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Review

Nitrogen Economy and Nitrogen Environmental Interactions in Conifers

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Abstract: Efficient acquisition, assimilation and economy of nitrogen are of special importance in trees that must cope with seasonal periods of growth and dormancy over many years. The ability to accumulate nitrogen reserves and to recycle N determine to a great extent the growth and production of forest biomass. The metabolic relevance of two key amino acids, arginine and phenylalanine, as well as other processes potentially involved in the nitrogen economy of conifers are discussed in the current review. During their long life cycles, conifers not only cope with cyclical annual and long-term changes in the environment but also interact with other organisms such as herbivores and symbionts. The interactions of biotic and abiotic factors with conifer nitrogen metabolism will also be outlined in this review.

Keywords: conifers; metabolism; nitrogen recycling; arginine; phenylalanine; environment

1. Introduction

Nitrogen (N) availability in natural soils frequently limits plant growth and development. An inadequate supply of N results in plants with slow growth, depressed protein levels, poor yield and inefficient water use. Conversely, excessive N can also be detrimental to crop growth and quality. N-stressed plants often have greater disease susceptibility than properly nourished plants. N may also be a contaminant, resulting from industrial activity and overuse of fertilizers. For these reasons, extensive research has been conducted on managing this essential nutrient [1,2].

N economy is of special importance in trees that must cope with seasonal periods of growth and dormancy over many years. The productivity of most temperate forest ecosystems is limited by N availability and woody plants have developed adaptation mechanisms, such as mycorrhizal associations, to increase the efficiency of N acquisition and metabolic assimilation [3]. However, some regions have recently experienced dramatic increases in N deposition rates as a result of human activities [4,5]. As N deposition increases, it may act as a fertilizer to increase forest productivity; as such, most studies have focused on evaluating the uptake of inorganic N sources. Unlike many other plants, conifers prefer ammonium over nitrate as a N source [6]. However, several lines of evidence suggest that amino acids may act as a potential N source for a number of plant species, including conifers [7]. This is because N from amino acids may be present in concentrations similar to those of inorganic N, at least in unmanaged systems [8]. If the supply of N continues to increase, then complex alterations to soil and plant biogeochemistry may occur. These changes can affect productivity, competition, and microbial community structure [9]. As water and other nutrients become more limiting relative to N, ecosystems may approach "N saturation" [10]. When N deposition increases, it leads to forest decline and soil acidification. Furthermore, human disruption of the global N cycle

in ecosystems is considered an important factor underlying climate change [9]. Conversely, when N deposition decreases, a tree's nutrient status will rapidly shifts towards N limitation. In addition to efficient incorporation of N from soil, effective mechanisms are required for N assimilation, storage, mobilization and recycling. One such mechanism, the translocation of nutrients from senescing tissues, is a key aspect of N nutrient economy in trees. Indeed, the ability of forest trees to store and internally redistribute N resources is a fundamental element of N economy in forest trees [11].

Forest N economy has considerable impact on several important tree attributes, including growth rate, disease and pest resistance, climate change and adaptability, tree form and wood fiber quality. N economy has a special importance in conifers, the most abundant group of gymnosperms that include trees of great economical and ecological importance such as pines, spruces and firs. Coniferous forests dominate large ecosystems in vast regions of the northern hemisphere and represent a main source of wood for the humanity [12]. Furthermore, conifers have evolved distinct responses at developmental, metabolic and physiological levels since their evolutionary divergence from a common ancestor shared with angiosperms [13]. Recent efforts to understand N economy in conifer trees and its applicability, as well as the interaction between N metabolism and the environment, will be reviewed in this article. The aim of this review is to provide a general overview of the research undertaken on this topic, as well as the identification of major areas of interest to develop further studies in a near future.

2. Efficiency of Nitrogen Use, Growth and Biomass Production in Forest Trees

Managed and unmanaged forests provide sustainable biomaterials, primarily wood and wood-derived products, for construction, paper production and bioenergy feedstocks. Mineral nutrition is pivotal for wood formation in growing trees and is thus a crucial factor for optimizing biomass production [14]. The N requirements of trees are satisfied via the uptake of new N and the remobilization of stored N. Plant species have the ability to store, remobilize and recycle N resources but the seasonal N cycling represents an acquisition of the woody/perennial plant lifestyle [15].

Forest productivity results in non-negligible amount of biomass that could be utilized for bioenergy and biopolymer production. This productivity is largely related to tree growth, which is a complex phenomenon that integrates many physiological processes. It is well documented that N availability is one of the most important factors affecting tree growth [11]. However, when studying N economy, cycling and storage, the integration of N with other nutrients, particularly carbon, must also be considered.

The efficiency of N uptake, allocation, residence time and final use is defined as Nitrogen Use Efficiency (NUE) [16]. In woody/perennial plants, two components must be considered when calculating NUE: (1) the mean residence time of N within a plant in terms of biomass in years; and (2) the instantaneous rate of carbon fixation per unit of N in the plant, which can be considered the dry matter produced per unit of N in tree biomass per unit of time [17]. Early studies of NUE in conifer populations growing at different sites indicated that NUE decreases with increasing N availability [18]. In environments where N is the primary limiting factor of plant growth, the dynamics of perennial plant populations are determined to a large extent by the balance between the uptake and the loss of N.

The distribution of biomass among leaves, stems and roots in forest trees is often influenced by environment, nutrition, developmental stage, and genetic background. However, patterns of whole-tree distribution of dry mass vary in response to soil water content, N availability, and as a function of developmental stage. Excessive N fertilization or atmospheric N deposition in coniferous forests greatly increases the content of arginine in needles and wood. It has been shown that N leaching in coniferous forests can be identified by analyzing arginine levels [19]. Long term, excess N is sequestered by storage proteins, which accumulate in seeds and vegetative organs. Vegetative Storage Proteins (VSP) responds positively and rapidly to increased N availability, and the transcription patterns of starch biosynthetic genes reflect photosynthetic trends [20]. Continual adjustments to C and N metabolism occur in response to N availability, contributing to changes in whole-plant process that influence overall growth and development [21]. The transcription patterns of genes involved

in N storage (VSP accumulation under excess N) and C storage (starch accumulation under limiting N conditions) show opposite trends for C and N partitioning as a function of resource availability. N availability affects wood properties by modifying wood density, altering cell wall thickness and influencing fiber length [22]. At the molecular level, a number of studies have shown that specific genes are differentially regulated in stems [23] and roots [24].

Forest biotechnology can be used to identify several desirable traits that contribute to NUE improvement. In combination with traditional selection and breeding, forest biotechnology can be used to implement the production of the most desirable families of trees with better yield and NUE. This accelerates the process of and thereby shortens the time needed for tree breeding, testing and plantation establishment. Over the past decade, advances in forest genomics have facilitated our understanding of the structure, function and evolution of tree genomes, including conifer genomes in the last few years [25,26]. These recent advances have the potential to enhance traditional tree breeding and will undoubtedly contribute to increase forest productivity.

3. Relevant Metabolic Processes Related to N Economy in Conifers

As described above, N availability strongly limits plant growth and biomass production. Consequently, plants have evolved efficient mechanisms to optimize N uptake. Plants have also evolved mechanisms to optimize nitrogenous resources by engaging in N storage and recycling at different stages of development and in response to different environmental conditions. In herbaceous plants, assimilation of N associated to photosynthesis is an essential component of the efficient use of nitrogenous nutrients. In contrast, the capacity to accumulate N reserves and engage in N recycling is of critical importance to woody plants; these processes determine the growth and production of forest biomass. Conifer N storage, mobilization and recycling have been previously reviewed [27,28]. In the present work, the metabolic relevance of two key amino acids, arginine and phenylalanine, as well as other processes potentially involved in N economy in conifers will be discussed.

3.1. Arginine Metabolism

The amino acid arginine has the highest N to carbon ratio and is therefore particularly suitable for N storage and transport in living organisms [29,30]. Arginine biosynthesis is a relatively unexplored area of plant research. The genes and enzymes involved arginine biosynthesis have been identified in *Arabidopsis* [31]; however, many steps of the arginine biosynthetic pathway, that is mainly located in the plastid, remain poorly characterized (see Winter *et al.* [30] for a recent comprehensive review). Arginine biosynthesis undergoes feedback regulation via inhibition of the enzyme N-acetylglutamate kinase [31]. When N is abundant, this inhibition is released by interaction with the N sensor protein PII [29,32]. Recently, it has been reported that PII controls, in a glutamine-dependent manner, the enzyme N-acetylglutamate kinase, the key step in arginine biosynthesis [33]. In pine seedlings, the gene encoding PII-like protein is expressed in different organs, and in adult trees PII-like protein transcripts are particularly abundant in developing xylem, suggesting a role for PII in the regulation of N metabolism during wood formation (Cánovas, F.M., Universidad de Málaga, Spain, unpublished, 2016). As arginine is a key amino acid for N storage and mobilization, arginine metabolism in conifers warrants special attention (Figure 1). In conifer seeds, most of the reserve proteins are located in the megagametophyte, a maternally derived tissue surrounding the embryo [34]. These reserve proteins are markedly enriched in amino acids with a low C/N ratio making them especially appropriate for storage and transport of N [30]. Arginine constitutes a large portion of the amino acid pool in these storage proteins [35] and therefore arginine biosynthesis is likely a relevant metabolic pathway during pine embryogenesis. However, only limited knowledge is currently available on the expression of key genes involved in arginine biosynthesis and the transcriptional regulation of this process in conifers. In last few years, the assembly of pine and spruce transcriptomes [36,37] and genomes have been reported [38,39] and these new resources will facilitate the identification and functional characterization of genes involved in arginine metabolism.

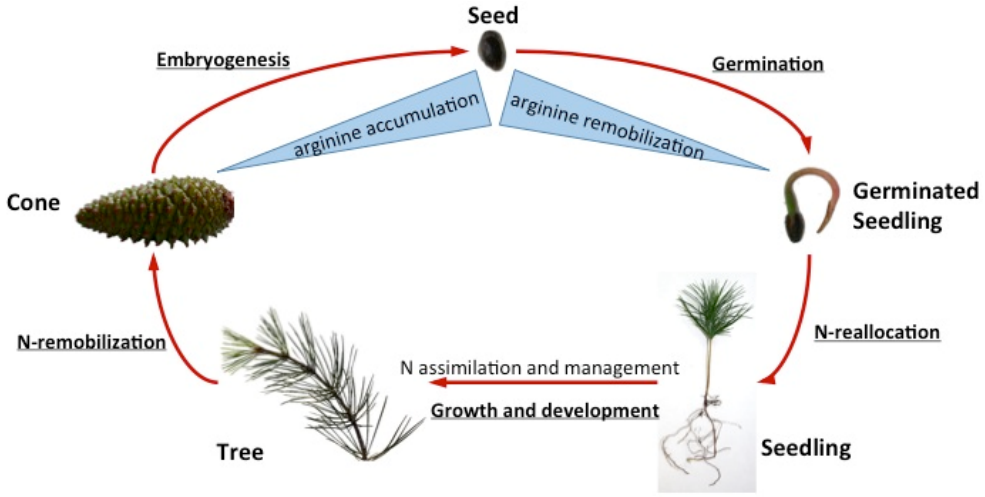

Figure 1. Nitrogen assimilation, storage and remobilization in maritime pine.

Following germination, amino acids, particularly arginine, are released from the hydrolysis of storage proteins and mobilized to the embryo in a process that is finely synchronized with the emergence of the radicle [35]. In this way, N reserves contained in the megagametophyte support the early stages of plant development before photosynthetic machinery is functional. This mobilization of reserves during germination depends on the activation and synthesis of key enzymes, including those involved in the proteolytic hydrolyzation of reserve proteins. High accumulation of arginine in the embryo is accompanied by increased arginase activity [35,40]. Arginase activity and expression of the corresponding transcripts, are localized to expanding cotyledons that are in direct contact with the megagametophyte. Through this activity, arginine is converted to ornithine and urea. The subsequent hydrolysis of urea by urease is an important source of ammonium for early seedling development [36]. Suárez *et al.* [41] have proposed that cytosolic glutamine synthetase (*GS1b*) in maritime pine is responsible for the channeling of ammonium to glutamine, thereby resulting in the synthesis of other N compounds in germinating seeds. The other metabolic product of arginine catabolism, ornithine, has been proposed to be channeled by ornithine-δ-aminotransferase for the synthesis of glutamate, which is required for glutamine and asparagine biosynthesis during pine germination [42].

3.2. *Phenylalanine Metabolism*

Phenylalanine is a precursor in the biosynthesis of phenylpropanoids, a diverse and complex family of organic compounds. This family includes compounds of enormous importance for plant defence, pigmentation and structural support such as lignins, lignans, flavonoids or suberins. The metabolism of these compounds is particularly important in trees since it is estimated that nearly 30%–40% of photosynthetically fixed carbon is channeled through phenylalanine for the biosynthesis of lignin during wood formation.

In plants, phenylalanine is synthesized within plastids via the shikimate pathway, which is followed by two alternative post-chorismate routes that use arogenate or phenylpyruvate as intermediates [43–45]. Then, in the first step of the phenylpropanoid pathway, which is catalyzed by phenylalanine ammonia lyase (PAL), phenylalanine is converted into trans-cinnamic acid with the concomitant release of a molecule of ammonium. In conifer trees, massive metabolic flux through this pathway is required for the biosynthesis of lignin during wood formation. Cells undergoing active lignification require the functioning of efficient N-recycling machinery to avoid severe N deficiency. Lignifying cells in the presence of ^{15}N-phenylalanine results in the accumulation of labelled glutamine and glutamate. The addition of GS inhibitors results in $^{15}NH_4^+$ accumulation, while the addition of glutamate synthase (GOGAT) inhibitors results in ^{15}N-glutamine accumulation [46]. These findings

strongly support the presence of an active N-recycling process that is mediated by the GS/GOGAT pathway. The resulting glutamate is subsequently used as amino donor for the biosynthesis of the phenylalanine precursor arogenate through a reaction catalyzed by prephenate aminotransferase (PAT). The arogenate pathway for phenylalanine biosynthesis occurs in the plastid, while phenylalanine deamination occurs in the cytosol, and N recycling via the GS/GOGAT cycle involves enzymes that are located in both subcellular compartments (Figure 2). Therefore, the actions of both a glutamine translocator and a phenylalanine transporter are necessary in lignifying plant cells (Figure 2). The existence of a functional glutamine/glutamate translocator in pine cells has recently been reported [47]. The specificity and activity of this translocator facilitate glutamine import into the chloroplast and glutamate export to the cytosol, two processes that prevent N loss from this essential pathway. The activity of a phenylalanine transporter in the plastid membrane is also necessary to satisfy the great demand for phenylalanine in the cytosol of cells undergoing lignification; however, the molecular and kinetic characteristics of this amino acid transporter have not been reported to date.

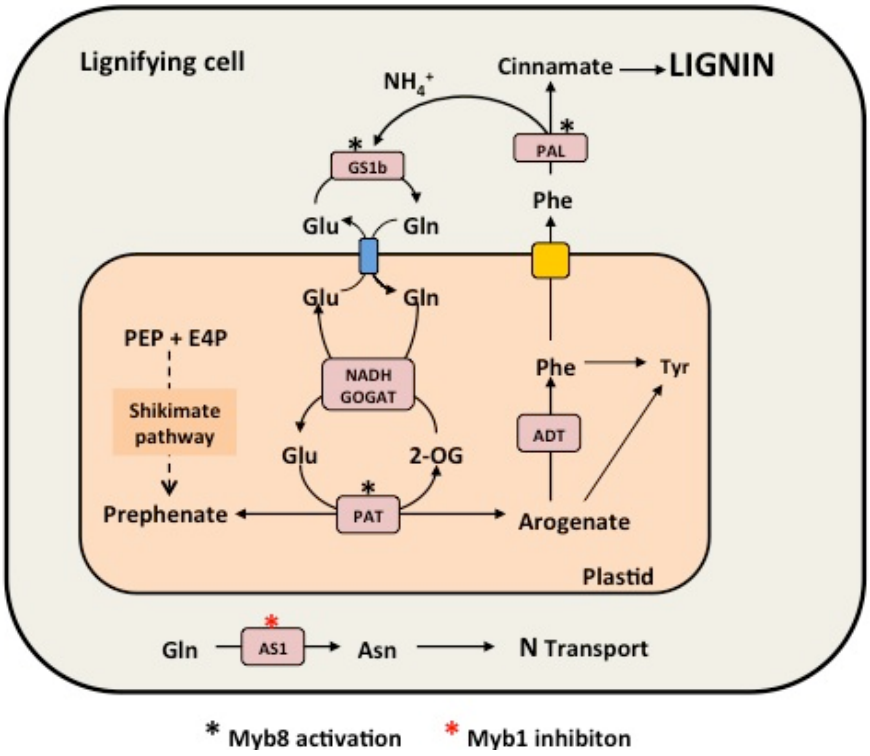

Figure 2. Phenylalanine metabolism and the associated nitrogen recycling (redrawn from Craven-Bartle *et al.* [46]. PEP, phosphoenolpyruvate; E4P, erytrose 4-phosphate; *GS1b* (glutamine synthetase 1b); PAL, Phenylalanine ammonia lyase; NADH GOGAT, NADH-dependent glutamate synthase; ADT, arogenate dehydratase; AS1, asparagine synthetase. Transcriptional regulation of specific metabolic steps is indicated by asterisks: Myb 8 activation (*); Myb 1 inhibition (*).

The transcriptional regulation of phenylalanine metabolism has recently been reported in maritime pine. The transcription factor Myb8 co-regulates the expression of genes involved in prephenate amination, phenylalanine deamination and N recycling [48]. Furthermore, it has been shown that asparagine synthetase expression is downregulated by Myb proteins in maritime pine preventing the biosynthesis of asparagine (which would divert N to other metabolic fates) [49]. Consequently, ammonium assimilated by the GS/GOGAT cycle must be redirected towards arogenate synthesis to maintain phenylalanine supply in lignifying cells (Figure 2).

3.3. Biosynthesis of Lignins and Lignans

Coniferyl alcohols are precursors for the biosynthesis of lignins and lignans in conifers [50]. The biosynthesis of these alcohols involves methylation reactions in which S-adenosylmethionine (SAM) acts as donor of methyl units. Villalobos *et al.* [51] have recently shown that the expression of a set of genes involved in the biosynthesis of lignins and lignans in compression wood of maritime pine is concomitant with the up-regulation of SAM metabolism genes. Within the set of genes that are required to ensure a continuous supply of SAM is the gene encoding for the H-protein subunit of the mitochondrial glycine decarboxylase complex (GDC). It has been proposed that GDC-mediated catabolism of glycine to serine is required to prevent the putative accumulation of glycine, which could result in the cytosolic serine hydroxymethyltransferase reaction favoring the formation of serine, thereby inhibiting SAM synthesis. GDC activity involves the release of ammonium, resulting in a direct correlation between monolignol biosynthesis, SAM biosynthesis, and the release of ammonium. Interestingly, the expression of genes involved in the biosynthesis of phenylpropanoids and SAM is strongly correlated with *GS1b* expression suggesting that *GS1b* is directly involved in the recycling of ammonium released by PAL and GDC during secondary growth. Fd-GOGAT levels in pine stems are very low suggesting that NADH-GOGAT is more likely involved in the reassimilation of ammonium in lignifying xylem cells [52].

4. Other Processes in N Recycling

Trees have the ability to store resources, which is as a fundamental aspect of their N economy. The N that is present in senescing conifer needles must be efficiently recycled and transported into perennial tissues. Evidence that autophagy is involved in N remobilization during leaf senescence has been recently established [53]. In senescing leaves of herbaceous plants, a parallel reduction in total free amino acid and plastid enzymatic activities involved in ammonium assimilation is observed during this stage compared to young leaves. This reduction in the levels of free amino acids is related both to the export of amino acids to sink tissues and to amino acid catabolism, which maintains mitochondrial respiration in the absence of sugars. As a result of amino acid catabolism, increased ammonium levels are detected throughout the senescence process and need to be reassimilated, as glutamine or asparagine, the predominant transport forms in the phloem [53]. Little is known about autophagy in perennial plants and its potential role in nutrient homeostasis. Recent reports have shown that autophagy is involved in suspensor cell differentiation during conifer embryogenesis [54], and autophagy-related genes have been associated with cell death processes [55]. However, further research is needed to uncover the potential role of autopaghy in N recycling during needle senescence and to determine how autophagy may contribute to the maintenance of N economy in conifer trees in response to biotic and abiotic stresses.

As winter approaches, forest species stop active growth and enter a period of dormancy in which they accumulate N via VSP. In the following spring, this accumulated N is mobilized to reinitiate growth and development. In poplar, the bark-storage protein family comprises the major set of VSP, and the role in this family in the N economy in poplar has been well documented [11]. VSP proteins have also been identified in conifers [56,57]. Other proteins, such as rubisco, are considered as unconventional storage proteins by contributing to N-reserve formation in summer and N recycling in autumn [57,58]. Interestingly, Pettengill *et al.* [59] have recently suggested that nucleoside phosphorylases are involved in ecophysiological adaptation for inter- and intra-seasonal N storage and cycling in poplar. This suggests that enzymes involved in nucleotide metabolism have a potential role of in N-recycling and that there is a connection between plant N economy and the metabolism of nucleic acids. This topic warrants special attention in future studies on N metabolism in conifers.

In the spring, N remobilization is accompanied by an important influx of amino acids into the xylem sap. Depending on the tree species, the predominant molecules used for N-transport vary betwwen asparagine, glutamine, citrulline and arginine, which is the most prevalent [60]. Regardless

of the amino acid used for N transport, glutamine is required for its biosynthesis; therefore, glutamine biosynthesis is an essential step in N remobilization [27].

5. Interaction between Nitrogen Metabolism and Environment

Conifers forests are comprised of species that grow in different climates and altitudes. These species have wide geographical distribution and long lifespans. One such species, *Pinus longaeva* is the longest lived individual on Earth (4000–5000 years), [12]. These characteristics indicate the great capacity of conifers to acclimatize and adapt to different environmental conditions. During their long life cycles, conifers not only cope with cyclical annual and long-term changes of various environmental stimuli but also must interact with other organisms, such as herbivores and symbionts. Thus, conifers have developed a range of responses to facilitate their acclimatization and adaptation to variable conditions [61]. Both terms, acclimatization and adaptation, are used in the text according to the definitions recommended by Giordano [62]. N metabolism is also affected by these responses and environmental adaptations (Figure 3). In the following section, we detail the abiotic and biotic conditions that can affect N metabolism.

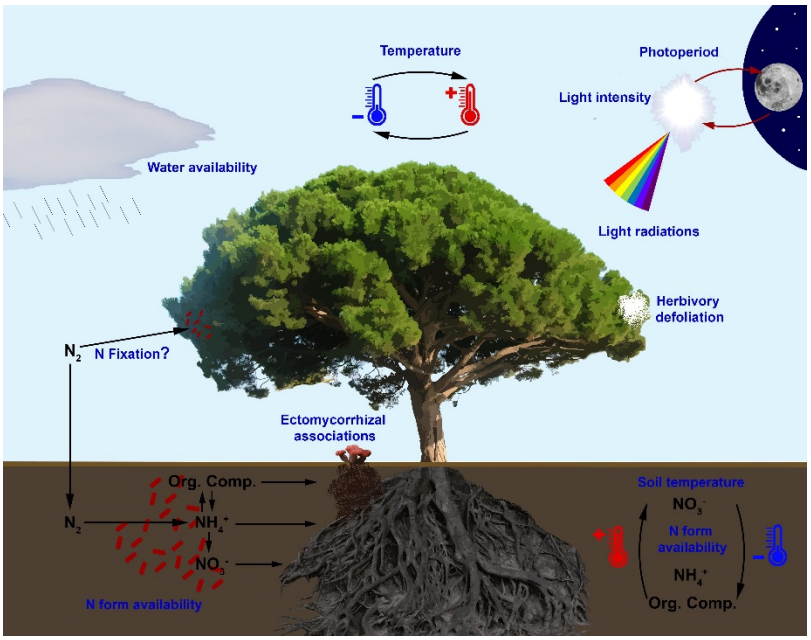

Figure 3. Interaction between nitrogen metabolism and environment.

5.1. Abiotic Interactions

Plants must develop specific responses to overcome climate changes, such as temperature shifts between seasons. At the metabolic level, these responses can be easily identified because the metabolic rates of all organisms positively correlate with temperature [63]. Conifers growing in cold conditions often exhibit increased respiration rates and accumulate N in their needles [64]. At the biochemical level, changes in the composition of lipids or carbohydrates are evident, as well as in the accumulation of N metabolites and proteins with cryoprotective functions [65]. One of the better-studied responses to temperature in conifers is their cold/freeze acclimatization process. Because conifer forests dominate large boreal regions [12], these plants have developed a unique ability to acclimatize to extremely low temperatures. The tissues of some species, such as *Picea obovate*, a boreal conifer species that can survive at extremely low temperatures, are able to survive immersion in liquid N [65]. This type of extreme tolerance generally evolves following exposure to sequential stages of cold acclimatization initiated by short photoperiods and non-freezing low temperatures [66]. Although cold tolerance is generally triggered by non-freezing low temperatures, it can also be induced by mild drought and

short photoperiods [66]. It has been proposed that acclimatization to cold or freezing temperatures in plants is not dependent on the synthesis of new compounds but rather on the transient accumulation of certain compounds, including N-containing compounds such as amino acids or proteins [67]. In conifers, some amino acids, such as tryptophan and proline, have been identified as osmolite solutes that exert cryoprotectant functions during cold acclimatization [65,68,69]. The use of tryptophan in these plants is extremely interesting because its synthesis is linked to the secondary metabolism, which is especially well developed in conifers. Tryptophan biosynthesis begins with chorismate, the final metabolic product of the shikimate pathway [43,70]. Furthermore, tryptophan can also act as a precursor for the synthesis of the plant hormone auxin, which is essential for growth and development as well as for responses to a range of environmental factors, including cold [70–72]. Other amino acids have roles in this response, but they do not act directly as compatible solutes. For example, γ-aminobutiric acid (GABA) has a possible role in stress acclimatization acting as a signaling metabolite [64]. Conversely, although conifers exhibit seasonal changes in the arginine levels [68], the role of arginine in acclimatization to cold and freezing temperature appears to be related to the production of polyamines [68]. Additionally, other N metabolites, such as glycine betaine can act as cryoprotectants [67]; however, the role of glycine betaine has not been completely elucidated. In the needles of *Pinus pinaster*, a western Mediterranean tree conifer species, glycine betaine has no defined seasonal accumulation pattern; rather, its amount is highest in younger needles and decrease as needle age. In this conifer species glycine betaine levels are well correlated with methionine and choline levels and the expression of genes involved in tetrahydrofolate metabolism [73]. These findings are consistent with the suggestion that glycine betaine is associated with S-adenosyl-methionine metabolism in conifers [74].

N metabolism can also contribute to acclimatization driving amino acids into the synthesis of proteins with cryoprotective properties, such as dehydrins [75,76]. In *P. obovate,* a specific pattern of protein accumulation has been observed during acclimatization to cold seasons. Dehydrins may have an important role in facilitating tolerance to extremely low temperature [77,78].

In addition, low temperatures can affect N acquisition by plants. Soil temperature is an important regulatory factor associated with N source availability and uptake. Low temperatures provoke decreases in mineralization and nitrification rates, resulting in ammonium and amino acids becoming the predominant forms of N in soil. As such, these compounds become the preferred N forms for uptake [79,80]. Under these conditions, general metabolic rate decreases [63], which also reduces N uptake. However, when the temperature increases, N uptake, mainly nitrate increases [80]. In this context, temperature may be an important factor controlling the uptake of inorganic N and consequently may affect plant adaptations to the environment. While trees adapted to cold climates usually prefer NH_4^+ over NO_3^-, trees in warmer conditions generally prefer NO_3^-, which is the predominant N form available in their soils [81]. Additionally, it has been observed that increased temperatures lead to higher nitrate reductase (NR) activity in Scots pine needles [82], which is consistent with improved NO_3^- uptake at warmer temperatures. This differential preference for N forms depending on soil temperature has been observed in different conifer species from contrasting environments in North America [80].

Other environmental factors affecting N metabolism are light and photoperiod. Typically, light can affect the expression of genes involved in N assimilation and management. In pine, a gene encoding cytosolic glutamine synthetase (*GS1a*) is light-regulated and potentially involved in the reassimilation of NH_4^+ that is released in the photorespiration pathway [73,83]. Conversely, the expression of the gene encoding asparagine synthetase *(AS1)* is repressed by light and induced in the dark, when sucrose levels are low. This gene facilitates the biosynthesis of asparagine, an amino acid with a lower C/N ratio than glutamine [49,84]. On the whole-organ level, light gradients can induce changes in N allocation in leaves resulting in improved leaf photosynthetic capacity and photosynthetic NUE (PNUE) [85,86]. As such, it has been suggested that PNUE is associated with geographic clines in white spruce [87]. Interesting latitudinal clines have also been observed in the expression and

allele frequency of genes controlling photoperiod and light responses in conifers [88–90]. In trees, N metabolism can also respond to shading. In the conifer *Cunninghamia lanceolata*, PNUE increases in sunlit leaves compared to shaded leaves [91]. In angiosperm trees, reallocation of leaf N has been observed in shaded conditions [85,92]. There is a trade-off in the allocation of leaf N between water-soluble (mainly rubisco) and water-insoluble proteins. It appears that in sunlit leaves the allocation of N into soluble proteins increases, resulting in improved PNUE [85,92]. However, in evergreen conifers, the relationship between leaf N concentration and photosynthesis rate is either very weak or non-existent [93]. In fact, a negative response of leaf N concentration to light has been observed in evergreen conifers under high N fertilization [94]. It has been proposed that the faster growth of trees in well-lit and highly fertilized conditions dilutes the effects of leaf N concentration [94].

Specific types of radiation, such as UV light, can also affect N metabolism. As an example, the UV-B rays may affect N concentration in plants and interfere with the utilization of N by soil microorganisms [95,96]. In conifers, little is known regarding the effect of UV radiation on N metabolism, although the inhibition of nitrate reductase (NR) activity in *Picea asperata* needles has been observed under enhanced UV-B radiation [97]. However, NR activity was also reduced in the needles of *Pinus sylvestris* saplings submitted to conditions of UV exclusion [82]. Interestingly, free amino acid content has been observed to increase in the needles of plants under UV exclusion [81]. This suggests that excess N is not used for the production of stress proteins and secondary metabolites. The compositions of secondary metabolites such as flavonoids or other phenolic compounds, can change depending on UV radiation [98,99]. N metabolism in conifers has a crucial role in the production of these compounds because the amino acid phenylalanine is a precursor for the biosynthesis of these metabolites [28,100].

Other environmental factors, such as gravity, may modulate N metabolism in conifers. When trees are grown over slopes, their stems tend to grow in a non-vertical orientation, and reaction wood is formed to compensate for tilt [101]. This results in the activation of N metabolism in association with the synthesis of phenylpropanoids and the reassimilation of NH_4^+ released by phenylalanine ammonia-lyase activity during the initial steps of lignin biosynthesis [48,51]. Genes involved in N metabolism, such as *GS1b*, which encodes glutamine synthetase, and *PAT,* which encodes prephenate aminotransferase, are upregulated in compression wood (reaction wood in conifers), which contains more lignin than normal wood [48,51].

5.2. Biotic Interactions

In addition to the physical and chemical environmental conditions that affect plant life, the relationships between plants and other organisms also affect plant N metabolism in both beneficial and detrimental ways. For example, defoliation by herbivores induces the reallocation of N. In *Pinus nigra*, needle defoliation by the pine processionary moth is compensated by enhanced photosynthesis in the remaining foliage to reallocate N [102]. Several conifer genes that are related to defence against pathogenic fungi and are structurally similar to antimicrobial peptides (AMP1) have been reported [103,104]. AMP1 expression is upregulated in *P. sylvestris* following infection with the pathogenic fungus *Heterobasidion annosum* [103], and recombinant western white pine AMP1 was found to inhibit fungal growth [105]. More recently the role of AMP1 in maritime pine has been linked to N acquisition. Excess ammonium induces the expression of AMP1 [24], and functional analyses performed with purified protein supports the function of AMP1 in the regulation of ammonium ion flux into pine roots [106]. These results highlight the connections between pathogen attack and N metabolism, a topic that warrants further research efforts.

Among beneficial biotic interactions, the well-known association between conifers and ectomycorrhizal (ECM) fungi contributes to more efficient N acquisition by conifers roots [107,108]. ECM fungi have a higher capacity to take N from soil than arbuscular mycorrhizal fungi [108]; as such, they influence soil characteristics, such as microbe growth and carbon accumulation [109]. In addition, the N form preferences of conifers may be affected by N uptake in associated ECM fungi [110], which

may lead to the allocation of N from organic decomposition and from arthropods predated by ECM fungi, such as *Laccaria bicolor* [111]. These associations are strong because each population of conifer species has specific affiliations with local ECM fungi, and the growth of trees in new environments or locations may be seriously affected by the lack of a precise ECM fungal variant [112]. As a classical view, it has been proposed that N is transferred from fungi to plants as amino acids, mainly arginine, although it is now suggested that mycorrhizal fungi directly transfer ammonia and peptides into plant roots [108,113]. In support of both hypotheses, *P. sylvestris* exhibits enhanced uptake and assimilation of NH_4^+ and arginine relative to NO_3^- [114]. Ammonium availability (whether excess or deficiency) can cause harmful effects on ECM colonization and alter gene expression in maritime pine roots [115]. It has been found that the ammonium regulated AMP1 protein [24,106] is also upregulated at the early stages of ectomycorriza formation and therefore might be involved in the regulation of N transfer from ectomycorrizal fungi to plant hosts [115]. Following transfer at the mycorrhizal interface, N is incorporated into the amides glutamine and asparagine [115]. These findings are interesting because *P. pinaster* trees associated to ECM fungi prefer ammonium as a N source for uptake [116]. Finally, a recent report describing possible atmospheric N fixation from endophyte microbiota on conifer needles is worth of mention [117]. These interesting findings open new and exciting fields for the study of N metabolism of conifers.

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