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eXtra Botany

Viewpoint

Nodulating another way: what can we learn from lateral root base nodulation in legumes?

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Certain legumes provide a special pathway for rhizobia to invade the root and develop nitrogen-fixing nodules, a process known as lateral root base (LRB) nodulation. This pathway involves intercellular infection at the junction of the lateral roots with the taproot, leading to nodule formation in the lateral root cortex. Remarkably, this LRB pathway serves as a backbone for various adaptative symbiotic processes. Here, we describe different aspects of LRB nodulation and highlight directions for future research to elucidate the mechanisms of this as yet little known but original pathway that will help in broadening our knowledge on the rhizobium–legume symbiosis.

Research on two model legumes, *Medicago truncatula* and *Lotus japonicus*, and—to a lesser extent—two major crop species, *Glycine max* (soybean) and *Phaseolus vulgaris* (common bean), has greatly advanced our understanding of the intricate molecular mechanisms of symbiosis whereby Nod factors secreted by rhizobia trigger the curling of growing root hairs and the subsequent development of an intracellular infection thread. The latter guides the bacteria towards the distantly induced nodule primordium that will emerge from the root cortex (Roy *et al.*, 2020).

Members of certain legume lineages such as Dalbergioids (e.g. *Arachis* and *Aeschynomene*), Mimosoids (e.g. *Neptunia*), and Galegoids (e.g. *Sesbania*) deviate from this common pattern by producing root nodules at the junction of tap and lateral roots, a process named LRB nodulation (Box 1). The emergence of lateral roots causes a crack in the taproot cortex, which

is why rhizobial infection at this level was originally called ‘crack-entry’. Rhizobia infect the base of the lateral root, often hidden within the taproot cortex, initiating nodule formation from the lateral root cortex. Tufts of axillary root hairs may be present at the base of lateral roots. However, infection threads have never been observed in these, or in the outer root cell layers. This indicates that in LRB nodulating legumes, the initial stage of rhizobial infection is intercellular. Subsequent steps vary among legume species but culminate in the internalization of rhizobia in a nodule primordium (Ibáñez *et al.*, 2017). In this Viewpoint, we provide a critical overview of current knowledge on LRB nodulation.

The LRB pathway is the standard mechanism for symbiotic engagement in certain legumes

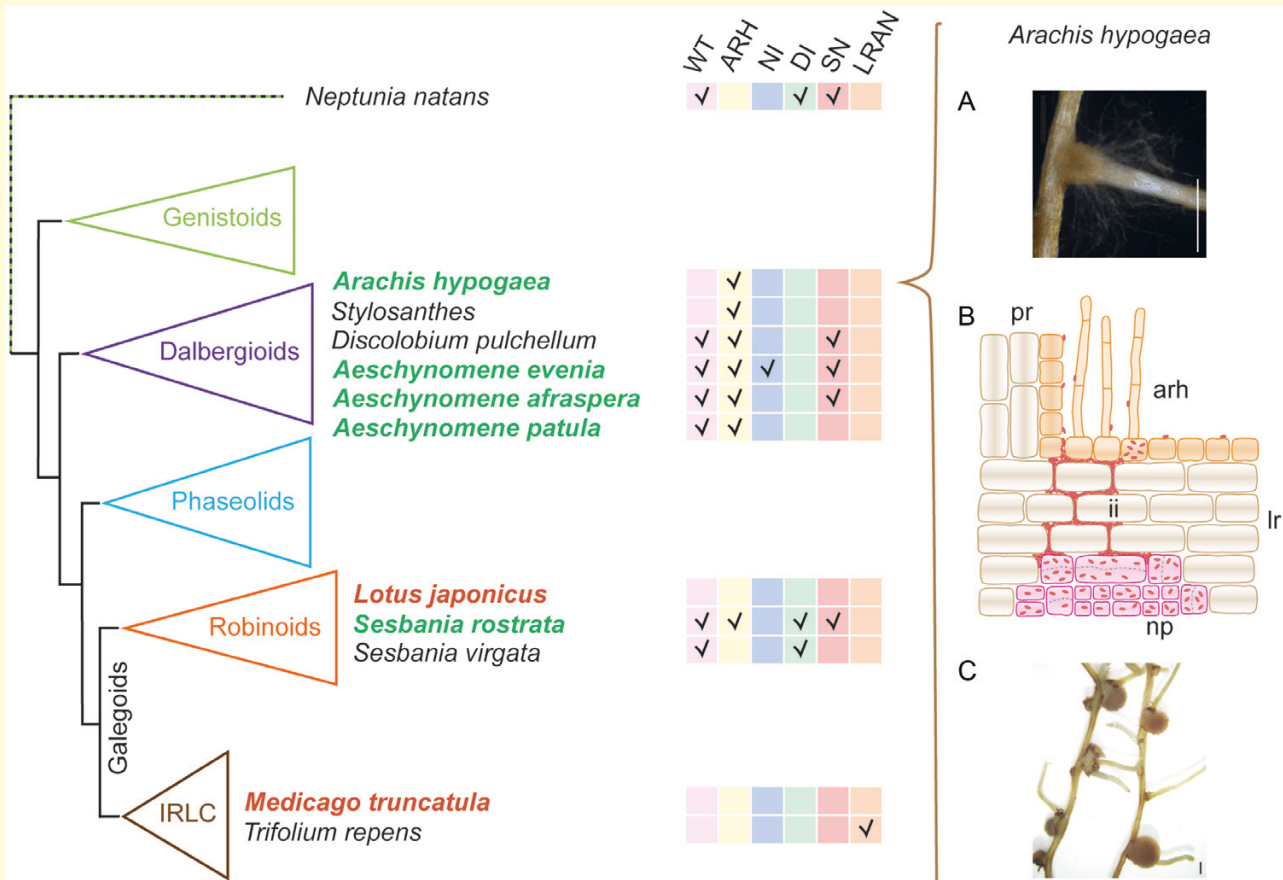
In Dalbergioid legumes, the LRB pathway is a regular invasion mode that leads to nodulation formation. This process has been observed in *Aeschynomene*, *Arachis*, and *Stylosanthes* species (Box 1) (Ibáñez *et al.*, 2017). Among these, peanut (*Arachis hypogaea*), a tetraploid (4×) species, stands out as particularly economically important. Peanut nodulation has been a long-standing research topic that is now fostered by genomic and transcriptomic resources developed for this crop (Sharma *et al.*, 2020; Raul *et al.*, 2022).

In peanut, *Bradyrhizobium* symbionts enter the root at the base of axillary root hairs, where the cell wall appears to be loosely constructed. Then they progress through the middle

Box 1. Occurrence and basal mechanism of LRB nodulation in legumes

Species using LRB nodulation are scattered throughout the legume family. While *N. natans* stands as the single representative in the Caesalpinoid subfamily (dashed line), multiple species are found in the Dalbergioid, Robinoid, and IRLC clades of the Papilionoid subfamily (continuous lines). LRB-nodulated legume species show diverse traits absent in the two legume models, *L. japonicus* and *M. truncatula* (represented in orange), such as water tolerance (WT), axillary root hairs (ARH), Nod factor-independent activation (NI), dual root infection (DI), stem nodulation (SN), and lateral root-associated nodules (LRANs). In the *A. hypogaea* (peanut) model, the LRB pathway serves as the regular mode of nodulation. Typical features include (A) the presence of tufts of axillary root hairs (arh) at the base of lateral roots, where (B) intercellular infection (ii) and cell invasion by *Bradyrhizobium* leads to (C) the development of a nodule primordium (np) at the junction of the lateral root (lr) and the primary root (pr). Features of the LRB infection process in species depicted in green are detailed through Boxes 1, 2, and 3.

Left: schematic representation of the legume family; adapted from Chaintreuil *et al.* (2016). Right: images of axillary root hairs, invasion mode, and nodules of *A. hypogaea* plants; adapted from Bhattacharjee *et al.* (2022), with permission from John Wiley and Sons. Scale bars: (A, C) 1000 µm.



lamella between cortical cells. Finally, the cell wall of certain cortical cells is degraded locally, allowing a direct contact of the bacteria with the plasma membrane, whereafter they are subsequently endocytosed (Sharma *et al.*, 2020). The process observed in peanut represents a simple model of intercellular infection leading to LRB nodulation (Box 1) (Quilbé *et al.*, 2022a). Functional studies have shown that *AhNFR5* (*Nod*

Factor Receptor 5) and several symbiotic signaling genes such as *AhCCaMK* (*Ca²⁺/Calmodulin dependent Kinase*), *AhCYCLOPS*, *AhLHK1* (*histidine kinase 1*), and *AhNSP2* (*Nodulation Signaling Pathway 2*) play a key role in peanut nodulation (Sharma *et al.*, 2020; Shu *et al.*, 2020; Peng *et al.*, 2021).

Promoter activity studies on symbiotic genes in peanut revealed specific gene expression at the lateral root base and

nodule primordia (e.g. *AhNIN*, *Nodule Inception*) or during *Bradyrhizobium* invasion (e.g. *AhNGH*, *Nodule enhanced Glycosyl Hydrolase*) (Bhattacharjee *et al.*, 2022). This study provides evidence that in peanut the LRB plays a key role in nodule initiation and identifies a few relevant markers for studying intercellular infection. However, the role of axillary root hairs in peanut nodulation is unclear because their development depends on the symbiotic signaling gene *AhNSP2*, while *AhNIN* and *AhNGH* are not expressed at their level (Peng *et al.*, 2021; Bhattacharjee *et al.*, 2022).

The LRB pathway supports a unique symbiosis that is independent on Nod factors

Aeschynomene is another genus of Dalbergioid legumes that has intrigued scientists due to the water-tolerant nature of many of its species and the presence of stem nodules. This water-logged ecology has potentially prompted the emergence of a unique symbiosis with photosynthetic bradyrhizobia that does not rely on Nod factor recognition (Box 1). To investigate and compare the two symbiotic processes present in *Aeschynomene*, the species *A. indica* (hexaploid, 6 \times , Nod factor-independent) and *A. afraspera* (octoploid, 8 \times , Nod factor-dependent) were initially used as working models. The Nod factor-independent process is associated with adaptations in the LRB pathway, which are observed through (i) changes in bacterial chemoattraction and colonization at the axillary root hair level and (ii) cortical cell infection occurring initially via plant cell collapses followed by chronic bacterial internalization (Bonaldi *et al.*, 2011). The precise modality of this internalization remains to be disclosed.

For the purpose of genetic tractability, the *Aeschynomene* species *A. evenia* (diploid, 2 \times) and *A. patula* (2 \times) are now being used to compare the Nod factor-independent and Nod factor-dependent symbiosis (Box 2) (Brottier *et al.*, 2018). For *A. evenia*, both gene- and mutant-based approaches have been developed (Fabre *et al.*, 2015; Quilbé *et al.*, 2021). So far, it has been demonstrated that the Nod-independent symbiotic signaling pathway present in *A. evenia* contains as components *AeSYMRK* (*Symbiosis Receptor-like Kinase*), *AePOLLUX*, *AeCCaMK*, *AeCYCLOPS*, *AeLHK1*, *AeNSP2*, and *AeNIN*. The identification of *AeCRK*, which encodes a cysteine-rich receptor-like kinase required for *A. evenia* nodulation, is expected to contribute to the understanding of how photosynthetic bradyrhizobia trigger Nod factor-independent symbiosis (Quilbé *et al.*, 2021, 2022b).

As observed in peanut, *AeNSP2* is required for the development of axillary root hairs, and the symbiotic signaling pathway seems to be implemented at the axillary root hair level in *A. evenia* (Quilbé *et al.*, 2022b). In contrast, the recently identified *AeORM1* (*Orosomucoïd protein 1*) gene involved in sphingolipid biosynthesis appears to be important for the symbiotic events occurring in the lateral root cortex (Nouwen *et al.*, 2024).

Some infection thread-using legumes can also use the LRB pathway

Certain legume species can exhibit a mixed invasion mode by using both root hair and LRB infection routes. One such example is white clover (*Trifolium repens*), which form nodules in both the young and mature zones of the main root. In the young zone, nodules emerge directly from the main root, while in the mature zone, nodules are located at the site of lateral root emergence and are then referred to as lateral root-associated nodules (LRANs) (Box 1) (Mathesius *et al.*, 2000).

Flexibility in the invasion mode is also observed in semi-aquatic *Sesbania* and *Neptunia* species (Box 1). This flexibility has been most intensively studied using *Sesbania rostrata* (2 \times) as a model plant. Under well-aerated conditions, *Azorhizobium caulinodans* bacteria invade and form nodules on the roots of *S. rostrata* roots through the typical root hair infection mechanism. However, under submerged conditions, nodules develop at the base of lateral roots through an intercellular infection mechanism (Box 2). Ethylene plays a crucial role in this process by inhibiting root hair formation and mediating LRB nodulation. *Azorhizobium caulinaudans* bacteria induce the development of axillary root hairs at the LRBs, but these remain uninfected. Instead, the bacteria penetrate the plant via a neighboring fissure in the lateral root epidermis, subsequently inducing localized cortical cell death. The latter results in the formation of an infection pocket that may function as a Nod factor signaling center for subsequent infection thread formation and inception of a nodule primordium (Goormachtig *et al.*, 2004).

Further studies demonstrated that Nod factors are sufficient to induce the development of axillary root hairs. Intriguingly, infection pocket formation appears to be dependent on Nod factor recognition, rather than the signaling genes *SrSYMRK* and *SrCCaMK* (Capoen *et al.*, 2010). These findings highlight *S. rostrata* as a valuable symbiotic model, enabling study of both LRB nodulation and root hair infection mechanisms within a single plant.

Stem nodulation is controlled by the LRB pathway

The stems of water-tolerant legumes produce dormant root primordia that can develop into adventitious roots when immersed in water, or transform into stem nodules after infection with rhizobia (Box 1). An *S. rostrata* mutant lacking stem-located root primordia has been isolated (Boivin *et al.*, 1997). This mutant only formed root nodules, indicating the requirement for dormant root primordia for stem nodulation. Stem nodulation is abundant in *A. afraspera* and *S. rostrata*, moderate in *A. evenia*, and absent in *A. patula* (Box 3) (Brottier *et al.*, 2018), strongly correlating with the degree of protrusion of dormant root primordia, which impacts bacterial accessibility (Boivin *et al.*, 1997).

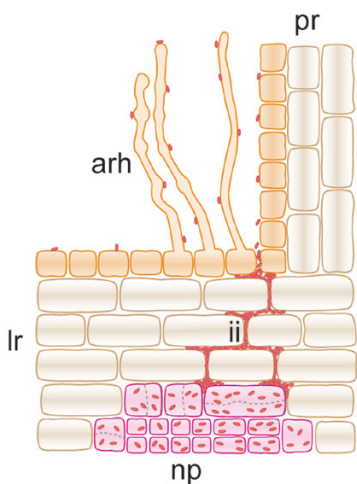
Both *A. afraspera* and *S. rostrata* develop stem nodules from adventitious root primordia, using the LRB pathway similar to root nodulation, but they lack axillary hairs at their base

Box 2. Water-tolerant legumes have variation in the rhizobial infection mode

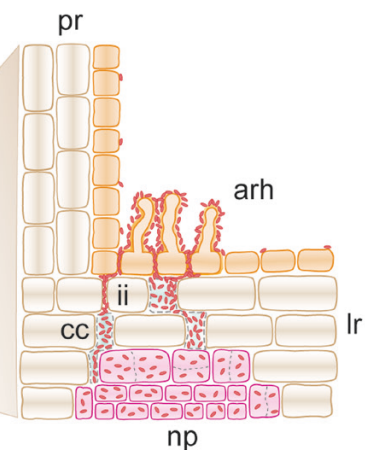
Water-tolerant legumes, exemplified by the *Aeschynomene* and *Sesbania* model species, exhibit variations in the basal LRB pathway for root nodulation under flooding conditions. (A) *A. patula* and *A. evenia* display Nod factor-dependent and Nod factor-independent symbioses, respectively, with *Bradyrhizobium* strains that either produce Nod factors or do not. In contrast to the long and thin axillary root hairs (arh) of *A. patula*, those of *A. evenia* are bulbous and densely covered with mucilage, which is heavily colonized by *Bradyrhizobium*. In both species, LRB infection is intercellular (ii) and leads to nodule primordium (np) formation. However, plant cell collapse (cc) is consistently observed in the outer cortex of *A. evenia*. Axillary root hair chemotaxis and cortical plant cell collapse are hypothesized components of the Nod-independent symbiosis. (B) *S. rostrata* employs dual infection mechanisms depending on root growth conditions. In well-aerated roots, *A. caulinodans* induces root hair curling (rhc) and formation of infection threads (it) that guide and release bacteria into the nodule primordium (np). Under hydroponic conditions, lateral root bases (LRBs) become infection sites. There, *A. caulinodans* induces axillary root hair (arh) development and penetrates the LRB cortex. Initial intercellular infection (ii) leads to formation of infection pockets (ip) from which infection threads (it) emerge. This switch in infection mechanism may be due to inhibited root hair formation by the gaseous plant hormone ethylene under waterlogging.

A Activation-dependent infection mode in *Aeschynomene* sp.

A. patula (Nod factor-dependent)

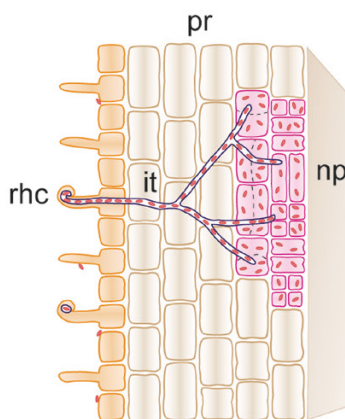


A. evenia (Nod factor-independent)

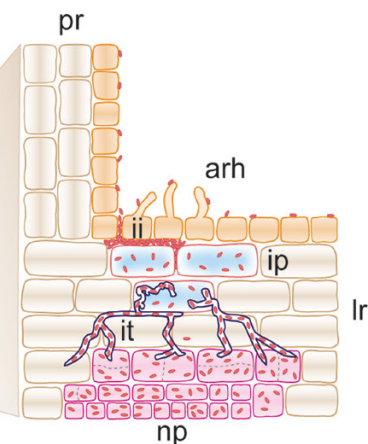


B Conditional infection mode in *Sesbania rostrata*

S. rostrata (Aerated roots)

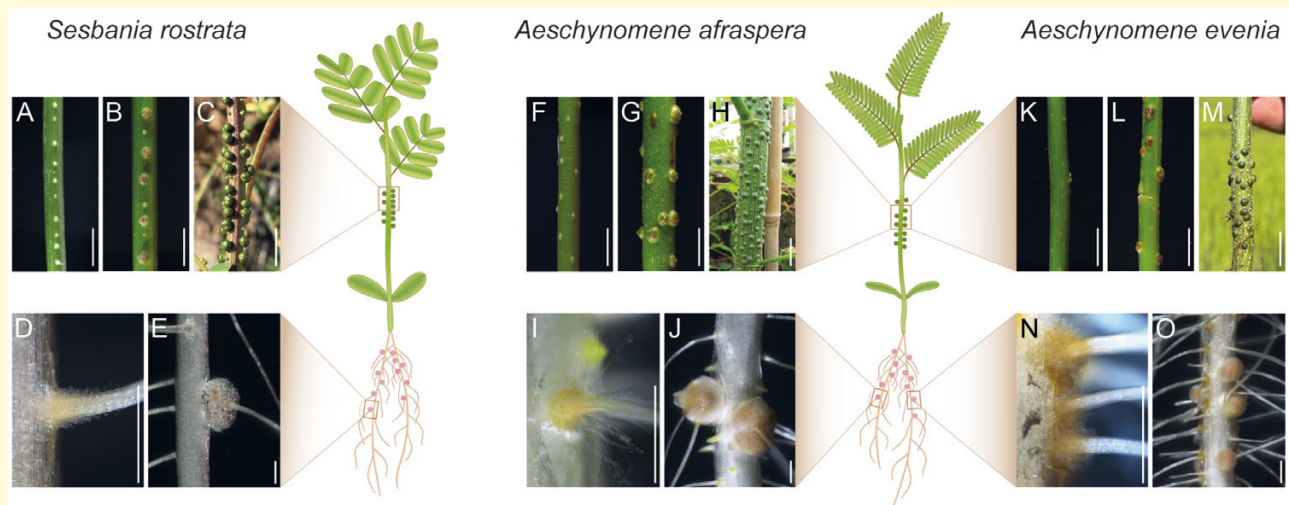


S. rostrata (Flooded roots)



Box 3. Dual root and stem nodulation in water-tolerant legumes

Many water-tolerant legumes have adventitious root primordia on their stem, enabling both root and stem nodulation. The *Aeschynomene* and *Sesbania* models demonstrate this dual capability. In *S. rostrata*, adventitious root primordia are arranged in straight vertical rows (A), whereas in *Aeschynomene* spp., they form spiral-shaped rows winding around the stem. (F and K) These dormant root primordia serve as pre-determined nodulation sites on the stem, both in greenhouse conditions and in the field. Stem nodulation depends on the LRB pathway, similar to root nodulation, but with notable distinctions: firstly (A, F, and K versus D, I, and N), dormant adventitious root primordia lack axillary root hairs; secondly (B, G, and L versus E, J, and O), stem nodules have active chloroplasts exposed to sunlight; finally (C, H, and M), nodulation levels can be profuse, covering almost the entire stem in species such as *S. rostrata* and *A. afraspera*. These differences in structure, function, and number between root and stem nodulation indicate distinct processes between them.



(Box 3) (Ladha *et al.*, 1992). Although the different stages of stem nodulation in *A. evenia* are yet to be described, symbiotic genes such as *AePOLLUX*, *AeCCaMK*, *AeCYCLOPS*, *AeNSP2*, *AeNIN*, and *AeCRK* are necessary for both root and stem nodulation (Quilbé *et al.*, 2021).

Stem nodules are physiologically distinct from root nodules due to the presence of functional chloroplasts in their cortex. These chloroplasts are capable of photosynthesis, and in *Aeschynomene* spp. they can also work together with the photosynthetic *Bradyrhizobium* symbionts to provide energy for rhizobial nitrogen fixation (Ladha *et al.*, 1992; Giraud and Fleischman, 2004). In *Aeschynomene* and *S. rostrata*, this may explain the reported higher nitrogen-fixing activity and reduced sensitivity to soil or mineral nitrogen in stem nodules compared with root nodules (Hungria *et al.*, 1992). Characterization of new stem-nodulating *Aeschynomene* species is expected to shed more light on the peculiar biology of stem nodules (Manantsoa *et al.*, 2024).

Outlook

The LRB pathway is a relatively rare route of infection and nodule formation found in Dalbergioid species and commonly seen in water-tolerant legumes. Peanut (4×) is an excellent

example of a legume that uses the regular LRB pathway. *Aeschynomene evenia* (2×) is a suitable model for understanding the Nod factor-independent symbiosis and stem nodulation, which can be compared with the Nod factor-dependent *A. patula* (2×) and other highly stem-nodulated *Aeschynomene* species. Finally, *S. rostrata* (2×) is an original model due to its versatile infection routes and dual root/stem nodulation. Therefore, studying these three symbiotic systems can help us address several key questions. (i) What is the lock-and-key system of the Nod factor-independent symbiosis? (ii) What role do axillary root hairs play in LRB nodulation? (iii) How is intercellular infection mediated by the host plant? (iv) How does intercellular infection result in intracellular rhizobia accommodation? (v) What makes the LRB competent for nodulation? (vi) What characterizes the biology of stem nodules?

Comparative gene analysis has revealed the extent of genetic conservation with root hair-infected legumes. In *A. evenia*, a mutant-based approach also identified genes that are absent or not known to be required for nodulation in model legumes. As *S. rostrata* is a diploid species, it has great potential for a similar forward genetic project. To support these genetic and functional analyses, it is warranted to re-examine the cellular events occurring during LRB nodulation using advanced microscopy and cell imaging techniques. Combined with single-cell

transcriptomics, this would provide a resolute monitoring of the early symbiotic responses.

In-depth study of these LRB-nodulated legumes will undoubtedly help to decipher the different ways by which legumes establish symbiosis with rhizobia. The knowledge gained on LRB nodulation will be relevant for agriculture. Rhizobia are beneficial root endophytes for cereals, and their infection route at the emergence of lateral roots is similar to LRB infection in legumes. Additionally, the photosynthetic activity of stem nodules and some *Bradyrhizobium* strains may reduce competition for energy between the nodules (N reduction) and the rest of the plant (C reduction). In the ambitious quest to transfer the ability to fix nitrogen to cereals, it is important to consider the above-mentioned characteristics as important factors.

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Author contributions

NHA, NN, and JFA: conceptualization and writing the manuscript; NHA: designing the figures.

Conflict of interest

The authors declare no conflicts of interest.

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Keywords: *Aeschynomene*, axillary root hairs, crack-entry, intercellular infection, legumes, LRB nodulation, Nod factor-independent symbiosis, peanut, *Sesbania*, stem nodulation.

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