

In Vitro Evaluation of Salt Tolerance and Melatonin-Mediated Stress Amelioration in Wild Potato (*Solanum okadae*)

Amar Hundare

a.hundare@soton.ac.uk

University of Southampton - Highfield Campus: University of Southampton <https://orcid.org/0000-0003-2055-3123>

Aneesh Lale

University of Nottingham Division of Plant and Crop Sciences: University of Nottingham School of Biosciences

Neelu Joshi

DY Patil University Deemed to be University

Timothy Robbins

University of Nottingham Division of Plant and Crop Sciences: University of Nottingham School of Biosciences

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Abstract

Salinity is a critical abiotic stress influencing agricultural crop productivity in arid and semiarid environments. This study reports in vitro evaluation of NaCl tolerance of wild diploid potato species *Solanum okadae* and the effect of exogenous MT application on reducing the salt stress effects. In vitro grown plantlets stressed with 50, 100, 150, and 200 mM NaCl had stress tolerance indices of 84.67%, 76.88%, 81.84%, and 67.94% respectively. Compared to the control, 200 mM NaCl had severe adverse effects on growth parameters and decreased total chlorophyll and K^+ content by 88.12% and 25.91%, respectively and increased Na^+ content by 50.17-fold. The rise in catalase activity, Ca^{2+} and Mg^{2+} contents was 1.08-fold, 1.59-fold and 1.25-fold respectively. Melatonin (100 μ M) treatment mitigated the growth inhibition caused by 200 mM NaCl, as demonstrated by an increase of 17.28%, 167%, 25.39%, 36.36% and 6.72% in fresh weight, shoot length, total chlorophyll, chlorophyll b and K^+ content along with decreased Na^+ content and catalase activity by 1.79-fold and 1.08-fold, respectively. This study demonstrates that *Solanum okadae* exhibits inherent salt tolerance, which can be further enhanced by exogenous melatonin application. Melatonin treatment improved physiological and biochemical responses under high salinity, indicating its role as an effective stress ameliorant. These findings suggest that *S. okadae* holds promise for use in potato breeding programs aimed at developing salt-tolerant cultivars and for advancing our understanding of the genetic and molecular mechanisms of salinity tolerance in potato.

1. Introduction

Salinity is a significant environmental stress factor that frequently poses a serious obstacle to the production of crops (Peethambaran et al. 2018). The most common salt that causes toxicity and is responsible for saline soils is NaCl. It affects the growth and productivity of crops by inducing ion imbalance and osmotic and oxidative stress (Jiang et al. 2021). In response, plants have developed several adaptive mechanisms including ion compartmentalization, osmolyte synthesis and antioxidative metabolism, which depend upon activation of molecular processes involved in stress recognition, signal transduction, and expression of stress-related genes and metabolites (Gupta and Huang, 2014). Growth, physiological and biochemical traits are generally measured to screen for salt tolerance. Besides, the stress tolerance index (STI) is another indicator of salt tolerance (Fernandez 1993).

Melatonin (MT, N-acetyl-5-methoxytryptophan), a lipophilic and hydrophilic physiological regulator, plays a significant role in plants' response to abiotic stress by inducing stress responsive pathways (Arnao et al, 2022). Exogenous application of MT has emerged as an effective strategy to mitigate salt stress in various plants, including tomato, rice and cotton (Zhou et al. 2016; Liang et al. 2017; Zhang et al. 2021). It works by regulating the processes such as antioxidant metabolism, photosynthesis, polyamine metabolism, ionic balance, and NO signalling etc.

Under in vivo and in vitro conditions, *S. tuberosum* is categorised as a moderately salt sensitive crop, with a threshold electrical conductivity (EC) level of 1.7 dS/m, as its tuber yield may decline at field

salinity EC levels of 2.0–3.0 dS/m (approximately 22–33mM NaCl) (Shrestha et al. 2023). In the reproductive phase, salinity stress can alter the normal K^+/Na^+ ratio in the leaves, adversely affect the ultrastructure of the leaves, weaken photosynthesis in the leaves and hamper the transfer of carbon from the vegetative tissues to the tubers, as is the quantity and size of the tubers, consequently (Levy & Veilleux, 2007). To control this loss, application of stress ameliorating agents to the potato crop could be an efficient, cost-effective and eco-friendly way. *In vitro* system is generally preferred for salt tolerance studies as it can provide quicker, reproducible and more accurate assessments of plant growth considering the environmental variations in conventional field trials (Banu et al. 2015). However, there are limited reports on the use of such system in potato. In one instance, Zaki & Radwan (2022) performed *in vitro* evaluation of diploid potato *Solanum chacoense* Bitt and identified its clones 'A-6', 'C-8', and 'D-2' as highest salt tolerant. They also determined the effect of exogenous applications of salicylic acid, proline and glycine betaine to reduce salt stress injury in the potatoes. Other instances include *in vitro* screening for salt tolerance (Ahmed et al. 2020) in commercial potato varieties like Innovator, Lady Claire, Kennebec, Burren, Marabel, Hermes and evaluating the effect of MT in relieving salt stress symptoms (Efimova et al. 2023) in *Solanum tuberosum* cv. Lugovskoi. Wild potato species serve as crucial genetic resources for enhancing abiotic stress tolerance in cultivated varieties. *Solanum okadae*, a wild diploid species native to Bolivia, is known for its resilience to environmental stresses like drought (Watanabe et al. 2011) and its compatibility with *Solanum tuberosum* (Camadro et al. 2008), making it an ideal candidate for pre-breeding efforts. These wild relatives often harbour unique physiological and biochemical mechanisms—such as improved ion homeostasis, antioxidant regulation, and osmotic adjustment—that are not present or are diminished in modern cultivars. Despite this potential, studies evaluating salt tolerance in wild diploid potato species remain limited. Such investigations are essential, as they could contribute significantly to the development of salt-tolerant potato lines through conventional or molecular breeding strategies. Therefore, our study aimed to evaluate the salt tolerance of *S. okadae* under *in vitro* conditions and to investigate the potential of exogenously applied melatonin (MT) in ameliorating salt-induced stress symptoms.

2. Materials and methods

2.1 Plant material

True seeds of *Solanum okadae* (accession: OKA7129) acquired from the Commonwealth Potato Collection curated by The James Hutton Institute (Invergowrie, Scotland), were germinated in the greenhouse at Plant and Crop Science department, University of Nottingham, UK. From a 4-week-old seed-derived plant, nodal explants were collected to establish shoot cultures (Hundare A, 2022). Explants were washed under running tap water, surface sterilised using 70% ethanol for 1 minute, followed by a 10-minute treatment with 5% NaOCl, and were rinsed thrice with sterile distilled water. Subsequently, explants (2 cm) were cultured on solidified Murashige and Skoog (MS) medium containing 8 g/L agar, 30 g/L sucrose along with 3 mg/L BA (6-benzylaminopurine), 2 mg/L NAA (1-Naphthaleneacetic acid) and 1 mg/L KIN (kinetin). The pH was adjusted to 5.7 ± 0.1 prior to the addition

of agar and subsequent autoclaving at 121°C and 15psi for 20 minutes. Tissue excision, implantation, and transition were conducted under sterile conditions. Cultures were incubated in culture room with a temperature of $22 \pm 2^\circ\text{C}$, 16-h photoperiod and $\sim 30 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and sub-cultured every week on fresh media to obtain shoot cultures.

Melatonin (MT) (Sigma-Aldrich, St. Louis, USA) was dissolved in 0.1% (v/v) ethanol to a stock concentration of 10 mM and diluted with sterile water to obtain a final working concentration of 100 μM . The final ethanol concentration in the culture medium did not exceed 0.1%.

2.2 Experimentation

Five individual stem nodes derived from *Solanum okadae* shoot cultures were placed in Magenta boxes containing MS medium supplemented with seven different treatments: (i) NaCl at concentrations of 0 (control), 50, 100, 150, and 200 mM and (ii) 200 mM NaCl combined with 100 μM MT. Cultures were maintained under standard growth room conditions ($22 \pm 2^\circ\text{C}$, 16-h photoperiod, $\sim 30 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity). All measurements were taken 30 days after inoculation. No pre-treatment or acclimation phase was applied before the start of the experiment.

The selected NaCl concentrations (50–200 mM) were based on previous in vitro studies in potato, which reported growth inhibition at ≥ 100 mM. The 200 mM level was used to represent severe salinity stress, as it reliably induces physiological and biochemical responses without causing complete lethality (Zaki & Radwan, 2022). The melatonin (MT) concentration of 100 μM was selected based on multiple studies demonstrating its effectiveness in improving plant survival and reducing oxidative damage under salt and heat stress conditions in tomato and cotton (Martínez et al., 2018).

2.3 Measurement of Growth Traits

Shoot height (SH, cm), root length (RL, cm), number of shoots (SN), roots (RN) and leaves (LN), fresh weight (FW, g) and dry weight (DW, g) were measured for plantlets from control and each treatment. The percent Plant water content (PWC) was calculated using the equation (Rashid et al, 2019):

$$\text{PWC} = ((\text{FW} - \text{DW})/\text{FW}) \times 100$$

2.4 Photosynthetic Pigments

Determination of carotenoids (Car), chlorophyll a (Chl a) and b (Chl b) were conducted following the methodology established by Arnon (1949) and Lone et al. (2018). 100 mg of leaf sample was crushed in 10 ml of 80% acetone using mortar and pestle followed by centrifugation at 5000 rpm for 5 minutes. The supernatant was transferred to a fresh tube following the absorbance (A) of the solution at 480nm, 645 nm and 663 nm against the blank (acetone) using a spectrometer (CE 2041 UV/VIS, Buck Scientific, Inc.). Chl a, Chl b, total chlorophyll (total Chl), and Car contents were expressed as micrograms per gram of fresh weight ($\mu\text{g/g}$ FW). The concentrations were calculated using the following equations:

- Total chlorophyll = $20.2(A_{645}) + 8.02(A_{663})$
- Chlorophyll a = $12.7(A_{663}) - 2.69(A_{645})$
- Chlorophyll b = $22.9(A_{645}) - 4.68(A_{663})$
- Carotenoid = $[A_{480} + \{(0.114 \times A_{663}) - (0.638 \times A_{645})\}] \times V / 1000 \times W$

V = Volume of acetone and W = Weight of sample in mg.

2.5 Proline determination

The proline concentration in the extracts was determined following the protocol of Stoleru et al. (2019). Fresh leaves (500 mg) were homogenised in 3% (w/v) 5-Sulfosalicylic acid and centrifuged at 4,000 g for 10 minutes at 4°C. The supernatant was mixed with acid ninhydrin and glacial acetic acid, heated for 60 minutes at 100°C, subsequently cooled, and then 4 mL of toluene was added before measuring the absorbance at 520 nm, using a spectrophotometer and toluene as the blank.

2.6 CAT activity assay

Leaf tissue (200 mg) was homogenised in 3 ml of 100mM PBS buffer (pH 7.8), centrifuged at 10,000x g for 20 min at 4°C and the supernatant collected and kept on ice. For determining catalase activity (Chen & Zhang, 2016), the supernatant was mixed with 100 mM PBS buffer (pH 7.8) containing 30% H₂O₂ and the change in absorbance at 240 nm was recorded for 1 min using a spectrophotometer. One unit of CAT activity was quantified as the amount of enzyme required to catalyse the decomposition of 1 μmole of H₂O₂ per minute at pH 7.0 and it was expressed as U/mg protein

2.7 Histochemical detection of Peroxide (H₂O₂)

H₂O₂ in leaves was detected histochemically by staining the leaf samples with 1 mg/mL DAB (3,3-diaminobenzidine) as described by Daudi & O'Brien (2012). Leaves were then mounted on clean glass slides and covered with coverslip and visualized under microscope (LAS AF, Leica Microsystems) on a plain white background under uniform lighting.

2.8 Elemental profiling using ICP-MS

The ionomics analysis of samples was carried out as described (Danku et al. 2013). Whole plantlets were harvested and rinsed twice in deionised water and quickly sealed. Samples were dried at 80°C for 24h and then ground. Ions were extracted from samples in 0.5% HNO₃. Na⁺, K⁺, Ca²⁺ and Mg²⁺ were assayed using inductively coupled plasma mass spectrometry (ICP-MS) (PerkinElmer NexION 2000 equipped with an Elemental Scientific Inc. auto-sampler and the Syngistix software in collision mode (He)). The concentrations of minerals were expressed in mmol/g on a dry weight basis.

2.9 Salt Tolerance Trait Index (STTI)

To evaluate the limit of salt tolerance (i.e. maximum salt concentration in which in vitro plantlets could survive) and check the effect of MT on stressed plantlets, average of each trait (morphological and

biochemical) as STI was estimated using the following equation (Kim et al. 2018):

$$STTI = \frac{\text{Value of trait under stressed condition}}{\text{Value of trait under no stress}} \times 100$$

For each salt concentration, average STTIs were computed separately for (i) morphological parameters, (ii) pigment traits, (iii) ionic concentrations, and (iv) biochemical traits. No weighting was applied to individual traits. Trait values were not normalized across datasets. The rationale for selecting these parameters was based on their known involvement in salt stress responses and their ease of quantification under in vitro conditions.

2.10 Statistical analysis

For each treatment, 30 explants were used, distributed across six magenta boxes (five explants per box), with each box considered one experimental unit. Experiments were conducted in a randomized design with three independent runs. All measurements were taken from individual explants, and data were presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using SPSS software (version 21.0, SPSS Inc., Chicago, USA). Differences between treatments were assessed using one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) at a significance level of $P < 0.05$.

3. Results

3.1 Growth parameters

All NaCl concentrations except 50 mM significantly declined shoot length, number of shoots, number of leaflets, number of roots, root length, fresh weight, and dry weight (Table 1). Also leaf necrosis was seen at higher NaCl levels. Severe salt stress (200 mM) resulted in reduction in fresh and dry weight, leaf number, and shoot length by 64%, 83.33%, 64.94%, and 82.67%, respectively, alongside a 100% decrease in the number of shoots, roots, and root length compared to control. Although root and shoot numbers were reduced to near zero in some replicates at 200 mM NaCl, plantlets retained some physiological activity as evidenced by measurable proline content, CAT activity, and ionic changes, suggesting survival under stress rather than complete lethality. The PWC increased with increase in the salt concentration with the highest value recorded at 200 mM NaCl stress (86.46%) (Table 1). Under 200 mM NaCl stress, MT application not only increased the fresh weight, shoot length and leaf number by 17.28%, 167% and 90.09%, but also promoted shoot and root formation compared to untreated stressed plantlets.

Table 1

Effect of various NaCl concentrations (mM) and Melatonin (MT) treatment on different morphological parameters and photosynthetic pigments of *Solanum okadae* in vitro

Treatment	0	50	100	150	200	NaCl + MT
FW (g)	2.25 ± 0.6 ^a	1.50 ± 0.1 ^b	1.32 ± 1.2 ^{bc}	1.10 ± 0.7 ^{bc}	0.81 ± 0.3 ^c	0.95 ± 0.4 ^c
DW (g)	0.18 ± 0.0 ^a	0.14 ± 0.0 ^a	0.08 ± 0.0 ^{bc}	0.06 ± 0.0 ^{bc}	0.03 ± 0.0 ^{ab}	0.10 ± 0.0 ^{bc}
PWC (%)	85.38 ± 0.9 ^a	85.83 ± 1.5 ^a	85.92 ± 2.9 ^a	86.26 ± 2.3 ^a	86.46 ± 1.2 ^a	85 ± 1.2 ^a
LN	9.50 ± 4.35 ^{ab}	6.67 ± 2.1 ^a	5.83 ± 0.9 ^{ab}	4.33 ± 2.0 ^{ab}	3.33 ± 1.7 ^b	6.33 ± 2.3 ^{ab}
SN	11.17 ± 4.2 ^a	4.67 ± 4.0 ^b	3.33 ± 3.8 ^b	0.33 ± 0.5 ^c	0.00 ± 0 ^c	1.23 ± 1.1 ^{bc}
SH (cm)	7.3 ± 0.3 ^a	7.2 ± 0.4 ^a	2.30 ± 0.2 ^d	1.9 ± 0.7 ^d	1.2 ± 0.3 ^d	3.0 ± 0.2 ^b
RN	3.50 ± 0.8 ^b	3.40 ± 1.5 ^a	2.00 ± 1.0 ^b	0.0 ^c	0.0 ^c	1.19 ± 0.2 ^c
RL (cm)	1.30 ± 0.0 ^c	1.4 ± 0.1 ^{bc}	1.7 ± 0.2 ^a	0.0 ± 0.0 ^d	0.00 ± 0 ^d	1.1 ± 0.1 ^{ab}
Total Chl	37.74 ± 5.4 ^a	6.42 ± 2.6 ^c	4.60 ± 1.2 ^d	11.81 ± 3.6 ^b	4.48 ± 1.6 ^d	5.58 ± 1.9 ^c
Chl a (µg/g FW)	14.44 ± 3.2 ^a	4.59 ± 1.1 ^b	3.02 ± 1.6 ^c	4.86 ± 1.5 ^b	2.17 ± 1.3 ^d	2.08 ± 1.1 ^d
Chl b (µg/g FW)	23.31 ± 4.4 ^a	1.56 ± 0.1 ^e	1.58 ± 0.8 ^e	7.22 ± 2.4 ^b	2.31 ± 1.1 ^d	3.51 ± 0.4 ^c
Car (µg/g FW)	1.53 ± 1.1 ^a	1.38 ± 0.4 ^c	2.60 ± 1.4 ^d	3.75 ± 0.7 ^e	4.20 ± 1.1 ^b	2.69 ± 0.2 ^d
All values represent the means ±S.E. Different letters within a column indicate significant differences at P< 0.05 by Duncan's multiple range test. Fresh weight: FW, Dry weight: DW, shoot height: SH, Root length: RL, Shoot number: SN, Root number: RN, Leaf number: LN, Chl: chlorophyll, Car: carotenoid						

3.2 Photosynthetic pigments

Chlorophyll (Chl) content in the leaves of stressed plantlets significantly declined compared to control (Table 1). The decline was dose-dependent till 150 mM NaCl where highest levels of chlorophyll a, b and total Chl (4.86 ± 1.5 µg/g FW, 7.22 µg/g FW, 11.81 ± 3.6 µg/g FW, respectively) were recorded. A reverse trend was noted for carotenoid content with the highest level (4.20 ± 1.12 µg/g FW) recorded in plantlets

growing in 200 mM NaCl. Application of MT under 200 mM NaCl showed a significant increase in total chlorophyll (24.55%) and chlorophyll b (51.94%) content and decrease in carotenoid (35.95%) compared to untreated stressed in plantlets.

3.3 Ionic content

The analysis revealed a rise in Na^+ , Ca^{2+} and Mg^{2+} and decline in K^+ levels with salt stress compared to the control (Fig. 1). The rise was highest for Na^+ (50.18-fold) and Ca^{2+} (1.59-fold) at 200 mM NaCl and for Mg^{2+} (2.12-fold) at 150 mM, over control. Slight decline in K^+ level (0.74-fold) was recorded at 200 mM NaCl. MT treatment at 200 mM NaCl notably enhanced the concentrations of K^+ (6%), Ca^{2+} (13%), and Mg^{2+} (17%), while also leading to a significant reduction in Na^+ (44%) concentrations compared to untreated stressed plantlets.

3.4 CAT activity, H_2O_2 and Proline content

NaCl stress significantly elevated CAT activity and proline content, with a highest rise of 7.77% in CAT activity at 200 mM NaCl and 27% of proline content at 150 mM NaCl, over control was recorded (Fig. 2). DAB staining of the leaf tissues of stressed plantlets showed small dark spots under the microscope, indicating increased contents of H_2O_2 (Fig. 3). Supplying exogenous MT helped in lowering the H_2O_2 content and ultimately lowered the CAT activity by 7.09% and proline content by 34% compared to untreated stressed plantlets.

3.5 Salt Tolerance Trait Index (STTI)

The highest STI for morphological traits (89.76%) and photosynthetic pigments (31.29%) was recorded at 50 mM NaCl whereas it was highest at 150 mM NaCl for ionic content (145.78%) and for CAT activity, proline and protein content (108.01%) (Table 2). Plantlets growing in 150 mM NaCl had the highest overall stress tolerance index (81.84%). At 200 mM NaCl stress, exogenous MT treatment significantly enhanced the STI for morphological parameters and ionic content from 38.58–63.31% and 103.42–118.70%, respectively.

Table 2

Salt tolerant trait indices (STTI, %) for various traits of in vitro raised *Solanum okadae* under NaCl concentrations (mM) and Melatonin (MT) treatment

Treatment	Morphological	Photosynthetic pigments	Ionic content	CAT activity & proline content	Mean STTI
50 mM NaCl	89.76 ^a	31.29 ^a	101.17 ^c	98.44 ^{ab}	80.16 ^a
100 mM NaCl	78.44 ^b	21.72 ^{bc}	107.48 ^c	99.87 ^{ab}	76.88 ^b
150 mM NaCl	43.81 ^d	29.75 ^b	145.78 ^a	108.01 ^a	81.84 ^a
200 mM NaCl	38.58 ^d	28.19 ^b	103.42 ^c	101.55 ^{ab}	67.94 ^c
200 mM NaCl + 100 μ M MT	63.31 ^c	23.22 ^{bc}	118.70 ^b	90.55 ^c	73.94 ^b
All values represent the means $\pm$ S.E. Different letters within a column indicate significant differences at P< 0.05 by Duncan's multiple range test.					

4. Discussion

The present study investigated the effects of NaCl stress and stress reliever MT in *S. okadae* under in vitro conditions. In vitro screening of potato genotypes for water stress tolerance has been proposed as an efficient alternative to field-based testing (Gelmesa et al. 2017) and results were found to be like those observed in field conditions (Rashid et al. 2019).

4.1 Morphological and Growth Responses

Salinity stress exerted a profound impact on the morphology and biomass accumulation of *S. okadae* plantlets. All tested NaCl concentrations significantly inhibited shoot length, root length, number of shoots, leaves, and overall biomass. This aligns with previous studies indicating that salinity adversely affects cell elongation and division due to osmotic and ionic stress (Sudharsan et al., 2012; Zaman et al., 2015). The inhibition of leaf formation and root development under high salt conditions could be attributed to energy diversion towards osmotic regulation and ion homeostasis, as maintaining cellular equilibrium becomes metabolically expensive under saline stress (Biswa et al., 2017; Evers et al., 1999).

Interestingly, plant water content (PWC) remained statistically unaltered across all treatments. This contrasts with reports in *S. tuberosum* and *S. chacoense*, where PWC decreased under salt stress (Zaki & Radwan, 2022). The maintenance of PWC in *S. okadae* suggests that this species possesses an intrinsic ability for osmotic adjustment, potentially involving effective solute accumulation, xylem water transport, and stomatal regulation (Sack et al., 2018). Such mechanisms allow the retention of cellular turgor and continued metabolic function, preventing premature senescence and supporting survival at 150 mM NaCl—a level at which many cultivars fail.

At a mechanistic level, salinity-induced reductions in turgor pressure result from progressive dehydration, leading to membrane destabilization and loss of cell viability (Tyree et al., 2003; Chaturvedi et al., 2014). Plants that prevent this through osmotic regulation, such as by maintaining water potential equilibrium between cells and xylem, can continue physiological processes under stress. Our data suggest that *S. okadae* may achieve this via solute accumulation and cellular compartmentalization, maintaining turgidity and delaying growth cessation.

Exogenous application of MT (100 μ M) further promoted shoot and root development under 200 mM NaCl stress, with significant increases in shoot length, root length, shoot number, and leaf count compared to untreated salt-stressed plantlets. This supports melatonin's known role in promoting cell division and elongation under abiotic stress, potentially through the modulation of auxin pathways, ROS homeostasis, and stress hormone signalling (Tan & Reiter, 2020).

While MT did not significantly affect fresh weight, dry weight, PWC, or root number compared to salt-only treatments, the improvements in above-ground morphological traits suggest selective reinforcement of growth-related processes. This indicates that MT may preferentially target shoot meristem activity and leaf expansion, possibly by stabilizing cytoskeletal elements or enhancing carbon assimilation efficiency. These findings align with those of Altaf et al. (2022), who demonstrated that MT-treated tomato seedlings under drought stress exhibited superior morphological traits due to improved antioxidant status and hormone balance.

Taken together, these results indicate that MT confers partial protection to growth-related traits under salt stress in *S. okadae* by enhancing cellular integrity, maintaining hormonal signalling, and promoting shoot meristem activity—mechanisms that collectively improve growth performance despite osmotic and ionic constraints.

4.3 Photosynthetic Apparatus and Pigment Stability

Salinity imposes multifaceted stress on the photosynthetic machinery by disrupting pigment synthesis, degrading chlorophyll, altering stomatal behaviour, and reducing gas exchange. It inhibits nitrogen and magnesium uptake—both crucial for chlorophyll biosynthesis—thereby compromising photosynthetic efficiency (Kaya et al., 2020; Sarker et al., 2019). Moreover, salinity-induced stomatal closure reduces internal CO₂ concentration, and lower stomatal density further impairs photosynthesis (Charfeddine et al., 2019).

In the present study, increasing NaCl concentrations led to a significant reduction in chlorophyll a, b, and total chlorophyll content in *S. okadae* plantlets, indicating stress-induced pigment degradation. These results are consistent with previous reports in both cultivated and wild potato species (Daneshmand et al., 2010) and tomato (Juan et al., 2005). The reduction in chlorophyll under salt stress is linked to elevated chlorophyllase activity and ion toxicity-induced oxidative stress, which accelerates pigment breakdown (Sultana et al., 1999).

Interestingly, at 150 mM NaCl, *S. okadae* showed a relative increase in chlorophyll content compared to other stress levels, suggesting an adaptive response. This mirrors findings in salt-tolerant genotypes of rice and pea where chlorophyll accumulation serves as an indicator of resilience (Misra et al., 1997; Hernandez et al., 2000). The capacity to retain or even upregulate chlorophyll under moderate stress suggests that *S. okadae* activates compensatory mechanisms to maintain photosynthetic capacity, likely involving antioxidant protection of chloroplast structures and regulatory control over pigment biosynthetic genes.

Chlorophyll b, primarily located in the light-harvesting complexes of photosystem II (PSII), increased under stress in this study. This may indicate a structural adaptation to improve light absorption and energy transfer under suboptimal conditions (Kitajima & Hogan, 2003). Given that PSII is highly sensitive to salinity-induced photoinhibition (Kolomeichuk et al., 2020), the maintenance of Chl b content in *S. okadae* may contribute to preserving photosynthetic efficiency under salt stress.

Carotenoids, essential for photoprotection and ROS scavenging, increased with rising NaCl concentrations. Their role in quenching singlet oxygen, stabilizing thylakoid membranes, and dissipating excess light energy is vital under abiotic stress. The increase observed here aligns with findings in *Amaranthus tricolor* and *Solanum acaule* (Wang & Nii, 2000; Daneshmand et al., 2010) and underscores the contribution of carotenoids to stress resilience by preventing lipid peroxidation and preserving photosystem integrity.

Regarding melatonin application, MT-treated *S. okadae* plantlets at 200 mM NaCl exhibited a marked increase in total chlorophyll and Chl b content. This suggests that MT stabilizes the photosynthetic apparatus, likely through upregulation of chlorophyll biosynthesis genes (*CHLH*, *CHLM*, *PORA*) and protection of PSII (Arnao & Hernández-Ruiz, 2019). However, Chl a and carotenoid levels were not significantly affected by MT in this study, which contrasts with findings in tomato (Altaf et al., 2021) and cucumber (Wang et al., 2015), where MT enhanced both Chl a and carotenoids. The variation could be due to species-specific responses or differential sensitivity of pigment types to MT regulation.

In terms of underlying processes, the observed effects may be linked to chloroplast-localized melatonin biosynthesis. Chloroplasts, originating from endosymbiotic cyanobacteria, retain the machinery for melatonin production, including the key enzyme N-serotonin acetyltransferase (SNAT) (Tan et al., 2013; Back et al., 2016). Under salinity, stress-induced ROS accumulation triggers melatonin synthesis within the chloroplast, providing local antioxidant defence and stabilizing pigment-protein complexes (Murch & Erland, 2021). Therefore, the MT-induced increase in Chl b and total Chl under salt stress in *S. okadae* likely reflects a coordinated response involving both enhanced pigment biosynthesis and ROS mitigation within the chloroplast.

In summary, *S. okadae* exhibits a unique capacity to retain or recover chlorophyll under moderate salt stress, likely through regulatory and structural adjustments in pigment biosynthesis and light-harvesting complexes. Melatonin enhances this adaptive capacity by protecting chloroplast function, mitigating

ROS damage, and preserving PSII integrity, contributing significantly to photosynthetic resilience under salinity.

4.3 Mineral Composition

Salt stress, often described as hyper-ionic stress, disrupts plant cellular ion homeostasis primarily through excessive Na^+ accumulation, which inhibits K^+ uptake and alters the K^+/Na^+ balance—crucial for enzymatic activity and protein synthesis (Ishikawa & Shabala, 2019; Maathuis & Amtmann, 1999). In this study, increasing NaCl concentrations elevated Na^+ levels while reducing K^+ levels in *S. okadae*, although K^+ remained relatively stable at lower stress levels (50 mM), indicative of intrinsic regulatory mechanisms.

Notably, MT application at 200 mM NaCl significantly reduced Na^+ accumulation and enhanced K^+ , Ca^{2+} , and Mg^{2+} concentrations, thereby improving the cytosolic K^+/Na^+ ratio. This suggests that MT confers salt tolerance by modulating ion transport and compartmentalization pathways. On a cellular level, melatonin may upregulate tonoplast-localized Na^+/H^+ antiporters (*NHX1*) and K^+ channels (*AKT1*), as demonstrated in other species like apple, maize, and rapeseed (Li et al., 2011; Jiang et al., 2016; Zhao et al., 2018). These proteins mediate Na^+ efflux and K^+ influx to restore ionic equilibrium under salt stress. Additionally, MT likely influences ion flux through ABA and Ca^{2+} -mediated signalling, as seen in the upregulation of Ca^{2+} transporters and SOS pathway components (e.g., *SOS1*, *SOS2*) in other model systems (Vafadar et al., 2020).

The observed increase in Ca^{2+} content under salt stress in *S. okadae* may reflect the activation of Ca^{2+} -dependent signalling pathways. Ca^{2+} sensors such as calmodulin (CaM), CBLs, and CDPKs activate downstream targets like non-selective cation channels (NSCCs), cyclic nucleotide-gated channels (CNGCs), and Ca^{2+} -ATPases, contributing to ionic balance and stress signal transduction (Conde et al., 2011; Adams & Shin, 2014).

Likewise, Mg^{2+} levels increased under salt stress, particularly at 150 mM NaCl, aligning with chlorophyll enhancement observed under the same treatment. As Mg^{2+} is integral to chlorophyll structure and enzyme function, its accumulation may support both photosynthesis and protein synthesis under stress (Gerendás & Führes, 2013).

Together, these results suggest that *S. okadae* employs a coordinated ionic stress mitigation strategy involving selective ion uptake, intracellular sequestration, and signalling crosstalk. Melatonin strengthens this mechanism by enhancing ion homeostasis, reducing ionic toxicity, and modulating transporter gene expression. Future studies should validate the expression of key transporters (*NHX1*, *HKT1*, *AKT1*, *SOS1/2*) in *S. okadae* to substantiate the proposed pathways and further elucidate the melatonin-mediated ionic regulatory network under salt stress.

4.3 Ionic Composition

Salt stress imposes hyper-ionic conditions in plant tissues, primarily due to elevated sodium accumulation and disrupted potassium uptake. In potato, the accumulation of Na^+ in shoot tissues and the antagonistic suppression of K^+ uptake under high salinity disrupt cellular ion balance, affecting membrane integrity, enzymatic activity, and inducing oxidative stress through reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide (Ishikawa & Shabala, 2019). The maintenance of cytosolic K^+/Na^+ homeostasis is thus a critical determinant of salinity tolerance.

In the present study, salt stress led to a clear increase in Na^+ concentrations and a progressive decline in K^+ levels in *S. okadae* plantlets, particularly beyond 50 mM NaCl, consistent with observations in *S. tuberosum* and *S. chacoense* (Zaki & Radwan, 2022; Altaf et al., 2021). Interestingly, despite significant Na^+ accumulation, the K^+ concentrations in *S. okadae* remained relatively stable across treatments, suggesting an intrinsic capacity to buffer Na^+ toxicity and preserve K^+ -dependent metabolic processes. This trait is associated with higher salt tolerance and has been attributed to active ion transport and compartmentalization mechanisms (Maathuis & Amtmann, 1999).

MT application under 200 mM NaCl significantly reduced Na^+ levels and improved K^+ retention, increasing the $[\text{K}^+]/[\text{Na}^+]$ ratio, a recognized marker of salinity tolerance (Flowers & Läuchli, 1983). On a genetic level, melatonin is known to regulate key transporter genes, including *NHX1* and *AKT1*, facilitating Na^+ sequestration into vacuoles and enhanced K^+ uptake (Jiang et al., 2016). In *S. okadae*, the improvement in ionic balance by melatonin likely reflects the upregulation of these ion transport systems, possibly through ABA-dependent or Ca^{2+} -mediated signalling cascades (Kaya et al., 2019). This is further supported by increased Ca^{2+} concentrations in MT-treated plants, which are known to activate SOS pathway components (e.g., *SOS3*, *SOS2*) that regulate *SOS1*-mediated Na^+ efflux (Qiu et al., 2002).

The Ca^{2+} influx observed in MT-treated plants may also trigger downstream signalling through calmodulin, CDPKs, and CBL–CIPK modules, enhancing the expression or activity of plasma membrane and tonoplast transporters (Conde et al., 2011). These interactions likely contribute to reduced cytosolic Na^+ concentrations and stable cellular function under high salt.

Magnesium, a key structural and enzymatic cofactor, also increased significantly under salt stress, particularly at 150 mM NaCl, correlating with elevated chlorophyll content and improved growth. This may reflect effective uptake and utilization of Mg^{2+} in *S. okadae*, possibly supported by reduced antagonism from Ca^{2+} at the root interface (Grattan & Grieve, 1999). The positive association between Mg^{2+} and photosynthetic capacity highlights its contribution to salinity tolerance by supporting chlorophyll biosynthesis, protein stability, and enzymatic reactions (Chen et al., 2018; Ishfaq et al., 2021).

MT enhanced Mg^{2+} and Ca^{2+} levels in stressed plants, which may further stabilize membranes, regulate ROS levels, and promote photosynthesis. These results align with earlier findings in wheat and cotton where melatonin increased K^+ and Ca^{2+} and suppressed Na^+ accumulation under salt and metal stress (Jiang et al., 2021; Kaya et al., 2019). Melatonin's role in ion regulation appears to extend beyond direct transporter activation to include crosstalk with hormonal and redox signalling, influencing both transporter expression and ion partitioning.

Additionally, MT-mediated protection of ion homeostasis may involve vacuolar compartmentalization of Na^+ via V-ATPase–driven NHX-type antiporters, preventing cytosolic ion toxicity while maintaining osmotic balance (Yang et al., 2010; Yuan et al., 2016). The coordination between Ca^{2+} signalling and MT may facilitate long-distance stress signalling, as reported in *Dracocephalum kotschyi* (Vafadar et al., 2020), further underscoring the integrative role of melatonin in systemic salt tolerance.

In summary, *S. okadae* exhibits a robust capacity for ion homeostasis under salt stress, which is significantly enhanced by melatonin through modulation of key transporter systems (*NHX1*, *AKT1*, *SOS1*), signalling elements (Ca^{2+} /CaM, ABA), and cytosolic $[\text{K}^+]/[\text{Na}^+]$ maintenance. These results move beyond phenotypic observations to provide mechanistic insight into the molecular and physiological pathways through which melatonin confers salt tolerance in *S. okadae*. Future research should target the expression analysis of ion transporter and signalling genes to validate the regulatory network underlying MT-mediated salt stress resilience.

4.4 Proline Accumulation

Proline is one of the most critical osmolytes accumulated in plants in response to salinity and other abiotic stresses. Beyond its traditional role in osmotic adjustment, proline functions as a molecular chaperone, ROS scavenger, redox buffer, and signalling molecule (Sharma et al., 2011). The accumulation of proline is typically regulated by increased expression of biosynthetic genes such as *P5CS* and *P5CR*, and/or repression of degradation pathway genes like *PDH* and *P5CDH* (Marco et al., 2015).

In the present study, proline concentration increased significantly in *Solanum okadae* plantlets at 150 mM NaCl, indicating its functional role in osmotic protection and stress adaptation. The elevated proline levels suggest either enhanced biosynthesis through the glutamate pathway or suppressed degradation, consistent with metabolic shifts under moderate salt stress. This accumulation likely aids in ROS detoxification and cellular homeostasis, particularly in leaf tissues where photosynthetic activity must be preserved (Silva-Ortega et al., 2008).

Interestingly, under severe salt stress (200 mM NaCl), proline levels declined markedly, which contrasts with the common expectation of continued accumulation. This reduction may signify a threshold beyond which the metabolic cost of osmolyte synthesis becomes unsustainable, or it could indicate activation of catabolic pathways due to cellular damage or altered hormonal signalling. These findings challenge the notion that proline accumulation is linearly correlated with tolerance and support the view that proline dynamics are tightly regulated in a context-dependent manner.

Melatonin application further modulated proline responses. At 200 mM NaCl, exogenous MT significantly decreased proline content relative to NaCl-only treatment. This decline suggests a shift in stress mitigation strategy from osmolyte-based protection to melatonin-mediated ROS detoxification. MT's antioxidant properties likely reduce the cellular need for proline accumulation as a secondary defence. This is consistent with observations in *Brassica juncea* and fenugreek, where MT decreased

proline under drought or salt stress while still improving plant tolerance (Park et al., 2021; Zamani et al., 2019).

Functionally, MT may regulate proline metabolism by downregulating *P5CS* or upregulating *PDH*, possibly through redox or hormone-mediated signalling pathways, including interactions with ABA and nitric oxide (NO). The reduced proline content in MT-treated *S. okadae* under high salt stress thus reflects an integrated regulatory network where MT substitutes for or suppresses traditional osmotic stress responses by enhancing antioxidant capacity and maintaining ion homeostasis. This underscores the potential of melatonin as a key regulator of cellular reprogramming under abiotic stress in wild potato genotypes.

4.5 Catalase Activity and Hydrogen Peroxide (H₂O₂) Accumulation

Under salt stress, the overproduction of reactive oxygen species (ROS), particularly hydrogen peroxide (H₂O₂), can overwhelm the plant's antioxidant defences, leading to lipid peroxidation, protein oxidation, membrane instability, and impaired cellular metabolism (Yazici et al., 2007). In this study, *Solanum okadae* exhibited a salt concentration-dependent increase in catalase (CAT) activity, indicating the activation of its enzymatic ROS-scavenging system in response to oxidative stress. This adaptive response mirrors that observed in other salt-tolerant species such as *Cassia angustifolia*, *Sesamum indicum*, and *Zea mays* (Agarwal & Pandey, 2004; Koca et al., 2008; de Azevedo Neto et al., 2006) and aligns with reports in salt-tolerant potato genotypes where antioxidant enzyme activities increased proportionally with stress severity (Sanwal et al., 2022).

Catalase, one of the primary ROS-detoxifying enzymes, decomposes H₂O₂ into water and oxygen, thereby preventing oxidative damage to organelles and maintaining redox balance. The observed increase in CAT activity in *S. okadae* under salinity highlights its potential for maintaining cellular homeostasis during stress, supporting its classification as a salt-tolerant species.

Exogenous melatonin further modulated the oxidative response. Melatonin treatment led to a significant reduction in H₂O₂ accumulation, as evidenced by histochemical DAB staining, accompanied by a moderate decrease in CAT activity. This inverse relationship between ROS content and CAT activity in MT-treated plantlets suggests that melatonin not only enhances antioxidant enzyme expression but also acts directly as a potent ROS scavenger. As a multifunctional antioxidant, melatonin's amphiphilic structure enables it to neutralize ROS such as H₂O₂ and superoxide radicals across various subcellular compartments (Szafrńska et al., 2016).

At the level of physiological mechanism, MT application may prime antioxidant defences through both transcriptional regulation and feedback inhibition. MT has been shown to upregulate genes encoding *SOD*, *CAT*, and *APX* under salinity in model systems like *Arabidopsis* and wheat (Chen et al., 2018; Ke et al., 2018), while also downregulating ROS production by stabilizing plasma membranes and suppressing Na⁺ uptake—factors that otherwise exacerbate oxidative stress. The reduced H₂O₂ content observed in

MT-treated *S. okadae* may thus be attributed not only to enhanced enzymatic detoxification but also to the upstream regulation of ion homeostasis and membrane integrity.

Furthermore, MT has been reported to increase the expression of *TaSNAT*, a key gene in its biosynthesis pathway, promoting endogenous MT accumulation under salt stress (Ke et al., 2018). This endogenous elevation could create a feedback loop that enhances systemic ROS tolerance without triggering excessive antioxidant enzyme activity, thereby conserving energy for growth and development.

Taken together, the findings from this study demonstrate that melatonin confers oxidative stress protection in *S. okadae* through a dual mechanism: (1) direct scavenging of ROS, and (2) transcriptional modulation of enzymatic antioxidant systems. These results extend beyond confirmatory observations by illustrating how melatonin orchestrates redox homeostasis and contributes to the broader physiological adaptation of *S. okadae* under high salinity stress.

4.6 Salt Tolerance Trait Index (STTI)

Salt stress disrupts multiple physiological processes in plants, including water balance, ion homeostasis, redox stability, and hormonal regulation. Plants adapt by activating a suite of interconnected mechanisms such as hormonal signalling, ion exclusion and compartmentalization, osmotic adjustment, and detoxification of reactive oxygen species (ROS) (Rana et al., 2019). In wild relatives and landraces, these responses tend to be more robust and integrated than in commercial cultivars, which have undergone intensive selection for yield and marketability at the expense of abiotic stress resilience (Morton et al., 2019). For instance, landraces of tomato have shown superior salinity tolerance compared to commercial hybrids, linked to higher K^+/Na^+ ratios and improved ion regulation (Massaretto et al., 2018).

The Salt Tolerance Trait Index (STTI) was employed in this study as a trait-specific metric to evaluate the relative tolerance of *Solanum okadae* to different salt concentrations and to assess the effect of exogenous MT. The STTI provided insights into how different functional traits respond to salt stress, revealing that *S. okadae* tolerates salinity through distinct physiological mechanisms at different salt levels.

At 50 mM NaCl, *S. okadae* exhibited the highest STTI for morphological traits (89.76%) and photosynthetic pigments (31.29%), indicating that early stress conditions allow the plant to maintain growth and pigment stability. This suggests efficient osmotic adjustment and possibly minimal ion toxicity at lower salt levels. According to the overall STTI analysis, growth and pigment traits were also most stable at this concentration. These findings support earlier studies by Sanwal et al. (2022), who associated salt tolerance in potato genotypes with sustained photosynthetic activity, osmolyte accumulation, and antioxidant enzyme activity.

At 150 mM NaCl, the highest STTI values were observed for ionic content (145.78%) and biochemical traits such as CAT activity, proline, and protein content (108.01%). This implies that under moderate-to-high stress, the plant shifts to a biochemical and ionic adaptation mode—engaging antioxidant defences,

osmoprotectant accumulation (e.g., proline), and enhanced ion regulation (e.g., K⁺ retention and Na⁺ sequestration). The strong ionic STTI values suggest the activation of efficient transport or compartmentalization mechanisms, potentially involving vacuolar Na⁺ sequestration or membrane-bound ion channels. Such responses may be mediated by upregulation of genes like *SOS1*, *NHX1*, and *CAT*, which are known to contribute to ion compartmentalization and ROS detoxification in salt-tolerant species.

The highest overall STIT value (81.84%) at 150 mM NaCl further supports that this concentration represents an upper physiological threshold where *S. okadae* can still maintain integrated functional performance across diverse traits. This aligns with the notion that wild potato species possess adaptive traits that are often lost during domestication (Morton et al., 2019), yet in rare cases, such traits have been retained or reintroduced through breeding (Chourasia et al., 2021). Thus, *S. okadae* demonstrates a high potential as a donor for salt tolerance traits in potato improvement programs.

At 200 mM NaCl, STTI values dropped for morphological and pigment traits, indicating substantial growth suppression and pigment degradation. However, relatively high STTI values for ionic content (103.42%) and antioxidant-related traits (101.55%) were still observed, suggesting that the plants, though morphologically impaired, retained physiological activity. This was further supported by measurable levels of proline and CAT activity, indicating sustained biochemical adaptation in response to severe stress.

By quantifying STTI across functional trait categories and under melatonin treatment, this study provides a mechanistic understanding of how *S. okadae* manages salinity stress and highlights trait-specific physiological strategies that may be exploited for crop improvement. Rather than merely confirming prior knowledge, this approach contributes new insights into the specific adaptive pathways activated in a wild diploid potato species under salt stress, laying the foundation for targeted breeding and molecular validation efforts.

5. Conclusion and Future Prospects

This study highlights the inherent salt tolerance of *Solanum okadae*, a wild diploid potato species, and demonstrates how this resilience can be further enhanced through the exogenous application of melatonin. Even at high salinity levels (up to 200 mM NaCl), *S. okadae* maintained physiological activity, suggesting robust intrinsic mechanisms for osmotic adjustment, ion homeostasis, and oxidative stress mitigation. The application of melatonin significantly improved shoot and root development, chlorophyll retention, and ionic balance while reducing oxidative damage markers such as hydrogen peroxide and catalase activity. These results underscore the dual advantage of utilizing *S. okadae* as a pre-breeding resource for salt tolerance traits and melatonin as an effective biostimulant to bolster stress responses under saline conditions.

Looking ahead, further studies are warranted to dissect the molecular and genetic bases of both *S. okadae*'s natural salt tolerance and melatonin-mediated stress alleviation. Transcriptomic and

metabolomic profiling could reveal key regulatory genes and pathways involved in these processes. Field-based evaluations will also be crucial to confirm the relevance of these findings under agronomic conditions. Moreover, exploring the integration of melatonin biosynthesis genes into cultivated varieties via genetic engineering, and studying its interaction with other plant hormones, could broaden its application in crop improvement. The combined use of resilient wild species and plant-derived signalling molecules like melatonin offers a promising route to enhance stress tolerance and ensure crop productivity in increasingly saline environments.

Declarations

Conflict of interest:

The authors declare that they have no conflict of interest, either financial or otherwise, in this work.

Author contributions

Amar H: Conceptualization, experimental execution, data collection and analysis and writing original draft of the manuscript. **Aneesh L:** Histochemical analysis, manuscript reviewing and editing. **Neelu J:** Data analysis, manuscript reviewing and editing. **Tim R:** Manuscript reviewing and editing. The final manuscript was read and approved by all authors.

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Figures

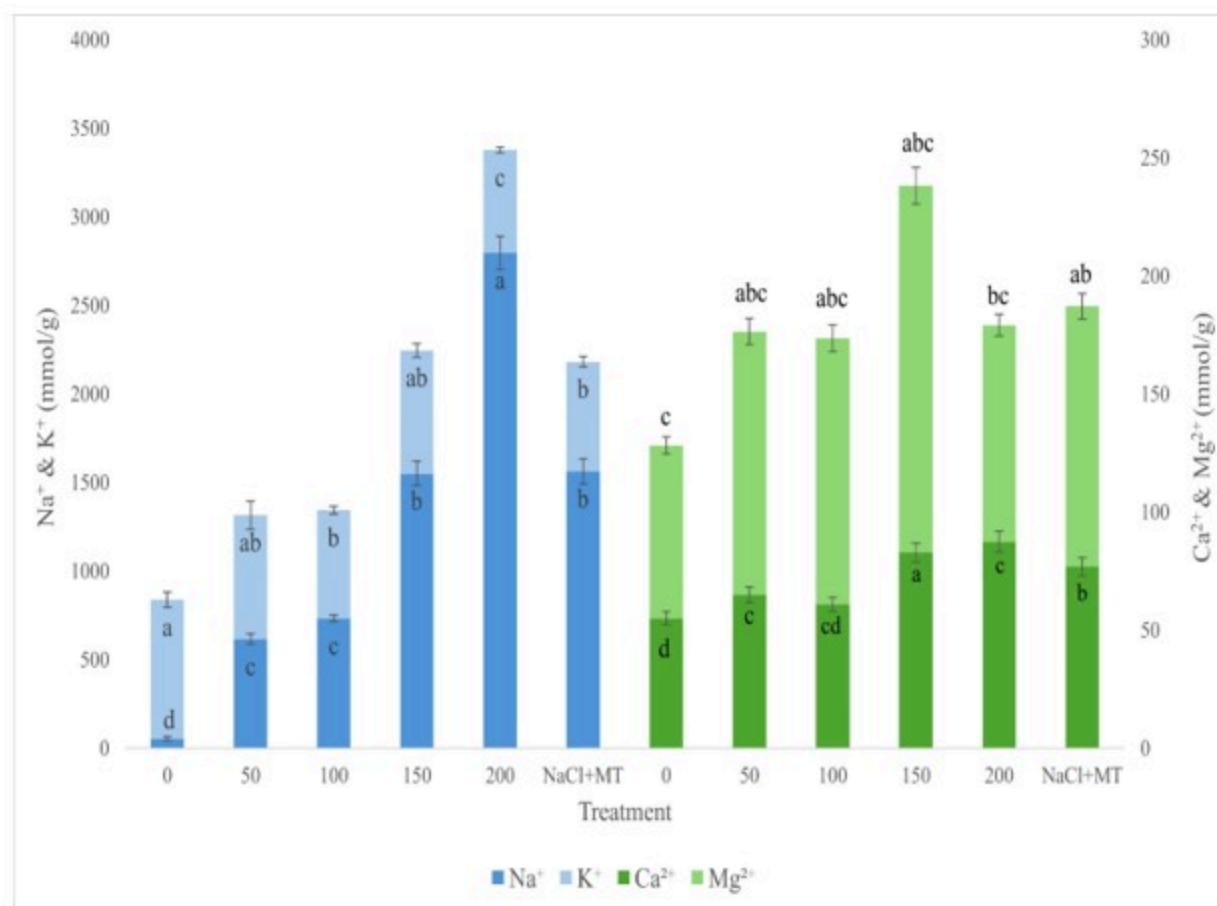


Figure 1

Effect of various NaCl concentrations (mM) and melatonin (MT) treatment on Na⁺, K⁺, Ca²⁺ and Mg²⁺ content of *Solanum okadae*. Means with different letters are significantly different (P < 0.05)

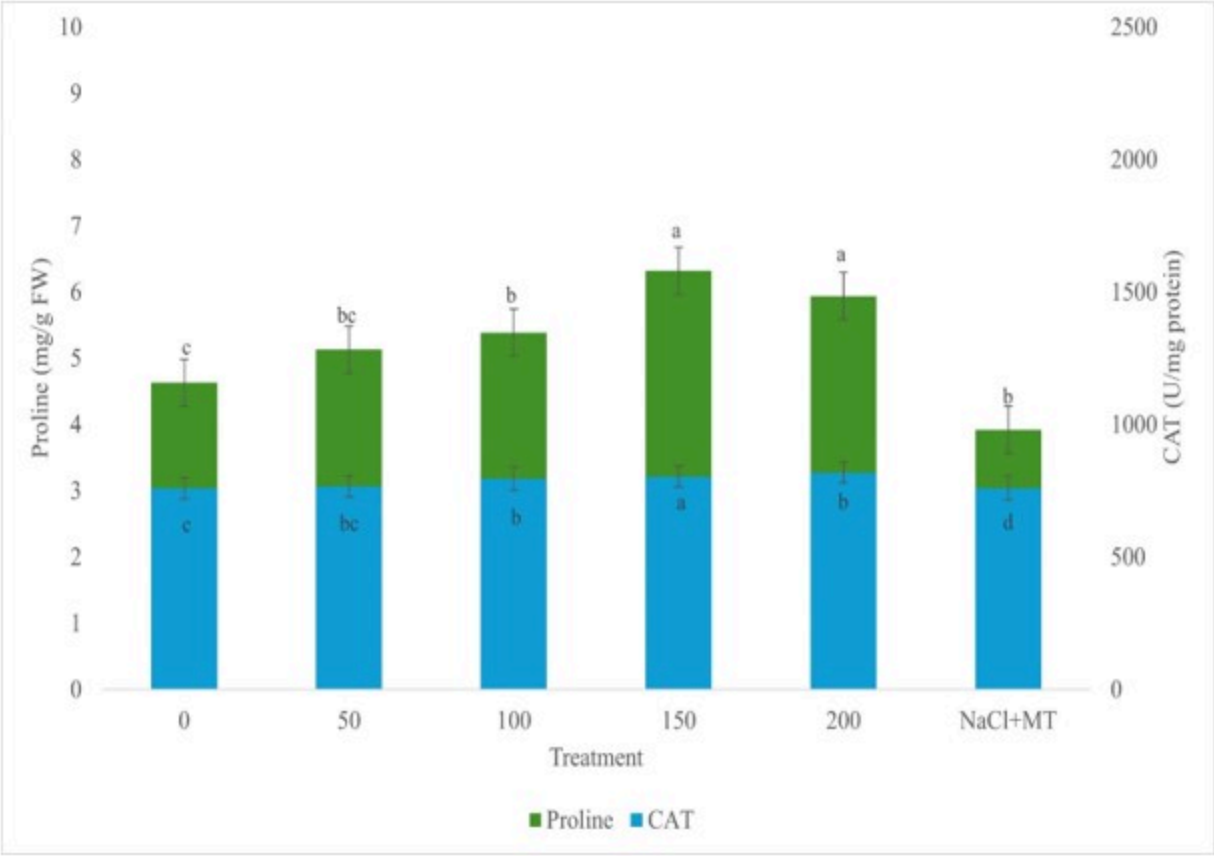


Figure 2

Effect of various NaCl concentrations and melatonin (MT) treatment on proline content and CAT activity of *Solanum okadae*. Means with different letters are significantly different (P < 0.05)

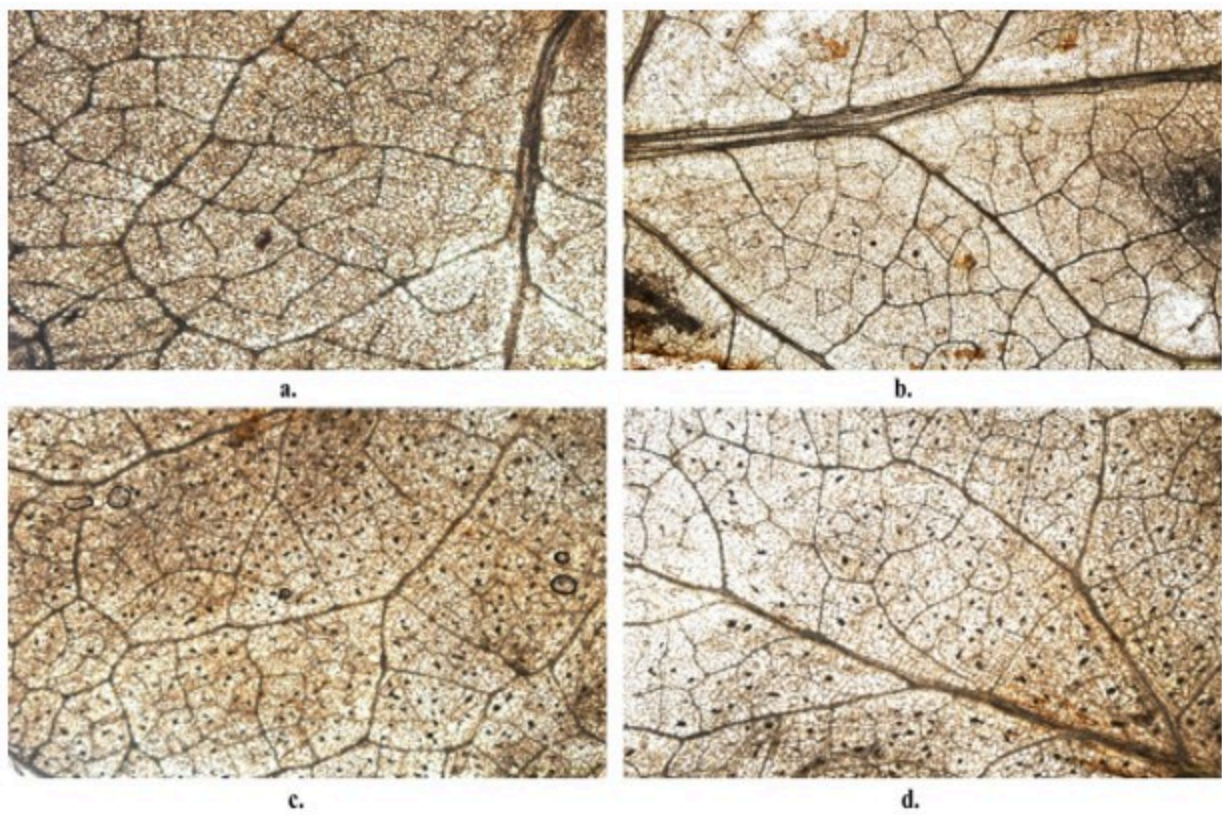


Figure 3

Visualization of H₂O₂ microbursts in response to salt stress using DAB staining. (a.) Control, (b.) 50 mM NaCl (c.) 200 mM NaCl and (d.) 200 mM NaCl+100 μM melatonin