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ORIGINAL ARTICLE

Influence of nitrogen starvation on physiology and biochemistry of red and green *Beta vulgaris* leaves: insights into salt stress

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Abstract

Main conclusion **Nitrogen starvation stimulates betacyanin accumulation and shared stress responses in *Beta vulgaris*, despite nitrogen costs, whereas salt stress reduces pigments and antioxidants, indicating stress-specific, genotype-dependent regulation and unresolved nitrogen sourcing.**

Abstract Betacyanins, unlike nitrogen-free anthocyanins, are betalamic immonium derivatives containing core nitrogen and are red-violet pigments found only in plants of the Caryophyllales order, possibly evolving to replace anthocyanins in this order. Their biosynthesis is influenced by endogenous and environmental factors like drought, salinity, and nutrient deficiency, yet their regulation under combined stresses remains poorly understood. This study investigated the role of betacyanins in *Beta vulgaris* (green- and red-leafed genotypes) under nitrogen starvation (NSt), salinity stress (SS), and their combination. Plants were assessed for biometric traits, gas exchange, photosynthetic pigments, betacyanin content, oxidative stress markers, antioxidant metabolites, and ion accumulation. NSt promoted betacyanin accumulation in green plants but not in red ones, highlighting a genotype-dependent response. In contrast, salinity reduced pigment levels and impaired photosynthetic traits in both genotypes. The combined stress resulted in intermediate responses, suggesting an interaction between nitrogen availability and salinity effects. Overall, our findings indicate that NSt and SS differentially regulate betacyanin accumulation, with responses strongly influenced by genotype, and point to a complex relationship between nitrogen metabolism and pigment biosynthesis under abiotic stress conditions.

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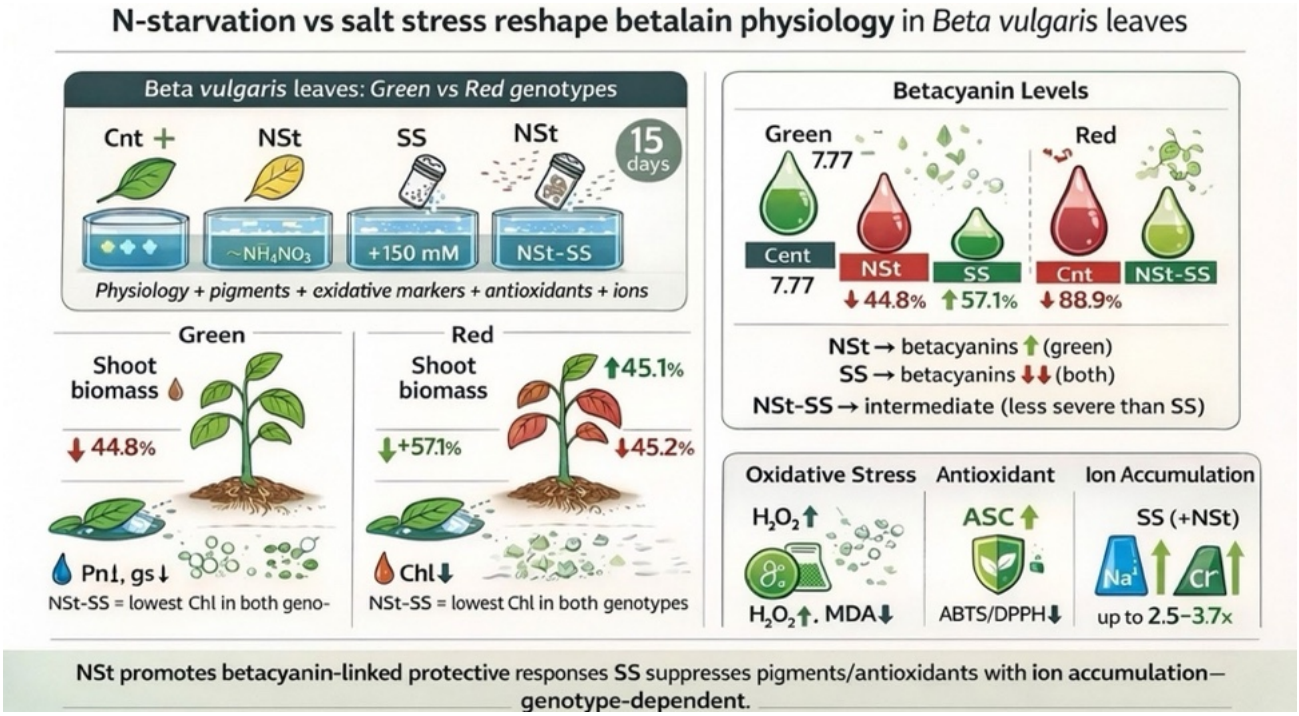
Graphical abstract



Keywords Antioxidant responses · Ascorbate · Betacyanins · Beetroot · Glutathione · Oxidative stress · Red and green genotypes

Abbreviations

ABTS	3-Ethylbenzothiazoline-6-sulfonic acid
ASC	Ascorbate
CYP76AD	Cytochrome p450 76AD
DHA	Dehydroascorbate
DODA	3,4-Dihydroxyphenylalanine 4,5-dioxygenase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
g_s	Stomatal conductance
GSH	Reduced glutathione
GSSG	Oxidised glutathione
MDA	Malondialdehyde
NSt	N starvation
P_n	Net photosynthetic rate
SS	Salt stress

Introduction

In the intricate tapestry of plant physiology and biochemistry, betacyanins emerge as pivotal metabolites with multifaceted roles, particularly in the face of environmental stresses (Nakashima et al. 2011; Jain et al. 2015; Jain and Gould 2015; Li et al. 2019, 2020; Davies et al. 2022). These water-soluble pigments, derived from tyrosine and containing two nitrogen (N) atoms in their structure, are unique to plants in the order of Caryophyllales (Gandía-Herrero and García-Carmona 2013; Timoneda et al. 2019). Notably, research indicates that no plant species produces both anthocyanins and betacyanins simultaneously, underscoring their mutual exclusivity (Gandía-Herrero and García-Carmona 2013; Timoneda et al. 2019). This exclusivity suggests that betacyanins may substitute for anthocyanins, but do not act in conjunction with them. Furthermore, betacyanins may offer the unique advantages to enhance plant fitness in specific environmental conditions, such as enhanced photoprotection and antioxidant capacity in high-light, drought, or saline habitats. In addition, as nitrogen-containing pigments derived from tyrosine, they may differ from anthocyanins in their metabolic cost and potentially contribute to nitrogen economy within the plant (Sakuta 2014; Jain and Gould 2015).

Among the adverse environmental conditions, soil nutrient availability poses a significant challenge to the synthesis of secondary metabolites in plants. Given the structural characteristics of betacyanins, various authors have attempted to understand the influence of N availability in betacyanin biosynthesis in beetroots and other Chenopodiaceae species (Salahas et al. 2011; Bascuñán-Godoy et al. 2018a, b; Sitompul and Zulfati 2019). Analysis of three genotypes of *Chenopodium quinoa*, distinguished by varying the N use

efficiency, revealed that the most sensitive genotype to N fluctuation, exhibited an increase in betacyanin content when cultivated at low N concentrations (Bascuñán-Godoy et al. 2018b).

However, this genotype also showed a decrease in the net photosynthetic rate (P_n) and other photosynthetic parameters, together with an increase in leaf NH_4^+ content. This increase was likely due to the photorespiration process, which releases a significant amount of NH_4^+ as an alternative electron sink under stress conditions and limited N sources (Bascuñán-Godoy et al. 2018a). Similarly, an increase in betacyanin content was observed in the leaves of *Beta vulgaris* cv. Ayumi 04 when cultivated with low N administration (Sitompul and Zulfati 2019). An increase in betacyanin content was noted in *B. vulgaris* ssp. *vulgaris* plants, with a 262% increase in leaves and a 225% increase in roots, when cultivated in hydroponics under nitrogen-starved conditions (Salahas et al. 2011). Moreover, a severe restriction in the rates of net CO_2 assimilation, stomatal conductance, and chlorophyll concentrations due to N starvation was observed, indicating that primary metabolism was severely limited by low N availability (Salahas et al. 2011).

Although the function of betacyanin under different abiotic stresses, such as N starvation (NSt), remains poorly explored, their role under salt stress (SS) has been deeper investigated. There are several findings regarding the relationship between SS exposure and betacyanin accumulation, as well as between betacyanin accumulation and the capability of a plant to tolerate other abiotic stressors (Jain et al. 2015; Vasconcellos Vilas Boas et al. 2016; Sarker and Oba 2018; Causin et al. 2020; Zhou et al. 2021). To our knowledge, all studies published to date have investigated betacyanin responses to individual stress factors, such as nitrogen deficiency or salinity, separately. No previous study has evaluated the effects of NSt in combination with another abiotic stress, nor examined how these responses may differ between genotypes with contrasting basal betacyanin accumulation. The recent observations suggest that these pigments may function to increase water use efficiency under drought or salinity, or to prevent oxidative damage when plant tissues are subjected to osmotic imbalance (Zhou et al. 2021; Cai et al. 2025). The high antioxidant capacity of betacyanins has been extensively documented, as reviewed by Bastos and Schliemann (2021). An increasing body of evidence suggests that this functional property is actively exploited by leaf cells to mitigate oxidative stress through the scavenging of reactive oxygen species (ROS). However, contrasting responses have also been observed, with marked reductions in betacyanin content under stress in species such as *Chenopodium quinoa* and *Amaranthus tricolor* (Sarker and Oba 2018; Causin et al. 2020), highlighting the complexity and species-specific nature of their regulation. Moreover, a key unresolved issue concerns the apparent paradox that betacyanins, being nitrogen-containing molecules, can accumulate under nitrogen-limiting conditions. To date, the relationship between nitrogen availability, internal nitrogen allocation, and stress-induced pigment biosynthesis has not been clearly elucidated, particularly in a genotype-dependent context. Consequently, many questions remain open regarding the integration of nitrogen metabolism and the physiological role of betacyanins in response to abiotic stress. The combined assessment of NSt and salinity in conjunction with genotype-specific differences in basal betacyanin content represents a novel framework that has not been previously explored in literature. For this reason, this study aimed to delve deeper into understanding the molecular responses activated in red-leafed and green-leafed *B. vulgaris* plants capable of accumulating betacyanins under conditions of SS and NSt and the combination of stress conditions (NSt-SS).

This study tests the hypothesis that nitrogen starvation and salt stress differentially regulate betacyanin accumulation and antioxidant responses in *Beta vulgaris* in a genotype-dependent manner, and that, despite the nitrogen requirement of betacyanin molecules, nitrogen starvation may paradoxically promote their biosynthesis through internal nitrogen remobilization mechanisms.

Material and methods

Plant material and experimental design

Seedlings of *Beta vulgaris* L. cv. Bull's blood (with red leaves; referred to as red) and *B. vulgaris* L. cv. Robuschka (with green leaves, referred to as green) were cultivated in a *floating system* in 50-L tanks for two weeks after being transplanted outdoor on October 3rd, 2022 at the Department of Agriculture, Food and Environment, University of Pisa (43,704,672°N, 10,427,292°E). Six tanks were utilized for each beetroot cultivar under investigation, with each tank containing 16 seedlings spaced 4 cm apart in the row and 11 cm between the rows. Each tank was aerated for 30 min every 6 h. The growing conditions were: 18.2 °C daily mean temperature, 80.5% humidity and 118.0 W m^{-2} daily mean solar light radiation. The nutrient solution for plants grown under nitrogen control condition (Cnt) is composed of: 0.78 mM NO_3^-, 1.0 mM PO_4^{3-}, 5 mM K^+, 8 mM Ca^{2+}, 1.0 mM Mg^{2+}, 4.1 mM SO_4^{2}, 18.9 mM Cl^-, 20.0 µM Fe^{2+}, 30.0 µM BO_3^-,1.0 µM Cu^{2+}, 11.5 µM Zn^{2+}, 10.0 µM Mn^{2+}, 1.0 µM Mo^{3+}, 10 mM NH_4NO_3; EC and pH were 3.02 dS m^{-1} and 5.8, respectively. Plants subjected to NSt were grown using the same nutrient solution but in the absence of 10 mM NH_4NO_3.

Starting from the transplanting, one tank for each N treatment (Cnt and NSt) was gradually enriched with 150 mM NaCl to induce SS. The use of 150 mM NaCl in this study

falls within the range commonly applied in experimental studies on *B. vulgaris* and related species to induce measurable physiological responses within the experimental timeframe considered (Yolcu et al. 2021b). The experimental design is represented in Fig. 1.

Physiological measurements and sampling were carried out after 15 days of treatment. The chosen sampling time reflects a condition in which plants exhibit well-defined phenotypic and physiological adjustments, although avoiding severe damage or the activation of cell death-related pathways. Leaves were sampled, immediately frozen in liquid nitrogen, and stored at − 80 °C until biochemical analyses were conducted. In addition, another number of leaves was sampled, dried at 105 °C in a ventilated oven (Universal Oven UN30; Memmert GmbH, Schwabach, Germany) and stored at room temperature until the determination of N, sodium (Na) and chloride (Cl) elements.

Biomass and biometric measurements

For measuring the fresh biomass, the aerial part (leaves and shoots) and roots were separated and weighed individually. Their biomass was then recorded. For dry weight (DW) determinations, both aerial parts and roots were dried at 105 °C until a constant weight was achieved. Leaf thickness was measured using a high precision digital thickness gauge, although leaf area index (LAI) and root length measurements were performed using the ImageJ software (version 1.52t).

Gas exchange

Leaf gas exchanges were measured using a portable infrared gas analyser LI-6800 System (Li-Cor, Lincoln, NE, USA). Gas exchange measurements were conducted on fully expanded leaves from 11:00 a.m. to 1:00 p.m. at ambient light intensity of 1500 µmol m^{-2} s^{-1}. Inside the leaf chamber, the CO_2 concentration was set to 400 µmol^{-1} through a CO_2 mixer, and the flow rate was 500 µmol s^{-1}. Once the steady state was reached, P_n, stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were recorded.

Chlorophyll (Chl) and carotenoid (Car) content determination

Chl (*a* and *b*) and Car content in the leaves of each beetroot cultivar and each treatment was determined spectrophotometrically as described by Porra et al. (1989). Briefly, 0.1 g of leaf material was added to 4 mL acetone 80% (v/v) and subjected to shaking until complete discoloration occurred. The absorption of the obtained solution was measured at 663 nm, 648 nm, and 470 nm using a spectrophotometer (Ultrospec 2100 Pro; Biochrom, Cambridge, UK). Chl and Car content of the plant extracts was calculated by the following equations:

$$Chla = 12.25 \times Abs_{663} - 2.55 \times Abs_{648} \left(\mu g\, mL^{-1}\right),$$

$$Chlb = 20.31 \times Abs_{648} - 4.91 \times Abs_{663} \left(\mu g\, mL^{-1}\right),$$

$$Car = \left[\left(1000 \times Abs_{470} - 1.82 \times Chla\right) - \left(85.02 \times Chlb\right)\right] / 198 \left(\mu g\, mL^{-1}\right).$$

Chl and Car content was expressed as µg g^{-1} fresh weight (FW).

Betacyanin determination

For the betacyanin extraction, 100 mg of fresh shoot material was homogenized in a mortar with 1 mL 80% (v/v) aqueous methanolic solution. The mixture was centrifuged for 10 min at 20,000 g. The supernatant was collected and used as extract in the betacyanin content determination and antioxidant activity assays.

Betacyanin quantification was carried out using a Shimadzu Prominence LC-20A chromatograph (Shimadzu, Milan, Italy) consisting of two LC-20 AD XR pumps, SIL-10ADvp, a CTO-20 AC column furnace, and a DGU-20 A3 degasser coupled with SPD-M10Avp PDA detector. Results were acquired through the Shimadzu LC solution Ver. 3.7 (Shimadzu, Version 3.7) program. Analyses were performed using an Ascentis® Express C18 (15 cm × 4.6 mm, 2.7 µm) and a mobile phase was composed of water acidified with 0.1% of formic acid (A) and acetonitrile (B), with a flow rate of 1 mL min^{-1}. The elution gradient was carried out as follows: 0 min 3%B; 10 min 10%B; 11 min 100%B. The injection volume was 2 µL. The data were acquired with PDA in the range between 250 and 600 nm and the chromatograms were extracted at 538 nm. A calibration curve was constructed using betanin (red beetroot extract diluted with dextrin).

RNA extraction and gene expression analysis

Leaf samples of *B. vulgaris* were harvested, immediately frozen in liquid nitrogen, and stored at − 80 °C until analysis. The total RNA was extracted using the NucleoSpin® RNA Plant kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions. To remove residual genomic DNA contamination, RNA samples were treated with DNase I (Ambion). RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

First-strand cDNA synthesis was performed from 1 µg of total RNA using oligo(dT) primers and reverse transcriptase (Applied Biosystems, Waltham, MA, USA), following the manufacturer's recommendations. Quantitative real-time PCR (RT-qPCR) was carried out using SYBR® Green chemistry on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Gene-specific primers were designed using Primer-BLAST (NCBI) and are here indicated:

Gene	Accession number	Forward primer (5'→3')	Reverse primer (5'→3')
DODA	XM_010697494.4	CAAGTATCC AGCTCC TGGGG	TTTTTCGTC AGTTTCCGC CG
CYP76AD1	XM_010697501.4	GCCGTCCAT TTGCTTTCT CC	TAAAGCTGT TGCACCTTC GC
ACTIN	HQ656028.1	TTTCGGGCA TTATGG CATGT	GAAGCAAAG GTGTGACTC AGC

The expression levels of key genes involved in betacyanin biosynthesis, *DODA* (XM_010697494.4) and *CYP76AD1* (XM_010697501.4), were quantified. Relative transcript abundance was calculated using the $2^{-\Delta Ct}$ method, with *ACTIN* (HQ656028.1) used as the internal reference gene for normalization.

Oxidative stress markers

Hydrogen peroxide (H_2O_2) was measured according to Sabetta et al. (2019). Briefly, 300 mg of leaf materials were quickly ground in liquid nitrogen and homogenized in 40 mM Tris–HCl, pH 7.0, in the presence of 20 µM of 2′,7′-dichlorofluorescein diacetate. The samples were incubated for 1 h in the dark at room temperature under continuous shaking. The H_2O_2 levels of the extracts were measured with a spectrofluorometer (Jasco Global FP8200, Tokyo, Japan; excitation λ = 495 nm; emission λ = 530 nm) and normalized for g FW.

Lipid peroxidation level was assayed following the method described by Hodges et al. (1999) with minor modifications. In brief, 200 mg of leaf material were homogenized with 4 mL of 0.1% (w/v) trichloroacetic acid (TCA), and the homogenate was centrifuged at 6000 *g* for 10 min at 4 °C. A volume (1 mL) of supernatant was added to a test tube containing 1 mL of either (i) –TBA solution composed of 80% (v/v) ethanolic solution and 20% (w/v) TCA, or (ii) + TBA solution composed of the same solution with the addition of 0.5% (w/v) thiobarbituric acid (TBA). Each mixture was incubated at 90 °C for 30 min, and the reaction was stopped by placing the tubes on ice.

Malondialdehyde (MDA) equivalents were determined by measuring spectrophotometric absorbance at 532 nm, with any potential interference from other compounds assessed at 440 and 600 nm. The calculation of MDA equivalents considered the MDA extinction coefficient of 157,000 M^{-1} cm^{-1}, following the equation corrected by Landi (2017). The level of lipid peroxidation was expressed as mmol MDA equivalents per gram of FW (mmol MDA g^{-1} FW).

Antioxidant activity assays

Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Brand-Williams et al. 1995) and the 2,29-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay (Re et al. 1999).

For the DPPH assay, 10 µL of shoot extract obtained for the betacyanin content determination were mixed with 990 µL of 80% (v/v) aqueous methanolic solution containing $3.12*10^{-5}$ M DPPH. After 30 min of incubation in the dark, the absorbance at 515 nm was recorded at time zero and after incubation.

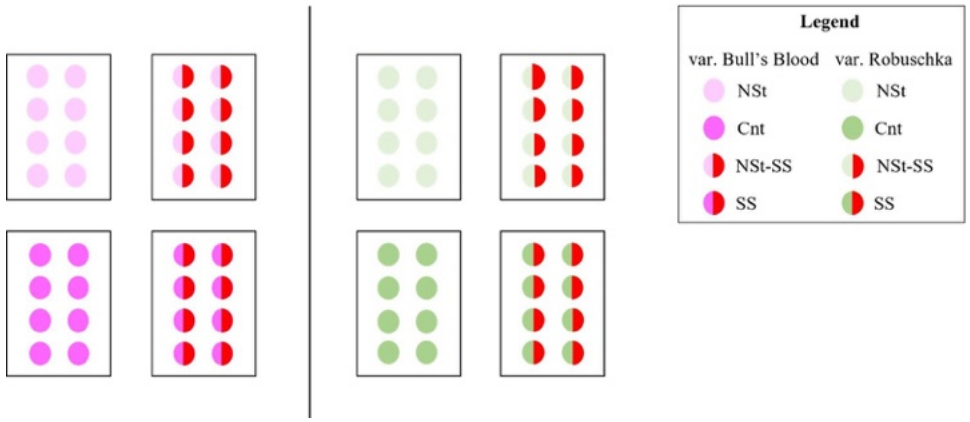


Fig. 1 Experimental design employed to test the role of betacyanins in two genotypes of beetroot plants subjected to N starvation (NSt), salt stress by adding 150 mM NaCl (SS) and a combined stress of nitrogen starvation and excess salt (NSt-SS). Plants fed with an optimal N content represented the control plants (Cnt)

For the ABTS assay, 50 µL of shoot extract were added to 950 µL of ABTS solution prepared dissolving 7 mM (w/v) ABTS and 2.5 mM (w/v) $K_2S_2O_8$ in Na-buffer (5 mM, w/v; pH 7.4). The kinetic of the reaction was spectrophotometrically followed for 90 s at 734 nm.

Trolox was used as a standard for calibration curve construction in the range 20–200 mg L^{-1} in both DPPH and ABTS assays. The results obtained from both antioxidant assays were expressed as mg Trolox equivalents per g FW (mg TE g^{-1} FW).

Determination of ascorbate and glutathione

For ascorbate and glutathione determinations, 0.3 g of fresh leaves were powdered with liquid nitrogen and homogenized with 6 volumes of 5% meta-phosphoric acid at 4 °C using mortar and pestle. The homogenates were centrifuged at 20,000 g for 15 min at 4 °C and the supernatants were collected and used for the analysis.

Reduced ascorbate (ASC) and dehydroascorbate (DHA) contents were determined following the method outlined by Kampfenkel et al. (1995) with slight adjustments. Briefly, to determine total ascorbate, DHA was first reduced to ASC using dithiothreitol (DTT), and the concentration of DHA was then calculated by subtracting the ASC content from the total ascorbate pool (which includes both ASC and DHA). For the total ascorbate quantification, a reaction mixture comprising 0.1 mL of the supernatant, 0.25 mL of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, and 0.05 mL of 10 mM DTT was prepared. Following a 10-min incubation at room temperature, 0.05 mL of 0.5% N-ethylmaleimide (NEM) was added to remove excess DTT. ASC was determined using a similar reaction mixture, except that 0.1 mL of water was added instead of DTT and NEM. Color development in both reaction mixtures was achieved by adding 0.2 mL of 10% TCA, 0.2 mL of 44% ortho-phosphoric acid, 0.2 mL of 4% 2,2'-dipyridil in 70% ethanol, and 0.1 mL 0.3% (w/v) $FeCl_3$. After vortexing, the mixture was incubated at 40 °C for 40 min, and the absorbance at 525 nm was measured. A standard curve was constructed based on ASC concentrations ranging from 0 to 100 µg mL^{-1}. Results were expressed as µmol ASC or DHA per g FW.

Reduced (GSH) and oxidized (GSSG) glutathione contents were determined following the method described by De Pinto et al. (1999) with minor modifications. Briefly, 0.4 mL of supernatant were added to 0.6 mL of 0.5 M Na-P phosphase buffer (pH 7.5). For the GSSG assay, the GSH was masked by adding 20 µL of 2-vinylpyridine to the supernatant, whereas 20 µL of H_2O were added in the aliquot utilized for total glutathione pool (comprising GSH plus GSSG) assay.

The resulting mixtures were incubated for 1 h at room temperature in the dark. GSSG and total glutathione content was measured in 1 mL of reaction solution constituted by 40 mM NADPH, 0.143 mM phosphate buffer Na-P (pH 7.5) containing 7.14 mM EDTA and 0.86 mM 5,5'-dithiobis(2-nitrobenzoic acid), 100 µL of the mixture obtained as described above. The reaction was started by adding 3 units of glutathione reductase and was monitored by measuring the change in absorbance at 412 nm for 90 s. GSH was estimated as the difference between the total glutathione and GSSG content. A standard curve for GSH within the range of 0–30 µM mL^{-1} was prepared. Results were expressed as µmol GSH or GSSG per g FW.

N, Na and Cl content

N content was determined with the Kjeldahl method with minor modifications using a TURBOTHERM® system for the digestion and a VAPODEST® 450 distillation unit for the distillation (Gerhardt Analytical Systems, Königswinter, Germany). Digestion was performed using concentrated sulfuric acid and H_2O_2 30% (v/v) aqueous solution for each sample. After the neutralization of the excess of sulfuric acid using a NaOH solution (30% w/v), the distillate is collected in boric acid and titrated potentiometrically with 0.1 N HCl. Results were expressed as g kg^{-1} DW.

Na determination was performed on mineralized samples with nitric acid 70% (v/v) and H_2O_2 30% (v/v) aqueous solutions, using an atomic absorption spectrometer Thermo Fisher Scientific ICE 3000. Cl concentrations were determined using an IonPac AG4A guard column and an IonPac AS4A analytical column of an ion exchange chromatograph (Dionex DX120; Dionex Corporation, Sunnyvale, CA, USA) with a conductivity detector as reported by D'Imperio et al. (2016). The eluent was constituted by 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$ with a flow rate of 2 mL min^{-1}. The suppressor was an anion micromembrane suppressor and the regenerant was constituted by 50 mM H_2SO_4 with a flow rate of 3–5 mL min^{-1}. The column storage solution was constituted by 100 mM NaOH and the background conductivity was 15–20 µS. Standard solutions of 0.5, 1, 2 and 4 ppm $CaCl_2$ (Sigma Aldrich, St. Louis, MO, USA) were injected as reference solutions. The extraction was obtained by adding dried and powdered material (200 mg) to 25 mL of Milli-Q water and incubating samples in a water bath, kept in agitation for 1 h at 60 °C. Na and Cl content results were expressed as mmol g^{-1} DW.

Statistical analysis

After checking the normality of distribution (Shapiro–Wilk test, 95% confidence interval) and the homoscedasticity by Bartlett test, a one-way ANOVA was carried out on biometric, physiological, and biochemical data using the abiotic

stress (NSt, SS and the combination) as variability factor. Significant differences among treatments were determined by LSD Fisher *post-hoc* test ($P \leq 0.05$). GraphPad software (GraphPad, La Jolla, CA, USA) was used for statistical analysis.

Results

Integrated biomass and biometric analyses revealed differential susceptibility of green and red beetroot plants to N starvation

Analysis of phenotypic parameters, including biomass and biometric measurements, showed that NSt significantly affected biomass accumulation in *B. vulgaris* (Table 1). In green beetroot plants, NSt induced a 44.8% reduction in shoot biomass whereas SS alone had no significant impact on biomass in either cultivar (Table 1). However, under NSt-SS, shoot biomass decreased in both green and red plants, resembling the NSt response. Root biomass under NSt and NSt-SS increased significantly with respect to Cnt (+ 80.4 and + 76.2%, respectively) in green plants but remained unchanged in red plants (Table 1).

DW is considered a crucial indicator of plant growth as it reflects the accumulation of water-free biomass. Monitoring DW allowed an assessment of the efficiency of the plant in converting resources into structural plant tissues, thus providing an insight into the overall health and productivity of the plant. Therefore, DW was monitored after exposure to the three growth conditions considered in the present experiment (Table 2). Shoot DW increased under NSt condition in both genotypes (green: + 38.2%, red: + 34.9% *vs* Cnt), indicating enhanced structural investment (Table 2). Conversely, the root DW decreased similarly in both green and red beetroot plants. This trend was also observed under the combined stress condition (NSt-SS), whereas no significant differences were noted under SS (Table 2).

Additional biometric measurements were used to evaluate the phenotypic responses of green and red beetroot plants to NSt, SS, and NSt-SS combine, including root length, leaf area index (LAI), and leaf thickness (Table 3). NSt and NSt-SS significantly increased root length in both green and red genotypes (+ 57.1% and + 66.2%, respectively, *vs* Cnt in green plants and + 45.2% and + 49.2%, respectively, *vs* Cnt in red plants). In red plants, NSt also led to a reduction in LAI, also when applied with SS. SS alone did not affect root length and LAI (Table 3). However, SS, both alone and combined, induced the highest leaf thickness in red and green plants (Table 3).

Gas exchanges showed a general reduction in the photosynthetic process under N starvation and salt stress treatments with no biochemical alterations under salt stress

Gas exchange parameters in leaves of green and red beetroot subjected to NSt, SS and NSt-SS are reported in Fig. 2. P_n was reduced in both cultivars under NSt, SS and NSt-SS (Fig. 2a, d). Similarly, g_s declined in both green and red plants under NSt, SS and NSt-SS (Fig. 2b, e). Intercellular CO_2 concentration (C_i) resulted negatively affected by SS in green plants (Fig. 2c), whereas an increase in C_i values was found in red plants subjected to NSt (Fig. 2f).

Photosynthetic pigments revealed a reduction in the combined treatment, and the betacyanin content was enhanced by N starvation

Pigment concentrations varied markedly in response to NSt and SS, with similar patterns observed in green and red beetroot (Table 4). In both cultivars, NSt consistently reduced chlorophyll levels, with the lowest values recorded under NSt-SS (green: –85.6%, red: –36.9% *vs* Cnt). SS also induced a reduction in chlorophyll levels in both green and red plants (–44.9% and –29% *vs* Cnt, respectively). Carotenoid content showed reductions in both NSt and NSt-SS treatments in green plants although in red plants a reduction exclusively in NSt-SS conditions (Table 4).

The applied treatments induced a different response in betacyanin accumulation (Table 4). In both green and red phenotypes, SS decreased betacyanin levels by 88.9% and 61.7%, respectively. In contrast, in green plants, NSt promoted an increase in betacyanin concentration by 70.8% relative to controls (Table 4). The combined stress had a negative effect in betacyanin concentration of red plants whereas an increase controls.

DODA expression is differentially regulated by N starvation and salt stress

The expression of *DODA*, a key gene involved in betacyanin biosynthesis, was strongly influenced by both genotype and treatment (Fig. 3a). Under control conditions, red plants showed higher *DODA* transcript abundance than green plants, consistent with their greater basal betacyanin accumulation. NSt markedly induced *DODA* expression in the green genotype, whereas only a slight increase was observed in red plants. In contrast, SS did not substantially affect *DODA* expression in green plants compared with Cnt, but clearly reduced transcript levels in red plants. Under the combined NSt-SS treatment, *DODA* expression increased compared with SS alone in the green genotype, although only a modest increase was detected in red plants. Overall,

these results suggest that *DODA* expression is mainly stimulated by NSt, whereas salinity tends to repress or to limit its induction, indicating a stress-specific transcriptional regulation of betacyanin biosynthesis.

The expression of *CYP76AD1*, another key gene involved in betacyanin biosynthesis, was significantly affected by genotype and treatment (Fig. 3b). Under control conditions, *CYP76AD1* transcript abundance was markedly higher in the red genotype than in the green genotype. NSt did not significantly alter *CYP76AD1* expression in either genotype compared with the respective controls. In contrast, SS strongly reduced transcript levels in red plants, whereas no significant changes were observed in the green genotype. Under the combined NSt-SS treatment, *CYP76AD1* expression increased relative to SS alone in both genotypes, with a more pronounced recovery in red plants. Nevertheless, transcript levels under NSt-SS remained lower than those recorded in red Cnt and NSt plants. Overall, *CYP76AD1* expression closely reflected the constitutive pigmentation differences between the two genotypes and appeared less responsive to N starvation than *DODA*.

Oxidative stress markers level was reduced by N starvation and by the combined treatment

Hydrogen peroxide (H_2O_2) and lipid peroxidation levels were differentially affected by NSt and SS in green and red phenotypes. In green plants, the highest H_2O_2 level was observed in SS plants, although NSt-treated plants (i.e., NSt and NSt-SS) showed similar results and lower when compared with Cnt and SS-treated plants (Fig. 4a). In red plants, H_2O_2 accumulation was lower in plants subjected to NSt treatment (both NSt and NSt-SS) with respect of Cnt plants and the lowest values were recorded in plants exposed to combined stress (Fig. 4c).

Table 1 Shoot and root biomass in green and red *Beta vulgaris* plants grown under three different conditions: nitrogen starvation (NSt), excess salt (SS), and a combined stress of nitrogen starvation and excess salt (NSt-SS)

	Shoot biomass (g plant^{-1})		Root biomass (g plant^{-1})	
	Green	Red	Green	Red
Cnt	5.24 ± 1.46 a	7.92 ± 2.19 a	0.69 ± 0.11 b	1.01 ± 0.63
NSt	2.89 ± 1. 30 b	2.84 ± 0.93 b	3.52 ± 0.26 a	1.76 ± 0.74
SS	4.60 ± 0.69 a	8.17 ± 2.96 a	0.41 ± 0.05 b	1.01 ± 0.86
NSt-SS	2.93 ± 0.35 b	3.79 ± 1.18 b	2.90 ± 1.05 ab	1.54 ± 0.94

Means (± SD, $n = 5$) were subjected to a one-way ANOVA with the abiotic stress as the variability factor among the same variety. For each parameter means, followed by different letters indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test. Lack of letters means the absence of significance of the F ratio following ANOVA analysis

Table 2 Shoot and root dry weight in green and red *Beta vulgaris* plants grown under three different conditions: nitrogen starvation (NSt), excess salt (SS), and a combined stress of nitrogen starvation and excess salt (NSt-SS)

	Shoot dry weight (g 100 g^{-1})		Root dry weight (g 100 g^{-1})	
	Green	Red	Green	Red
Cnt	8.65 ± 0.95 b	6.48 ± 0.45 b	7.84 ± 1.89 a	7.56 ± 2.31 a
NSt	14.00 ± 2.53 a	9.96 ± 0.90 a	5.09 ± 1.10 b	5.73 ± 1.23 b
SS	9.26 ± 0.30 b	6.62 ± 0.42 c	7.30 ± 0.64 a	8.88 ± 2.13 a
NSt-SS	14.58 ± 1.41 a	9.25 ± 0.80 a	4.99 ± 0.97 b	5.93 ± 1.15 b

Means (± SD, $n = 5$) were subjected to a one-way ANOVA with the abiotic stress as the variability factor, among the same variety. For each parameter means followed by different letters indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test

The behavior of lipid peroxidation mirrored those of H_2O_2 in both cultivars. In green plants resulted lower in NSt (both NSt and NSt-SS) leaves than in Cnt plants and the lowest level was found in NST-SS plants (Fig. 4b). In red plants, the pattern was like that of green plants, even though no significant differences in MDA levels were observed in NSt and NSt-SS leaves (Fig. 4d). It is also interesting to underline that mean values of the lipid peroxidation in the red cultivar resulted in lower values as compared to the green cultivar ($P \leq 0.05$).

Antioxidant metabolism was particularly affected by salt stress in green plants and not compromised by N starvation

Antioxidant responses to NSt and SS differed between green and red phenotypes (Fig. 5). In green leaves, SS induced a reduction in antioxidant activity, independently of the used assay (i.e., DPPH or ABTS; Fig. 5a and b). In red leaves, NSt induced an increase in antioxidant activity assayed by ABTS (Fig. 5d) when compared with controls, whilst no significant differences were found in antioxidant activity assayed by DPPH (Fig. 5c).

Analysis of antioxidant metabolites revealed that, in green plants, the total ascorbate content resulted in higher values in NSt plants when compared with Cnt plants and the combined NSt-SS induced a reduction in total ascorbate content when compared with Cnt and no significant difference between SS and Cnt plants (Fig. 6a). Even though DHA content was similar between the treated green leaves (NSt, SS and NSt-SS), the ASC content reported the highest value in NSt plants (Fig. 6a). Similarly, in red plants, the highest total ascorbate content was found in NSt plants whilst the other treatments did not induce difference in the level of total ascorbate as compared with the controls (Fig. 6c). DHA content reported the lowest value in NSt-SS plants even though no different with the Cnt leaves, whilst ASC content reported

Table 3 Root length, leaf area index (LAI) and leaf thickness in green and red *B. vulgaris* plants grown under three different conditions: nitrogen starvation (NSt), excess salt (SS), and a combined stress of nitrogen starvation and excess salt (NSt-SS)

	Root length (cm)		LAI (cm^2)		Leaf thickness (mm)	
	Green	Red	Green	Red	Green	Red
Cnt	12.70 ± 2.05 c	19.08 ± 5.72 b	23.30 ± 3.05 a	34.53 ± 6.81 a	0.42 ± 0.02 b	0.41 ± 0.05 b
NSt	29.6 ± 5.55 b	34.8 ± 5.76 a	19.47 ± 4.38 a	12.05 ± 1.93 c	0.47 ± 0.05 ab	0.44 ± 0.03 b
SS	12.90 ± 2.56 c	12.90 ± 5.24 b	21.21 ± 2.35 a	30.54 ± 4.91 a	0.50 ± 0.06 a	0.48 ± 0.02 ab
NSt-SS	37.60 ± 6.80 a	37.60 ± 9.69 a	19.55 ± 4.58 a	20.49 ± 2.96 b	0.52 ± 0.04 a	0.52 ± 0.04 a

Means (± SD, $n = 5$ for root length and leaf thickness and $n = 3$ for LAI) were subjected to a one-way ANOVA with the abiotic stress as the variability factor. For each parameter means followed by different letters indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test. Lack of letters means the absence of significance of the F ratio following ANOVA analysis

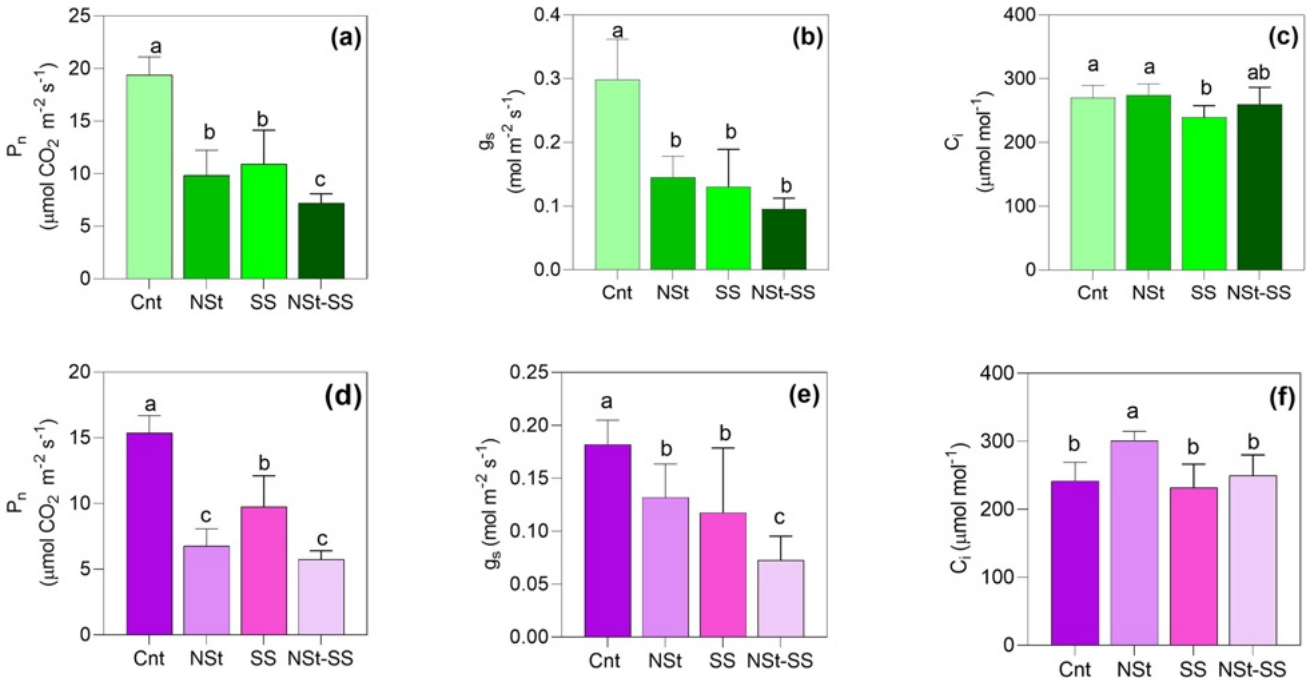


Fig. 2 Net-photosynthesis (P_n; **a, d**), stomatal conductance (g_s; **b, e**) and intercellular CO_2 concentration (C_i; **c, f**) in green (**a**, **b**, **c**) and red (**d**, **e**, **f**) *Beta vulgaris* plants grown under three different conditions: nitrogen starvation (NSt), excess salt (SS), and a combined stress of nitrogen starvation and excess salt (NSt-SS). Means (± SD; $n = 7$) were subjected to a one-way ANOVA with the abiotic stress as the variability factor. For each parameter means followed by different letters indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test

the highest value in NSt plants and similar results among the other treatments and controls, as seen for the total ascorbate content (Fig. 6c).

Glutathione profiles further highlighted distinct stress responses of *B. vulgaris* phenotype (Fig. 6b and d). In green plants, the highest total glutathione content was found in the combined treatment and the lowest in NSt plants (Fig. 6b). The GSSG content resulted in lower values in NSt treatment (alone or combined with salt treatment) than in Cnt plants. Conversely, GSH content resulted in higher levels in NSt-SS plants than controls, whilst the other treatments induced a similar GSH content to Cnt plants (Fig. 6b). In red plants, the total glutathione content resulted in lower values in plants under NSt and NSt-SS treatments when compared to Cnt plants, with a slight reduction also under SS. GSH level was lower in NSt and NSt-SS leaves when compared to controls. Similarly, GSSG level resulted in lower levels in NSt and NSt-SS plants and like controls in SS plants (Fig. 6d).

N, Na and Cl content

Table 5 shows the N, Na and Cl content in leaves from red and green plants subjected to N management and SS treatment. In green plants, N uptake was reduced by all treatments

Table 4 Total chlorophyll, carotenoid and betacyanin content in green and red *B. vulgaris* plants grown under three different conditions: nitrogen starvation (NSt), excess salt (SS), and a combined stress of nitrogen starvation and excess salt (NSt-SS)

	Chlorophylls (μg g^{-1} FW)		Carotenoids (μg g^{-1} FW)		Betacyanins (mg g^{-1} FW)	
	Green	Red	Green	Red	Green	Red
Cnt	429.55 ± 92.29 a	231.93 ± 58.88 a	58.21 ± 7.91 a	31.27 ± 7.51 a	7.77 ± 0.23 c	54.77 ± 6.58 a
NSt	133.44 ± 24.22 c	171.45 ± 40.26 b	22.44 ± 4.84 c	28.35 ± 3.24 ab	26.58 ± 0.62 a	55.29 ± 0.99 a
SS	236.46 ± 49.74 b	164.54 ± 20.76 c	33.89 ± 13.56 b	31.87 ± 3.54 a	0.86 ± 0.09 d	20.98 ± 4.16 c
NSt-SS	62.02 ± 19.92 d	146.02 ± 34.85 d	24.14 ± 9.53 c	23.31 ± 8.21 b	15.90 ± 3.67 b	35.13 ± 4.33 b

Means (± SD, $n = 9$ for chlorophylls and carotenoids and $n = 3$ for betacyanins) were subjected to a one-way ANOVA with the abiotic stress as the variability factor among the same variety. For each parameter means followed by different letters indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test

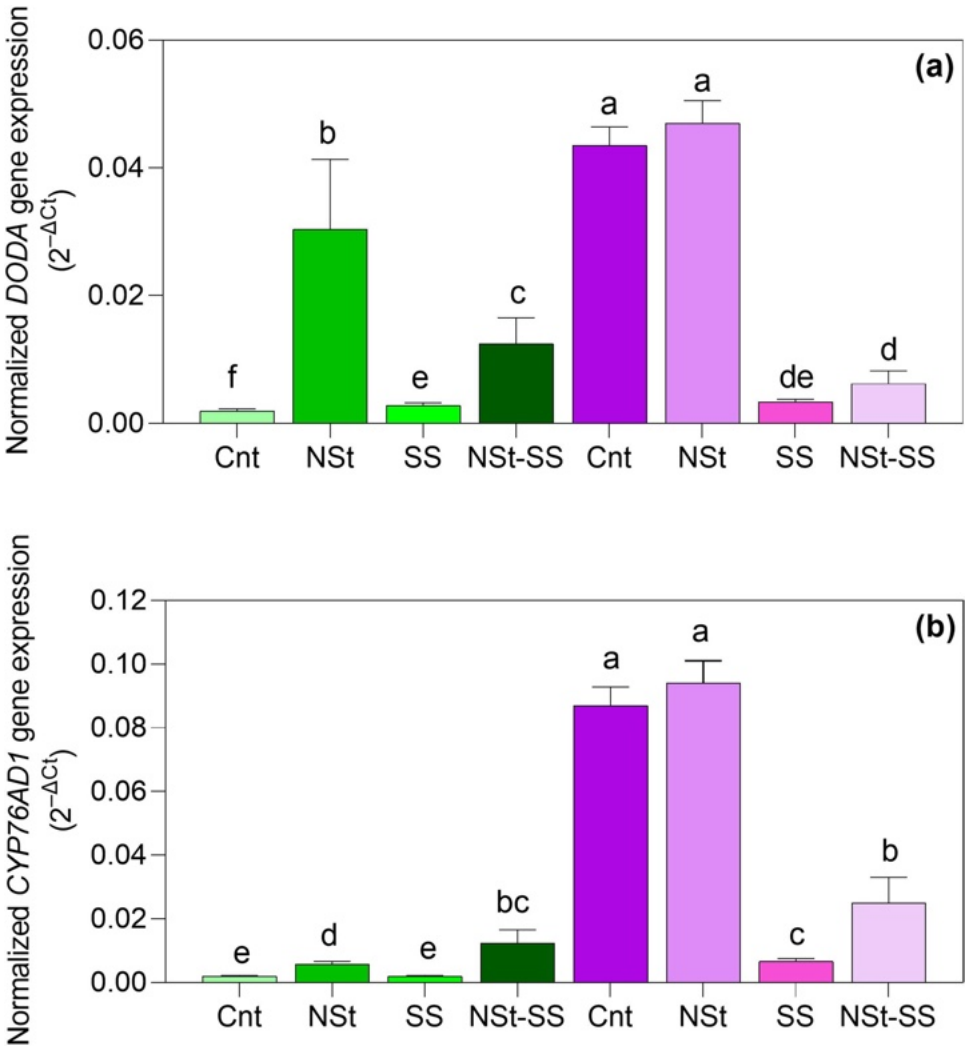


Fig. 3 **a, b** Relative expression of genes involved in betacyanin biosynthesis in green- (green bars) and red- (purple bars) leafed *Beta vulgaris* plants subjected to control conditions (Cnt), nitrogen starvation (NSt), salt stress (SS), and combined nitrogen starvation and salt stress (NSt-SS). *DODA* (**a**) and *CYP76AD1* (**b**) gene expression. Transcript abundance was determined by RT-qPCR and normalized against ACTIN using the $2^{-\Delta Ct}$ method. Means (± SD; $n = 3$) were subjected one-way ANOVA with the abiotic stress as the variability factor. Means followed by different letters indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test

(NSt, SS and NSt-SS) with the lowest content in NSt treatment (–67.0% *vs* Cnt). Similarly, in red plants, the N uptake was lower in NSt and NSt-SS plants when compared with Cnt and SS plants (Table 5). In both green and red cultivars, Na and Cl contents were obviously increased by SS, with a slight difference between SS and NSt-SS treatment in Cl uptake by green plants (Table 5).

Discussion

Soils are becoming increasingly affected by salinity due to climate change and reduced rainfall patterns (Yadav et al. 2011; Corwin 2021) as well as deficiencies in essential nutrients such as N, P, and K (Graham et al. 2008; White and Brown 2010). These conditions have a detrimental impact on plant growth and productivity (Fageria 2001; Yadav et al. 2011). *B. vulgaris* represents a promising species for its tolerance to both salinity and N deficiency (Shaw et al. 2002; Yolcu et al. 2021a). This species is known for its ability to synthesize betacyanins, nitrogenous pigments that could be involved in many aspects such as enhancement of salt tolerance regulating osmotic pressure, scavenging ROS but also stabilizing cellular structures in oxidative stress conditions in other salt-tolerant species (Jia et al. 2021). However, the role of these pigments in stress tolerance remains not fully understood. In this context, this study provides comprehensive insights into the physiological and biochemical responses of *B. vulgaris* (green and red cultivars) to SS. Moreover, the presence of N in betacyanin structure has prompted an investigation into physiological responses. To understand the responses and/or defensive mechanisms involved in this species under SS and N starvation, we utilized two genotypes of *B. vulgaris* – one naturally red, rich in betacyanins, and the other with green leaves but still capable of biosynthesizing and accumulating betacyanins. The differential responses observed between the two cultivars underline the importance of genotype-dependent mechanisms in stress tolerance. Notably, N availability emerged as a key determinant of plant performance, affecting growth, gas exchange, antioxidant capacity, and pigment metabolism more profoundly than SS.

N starvation compromises photosynthetic performance without affecting the integrity of the antioxidant system

In this study, the most prominent observation is the increase in root biomass in green NSt plants, which is attributed to the elongation of the root length in green beetroot. The root elongation was also observed in the red NSt plants, even though no significant differences were reported in terms of root biomass. The observation of the root elongation in plant subjected to N deficiency aligns with findings from other studies that have examined the impact of N starvation in plant species distinct from *B. vulgaris* or not belonging to the Caryophyllales order (Tian et al. 2020; Wang et al. 2020; Lopez et al. 2023). For example, Wang et al. (2020) observed the promotion of rice root growth when plants

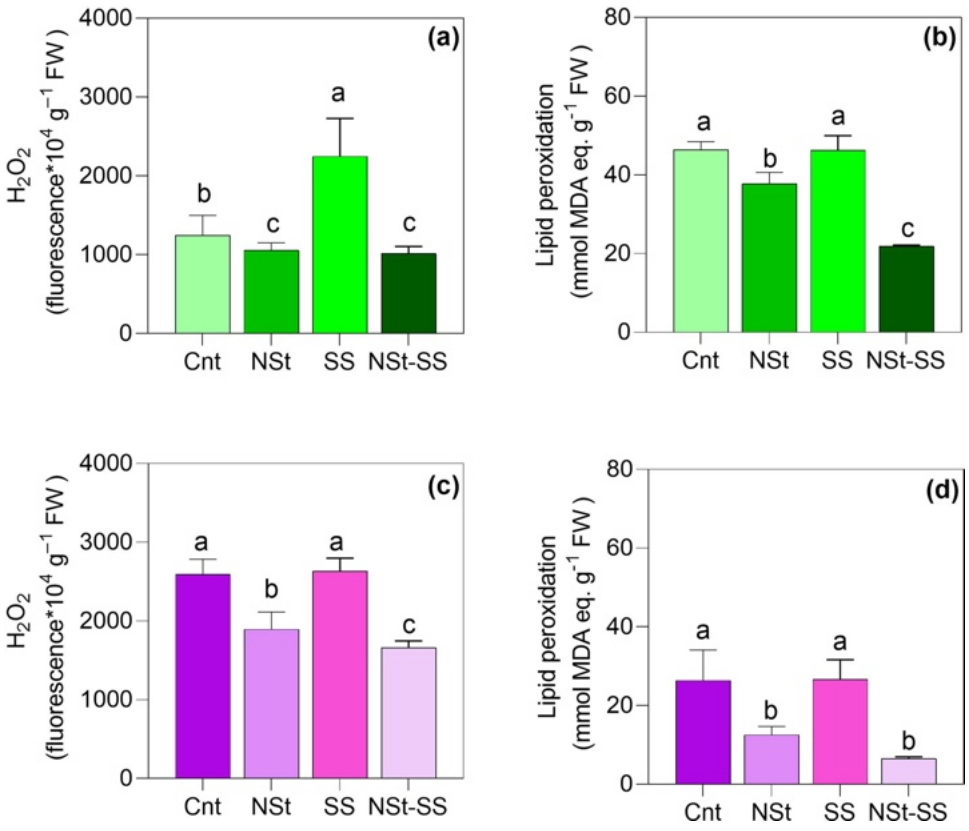


Fig. 4 Hydrogen peroxide (H_2O_2; **a**, **c**) and lipid peroxidation (**b**, **d**) in green (**a**, **b**) and red (**c**, **d**) *B. vulgaris* plants grown under three different conditions: nitrogen starvation (NSt), excess salt (SS), and a combined stress of nitrogen starvation and excess salt (NSt-SS). Means (± SD; $n = 9$ for H_2O_2 and $n = 3$ for lipid peroxidation) for H_2O_2 and for lipid peroxidation were subjected to a one-way ANOVA with the abiotic stress as the variability factor. For each parameter means followed by different letters indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test

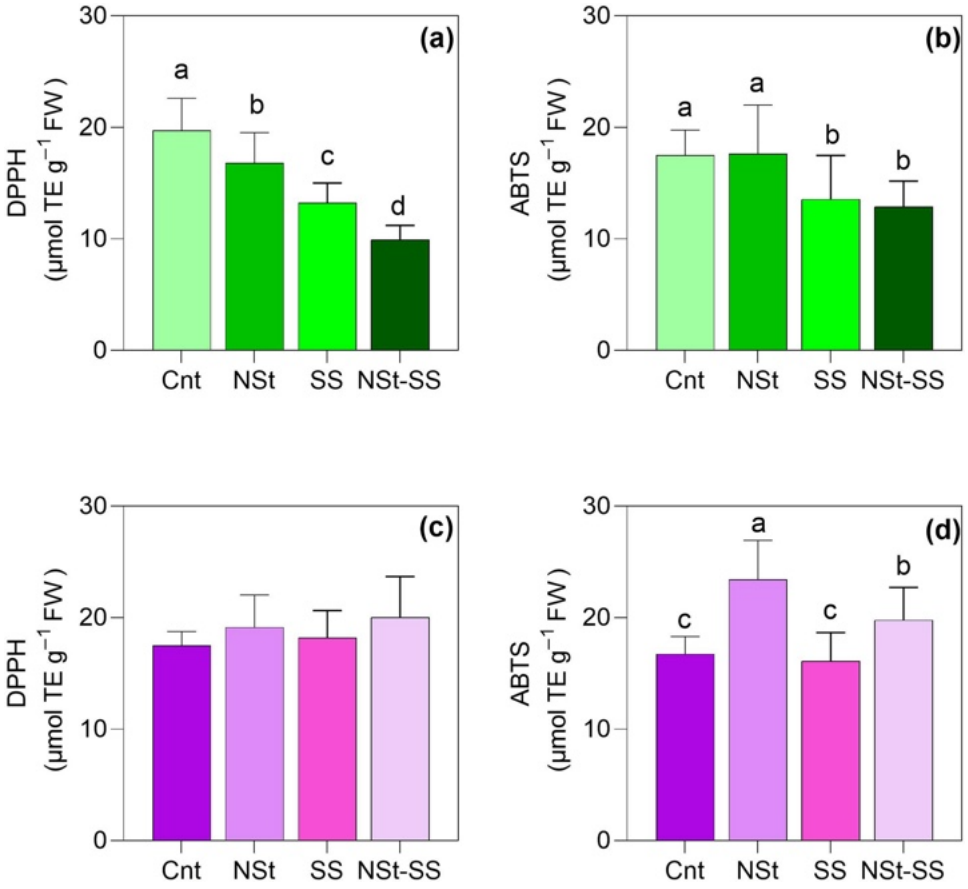


Fig. 5 Antioxidant activity measured with DPPH (**a**, **c**) and ABTS assays (**b**, **d**) in green (**a**, **b**) and red (**c**, **d**) *B. vulgaris* plants grown under three different conditions: nitrogen starvation (NSt), excess salt (SS), and a combined stress of nitrogen starvation and excess salt (NSt-SS). Means (± SD; $n = 9$) were subjected to a one-way ANOVA with the abiotic stress as the variability factor. For each parameter means followed by different letters indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test. Lack of letters means the absence of significance of the F ratio following ANOVA analysis

were subjected to N starvation (0 mM N) since this strong deficient N condition can decrease the cytokines content, thereby enhancing the root meristem cell proliferation rate and promoting root cell elongation by up-regulating cell elongation-related genes. Sun et al. (2020) reviewed the possible mechanisms able to induce root cell division and expansion in plants grown at low N condition, although the mechanisms that regulate cell division and/or elongation are not still clear. However, these authors confirmed that high cytokines level in the roots is not favorable for root elongation, confirming the findings of Wang et al. (2020).

Another evident result was the reduction in shoot biomass in NSt leaves in both genotypes. In red plants, this result was attributable to the reduction in leaf area, whilst in green plants, the explanation result was more difficult since the leaf area index (LAI) did not change between NSt and Cnt plants. However, the reduction in shoot biomass can be explained by the fact that N is responsible for the vegetative growth and consequently for plant productivity (Below 2001).

Moreover, NSt treatment induced a reduction in P_n and g_s in both *B. vulgaris* genotypes (Mu and Chen 2021). These results are in accordance with findings by Bascuñán-Godoy et al. (2018a, 2018b) who noted a P_n and g_s decrease in three genotypes of *C. quinoa* exposed to a low N supply (50 mM). These authors observed that these declines were accompanied by a rise in the NH_4^+ concentration in leaves, possibly attributed to the photorespiration process, process that can also to be related to the reduction in CO_2 assimilation. Interestingly, despite the g_s decrease in leaves of red and green NSt plants, the intercellular CO_2 concentration (C_i) did not report any significant difference in green plants, and a slight increase was observed in red plants. This phenomenon might be explained by biochemical limitations in CO_2-consuming pathways such as the Calvin-Benson-Bassham cycle, in addition to the stomatal limitation. Moreover, a strong decrease in chlorophyll and carotenoid content was observed in line with the reduction in photosynthetic process in NSt conditions in both genotypes, as well reported in literature (Giorgi et al. 2009; Prinsi et al. 2020). Despite the decrease in the photosynthetic process and in the photosynthetic pigment concentrations, the level of H_2O_2 and MDA, indicative of oxidative stress, was lower than the controls in both genotypes subjected to N starvation. On the other hand, total ASC and its reduced form increased in NSt treated leaves to indicate the activation of this antioxidant molecule in contrasting oxidative stress. On the other hand, glutathione metabolism displayed a complex pattern. In green plants, NSt reduced total glutathione content whilst in red plants, both NSt and NSt-SS decreased glutathione pools. Therefore, it is supposed that N starvation induces

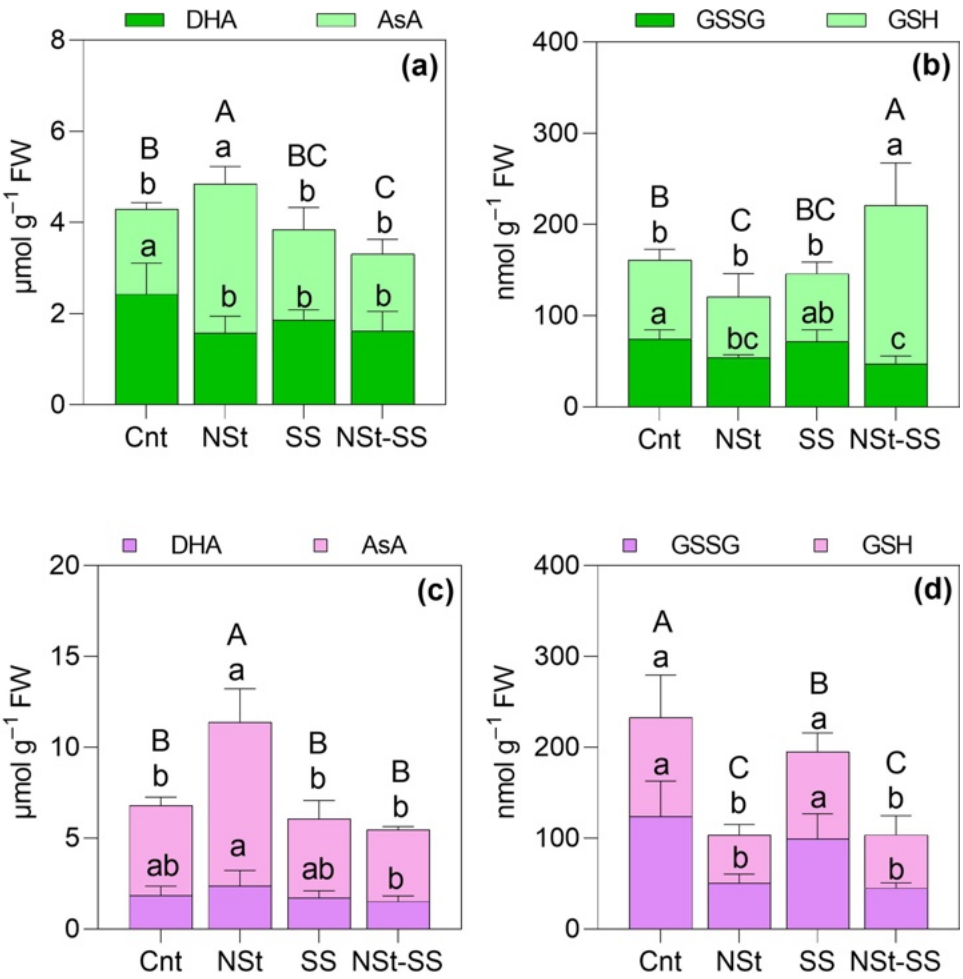


Fig. 6 Reduced (ASC; **a**) and oxidized (DHA; **c**) forms of ascorbate and of glutathione (GSH and GSSG; **b**, **d**) in green (**a**, **b**) and red (**c**, **d**) *B. vulgaris* plants grown under three different conditions: nitrogen starvation (NSt), excess salt (SS), and a combined stress of nitrogen starvation and excess salt (NSt-SS). Means (± SD, $n = 9$) were subjected to a one-way ANOVA with the abiotic stress as the variability factor. Different lowercase letters for the factor indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test for DHA or GSSG and ASC or GSH, separately. Different capital letters indicate significant differences in the total ascorbate or glutathione content utilizing the same statistical analyses

Table 5 N, Na and Cl content in green and red *B. vulgaris* plants grown under three different conditions: nitrogen starvation (NSt), excess salt (SS), and a combined stress of nitrogen starvation and excess salt (NSt-SS)

	N (g kg^{-1} DW)		Na (mmol g^{-1} DW)		Cl (mmol g^{-1} DW)	
	Green	Red	Green	Red	Green	Red
Cnt	51.72 ± 1.04 a	53.28 ± 4.03 a	0.40 ± 0.10 b	0.37 ± 0.02 b	0.52 ± 0.05 c	0.82 ± 0.10 b
NSt	17.11 ± 2.25 d	20.59 ± 3.45 b	0.30 ± 0.05 b	0.22 ± 0.02 c	0.84 ± 0.11 c	0.86 ± 0.09 b
SS	47.58 ± 2.20 b	49.64 ± 3.38 a	0.89 ± 0.17 a	1.07 ± 0.04 a	1.88 ± 0.35 b	3.14 ± 0.18 a
NSt-SS	28.23 ± 0.84 c	26.01 ± 1.49 b	1.03 ± 0.11 a	0.95 ± 0.13 a	3.03 ± 0.43 a	3.26 ± 0.76 a

Means were subjected to a one-way ANOVA with the abiotic stress as the variability factor among the same variety. For each parameter means followed by different letters indicate significant differences at $P \leq 0.05$ using the *post-hoc* LSD test

positive responses in ascorbate turn over in *B. vulgaris* leaves of both genotypes, repairing partially the antioxidant system affected by the starvation. Differently, following the Foyer-Halliwell-Asada cycle, the turnover of glutathione is negatively affected by the starvation treatment. However, the low oxidative stress induced by N starvation can explain the little necessity of repairment induced by ascorbate, avoiding the involvement of glutathione system.

The decrease in oxidative stress markers in NSt plants agrees with findings by Bascuñán-Godoy et al. (2018a). These authors found a reduction in lipid peroxidation level in three genotypes of *C. quinoa* treated with a low N supply (50 mM). These authors explain this result, affirming that plants grown at N deficiency conditions (with poor investment in photosystem proteins) might have reduced light energy capture capacity, thus a lower probability of producing ROS (Bascuñán-Godoy et al. 2018a). These findings are

consistent with the observed accumulation of ASC under NSt in both phenotypes, pointing to its key role in ROS detoxification. However, this aspect needs further in-depth investigation to understand the low levels in oxidative markers in treated samples of red genotype.

Finally, NSt obviously reduced the N accumulation in leaves of both green and red genotypes and an accumulation in Cl and Na ions in salted treated plants. In particular, NSt-SS led to the uptake and higher accumulation of Cl due to the great selection of membrane ion channels for Cl ion rather than nitrate (Pérez-Jiménez et al. 2019). On the other hand, Pérez-Jiménez et al. (2019) observed an increased uptake of Cl ion in pepper leaves and a reduction of nitrate accumulation in NO_3^- fed plants. The authors suggested that Cl can use the membrane nitrate channel, augmenting its uptake in leaves (Colmenero-Flores et al. 2019). Indeed, other authors observed that salinity decreased nitrate uptake in some halophyte (Rubinigg et al. 2003; Song et al. 2009) or non-halophyte (Aslam et al. 1984; Peuke et al. 1996) species, attributing this pattern to a competition between nitrate and Cl for certain transport systems, which are proposed to play significant roles in uptake or the xylem loading of both anions (Bottacin et al. 1985; Cerezo et al. 1997; Köhler and Raschke 2000).

Low impact of salt stress on green- and red-leafed *B. vulgaris* genotypes

An interesting finding is that SS did not induce significant differences in terms of plant biomass in both genotypes. This aspect suggests the adaptability to salinity of plant species belonging to the Caryophyllales order such as *Suaeda salsa* (Li et al. 2020), *Salicornia* spp. (Ventura and Sagi 2013), *B. maritima* (Gholipor et al. 2022), *Amaranthus* sp. (Bhargava and Srivastava 2020), *C. quinoa* (Adolf et al. 2013). Some of these species such as *Salicornia* spp., are named halophytes because of their strictly dependence on Na in their physiology since this element is commonly substituted to potassium in its essential functions such as stomatal opening and closure (Negrão et al. 2017).

However, SS induced significant effects on physiological responses, reducing P_n and g_s in both genotypes after 15 days of NaCl 150 mM treatment. Salt stress induced both stomatal and biochemical limitation to CO_2 uptake in green genotype as evidenced by the reduction in intercellular CO_2 concentration. These responses at physiological level agree with the findings of other authors who analyzed plants belonging to the Caryophyllales order (Dadkhah and Rassam 2017; Skorupa et al. 2019). For instance, Dadkhah and Rassam (2017) observed a reduction in P_n and g_s in two sugar beetroot cultivars exposed to a range from 50 to 350 mM salinity (NaCl and $CaCl_2$ in a 5:1 molar ratio) for 48 h, and the reductions were closely related to the salt concentration increase. Skorupa et al. (2019) observed a reduction in transpiration rate and P_n in *B. vulgaris* and *B. maritima* subjected to SS or salt shock, evidencing, through the transcriptomic analysis, the silencing of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit and light-harvesting Chl *a-b* binding protein (LHCB) genes under SS and the down regulation of LHCBs and photosystem genes under salt shock.

The imposition of SS induced also a reduction in chlorophyll and carotenoid contents in both genotypes as already reported in the same species by Shokohian and Omidi (2021). Salt stress determined an increase in H_2O_2 level in green plants even though no changes in lipid peroxidation were observed, indicating no damage in the glycolipids and phospholipids bilayer belonging to cell membranes (Smirnoff et al. 2019). Clearly, the unchanged GSH/GSSG ratio recorded in this genotype under SS is related to preserving membranes to oxidative stress and, at the same time, to confirming the lack of the involvement of glutathione system in the antioxidant responses of the *B. vulgaris* genotypes under investigation, already evidence in the N starvation treatment (Hasanuzzaman et al. 2017). However, even though ascorbate turnover increased in both genotypes, this antioxidant molecule could not contain H_2O_2 content, differently than observed for the N starvation treatment. The effect of salinity decreased the oxidative stress level of salted plants when grown in deficiency of N in terms of lipid peroxidation in green plants and of both H_2O_2 and MDA levels in red plants, confirming the results already highlighted for N starvation in which no cell damage was induced by this treatment. These results indicate that beetroot shows a pattern evidenced for halophyte species in terms of oxidative stress (Yildiztugay et al. 2014).

Salt treatment induced an accumulation of Na and Cl elements in both red and green beetroots. This accumulation resulted in accordance with results obtained by other authors in species belonging to Caryophyllales order (Orsini et al. 2011; Adolf et al. 2013). However, the high accumulation of these ions can explain the decrease of P_n and photosynthetic pigments as well as the high H_2O_2 presence due to the toxicity of these elements for the plant cells and the difficulties of the antioxidant responses to scavenge this oxidative stress condition (Flowers et al. 2015; Shelke et al. 2019).

Low impact on oxidative markers by combined NSt-SS treatment on green- and red-leafed *B. vulgaris* genotypes

Regulation of nitrogen metabolism is a critical point for salt tolerance, and the combination of salinity and N metabolism is very complex and influences plant metabolism and physiology (Nazir et al. 2023). The combination of N starvation and salt stress did not influence the response of the two genotypes of *B. vulgaris* in terms of root biomass, being the mean values similar to the recorded in NSt plants and it was related to a strong increase in root length in both genotypes. Plants subjected to both stresses evidenced also the strong constraint to CO_2 uptake that was attributed to both stomatal and biochemical limitations, reporting very low values of g_s and the value of intercellular CO_2 concentration similar to Cnt. The reduction of g_s in NSt-SS leaves could be represented a mechanism that contributes to the maintenance of leaf turgor and, in this way, the survival in stress conditions. It is well known as salinity stress influences carbon and nitrogen metabolism but also the uptake of some other nutrients such as calcium and potassium (Iqbal et al. 2015). However, in the present experiment, green genotype evidenced a strong increase in chloride uptake when salinity stress was applied on N-deficiency plants. Nitrate (NO_3^-) and chloride (Cl^-) share similar physicochemical properties, resulting in competition for membrane transport systems. Furthermore, as major inorganic anions, both contribute to charge balance and cellular osmoregulation (Colmenero-Flores et al. 2019).

However, in leaves of the two genotypes subjected to both stresses, a significant decrease in oxidative stress was evidenced by the decrease in H_2O_2 and MDA-by-products level. This evidenced the capacity of this species to activate antioxidant systems to counteract the ROS production as already reported by Lucas et al. (2024). Relative to Cnt- and NSt-treated plants, N starvation significantly declined the generation of H_2O_2 and lipid peroxidation induced by salt stress as already reviewed by Zhang et al. (2017), even though no clear explanations are reported in scientific literature. Clearly, the most important mechanism activated by the plant to contrast the negative effects of salinity involves ionic homeostasis but also the regulation of the scavenging mechanism of ROS (Hasanuzzaman et al. 2021). In particular, the ascorbate–glutathione pathway plays a key role in detoxifying ROS and in particular H_2O_2 (Hasanuzzaman et al. 2019) but also scavenge other active species of oxygen. Unlike the effects of the individual treatments (NSt or SS), the combined stress condition elicited a different antioxidant response. In the green genotype, glutathione was the only antioxidant that increased substantially, owing primarily to the accumulation of reduced glutathione (GSH). At the same time, ascorbate turnover declined, in agreement with the findings of Li et al. (2008) in apple plants, suggesting a shift in redox buffering from the ascorbate to the glutathione system under combined stress. Differently to the single treatments, the combination induced a complete activation of the antioxidant system involving both ascorbate and glutathione turnover, with a modulation of glutathione metabolism and a reorganization of the antioxidant network.

Betacyanins affect plant responses to N starvation more than salt stress

A key finding of this study is the contrasting response of the two genotypes to N starvation in terms of betacyanin accumulation. Although NSt induced a marked increase in betacyanin content in the green genotype, no significant change was observed in the red genotype. This pattern results in accordance with the results of other authors, showing an increase of these purple molecules under N-deficiency stress (Salahas et al. 2011; Bascuñán-Godoy et al. 2018a, b; Sitompul and Zulfati 2019). The accumulation of betacyanins could be a form of nitrogen storage by the plant that is experiencing N deficiency, thus having it as a reserve for subsequent plant growth and survival.

An intriguing aspect of our results is the increase in betacyanin content in the green genotype under N starvation, despite the marked reduction in total leaf N (Table 5). This apparent paradox suggests that betacyanin biosynthesis under N limitation is not directly dependent on external N availability, but rather on internal N remobilization and metabolic reprogramming. Indeed, N starvation is known to enhance protein turnover and activate recycling pathways that release NH_4^+, partly derived from photorespiration and other catabolic processes (Bascuñán-Godoy et al. 2018a, b). This N may be reassimilated and preferentially allocated to the synthesis of stress-related metabolites, including betacyanins. At the same time, the strong reduction in photosynthetic activity and chlorophyll content observed under NSt treatment indicates a downregulation of primary metabolism, which may redirect carbon and nitrogen fluxes toward secondary metabolism. In this context, betacyanin accumulation may represent a stress-adaptive strategy contributing to redox homeostasis, although also acting as a transient sink or buffering pool for nitrogen under limiting conditions. However, the absence of a similar response in the red genotype further indicates that this mechanism is strongly genotype-dependent and likely influenced by differences in basal pigment levels and metabolic regulation. Although the present data supports a link between nitrogen remobilization and pigment accumulation, further studies integrating isotope tracing

and molecular analyses will be necessary to fully elucidate the underlying mechanisms.

The analysis of *DODA* expression provided molecular support for the observed changes in betacyanin accumulation. *DODA* encodes a key enzyme of the betalain biosynthetic pathway, catalyzing the formation of betalamic acid, the central precursor of all betalains including betacyanins (Gandìa Herrero and Garcìa Carmona 2013). In green plants, NSt strongly induced *DODA* expression, paralleling the increase in betacyanin content, suggesting that pigment accumulation is actively regulated at the transcriptional level (Fig. 3a). Conversely, salinity reduced *DODA* expression, particularly in the red genotype, consistently with the decline in betacyanin concentration. Similar reductions in betacyanin accumulation under SS have been reported in quinoa and other betacyanin-producing species (Sarker and Oba 2018; Causin et al. 2020). Overall, these results indicate that N starvation promotes, whereas salinity limits, the activation of the betalain biosynthetic pathway in *B. vulgaris.*

A similar trend was observed for *CYP76AD1*. Unlike *DODA*, *CYP76AD1* expression was only marginally affected by NSt, suggesting that this step of the pathway is less responsive to N availability (Fig. 3b). Nevertheless, red plants consistently exhibited much higher transcript levels than green plants, supporting the existence of a constitutively active betacyanin biosynthetic machinery in this genotype. SS markedly reduced *CYP76AD1* expression, particularly in red plants, in agreement with the decline in betacyanin content. The partial recovery observed under combined NSt-SS conditions indicates that NSt signaling may alleviate, at least in part, the inhibitory effect of salinity on betacyanin biosynthesis. Taken together, the expression patterns of *DODA* and *CYP76AD1* suggest that the reduction in pigment accumulation under salinity is associated with transcriptional downregulation of key biosynthetic genes, whereas the stimulation of betacyanin accumulation under nitrogen starvation is primarily linked to the induction of *DODA*.

The induction of betacyanins accumulation in green plants under N limitation may reflect stress-driven metabolic reprogramming, in which antioxidant metabolites, such as ASC and GSH, contribute to maintaining redox balance and protecting cellular structures. Overall, these results highlight that the regulation of betacyanin accumulation is strongly genotype-dependent and caution against generalizing responses across phenotypes with distinct basal metabolic states.

Moreover, SS induced a reduction in betacyanins in both genotypes, even though red plants showed a higher betacyanin accumulation when subjected to salinity and N deficiency when compared with those subjected exclusively to salinity. The betacyanin reduction in SS plants results in accordance with the findings of other authors (Jain et al. 2015; Vasconcellos Vilas Boas et al. 2016; Sarker and Oba 2018; Causin et al. 2020). Causin et al. (2020) observed a significant reduction in the accumulation of betacyanins in seedlings of three quinoa genotypes exposed to 300 mM NaCl. This suggests that the synthesis and/or catabolism of these pigmented molecules may vary among different plant species and/or phenological stages within the same individual. This proposition aligns with the findings of Jain et al. (2015), who investigated two distinct morphs of *Disphyma australe* and determined that, although both morphs possessed the genetic capability to biosynthesize betacyanins, one did not induce pigment accumulation in response to SS.

Interaction between N starvation and salinity in *B. vulgaris:* evidence of antagonistic regulation

The analysis of the combined NSt–SS treatment in *B. vulgaris* reveals a response pattern that cannot be interpreted as a simple additive effect of the two single stresses but rather suggests a non-additive and predominantly antagonistic interaction. In the green genotype, N starvation strongly stimulated betacyanin accumulation, whereas salinity alone markedly reduced pigment levels; under combined conditions, betacyanin content was intermediate, indicating that the promotive effect of NSt is partially counteracted by SS. This trend is consistent with the concomitant physiological responses observed in this study, where NSt induced a strong downregulation of photosynthetic activity and nitrogen content, although SS imposed additional ionic and osmotic constraints without significantly affecting biomass.

Such antagonistic behavior likely reflects competing metabolic priorities. N starvation appears to trigger a reprogramming of metabolism toward the synthesis of secondary metabolites, including betacyanins, possibly supported by internal nitrogen recycling and reduced investment in primary metabolism. Conversely, salinity is known to impose energetic and metabolic costs associated with ion homeostasis, osmotic adjustment, and maintenance of cellular integrity, which may limit the allocation of resources to nitrogen-demanding compounds such as betacyanins (Fernandes et al. 2021). In this context, the intermediate pigment levels observed under combined stress conditions suggest that plants prioritize essential survival processes over the accumulation of secondary metabolites.

This interpretation is further supported by the observed modulation of oxidative stress markers and antioxidant systems. Under NSt, reduced levels of H_2O_2 and lipid peroxidation were associated with increased ascorbate content, whereas SS alone led to increased oxidative pressure in the green genotype. Under combined stress, the attenuation of oxidative markers, together with the modulation

of glutathione metabolism, indicates a reorganization of the antioxidant network that may reduce the need for high betacyanin accumulation as a protective strategy.

More broadly, these findings align with recent evidence showing that plant responses to combined abiotic stresses are not predictable from single-stress experiments, as they involve complex crosstalk between signaling pathways and metabolic trade-offs (Suzuki et al. 2014; Pandey et al. 2017; Zandalinas et al. 2018). In this framework, the NSt–SS combination in *B. vulgaris* represents a clear case of non-additive interaction, in which salinity partially suppresses the metabolic reprogramming induced by nitrogen limitation. This highlights the importance of considering stress combinations when evaluating the functional role of secondary metabolites, as their regulation emerges from the integration of multiple, and sometimes conflicting, physiological constraints.

Conclusion

Betacyanins, distinguished pigments produced solely by plants within the Caryophyllales order, exhibit similarities to anthocyanins owing to their red hue and glycosylated composition. However, the N incorporation into their molecular structure contradicts their tendency to accumulate in plants thriving in N-deficient soils. The decrease in betacyanin accumulation induced by SS distances them from the ecological defense and antioxidant role attributed to anthocyanins. In the present experiment, two *B. vulgaris* genotypes, one naturally red and one green-leafed but capable of accumulating betacyanins in their leaves, exhibited similar responses in terms of production and antioxidant system under SS and N starvation. Both genotypes showed an increase in root length in N starved plants. Moreover, they showed a reduction in P_n and g_s values of N starved plants, characterized by an increase in betacyanin accumulation. If N deficiency showed interesting and stimulating results in terms of future research, SS induced a reduction in betacyanin content and antioxidant responses in both analyzed genotypes. Therefore, N starvation can be considered an abiotic stress able to stimulate betacyanin accumulation in plants belonging to the Caryophyllales order, whilst SS induces species specific responses depending on cultivar/species/genotype salt sensitivity. However, the betacyanin accumulation in N starved plants stimulates the curiosity to investigate the origin of the N present in their molecule.

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Data availability Data will be made available on request.

Declarations

Conflict of 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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