

eXtra Botany

Virtual Issue Editorial

Symbiotic nitrogen fixation: a launchpad for investigating old and new challenges

Legumes establish symbiotic relationships with nitrogen-fixing bacteria to convert atmospheric nitrogen into usable forms within root nodules, a process regulated by complex signalling, gene expression, and energy balance. In this Virtual Issue, research articles and reviews that discuss the latest plant-oriented advances in biological nitrogen fixation were collected to provide an updated view of the biochemical and molecular aspects of different steps of the nodulation process.

The symbiotic association between plants (mostly legumes) and microbial nitrogen (N₂)-fixing partners is a multistep mutualistic relationship, in which plants provide a niche (represented by the nodule organ) for the reduction of atmospheric N₂ into biologically useful forms through the process called symbiotic nitrogen fixation (SNF). The establishment of this symbiosis involves chemical and physical interactions between partners, as well as complex signalling and regulatory processes underlying major changes in gene expression, at the same time suppressing plant defence responses (Roy *et al.*, 2020). Nodule organogenesis and N₂ fixation are very expensive processes supported by photosynthate products, which must be strictly regulated to maintain a convenient balance between the influx of N and the efflux of organic carbon. Despite the remarkable progress in this field, much remains to be learned about regulatory networks governing nodule organogenesis and functioning and the molecular/physiological bases that prevent non-legume plants from establishing SNF. This Virtual Issue stems from the 15th European Nitrogen Fixation Conference (ENFC), held in Naples, Italy on August 31–September 3 2023, where the newest research and concepts related to SNF were widely discussed.

The approach we chose for constructing this Editorial is to describe the contributions to the Virtual Issue in the chronological order in which the different phases of nodulation, as discussed in the articles, occur throughout the process (Box 1).

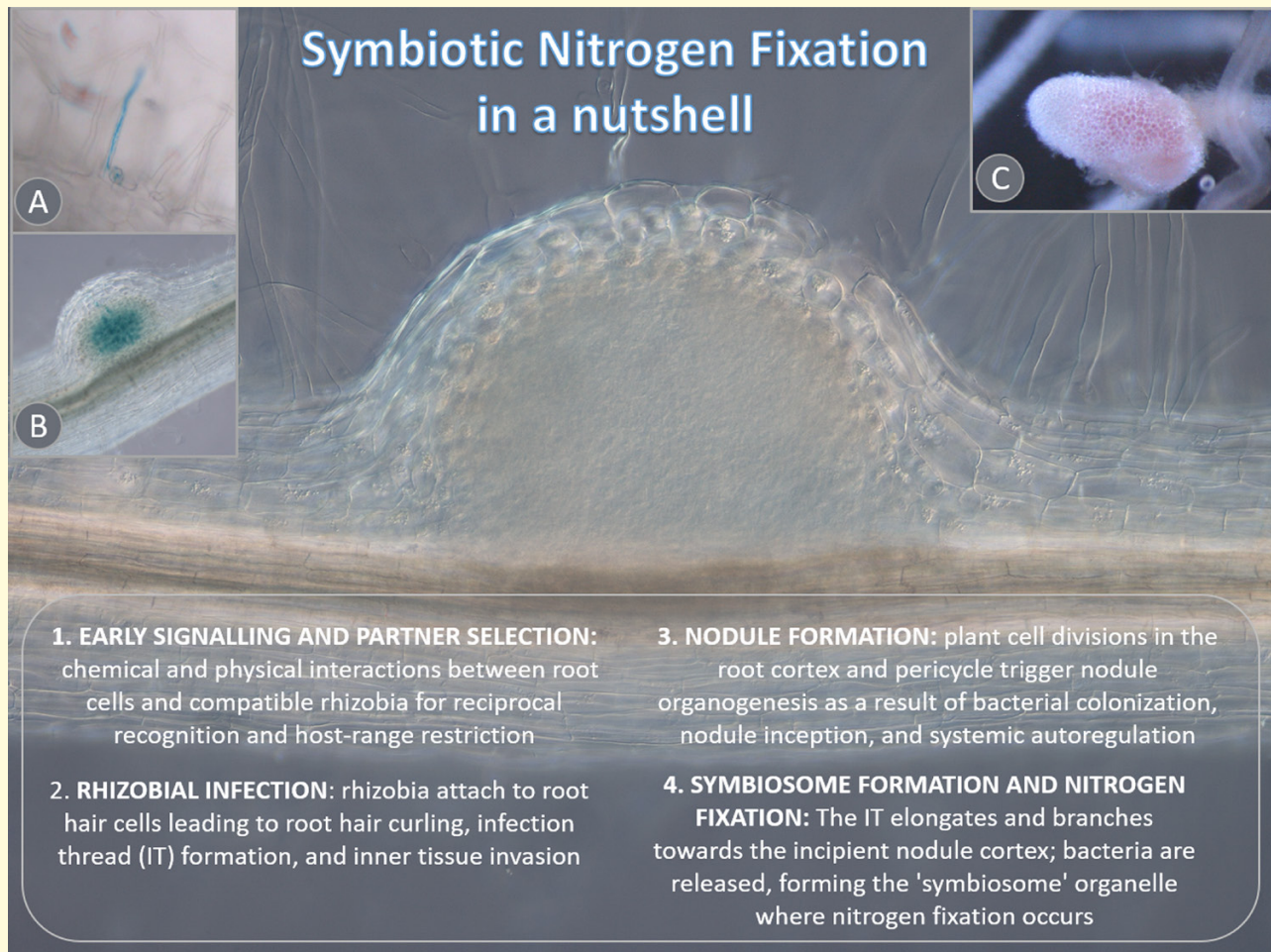
Jacott and del Cerro (2024) described the intimate liaison between the host plant and the bacterial endosymbiont, which initiates in the rhizosphere, where diffusible signals are

reciprocally exchanged between the partners. The crucial role played by Ca²⁺ signalling in the early stages of legume–rhizobium interactions has been firmly established through almost 30 years of research, after the pioneering work by Sharon Long’s lab (Ehrhardt *et al.*, 1996). In particular, Nod factor-triggered nuclear Ca²⁺ spiking has been shown to encode key information to trigger the subsequent cascade of events leading to SNF-specific gene expression (Charpentier, 2018). Spatially and temporally distinct Ca²⁺ elevations have also been demonstrated to underlie the discrimination of plant symbiosis- and immunity-related pathways in response to fungal signals (Binci *et al.*, 2024). Jacott and del Cerro (2024) in this issue report recent advances in current understanding of the regulatory mechanisms and interplay of the ion channels CNGC15–DMI1 in the generation of nuclear Ca²⁺ oscillations during plant root endosymbiosis with both rhizobia and arbuscular mycorrhizal fungi. Moreover, the authors highlight novel discoveries on additional functions of the Ca²⁺-permeable channel CNGC15 in processes beyond symbiosis, such as nitrate signalling and root apical meristem development (Tipper *et al.*, 2023).

After the reciprocal recognition of the symbiotic partners, the intracellular infection thread structure originates in a root hair cell, where an inverted tip growth of the plant cell wall creates a plasma membrane-delimited, cytoplasmic tunnel that enables bacterial invasion into host cells during the formation of N₂-fixing root nodule primordia. Gao *et al.* (2024) in this issue review recent advancements in our understanding of the infection process, focusing on the connection between newly identified mitotic factors and cytokinesis mechanisms involved in intracellular infection with the ‘infectosome complex’ that drives the polar growth of the infection thread. The authors cite the initial report of a possible link between mitosis and rhizobial infection (Yang *et al.*, 1994), leading to the crucial functional characterization of the *Medicago truncatula* *Aurora Kinase 1* gene (*AUR1*). This gene has been shown to play a role in the infection process, with multiple branched infection threads forming within the root hairs of *aur1* mutant roots after inoculation with *Sinorhizobium meliloti* (Gao *et al.*, 2022). The TPXL–AUR1–MAP65 complex operates within the pre-infection thread, potentially mediating microtubule bundling

Box 1. The main steps in symbiotic nitrogen fixation

Symbiotic nitrogen fixation refers to the biological process occurring primarily in legume plant species where specialized structures called nodules form from the roots in response to symbiotic bacteria interactions. These nodules host nitrogen-fixing bacteria, most commonly from the genus *Rhizobium* or closely related genera, in a mutualistic relationship. This multistep process involves complex interactions between root tissues and rhizobia, including early signalling for reciprocal recognition and host-range restriction, rhizobia infection through root hairs, hormonal and systemic signalling for nodule formation, and the establishment of symbiosomes for nitrogen fixation. (A) Rhizobia attach to root hair cells, leading to root hair curling, and formation of the infection pocket and infection thread (highlighted by the β -galactosidase activity of the *Rhizobium* strain carrying a constitutively expressed *lacZ* gene). (B) Infection thread elongation and invasion of the divided cells of the nodule primordium. (C) A *M. truncatula* mature nodule with the N_2 -fixing zone characterized by the high content of leghaemoglobin. In the background a symbiotic nodule primordium at its early stages of formation is shown (the included images are courtesy of Giovanna Frugis and Maurizio Chiurazzi).



to support infection thread formation. The authors also propose a possible scenario involving kinesins and the mitotic transcription factor MYB3R1 in coordinating cell cycle responses during rhizobial infection; however, no direct functional correlations have been reported. In their review, [Gao et al. \(2024\)](#)

provide a chronological overview of the identification of genes (*LIN*, *CERBERUS*, *VPY*, and *RPG*) involved in forming the infectosome complex, localized at the tip of the growing infection threads. Phenotypic characterization of specific mutants has revealed various defects in the infection thread elongation

process (Arrighi *et al.*, 2008; Yano *et al.*, 2009). The infectosome complex is likely to function as a cargo involved in the exocytotic secretion and delivery of vesicles loaded with material necessary for the infection thread formation. The exact nature of the material delivered by the infectosome remains a topic of debate, as it could involve lipid vesicles themselves or materials required for cell wall modification and growth.

Some legumes deviate from the above-described, conventional root hair-mediated infection pattern, by producing root nodules at the junction of the tap and lateral root, a process known as lateral root base (LRB) nodulation. In this issue, the Viewpoint by Horta Araújo *et al.* (2024) provides a critical overview of current knowledge of LRB nodulation. Elucidating the mechanisms underlying this little-known special pathway may increase our understanding of the rhizobium–legume symbiosis. Indeed, from what may look like an oddity, we can learn the fundamentals of rules of engagement for rhizobium–legume symbiosis, which may vary among different legume lineages. Intriguingly, certain legume species show mixed invasion modes (i.e. both root hair and LRB infection routes, indicating flexibility in the rhizobial invasion mode). From this point of view, *Sesbania rostrata* lends itself as a valuable symbiotic model that enables the study of both the LRB nodulation and root hair infection mechanism within a single plant. In general, studying LRB-nodulated legumes may help decipher the different ways by which legumes establish symbiosis with rhizobia.

Argirò *et al.* (2024) investigated the crosstalk between external (nutrient availability) and internal (distribution of hormones and miRNAs) signalling pathways controlling nodule organogenesis. They used a split-root assay in *M. truncatula* to identify potential shoot-to-root systemic signals modulated by N availability. At N satiety, the systemic accumulation of cytokinin *trans*-zeatin in both shoots and roots was observed. Moreover, two miRNA families showed a coordinated accumulation pattern, both dependent on the Compact Root Architecture 2 (CRA2) signalling pathway. The miR2111 family accumulated systemically under N deficit in both shoots and non-treated distant roots, whereas miR399, related to inorganic phosphate (Pi) acquisition, accumulated in shoots and roots of plants under N satiety. These results suggest a crosstalk between Pi and mineral N acquisition. Taken together, this work highlights a finely tuned coordination of systemic signalling pathways integrating plant symbiotic nodulation, root development, and N and Pi nutrition, and identifies cytokinins and the miR399 as candidate shoot to-root systemic signals regulating root growth and potentially nodulation in plants at N satiety.

Nodules are heterogeneous organs composed of various cell types with distinct functions. Single-cell RNA sequencing (scRNA-seq) technology is rapidly becoming a crucial tool for exploring the differentiation trajectories of nodule cells. Establishing a cellular atlas that combines single-cell and spatial transcriptomic data across different stages of nodulation is

invaluable for addressing long-standing questions about cellular composition and nodule development. Furthermore, these studies provide powerful insights for biotechnological approaches aimed at enhancing N₂ fixation performance and transferring symbiotic N₂ fixation potential to non-legume crops. In their review, Pereira *et al.* (2025) provide examples of how scRNA-seq technology is offering significant insights into studying the root nodule symbiosis process. Specifically, the authors thoroughly discuss recent findings that reveal apparently common regulatory programmes in determinate and indeterminate nodules, which govern infection processes and nodule function. Distinct transcriptional cell response mechanisms control the early stages of infection following mutual recognition between the symbiotic partners. During the canonical root hair entry process, only a few epidermal root hairs in the susceptible zone that physically encounter rhizobia respond to the microbial partner by becoming infected. Of these, only a small fraction progress to infect the dividing cortical cells of the nodule primordia. The use of specific infection marker genes in *M. truncatula*, *Lotus japonicus*, and *Glycine max* has revealed conserved profiles of expression in spatially separated cells involved in different stages of the infection process (Frank *et al.*, 2023; Liu *et al.*, 2023; Pereira *et al.*, 2024). Another example is the identification of specialized expression profiles in the infected and uninfected cells within developing N₂-fixing nodules using scRNA-seq technology. These results have traced the functional compartmentalization of infected and uninfected cells and their respective contributions to N assimilation through specific pathways involved in determinate and indeterminate nodules (Cervantes-Pérez *et al.*, 2022; Wang *et al.*, 2022; Ye *et al.*, 2022; Sun *et al.*, 2023).

Nodule functioning is also the phase of the SNF investigated by Minguillón *et al.* (2024) in their research article included in this issue. N₂ fixation is highly demanding for legume plants, as a substantial amount of photosynthates must be allocated to the nodule ‘sink’ organs to support the action of the bacterial nitrogenase. To optimize plant growth, a balance between photosynthate investment and the N returned by fixation must be maintained. In other words, N starvation is essential for both nodulation and N₂ fixation because, when N is readily available, plants prefer to absorb it directly from the soil rather than undertake the energetically costly fixation process. For this reason, studies on the regulatory role of nitrate in nodulation have primarily focused on identifying the signalling pathways and factors involved in inhibitory mechanisms triggered by high external nitrate levels (Omrane and Chiurazzi, 2009; Cabeza *et al.*, 2014). However, recent studies suggest a complex network of nitrate transport in mature nodules, highlighting a positive role for nitrate in nodule functionality under low external nitrate conditions (Wang *et al.*, 2020; Vittozzi *et al.*, 2021). Leghemoglobins (Lbs) are essential for efficient N₂ fixation in nodules. Lbs, which can constitute up to 40% of nodule proteins, exhibit an extremely fast rate of O₂ association and a relatively slow rate of O₂ dissociation. This enables them to

buffer free O₂, maintaining levels compatible with N₂ fixation, thereby contributing to solve the so-called ‘nodule oxygen paradox’. Minguillón *et al.* (2024) explored the regulation of the entire set of haemoglobins (Glbs) in *L. japonicus*, including three Lbs and six Glbs (from classes 1, 2, and 3), throughout nodule development. Their analysis, conducted in young (2 weeks post-inoculation), mature (4–6 weeks post-inoculation), and senescent nodules (8–10 weeks post-inoculation) under various nitrate regimes and genetic backgrounds revealed distinct regulatory patterns. The specific nitrate response profiles of haemoglobin gene expression aligned with regulation by the transcription factor NLP4, a critical component in the mature nodule nitrate response pathway (Nishida *et al.*, 2018, 2021). This extensive analysis also highlighted a strong connection between Lb deficiency and nitro-oxidative stress (Minguillón *et al.*, 2024). Additionally, the observed accumulation of nitric oxide (NO) in the nodules of Lb-deficient mutants grown in the absence of nitrate led the authors to suggest a potential nodular nitrate-independent pathway for NO biosynthesis (Pathak *et al.*, 2024).

Tracing the evolution of the nodulation capacity in plants is a hot topic in the field of SNF. An interesting research paper discusses the evolution of nodulation in the large genus *Chamaecrista* (Casaes *et al.*, 2024), that belongs to the legume subfamily *Caesalpinioideae* and comprises shrubs, subshrubs, and tree species. The *Caesalpinioideae* subfamily is characterized by the confinement of their rhizobial symbionts within cell wall-bound ‘fixation threads’ (FTs). However, in the genus *Chamaecrista*, only tree species have FTs, whereas shrubs and subshrubs house their rhizobial bacteroids within symbiosomes (SYM-type nodules), being the only nodulated caesalpinoid genus with species in temperate regions. In this study, evolutionary relationships between *Chamaecrista* growth habit, habitat, nodule bacteroid type, and rhizobial genotype are analysed in 30 *Chamaecrista* species from Bahia, Brazil. Tree species predominantly form FTs, smaller shrubs and subshrubs form SYM-type nodules, but intermediate FT–SYM nodules were observed in some treelets and large shrubs, representing a transitional stage. Using data from various sources, including unpublished anatomical data and literature, the authors found that the distribution of FTs and symbiosomes is not random but co-evolved with their environments and specific rhizobial symbionts. FTs have thinner walls with less unesterified pectin, probably optimizing nutrient and O₂ exchange between rhizobia and the host. SYM-type nodules allow for higher rhizobial packing and may be more efficient in N₂ fixation. These findings allow the authors to suggest implications of nodule diversity in adaptations to growth habits and biomes, as well as ecological flexibility and the ability to form symbioses outside native ranges. Overall, *Chamaecrista* species evolution reflects adaptive responses to habitat changes and nutrient availability, with significant implications for its diversification and N₂-fixing strategies, thus constituting a very interesting system for evolutionary studies of N₂ fixation.

Conclusions

In the context of developing tools capable of reducing the impact of nitrogen fertilization in intensive agriculture, transferring the nodulating and nitrogen-fixing capacity to crops of agricultural interest remains a fundamental goal of studies on SNF. During the 15th ENFC, the presentation and discussion of data on: (i) new methodological approaches capable of unravelling specific cellular expression profiles during the symbiotic interaction, thereby identifying new crucial markers for the various phases of the nodulation process; (ii) the discovery and genomic characterization of new forms of symbiotic association between cereals and diazotrophic bacteria; (iii) attempts to express a functional bacterial nitrogenase in plant cells; and (iv) mechanisms controlling the proper energy balance of SNF and responses to environmental stresses have certainly represented significant advances toward realizing the dream of generations of SNF biologists.

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Conflict of interest

The authors declare no competing interests.

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