

Review

# Enhancing *Rhizobium*–Legume Symbiosis and Reducing Nitrogen Fertilizer Use Are Potential Options for Mitigating Climate Change

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**Abstract:** This review article explores the impact of nitrogen fertilizers on the symbiotic relationship between *Rhizobium* bacteria and legume plants. Nitrogen fixation has the potential to address the global protein shortage by increasing nitrogen supply in agriculture. However, the excessive use of synthetic fertilizers has led to environmental consequences and high energy consumption. To promote sustainable agriculture, alternative approaches such as biofertilizers that utilize biological nitrogen fixation have been introduced to minimize ecological impact. Understanding the process of biological nitrogen fixation, where certain bacteria convert atmospheric nitrogen into ammonia, is crucial for sustainable agriculture. This knowledge helps reduce reliance on synthetic fertilizers and maintain soil fertility. The symbiotic relationship between *Rhizobium* bacteria and leguminous plants plays a vital role in sustainable agriculture by facilitating access to atmospheric nitrogen, improving soil fertility, and reducing the need for chemical fertilizers. To achieve optimal nitrogen fixation and plant growth, it is important to effectively manage nitrogen availability, soil conditions, and environmental stressors. Excessive nitrogen fertilization can negatively affect the symbiotic association between plants and rhizobia, resulting in reduced soil health, altered mutualistic relationships, and environmental concerns. Various techniques can be employed to enhance symbiotic efficiency by manipulating chemotaxis, which is the ability of rhizobia to move towards plant roots. Plant-specific metabolites called (iso)flavonoids play a crucial role in signaling and communication between legume plants and rhizobia bacteria, initiating the symbiotic relationship and enhancing nitrogen fixation and plant growth. Excessive nitrogen fertilizer application can disrupt the communication between rhizobia and legumes, impacting chemotaxis, root exudation patterns, nodulation, and the symbiotic relationship. High levels of nitrogen fertilizers can inhibit nitrogenase, a critical enzyme for plant growth, leading to reduced nitrogenase activity. Additionally, excessive nitrogen can compromise the energy demands of nitrogen fixation, resulting in decreased nitrogenase activity. This review discusses the disadvantages of using nitrogenous fertilizers and the role of symbiotic biological nitrogen fixation in reducing the need for these fertilizers. By using effective rhizobial strains with compatible legume cultivars, not only can the amounts of nitrogenous fertilizers be reduced, but also the energy inputs and greenhouse gas emissions associated with their manufacturing and application. This approach offers benefits in terms of reducing greenhouse gas emissions and saving energy. In conclusion, this paper provides a comprehensive overview of the current understanding of the impact of nitrogen fertilizers on the symbiotic relationship between *Rhizobium* and legume plants. It also discusses potential strategies for sustainable agricultural practices. By managing nitrogen fertilizers carefully and improving our understanding of the symbiotic relationship, we can contribute to sustainable agriculture and minimize environmental impact.

**Keywords:** nitrogen fertilizer; *Rhizobium*; legume; symbiotic interactions; nodulation; nod factors; (iso)flavonoids; molecular dialogue



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## 1. Introduction

### 1.1. Nitrogen Resources: Tackling Protein Scarcity Globally

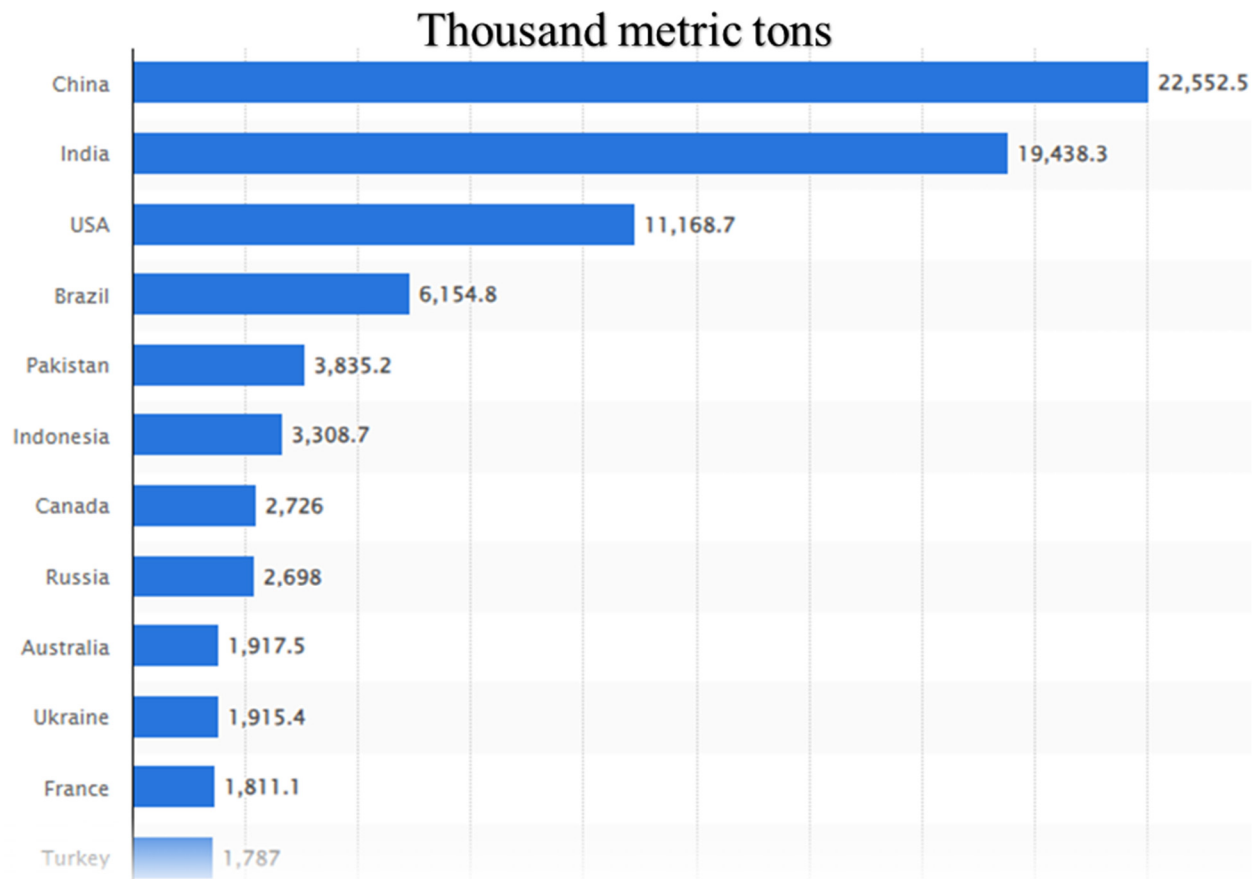
Addressing global protein scarcity has been a persistent challenge in human nutrition throughout history [1,2]. This scarcity primarily arises from the limited availability of nitrogen, which is essential for protein production. To meet the increasing protein demand, it is crucial to ensure an ample supply of nitrogen. This is predominantly achieved through biological and chemical nitrogen fixation processes [3,4]. Fortunately, significant progress has been made in the past four decades in exploring diverse nitrogen resources in nature for agricultural purposes. Consequently, the daunting task of fulfilling protein requirements for the rapidly growing global population appears less formidable [5,6]. Encouragingly, there are numerous opportunities to cleverly manipulate various biological nitrogen fixation processes to significantly increase protein yield [7].

### 1.2. Crop Production and Environmental Consequences of Nitrogen Fertilizer Usage

Nitrogen, also known as  $N_2$ , is a vital element that makes up 78% of Earth's atmosphere [8]. It plays a crucial role in plant growth, with plants requiring larger amounts of nitrogen compared to other elements [9,10]. However, plants cannot directly use nitrogen gas due to its stability and strong triple bond between nitrogen atoms. They need nitrogen to be converted into reduced forms, which they obtain from various sources such as ammonia or nitrate fertilizers, organic matter decomposition, natural processes like lightning, and biological nitrogen fixation [11]. The production of fertilizers, insecticides, irrigation, and machinery for the green revolution heavily relies on fossil fuels, with approximately 80% of the world's fossil energy being used [12,13]. Over the past four decades, global nitrogen fertilizer usage has significantly increased, contributing to over half of the energy consumed in agriculture [14,15]. The manufacturing process for nitrogen fertilizer using the Haber-Bosch process alone emits approximately 465 teragrams of carbon dioxide annually, making it a significant source of greenhouse gas emissions [16–18]. The nitrogen fertilizer industry has been found to contribute up to 1.2% of total greenhouse emissions resulting from human activities [12,19–21]. Additionally, the nitrification and denitrification processes in the soil release substantial amounts of nitrous oxide ( $N_2O$ ), accounting for approximately 1.5% of total greenhouse emissions in agricultural systems [16,22]. The Intergovernmental Panel on Climate Change (IPCC) and the International Fertilizer Industry Association (IFA) have recognized the impact of the fertilizer industry's emissions.

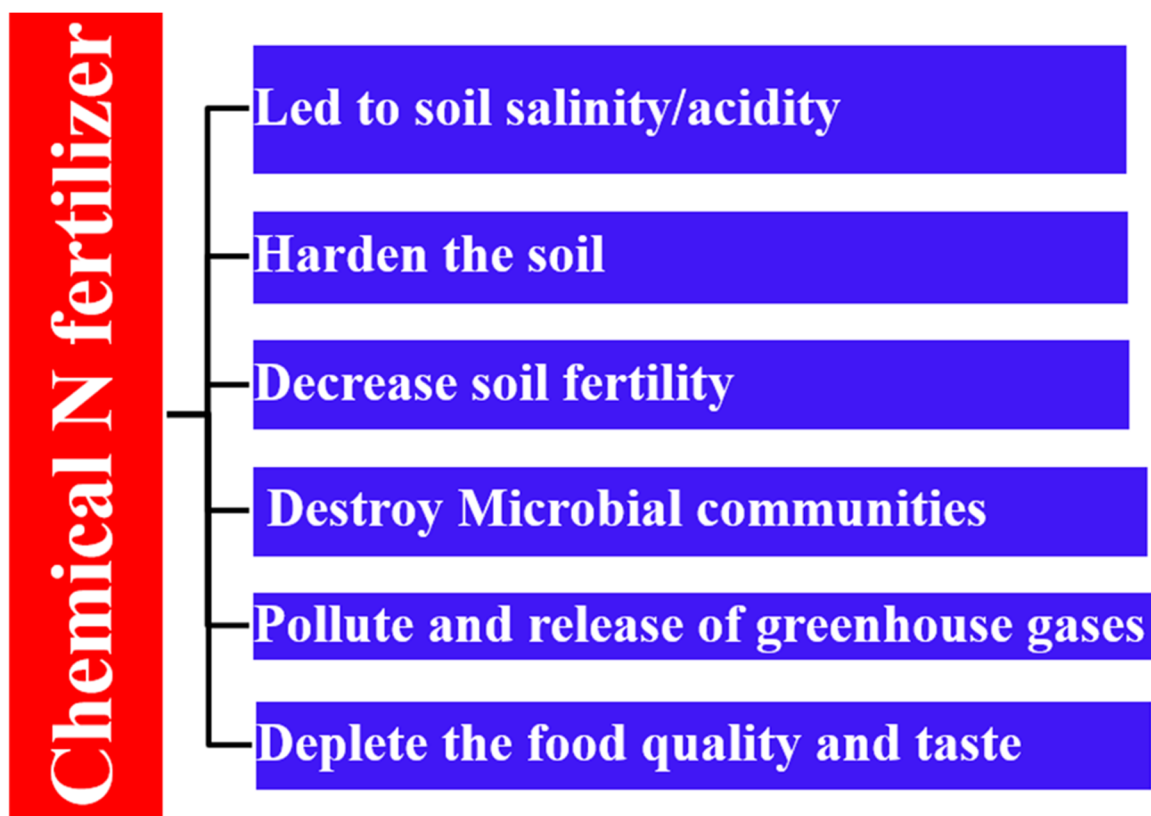
The fertilizer industry heavily relies on energy-intensive technologies for agricultural production, including the manufacture of nitrogen fertilizers and pesticides. Global nitrogen fertilizer consumption reached approximately 108 million tons in 2019 and slightly increased to 110 million tons in 2020–2021 (Figure 1), with a projected annual growth rate of 4.1% until 2025–2026 [23]. However, the scarcity of fossil energy is a significant challenge that the world may face [24,25]. The excessive use of nitrogen fertilizer can have significant environmental consequences. One major issue is nutrient runoff, where high levels of nitrogen and phosphorus from synthetic fertilizers can be washed into nearby water bodies, causing eutrophication, harmful algal blooms, oxygen depletion, and disruption in aquatic ecosystems [26–28]. Another problem is soil degradation. Synthetic fertilizers primarily focus on macronutrients like nitrogen, phosphorus, and potassium, neglecting other essential micronutrients. This imbalanced nutrient application can deplete soil organic matter, damage its physical structure, decrease beneficial microbial activity, and reduce overall fertility over time [29–31]. Biodiversity loss is also a concern (Figure 2). Nutrient runoff leading to eutrophication can harm aquatic life, resulting in a decline in fish populations and other species. Moreover, the loss of soil fertility due to synthetic fertilizers can negatively affect soil organisms crucial for maintaining healthy soil and biodiversity, such as earthworms, beneficial insects, and microorganisms [32]. The production and distribution of synthetic fertilizers contribute to greenhouse gas emissions and air pollution. This energy-intensive process relies on fossil fuels and releases carbon dioxide ( $CO_2$ ), nitrogen oxides ( $NO_x$ ), and methane ( $CH_4$ ), exacerbating climate change [33]. In addition to their environmental

impacts, synthetic fertilizers are expensive to produce. The entire production cycle, including raw material extraction, chemical production, transportation, and packaging, requires substantial energy inputs and contributes to high energy consumption and associated environmental impacts [34].



**Figure 1.** Worldwide nitrogen fertilizer consumption in 2021 by country.

To mitigate the consequences of unsustainable agricultural practices, several sustainable alternatives have been developed. These include organic farming, crop rotation, cover cropping, and the use of natural fertilizers such as compost and manure. By reducing reliance on synthetic fertilizers, these practices promote environmental sustainability. Additionally, alternatives like biofertilizers and biopesticides have been embraced in modern agriculture. These options help alleviate energy consumption, greenhouse gas emissions, and negative impacts of excessive nitrogen waste in agroecosystems [35,36]. Biofertilizers and biopesticides encourage biological nitrogen fixation, a process facilitated by microorganisms that significantly contribute to the nitrogen cycle and overall nitrogen balance. Global terrestrial biological nitrogen fixation is estimated to range from 52 to 130 teragrams (Tg) of nitrogen per year [37–39]. Biological nitrogen fixation aligns with the principles of green engineering as it relies on renewable sunlight and has minimal ecological impact [40,41]. However, it is important to acknowledge that these strategies may have practical and economic limitations depending on specific agricultural systems and contexts. To achieve more sustainable nitrogen management in agriculture, a combination of approaches tailored to local conditions, supported by research and education, is necessary.



**Figure 2.** Harmful effects of excessive use of chemical nitrogen fertilizers in the long term.

## 2. Biological Nitrogen Fixation Systems

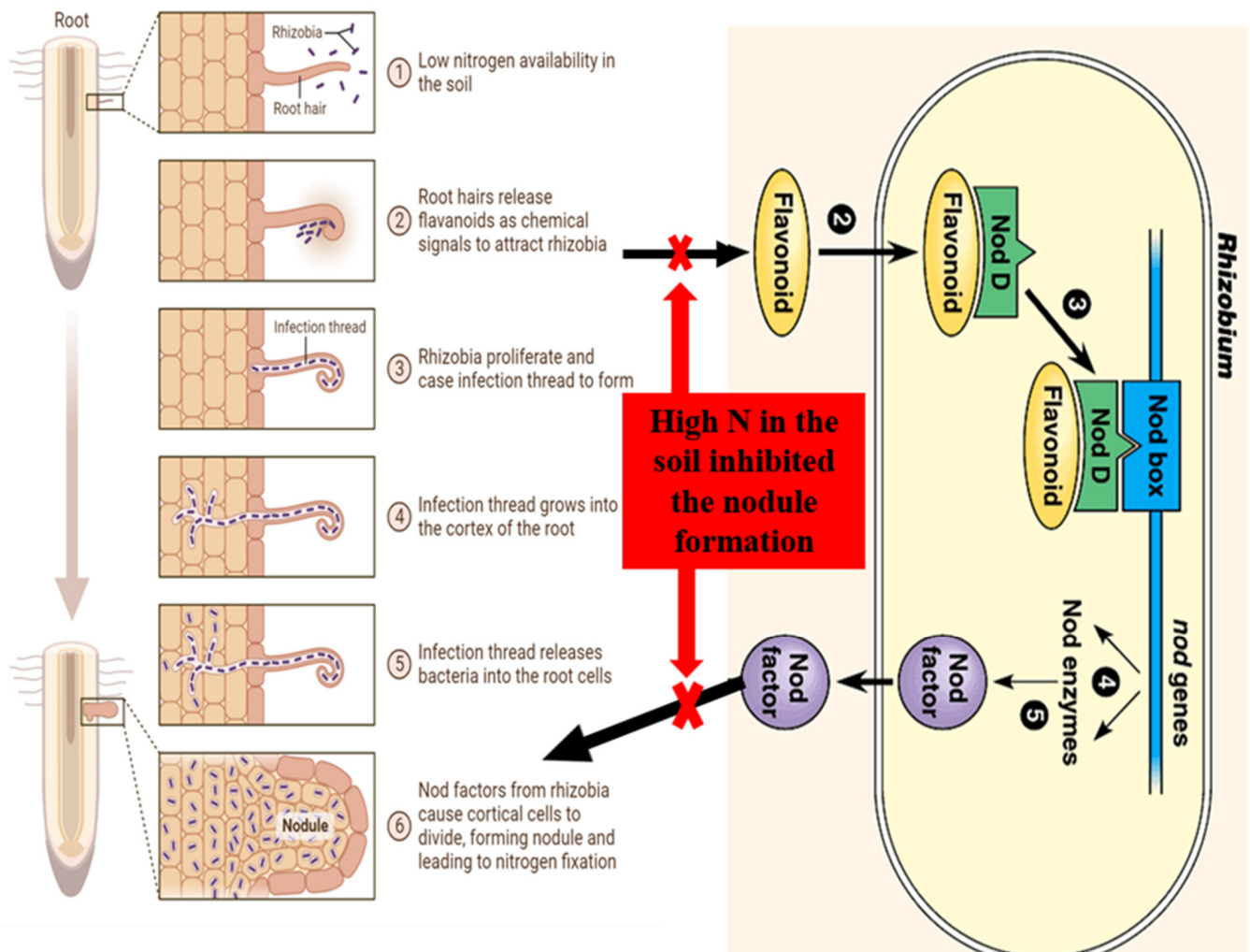
Biological nitrogen fixation is a critical process in the global nitrogen cycle, wherein certain prokaryotic organisms possess the necessary genetic information to produce nitrogenase. This enzyme converts atmospheric nitrogen ( $N_2$ ) into ammonia ( $NH_3$ ), which can be further transformed into various organic forms of nitrogen. Three primary strategies for nitrogen fixation occur in terrestrial ecosystems [42]: 1. Free-living bacteria. These bacteria exist both in bulk soil and on the surfaces of plants. They include heterotrophic bacteria, such as *Azotobacter*, and autotrophic cyanobacteria. Heterotrophic bacteria obtain energy from organic compounds, while cyanobacteria can utilize sunlight as an energy source [43,44]; 2. Associative or ectosymbiotic bacteria. These bacteria live in close association with plant roots in the rhizosphere (soil surrounding roots), phyllosphere (surface of leaves), or intercellular spaces of the root cortex. Examples include *Azospirillum* and cyanobacteria. These bacteria colonize plant surfaces and benefit from the organic carbon exuded by plants while providing fixed nitrogen to their host [45,46]; 3. Symbiotic bacteria. Symbiotic nitrogen-fixing bacteria form specialized associations with host plants. They establish mutualistic relationships with legume or nonlegume plant species, forming structures known as root nodules. Inside these nodules, bacteria such as *Rhizobium* (legume plants) or *Frankia* (nonlegume plants) convert nitrogen gas into ammonia, which is then used by the plant as a nitrogen source. This symbiotic association benefits both the bacteria and the host plant, with the plant providing carbohydrates to the bacteria in return [47,48]. Understanding biological nitrogen fixation is crucial for sustainable agriculture and ecosystem functioning. It reduces reliance on synthetic nitrogen fertilizers and helps maintain soil fertility. Additionally, it is a vital process for the nitrogen requirement of many plants and contributes to the overall availability of nitrogen in terrestrial ecosystems.

### *Rhizobium–Legume Symbiotic Relationship and Environmental Stress*

The *Rhizobium*–legume symbiotic relationship is a crucial association between leguminous plants and nitrogen-fixing bacteria called rhizobia. Rhizobia play a vital role in nitrogen fixation and sustainable agriculture [49]. They colonize legume roots, forming nodules, where they convert atmospheric nitrogen into a plant-usable form through nitrogen fixation [50]. In return, legumes provide energy to the rhizobia through photosynthesis. Nitrogen fixation is significant for sustainable agriculture as it enhances soil fertility by converting inaccessible atmospheric nitrogen into a usable form. This reduces reliance on synthetic fertilizers, which have adverse environmental impacts [51]. The symbiosis between rhizobia and legumes also promotes leguminous crop growth and development, leading to improved crop yield. Moreover, this relationship contributes to sustainable agriculture by reducing the need for chemical fertilizers, mitigating soil degradation, and enhancing overall soil health. Legume plants can serve as valuable cover crops or be integrated into crop rotations, thus enhancing the sustainability and productivity of agricultural systems [52–54]. Several environmental conditions can negatively affect symbiotic nitrogen fixation in legumes, such as nitrogen availability, soil acidity, salinity, and low soil temperature [55–57]. These factors can impact various aspects, including rhizobial survival in the soil, the infection process, nodule development, nodule function, and indirect effects on host plant growth [58–60]. Nitrogen availability plays a crucial role in legume–rhizobia symbiosis, as higher doses of nitrogen fertilizer can hinder successful symbiotic establishment [60]. When the soil has a high nitrogen content, especially during the period between seed inoculation and germination, it presents challenges for establishing functional symbiosis [61,62]. Excessive nitrogen in the soil can reduce the reliance of plants on nitrogen fixation and limit root nodule development. Soil acidity and salinity can also impede symbiotic nitrogen fixation by creating unfavorable conditions for rhizobial survival and root infection. This hampers nodule development and results in fewer functional nodules [63]. Additionally, acidic or saline conditions negatively affect host plant growth, further affecting the nitrogen-fixing performance. Low soil temperature is another environmental condition that hinders symbiotic nitrogen fixation, particularly in legumes, including tropical species [64–66]. Cold temperatures inhibit rhizobial metabolic activity, impairing their nitrogen-fixing capacity and symbiotic establishment with plants. It is important to note that these environmental factors not only directly affect legume–rhizobia symbiosis but also indirectly affect nitrogen fixation by influencing host plant growth [67,68]. Unfavorable conditions limit overall plant growth, decrease photosynthesis, reduce carbon allocation to the roots, and ultimately compromise the energy and resources available for nitrogen fixation. Understanding and managing these environmental conditions are critical to facilitate successful nitrogen fixation and ensure optimal plant growth and agricultural productivity in legumes.

### **3. Effects of N Fertilizer on *Rhizobium*–Legume Molecular Signaling**

In the symbiotic relationship between legumes and rhizobial bacteria, several molecular signals are involved in the recognition and initiation processes. These signals facilitate the establishment of a beneficial relationship between legume plants and rhizobial bacteria by guiding their interactions and ensuring successful symbiosis (Figure 3). The key players in this process are as follows:



**Figure 3.** Reciprocal molecular communication between *Rhizobium* and legume in the presence of low nitrogen in the soil.

### 3.1. Isoflavonoids

(Iso)flavonoids are compounds that are released by legume roots and serve as chemoattractants for rhizobia (Figure 3). These specific flavonoids are primarily found in legumes [69,70]. They are synthesized in response to various biotic and abiotic stimuli, including rhizobia. Once synthesized, these compounds are released from the roots into the rhizosphere, which is the region surrounding the roots [71]. One of the main functions of (iso)flavonoids is to modulate the expression of genes involved in the nodulation process, thereby initiating *Rhizobium*–legume symbiosis [72]. Flavonoids released by legume plants into the soil attract rhizobia, initiating a complex molecular dialogue between them [73]. This molecular dialogue not only ensures symbiotic compatibility between rhizobia and their respective host plants but also dictates the type and structure of the nodules formed [74]. Furthermore, (iso)flavonoids have been shown to enhance the competitiveness of rhizobia, enabling them to outcompete other soil microorganisms [75–79]. Several studies have investigated the influence of nitrogen fertilizers on (iso)flavonoid secretion in legumes. Some studies have shown that the application of nitrogen fertilizers leads to a reduction in the production and secretion of (iso)flavonoids [80]. High nitrogen availability appears to suppress the genes responsible for (iso)flavonoid biosynthesis [81,82]. Reduced production of (iso)flavonoids can have detrimental effects on legume–*Rhizobium* symbiosis. A decrease in (iso)flavonoid secretion may hinder proper rhizobial colonization and, subsequently, nitrogen fixation. This, in turn, may compromise the ability of the plant to meet its nitrogen requirements, leading to a greater reliance on synthetic fertilizers. The direct supply

of nitrogen fertilizer to the root system decreased daidzein and genistein levels in soybean roots [83,84]. Furthermore, (iso)flavonoids have been shown to possess antimicrobial properties that protect legume plants from various pathogens. The reduction in (iso)flavonoid secretion due to nitrogen fertilizers may render legumes more vulnerable to diseases and infections, necessitating the use of additional pesticides or fungicides [85]. Although nitrogen fertilizers undoubtedly enhance plant growth and productivity, it is crucial to consider their impact on intricate legume–*Rhizobium* symbiosis. Lyu et al. [86] found that nitrogen fertilizer hampers nitrogen fixation in soybean nodules at an early stage. This inhibition also affects isoflavone levels in the roots and root exudates of soybean plants. The researchers observed that changes in nodule nitrogen fixation, caused by unilateral nitrogen supply or isoflavones, correlate with fluctuations in isoflavone concentrations in the roots and root exudates.

The reduction in (iso)flavonoid secretion induced by nitrogen fertilizers can disrupt the delicate balance required for successful symbiosis (Figure 3). This could lead to greater reliance on synthetic fertilizers and agrochemicals, reinforcing a cycle of dependence that hampers sustainable agricultural practices. Future research should focus on developing innovative strategies that maximize nitrogen utilization while preserving the integrity of legume–*Rhizobium* symbiosis and the production of beneficial (iso)flavonoids [87].

### 3.2. Nod Factors

Nod factors are lipochitooligosaccharides produced by rhizobia, bacteria that respond to (iso)flavonoids by secreting nod factors as signaling molecules. Nod factors induce various responses in legume plants, including root hair deformation, curling, and the initiation of nodule formation, as shown in Figure 3 [88–90]. (Iso)flavonoids act as chemoattractants, guiding rhizobia towards the roots [91]. Bacterial chemoreceptors perceive (iso)flavonoids, initiating chemotaxis towards the chemoattractant source [92]. The specificity of the symbiosis lies in the ability of (iso)flavonoids to activate specific nod factor receptors on root hairs. The binding of nod factors to these receptors triggers a signal transduction pathway. The interaction between root hairs and rhizobia leads to the formation of infection threads, specialized structures that allow bacteria to penetrate root tissues [93,94]. Infection threads provide a protected pathway for rhizobia to move towards the inner regions of the root [95,96]. Within the root, rhizobia colonize nodule primordia and induce their differentiation into mature nodules. Infection threads guide rhizobia towards the nodule primordia, where they undergo morphological changes [97]. The nodule meristem, formed through host plant cell division, provides a continuous source of new cells for nodule formation and growth. Rhizobia within nodules differentiate into bacteroids, highly specialized forms enclosed within symbiosomes derived from host plant cells [98]. Symbiosomes facilitate nutrient exchange. Nitrogen fixation occurs within nodules, converting atmospheric nitrogen into a usable form. The plant supplies carbon sources and essential nutrients to rhizobia. High levels of nitrogen fertilization can negatively impact nod factor production, leading to decreased nodule formation and effectiveness [99]. Excessive nitrogen availability suppresses nod factor synthesis genes [100]. This reduction in nod factor production affects symbiosis and overall nitrogen fixation [101]. The impact of nitrogen fertilizers varies based on factors such as fertilizer type, concentration, soil conditions, rhizobial strain, and the leguminous plants involved. Timing of fertilizer application is crucial, with excessive nitrogen during early stages having more detrimental effects [102]. To ensure sustainable agriculture and efficient nitrogen fixation, proper nitrogen fertilizer management is essential. By optimizing nod factor production, nodulation, and nitrogen fixation while minimizing negative effects, a balanced and beneficial relationship can be maintained [103].

### 3.3. Nodulation Receptor Kinases (NORKs)

Nodulation Receptor Kinases (NORKs) are a group of receptor proteins found in the root hairs of leguminous plants [104]. They play a crucial role in the symbiotic relationship between legumes and nitrogen-fixing bacteria, known as rhizobia [105]. NORKs are

responsible for recognizing and binding to specific signaling molecules called nod factors, which are produced by rhizobia. This recognition and binding event initiate a series of downstream signaling events that trigger physiological and developmental responses in both legume plants and rhizobia [106]. The signaling cascade initiated by NORKs leads to nodule formation on the roots of leguminous plants [107]. NORKs belong to a larger family of receptor proteins called Receptor-Like Kinases (RLKs). RLKs are transmembrane proteins with a receptor domain on the extracellular side of the cell membrane and a kinase domain on the intracellular side. This dual nature allows RLKs to perceive external signals, such as nod factors, and transmit them to the cell by phosphorylating downstream proteins [108]. The precise mechanisms by which NORKs transmit these signals are still being investigated, but they are believed to interact with other proteins and enzymes to relay nod factor signals into the cell [109]. Research has shown that genetic mutations that disrupt the function of NORKs result in impaired nodulation in legumes. In contrast, the overexpression of NORKs has been shown to promote increased nodulation and nitrogen fixation efficiency [110,111]. These findings highlight the critical role of NORKs in the establishment of a successful symbiotic relationship between legumes and rhizobia [112,113]. When excess nitrogen fertilizer is applied, it can inhibit the activity of NORKs and interfere with the nodulation process [114]. The exact mechanism underlying this inhibition is not fully understood, but studies have suggested that high levels of nitrogen can disrupt the delicate balance of phytohormones involved in nodule development [115]. Additionally, excessive nitrogen can lead to an overabundance of nitrate in plants, which has been linked to the repression of NORK genes. The inhibition of NORKs by nitrogen fertilizer can have significant implications for leguminous crops. Nodules play a vital role in enhancing nitrogen availability and reducing the need for synthetic fertilizers. Without proper nodulation, plants may struggle to meet their nitrogen requirements, leading to reduced growth, lower yields, and increased dependence on external nitrogen inputs. Understanding the molecular mechanisms of NORKs and their role in nodulation is crucial for enhancing nitrogen fixation in agriculture and reducing reliance on synthetic fertilizers.

### *3.4. Calcium Spikes Play a Crucial Role in Symbiotic Signaling*

Calcium spikes play a crucial role in symbiotic signaling and the modulation of gene expression in interactions between legumes and rhizobia [116]. These spikes are triggered by the recognition of nod factors by nodulation receptor kinases (NORKs) in the root hairs, leading to oscillations in calcium levels [117]. The frequency and pattern of these spikes are vital for accurate signaling and changes in gene expression during symbiosis [117]. Calcium spikes act as second messengers in the signaling pathway, transmitting the perception of nod factors from the membrane to the nucleus [118]. They function as a “calcium clock”, regulating the timing and duration of subsequent events [119]. The precise mechanism behind calcium spiking is currently under investigation, but it is known that NORKs play a crucial role in initiating the oscillations [120]. When nod factors bind to NORKs, specific channels or pumps in the plasma membrane are activated, resulting in the influx or release of calcium ions into the cytosol. This leads to temporary increases in calcium levels, known as calcium spikes. These spikes are associated with the activation of symbiotic genes, promoting the expression of nodulation-related proteins and transcription factors [121]. Calcium spikes also regulate other aspects of nodulation, such as the modulation of calcium-dependent protein kinases (CDPKs) and calcium-dependent protein phosphatases (PP2Cs). These calcium-dependent proteins further regulate signaling pathways and coordinate molecular events during nodulation [122]. Additionally, calcium spikes play a critical role in legume–rhizobia interactions by triggering rapid calcium oscillations in root hairs upon the recognition of nod factors [123]. They act as second messengers in the signaling cascade. The patterns of calcium spiking during symbiotic signaling can be influenced by nitrate [124,125]. The impact of nitrate on calcium spiking varies depending on the plant species and specific circumstances. In some legume–rhizobia symbioses, nitrate inhibits calcium spiking, preventing nodulation and nitrogen fixation [126]. Alternatively,



in certain mycorrhizal symbioses, nitrate enhances calcium spiking, which is crucial for the establishment and functioning of the symbiosis [127–129]. Overall, understanding the mechanisms of calcium spiking and its impact on gene regulation is essential for comprehending the complex molecular processes involved in symbiotic nitrogen fixation. Further research is needed to fully unravel the molecular mechanisms and complexities of nitrate's role in symbiotic signaling pathways.

### 3.5. Cytokinins and Auxins

Cytokinins and auxins are vital plant hormones involved in the nodulation process [130–134]. Cytokinins stimulate cell division and growth, while auxins drive swelling and deformation, leading to the formation of nodules [135]. During nodulation, leguminous plants recognize nod factors, which trigger the production of cytokinin in the root hairs and cortical cells. Increased cytokinin levels promote cell division in the root cortex, forming a nodule primordium. Cytokinins also assist in the swelling and deformation of root hairs, facilitating rhizobial infection [134,136]. On the other hand, auxins play a crucial role in nodulation by promoting cell elongation and differentiation [131]. Nod factor signaling triggers the synthesis and redistribution of auxins in the root hairs and cortical cells [131]. Auxins contribute to the deformation of root hairs, aiding rhizobial infection [137]. They also promote the growth of nodule primordia by stimulating cell elongation and division in the proliferating zone [130]. The interaction between cytokinins and auxins is vital for nodule formation, as they work together to regulate cell division, elongation, and differentiation, ensuring proper nodule growth [138]. Furthermore, cytokinins and auxins influence the expression of genes related to nodulation, including those involved in nitrogen fixation and nutrient transporters [139]. Precise regulation of hormone levels is necessary for optimal nodulation and nitrogen fixation [140]. It is important to consider that manipulating cytokinins and auxins to improve legume crop productivity requires caution [141]. Excessive nitrogen fertilizer can disrupt hormone production and inhibit nodulation [142,143]. High nitrate levels negatively impact nod factor signaling and cytokinin production, thereby reducing nitrogen fixation [144]. Excessive auxin can also hinder nodule development [145]. Understanding the roles and regulation of cytokinins and auxins in nodulation provides valuable insights into symbiotic nitrogen fixation and agricultural practices. By manipulating these hormones, researchers have the potential to enhance nitrogen fixation, increase crop yield, and reduce reliance on nitrogen fertilizers [146].

### 3.6. Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) play a vital role in the symbiotic relationship between legumes and rhizobia [147–150]. ROS are produced when legumes detect rhizobia through nod factors, initiating a signaling cascade [151]. These ROS molecules act as signaling molecules themselves, coordinating a complex dialogue that promotes the symbiotic relationship [152]. Receptor proteins on legume root cells recognize nod factors and activate enzymes involved in ROS production [153]. ROS, including hydrogen peroxide ( $H_2O_2$ ), superoxide ( $O_2^-$ ), and hydroxyl radicals ( $OH^\bullet$ ), have both positive and negative effects within cells. In the legume–rhizobia symbiosis, ROS production is crucial for recognition, cell wall modifications, and infection thread formation [154–156]. ROS also regulate gene expression, defense responses, and root nodule formation [157–159]. While ROS are important, excessive production can cause oxidative damage to both the legume host and rhizobia. Therefore, the ROS signaling pathway is tightly regulated [160]. Understanding the role of ROS in legume–rhizobia symbiosis provides insights into the molecular mechanisms and potential for enhancing crop productivity through improved nitrogen fixation. Nitrogen fertilizer can influence ROS levels in plants. Excessive nitrogen fertilization disrupts nutrient balance and metabolic activity, leading to elevated ROS levels and oxidative stress [161,162]. Imbalanced nitrogen fertilization can impair nodule formation, nitrogen fixation efficiency, and the symbiotic relationship. Optimal nitrogen management is crucial

for maintaining balanced ROS homeostasis and a healthy symbiosis [162]. Further research is needed to assess the impact of nitrogen fertilizers on antioxidant enzyme activity to mitigate the detrimental effects of ROS on symbiosis.

#### 4. Effects of N Fertilizer on Rhizobial Motility

Motility in rhizobia refers to their ability to actively move in response to external stimuli, usually by using flagella. Rhizobia are bacteria that form mutually beneficial relationships with legume plants, helping with nitrogen fixation [163–165]. However, excessive nitrogen fertilization can have negative effects on rhizobial populations, motility, and their symbiotic relationship with legume plants. Studies have shown that high nitrogen levels can reduce the density, motility, and diversity of free-living rhizobia, which can harm soil health and plant productivity [166]. The effects of excessive nitrogen fertilization include population dynamics, reduced motility, inhibition of nodulation, altered mutualistic relationships, and environmental concerns [163]. Excess nitrogen can change soil pH, inhibiting rhizobial growth [167,168]. Additionally, high nitrate levels can provide an alternative nitrogen source for plants, reducing their reliance on rhizobial symbiosis and further impacting *Rhizobium* populations [169,170]. High ammonium concentrations from excessive nitrogen fertilization can decrease rhizobial motility, impairing their ability to effectively colonize plant roots [171]. Managing nitrogen fertilizer application is crucial to ensure optimal nodulation and symbiotic nitrogen fixation in legume crops while minimizing negative effects on rhizobia and the environment [172,173]. The contribution of motility to symbiotic recognition is essential in establishing and maintaining beneficial relationships between organisms [174–176]. By manipulating flagellar biosynthesis and chemotaxis-related genes, scientists aim to enhance *Rhizobium* motility, resulting in better root colonization, improved nitrogen fixation, and increased crop productivity. Exploiting *Rhizobium* motility for improved symbiotic association holds great potential for sustainable agriculture and a greener future.

#### 5. Effect of N Fertilizer on Root-Hair Curling, Infection Thread Formation and Nodulation

Extensive research has been conducted on the impact of nitrogen fertilizer on various aspects of legume growth, including root-hair curling, infection thread formation, and nodulation. Leguminous plants rely on symbiotic nitrogen fixation with rhizobia to meet their nitrogen requirements. However, excessive use of nitrogen fertilizer can disrupt this symbiotic association [59,177–180]. The effect of nitrogen fertilizer on root-hair curling and rhizobial infection varies depending on factors such as plant species, soil conditions, and the timing, form, and amount of nitrogen application [59,181]. Abdel Wahab et al. [59] reported that nitrogen fertilizers can hinder multiple stages of legume nodulation, including root-hair infection. Root hairs play a crucial role in the interaction between legume roots and rhizobia [182]. High concentrations of nitrogen fertilizers, particularly nitrate, can significantly reduce root elongation and curling, negatively impacting the ability of rhizobia to colonize and infect the roots [183,184]. The addition of nitrate or urea has been found to have significant effects on root-hair curling, infection thread formation, and nodulation in various plant species. For example, it reduces root-hair curling in *Medicago sativa* and inhibits both curling and infection thread formation in soybean [185]. Nitrogen fertilizers can directly impact nodule development, leading to the abortion of infection. Additionally, their presence can reduce the proliferation and multiplication of free-living rhizobia in the soil, delaying or inhibiting nodule formation [186–189]. Nitrogen fertilizer application also leads to premature nodule senescence and feedback inhibition of nitrogenase activity [190–196]. The inhibitory effects of nitrogen fertilizers on nodulation are likely plant-mediated, and different strains of rhizobia exhibit varying degrees of tolerance to these effects [197,198]. Furthermore, the form of nitrogen also plays a role in its suppressive effects on nodulation, with nitrate being more inhibitory compared to ammonia or urea [195]. Early application of nitrate during the growth season has been found to

inhibit nodulation in soybean. Changes in rhizosphere conditions caused by nitrate can affect the composition of the root cell wall, inhibiting bacterial attachment and invasion of root hairs and ultimately reducing nodulation [199,200]. The concentration of nitrate in the rhizosphere is a key factor in inhibiting nodule initiation, while absorbed nitrate has a greater impact on nodule development [201]. Nitrogen levels exceeding certain thresholds have been found to negatively affect nodule development in *Phaseolus vulgaris*. Additionally, the addition of nitrate suppresses further nodule development in plants that have already formed nodules [202]. The timing of nitrogen application also influences nodulation patterns. Applying nitrogen during rhizobial infection phases limits nodule formation while applying it after nodule formation inhibits nodule development [59]. Nitrate inhibits nodule development and impairs established nodule activity by depriving photosynthates and utilizing saccharides in nitrate assimilation [203]. The sensitivity of nodule development to nitrogen fertilizers is observed in various plant species, including *Lens esculenta*, *Vigna unguiculata*, and soybean [185,204,205]. Nitrogen fertilizer application restricts primary root nodulation, resulting in decreased total nodule mass. However, foliar application of nitrogen fertilizers has been found to have less suppressive effects on nodulation and nitrogenase activity compared to soil treatments [206]. Reports suggest that the detrimental effects of nitrogen fertilizer on nodulation in secondary roots of common bean and soybean can be mitigated by foliar application [207,208]. The application of nitrogen fertilizers, especially in high concentrations or in the form of nitrate, negatively affects the symbiotic association between rhizobia and leguminous plants. The specific effects on root-hair curling, rhizobial infection, and subsequent nodulation play a significant role in inhibiting nodulation and nitrogen fixation in leguminous plants. It is evident that the use of nitrogen fertilizers on legumes can hinder nodulation by rhizobia rather than improve soil fertility. The response of the symbiotic relationship to the addition of nitrogen depends on various factors, such as the timing, amount, and form of nitrogen, as well as the specific legume species being studied.

## 6. Effect of Nitrogen Fertilizers on Nodule Physiology

### 6.1. Nodule Nitrate Reductase

Early studies revealed that the addition of nitrate or ammonium to soybean plants resulted in a decrease in nitrate reductase activity in nodules [209]. Further research investigated the effects of nitrate on symbiotic properties using nitrate-reductase-deficient mutants of cowpea rhizobia and *Rhizobium trifolii* [210]. The study found that nitrate inhibited initial nodulation. It has been found that nitrate inhibits nitrogen fixation in cowpea and lupine nodules, irrespective of the presence or absence of nitrate reductase activity [211]. A significant reduction in nodule weight was observed in soybean plants exposed to high concentrations of nitrate, as reported by Streeter [212]. It is worth noting that the inclusion of sucrose has been found to enhance nitrogenase activity and decrease nitrite accumulation [213]. The study conducted by Sekhon et al. [214] investigated the in vivo nitrate reductase activity of summer moong. It was found that a nitrogen concentration of  $6 \text{ mg kg}^{-1}$  during the preflowering stage resulted in the highest nitrate reductase activity. The Minchin group discovered that a short exposure to nitrate significantly improved the nodules' ability to resist oxygen diffusion [215]. Becana et al. [216] indicated that short-term exposure to 10 mM nitrate did not lead to significant accumulation of nitrite in legume nodules. The decrease in nitrogenase activity was not due to toxic levels of nitrite. The buildup of harmful levels of nitrite only occurred with prolonged exposure to nitrate, which was counteracted by bacteroid and cytosol nitrate reductase activity. Many studies demonstrated that the periplasmic nitrate reductase of bacteroid plays a crucial role in the formation of nitrosylhaemoglobin in soybean nodules, leading to the inhibition of nitrogen fixation [217–221]. It has been shown that nitrate reductases in legumes and bacteroids contribute to the production of nitric oxide in nodules during nitrogen fixation [222]. Additionally, research has shown that bacteria play a crucial role in the detoxification of nitric oxide, which helps prevent premature nodule senescence and

promotes efficient symbiosis [223]. The significance of nitrate reductases and hemoglobins in the regulation of nitric oxide accumulation and the control of nitrogen-fixing symbiosis has been emphasized [224]. They also highlighted the need for further investigation into the coordination of regulatory systems between plants and bacteria at various stages of the symbiotic interaction [225].

## 6.2. Leghemoglobin

The first plant hemoglobin was discovered in soybean nodules [226]. It has been demonstrated that this hemoglobin forms a reversible compound with molecular oxygen [227]. Hemoglobin in *Pisum sativum* nodules was also discovered, and its content was found to be related to nitrogen fixation. This hemoglobin was named “leghemoglobin” for legume nodules by Virtanen and Laine [228]. However, the role of this protein in facilitating oxygen diffusion to the bacteroids has been firmly established [229,230]. Hemoglobin was also discovered in the nodules of *Parasponia andersonii*, a nonlegume plant belonging to the Cannabaceae family [231]. These nodules form a symbiotic relationship with bradyrhizobia. Hemoglobin was also found in the nodules of actinorhizal plants such as *Casuarina glauca*, *Myrica gale*, and *Alnus glutinosa* [232]. The researchers have indicated that these plants establish a symbiotic connection with the actinobacterium *Frankia*. These discoveries suggest that there are hemoglobins present in these plants, apart from the commonly known leghemoglobin, which play a role in symbiotic relationships.

The presence and function of multiple legume hemoglobins in nodules are still not fully understood [233–235]. In soybean nodules, there are four major leghemoglobins (a, c1, c2, and c3) and four minor leghemoglobins (b, d1, d2, and d3). As the nodule matures, the ratio of lba/lbc3 increases, indicating the role of lba in regulating oxygen as the nodule structure becomes more complex [236]. Pea nodules have two major leghemoglobins (lbi and lbiv) and three minor leghemoglobins. The lbi/lbiv ratio decreases with nodule age [237]. The oxygen-binding affinities differ between lb types, suggesting that changes in lb proportions during nodule development optimize nitrogen fixation efficiency [230,237]. Research on pea leghemoglobin indicates spatial and temporal regulation of leghemoglobin expression, but their specific roles remain unknown [238]. Limited studies have investigated factors other than aging that influence leghemoglobin gene expression, such as the impact of nitrate supply on leghemoglobin abundance in mung bean plants [233]. These variations observed in soybean, pea, and mung bean nodules may be attributed to different rates of leghemoglobin biosynthesis.

The excessive use of nitrogen fertilizer in agriculture has been found to disrupt the nodulation and nitrogen fixation processes, leading to the deterioration of nodules and the production of green leghemoglobins [239]. These green leghemoglobins are associated with the breakdown of leghemoglobin complexes and their interaction with reactive nitrogen species [240,241]. It has been shown that the periplasmic nitrate reductase of bacteroids plays a vital role in inhibiting nitrogen fixation, resulting in the production of nitrosyl-leghaemoglobin [217]. However, the specific role of green leghemoglobins in nodule aging remains unclear. Therefore, further investigation into the content and synthesis of leghemoglobin is necessary to gain a better understanding of the effects of high nitrogen supply on biological nitrogen fixation. This can be achieved by identifying the gene and protein sequences of different legume species. A recent study has revealed that efficient biological nitrogen fixation depends on leghemoglobin to regulate oxygen pressure in nodules. However, this crucial process is impeded by an excessive supply of nitrogen. Surprisingly, a comprehensive investigation into the genes responsible for encoding leghemoglobin in legumes has not been documented yet. Further research in this field could provide valuable insights for nutrient management and enhancing legume yield under varying nitrogen availability.

### 6.3. Nitrogenase Activity

Nitrogenase is an important enzyme in the reduction of atmospheric nitrogen to ammonia. It consists of two components: metal protein FeP and molybdenum-metal protein MoFeP. Nitrogenase requires ATP hydrolysis and electron and proton transfer for substrate reduction. The FeP cycle involves ATP hydrolysis for electron transfer, while the MoFeP cycle transfers electrons to the iron–molybdenum cofactor (FeMoco) for nitrogen binding and reduction. Interactions between FeP and MoFeP are important for electron transfer and conformational changes, but their exact nature is unknown [242].

Studies have been conducted over several decades to understand the inhibition of nitrogen fixation by mineral nitrogen. This is of increasing importance as environmentalists and agricultural scientists seek ways to reduce fertilizer use in field crops. If we can manipulate symbiosis to overcome this inhibition, legumes could increase the amount of nitrogen derived from nitrogen fixation, which would have a greater impact on soil nitrogen levels. Several hypotheses have been proposed to explain the mechanism of nitrate inhibition of nitrogenase activity in legumes. These include changes in plant carbohydrate distribution, resulting in energy and carbon deficiencies in nodules [203], inhibition of nitrogenase or leghemoglobin synthesis [243,244], inhibition of nitrogenase or leghemoglobin activity by nitrite [245,246], and inhibition of nitrogenase activity by the products of nitrogen fixation [247]. These factors include exposure of nodulated roots to nitrate [248–250]. Understanding these mechanisms is important for reducing crop fertilization and increasing the amount of plant nitrogen from nitrogen fixation. Inhibition of leghemoglobin synthesis and ammonia assimilating enzymes in nodules has been found to contribute to this process [215]. It has been determined that the decrease in nitrogenase activity in peas exposed to ammonium nitrate is attributed to a decline in leghemoglobin synthesis [244]. However, another study suggests that the inhibition of nitrogenase activity by nitrate is primarily caused by insufficient carbohydrates and the toxicity of nitrite [251]. The availability of oxygen to bacteria in the nodule cortex has also been emphasized as an important factor in this inhibition [251,252]. Further studies have explored the effect of nitrate restriction on nitrogenase activity in soybean, suggesting that asparagine and its metabolites may be involved in regulating this process [253]. Changes in asparagine levels and metabolism in shoots and roots may influence this mechanism.

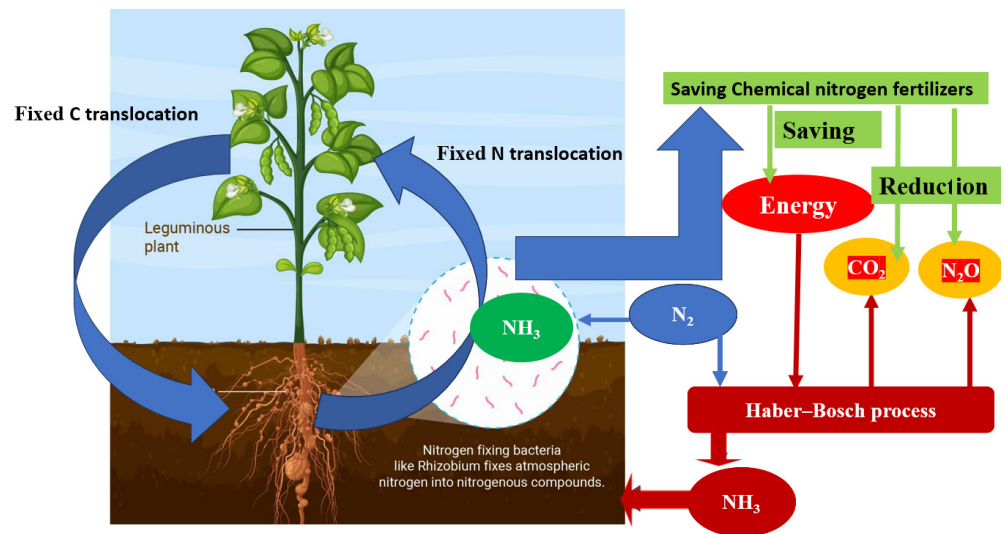
Several studies have highlighted the toxic or inhibitory effect of nitric oxide (NO) during the symbiosis process [254]. NO has been found to strongly inhibit the activity of nitrogenase in vitro [246]), and this has been confirmed by in vivo studies [255,256]. Furthermore, NO has been shown to inhibit both the activity and expression of bacterial nitrogenase in soybeans [218]. Given these findings, it is conceivable that gaining a deeper understanding of the signaling, transport, and interactions of soil nitrogen during nodule development will enable us to enhance the efficiency of symbiotic nitrogen fixation in legume crops, thereby contributing to sustainable agriculture.

## 7. Mitigation Strategies

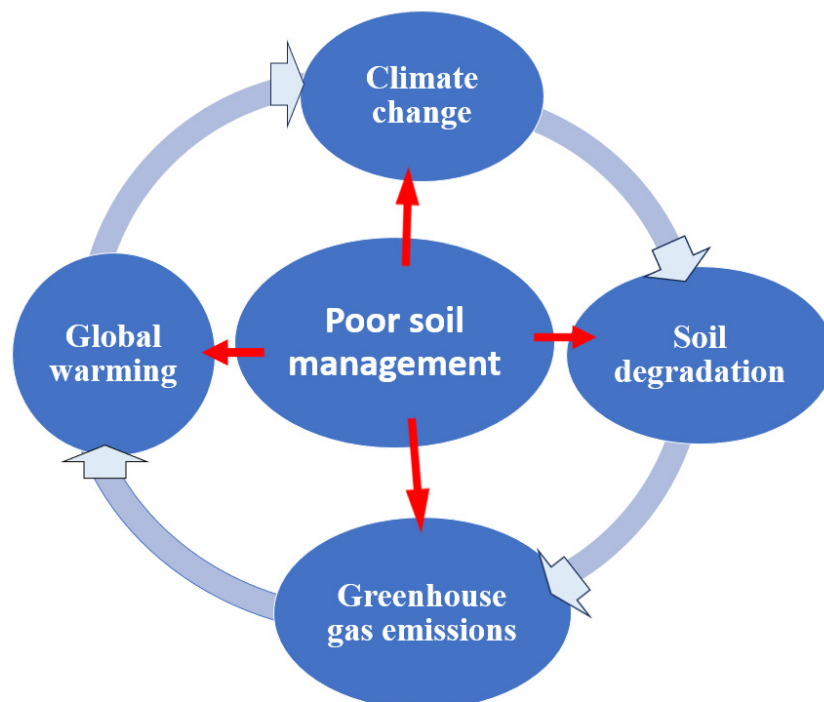
To mitigate climate change, it is crucial to find sustainable solutions for reducing nitrogen fertilizer use. One promising approach is through the utilization of *Rhizobium*–legume symbiosis. By harnessing this natural symbiosis, farmers can reduce their reliance on synthetic nitrogen fertilizers. This is significant because the production and application of nitrogen fertilizers contribute to greenhouse gas emissions, particularly nitrous oxide, which is a potent greenhouse gas (Figure 4). To encourage the adoption of this approach, education and outreach programs should be implemented to raise awareness among farmers about the benefits of *Rhizobium*–legume symbiosis. Additionally, research and development efforts should focus on optimizing the efficiency of nitrogen fixation in legume crops and identifying suitable *Rhizobium* strains for different soil conditions. Reducing nitrogen fertilizer use through *Rhizobium*–legume symbiosis is a promising strategy for climate change mitigation [257,258]. To enhance the symbiotic relationship between *Rhizobium* and legumes and reduce reliance on nitrogen fertilizers for climate change mitigation, several

strategies can be implemented. Selecting effective rhizobial strains: To enhance nodulation and nitrogen fixation efficiency, it is crucial to carefully select specific rhizobial strains. This can be accomplished by screening and choosing strains that have a higher compatibility with legume plants. This approach brings numerous advantages in addressing climate change, such as decreased fertilizer usage, improved soil health and carbon sequestration, enhanced plant resilience, and economic benefits for farmers [123,259]. Crop rotation and intercropping: Rotating legume crops with nonleguminous crops to break pest and disease cycles and promote nitrogen cycling in the soil. Intercropping legumes with other crops can enhance nutrient cycling and reduce the need for nitrogen fertilizers. Using cover crops: Planting cover crops, especially leguminous plants, during fallow periods can enhance soil health and increase the availability of nitrogen [260]. These cover crops can fix atmospheric nitrogen, making it accessible to subsequent crops [261]. Improving soil fertility: Employing practices such as organic matter addition, composting, and proper nutrient management to enhance soil fertility [262]. Organic fertilizers, crop residue incorporation, and balanced nutrient application can reduce the need for nitrogen fertilization. Soil fertility is crucial for maximizing carbon sequestration, promoting nutrient cycling, improving water retention, supporting biodiversity, and reducing erosion [263,264]. Fertile soils contribute to resilient and sustainable agricultural systems that help mitigate the negative effects of climate change [265]. Proper soil management: Practice good soil management techniques to create a favorable environment for *Rhizobium*–legume symbiosis and microbial communities [266]. Maintain optimal soil pH and proper drainage, and avoid excessive use of nitrogen fertilizers, which can hinder nodulation and nitrogen fixation. Healthy soils are crucial for storing carbon and mitigating climate change. When managed sustainably, soils can sequester carbon and reduce greenhouse gas emissions. However, poor soil management and unsustainable agricultural practices can release carbon into the atmosphere, contributing to climate change (Figure 5). The conversion of grassland and forestland to cropland and grazing lands has led to significant soil carbon losses worldwide. By restoring degraded soils and adopting soil conservation practices, we can decrease greenhouse gas emissions, increase carbon sequestration, and build resilience to climate change [267]. Land use, land-use change, vegetation cover, and soil management strongly influence the processes and emissions of greenhouse gases in the soil. Managing soil organic carbon stocks in the upper soil layers can help reduce greenhouse gas concentrations in the atmosphere [268]. Optimizing inoculation techniques: Ensuring the proper inoculation of legume seeds with efficient rhizobial strains. This involves using appropriate inoculant formulations and ensuring proper seed coating or inoculant application to improve symbiosis establishment. Factors such as bacterial strains, inoculant formulation, application methods, and environmental conditions all play a role in successful inoculation. By improving our understanding and application of these techniques, we can maximize the benefits of symbiotic nitrogen fixation and contribute to climate change mitigation efforts [269]. Improving farming practices: Adopting conservation agriculture practices, including reduced tillage, mulching, and water management techniques. These practices enhance soil structure, increase soil organic matter, and reduce nitrogen losses, maximizing the benefits of *Rhizobium*–legume symbiosis [270]. By adopting and implementing these improved farming practices, agricultural systems can become more resilient, efficient, and sustainable. This, in turn, helps to reduce the negative impacts of climate change on food production, ecosystems, and the environment. It is crucial to adopt these approaches to preserve nature and its benefits to humanity while also ensuring the availability of good food at reasonable prices and protecting the environment for our survival [271]. Integrated nutrient management: Combining *Rhizobium* inoculation with judicious application of mineral fertilizers to optimize nitrogen availability. This integrated approach ensures adequate nutrient supply while minimizing the use of synthetic fertilizers [272–274]. Rhizobial inoculant quality control: Verify the effectiveness of commercially available rhizobial inoculants through field trials or consultation with experts [275–277]. Use commercially available inoculants or isolate local strains proven to be effective in your specific region [278]. Research findings indicate

that native rhizobia have a greater positive impact on plant growth, nitrogen fixation, and reduction in greenhouse emissions in sun hemp crops within Florida citrus orchards, compared to commercial rhizobia inoculants [279]. Maintain proper storage conditions, follow application instructions, and use fresh inoculants to maximize efficacy. Genetic improvement: Employ breeding programs to develop legume cultivars with improved compatibility with specific *Rhizobium* strains. This can be achieved through marker-assisted selection or genetic engineering, enhancing the signaling and recognition process between legumes and rhizobia [258,280]. It is important to note that the effectiveness of these strategies can vary depending on factors such as soil type, climate, legume species, and management practices. Therefore, it is advisable to adapt these strategies based on local conditions and consult with agricultural experts for specific recommendations.



**Figure 4.** Benefits of *Rhizobium*–legume symbiosis include energy savings and reduced greenhouse gas emissions resulting from decreased use of chemical nitrogen fertilizers.



**Figure 5.** Poor soil management practices and greenhouse gas emissions.

## 8. Future Prospects

Our understanding of the *Rhizobium*–legume symbiosis has improved, but much remains unknown. The impact of nitrogen fertilizers on this symbiosis has significant implications for the environment. Further research is needed to uncover the mechanisms of nitrogen fertilizer effects, study rhizobial strain dynamics, and evaluate carbon resource allocation in relation to nitrogen fixation. Exploring the role of (iso)flavonoids in nodulation and nitrogen fixation by nitrogen fertilizers is crucial. Utilizing *Rhizobium* motility for an improved symbiotic association has the potential for sustainable agriculture. Enhancing the efficiency of symbiotic nitrogen fixation in legume crops could be achieved by gaining a deeper understanding of the signaling, translocation, and interactions of soil nitrogen during nodule development. Addressing these research gaps will contribute to sustainable agricultural practices. Technological breakthroughs in microbial communication networks are expected. Optimizing nitrogen fixation management is crucial for global protein shortages. By embracing sustainable alternatives and carefully managing nitrogen fertilizer application, we can promote sustainable agriculture while minimizing detrimental impacts on the environment and productivity.

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