

Nano-enabled strategies to enhance biological nitrogen fixation

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Increasing the capacity of biological nitrogen fixation (BNF) is an effective strategy to enhance food security while simultaneously reducing the carbon and nitrogen footprint of agriculture. Nanotechnology offers several pathways to enhance BNF successfully.

Nitrogen is the most limiting essential element for plant growth. Although 78% of the air is nitrogen, terrestrial plant species have no evolved pathways for direct acquisition and utilization of nitrogen for growth. However, legume plants such as soybean (*Glycine max*), peas (*Pisum sativum*), and beans (*Phaseolus*, *Vigna* and *Cajanus* species) form a symbiotic relationship with certain bacteria that can fix the ubiquitous nitrogen in the environment into ammonia, allowing them to utilize it. This process is called biological nitrogen fixation (BNF). Prior to the development of synthetic nitrogen fertilizer production through the energy-intensive Haber–Bosch process, BNF was the primary source for replenishing bioavailable nitrogen on agricultural land¹. However, synthetic nitrogen fertilizers are widely used today to supplement soil fertility, despite their low delivery and use efficiency by crops. This ultimately contributes significantly to greenhouse gas (GHG) emissions, ammonia volatilization and loss of reactive nitrogen into the water from land. Continued increases in nitrogen fertilizer application will further jeopardize climate stability through the excessive release of potent GHGs (including N₂O, which is 300 times more potent than CO₂ on a 100-year scale) and intensive consumption of fossil fuels². N₂O is also the key contributor to ozone depletion in the 21st century. Therefore, reducing nitrogen fertilizer application is a critical strategy for mitigating food insecurity and global warming.

Enhanced BNF in soybeans offers an unparalleled opportunity to reduce nitrogen fertilizer use and increase crop yields. Soybean is one of the four major food crops, facilitating the fixation of 25 Tg of nitrogen in 2018 and accounting for 70% of the legume crop production³. Biological nitrogen fixation in soybeans can also be used for intercropping strategies (that is, growing two or more crops in proximity) to improve soil fertility and subsequent yields⁴. In addition, soybean is an economical and high-quality source of plant protein in the human diet. Also, it contains essential nutrients such as unsaturated fatty acids, phospholipids, B vitamins and minerals that have great potential to improve the quality of human diets⁵. Plant-based protein diets hold the promise of halving reactive nitrogen use globally⁶. However, the natural BNF systems are plagued by several shortcomings, including the environmental sensitivity of nitrogenase (damage to nitrogenase by O₂ and stress-induced reactive oxygen species, ROS), the high energy consumption of BNF processes, lack of essential minerals in

intensively cultivated soils which forms part of the nitrogenase enzyme (for example, Mo) and the weak competitiveness of nitrogen-fixing bacterial species⁷. Current methods to improve the efficiency of soybean BNF need to be sufficiently effective. Gene editing may offer some potential for enhancing BNF; however, delivery at the field scale, unfavourable government regulations, and general public unease toward these approaches are significant barriers to overcome. In addition, single trait improvement by gene editing is probably insufficient to support the effectiveness of BNF, specifically when plants are exposed to biotic or abiotic stresses and particularly in soils with poor health (Fig. 1).

Conversely, beneficial microorganisms enhance BNF through various mechanisms, including hormone regulation and nutrient delivery. Still, their effectiveness is constrained by variability among soybean cultivars, environmental conditions and logistical delays between inoculation, planting and harvest. Plant growth regulators such as phytohormones, hydrogen sulfide, and isoflavones can promote nodulation and BNF efficiency when added exogenously but are highly influenced by factors such as concentration and application method as well as costly at field scale. Thus, a significant gap between soybean BNF enhancement strategies and their practical application still exists, and the development of novel strategies that can substantially and sustainably enhance BNF is needed.

Nano-enabled strategies for improving BNF

In the past decade, the application of nanotechnology in agriculture has expanded rapidly with significant advances in the synthesis and characterization of nanomaterials and a more mechanistic understanding of nanomaterial–plant interactions. The ability to design and fine-tune nanomaterials for highly specific and even responsive behaviour offers tremendous potential as a sustainable strategy for agricultural improvement⁸. The development of such functional nanomaterial platforms to enhance soybean BNF could offer significant benefits on multiple levels, as described below (Fig. 1).

Targeted delivery mechanism. Nanomaterials have been developed as a gene delivery platform to target specific organelles (chloroplasts and mitochondria)⁹. The small size and tuneable surface chemistry of nanomaterials provide unique opportunities for effective and precise delivery platforms across plant cell walls and membranes⁹. The pathways by which nanomaterials enter cells are predominantly a mechanical process unlikely to be affected by species differences that often limit CRISPR, an important genetic engineering technology¹⁰. Notably, organelle-specific gene delivery by nanomaterials with soybean achieves independent delivery of genes to avoid integration into the genome, so soybean may not require labelling as a genetically modified organism (GMO), which could be significant in risk communication with regulators and the general public.

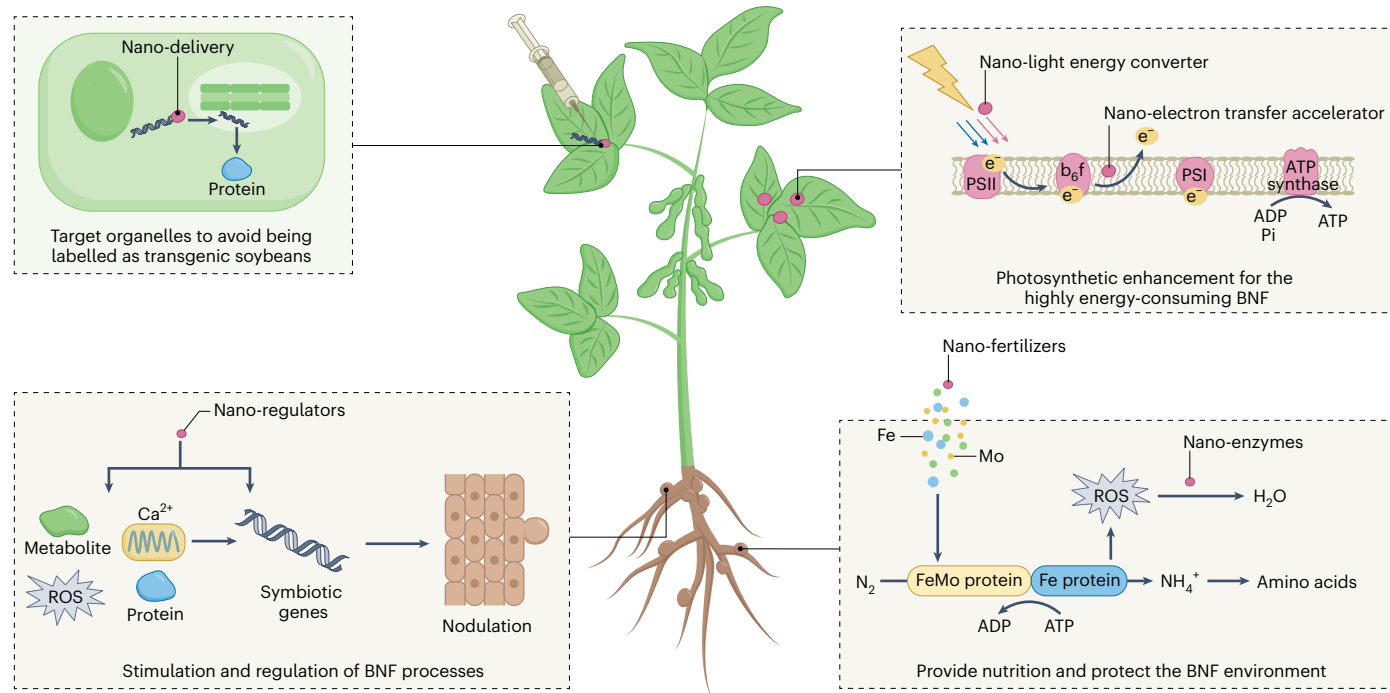


Fig. 1 | Nanotechnology-based BNF enhancement strategies. The intentional design of nanomaterials can be used as delivery platforms to target specific organelles, enhance photosynthesis, modulate BNF processes including nodulation, and provide nutrients and scavenge reactive oxygen species to support efficient BNF.

Nanomaterials can also serve as a precise delivery platform for analytes beyond genetic material, maximizing efficacy and reducing both chemical usage and environmental impact. For example, nanomaterials could be loaded with ROS/O₂ scavengers targeted for activity in proximity to oxygen-sensitive nitrogenase in the cytoplasm, with photosynthetic enhancers that can facilitate electron capture or transport to improve photosynthetic efficiency in support of highly energy-consuming BNF, or with signalling substances (ROS, Ca²⁺, or autoinducers) targeted at promoting nodulation and enhancing BNF. The intentional and tuneable design of nanomaterials is critical to achieving a precise delivery function. The innate molecular recognition mechanisms that plant tissue/cell/subcellular compartments have for biomolecules can be used to guide nanomaterials to target sites. For example, nano-Au coated with the stomatal affinity protein LMS-M can target stomata to prevent pathogens from entering plants¹¹. Sequence recognition function-guided peptide-modified nanomaterials can overcome plant biological barriers and deliver chemicals to chloroplasts to enhance plant photosynthesis¹². The size, morphology, and surface properties of nanomaterials are essential factors affecting the pathway and efficiency of their uptake and transport in plants. For example, as a dicotyledonous plant, the xylem of soybean contains more negatively charged groups. Therefore, positively charged nanomaterials are more easily trapped in the soybean xylem, which enhances the transport of nanomaterials in soybean¹³. However, the intentional design of nanomaterials is complex, and multiple aspects need to be considered prior to synthesis, such as the intended plant species, environmental conditions, the type and properties of nanomaterials (including nanomaterial stability and in-plant transformation), cost and scale up concerns, and the potential unanticipated toxicity to target or non-target species. An orthogonal approach

using artificial intelligence/machine learning, nanoinformatics and data-driven models such as quantitative structure–activity relationship (QSAR), quantitative structure–property relationship (QSPR) models, and lipid exchange envelope penetration (LEEP) prediction models will collectively facilitate the design of nanomaterials with precise delivery function^{14,15}.

Precision release mechanism. Tuneable nanomaterial design also enables the precise release of analytes, which could be of tremendous value for improving the efficiency of soybean utilization of fertilizers, pesticides and growth regulators and the success of gene editing. Soybeans have highly specific nutritional requirements to support BNF. Thus, the emphasis on improving nitrogen use efficiency in soybean should focus on optimizing BNF, which will not only minimize the nitrogen requirement but also maximize the efficiency of other nutrients. For example, soybean has a low nitrogen requirement in early growth. Excessive nitrogen fertilization at this point, particularly ammonium, which is not uncommon, can inhibit nodule formation and the initiation of BNF processes. Furthermore, BNF efficiency decreases during the filling period and nitrogen fertilizer application at this stage can delay nodule senescence and increase yield¹⁶.

Conversely, soybean nodulation and BNF processes have high phosphorus requirements due to energy conversion and protein and lipid synthesis¹⁶. Soybean nodules are also significant metal sinks and plants must supply iron, molybdenum, magnesium, nickel, cobalt, calcium and other elements necessary for the robust synthesis of nitrogenase; thus, overall plant nutrition is critical to initiating and sustaining BNF. Therefore, given these specific nutritional requirements for nitrogen, phosphorus and metal elements during

different growth periods, nanomaterial carriers can be designed for precise nutrient release as a function of required time, rate and amount; this approach would greatly improve the efficiency of nutrient utilization while simultaneously reducing resource waste, environmental damage and GHG emissions. This can be achieved by designing responsive nanomaterials that release cargo when triggered to do so by exogenous signals such as temperature, light, ROS, water, ionic strength, pH or redox potential¹⁷. Moreover, metal-based nanomaterials (such as Fe-based nanomaterials) have unique and tunable surface properties themselves. Those properties could be utilized directly in the absence of a carrier to promote the synthesis of the nitrogenase in soybean by enhancing overall plant nutrition¹⁸.

Regulation mechanism of nitrogen fixation in soybean. Soybean BNF involves various physiological and biochemical processes, including signalling, gene expression, energy conversion, and nutrient assimilation and metabolism. Nanomaterial modulation of plant physiological and biological processes such as photosynthesis, redox homeostasis and gene/protein expression, among others, have been reported in several recent studies^{8,18}. Applying these approaches to modulate soybean BNF should prove to be highly effective. In addition, an indirect pathway would be to increase energy production to support the BNF process. For example, nanomaterials with unique electronic, optical and catalytic properties, such as carbon dots (CDs) and conjugated polymer nanomaterials (CPNs), can act as electron transfer accelerators or photoconverters to improve the efficiency of electron transfer or utilization of sunlight in chloroplasts, thereby enhancing photosynthesis and providing greater energy for BNF given that 16 ATPs are consumed in fixing a mole of ammonia^{19,20}.

Another key strategy is to modulate the signalling molecules involved in the BNF process, such as ROS Ca^{2+} , autoinducers or the genes that regulate the nodulation process. It has been reported that certain nanomaterials may induce the production of ROS in amounts that cause them to be recognized by cells as signalling molecules, subsequently changing Ca^{2+} concentration or releasing trace amounts of metals. For example, ferromanganese nanomaterials induced the production of trace ROS that were recognized as signalling molecules which subsequently enhanced soybean nodulation and BNF efficiency²¹. Functionalized multi-walled carbon nanotubes can upregulate the expression of *Nif*, a key gene for nodule development, resulting in increased nodule number and nitrogenase activity²². However, the underlying mechanisms by which nanomaterials can mediate these processes and the spatial and temporal dynamics of plant and BNF response are still largely unknown. Importantly, this lack of understanding complicates nanomaterials design. Future work needs to include orthogonal studies to elucidate these molecular pathways and combine this knowledge with nanoinformatics-related models that can feed back into more intentional nanomaterial design.

Protection of BNF mechanism from environmental stress. Soybean production is severely challenged by environmental and climate stress; e.g., drought stress alone can depress the annual yield by up to 40%. Oxidative burst, the rapid release of excessive ROS, is a major cause of plant damage and death under stress conditions. ROS accumulation can cause rapid nodule senescence and loss of BNF function. Mitigating excessive ROS could be an effective strategy for protecting the BNF environment and enhancing the resilience of soybean to stressors. We argue that nanozymes, a group of nanomaterials with

intrinsic enzyme-like activities that can capture ROS and have been extensively studied in biomedicine²³, can be adopted in agriculture. Unlike current genetic manipulation or conventional breeding techniques for enhancing plant resilience, the effectiveness of which is highly constrained by plant species and environmental factors, nanozymes might offer a robust strategy based on ROS-capturing mechanisms. This has been reported in some early studies; for example, nano- CeO_2 , a well-known nanozyme, can improve plant growth under various stresses such as nitrogen deficiency, high salinity, heat, and dark or chilling^{24,25}. These results come from short-term pilot studies, and further work is needed to determine whether the nanozymes can maintain long-term plant functionality. A more recent life-cycle study suggests that nano- Fe_2O_3 upregulated the expression of genes related to phytohormone synthesis, which promoted the development of nodules and delayed senescence, subsequently resulting in a 13.7% increase in soybean yield¹⁸. Fe_2O_3 is not only a nanozyme but also includes the essential plant nutrient (Fe); this characteristic allows protection of the nodule by capturing the ROS and providing an essential nutrient for the plants. Many other nanozymes, such as Fe_3O_4 , MoS_2 , Mn_2O_3 and CoFe_2O_4 , may have this dual functionality and, thus, could enhance soybean BNF and plant resilience to environmental stressors arising from climate change.

Potential to extend to a broader range of plant species

The host specificity of rhizobia is limited to leguminous species, which places an obvious and significant restriction on the broader application of BNF in agriculture. Developing nitrogen-fixing cereal crops has been a long-term goal of agricultural scientists. One potential strategy is to transfer and express the specific genes (for example, *nif*) from nitrogen-fixing bacteria into cereal crops; however, this has long been regarded as technically infeasible due to the difficulties of transferring large gene clusters and expressing the genes derived from prokaryotic bacteria in a eukaryotic plant. Alternative strategies have been pursued by targeting organelles such as chloroplast and mitochondria rather than engineering the whole plant cell. However, this leads to another challenge: getting the genes targeting the organelles through the intracellular double membrane structure. Several studies have shown that nanotechnology can overcome this challenge by enabling targeted delivery with nanomaterials that possess a bio-recognition motif or enable penetration using a lipid exchange envelope penetration (LEEP) method, thus delivering the gene directly into the chloroplast.

Another strategy that could be effective, aside from genetically engineering the plant, is to boost the nitrogen fixation of endophytic nitrogen-fixing bacteria. These bacteria reside in the intercellular spaces of plant cells, which contain non-structural carbohydrates, amino acids, and inorganic nutrients that can provide the necessary nourishment for the bacteria to grow. For example, the lower stem of rice is known as an active site for nitrogen-fixation by endophytic bacteria²⁶. The proposed mechanisms for soybean may also be applied to cereal crops by enabling targeted delivery of nutrients or ROS scavengers to the bacterial colonies, thereby protecting from stress damage or enhancing plant growth, thus indirectly increasing BNF.

Outlook

It is now clear that certain aspects of climate change are proceeding more rapidly than previously predicted. As such, efforts to reduce GHG emissions from agriculture, among the largest GHG-emitting sectors, must be implemented. Enhancing BNF in soybeans can be an essential

strategy to reduce fuel use for ammonia production as a fertilizer and subsequent GHG emissions along the production-use chain of synthetic nitrogen fertilizer. It may also improve soil carbon sequestration capacity and food security. Nanomaterials can serve as a robust and flexible toolbox, given the ability to manipulate and tune their design according to the specific application needs for achieving precise delivery and plant growth regulation. The intentional design of nanomaterials for BNF enhancement can be achieved by understanding soybean physiological characteristics, nutritional requirements, and soybean–nanomaterial interaction mechanisms. Importantly, this strategy has the potential to be extended to non-legume crops. However, factors such as species differences, climate change (for example, extended droughts, intense precipitation and so on), and environmental and biological material transformations complicate the design of nanomaterials and the assessment of any potential environmental and human health risks. Therefore, nanomaterials must be designed according to safe and sustainable design principles. Machine learning- and artificial intelligence-based nanoinformatics can integrate physicochemical properties of nanomaterials (size, morphology, surface structure, chemical composition and crystal structure) and environmental factors (species, soil properties and climatic conditions) into predictive models for intentional nanomaterial design and safe application. This approach can facilitate the optimization of nanomaterials at the design stage to achieve specific functions and minimize unforeseen negative consequences. However, predictive modelling requires data for model training. Thus, it is necessary to determine the long-term fate of nanomaterials and the impact of these strategies over multiple growing seasons in complex agroecosystems.

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Competing interests

The authors declare no competing interests.