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Posted Date: 23 August 2023

doi: 10.20944/preprints202308.1575.v1

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Review

Nitrogen Journey in Plants: from Uptake to Metabolism to Stress response and Microbe Interaction

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Abstract: Plant uptake and assimilation of essential nitrogen compounds from the soil are mainly in the form of nitrate and ammonium ions. Plant nitrate transporters (NRTs) play a crucial role in nitrate uptake from the soil into the roots. NRT structural analysis reveals the mechanisms by which these transporters function. Similarly, plant ammonium transporters (AMTs) mediate the uptake of ammonium ions, and their structural analysis has provided valuable information on their mechanisms. After nitrogen uptake by plant roots, the nitrogen metabolism pathway involves the incorporation of nitrogen into organic compounds. Glutamine synthetase (GS) and glutamate synthase (GoGAT) are the master players in this pathway. They work together to convert ammonium ions into glutamine and glutamate, respectively. Different isoforms of GS and GoGAT exist, enabling plants to fine-tune nitrogen metabolism based on environmental cues. Under severe abiotic stress conditions, nitric oxide (NO) has been found to enhance plant survival under drought. Furthermore, the interaction between salinity stress and nitrogen availability in plants has been studied, with NO identified as a potential mediator of responses to salt stress. On the other hand, excessive use of nitrate fertilizers can lead to health and environmental issues. Therefore, alternative strategies, such as establishing nitrogen fixation in plants through diazotrophic microbiota, have been explored to reduce reliance on synthetic fertilizers. Ultimately, genomics can identify new genes related to nitrogen fixation, which could be harnessed to improve plant productivity. By deciphering the genetic basis of nitrogen-fixing traits, researchers aim to develop crops that efficiently utilize nitrogen from the environment.

Keywords: Nitrogen uptake; nitrogen metabolism; plant stress response; plant-microbe interaction

Introduction

Soil erosion, compaction, acidification, and contamination are significant factors causing soil degradation affecting 50% of agricultural land and food production [1–3]. Consequently, 75 billion tons of fertile soil are lost worldwide due to degradation annually [4]. Moreover, massive amounts of synthetic agro inputs to fertilize crops used by farmers harm environmental health [5,6]. In this sense, applying nitrogen fertilizers has increased sharply by 7.4 times compared with crop productivity, which only increased by 2.4 times, indicating that crops have reduced their ability to

use N efficiently [7], increasing food insecurity [8,9]. Therefore, innovative and sustainable strategies in the agro-biotechnological sector have received more attention from botanical scientists worldwide over the last century to solve food security challenges and develop environment-friendly products [10]. Throughout time, microbial diversity and biomass have been assessed as partners in the plant-microbe association in maintaining soil multifunctionality [11].

Nitrogen uptake

Nitrogen (N_2) is an essential macronutrient for plants, and it can be acquired from the soil in various forms. The most common inorganic forms are nitrate (NO_3^-) and ammonium (NH_4^+), which are transported across the root plasma membrane by different families of transporters. Four families of transporters mediate nitrate uptake: NRT1, NRT2, CLC-1, and SLAC1/SLAH. These transporters have distinct characteristics in affinity, capacity, regulation, and localization [12]. Ammonium uptake is facilitated by AMTs, high-affinity transporters expressed mainly in the root hairs and epidermis [13]. Ammonium is the dominant form of N in flooded or acidic soils, and AMT-mediated acquisition is crucial for the N demand of plants growing in such conditions [14]. Besides inorganic N, plants can also absorb organic N in the form of amino acids (AAs), which are abundant in soils that receive organic amendments such as manure or compost [15]. Several AAs transporters have been identified in plant roots, including AAP1, AAP5, ProT2, LHT1, and LHT6 [16,17]. These transporters have different substrate specificities and expression patterns, contributing to the uptake of a range of amino acids from the soil solution [18].

Plant nitrate transporters (NRTs)

Plants acquire nitrate (NO_3^-) from the soil through various transporters that belong to different families [19]. The NRT1 and NRT2 families are the primary transporters involved in NO_3^- uptake, and they have different roles in plant growth and seed development. The NRT1 family is a large and diverse group of transporters in the NPF family. In *Arabidopsis*, there are 53 NRT1 genes, while 93 in rice [20]. These genes can be classified into 8–10 subfamilies; NRT1.1 and NRT1.2 are responsible for NO_3^- root uptake. The NRT1 family can transport NO_3^- and other substrates such as hormones, nitrite, amino acids, peptides, chloride, glucosinolates, and jasmonate-isoleucine [21]. The NRT2 family is a smaller, more specific group of transporters expressed mainly under low NO_3^- conditions. In *Arabidopsis*, four NRT2 transporters (NRT2.1, NRT2.2, NRT2.4, and NRT2.5) function in nitrate influx and account for 95% of nitrate uptake under low NO_3^- concentrations [22]. However, NRT2.1 and NRT2.2 are significant members of the NRT2 family for nitrate uptake. The NRT2 family has different spatial and temporal expression patterns, and they transport NO_3^- from different sources to different tissues. For example, NRT2.4 and NRT2.5 absorb NO_3^- from the soil root hairs, while NRT2.1 and NRT2.2 transport it from the apoplast to the cortex and endodermis cells. Moreover, NRT2.5 is induced by long-term starvation and enhances NO_3^- uptake from both shoots and roots of mature plants [22,23]. In addition to the NRT2 family, another transporter family called NRT3 is also involved in NO_3^- transport in plants. The NRT3 family consists of two members: NRT3.1 and NRT3.2. These transporters form a complex with NRT2 transporters and regulate their activity and stability [24]. The NRT1.1 transceptor is a crucial component of NO_3^- signaling in plants, as it can sense the external NO_3^- concentration and modulate the root growth accordingly [25]. The activity and specificity of NRT1.1 are regulated by two CIPKs, CIPK8 and CIPK23, which interact with NRT1.1 and mediate low- and high-affinity responses, respectively [26]. At high NO_3^- concentrations, NRT1.1 activates the expression of ANR1, a transcription factor that promotes lateral root growth and proliferation [27]. At low NO_3^- concentrations, NRT1.1 inhibits lateral root development by controlling auxin levels and meristem activation [28]. To transmit the NO_3^- signals from NRT1.1 to the nucleus, calcium acts as a secondary messenger that modulates the expression of NO_3^- responsive genes. Three calcium-dependent protein kinases (CPK10, CPK30, and CPK32) and their partner CBL are involved in this process [29]. After being sensed and transported by NRT1.1, NO_3^- is assimilated into amino acids in different plant tissues. Several other NRT transporters are involved in the loading and unloading NO_3^- from the xylem and phloem, which affects the distribution and availability of

NO_3^- within the plant. For example, NRT1.5, NRT1.8, and NRT1.9 are expressed in the xylem and phloem, and they function in the influx/efflux, removal, and loading of NO_3^- from/to the root vascular tissues, respectively [29,30]. Several studies have shown that overexpressing NRTs in different plant species can improve nitrogen use efficiency (NUE), which is the ratio of plant biomass or yield to nitrogen input. However, the effects of NRT overexpression may depend on the tissue specificity, nitrate concentration, and interaction with other genes. For instance, only NRT1 transporters have been successfully overexpressed in both leaves and roots to enhance NUE, while the role of NRT2 transporters in this process is still unclear. Recently, a chimeric NRT1 transporter, AtNC4N, was overexpressed in the phloem of old leaves in Arabidopsis, rice, and tobacco, and it increased nitrate uptake and NUE under low nitrate conditions [31]. Another NRT1 transporter, OsNRT1.1A (OsNPF6.3), was found to improve NUE, flowering time, yield, and maturity in rice [32]. Several other NRTs, such as OsNRT1.1B (OsNPF6.3B), OsNRT2.1, OsNRT2.3a/b, OsPTR9, OsAMT1.1, and qNGR9, have also been reported to enhance NUE under high or low nitrate levels in rice [32–35].

Moreover, some genes that regulate or interact with NRTs have been identified as potential targets for improving NUE in plants. For example, the transcription factor OsNAC42 activates the expression of several NRTs and increases nitrate assimilation and NUE in rice. The nitrate reductase gene OsNR2 converts nitrate to ammonium, affecting the expression of NRTs and other nitrogen-related genes in rice. The nitrate transporter OsNPF4.5 is regulated by a microRNA (miR827) and modulates nitrate distribution and remobilization in rice. These studies demonstrate the complexity and diversity of nitrate transport and signaling in plants and provide valuable insights for improving NUE and crop production [36].

NRT Structural analysis

The nitrate transporter system includes three distinct genes: NRT1, NRT2, and NRT3; they have different structural conformations (**Figure 1A-C**). As a result, Pfam database collects these genes under distinct families: PF00854 for NRT1, PF07690 for NRT2, and PF16974 for NRT3 [37]. PF00854 refers to the proton-dependent oligopeptide transporter (POT) family (also known as the peptide transporter (PTR) family) and is a class of energy-dependent transporters discovered in organisms ranging from bacteria to humans [38]. However, some family members are nitrate permeases, while others are in histidine transport [39]. The NRT1 transporter adopts a standard major facilitator superfamily (MFS) fold, which is described by 12 TMHs with a pseudo 2-fold axis connecting the N-terminal (TMH1–6) and C-terminal (TMH7–12) domains [40].

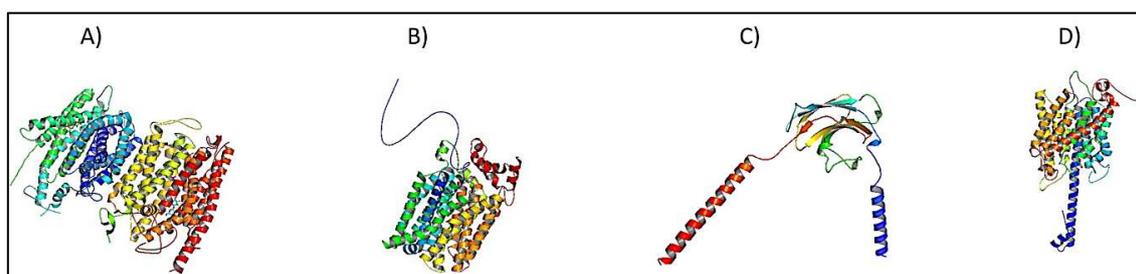


Figure 1. Secondary structure of nitrogen plant transporters families. A) NRT1 family is represented by 4OH3; B) alphafold model of Q9LMZ9 represents NRT2 family; C) NRT3 family is represented by the alphafold model of Q9FGS5; D) alphafold model of P54144 represents AMT1 family.

NRT2 members belong to the PF07690 (MSF) family, representing the largest family of secondary transporters with members ranging from Archaea to Homo sapiens. MFS proteins target a diverse range of substrates in both directions across the membrane, including ions, carbohydrates, lipids, amino acids and peptides, nucleosides, and other small molecules, in many cases catalyzing active transport by converting the energy stored in a proton electrochemical gradient into a concentration gradient of the substrate [41]. NRT3 candidates lie under the PF16974 family, which contains a C-terminal transmembrane domain required for high-affinity nitrate absorption [42,43]. NRT3 is

implicated in the inhibition of lateral root initiation in the presence of high sucrose-to-nitrogen ratios in the medium. As a result, the NRT2 and NRT3 component systems are believed to be implicated in the signaling pathway that integrates nutritional inputs for the regulation of lateral root architecture [44]. In *Arabidopsis*, the functional unit of the high-affinity nitrate influx complex is most likely a tetramer composed of two NRT2 and NRT3 subunits each [45].

Plant ammonium transporters (AMTs)

In most natural and agricultural environments, ammonium and nitrate are the two principal inorganic nitrogen sources absorbed by higher plant roots [46,47]. Because ammonium absorption requires less energy than nitrate assimilation, ammonium is the preferred form of nitrogen uptake when plants are nitrogen deficient [48,49]. Although ammonium uptake is more effective, too much ammonium in the plant can be hazardous [50–52]; thus, the ammonium uptake system in plants receives special attention [53–55]. Recent research indicates that AMTs also play a role in a variety of other physiological processes, such as transporting ammonium from symbiotic fungi to plants [56,57], delivering ammonium from roots to shoots [58], transmitting ammonium in leaves and reproductive organs [59–61], and encouraging resistance to plant diseases through ammonium transport [62,63]. Aside from being a transporter, many AMTs are essential for root growth in the presence of ammonium [54,64–66]. To avoid the adverse effects of insufficient or excessive ammonium nitrogen intake on plant growth and development, AMTs activities are fine-tuned not only at the transcriptional level through the involvement of at least four transcription factors [67–70] but also at the protein level through phosphorylation [71,72], pH [73], and endocytosis [74].

AMTs Structural analysis

The ammonium transporter domain (PF00909) comprises two structural copies of five helices each, plus one extra C-terminal helix (**Figure 1D**). It has been defined as an 11-times-spanning membrane channel. An AMT is composed of 11–12 transmembrane spots, with feature sequences "D (F Y W S) A G (G S C) X2 (L I V) (E H) X2 (G A S) (G A) X2 (G A S) (L F)" at transmembrane spot 5 and "D D X (L I V M F C) (E D G A) (L I V A C) X3 H (G A L I V) X2 (G S) X (L I V A W) G" at transmembrane region 10 [54]. AMTs can be divided into two subtypes: AMT1 and AMT2 or methylammonium permeases (MEP) [54,58,75].

(KEGG;M00528), Complete nitrification, complete ammonia oxidation (comammox) (KEGG;M00804) and Anammox (KEGG;M00973) (**Figure 2**).

Moreover, each sub-pathway from these previous sub-pathways consists of a list of genes and their encoded enzymes that are involved in the various biogenic processes in the nitrogen cycle. The nitrogen fixation path consists of Nitrogenase molybdenum-iron protein alpha chain (NIFD), Vanadium-dependent nitrogenase alpha chain (VNFD), and Nitrogenase delta subunit (ANFG) genes [86–88], see (**Figure 2**) and Table 1.

Table 1. List of genes and enzymes involved in the nitrogen cycle.

No.	KEGG ID	Gene Symbol	Gene Name	EC number	Reference
1	K04561	NORB	Nitric oxide reductase subunit B	1.7.2.5	Heiss et al., 1989; Suzuki et al., 2006
2	K15877	CYP55	Fungal nitric oxide reductase	1.7.1.14	Kizawa et al., 1991; Zhang and Shoun, 2008
3	K00376	NOSZ	Nitrous-oxide reductase	1.7.2.4	Coyle et al., 1985; Arai et al., 2003; Zumft and Kroneck, 2007
4	K02586	NIFD	Nitrogenase molybdenum-iron protein alpha chain	1.18.6.1	Joerger et al., 1989; Fani et al., 2000
5	K22896	VNFD	Vanadium-dependent nitrogenase alpha chain	1.18.6.2	Joerger et al., 1990
6	R05186	NIFF	Nitrogenase	1.19.6.1	Deistung et al., 1985
7	K01455	FORMAMIDASE	Formamidase	3.5.1.49	
8	K20935	HDH	Hydrazine dehydrogenase	1.7.2.8	Kartal et al., 2011
9	K20932	K20932	Hydrazine synthase subunit	1.7.2.7	1.7.2.7
10	K01672	CA	Carbonic anhydrase	4.2.1.1	Breton, 2001; Pastorekova et al., 2004
11	K01725	CYNS	Cyanate lyase	4.2.1.104	Harano et al., 1997
12	K00368	NIRK	Nitrite reductase (NO-forming)	1.7.2.1	Cantera and Stein, 2007
13	K02575	NRT2, NARK, NRTP, NASA	MFS transporter, NNP family, nitrate/nitrite transporter	-----	Noji et al., 1989
14	K15576	NRTA, NRTB, NRTC, NASD	Nitrate/nitrite transport system substrate-binding protein	7.3.2.4	Maeda and Omata, 2009
15	K00370	NARG, NARZ, NXRA	Nitrate reductase/nitrite oxidoreductase, alpha subunit	1.7.5.1 1.7.99.-	Blasco et al., 1990; Sohaskey and Wayne, 2003
16	K10534	NR	Nitrate reductase (NAD(P)H)	1.7.1.1 1.7.1.2 1.7.1.3	Okamoto and Marzluf, 1993
17	K00367	NARB	Ferredoxin-nitrate reductase	1.7.7.2	Rubio et al., 1996
18	K02567	NAPA	Nitrate reductase (cytochrome)	1.9.6.1	Stolz and Basu, 2002; Jepson et al., 2007
19	K17877	NIT-6	Nitrite reductase (NAD(P)H)	1.7.1.4	Exley et al., 1993
20	K00366	NIRA	Ferredoxin-nitrite reductase	1.7.7.1	Takahashi et al., 2001
21	K00362	NIRB	Nitrite reductase (NADH) large subunit	1.7.1.15	Harborne et al., 1992
22	K03385	NRFA	Nitrite reductase (cytochrome c-552)	1.7.2.2	Einsle et al., 1999
23	K00459	NCD2, NPD	Nitronate monooxygenase	1.13.12.16	Gadda and Francis, 2010
24	K19823	NAO	Nitroalkane oxidase	1.7.3.1	Daubner et al., 2002

Cont. Table 1: List of genes and enzymes involved in the nitrogen cycle.

No.	KEGG ID	Gene Symbol	Gene Name	EC number	Reference
25	R00143		Hydroxylamine reductase (NADH)	1.7.1.10	
26	K05601	HCP	Hydroxylamine reductase	1.7.99.1	Wolfe et al., 2002
27	K10535	HAO	Hydroxylamine dehydrogenase	1.7.2.6	Hommes et al., 2001
28	R10230		Hydroxylamine oxidase (cytochrome)	1.7.3.6	Wehrfritz et al., 1996

29	K10944	PMOA-AMOA	Methane/ammonia monooxygenase subunit A	1.14.18.3 1.14.99.39	Norton et al., 2002
30	K00926	ARCC	Carbamate kinase	2.7.2.2	Durbecq et al., 1997
31	K01948	CPS1	Carbamoyl-phosphate synthase (ammonia)	6.3.4.16	Haraguchi et al., 1991
32	K00260	GUDB, ROCG	Glutamate dehydrogenase	1.4.1.2	Belitsky and Sonenshein, 1998
33	K00261	GLUD1_2, GDHA	Glutamate dehydrogenase (NAD(P)+)	1.4.1.3	Frigerio et al., 2008
34	K00262	E1.4.1.4, GDHA	Glutamate dehydrogenase (NADP+)	1.4.1.4	Riba et al., 1988
35	K01915	GLNA, GLUL	Glutamine synthetase	6.3.1.2	Newsholme et al., 2003
36	K01501	E3.5.5.1	Nitrilase	3.5.5.1	Zhu et al., 2008
37	K00265	GLTB	Glutamate synthase (NADPH) large chain	1.4.1.13	Sonawane and Röhm, 2004
38	K00264	GLT1	Glutamate synthase (NADH)	1.4.1.14	Lancien et al., 2002
39	K00284	GLU, GLTS	Glutamate synthase (ferredoxin)	1.4.7.1	Coschigano et al., 1998
40	K00372	NASC, NASA	Assimilatory nitrate reductase catalytic subunit	1.7.99.-	Ogawa et al., 1995; Lin et al., 1993
41	K26139	NASD, NASB	Nitrite reductase [NAD(P)H] large subunit	1.7.1.4	Ogawa et al., 1995
42	K15864	NIRS	Nitrite reductase (NO-forming) / hydroxylamine reductase	1.7.2.1 1.7.99.1	Rees et al., 1997; Hole et al., 1996
43	K00531	ANFG	Nitrogenase delta subunit	1.18.6.1	Joerger et al., 1989
44	K04561	NORB	Nitric oxide reductase subunit B	1.7.2.5	Heiss et al., 1989; Suzuki et al., 2006

In this sub-pathway these previous genes were used to convert N_2 to NH_3 by oxidoreductases reaction, and this NH_3 molecules are widely used in different vital pathways for living organisms such as, Arginine biosynthesis (KEGG;map00220), Purine metabolism (KEGG;map00230), Alanine, aspartate and glutamate metabolism (KEGG;map00250), Glycine, serine and threonine metabolism (KEGG;map00260), Cyanoamino acid metabolism (KEGG;map00460), Glyoxylate and dicarboxylate metabolism (KEGG;map00630), Lipoic acid metabolism (KEGG;map00785), Nitrogen metabolism (KEGG;map00910), Metabolic pathways (KEGG;map01100), Biosynthesis of secondary metabolites (KEGG;map01110), Microbial metabolism in diverse environments (KEGG;map01120), Carbon metabolism (KEGG;map01200), Biosynthesis of amino acids (KEGG;map01230), Proximal tubule bicarbonate reclamation (KEGG;map04964), Collecting duct acid secretion (KEGG;map04966), Protein digestion and absorption (KEGG;map04974), Vibrio cholerae infection (KEGG;map05110) and Epithelial cell signaling in Helicobacter pylori infection (KEGG;map05120) [86–88].

In addition, the Anammox path consists of Hydrazine dehydrogenase (HDH), Hydrazine synthase subunit (K20932), and Nitrite reductase (NO-forming) (NIRK) genes, and these previous genes can convert NO_2 to N_2 or NH_3 using nitrite reductase enzyme [89,90] (**Figure 2**) and Table 1. In that context, the Denitrification path consists of four genes Nitrous-oxide reductase (NOSZ), Nitrite reductase (NO-forming) (NIRK), Nitrate reductase (cytochrome) (NAPA), and nitric oxide reductase subunit B (NORB) [91–93]. In this sub-pathway, the Denitrification genes convert NO_3 to N_2 through NO_2 , NO , and NO_2 . Furthermore, Assimilatory and dissimilatory nitrate reduction paths have different genes, such as Nitrate reductase/nitrite oxidoreductase, alpha subunit (NARG, NARZ, and NXRA), Nitrate reductase (cytochrome) (NAPA), Nitrite reductase (NADH) large subunit (NIRB), Nitrite reductase (cytochrome C-552) (NRFA), Ferredoxin-nitrate reductase (NARB), Nitrate reductase (NAD-(P)-H) (NR), Assimilatory nitrate reductase catalytic subunit (NASC and NASA), Nitrite reductase (NAD-(P)-H) (NIT-6), Ferredoxin-nitrite reductase (NIRA) and Nitrite reductase [NAD(P)H] large subunit (NASD and NASB) that can convert NO_2 to NH_3 [94–97].

Function and regulation of glutamine synthetase and glutamate synthase in nitrogen metabolism

In various biological systems, the interconversion of glutamate and glutamine plays a central role in nitrogen assimilation, transport, and recycling. Glutamine synthetase (GS) and glutamate synthase or glutamine 2-oxoglutarate aminotransferase (GOGAT) are vital enzymes orchestrating these interconversions, ensuring optimal nitrogen utilization, and maintaining nitrogen homeostasis.

The process of nitrogen uptake by plants from the soil for growth has been well studied. Plants acquire nitrogen in either ammonium or nitrate. Nitrate is converted into ammonium, then combined with glutamate to be assimilated into glutamine [98]. The principal site of ammonium assimilation is the roots, where the enzyme GS facilitates this conversion [99,100]. Following this, one of the amino groups from glutamine is transferred to 2-oxoglutarate (2-OG) to synthesize glutamate. This reaction is catalyzed by GOGAT (**Figure 3**). The coordinated action of GS and GOGAT is responsible for these interconnected processes. The GS/GOGAT cycle is the plants' central pathway for ammonium assimilation [101–103]. Experimental studies involving reverse genetic analysis have demonstrated that the absence of genes encoding these ammonium-responsive GS/GOGAT isoenzymes reduces ammonium assimilation, particularly in the roots, resulting in impaired growth [104–107]. These findings suggest that the ammonium-responsive forms of GS/GOGAT have a central role in the primary assimilation of ammonium within the roots [108]. As the expression of these isoenzyme-encoding genes increases with ammonium availability, it is plausible to infer that plants possess a regulatory transcriptional network that modulates their gene expression in response to ammonium availability [105].

Plant GS (EC6.3.1.2), a highly conserved enzyme, catalyzes glutamine synthesis from glutamate and ammonium ions, conserving the nitrogen atom in an organic molecule. This conversion is fueled by the energy released through the hydrolysis of ATP to ADP, making GS a crucial component of nitrogen metabolism across different life forms [109], in addition, the GOGAT is the critical enzyme in synthesizing glutamate, [101]. This reaction involves transferring the amide amino group of glutamine to 2-oxoglutarate through a reductant-driven process, yielding two glutamate molecules [110]. Within the realm of plants, this enzyme exists in two distinct variants: one employs reduced ferredoxin (Fd) as the electron contributor (EC 1.4.7.1), while the other employs NADH as the electron contributor (EC 1.4.1.14). The Fd-dependent form of the enzyme is notably abundant within the chloroplasts of photosynthetic tissues. In these regions, it utilizes light energy directly to serve as a reductant source.

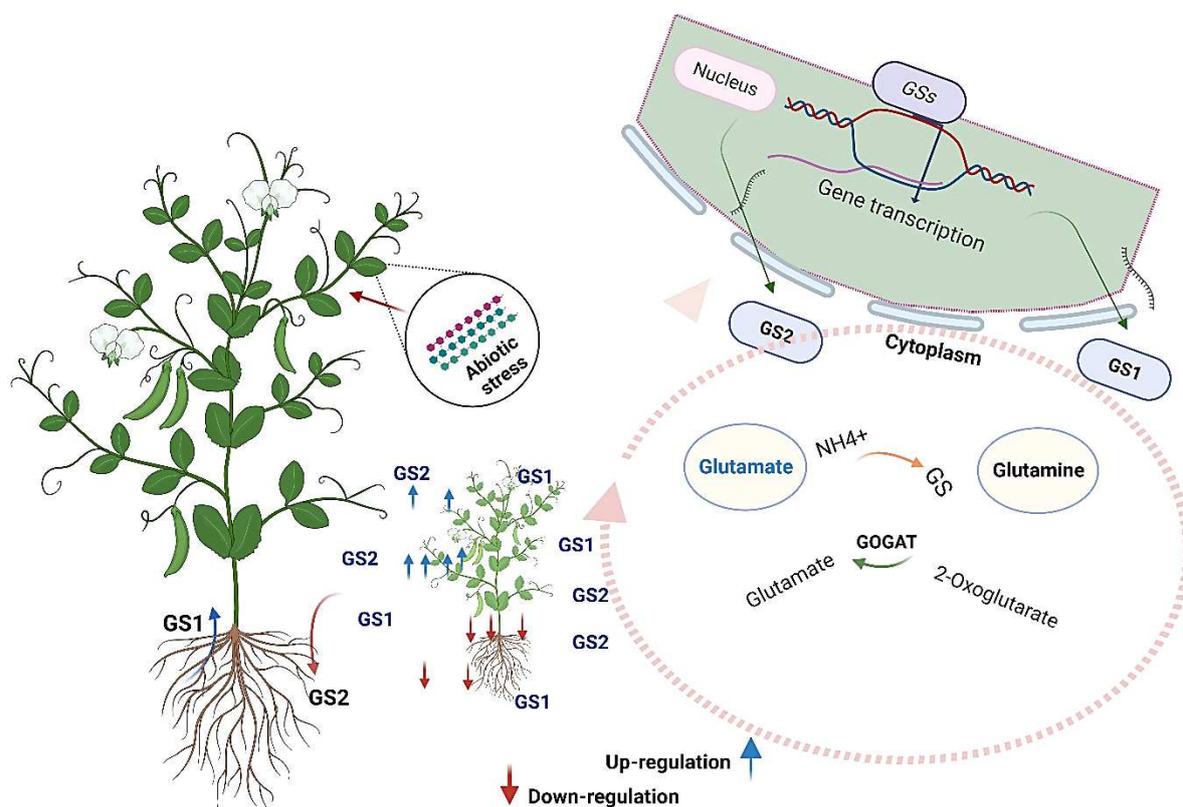


Figure 3. The functional role of Glutamine synthetase (GS) isoforms and associated in response to abiotic stress. GS catalyzes the synthesis of glutamine from glutamate and ammonium ions and acts as a critical enzyme in the nitrogen metabolic pathway in organisms.

Conversely, the NADH-dependent variant, also present within plastids, is primarily concentrated in non-photosynthesizing cells. The reductant is sourced from the pentose phosphate pathway [111]. Notably, the expression of the Fd- and NADH-dependent forms of glutamate synthase seems to exhibit dissimilar patterns across distinct plant tissues. It has been suggested that in most plants, two genes encode each form of glutamate synthase [110].

Isoforms of GS and GOGAT

According to subcellular distribution patterns, investigations into plant GS enzymes have identified two main categories. The initial group, designated as GS1, is primarily situated within the confines of the cytoplasm. Conversely, the second group, GS2, is predominantly found within plastid structures [112,113]. Various multigenic families are responsible for encoding multiple isoforms of GS1, whereas the plastidial GS2 arises from a limited number of nuclear genes [114]. Typically, GS2 is linked to the process of (re)assimilation of NH_4^+ in leaves, whereas GS1 is associated with recycling nitrogen within the plant. However, the relative activity levels exhibited by GS1 and GS2 are subject to variation based on species, specific plant organs, nitrogen sources, developmental stages, and prevailing environmental conditions, including abiotic stress factors. This variability underscores the intricate involvement of these isozymes, reflecting a multifaceted role [113,115]. In wheat research, a comprehensive exploration has unveiled four distinct variations of GS genes: GS1, GS2, GSr, and GS_e [112]. Employing advanced high-performance liquid chromatography (HPLC) methods, [116] successfully separated GS and discovered two subtypes in leaves: cytoplasmic subtype GS1 and chloroplast subtype GS2. Both subtypes' content and activity fluctuate in tandem with developmental processes. The subcellular localizations of GSr and GS_e align with the cytoplasmic compartment [117]. In addition, the advancement of genome sequencing has unveiled various isoenzymes of GS/GOGAT present in plants. Among these, specific isoenzymes, such as GS1;2 and NADH-

GOGAT1 from rice [118], as well as GLN1;2 and NADH-GOGAT (GLT1) from *Arabidopsis* [119,120], are expressed in plant roots in response to ammonium supply. These isoenzymes' expression at transcript and protein levels highlights their significance in ammonium assimilation.

Biological function of GS in response to abiotic stress

The pivotal function of GS within the nitrogen metabolism pathway of plants has been extensively investigated. Many research endeavors have underscored GS's capacity to regulate nitrogen levels within plants, exerting an indirect influence over diverse photosynthesis and physiological metabolic processes. Consequently, these regulatory actions have far-reaching implications on the trajectory of growth, development, crop yield, and overall quality of the produce. Nevertheless, the intricate diversity exhibited by GS isoforms implies a multifaceted role for GS in navigating through various dimensions of abiotic stress tolerance [113,121]; for instance, in rice plants exposed to salinity stress, GS1 increased in root at seedling stage and old leaves with no change in young leaves, while GS2 was down-regulated in old leaves and young leaves [122]. Under drought stress, GS1 and GS2 exhibited differential expression among different plant organs and species, with a prominent increase of GS1 in roots and leaves, while the total GS activity showed a decrease in the potato root [123,124]. A comparative proteomic analysis under drought stress conditions in wheat was studied [125]. They found that drought-tolerant wheat varieties exhibited a significant upregulation of GS2 expression, indicating its potential role in enhancing drought tolerance. Similar results have been obtained by [126], who explored GS activity in wheat leaves and observed that older leaves had lower GS activity than flag leaves in drought-tolerant and sensitive varieties under normal conditions. During drought stress, GS activity in sensitive varieties' flag leaves decreased compared to tolerant varieties. This suggested that GS activity could indicate drought stress sensitivity and tolerance, with elevated cytoplasmic-to-plastid GS ratio and premature senescence as markers. Exposure to heavy metals such as Cadmium, Molybdenum, copper, and Aluminum led to the down-regulation of GS and decreased its total activity in different plant species, including rice, soybean, tomato, and tea [127–130]

Nitrogen-induced resilience in plants against drought stress

Drought stress represents one of the most significant abiotic stresses that significantly damages crops worldwide, particularly in arid and semi-arid regions. Crops are constantly exposed to drought and temperature pressures during the growing season in arid and semiarid regions [131]. Plants adapt to drought via avoidance (stomata adjustment to reduce transpiration rate) and tolerance (antioxidants and osmolytes production) of drought [132] (**Figure 4**). The concentrations of NO_3^- and NH_4^+ have remarkable impacts on plants under drought conditions; the application of NH_4^+ alleviates the effect of drought on plant development, while NO_3^- has the opposite impact [13]. Nitrogen fertilizers might enhance the physiological responses to drought stress by elevating the concentrations of N and chlorophyll and promoting PSII photochemical activity [133]. It has been shown that adding N increases water use efficiency (WUE) by increasing plant dry mass and decreasing water loss [132].

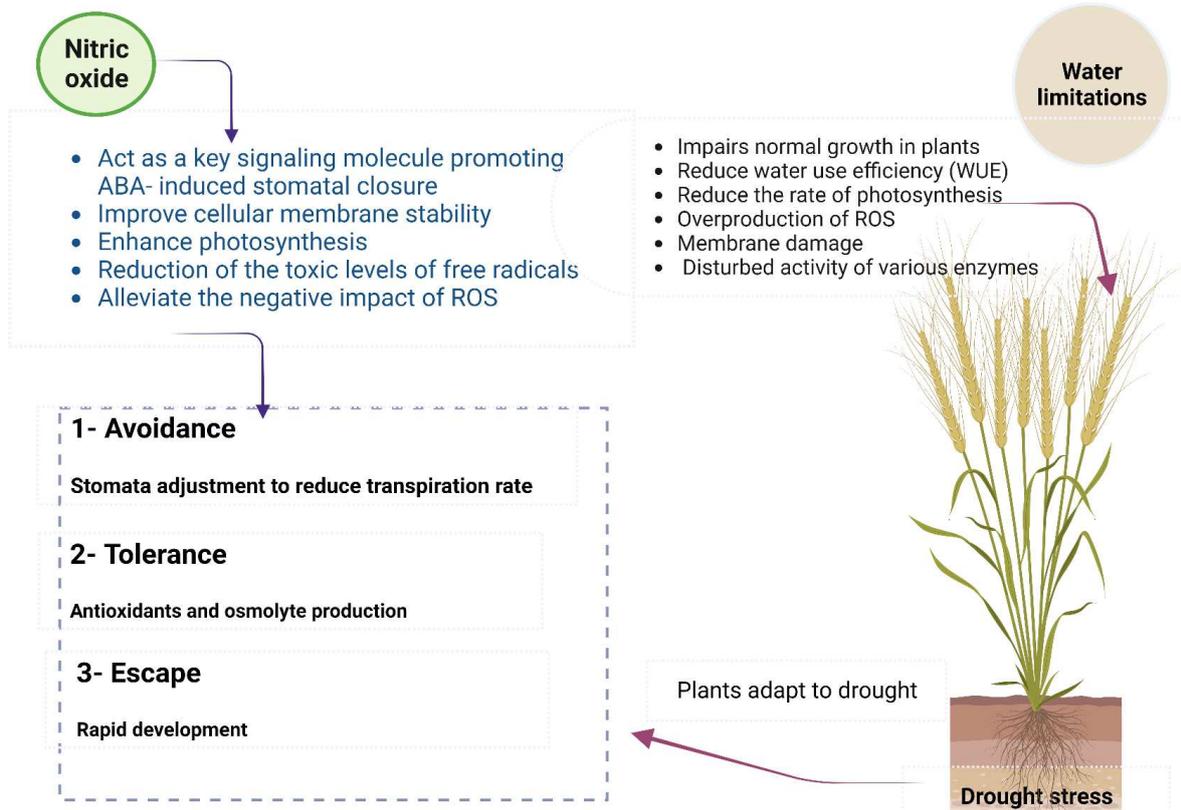


Figure 4. Effect of drought stress on plant growth and activation of defense strategies by nitric oxide. When a plant is exposed to drought, it shows physiological and morphological changes to improve tolerance or avoid stress.

Additionally, nitrogen may lessen the drought's inhibitory effects on photosynthesis and prevent C starvation [134]. On the other hand, low N availability can make plants more sensitive to dehydration and cause protein degradation, which lowers the amount of N-containing osmolytes like proline [132,134]. Nitrogen supply enhances osmotic adjustment and the activity of antioxidant enzymes via proline accumulation induction to mitigate the physiological harm from drought stress. However, the nitrogen effects depend on the ionic form of nitrogen supplied [135]. NH_4^+ is essential in promoting plant drought tolerance by increasing root numbers (surface area) and subsequently improving and facilitating water uptake. Also, decreased aerenchyma development may contribute to NH_4^+ promoted drought tolerance [13]. The influence of NO_3^- on drought responses is linked to NO_3^- transport/assimilation in plants. Numerous NO_3^- transport/assimilation-related genes, such as NRT2.5, GS, and GOGAT, are repressed in response to drought stress. Plants respond better to drought when genes involved in NO_3^- absorption or assimilation pathways are disrupted [13]. Drought stress and N deficiency have similar plant physiological responses. They reduce photosynthesis and use similar adaptive mechanisms such as osmolyte accumulation, longer roots, induction of antioxidative enzymes, reduction of photosynthesis and stomatal conductance, and induction of reactive oxygen species (ROS) [136]. Therefore, a high nitrogen concentration enhances root growth and photosynthetic traits, alleviates drought stress via stomatal conductance, and increases the photosynthetic rate [137].

Additionally, at high nitrogen supply, more nitrogen forms soluble nitrogenous molecules like free amino acids such as proline, which function as osmotica to balance the water status under abiotic stress [138]. Plant nutrient management is crucial to promote abiotic stress tolerance in cotton plants. The use of nitrogen fertilizer either in the soil or foliar spray can decrease the toxicity of ROS caused by abiotic stresses through the increase the peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) levels in plant cells [131,139]. These enzymes improve the photosynthetic rate,

promoting stress tolerance by scavenging ROS and reducing photooxidation to preserve chloroplast membrane integrity [139–141]. Applying nitrogen fertilizers (N_{60} kg.ha⁻¹) can increase the tolerance of drought stress in wheat plants combined with the highest yield [131].

Nitric oxide enhances plant survival under drought stress

Nitric oxide (NO) is a highly reactive, redox-active nitrogen species (RNS) formed in living cells under normal and stressful biotic and abiotic conditions. NO may function as a detoxifier and lessen adverse effects when a plant's ROS content reaches dangerous levels [142]. It was reported that the synthesis of NO increased in plant cells under drought stress conditions, and it has been suggested that this may help enhance plants' acclimation responses to cope with water limitation [143]. Drought stress enhanced NO synthesis in bean, pea, tobacco, cucumber, grapevine, and rice plants. Also, the activity of NO synthase (NOS)-like and NO release rate have increased under dehydration conditions [144]. NO has a vital role in respiratory function, called pathways of electron transport in mitochondria; it adjusts ROS and therefore activates defense strategies via promoted antioxidant formation in plants under various abiotic stresses [142]. NO plays a crucial role in mediating hormonal activities, interacting with ROS, and influencing gene expression as well as protein activity during sensing and signaling processes that enable plants to respond to and survive in environmental stress circumstances. Exogenously supply of NO increased drought tolerance in various plants such as maize, rice, and cucumber due to the increase of antioxidant defense, which acts to scavenge ROS, improve cellular membrane stability, and preserve photosynthesis as well as water status [144,145].

Drought induces the synthesis of abscisic acid (ABA), which regulates several critical plant processes, like seed germination and stomatal conductance, with the help of signaling molecules like NO and ROS [146]. NO plays an essential role in hormonal signaling pathways, where NO acts as a critical signaling molecule promoting ABA-induced stomatal closure in case of hyperosmotic stress [147]. NO is able to reduce the toxic levels of hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and lipid-free radicals (R^*). Moreover, NO can alleviate the negative impact of ROS through lipid peroxidation, enhancing photosynthesis as well as the expression of antioxidant enzymes via mitogen-activated protein kinase (MAPK) and other signaling pathways [142]. It was found that exogenous application of NO caused a significant increase in seedling growth of cucumber by decreasing seedlings injury, enhancing the antioxidant activities and chlorophyll and free proline contents under stress conditions. Moreover, an exogenous supply of NO in wheat markedly preserved high relative water content via decreasing transpiration, increasing K content, and mitigated cell membrane damage [148] (Figure 4).

Salinity stress and nitrogen in plant

Salinity stress is one of the harmful abiotic stress elements that impact the metabolism and productivity of crop plants worldwide. Salinity increases represent global hazards and threats to food security, resulting in the remarkable conversion of agricultural arable soil into unproductive land [149]. The high level of salt ions in agricultural soil may be harmful to plants causing salt stress, where sodium (Na^+) and chlorine (Cl^-) concentrations increase in saline soil and affect the natural physiological process in plants [150]. The most efficient way to improve plant development under salt stress is to apply nitrogen (N) due it is considered one of the most influencing plant growth macronutrients in saline environments. Nitrogen is a necessary, powerful nutrient that mediates various molecular, physiological, and cellular responses essential to plant survival as well as different signal transduction pathways linked to plant defense systems and salt-stress tolerance [151,152]. Moreover, salt stress affects ammonification and nitrification in the soil due to chloride (Cl^-) competes with nitrate (NO_3^-), ammonium (NH_4^+) competes with sodium (Na^+) and creates ion toxicities and inequalities, which can limit the processes of N uptake, transport, and assimilation [153,154]. Salt stress has inhibited various enzyme activities that contribute to N assimilation in plants such as maize, tomato, rice, cowpea, and mung bean, reducing N uptake and utilization [155].

Interestingly, it was reported that the interaction between salt stress and N had an impact on plant growth, and the effect of N on salt-exposed plants was dependent on the N rate [150]. In the

case of maize, excessive N treatment can successfully mitigate the deleterious effects of salt stress [156]. Similar findings in tomatoes showed that supra-optimum N was better than optimum N in reducing salt stress [138]. Under salinity stress, the application of N caused a significant increase in N and K uptake and decreased Na accumulation in wheat seedlings. Furthermore, N application can protect wheat seedlings in saline conditions via the upregulation of osmolytes, antioxidant system, and secondary metabolite accumulation [149]. In contrast, excessive N application exacerbated the negative effect of salt stress on wheat and rice, while moderate N supply could ameliorate the deleterious effect of salt stress. Therefore, applying an appropriate N rate is the key to mitigating the adverse effects caused by salt stress [150].

On the contrary, it was reported that the application of NO_3^- has resulted in an increase of Na^+ uptake and loading of the sodium into the xylem, increasing root inhibition caused by the salinity. The co-transportation of Na^+ and NO_3^- was suggested based on NO_3^- dependent Na^+ uptake data at various Na^+ concentrations [13]. Similarly, NO_3^- -dependent transport mechanisms in salt-stress environments encourage Na^+ ion uptake and loading into the xylem, which may represent a main pathway for Na^+ accumulation in *Arabidopsis* shoots [151].

Nitric oxide and salt stress

It has been demonstrated that nitric oxide can act as a signal to induce the resistance of salt stress by raising the K: Na ratio in plant cells, which is based on an increase in the H1-ATPase activity of plasma membrane in calluses from maize seedlings, reed (*Phragmites communis*) plants, and poplar trees [144]. Exogenous NO can improve salt tolerance under salinity conditions by stimulating the K^+/Na^+ ratio, the Na^+/H^+ antiport in the tonoplast, and proton-pump activity [157]. Under salt stress, NO improve the absorption and translocation of several macro- and micronutrients, including K, Fe, Mg, and Zn, which improve respiration and chlorophyll biosynthesis [158]. The uptake of mineral nutritional elements such as nitrogen (N), zinc (Zn), iron (Fe), potassium (K), and calcium (Ca) was improved as a result of NO application and thereby diminished the harmful effect of high salinity on strawberry plants [159]. The exogenous NO supply can protect chickpea plants from salt stress-induced oxidative harm by promoting the biosynthesis of photosynthetic pigment, and antioxidant enzymes, osmolytes accumulation, and thereby improving plant development under saline stress [160]. Furthermore, under salt stress conditions, exogenous NO treatment enhanced wheat seed germination by converting starch to sugars, where the application of NO led to elevate in the effectiveness of α - and β -amylase enzymes [158,161]. Under salt stress, NO treatment boosted plant development and demonstrated a protective ability against salinity-triggered oxidative stress by increasing the effectiveness of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) enzymes in *Triticum aestivum* [159].

Nitrate fertilizers have hazardous health issues

Nutrient deficiency and/or availability are crucial to plant production [162,163]. One of the severe issues the world faces is the adverse effects of fertilizers on the environment. Farmers have been using fertilizers since ancient times, but long-term use has affected soil fertility adversely. Due to over-fertilization in many agricultural areas, environmental pollution was established [164]. The seriousness of N pollution is a critical factor affecting food sustainability. For example, Nitrate pollution in groundwater is a severe problem worldwide, affecting animal and human health. In addition, Nitrate leaching decreases N availability for crops and can cause water pollution. In addition, high rates of N_2 fertilizer application to crops led to low N fertilizer use efficiency and reduced N availability by Nitrate leaching, which may cause groundwater nitrate pollution [165]. In general, the improper application of fertilizers damages the farmlands, leading to soil and water pollution. It also causes heavy metals contaminations and soil erosion. Nitrous oxide and methane lead to greenhouse gas (GHG) emissions and reduce food quality.

While ammonium sulfate cause soil pollution, nitrogen oxides and ammonia cause air pollution. Nitrate is the final breakdown product of the nitrogen applications accumulating in groundwater, contaminating drinking water [166,167]. Since Nitrate levels above 10 mg L^{-1} N cause

methemoglobinemia which is fatal. The U.S. Environmental Protection Agency set human drinking water to maintain nitrate levels below a Maximum Contaminant Level (MCL) at a critical value of 10 mg L⁻¹ nitrate-nitrogen N [168]. In Argentina, the national food law set the limit for human consumption at 11.3 mg L⁻¹ NO₃⁻-N. Consumption of nitrate-rich water has been associated with methemoglobinemia (Blue Child Syndrome) and different forms of cancer [169–171]. Furthermore, diabetes, adverse reproductive outcomes (especially neural tube defects), and thyroid conditions are related to nitrate-contaminated drinking water.

A notable study by Maghanga et al. (2013) was conducted to monitor changes in surface water nitrate levels in ten rivers within a Kenyan tea plantation for three years. Water samples were obtained before and after fertilizer application in 2004, 2005, and 2006. For three years, there was no established trend between surface water nitrate levels and the time of fertilizer applications. However, the highest nitrate-nitrogen levels were in river Temochewa in 2005 during the first fertilizer applications (4.9 mg L⁻¹ to 8.2 mg L⁻¹). Furthermore, fertilizer application increased nitrate levels, and the study indicated that initial nitrate-nitrogen levels in most rivers were high, causing surface water contamination [172].

Establishing nitrogen fixation in plants by diazotrophic microbiota

Rapid human population growth and declining agricultural soils intensify resource competition, affecting human food hunger and sustainable agriculture systems worldwide [173]. Hence, developing alternative strategies to increase the current food demand is an inevitable need [174]. Accordingly, crop productivity is the pillar of nutritional food security and heavily depends on applying nitrogen fertilizers [175]. Nitrogen element is a critical requirement for crop productivity; While applying N fertilizers consumes vast amounts of energy, the excess harms environmental health. In addition, the expensive cost, environmental hazards, soil fertility reduction, and other negative consequences [176,177]. Therefore, increasing plant N use efficiency (NUE) is essential for developing sustainable agriculture [178].

Ultimately nitrogen fixation has multiple benefits through improving the yields of landraces that often receive little attention from breeders and empowering farmers using a sustainable and less expensive strategy. Thus, this technology can improve farmers' nutrition by enhancing access to indigenous microbial biodiversity, income, and resilience. Additionally, it reduces the need for fossil fuels requiring synthetic nitrogen fertilizers [179,180]. Overall, the symbiotic relationship between leguminous plants and *rhizobia* is a powerful adaptation strategy that enables these plants to reduce their dependence on synthetic nitrogen fertilizers and thrive in nitrogen-deficient soils. The ability to fix atmospheric dinitrogen (N₂) and transfer it to legumes has a distinct advantage over non-leguminous plants in nitrogen-stressed environments, contributing to their ecological significance and agricultural importance in sustainable farming practices [181,182]. Taking together, improving the microbial activities (*Rhizobium* and *Bacillus* spp.) that contribute positively to boosting plant growth to sustain and increase food production under diverse climate situations is necessary [183]. In that sense, several beneficial mutualistic microbes have been discovered; however, their dependable utilization as biofertilizers in soil conditions is still challenged. Thus, improving the microbial inoculants, exploring their diversity under variable conditions *via* testing *in vitro*, and improving their performance in the external habitat could lead to understanding the knowledge gap between the microbial performance *in vitro* and the external habitat. Besides, biofertilizers have the advantage of being eco-friendly and cheap [184]. Applying mineral fertilizers at site-specific doses, increasing organic waste in agriculture, and bio-fertilizers with nitrogen-fixing bacteria and legumes in crop rotation seem more environmentally sustainable. In addition, N supply is necessary to provide N required for nucleotide and protein biosynthesis with other functions, i.e., carbon fixation capacity [185].

Bacterial endophytes associated with crops, i.e., corn and wheat, can produce various sugar compositions, such as arabinose [186]. They stimulate the production of a thick gelatinous layer (mucilage) around the root systems of these crops. This layer allows crops to better uptake fertilizer by forming a continuous boundary layer between roots and soil particles, protecting the root system

from drought, and supporting other microbes (Figure 5). Most interestingly, mucilage structures associated with complex microbiota contribute to nutrient acquisition play a vital role in plant growth and defence [180]. Since inorganic fertilizers are produced from fossil fuels and are estimated to use 1% to 2% of the global energy supply, the structure of a complex polysaccharide (e.g., fucose, arabinose, and xylose) comprising the mucilage might provide the energy source to fuel nitrogenase activity [187].

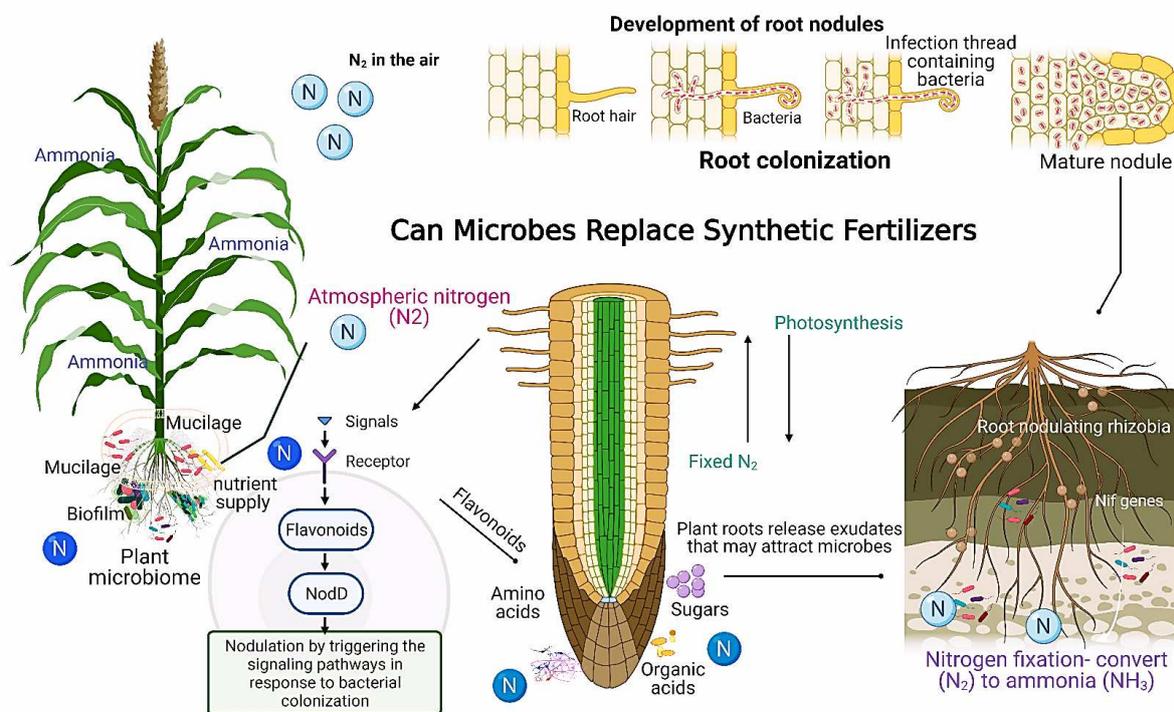


Figure 5. A simplified model represents the microbial beneficial effects and the signal pathways involved in modulating the effects on nitrogen availability. Nitrogen source limitation also increases ammonium and nitrate uptake activity as a promising strategy to achieve the sustainability of agriculture. The availability of nitrogen is one of the critical elements in the process of biological nitrogen fixation that converts atmospheric nitrogen (N_2) to ammonia (NH_3) via a nodule structure.

Remarkably, a novel concept related to the protective plant strategy under stress [188] regarding nitrogen fixation in an indigenous maize landrace grown in nitrogen-stressed fields near Oaxaca, Mexico, was recently reported. High levels of a complex nitrogen-fixing microbiome were associated with the abundant production of a sugar-rich mucilage-supported nitrogen fixation [180]. They hypothesized a general mechanism of N_2 fixation for cereals using mucilage structure to support associations with microbial diazotrophs. *Setaria viridis* and *Setaria italica* had a significant amount of fixed nitrogen associated with *Azospirillum brasilense*, contributing to growth promotion [189,190]. Several factors, such as plant root communities, plant genotypes, plant growth, and root microbe associations, considerably influence the microbial abundance and the quantity of mucilage exudation that modify the rhizodeposition [191,192]. In this context, mucilage exudation significantly increased under drying environmental conditions. In addition, mucilage secreted from plants grown in water-stressed soil had a higher contact angle than mucilage from plants grown in wet soil [180,191]. This research will open a new avenue in cereal crop-microbe interactions by transferring atmospheric nitrogen with significant economic and environmental benefits to farmers.

Moreover, reducing the chemical N fertilizer application by modifying N_2 -fixing bacteria in the microbial structure of cereal crop rhizospheres will improve the N-fixing efficiency. Improving the sustainability of biologically fixed N_2 to cereal crops is a future need to enhance crop productivity, representing a significant breakthrough in N-fixation research. To achieve this goal, engineering key

regulators of N₂-fixing by activating the symbiotic signaling pathway between diazotrophic bacteria and cereal crops is a promising strategy for sustainable development [181].

Fortunately, establishing new Omics techniques, such as metabolomics, metagenomics, metatranscriptomics, and metaproteomics, will help in-depth research and identify the rhizospheric microbes using next-generation sequencing approaches [193]. Accordingly, manipulating the promising microbes that replace synthetic fertilizers and improve food security independently is a solution. *Rhizobium*-plant symbiosis is now becoming challenging with diverse genetic modifiers of the symbiosis relationship, not only in the genomic variability of partners but also in the soil microbiota. In that sense, there is a future direction to improve the specificity of the partnership during symbiotic colonization using different methods (e.g., axenic cultures) by eliminating other microbes. Therefore, identifying and developing bioinoculant strains and genotypes by adapting and evolving elite strains in both lab-scale analyses and trials to open-field applications to increase crop yield becomes crucial. Considering the human–microbe interaction to find non-pathogenic microbes (especially in the *rhizobia* genomes) that can colonize their host plant in field conditions.

Can microbes replace synthetic fertilizers?

Variable climatic conditions, abiotic stressors, and imbalance in nitrogen cycling are the threatening factors that limit crop production and sustainable agriculture. Fertile soil is a primary component in the backbone of sustainable agriculture; however, land degradation and rapid desertification cause an estimated global loss of 24 billion tons of fertile arable land [194]. Noticeably, eutrophication and decline of soil fertility result from massive application of chemical fertilizers, causing significant environmental concern [195]. Therefore, the production and application of microbial fertilizers are increasing globally due to the negative impacts of the overuse of chemical-based fertilizers [196,197]. Moreover, with the limited N resources and increasing agricultural demands for nitrogen supplies, there is a need to discover diverse mutualistic interactions between plant roots and rhizosphere microbiome (**Figure 5**).

Soils are habitats for diverse microorganisms, either harmful (pathogenic) or beneficial (symbiotic) to plants, comprising organic substances, water, and nutrients. Food production requires essential nutrients, metabolites, and water that the soil provides to plants. Thus, soil fertility is a vital natural resource from which most global food is produced, directly related to the quantity and quality of produced food [173,198,199]. Besides the nutrient resources in the soil, plants attract microbes via root exudates, root border cells, and mucilages that serve as food for the rhizosphere microbiome and their assembly [200]. Interestingly, soil contains an extensive reservoir of microorganisms (1×10^9 microbial cells g⁻¹ dry soil) and microbial diversity (1×10^5 microbial species g⁻¹ dry soil) [201,202]. Rhizospheric microbes are crucial in diverse biological processes, including soil structure, climate regulation, disease control, and organic matter decomposition. Rhizosphere microbiome plays a functional role in plant growth *via* various aspects, including nutrient uptake, cycling water availability, and the formation of stable aggregates to reduce the risks of soil erosion [203–205]. In addition, these microbes can improve plant health and serve as biocontrol agents against plant pathogens as well as biofertilizers (eco-friendly alternatives to hazardous synthetic fertilizers) (i.e., *Rhizobium*, *Azotobacter*, *Bacillus subtilis*) [184,206]. Accordingly, the functional role of these microbial communities has had enormous efforts from botanical scientists over two decades to eliminate hunger and improve soil fertility by reducing synthetic fertilizers [207–210]. Protein (amino acid) deficiencies, primarily in Africa and South Asia, negatively impact people globally [211]. In addition, metabolites secreted by plant roots, such as flavonoids, benzaldehyde, biochanin A, salicylic acid, terpenoids, and tryptophan.

Furthermore, some bacterial metabolites in this signaling help build a beneficial association between microbes and their host. For example, 2,4-diacetyl phloroglucinol, hydrogen cyanide (HCN), indole-acetic-acid (IAA), lipopeptides, phenazine-1-carboxamide salicylic acid, and siderophores play a vital role in the plant-microbe signaling and promoting the bacterial colonization [212,213]. For example, N-fixer microbes convert nitrogen from the atmosphere into reactive nitrogen that plants can use in the natural rhizosphere as an alternative to chemical fertilization practices. Nitrogen

is required to synthesize chlorophyll and other amino acids during crop production. Currently, nitrogen starvation is expected during fuel price increases, affecting synthetic fertilizer manufacture [214,215].

Consequently, synthetic nutrients will be unaffordable for million smallholder farmers worldwide [216]. Fortunately, legume plants represent a solution, including fava beans, soybeans, lentils, and cowpea. They recruit symbiotic *rhizobia* bacteria from the soil into specialized root organs called nodules (N₂-fixation factory) through biological nitrogen fixation (BNF) (**Figure 5**). Nitrogen fixation is an eco-friendly and manageable strategy by harnessing nitrogen-fixing endophytic and free-living rhizobacteria to increase nitrogen levels in agricultural land. Numerous studies revealed that some beneficial microbes, i.e., *Rhizobium*, *Pseudomonas*, *Azospirillum*, and *Bacillus*, positively impacted crops by enhancing above and belowground biomass and could play vital roles in achieving sustainable agriculture outcomes [217]. Biological nitrogen fixation converts dinitrogen (N₂) to the fixed form of ammonia (NH₃), essential for crop productivity, as fixed nitrogen is a critical component of many biological molecules [218].

The biologically fixed amounts of atmospheric nitrogen into ammonia inside root nodules by legumes account for about 65% of nitrogen utilization in global agriculture *via* mutualistic symbiotic relationships with nitrogen-fixing *rhizobia*. *Rhizobia* can remain viable in mature nodules [219]. Accordingly, this process allows legume plants to grow well in N-deficient soils, eliminating N fertilizer application [220]. The inoculation of *rhizobia* strains (native or commercial) was studied on the growth and nodulation of three cowpea (*Vigna unguiculata*) genotypes in semiarid regions of Kenya. Nitrogen (N₂) is one of the limiting nutrients in semiarid regions, adversely affecting plant growth. Field trials were performed in a randomized complete block design with three replications, and the symbiotic efficiency (SE) of *Rhizobium* isolates was evaluated. In the field, *rhizobia* inoculation significantly ($P < 0.05$) increased nodulation and shoot DW compared to the uninoculated controls. Interestingly, *Rhizobia* inoculation significantly increased yields, whereas inoculation with native isolates recorded a 22.7% increase compared to uninoculated control in the first season and a 28.6% increase in yield in the second season. This study concluded that the efficient native *rhizobia* in smallholder farms with a promising potential to improve cowpea yield under a changing climate. In particular, *R. tropici* clone H53, *Mesorhizobium* sp. WSM3874 and *R. pusense* strain Nak353 showed more superiority in all the tested parameters. However, *rhizobia* inoculants improved nodulation and crop yield; cowpea genotypes did not prefer any inoculants. A practical and sustainable biofertilizer approach will empower cowpea production in subsistence farmers and establish effective symbiosis among legumes [221]. A recent study revealed that plant exudates and chemotaxis play a role in the development of bacterial biofilms in the soil, and creating microaerophilic conditions improves the nitrogen-fixing efficacy of diazotrophic bacteria in the soil, supplying plants with ammonia. In addition, plants support a diverse bacterial signal on the root surface or inside the tissues [222]. Flavonoids released by plant roots into the rhizosphere are crucial in regulating interactions between plants and microbes and controlling bacterial colonization [223]. The biofilm formation in the nitrogen-fixing bacterium *Gluconacetobacter diazotrophicus* [224] exhibited enhanced flavonoid compounds. This study also hypothesized that manipulating flavonoid synthetic pathways leads to the induction of biological nitrogen fixation in cereals through biofilm formation in soil diazotrophs [225,226]. Furthermore, flavonoid compounds are critical to the symbiotic relationship between legumes and *rhizobia* via inducing rhizobial Nod genes that signal nodule formation [227]. Biofilm formation (multicellular communities of microorganisms) is critical to the nitrogenase activity, which supported a free-living rhizospheric bacterium *Pseudomonas stutzeri* A1501, and *Azospirillum brasilense* to fix nitrogen under nitrogen-limiting conditions. Moreover, a positive correlation was observed between the nitrogen-fixing efficacy and the biofilm production of *Gluconacetobacter diazotrophicus*. Most interestingly, N-limiting signals very likely induce the production of exopolysaccharides which may function as a barrier to block excess oxygen in the air and provide a suitable microaerobic condition for bacteria inside the EPS to fix nitrogen [228,229]. A large amount of exopolysaccharide was reported in response to N limitation by cellulolytic bacteria (e.g., *Bacillus*, *Pedobacter*, *Chryseobacterium*, and *Flavobacterium* [230]. Therefore, exopolysaccharide production could be a

general response of bacteria to N-limiting growth conditions. They hypothesized biofilm formation is a competitive carbon and nitrogen acquisition strategy and provides valuable insights linking nitrogen inputs to carbon sequestration. Applying the symbiotic association between *rhizobia* and leguminous plants is a significant research area to reduce the dependency on chemical fertilizers. *Bradyrhizobium*, *Azospirillum*, and *Rhizobium* had high plant growth-promoting capacity, N-fixing efficiency, and resistance to heavy metals (e.g., Cd contamination), indicating abundant functional microbial resources in extreme soil environments [231,232] (**Figure 5**).

Inoculation of *Azospirillum*, *Burkholderia*, and other nitrogen-fixing bacteria had little success in the field [233]. Thus, the evolution of a compatible host for the indigenous nitrogen-fixing bacteria is critical for the survival rate, colonizing the host, and improving nitrogenase activity.

In response to environmental pressure (e.g., nitrogen stress), leguminous plants may allocate more energy and resources for nodule formation by secreting phenolics (such as *flavonoids*) to induce the expression of rhizobial nodulation genes. These phenolics act in a pathway with the bacterial activator protein NodD to regulate gene expression that codes for Nod-factor synthesizing proteins as plant stimulants [234]. Legume crops may exhibit changes in the efficiency of nitrogen fixation within the nodules in response to nitrogen stress to maximize the nitrogen-fixing capacity of *rhizobia* through various molecular and physiological processes. In addition, plants elaborately adjust their growth and nitrogen uptake. *Rhizobia* and the host legume plant exchange various molecular signals to maximize symbiont performances; some are essential for symbiosis (such as *flavonoids* and Nod factors), while others influence the association [235]. Thus, we advocate more future research efforts to explore the elite rhizosphere microbiota under fluctuating climatic conditions and limited economic resources (**Figure 5**).

Arctic N cycling microbiota

By the end of this century, arctic land areas are expected to warm by 5.6–12.4 °C [236], resulting in significant permafrost thawing [237,238] and significant alterations in ecological functioning [239]. Massive nitrogen stocks (>67 billion tons) [240] deposited in permafrost thousands of years ago may now be available for breakdown, resulting in the release of nitrous oxide (N₂O) into the atmosphere. N₂O is an influential greenhouse gas, over 300 times more powerful than CO₂ in terms of warming the climate. While the dynamics of carbon in the Arctic are well understood, the fact that Arctic soils store massive amounts of nitrogen has gotten little attention thus far [241]. Microorganisms can convert nitrogen compounds as reactive and harmful as nitric oxide or as inert and harmless as dinitrogen gas. In brief, a dinitrogen gas molecule is first fixed to ammonia, which then transforms into organic nitrogen (biomass). Ammonification, the decomposition of organic nitrogen, produces a molecule of ammonia, which is then oxidized to nitrate *via* nitrification (NH₄⁺ to NO₂⁻ to NO₃⁻) and eventually transformed back into a molecule of dinitrogen gas *via* denitrification (NO₃⁻ to NO₂⁻ to NO to N₂O to N₂) or anaerobic ammonium oxidation (anammox; NO₂⁻ + NH₄⁺ to N₂) [242]. In addition to methane and carbon dioxide fluxes, warming arctic soils release nitrogen, which can increase nitrous oxide (N₂O) emissions, another potent greenhouse gas. Microbial mechanisms such as denitrification have a crucial role in controlling N₂O release. The significance and amount of N₂O released from Arctic soils are unknown, as are the bacteria responsible for greenhouse gas emissions.

Genomic approaches have a role in identifying new genes to improve nitrogen fixation

The identification of new genes involved in nitrogen fixation, a complete investigation of the gene organization of nitrogen-fixing species, and the discovery of novel diazotrophic species have all been made possible by genomics. This section of the review discusses the essential traits of nitrogen-fixing genomes as well as techniques for employing genome scanning to find and examine the genomes of new diazotrophs [243]. These diazotrophs are bacteria that can convert atmospheric nitrogen gas into ammonia, a more beneficial form of nitrogen. For example, *Klebsiella pneumoniae* and *Azotobacter vinelandii* may develop without external sources of fixed nitrogen [244,245]. The primary genes involved in nitrogen fixation were addressed. In order to explore nitrogen-fixing at the genetic, biochemical, and biophysical levels, we also evaluate methods for genome modification.

Additionally, one of the four types of nitrogenase enzymes is responsible for reducing nitrogen to ammonia. (EC 1.18.6.1; EC 1.19.6.1), which are generated by specific bacteria. All diazotrophs examined thus far employ at least one of these enzymes. The first three forms of nitrogenase are linked architecturally, phylogenetically, and mechanistically; however, they differ in that either vanadium or molybdenum comprise the heterometal component or possess a homometal cofactor that solely comprises iron. The fourth form of nitrogenase, a superoxide-dependent, molybdopterin-containing nitrogenase, was discovered in a single species, *Streptomyces thermoautotrophicus*. The genomic sequence of *S. thermoautotrophicus* and the amino acid sequence of this fourth kind of nitrogenase has not yet been established [246,247]. The fact that the inventory and distribution of species encoding for the fourth kind of nitrogenase are unknown highlights how few diazotrophs' genomes have been sequenced. *A. vinelandii* is one of the most extensively researched species among diazotrophs with sequenced genomes. In an aerobic culture, the free-living bacteria *A. vinelandii* can fix nitrogen. It has served as a model for nitrogen fixation for decades due to several factors, including (i) its genetic adaptability, (ii) its capacity to fix nitrogen during aerobic growth, (iii) its nutritional flexibility—which is demonstrated by its capacity to fix nitrogen via three different pathways and its capacity to adapt its metabolism to a variety of nutrients and media additives—(iv) and (v) the genome's comprehensive sequencing and manual editing [248,249]. *A. vinelandii* has a single circular chromosome with 5,365,318 base pairs in its genome, which is expected to code for 5051 proteins [249]. The diazotroph *Pseudomonas stutzeri* is the closest companion of *A. vinelandii* with a sequenced genome [250]. Even though *P. stutzeri* may grow aerobically and can only fix nitrogen in microaerobic conditions [251,252]. Alternatively, in ambient oxygen concentrations (20% O₂), *A. vinelandii* can catalyze nitrogen fixation, an oxygen-sensitive process [253,254]. Its respiratory protection system makes it feasible for it to undertake two incompatible biological processes, oxidative phosphorylation, and nitrogen fixation, concurrently. *A. vinelandii* may modify its respiration rate to maintain a low amount of cytoplasmic oxygen while nitrogen-fixing is taking place [255]. Five terminal oxidases were found in the genome, along with additional NADH oxidoreductases and other respiratory complexes that provide electrons to terminal oxidases, raising oxygen consumption [249]. Some of these genes, such as *cydAB I*, have been linked to respiratory protection and are crucial for aerobic nitrogen fixation [246,247].

The Discovery of novel nitrogen-fixing organisms and the quantity and kind of nitrogenases coding in these species may also be targeted by genome sequencing utilizing *NifD* sequences as queries [256–258]. The genomes of diazotrophs also encode for other proteins comparable to *NifD*, in addition to the *Nif/Vnf/AnfD* sequences [258]. The discovery of new genes involved in nitrogen fixation in *A. vinelandii* and other diazotrophs might improve the efficacy of nitrogen fixation.

Conclusion

Nitrogen is an essential macronutrient required for the growth and development of all living organisms. The most common inorganic forms are nitrate (NO₃⁻) and ammonium (NH₄⁺), transported across the root plasma membrane. However, applying nitrogen fertilizers is necessary for plant growth and productivity, but massive amounts of synthetic agro inputs to fertilize crops used by farmers harm environmental health. In this sense, improving the use of N efficiency by plants is a promising strategy to reduce the negative impact of using synthetic fertilizers. Bacterial endophytes play a crucial role in the nitrogen cycle by converting atmospheric nitrogen gas into ammonia, a more beneficial form of nitrogen via biological nitrogen fixation (BNF). Nitrogen fixation is an eco-friendly and manageable strategy to increase nitrogen levels in agricultural land. Thus, improving nitrogen bacterial production by genome editing could replace synthetic fertilizers to reduce dependency on conventional chemical fertilizers. In addition, discovering new plant microbiomes and understanding their structure, abundance, and diversity will open a new avenue to better understanding the agricultural scenario to sustain global food security under limited nitrogen conditions.

Author Contributions: All authors listed have made substantial, direct, and intellectual contributions to the work and approved it for publication.

Funding: Ze-Chun Yuan's lab appreciates the supports from Agriculture and Agri-Food Canada A-base project funds (J-002488 and J-003105), Mitacs fund, the Natural Sciences and Engineering Research Council of Canada (NSERC) Strategic Partnership Grant as well as the support from Ontario Greenhouse and Vegetable Growers Association.

Conflicts of Interest: The authors declare no conflict of interest.

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