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Quantifying Soil–plant Nutrient Dynamics in Nitrogen-fixing Trees and Shrubs under Soil Amendments and Bio-inoculants: A Critical Appraisal of Measurement Methods in Containerised Nursery and Field Systems

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Abstract

Nitrogen-fixing trees and shrubs are planted widely in nursery-raised restoration programmes, mixed-species plantations and agroforestry systems, and soil or substrate amendments and bio-inoculants are applied routinely to improve their nutrition. Confidence in the reported effects of these interventions rests on measurement systems whose validity conditions the interventions themselves disturb, and this dependence has not been examined systematically. This critical narrative review appraises the methods used to quantify soil–plant nutrient dynamics in woody nitrogen-fixing systems across containerised nursery and field domains. Literature was identified in open scholarly indexes and agricultural and biomedical databases, supplemented by citation searching and institutional technical sources, for the period 1985 to 14 June 2026, and was selected and appraised for its bearing on measurement validity rather than for effect estimation. Five arguments emerge. Methods for estimating symbiotic nitrogen input each hold one quantity constant, and amendments or inoculation alter that quantity, with predictable directions of bias. Extraction-based measures of nutrient availability lose their calibration to plant uptake when sorptive amendments are present, and the best-documented case shows soil test results and plant responses moving in opposite directions. Diffusion-based and sink-based measures of phosphorus availability outperform conventional extraction in high-sorption soils, which is the soil class most relevant to restoration planting. Colonisation percentages, functional gene abundances and acetylene-dependent activity are surrogates whose conversion to nutrient fluxes is neither linear nor stable, yet they are frequently reported as fluxes. Containerised and field systems are distinct measurement domains rather than two scales of one system, so container results support statements about mechanism and capability but not about magnitude. Major unresolved questions concern host-specific isotopic fractionation parameters for the species in widest use, the separation of inoculant establishment from inoculant effectiveness, and the persistence of nursery treatment effects after outplanting. A substantial and identifiable portion of the apparent variability in this literature is methodological rather than biological, and most remedies require changes to design and reporting rather than new instrumentation.

Keywords: *Biological nitrogen fixation; bio-inoculants; containerised nursery production; isotopic methods; nutrient availability assessment; phosphorus dynamics; soil amendments.*

1. Introduction

Nitrogen-fixing trees and shrubs occupy a distinctive position in applied soil science because they are simultaneously recipients and modifiers of the nutrient supply they are measured against. Species such as *Acacia mangium*, *Leucaena leucocephala*, *Gliricidia sepium*, *Dalbergia sissoo*, *Prosopis alba*, *Faidherbia albida*, *Casuarina equisetifolia* and *Alnus* spp. are deployed in restoration plantings, mixed-species plantations, agroforestry parklands and fuelwood systems precisely because they import atmospheric nitrogen into the plant–soil system, alter the availability of phosphorus and base cations, and change the isotopic and chemical baselines of the soils in which they grow (Herridge *et al*., 2008; Perakis & Pett-Ridge, 2019). Symbiotic dependence in these taxa is not fixed. It responds to soil mineral nitrogen, phosphorus supply, water status and microbial partner identity, which means that any treatment intended to improve nutrition also shifts the proportion of plant nitrogen derived from the atmosphere (Reddell *et al*., 1997; Sayed, 2011). Quantification is therefore not a neutral act of observation. The measurement system and the treatment interact.

A recent global compilation of terrestrial biological nitrogen fixation measurements explicitly records quantification method alongside fixation rates, reinforcing the need to distinguish measurements generated by different approaches before synthesis or upscaling (Reis Ely *et al*., 2025).

Two classes of intervention dominate contemporary practice. Soil and substrate amendments, including biochar, compost, vermicompost, farmyard manure, lime and sparingly soluble phosphate sources, are applied to modify retention, pH, cation exchange and organic matter status. Bio-inoculants, including rhizobia, *Frankia*, arbuscular mycorrhizal fungi, phosphate-solubilising microorganisms and other plant growth-promoting bacteria, are applied to establish or intensify the symbioses on which woody nitrogen fixers depend. Both classes are widely reported to raise seedling nutrient concentration, nodulation and growth in containerised nurseries, and both are recommended for field establishment on degraded substrates (Muthukumar & Udaiyan, 2010; Salto *et al*., 2020; Simiele *et al*., 2022). The evidence base for these claims is nonetheless assembled from measurements whose validity conditions are, in several important cases, violated by the very treatments under test.

Recent meta-analytic evidence also indicates that microbial inoculation can alter the structure and bacterial composition of resident soil microbial communities, with responses conditioned by nutrient status and environmental conditions; this supports treating inoculation as a change to the biological reference state rather than as an isolated input (Li *et al*., 2024).

Three specific tensions recur. First, carbonaceous and organic amendments alter the behaviour of the extractants and isotopic baselines used to define nutrient availability. Field-aged biochar retains nitrate in forms that standard salt extraction fails to recover, yet that retained nitrogen is subsequently taken up by plants, so the same soil can be classified as nitrogen-poor by one operational definition and nitrogen-supplying by another (Haider *et al*., 2016, 2020). Amendments also change soil and plant nitrogen isotope signatures, which is the quantity on which the most widely used field estimates of biological nitrogen fixation depend (Craswell *et al*., 2021). Second, inoculation changes the reference condition against which treatment effects are computed. Non-inoculated controls in field soils and in non-sterilised substrates are rarely free of indigenous propagules, and inoculant strains frequently fail to occupy nodules in competition with resident populations, so a null growth response is ambiguous between biological ineffectiveness and failure of establishment (Mendoza-Suárez *et al*., 2021; O'Callaghan *et al*., 2022). Third, the containerised nursery is not a small field. Finite rooting volume, engineered substrates, irrigation-driven leaching and controlled-release fertiliser dissolution redefine the boundary conditions of the nutrient system, and measured nutrient availability in pots diverges systematically from that of the parent soil (Dalling *et al*., 2013; Huett, 1997; Poorter *et al*., 2012).

In biochar-amended soils, recent evaluation of portable X-ray fluorescence against conventional extraction methods showed that predictive performance differed among nutrients and experimental conditions, further illustrating the method dependence of nutrient-availability estimates under amendment (Antonangelo & Zhang, 2024).

Recent review evidence on diffusive gradients in thin films further shows that nutrient-bioavailability assessment can differ from conventional chemical extraction and that DGT measurements are commonly evaluated against nutrient accumulation in plants (Senila & Kovacs, 2024).

Existing syntheses address parts of this problem but not its measurement core. Reviews of isotopic methodology for biological nitrogen fixation are thorough for annual crops and give woody perennials comparatively brief treatment, and the most comprehensive appraisal of the natural abundance technique for woody perennials predates the current generation of amendment research (Boddey *et al*., 2000; Chalk & Craswell, 2018). Methodological reviews of soil phosphorus and nitrogen availability assessment are strong on analytical principles but are not written for systems in which the plant itself supplies nitrogen and modifies rhizosphere chemistry (Kruse *et al*., 2015; Negassa & Leinweber, 2009). Meta-analyses of amendment and inoculant effects pool outcomes across heterogeneous measurement protocols without asking whether the protocols are commensurable (Farhangi-Abriz *et al*., 2024; Ross & Emery, 2025; Thilakarathna & Raizada, 2017). Nursery-oriented reviews concentrate on seedling quality outcomes rather than on the inferential status of the nutrient measurements used to explain them (Simiele *et al*., 2022). What has not been assembled is a measurement-centred critical appraisal that asks, for each quantification approach in routine use, whether its assumptions survive the introduction of amendments and inoculants in woody nitrogen-fixing systems, and how far conclusions drawn in containers can be transferred to the field.

1.1 Scope, Research Gap and Objectives

This review evaluates the methods used to quantify soil–plant nutrient dynamics in nitrogen-fixing trees and shrubs grown under soil or substrate amendments and bio-inoculants, in containerised nursery production and in field plantings. Coverage extends to methods for estimating symbiotic nitrogen inputs, soil nutrient availability and transformation, nutrient loss pathways, the abundance and activity of microbial mediators, plant nutrient status and content, and the upscaling of seedling measurements to stand level. The review is organised by measurement domain rather than by species, amendment product or geographical region, and it treats nitrogen and phosphorus as the principal elements while addressing base cations where the evidence permits.

The gap addressed is specific. Appraisals of quantification methods and appraisals of amendment and inoculant efficacy have developed largely in parallel, so the question of whether the standard measurement toolkit remains valid when amendments modify extractant behaviour and isotopic baselines, and when inoculants modify the biological reference state, has not been examined systematically for woody nitrogen-fixing systems. The parallel question of whether container-based nutrient measurements support field inference in these systems has likewise been left implicit.

Four objectives follow. The first is to set out the measurement architecture of nutrient dynamics in woody nitrogen-fixing systems and to distinguish pools, fluxes, surrogates and diagnostic indices. The second is to appraise critically each principal quantification approach, stating its assumptions, the conditions under which those assumptions fail, and the direction of the resulting bias. The third is to identify where the literature is internally inconsistent and to explain the methodological reasons for disagreement rather than attributing it to biological variation alone. The fourth is to specify a defensible minimum measurement and reporting set, and a prioritised research agenda, for studies of amendments and bio-inoculants in these systems.

The review excludes herbaceous grain and forage legumes except where their methodological literature is directly transferable, non-symbiotic free-living diazotrophy as a primary subject, genetic improvement of symbiotic partners, economic appraisal of amendment supply chains, and the wider carbon-sequestration literature that does not bear on nutrient measurement.

2. Methods for Literature Selection

2.1 Review Design and Rationale

A critical narrative design was selected because the governing question is evaluative rather than effect-estimating. The literature relevant to this review spans isotope geochemistry, soil chemistry, microbial ecology, nursery silviculture and agroforestry, and the studies within it do not share a common outcome definition, exposure definition or reporting convention. A systematic review protocol would require the eligible studies to be commensurable in exactly the respect that this review contests, and a meta-analysis would pool effect sizes derived from measurement protocols whose comparability is the object of appraisal. Narrative synthesis was therefore the appropriate design, and the standardisation deficits that make quantitative pooling unsafe are themselves treated here as findings. The looser structure of a narrative review does not remove the obligation of transparency, and recent methodological commentary on inoculation research makes the reporting expectations explicit, requiring disclosure of inoculum identity, viability, application rate, substrate characterisation and control condition before a treatment effect can be interpreted (de-Bashan & Nannipieri, 2024; Novak *et al*., 2025). The search, eligibility and appraisal procedures described below were applied in that spirit, and each judgement of methodological adequacy is stated with its reasons rather than reduced to a score.

2.2 Information Sources

Searching was conducted in AGRIS, Crossref, the Directory of Open Access Journals, Europe PMC, Google Scholar, OpenAIRE and Semantic Scholar. AGRIS was used to reach agricultural, forestry and nursery literature, including regionally published work on

tropical nitrogen-fixing tree nurseries that is unevenly represented in biomedical and multidisciplinary indexes. Europe PMC was used for microbial ecology and plant–microbe interaction literature. Crossref was used both for retrieval and for bibliographic confirmation. Institutional technical literature was retrieved from the publication repository of the International Atomic Energy Agency, which issues guidance on isotopic methods for nitrogen management. Database searching was supplemented by backward citation searching of reference lists, forward citation searching of pivotal methodological papers, related-article searching, and targeted checking for corrections and expressions of concern attached to the retained records.

2.3 Search Strategy and Search Period

Five concept blocks were developed from the review question and combined with Boolean operators, using controlled and free-text terms with truncation where the interface allowed it. The blocks covered the host system, the growing domain, the intervention, the measurement or quantification approach, and the nutrient process or outcome. Representative strings are reproduced in Table 1. Blocks were combined with the AND operator and terms within blocks with the OR operator; the measurement block was also run against the host block alone, without the intervention block, so that methodological papers of general soil science relevance were retrieved and appraised for transferability. The search period extended from 1985 to 14 June 2026. The starting year reflects the consolidation of field isotopic methodology for multipurpose woody legumes and of vector-based nutrient diagnosis for containerised seedlings, the two measurement traditions on which this literature continues to rest.

The search architecture is summarised below, and its structure clarifies an asymmetry in the retrieved evidence. Concept blocks concerning the host system, the intervention and the outcome returned large and rapidly growing sets, whereas the measurement block returned a much smaller set in which methodological appraisal specific to woody nitrogen-fixing hosts was sparse and concentrated in isotope-based nitrogen work. Most measurement-critical evidence therefore had to be drawn from general soil and plant nutrition methodology and assessed for transferability, a dependency that shapes the confidence statements made throughout this review.

Table 1. Search concept blocks, representative search terms and the analytical purpose of each block

Block	Concept	Representative terms and operators	Analytical purpose	Supporting references
1	Host system	"nitrogen-fixing tree*" OR "N2-fixing tree*" OR "leguminous tree*" OR actinorhizal OR *Acacia* OR *Leucaena* OR *Gliricidia* OR *Casuarina* OR *Faidherbia* OR *Prosopis* OR *Dalbergia* OR *Alnus* OR *Robinia* OR *Sesbania*	Restricts retrieval to woody symbiotic systems in which host-supplied nitrogen alters the measurement baseline	Herridge *et al*. (2008); Sayed (2011)
2	Growing domain	nursery OR container* OR polybag OR "root trainer" OR "growing medium" OR substrate OR plantation OR "mixed-species" OR agroforestry OR field	Separates the containerised and field measurement domains, which impose different boundary conditions	Huett (1997); Poorter *et al*. (2012)
3	Intervention	biochar OR compost OR vermicompost OR "farmyard manure" OR "organic amendment" OR lime OR "rock phosphate" OR inoculat* OR rhizobi* OR *Frankia* OR "arbuscular mycorrhiza*" OR "phosphate-solubilis*" OR "plant growth-promoting"	Captures both amendment and bio-inoculant classes, including combined application	Ross & Emery (2025); Simiele *et al*. (2022)
4	Measurement approach	"15N" OR "natural abundance" OR "isotope dilution" OR "B value" OR "acetylene reduction" OR ureide OR "pool dilution" OR "diffusive gradients in thin films" OR "ion exchange membrane" OR microdialysis OR "sequential fractionation" OR Olsen OR Mehlich OR qPCR OR *nifH* OR colonisation OR zymography OR "vector analysis" OR allometr* OR lysimeter	Identifies the quantification methods whose assumptions are the object of appraisal	Chalk & Craswell (2018); Kruse *et al*. (2015)
5	Process or outcome	"nutrient dynamics" OR "nutrient uptake" OR "nutrient use efficiency" OR mineralis* OR immobilis* OR availability OR "nitrogen transfer" OR leaching OR "Ndfa" OR "nutrient budget"	Restricts to studies reporting nutrient pools, fluxes or derived efficiency terms rather than growth alone	Ladha *et al*. (2022); Nygren & Leblanc (2015)

Note. Asterisks within search terms denote truncation. Blocks were combined with AND and terms within blocks with OR. Block 4 was also run against Block 1 alone to retrieve methodological literature of general relevance for appraisal of transferability. The listed references illustrate the block concept and are not an exhaustive account of retrieval for that block

2.4 Eligibility Criteria

Records were eligible when they were peer-reviewed journal articles, or authoritative technical publications of recognised scientific or intergovernmental bodies, published between 1985 and 14 June 2026 in English, and when they reported or critically appraised the measurement of soil or plant nutrient pools, fluxes, transformation rates, symbiotic nitrogen inputs, microbial mediators or

nutrient status in woody nitrogen-fixing systems, or in methodological domains from which transfer to such systems could be justified explicitly. Studies of herbaceous legumes were eligible only where the methodological finding was general, such as appraisals of isotopic fractionation, pool dilution assumptions or inoculant competition. Records were excluded when they were promotional or trade material, patents used as evidence of effectiveness, non-scholarly commentary, conference abstracts without methodological detail, or preprints in areas where peer-reviewed evidence was available, and when bibliographic identity could not be confirmed. Older foundational work was admitted where it defined a method still in use.

2.5 Screening, Duplicate Handling and Study Selection

Titles were screened first for relevance to the measurement question, then abstracts for the presence of a quantification method or of a methodological appraisal, then full texts for the adequacy with which the method and its assumptions were described. Duplicates arising from overlapping index coverage were identified by digital object identifier where available and otherwise by matching author, title, journal and year, and the record with the most complete metadata was retained. Where a preprint and a subsequent peer-reviewed version of the same work were retrieved, only the peer-reviewed version was retained. Records whose full text could not be obtained were retained only when the accessible metadata and abstract supported the specific claim for which the work was cited, and were otherwise set aside. No numerical screening tally is reported, because selection was purposive and iterative rather than a single documented sequence of exclusions.

2.6 Critical Appraisal and Evidence Synthesis

Retained studies were appraised against criteria appropriate to their design and claim. For method-comparison studies these were the presence of an independent reference measurement, the range of soils or substrates covered, the treatment of analytical replication and detection limits, and whether calibration was internal or external to the dataset. For amendment and inoculant experiments these were the characterisation of the amendment or inoculum, the viability and identity of applied organisms, the adequacy of the control condition, the rooting volume and substrate description, the duration relative to the process claimed, the level at which replication was applied, and whether nutrient results were reported as content, concentration or both. For isotopic studies these were the justification of the reference plant or reference pool, the derivation of isotopic fractionation parameters, and the reporting of measurement uncertainty relative to the isotopic difference being resolved. Formal risk-of-bias instruments were not applied, because no recognised tool covers this combination of designs and because the reporting detail required to apply one is frequently absent.

Themes were constructed around measurement domains and around the assumptions that amendments and inoculants place under strain, rather than around products or species. Conflicting findings were retained and interpreted by asking whether the disagreement could be attributed to a difference in operational definition, in measurement domain, in duration, or in the biological reference state, before any biological explanation was accepted. Confidence statements in the sections that follow are graded qualitatively according to the number of independent method-comparison studies available, the breadth of soils and hosts covered, and the consistency of direction across studies.

3. The Measurement Architecture of Nutrient Dynamics in Woody Nitrogen-Fixing Systems

3.1 Pools, Fluxes, Surrogates and Diagnostic Indices

The phrase nutrient dynamics is used across this literature to denote at least four categories of quantity, and the conflation of these categories accounts for a substantial share of apparent disagreement between studies. The first category comprises pools, which are state variables such as soil total nitrogen, extractable ammonium and nitrate, sequentially extracted phosphorus fractions, and plant tissue nutrient content. The second comprises fluxes, which are rates such as gross and net nitrogen mineralisation, phosphorus mineralisation and immobilisation, symbiotic nitrogen input per unit time, nutrient uptake per plant per interval, and leaching loss. The third comprises surrogates, which are quantities correlated with a flux but not equal to it, including nitrogenase-dependent acetylene reduction, relative ureide abundance in xylem sap, nodule number and mass, arbuscular mycorrhizal root colonisation percentage, and the abundance of functional genes. The fourth comprises diagnostic indices, which are constructed from pools in order to support a management decision, and which include nutrient use efficiency terms, vector diagnosis displacement in the concentration–content–mass plane, and critical concentration comparisons.

Confusion between these categories has direct consequences. A study reporting that an inoculant raised root colonisation and nodule number has measured surrogates; it has not measured a nitrogen flux, and the relationship between the surrogate and the flux is neither linear nor stable across nutrient supply levels. A study reporting higher extractable phosphorus under an amendment has measured a pool that is operationally defined by the extractant, and whether that pool corresponds to plant-accessible phosphorus in the presence of that amendment is a separate empirical question (Gu & Margenot, 2021; Six *et al*., 2013). A study reporting higher tissue nitrogen concentration in inoculated seedlings may be reporting a dilution effect in slower-growing plants rather than an increase in acquisition, which is precisely the ambiguity that content-based vector diagnosis was developed to resolve (Imo & Timmer, 1997; Timmer, 1997).

A further structural feature of these systems is that the host modifies several pools simultaneously. Symbiotic nitrogen input raises the nitrogen status of litter and rhizodeposits, which changes subsequent mineralisation; increased nitrogen supply raises phosphorus demand, which changes rhizosphere acidification and phosphatase expression; and nitrogen-fixing trees have been shown to draw more heavily on rock-derived nutrient sources than co-occurring non-fixers, which alters the interpretation of base cation and phosphorus budgets in mixed stands (Perakis & Pett-Ridge, 2019; Spohn & Kuzyakov, 2013). Measurement designs that treat the

soil as an independent supplier and the tree as a passive recipient therefore misrepresent the causal structure of the system they sample.

3.2 Why Containerised and Field Systems Impose Different Measurement Logics

The containerised nursery and the field planting are distinct measurement domains, not two scales of one domain. In a container the rooting volume is finite and typically explored completely, so the plant is not sampling a spatially heterogeneous soil but exhausting a bounded reservoir; the substrate is commonly an engineered mixture of peat, coir, bark, perlite or biochar whose cation exchange, buffering and hydraulic behaviour differ fundamentally from mineral soil; nutrient supply is frequently dominated by controlled-release fertiliser prills whose dissolution is temperature and moisture dependent; and irrigation imposes a repeated drainage flux that carries nutrients out of the system at rates that have been measured directly and can account for a large share of applied nutrients (Huang & Gu, 2019; Huett, 1997). Pot volume alone exerts a systematic and quantitatively large influence on plant growth, which propagates into any nutrient content measure derived from biomass (Poorter *et al*., 2012). The chemistry of the pot environment also diverges from that of the source soil during the experiment itself, with measurable changes in nutrient availability attributable to the container rather than to the treatment (Dalling *et al*., 2013). Interpretation of root responses in pots is also sensitive to the assumptions the analyst brings, because the same root distribution is consistent with different foraging models depending on whether pot volume is treated as a constraint or as a resource (McNickle, 2020).

Field systems invert most of these properties. Rooting volume is effectively unbounded on the timescale of a seedling establishment trial, soil properties vary spatially at scales comparable to the plot, nutrient supply is dominated by mineralisation of native organic matter, and loss pathways include gaseous and lateral routes that are difficult to close. The consequence for inference is that the two domains answer different questions. Container work can establish that a substrate amendment or an inoculant is capable of altering seedling nutrient status under defined conditions; it cannot establish the magnitude of that alteration in the field, and comparisons between controlled and field environments across a wide range of plant responses show differences large enough to reverse conclusions (Poorter *et al*., 2016). The inferential rule that follows, and that much of this literature does not observe, is that container experiments support statements about mechanism and capability, and field experiments are required for statements about magnitude and persistence.

The distinctions drawn above are summarised in Table 2, which separates the quantity measured from the operational definition and from the inferential claim it can support. Its main implication is that several quantities routinely treated as interchangeable are not, and that the container and field columns differ not in precision but in what the measurement means.

Table 2. Conceptual distinctions among quantities used to describe nutrient dynamics, their operational definitions, and the inferential claims each supports

Quantity class	Representative measures	Operational definition	Claim supported	Principal constraint	Supporting references
Pool	Extractable ammonium and nitrate; Olsen or Mehlich phosphorus; sequentially extracted phosphorus fractions; tissue nutrient content	Quantity recovered by a specified reagent, contact time and soil-to-solution ratio, or by digestion of tissue	Relative comparison between treatments analysed by the identical protocol	Recovery is reagent-defined and can be altered by the amendment itself	Gu & Margenot (2021); Haider *et al*. (2016); Negassa & Leinweber (2009)
Flux	Gross and net nitrogen mineralisation; phosphorus mineralisation and immobilisation; symbiotic nitrogen input; leaching loss	Rate of transfer between defined pools over a stated interval, usually from isotopic dilution or mass balance	Process-level statement about supply or loss	Requires assumptions about pool homogeneity and tracer behaviour that are rarely tested	Braun *et al*. (2018); Li & Margenot (2026); Schimel (1996)
Surrogate	Acetylene reduction rate; relative ureide abundance; nodule number and mass; mycorrhizal colonisation percentage; functional gene abundance	Measured property correlated with, but not equal to, the flux of interest	Evidence that a mechanism is present and responsive	Surrogate-to-flux conversion is species and condition dependent and often uncalibrated	Herridge *et al*. (1996); Soper *et al*. (2021); Sun & Tang (2012)
Diagnostic index	Nutrient use efficiency terms; vector diagnosis displacement; critical concentration comparison	Derived ratio or graphical displacement computed from pools and biomass	Management interpretation of nutritional adequacy or limitation	Interpretation depends on whether concentration, content and biomass are reported jointly	Imo & Timmer (1997); Park *et al*. (2015); Timmer (1997)
Domain-conditional measure	Any of the above obtained in containers	Quantity measured within a bounded engineered substrate under irrigation	Statement about mechanism and capability	Magnitude is not transferable to field soils without calibration	Dalling *et al*. (2013); Poorter *et al*. (2012, 2016)

Note. The final row is not a separate quantity class but a qualification that applies to every class when measurement occurs in containers

4. Quantifying Symbiotic Nitrogen Inputs Under Amendments and Inoculation

4.1 The Total Nitrogen Difference Method and the Reference Plant Problem

The oldest field approach compares the nitrogen accumulated by the fixing species with that accumulated by a non-fixing reference grown under the same conditions, attributing the difference to atmospheric fixation. The method requires that the reference plant absorb soil nitrogen from the same pools, over the same period, with the same efficiency as the fixing species. For woody perennials this requirement is severe. Root architecture, rooting depth, phenology, mycorrhizal status and lifespan differ between a nitrogen-fixing tree and any available non-fixing tree, and the difference persists over the multi-year intervals across which tree nitrogen accumulation is integrated (Boddey *et al*., 2000). Allometric work on paired fixing and non-fixing species confirms that biomass–diameter relationships themselves differ between the two functional groups, so the reference plant introduces error into the biomass term as well as the nitrogen concentration term (Carreras Pereira *et al*., 2023).

Amendments aggravate the problem in a specific direction. If an amendment raises soil nitrogen supply, the reference plant takes up more soil nitrogen while the fixing species partially substitutes soil nitrogen for fixation, so the measured difference contracts and fixation appears to fall even when absolute fixation is unchanged. If an amendment raises phosphorus supply and phosphorus limits nodule function more strongly than it limits the reference species, the difference expands. Neither outcome is interpretable without independent evidence on symbiotic dependence, which the method does not provide. The approach retains value for estimating net nitrogen accretion in a stand over long intervals, and for that purpose it is arguably more robust than short-term isotopic estimates, but it should not be used to attribute treatment effects to changes in fixation.

4.2 Isotopic Enrichment and Isotope Dilution

Labelling the soil with a nitrogen-15 enriched source and comparing the isotopic composition of the fixing species with that of a reference raises the isotopic contrast well above analytical uncertainty and in principle removes the sensitivity to small natural signals. The assumptions are nonetheless demanding: the labelled and native soil nitrogen pools must remain isotopically uniform over the uptake period, and the fixing and reference species must access them in the same proportions through time. In woody systems the uptake period is long relative to the rate at which added label is diluted by mineralisation of native organic nitrogen, so the isotopic composition of the plant-available pool declines through the experiment, and the two species integrate a moving signal at different rates (Chalk & Craswell, 2018). Guidance issued for isotopic nitrogen work in agricultural systems sets out the conditions under which enrichment approaches remain defensible and emphasises repeated characterisation of the labelled pool rather than a single initial value (International Atomic Energy Agency, 2008).

The interaction with amendments is direct and under-appreciated. Organic amendments add a large, isotopically distinct, mineralisable nitrogen pool, which accelerates dilution of the applied label and changes its trajectory. Carbonaceous amendments add a sorptive phase that retains mineral nitrogen and releases it on a different timescale, so the isotopic composition of the solution pool and that of the exchangeable pool diverge. Isotopic tracing in biochar-amended soils has been reviewed specifically, and the conclusion relevant here is that biochar alters the distribution of applied label among pools in ways that require explicit accounting rather than assumption (Craswell *et al*., 2021). Comparative work indicates that enrichment-based and natural-abundance-based estimates of symbiotic dependence do not always agree, which constrains the practice of treating one as a validation of the other (Chalk *et al*., 2016).

4.3 Natural Abundance, the Isotopic Baseline and the Fractionation Parameter

The natural abundance method exploits the small enrichment of soil nitrogen in nitrogen-15 relative to atmospheric nitrogen, estimating the proportion of plant nitrogen derived from the atmosphere from the isotopic composition of the fixing species, of a reference integrating soil nitrogen, and of the same fixing species grown without soil nitrogen, the last supplying the isotopic fractionation parameter conventionally termed the B value. Its attraction for woody systems is that it requires no soil manipulation and can be applied retrospectively in existing plantations. Its weakness is arithmetic: when the isotopic difference between the reference plant and the fixing species is small relative to spatial variability and analytical uncertainty, the estimate becomes unstable, and in some forest legume settings the resulting estimates have been shown to be too imprecise to support conclusions about treatment effects (Boddey *et al*., 2000; Gehring & Vlek, 2004).

The fractionation parameter is the most frequently mishandled term. It is species specific, and within a species it varies with growth stage, with the nitrogen contribution of the seed, and with the tissue sampled, so that values borrowed from another species or another growth stage propagate substantial error into the estimate of atmospheric dependence and into nitrogen balances constructed from it (Nebiyu *et al*., 2014; Rose *et al*., 2018). Published values for tree species are comparatively scarce, and studies of woody systems frequently adopt a value from a herbaceous legume or assume a single figure across a rotation. This practice is a more serious source of error than the sampling design attention it usually receives.

Amendments and inoculants disturb both ends of the calculation. Organic amendments, composts and manures carry their own isotopic signatures and alter the isotopic composition of the soil nitrogen actually taken up, which changes the reference plant value in a direction that depends on the amendment and on the extent of nitrification and gaseous loss that follow application (Craswell *et al*., 2021). Inoculation changes the microsymbiont population, and because the fractionation parameter is a property of the host–microsymbiont combination rather than of the host alone, inoculation with an unfamiliar strain in principle changes the parameter that is being held constant. Studies of nitrogen fixation in mixed *Acacia–Eucalyptus* plantations illustrate what a careful application looks like, combining natural abundance estimates with stand nitrogen accumulation and reporting that symbiotic dependence

differed between mixture and monoculture, which is a statement about the system rather than about the method (Paula *et al*., 2018). The same research programme used isotopic labelling to demonstrate short-term belowground transfer of fixed nitrogen between species and later showed that nutrient deficiency increased the transfer rate, results that depend on tracer addition rather than natural abundance and that would be undetectable by natural abundance alone (Oliveira *et al*., 2021; Paula *et al*., 2015). Agroforestry applications in cocoa systems with *Gliricidia sepium* shade trees, and in silvopastoral systems with legume trees and associated grasses, have used the same family of methods to estimate both fixation and transfer, with the estimates of transfer proving more sensitive to assumptions than the estimates of fixation (Kaba *et al*., 2019; Nygren & Leblanc, 2015; Sierra & Nygren, 2006). Where transfer occurs through fungal pathways, tracer work in smallholder fields has shown that mycorrhizal mycelium mediates a measurable share of tree-derived nitrogen reaching an associated crop, and that the identity of the mycelium maintaining the pathway matters for the quantity transferred (Dierks *et al*., 2022, 2024).

4.4 Activity-Based Surrogates

The acetylene reduction assay substitutes acetylene for atmospheric nitrogen and measures ethylene production as a proxy for nitrogenase activity, converting to a nitrogen fixation rate by a theoretical electron allocation ratio. Two problems limit its use for quantification. The conversion ratio is not a constant and has been shown to depart from the conventional value across systems, so rates computed with a fixed ratio carry an unquantified multiplicative error (Soper *et al*., 2021). Detachment of nodules or roots and exposure to acetylene also perturb the system being measured, so the assay reports a transient and partly artefactual activity rather than a sustained rate. Methodological development has addressed part of this by measuring nitrogenase-associated gas exchange continuously and non-destructively at the whole-plant scale, which removes the detachment artefact and allows repeated measurement of the same individual, although the approach remains confined to plants small enough for whole-plant chambers and therefore to the nursery domain (Bytnerowicz *et al*., 2019).

The relevance to amendment and inoculant research is that the assay is frequently the only fixation-related measurement reported in nursery experiments, where it is presented alongside nodule counts as evidence of enhanced fixation. Studies of rhizobial inoculation in *Dalbergia sissoo* seedlings, for example, report nodulation and nitrogenase activity responses to inoculant isolates and to mineral nitrogen supply, which establishes that the symbiosis responded but does not quantify the nitrogen accrued (Dhiman *et al*., 2022). Interpreting such data as a fixation flux overstates what the measurement can bear, and the overstatement is systematic rather than occasional in this literature.

4.5 Solute-Based Surrogates

In legumes that export fixed nitrogen predominantly as ureides, the relative abundance of ureides among xylem solutes indexes symbiotic dependence. Evaluation across six tree legume species established that the relationship is species specific and requires independent calibration, and that species differ in whether they are ureide exporters at all, which restricts the method to a subset of the taxa of interest (Herridge *et al*., 1996). Parallel work on shrub legumes subjected to repeated pruning combined isotopic and ureide approaches and found the two broadly concordant under some conditions, while also showing that pruning, by altering the sink demand for nitrogen, changes the solute composition independently of fixation (Peoples *et al*., 1996). Actinorhizal genera such as *Casuarina* and *Alnus* fall outside the method entirely, since they do not use the ureide export pathway, and reviews of *Casuarina–Frankia* nutrition accordingly rely on nodulation, growth and isotopic evidence rather than on solute composition (Sayed, 2011).

The practical consequence is a taxonomic bias in the measurement base. Ureide methods are cheap and field-portable, so they are applied where they can be applied, and the species for which calibrated estimates of symbiotic dependence exist are not a representative sample of the species used in nurseries and restoration plantings.

4.6 How Amendments and Inoculants Destabilise each Approach

The common structure of the failures described above is that each method holds one quantity constant while the treatment changes it. The difference method holds reference plant uptake constant; amendments change soil nitrogen supply and therefore reference uptake. Enrichment methods hold the isotopic composition of the plant-available pool constant or predictably decaying; organic and carbonaceous amendments change both its magnitude and its trajectory. Natural abundance methods hold the reference plant signature and the fractionation parameter constant; amendments change the former and inoculation can change the latter. Activity assays hold the electron allocation ratio constant; it varies. Solute methods hold the export pathway constant; pruning, nitrogen supply and taxonomy vary it.

The direction of bias is not random. Amendments that increase soil nitrogen supply will tend to produce underestimates of fixation by the difference method and by natural abundance, because both rely on a contrast that soil nitrogen erodes. Amendments that retain mineral nitrogen without releasing it to the extractant, as field-aged biochar does, will tend to produce overestimates of symbiotic dependence, because the soil appears nitrogen-poor while the plant is in fact accessing retained nitrogen (Haider *et al*., 2016, 2020). Reported instances of amendments increasing the proportion of plant nitrogen derived from the atmosphere should therefore be treated with particular caution when the amendment is sorptive and when the isotopic baseline was characterised once at the start of the experiment. Meta-analytic evidence that biochar increases nitrogen fixation in legumes is consistent with a genuine effect mediated by improved phosphorus and micronutrient supply and by relief of soil constraints, and it is also consistent in part with the measurement artefact just described; the primary studies pooled do not generally contain the information needed to separate the two (Farhangi-Abriz *et al*., 2024).

Table 3 sets out, for each approach, the assumption at risk and the expected direction of bias under amendment and inoculation. Its practical implication is that no single method is adequate on its own in amended systems, and that the combination of a tracer-based estimate with an independent nitrogen accumulation estimate is the minimum defensible design where a fixation claim is central.

Table 3. Comparative appraisal of methods for quantifying symbiotic nitrogen input in woody hosts, with the assumption placed at risk by amendments and inoculation

Method	Quantity obtained	Critical assumption	Effect of amendments	Effect of inoculation	Appropriate use	Supporting references
Total nitrogen difference	Net nitrogen accretion attributable to the fixing species	Reference plant accesses the same soil nitrogen with the same efficiency over the same period	Changed soil nitrogen supply alters reference uptake and host substitution, contracting or expanding the difference	Minor, except where inoculation changes growth rate and therefore soil nitrogen depletion	Long-interval stand nitrogen accretion; not for attributing treatment effects to fixation	Boddey *et al*. (2000); Carreras Pereira *et al*. (2023)
Isotopic enrichment and isotope dilution	Proportion of plant nitrogen derived from the atmosphere; tracer recovery	Labelled pool remains uniform and is accessed identically by both species	Organic amendments accelerate label dilution; sorptive amendments redistribute label among pools	Limited direct effect on the tracer, but altered uptake pattern changes integration	Short-interval flux and transfer studies with repeated pool characterisation	Chalk & Craswell (2018); Craswell *et al*. (2021); International Atomic Energy Agency (2008)
Natural abundance	Proportion of plant nitrogen derived from the atmosphere	Reference plant signature and host fractionation parameter are known and stable	Amendment-derived nitrogen shifts the soil signature; sorptive retention can inflate apparent symbiotic dependence	Changed microsymbiont alters the fractionation parameter that is assumed fixed	Retrospective estimation in established stands where the isotopic contrast is large	Gehring & Vlek (2004); Nebiyu *et al*. (2014); Paula *et al*. (2018); Rose *et al*. (2018)
Acetylene reduction	Instantaneous nitrogenase-dependent activity	Electron allocation ratio is constant and detachment does not perturb activity	Indirect, through changes in nodule function and carbon supply	Direct and intended, but the surrogate does not convert to a flux	Comparative screening of symbiotic responsiveness within one experiment	Bytnerowicz *et al*. (2019); Soper *et al*. (2021)
Xylem ureide relative abundance	Index of symbiotic dependence in ureide-exporting legumes	Species-specific calibration holds and export pathway is unchanged	Changed mineral nitrogen supply alters solute composition independently of fixation	Limited, provided the host remains the same	Field screening in calibrated ureide-exporting legume species	Herridge *et al*. (1996); Peoples *et al*. (1996)
Continuous whole-plant gas exchange	Repeated non-destructive activity and coupled carbon exchange	Chamber conditions represent growing conditions	Indirect	Direct and repeatable on the same individual	Nursery-scale mechanistic work and calibration of destructive assays	Bytnerowicz *et al*. (2019)

Note. Direction of bias is stated for the amendment classes discussed in the text, principally carbonaceous and organic amendments; the entries are not intended to apply to liming or to soluble mineral fertiliser without further qualification

5. Quantifying the Soil Supply: Availability, Transformation and Loss

5.1 Chemical Extraction and amendment-induced Artefacts

Routine assessment of nutrient availability rests on chemical extraction, in which a reagent of specified composition is equilibrated with soil for a specified time and the recovered nutrient is reported as the available fraction. The approach is an operational definition rather than a measurement of a physical quantity, and its usefulness derives from empirical calibration against plant response in particular soil groups. Amendments break that calibration in two distinct ways. They change the solid phase with which the reagent equilibrates, and they introduce a new sorptive or soluble phase whose interaction with the reagent has not been calibrated.

The best-documented case concerns nitrate and ammonium in biochar-amended soils. Field-aged biochar retains mineral nitrogen in forms that conventional potassium chloride extraction recovers incompletely, so extractable nitrogen underestimates the quantity present, and the discrepancy increases with the residence time of the biochar in the soil (Haider *et al*., 2016). Subsequent work

demonstrated that the nitrogen retained in this way is subsequently available to plants, which establishes that the extraction result rather than the soil is at fault (Haider *et al*., 2020). Methodological comparison of extraction procedures for biochar confirms that recovery depends on the extractant and on the extraction regime, and that no single protocol has been shown to recover the plant-relevant pool across biochar types (Walsh *et al*., 2019). The practical implication for nursery and field experiments is uncomfortable. A biochar treatment can reduce measured extractable nitrogen while increasing plant nitrogen uptake, and studies reporting that pattern have sometimes interpreted it as evidence of enhanced uptake efficiency when the more parsimonious explanation is an extraction artefact. Meta-analytic summaries of biochar effects on the soil nitrogen cycle report heterogeneous and sometimes opposing effects on mineral nitrogen pools, and part of that heterogeneity is attributable to the measurement problem rather than to the underlying process (Liu *et al*., 2018; Zhang *et al*., 2021).

Phosphorus presents a parallel difficulty with a different mechanism. Alkaline extractants used for calcareous and neutral soils dissolve phosphorus associated with carbonates and with freshly added organic amendments, and organic amendments supply phosphorus in forms whose recovery differs between reagents. Where an amendment raises pH, the reagent appropriate to the unamended soil may no longer be appropriate to the amended soil, so a treatment-by-method interaction is embedded in the design. Studies that apply a single extractant across amendment treatments and interpret the resulting differences as availability differences are therefore reporting a composite of availability and reagent behaviour.

5.2 Diffusion-Based and Sink-Based Measures

Methods that impose a sink and measure the rate at which the soil resupplies it are conceptually closer to the plant uptake process than equilibrium extraction, because they integrate concentration, buffering and diffusive transport. Three variants are relevant. Diffusive gradients in thin films deploy a binding gel behind a diffusive layer and yield a flux-like measure of resupply. Ion-exchange membranes and resins provide a lower-cost sink that can be deployed in situ over days to weeks. Microdialysis samples the diffusive flux of solutes to a small probe at high temporal resolution and with minimal disturbance.

Comparative evidence favours the diffusion-based approach for phosphorus in several soil groups. In highly weathered tropical soils assessed against isotopic dilution, diffusive gradients in thin films tracked isotopically exchangeable phosphorus more closely than conventional tests, and in a companion study using maize and rice the same technique predicted crop response to applied phosphorus more consistently than the conventional tests across a soil set spanning contrasting sorption capacities (Six *et al*., 2012, 2013). Work in temperate arable soils reached a convergent conclusion when comparing the technique with a widely used extraction procedure (Hill *et al*., 2021). The convergence of these results across climatic zones and crops is one of the firmer findings in the soil availability literature, and it has a direct bearing on the topic of this review, because the soils on which nitrogen-fixing trees are planted for restoration are frequently the high-sorption, low-phosphorus soils in which conventional tests perform least well.

Sink-based nitrogen measures are less settled. Direct comparison of in situ ion-exchange membranes with extraction in tundra soils found that the two approaches describe different aspects of nitrogen supply and are not interchangeable, with membrane results reflecting resupply over the deployment period and extraction results reflecting the instantaneous pool (Gu & Grogan, 2020). Microdialysis-derived diffusive flux measurements provide a defensible index of nitrogen availability at high temporal resolution, and their principal contribution has been to show that the flux available to a small sink is often far smaller than extractable pools would suggest (Shaw *et al*., 2014). Neither technique has been calibrated against plant uptake in woody nitrogen-fixing systems, and neither has been evaluated in engineered nursery substrates, where the diffusive properties differ markedly from mineral soil. The transfer of these methods into the nursery domain is a clear opportunity, but it has not yet been made, and claims about their superiority in that domain would at present be unsupported.

5.3 Phosphorus Speciation and Exchange

Phosphorus dynamics are central to this topic because symbiotic nitrogen fixation is phosphorus demanding, and because amendment and inoculant strategies are frequently justified by phosphorus mobilisation. Experimental work on *Casuarina cunninghamiana* established that seedlings dependent on symbiotic fixation had a higher phosphorus requirement than seedlings supplied with adequate fertiliser nitrogen, which places phosphorus measurement at the centre of any credible account of nitrogen dynamics in these systems (Reddell *et al*., 1997). Species also differ in phosphorus acquisition and use efficiency, and those differences develop over time rather than being fixed at establishment, so single-harvest comparisons of nitrogen-fixing tree legumes can misrank species (Dikand Kadiata & Lumpungu, 2003).

Sequential fractionation remains the most widely used descriptive tool for soil phosphorus. Its limitations are well characterised: the fractions are operationally rather than mineralogically defined, reagent selectivity is imperfect, and the mapping from fraction to plant availability is inferred rather than demonstrated (Negassa & Leinweber, 2009). Methodological commentary has argued that the procedure retains value for comparative work provided its outputs are not reinterpreted as chemical species, and that the frequent practice of naming fractions after presumed mineral associations invites overinterpretation (Gu & Margenot, 2021). Spectroscopic and isotopic methods provide the independent information that fractionation lacks, and a comprehensive appraisal of innovative approaches in soil phosphorus research sets out both their capabilities and the interpretive limits that persist when they are applied to complex field soils (Kruse *et al*., 2015). Isotopic exchange approaches provide a further and largely independent line of evidence, and recent comparison of isotope pool dilution and isotopic exchange kinetics frameworks for estimating phosphorus mineralisation and immobilisation shows that the two frameworks yield different rate estimates from the same soils, which constrains the practice of citing one framework in support of the other (Li & Margenot, 2026).

For amendment research the implication is that a change in a fractionation profile is weak evidence about plant-available phosphorus, especially where the amendment itself contributes phosphorus in forms that partition differently among reagents. Where the claim concerns availability to the tree, a sink-based measure or an isotopically calibrated measure is required.

5.4 Gross and Net Transformation Rates

Net mineralisation measured by incubation reports the balance of production and consumption and is therefore insensitive to large offsetting gross fluxes. Isotope pool dilution resolves gross rates by labelling a product pool and following the decline in its isotopic enrichment. The assumptions are that the labelled pool is homogeneous and accessible, that the label is not remineralised within the measurement interval, and that added label does not stimulate the processes measured. Each has been questioned. An early critique set out the arithmetic consequences of non-uniform labelling and of remineralisation for mineralisation and immobilisation estimates (Schimel, 1996). Full tracer accounting across pools subsequently confirmed that several of the standard assumptions are violated in practice and quantified the resulting bias, concluding that gross rates derived without complete recovery accounting should be treated as conditional (Braun *et al*., 2018).

Amendments interact with these assumptions in a structured way. Sorptive amendments create a pool that exchanges with the labelled pool on an intermediate timescale, which is precisely the condition under which pool dilution estimates are biased. Organic amendments supply labile carbon and nitrogen that stimulate both mineralisation and immobilisation, so the interval over which the measurement is valid contracts. Isotopic work in biochar-amended soils has been reviewed with this in mind, and the recommendation that emerges is for explicit mass balance across solution, exchangeable and sorbed pools rather than reliance on solution-phase dilution alone (Craswell *et al*., 2021). Very few studies of nitrogen-fixing trees under amendments report gross transformation rates at all, and those that report net mineralisation seldom state the incubation conditions in enough detail for the result to be compared across studies.

Table 4. Methodological limitations in soil-side quantification, by amendment class, with consequence for inference

Measurement	Amendment class that triggers the limitation	Mechanism of the limitation	Consequence for inference	Available mitigation	Supporting references
Salt-extractable ammonium and nitrate	Carbonaceous amendments, particularly field-aged biochar	Retention of mineral nitrogen in forms incompletely recovered by the extractant	Availability underestimated; plant uptake and soil test diverge; apparent uptake efficiency inflated	Report plant uptake alongside extraction; compare extraction regimes; avoid cross-treatment calibration transfer	Haider *et al*. (2016, 2020); Walsh *et al*. (2019)
Alkaline and dilute-acid phosphorus extraction	Liming materials, composts, sparingly soluble phosphate sources	Change in soil pH and in the phosphorus forms presented to the reagent	Treatment-by-method interaction embedded in the comparison	Use a sink-based or isotopically calibrated measure where availability is the claim	Hill *et al*. (2021); Six *et al*. (2012, 2013)
Sequential phosphorus fractionation	Organic and mineral phosphorus amendments	Operationally defined fractions; imperfect reagent selectivity; amendment phosphorus partitions differently	Fraction shifts cannot be read as availability or as mineral speciation	Pair with spectroscopic or isotopic evidence; report fractions as operational	Gu & Margenot (2021); Kruse *et al*. (2015); Negassa & Leinweber (2009)
Isotope pool dilution for gross nitrogen and phosphorus rates	Sorptive and labile organic amendments	Intermediate-timescale exchange with a sorbed pool; stimulation of the measured processes	Gross rates biased by an amount that is not estimable from the data reported	Full tracer accounting across solution, exchangeable and sorbed pools; shorten the interval	Braun *et al*. (2018); Craswell *et al*. (2021); Li & Margenot (2026); Schimel (1996)
Net mineralisation by incubation	All classes	Offsetting gross fluxes are invisible; incubation conditions differ from field or container conditions	Direction of net change reported without the process explanation	Report incubation conditions fully; interpret as an index rather than a rate	Braun *et al*. (2018); Schimel (1996)
Leaching and drainage loss	All classes in containers	Term is measurable at the container boundary but usually omitted	Budgets left unclosed; retention and uptake effects confounded	Collect and analyse leachate as standard in substrate amendment trials	Huang & Gu (2019); Huett (1997); Simiele *et al*. (2022)

Note. Amendment classes are those represented in the retrieved literature. Absence of a class from a row indicates absence of evidence for that combination rather than evidence that the limitation does not arise

5.5 Loss Pathways and the Container Boundary

Closure of a nutrient budget requires loss terms, and in containerised production the dominant loss is drainage. Direct measurement in containerised nursery systems established that nutrient leaching accounts for a substantial share of applied nutrients and that the

share depends on irrigation management and fertiliser placement rather than on plant demand alone (Huett, 1997). The container therefore has a well-defined lower boundary across which flux can be measured, which is an analytical advantage that the field domain lacks and that this literature under-exploits. Where leachate is collected and analysed, a nursery experiment can close a nitrogen and phosphorus budget to a degree that is impossible in the field, and it can distinguish an amendment that increases retention from an amendment that increases uptake. Substrate amendment studies that report growth and tissue nutrient status but not leachate composition discard this opportunity. Reviews of biochar in container substrates describe changes in water-holding capacity, cation exchange and nutrient retention that would be expected to alter drainage losses, yet the leaching term is measured in a minority of the underlying studies (Huang & Gu, 2019). The same gap appears in nursery work combining biochar and compost for restoration planting stock, where seedling responses are documented in detail and the fate of unrecovered nutrients is not (Simiele *et al*., 2022).

In the field, loss pathways are harder to quantify and are correspondingly neglected. Gaseous losses following the addition of nitrogen-rich organic amendments to soils under nitrogen-fixing trees are rarely measured, and lateral and deep drainage losses are rarely constrained. Estimates of the contribution of biological nitrogen fixation to agricultural systems at large scales consequently depend on a measurement base that is thin with respect to losses, and the authors of such estimates have been explicit about that dependence (Herridge *et al*., 2008; Ladha *et al*., 2022).

The recurring limitations identified across this section are collected in Table 4, which groups them by the amendment class that triggers them and states the consequence for inference. Table 4 makes clear that the problems are concentrated in sorptive and organic amendments, and that they affect the soil-side measurements more severely than the plant-side measurements.

6. Quantifying the Biological Mediators

6.1 Colonisation and Abundance Metrics for Mycorrhizal Symbionts

Arbuscular mycorrhizal colonisation is almost always reported as a percentage of root length or of intersections colonised, determined microscopically after clearing and staining. Direct comparison of four routine procedures on the same material showed that the estimates they produce differ systematically, so the numerical value of colonisation is method dependent and cross-study comparison of percentages is unsound (Sun & Tang, 2012). Biochemical alternatives based on signature lipids provide a quantity proportional to fungal biomass rather than to root length occupied, and comparison with microscopic assessment demonstrated that the two describe different properties and correlate imperfectly (Sharma & Buyer, 2015). Quantitative polymerase chain reaction approaches permit absolute quantification of fungal genetic material within roots and have been validated across several crop hosts, offering better reproducibility than visual scoring at the cost of requiring taxon-specific primers and careful normalisation (Corona Ramírez *et al*., 2023). Image-based automated scoring is developing quickly and has been reviewed recently, with the principal unresolved issue being the availability of annotated training material that spans host taxa and staining protocols (Bouvette *et al*., 2026).

None of these approaches measures nutrient transfer. The distinction matters because amendment effects on colonisation and on nutrient transfer need not share a sign. Meta-analytic synthesis across a global dataset found that biochar, compost and manure altered root colonisation rates, with the direction and magnitude depending on amendment type and dose (Wang *et al*., 2026). A narrative review of arbuscular mycorrhizal fungi in biochar-amended soils reached a compatible conclusion and emphasised that responses are conditioned by biochar feedstock, pyrolysis temperature and application rate, which are inconsistently reported (Rosenthal & Gunn, 2025). Interpreting a colonisation decline under a nutrient-enriching amendment as functional damage is unwarranted, since reduced colonisation is the expected response to relief of nutrient limitation, and the functional question requires a transfer measurement. In nursery work on nitrogen-fixing hosts, studies that combine inoculation with fertilisation have found that colonisation can be maintained under intensive nutrient loading, so the assumed antagonism between fertilisation and mycorrhizal establishment is not general (Idol & Diarra, 2017). Experiments on *Prosopis alba* similarly reported that mineral fertilisation and arbuscular mycorrhizal inoculation interacted positively for nursery growth and drought tolerance, which indicates that the interaction requires empirical determination in each substrate rather than assumption (Salto *et al*., 2020).

6.2 Molecular Abundance, Nodule Occupancy and the Activity Gap

Molecular quantification of diazotroph or symbiont abundance, and of functional gene copy number, is now routine and is frequently presented as evidence for enhanced nitrogen fixation potential. The inferential gap between gene abundance and process rate is substantial, and the more defensible use of molecular data in this context is to answer questions about identity and establishment rather than about rate. For inoculant research the decisive question is whether the applied strain occupied nodules, and molecular strain tracking answers it directly. Methodological advances now permit simultaneous assessment of rhizobial competitiveness and the nitrogen-fixing performance of individual nodules, which separates the establishment question from the effectiveness question for the first time in a single assay (Mendoza-Suárez *et al*., 2020). Synthesis of the occupancy and persistence literature makes clear that competition with resident populations is the dominant cause of inoculation failure in soils with established rhizobial communities, and that inoculant effectiveness measured in sterile or near-sterile substrate is a poor predictor of field outcome (Mendoza-Suárez *et al*., 2021).

This distinction explains a pattern that otherwise appears as unexplained variability. Meta-analysis of rhizobial inoculation trials found responses that were positive on average but highly heterogeneous, with the largest responses where indigenous populations were small or absent (Thilakarathna & Raizada, 2017). Broader synthesis of microbial inoculation across plant traits identified both biological and experimental factors as determinants of measured effectiveness, with experimental factors including substrate

sterilisation, inoculum dose and assessment timing contributing appreciably to the variance (Azarbad & Junker, 2024). Appraisal of the inoculant field as a whole reaches the same place from a different direction, identifying formulation, survival and establishment as the binding constraints and noting that these are seldom verified in the experiments that report inoculant effects (O'Callaghan *et al*., 2022). Nursery experiments on nitrogen-fixing trees rarely report inoculum viability at application or strain occupancy at harvest. Work on *Casuarina equisetifolia* that applied bioinoculant combinations under tropical nursery conditions reported nutrient utilisation and growth responses of the kind that would follow from successful establishment, and the absence of occupancy verification in this literature means that negative results remain uninterpretable (Muthukumar & Udaiyan, 2010).

6.3 Enzyme Assays and Rhizosphere Imaging

Potential enzyme activity assays, conducted at substrate saturation and controlled pH, are widely used as indices of nutrient cycling capacity. Their interpretation requires care that is often absent. Methodological commentary has set out the basic requirements, including appropriate buffering, correction for substrate and product interference, and refraining from converting potential activities into field process rates, and has documented how often these are neglected (Margenot & Wade, 2023). In amended soils the interference problem is acute, because carbonaceous amendments adsorb both fluorogenic substrates and reaction products, and composts contribute their own enzyme activity independently of the soil microbial community. A reported increase in phosphatase activity under a compost amendment may therefore originate in the amendment rather than in a change to soil biological function.

Imaging methods provide spatial information that bulk assays cannot. Soil zymography maps the two-dimensional distribution of enzyme activity around roots and has developed rapidly over the past decade, with quantitative interpretation now the principal methodological frontier (Bilyera & Kuzyakov, 2024). Coupling of zymography with isotopic imaging of carbon allocation demonstrated that phosphatase activity around roots is partitioned between microbial and root origins in a way that depends on phosphorus availability, which is directly relevant to phosphorus mobilisation claims made for inoculants (Spohn & Kuzyakov, 2013). These techniques have been applied almost exclusively to herbaceous species in flat rhizoboxes. Their extension to woody seedlings in containers is technically feasible and would address the specific question of whether an inoculant changes the spatial organisation of nutrient mobilisation, rather than only its bulk magnitude. Standardisation of the root-side measurements on which such work depends has been addressed comprehensively for root ecology, and the sampling and trait conventions set out there are applicable to nursery seedling work, where root system description is frequently reduced to a single mass value (Freschet *et al*., 2021).

7. Quantifying the Plant Side: Status, Content and Interpretation

7.1 Elemental Analysis and Analytical Comparability

Plant nutrient content is the quantity on which most inferences about uptake ultimately rest, and it is the product of a biomass measurement and a concentration measurement, each with its own error structure. Concentration determination for nitrogen by high-temperature combustion and by wet digestion are not perfectly interchangeable, and for phosphorus and cations the digestion procedure determines recovery from resistant tissue fractions. Where an experiment reports concentrations only, the uptake claim is not supported, because concentration responds to both acquisition and growth dilution. This is not a minor reporting preference. In nursery experiments with strong growth responses, concentration and content frequently move in opposite directions, and the choice of which to report determines the conclusion.

Analytical comparability across studies is further limited by inconsistent reporting of tissue selection, of the developmental stage at sampling, and of whether roots were included. In woody seedlings a large share of nutrient content resides in roots, and the root fraction is the part most affected by container volume, so exclusion of roots biases content comparisons in a direction that depends on the treatment. Standardised conventions for root sampling and processing exist and would resolve much of this inconsistency if adopted (Freschet *et al*., 2021).

7.2 Content, Concentration and Vector Diagnosis

Vector diagnosis was developed precisely to resolve the concentration–content ambiguity by plotting relative nutrient concentration, relative nutrient content and relative dry mass together, so that treatment effects are classified as deficiency, sufficiency, dilution, antagonism or toxicity according to the direction of displacement. Applied to *Prosopis* seedlings under contrasting fertilisation, the approach distinguished genuine nutrient limitation from growth dilution in a way that concentration data alone could not (Imo & Timmer, 1997). It has since been combined with morphological indices to strengthen interpretation of fertilisation response in nursery stock (Park *et al*., 2015). The related practice of exponential nutrient loading, in which nutrient supply increases through the growing period to build internal reserves without excess early growth, was introduced with the explicit aim of manipulating content rather than concentration, and it provides the clearest demonstration that these two quantities are separately manipulable (Timmer, 1997).

Adoption of vector diagnosis in amendment and inoculant research on nitrogen-fixing trees is limited, and its absence is consequential. Where an inoculant increases biomass and reduces tissue nitrogen concentration, vector diagnosis would classify the response as dilution and direct attention to whether nitrogen supply had become limiting; the more common reporting practice, which presents the concentration decline without the content calculation, invites the opposite conclusion. Studies of nursery mycorrhizal inoculation that report colonisation, growth and nutrient uptake jointly are correspondingly more interpretable than those reporting concentration alone (Quoreshi & Khasa, 2008).

7.3 Non-Destructive Optical Proxies

Chlorophyll meters and reflectance indices offer repeated non-destructive estimation of nitrogen status, which is attractive in nursery experiments where destructive sampling limits the number of measurement occasions. Evaluation in tropical tree seedlings at the nursery stage established relationships between reflectance and absorbance chlorophyll indices and digital image components, and also established that the relationships are species specific and require local calibration (do Amaral *et al*., 2019). The constraint for the present topic is that the proxy responds to chlorophyll, and the chlorophyll–nitrogen relationship shifts with phosphorus and micronutrient status, with leaf thickness and with shading, all of which amendments and inoculants alter. Optical proxies are therefore appropriate for tracking the trajectory of a treatment within an experiment after calibration against destructive measurements on the same material, and inappropriate as a substitute for tissue analysis when the treatments differ in more than nitrogen supply.

Table 5. Cross-cutting design and reporting problems in amendment and bio-inoculant research on nitrogen-fixing woody species, with evidence-supported remedies

Problem	How it appears in the literature	Why it undermines inference	Remedy supported by the evidence	Priority	Supporting references
Concentration reported without content	Tissue nutrient percentages presented as evidence of uptake	Concentration confounds acquisition with growth dilution and can move opposite to content	Report concentration, content and dry mass jointly and apply vector diagnosis	Immediate	Imo & Timmer (1997); Park *et al*. (2015); Timmer (1997)
Unverified inoculant establishment	Inoculant treatments without viability counts at application or occupancy data at harvest	Null results are ambiguous between biological ineffectiveness and failure to establish	Report viable counts at application and strain occupancy at harvest	Immediate	Mendoza-Suárez *et al*. (2020, 2021); O'Callaghan *et al*. (2022)
Inadequate control condition	Non-inoculated controls in non-sterile substrate or field soil with resident symbionts	Treatment contrast is diluted by an unmeasured background inoculum	Characterise the resident population and report substrate treatment history	Immediate	Azarbad & Junker (2024); Thilakarathna & Raizada (2017)
Amendment-specific extraction bias ignored	A single extractant applied across amendment treatments	Availability differences are confounded with reagent behaviour	Validate extraction against plant uptake within the amended system, or use sink-based measures	Immediate	Haider *et al*. (2016, 2020); Six *et al*. (2012, 2013); Walsh *et al*. (2019)
Unreported amendment characterisation	Biochar or compost identified only by name and rate	Responses are conditioned by feedstock, pyrolysis temperature, maturity and nutrient content	Report full physicochemical characterisation of the amendment batch used	Immediate	Huang & Gu (2019); Rosenthal & Gunn (2025); Wang *et al*. (2026)
Surrogate presented as flux	Nodule counts, colonisation percentage or acetylene reduction reported as fixation	The surrogate-to-flux relationship is uncalibrated and condition dependent	State the surrogate as such, or pair it with an isotopic or accretion-based estimate	Immediate	Soper *et al*. (2021); Sun & Tang (2012)
Unclosed nutrient budget in containers	Growth and tissue data without leachate analysis	Retention and uptake effects of the amendment cannot be separated	Collect and analyse drainage from containers as a standard term	Short term	Huett (1997); Simiele *et al*. (2022)
Isotopic parameters borrowed	Fractionation parameter taken from another species or growth stage	Error propagates directly into the estimate of atmospheric dependence	Determine the parameter for the host and growth stage, or report sensitivity to its range	Short term	Nebiyu *et al*. (2014); Rose *et al*. (2018)
Single harvest and short duration	One destructive harvest within a nursery cycle	Rankings of species and treatments change with time since establishment	Include repeated harvests spanning at least one seasonal cycle	Short term	Dikand Kadiata & Lumpungu (2003); Poorter *et al*. (2016)
Container results generalised to field	Nursery magnitudes cited as expected field magnitudes	The two domains differ in boundary conditions, not only in scale	Confine container results to mechanism and capability; measure magnitude in the field	Longer term	Dalling *et al*. (2013); McNickle (2020); Poorter *et al*. (2012, 2016)

Note. Priority denotes the feasibility of adoption rather than the magnitude of the problem. Immediate items require only changes to reporting or to existing protocols; short-term items require additional measurement within existing designs; the longer-term item requires new field experiments

7.4 Allometry and Upscaling

Translating seedling or tree measurements into stand-level nutrient stocks requires allometric relationships, and the uncertainty introduced at this step is frequently larger than the measurement uncertainty it propagates. Purpose-built allometric work on fixing and non-fixing species at the same age and site established that biomass–dimension relationships differ between the two functional groups, which means that applying a generic equation across a mixed planting introduces a systematic and directional error into any comparison of nutrient accumulation between fixers and non-fixers (Carreras Pereira *et al*., 2023). Stand nitrogen accumulation estimates in mixed *Acacia–Eucalyptus* systems depend on species-specific equations for exactly this reason, and the fixation estimates derived from them inherit the allometric uncertainty (Paula *et al*., 2018).

The nursery-to-field transition compounds the problem, because seedling allometry at the point of planting bears no fixed relationship to allometry after establishment, and nursery nutrient content is depleted or retranslocated during the establishment period. Claims that nursery amendment or inoculation improves field nutrition therefore require field measurement; they cannot be derived from nursery content plus an assumed retention fraction.

The cross-cutting problems identified in this and the preceding sections are assembled in Table 5 with the remedy that the evidence supports in each case. Table 5 is organised by problem rather than by method because most of these problems recur across several methods, and its central implication is that the majority are reporting and design failures that could be corrected without new instrumentation.

8. Design, Inference and Transferability

8.1 Container Artefacts and the Nursery-to-Field Discontinuity

The containerised nursery is the dominant experimental setting for amendment and inoculant research on nitrogen-fixing trees, for reasons of cost, control and the practical relevance of planting stock quality. The inferential price is a set of artefacts that are quantitatively large and directionally predictable. Rooting volume exerts a systematic effect on growth, which propagates into every content-based nutrient measure and into every efficiency ratio computed from it (Poorter *et al*., 2012). The chemistry of the container environment diverges from that of the parent soil during the experiment, so nutrient availability measured in pots is a property of the pot and not of the soil from which the substrate was drawn (Dalling *et al*., 2013). Root system responses are ambiguous between competing interpretations unless the assumptions about pot volume are made explicit, which they seldom are (McNickle, 2020). Comparison of plant performance between controlled environments and the field across a wide range of responses shows differences large enough to change the ranking of treatments (Poorter *et al*., 2016).

For inoculant research the discontinuity is sharper still, because container substrates are frequently sterilised or are inherently low in resident symbionts, which is the condition under which inoculation effects are largest and under which they predict field outcome least well (Mendoza-Suárez *et al*., 2021; Thilakarathna & Raizada, 2017). The evidence that would resolve the transferability question consists of experiments in which the same inoculant and the same measurement protocol are applied across the transplant boundary, with occupancy and nutrient status assessed in both domains. Work on nursery mycorrhizal inoculation of poplar species that followed colonisation, growth and nutrient uptake through nursery production demonstrates the feasibility of the nursery component, and the field continuation of such designs is the part most often missing (Quoreshi & Khasa, 2008).

8.2 Factorial Inadequacy in Amendment and Inoculant Research

Amendments and bio-inoculants are commonly applied together in practice, and the rationale for doing so is that the amendment provides habitat, carbon and nutrient conditions that support the inoculant. Testing that rationale requires factorial designs with both factors at more than one level, and the primary literature is dominated by designs that compare a combined treatment with an untreated control. Meta-analytic evidence that microbial inocula enhance the effects of biochar on productivity and soil properties is consistent with a genuine interaction, and the pooled studies are largely unable to distinguish interaction from additivity because they lack the single-factor arms required (Ross & Emery, 2025). Synthesis of arbuscular mycorrhizal responses to biochar, compost and manure reaches an analogous position, reporting dose and amendment-type dependence that can only be described, not mechanistically resolved, from the available designs (Wang *et al*., 2026).

Where factorial designs have been used in nursery work on nitrogen-fixing hosts, the results indicate that the interaction cannot be assumed. Mineral fertilisation and arbuscular mycorrhizal inoculation acted together to improve *Prosopis alba* nursery growth and drought tolerance, indicating positive interaction under those conditions (Salto *et al*., 2020). Intensive nutrient loading proved compatible with the maintenance of mycorrhizal colonisation in tree seedlings, contradicting the expectation that high nutrient supply suppresses the symbiosis to functional irrelevance (Idol & Diarra, 2017). Rhizobial isolates and mineral nitrogen supply interacted in their effects on nodulation and nitrogenase activity in *Dalbergia sissoo* seedlings, with the direction depending on nitrogen rate (Dhiman *et al*., 2022). The generalisation supported by these results is narrow but useful: the sign of the amendment-by-inoculant interaction is not predictable from the separate effects, and designs that cannot estimate it cannot support the mechanistic claims routinely made from them.

8.3 Reporting Deficits and Reproducibility

The recurring obstacle to synthesis in this field is missing information rather than conflicting information. Amendment batches are identified by name and rate without characterisation, although feedstock, pyrolysis temperature, maturity and nutrient content

determine the response (Huang & Gu, 2019; Rosenthal & Gunn, 2025). Inoculum identity, viable count at application and occupancy at harvest are rarely reported, although these determine whether the treatment was delivered (O'Callaghan *et al*., 2022). Substrate composition, container volume and irrigation regime are frequently incomplete, although each independently alters nutrient dynamics (Huett, 1997; Poorter *et al*., 2012). Isotopic studies report estimates of atmospheric dependence without the fractionation parameter used or its provenance (Rose *et al*., 2018). Explicit recommendations for the conduct and reporting of plant growth-promoting bacterial inoculation studies have been issued and cover most of these items directly (de-Bashan & Nannipieri, 2024). Standardised protocols developed for plant–microbiome research address the deeper problem of cross-laboratory comparability by specifying the growth system as well as the measurements, and their adoption in nursery research on woody hosts would create the conditions for quantitative synthesis that do not currently exist (Novak *et al*., 2025). The appraisal of experimental determinants of inoculation effectiveness reinforces the point by showing that method-related factors account for an appreciable share of between-study variance, which means that better reporting is not merely tidier but would reduce apparent biological heterogeneity (Azarbad & Junker, 2024).

Statistical practice adds a further constraint. Replication in nursery experiments is often applied at the level of the tray, bench or irrigation zone while analysis proceeds at the level of the individual seedling, which inflates apparent precision. Container experiments with small numbers of large containers are particularly exposed, because the container is the unit to which treatment was applied. This issue is seldom discussed in the primary literature on this topic, and its effect is to make marginal treatment effects appear better supported than the design allows.

9. Future Directions

The first priority is the calibration of soil availability measurements within amended systems rather than across them. The unresolved problem is that extraction-based availability indices lose their relationship to plant uptake when a sorptive or organic amendment is present, and current evidence establishes the existence of the problem for nitrogen in biochar-amended soils without providing a corrected procedure (Haider *et al*., 2016, 2020; Walsh *et al*., 2019). The appropriate design is a factorial comparison of extraction, sink-based and isotopically calibrated measures against measured plant uptake, conducted across a graded series of amendment rates and residence times, with a woody nitrogen-fixing host and a non-fixing comparator grown in the same substrates. The outcome measure should be the strength and stability of the relationship between the soil measure and plant nutrient content rather than the absolute value of either. Diffusion-based measures are the most promising candidates on the strength of their performance for phosphorus in high-sorption soils, and the immediate task is to establish whether that performance extends to engineered nursery substrates and to nitrogen (Gu & Grogan, 2020; Hill *et al*., 2021; Shaw *et al*., 2014; Six *et al*., 2012, 2013).

The second priority is the determination of host-specific isotopic fractionation parameters for the woody species in widest use, and the characterisation of their variation with growth stage and with microsymbiont identity. Current estimates of atmospheric dependence in these species rest on parameters borrowed from other taxa, and the resulting error is large enough to account for reported treatment effects in its own right (Nebiyu *et al*., 2014; Rose *et al*., 2018). The required work is straightforward if unglamorous: nitrogen-free culture of each host with defined microsymbionts, sampled at several growth stages, with separate determination for shoot, root and whole-plant material. Priority should follow deployment, beginning with the *Acacia*, *Leucaena*, *Gliricidia*, *Dalbergia* and *Casuarina* species that dominate nursery production, and for actinorhizal hosts the parameter should be determined across *Frankia* strains, since the review evidence on *Casuarina* symbiosis indicates strong strain effects on nodulation and growth (Sayed, 2011).

The third priority is the routine separation of establishment from effectiveness in inoculant experiments. The problem is that a null result currently carries no information, and the available strain-tracking methodology resolves it (Mendoza-Suárez *et al*., 2020, 2021). The design requirement is modest: viable counts at application, occupancy assessment at harvest, and characterisation of the resident population in the substrate or soil used. Where this is done, the heterogeneity observed in inoculant meta-analyses should resolve into interpretable patterns, since indigenous population size is already identified as a major moderator (Thilakarathna & Raizada, 2017). The outcome measures that matter for this topic are occupancy proportion, nodule-level effectiveness and plant nutrient content, assessed jointly.

The fourth priority is the design of experiments that cross the transplant boundary with both the treatment and the measurement protocol intact. The unresolved question is whether nursery amendment and inoculation effects on nutrient status persist after outplanting, and the current evidence base cannot answer it because nursery and field studies are conducted by different groups with different protocols. The required study follows a single cohort of seedlings from nursery to at least two growing seasons in the field, with identical nutrient measurements at both stages, species-specific allometry for upscaling, and sufficient field replication at the plot level (Carreras Pereira *et al*., 2023; Poorter *et al*., 2016). Such studies are expensive, which argues for concentrating them on a small number of species and amendment combinations of demonstrated nursery efficacy rather than for further proliferation of short nursery trials.

The fifth priority is longer term and concerns the spatial organisation of nutrient acquisition. Rhizosphere imaging methods now permit mapping of enzyme activity and nutrient depletion around roots, and coupling these with isotopic imaging has already shown that the origin of phosphatase activity depends on phosphorus supply (Bilyera & Kuzyakov, 2024; Spohn & Kuzyakov, 2013). Extension to woody seedlings in containers would test whether inoculants and amendments change where nutrient mobilisation occurs, which is the mechanistic question that bulk assays cannot address and which bears directly on how amendments should be placed in nursery containers and in planting pits. Automated image-based quantification of symbiont colonisation would reduce the operator dependence that presently limits comparability of colonisation data, and the principal constraint identified is the availability of annotated material spanning host taxa (Bouvette *et al*., 2026).

Two further needs cut across these priorities. Nutrient budgets in container experiments should be closed by routine leachate analysis, which converts a currently confounded comparison into a decomposition of amendment effects into retention and uptake components (Huett, 1997). The measurement base for gross transformation rates in amended soils should be rebuilt with full tracer accounting, because the assumptions of pool dilution are violated in precisely the conditions that amendments create (Braun *et al*., 2018; Craswell *et al*., 2021; Li & Margenot, 2026; Schimel, 1996). Neither requires new instrumentation, and both would improve the interpretability of studies already being conducted.

10. Conclusions

The methods used to quantify soil–plant nutrient dynamics in nitrogen-fixing trees and shrubs are, for the most part, sound within the conditions for which they were developed, and the difficulty addressed in this review is that soil amendments and bio-inoculants systematically move experiments outside those conditions. Each principal method holds one quantity constant, and the interventions under test alter that quantity. The reference plant assumption of difference-based estimation is disturbed by any amendment that changes soil nitrogen supply. The isotopic baseline on which enrichment and natural abundance estimates depend is disturbed by organic and carbonaceous amendments, and the host fractionation parameter on which natural abundance estimates depend is a property of the host and microsymbiont together, so inoculation is capable of changing it. Extraction-based availability indices lose their calibration to plant uptake when sorptive amendments are present, and the best-documented case shows the soil test and the plant response moving in opposite directions. Colonisation percentages, gene abundances and acetylene-dependent activity are surrogates whose conversion to nutrient fluxes is neither linear nor stable. The conclusion that follows is conditional rather than dismissive: the direction of many reported effects is probably reliable, the magnitudes are frequently not, and the attribution of effects to specific processes is often weaker than the reporting implies.

Confidence varies markedly across the evidence reviewed. It is highest for the conclusion that diffusion-based measures of phosphorus availability outperform conventional extraction in high-sorption soils, which is supported by independent studies in contrasting climatic zones with plant response as the criterion. It is high for the conclusion that standard extraction underestimates mineral nitrogen retained by aged biochar and that the retained nitrogen is plant-available, since the two findings come from complementary designs. It is moderate for the conclusion that inoculation outcomes are governed principally by establishment in the face of resident populations, which is well supported in herbaceous systems and inferred for woody hosts. It is low for any quantitative statement about the magnitude of amendment or inoculant effects on symbiotic nitrogen input in field-grown nitrogen-fixing trees, because the isotopic parameters required are largely undetermined for these species and the measurement base is thin.

The containerised nursery and the field planting emerge as distinct measurement domains rather than as two scales of the same system. The container offers a closed lower boundary across which loss can be measured and a controlled substrate in which mechanism can be isolated, and it imposes a finite rooting volume, an engineered substrate chemistry and an irrigation-driven drainage flux that make its magnitudes non-transferable. Recognising this asymmetry would improve the field immediately, because it identifies the claims that container work can and cannot support and it directs field experimentation towards the questions that only field experimentation can answer.

The broader significance of this synthesis is that the apparent variability of amendment and inoculant effects in woody nitrogen-fixing systems is not solely biological. A substantial and identifiable portion of it arises from operational definitions that differ between studies, from surrogates reported as fluxes, from control conditions that are not nutrient-neutral or symbiont-free, and from measurement domains that are treated as interchangeable. These are tractable problems. Most of the remedies identified require changes to reporting and to experimental design rather than new instruments, and their adoption would make quantitative synthesis possible in a field where it is at present unsafe.

11. Limitations

This review is a critical narrative synthesis, and it therefore carries the interpretive and selection biases intrinsic to that design. Literature was selected purposively for its bearing on measurement validity, judgements of methodological adequacy were made by a single analytical route without independent duplicate appraisal, and the emphasis placed on particular studies reflects reasoned argument rather than a pre-specified weighting rule. A different reviewer applying the same criteria could reasonably assemble a partly different evidence base and reach somewhat different confidence judgements, particularly in the sections where the evidence consists of a small number of method-comparison studies.

Access was restricted to freely available scholarly indexes and to openly accessible or publicly abstracted records, so literature indexed only in subscription citation databases, and full texts behind paywalls for which sufficient information could not be obtained, may be under-represented. The search was confined to English-language publications, which is likely to have reduced coverage of nursery and plantation research published in national languages in the tropical regions where these species are most widely planted, and where nursery practice with amendments and bio-inoculants is most active. Regional under-representation of this kind cannot be quantified from the material retrieved.

The evidence base itself constrains what could be concluded. Methodological appraisal specific to woody nitrogen-fixing hosts is sparse, so a considerable share of the argument rests on transfer from general soil and plant nutrition methodology and from herbaceous legume research, and the justification for each transfer is argued rather than demonstrated experimentally. Study designs are heterogeneous in duration, container volume, substrate, amendment characterisation and outcome definition, which is one of the findings of this review and simultaneously a limit on it, because it precluded quantitative synthesis and any formal assessment of publication bias. Long-term evidence is scarce, with most nursery studies confined to a single production cycle and most field

studies to the establishment phase, so statements about persistence are weakly supported. The literature is also geographically concentrated, with tropical plantation isotopic work drawn from a small number of research programmes and sites, which limits generalisation across soil types and climates. Finally, several of the methods appraised are developing quickly, and appraisals of rapidly moving techniques date faster than appraisals of established ones.

Declaration of AI Use

This manuscript was prepared through the intellectual contributions of the author(s) to the study design, data analysis, interpretation of results, and scholarly content. Grammarly and ChatGPT were used solely to assist with grammatical refinement and reference formatting during manuscript revision. The author(s) accept full responsibility for the accuracy, integrity, and final content of the manuscript.

Competing Interests

Authors have declared that no competing interests exist.

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