Enhancing salt tolerance in a hybrid poplar *(Populus nigra x maximowiczii)* through exogenous putrescine application: Growth, physiological, and biochemical responses

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Abstract

Introduction: Polyamines regulate plant growth and development under normal conditions and protect plants from a multitude of biotic and abiotic stresses. However, little information is available about how polyamines improve salt tolerance.

Methods: This study was conducted to investigate whether exogenous polyamine application has the potential to maintain poplar growth and yield under a short duration of salt stress. We analyzed the trends in growth and physiological and biochemical responses of hybrid poplar (*Populus nigra x maximowiczii,* clone NM6) plants to salt stress by foliar application of putrescine (a diamine). Leaves of young greenhouse-grown plants were sprayed with 1 mM putrescine on several days following one-time application of 100 mM and 200 mM NaCl via roots. For measurement of growth, plant height, stem diameter, and leaf number for each plant were recorded throughout the experiment. Studies on the physiological and biochemical trends included gas exchange, total chlorophyll, carotenoids, soluble sugars and proteins, amino acids, polyamines, and relative water content in leaves.

Results: Putrescine spray on the salt-treated plants caused a significant increase in chlorophyll a & b, carotenoids, and soluble proteins on a fresh weight basis. Putrescine application caused a significant increase in fructose, glucose, and galactose in all plants, but putrescine spray had a variable impact on the sucrose content of 100 mM NaCl-treated plants. Based on metabolic responses, plants treated with 100 mM NaCl fared better when sprayed with putrescine than those treated with 200 mM NaCl.

Discussion: In conclusion, the results indicate that putrescine did reverse the effects of salt in many ways, and there were trends indicating that sprayed putrescine promotes salt tolerance in young poplar plants.

1 Introduction

Polyamines (PAs) are emerging as critical regulators of plant adaptation to abiotic stress (Minocha et al., 2014, Alcázar et al., 2006, Blázquez, 2024, Atabayeva et al., 2020, Zhao et al., 2024, Pál et al., 2018, Tabur et al., 2024), particularly in mitigating the detrimental effects of salt stress on growth and metabolism (Borromeo et al., 2023, Antoniou et al., 2021, Raziq et al., 2022, Shao et al., 2022). Polyamines like putrescine (Put), spermidine (Spd), and spermine (Spm) are derived from various amino acids (AAs), and influence fundamental processes such as seed germination, photosynthesis, root architecture, and stress signaling (Alcázar et al., 2010, Pál et al., 2021, Pál et al., 2018, Blázquez, 2024). While extensive studies have explored PA functions in several model crop species (Wuddineh et al., 2017, Sundararajan et al., 2021, Pál et al., 2025, Imran et al., 2021, Antoniou et al., 2021, Borromeo et al., 2023), their role in woody plants- particularly fast-growing poplars, remains unexplored despite their significant ecological and economic importance (Zalesny Jr et al., 2019). Woody plants, including poplars, face unique challenges in research due to their long-life cycles and complex anatomical structures, and ethical considerations in genetic studies (Neale and Kremer, 2011, Chen et al., 2021). Understanding the stress adaptation mechanisms in woody perennial plants is crucial for sustainable forestry and climate resilience, especially in the face of global warming and increasing soil salinity.

Abiotic stresses such as salinity, drought, and extreme temperatures impose several constraints on plant growth and productivity (Cramer et al., 2011, Parihar et al., 2015, Zhang et al., 2020a, Zhang et al., 2023). Salt stress in particular, disrupts water uptake, ion homeostasis, photosynthesis, and carbon (C) and nitrogen (N) metabolism, leading to reduced growth, delayed development, and premature senescence (Hanson et al., 2016, Sun et al., 2022, Bargmann et al., 2009, Dinneny, 2015, Calhoun et al., 2023). The accumulation of chloride ions further exacerbates these effects by interfering with nutrient absorption, impacting chlorophyll content, root-shoot ratio, and reproductive development (Cramer et al., 2011, Amiour et al., 2012, Parihar et al., 2015, Zhang et al., 2020a, Kumar et al., 2023, Magallon and Dinneny, 2019). Azimi et al. (2021) reported that N deficiency is a major consequence of salt stress, reducing leaf area and inducing chlorosis, which hinders plant productivity. While N fertilization is a common approach to mitigate these effects, excessive application of N contributes to environmental hazards such as water pollution and algal blooms, necessitating sustainable stress mitigation strategies (Li et al., 2021, Zhang et al., 2020a).

Polyamines have been recognized as key regulators of abiotic stress responses, accumulating in plants subjected to salinity, heavy metals, drought, and temperature extremes (Alcázar et al., 2010, Mohapatra et al., 2010, Pál et al., 2018, Malik et al., 2022, Wang et al., 2024). They play a vital role in modulating carbon metabolism, stabilizing membrane integrity, scavenging reactive oxygen species (ROS), and regulating osmotic balance (Majumdar et al., 2013, Pál et al., 2021, Pál et al., 2025). Polyamines can also enhance N assimilation, thereby improving chlorophyll

synthesis and overall plant growth (Alcázar et al., 2010, Tamang et al., 2021). Several studies have demonstrated that exogenous PA application improves plant resilience by modulating hormone signaling like abscisic acid, gene expression (Paul et al., 2018), and enzymatic responses under salt stress and others (Borromeo et al., 2023, Xiong et al., 2018, Wang et al., 2024). Seed priming with PAs has been reported to enhance photosynthetic pigments, proline accumulation, root growth and biomass production in crops such as tomato (Borromeo et al., 2023), rice (Mahmud et al., 2024, Zhang et al., 2024), rapeseed (ElSayed et al., 2022), and soybean (Sheteiwy et al., 2021) under salinity stress. Additionally, exogenous PA treatments have been shown to increase soluble sugars, proteins, and amino acid accumulation, enhancing abiotic stress tolerance in economically significant plants like grapevine, ginseng, rice (Sundararajan et al., 2021), and tomato (Raziq et al., 2022). Recent studies have also shown that exogenous spermidine (Spd) treatment increased resistance to fusariosis in flax by suppression of PA metabolism (Augustyniak et al., 2025). However, despite these promising findings, studies in woody perennials remain limited, particularly in fast-growing trees like poplars.

Poplars (*Populus* spp.) are among the fastest-growing perennial tree species, widely distributed across North America, China and India (Dickmann, 2001, Hawkins et al., 2003, Plomion et al., 2016, Zalesny Jr et al., 2019, Buell et al., 2023); and serve as key resources for sustainable forestry, bioenergy production, and lumber industry (Yi et al., 2022). Poplars exhibit notable stress tolerance, including resistance to salt and heavy metal contamination (Zalesny Jr et al., 2019, Lin et al., 2023), making them ideal candidates for genetic and physiological research (Guerra et al., 2011). Several varieties of poplars have shown potential to be grown in marginal sandy lands (Ghezehei et al., 2021). Advances in 'omics' technologies and the sequencing of poplar genome (Ma et al., 2019) have enabled deeper insights into their stress response pathways. Notably, transgenic studies in hybrid poplars have identified genes like *NAC13,* which enable salt tolerance (Zhang et al., 2019). Beyond traditional transcriptomics, the emergence of single-cell RNA sequencing (scRNA-seq) has revolutionized poplar research by providing high-resolution gene expression profiles of vascular system and wood plasticity under drought stress (Gomez-Soto et al., 2025, Conde et al., 2022). Additionally, some studies have applied scRNA-seq to dissect root adaptations to soil compaction and salt stress, revealing cell-type-specific regulatory mechanisms (Zhu et al., 2025, Liu et al., Li et al., 2024). Recent computational advances, such as orthologous marker genes groups (OMGs), have further enabled cross-species comparison of conserved cell types, expanding the potential for identifying PA-mediated functions across plant taxa at single-cell resolution (Chau et al., 2025). Although not employed in this study, such approaches offer promising avenues for future research on PA specific stress responses in woody perennials. Understanding how PAs contribute to salt stress tolerance at tissue- or cell-specific resolution could bridge a critical knowledge gap in tree physiology and enhance strategies for improving stress resilience in forestry species.

Among poplar hybrids, *Populus nigra x maximowiczii* (NM6 clone) is widely cultivated in North America due to its fast growth, high biomass yield, asexual propagation capacity, and genetic stability (Labrecque and Teodorescu, 2005, Guerra et al., 2011). Zalesny Jr et al. (2019) also reported that the NM6 clone was more salt-tolerant than other willow and poplar species. Yet no studies have investigated the physiological and biochemical effects of exogenous PA application in mitigating salt stress in NM6 poplars. Given the increasing soil salinity issues affecting

127 forestry and agricultural productivity, understanding how PA treatments can enhance stress
128 tolerance in NM6 poplars is of scientific as well as economic significance.

129 To address this research gap, we investigated the role of exogenous PA application in
130 conferring salt stress tolerance in young NM6 poplars. Using a controlled greenhouse
131 experiment, we assessed the physiological and biochemical responses of NM6 poplar plants
132 to NaCl-induced stress and examined whether foliar-application of Put could alleviate its adverse
133 effects. Specifically, we aimed to answer the following research questions: 1. How does NaCl
134 stress affect the physiological and metabolic processes in NM6 poplar plants? 2. Can exogenous
135 application of Put mitigate NaCl-induced stress; and if so, through which biochemical and
136 physiological pathways? We hypothesize that Put treatment will enhance salt stress tolerance in
137 NM6 poplars by improving plant growth, photosynthetic efficiency, and metabolic stability
138 (soluble sugars, AAs, and PAs) thereby promoting growth and stress resilience. This study
139 provides novel insights into PA-induced stress mitigation strategies in woody plants and expands
140 current knowledge on sustainable approaches to enhance abiotic stress resilience in forestry
141 species.

142

2 Materials and methods

2.1 Plant Material and Growth Conditions

This study utilized young cuttings of hybrid poplar (*Populus nigra x maximowiczii* - NM6) plant, collected from a 5-year-old, healthy tree at the University of New Hampshire (UNH) Kingman Farm. The cuttings were rooted and maintained at the UNH MacFarlane greenhouse for one growing season of (mid-April to late June) before the initiation of experiment.

During the initial growth phase, cuttings (~15 cm in height, 0.6 cm stem diameter) were kept under mist for two weeks in 17.78 cm grow tubes containing PRO-MIX soil with Mycorrhizae**.** The grow tubes were placed in trays to retain excess water, ensuring consistent soil moisture, and plants were watered regularly. After 2 months, well-established plants (~30–38 cm in height with 9–10 leaves) were selected and transferred to 33 cm pots filled with a 1:1 mixture of vermiculite and perlite**.**

The experiments were conducted from July to September 2021 in a controlled greenhouse environment with natural sunlight and a 16-hour photoperiod**.** The temperature in the greenhouse ranged between 22°C and 24°C**,** with a relative humidity of 70%. Plants were irrigated and fertilized twice daily using an automated drip-line irrigation system with Jack's Pure Water LX - Professional fertilizer (J.R. Peters Inc., https://www.jrpeters.com/17-4-17-pure-water-lx). Each plant received 200 mL of water at 08:00 AM and 2:00 PM**,** with additional watering in the evening on with water, if needed.

2.2 Experimental Design

A nonrandomized block design was used in this study, ensuring that plants remained in fixed positions throughout the experiment. Salt treatment with sodium chloride (NaCl) was initiated 21 days after transplanting the cuttings into 33 cm pots, when the plants were approximately 3 months old from the initial collection of cuttings. A total of 36 plants were assigned to 6 treatment groups (n = 6 per treatment): treatment A (control- no NaCl, no Put), treatment B (100 mM NaCl), treatment C (200 mM NaCl), treatment D (control + 1 mM Put foliar spray), treatment E (100 mM NaCl + 1 mM Put foliar spray), and treatment F (200 mM NaCl + 1 mM Put foliar spray).

Putrescine was purchased from Sigma-Aldrich as Put dihydrochloride (≥98% purity, molecular weight = 161.07). A 0.5% Silwet solution was added as a surfactant to enhance the penetration of the 1 mM Put solution. To ensure uniform salt application, drip irrigation was halted 18 hours before salt treatment**.** Salt solutions were applied via root irrigation by pouring 200 mL of NaCl solution per pot at the time of treatment initiation (Day 0) and again 6 hours later. Drip irrigation resumed 6 hours after the final salt application**.** Saucers were placed beneath the pots to prevent water leakage.

For treatments receiving Put (D, E, and F), 1 mM Put foliar spray was applied immediately after salt treatment**.** Each plant received approximately 30 mL of Put per spray session, applied only to the leaves while the soil was carefully covered with plastic sheets to prevent contamination. After spraying, excess solution was manually tapped off the leaves**.** The foliar spray was applied on days 0, 3, 6, and 13.

Leaf samples (~6th leaf from the top) were collected on days 3, 6, 13, and 20 for physiological and biochemical analyses.

188 **2.3 Sample Collection**
189 Plant samples including the remaining leaves and root tissues**,** were collected at various time
190 points before and after treatment application for physiological and biochemical analyses.
191
192 **2.3a Leaf Sampling**
193 Zero-time leaf discs were collected 1 day prior to stress treatments (day 0), which served as a
194 baseline measurement. Sampling was performed from 1representative plant per treatment group.
195 Leaves were washed with fresh water, pat-dried between 2 layers of paper towels, and
196 subsequently collected using specific extraction solutions or buffers (Supplemental Table 1).
197 For post-treatment collections, the fully expanded 6th leaf from the top was sampled. Leaf discs
198 (6.35 mm diameter) were obtained using a standard paper punch and stored in the
199 appropriate solvents or buffers as per day 0 collection.
200 Physiological traits like relative water content (RWC) and chlorophyll content were measured
201 using leaf discs. Gas exchange measurements were taken with LICOR-6400 directly on the plant
202 leaves. Biochemical assays like soluble sugars, PAs, AAs, and total protein were done with leaf
203 discs. All samples (except leaf discs for biomass measurements) were stored at -20 ºC until
204 further analyses.
205
206 **2.3b Roots Sampling**
207 Root samples were collected at the time of harvesting. To ensure cleanliness, roots were
208 thoroughly washed under running tap water to remove residual vermiculite and perlite.
209 Fine secondary roots (2–3 mm in diameter) were carefully snipped from the main root using
210 sterilized scissors. Approximately 100 mg of fresh root tissue was immediately placed into 2 mL
211 microfuge tubes containing respective extraction solutions (Supplemental Table 1) for further
212 biochemical analyses, including PA and AA quantification.
213
214 **2.4 Plant Growth Parameters**
215 Plant growth rate was assessed based on changes in height and stem diameter over time.
216 Measurements were recorded on days 0, 15, 30, and 45 post-treatments with salt. Stem basal
217 diameter was measured using a digital caliper, with a consistent reference point marked near
218 the base of the stem to ensure accuracy across measurement intervals. Growth in height and stem
219 diameter was expressed as a percentage increase using the following formulas:
220 Increase in height (%) = [(Height on next day - height on previous day)/ Height of day 1] * 100
221 Increase in diameter (%) = [Diameter on next day – diameter on previous day)/ Diameter of day
222 1] * 100
223
224 **2.5 Soluble Sugars**
225 Soluble sugars were quantified following a modified protocol from Blagden et al. (2022). Leaf
226 discs (50 ± 2 mg FW) was incubated at 65°C for 30 min in 1 mL of 80% ethanol. The extract
227 was allowed to cool to room temperature for 5 min, followed by vortexing at medium speed for 2
228 min. After centrifugation at 13,000 × g for 8 min, the supernatant was filtered into
229 an autosampler vial using a 0.45 µm nylon syringe filter (Pall Corp., Port Washington, NY) fitted
230 onto a 3 mL syringe (Becton, Dickinson and Company, Franklin Lakes, NJ).
231 Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) was used for sugar
232 quantification based on external standard curves. The mobile phase consisted of 80% acetonitrile
233 (ACN) and 20% double-distilled water (ddH_2O), with a flow rate of 2 mL min^{-1}. The injection

volume was 40 µL into a 200 µL loop, and separation was performed using a Phenomenex Luna-NH_2 analytical column (5 µm, 100 Å, 250 × 4.6 mm, #00G-4378-EO).
A total of 11 sugars were quantified: xylose, arabinose, fructose, mannose, glucose, galactose, sucrose, trehalose, rhamnose monohydrate, maltose monohydrate, and raffinose pentahydrate. Detection was performed using a Shimadzu RID-10A refractive index detector (RID) set at 30°C (Shimadzu Scientific Instruments Inc., Columbia, MD). The total run time was 15 min, including column washing and stabilization between injections. The column temperature was maintained at 25°C throughout the analysis.
Chromatographic data were processed using Perkin Elmer TotalChrom software (version 6.2.1). Peaks were identified by matching retention times with known sugar standards. An 8-point external standard curve (3 mg mL^{-1}) was generated for individual sugar quantification (Supplemental Table 2). Unresolved sugar peaks were quantified as combined concentrations based on their summed peak areas, including xylose + arabinose, glucose + galactose, and trehalose + maltose.

2.6 Polyamines and Amino acids

For the quantification of different AAs and PAs, approximately 40±2 mg fresh leaf discs were collected in 5% perchloric acid ($HCLO_4$) at a ratio of 1:25 (w:v) in 2 mL microfuge tubes. The samples were freeze-thawed three times and processed for dansylation following a modified protocol from Minocha et al. (1994), (Minocha and Long, 2004). After the final thawing, the samples were vortexed at high speed for 2 min and centrifuged at 14,000 × g for 8 min.
For each sample and external standards, 20 µL of an internal standards mix containing 0.05 mM α-methyl-DL phenylalanine (for AAs) and 0.05 mm heptane diamine (for PAs), both dissolved in 5% $HCLO_4$, was added to each tube. Subsequently, 100 µL of the freeze-thawed extract was combined with 100 µL of 2.691 M sodium carbonate and 100 µL of freshly prepared dansyl chloride (20 mg/mL in acetone). The mixture was vortexed and incubated at 60°C for 30 min. After incubation, the microfuge tubes were cooled at room temperature for 3 min, followed by the addition of 45 µL glacial acetic acid to terminate the reaction. Acetone was evaporated using a speed-vac for 10 min, and 1735 µL methanol was added to the mixture.
The methanol extract was filtered using a 0.45 µm nylon syringe filter fitted onto a 3 mL syringe before transferring the solution into autosampler vials. Separation of AAs and PAs was performed using a 15 cm column (Phenomenex Synergi Hydro-RP 80 Å, LC Column 150 x 4.6 mm, 4 µm). Quantification was achieved using a fluorescence detector (Series 200 PerkinElmer) set at 340 nm for excitation and 510 nm for emission. Relative quantification of AAs and PAs was performed using an external standard curve (Supplemental Table 3). Chromatographic data were analyzed using Perkin Elmer TotalChrom software (version 6.2.1), incorporating a multiplication factor within the software to determine the concentration of each component in nmol g^{-1} FW of tissue.

2.7 Total Soluble Proteins

Total soluble protein content was quantified following a modified protocol from Minocha et al. (2019). Fresh leaf discs (50 ± 2 mg FW) were extracted in a freshly prepared 100 mM Tris buffer (pH 8.0) containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM ethylenediaminetetraacetic acid (EDTA), and 10% (v/v) glycerol. The extraction was performed using a three-cycle freeze-thaw method.

The extract was centrifuged at 13,000 × g for 5 min, and the supernatant was used for total soluble protein quantification following the Bradford (1976) method. The analysis was conducted using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin (BSA) as the standard. Absorbance was recorded at 595 nm using a spectrophotometer. A standard curve (0.1–0.5 mg/mL) was generated to quantify protein concentrations in the samples.

2.8 Relative Water Content

Relative water content (RWC) was determined by measuring the (FW) of the leaf tissue immediately after collection. For DW, the leaf samples were incubated in an oven at 70°C for 48 hours. The samples were reweighed after 24 hours to confirm that no residual moisture remained. Relative water content was calculated using the following formula:

$$RWC (\%) = [(FW - DW)/FW] * 100$$

2.9 Chlorophyll Content

Chlorophyll content was quantified following the method described by Minocha et al. (2009). Two leaf discs (8–13 mg FW) were transferred into 2 mL microfuge tubes containing 1 mL of 95% ethanol. The tubes were incubated in a water bath at 60°C for 16 hours, wrapped with aluminum foil to prevent chlorophyll degradation.

Following incubation, the samples were vortexed for 1 min, centrifuged at 13,000 × g for 5 min, and absorbance readings were recorded at A_{664}, A_{649}, and A_{470} nm using a spectrophotometer. The total chlorophyll, chlorophyll a, chlorophyll b, and carotenoid contents were calculated using the equations described by Lichtenthaler (1987):

1. Total chlorophyll (a + b) = $[(22.24(A_{649}) + 5.24(A_{664})) * \text{volume of ethanol(mL)}]/\text{FW of tissue (mg)}$
2. Chlorophyll a $=[(13.36(A_{664}) - 5.19(A_{649})) * \text{volume of ethanol (mL)}]/ \text{FW of tissue (mg)}$
3. Chlorophyll b $= [(27.43(A_{649}) - 8.12(A_{664}) * \text{volume of ethanol (mL)}]/ \text{FW of tissue (mg)}$
4. Total carotenoids $=[(4.8(A_{470}) - 12.7(A_{649}) + 3.65(A_{664}) * \text{volume of ethanol (mL)}]/ \text{FW of tissue (mg)}$

2.10 Leaf Gas Exchange

Gas exchange parameters, including photosynthetic rate (P_n), transpiration rate (E), and stomatal conductance (g_s) were measured on a fully expanded 6th leaf from the apex for each plant. Measurements were taken on 3–4 plants per treatment using a portable photosynthesis system (LI-6400/XT, Li-COR Biosciences, Lincoln, NE, USA).

Gas exchange was recorded between 09:00 and 12:00 under a photosynthetic photon flux density (PPFD) of 1000 µmol m^{-2} s^{-1} provided by a red-blue LED light source (6 cm^2 chamber). Airflow was maintained at 500 µmol s^{-1}, and reference CO_2 concentrations were set to 400 µmol mol^{-1}. The block temperature was maintained at 25°C. Leaf humidity was not actively controlled but ranged between 50–60% during measurements.

2.11 Statistical Analysis

All statistical analyses were conducted with JMP Pro 18 (www.jmp.com/en_us/home.html). Prior to analysis**,** log transformation was applied to all raw data, and statistical evaluations were based on the log-transformed values. Data were analyzed using a two-way analysis of variance (ANOVA) to assess statistically significant differences between treatments within each time

point. Post-hoc comparisons were performed using Tukey's test, with statistical significance set at $p < 0.05$. Graphical figures of the data were generated using the ggplot2 package, and both heatmap and correlation plots were produced with the pheatmap and corrplot packages in RStudio (R version 4.4.1).

3 Results

3.1 Effects of Put spray on Plant Growth and Morphological Symptoms

Changes in height and stem diameter were measured for various days till 45 days after salt treatment. Figure 1 (A, B) indicates that the combination of NaCl with Put enhances growth in terms of both stem length and diameter compared to NaCl treatments alone. Our data suggest that Put had a growth-promoting effect on plants under salt stress, leading to better overall growth metrics. Morphological symptoms in the salt-treated plants began to appear 13 days after salt exposure. Leaf morphology analysis **(**Fig. 1C) under salt stress revealed that 100 mM NaCl and 200 mM NaCl caused more severe chlorosis and necrosis compared to the control (A). However, the application of 1 mM Put reduced these stress symptoms in salt-treated plants. Putrescine-treated leaves displayed a healthier appearance, with markedly less chlorosis and necrosis, highlighting its protective effect under salt-induced damage.

3.2 Effects of Put spray on Gas exchange, chlorophyll and total soluble protein

Leaf gas exchange was measured in the 6th fully matured leaf at 7 and 35 days after NaCl treatment. On day 35, g_s was significantly higher in control plants compared to 100 mM NaCl-treated plants under Put spray (Fig. 2A). Transpiration rate (E) was significantly lower in control vs. 100 mM NaCl under Put at the same time point (Fig. 2C). Net photosynthesis (Pn) remained consistently higher under salt stress vs. control, though differences were not statistically significant (Supplementary Fig. 1C). Carotenoid was significantly higher in Put-sprayed plants *vs.* unsprayed for 200 mM NaCl on the 3rd day (Fig 2B). Total soluble protein was significantly different under salt stress compared to control on 13th day (Fig 2D). These results indicate that Put application mitigates the adverse effects of salinity on gas exchange and pigment stability, while modulating protein accumulation in a stress- and time-dependent manner.

3.3 Soluble sugar content

Fructose content significantly increased under 200 mM NaCl at 6th day (Fig. 3A). Fructose was significantly higher in Put-sprayed plants *vs.* unsprayed for 100 mM NaCl on the 6th day. Glucose and galactose were non-separable in our HPLC system; hence are represented together. Glucose+galactose levels were significantly lower in 200 mM NaCl treated plants compared to Put sprayed control plants on the 6th day (Fig. 3B). Glucose + galactose levels were significantly lower in 200 mM NaCl-treated plants at 6th and 13th day. Sucrose levels were significantly lower in Put-sprayed plants *vs.* unsprayed for 100 mM NaCl on the 6th day (Fig. 3C). Sucrose was significantly higher for 200 mM NaCl on the 13th day. Sucrose was also significantly higher in Put-sprayed plants than those unsprayed for control and 100 mM NaCl on the 13th day. Our results also showed that log sucrose contents were also significantly higher in 100 mM NaCl treated plants compared to the control plants. These findings suggest Put may help regulate sugar accumulation under salt stress in a time- and sugar-dependent manner.

3.4 Amino acids and Polyamines content in leaves

Plants under 100 mM NaCl treatment, has significantly higher Gln, Ser, Pro and Ala content (Fig. 4A, B, 5A, B) on Day 6. Foliar spray of Put also increased Gln, Ser, and GABA levels in NaCl-treated plants (Fig. 4). Gln and Ser was significantly higher for Put-sprayed plants 200 mM NaCl compared to control on Day 13 (Fig. 4A, B). Additionally, His and Glu was significantly

373 higher in 200 mM NaCl compared (both unsprayed and sprayed) to control on Day 6
374 (Supplemental Fig.2). Foliar spray of Put significantly changed endogenous Spd content under
375 200 mM NaCl on Days 6 and 13 (Fig. 5C). However, there was varied level of Put and Spm
376 content under salt treatment, but they were not significantly different after LogNormal
377 transformation (Supplemental Fig. 1A, B). These findings suggest that Putrescine application
378 influences AAs accumulation under salt stress in a time- and AA dependent manner.

379 Amino acids and PAs content of the roots were analyzed (with HPLC) only at 21 days after NaCl
380 treatment at the termination of the study. Salt significantly increased Gln, Orn, Ser,
381 Arg+Thr+Gly, Phe+Cys accumulation (in both concentrations of NaCl) compared to the control
382 (Fig. 6A, B). Polyamines like Spd was significantly reduced in salt exposure roots (Fig. 6C).
383 These findings strongly suggest that salt stress influences AAs and PAs accumulation.

384

385 **3.5 Combined analysis of polyamine metabolic cycle in roots**

386 To better understand the metabolic coordination among AAs and PAs in roots under salt stress,
387 we conducted a correlation analysis based on their relative contents across control, 100 mM
388 NaCl, and 200 mM NaCl treatments (Fig. 7A). The heatmap revealed 2 major metabolite
389 clusters: one comprising Gln, Pro, Ser, GABA, Put and another dominated by Spm, Spd, and
390 Ala. Glutamine showed strong positive correlations with Ala, Ser, and Arg+Thr+Gly, but was
391 significantly negatively correlated with Spd and Lys ($p < 0.05$). Similarly, Gly, Ser, and GABA
392 were significantly positively correlated with Gln (Fig. 7B). These findings suggest that
393 increasing salt concentrations may shift nitrogen metabolism in roots, reducing amino acid
394 accumulation while enhancing PA synthesis, possibly as part of a salt stress adaptation response.
395
396

4 Discussion

Soil salinity is a growing global threat to agriculture and forestry, significantly reducing crop yields and limiting the availability of arable land. Salt stress adversely affects plants by limiting growth, water uptake, inducing toxic ion accumulation, and triggering oxidative stress (Hasanuzzaman et al., 2013, Safdar et al., 2019, Rady et al., 2023, Sun et al., 2022, Dinneny, 2015), which hampers plant growth and productivity (Liu et al., 2023, Rodríguez Coca et al., 2023, Zhang et al., 2023, Sun et al., 2022). The primary symptoms of salinity stress are stunted plant growth, leaf burning, chlorosis, leaf rolling, reduced water uptake, nutrient deficiency, stomatal closure, photosynthesis inhibition through reduced leaf area, and altered metabolism (Hasanuzzaman et al., 2013, de Oliveira et al., 2013, Sun et al., 2022, Calhoun et al., 2023). Plants tolerate salt by reprogramming their physiological, molecular, and biochemical responses (Gupta and Huang, 2014, Yang and Guo, 2018, Raza et al., 2022). Different salinity levels have varying effects on plants, and the physiological and biochemical responses may also differ in different plants (Nguyen et al., 2018, Ding et al., 2010, Zhao et al., 2021). It has been studied by Ding et al. (2010) that salt-tolerant plants like *P. euphratica* Oliv had high up-regulation of antioxidant enzymes compared to salt-sensitive *P. popularis 35-44*. It has been found by Zalesny Jr et al. (2019) that salt-tolerant *P. nigra X maximowiczii* (NM2 and NM6 hybrid clones) had superior mineral uptake capacity from high-salinity soils than in low-salinity soils. Seed priming or exogenous treatment with various hormones or nitrogenous compounds can help mitigate plant salt stress damage when the plants grow up (Ma et al., 2012, Zafar et al., 2022, Eisa et al., 2023, Srivastava et al., 2021).

Polyamines like Put, Spd have been widely studied for their protective roles in alleviating abiotic stress in crop plants (Nahar et al., 2016, Alcázar et al., 2020, Ma et al., 2022, Xiong et al., 2018, Blázquez, 2024). High tolerance to salt stress due to foliar Put application has been reported in many plant species (Supplemental Table 4). Despite the growing interest in polyamines as potential bio stimulants, their role in salt stress tolerance in woody perennials, particularly poplar, remains largely unexplored. In this study, we investigated the effects of exogenous Put application on hybrid poplar under salt stress, aiming to elucidate its potential as a non-transgenic, physiological intervention for enhancing stress resilience.

4.1 Effect of putrescine on physiological parameters

Visible stress symptoms, such as leaf tip burn, chlorosis, and browning, began to appear in NM6 poplar plants after 13 days of exposure to 100 mM NaCl. Interestingly, 200 mM NaCl-treated plants exhibited milder visual symptoms, with less leaf burn and chlorosis. Plants treated with Put alone developed brown spots near the leaf tips but maintained darker green pigmentation overall. However, Put-sprayed plants under salt stress exhibited more severe symptoms, with signs of salt-burn appearing in the middle parts of the leaves. In addition to leaf symptoms, salt-treated plants also exhibited slower growth and gradual leaf wilting, similar to previous findings in salt-stressed cotton reported by Cao et al. (2020) and microalgae Calhoun et al. (2023). Notably, the plants treated with 100 mM NaCl and sprayed with Put demonstrated the most vigorous growth among all treatment groups, suggesting a possible protective or growth-promoting effect of Put under moderate salt stress. Abiotic stress is known to induce long-term changes in gene expression, contributing to the development of molecular stress memory (Wang et al., 2014, Georgii et al., 2019). Wang et al. (2014) reported that *Zea mays* under prolonged drought stress showed enhanced photosynthetic activity at later stages. A similar pattern was

observed in our study, where NM6 poplars exhibited higher net photosynthesis rates after 35 days of salt exposure compared to the early (7-day) stress period. Furthermore, after 35 days, Put-sprayed plants showed higher stomatal conductance and transpiration rates than unsprayed controls under salt stress, aligning with previous observations in *Camellia sinensis* and *Cucumis sativus* by Xiong et al. (2018) and Shen et al. (2019). These findings suggest that Put may enhance physiological resilience over time by modulating long-term stress responses and photosynthetic recovery. Salt stress also affected the total soluble protein content. We observed a significant reduction in protein levels under salt, likely due to increased protein degradation or decreased synthesis, which could account for the concurrent rise in free amino acid pools. However, Put spray under 100 mM NaCl restored protein levels, while under 200 mM NaCl, sprayed plants had lower protein content than unsprayed counterparts. Putrescine treatment did not affect protein levels in non-stressed plants, indicating its stress-dependent influence. Relative water content (RWC) also increased after 13 days of salt exposure, though the Put spray did not affect RWC under stress. This suggests that changes in soluble protein are more likely due to active metabolic regulation than water availability. Similarly, the FW/DW ratio increased under salt, reflecting possible osmotic adjustment.

4.2 Effect of putrescine on soluble sugars

Liang et al. (2018), Liu et al. (2022) and Zhang et al. (2024) reported that one of the most common adaptive responses to abiotic stress in plants such as *Arabidopsis thaliana*, *Populus euphratica*, *Morus multicaulis* and *Oryza sativa* is the accumulation of soluble sugars. This osmotic adjustment is a well-documented mechanism in various species including Tartary buckwheat, where sugars act as key osmolytes. In our study, we observed a significant increase in fructose and sucrose levels following 6 days of salt stress, consistent with these findings. In contrast, glucose + galactose levels were significantly reduced, indicating a differential modulation of carbohydrate metabolism under salt exposure. These shifts may be part of a stress-induced self-regulatory mechanism, wherein sugars contribute to osmotic balance, protect membrane stability, and support energy supply for metabolic adaptation. It has been previously established that such osmolyte accumulation also aids in maintaining high intracellular K^+ and favorable Na^+/K^+ ratios, while preventing dehydration and oxidative damage (Liu et al., 2022, Azeem et al., 2023). Moreover, Put-sprayed NM6 plants exhibited significantly enhanced sugar accumulation under salt stress compared to unsprayed controls. This aligns with earlier findings in *Cucumis sativus* by Yuan et al. (2015), suggesting that exogenous polyamines may regulate carbon metabolism under salt stress through crosstalk between the PA and sugar synthesis pathways. Therefore, our results indicate that Put may contribute to sugar-mediated salt tolerance in poplar by enhancing osmotic adjustment and supporting cellular metabolism under stress.

4.3 Effect of putrescine on amino acids and polyamines

Polyamines (PAs) are small nitrogen-rich compounds known to mitigate salt-induced damage in plants through diverse biochemical and physiological mechanisms (Saha et al., 2015, Choudhary et al., 2023, Alcázar et al., 2010). Under salt stress, plants often accumulate high levels of PAs, which help reduce lipid peroxidation, stabilize membrane integrity and ionic homeostasis, and regulate stress-responsive genes (Alcázar et al., 2020, Minocha et al., 2014). The biosynthesis of PAs is upregulated through key enzymes such as arginine decarboxylase (ADC) and ornithine

decarboxylase (ODC) (Attia et al., 2022, Tailor et al., 2019). Following their synthesis from methionine, S-adenosylmethionine (SAM) is converted to decarboxylated SAM (dcSAM), which acts as a precursor for spermidine (Spd) and spermine (Spm) production via SPDS and SPMS, respectively (Groppa and Benavides, 2008). Notably, Spd can be back-converted to putrescine (Put), a process that generates hydrogen peroxide (H_2O_2) as a byproduct, further linking PA metabolism to oxidative signaling (Moschou et al., 2012).

In this study, salt stress induced time-specific increases in Put and Spm content in the leaves, especially under 100 mM NaCl, while Spd levels decreased over time. The accumulation increased under salt in the roots, whereas Spd levels declined, and Spm was not detected. These responses are consistent with prior studies showing that PA profiles under salt stress are species-specific and dynamically regulated (Zhang et al., 2020b, Huo et al., 2020, Baniasadi et al., 2018, Ghalati et al., 2020, Shu et al., 2015). The importance of Spm in stress adaptation is further supported by studies in *Arabidopsis*, where Spm synthase mutants were more salt-sensitive (Marco et al., 2019).

Polyamine catabolism is tightly linked to amino acid (AA) metabolism through shared intermediates and signaling molecules. Several studies have shown that PAs act as stress messengers influencing the accumulation of stress-related amino acids such as GABA, Ala, and Pro (Verslues et al., 2023, Gupta et al., 2013, Marco et al., 2019, Singh and Roychoudhury, 2020, Srivastava et al., 2021). Putrescine spray under salt stress further influenced AA profiles. In our study, Glu, Ser, Ala, His, Arg+Thr+Gly, Gln, GABA, and Pro increased in the leaves following salt exposure, particularly after 6 days, indicating a time-dependent AA response. In the roots, salt treatment led to elevated levels of Gln, Arg+Thr+Gly, Phe+Cys, Orn, His, Ser, and Pro. The accumulation of these AAs may reflect a metabolic shift supporting osmoprotection and nitrogen storage in poplar under salt stress (Srivastava et al., 2021, Verslues et al., 2023, Wilhelmi et al., 2025). A notable decline in Lysine in roots under 200 mM NaCl may be attributed to the translocation of these AAs to aerial tissues, as previously reported in *Camellia sinensis* (Hijaz et al., 2018). Interestingly, Gln was positively correlated with GABA, Gly, and Serine in roots, suggesting that enhanced Gln biosynthesis under salt stress enhance precursors for producing other AAs. Notably, the interaction between AA metabolism and the TCA cycle may contribute to enhanced salt tolerance, as Ali et al. (2019) suggested, providing a metabolic basis for the coordinated changes observed in our study.

5 Conclusions

This study demonstrates that *Populus nigra* × *maximowiczii* (clone NM6) exhibits adaptive physiological and metabolic responses under salt stress, particularly when treated with exogenous Put. Under 100 mM NaCl: a level that reflects marginal salinity; Put-treated plants showed improved biomass accumulation, enhanced conductivity rates, better maintenance of relative water content (RWC) and fresh/dry weight ratio (FW/DW) and increased soluble protein and amino acid levels. These results suggest improved osmotic adjustment and metabolic stability, contributing to greater salt stress resilience. Given its performance under moderate salinity, NM6 poplar shows promise as an alternative species for marginally saline soils, mainly when supported by low-cost, non-transgenic interventions such as Put application. Due to the unique role of deep-rooting system, soil-binding capacity, and faster growth, NM6 offers significant potential for land restoration, sustainable silviculture, and bioenergy production in marginal sandy land. Future strategies integrating physiological treatments with genetic improvements could further enhance their role in saline land management.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contribution

SK, RM and SC conceived and designed the experiment. SK, MW, MG, RM, SL collected the samples. RM supervised and provided resources for HPLC. SK, SL, analyzed the data. SK wrote the manuscript. SM and RM supervised the results and analysis. SM supervised and edited the draft manuscript.

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Generative AI statement

The author(s) declare that some Generative AI was used for checking grammatical statement for the creation of this manuscript.

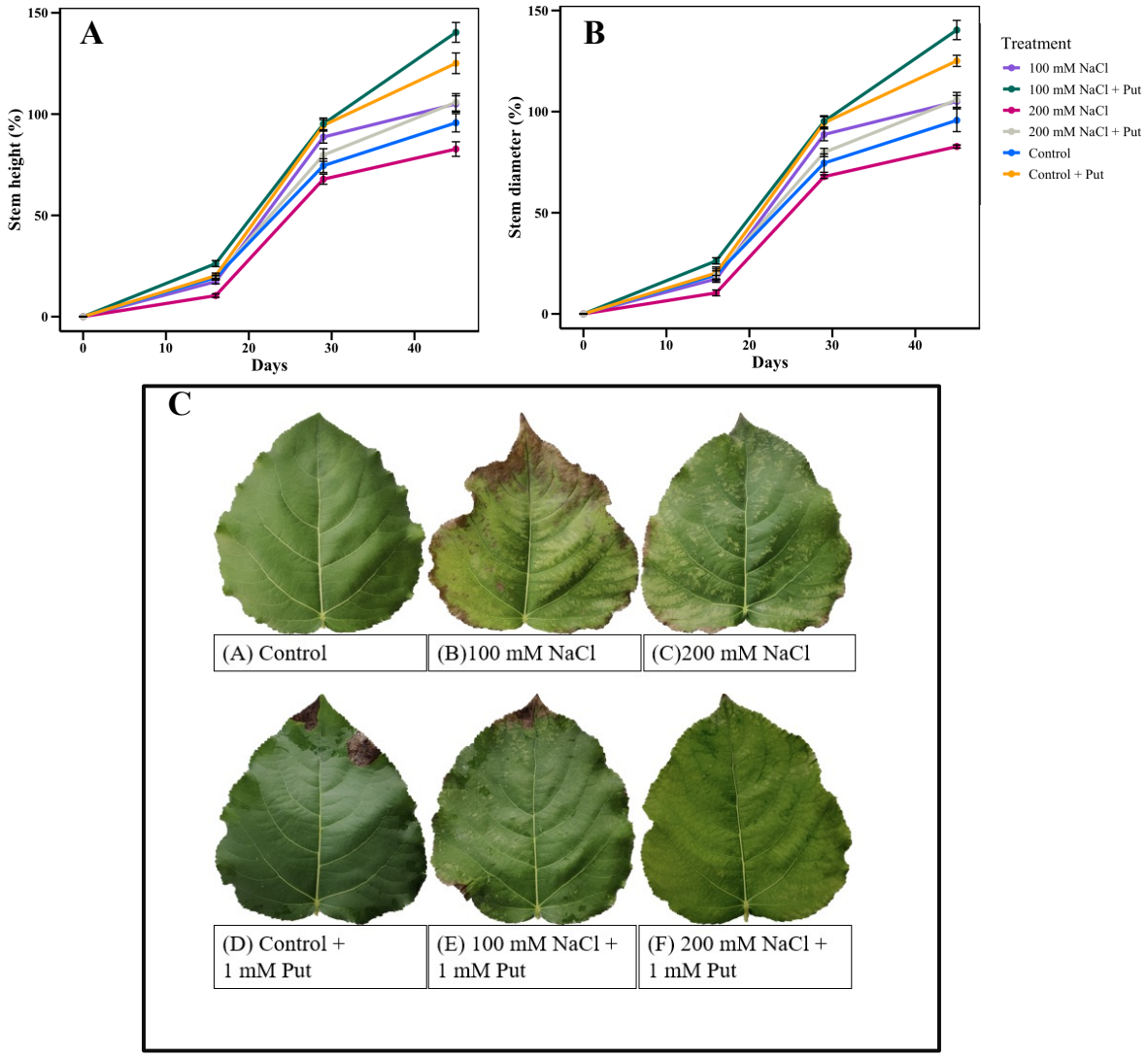


Figure 1: The effect of two different concentrations of NaCl (± Putrescine spray) on the growth and morphology of hybrid poplar NM6 plants. (A, B) Average stem length and stem diameter changes over 45 days of salt treatment. (C) Morphological symptoms of leaves under different treatments**:** (A) Control, (B) 100 mM NaCl, (C) 200 mM NaCl, (D) Control + 1 mM Put, (E) 100 mM NaCl + 1 mM Put, (F) 200 mM NaCl + 1 mM Put. Data represent mean ± SE (n = 6).

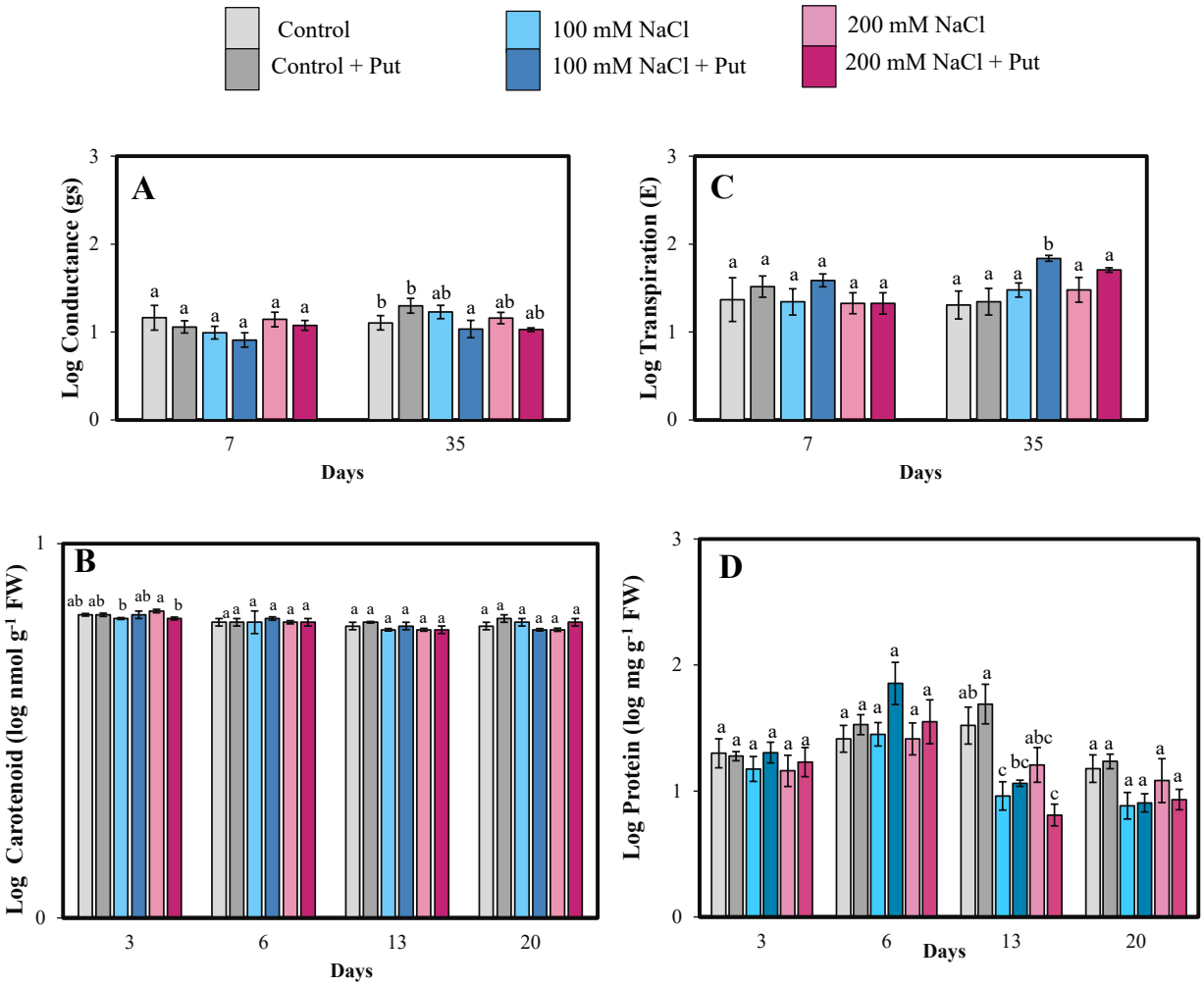


Figure 2. Effect of two different concentrations of NaCl (± Putrescine spray) on physiological accumulation in hybrid poplar NM6 leaves over time. (**A**) Log Conductance, (**B**) Log Carotenoid, (**C**) Log Transpiration, and (**D**) Log Protein measured at 3, 6, 13, and 20 days after salt treatment. Different letters indicate statistically significant differences ($p < 0.05$) among treatments. Data represent mean ± SE (n = 5).

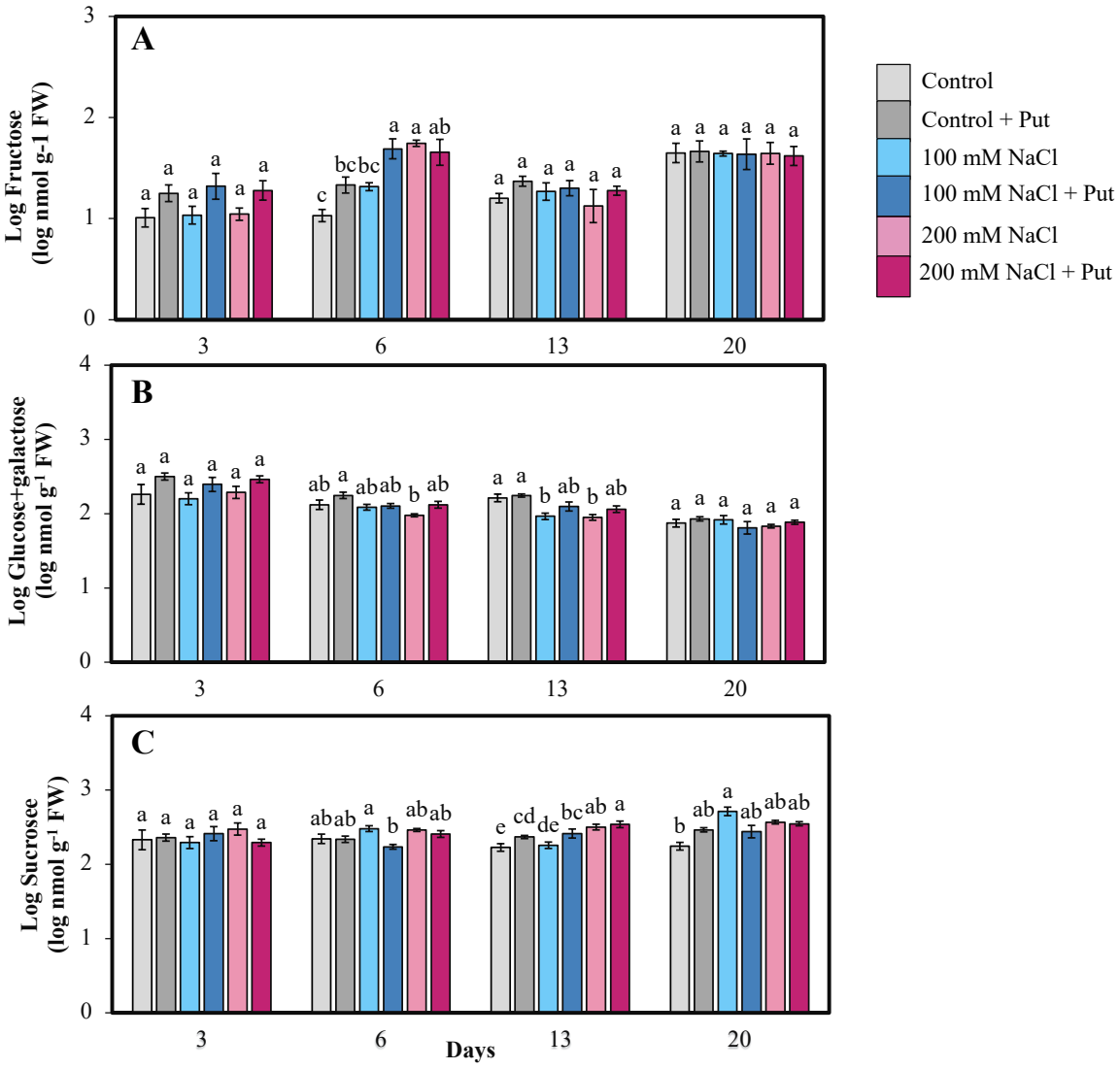


Figure 3: Effect of two different concentrations of NaCl (± Putrescine spray) on soluble sugar accumulation in hybrid poplar NM6 leaves over time. (**A**) Log Fructose, (**B**) Log Glucose + Galactose, and (**C**) Log Sucrose measured at 3, 6, 13, and 20 days after salt treatment. Different letters indicate statistically significant differences ($p < 0.05$) among treatments. Data represent mean ± SE ($n = 5$).

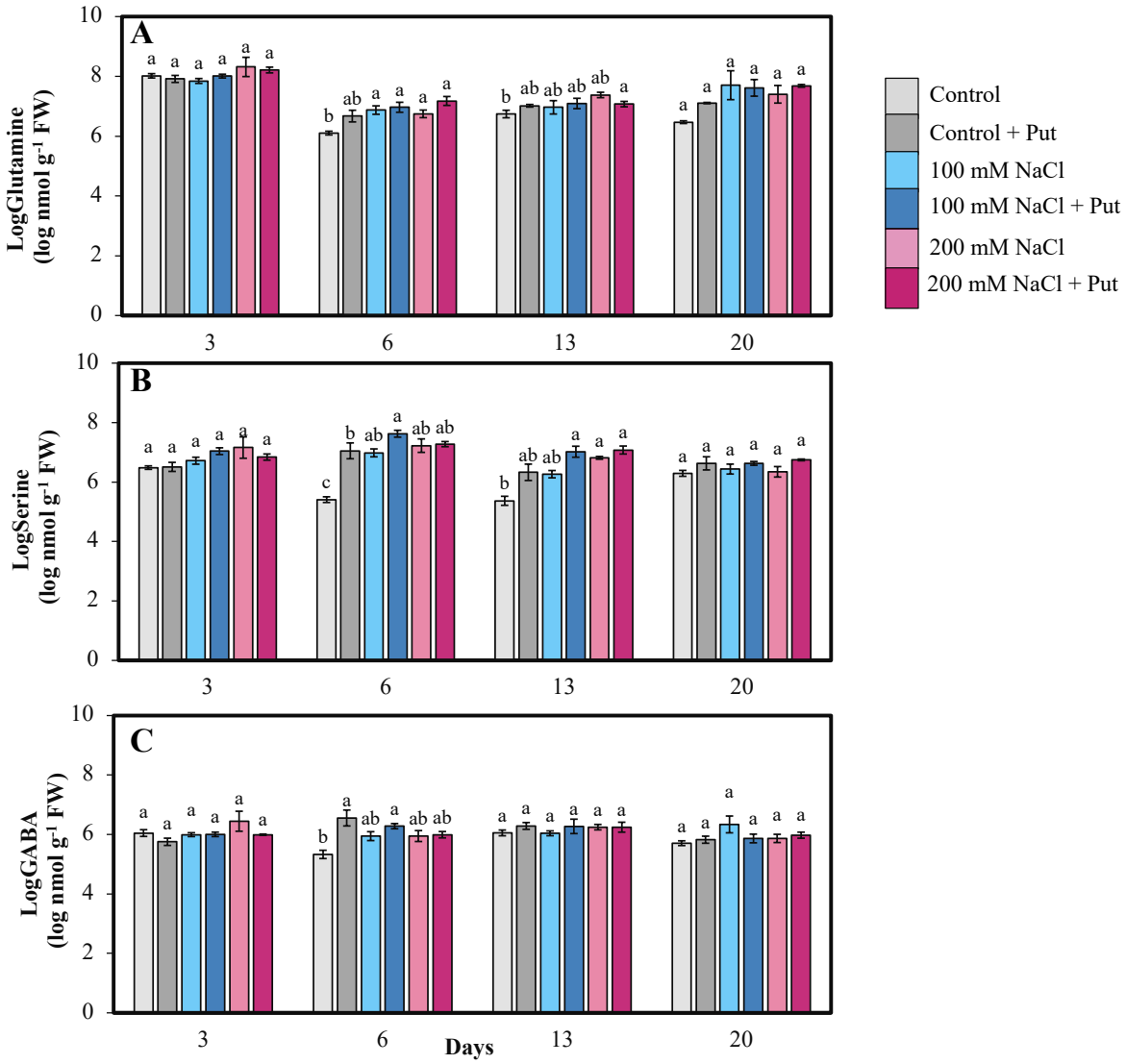


Figure 4: Effect of two different concentrations of NaCl (± Putrescine spray) on amino acids accumulation in hybrid poplar NM6 leaves over time. (**A**) Log Glutamine, (**B**) Log Serine, and (**C**) Log GABA measured at 3, 6, 13, and 20 days after salt treatment. Different letters indicate statistically significant differences ($p < 0.05$) among treatments. Data represent mean ± SE (n = 5).

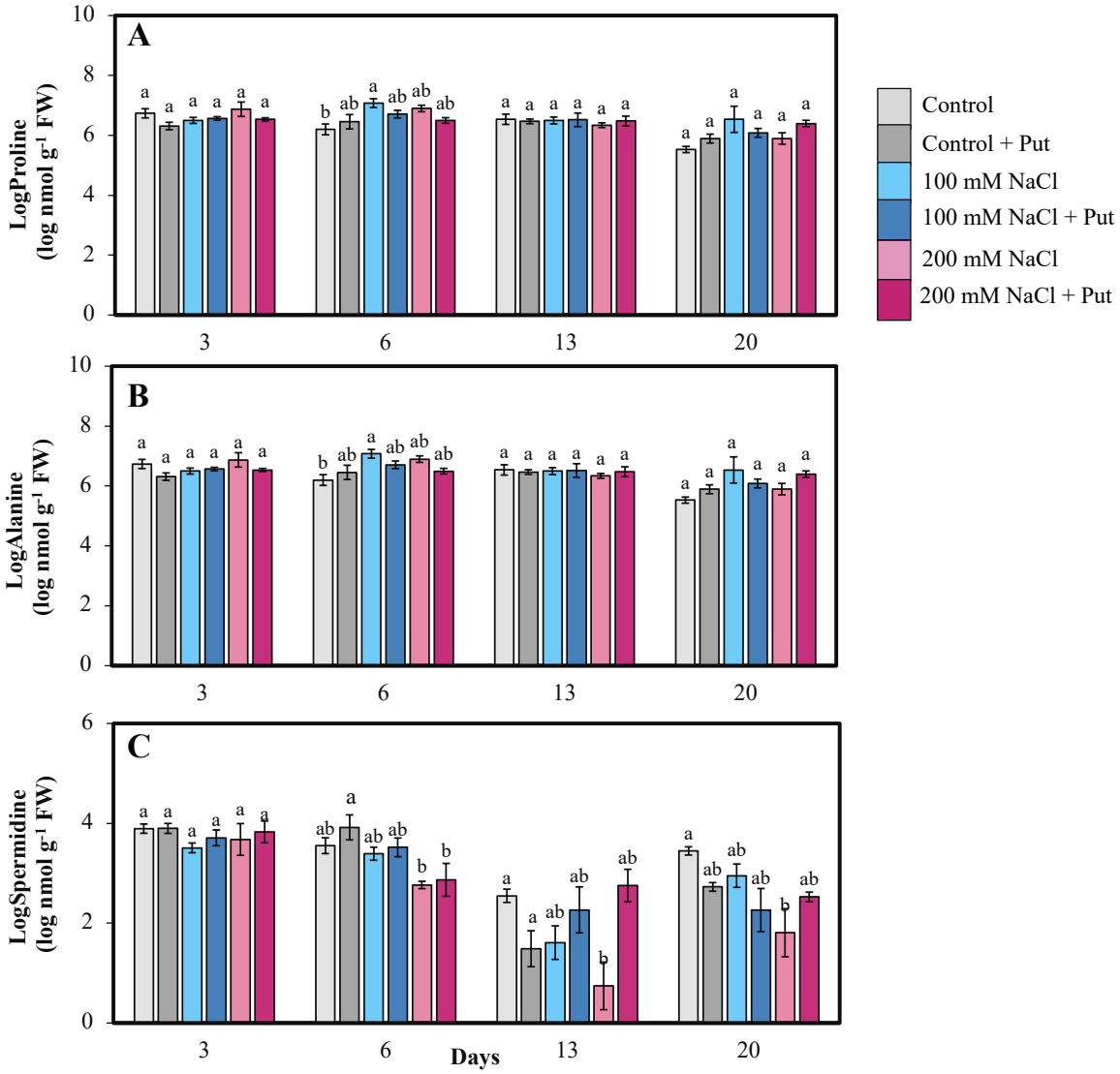


Figure 5: Effect of two different concentrations of NaCl (± Putrescine spray) on amino acids and polyamines accumulation in hybrid poplar NM6 leaves over time. (**A**) Log Proline levels, (**B**) Log Alanine levels, and (**C**) Log Spermidine levels measured at 3, 6, 13, and 20 days after salt treatment. Different letters indicate statistically significant differences ($p < 0.05$) among treatments. Data represent mean ± SE (n = 5).

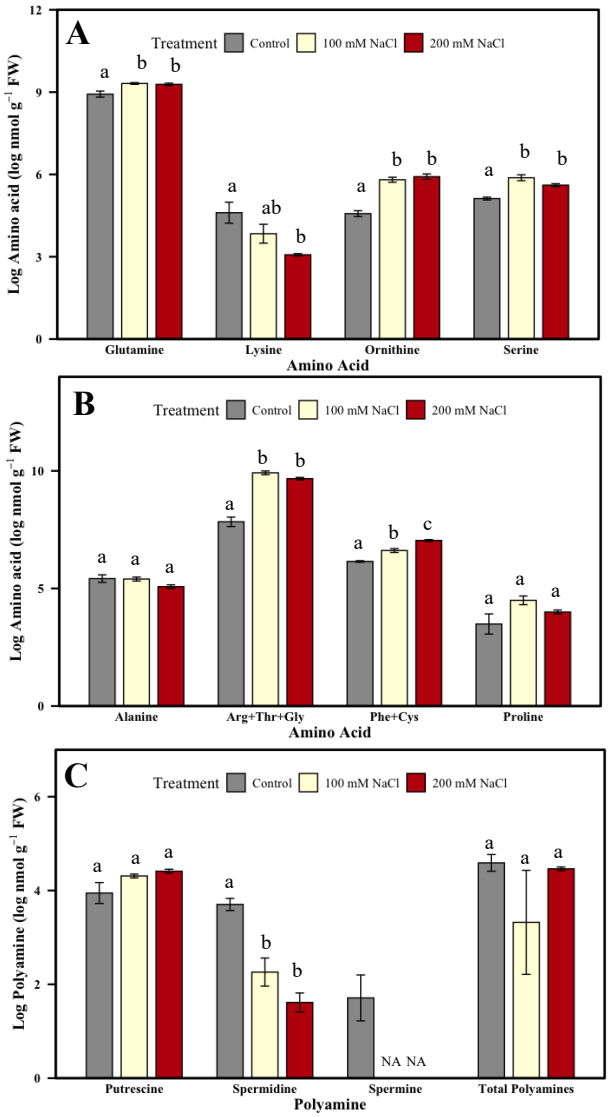


Figure 6: Effect of two different concentrations of NaCl on amino acids and polyamines accumulation in hybrid poplar NM6 roots. (**A, B**) Log Amino acids levels, (**C**) Log Polyamines levels measured at 21 days after salt treatment. Different letters indicate statistically significant differences ($p < 0.05$) among treatments. Data represent mean ± SE ($n = 4$).

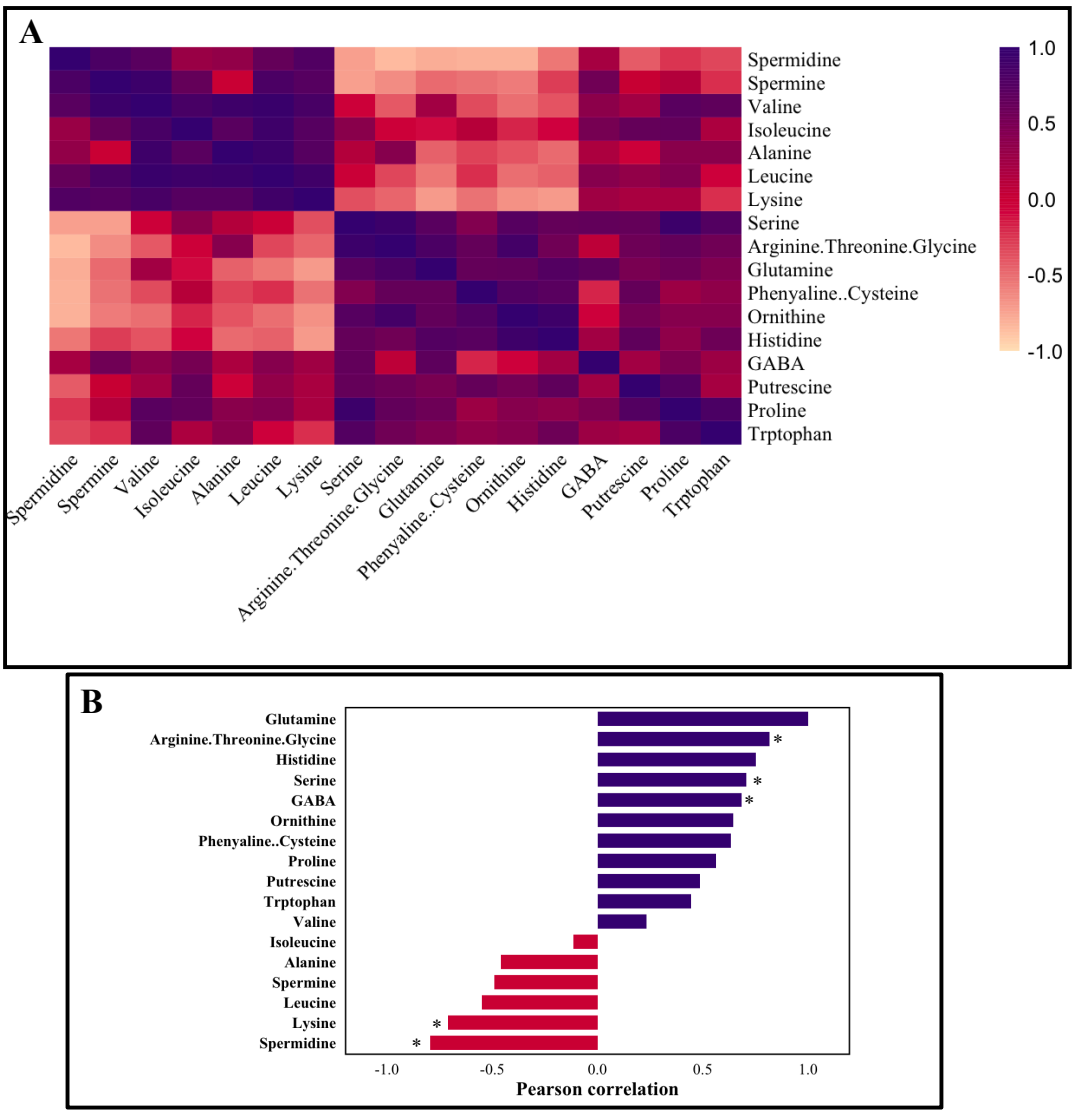
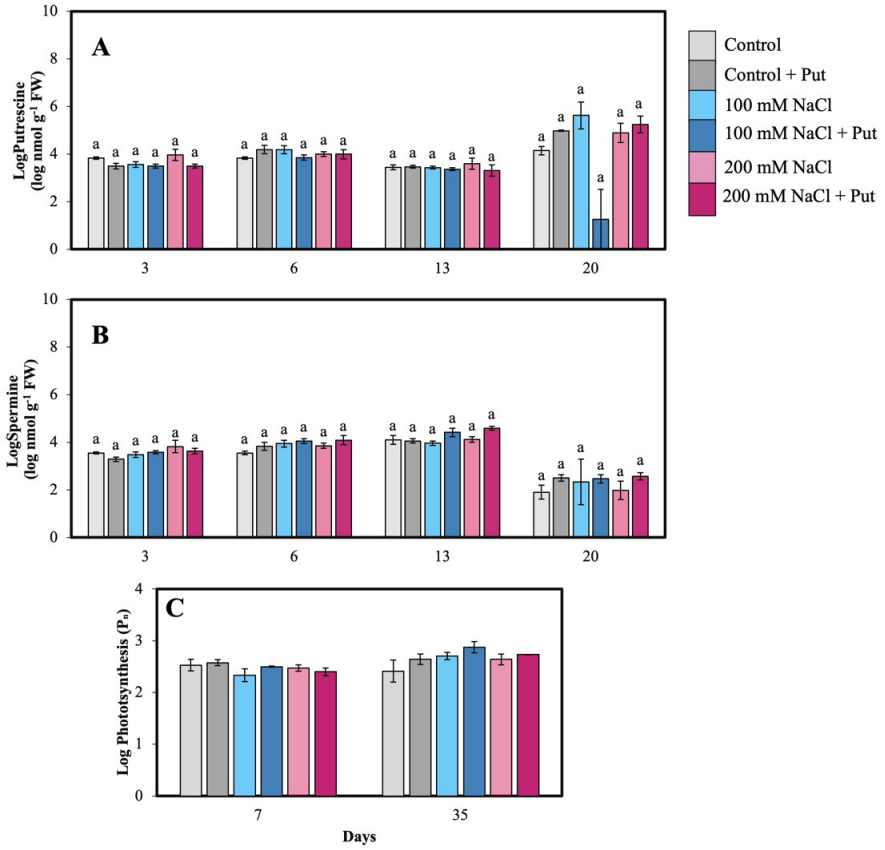
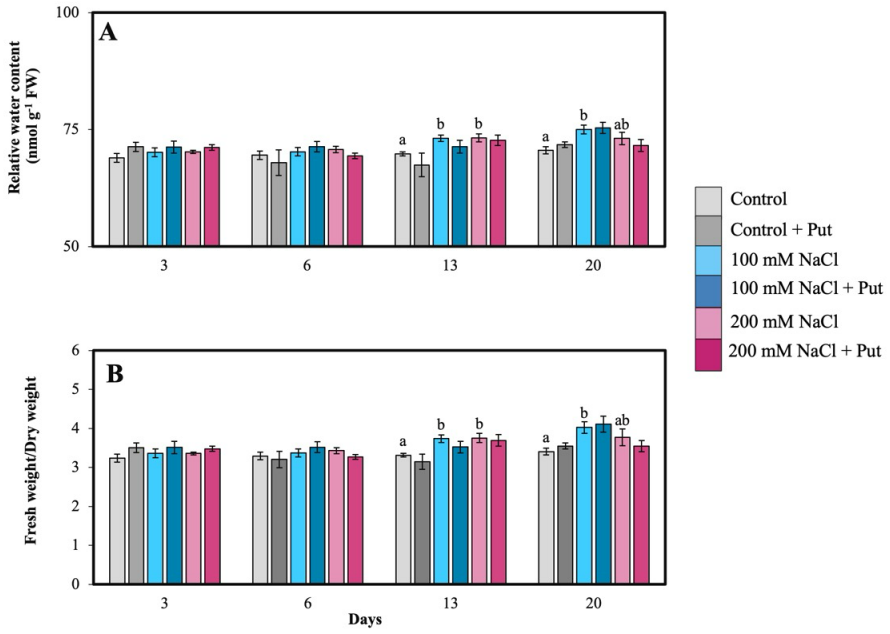


Figure 7. (A) Correlation coefficients of amino acids and polyamines analyzed in the poplar roots treated with two different concentrations of salt. (B) Pattern correlation analysis with Glutamine. The graph reports the significant features detected and ordered according to their correlation coefficient when correlated with Glutamine. The purple color represents a positive correlation, and the red color represents a negative correlation. Correlation distance- Pearson r. Correlation significant at *$p < 0.05$.

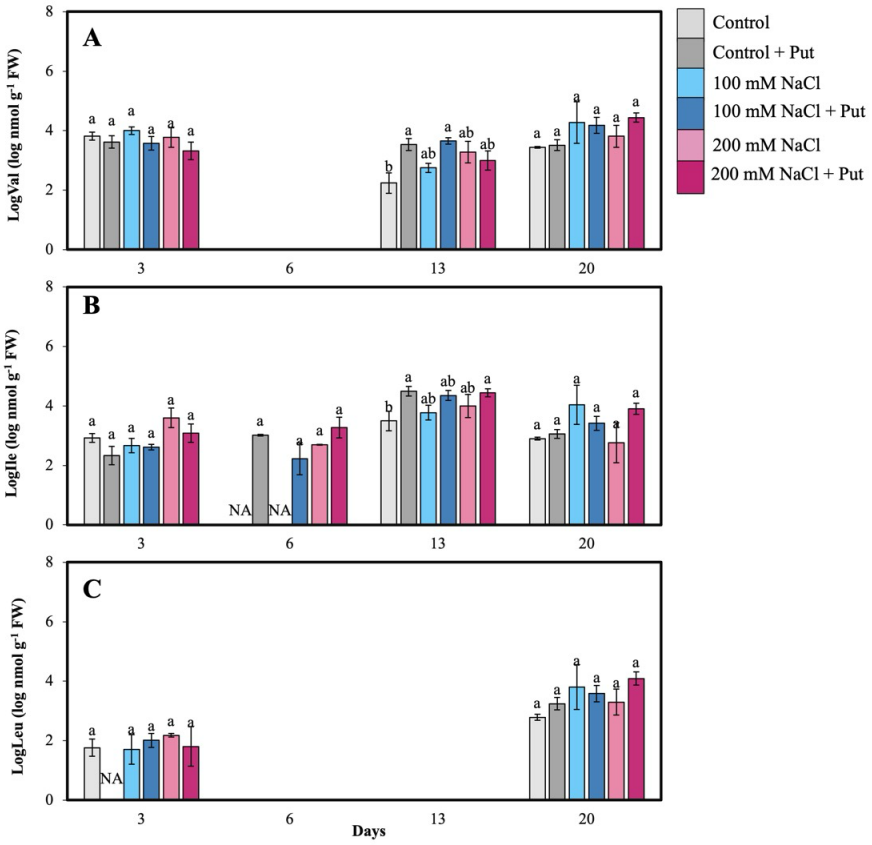
Supplementary Figures



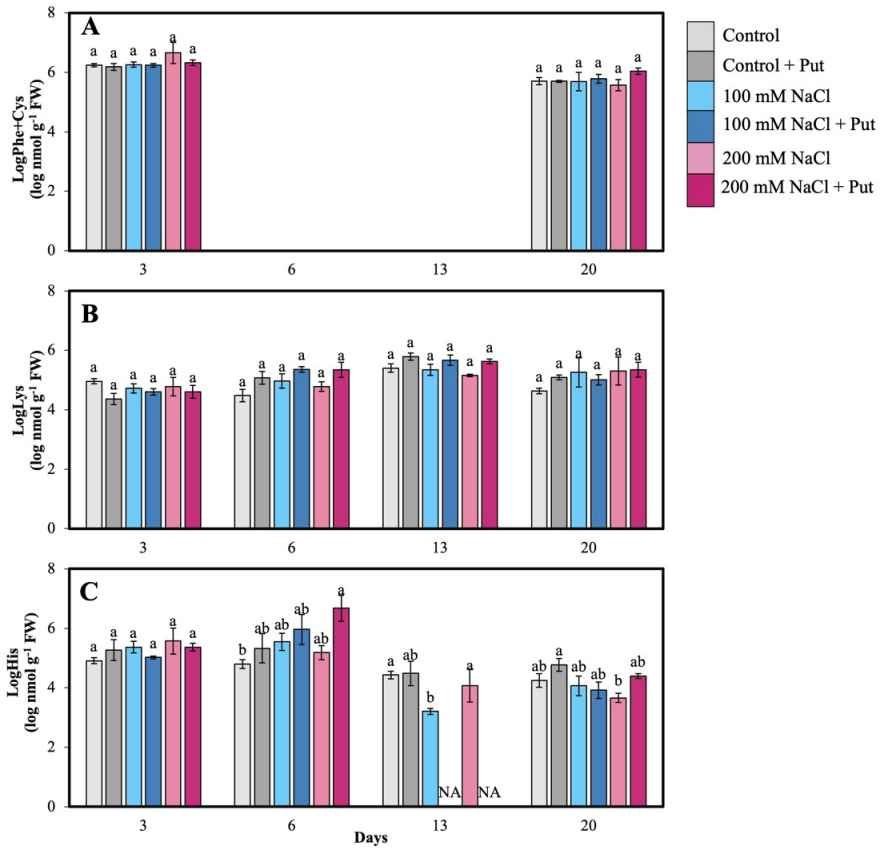
Supplementary Figure 1: Effect of two different concentrations of NaCl (± Putrescine spray) on amino acids and polyamines accumulation in hybrid poplar NM6 leaves over time. (**A**) Log Putrescine levels, (**B**) Log Spermine levels, and (**C**) Log Photosynthesis levels measured at several days after salt treatment. Different letters indicate statistically significant differences ($p < 0.05$) among treatments. Data represent mean ± SE (n = 5).



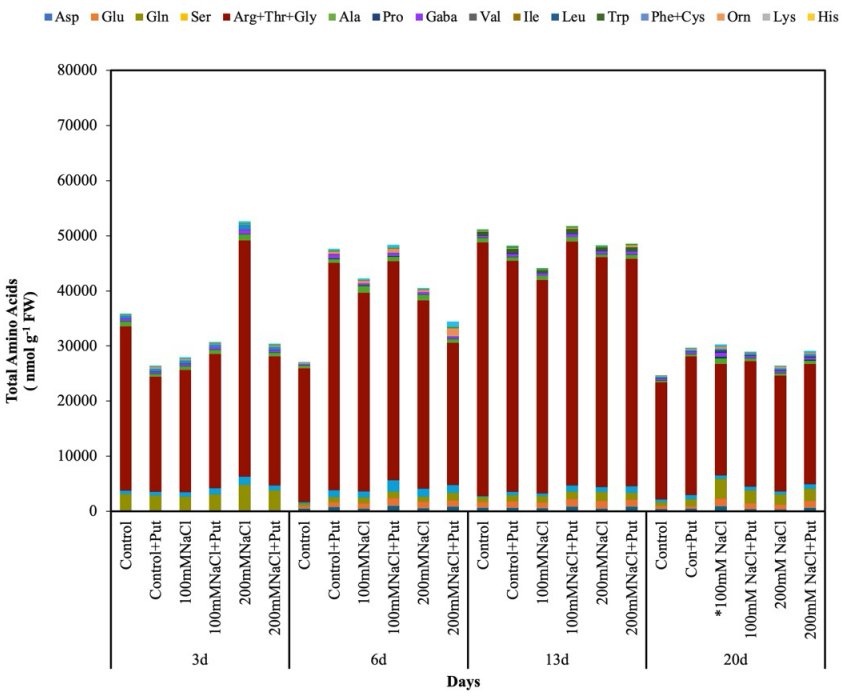
Supplementary Figure 2: Effect of two different concentrations of NaCl (± Putrescine spray) on amino acids and polyamines accumulation in hybrid poplar NM6 leaves over time. (**A**) Relative water content, and (**B**) Fresh weight/Dry weight levels measured at several days after salt treatment. Different letters indicate statistically significant differences ($p < 0.05$) among treatments. Data represent mean ± SE (n = 5).



Supplementary Figure 3: Effect of two different concentrations of NaCl (± Putrescine spray) on amino acids and polyamines accumulation in hybrid poplar NM6 leaves over time. (**A**) Log Valine levels, (**B**) Log Isoleucine levels, and (**C**) Log Leucine levels measured at several days after salt treatment. Different letters indicate statistically significant differences ($p < 0.05$) among treatments. Data represent mean ± SE (n = 5).



Supplementary Figure 4: Effect of two different concentrations of NaCl (± Putrescine spray) on amino acids and polyamines accumulation in hybrid poplar NM6 leaves over time. (**A**) Log Phenylalanine+Cysteine levels, (**B**) Log Lysine levels, and (**C**) Log Histidine levels measured at several days after salt treatment. Different letters indicate statistically significant differences ($p < 0.05$) among treatments. Data represent mean ± SE (n = 5).



Supplementary Figure 5: Effect of two concentrations of NaCl (± Putrescine spray) on total free amino acid accumulation in hybrid poplar NM6 leaves over time. Stacked bars show the concentrations (nmol g^{-1} FW) of individual amino acids measured at 3, 6, 13, and 20 days after salt treatment.

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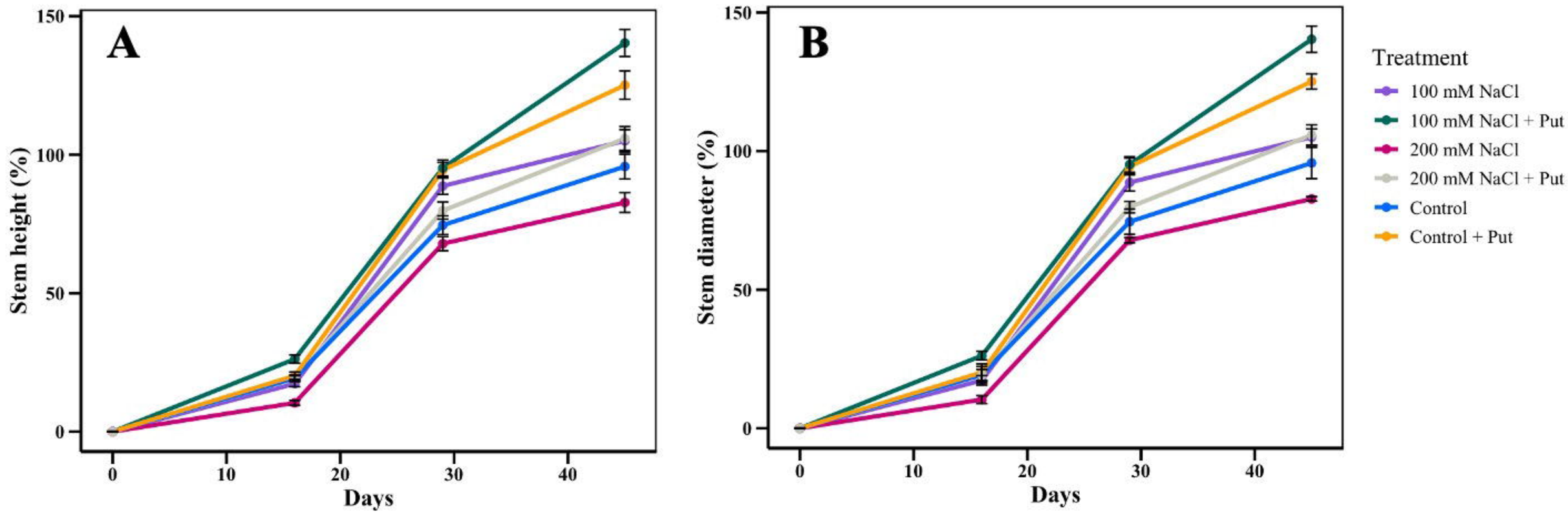
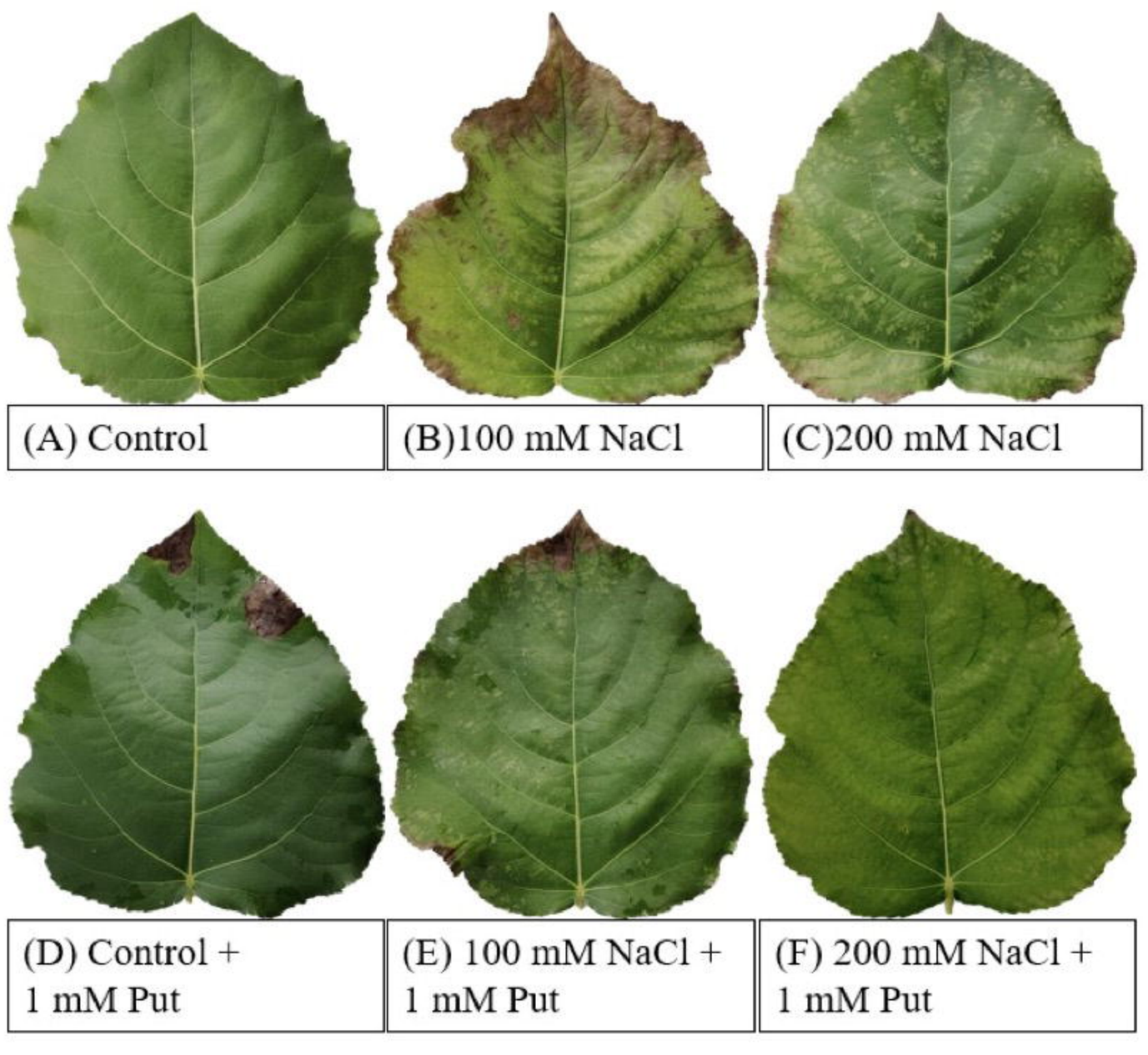
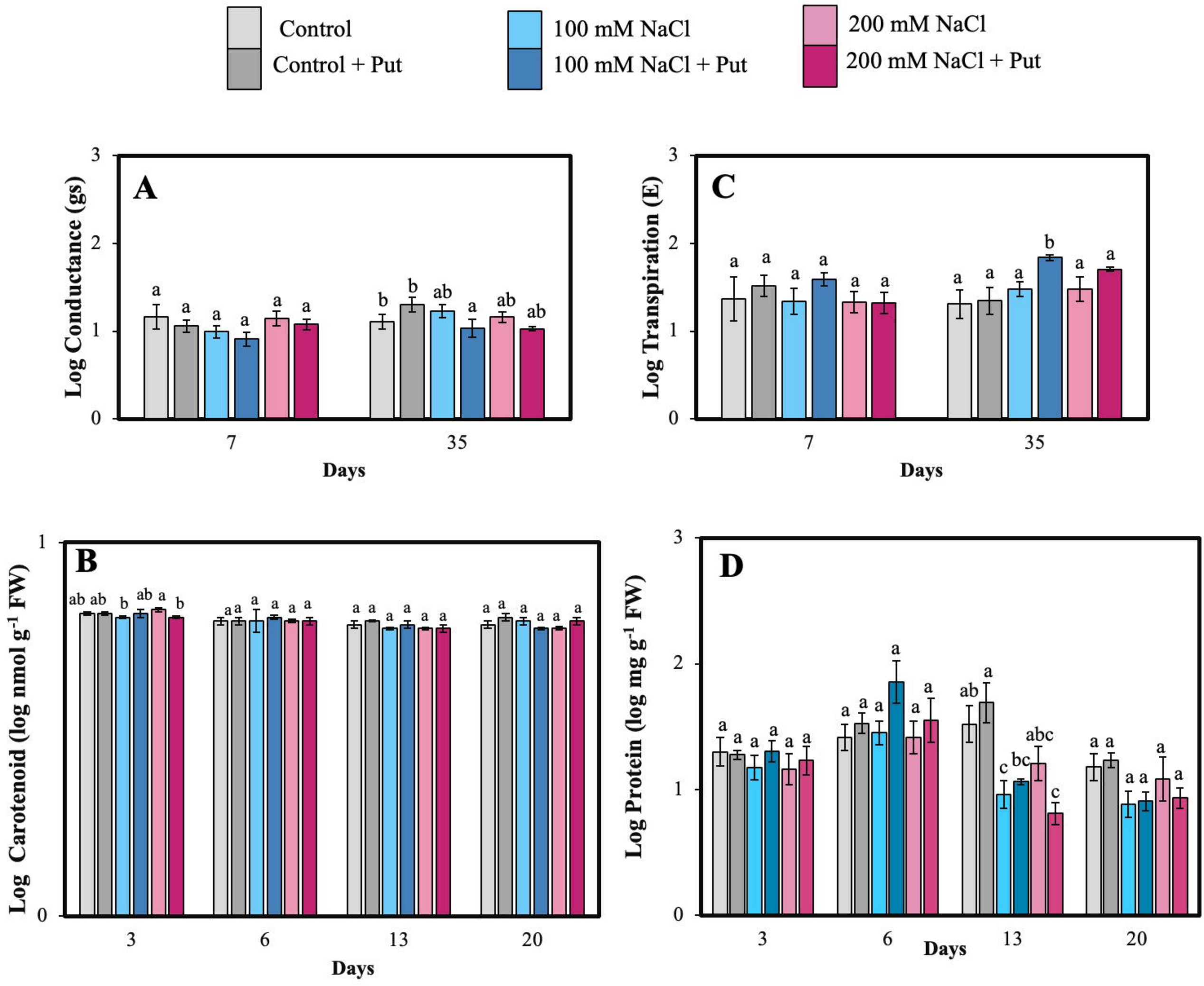
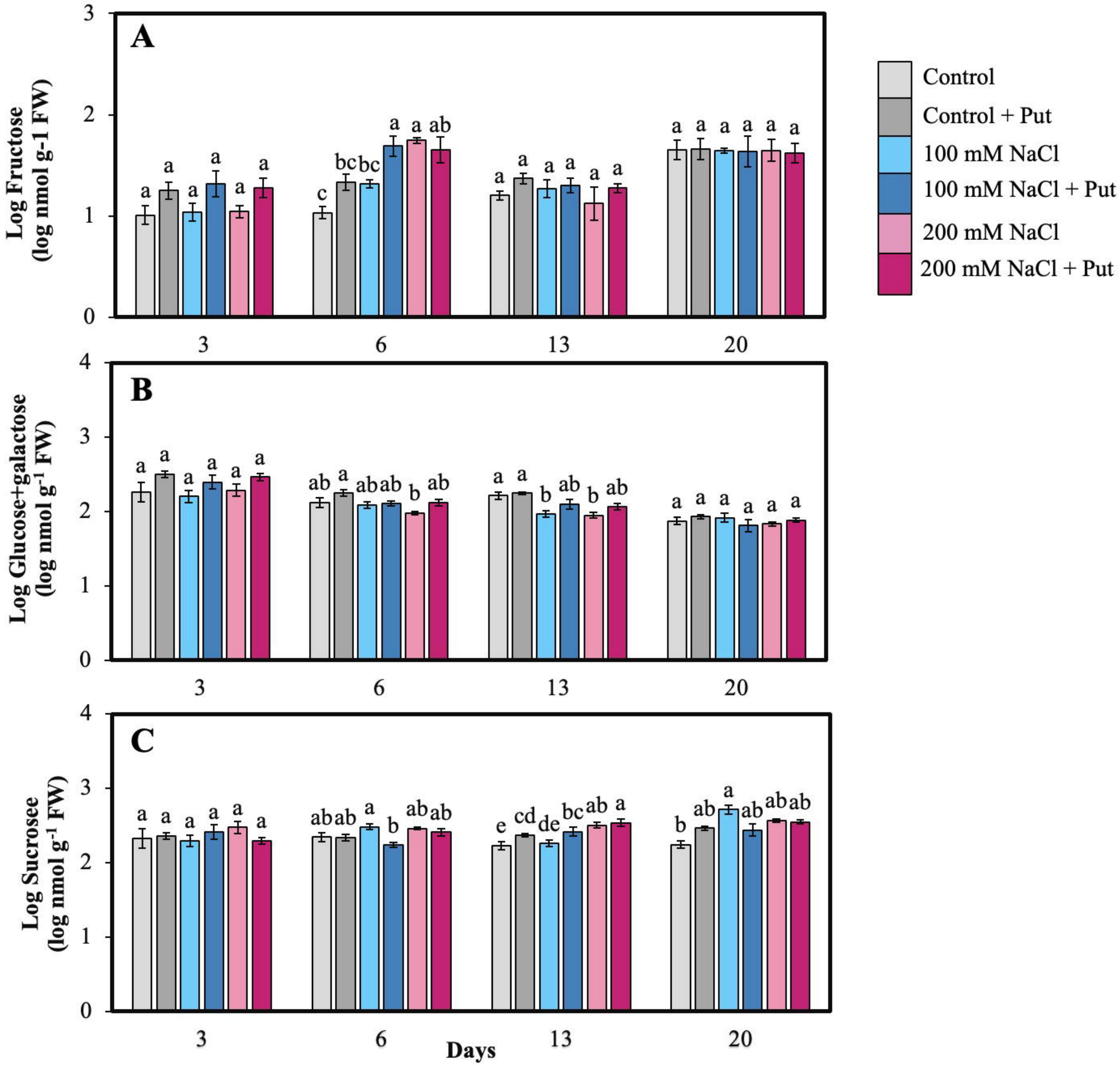
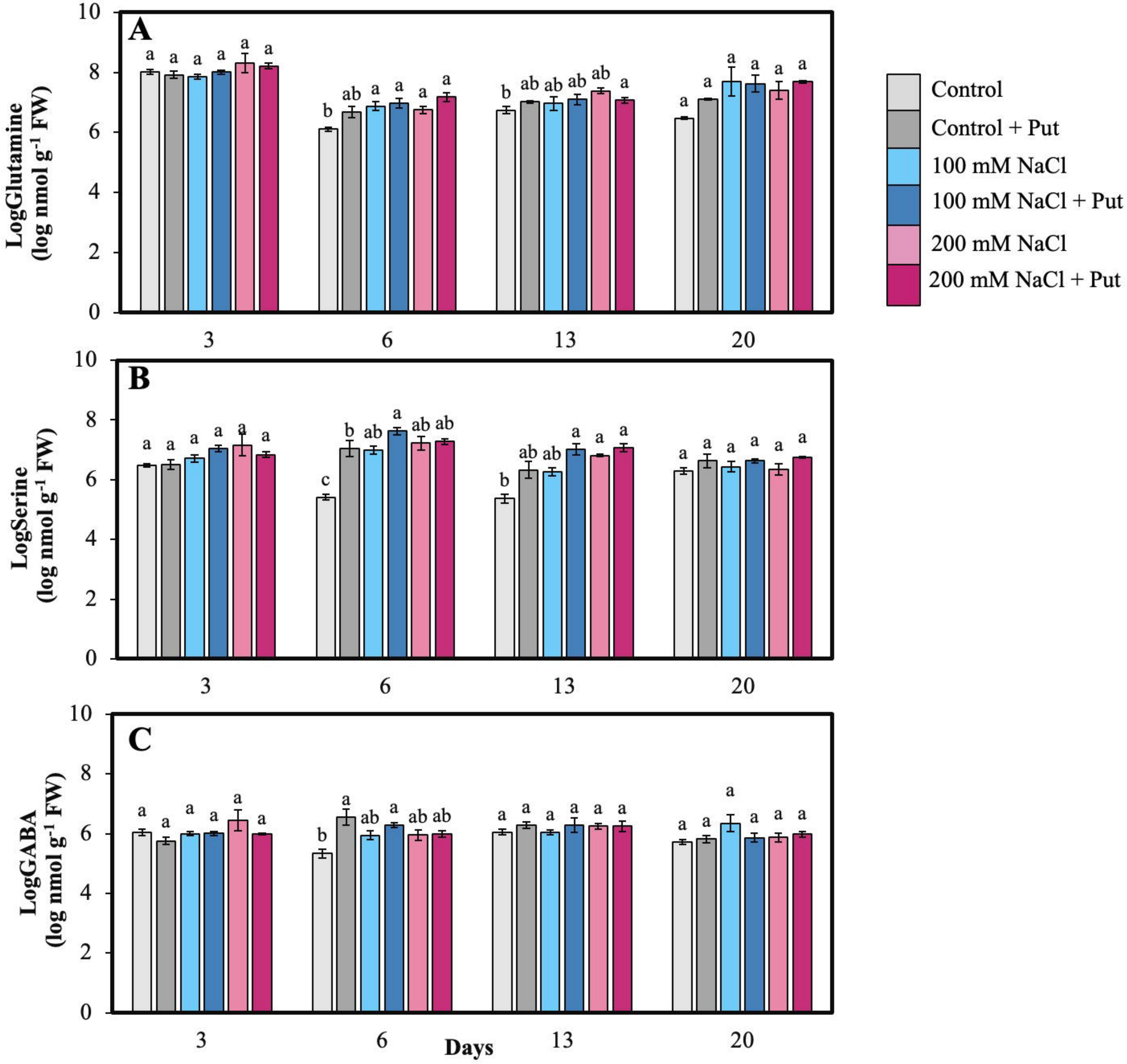
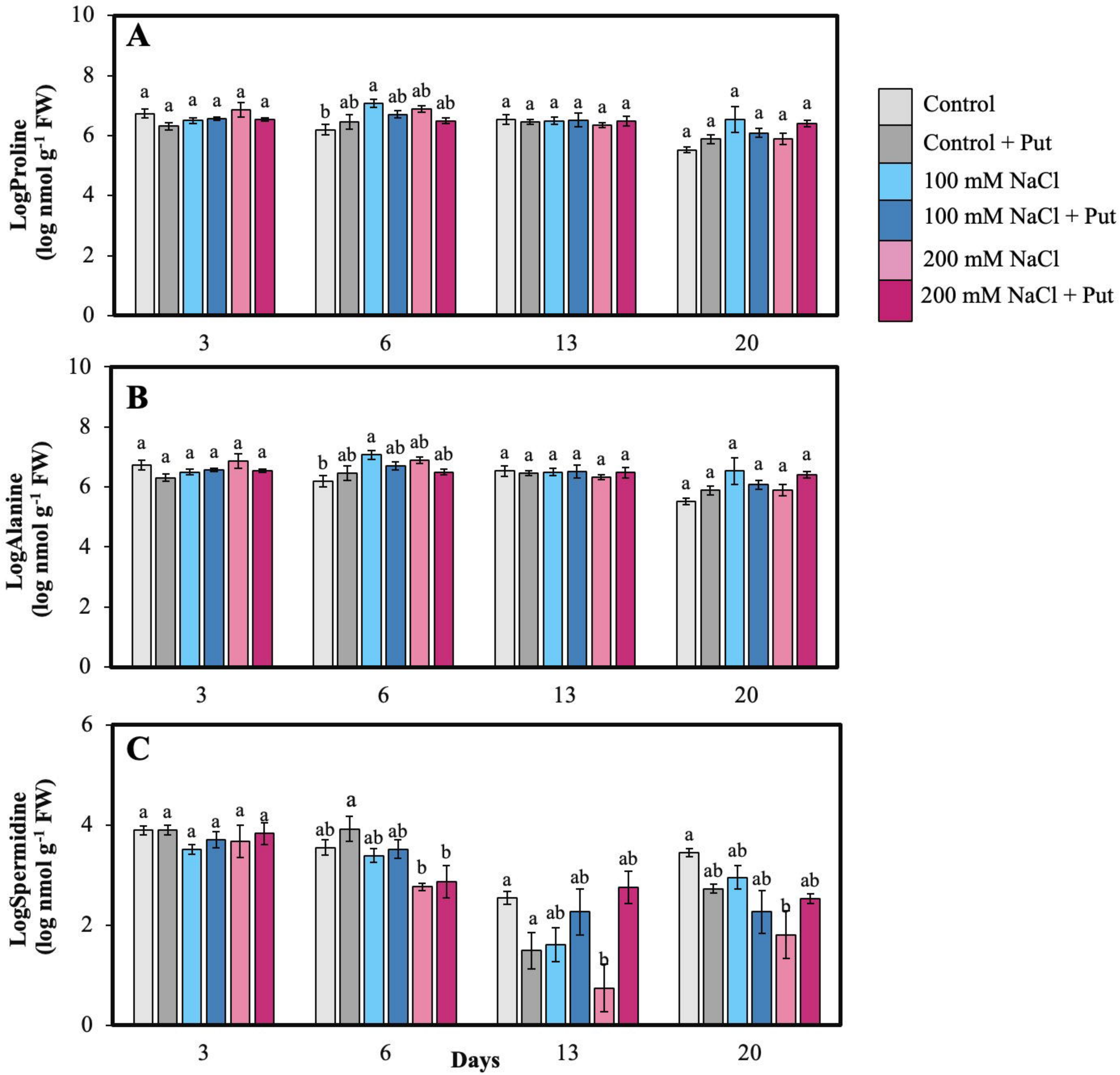

A
Stem height (%)
Days
B
Stem diameter (%)
Days
Treatment
100 mM NaCl
100 mM NaCl + Put
200 mM NaCl
200 mM NaCl + Put
Control
Control + Put

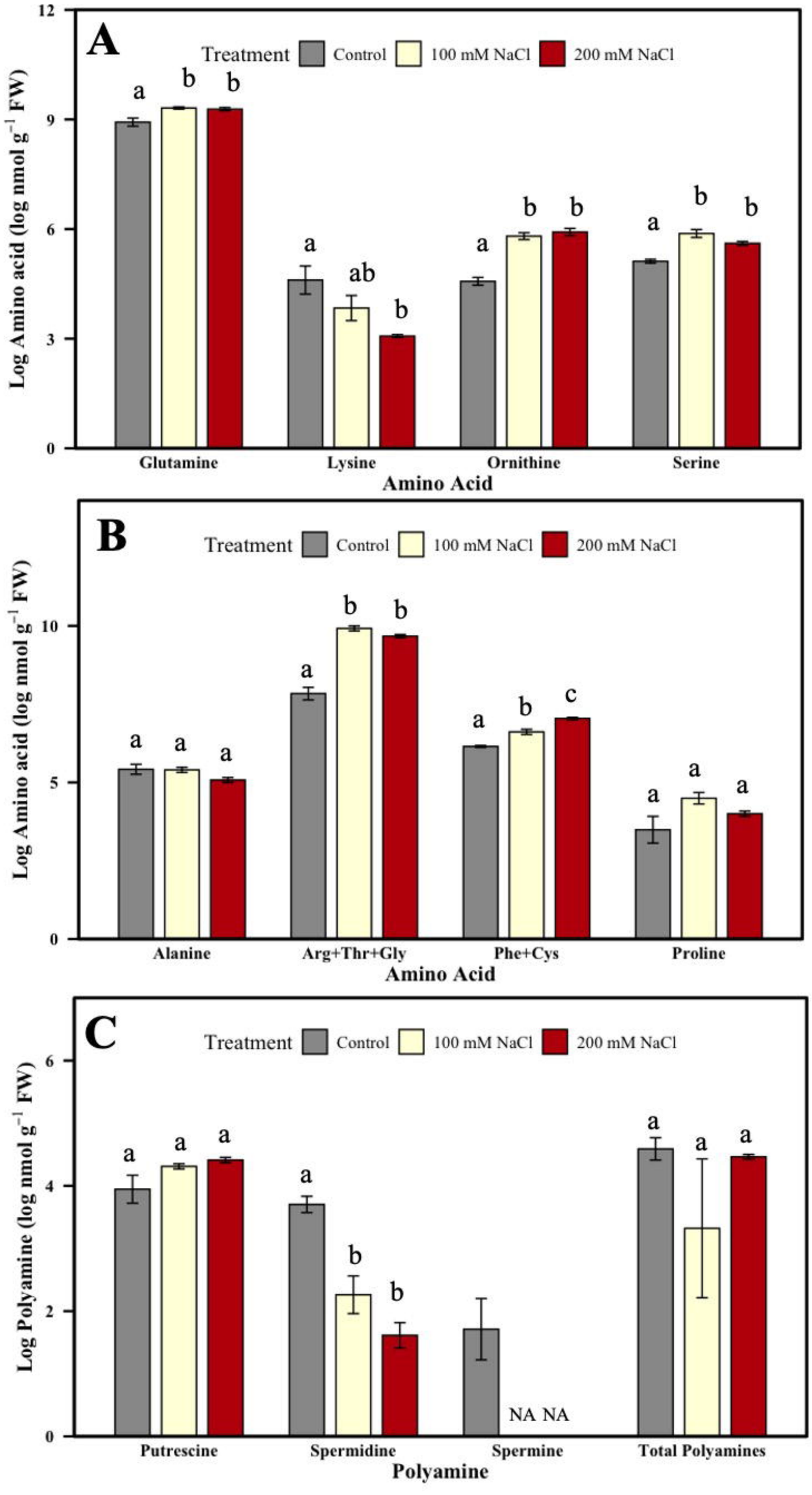

C
(A) Control
(B)100 mM NaCl
(C)200 mM NaCl
(D) Control + 1 mM Put
(E) 100 mM NaCl + 1 mM Put
(F) 200 mM NaCl + 1 mM Put


Control
Control + Put
100 mM NaCl
100 mM NaCl + Put
200 mM NaCl
200 mM NaCl + Put
A
Log Conductance (gs)
Days
B
Log Carotenoid (log nmol g-1 FW)
Days
C
Log Transpiration (E)
Days
D
Log Protein (log mg g-1 FW)
Days


A
Log Fructose
(log nmol g-1 FW)
B
Log Glucose+galactose
(log nmol g-1 FW)
C
Log Sucrosee
(log nmol g-1 FW)
Days
Control
Control + Put
100 mM NaCl
100 mM NaCl + Put
200 mM NaCl
200 mM NaCl + Put


A
LogGlutamine
(log nmol g-1 FW)
B
LogSerine
(log nmol g-1 FW)
C
LogGABA
(log nmol g-1 FW)
Days
Control
Control + Put
100 mM NaCl
100 mM NaCl + Put
200 mM NaCl
200 mM NaCl + Put


A
B
C
LogProline (log nmol g-1 FW)
LogAlanine (log nmol g-1 FW)
LogSpermidine (log nmol g-1 FW)
Days
3
6
13
20
Control
Control + Put
100 mM NaCl
100 mM NaCl + Put
200 mM NaCl
200 mM NaCl + Put


A
Treatment
Control
100 mM NaCl
200 mM NaCl
Log Amino acid (log nmol g^{-1} FW)
Glutamine
Lysine
Ornithine
Serine
Amino Acid
B
Alanine
Arg+Thr+Gly
Phe+Cys
Proline
C
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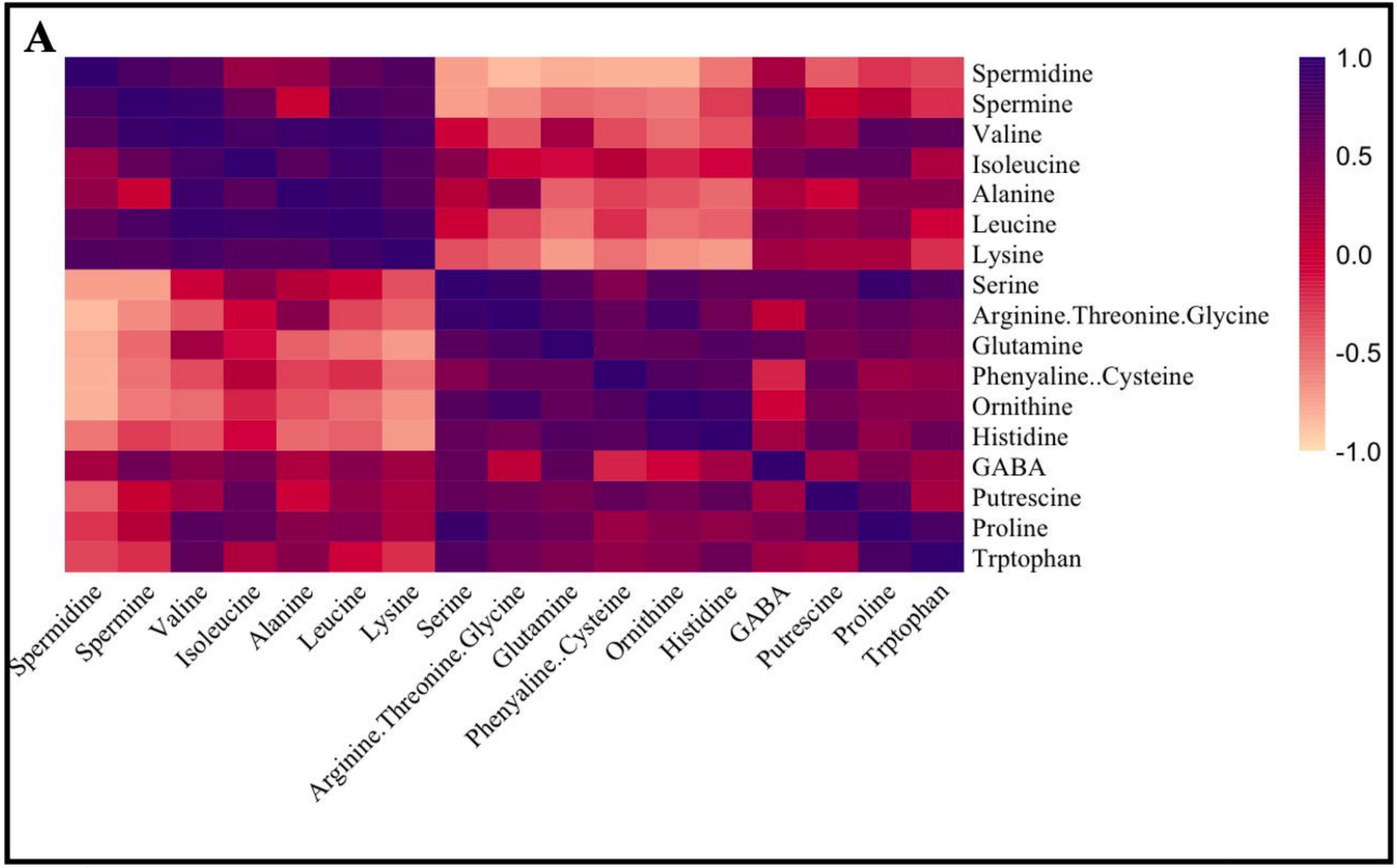
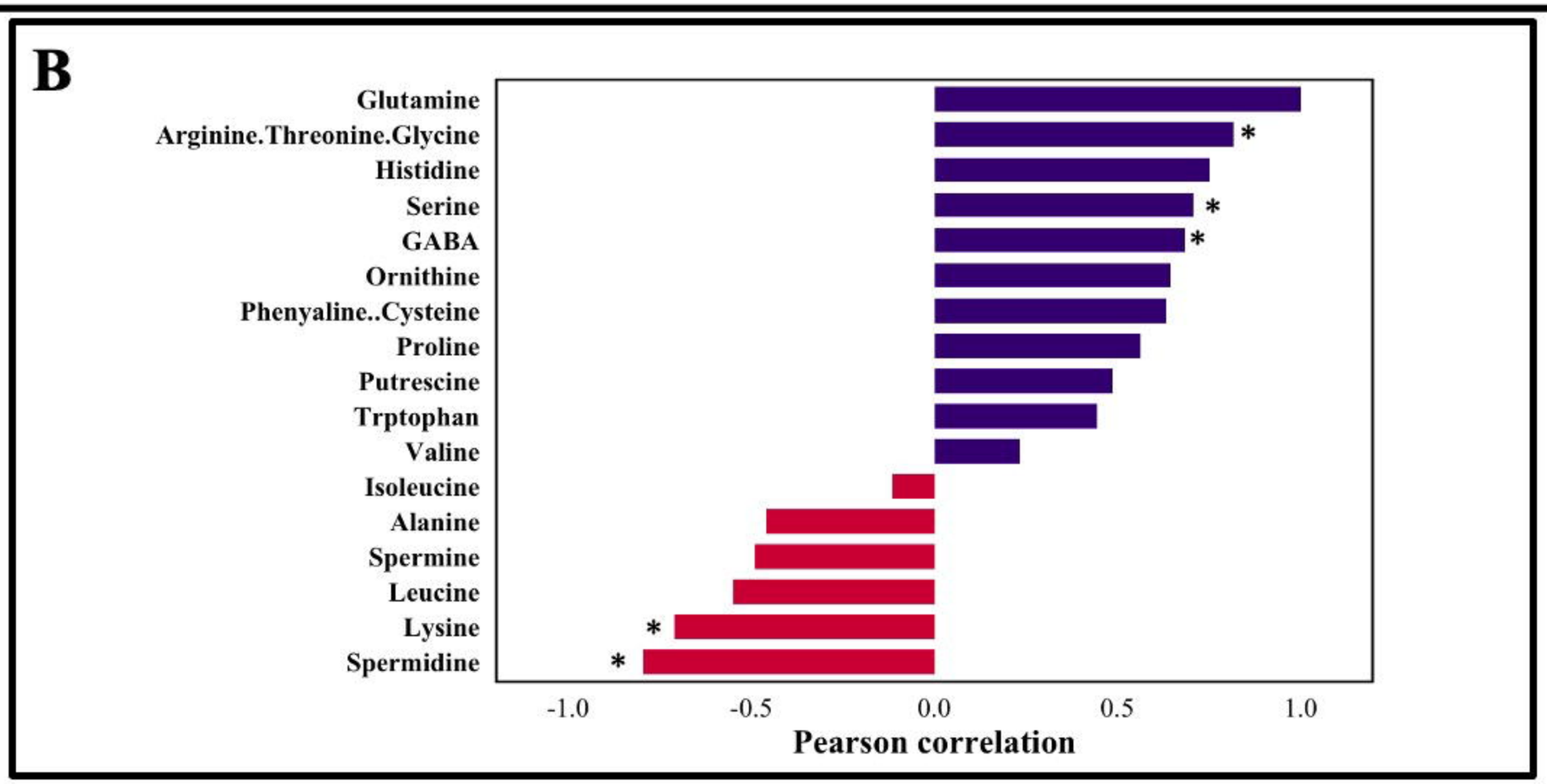
Spermidine
Spermine
Total Polyamines
Polyamine


A
Spermidine
Spermine
Valine
Isoleucine
Alanine
Leucine
Lysine
Serine
Arginine.Threonine.Glycine
Glutamine
Phenyaline..Cysteine
Ornithine
Histidine
GABA
Putrescine
Proline
Trptophan
1.0
0.5
0.0
-0.5
-1.0


B
Glutamine
Arginine.Threonine.Glycine
Histidine
Serine
GABA
Ornithine
Phenyaline..Cysteine
Proline
Putrescine
Trptophan
Valine
Isoleucine
Alanine
Spermine
Leucine
Lysine
Spermidine
*
-1.0
-0.5
0.0
0.5
1.0
Pearson correlation