



Emerging insights into nitrogen assimilation in gymnosperms

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Abstract

Key message The current status of molecular regulation of nitrogen assimilation and recent advances made in gymnosperms are reviewed.

Abstract Gymnosperms are a heterogeneous and ancient group of seed plants that includes conifers, ginkgos, cycads and gnetophytes. Molecular studies on extant gymnosperms have been constrained by some discouraging features for experimental research such as their long life cycles, large sizes, complex megagenomes and abundant phenolic compounds in their woody tissues. However, the development of high-throughput sequencing and refined multiomics technologies in the last few years has allowed to explore the molecular basis of essential processes in this ancient lineage of plants. Nitrogen is one of the main limiting factors determining vascular development and biomass production in woody plants. Therefore, nitrogen uptake, metabolism, storage and recycling are essential processes for fundamental gymnosperm biology. Here, recent progress in the molecular regulation of nitrogen assimilation in gymnosperms is reviewed and some future perspectives on this topic are outlined.

Keywords Seed plants · Gymnosperms · Conifers · Nitrogen acquisition · Nitrate · Ammonium · Glutamine synthetase · GS isoenzymes

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Introduction

Gymnosperms (*Gymnospermae*) include a wide range of vascular and seed-producing plants that diverged from angiosperms approximately 300 million years ago (Clarke et al. 2011). Currently, this ancient and widespread plant lineage comprises approximately 1000 different species (Wang and Ran 2014), a number considerably lower than the 370,000 species estimated for angiosperms (Kew 2016). It was initially thought that they represented a group of relict plants, however, recent findings indicate that gymnosperms have been subjected to pulses of extinction and expansion throughout their evolution and possibly still are occurring today (Davis and Schaefer 2011). In fact, it has been estimated that 40% of the species are threatened and at high risk of extinction, which is a great concern for conservation science (Forest et al. 2018). Gymnosperms comprise four main lineages of seed plants: conifers, cycads, ginkgos and gnetophytes (Crisp and Cook 2011).

Within gymnosperms, conifers constitute the most numerous and representative group both from an economic and ecological point of view, consisting of more than 600

species with a wide distribution worldwide (Farjon 2017). Conifers cover vast extensions of land mainly in the Northern Hemisphere, where they are the main constituents of the forests of North America and Eurasia. Therefore, conifers are of extraordinary ecological relevance because they play an essential role in global carbon fixation and the maintenance of biodiversity. In contrast, the areas occupied by this group of seed-bearing land plants are much smaller in the Southern Hemisphere, although paradoxically, it is in these particular regions of the planet where the greatest biodiversity of coniferous species is found (Farjon 2017).

The distribution of conifers shows great versatility in terms of adaptation, with species located both at sea level (Burban and Petit 2003) and in the Himalayas (Farjon 2018). Conifers are very often present in suboptimal climates and/or poor soils for the growth of most plant species, mainly angiosperms, likely reflecting an environmental adaptation developed throughout their long evolutionary history (Farjon 2018). One important feature supporting their wide distribution is the symbiotic interaction established between the roots of conifers and fungi, forming ectomycorrhizal systems that can enhance the capacity to capture water and nutrients by up to ten thousand times (Taylor and Alexander 2005; Martin et al. 2016; Farjon 2018). From an economic point of view, conifers are the largest contributors of raw material for wood industries worldwide, as they provide higher yields due to better growth and more predictable wood shapes and sizes compared to angiosperms (Farjon 2018). It is also worth mentioning the use of conifers as ornamental plants, a practice initiated during the XIX century, and the demand for seeds of different types of conifers to decorate gardens and parks.

Most gymnosperms are woody plants with long lifespans and life cycles, large sizes, and the ability to produce large amounts of phenolic secondary metabolites (De la Torre et al. 2014; Cañas et al. 2019). All these characteristics have hampered molecular biology studies in members of this ancient lineage of plants. In addition, the enormous magnitude and the highly repetitive nature of their genomes (15–30 Gb) have largely limited the structural and functional genomics of gymnosperms (De La Torre et al. 2014; Cañas et al. 2019). The increasing capacity and lower costs of next generation sequencing (NGS) technologies after the completion of the human genome project have facilitated the characterization of the genomes of a growing number of plant species and have also paved the way for the genome assembly of several conifer species, such as *Picea abies* (Nystedt et al. 2013), *Pinus taeda* (Zimin et al. 2014), *Picea glauca* (Warren et al. 2015) and *Pinus lambertiana* (Stevens et al. 2016). The recent development of single-molecule sequencing technologies such as PacBio and Oxford Nanopore has provided much longer reads, overcoming the inherent difficulties for the characterization of highly

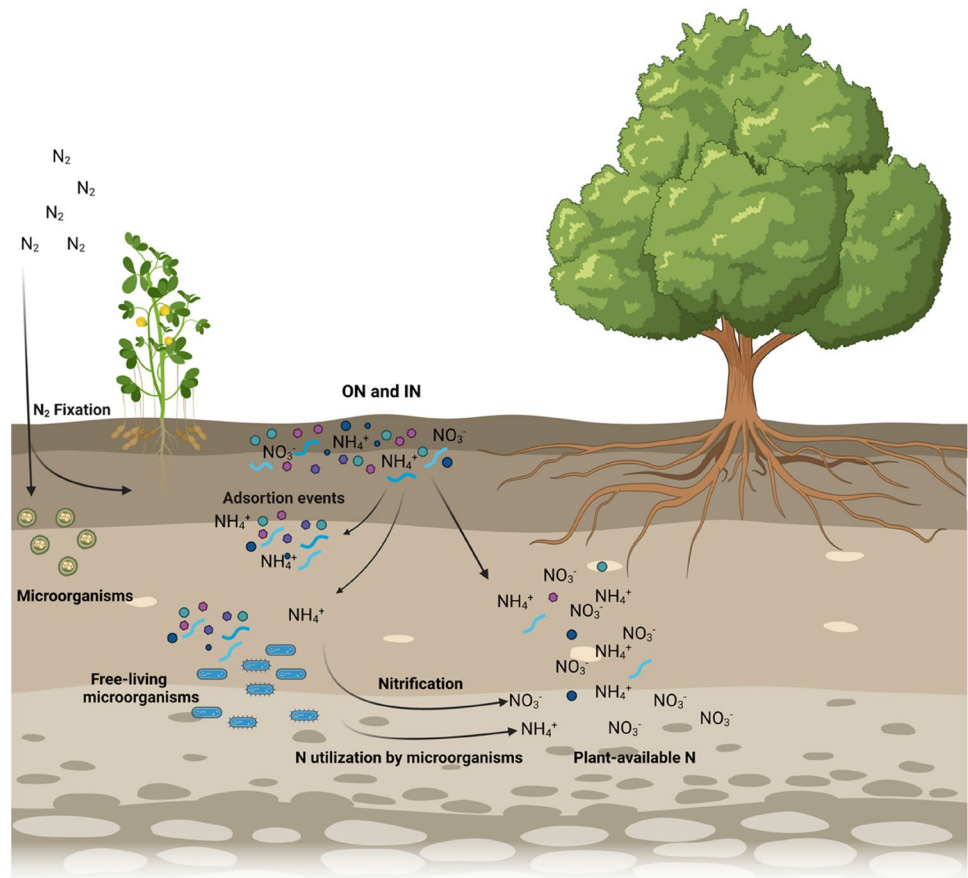
repetitive gymnosperm megagenomes. These advanced techniques in combination with conventional NGS have permitted the genome assemblies of an increased number of gymnosperm species during the last few years covering representatives not only for conifers (Scott et al. 2020; Xiong et al. 2021; Niu et al. 2022; Neale et al. 2022) but also for cycads (Liu et al. 2022), ginkgo (Liu et al. 2021) and gnetophytes (Wan et al. 2021). All these genomic resources are of paramount significance for the development of functional genomics studies that will be able to provide new insights into fundamental plant biology from an evolutionary perspective. These new advances have recently been used to further study the molecular basis of nitrogen (N) assimilation and metabolism in gymnosperms. In this work, a review of studies in this research area is presented and discussed.

Nitrogen availability for plant nutrition

N is an essential element as constituent of the main biomolecules of paramount importance for life, such as nucleic acids and proteins as well as a wide range of primary and secondary metabolites (Kishorekumar et al. 2020). In natural environments, N is one of the main limiting factors for plant growth determining raw material production and crop yield (Hirel and Krapp 2021). Moreover, N is also involved in plant architecture and resistance against environmental stresses (Kishorekumar et al. 2020). Although N is the most abundant element in the atmosphere, only diazotrophic microorganisms, either free-living or associated with plants, can fix atmospheric dinitrogen (N₂) (Sharma et al. 2021). Therefore, the major sources of N for plants are found in soil (Shafreen et al. 2021), where a substantial part of the available N is indeed provided by microorganisms (Courty et al. 2015; Verzeaux et al. 2017).

In soils, N can be found in both organic and inorganic forms (Fig. 1). Plants have evolved N uptake mechanisms to adapt to their own environments, thus allowing the incorporation of organic N compounds such as peptides and amino acids and inorganic N molecules such as nitrate and ammonium (Hirel and Krapp 2021; Farzadfar et al. 2021). Furthermore, some of these mechanisms allow plants to uptake N from soils that are poor in nitrogenous compounds, while others protect them from toxicity due to eventual high N availabilities (Nacry et al. 2013). Several studies have shown that, especially under low levels of inorganic N, plants can efficiently take up organic N such as amino acids or peptides (Moran-Zuloaga et al. 2015). Indeed, the production rates of organic N compounds have been found to be higher than the rates from N mineralization in forest ecosystems (Näsholm et al. 2009). Nevertheless, the high competition for these organic N forms, mostly used by microorganisms, together

Fig. 1 Plant-available N sources. *ON* organic N, *IN* inorganic N



with the adsorption process by the soil, does not allow plants to fulfill their requirements, even though those compounds are a great source of N for different plant species in several ecosystems (Näsholm et al. 2009; Franklin et al. 2017; Lim et al. 2022).

Nitrate and ammonium are the two main forms of inorganic N in soils, mostly produced by microbial mineralization of organic compounds (Bernard and Habash 2009). The relative abundance of these substrates in soil depends on several factors, such as environmental conditions and the chemical nature of the soil (Esteban et al. 2016), thus producing a concentration range that goes from micromolar to molar (Britto and Kronzucker 2006).

The importance of N in crop production led to the “Green Revolution” after the Second World War, when the use of N fertilizer in agriculture suffered a huge increase to keep enough food incoming for a constantly growing population (Galloway et al. 2013; Nacry et al. 2013). The massive use of fertilizers has a negative impact on the biosphere and environment. Over the 50–75% of the N supplied by fertilizers is lost by leaching into the soil or released as N gases to the atmosphere (Hirel et al. 2011; Cameron et al. 2013). Excess N can also lead to eutrophication of aquatic and terrestrial habitats (Harding et al. 2019), an increase

of N reactive species input into the environment (Canfield et al. 2010) and other environmental problems (Hirel et al. 2011), which have repercussions on the biodiversity of the ecosystems (de Graaf et al. 1998).

Therefore, the study of the mechanisms involved in plant N use efficiency (NUE) has gained increased importance in recent decades, particularly those focusing on crop yield improvement, as well as the attenuation of the environmental impact due to extensive agricultural activities (Liu et al. 2022). Biotechnological advances, together with crop and forest management strategies, are required for this purpose.

Assimilation of nitrate and ammonium

In plants, any form of inorganic N is first reduced to ammonium and then assimilated into organic molecules. Nitrate is first reduced to nitrite in the cytosol by nitrate reductase (NR, EC 1.7.1.1) using NADH as reductant. Afterward, nitrite is reduced to ammonium by nitrite reductase (NiR, EC 1.7.7.1), a plastid-located ferredoxin-dependent enzyme (Hirel and Krapp 2021). As nitrite and ammonium are toxic molecules for the plant, their assimilation must be well coordinated in response to N demand and supply (Wang et al.

2018). Ammonium is assimilated by glutamine synthetase (GS, EC 6.3.2.1) to produce glutamine in an ATP-dependent reaction (Heldt and Piechulla 2011). Finally, glutamate synthase (GOGAT, EC 1.4.1.14; EC 1.4.7.1) uses glutamine, along with 2-oxoglutarate and reducing power, in the form of either of ferredoxin (Fd) or NADH, to produce two molecules of glutamate. One of these two molecules will be used as a substrate of the GS/GOGAT cycle, and the other molecule is the net product of the reactions of these two enzymes during N assimilation (Fig. 2) (Bernard and Habash 2009; García-Gutiérrez et al. 2018).

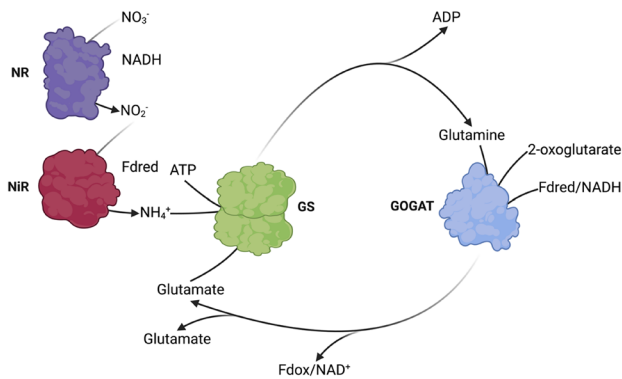
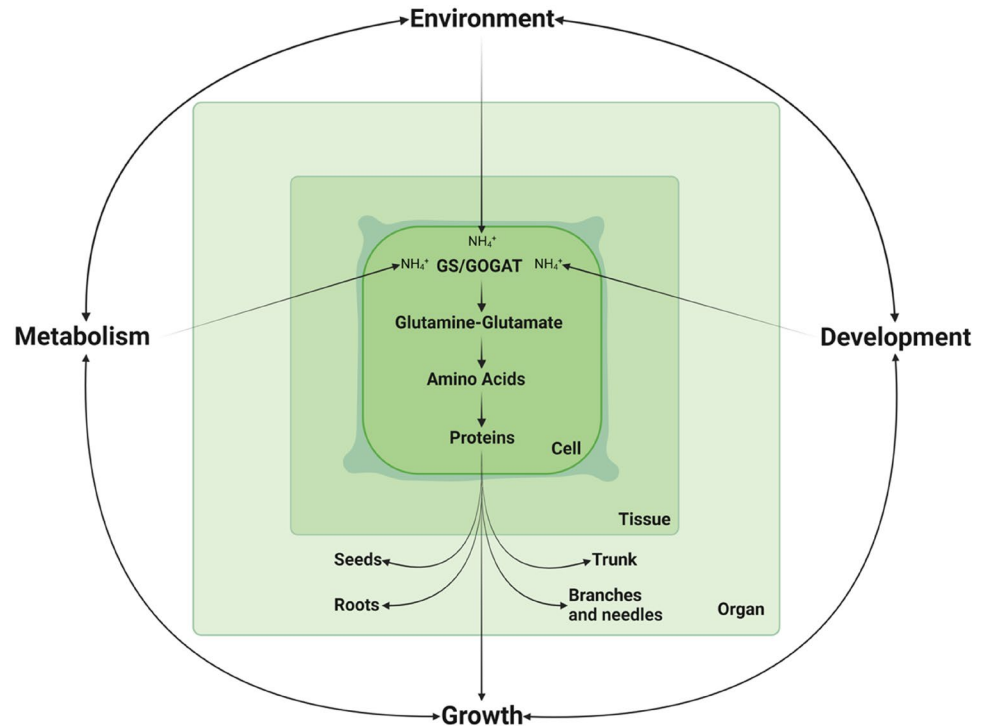


Fig. 2 Scheme of the nitrate reduction and ammonium assimilation by the glutamine synthetase/glutamate synthase cycle. Nitrate reductase (NR); nitrite reductase (NiR); glutamine synthetase (GS); glutamate synthase (GOGAT)

Fig. 3 Involvement of glutamine synthetase in different functional processes of trees. GS glutamine synthetase, GOGAT glutamate synthase



The GS/GOGAT cycle is the major pathway for the incorporation of inorganic N into organic N (Hirel and Krapp 2021). Some studies using ^{15}N and mutants deficient in GS and GOGAT have shown that 95% of ammonium in plants is assimilated via GS (Lea and Ireland 1999). This cycle is also one of the main links between carbon and N metabolism as it allows the assimilation of N into carbon skeletons using 2-oxoglutarate directly provided through the Krebs cycle (Hirel and Krapp 2021). Glutamine and glutamate are used as precursors for the biosynthesis of all N-containing molecules in plants, such as amino acids, proteins, nucleic acids, chlorophylls and secondary metabolites (Fig. 3) (Forde and Lea 2007; Bernard and Habash 2009). In addition, these amino acids are also used to transport organic N to developing and storage organs (Tegeder and Masclaux-Daubresse 2018). Therefore, the GS/GOGAT cycle has a main role in NUE, emphasizing GS activity, whose complex regulation and importance in N remobilization, yield, grain production and growth rate have been reiterated by some studies focused on quantitative trait loci (QTL) and using different types of crop plants exhibiting contrasting NUE (Gallais and Hirel 2004; Obara et al. 2004; Habash et al. 2007; Fontaine et al. 2009; Cañas et al. 2012; Kaminski et al. 2015). Consequently, GS probably remains the most studied enzyme in terms of NUE enhancement in monocot and dicot plants, but also in trees (Castro-Rodríguez et al. 2016a; Hirel and Krapp 2021) (Fig. 3).

In plants, there are different isoforms of both enzymes. Regarding GOGAT, two different plastid-located isoenzymes

can be found, NADH-GOGAT and Fd-GOGAT. These enzymes play a key role in primary N assimilation and N recycling. Fd-GOGAT mainly acts in photosynthetic tissues, whereas NADH-GOGAT acts in nonphotosynthetic tissues (Bernard and Habash 2009; García-Gutiérrez et al. 2018). In conifers, independent of the N source taken up from soil, ammonium is the ultimate form to be incorporated into amino acids through GS1a and Fd GOGAT in photosynthetic tissues, and GS1b and NADH-GOGAT in nonphotosynthetic tissues (Avila et al. 2022a). The identification of a glutamine translocator in isolated chloroplasts from maritime pine (*P. pinaster*) supports the function of the compartmentalized GS/GOGAT cycle in conifer cells (Claros et al. 2010).

Glutamine synthetase: a key enzyme in nitrogen metabolism of land plants

Studies over the past two decades have provided a very deep understanding of the GS phylogeny. Three GS superfamilies have been identified thus far, namely, GSI, GSII and GSIII, and all these enzymes are differentiated by the number of subunits, molecular size and kingdom distribution (Goshroy et al. 2010). The GSI superfamily is predominantly present in prokaryotes, although its presence in mammals and plants has also been reported (Mathis et al. 2000; de Carvalho Fernandes et al. 2022). The GSII superfamily was described as a characteristic group of *Eukarya* and some *Bacteria*, such as *Proteobacteria* and *Actinobacteria* (James et al. 2018). However, this superfamily appears also to be present in *Euryarchaeota*, a phylum of the domain *Archaea*, in public sequence databases. Finally, the GSIII superfamily has been found and described in bacteria, including cyanobacteria (James et al. 2018), and some eukaryotes such as diatoms and other heterokonts, thus suggesting the presence of GSIII gene in the nucleus of early eukaryotes (Robertson et al. 2006). Different works support that these GS superfamilies appeared prior to the divergence of prokaryotes and eukaryotes (Robertson et al. 2006).

In plants, GS activity is carried out by members of the GSII superfamily (James et al. 2018), which have been described to be holoenzymes with octameric and decameric tridimensional structures in different organisms (Eisenberg et al. 2000; Llorca et al. 2006; Unno et al. 2006; Krajewski et al. 2008; He et al. 2009). Two main GSII clades have been identified in the *Viridiplantae* group: eukaryotic origin GSII (GSIIe) and eubacterial origin GSII (GSIIb). It has been hypothesized that GSIIb arose as a result of horizontal gene transfer (HGT) that took place after the prokaryote and eukaryote divergence, which indeed constitutes a sister group with GSII from γ -proteobacteria (Ghoshroy et al. 2010). Regarding GSIIe, its importance in plant growth and development has led to extensive studies of these enzymes

in vascular plants, particularly in crops including trees (Plett et al. 2017; Cánovas et al. 2018; Mondal et al. 2021).

It is generally indicated that angiosperms present two groups of nuclear *GSIIe* genes, one coding for cytosolic proteins (GS1) and a second coding for a plastidic GS (GS2), each playing nonredundant physiological roles within the plant (Ghoshroy et al. 2010; Hirel and Krapp 2021). Usually, there are different cytosolic isoforms encoded by a small multigene family, while only one nuclear gene encodes the plastid isoform (James et al. 2018) with some exceptions such as in *M. truncatula* and poplar (*Populus trichocarpa*) where multiple *GS2* genes have been detected (Seabra et al. 2010; Castro-Rodríguez et al. 2011).

Phylogenetic analyses suggested that *GS2* may have arisen as a result of a gene duplication of a *GS1* gene (Biesiadka and Legocki 1997) 300 million years ago, before the divergence between monocotyledons and dicotyledons (Bernard and Habash 2009). Interestingly, the presence of a *GS2* was reported in the gymnosperms *Ginkgo biloba* (García-Gutiérrez et al. 1998) and *Cycas revoluta* (Valderrama-Martín et al. 2022), but no biochemical, molecular or microscopic analysis has allowed the detection of plastid isoforms in conifers (Cánovas et al. 2007; Avila et al. 2022a) and no *GS2* genes have been found in their fully sequenced genomes (Nystedt et al. 2013; Zimin et al. 2014; Warren et al. 2015; Stevens et al. 2016; Neale et al. 2017; Scott et al. 2020). Instead, conifers present two well-differentiated families of cytosolic GS isoforms, GS1a and GS1b, with different molecular and kinetic properties (Ávila-Sáez et al. 2000; de la Torre et al. 2002; Avila et al. 2022a). These GS1 lineages are found in all gymnosperms and in basal groups of angiosperms and Magnoliidae, such as *Amborella trichopoda* and *Magnolia grandiflora* (Valderrama-Martín et al. 2022).

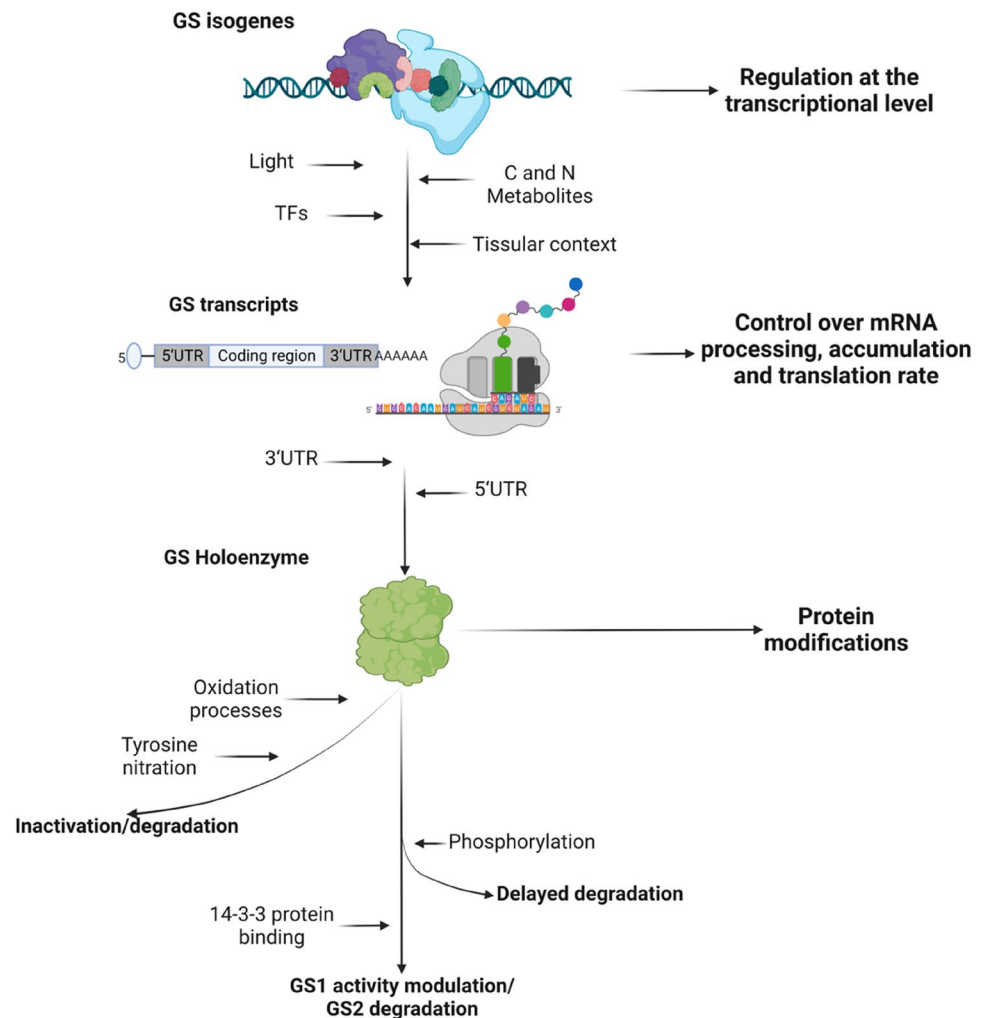
In gymnosperms and angiosperms, both the synthesis and relative activity of each GS isoenzyme are species specific, but its expression is also regulated according to nutritional status, tissue, developmental stages, and environmental conditions (Cánovas et al. 2007; Bernard and Habash 2009; Mondal et al. 2021) in which transcription factors play a key role (Thomsen et al. 2014). Many *cis*-acting elements have been identified as potential binding sites of distinct transcription factors regulating the expression of plant GS genes (Mondal et al. 2021). In pine (*Pinus pinaster*), several members of the Myb family (Myb 1, Myb 4 and Myb8) are involved in the transcriptional regulation of the *GS1b* gene during lignification (Gómez-Maldonado et al. 2004b; Craven-Bartle et al. 2013). In this context, it is also interesting to mention that a single transcription factor, PpDof 5, differentially regulates the genes encoding GS1a and GS1b isoforms suggesting that the correct spatial distribution of the isoforms in the tree is transcriptionally controlled (Rueda-López et al. 2008).

A wide variety of regulatory mechanisms at different levels have been observed on GS (Fig. 4), thus showing the precise and complex regulation of this enzyme. The transcription rate of *GS* isogenes from angiosperms (Oliveira and Coruzzi 1999) and gymnosperms (Cantón et al. 1999; Gómez-Maldonado et al. 2004a) has been shown to be affected by light, and by carbon levels (Oliveira and Coruzzi 1999). N metabolites such as glutamate can directly upregulate GS1 gene expression (Masclaux-Daubresse et al. 2005) as well as the cellular ratio of glutamine/glutamate (Watanabe et al. 1997). Furthermore, nitrate positively regulates GS2 expression in the leaves of maize (*Zea mays*) (Sakakibara et al. 1997) and ammonium has been previously reported to upregulate GS1 expression in the roots of maritime pine (Ortigosa et al. 2022). At the same time, nitrate seems to reduce the accumulation of GS1 transcripts in leaves at the posttranscriptional level (Ortega et al. 2001). Indeed, the GS1 transcript accumulation in *Medicago sativa* by

N/C metabolites has been shown to be mediated through the 3'UTR of these transcripts (Ortega et al. 2006). However, studies suggest that is probably glutamine or a product of glutamine metabolism, but not nitrate, the molecule mediating this 3'UTR-turnover (Simon and Sengupta-Gopalan 2010). In addition to regulation at the transcriptional level, GS has also been reported to be regulated by different protein modifications such as oxidation (Ortega et al. 1999), phosphorylation and binding to 14-3-3 proteins (Finnemann and Schjoerring 2000; Lima et al. 2006a, b) and tyrosine nitration (Melo et al. 2011), which produce different effects on GS isoenzymes (Fig. 4). Recently, it has been shown that GS2 phosphorylation specifically regulates plant growth and defense (Ding et al. 2022).

GS2 gene encodes a 44–45 kDa polypeptide, which is indeed larger than the GS1 polypeptide (38–40 kDa), mainly due to the presence of the plastid targeting peptide in the N-terminus and a C-terminal extension

Fig. 4 GS regulatory mechanism. Factors such as light, metabolites, even the cellular type or the tissue could determine which *GS* gene is going to be transcribed and the rate of transcription. The 3'UTR is also responsible for the regulation of this enzyme at the post-transcriptional level. The 5'UTR of the mRNA determines the context of the start codon and it is believed to bind different mRNA, determining this way the translation rate. Post-translational protein modifications such as phosphorylation of serine residues, nitration of cysteine residues and nitration of tyrosine residues, determines de activity of the different isoforms or mark them for degradation



of approximately 16 amino acids. The function of this C-terminal extension remains unknown, but it has been described to be important for the GS2-glutamate interaction in *Medicago truncatula* (Ferreira et al. 2017). This isoform is mainly expressed in photosynthetic tissues of angiosperms associated with the chlorophyll-containing parenchyma (Castro-Rodríguez et al. 2015). This expression pattern suits his role in the assimilation of ammonium from photorespiration and nitrate reduction (Tegeader and Masclaux-Daubresse 2018).

On the other hand, although GS1 isoform expression is mainly related to vascular tissues, the expression patterns of the different GS1 isoenzymes cover the entire plant (Lea and Mifflin 2018). GS1 isoforms are predominantly implicated in primary N assimilation in roots, remobilization, and recycling (Thomsen et al. 2014). These isoforms have also been described as a key component of plant NUE with roles in processes such as senescence (Thomsen et al. 2014), amino acid catabolism and different stress responses (Bernard and Habash 2009). Moreover, the direct implication of some GS1 isoenzymes in developmental processes such as grain and biomass production has been demonstrated in many plant species (Martin et al. 2006; Jing et al. 2004; Funayama et al. 2013; Bao et al. 2014; Guan et al. 2015; Urriola and Rathore 2015; Cánovas et al. 2018; Gao et al. 2019; Ji et al. 2019; Wei et al. 2021; Fujita et al. 2022).

Previous works on *GS1a* in conifers have determined a similar expression pattern of this gene to that exhibited by *GS2* in other plants. This cytosolic enzyme has also been found to be associated with the chlorophyll-containing parenchyma of photosynthetic organs (Ávila et al. 2001) and, as well as *GS2* from angiosperms, its expression is upregulated by light (Cantón et al. 1999; Gómez-Maldonado et al. 2004a). In this sense, *GS1a* has been proposed to fulfill the role of *GS2* in gymnosperms (Cantón et al. 1999; Valderrama-Martín et al. 2022). For its part, conifer *GS1b* is phylogenetically more related to cytosolic isoforms from angiosperms than to coniferous *GS1a* (Ávila Sáez et al. 2000). The GS1b isoform is ubiquitously expressed, and its expression is also related to vascular tissues (Ávila et al. 2001). GS1b has been proposed to play a role in N remobilization between source and sink organs during the active growth period (Suárez et al. 2002). Furthermore, studies in pine suggest a role of GS1b in the canalization of ammonium to glutamine during embryogenesis, seed germination and early developmental stages of seedlings (Ávila et al. 2001), a hypothesis that is supported by its expression patterns in different developmental stages of zygotic and somatic pine embryos (Pérez-Rodríguez et al. 2005; Avila et al. 2022b). The upregulated expression of *GS1b* in reaction wood, together with its association with vascular tissues,

indicates that this gene is also involved in ammonium reassimilation during lignin biosynthesis (Cantón et al. 2005; Craven-Bartle et al. 2013). Recently, a new GS1b (GS1b.2) isoenzyme has been identified and characterized that appears to be restricted to the *Pinus* and *Picea* genera (Valderrama-Martin et al. 2023).

GS1b in angiosperms is encoded by a multigene family, while GS1b in conifers is usually encoded by a single gene (James et al. 2018). However, the identification of an additional variant in *Pinus* and *Picea* suggests that a diversification process of *GS1b* genes (*GS1b.1* and *GS1b.2*) is currently ongoing in this group of gymnosperms. In some angiosperms, the expansion of this family has led to different isoforms with overlapping functions within the plant (Castro-Rodríguez et al. 2015). In pine, *PpGS1b.2* probably arose via gene duplication of the already well-characterized *GS1b.1* gene (Valderrama-Martin et al. 2023). Based on localized *PpGS1b.2* expression, a role in developing tissues has been proposed for this enzyme, similar to some GS1b isoforms in other plants (Bao et al. 2014; Guan et al. 2015; Urriola and Rathore 2015; Gao et al. 2019; Ji et al. 2019; Wei et al. 2021; Fujita et al. 2022). Taken together, these findings imply evolutionary convergence since a new GS1b isoform arose in a process of neofunctionalization likely to meet different metabolic needs, independent of the GS1b from angiosperms.

Notably, even though these two GS1b paralogs (GS1b.1 and GS1b.2) are quite similar in protein sequence, they present differences in their molecular characteristics (Valderrama-Martin et al. 2023). Therefore, both enzymes have been shown to present differences in their thermostability, with GS1b.1 being more thermostable than GS1b.2. Although these isoenzymes showed optimal activity at 42 °C, both reached their maximum activity at different pH levels (6.5 and 6 for GS1b.1 and GS1b.2, respectively). Moreover, regarding the behavior toward substrates and the kinetic properties, they have also been shown to present differences. Considering all the above results, two hypotheses have been suggested: (a) GS1b.2 could supplement GS1b.1 activity in developing tissues that exhibit a high demand for glutamine; (b) GS1b.2 could play a specific role, still unknown, in certain developing tissues. *In silico* studies over the promoter have also revealed differences in the regulatory region of *PpGS1b.1* and *PpGS1b.2* also supporting this idea (Valderrama-Martin et al. 2023). Nevertheless, additional comparative studies of both isogenes including the functional characterization of their regulatory regions will be required to shed light on this topic.

Evolution of glutamine biosynthesis in seed plants

GS is a key gene in N metabolism in the vast majority of organisms, as can be inferred by its wide distribution in all kingdoms (Ghoshroy et al. 2010). It is also one of the oldest known genes, which is why some authors have described it as a molecular clock with excellent potential for phylogenetic analyses (Pesole et al. 1991).

Numerous reports have been made about the evolution of glutamine synthetase, not only in plants but also regarding the evolution of this gene family within eukaryotes (Ghoshroy et al. 2010). Phylogenetic analyses of different protein and nucleic acid sequences have allowed a better understanding of the evolution of this family of genes in seed plants (Valderrama-Martín et al. 2022). The results of these analyses clearly suggest the occurrence of 3 well-differentiated GS families within seed plants: GS1a, GS1b, and GS2 (Valderrama-Martín et al. 2022). This hypothesis is also supported by phylogenetic analyses carried out including GS1b.2 sequences (Valderrama-Martín et al. 2023). The emergence of tracheophytes during the evolution of land plants likely led to the evolution of a cytosolic GS (GS1) located in vascular cells able to synthesize glutamine for N transport and N distribution to different tissues and organs. Another role of this GS1 enzyme should be the re-assimilation of the ammonium released in lignin biosynthesis, quantitatively the most important metabolic fate of phenylalanine biosynthesis in trees (Craven-Bartle et al. 2013; Pascual et al. 2016; El-Azaz et al. 2022). A duplication of this ancestral gene and neofunctionalization of young duplicates could possibly lead to the appearance of GS1b and GS1a isoforms in gymnosperms (Fig. 5).

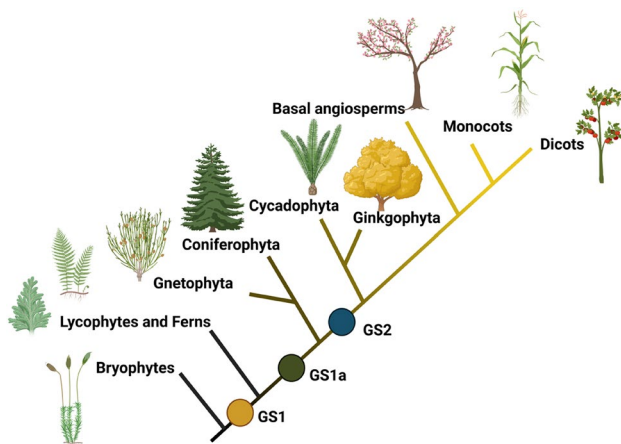


Fig. 5 Proposed scheme for the evolution of GS isoforms in land plants. *GS1* cytosolic GS, *GS1a* cytosolic GS located in photosynthetic cells, *GS2* chloroplasmic GS

GS1a is specifically located in the cytosol of conifer photosynthetic cells (García-Gutiérrez et al. 1998; Ávila et al. 2001), and it was proposed to be involved in the re-assimilation of ammonium released from photorespiration and the primary assimilation of ammonium from nitrate reduction (Cánovas et al. 2007; Avila et al. 2022a), therefore fulfilling the role of GS2 in angiosperms (Avila et al. 2022a; Valderrama-Martín et al. 2022). On the other hand, it is well established that enzymes in the GS1b group have a role in primary assimilation, re-assimilation and remobilization of N, as well as in developmental processes, amino acid catabolism, senescence and stress response (Thomsen et al. 2014; Ji et al. 2019; Wei et al. 2021; Fujita et al. 2022).

Recent reports have noted that the groups of *Cycadopsida* and *Ginkgoopsida* may form a monophyletic clade in gymnosperms (Wu et al. 2013; One Thousand Plant Transcriptomes Initiative 2019). In line with this, the new insights into nitrogen assimilation reported here support this assumption, but in addition, these data could indicate that the monophyletic clade conformed by *Cycadopsida* and *Ginkgoopsida* may be more related to angiosperms than previously thought (Valderrama-Martín et al. 2022). The most parsimonious hypothesis resulting from phylogenetic analyses indicates that the *GS2* gene must have been absent in a common ancestor of gymnosperms and angiosperms and probably evolved, as a result of a duplication of the *GS1a* gene and the acquisition of a plastid transit peptide, in a common ancestor of *Cycadopsida/Ginkgoopsida* and angiosperms (Fig. 5). This hypothesis also fits well with the overlapping roles previously proposed for GS1a and GS2 (Cantón et al. 1999; Avila et al. 2022a).

Nitrogen assimilation and subcellular localization of glutamine biosynthesis

The presence of this enzyme in the chloroplast confers additional advantages to the plant since more efficient nitrate assimilation likely allowed angiosperms to colonize new ecological niches that were rich in nitrate, different from those ecosystems populated by conifers in which ammonium was the major N source. In concordance, although conifers are widely distributed throughout the world, these plants are mostly present in boreal zones where temperature hinders nitrification. Moreover, several studies have previously reported a preference of conifers for ammonium over nitrate (Warren and Adams 2002; Boczulak et al. 2014; Ortigosa et al. 2020). However, recent studies have shown that the concentrations of both nitrate and ammonium are equalized with depth in the soils, and adult trees of these species can use nitrate as well as ammonium (Zhou et al. 2021). In addition, there are gymnosperm species such as *P. pinaster*, that are autochthonous from warm places where nitrification

can take place and soils are probably richer in nitrate. Indeed, previous works have shown a strict regulation of nitrate uptake (Ortigosa et al. 2020), which is interesting considering the fact that the NRT3 (component of the nitrate high-affinity transport system) family has been expanded in this conifer species and that this phenomenon has not taken place in angiosperms (Castro-Rodríguez et al. 2017). Unfortunately, the above studies are restricted to conifers and no similar information is available for other gymnosperms. In this context, it would be of great interest to know the nutritional preference of cycads and ginkgo for nitrate and ammonium to determine how the assimilation of inorganic N is related to the appearance of GS in plastids. Likewise, the investigation of the NRT3 family in the different groups of gymnosperms deserves particular attention to further understand the molecular basis of N assimilation in this ancient lineage of plants.

Independent of whether the presence of a plastid GS will be an evolutionary advantage in plants or not, the following question arises: why do conifers and gnetophytes conserve GS1a and not present a plastid GS isoform? The recent report of toxicity phenotypes related to GS activity in the chloroplast may shed light on the above question. Studies carried out by Hachiya et al. (2021) in *Arabidopsis* suggested a toxic effect resulting from excessive ammonium assimilation in the chloroplast by GS2, probably linked to the acidification effect related to this enzyme activity. The homeostasis of the pH in the chloroplast is essential and excessive acidification of the stroma could lead to problems

with the photosynthetic apparatus (Kuvykin et al. 2009; Tikhonov 2013), which is necessary to produce the reducing power required for N and carbon assimilation. Therefore, the assimilation of ammonium in the cytosol of conifer photosynthetic cells would prevent these toxic effects.

In line with this, a conflict between nitrate assimilation and CO₂ fixation has been previously discussed (Bloom 2015). Rubisco is the enzyme in charge of catalyzing the combination of CO₂ and ribulose-1,5-bisphosphate (RuBP) to produce two molecules of 3-phosphoglycerate (3-PGA), which are further used for the biosynthesis of fructose-6-phosphate. However, this enzyme also catalyzes a second reaction where O₂ is used instead of CO₂ producing one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG), thus leading to the restoration of RuBP from 2-PG in a process considered to be energy-wasting and releasing ammonium that should be efficiently reassimilated: photorespiration (Eisenhut et al. 2015, 2019) (Fig. 6). Although the increase in CO₂ concentration in the atmosphere produces a consequent decrease in photorespiration, which would be considered favorable, several works have reported that C3 plants are impaired in their ability to assimilate nitrate (Bloom et al. 2012). Different hypotheses stand up to this conflict, nevertheless, the hypothesis that best fits the decrease in sugar content under nitrate nutrition would be a competence for reducing power between the NR and NiR enzymes and the enzymes from the Calvin–Benson Cycle (Bloom 2015). Thus, the photorespiration process somehow acts as a regulator of the cellular redox

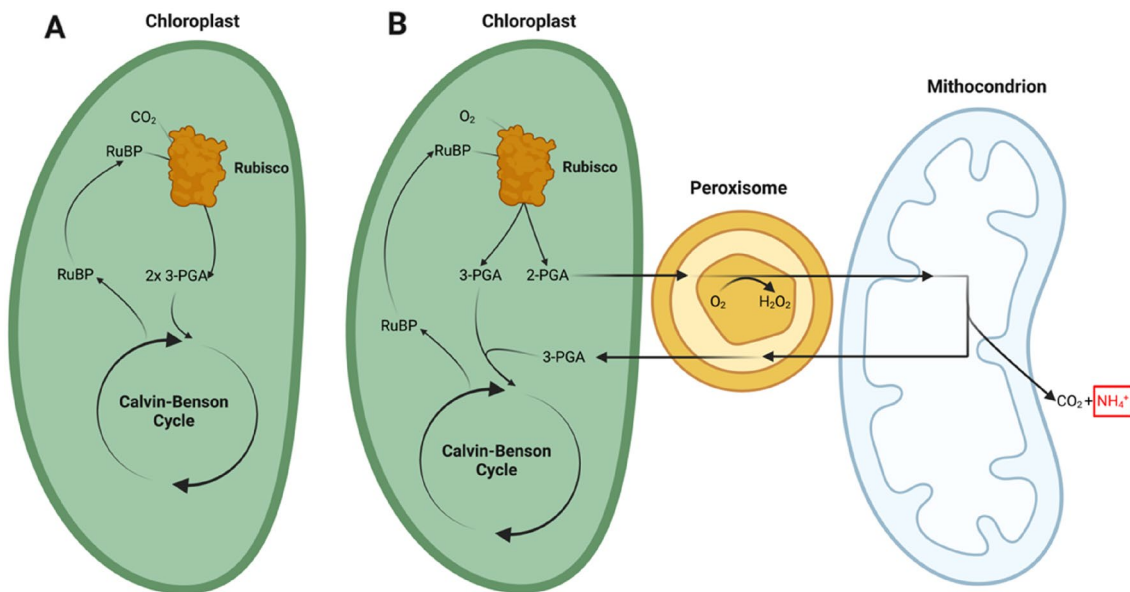


Fig. 6 Schematic representation of the carboxylase (A) and oxygenase activity of the rubisco enzyme and the photorespiratory process through the chloroplast, peroxisome and mitochondria (B). Ribulose-1,5-bisphosphate (RuBP); 3-phosphoglycerate (3-PGA); 2-phos-

phoglycolate (2-PG). Ammonium released in the mitochondria (red square) should be efficiently reassimilated by GS either in the cytosol (gymnosperms) or in the chloroplast (angiosperms). Otherwise nitrogen deficiency would occur

homeostasis allowing nitrate assimilation to occur. This phenomenon takes place both in gymnosperms and angiosperms. In gymnosperms, ammonium released in photorespiration will be assimilated via GS1a in the cytosol, while in angiosperms, this ammonium needs to be transported to the chloroplast and assimilated via GS2. It is unknown whether ammonium exchange across the inner membrane envelope is a passive or active process (Eisenhut et al. 2015). However, the pine ammonium transporters (AMT) lack a plastid target peptide and are specifically located in the plasma membrane of both root and leaf cells (Castro-Rodríguez et al. 2016b). Similarly, it is also unclear how nitrite is transported into the chloroplast for NiR activity, an essential step for nitrate assimilation. The elucidations of the mechanisms involved in the transport of nitrite and ammonium are therefore potential areas deserving further investigation efforts.

Taken together, the above data suggest that the subcellular localization of the GS enzyme, one of the main links between carbon and N metabolism, may be relevant for the metabolic regulation of photorespiration and inorganic N assimilation. Bloom (2015) also showed that those plants irrigated with ammonium did not present any growth problems under high CO₂ or low O₂ conditions when compared with controls, thus supporting a conflict with nitrate assimilation. The high amounts of reducing power required in nitrate assimilation can also explain differences in biomass in pine seedlings irrigated with ammonium and with nitrate. Ortigosa et al. (2020) showed a higher accumulation of biomass in plants irrigated with ammonium than in those irrigated with nitrate. Metabolite analyses showed a decrease in the amount of carbohydrates in plants irrigated with nitrate when compared with plants irrigated with ammonium, which could be associated with a limited availability of carbon for such metabolic processes due to the higher requirement of reducing power by nitrate reduction. In contrast, pine seedlings irrigated with ammonium presented considerably higher concentrations of sugars such as sucrose, D-fructose and D-glucose (Ortigosa et al. 2020). Free sugars are required for N assimilation to provide carbon skeletons, thus explaining the occurrence of high levels of these monosaccharides and disaccharides when ammonium is the main N source since it is directly assimilated in roots. Nitrate is mainly stored long-term in the vacuoles and photoassimilated in smaller quantities at a time, avoiding the excessive consumption of reducing power and therefore requiring lower levels of carbon skeletons. In this respect, the preference of conifers for ammonium nutrition over nitrate nutrition may be due to a more efficient assimilation of ammonium itself, a preference that is caused by the higher tolerance of these plants for ammonium.

Author contribution statement JMVM, RAC and FMC have written the initial version of the manuscript; FO, FRC and CA made additional contributions and edited the text.

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Declarations

Conflict of interest The authors have no conflict of interest.

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