Genes Underlying Associative Biological Nitrogen Fixation in Maize: Host Regulation, Diazotrophic Metabolism, and Breeding Opportunities. A Critical Narrative Review

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ABSTRACT

Background. Maize does not form a legume-like nitrogen-fixing symbiosis but it associates with diazotrophic microorganisms whose activity may be supported by specific plant traits. Research on aerial-root mucilage raises an important question: whether maize genes that favor microbial nitrogen fixation also increase nitrogen acquisition by the crop.

Objective. This review examines maize genes and microbial nitrogenase systems associated with associative biological nitrogen fixation, distinguishing genetic function, microbial nitrogen fixation, nitrogen transfer and agronomic benefit.

Methods. A critical narrative review of primary studies and curated bibliographic sources available through October 2026 assessed genetic evidence, measured phenotypes, isotope tracing and relevance to field nitrogen nutrition.

Results. The genes *ZmSBT3* and *ZmAco3* are functionally characterized regulators of aerial-root mucilage exudation, but neither has been demonstrated to increase isotope-measured atmospheric nitrogen recovery by maize. Disruption of *ZmSBT3* increased mucilage exudation, while knock out of *ZmAco3* it, but neither significantly changed measured nitrogenase activity. Genetic mapping has identified additional candidate genes associated with aerial-root morphology and hydration responses, although their contributions to nitrogen transfer remain unresolved. The Sierra Mixe maize landrace provides isotope-based evidence of associative nitrogen fixation under specific environmental conditions. Findings from other landraces and engineered diazotrophs vary with nitrogen fertilization, reference plants and experimental design. Importantly, microbial nitrogen fixation, transfer of newly fixed nitrogen to maize and fertilizer-equivalent crop responses represent distinct processes requiring independent experimental verification.

Conclusions. Advancing associative nitrogen fixation in maize requires demonstrating causal relationships between host alleles, microbial nitrogenase activity and isotope-defined nitrogen recovery. Increased mucilage exudation or microbial fixation alone cannot establish improved crop nitrogen nutrition or productivity. Rigorous evaluation should integrate matched maize genotypes, nitrogenase-competent and deficient bacterial strains, isotope tracing and replicated field experiments across contrasting environments. Such approaches will clarify whether genetic manipulation of maize-associated microbial habitats can deliver meaningful and reproducible agronomic benefits.

Keywords: Associative nitrogen fixation; aerial root mucilage exudation; ZmSBT3; ZmAco3; nif genes.

INTRODUCTION

An Important Distinction Between Host Traits and Biological Fixation

Nitrogen availability remains a major constraint to maize production, particularly in nutrient-depleted soils and regions where fertilizer costs limit crop productivity. Diazotrophic bacteria and archaea can reduce atmospheric

dinitrogen to ammonia, providing a possible source of nitrogen beyond soil reserves and applied fertilizer (Nyanapah et al., 2026). Maize, however, lacks the specialized root nodules through which legumes maintain an intimate rhizobial symbiosis. Its associations with diazotrophs occur in the rhizosphere, root endosphere, and other plant-associated habitats. One unusual habitat, the mucilage surrounding aerial roots of certain landraces, has brought renewed attention to the prospect of associative biological nitrogen fixation (A-BNF) in this cereal (Van Deynze et al., 2018; Bloch, Ryu, et al., 2020).

Two genomes contribute to this association, but the tasks they perform are different. Maize genes influence root architecture, mucilage production, carbon release and aspects of microbial recruitment. By contrast, microbial genes encode the nitrogenase machinery and its regulation. An effective association must overcome an additional constraint: biologically fixed nitrogen must be released from or otherwise made available by microbial cells, transferred to maize tissues, and contribute meaningfully to plant nutrition. Mucilage volume, diazotroph abundance, nifH gene detection, acetylene reduction, biomass gains and atmospheric nitrogen recovered in the plant are therefore different measurements of the trait. Treating them as interchangeable obscures where the proposed benefit has actually been demonstrated (Unkovich et al., 2008; Higdon et al., 2020).

The Sierra Mixe maize landrace from Oaxaca, Mexico was the one that brought this distinction to light. Van Deynze et al. (2018) described the presence of extensive mucilage bearing aerial roots, associated diazotrophs and isotope-based evidence of atmospheric N in the plants grown under the conditions studied. Subsequent work has linked mucilage exudation to root cap and border cell development, identified genetic variation amongst the modern maize inbreds, functionally characterized the genes ZmSBT3 and ZmAco3 and mapped the aerial root traits in the Sierra Mixe-derived material (Pankievicz et al., 2022; Gao et al., 2023; Zhao et al., 2025; Laspisa et al., 2026). A separate line of study has focused on altering nitrogenase regulation in the maize-associated diazotrophs rather than in the maize host (Bloch et al., 2020; Martinez-Feria et al., 2024). These lines of work are complementary but have different endpoints.

The central question is therefore much more focused than simply whether maize can harbor nitrogen fixing microbes. Scholars should ask which host alleles and which microbial genes have been examined, what nitrogen has been tracked into the crop, and under what circumstances does an association lead to an improvement in field performance. This review focuses on these questions without automatically conferring fixation-gene status on a candidate root gene simply because it changes the environment in a way favorable to the microbes.

The review approach and evidence evaluation

Research into associative biological nitrogen fixation (A-BNF) in maize is inherently multidisciplinary, with evidence drawn from a number of different disciplines. Root anatomical and microbiological studies can identify the conditions that are conducive to the formation of diazotrophic associations. Genetic and biochemical work can go on to establish the underlying molecular mechanisms. Isotope work and field studies have to answer the real question of whether or not the association is in fact supplying nitrogen to the plant. Such distinction is important. A change in gene expression following root hydration does not prove that the gene is doing anything, any more than a reproducible phenotype in a transgenic plant would prove that it was fixing nitrogen. Nitrogenase activity itself is not a measure of how much nitrogen is actually being fixed and made available to the plant. Our assessment of the evidence therefore considers the experimental evidence, the genetic material, and the controls, as well as the environmental conditions under which each finding was made. We pay particular attention to studies that report contradictory or statistically non-significant results, as these determine which mechanisms are established, which remain merely possible, and which still lack evidence of providing a nutritional benefit to the plant.

Review design and source identification

This structured critical narrative review followed the SANRA principles of a clearly defined aim, transparent source identification, appropriate referencing and critical scientific reasoning (Baethge et al., 2019). We searched PubMed Central, AGRIS, Google Scholar, journal-publisher websites and institutional repositories for empirical and methodological literature available up to 30 October 2026. No lower publication-year cutoff was prespecified. Backward citation searching of relevant primary papers was used to locate foundational

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investigations and connected studies. The searches combined maize (*Zea mays*), associative or biological nitrogen fixation, aerial roots, mucilage, ZmSBT3, ZmAco3, root QTL, GWAS, diazotrophs, nitrogenase and nif genes with isotope tracing, acetylene reduction and nitrogen transfer. Comparative studies of other cereals were considered where they could inform the biological interpretation of maize observations.

The following Boolean formulations reconstruct the principal documented search concepts and are provided for transparency: (1) ("maize" OR "Zea mays") AND ("associative nitrogen fixation" OR "biological nitrogen fixation" OR diazotroph* OR nitrogenase); (2) ("maize" OR "Zea mays") AND ("aerial root*" OR mucilage OR "border cell*") AND ("nitrogen fixation" OR "15N2" OR "15N" OR "nitrogen transfer"); (3) ("maize" OR "Zea mays") AND (ZmSBT3 OR ZmAco3 OR QTL OR GWAS OR "mucilage exudation"); and (4) ("maize" OR "Zea mays") AND (nifH OR nifA OR nifL OR "engineered diazotroph*" OR "acetylene reduction" OR "isotope dilution"). Terms were adapted conceptually to each source; these formulations are not represented as verbatim saved database search histories, because platform-specific search records and export files were not archived. The original protocol also identified Web of Science Core Collection, Scopus and CAB Abstracts, but reproducible searches of those subscription databases were not completed. Accordingly, the evidence base is selective rather than exhaustive, and no PRISMA screening counts or comprehensive-study-coverage claim is made.

Eligibility and critical interpretation

We focused on the primary studies that actually looked at maize gene function, root development, mucilage production, microbial nitrogenase activity or the assimilation of atmospheric N into plant tissue. The studies on plant growth promotion and microbial community composition were used as supporting evidence only and their results were not considered as direct evidence of A-BNF. We used work on legume symbioses and nitrogenase biochemistry to explain only the relevant mechanisms, without assuming they would be identical in maize.

Eligible core studies reported original maize data on host-gene perturbation, aerial-root or mucilage traits, root-associated diazotrophs, nitrogenase activity, isotope-based atmospheric N acquisition, or crop responses to diazotrophic inoculation. Studies of sorghum, legume symbiosis and nitrogenase biochemistry, together with methodological reviews, were included only for comparative or interpretive context. Records lacking empirical information relevant to these questions, duplicate reports of the same observations, and studies reporting general plant growth promotion without evidence of fixation were not used to support a claim of atmospheric N transfer. Microbiome-only studies could inform microbial recruitment but were not treated as evidence of host N acquisition. Eligibility decisions were made by critical reading of the available papers, rather than a formally logged, independently replicated screening process.

To separate out the biological processes under investigation from the conclusions that can be drawn from them we have grouped the evidence into five classes (Figure 1A). Class A is direct evidence that variation in a specific maize allele changes the plant's ability to take up atmospheric nitrogen, as demonstrated by appropriate isotope work. Class B are maize genes which have been functionally validated for traits associated with A-BNF (e.g., mucilage exudation) but which have not been shown to have any effect on the plant's ability to take up atmospheric nitrogen. Class C are candidate genes identified via QTL, GWAS, gene expression or orthology but have not been functionally validated for the trait in question. Class D are microbial genes whose role in nitrogenase function has been established through genetic or biochemical work, although the transfer of the fixed nitrogen to the plant has not been demonstrated. Class E are studies of root development, microbial community structure and the like which provide biological plausibility but which do not establish a causal contribution to fixation.

These categories describe the nature and extent of the evidence available rather than defining a hierarchy of study quality. Functional validation of a maize gene involved in mucilage exudation (Class B) is not, by itself, sufficient to validate isotope-based evidence that the same gene is involved in increased atmospheric nitrogen acquisition by maize (Class A). Likewise established microbial nitrogenase activity (Class D) is not in itself sufficient to demonstrate nitrogen transfer to the host.

The unit of classification was the specific result rather than the publication as a whole. For each relevant result, we considered the biological compartment measured, genetic manipulation or association tested, assay used,

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control material and claimed endpoint. Host-gene perturbation experiments without isotope-defined plant N recovery were assigned to Class B; positional or expression candidates to Class C; functional microbial nitrogenase studies to Class D; and descriptive developmental or community findings to Class E. Class A was reserved for experiments showing that a defined maize allele causes a change in plant recovery of atmospheric N, a criterion not met by the studies reviewed. A paper could consequently contribute findings to more than one category. Plant isotope evidence without a matched host-allele test, such as the Sierra Mixe studies, was discussed separately and was not labelled Class A. No independent dual classification, inter-reviewer agreement analysis, or pre-specified consensus-adjudication procedure was documented; the assignments are therefore the interpretive synthesis of the review authors. Figure 1B maps the main studies to the corresponding endpoints and shows where causal evidence remains missing.

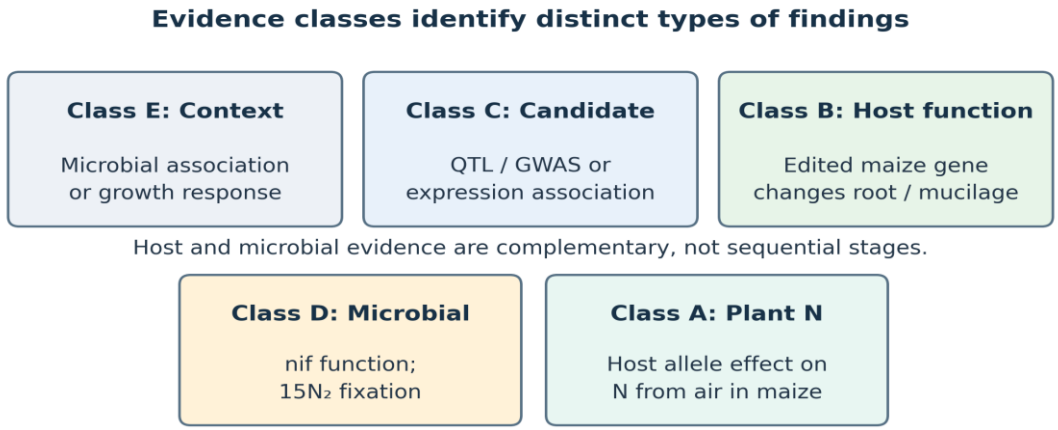

Figure 1A. Complementary evidence classes for host traits, microbial nitrogenase function and isotope-defined nitrogen transfer to maize (Categories are not sequential steps or a universal ranking of study quality).

Biological basis and compartments of associative BNF in maize

Associative N fixation in maize occurs without the specialized nodules found in legumes. Diazotrophs come into contact with the plant in several spheres (rhizosphere soil, internal root tissues, and aerial root mucilage), and each represents a different ecological context. Access to plant carbon, water, oxygen, and attachment surfaces, in turn, determines whether diazotrophs can persist and express active nitrogenase. Aerial root mucilage is particularly interesting because its hydrated, carbohydrate-rich matrix can support very dense microbial communities. However, a suitable habitat is only the first step; each case must demonstrate how fixed nitrogen is delivered to the plant and its actual contribution to maize nutrition. Therefore, studies of aerial-root mucilage should be considered alongside, rather than simply extrapolated to, rhizosphere and endosphere associations.

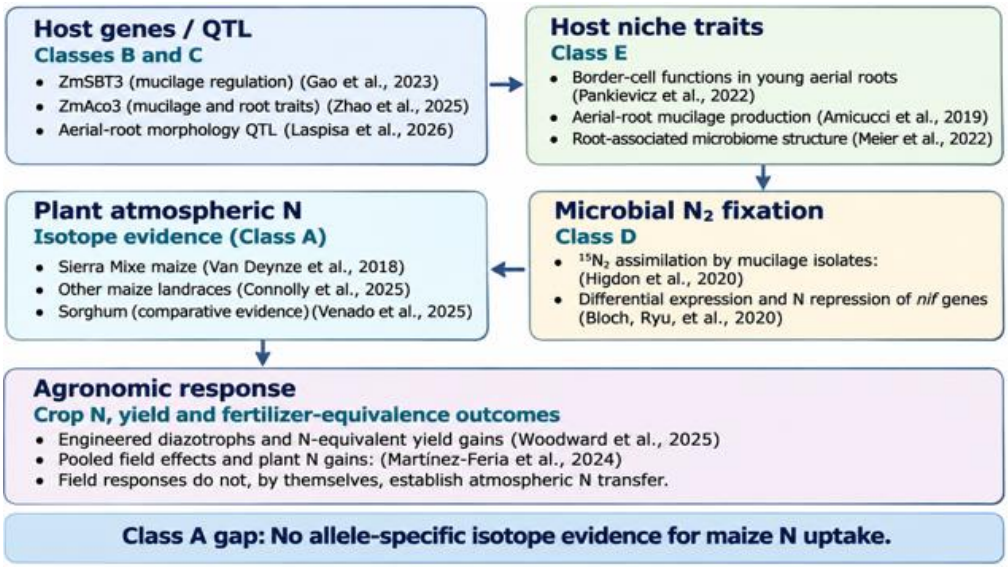

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Figure 1B. Evidence map connecting maize host genes, root-associated habitats, microbial nitrogenase activity, atmospheric N recovery and field response. Studies are placed by the outcome they measured, not by a universal ranking of quality. Class A requires an allele-specific causal effect on isotope-defined plant atmospheric N recovery and remains unestablished; observed plant nitrogen derived from the atmosphere (Ndfa) without a host-allele test does not meet this criterion.

Aerial-root mucilage as a diazotrophic niche

Sierra Mixe maize produces numerous aboveground nodal roots. After rain or wetting, these roots exude a viscous, carbohydrate-rich mucilage that holds water and provides a substrate for colonizing microbes. Van Deynze et al. (2018) found putative diazotrophs, acetylene reduction activity, bacterial $^{15}N_2$ incorporation and isotope evidence, which were consistent with atmospheric N_2 being a source of nutrition for the host. Across sites, years, and assessment methods, the proportion of plant N derived from atmospheric N_2 varied from 29% to 82%. These are values for particular plants and particular experimental conditions. They are not an average for maize as a species and are certainly not a prediction of what an elite inbred would obtain under field conditions where fertilizer is applied.

The 29–82% range reported by Van Deynze et al. (2018) combines estimates from different field years, locations and isotope approaches, rather than repeated determinations of a fixed genotype-specific N contribution. Natural-abundance ^{15}N estimates depend on the isotopic contrast between maize and non-fixing reference plants, the assumed B value, and whether both acquire soil N from comparable pools. Differences in rooting depth, phenology, mineral N availability and sampling time can alter this comparison even without a change in fixation. Soil ^{15}N-enrichment or dilution approaches increase the isotopic contrast but still depend on comparable soil-N acquisition by the test and reference plants. By contrast, $^{15}N_2$ gas feeding can demonstrate fixation directly in the labelled microbial compartment or transfer into plant tissue, depending on what is sampled, but short exposures do not quantify season-long whole-plant N acquisition. The high and low estimates must therefore be interpreted against their respective controls and sampling periods, not combined into a single landrace-level expectation (Van Deynze et al., 2018; Unkovich et al., 2008).

Comparative evidence reinforces this caution. In replicated Ohio field experiments involving 21 landraces and three improved maize varieties, Connolly et al. (2025) estimated approximately 21–46% nitrogen derived from the atmosphere (Ndfa) in unfertilized material and 0–26% in fertilized material when sunflower was used as the non-fixing reference. Results differed when a conventional maize reference was substituted, showing that both fertilizer management and reference choice materially affect interpretation. In sorghum, Venado et al. (2025) combined acetylene reduction, $^{15}N_2$ feeding and isotope dilution, estimating up to about 43% Ndfa in greenhouse-grown accessions bearing mucilaginous aerial roots. Their isotope and non-fixing bacterial controls strengthen the evidence for transfer in that experimental system, but the value cannot be extrapolated to maize or treated as field fertilizer replacement. Across these studies, the presence of mucilage-associated fixation is more persuasive than any universal percentage of crop N supplied by the association.

Mucilage chemistry matters because N_2 fixation uses ATP and reducing power. Amicucci et al. (2019) characterized a complex polysaccharide, while Higdon et al. (2020) characterized the carbohydrate-active enzymes and transport systems in the mucilage-derived microbes and showed $^{15}N_2$ incorporation by the isolates in vitro. This is consistent with a plausible C-N interaction. However, it does not reveal how much of the newly fixed N is released from the microbial cells, how efficiently maize takes it up, or how much of it makes it to the grain. Those are separate experimental questions.

Border cells, root developmental stage and environmental activation

Aerial roots' ability to support diazotrophic activity is developmental and moisture-dependent. Pankievicz et al. (2022) found greater mucilage exudation and higher nitrogenase activity in young, relatively thick aerial roots than in older roots. Water exposure led to increased exudation via root cap and border cell activity. Transcriptomic analysis revealed changes in genes involved in polysaccharide metabolism and nitrogen metabolism. The mucilage exudation is therefore a function of the interaction between root development and the environment rather than a simple function of root anatomy. These transcriptional responses are not necessarily

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indicative of any regulatory role in nitrogen fixation. The changes in gene expression may simply reflect hydration, tissue maturation, or metabolic adjustment to exudation. Distinguishing between these possibilities will require experiments that consider root age, diameter, and developmental stage as well as the timing and duration of the rainfall or artificial wetting.

Distinguishing between aerial root mucilage and belowground microbial habitats is just as important. The diazotrophs in the rhizosphere and root endosphere are exposed to very different sources of carbon, very different oxygen conditions and very different opportunities for colonization and for nitrogen exchange. Genetic changes that increase mucilage production or expand the aerial root habitat may have little effect on nitrogen fixation associated with belowground roots. It is unclear whether these two spheres operate independently, complement one another, or depend on environmental conditions. To determine their relative contributions requires measurements of nitrogenase activity and atmospheric N transfer to maize in each compartment, ideally under very similar genetic and environmental conditions.

Maize host genes

Maize genotypes differ in aerial-root architecture, mucilage exudation, carbon allocation and the microbial communities associated with their roots. Several of these host traits have a measurable genetic basis. By contrast, the inheritance of atmospheric nitrogen actually recovered by maize remains poorly resolved. Inferring a polygenic architecture for realized A-BNF from the many quantitative trait loci reported for root morphology alone would be misleading: as these are different phenotypes with different causal requirements.

Association mapping, quantitative trait locus (QTL) analysis and transcript profiling have supplied candidate geenes for root and mucilage traits (Table 1). Their value lies in identifying testable hypotheses, not in establishing nitrogen-transfer genes. We therefore consider separately the maize genes with functional perturbation evidence, the loci supported by mapping or expression, and developmental genes that may alter microbial habitat without directly affecting fixation.

***ZmSBT3*: a functionally tested negative regulator of mucilage exudation**

Gao et al. (2023) looked at 258 maize inbred lines and found significant genetic variation in the amount of mucilage secreted by the aerial roots. HN5-724 had very high diazotrophic populations and high nitrogenase activity. A Genome-Wide Association Study (GWAS) across three environments identified 17 candidate genes. Functional analysis then established *ZmSBT3* (a subtilisin-like serine protease) as a negative regulator of mucilage exudation. Two independent CRISPR/Cas9 knockout lines in the B104 background produced more mucilage than the wild type, though the increase in nitrogenase activity was not statistically significant. Under nitrogen-free, sterile sand conditions, inoculation with a defined diazotrophic isolate led to greater bacterial colonization, greater biomass and greater total plant N in the knockout lines. The findings therefore show that *ZmSBT3* has an effect on a host trait related to diazotrophic colonization. However, the mechanism by which the increased plant N is achieved is still unknown.

The distinction between mucilage production and acquisition of atmospheric nitrogen is of course key to interpreting these results. More mucilage may create a more conducive environment for diazotrophs, but greater microbial colonization does not necessarily equate to greater nitrogen fixation or transfer to maize. The difference in total plant nitrogen alone does not reveal the source of that nitrogen without isotope work. *ZmSBT3* should therefore be viewed as a functionally validated regulator of mucilage exudation rather than as a proven determinant of A-BNF. A causal link would have to be established via comparative $^{15}N_2$-tracing experiments using wild-type plants, independent knockout lines and complemented lines as well as nitrogenase-competent and nif-deficient bacterial controls. That would be the only way to determine whether altered *ZmSBT3* function has an effect on microbial colonization, nitrogenase activity, transfer of newly fixed nitrogen to maize, or on nitrogen acquisition via alternative routes.

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***ZmAco3*: a positive contribution to mucilage exudation**

Zhao et al. (2025) investigated genetic variation in aerial root mucilage exudation across a range of maize populations under differing nitrogen regimes. They identified 15 QTL, 68 QTNs and 59 candidate genes associated with mucilage exudation. When they separated out the constitutive exudation from the exudation responsive to low nitrogen, they found 8 QTL, 16 QTNs and 19 candidate genes. There was functional evidence from an ethyl methanesulfonate (EMS)-derived loss-of-function mutation in *ZmAco3,* which introduced a premature termination codon and resulted in reduced mucilage exudation compared to the baseline genotype. This points to a role for *ZmAco3* in the regulation of mucilage production and reveals genetic variation in its response to nitrogen availability. The QTL identified are associated with variation in a host phenotype related to diazotrophic colonization rather than with variation in the ability to acquire atmospheric nitrogen.

Zhao et al. (2025) also observed a smaller increase in total plant N following diazotroph inoculation in the *zmAco3* mutant (though the contribution of atmospheric N to maize uptake remained unquantified by the absence of isotope tracing). The results are a rather interesting contrast to those seen with *ZmSBT3* where disruption of the gene leads to increased mucilage exudation, whereas *ZmAco3* disruption reduced mucilage. The two genes thus appear to have very different effects, but that does not tell us whether they are acting through independent pathways, interacting pathways, or shared pathways. It also does not mean that increased mucilage exudation necessarily improves N_2 fixation either, as we would have to consider the cost of secreting the mucilage and the efficiency of N transfer to the host. Therefore,the breeding value of these genes will only be ascertainable through comparisons in matched genetic backgrounds, analysis of their combined effects, and direct measurement of the incorporation of atmospheric N into maize tissues.

Mapped aerial-root QTL and provisional positional candidate genes

Laspisa et al. (2026) examined the genetic architecture of aerial maize root morphology in three segregating populations derived from the Sierra Mixe landrace. They found 37 QTLs associated with the number of nodes with aerial roots, roots per node, and root size. The broad sense heritability estimates were between 0.65 and 0.83 and the QTLs collectively explained 23-51% of the phenotypic variation (depending on population and trait). This shows there is a considerable amount of genetic variation in aerial root architecture and provides a foundation for identifying developmental traits that might be involved in the establishment of diazotrophic associations. Their relevance to A-BNF is, of course, entirely indirect. Neither the heritability estimates nor the proportions of phenotypic variation explained can be taken as measures of the genetic control of atmospheric nitrogen uptake, which was not in fact measured at all.

Six of the previously characterized/annotated maize genes were found to lie within the mapped QTL intervals: *ZmARF4*, *ZmD1/ZmGAOX3*, *ZmCCT10*, a NAC transcription factor (Zm00001eb096740), *ZmABF2* and *ZmEpc1*. Given their known or predicted roles in plant development these genes would be reasonable candidates for further study. It is important to stress that colocalization with a QTL does not prove that a particular gene is responsible for the phenotypic variation observed. Nor does it prove that the gene has any effect on diazotrophic colonization, nitrogenase activity, or the transfer of fixed nitrogen to maize (Laspisa et al., 2026). All of those relationships would require functional validation beyond mere positional colocalization.

Interpretation is further muddied by the genetic links between aerial root development, flowering time, plant height, and duration of vegetative growth. A locus that leads to increased aerial root production could be having a more general effect on plant development rather than being specifically responsible for an adaptation that would facilitate diazotrophic activity. For instance, a later maturing plant may simply have more aerial root-bearing nodes as a result of its extended vegetative phase. Such an effect could lead to an apparent link between root architecture and A-BNF potential without any actual increase in N_2 fixation. Fine mapping, near-isogenic comparisons, and targeted allelic substitutions would be useful in separating out the direct effect on root development from the effect on general development. This would have to be combined with isotope-based measurements of N transfer to determine whether the loci in question are of any real value for improving A-BNF or simply result in a change in root architecture.

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Established maize root-development regulators as contextual genes

The maize rtcs gene, which encodes an auxin-responsive LOB-domain transcription factor, is indispensable for the initiation of seminal and shoot-borne roots (Taramino et al., 2007). Its paralog, ***rtcl***, also contributes to the development of shoot-borne root systems, although with partially distinct developmental functions (Xu et al., 2015). Functional evidence for both genes is strong, arising from genetic and phenotypic analyses that directly link gene disruption to impaired root formation. Their importance to root architecture makes them relevant to the ecological context in which associative diazotrophs establish and persist, since root system structure influences the availability and distribution of microbial niches. However, evidence linking *rtcs* or *rtcl* to atmospheric nitrogen acquisition is lacking. Neither gene has been shown to alter nitrogen fixation rates, the transfer of fixed nitrogen to the host, or the quantity of atmospheric nitrogen recovered by maize. This distinction is important because genes that shape root development do not necessarily influence the biological processes required for realized A-BNF. Rather, ***rtcs*** and ***rtcl*** exemplify a broader category of developmentally important loci whose effects on diazotrophic interactions are biologically plausible, but have not been demonstrated experimentally. They should therefore be regarded as genes that influence potential microbial habitat formation rather than as validated A-BNF genes

Broader host control of root-associated microbial consortia

Variation in microbiome composition across maize genotypes is well established, but the biological significance of that variation will depend on the function under consideration rather than on the consortia composition. Evidence from field studies show that host genetics plays a role in the assembly of rhizosphere microbial communities. Peiffer et al. (2013) showed genotype-associated differences in rhizosphere composition in the field, and Walters et al. (2018) demonstrated that a number of microbial taxa are heritable across a large population of maize lines. Meier et al. (2022) went on to show that the effect of host genotype on certain rhizosphere taxa was actually greater under low nitrogen than under higher nitrogen. All of this evidence points to the conclusion that the maize genotype influences microbial recruitment and that the magnitude of that influence is dependent on the environment (in this case nutrient availability).

What is still unsettled is whether the compositional changes actually result in any difference in nitrogen acquisition. The mere presence of certain microbial taxa at different abundances across genotypes does not, by itself, prove they are involved in A-BNF. We still need evidence that the taxa that are enriched in particular genotypes are actually fixing atmospheric N, releasing plant-available N, and making a real contribution to maize N nutrition. The literature currently supports a much stronger case for genetic control of microbiome assembly than for genetic control of realized atmospheric N acquisition. Blurring the two together risks ascribing functional significance to microbial community patterns that may be ecologically interesting but nutritionally trivial.

This lacuna should be closed via experiments that bring together host genetics, strain-level characterization of the microbiota, and direct measurements of nitrogen flux all within the same experimental system. Isotope tracing is obviously of immense value as it can tell you whether the microbe is actually transferring nitrogen to the plant or not. Equally important is the measurement of root biomass, architecture, and carbon allocation. A host genotype that produces a larger root system, or a more carbon-rich root system, may simply support a larger microbial population regardless of any direct contribution to nitrogen fixation. In that scenario, an increase in the abundance of a given taxon could be entirely attributable to a larger root system or more carbon available to the microbe, rather than any direct effect on plant nitrogen status. The most robust way of separating out the microbial response to plant phenotype from the microbial processes that are actually improving plant nitrogen status is to combine the functional isotope data with a detailed characterization of the root traits.

Microbial genes directly responsible for nitrogenase activity

Nitrogenase catalysis is a microbial function. Its structural genes, maturation machinery and regulatory networks determine whether diazotrophs can reduce atmospheric N_2 under the oxygen, carbon and nitrogen conditions encountered near maize roots (Table 2). The core components are conserved sufficiently to make comparative work useful, but the organization and regulation of these genes differ among taxa. That variation helps explain

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why two isolates with recognizable *nif* genes need not perform equally in mucilage, rhizosphere soil or N-fertilized fields. The relevant genetic question is therefore broader than whether a nitrogenase marker is present: can a particular strain express a functional system, obtain sufficient energy and deliver fixed nitrogen beyond its own biomass?

Table 1. Genes and genetic regions associated with associative biological nitrogen fixation in maize: findings and strength of the evidence

Gene or locus	**What the research found**	**Has increased N_2-derived nitrogen in maize been demonstrated?**	**Evidence class** [a]	**Citation(s)**
ZmSBT3	Disabling this gene increased aerial-root mucilage and total plant nitrogen. The increase in measured nitrogenase activity was not statistically significant.	No. Greater atmospheric N_2 recovery by maize has not been shown using isotope tracing.	B	Gao et al. (2023)
ZmAco3	A loss-of-function mutation reduced aerial-root mucilage. The nitrogen response following bacterial inoculation also declined.	No. Direct isotope evidence of increased nitrogen transfer to maize is lacking.	B	Zhao et al. (2025)
37 aerial-root QTL	Genetic regions were linked to the number of aerial-root-bearing nodes, roots per node, and root size in Sierra Mixe-derived maize.	No. The mapped traits were root characteristics, not nitrogen fixation or plant N_2 uptake.	C	Laspisa et al. (2026)
ZmARF4, ZmD1, ZmCCT10	These genes lie within aerial-root QTL regions and have known or proposed roles in plant development.	No. Their effects on associative N_2 fixation or transfer have not been established.	C / E	Laspisa et al. (2026); developmental studies not specified in source table
NAC transcription factor, *ZmABF2, ZmEpc1*	Candidate genes overlap aerial-root QTL regions and may influence root growth or development.	No. An effect on atmospheric nitrogen recovery has not been demonstrated.	C / E	Laspisa et al. (2026)
rtcs, rtcl	Functionally studied genes involved in the formation of shoot-borne roots.	No. A contribution to associative biological nitrogen fixation has not been established.	E	Taramino et al. (2007); Xu et al. (2015)
Host-associated microbiome QTL / associations	Maize genotype is associated with differences in root-zone microbial communities under different nitrogen conditions.	No. Changes in microbial composition do not demonstrate transfer of fixed N_2 to maize.	C / E	Meier et al. (2022)

[a] **Evidence classes:** B, gene function tested experimentally; C, candidate gene or genetic association; E, developmental or microbial-context evidence. Combined classes indicate more than one type of evidence. These classes do not establish that a gene increases nitrogen acquired from the atmosphere.

Structural and assembly genes

The classic molybdenum-dependent nitrogenase system is built around a core set of genes that encode the proteins that perform nitrogen reduction and those that assist in enzyme assembly. The dinitrogenase reductase protein (Fe protein) is encoded by the *nifH* gene and the catalytic Dinitrogenase (MoFe) protein is made up of subunits encoded by the genes *nifD* and *nifK*. A further set of genes (*nifB, nifE and nifN*) are involved in the synthesis and incorporation of the FeMo cofactor and *nifU* and *nifS* are involved in Fe-S cluster biogenesis in many of the diazotrophs. Other genes (e.g. *nifV*) are required in some but not all taxa. Thus, the genetic basis of nitrogen fixation differs among diazotrophs. Comparative genomic analyses have revealed considerable variation in gene organization, accessory functions and regulatory networks. It is therefore highly unlikely that any one operon model will be sufficient to describe the functional requirements of all the maize-associated diazotrophs (Higdon et al., 2020; Harwood, 2020).

The genetic diversity of the *nif* genes has important implications for the interpretation of molecular surveys. Detecting a *nifH* sequence is frequently taken as indicative of the potential for nitrogen fixation, as the gene is found in a wide range of diazotrophs and encodes a key component of the nitrogenase complex. The presence of a *nifH* homolog does not, by itself, indicate nitrogen fixation under the study conditions, nor does it reveal the extent of nitrogen fixation that may be occurring. The interpretation is further muddied by the existence of homologous sequences which have non-canonical functions and by the methodological constraints of PCR-based surveys. Primer sets commonly used to amplify *nifH* may fail to amplify divergent lineages, which will inevitably bias estimates of diazotroph diversity and abundance (Gaby & Buckley, 2012). Ultimately, molecular detection alone is not a sufficient basis for judging the functional contribution of a microbial community to nitrogen acquisition.

There is a much more robust evidentiary basis for linking gene content to biological activity via a number of independent and convergent lines of evidence. It may be argued that nitrogenase genes are being expressed, that enzyme activity is present, and that atmospheric nitrogen is being incorporated into biomass. The strongest evidence for fixation comes from the use of $^{15}N_2$ labeling, which allows tracking the newly fixed nitrogen through the biological system. However, one has to be careful about where the label is found. Detection of the isotope in bacterial metabolites is evidence of fixation occurring in the microbial compartment, but that in itself is not evidence of nutritional benefit to the host plant. The detection of the label in maize tissue is evidence of a different phenomenon; the fixed nitrogen has been taken up by the plant.

Regulatory genes and nitrogen-dependent repression

A-BNF is costly for the diazotrophic bacteria involved. Nitrogenase's oxygen sensitivity adds another constraint on its activity. Many of the diazotrophs therefore regulate both the synthesis and activity of nitrogenase in response to nitrogen availability and other environmental conditions. In many of the Proteobacteria, the transcription of nitrogen fixation genes is activated by *NifA*, though the activity of the gene can be subject to modulation by *NifL* and other regulatory systems, which in turn can vary in their organization and response between taxa. Regulation of nitrogenase is only one aspect of the problem of harnessing A-BNF for crop nutrition. Fixed nitrogen may well remain in the bacterial biomass, and not be available to the host plant at all. It is therefore not sufficient to have high levels of nitrogenase activity to conclude that a bacterial association is making a significant contribution to the plant's nitrogen acquisition. The amount of fixed nitrogen that is actually transferred to the plant is the more relevant parameter in terms of its agronomic value.

Bloch et al. (2020) looked at this constraint by genetically modifying a maize root-associated isolate of *Kosakonia sacchar*i. They targeted the regulatory and metabolic pathways that govern nitrogen-sensing, N fixation, and ammonium assimilation with the intention of maintaining nitrogenase activity and increasing ammonium exudation under conditions that would normally suppress fixation. The modified bacteria showed

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increased *nif*-associated activity and ammonium exudation in both the laboratory and in the plant-associated systems tested. This provides evidence that bacterial nitrogen regulation can be engineered to promote extracellular ammonium release (although, ammonium exudation does not necessarily equate to nitrogen uptake by the maize). The study focused on engineering the bacterial symbiont rather than identifying or modifying a maize nitrogen fixation gene. Its agronomic value will therefore depend on whether the engineered strains can consistently colonize maize roots, maintain the modified phenotype, outcompete the indigenous microbiota, and deliver a measurable amount of nitrogen under field conditions, particularly where the application of inorganic nitrogen would otherwise suppress fixation.

Alternative nitrogenases and mucilage carbon metabolism

Nitrogenase diversity in diazotrophic bacteria extends beyond the well-known molybdenum-dependent enzyme. Some diazotrophs have alternative vanadium-dependent (vnf) or iron-only (anf) nitrogenases that differ in their metallocofactor, catalytic properties, and regulatory requirements (Harwood 2020). This diversity makes it very difficult to identify and functionally characterize the N_2-fixing bacteria associated with maize aerial root mucilage. In a study of nearly 600 mucilage-associated bacterial isolates, Higdon et al. (2020) found substantial variation in nitrogen fixation gene configurations, including non-canonical *nif* signatures, and demonstrated $^{15}N_2$ assimilation by the cultured organisms. The study showed high genetic heterogeneity within the mucilage-associated bacterial community and that at least some cultured isolates could fix N_2. However, they did not show that the non-canonical sequences encode a novel nitrogenase system. Sequence divergence alone does not prove the existence of a novel biochemical pathway. Functional interpretation requires evidence of nitrogenase expression and activity together with physiological characterization under defined metal and oxygen conditions. This is particularly relevant to microbial prospecting, where genomic novelty may identify a candidate of interest without necessarily demonstrating a contribution to N_2 fixation.

The ability to exploit carbon derived from mucilage is another potential source of functional variation in these bacteria. The capacity to utilise the complex carbohydrates present in maize aerial root mucilage (Amicucci et al. 2019) is influenced by the presence of glycoside hydrolases, carbohydrate transporters and enzymes of central carbon metabolism. Carbon metabolism will provide the ATP and reducing power required for nitrogenase activity, but the ability to metabolise mucilage carbohydrates does not in itself confer an advantage in nitrogen fixation. That advantage would only be conferred if there was a co-ordination of substrate utilisation, respiratory metabolism, oxygen regulation and nitrogenase expression. Genes involved in carbohydrate acquisition and metabolism should be separated from those directly involved in nitrogen fixation, and their functional significance established by experiment rather than inferred from genome annotation. A useful comparative approach would be to look at carbohydrate utilisation, oxygen tolerance, nitrogenase expression and $^{15}N_2$ assimilation in the same strains to determine whether there is a correlation between metabolic capacity and fixation activity. Subsequent work with maize would then be required to establish whether strains physiologically well-adapted to the mucilage environment actually transfer measurable quantities of fixed nitrogen to the host (Higdon et al. 2020; Amicucci et al. 2019). Such evidence would be key to distinguishing adaptation to the mucilage environment from a real contribution to the host's nitrogen nutrition.

Genetic and environmental regulation of realized N transfer

Nitrogen fixed by a bacterium becomes agriculturally useful only if it reaches the plant in an available form and in meaningful quantities. Release from microbial cells, transport through the root-associated environment and uptake by maize each impose a possible limitation. These steps respond differently to fertilizer nitrogen, oxygen, water, host carbon supply and competition among microbes. A positive assay for nitrogenase activity is therefore compatible with no measurable improvement in plant nitrogen. For that reason, assessing A-BNF as a breeding target requires simultaneous measurements of microbial function, plant nitrogen recovery and crop performance, rather than extrapolation from any one of them.

Nitrogen fertilizer creates both biological and agronomic trade-offs

Nitrogen availability is a physiological constraint on A-BNF in maize. Mineral N can suppress nitrogenase expression and activity in many unmodified diazotrophs, but severe N deficiency will limit plant growth and the

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supply of photosynthates required to maintain a population of metabolically active bacteria. Additionally, moderate N availability may support host growth without necessarily promoting fixation.

Table 2. Major bacterial gene modules relevant to associative N-fixation in maize

Gene group	**Main established function**	**Evidence requirement and limitation**
nifH; nifD; nifK	Electron transfer and Mo-nitrogenase catalytic subunits.	Expression and $^{15}N_2$ fixation required; DNA presence alone is insufficient.
nifB; nifE; nifN	Cofactor assembly and maturation of active nitrogenase.	Function/necessity depends on genomic context.
nifU; nifS; nifV and accessory genes	Fe–S assembly and auxiliary cofactor pathways in applicable taxa.	Do not assume every strain has an identical accessory set.
nifA; nifL and N regulatory pathway genes	Expression response to nitrogen, oxygen and cellular metabolic status.	Edited bacterial strains can decouple repression (Bloch et al., 2020).
vnf; anf systems	Alternative metal-dependent nitrogenase systems.	Confirm full structural modules and biochemical activity.
*CAZ*ymes, sugar uptake, energy metabolism	Mucilage substrate utilization and reductant supply.	Indirect enabling functions; not nitrogenase genes.

The response is further complicated by variation in both host and bacterial regulation. Zhao et al. (2025) found that many modern maize lines increase aerial root mucilage exudation under low-N conditions, indicating that the host's carbon allocation to the mucilage environment responds to N availability. Bloch et al. (2020) showed that modifying the bacterial nitrogen-sensing and assimilation pathways could reduce the repression of nitrogen fixation associated with available fixed N. These are two distinct aspects of the association, and the studies do not tell us how mucilage production, bacterial fixation, and N transfer respond when host genotype, bacterial regulatory characteristics, and fertilizer level vary simultaneously. A factorial experiment with all of these factors would be needed to determine whether increased mucilage exudation under N limitation is sufficient to support fixation to the extent that it compensates for the reduced host growth and carbon supply.

Field evidence is the basis for assessing whether associative N_2 fixation can meaningfully contribute to maize nutrition under farm conditions. In a set of experiments looking at *Azospirillum brasilense* and *Bacillus subtilis* inoculation in combination with various rates of N fertiliser, Galindo et al. (2024) reported isotope-based estimates of atmospheric N contributions of 25-50 kg N ha^{-1} for the treatment combinations assessed, with smaller responses under high N inputs. The implication is that the inoculation effect may be dependent on the fertiliser regime. The magnitude of the response however cannot be attributed to any particular maize allele or bacterial mechanism without further evidence. The interpretation of the results is also contingent upon the assumptions made in the isotope-based measurements, particularly the choice of reference plants, the variation in soil N availability and the differences in N uptake between the treatments. These are all factors which must be considered when comparing such estimates across studies as the differences in the apparent level of biological N_2 fixation may in fact be due to the experimental design and local conditions rather than any inherent difference in the N_2-fixing capacity of the associations. It will therefore be necessary to have evidence that the estimated inputs of atmospheric N translate into improved crop N status, improved fertiliser use efficiency or improved yield under field conditions.

Field tests of engineered diazotrophs show promise and uncertainty

Engineered diazotrophs have shown potential to improve maize N nutrition, but performance varies across studies, and no consistent estimate exists of their ability to replace synthetic N fertilizer. Woodward et al. (2025)

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assessed a *Klebsiella variicola–Kosakonia sacchari* inoculant over four site-years and reported fertilizer equivalent estimates of 38.5 kg N ha^{-1} at V8 and 12.1 kg N ha^{-1} at R1 maize developmental stages based on crop response regressions. Mean grain yield was increased by approximately 110 Kg ha^{-1} and isotope data were consistent with a bacterial contribution to plant N acquisition. Again, it is important to note that the fertilizer equivalent derived from crop response cannot be taken as a direct measure of N_2 fixation. The improved N uptake or crop growth could be due to a number of other processes.

Martinez-Feria et al. (2024) found less consistent results under field conditions. Their pooled estimate of the inoculation-associated nitrogen rate was 21.2 kg N ha^{-1} by VT–R1, with a 95% CI of (−0.16 to 42.6 kg N ha^{-1}), which includes zero, so the estimate is not statistically significant. In one of the 2021 experiments, Ndfa was 8.2% ($p = 0.08$), which is at the exploratory significance level ($\alpha = 0.10$) but not the standard $\alpha = 0.05$ level. The experiments showed no significant isotope dilution. This does not rule out some contribution from associative N_2 fixation, nor does it establish a consistent fertilizer replacement rate. Some variation may be due to differences in field conditions, nitrogen availability, and/or inoculant performance, though we do not know how much each factor contributed. More work is therefore needed across a range of environments and fertilizer regimes to determine whether the benefits observed are consistent enough to warrant any recommendations for reduced nitrogen inputs.

Water, oxygen, developmental timing and microbial establishment

Maize aerial root mucilage provides a dynamic diazotroph habitat whose suitability for N_2 fixation varies with environmental conditions and root development. Nitrogenase activity is sensitive to O_2 and can be inhibited or irreversibly damaged by exposure to O_2 (Lery et al., 2010). However, respiration by the microbes, O_2 diffusion and mucilage hydration may all lead to local conditions that are conducive to fixation. Rainfall influences mucilage production and root age affects the duration of mucilage exudation (Van Deynze et al., 2018; Pankievicz et al., 2022). The temporal and physiological constraints of this system make selection for aerial root traits a difficult proposition. Simply increasing the number of aerial roots may provide limited benefits if the timing of mucilage production does not coincide with rainfall events, if suitable diazotrophic populations are absent, or if the crop demand for nitrogen is not substantial at that time. The agronomic value of aerial root development therefore lies in the timing and duration of the conditions that are conducive to fixation rather than in the number of aerial roots per se.

Plant-microbe compatibility leads to further variation in the effectiveness of A-BNF. A bacterium may have the genes for functional nitrogenase yet colonize mucilage poorly, be outcompeted by the resident microbial community, or actively suppress fixation when mineral N is available. On the other hand, successful colonization does not necessarily equate to nitrogen transfer as the bacteria may fix the nitrogen and retain the vast majority of it for their own growth and metabolism. Microbial abundance alone is therefore a poor indicator of the contribution of the diazotrophs to maize N nutrition. These differences will only be resolved through long-term measurements of strain persistence, nitrogenase expression, and fixation activity, alongside isotope tracing to separate the N retained in microbial biomass from the N actually transferred to the plant.

Evaluating the evidence of A-BNF: methods and interpretive limits

Methods used to study A-BNF often answer different questions even though they are discussed as if they all measure the same phenomenon. Sequencing reveals which microbes are present or which genes may be present; acetylene reduction yields information about a nitrogenase-associated reaction; ^{15}N methods can reveal the movement of atmospheric nitrogen into microbial or plant pools; growth and yield trials show the crop response. The evidence becomes compelling when the various measurements are brought together in the same causal design. A single assay may be perfectly valid in a technical sense but still not be sufficient to support a claim about maize nitrogen nutrition. Table 3 summarizes the inferences each method can support and the conclusions it cannot establish on its own.

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Acetylene reduction and molecular detection

Acetylene reduction measures the conversion of acetylene to ethylene by nitrogenase and is a practical measure of enzyme activity (Unkovich et al., 2008). Its relationship to actual N_2 reduction varies with substrate conditions, organism, and assay conditions. The reaction does not show whether any fixed nitrogen enters the maize. The same applies to the detection or expression of *nifH*: this is a marker for gene presence or transcriptional activity; it does not prove that any nitrogen has been contributed to the crop nutrition. Primer bias and differences between the compartments sampled will also make comparisons between studies difficult (Gaby & Buckley, 2012).

Direct $^{15}N_2$ incorporation and host–microbe transfer

Direct $^{15}N_2$ labeling can be used to establish the incorporation of atmospheric N into bacterial biomass, extracellular metabolites, or host tissue. Interpreting the results depends on where the label is found. If the label is found exclusively in the bacterial fraction, this indicates microbial fixation, not transfer to the maize. The experiment should measure the microbial and plant fractions separately, account for background ^{15}N and contamination by fixed N, and consider variation in biomass and gas purity. A more robust test of the association in terms of crop nutrition would be to look at enrichment in the shoots and grain, as well as total plant N accumulation.

Natural abundance, dilution methods and the definition of Ndfa

The ^{15}N isotope dilution and natural abundance techniques both determine the proportion of plant N of atmospheric origin, but each method does so differently. The dilution method does it by comparing the enrichment of the test plant to that of a non-fixing reference plant after addition of labeled soil N. The natural abundance method compares the isotopic signature of the test plant to that of the reference and an appropriate the stable nitrogen isotope composition value (B value). In both cases, the reference and test plants must uptake their N from the same soil pool. If there is a difference in rooting depth, timing of uptake, or in access to different N sources, then that condition is breached, which can result on a false appearance of fixation. That uncertainty can be reduced through repeated measurements and complete N budgets, but it cannot be eliminated (Unkovich et al., 2008; Connolly et al., 2025).

For the ^{15}N isotope-dilution method, percentage nitrogen derived from the atmosphere (%Ndfa) is estimated as:

$$\%Ndfa = 100 \times [1 - (E_{test} / E_{reference})]$$

Here E denotes atom % ^{15}N excess in a plant sample after labeled soil N was applied. For the natural-abundance method, the corresponding calculation is:

$$\%Ndfa = 100 \times [(\delta^{15}N_{reference} - \delta^{15}N_{test}) / (\delta^{15}N_{reference} - B)]$$

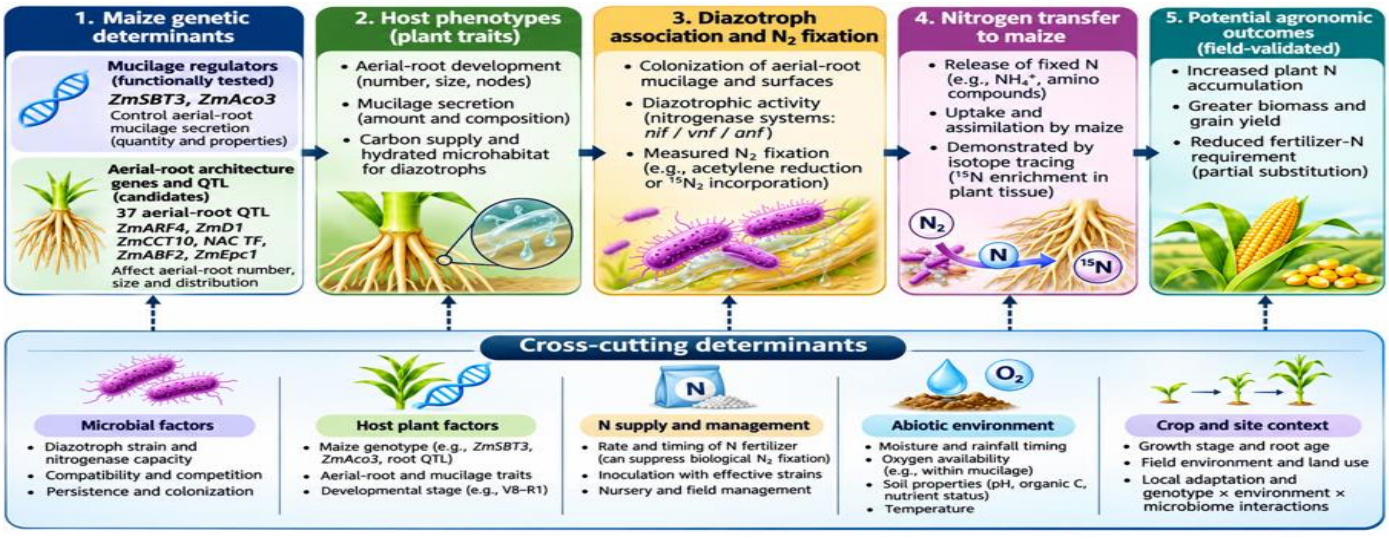

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Figure 2. Conditional genetic pathways linking maize host traits, diazotroph associations, nitrogen transfer and crop response. The arrows represent hypothesized links in a multistep process rather than evidence that the full sequence has been established. Solid arrows represent main pathways while dashed arrows depict conditional or incompletely validated steps.

The B denotes the stable nitrogen isotope composition ($\delta^{15}N$) signature expected when the test plant obtains all its nitrogen through atmospheric fixation under the relevant conditions. Both calculations depend on a credible reference with access to equivalent soil-N pools. Multiplying %Ndfa by total plant N estimates atmospheric nitrogen in the sampled biomass; it is not a direct prediction of fertilizer savings or a guarantee of yield response.

The genotype validation experiment

To establish host-gene causality, the most informative experiment is one that crosses near-isogenic maize alleles with defined microbial strains and nitrogen regimes. Wild-type plants, independent edited events, and complemented lines should be compared with a nitrogenase-competent inoculant, a matched *nif*-deficient control and, where relevant, no inoculant. Mucilage, colonization and microbial activity must be measured alongside ^{15}N recovered in maize. This arrangement can distinguish increased fixation from improved transfer or altered uptake of pre-existing soil nitrogen. Replication across seasons and representative field conditions is then required to determine whether an allele has practical value.

Table 3. What each measurement associated with biological N-fixation in maize can and cannot establish

Measurement	**Supports**	**Does not establish alone**
Aerial-root number or mucilage volume	Host niche phenotype and substrate opportunity.	Nitrogenase activity or atmospheric N uptake.
16S/shotgun community composition	Taxa or putative genetic potential.	Activity or transfer to host.
nifH PCR or transcript abundance	Presence/expression of nitrogenase marker.	Complete functional system or N flux.
Acetylene reduction	Nitrogenase-associated catalytic activity.	Plant N derived from atmospheric N_2.
Microbial $^{15}N_2$ incorporation	Microbial fixation and labeled metabolites.	Amount entering maize tissues.
Plant $^{15}N_2$ enrichment or appropriate dilution	Atmospheric N in plant under tested assumptions.	Causal maize allele effect unless genotypes compared.
Yield or total plant N response	Agronomic benefit.	Fixation contribution without source attribution.

Comparative synthesis: what has and has not been demonstrated

Evidence for A-BNF in maize is highly study-dependent and varies dramatically across the different stages of the proposed mechanism. In Sierra Mixe maize, nitrogenase assays and plant isotope signatures provided evidence of atmospheric N contribution under the experimental conditions (Van Deynze et al. 2018). Functional evidence supports the role of *ZmSBT3* and *ZmAco3* in mucilage-related traits. Parallel QTL studies on aerial roots have identified genomic regions associated with the morphological variation (Figure 3). These are all elements of the proposed mechanism, but none of the studies demonstrate a causal link from a particular maize genotype through altered mucilage and, hence, microbial fixation, to a reproducible increase in the amount of

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atmospheric N recovered in grain as isotope-derived. This is the key point that underpins the limitation of the current evidence in interpreting variation in aerial root or mucilage traits as variation in A-BNF capacity.

Evidence from microbial genetics is far more direct with respect to the biochemical machinery of nitrogen fixation (Table 4). Work on maize-associated diazotrophs has identified nitrogenase-related genes and shown $^{15}N_2$ incorporation into microbial metabolites. Alterations to bacterial regulatory pathways have also been shown to increase *nif*-associated activity and ammonium release under controlled conditions (Higdon et al., 2020; Bloch et al., 2020). The agronomic results have been less consistent. Some field trials have reported positive fertilizer equivalent responses and isotope evidence of nitrogen contribution, though these effects have not been replicated in all site-years (Woodward et al., 2025; Martinez-Feria et al., 2024). These findings highlight the difference in the quality of the evidence: demonstrating fixation or ammonium release establishes the bacterium's capacity to fix nitrogen, whereas demonstrating a contribution to crop nutrition requires evidence of nitrogen transfer and recovery by the plant. Therefore, the functional characterisation of an engineered diazotroph in itself cannot be taken as proof of a predictable reduction in the amount of N fertiliser required by maize.

The importance of aerial root mucilage should also be examined more closely. Mucilage provides a good habitat for some of the diazotrophs, but nitrogen fixation in the rhizosphere and root endosphere may also make a contribution to the plant's overall nitrogen uptake. We do not know how significant this is, and it may differ in traditional landraces compared with modern breeding material. Focusing solely on the amount of aerial root or on the amount of mucilage it produces may therefore miss other host traits that will have an influence on the recruitment, persistence and nitrogen transfer of the associated microbes. Breeding evaluations need to separate the morphological traits that promote microbial colonization from the functional traits that contribute to the plant's nitrogen nutrition. Measuring strain persistence, nitrogenase activity, isotope-derived recovery of atmospheric nitrogen, and maize grain yield would be a much better basis for selecting useful host-microbe interactions. Aerial root number may be a trait of interest, but its value in improving nitrogen use efficiency (NUE) will only be apparent in relation to actual nitrogen acquisition and crop performance.

Breeding and microbiome-engineering opportunities

Improving A-BNF requires two co-evolving but separable breeding efforts: maize must provide an appropriate habitat for diazotroph recruitment and activity, and the associated microbes must be able to fix and release nitrogen to the maize under field conditions. The key host traits are aerial root form, mucilage production, and carbon allocation. The microbial side of the equation concerns colonization, nitrogenase regulation, and nitrogen release. Neither effort alone will be sufficient. A maize allele can increase mucilage without necessarily improving nitrogen acquisition, and a very active isolate may fail to persist and thrive on an elite genotype. The real aim is to find a reproducible host-microbe combination that increases plant N recovery without detrimental effects on other agronomic traits.

Host breeding should target a multicomponent phenotype

Heritable aerial-root variation and the functional results for *ZmSBT3* and *ZmAco3* suggest that host selection is a promising research direction (Gao et al., 2023; Zhao et al., 2025; Laspisa et al., 2026). A good first step would be to introduce favorable mucilage and root-architecture alleles into elite genetic backgrounds and test how they are expressed. These lines would be improved for a specific habitat trait, but not for N fixation. When selecting, it is also important to consider lodging resistance, flowering time, biomass distribution, root function, and grain yield: greater mucilage exudation or more aerial roots can increase developmental and carbon costs, and water stress might prevent the desired niche from forming.

Host-by-strain matching and synthetic microbial communities

A diazotroph that performs well on one maize genotype may perform differently on another, as exudate chemistry, attachment surfaces, and resident microbial competitors all vary. Host selection and strain screening therefore need to go hand in hand. A useful consortium must colonize reliably, maintain nitrogenase activity under prevailing local O_2 and moisture conditions, and release N that the plant can take up. Randomly adding

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strains may increase competition or even reduce formulation stability. Strain-resolved genomic checks and direct $^{15}N_2$ transfer assays should precede any broad field deployment.

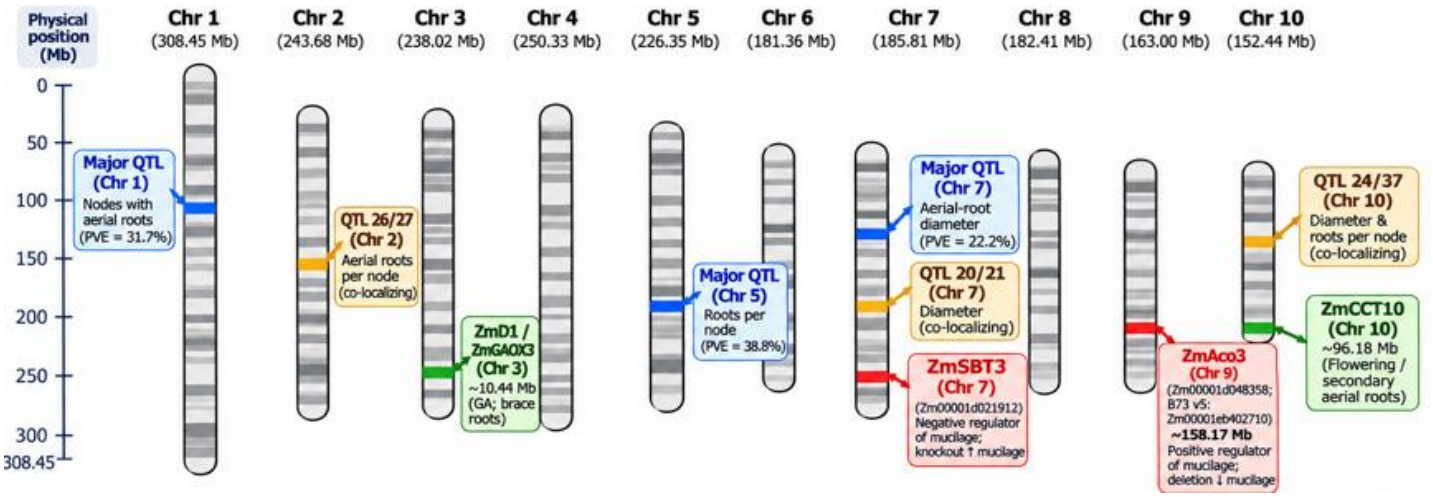

Figure 3. Chromosomal distribution of quantitative trait loci (QTLs) and candidate genes associated with aerial-root development, mucilage exudation, and root architecture in maize (Zea mays L.). The ten chromosomes are drawn proportionally using physical lengths from the B73 RefGen_v5 reference genome. Blue and orange markers indicate major and overlapping QTLs associated with aerial-root number, diameter, and distribution, whereas green markers identify positional candidate genes implicated in root development and hormonal regulation. Red markers highlight ZmSBT3 and ZmAco3, which have been functionally implicated in mucilage exudation.

Table 4. Selected primary-study evidence and warranted conclusions

Study	**Design and key finding**	**Warranted conclusion / important limitation**
Van Deynze et al. (2018)	Field isotope methods and mucilage microbial assays; reported 29–82% plant Ndfa in Sierra Mixe settings.	Context-specific isotopic association, not cloned host allele or elite-maize average; reference assumptions matter.
Gao et al. (2023)	258 inbreds; GWAS and ZmSBT3 CRISPR knockout increased mucilage; nitrogenase difference was nonsignificant.	Host gene affects mucilage, but no demonstrated increase in isotope-derived plant N from air.
Zhao et al. (2025)	15 QTL, 68 QTNs, 59 candidates; ZmAco3 EMS loss-of-function mutant reduced mucilage.	Causal mucilage effect; no directly established host nitrogen transfer.
Laspisa et al. (2026)	Three populations, 37 aerial-root QTL, broad sense heritability of 0.65–0.83.	Genetic architecture for root morphology, not for fixation itself.
Higdon et al. (2020)	Genome analysis and $^{15}N_2$-labeled metabolites in mucilage isolates.	Bacterial fixation and metabolic potential; plant transfer unmeasured in isolate tests.
Bloch et al. (2020)	Edited K. sacchari regulatory pathways; increased N-related activity under available N.	Validated microbial genetic regulation; field agronomic benefit separately assessed.

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Martinez-Feria et al. (2024)	Multi-year field trials; pooled 21.2 kg N ha^{-1} by VT–R1 (95% CI, −0.16 to 42.6); some null site-years.	Wide uncertainty includes zero; effects cannot define universal fertilizer substitution.
Woodward et al. (2025)	Four site-years; response-regression fertilizer equivalents 38.5 (V8) and 12.1 (R1) kg N ha^{-1}; mean yield gain 0.11 Mg ha^{-1}.	Inferred fertilizer equivalence differs from isotope-measured atmospheric nitrogen.

Genome editing and microbial regulation

Genome editing offers several alternative approaches to studying and improving A-BNF in maize. In the host plant, targeted mutations can determine whether candidate alleles affect mucilage production and, more importantly, whether these changes increase the amount of N_2 the plant takes up from the atmosphere. In the diazotrophic bacteria, editing of the pathways that regulate nitrogen sensing, ammonium assimilation and nitrogenase activity may be used to maintain fixation in the face of conditions which would normally lead to its suppression, such as in the abundance of soil N. or oxygen-rich environments The combination of these approaches could lead to an improved level of host-microbe compatibility, though the extent to which this would be the case has not been demonstrated experimentally. For agricultural application, genetic stability, ecological safety, and regulatory compliance would also need to be addressed. The claim that A-BNF can replace or supplement fertilizer would ultimately require field data showing a reproducible contribution of isotope-derived N to crop N accumulation, grain yield, and fertilizer response across a range of environments (Bloch et al., 2020; Martinez-Feria et al., 2024).

Participatory and access-and-benefit considerations

The scientific value of Sierra Mixe maize is entirely predicated upon its origin in the Indigenous farming systems of Oaxaca, Mexico, where it has been the subject of generations of farmer selection and stewardship. The initial work in this area established formal access and benefit-sharing arrangements with respect to the research (Van Deynze et al., 2018; Higdon et al., 2020). Any subsequent breeding, genetic characterization, or commercial development of the material will need to be in accordance with the terms of the access and benefit-sharing agreements in place. This is not simply a matter of regulatory compliance. The very traits that were the subject of scientific interest arose in a farming system that was itself the product of local knowledge, selection, and long-term conservation. Recognizing and rewarding the contributions of the farming systems from which germplasm originates is therefore fundamental to the ethical use of genetic resources and the fair and equitable sharing of benefits derived from their research and development.

New evidence from landrace comparisons and related cereals

A-BNF in maize research has moved on from the initial Sierra Mixe observations, but the wide distribution of mucilage-bearing roots in maize should not be taken as evidence of equally strong nitrogen transfer. The number of aerial roots, exudation, microbial colonization, and isotope-based estimates vary across the range of maize landraces and related cereals. These traits may share some developmental or ecological characteristics but will differ in their genetic control and expression in the field. The issue is whether the same habitat leads to the same mechanism and whether it delivers the same amount of nitrogen to the host. Landrace and cross-species work is most useful in sorting out these possibilities rather than simply taking every mucilage-bearing root as evidence of the same fixation phenotype.

Diversity beyond Sierra Mixe: direct evaluation of maize landraces

Despite being the clearest example of a combination of traits associated with aerial root-based nitrogen fixation, Sierra Mixe maize remains poorly understood, and it is still unclear how widespread this trait combination is among maize populations. Connolly et al. (2025) looked at 21 maize landraces and three improved varieties in two replicated field trials in Ohio (with and without N fertilizer). They examined aerial root traits and used ^{15}N natural abundance to estimate Ndfa. Root development and early growth varied considerably among accessions, and some Ndfa estimates were as high as 36%. The estimates depended heavily on the non-fixing reference (i.e.,

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whether sunflower or commercial maize was used), the N treatment, and the accession considered. A difference that was significant under one reference comparison was not necessarily significant under another. The study did report substantial variation in root morphology and in reference-sensitive isotope estimates; it did not establish any fixed intrinsic rates of N fixation for the individual landraces.

Landraces exhibiting contrasting aerial root phenotypes provide valuable material for genetic mapping studies, especially when traits such as maturity, plant vigor, and overall plant size are carefully documented. Without accounting for these characteristics, selection for a greater number of nodal roots may inadvertently favor genotypes with longer vegetative growth periods rather than enhanced nitrogen-fixing potential. A more comprehensive phenotyping framework should therefore include measurements of root emergence dynamics, root wetting capacity, root cap integrity, and patterns of microbial colonization. Such assessments should also quantify nitrogen transfer over time while controlling for soil-derived nitrogen uptake to distinguish A-BNF from other nitrogen acquisition pathways.

Developmental architecture, root cap integrity and a transient microbial niche

Pankievicz et al. (2022) linked mucilage exudation to young aerial roots that had intact, viable root caps and border cells. This implies that the ability to maintain a mucilage-associated microbial community is developmentally regulated. Water exposure stimulated exudation and the expression of genes associated with exudate polysaccharides varied with treatment and maize developmental stage. It would therefore appear that both root age and hydration affect the amount of mucilage available as a microbial habitat, though the effect on A-BNF needs direct testing. Sampling plants only at maturity may therefore miss the period of significant mucilage exudation and potentially significant nitrogenase activity. For reliable field characterization, it would be necessary to account for the developmental age of the root cohort (i.e. the age of the roots), root diameter, root tip integrity, rainfall or irrigation events, and temporal changes in mucilage exudation. Repeated sampling would be required to separate out genotypic differences in exudation capacity from variation due to root development and environment.

Aerial roots also have a structural role, which makes their assessment as traits relevant to A-BNF rather tricky. When they are in contact with the soil surface, they may enhance plant anchorage, while those that remain above ground will be subject to very different moisture regimes and microbial environments. Selection for increased aerial root node number could therefore have an effect on plant architecture, lodging resistance and carbon allocation without necessarily having any effect on N_2 acquisition by maize. The duration of mucilage exudation, its hydration and its ability to support metabolically active diazotrophs may therefore be more informative than root number per se. These characteristics should be analyzed in conjunction with the structural functions of the aerial roots, particularly where selection for the creation of a microbial habitat may have an effect on other agronomic traits. It would be useful to map root number, developmental timing, mucilage characteristics and anchorage characteristics separately to determine their genetic links and to identify potentially adverse trait associations which might be masked by a composite morphological score (Pankievicz et al., 2022; Laspisa et al., 2026).

What the evidence from sorghum roots contributes, and what it cannot establish for maize

Related grasses provide a good test of whether the Sierra Mixe maize phenotype reflects a broader ecological mechanism. Venado et al. (2025) examined aerial root mucilage with diazotrophic communities in several sorghum (*Sorghum bicolor*) accessions and combined microbial isolation with acetylene reduction, $^{15}N_2$ feeding, and isotope dilution. The contribution of atmospheric N varied between accessions and between the microbial communities. This is of relevance to the maize work as sorghum also has exposed nodal roots, border cells and a hydrated, carbohydrate-rich environment. It does not, however, establish that the two species have the same causal genes.

Conserved gene annotations and synteny can help prioritize experiments. However, an ortholog, by itself, is not evidence of a conserved function. A proper maize-sorghum comparison would involve knocking out the candidate genes in each species and then measuring the resulting mucilage chemistry, microbial activity and

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$^{15}N_2$ incorporation under the same soil nitrogen and water regimes. That would test for conservation of function rather than infer it from similar anatomy (Venado et al. 2025; Laspisa et al. 2026).

Deployable breeding targets

A long candidate gene list is not a breeding program. GWAS, QTL, and transcript profiling have identified genes that may be involved in aerial root formation, mucilage exudation, or microbial association. Very few of these have been manipulated, and none in this evidence set have been shown to increase the isotope-tagged atmospheric N that maize recovers. The pathway to deployment is long and requires a series of increasingly rigorous tests: a reproducible genetic association, a causal effect on the putative host trait, a microbial response, and finally a benefit in N and yield. This distinction matters when selecting markers. An allele that increases exudation is not a technology that can currently be sold or recommended as a fixation allele.

Transcriptomic candidates for mucilage synthesis and hydration responses

Beyond *ZmSBT3 and ZmAco3*, most reported mucilage loci remain expression or positional candidates (Table 5). Pankievicz et al. (2022) found relatively abundant transcripts for four putative UDP-glycosyltransferase-associated genes, Zm00001d043166, Zm00001d033320, Zm00001d016091 and Zm00001d042740, in stage 2 aerial roots, which generally secreted less mucilage than stage 1 roots. Therefore, greater expression of mucilage chemistry genes cannot be taken as evidence that they promote exudation. Two aquaporin-associated loci, Zm00001d010410 and Zm00001d037779, responded to hydration, but neither has a demonstrated causal role in mucilage production. Root stage and alignment of Sierra Mixe reads to a B73 reference may also influence the expression contrasts (Pankievicz et al., 2022).

Exudation volume may be a poor predictor of microbial benefit when mucilage chemistry differs. Amicucci et al. (2019) described a complex Sierra Mixe polysaccharide containing a fucosylated and xylosylated galactose backbone and additional branches. Bacteria can exploit such polymers only if they possess compatible hydrolases, uptake systems and metabolic capacity. Two genotypes that produce the same mucilage mass could therefore support different microbial communities, carbon flux and nitrogenase activity. Polymer structure, pH and oxygen diffusion are potential host-associated properties that deserve measurement in their own right.

Functional tests should look at chemistry and exudation kinetics rather than mucilage volume per se. For the candidate glycosyltransferases, mutants or repaired alleles in a common background should be used to separate biosynthetic function from the response to developmental decline. The aquaporins that are hydration-responsive would be tested via wetting and drying cycles. The resulting host phenotypes would then be sequenced for colonization, *nif* expression, microbial $^{15}N_2$ assimilation, host ^{15}N recovery, and grain traits. The older Zm00001d identifiers would need to be matched to the correct B73 assembly and Zm00001eb models before any editing or marker design (Pankievicz et al., 2022; Amicucci et al., 2019).

Nitrogen-responsive alleles and reaction norms

Factorial experiments are needed to assess these relationships among aerial root development, microbial colonization, A-BNF, and plant N acquisition, with the factors being contrasting maize genotypes, defined bacterial inocula, low and moderate N supplies, representative field fertilizer rates, and differing water regimes. This would enable the examination of genetic, N, and water effects (and their interactions) on mucilage production, nitrogenase activity, Ndfa, and grain yield. Direct measurement of Ndfa is particularly important, as increased total plant N could result from improved uptake of soil N or greater biomass accumulation without increased S-BNF. Zhao et al. (2025) identified 15 QTL, 68 QTNs and 59 candidate genes associated with mucilage exudation traits. These are clearly genetic targets for further study, but their contribution to A-BNF has not been established. It will be necessary to functionally characterize the candidate loci and determine if their effect on mucilage exudation is, in fact, associated with increased acquisition of N from the atmosphere.

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Nitrogen assimilation genes as downstream modifiers, not nitrogenase genes

Maize transport and assimilation genes may affect how the crop captures nitrogen released by diazotrophs, but their role in associative N transfer remains unclear. Pankievicz et al. (2022) reported expression or hydration-related responses in *ZmAMF1.1, ZmAMT1.3, ZmNRT3.1B*, and *ZmNRT2.5,* as well as candidate glutamine synthetase (Zm00001d017958) and glutamate dehydrogenase (Zm00001d016419) genes. These loci are all consistent with normal ammonium or nitrate transport and metabolism. They do not represent a pathway for nitrogen to be traced from the diazotrophs into the maize. The more general nitrogenuse efficiency (NUE) pathways described by Li et al. (2025) are physiologically relevant, but they must be kept distinct from the proven pathway for atmospheric-N transfer.

Testing the putative N transport gene requires a microbial strain known to take up $^{15}N_2$ and an isogenic maize N transporter allele under the same mineral-N conditions. Separate measurements of ^{15}N in the bacterial biomass, roots, shoots, and grain would then allow the determination of where the label accumulates. Further work would be required to determine whether uptake is of released ammonium, organic N, or N released through microbial turnover. Improved NUE without source tracing cannot be ascribed to fixation (Higdon et al., 2020; Li et al., 2025).

Genome assembly, gene identity and causal variant requirements

Candidate gene lists are a complete mess of old *GRMZM* identifiers, B73 gene models, aliases, and genes included simply because they fall within broad QTL regions. For any gene to be a candidate, it needs to have a specific genome assembly, a specific physical coordinate, a specific transcript identity, and a documented variant. A nearby developmental locus or a structural variant could drive the association, and the candidate gene itself might not be causal. Fine mapping is absolutely required to sort out any maturity and ancestry effects. Complementation, allelic series, and reciprocal editing can then be used to determine whether the variant in question causes the phenotype.

The Sierra Mixe populations examined by Laspisa et al. (2026) showed heritable aerial root morphology, not heritable isotope-derived Ndfa. Potential determinants of the latter (i.e., the isotope-derived Ndfa) include microbial habitat, carbon provision, transfer efficiency, and host N capture. These could be genetically independent, or they could interact. Direct mapping of Ndfa is required before describing any mucilage-associated interval as an atmospheric N acquisition locus, with adjustments for maturity, plant size, root traits, and fertilizer supply.

Microbial engineering and the limits of fertilizer-equivalence claims

Genetic engineering of the diazotrophs offers a more direct route to modifying nitrogenase regulation for improved A-BNF than conventional maize breeding. Martinez-Feria et al. (2024) examined modified *K. variicola* and *K. sacchari* using a range of $^{15}N_2$ feeding experiments, field N measurements, and isotope dilution. The authors observed little or no evidence of N transfer in some of the site-years. This illustrates the difficulty of extrapolating from controlled environment measurements of A-BNF to actual agronomic performance. Even if engineered bacterial strains exhibit enhanced nitrogen-fixation capacity, the magnitude and consistency of their contribution to maize N nutrition remain insufficiently quantified to establish a reliable fertilizer replacement value.

Agronomic evaluation is required to determine whether the N supplied by engineered diazotrophs actually adds to plant N accumulation and grain yield. Isotopic evidence of atmospheric N uptake is useful, but the amount of N transferred may be too small, poorly matched to the crop's N demand at any given time or too variable across environments to have any impact on yield. The field trials therefore need to look at N recovery and grain yield across a range of soils, fertilizer regimes and growing seasons, taking into account the cost of inoculant production, storage and deployment. The strain itself, its genetic stability and its ecological safety also need to be confirmed, especially for modified bacteria which may persist in the environment or interact with the indigenous microbial community. The practical value of the engineered inoculant will ultimately depend on

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whether it delivers a consistent and economically viable contribution to N in real-world farming situations (Martinez-Feria et al., 2024; Woodward et al., 2025).

A decisive factorial study for host gene, microbial genotype and nitrogen transfer

Directly comparing the published *ZmSBT3* knockout in B104 with the *ZmAco3* mutants generated in different genetic backgrounds would be confounding, as it would mix gene-specific effects with broader genomic variation. A much more rigorous approach would be to generate independent mutant events for each target gene and evaluate them alongside appropriate complemented or precisely repaired control lines within a common maize genetic background. To strengthen the validity and generalizability of the findings, the results should ideally be replicated and confirmed in a second genetic background. The genotypes would be grown under defined N and water regimes and inoculated with a well-characterized diazotroph, its matched n*if*-deficient derivative, and a non-inoculated control. Aerial root morphology and mucilage composition would then be assessed, as would bacterial colonization, *nif* expression, and nitrogenase activity to establish the physiological consequences of the mutation. The effect on atmospheric N_2 uptake would subsequently be determined by direct $^{15}N_2$ tracing, followed by measurement of grain N accumulation and yield.

The experimental design should distinguish between changes in the microbial habitat and changes in nitrogen fixation and nitrogen transfer. Increased mucilage production in a mutant would indicate an effect on establishment of the potential microbial niche, whereas increased nitrogenase activity would indicate an effect on the capacity for bacterial fixation. Very strong evidence for a host allele affecting A-BNF would be observing greater recovery of atmospherically derived ^{15}N in maize carrying the favorable allele when inoculated with a nitrogen-fixing strain. This effect should be absent or substantially reduced when the plants are inoculated with a corresponding *nif*-deficient strain to demonstrate that the response is specifically linked to nitrogen fixation. Restoration of the phenotype by genetic complementation would be further evidence of a causal relationship. Such experiments would separate nitrogen acquired via biological fixation from changes in conventional soil N uptake and would allow determination of whether the candidate gene influences microbial activity, nitrogen transfer, or both.

Temporal variation in mucilage exudation and bacterial activity is another issue. Exudation following rain may be transient, and so a single date measurement may not be sufficient to capture periods of high N fixation. Repeated observations would need to be made on defined aerial root cohorts through development and would need to quantify the amount of atmospheric N taken up, retained, and potentially remobilized into the grain. Statistical analysis would have to take into account genotype, bacterial strain, N supply, water availability, and environment and their interactions within the randomized design. Where destructive sampling is required, matched replicate cohorts would be necessary to preserve the temporal structure of the experiment. Effect sizes and confidence intervals (including non-significant and negative responses) should be reported as this would give a far more robust assessment of genetic effects than simply highlighting the treatment combinations that give a statistically significant positive response.

Research priorities and experiments needed for causality

The unanswered question is not whether maize has the genetic variation for the structures that house the diazotrophs (it does). The question we still need to answer is whether a particular maize allele leads to additional nitrogen being transferred into the crop from atmospheric N_2 via the microbes, and whether that additional nitrogen actually translates into improved agronomic performance. The right genetic studies and isotope experiments need to be designed to answer that very same question rather than be two separate research efforts.

The priority is to go right back to the host-microbe-nitrogen pathway (Figure 2) and test it in full with the direct isotopes (Table 6). The matched alleles should be compared across mucilage, colonization, enzyme activity, atmospheric N recovery, total N uptake, and grain yield regimes. Fine mapping can then be used to separate the aerial root QTL from any linked effects of maturity or vigor. The work on *ZmSBT3, ZmAco3*, and the candidate regulators should establish the response to water and N supply and determine whether the positive effects can be stacked without incurring unacceptable carbon or developmental penalty. The microbiome work needs to reach the strain and function level rather than rely only on taxonomic profiles. The multi-environment trials need

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to include both low-input and higher-fertility systems, as inoculant establishment and the degree of nitrogen repression may differ substantially between them. The follow-up has to run through to grain filling, since only then can we determine whether any atmospheric N obtained early on makes it to harvest and whether fertilizer can be reduced without compromising yield stability.

A practical breeding sequence would start with low-cost screening for root and mucilage traits, move on to near-isogenic tests with characterized diazotrophs and isotope tracing, and progress to the next stage only with lines that demonstrably provide nitrogen and grain yield benefits. Selection on mucilage alone should remain a preliminary stage and not be the claim made with a released cultivar.

Limitations of the evidence and of this review

This is a focused critical narrative review rather than a full systematic synthesis. The literature search was not supported by subscription database exports or a formal study selection dataset, so we cannot produce a study selection flow diagram or properly account for how many studies were actually included. We have used evidence from non-maize systems to describe the mechanisms involved, without treating that as direct evidence of N_2 fixation in maize occurring in the field. There is a significant imbalance in the amount of evidence available along the proposed causal chain. We have far more data on aerial root traits, mucilage characteristics, and the associated microbiota than we have on atmospheric N uptake, plant N recovery, and grain N accumulation. The comparisons between studies are further complicated by differences in reference plants, isotope methodology, inoculant treatments, and sampling regimes. Ultimately, we can identify likely mechanisms and research directions, but we cannot show that A-BNF makes a significant contribution to maize N requirements. The literature available is also potentially subject to selective reporting, particularly where the reporting of favorable mucilage phenotypes or positive inoculation responses is likely to be more extensive than that of null or inconsistent results. Access to some of the proprietary strain development data is restricted and there is an uneven availability of microbial genome sequences which in turn hampers independent validation. Non-canonical *nif* annotations add to the uncertainty and candidate gene identifiers will need to be validated against a specific reference genome assembly prior to their use in marker development or genome editing. All of this underlines the importance of transparent reporting of data availability, funding, and any intellectual property interests. Research credibility is still judged based on experimental design, appropriate controls, methodological transparency, reproducibility, and the degree to which the evidence supports the conclusions. Neither commercial sponsorship nor public funding of research guarantees research quality.

Table 5. Additional maize candidate pathways and the experiments needed to establish their contribution to associative BNF

Genetic target or system	Evidence currently available	Discriminating next experiment
Zm00001d043166, Zm00001d033320, Zm00001d016091, Zm00001d042740 (UDP-glycosyltransferase-related)	Higher expression in stage 2 roots with generally lower mucilage; may be stage-related rather than positive exudation regulators (Pankievicz et al., 2022).	Edit or replace alleles; test mucilage structure and microbial $^{15}N_2$-to-host transfer.
Zm00001d010410 and Zm00001d037779 (aquaporin-related)	Expression responds to hydration; direct effect on mucilage release is untested (Pankievicz et al., 2022).	Perturb genes; quantify exudation kinetics after watering and subsequent nitrogen transfer.
Low-N responsive exudation QTL and QTN	Mapping and G×E associations for mucilage, not direct nitrogen transfer (Zhao et al., 2025).	Estimate genotype × N response for mucilage, Ndfa and yield in fine-mapped families.

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ZmAMF1.1, ZmAMT1.3, ZmNRT2.5, ZmNRT3.1B; GS/GDH candidates	Transcriptome or annotated nitrogen-transport/metabolism roles; no specific demonstration of microbially fixed N uptake.	Test host alleles with a $15N_2$-fixing diazotroph, matched nif-deficient strain and root-to-grain N tracing.
Sierra Mixe-derived aerial-root QTL	Mapped architecture loci with H2 0.65-0.83 for root traits (Laspisa et al., 2026).	Use near-isogenic lines to test Ndfa independent of maturity, vigor and root number.
Diazotrophic nif regulatory alleles	Biochemical and greenhouse evidence; mixed field isotope outcomes (Martinez-Feria et al., 2024).	Replicated yield and 15N-derived N budgets across N fertility, soils, and seasons.

Table 6. Priority experiments required to advance candidate genes toward breeding

Priority question	**Recommended experiment**	**Critical endpoint**
Does ZmSBT3 change realized A-BNF?	Independent knockout, complemented, and wild-type maize; nif+ versus nif− inocula; $^{15}N_2$ tracing.	Genotype-dependent atmospheric N recovered in maize tissues and grain.
Does ZmAco3 alter N transfer?	Isogenic deletion and allelic repair under water × nitrogen treatments; mucilage metabolomics.	Mucilage, colonization, bacterial N fixation and plant ^{15}N transfer.
Are aerial-root QTL causal?	Fine map and introgress QTL into elite backgrounds; control maturity and plant stature.	Reproducible root effects and subsequently maize Ndfa.
Which bacteria donate N efficiently?	Strain-resolved nif genetics, transcriptomics, secreted ^{15}N metabolites, host labeling.	Fixed-N release and plant capture.
Is a fertilizer-N substitution robust?	Replicated multi-environment N rate × inoculant × host genotype trials.	Grain yield, N balance, isotope-based Ndfa and economics.

CONCLUSIONS

Maize genes control the habitats that support diazotrophs, but microbial genomes contain the machinery to reduce atmospheric N_2. The strongest evidence for host gene function comes from mucilage exudation: *ZmSBT3* and *ZmAco3* have functional support, although disrupting *ZmSBT3* did not significantly increase measured nitrogenase activity. No causal link has been established between aerial root QTL, hydration-responsive genes, or nitrogen transport candidates and isotope-defined uptake of atmospheric nitrogen. Landrace isotope estimates depend heavily on the choice of reference plant and nitrogen regime. For the microbes themselves, nitrogenase biology is well established, but field N delivery and fertilizer replacement remain highly variable. The real breakthrough will come from testing matched host alleles with competent and *nif*-deficient strains, direct $^{15}N_2$ labeling, whole-plant N budgets, and replicated grain yield trials. Until those data are in, mucilage-regulating alleles should be described for the traits they demonstrably affect rather than as proven fixation alleles in elite maize.

For breeding, the immediate priority is to test mucilage and root-architecture alleles for their effects on isotope-defined N recovery, yield and stability across varying fertilizer and water regimes before they are used as selection targets. A reproducible association could eventually reduce dependence on synthetic N inputs, but no general fertilizer-replacement claim is warranted from the present evidence. Engineered diazotrophs raise additional questions about environmental persistence, genetic stability, biosafety assessment and proportionate regulation before agricultural release. Because valuable aerial-root traits have been documented in Indigenous

landrace germplasm, germplasm access and downstream commercialization must also respect applicable access-and-benefit-sharing arrangements and the contributions of farming communities. Assessed together, these considerations locate the potential bioeconomic value of A-BNF in verified N delivery and equitable stewardship, rather than in the presence of promising genes or microbes alone.

Declarations

The authors declare no conflicts of interest. This narrative synthesis uses the cited publications. The protocol and targeted search documentation are descriptive and do not constitute a full systematic dataset screening.The review did not generate an original experimental dataset. Evidence was drawn from the published studies and repositories identified in the reference list. A comprehensive database-search export, dual-screening record and systematic-review selection dataset were not generated.

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