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Nitrogen source and availability associate to mitochondrial respiratory pathways and symbiotic function in *Lotus japonicus*

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ABSTRACT

Legumes form symbioses with nitrogen-fixing bacteria, well studied metabolically but less so in terms of respiration. Symbiotic nitrogen fixation demands high respiratory ATP and carbon skeletons, linking nitrogen assimilation and both NADH- and ATP-dependent process to mitochondrial respiration. The plant mitochondrial electron transport chain contains two terminal oxidases that differentially fractionate against ¹⁸O, providing estimations *in vivo* of the energy efficiency of respiration. The regulation of N₂ fixation by plant respiratory parameters remains unknown. To investigate the regulatory interactions of these two metabolic processes, we tested the effect of different plant N status and sources on respiratory parameters and nutrition in *Lotus japonicus*. Plants were grown with two levels of KNO₃ fertilization (5 mM and 25 mM) and with the N₂ fixing symbiotic bacteria *Mesorhizobium loti*, which induced the formation of root nodules (NP). Additionally, we characterized roots containing non-fixing nodules by growing plants that display spontaneous nodule formation (*snf*) (SNF). We evaluated the natural abundances of ¹³C and ¹⁵N, and ¹⁸O discrimination during respiration in leaves and roots using isotope-ratio mass spectrometry. NADH and nutrient content were measured using ultra-performance liquid chromatography and inductively coupled plasma spectrometry. We observed that cytochrome *c* oxidase activity was higher in nodule roots capable of nitrogen fixation than in plants fertilized with high availability of nitrate, and that nitrogen status strongly associates to respiratory parameters. These findings highlight the role of cytochrome *c* oxidase in meeting the carbon and energy demands of symbiotic nitrogen fixation.

1. Introduction

Nitrogen (N) is a key component of DNA, proteins, and chlorophyll in plants (de Bang et al., 2021). Along with carbon (C), the biogeochemical pathways of N provide the raw materials for biosynthesis of nucleotides, amino acids, chlorophyll and enzymes, determining the stoichiometry of other nutrients within plants and driving physiological processes linked to growth, such as photosynthesis and respiration (Ågren, 2004; Körner, 2013; Igamberdiev and Kleczkowski, 2018). It is well known that increasing N levels in plants accelerates biomass allocation by enhancing both respiration and C assimilation (Niklas et al., 2005; Reich et al., 2006; Ågren et al., 2012; Khan et al., 2022). However, excessive

fertilization negatively impacts natural nutrient cycling and induces metabolic changes that disrupt the redox balance in plants (Yuan and Chen, 2015). Mass spectrometry enables the analysis of ¹³C and ¹⁵N abundances (δ¹³C and δ¹⁵N) in dry tissues, serving as indicators of processes related to C and N metabolism under varying nutrient levels. Analyses of δ¹⁵N are particularly useful for identifying whether N in plants is derived from symbioses with soil microorganisms (Farquhar et al., 1989; Craine et al., 2015; Busch et al., 2020). It is well established that higher N availability increases δ¹³C and decreases δ¹⁵N in plant bulk dry matter due to its positive effects on carboxylation efficiency (Dercon et al., 2006; Craine et al., 2015; Wang et al., 2017). However, there is a lack of data on ¹⁸O fractionation values in plants grown under

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different N levels and sources. Most studies focus on CO₂ production, which reflects the functioning of tricarboxylic acid cycle reactions but does not account for variations in the energy efficiency of respiration. These variations may depend on the energetic status and redox state of the plant (Del-Saz et al., 2018).

During respiration, substrates are oxidized to generate energy in form of ATP and NAD(P)H. The mitochondrial electron transport chain (mETC) includes two terminal oxidases that fractionate differently against ¹⁸O, enabling the calculation of their *in vivo* activities. These activities are biologically significant, providing metabolic flexibility when the energy status is decoupled from biosynthetic needs (Ribas-Carbó et al., 2005; O'Leary et al., 2019). The cytochrome c oxidase pathway (COP) is the primary pathway that couples mETC to ATP synthesis and fractionates less against ¹⁸O compared to the alternative oxidase pathway (AOP), which plays a critical role in maintaining redox homeostasis. Notably, the AOP is characterized by high CO₂ production without ATP synthesis (Henriksson et al., 2019). Analyses of the *in vivo* fractionation against ¹⁸O during respiration in plant organs are essential for predicting patterns of respiratory energy efficiency and, consequently, productivity (Del-Saz et al., 2018; Del-Saz and Hernandez-Montes, 2025). To date, the *in vivo* ¹⁸O fractionation has only been characterized in plants under different N status and sources (e.g., NO₃, NH₄⁺) (Jéréz et al., 2023). However, studies combining $\Delta^{18}\text{O}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values remain scarce. Only one study has integrated these parameters to identify an adaptive role for AOP, demonstrating its ability to support plant growth at low CO₂ levels in *Arabidopsis* (González-Meler et al., 2009). Applying this combination of isotope-ratio mass spectrometry (IRMS) analyses to plants grown under varying N availability could facilitate a better understanding of the integration of element cycling (C and N) with respiratory parameters (COP and AOP). This approach is particularly relevant for crops with access to different N sources, as it could help optimize N fertilization strategies to enhance the energy efficiency of respiration.

Symbiotic interactions with nitrogen fixing bacteria represent a sustainable method for enhancing crop productivity. Rhizobial associations affect the nitrogen (N) status of host plants (legumes), with the forms of N transported from roots to shoots differing significantly from those in plants receiving nitrate fertilizers (McKey, 1994; Kaschuk et al., 2009). Legumes roots containing nodules rely on nitrogenase activity to reduce atmospheric dinitrogen (N₂) to ammonia, which is metabolized and exported as fixed-N compounds to the leaves. These compounds are then incorporated into photorespiratory metabolic pathways, contributing to NADH accumulation in mitochondria (Ortiz et al., 2020; Schulte et al., 2021). Although $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ have been extensively studied in legume-rhizobia interactions (Lazali et al., 2014; Oteng-Frimpong and Dakora, 2018; Yahaya et al., 2019; Yuan et al., 2022), ¹⁸O discrimination during respiration and the *in vivo* activities of COP and AOP have not yet been characterized in roots containing nodules. Compared to fertilized plants under similar N status, the energy efficiency of respiration in nodulated roots may differ because N₂-fixing symbionts act as an additional C sink in legumes (Parvin et al., 2020). This can influence respiration rates due to changes in C fluxes. In nodule bacteroids, the nitrogenase reaction requires ATP and C skeletons, which are derived from photosynthetic sucrose (Naya et al., 2007; Vance, 2008; Schulte et al., 2021). The oxidation of these C skeletons in roots is linked to NADH generation in mitochondria, which is dissipated during oxygen consumption (Millar et al., 1997; Shane et al., 2004; Del-Saz et al., 2018). Given its role in ATP synthesis, COP is expected to have an *in vivo* role in roots with functional nodules. Conversely, AOP may play a more prominent role in non-inoculated roots under conditions of N excess and/or N deficiency, acting as a mechanism to maintain redox homeostasis. Therefore, creating different scenarios of N availability to characterize respiratory parameters in both non-N₂- and N₂-fixing plants is crucial. In this context, using genetic mutants of legumes (e.g. *Lotus japonicus*), with alterations in nodulation genes or redox regulation, offers a powerful approach to dissect the molecular coordination

between mitochondrial function and symbiotic N₂ fixation, as highlighted by recent functional genomic studies (Yamaya et al., 2023; Boussageon et al., 2023; Shimoda et al., 2024). Such studies could provide new insights into the regulation of respiratory parameters by plant N status and symbiotic interactions.

Legumes are excellent candidates for characterizing the *in vivo* activities of respiratory pathways under different scenarios of N availability due to their high levels of alternative oxidase (AOX) and their ability to establish symbiosis with soil bacteria (Adams et al., 2016; Sweetman et al., 2019). Moreover, *L. japonicus* is a well-studied nodulating legume and a model organism for investigating plant-microbe interactions, owing to its suitability for molecular genetic studies (Udvardi et al., 2005; Sato and Tabata, 2006; Mun et al., 2016; Li et al., 2020). In this study, we tested the effects of different plant N status on respiratory parameters by growing wild-type (WT) *L. japonicus* under three conditions: 5 mM, 25 mM KNO₃, and in symbiosis with *Mesorhizobium meliloti* (0 mM KNO₃). Additionally, to examine the effects of low N availability and explore the maintenance cost of nodules at the plant respiratory level, we used *L. japonicus* mutant plants with deregulated Ca²⁺/calmodulin-dependent kinase, which exhibit spontaneous nodule formation (*snf* mutations). These spontaneous nodules lack rhizobia, bacteroids, and infection threads (Tirichine et al., 2006). We evaluated $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in leaves and roots as proxies for functional symbiosis and nutrient cycling using isotope-ratio mass spectrometry (IRMS). We also used the oxygen isotope fractionation technique to calculate the *in vivo* activities of AOP and COP in both organs. Metabolic activity and nutrient economy were assessed by measuring NADH content and nutrient levels using ultra-performance liquid chromatography (UPLC) and inductively coupled plasma (ICP) spectrometry. We hypothesize that N status is strongly linked to respiration and that symbiotic N₂ fixation requires enhanced energy efficiency of respiration via COP, accompanied by improved nutrition. Furthermore, we sought to identify relationships that enable better integration of respiratory parameters with element cycling (C and N) and nutrient status.

2. Material and methods

2.1. Plant material

Seeds of *L. japonicus* WT (ecotype Gifu) and the spontaneous root nodule formation mutant (*snf1*) with a deregulated Ca²⁺/calmodulin-dependent kinase were supplied by Jens Stougaard lab. Seeds were scarified using a concentrated H₂SO₄ for 5 min. Then, seed were washed with distilled water, sterilized with 10 % of sodium hypochlorite for 10 min, and placed at 28 °C for 5 days in Petri dishes containing 0.8 % agar medium for germination. Seedlings were transferred to pots containing sterile substrate of vermiculite-perlite (3:1) mix, and grown in growth chamber for 30 days at 25 °C, 12/12 day/night cycle and photosynthetic photon flux density (PPFD) of 350 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Plants were irrigated with a modified Rigaud and Puppo (1975) nutrient solution. *L. japonicus* seedlings were inoculated with a stationary liquid culture of *Mesorhizobium loti* (MAFF303099) (10⁻⁹ cell/ml), grown in TY media for 24h at 28 °C in a rotary shaker at 150 rpm, when plants were transferred to pots and seven days after sowing. Inoculated plants were irrigated with the same nutrient solution which contains 0 mM of KNO₃ (NP). Non-inoculated *snf1* plants were irrigated with 1 mM of KNO₃ (SNF) to ensure both spontaneous nodulation and a minimum of plant growth. The 1 mM KNO₃ concentration does not stimulate but rather permits spontaneous nodule development in the *snf1* plant, as higher nitrate concentrations (>1 mM) are known to suppress nodule formation (Tirichine et al., 2006). Non-inoculated WT plants were irrigated with high (HN; 25 mM), and limited or moderate (LN; 5 mM) concentrations of KNO₃ (Fig. S1). All plants were irrigated twice a week. In order to avoid excessive chlorosis, and to increase the N status in *snf1* plants to the same level that in LN, one irrigation with 5 mM of KNO₃ at day 21 was applied when leaves became pale-green. The reason behind our

experimental design resides in the possibility to create two different N status among treatments, similar by pairs (HN and NP vs LN and SNF), that facilitate comparisons under contrasting scenarios of N availability and sources (Table S1). Leaves and roots were used for the *in vivo* respiration measurements. For NADH analysis, leaves of plants were immediately frozen in liquid nitrogen and stored at -80°C . For analyses of nutrients, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, plant tissues were dried at 70°C for 48 h prior to the analyses of ion concentration. Another set of plants were grown for measurements of the AOX capacity, N_2 fixation and plant biomass.

2.2. Oxygen-isotope fractionation and AOX capacity

For respiration measurements, leaves and roots of ten plants per treatment were darkened for 30 min and placed in a 3 ml stainless-steel closed cuvette at a constant temperature of 25°C . The air sampled from the cuvette were sequentially removed and fed into the mass spectrometer (Delta XPlus; Thermo LCC, Bremen, Germany). Changes in the $^{18}\text{O}/^{16}\text{O}$ ratios and oxygen concentration were obtained calculated the oxygen-isotope fractionation of both oxidases, and the electron partitioning through AOP (τ_a) as described in Del-Saz et al. (2017). Additionally, the end point fractionation values of both COP (Δ_c) and AOP (Δ_a) were performed for the calculation of COP activity (v_{COP}) and AOP activity (v_{AOP}). To obtain Δ_a , leaves and roots were treated with 10 mM KCN solution for 30 min. The values of Δ_a obtained in leaves and roots were $31.7 \pm 0.2\text{‰}$ ($n = 5$) and $27.7 \pm 0.2\text{‰}$ ($n = 4$), respectively. For calculation of Δ_c , leaves were treated with 25 mM SHAM solution for 30 min. The values of Δ_c obtained in leaves were $20.9 \pm 0.5\text{‰}$ ($n = 4$). In roots, an assumed value of 17 ‰ for the Δ_c was used for the electron partitioning calculations, as this has been shown to be fairly constant among the species examined up to date (Ribas-Carbo et al., 2005).

For measurements of the AOP capacity (V_{AOP}), leaves and roots of three plants per treatment were incubated in a solution with 10 mM KCN for 30 min. Oxygen uptake rates were measured in darkness using a liquid-phase Clark-type O_2 electrode (Rank Brothers, Cambridge, UK) in a solution containing 30 mM MES pH 6.2 and 0.2 mM calcium chloride. Measurements were performed at a constant temperature of 25°C .

2.3. Carbon and nitrogen isotopic composition

Carbon and nitrogen isotopic composition were determined in the same samples ($n = 8$) used for the calculation of AOX and COX activities. Dry leaf and root samples were ground into a fine powder with a mixer mill MM200 (Restsch®, Haan, Germany). All samples were combusted in an elemental analyzer (Thermo Flash EA 1112 Series, Bremen, Germany), and CO_2 and N_2 were directly injected into a continuous-flow isotope ratio mass spectrometer (Thermo-Finnigan Delta XP, Bremen, Germany) for isotope analysis. Peach leaf standards (NIST 1547) were run every seven samples. The standard deviation of the analysis was $<0.08\text{‰}$. Results are presented as δ vs. PDB (Pee Dee Belemnite) for C and as δ vs. atmospheric N_2 for N. The natural abundance of ^{15}N was also analyzed in nutrient solution containing nitrate. The percentage of plant nitrogen derived from the atmosphere (%Nd_{fa}) in NP was also calculated according to Shearer and Kohl (1988):

$$\% \text{Nd}_{\text{fa}} = \frac{\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{fix}}}{\delta^{15}\text{N}_{\text{ref}} - B} \times 100$$

where $\delta^{15}\text{N}_{\text{ref}}$ is the mean $\delta^{15}\text{N}$ value of a non-reference N_2 fixing plant, $\delta^{15}\text{N}_{\text{fix}}$ is the mean $\delta^{15}\text{N}$ value of NP, and the B value is the $\delta^{15}\text{N}$ of the legume wholly dependent on N_2 fixation for its N nutrition, and can be obtained from the smallest $\delta^{15}\text{N}$ values observed among measurements (Peoples et al., 2002).

2.4. Foliar NADH content

Leaves of *L. japonicus* plants ($n = 3-5$) were harvested and grounded to obtain 0.5 g for NADH measurements. The extraction and quantification were performed as proposed by Palma et al. (2019) with some modifications. Plant material was extracted in ammonium acetate buffer 5 mM pH 8.0, incubated at 85°C for 3 min, cooled on ice, and centrifuged 10 min at 5000g for 4°C . After centrifugation, the supernatant was filtered and used for NADH determination in an UPLC WATERS model ACQUITY equipped with a HSS T3 column ($1.8\text{ }\mu\text{m}$, $2.1\text{ mm} \times 100\text{ mm}$). NADH was eluted at a flow rate of 0.3 mL min^{-1} using an elution gradient prepared with 5 mM ammonium acetate buffer pH 6 (A) and methanol (B). The gradient profile expressed as (t [min]; %B), was: (0; 0 %), (2; 0 %), (3.5; 17 %), (6; 90 %), (6.10; 0 %). A peak of NADH was observed for the gradient elution (5; 62 %). Finally, NADH was detected with the following transitions: $m/z\ 666.35 \rightarrow 107.83$ (cone voltage 10 and collision energy 54) and $666.35 \rightarrow 135.93$ (cone voltage 10 and collision energy 56) using a Xevo TQ-S triple quadrupole mass spectrometer (Waters) in the positive electro-spray ionization (ESI) mode.

2.5. Element concentration, nitrogen fixation and biomass

The ion concentration was determined in the fine powder of leaves and roots ($n = 4$). Tissue concentrations and contents were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Agilent ICP 720-ES), carried out by the Analytical Service of the EEZ, CSIC, Granada, Spain.

Nitrogenase activity (E.C. 1.7.9.92) was measured in nodulated roots ($n = 4-6$) of SNF and NP according to Witty and Minchin (1998). Roots were placed in an open-flow system to measure the H_2 -evolution using an electrochemical H_2 sensor (QubitSystem Inc., Canada). Apparent nitrogenase activity (ANA, rate of H_2 production in air) was determined under $\text{N}_2:\text{O}_2$ (80 %:20 %) with a total flow of 0.4 L min^{-1} . Total nitrogenase activity (TNA) was determined under Ar: O_2 (79 %:21 %). Nitrogen fixation rate (NFR) was calculated as $(\text{TNA}-\text{ANA})/3$. Standards of high purity H_2 were used to calibrate the detector.

At harvest (30 days after sowing), the shoot and root systems of four replicates per treatment were separated and weighed to determine fresh weight (FW). Subsequently, the dry weight (DW) was also measured after drying at 70°C for 2 days, and total weight (TW) was calculated.

2.6. Statistical analysis

A one-way ANOVA were performed for all the parameters measured in this study. The independent variables were N availability (HN, LN, SNF, NP), and dependent variables were respiratory components (V_{C} , v_{Cyt} , v_{alt} , V_{alt}), $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, NADH and all nutrients. Homoscedasticity was tested before ANOVA analysis. The statistical analyses were performed in R 3.0, and differences between dependent variables were detected by a Tukey test (R core Team, 2021). In addition, Pearson coefficients were obtained with JMP®, Version 18.1.0 (SAS Institute Inc., Cary, NC, United States, Table 3) after to generate individual fold change data from the parameters corresponding to each treatment (HN, LN, NP, SNF), and to normalize each measurement of LN, HN and NP to the mean of SNF. Each correlation was calculated from four mean data points representing each treatment.

3. Results

3.1. Nitrogen fixation and biomass production

In NP roots, nodules showed apparent nitrogenase activity ($11.7 \pm 2.93\text{ }\mu\text{mol H}_2\text{ g}^{-1}\text{h}^{-1}$), total nitrogenase activity ($117.4 \pm 19.79\text{ }\mu\text{mol H}_2\text{ g}^{-1}\text{h}^{-1}$) and nitrogen fixation rate ($35.2 \pm 7.0\text{ }\mu\text{mol N}_2\text{ g}^{-1}\text{h}^{-1}$). In SNF nodules, neither apparent nitrogenase activity nor total nitrogenase

activity were observed and thus, nitrogen fixation rate was not detected (data not shown).

Shoot, root and total biomass showed significant differences between treatments (Tables 1 and 2). HN and LN showed the highest total accumulation of biomass among treatments, although LN showed both higher shoot biomass and lower root biomass than HN. SNF and NP showed similar total biomass although pale green leaves were observed in SNF.

3.2. Carbon and nitrogen isotopic composition

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ showed significant differences between treatments (Table 1, Fig. 1A–D). Values of $\delta^{13}\text{C}$ ranged from -30.14 ± 0.09 to -32.19 ± 0.14 ‰ in both leaves and roots. In SNF leaves, the $\delta^{13}\text{C}$ values were significantly lower than in LN and HN leaves, and similar to NP leaves (Fig. 1A). Among treatments, leaf $\delta^{13}\text{C}$ values showed minor variation, and were typical of well-watered plants, but root $\delta^{13}\text{C}$ values showed major variation. SNF roots showed the lowest $\delta^{13}\text{C}$ value, LN and NP showed similar values, and HN roots showed the highest abundance of ^{13}C (Fig. 1B). For $\delta^{15}\text{N}$, values among treatments were significantly different (Table 1, Fig. 1C and D), and within a rather wide range, roughly between -1.14 ± 0.08 and 12.31 ± 0.28 ‰ in both organs. In leaves and roots, the highest abundance of ^{15}N was found in LN, followed by SNF, showing HN and NP plants values close to atmospheric nitrogen (0 ‰), although statistically different (Fig. 1C and D). Note that, among treatments, we observed $\delta^{15}\text{N}$ negative values in leaves together with $\delta^{15}\text{N}$ positive values in roots, only in NP, which is commonly observed in nodulated legumes due to isotopic fractionation during nitrogen assimilation and the preferential transport of ^{14}N -enriched compounds to shoots (Evans, 2001). As a result, the leaves become ^{14}N -enriched ($\delta^{15}\text{N} < 0$ ‰) and the nodulated roots ^{15}N -enriched ($\delta^{15}\text{N} > 0$ ‰), a phenomenon consistent with our observations in NP. Both fractionation and preferential transport provide further evidence of active symbiotic N_2 fixation in NP plants and suggest distinct patterns of nitrogen partitioning among treatments. In NP leaves, %Ndfa was 98.4 and 98.0 when $\delta^{15}\text{N}_{\text{ref}}$ are the mean $\delta^{15}\text{N}$ values of LN and SNF, respectively. In NP roots, %Ndfa was 88.1 and 85.5 when $\delta^{15}\text{N}_{\text{ref}}$ were the mean $\delta^{15}\text{N}$ values of LN and SNF, respectively (data not shown). The smallest three replicates obtained among $\delta^{15}\text{N}$ values in leaves and roots were averaged to be selected as 'the B value' in each

Table 1

One-way ANOVA was performed for each variable to assess the effect of nitrogen treatment (HN, LN, SNF, and NP) in leaves and roots separately. Asterisks denote the level of statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; “—” indicates non-significant differences ($p > 0.05$) or cases where the test was not applicable for a given organ. The table indicates whether each variable was significantly affected by treatment but does not convey the direction or magnitude of the effect. Full mean values, standard errors, and post hoc comparisons are provided in Tables 2 and 3

Variable	Leaves	Roots
$\delta^{13}\text{C}$	***	***
$\delta^{15}\text{N}$	***	***
V_t	***	***
v_{cyt}	***	***
v_{alt}	***	***
V_{alt}	***	***
NADH	***	—
C	*	***
N	**	**
P	**	***
K	**	—
N:C	**	**
N:K	***	*
N:P	***	*
Biomass _{leaves}	***	—
Biomass _{root}	—	**
Biomass _{total}	***	—

Table 2

Effects of different nitrogen availability and source on shoot, root and total biomass in *Lotus japonicus*. HN (25 mM), LN (5 mM), SNF (1 mM), NP (0 mM). Values are mean $\pm$ SE of 4 biological replicates. Differences were assessed by One-way ANOVA for each measured variable. Treatments with different letters indicate statistical differences by post hoc Tukey Test ($p < 0.05$).

	Shoot (g)	Root (g)	Total (g)
HN	$0.127 \pm 0.019\text{b}$	$0.174 \pm 0.037\text{a}$	$0.302 \pm 0.056\text{a}$
LN	$0.184 \pm 0.015\text{a}$	$0.111 \pm 0.010\text{b}$	$0.294 \pm 0.023\text{a}$
SNF	$0.038 \pm 0.005\text{c}$	$0.073 \pm 0.005\text{b}$	$0.111 \pm 0.008\text{b}$
NP	$0.054 \pm 0.003\text{c}$	$0.071 \pm 0.006\text{b}$	$0.125 \pm 0.007\text{b}$

organ (-1.96 ‰ and 2.30 ‰ in leaves and roots, respectively, as a proxy of values typically found in legumes wholly dependent on N_2 fixation (Peoples et al., 2002). In addition, the $\delta^{15}\text{N}$ value of source potassium nitrate was 0.24 ‰, which is in the range of other studies that analyzed $\delta^{15}\text{N}$ in synthetic fertilizers (Bateman and Kelly, 2007; Cui et al., 2020).

3.3. Nutrient content and stoichiometry

Nitrogen availability significantly affected the content of C, N, K, P and other nutrients in laves and roots (Tables 1, 3 and 4). Unlikely LN and SNF, plants with high content of N in both organs (HN and NP) also showed small but significant increases in the content of C (Tables 3–4). In roots, NP showed the highest N content among treatments (Table 4). Regarding P content, N availability significantly affected the abundance of this nutrient, and NP presented the highest content in leaves among treatments, with higher content in roots than HN. The highest values of P were observed in roots of LN and SNF (Table 4). In leaves, HN and NP showed the highest K content (Table 3). Because of this, N availability significantly affected the stoichiometry of N:C, N:P, and N:K in leaves and roots (Tables 1, 3 and 4), showing important variability among treatments (≈ 2 -fold). In leaves and roots, HN and NP presented higher values of N:C, N:P, N:K, than LN and SNF (Tables 3–4). Interestingly, in roots, we observed similar K content across all treatments, indicating that differences in K content in leaves were not caused by varying levels of K in the fertilizer. Notably, KNO_3 fertilization was not applied to NP plants; however, NP leaves exhibited similar K content and N:K ratios to HN leaves, and NP roots showed a higher N:K ratio than HN roots (Tables 3–4). Additionally, N availability significantly affected the content of other nutrients such as Mn, Ni, S and Sr in both organs (Tables S2 and S3). In leaves and roots, Mn, Ni and Sr, were higher in LN and SNF than in HN and NP, while the amount of S was higher in HN and NP, with the highest value observed in NP (Tables S2 and S3).

3.4. Respiration, electron partitioning to alternative oxidase pathway, AOX capacity and NADH content

In leaves, respiratory parameters as V_t , v_{cyt} and v_{alt} were statistical related to N and C content (Figs. 2–3). In leaves, total rates of respiration reached around $30 \text{ nmol O}_2 \text{ g}^{-1}\text{DWs}^{-1}$ and the values of V_t , v_{alt} and v_{cyt} were significantly higher in HN and NP, than in LN and SNF (Table 3, Fig. 2A–C). Content of NADH showed a similar pattern than V_t , v_{alt} and v_{cyt} in leaves, being significantly higher in HN and NP compared to LN and SNF (Tables 1 and 3). In roots, V_t showed the maximum values around $26 \text{ nmol O}_2 \text{ g}^{-1}\text{DWs}^{-1}$ (Fig. 3A), and v_{cyt} value was the highest in NP (Fig. 3B). On the contrary, v_{alt} was higher in HN, followed by LN and SNF, with the lowest value observed in NP (Fig. 3C). In this sense, in NP roots, a high energy efficiency of respiration was observed compared to N fertilization. On the other hand, V_{alt} was higher in leaves of HN and LN than in SNF and NP, whilst in roots, it was higher in HN and NP (Fig. S1).

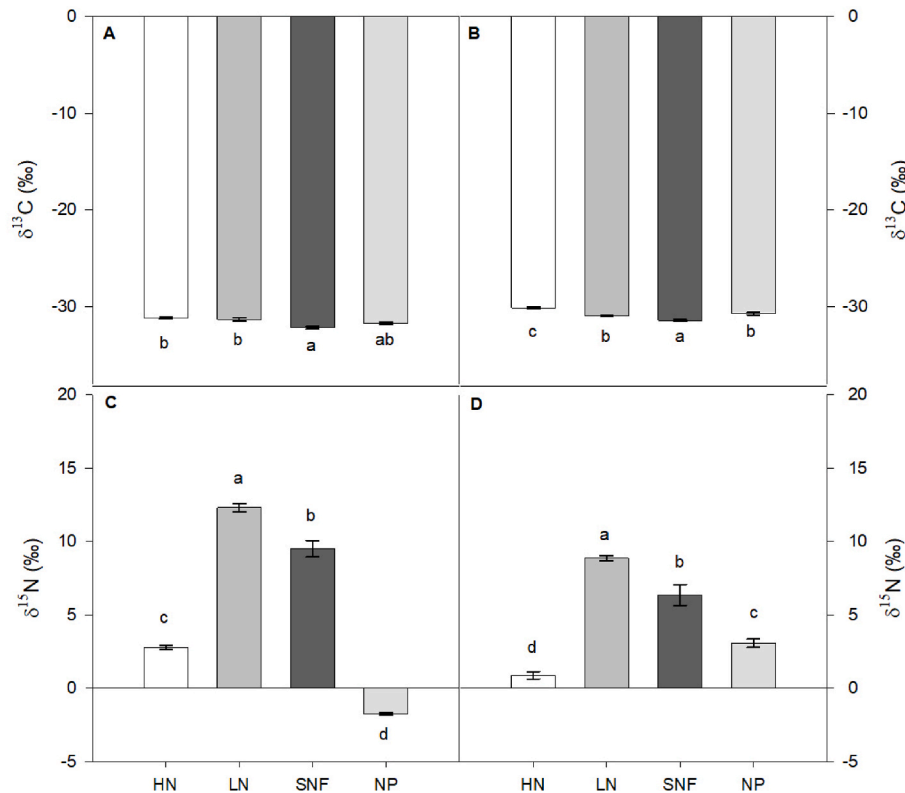


Fig. 1. Carbon and nitrogen isotopic composition in (A–C) leaves and (B–D) roots of *L. japonicus* plants grown under different N treatments. **HN** (25 mM), **LN** (5 mM), **SNF** (1 mM), **NP** (0 mM). Bars are means $\pm$ SE of 8 biological replicates. Differences were assessed by One-way ANOVA for each measured variable. Different letters represent statistical differences by post hoc Tukey’s test ($p < 0.05$).

Table 3
Content of Carbon (C), Nitrogen (N), Phosphorus (P), Potassium (K), C:N:P:K ratios and NADH content in leaves of *L. japonicus* plants grown under different N treatments. **HN** (25 mM), **LN** (5 mM), **SNF** (1 mM), **NP** (0 mM). Values are means $\pm$ SE of 4 biological replicates. Differences were assessed by One-way ANOVA for each measured variable. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

	C (g g ⁻¹)	N (g g ⁻¹)	P (mg g ⁻¹)	K (mg g ⁻¹)	NADH (mg FW ⁻¹)	N:C	N:P	N:K
HN	0.44 $\pm$ 0.003a	0.046 $\pm$ 0.001a	1.42 $\pm$ 0.108b	23.9 $\pm$ 1.09a	183.7 $\pm$ 14.7a	0.005 $\pm$ 0.000a	33.3 $\pm$ 2.14a	0.010 $\pm$ 0.001a
LN	0.42 $\pm$ 0.009b	0.017 $\pm$ 0.006b	1.23 $\pm$ 0.029b	17.3 $\pm$ 0.609b	74.3 $\pm$ 9.12b	0.002 $\pm$ 0.000b	13.7 $\pm$ 0.919c	0.005 $\pm$ 0.000b
SNF	0.41 $\pm$ 0.001b	0.016 $\pm$ 0.005b	1.50 $\pm$ 0.096 ab	17.9 $\pm$ 1.33b	76.3 $\pm$ 2.28b	0.002 $\pm$ 0.000b	10.7 $\pm$ 0.591c	0.005 $\pm$ 0.000b
NP	0.44 $\pm$ 0.002a	0.043 $\pm$ 0.001a	1.76 $\pm$ 0.049a	20.8 $\pm$ 0.604 ab	143.9 $\pm$ 16.8a	0.005 $\pm$ 0.000a	24.2 $\pm$ 0.749b	0.011 $\pm$ 0.000a

Table 4
Levels of Carbon (C), Nitrogen (N), Phosphorus (P), Potassium (K), C:N:P:K ratios in roots of *L. japonicus* plants grown under different N treatments. **HN** (25 mM), **LN** (5 mM), **SNF** (1 mM), **NP** (0 mM). Values are means $\pm$ SE of 4 biological replicates. Differences were assessed by One-way ANOVA for each measured variable. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

	C (g g ⁻¹)	N (g g ⁻¹)	P (mg g ⁻¹)	K (mg g ⁻¹)	N:C	N:P	N:K
HN	0.420 $\pm$ 0.006a	0.029 $\pm$ 0.001b	1.616 $\pm$ 0.269c	29.9 $\pm$ 3.54	0.068 $\pm$ 0.001b	18.73 $\pm$ 2.94a	0.99 $\pm$ 0.11b
LN	0.377 $\pm$ 0.190b	0.012 $\pm$ 0.004c	2.992 $\pm$ 0.136a	33.7 $\pm$ 0.308	0.031 $\pm$ 0.003c	3.99 $\pm$ 0.27b	0.35 $\pm$ 0.02c
SNF	0.372 $\pm$ 0.007b	0.011 $\pm$ 0.006c	2.872 $\pm$ 0.124a	28.5 $\pm$ 0.495	0.028 $\pm$ 0.001c	3.64 $\pm$ 0.09b	0.37 $\pm$ 0.01c
NP	0.420 $\pm$ 0.004a	0.044 $\pm$ 0.008a	2.268 $\pm$ 0.023b	28.0 $\pm$ 0.957	0.105 $\pm$ 0.290a	19.28 $\pm$ 0.41a	1.63 $\pm$ 0.01a

3.5. Associations between nitrogen status and respiratory parameters in leaves and roots

In order to gain insight into the influence of organ N content on the different acclimations to N availability, pairwise Pearson correlation coefficients between averaged values of all parameters studied in *L. japonicus* (except for V_{alt} , ANA, TNA, NFR and biomass parameters) were performed. Significantly, in leaves, we observed positive correlations between NADH, C and N content with respiratory parameters (V_t , v_{cyt} and v_{alt}), and negative correlations between Ca content and respiratory parameters (Table 5). We also observed significant positive

correlations between N and NADH, N and C. Significant negative correlations were observed between C and Ca, and between N and Ca. Significantly, in roots, we observed positive correlations between V_t and C, and between v_{cyt} and N, whilst negative correlations were showed between v_{cyt} and Mn, between v_{cyt} and Cu, and between V_t and $\delta^{15}N$. Similarly, a positive correlation was observed between Mn and Cu, and a negative correlation was observed between N and Mn (Table 5). These results suggest that N and C status are strongly associated with respiratory activity in both organs, although both direction and strength of correlations vary between leaves and roots. Note that correlation coefficients were consistently high, with values exceeding 0.95 across

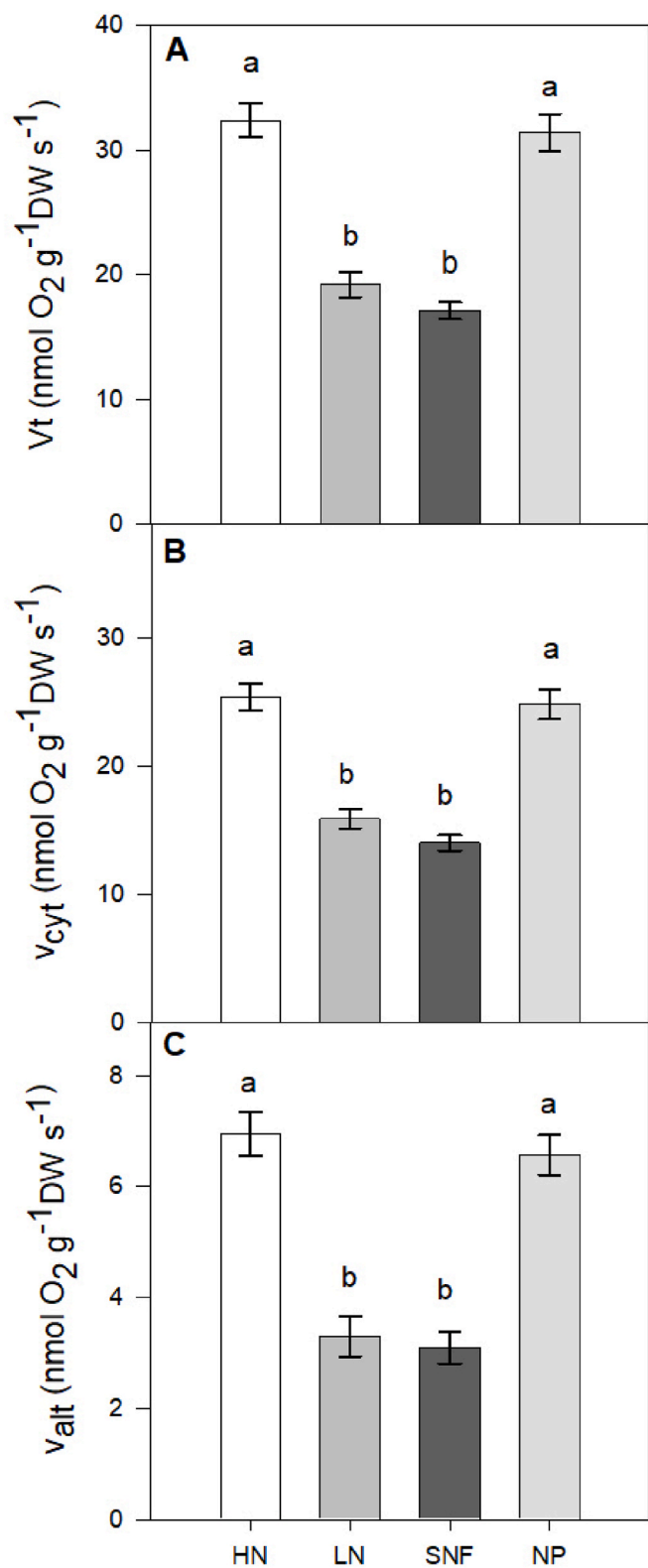


Fig. 2. A) Total respiration (Vt), B) COP activity (vcyt), C) AOP activity (valt), in leaves of *L. japonicus* plants grown under different N treatments. HN (25 mM), LN (5 mM), SNF (1 mM), NP (0 mM). Bars are means $\pm$ SE of 10 biological replicates. Differences were assessed by One-way ANOVA for each measured variable. Different letters represent statistical differences by post hoc Tukey's test ($p < 0.05$). Different letters indicate statistically significant differences between treatments ($p < 0.05$).

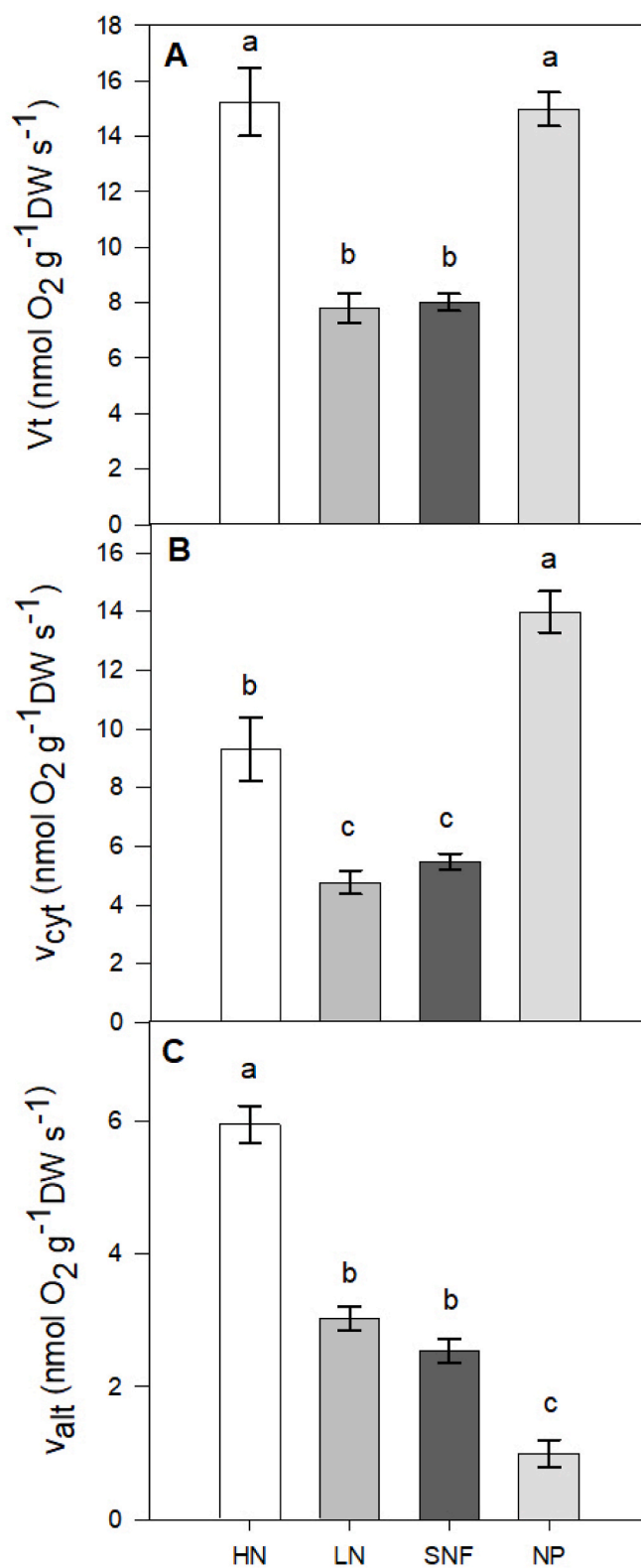


Fig. 3. A) Total respiration (Vt), B) COX activity (vcyt), C) AOX activity (valt), in roots of *L. japonicus* plants grown under different N treatments. HN (25 mM), LN (5 mM), SNF (1 mM), NP (0 mM). Bars are means $\pm$ SE of 10 biological replicates. Differences were assessed by One-way ANOVA for each measured variable. Different letters represent statistical differences by post hoc Tukey's test ($p < 0.05$).

Table 5

Direction and significance of Pearson correlations between *in vivo* respiratory parameters (V_t , v_{cyt} , v_{alt} , and NADH) and nutrient content (C, N, Ca, Mn, Cu) in leaves and roots of *L. japonicus* plants grown under different N treatments. “+” and “-” indicate positive and negative associations, respectively. Asterisks denote the level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Correlations were calculated using four averaged data points, each corresponding to one nitrogen treatment (HN, LN, NP, SNF).

Leaves	NADH	$\delta^{15}\text{N}$	Ca	C	N
V_t	+(*)	-	.(*)	+(**)	+(**)
v_{cyt}	+(*)	-	.(*)	+(*)	+(**)
v_{alt}	+(*)	-	.(*)	+(**)	+(***)
C	+	-	.(*)		+(***)
N	+(*)	-	.(*)	+(**)	
Roots	$\delta^{15}\text{N}$	C	N	Mn	Cu
V_t	.(*)	+(*)	+	-	-
v_{cyt}	-	-	+(**)	.(*)	.(*)
N	-	+		.(*)	-
Mn	+	-	.(*)		+(*)

significant associations likely reflecting overfitting due to the low number of data points. The significant correlations should therefore be interpreted as potential associations rather than regulatory relationships.

4. Discussion

Respiratory parameters and nutrient status (and stoichiometry) in *L. japonicus* are strongly affected by N availability and source (nitrate or symbiotic nitrogen fixation) (Tables 1, 3 and 4; Figs. 2–3). Compared to HN and NP, plants grown at 5 mM and 1 mM KNO_3 (LN and SNF) exhibited lower rates of V_t via COP in leaves and roots, along with reduced foliar NADH content. This suggests decreased ATP production in these treatments, as well as altered contents of other essential nutrients like C, P and K (Table 3; Figs. 2–3). These findings align with previous studies describing relationships between O_2 consumption, metabolic activity and N availability (Johnson, 1990; Bloom et al., 1992; Shane et al., 2004; Kornfeld et al., 2013), and are further supported by observed correlations (Table 5; Figs. 2–3). In leaves, N and C contents were positively correlated with V_t , v_{cyt} , v_{alt} , and NADH content. Bearing in mind that free NADH does not directly regulate mitochondrial metabolism (Kasimova et al., 2006), the positive correlations between NADH and respiratory parameters should be interpreted as associations that reflect the balance between reductant supply and protein demand. Under low N availability, plants reduce the demand for NADH and NAD^+ (and content) to limit amino acid production, involving reductions in metabolic activity (and respiration) to conserve N (Nunes-Nesi et al., 2010; Xue et al., 2022). In line with this, in roots, both C content and $\delta^{15}\text{N}$ correlated with V_t (Table 5), suggesting that respiratory C metabolism could limit amino acid synthesis under low N availability. This limitation may prompt plants to discriminate less against ^{15}N to maximize N assimilation and protein synthesis (Johnson, 1990; Evans and Clarke, 2019; Arnold et al., 2015; Perchlik and Tegeder, 2018).

As higher growth rates require more N to invest in proteins (Ågren, 2004; Yu et al., 2012), and since HN and NP treatments showed increases in the ratios of N:C, N:P, and N:K, which are often associated to growth (Ågren, 2004; Yu et al., 2012), we conducted an experiment on biomass accumulation to explore the costs and benefit of symbiotic N_2 fixation (Table 2). We observed that nodules imposed a biomass cost in nodulated roots (SNF and NP), but functional nodulation provided a benefit in NP, as green leaves developed and the root-to-shoot ratio was comparable to HN (Fig. S1; Table 2). Although photosynthesis was not evaluated, the chlorotic phenotype observed in SNF plants suggests a reduced chlorophyll content, which would limit light harvesting and C assimilation, thereby likely decreasing amino acid production, NADH

demand and respiratory capacity (Evans and Clarke, 2019) (Fig. S1). Additionally, LN and HN plants accumulated more biomass than NP, consistent with studies reporting that nitrate often stimulates biomass production due to the lower costs associated with nitrate assimilation compared to symbiotic N_2 fixation (Fotelli et al., 2011). LN plants achieved similar total biomass to HN, demonstrating comparable growth at 5 mM and 25 mM nitrate levels, in line with other studies (Li et al., 2020; Xing et al., 2022). Although a complete nitrate response curve was not performed in this study, the similar total biomass between 5 mM and 25 mM suggests that 5 mM was sufficient to support growth under our conditions (Table 2). In fact, differences in shoot biomass indicated that 25 mM nitrate is excessive for plant metabolism in this species, as it did not further stimulate growth, in line with previous studies (Pajuelo et al., 2002; Orea et al., 2005; Prosser et al., 2006). This suggests that growth respiration may be less predominant in HN than in LN when plants are 30 days old. The dry weight achieved by LN could result from higher C accumulation in structural components (e.g., cellulose) due to lower N:C and N:P ratios (Tables 2–3), which are linked to growth limitation by N content (Güsewell, 2004). On the other hand, the low dry weight and N content observed in SNF leaves suggest the presence of stress. In this scenario, maintenance respiration may predominate in plants with defective nodules. However, caution is necessary when interpreting these results, as the supplementation of the *snf1* plant with 1 mM KNO_3 (instead of 0 mM) during the last days of growth period could have underestimated the maintenance cost of nodules in SNF. However, based on respiration results and NADH content, the DW observed in SNF suggests that this cost may be related to higher respiratory demand and lower nutrient efficiency compared to LN treatment. In roots, we observed a negative correlation between Cu content and v_{cyt} , which may reflect the structural role of Cu in cytochrome c oxidase (COX). NP roots exhibited the highest COP activity and relatively lower Cu levels compared to other treatments (Fig. S3; Table 2). This observation may indicate that COX synthesis proceeds efficiently even under reduced Cu availability. A negative correlation was also found between Mn and N content in roots. Since Mn is a known cofactor of glutamine synthetase (GS), involved in N assimilation (Alejandro et al., 2020), this relationship could reflect a role for Mn in supporting N metabolism. Furthermore, the high Mn content and lower COP activity observed in LN and SNF coincided with the comparable biomass accumulation between LN and HN treatments, which is consistent with previous findings linking Mn availability to enhanced GS activity and growth (Pradhan et al., 2014).

We also observed that $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ are influenced by N availability and symbiotic N_2 fixation, with respiration via COP playing a crucial role for nitrogenase activity (Tables 3–4; Figs. 2–3). In NP, leaves $\delta^{15}\text{N}$ values were negative and close to atmN_2 , consistent with the presence of functional root nodules (Fig. 1C). In HN, due to the abundance of nitrate in the nutrient solution and the absence of root nodules, roots were less enriched in ^{15}N compared to the source nitrate, supporting the idea of greater discrimination against ^{15}N when N is abundant (Evans, 2001). Similarly, in SNF and LN, nitrate served as the primary N source, and its low and moderate availabilities (1 mM and 5 mM) resulted in reduced discrimination against ^{15}N , consistent with Evans (2001). Additionally, in roots, we observed differences in $\delta^{13}\text{C}$ values between HN and LN, and between NP and SNF (Fig. 2B), which align with the concept that $\delta^{13}\text{C}$ increases with higher N availability (Cordell et al., 1999; Clay et al., 2001; Ripullone et al., 2003; Choi et al., 2005; Plett et al., 2020). In both leaves and roots, SNF showed the lowest accumulation of ^{13}C , likely due to the low nitrate availability in the solution (1 mM). In contrast, NP plants were more ^{13}C -enriched, particularly in roots. This enrichment may be attributed to a CO_2 recycling mechanism involving C_4 -type photosynthesis in functional nodules, as part of a carbon-nitrogen positive feedback loop that enhances nodule metabolism (Fotelli et al., 2011; Ferguson et al., 2019). Moreover, NP plants accumulated more P than HN in both leaves and roots. This nutrient is essential not only for nodulation and N_2 fixation (Zhong et al., 2023) but also for ATP

synthesis and C accumulation. These observations could explain the differences in v_{cyt} and $\delta^{13}\text{C}$ between HN and NP. Kaschuk et al. (2009) reported that rhizobia inoculation stimulates photosynthesis by improving plant nutrition. Here, we describe that rhizobia inoculation also stimulates COP activity in roots through enhanced nutrient availability (Tables 3–5), likely to meet the ATP and C skeleton demands of nodules, thereby supporting nitrogenase activity in agreement with previous observations (diCenzo et al., 2020; Vittozzi et al., 2021). As a determinate nodule-forming species, *L. japonicus* exports asparagine rather than ureides, and its synthesis requires ATP. Through amino acid cycling within nodules (Lodwig et al., 2003) and COP activity, the TCA cycle in nodulated roots may support the ATP necessary to meet the energetic costs of N assimilation and asparagine export. In agreement, a proteomic study in soybean identified upregulation of genes related to complexes III and IV (COX) in nodules (Sin et al., 2024), suggesting improved respiratory yield and energy efficiency, patterns consistent with our observations.

We conclude that N status associates to respiratory metabolism and, consistent with our hypothesis, symbiotic N_2 fixation enhances the energy efficiency of respiration by increasing COP activity and improving nutrient status. Additionally, we demonstrate that the oxygen isotope fractionation technique, when combined with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses, can be valuable tool for identifying functional root nodules (Fig. S3 and S4). Furthermore, while an excess of N did not result in increased dry weight, our study suggests that higher biomass allocation may occur at N concentrations of approximately >5 mM. As carbon models at the ecosystem scale emphasize the importance of C sequestration in plants, we report that excessive N fertilization is associated with increased alternative respiration in both leaves and roots, which correlates negatively with C sequestration. Future studies involving detailed analyses of bacteroids and mitochondria, along with comprehensive photosynthetic and metabolic characterizations, would provide further insights into the metabolic connections underlying legume-rhizobia symbiosis.

CRedit authorship contribution statement

José Ortiz: Writing – original draft, Investigation, Formal analysis. **Carolina Sanhueza:** Investigation, Formal analysis. **Antonia Romero-Munar:** Investigation. **Sandra Sierra:** Investigation. **Francisco Palma:** Investigation. **Ricardo Aroca:** Writing – review & editing, Investigation. **Teodoro Coba de la Peña:** Writing – review & editing, Resources. **Miguel López-Gómez:** Writing – review & editing, Investigation. **Luisa Bascuñán-Godoy:** Writing – review & editing. **Néstor Fernández Del-Saz:** Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jplph.2025.154606>.

Data availability

Data will be made available on request.

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Corrigendum to “Nitrogen source and availability associate to mitochondrial respiratory pathways and symbiotic function in lotus japonicus” [J. Plant Physiol. 2025 (314), November 2025, 154606]

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In addition, the current affiliation of C. Sanhueza has been added. Consequently, the superscript letters indicating the author

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This correction does not affect the results, discussion, conclusions, or any other content of the published article. The authors apologize for this oversight and any inconvenience it may have caused.

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