



# Thriving on half: low nutritional demand in *Stephanopodium engleri* involves adjustments in photosynthetic apparatus functionality

Evandro Alves Vieira<sup>1</sup> · Luiz Palhares Neto<sup>2</sup> · Joana Patrícia Pantoja Serrão Filgueira<sup>1</sup> · Marina Ludmila Conon Salles<sup>1</sup> · Rafael Valadares<sup>1</sup> · Silvio J. Ramos<sup>1</sup> · Markus Gastauer<sup>1</sup> · Cecílio Frois Caldeira<sup>1</sup>

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## Abstract

Cultivating threatened plant species is vital for conservation, safeguarding against extinction by ensuring propagation and maintaining genetic diversity. Here, we conducted an integrative morphological, physiological, and quantitative proteomic analysis of *Stephanopodium engleri* plants grown under different substrates and nutritional sources. *Stephanopodium engleri* is a threatened tree endemic to the Iron Quadrangle, a Brazilian mining region. Different nutritional sources resulted in changes in the growth of *S. engleri*, with N, P, and micronutrients identified as the most limiting nutrients. The species exhibited maximum growth with half of the applied fertilization, suggesting a low nutritional requirement. The highest growth was related to adjustments in the photosynthetic apparatus and protein regulation, with no significant differences in morpho-anatomical traits. The most representative proteins displayed a diverse array of metabolic functions, particularly related to iron and zinc homeostasis, photosynthesis and energy pathways and reactive oxygen species signaling. Plants with lower carbon assimilation and growth exhibited down-regulation of these proteins. Our findings provide valuable insights into *S. engleri* physiology and adaptations, emphasizing the importance of minimum fertilization to produce high-quality seedlings. These results also support broader conservation efforts aimed at preventing the species from becoming extinct, including reinforcing natural populations, establishing new ones, and supporting the maintenance of germplasm banks and living collections.

**Keywords** Endangered species · Iron quadrangle · Plant growth · Nutritional sources · Proteome

## Introduction

Several soil types often have mineral nutrient imbalances compared to the plant nutritional requirements. Adequate nutrient supply ensures plant growth, development and yield by keeping the multiple physiological processes in optimal conditions (Lombardo et al. 2017). Essential nutrients, such as nitrogen (N), phosphorus (P), and potassium (K), are required in higher quantities and play crucial roles in plant metabolism (Khalofah et al. 2022). They support processes

like photosynthesis, nutrient uptake, and overall plant health (Fan et al. 2021). Balancing these nutrients in the soil is vital to maximize seedling production and maintain the health of natural ecosystems (Karthika et al. 2018). Inadequate supply of NPK usually exhibits stunted growth by limiting pigments content and quantum yield, thereby reducing carbon assimilation and distribution, and lipid peroxidation in chloroplasts (Soares et al. 2021). Together with macronutrients, micronutrients availability can be also decisive for plant growth and development. Micronutrients, such as iron (Fe) and manganese (Mn), are vital for key physiological functions, including chlorophyll biosynthesis, electron transport, cellular respiration, and carbohydrate metabolism. In addition, copper (Cu) and zinc (Zn) function as essential enzyme activators and cofactors, participating in a wide range of metabolic pathways in plant (Shao et al. 2021; Liu et al. 2021).

Plants developed adaptations to deal with adverse or favorable soil conditions, and several species can acclimate to a range of soils. This acclimation involves physiological

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✉ Cecílio Frois Caldeira  
cecilio.caldeira@itv.org

<sup>1</sup> Vale Institute of Technology Sustainable Development, 66055090, Belém, Brazil

<sup>2</sup> Department of Biology, Southwest Bahia State University, 45206-190, Jequié, Brazil

mechanisms, such as excretion of Fe-chelating molecules, reabsorption of elements from senescent leaves; anatomical changes, with the formation of more or longer root hairs when P supply is short; and morphological adjustments leading to root: shoot mass ratio increment when N or P are limiting plant growth (Lambers and Oliveira 2019). An essential physiological response in plants dealing with nutrient unbalancing involves safeguarding their photosynthetic machinery. Elevated N availability enhances N metabolism and bolster stress resilience within the photosynthetic apparatus by mitigating cell membrane damage and enhancing both energy dissipation and reactive oxygen species (ROS) scavenging (Zhong et al. 2018; Kartseva et al. 2021). Furthermore, the crosstalk between Fe and Cu has been documented as critical cofactors for components of the electron transport chain in the mitochondria and the chloroplast (Rai et al. 2021). Likewise, Fe interferes with Zn translocation and reduces the rate of its absorption (Gai et al. 2017; Chen et al. 2018) as also interfere remarkably in the complex cross-talks between N, P, calcium (Ca), magnesium (Mg), and sulfur (S), altering the plants nutritional homeostasis (Fan et al. 2021).

The proteomic approaches may help elucidate the mechanisms used by species to deal with nutrient excess or deficiency. In the case of Fe, the activity of ferric-chelate reductase in the roots is a reliable indicator of Fe uptake regulation (Rai et al. 2021). When Cu availability increases, the plants synthesize Cu-containing proteins instead of Fe-dependent ones. This shift reduces the internal demand for Fe and consequently downregulates ferric-chelate reductase activity through a feedback inhibition mechanism (Waters and Armbrust 2013; Rai et al. 2021). Additionally, nicotinamide (NA), a known chelating agent, has been shown to bind both Fe and Cu, thereby facilitating the Fe movement through the phloem (Chao and Chao 2022). The chelating function is important for maintaining redox homeostasis and CO<sub>2</sub> assimilation rates. A variety of proteins are associated with key physiological processes, such as photosynthesis (including the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, or Rubisco), ATP-dependent carboxylation, redox homeostasis and protein folding (Ahmad et al. 2016). Rubisco activase (RCA) ensures the continuous regeneration of ribulose-1,5-bisphosphate, thereby maintaining Rubisco activity and sustaining the photosynthetic carbon fixation cycle (Turek et al. 2020). Under N-deficient conditions, senescence of leaves is accelerated mainly due to degradation of Rubisco proteins (Feller et al. 2007). Indeed, studies on wheat have demonstrated that Mg sources promote RCA activity, thereby stabilizing Rubisco activation (Shao et al. 2021).

The interplay between various plant nutrients indicates that they influence each other's uptake, transport, and assimilation. The complex interactions between nutrients

remain poorly understood, requiring further research to uncover their dynamics and the signaling pathways activated by changes in their availability (Kumar et al. 2021). Despite the unfavorable nutritional characteristics for the growth of many species, semi-deciduous forests have high plant biodiversity, with a large number of rare and endemic species. Allied to the edaphoclimatic characteristics, these ecosystems are characterized by the fragmentation of the landscape due to the strong anthropization, which threatens the maintenance of its flora (Do Carmo and Jacobi 2016).

*Stephanopodium engleri* Baill. (Dichapetalaceae) is an endemic species of the underlying areas of Iron Quadrangle in Minas Gerais state, Brazil. It is a tree species considered extinct until 2010s and currently recognized as an “endangered” species due to the restriction of its occurrence and area of occupation (Ferreira et al. 2022). The lack of information on the biology of the species, combined with the increasing environmental degradation of its habitat, compromises its survival and requires the development of research aimed at its propagation and conservation. Consequently, understanding the species' nutritional requirements can enhance the effectiveness of ex situ conservation and reintroduction efforts within management programs, supporting population growth and reducing the risk of extinction.

In this study, we conducted an integrative morphological, physiological and quantitative proteomic analysis in plants of *S. engleri* grown under differential substrates and nutritional sources, aiming to provide a more in-depth and comprehensive understanding of its edaphic requirements, morphological and physiological adjustments, focusing on the roles of the proteome in the regulation of assimilatory metabolism. Therefore, we hypothesize: (i) the balance of macro- and micronutrients availability influences the net rates of CO<sub>2</sub> assimilation and plant growth; (ii) metal-chelating proteins, ion transporters and proteins linked to the assimilatory metabolism and energy pathway are up-regulated according to the nutritional source; (iii) changes in some structural attributes would provide better absorption or retention of nutrients.

## Materials and methods

### Plant material

Seeds of *S. engleri* were removed from fresh fruits harvested from areas of forest fragments (Semideciduous Seasonal Forest) near of Belo Horizonte, Brazil. The regional climate follows Köppen Aw, with a warm wet summers (November–April) and dry winters (May–October), average annual precipitation of 1300 mm and average annual temperature of 20 °C (Reboita et al. 2015). Access to the plants at these locations was kindly granted by Vale S.A. and Gerdau S.A.

Vouchers from these plants were previously deposited in the herbaria BHZB (11303), FLOR (78441), and CVRD (15967). The freshly fruits harvested were transported to the Plant Growth Laboratory of the Instituto Tecnológico Vale in Belém (PA), Brazil, where the experimental procedures were carried out. The seeds were sterilized (ethanol solution 70% for one minute and sodium hypochlorite 4% during five minutes) and placed to germinate in Gerbox<sup>®</sup> dishes (transparent plastic boxes of 11 × 11 × 3.5 cm L × W × H) containing filter paper saturated with distilled water and incubated at alternating temperatures of 28:22 °C with a 12:12 h photoperiod (50 μmol s<sup>-1</sup> m<sup>-2</sup> density of photon flux). After germination, the seedlings were transferred to trays with conical cells filled with a mixture of commercial organic substrate (Carolina soil<sup>®</sup>) and sterile sand. At this stage, the seedlings were cultivated in growth chamber (SGC 120, Weiss Technik, UK) under constant relative humidity (60%), photoperiod of 12:12 h for day:night and density of photon flux of 50 μmol m<sup>-2</sup> s<sup>-1</sup> and day: night temperature of 28: 22 °C.

### Plant growth conditions

After 30 days of cultivation, seedlings of uniform size were transferred to individual PVC pots of 2.3 L (30 × 10 cm height and diameter). To investigate the nutrient requirements of *S. engleri*, we cultivated these seedlings using a range of substrate compositions and fertilization treatments. This study aimed to evaluate plant performance—based on traits described below—across different growth media, determine the essentiality of individual macronutrients and micronutrients, and assess whether reduced fertilization levels could adequately support plant growth. The experimental design comprised nine treatments: (1) a commercial organic substrate (Carolina Soil<sup>®</sup>; CS); (2) a 1:1 volumetric mixture of CS and dystrophic Yellow Latosol (CS + S); (3) dystrophic Yellow Latosol without amendments, serving as a nutrient-poor control (S); (4) dystrophic soil supplemented with a complete fertilization regime including nitrogen (N), phosphorus (P), potassium (K), and micronutrients (NPK + M); (5) complete fertilization excluding micronutrients (NPK–M); (6) complete fertilization at half the standard content, including micronutrients (NPK ½); (7) fertilization with phosphorus, potassium, and micronutrients, excluding nitrogen (PK); (8) fertilization with nitrogen, phosphorus, and micronutrients, excluding potassium (NP); and (9) fertilization with nitrogen, potassium, and micronutrients, excluding phosphorus (NK).

The physical and chemical properties of the substrates were determined after plants cultivation. Soil samples were homogenized and sieved through a 4 mm mesh (Table S1); the pH was determined by a pH electrode in a 1:2.5 soil-to-water ratio, and the organic carbon was determined using the

potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) method. The available P, K, B, Zn, Fe, Mn, and Cu contents were determined using the Mehlich-1 method (0.05 mol L<sup>-1</sup> HCl + 0.0125 mol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub>), where P was determined by colorimetry, K was determined by flame photometry and the other elements were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES). Exchangeable Ca, Mg and Al were measured using atomic absorption spectrophotometry on 1 M KCl extracts with the addition of lanthanum oxide. The soil texture was determined as described by Kettler et al. (Kettler et al. 2001).

Due to the high acidity and reduced base saturation, 30 days before seedlings planting, the soil of all treatments using the dystrophic soil (except control) was treated with dolomitic limestone (Dimy<sup>®</sup>) to increase the base saturation to 60% (3.82 g of limestone per dm<sup>-3</sup> of soil). Treatments with complete fertilization received 100 mg N dm<sup>-3</sup> (CH<sub>4</sub>N<sub>2</sub>O, Sigma Aldrich), 200 mg P dm<sup>-3</sup> (NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, Sigma Aldrich) and 100 mg K dm<sup>-3</sup> (K<sub>2</sub>SO<sub>4</sub>, Sigma Aldrich). The micronutrients manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), boron (B) and molybdenum (Mo) were applied in amounts of 10 mg Mn dm<sup>-3</sup> (MnCl<sub>2</sub>, Sigma Aldrich), 5 mg Fe dm<sup>-3</sup> (6% FeEDDHA, Sigma Aldrich), 5 mg Zn dm<sup>-3</sup> (ZnSO<sub>4</sub>·7H<sub>2</sub>O, Sigma Aldrich), 1.5 mg Cu dm<sup>-3</sup> (CuSO<sub>4</sub>·5H<sub>2</sub>O, Sigma Aldrich), 0.5 mg B dm<sup>-3</sup> (H<sub>3</sub>BO<sub>3</sub>, Sigma Aldrich) and 0.15 mg Mo dm<sup>-3</sup> (Na<sub>2</sub>MoO<sub>4</sub>, Sigma Aldrich). Chemically, the studied latosol is desaturated with a dystrophic character, however, correction with limestone increased base saturation above 70%. The low cation exchange capacity corroborates the lower proportion of clay detected, while the pH is in the normal range. In general, the soil's nutritional concentration is low, with emphasis on the reduced values of nitrogen, sodium and potassium, and the low organic matter content.

### Environmental conditions

The plants (12 plants in each treatment) were grown for 120 days in greenhouse conditions with temperature and relative air humidity monitored every 15 min with a thermocouple connected to a datalogger (RHT10, Extech Instruments, Boston, USA). The daily air temperature varied between 25 and 37 °C, and the vapor pressure deficit varied between 0.4 and 2.5 kPa. The photoperiod was relatively constant throughout the year (12:12 h day:night, latitude approximately 1.45° South), and midday PPFD (photosynthetic photon flux density) was measured periodically (LI-190R, LICOR, Lincoln, NE, USA) and ranged from 900 to 1500 μmol m<sup>-2</sup> s<sup>-1</sup>. The water availability was held at 70% of the soil water retention capacity by replacing water loss from evapotranspiration with distilled water after pot weights monitoring every two days.

## Plant measurements

### Growth and biomass accumulation

Plant height and shoot diameter parameters were recorded monthly. Root and shoot biomass, gas exchange, foliar nutrients, anatomical traits, and proteomic profiling were performed at the end of the experiment. The shoot length (cm), stem diameter (mm), shoot dry mass (g), root dry mass (g), root/shoot ratio, specific leaf area ( $\text{m}^2$ ) and relative growth rate (RGR) were evaluated monthly in 10 plants between 0 and 120 days. To calculate root and shoot dry mass, samples were oven-dried at 65 °C for 72 h. Relative growth rate (RGR) was evaluated in eight plants per treatment and calculated according to the equation:  $(\log P2 - \log P1)/(t2 - t1)$ , where P2 and P1 correspond to the plant height between  $t2$  and  $t1$  (Jaqueti et al. 2016).

### Gas exchange

Instantaneous measurements of net carbon assimilation rates ( $A$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), stomatal conductance ( $g_s$ ,  $\text{mmol m}^{-2} \text{ s}^{-1}$ ), transpiration ( $E$ ,  $\text{mmol m}^{-2} \text{ s}^{-1}$ ), and the quantum yield of photosystem II (QY) were taken between 08:00 and 11:00 A.M., using an infrared gas analyzer (IRGA) model Li-6400 (Li-Cor-Nebraska, USA), under controlled conditions of  $\text{CO}_2$  concentration (400 ppm), PPFD of 1000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  and chamber temperature of 25 °C. During the experimental period, measurements were conducted in eight plants per treatment using the middle portion of the third fully expanded leaf as described above. Intrinsic water use efficiency ( $A/g_s$ ), ratio of intercellular to ambient  $\text{CO}_2$  concentration ( $C_i/C_a$ ), and instantaneous carboxylation rate ( $A/C_i$ ) were also estimated.

### Leaf nutrients content

Leaf nutrients content was determined in eight plants aleatory sampled from each treatment. The contents of N, P, K, Ca, Mg, S, Cu, Fe, Mn, B, and Zn were determined according to Gai et al. (2017). Nitrogen content was determined after sulfuric acid digestion by Kjeldahl's method. The P, K, Ca, Mg, S, Cu, Fe, Mn, and Zn contents were assessed by the nitric perchloric acid digestion method; P was dosed by the metavanadate colorimetric method; K was quantified by flame photometry (600 plus Fento, SP, Brazil); Ca and Mg were quantified by atomic absorption spectrophotometry (2380, Perkin-Elmer, Norwalk, CT USA); S was assessed by turbidimetry; Cu, Fe, Mn, and Zn were analyzed by atomic absorption spectrophotometry and B by colorimetry (Azomethine-H).

## Anatomical traits

Samples were collected from the middle region of the leaf blades of fully expanded leaves and roots 5 cm from the root apex were fixed in FAA (formalin, acetic acid, 70% alcohol) for 24 h. The material was isolated, dehydrated in an ethyl series, embedded in methacrylate (Historesin, Leica®) and sectioned in a rotary microtome (Leica RM 2245, Leica® Biosystems, Heidelberg Germany). The cross Sects. (5  $\mu\text{m}$  thick) were stained with toluidine blue, pH 4.0 (O'Brien et al. 1964). For stomatal characterization, the epidermal impression method was used according to Segatto et al. (2004). The samples were observed and photomicrographed under an optical microscope (DM750, Leica® Microsystems, Switzerland) coupled to a digital camera (Leica CH-9134, Leica® Microsystems, Switzerland). The images were analyzed with Image J software, previously calibrated. The root anatomical parameters evaluated were as follows: root periderm thickness (RPT), root cortex thickness (RCT), and root cortex diameter (RCD). In leaf blade, the epidermis thickness from the adaxial leaf side (ETAd), the epidermis thickness from the abaxial leaf side (ETAb), the palisade parenchyma thickness (PPT), and the spongy parenchyma thickness (SPT) were measured. The stomatal density (SD) of abaxial surface was calculated as the number of stomata per  $\text{mm}^2$  and the stomatal functionality.

## Protein isolation and proteome analysis

Leaves from three individual plants were sampled aleatory from each treatment and fast frozen in liquid nitrogen. Before protein isolation, samples were kept in deep freezer at -80 °C. Protein isolation, determination of protein concentrations, and further processing were performed according to Nascimento et al. (2018). The identification and quantification of proteins were performed in a nanoACQUITY UPLC® ultra-performance liquid chromatography (Milford, MA, USA), configured for fractionation in two dimensions as reported in Herrera et al. (2018). The chromatograph was coupled to a NanoLock ESI-Q-ToF SYNAPT G2-S (Waters) mass spectrometer. The acquisition ranged from 50 to 2000 Da, in MSE mode (data independent analysis) at a scan rate of 0.5 s and an interscan delay of 0.1 s.

The data were processed using the Progenesis QI software (Waters) for identification and quantification, using the Viridiplantae database from UniProt (UniProtKB/swiss-prot, uniprot.org). Protein identification was accepted if the probability of identifying peptides was greater than 90% and proteins with 95%. The significance levels of the differential abundances of proteins were determined by applying the ANOVA test ( $p < 0.05$ ). The functional annotation of proteins was performed using the OmicsBox v1.2.4 (bioBam) and Uniprot (UniProtKB/swiss-prot, uniprot.org).

Kyoto Encyclopedia of Gene and Genome (KEGG) pathway enrichment analysis for proteins was performed using the KOBAS software v3.0. (kobas.cbi.pku.edu.cn) with *Arabidopsis thaliana* as the background species for the analysis. The R package ggplot2 was used for the enriched KEGG pathways visualization. The PPI networks were predicted based on functional analysis using the software STRING (research tool for recovering genes/proteins in interaction) v11.0 (<http://string-db.org/>), using homologous proteins from the *Arabidopsis thaliana* as the background species.

## Data analysis

The soil type with different nutritional supplementation was considered a factor (independent variable), and the physiological, morphological, and biochemical changes were response variables (dependent variable). A completely randomized design was used, and all data were subjected to a one-way ANOVA ( $p < 0.05$ ) and compared by Tukey's test using R software (R Core Team 2018; <https://www.R-project.org>). The main correlations between assimilation and growth rates, as well as the interactions between macro- and micronutrients were demonstrated by the Debiased Sparse Partial Correlation (DSPC) algorithm analysis using the software MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca>). Proteomic data matrix was subjected to hierarchical clustering heatmap analysis and enrichment analysis using the Heatmapper online program (<http://heatmapper.ca/expression>).

## Results

### Nutrients and plant growth

Differential soil fertilization promoted changes in the initial growth of *S. engleri* (Fig. 1). The treatments of complete fertilization, half fertilization, and absence of K resulted in respective increases of 112%, 156%, and 158% in shoot length; 37%, 47%, and 33% in stem diameter; 442%, 379%, and 419% in shoot dry mass; and 229%, 293%, and 266% in root dry mass (Fig. 1A–D). While plants from the commercial substrate and mix of substrates treatments exhibited intermediate growth for most parameters, dystrophic soil (control treatment), absence of micronutrients, and absence of P treatments were the least promising for the *S. engleri* growth. Higher values of SLA were observed in plants grown in the absence of micronutrients (Fig. 1G).

### Gas exchanges

The treatments with the highest growth, namely complete fertilization, half fertilization, and absence of K, were

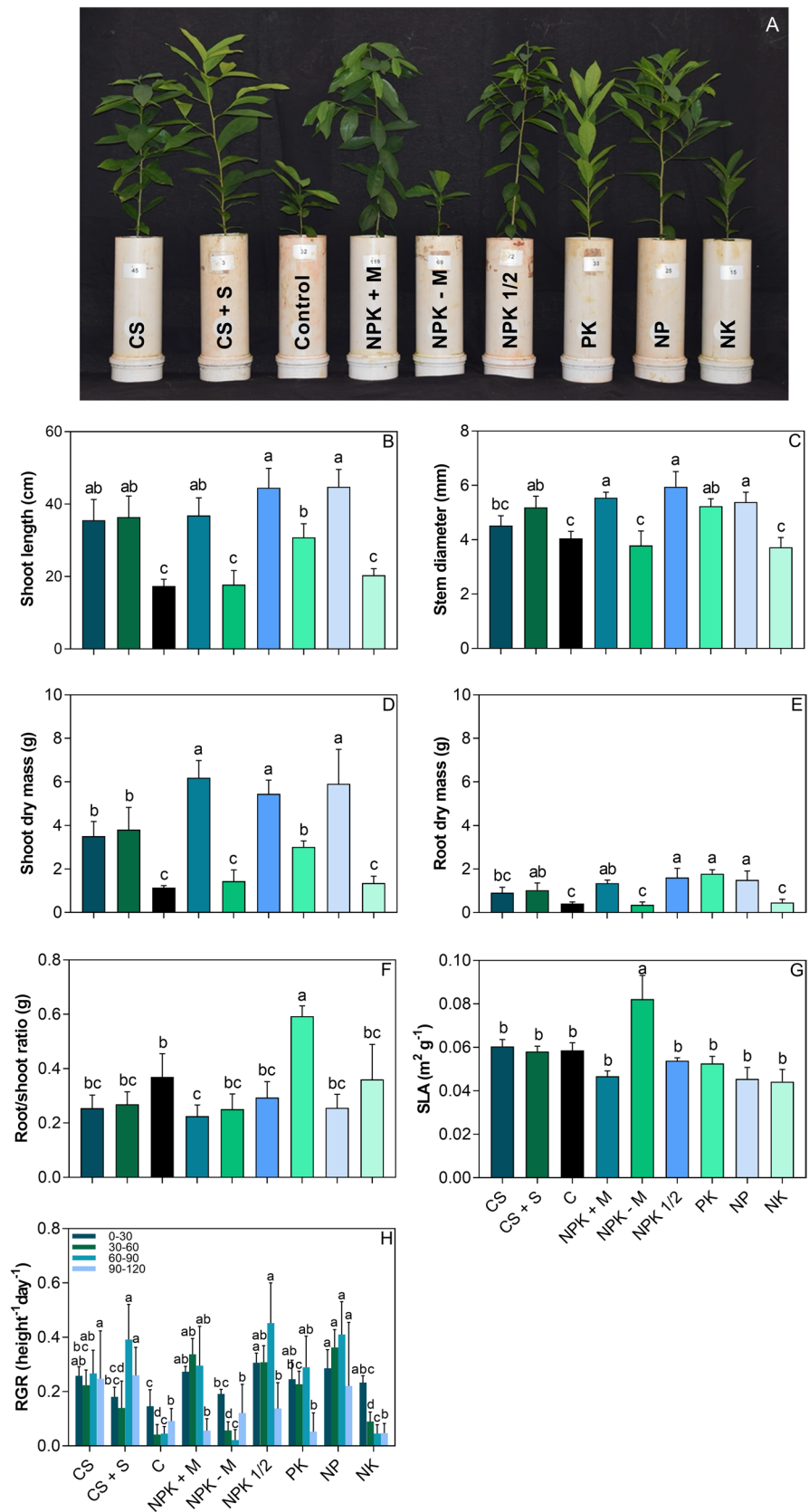
strongly associated with carbon assimilation. Notably, these treatments showed significant changes in several parameters, including a 28%, 59%, and 47% increment in photosynthetic rate ( $A$ ), a 152%, 87%, and 157% increase in the ratio of  $A/g_s$ , and a 73%, 86%, and 93% rise in  $A$  to intercellular  $CO_2$  concentration ( $A/C_i$ ), respectively. These treatments exhibited higher quantum yield of photosystem II (QY), with respective increases of 38%, 26%, and 19% (Fig. 2A, D–F). Conversely, treatments with lower growth rates showed reduced assimilation rates, notably the complete fertilization without micronutrients. Also, plants grown in absence of N, P, or K exhibited lower stomatal conductance ( $g_s$ ) and transpiration rate ( $E$ ) (Fig. 2B, C).

### Leaf nutrients content

Leaf N content did not change significantly between treatments, even with fertilization (Fig. 3A). While the use of CS contributed to high P contents and organic matter, plants grown on absence of micronutrients, absence of N and absence of K treatments showed similar contents of this nutrient in their leaves (Fig. 3B). For the K content, only the control treatment showed a significant difference with lower values (Fig. 3C). Higher Ca accumulation was detected in commercial substrate, absence of micronutrients and absence of P treatments, while the lowest contents were observed in treatments with higher RGR (Fig. 3D), with possible dilution effect due to greater accumulated biomass. Magnesium also accumulated more in the control treatment, followed by complete fertilization and commercial substrate (Fig. 3E), while S content did not change significantly between treatments (Fig. 3F).

Micronutrient contents exhibited distinct patterns across various fertilization treatments. Specially, B content significantly increased in treatments with complete fertilization but without N or P (Fig. 3G). Higher Cu accumulation was observed in the absence of P, showing significant differences from other treatments (Fig. 3H), while Fe values remained consistent across all fertilization treatments (Fig. 3I). Leaf Mn contents were the highest in commercial substrate and absence of N treatments, and the lowest in half fertilization and without N treatments (Fig. 3J). Plants subjected to the absence of P and the control treatments exhibited the highest Zn contents, while those in the complete fertilization treatment showed the lowest levels (Fig. 3K). Debiased Sparse Partial Correlation (DSPC) analysis revealed several notable correlations (Fig. 4), including positive associations between shoot dry mass and  $A$  ( $r=0.74$ ), shoot dry mass and  $A/g_s$  ( $r=0.74$ ), Ca and Mg ( $r=0.89$ ), Ca and Zn ( $r=0.73$ ), Zn and S ( $r=0.76$ ), Zn and Fe ( $r=0.74$ ), S and Fe ( $r=0.78$ ), and Fe and Mn ( $r=0.72$ ) (See Supplementary Material for further details).

**Fig. 1** Growth parameters of *Stephanopodium engleri* under different nutritional sources for 120 days. *RGR* relative growth rate, *SLA* specific leaf area (leaf area per unit dry weight), *CS* commercial substrate, *CS + S* commercial substrate + soil, *C* control, *NPK + M* NPK solution with micronutrients, *NPK - M* NPK solution without micronutrients, *NPK 1/2* NPK half-strength solution, *PK* nitrogen-free solution, *NP* potassium-free solution and *NK* phosphorus-free solution. Different letters indicate statistically significant differences between means by the Tukey's test ( $P < 0.05$ ). Bars show mean standard deviation (n 8 per treatment)



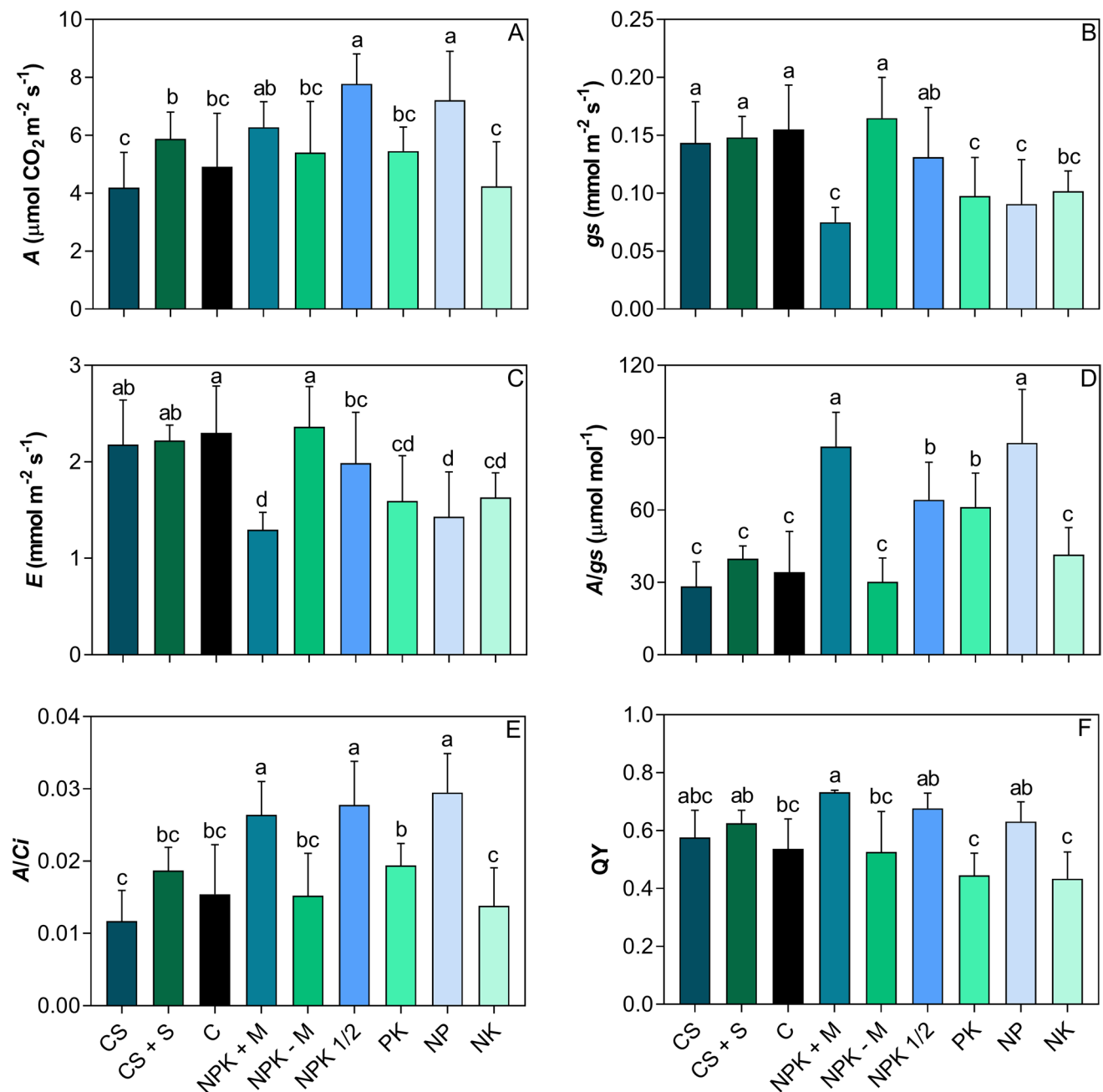


**Fig. 2** Gas exchange of *Stephanopodium engleri* under different nutritional sources for 120 days. **A** Liquid carbon assimilation ( $A$   $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ); **B** stomatal conductance ( $g_s$   $\text{mmol m}^{-2} \text{ s}^{-1}$ ); **C** transpiration rate ( $E$   $\text{mmol m}^{-2} \text{ s}^{-1}$ ); **D** intrinsic efficiency of water use ( $A/g_s$ ); **E** instantaneous carboxylation rate ( $A/C_i$ ); **F** ratio of intercellular to ambient  $\text{CO}_2$  concentration ( $C_i/C_a$ ); **G** quantum yield of photosystem II (QY); and **H** leaf temperature ( $^{\circ}\text{C}$ ). CS commercial substrate,

CS+S commercial substrate+soil, C control, NPK+M NPK solution with micronutrients, NPK-M NPK solution without micronutrients, NPK 1/2 NPK half-strength solution, PK nitrogen-free solution, NP potassium-free solution and NK phosphorus-free solution. Different letters indicate statistically significant differences between means by the Tukey's test ( $P < 0.05$ ). Bars show mean standard deviation ( $n$  8 per treatment)

### Anatomical changes in roots and leaves

Root periderm thickness (RPT) and root cortex thickness (RCT) were more developed in the absence of N and micronutrients treatments (Fig. 5A, B), while the

root cortex diameter (RCD) was also more expanded in the absence of N, commercial substrate, and P (Fig. 5C). While palisade parenchyma thickness (PPT) was higher in the control, the half fertilization, and the absence of P,

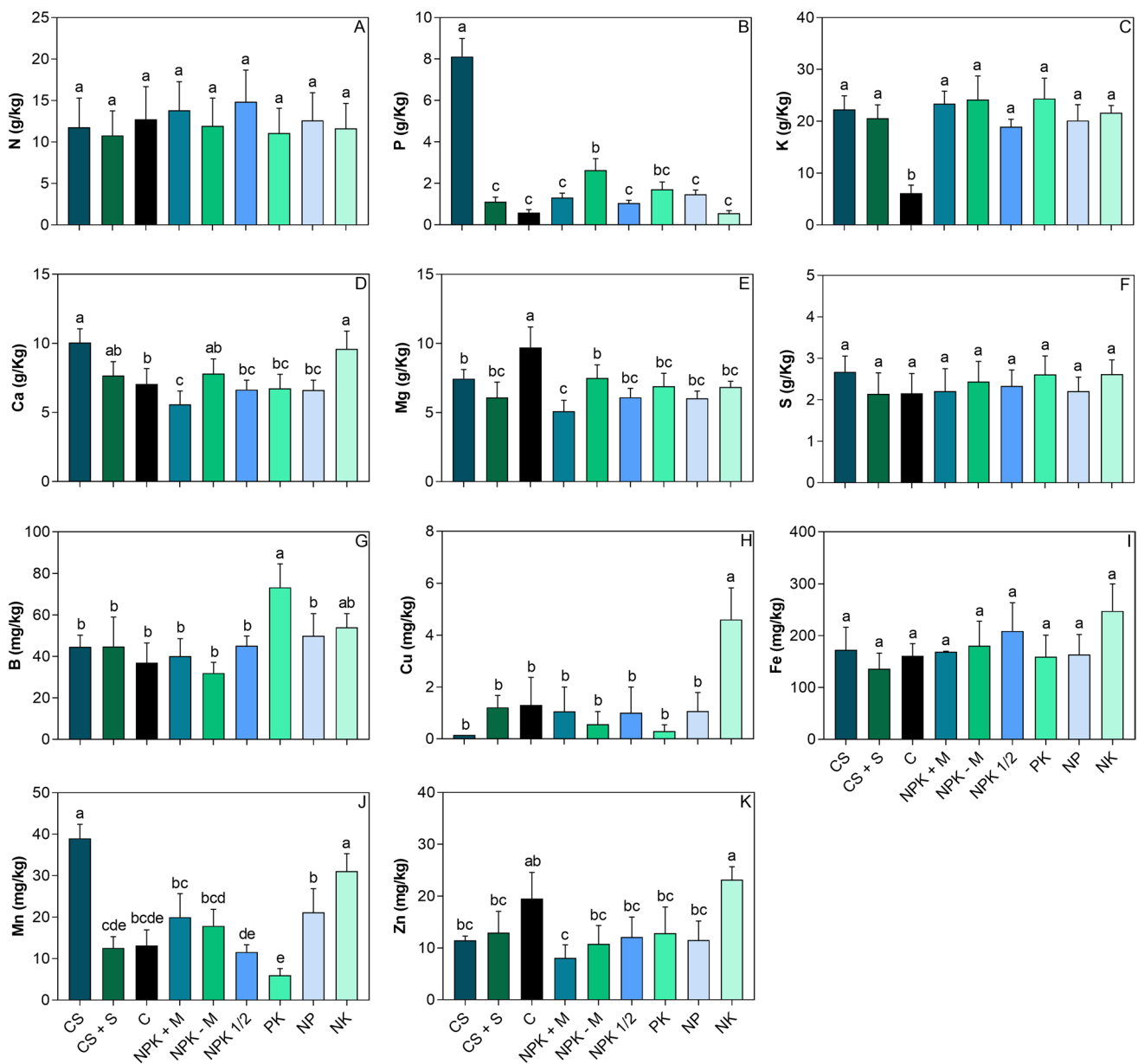


**Fig. 3** Macronutrients and micronutrients content in the dry mass of *Stephanopodium engleri* leaves under different nutritional sources for 120 days. CS commercial substrate, CS+S commercial substrate+soil, C control, NPK+M NPK solution with micronutrients, NPK-M NPK solution without micronutrients, NPK 1/2 NPK half-

strength solution, PK nitrogen-free solution, NP potassium-free solution and NK phosphorus-free solution. Different letters indicate statistically significant differences between means by the Tukey's test ( $P < 0.05$ ). Bars show mean standard deviation (n = 8 per treatment)

the treatments commercial substrate and the absence of N exhibited larger spongy parenchyma thickness (SPT) (Fig. 5D, E). Conversely, the control and absence of micronutrients treatments showed a negative effect for ETad, PPT, and SPT. Regarding the stomatal density (SD), higher values were observed in micronutrient absence, together with higher SLA and  $g_s$  and lower  $iWUE$  (Fig. 5F). See Fig. S1.

## Leaf proteome profiling

The proteomic analysis detected 3969 proteins in *S. engleri* leaves, and 1499 were initially quantified by the Progenesis QI software. From that, 52 proteins were present in all eight replicates of the nine treatments and quantified with at least 1 peptide (Fig. S2). Only these protein species were considered in this study, and identified proteins were analyzed by placing them in appropriate functional pathways. Based on

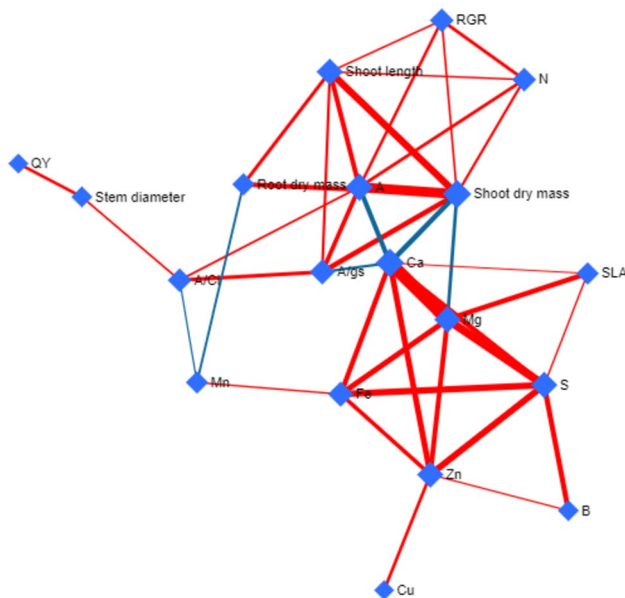


**Fig. 4** Network building between growth parameters, gas exchanges and nutritional composition based on Debiased Sparse Partial Correlation algorithm (DSPC) analysis of *Stephanopodium engleri* under different nutritional sources for 120 days. Nodes demonstrate reconstructing correlation-based networks, and the edge thickness is proportional to the strength of the shown correlation. Red lines indicate positive correlations, while blue lines show negative correlations

biological functions, the identified proteins were categorized into nine groups, with a predominant focus on biological and metabolic processes, particularly photosynthesis and energy pathways (Fig. 6A). Regarding molecular functions and localization of these proteins, the most prominent functions were DNA and nucleotide binding, with localization observed within the organelles, the cellular compartments, and the cell (Fig. 6B).

The partial least squares-discriminant analysis (PLS-DA) revealed that proteins with high variable importance in projection correlated with treatments fostering greater plant growth and the absence of P and micronutrients (Fig. S3). Treatments, such as complete fertilization, half fertilization, and absence of N and K, exhibited up-regulation of proteins associated with important nutrients like Ca and Zn and maintenance of Fe homeostasis (Q9SYP3, Q9ZQV9). These proteins also played roles in assimilatory metabolism and energy pathway (Q40073, P12860, O49213, Q67VU7, Q94B74), plant ROS signaling (Q9M9W9, Q9C5R8), nitrogen metabolism (Q6ZLB0), as well as in lipid transport (Q84M24) in a mixture of substrates (Fig. 6B). Conversely, treatments with lower assimilation and growth rates (control, absence of micronutrients, and absence of P) exhibited down-regulation of these proteins. Additionally, proteins linked to increased cellular expansion (Q7XR91), photosystem I activity and Mg transport (Q1ACL4), carbon uptake

(A2T345), Zn and Cu binding, methionine metabolism (Q9SRV5), and triterpenoid biosynthetic process (Q2XPU6) were up-regulated in these treatments. Notably, the absence of micronutrients treatment demonstrated an increase in the catabolism of ubiquitin-dependent proteins (Q9XIE8, Fig. 6B).

## Discussion

### Plants of *S. engleri* show efficient growth with less nutrients input

Our study demonstrates that *S. engleri* exhibits varying growth responses according to fertilization levels and substrates, even in dystrophic soil with low nutrient adsorption capacity. Complete and half of fertilization improved nutritional status, biomass production, and growth, with half fertilization being sufficient for maximum growth, comparable to complete fertilization. This reduced nutrient requirement of *S. engleri* aligns with findings in native species, which show biomass gains under half fertilization (Holdo 2013; Ramos et al. 2020; Caldeira et al. 2021). Using fewer nutrients, it is possible to enhance management efficiency, reduce costs, and minimize environmental impacts such as nutrient leaching and decrease the risk of invasive species proliferation (Booth et al. 2004), minimizing disturbances to native plant communities, supporting ecosystem stability while ensuring sustainable seedling production.

The addition of commercial substrate (CS) enriched the soil with nutrients and organic matter, promoting greater growth and biomass gain in *S. engleri*. Organic matter improves soil structure, water retention, and cation exchange capacity, reducing nutrient leaching (Nagase and Dunnett 2011). However, the absence of nitrogen (N) supplementation in the dystrophic soil reduced growth and shoot biomass allocation, likely due to decreased CO<sub>2</sub> fixation and carbon redistribution to *S. engleri* roots. Similarly, phosphorus (P) deficiency impaired growth as P is essential for photosynthesis, energy transfer, and carbohydrate metabolism (Srivastava 2012; Moura and Vieira 2020). Potassium (K) deficiency did not significantly affect growth in our study, possibly due to the short exposure time. However, K is known to influence photosynthetic metabolism and growth (Pettigrew 2008; Lu et al. 2016; Hu et al. 2023). The reduced growth of *S. engleri* in the absence of micronutrients confirms their importance for various metabolic functions. Micronutrient deficiencies also are critical for enzymatic activities and metabolic processes like photosynthesis and respiration (Magalhães et al. 2023). Also, Hantzis et al. (2018) demonstrated that Arabidopsis plants exposed to Fe deficiency for seven days exhibit unregulated enzyme activity in the S assimilation

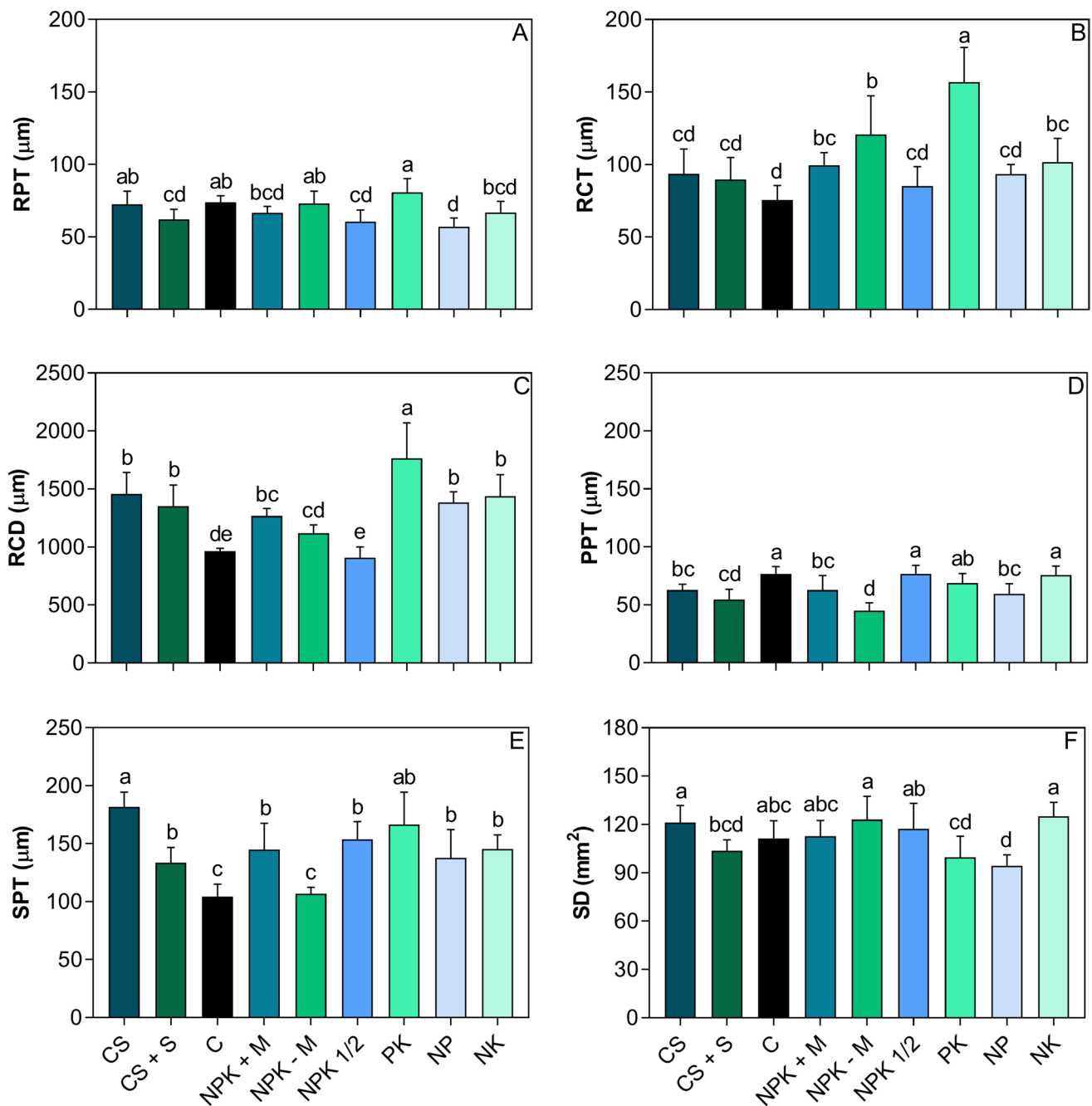


**Fig. 5** Anatomical traits on roots and leaves of *Stephanopodium engleri* under different nutritional sources for 120 days. **A** Root periderm thickness (RPT); **B** root cortex thickness (RCT); **C** root cortex diameter (RCD); **D** palisade parenchyma thickness (PPT); **E** spongy parenchyma thickness (SPT); and **F** stomatal density (SD). CS commercial substrate, CS+S commercial substrate+soil, C control, NPK+M NPK solution with micronutrients, NPK-M NPK solution without micronutrients, NPK 1/2 NPK half-strength solution, PK nitrogen-free solution, NP potassium-free solution and NK phosphorus-free solution. Different letters indicate statistically significant differences between means by the Tukey's test ( $P < 0.05$ ). Bars show mean standard deviation ( $n = 8$  per treatment)

pathway, while proteins involved in N assimilation are also affected. Thus, our findings with *S. engleri* confirm that even in low quantities, if unavailable, micronutrients can reduce energy metabolism and render growth unfeasible.

Regarding growth and nutritional content in leaves, the omission of N and K did not significantly alter the levels of these nutrients in the leaves of *S. engleri*. This suggests a dilution effect, whereby plants with greater biomass

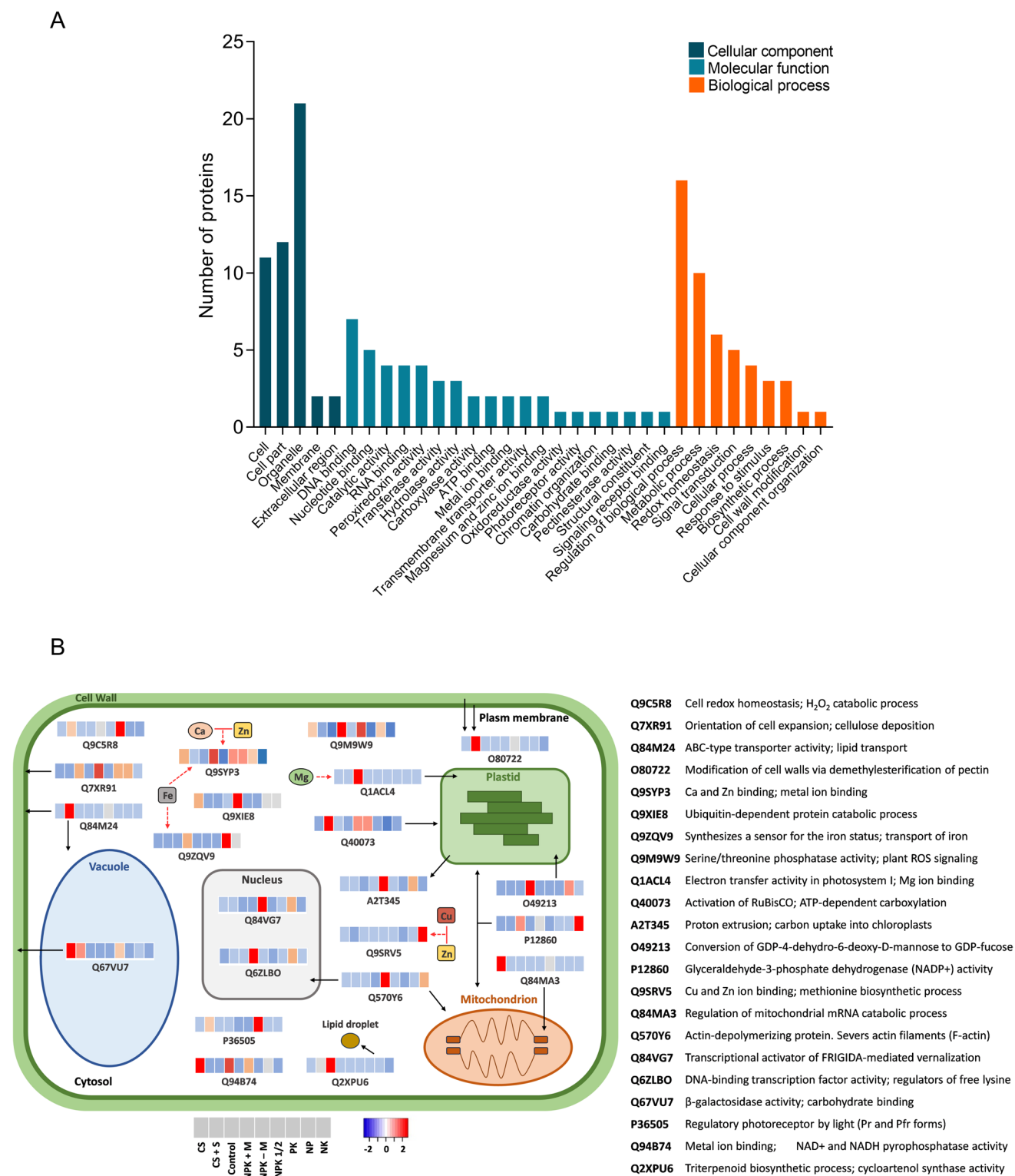


**Fig. 6** **A** Functional category distribution and protein subcellular locations of all identified proteins and **B** schematic diagram of differentially regulated proteins in leaves of *Stephanopodium engleri* under different nutritional sources for 120 days. The intensity of each protein peak for all samples represents the mean of each treatment. Colors represent abundance (red, significantly higher than control; blue, significantly lower than control; lighter, no significant changes)

by Tukey's test ( $p < 0.05$ ). Red dotted arrows indicate nutrient binding proteins. Undetected proteins are indicated in gray (See Fig. S1). CS commercial substrate, CS+S commercial substrate + soil, C control, NPK+M NPK solution with micronutrients, NPK-M NPK solution without micronutrients, NPK 1/2 NPK half-strength solution, PK nitrogen-free solution, NP potassium-free solution and NK phosphorus-free solution

accumulation also absorbed more nutrients. This indicates that nutrient content remained stable due to proportional uptake in relation to growth. However, a decline in nutritional quality is associated with the plant's inability to absorb nutrients at a rate that matches dry matter accumulation. As previously reported, there is a direct relationship between  $\text{CO}_2$  assimilation rates, stomatal conductance, and the dilution effect on nutrient content in tissues (Högy and Fangmeier 2009; Loladze 2014; Myers et al. 2014; Dong et al. 2018). Therefore, it is possible to consider that the content of certain nutrients in *S. engleri* plants was influenced by this effect.

### Assimilatory metabolism depends on nutrient supply

Proper nutritional supplementation works on the development of root structure and increases the flow rate of root xylem because of enhanced water absorption and the provision of essential nutrient elements. This leads to differences in assimilatory metabolism and growth rates, compared to plants not treated with fertilizers or lacking certain elements (Almadiy et al. 2023). The highest values of carbon assimilation,  $A/gs$ ,  $A/Ci$ , and QY on plants receiving all nutrients indicate that adequate supplementation improves the development of the species, while the absence of some nutrients causes the opposite effect. As observed here, N fertilization consistently enhances carbon assimilation in plants, likely attributable to heightened photosynthetic pigment contents (Martin et al. 2010; Wang et al. 2018; Kubar et al. 2022), thus emphasizing the pivotal role of N in bolstering photosynthetic capacity. Otherwise, the decrease in growth in the absence of P was certainly associated with the lower  $A$  rate,  $gs$ ,  $A/gs$ ,  $A/Ci$ , and QY. Phosphorus is another key element for growth as it forms part of nucleic acids and phospholipids of cell membranes. It also plays a central role in essential metabolism processes, including carbon assimilation, sugar transformation, and energy transfer (Valle-Romero et al. 2023).

Potassium fertilization is recognized to significantly enhance gross primary production (GPP) in plants by reducing stomatal and mesophyll resistance to carbon dioxide ( $\text{CO}_2$ ) diffusion and boosting the photosynthetic capacity within the leaves (Christina et al. 2015; Battie-Laclau et al. 2016). In this sense, the greater growth registered in *S. engleri* fertilized with complete fertilization, half fertilization, and absence of K would also have been directly related to an improvement in key steps of the plant carbon assimilation and energy transfer efficiency. Furthermore, the fact that the absence of K did not reduce gas exchange and QY and its content in the leaves in our study, it could be a result of their higher concentration in the soil. The decrease in the values of  $A$  rate,  $A/gs$ , and  $A/Ci$  in the absence of

micronutrients treatment may be related to the low levels of Zn and Mn. The important role of Fe, Zn, and Mn in maintaining the efficiency of electron transport between PSI and PSII has already been reported. This efficiency reduces as a function of the availability of these microelements (Roosta et al. 2018).

### Accumulation of Ca and Mg enhances responses to nutritional changes

The foliar Ca accumulation in absence of micronutrients detected in this study suggests its role in signaling for micronutrient deficiency. Calcium is well known for its function in sensing and responding to environmental fluctuations. According to Bang et al. (2021), variations in nutrient availability and transport are mediated by the  $\text{Ca}^{2+}$ -dependent CBL–CIPK pathway. For the absence of P, increased Ca levels may be associated to changes in membrane properties. High levels of  $\text{Ca}^{2+}$  promote the signaling and stabilization of cell walls, as well as stabilize cell membranes via the formation of bridges with phosphate and carboxylate groups in lipids and proteins (Kölling et al. 2019; De Bang et al. 2021). In the control treatment, the higher content of foliar Mg may be related to attenuating the  $\text{CO}_2$  assimilation deficit and intensified catalytic activity to obtain energy. When RuBisCo binds to  $\text{Mg}^{2+}$ , carboxylation is favored many times faster than oxygenation. The ability of  $\text{Mg}^{2+}$  to undergo ligand exchange is essential for ATP coupling with substrates and enzymes, facilitating phosphorylation, energy-demanding transport and ATP synthesis (Tränkner et al. 2018; De Bang et al. 2021). Thus, in nutrient-poor soil, the accumulation of Mg would improve assimilation rates and attenuate the energy deficit in *S. engleri*. Additionally, the increments of Fe, Zn, Cu, and Mn especially in the absence of P (NK) are related to the antagonism of these elements with P as already mentioned. Low P content causes Fe increment in the soil, which once increased, can induce Zn and Mn accumulation.

### Chelating proteins regulate the levels of Fe and Zn

The proteomic analysis in this study identified and quantified a large set of leaf proteins, with 90.3% showing significant changes across treatments. Most proteins were intracellular, reflecting adjustments in cytoplasm and organelles in response to varying nutrient sources. Notably, specific proteins were upregulated, indicating effective nutrient regulation in *S. engleri*. Putative F-box/Kelch-repeat protein (Q9SYP3) increased in five of nine treatments, while nicotianamine synthase 1 (Q9ZQV9) was upregulated in the absence of micronutrients and K. Q9SYP3, part of an E3 ubiquitin ligase complex, prevents toxic Fe overload by regulating Fe homeostasis. According to Rodríguez-Celma

et al. (2016), Fe binding to the N-terminal hemerythrin motifs modulates E3 ubiquitin ligase activity. FBXL5, containing a hemerythrin motif and F-box domain, regulates Fe homeostasis by degrading IRP2 upon Fe binding. In *Medicago truncatula*, Fe deficiency in the presence of  $\text{CaCO}_3$  has a stronger impact in the protein metabolism, leading to a greater decrease in the number of photosynthesis-related proteins (Rodríguez-Celma et al. 2016). The upregulation of Q9SYP3 in commercial substrate, complete fertilization, and half fertilization treatments suggests Fe accumulation triggered this regulatory mechanism, likely due to the species' low nutritional requirements.

Nicotianamine (NA), a major Fe chelator in phloem, facilitates Fe transport, and its deficiency disrupts Fe homeostasis in sink organs (Chao and Chao 2022). NA also chelates  $\text{Zn}^{2+}$  and other divalent cations. The upregulation of these proteins indicates efficient Fe and Zn regulation in *S. engleri* leaves, supported by nutrient concentrations in complete fertilization and absence of K treatment. The downregulation of 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase 2 (Q9SRV5), which catalyzes methionine synthesis from homocysteine, suggests its role in nicotianamine formation. Homocysteine is crucial for de novo methionine synthesis and S-adenosylmethionine recycling, serving as a precursor for nicotianamine, polyamines, and ethylene (Sobieszczyk-Nowicka et al. 2022). The increased abundance of Q9SRV5 in the absence of P treatment, alongside elevated Fe, Zn, Cu, and Mn levels, indicates enhanced transferase activity for nicotianamine production. This process likely maintained cation homeostasis in *S. engleri*, supporting growth and preventing oxidative damage under varying nutrient conditions.

### Increase of chlorophyll–protein complexes maintains photosynthesis and energy regulation

Regarding of assimilatory metabolism, the upregulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activase A (RCA, Q40073) in *S. engleri* grown in mixed substrates and half fertilization treatments highlights its role in enhancing  $\text{CO}_2$  assimilation and biomass allocation. RCA activates RuBisCO through ATP-dependent carboxylation, which is significantly enhanced by  $\text{Mg}^{2+}$  binding. According to Hazra et al. (2015),  $\text{Mg}^{2+}$  concentrations in the stroma vary with proton pumping across thylakoid membranes. As stromal pH rises,  $\text{Mg}^{2+}$  increases, providing electrostatic compensation and upregulating RCA activity to reactivate RuBisCO for  $\text{CO}_2$  fixation. The increase in RCA under absence of micronutrient suggests an adaptive response of *S. engleri* to maintain  $\text{CO}_2$  fixation and mitigate photoinhibition. Carbon assimilation homeostasis is supported by the electron transport chain, which relies on Fe-containing metalloproteins (Sebastian and Prasad 2015). In absence of

micronutrient conditions, cells reallocate resources, leading to the upregulation or degradation of specific polypeptides to adapt to unfavorable conditions (Kolesinski et al. 2017). The increased catalytic activity of GDP-L-fucose synthase 1 (O49213) in complete fertilization treatments indicates active Golgi-localized nucleotide sugar transport. GDP-Fuc transport is essential for xyloglucan fucosylation, a process critical for plant growth and development. Plants with reduced GFT1 expression exhibit severe growth impairments (Rautengarten et al. 2016). The regulation of these proteins ensures efficient sugar import for cell wall structuring, supporting growth in *S. engleri*.

Our findings showed the enhanced activity of nudix hydrolase 2 (NUDT2, Q94B74) in commercial substrate, complete fertilization, and half fertilization treatments suggests its role in mitigating oxidative stress caused by nutrient excess. NUDT2 asymmetrically hydrolyzes di-adenosine tetraphosphate (Ap4A) into ATP and AMP, a process critical for managing oxidative stress, apoptosis, DNA repair, transcription, and immune responses in chloroplasts (Ogawa and Yoshimura 2019). Phosphoprotein phosphatases, such as probable protein phosphatase 2C 34 (PP2C type, Q9M9W9), also play a key role in oxidative stress signaling (Neill 2002). PP2C was significantly upregulated in commercial substrate, complete fertilization, half fertilization, and absence of K treatments, indicating its involvement in maintaining redox homeostasis of *S. engleri*. According to Máthé et al. (2019), PP2C scavenges reactive oxygen species (ROS) generated by plasma membrane-associated NADPH oxidases (RBOHs) or systemic oxidative stress, which can lead to excess  $^1\text{O}_2$ ,  $\text{O}_2^{\bullet-}$ , and  $\text{H}_2\text{O}_2$  in chloroplasts. Thus, the higher rates of  $\text{CO}_2$  assimilation and growth observed in *S. engleri* in these treatments were partially sustained by effective ROS control mechanisms.

Interestingly, most of the upregulated proteins discussed earlier showed decreased abundance in treatments with reduced growth, such as control, absence of micronutrients, and absence of P. This reinforces the importance of nutrient availability for *S. engleri* development. Under unfavorable nutritional conditions, the abundance of COBRA-like protein 7 (COBL 7, Q7XR91), a growth-related protein involved in cellulose biosynthesis and cell expansion (Sato et al. 2010; Liu et al. 2013; Sangi et al. 2021), increased, particularly in control, absence of N and absence of micronutrients treatments. The reduced growth reported here is possibly a result of the lower QY and A rates observed in control and absence of micronutrients, which is corroborated by the up-regulation of photosystem I P700 chlorophyll apoprotein A1 (PSAA, Q1ACL4) and glyceraldehyde-3-phosphate dehydrogenase B (G3PB, P12860). According to Zhang et al. (2020), repression of PSAA and photosystem II Qb protein D1 impairs photosynthetic rates. Wang et al. (2016) reported that in Arabidopsis plants, the downregulation of

the PSA2 protein affected chloroplast structure and development in Zn-deficient leaves. Therefore, the upregulation of PSAA and G3PB in these treatments likely reflects an adaptive mechanism to enhance electron transfer activity in photosystem I of *S. engleri*, maintaining minimal CO<sub>2</sub> assimilation rates.

The increased activity of cycloartenol synthase (CAS1, Q2XPU6) observed in the control plants of our study indicates the increased biosynthesis of cycloartenol, a sterol that maintains the stability of chlorophyll–protein complexes. As reported by Babiychuk et al. (2008), thylakoids biogenesis depends on the extensive exchange of lipid precursors between the chloroplast and the endoplasmic reticulum (RE) by establishing contact sites between the outer plastid envelope and the sterol-rich ER membranes. Unbalanced or depletion of sterol composition compromises ER–plastid physical contacts, altering the number of chloroplasts and consequently photosynthesis. The protein adjustment mechanisms of the photosynthetic apparatus appear to be essential for the minimal maintenance of its functionality, thus allowing *S. engleri* plants to remain energetically active under unfavorable nutritional conditions.

Overall, this study provides an integrated approach to understanding the nutritional management of *S. engleri*, shedding light on the mechanisms it employs to cope with nutrient availability. As a threatened and range-restricted species with limited research available, our findings offer crucial insights for its conservation. Notably, the species' low nutrient requirements enable the production of healthy seedlings without physiological impairment, yielding both environmental and economic benefits. Additionally, these findings can guide field practices, optimizing planting strategies in its natural habitat and supporting ex situ conservation efforts to mitigate its risk of extinction.

## Conclusions

Although different nutritional sources caused changes in gas exchanges and growth rates, *S. engleri* plants grew substantially in complete fertilization, half fertilization, and absence of K, and exhibited acclimation to nutritional deficit. The absence of some macronutrients and micronutrients limits the development of biometric variables and the production of dry mass, where P, Ca, Mg, and micronutrients were the most limiting. Most of the common proteins increasing in abundance may have an intracellular origin and thus reflect cytoplasmic and organelle adjustments. These proteins have a diverse array of metabolic functions, especially related to Fe and Zn homeostasis, participation in photosynthesis and energy pathway, ROS signaling and preservation of functional structures. Our findings indicate that *S. engleri* plants maintain a tight adjustment of the photosynthetic apparatus

functionality to cope with nutrients shortages. Half of the fertilization is enough for the species to reach maximum growth. The applications of Ca, Mg, and micronutrients are essential for the development of *S. engleri*. Our findings are crucial to support field planting as adequate fertilization is essential to produce high-quality seedlings, thereby facilitating plans to reintroduce the species into their native environment. As half fertilization does not impair *S. engleri* growth, it may offer significant environmental and economic benefits. Furthermore, ex situ conservation strategies, such as captive breeding for reintroduction and population augmentation, are considered useful tools to help recover endangered species.

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**Author contributions** All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Evandro Alves Vieira, Luiz Palhares Neto, Joana Patrícia Pantoja Serrão Filgueira, Marina Ludmila Conon Salles, and Cecilio Frois Caldeira. The first draft of the manuscript was written by Evandro Alves Vieira and Cecilio Frois Caldeira, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data availability** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Conflict of interest** The authors declare that they have no competing interests.

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

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