeri* A1501 carries a nitrogen fixation cluster with high similarity to *Azotobacter vienelandii* AvOP, located in a genomic island [70], and can fix nitrogen under microaerobic conditions [71]. Moreover, *Pseudomonas* are metabolically versatile, capable of transforming a variety of complex aromatic compounds [72], which could contribute to the secondary metabolism of nodules. They are also able to catabolise and produce auxin [73,74], a hormone essential for nodule development [75]. Additionally, *Pseudomonas* have been identified as part of a minimal community inside *Medicago truncatula* nodules in which one of the members produces antimicrobial compounds *in planta* [18]. *Pseudomonas* themselves have a high potential to produce antimicrobials, as outlined in a previous section. Among these antimicrobials, phenazines are particularly noteworthy. These molecules could play dual roles, facilitating redox balancing and acting as terminal electron acceptors in the absence of oxygen or nitrate [76], potentially facilitating the survival of *Pseudomonas* in the microaerophilic nodule environment. Whether these or other traits are essential for survival inside nodules remains to be investigated.

Concluding remarks and future perspectives

Legumes hold significant potential for sustainable agriculture as they reduce the reliance on nitrogen fertilisers by forming a symbiosis with nitrogen-fixing rhizobia. Exploring the nodule microbiome presents opportunities to identify additional beneficial biological activities complementary to nitrogen fixation. Nodule endophytes promote plant growth and nodulation, alleviate abiotic stress, and protect against pathogens. However, the molecular basis of these beneficial activities remains unclear. Future research must focus on establishing causal links between *in vitro* traits and *in planta* phenotypes in a bid to use them as predictive tools for plant health promotion.

We propose *Pseudomonas* as a model to study the tripartite interactions among legumes, rhizobia, and nodule endophytes. *Pseudomonas* strains often promote plant growth, are genetically tractable and easy to culture, and belong to one of the most diverse bacterial genera, with over 300 species [77]. Focusing on *Pseudomonas* isolated from healthy nodules ensures compatibility with both rhizobia and the host, which is critical as *Pseudomonas* have a wide spectrum of antimicrobial activities [46–48]. Key steps in their study include: (i) creating targeted culture collections from hosts grown in different soils, aiming to co-isolate rhizobia with *Pseudomonas*; (ii) systematically evaluating plant growth and nodulation under controlled conditions; (iii) sequencing a broad diversity of isolates; (iv) performing genetic screens to identify genes associated to health-promoting traits; (v) establishing minimal synthetic communities with other bacteria like *Bacillus* and *Flavobacterium*; and (vi) using fluorescent tags to track bacterial distribution within nodules. Integrating these data through synthetic biology approaches could enable the engineering of optimised inoculant consortia tailored to specific legume crops. This comprehensive approach will help to address key open questions (see Outstanding questions) and harness the full potential of nodule microbiota.

Outstanding questions

Can legumes selectively recruit specific *Pseudomonas* from the rhizosphere? If so, what is the genetic basis for this process?

How can *Pseudomonas* promote nodulation with some rhizobia and decrease nodule numbers with others? Is this mediated by direct microbe–microbe interactions or through the modulation of the host's response to the rhizobia?

How do *Pseudomonas* colonise nodules? Do they rely on rhizobia-induced infection threads or do they enter independently?

What is the genetic makeup of *Pseudomonas* colonising nodules? Can a diverse group of *Pseudomonas* colonise nodules or is this limited to specific species and strains?

How do *Pseudomonas* adapt to the nodule environment? What mechanisms do they employ to survive in the microaerophilic nodule environment?

What is the host cellular response to nodule colonisation by *Pseudomonas*?

Are *Pseudomonas* mutualists that provide services to the host plant inside nodules or are they parasites that benefit from the symbiosis between legumes and rhizobia? Does metabolic exchange occur between nodule *Pseudomonas* and the host?

Do nodule *Pseudomonas* promote systemic immune protection?

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Declaration of interests

The authors declare no competing interests.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT3.5 in order to reduce the number of words. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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