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ROLES OF TRACE ELEMENTS IN RELATION TO ASSOCIATIVE AND SYMBIOTIC NITROGEN FIXATION

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Abstract

Trace elements including calcium (Ca^{2+}), copper (Cu), and zinc (Zn) play crucial roles in enzymatic catalysis, structural integrity, and maintaining redox balance. Their regulation significantly affects both microbial and plant metabolic processes, especially in systems involved in nitrogen fixation. Biological nitrogen fixation (BNF), occurring through associative and symbiotic pathways, offers a sustainable source of nitrogen for plants. This review compiles recent discoveries regarding the biochemical roles of Ca, Cu, and Zn, while also examining the differences between associative and symbiotic nitrogen fixation mechanisms, regulatory frameworks, and the interaction of trace metals with nitrogenase activity. Additionally, we highlight existing research gaps and suggest future avenues for enhancing nitrogen fixation through engineering.

Keywords: *Calcium; Copper; Zinc; Nitrogenase; Associative nitrogen fixation; Symbiotic nitrogen fixation*

1. Introduction

Trace elements including calcium (Ca^{2+}), copper (Cu), and zinc (Zn) play crucial roles in enzymatic catalysis, structural integrity, and maintaining redox balance. Their regulation significantly affects both microbial and plant metabolic processes, especially in systems involved in nitrogen fixation. Biological nitrogen fixation (BNF), occurring through associative and symbiotic pathways, offers a sustainable source of nitrogen for plants. This review compiles recent discoveries regarding the biochemical roles of Ca, Cu, and Zn, while also examining the differences between associative and symbiotic nitrogen fixation mechanisms, regulatory frameworks, and the interaction of trace metals with nitrogenase activity. Additionally, we highlight existing research gaps and suggest future avenues for enhancing nitrogen fixation through engineering.

2. Biochemistry of Trace Elements:

Calcium (Ca)

Calcium (Ca^{2+}) is well known as a **secondary messenger** in eukaryotes, mediating responses to stimuli via transient cytosolic changes (e.g., in leaves or root cells) (Kudla et al., 2018). It also has structural roles: in cell walls (middle lamella), stabilizing membranes, and binding to proteins like calmodulin.

In planta, Ca^{2+}-dependent protein kinases (CDPKs) regulate stress responses and metabolic pathways, including nitrogen assimilation. In microorganisms, Ca can modulate cell-wall stability and signaling under environmental stress.

Homeostasis is maintained via channels, pumps (Ca-ATPases), exchangers, and buffering proteins. Overaccumulation or deficiency disrupts metabolism and may influence uptake or competition of other metals (White & Broadley, 2003).

Copper (Cu)

Copper is a **redox-active** metal that cycles between Cu(I) and Cu(II). It is central in metalloenzymes such as **cytochrome c oxidase**, **Cu/Zn-superoxide dismutase (SOD)**, **laccases**, and **plastocyanin** (Yruela, 2005). It helps in electron transport, reactive oxygen species (ROS) detoxification, and oxidation-reduction reactions.

In nitrogen fixation, copper is indirectly important: it supports respiratory electron transport (energy generation) and oxidative stress defense in diazotrophs (Gupta et al., 2011). However, free Cu ions are toxic, catalyzing Fenton-type ROS generation; thus precise regulation (via Cu-transporters, chaperones, e.g. CTR1, ATP7A/B) is essential.

Zinc (Zn)

Zinc is a **non-redox** metal, but highly versatile. It stabilizes protein folds (e.g., zinc-finger domains), acts as structural elements, and is catalytic in many enzymes (e.g., DNA/RNA polymerases, carbonic anhydrase, alcohol dehydrogenase) (Broadley et al., 2007).

In plants, Zn is involved in auxin metabolism, membrane integrity, and chlorophyll synthesis. Zn deficiency impairs nucleic acid synthesis and nitrogen metabolism (Alloway, 2008). Cells buffer Zn via metallothioneins and control transport through ZIP and ZnT transporter families.

Integrated Interactions

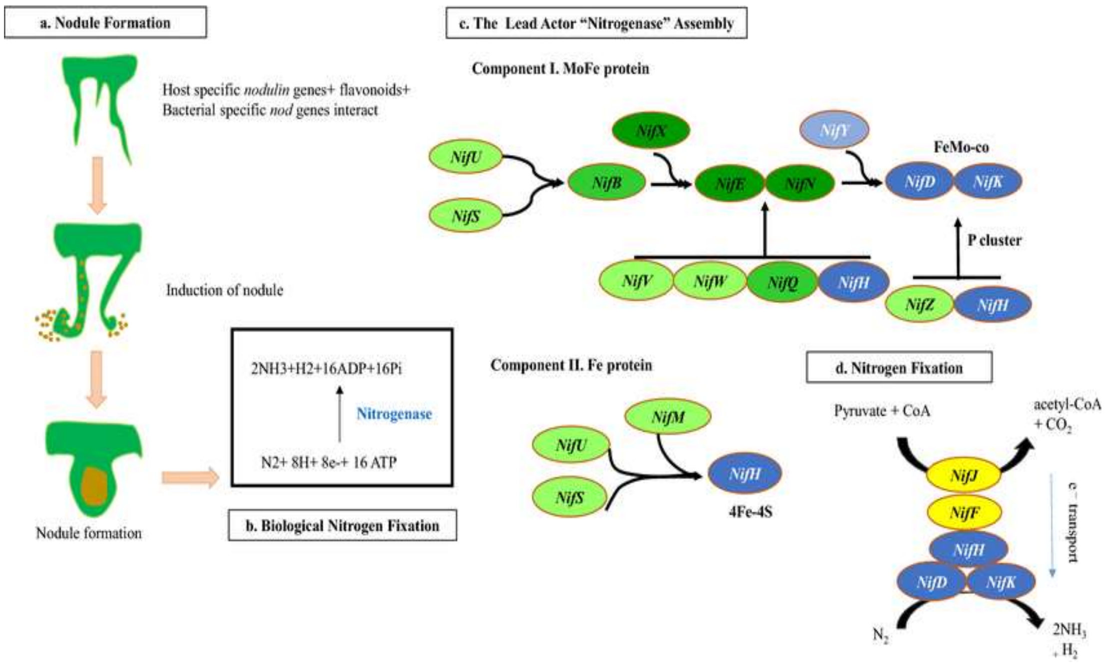
In living cells, Ca, Cu, and Zn homeostasis are not independent. For instance:

- Excess Ca may inhibit uptake of Zn or Cu by competitive binding sites.
- Metalloproteins may co-coordinate multiple metals (e.g., Cu/Zn-SOD).

- Disruption in one metal's balance often perturbs redox homeostasis, protein folding, and signaling networks (Jomova et al., 2012).

Hence, in nitrogen-fixing systems, the metal environment must support not only nitrogenase cofactors (Fe, Mo) but also maintain cellular systems that depend on Ca, Cu, Zn.

3. Biological Nitrogen Fixation: Mechanisms and Enzymology



The Nitrogenase Reaction

Nitrogenase catalyzes:

N 2+8 e −+8 H ++16 A TP → 2 NH 3+ H 2+16 A DP +16Π

It comprises two protein components:

1. **Fe-protein** (dimeric, ATP-binding)
2. **MoFe-protein** (heterotetramer with Mo–Fe–S clusters).

Alternative nitrogenases (V-dependent, Fe-only) also exist. Because nitrogenase is extremely **oxygen-sensitive**, fixation must occur under micro-aerobic or anaerobic conditions (Sun et al., 2011).

Diazotroph Lifestyles

Diazotrophs adopt various lifestyles:

- **Free-living**: e.g., *Azotobacter*, some cyanobacteria
- **Associative**: colonize root surfaces or rhizosphere (e.g., *Azospirillum*, *Herbaspirillum*)

- **Symbiotic**: intracellular in plant nodules (e.g., *Rhizobium* in legumes, *Frankia* in actinorhizal plants) (Rolic et al., 2015)

Each lifestyle differs in the degree of host interaction, oxygen control, nutrient exchange, and regulatory complexity.

4. Associative Nitrogen Fixation:

Definition and Significance

Associative nitrogen fixation refers to bacteria that fix N_2 in association with plant roots (rhizosphere or rhizoplane) without forming nodules. For example, *Azospirillum brasilense* and *Herbaspirillum seropedicae* provide nitrogen to grasses and cereals (Dobereiner et al., 1997). This mode is relevant for non-legume crops.

Mechanistic Integration

- Bacteria attach or colonize root surfaces, sometimes enter intercellular spaces but do not prompt organogenesis.
- Expression of nif genes (nifH, nifD, nifK) is regulated by oxygen, fixed nitrogen levels, and redox state.
- Carbon sources (e.g., root exudates such as malate, sugars) fuel bacterial metabolism, providing reducing power (NADH) and ATP for nitrogenase.
- Fixed nitrogen (NH_3, amino acids) leaks or is excreted and taken up by plants.

Strengths, Constraints, and Application

Associative fixation can extend benefits to cereals but is generally less efficient than symbiotic systems. Challenges include competition with other microbes, low energy yield, and regulation by nitrogen in soil. Use of such diazotrophs as biofertilizers is a major research focus (Frontiers, 2014).

Recent advances aim to engineer associative strains with improved oxygen tolerance, carbon efficiency, and competitiveness (Beck et al., 2019).

5. Symbiotic Nitrogen Fixation (Legume–Rhizobium Symbiosis):

Nodule formation and infection

- Legumes secrete **flavonoids** → rhizobia activate **nod genes** → produce **Nod factors**.
- Nod factors bind plant receptors, causing root hair curling and infection thread formation.
- Bacteria travel through infection threads, reach cortical cells, differentiate into **bacteroids** within nodules (Rhizobia–Host Interactions, 2011).

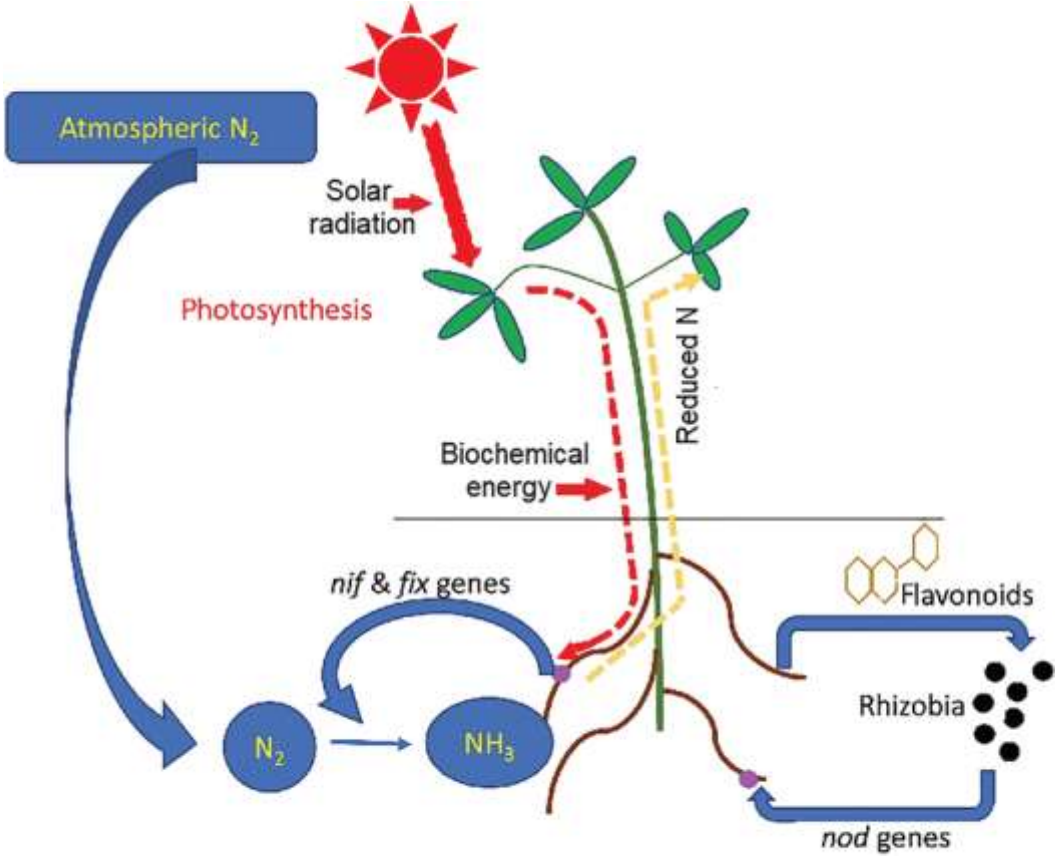
- In some legumes, **nodule-specific cysteine-rich peptides** direct differentiation (Symbiotic nitrogen fixation, 2014).

Nitrogenase activity inside nodules

- Plant supplies carbon (malate, succinate) to bacteroids, which oxidize it to generate ATP and reducing equivalents.
- **Leghemoglobin** binds O_2, maintaining low free O_2 to protect nitrogenase while supporting respiration (Udvardi & Poole, 2013).
- Ammonia enters plant metabolism (via glutamine synthetase/glutamate synthase).

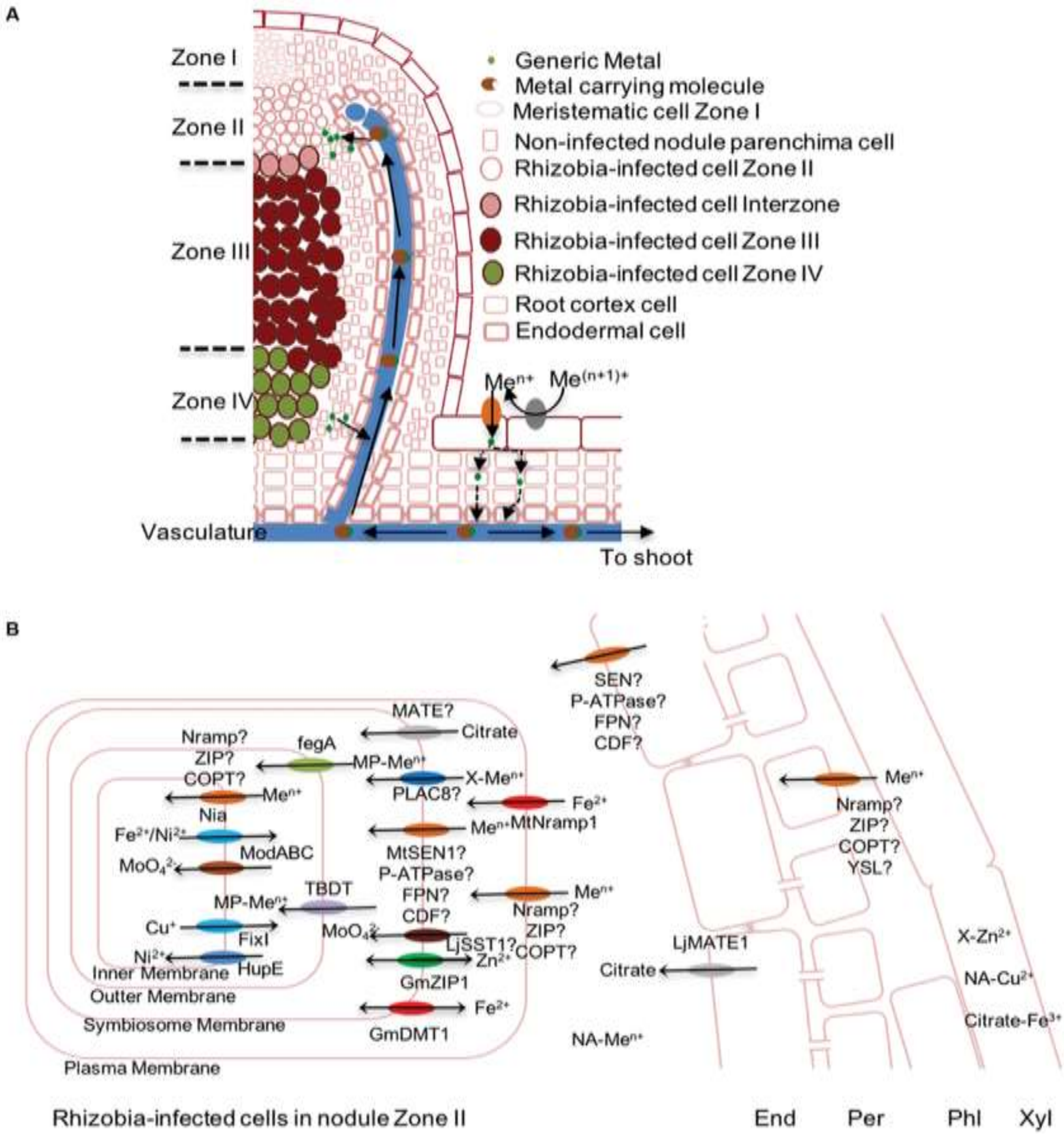
Regulation and limitations

- Autoregulation of nodule formation (AON) controls nodule number.
- Efficiency depends on oxygen diffusion, nutrient availability (P, Mo, Fe), and abiotic stress (salinity, heavy metals) (Effectiveness of nitrogen fixation, 2019).
- Feedback inhibition by fixed N is a common bottleneck.



Symbiosis in Non-Legumes & Engineering

- *Frankia* nodules in actinorhizal plants also require Ca, Cu, and Zn for signaling, ROS management, and enzyme regulation.
- Engineering cereals for endophytic nitrogen fixation demands precise delivery of these metals to bacterial endophytes for effective nitrogenase activity (Beck et al., 2019).



Metal transport in indeterminate nodules. (A) General overview of metal delivery and recovery to legume nodules. **(B)** Detail of transport process to deliver metals to symbiosomes in the nodule Zone II. Mt is used as a prefix to indicate *M. truncatula* proteins, Gm for *G. max*, and Lj for *Lotus japonicus*. X or MP are used to indicate an unkown metal chelator or a general metallophore, respectively. End stands for Endodermis, Per for Pericycle, Xyl for Xylem, Phl for Phloem, and Me^{n+} for a general metal.

6. Trace Metal Integration in Nitrogen Fixation:

While nitrogenase requires Fe, Mo, and sometimes V, Ca, Cu, and Zn influence the surrounding supporting systems:

- **Calcium (Ca^{2+}):** Regulates nodule formation, bacterial signaling, and nif gene activation.
- **Copper (Cu):** Supports electron transport, ROS detoxification, and ATP production. Excess is toxic, requiring tight homeostasis.
- **Zinc (Zn):** Stabilizes structural proteins and transcription factors controlling nitrogenase expression.

Key Insight: Disturbances in Ca, Cu, or Zn availability directly impair nitrogen fixation rates, nodule development, and efficiency of both associative and symbiotic systems (Hernandez et al., 2015; Gupta et al., 2011).

Understanding how these metals affect the entire fixation system (from gene regulation to energy metabolism) is crucial for optimizing BNF.

7. Future Prospects & Recommendations

- **Engineering nitrogen-fixing cereals**: synthetic biology, gene clusters, and microbiome design all converge here.
- **Optimized trace metal fertilization**: careful micronutrient amendments (nanoforms of Ca, Cu, Zn) to support both plant and microbial metabolism.
- **Omics integration**: transcriptomics, proteomics, and metallomics to dissect the metal-dependent regulatory networks in BNF.
- **Field trials and modeling**: quantify BNF contribution under varied soils, seasonal cycles, and metal regimes.

8. Conclusion

Ca, Cu, and Zn are indispensable for biochemical systems and indirectly modulate nitrogen-fixing metabolisms. Integrating knowledge of metal homeostasis with nitrogen fixation mechanisms offers promising routes toward sustainable agriculture. This review assembles a framework for future research, aiming at engineering robust nitrogen-fixing systems in diverse crops.

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