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PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES TO SALT AND CADMIUM STRESS AND IT'S AMELIORATION BY EXOGENOUS APPLICATION OF POLYAMINES IN HYBRID POPLAR (Populus nigra x maximowiczii, CLONE NM6)

Sanchari Kundu
University of New Hampshire, Durham

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By

SANCHARI KUNDU

B.SC. WEST BENGAL UNIVERSITY OF TECHNOLOGY, INDIA, 2014

M.SC. RAMAKRISHNA MISSION VIVEKANANDA UNIVERSITY, INDIA, 2016

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This thesis has been examined and approved in partial fulfillment of the requirements for the degree of MS in Integrative Biology by:

Thesis Director, Dr. Subhash C. Minocha, Emeritus
Department of Biological Sciences

Dr. Rakesh Minocha, Research Scientist
USDA-Forest Service
Affiliate Professor of Plant Biology and Natural Resources, UNH

Dr. Anissa Poleatewich, Assistant Professor
Agriculture, Nutrition, and Food Systems

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Original approval signatures are on file with the University of New Hampshire Graduate School.

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ABBREVIATIONS

ABA= Abscisic acid; ACN= Acetonitrile; Ala= Alanine; AA= Amino acid; APX= Ascorbate peroxidase; Arg= Arginine; AsA= Ascorbic acid; Asp= Aspartic acid; C= Carbon; Cd= Cadmium; Cys= Cysteine; Cu= Copper; DAPT= N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DFMO= Difluoro methyl ornithine; DNA= Deoxyribonucleic acid; ddH_2O= Double distilled water; DTPA= Diethylenetriamine pentaacetic acid; DW= Dry weight; E=Transpiration rate; EDTA= Ethylene diamine tetraacetic acid; GABA= γ-aminobutyric acid; Gln= Glutamine; Glu= Glutamate, Gly= Glycine; γ-EC= Gamma-glutamylcysteine; GSH= Glutathione; HEPPS= 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid; Hg= Mercury; His= Histidine; HPLC= High Performance Liquid Chromatography; HP= High putrescine; Ile= Isoleucine; CT= Leaf temperature; Leu= Leucine; Lys= Lysine; $MgCl_2$= Magnesium chloride; Met= Methionine; mBBR= Monobromobimane; MSA= Methanesulfonic acid; N= Nitrogen; NAC= N-acetyl-L-cysteine; NaCl= Sodium chloride; $NAHCO_3$= Sodium bicarbonate; ND= Not determined; NO_3^-= Nitrate; NT= Non-transformed; NUE= Nitrogen use efficiency; ODC= Ornithine decarboxylase; Orn= Ornithine; ODC= Ornithine decarboxylase; PLS-DA= Partial least squares discriminant analysis; PA= Polyamines; Pb= Lead; PC= Phytochelatin; PCA= Principal component analysis; Phe= Phenylalanine; P_n= Photosynthetic rate; Pro= Proline; Put= Putrescine; RID= Refractive index detector; RNA= Ribonucleic acid; ROS= Reactive oxygen species; Rubisco= Ribulose bisphosphate carboxylase; SE= Standard error; Ser= Serine; Spd= Spermidine; Spm= Spermine; g_s= Stomatal conductance; TCA= Tricarboxylic acid; TCEP= Tris(2-carboxyethyl)phosphine hydrochloride; TFA= Trifluoroacetic acid; Thr= Threonine; Trp= Tryptophan; TS= Threonine synthase; Val= Valine; Zn= Zinc.

ABSTRACT

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES TO SALT AND CADMIUM STRESS AND IT'S AMELIORATION BY EXOGENOUS APPLICATION OF POLYAMINES IN HYBRID POPLAR (*Populus nigra x maximowiczii,* CLONE NM6)

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Sanchari Kundu

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The importance of nitrogen metabolism in plants in response to abiotic stress is widely known. Plant growth is severely hampered by abiotic stress, and appropriate nitrogen supply helps in plant growth and improves stress tolerance. At the same time, the regulation of nitrogen metabolism depends on the stress intensity. Abiotic stress can occur due to natural calamities, improper use of hazardous chemicals from industries, and careless environmental dumping of these chemicals. Hence, soils contaminated with hazardous chemicals are a significant threat to the development and yield of plants. Plants are affected by various abiotic stresses, i.e., salt, drought, and heavy metal stress. In the presence of these stresses, plants respond via complex mechanisms that include morphological, physiological, biochemical, and molecular changes.

Small aliphatic amines like polyamines regulate plant growth and development, including plant metabolism. Polyamines like putrescine, spermidine, and spermine regulate several critical biochemical pathways, including the biosynthesis of amino acids and plant growth regulators. Under abiotic stress, polyamines protect plants from various detrimental effects. The interplay of polyamine and nitrogen is a critical factor in plant response to abiotic stress, as it connects nitrogen metabolism, carbon fixation, and other metabolic pathways. Some of these responses are short-lived and others long-term, affecting not only the physiology but also growth and biomass. However, little information about how polyamines interact with plants to maintain normal growth

in response to abiotic stress is available. So, it is essential to investigate the biochemical responses in cellular polyamines and amino acids in abiotic stress.

The goal of the study was to understand the physiological and biochemical effects of salt and cadmium, and their interactions with polyamines sprayed on the leaves in *Populus nigra x maximowiczii,* (clone NM6) young plants.

The two specific objectives of the study were: (1) to analyze the effects of two concentrations of NaCl on the physiology and biochemistry of poplar NM6 plants and potential mitigation of these effects by spray with putrescine; and (2) to analyze the effects of two concentrations of cadmium chloride on the physiology and biochemistry of poplar NM6 plants and potential mitigation of these effects by spray with spermidine. The results showed that plants had chlorosis, necrosis, slower growth because of abiotic stress. Exogenous putrescine application increased gas exchange & altered several metabolites under salt stress. The results also suggest a close relationship between amino acids, polyamines, and phytochelatins and their involvement in the cadmium detoxification mechanisms. Finally, it can be said that timely applications of exogenous spermidine via foliar spray could ameliorate the adverse effect of heavy metal stress in poplar NM6 plants. In conclusion, overall finding addressed multi-dimensional effect of the foliar spray of polyamines on poplar NM6 plants in response to abiotic stress.

CHAPTER 1: INTRODUCTION

For decades, natural calamities like forest fires and flash floods have imposed several stresses on plants, negatively affecting their ecological distribution, productivity, and resilience (Alcázar et al. 2006a, Minocha et al. 2014, Verslues et al. 2023). Stress is a condition that reduces growth and development of plants by disrupting metabolic responses, as these metabolic changes become unfavorable for the plants in a relatively short time (Levitt 1980, Larcher 1987, Lichtenthaler 1996). These stresses can broadly be classified into biotic (due to living organisms) and abiotic (due to physical factors). Abiotic stress can be due to a multitude of factors, e.g. excessive salt, heavy metals, drought, and flooding in the soil, radiation, and low/high temperatures in the environment. Abiotic stress can result in economic losses (as much as 50-70% loss) in yearly crop yields in many cases (Alcázar et al. 2010, Ciarmiello et al. 2011, Minocha et al. 2014, Hasanuzzaman et al. 2017, Verslues et al. 2023). On the other hand, Zalesny Jr et al. (2019) have argued that environmental stress effects could be relatively less on forest/hardwood trees; however, the costs could still be high. Studies have shown that forest trees have evolved complex mechanisms to cope with environmental stresses. These include modification in the leaf and root morphologies, activation of stress-specific genes that induce metabolic responses of polyamines (PAs) and other amines, protein activation (e.g. dehydrin), antioxidants production (enzymatic and nonenzymatic), and synthesis of osmoprotectants (e.g. sugars, proline) to cope with abiotic stress (Chen and Murata 2011, Suzuki et al. 2012, Zulfiqar et al. 2020, Colin et al. 2023, Verslues et al. 2023). However, with ever-increasing global warming and land degradation, forest trees face more extreme stress conditions, making them increasingly vulnerable to the changing environment (Pericolo et al. 2023). There has been a lack of understanding on how abiotic stress affects the physiology and biochemistry of forest trees compared to stress-sensitive crop plants because of

their long-life cycle, complex physiology, large size, difficulty in manipulation for their deep roots, and ethical reasons (Neale and Kremer 2011, Kremer et al. 2012, Cortés et al. 2020). Forest trees have beneficial roles in carbon sequestration, soil conservation, water and biodiversity conservation, and economic values (Zalesny Jr et al. 2019, Ameray et al. 2021, Verslues et al. 2023).

Studies have shown that in the presence of abiotic stress, plant growth is hampered due to reduced photosynthesis and water uptake, increased pathogen susceptibility, interference with hormonal balance, altered gene expression, and nitrogen (N) and carbon (C) metabolism (Cramer et al. 2011, Parihar et al. 2015, Zhang et al. 2020a). Abiotic stress can alter N and C metabolism in several ways by affecting the availability and uptake of nutrients and the allocation of resources into different pathways such as photorespiration (Tamang et al. 2021, Jalal et al. 2023). Valentine et al. (2010) showed that abiotic stress could reduce the efficiency of N fixation in legumes and limit the activity of N-fixing microorganisms in the rhizosphere. All these physiological and biochemical changes involve the expression of genes regulating N and C metabolism directly as well as via biochemical pathways connected to each other, and remarkably affecting the plant metabolic response (Alcázar et al. 2010, Mantri et al. 2012, Goel and Singh 2015, Jalal et al. 2023).

If the metabolic activity of the plant is altered significantly, it can lead to premature senescence and apoptosis (Hanson et al. 2016). Soils contaminated with heavy metals and salt (chloride ions - Cl^-) have shown to have harmful effects on the plants due to the toxic effects of these ions and several other antagonistic interactions with soil N like reduced N fixation and nutrient uptake, enzyme activities involved in N metabolism and altered protein synthesis (Grattan and Grieve 1998, Ashraf et al. 2018, Zhang et al. 2020a, Li et al. 2021b). It has been reported by Grattan and Grieve (1998) and Li et al. (2021b) that the presence of Cl^- ions hampers the nitrate

(NO_3^-) nutrition and reduce the nitrogen use efficiency (NUE). They also showed that the reduced NUE impairs N uptake and assimilation during abiotic stress and affects growth, development, and biomass accumulation in plants. Excessive amounts of N fertilizers are often applied to plants to counter these effects and achieve a better yield in the short run. However, there has been a major concern regarding the overuse of N, which has become a chronic environmental concern itself due to excessive leaching and accumulation in lakes, ponds and streams, causing microalgal blooms and toxicity (Gu et al. 2020, Agathokleous et al. 2022). Excessive Cl^- ions in the soil further aggravate this problem, as described by Gu et al. (2020) and Agathokleous et al. (2022). Although there have been studies on how Cl^- ions hamper overall plant growth and N promotes plant growth, the interaction of Cl^- ions with N uptake and assimilation in forest plants remains poorly understood.

Nitrogen deficiency hinders plant growth and photosynthesis due to reduced chlorophyll content and stomatal conductance (Cramer et al. 2011, Amiour et al. 2012, Parihar et al. 2015, Zhang et al. 2020a). Studies show that N is also a major component of cellular compounds like enzymes, proteins, alkaloids, flavonoids, polyamines (PAs), and amino acids (AAs) which are involved in stress tolerance and defense mechanisms (Gu et al. 2020, Li et al. 2021a).

Effects like reduced leaf area, chlorosis, alteration in the root-shoot ratio, short lateral branches, and reduced seed and fruit production due to less N availability, have been reported by Zhao et al. (2005) and Azimi et al. (2021), among others. On the other hand, Nunes-Nesi et al. (2010) ha d shown earlier that abiotic stress can also affect C metabolism by changing the C partitioning, altering starch and sugar metabolism, and activating photorespiration. As mentioned earlier, N deficiency in plants allows them to opt for photorespiration; hence, N and C metabolism are tightly linked with each other (Nunes-Nesi et al. 2010, Parihar et al. 2015, Zhang et al. 2020a).

Nitrogen and carbon metabolism in plants

Nitrogen is an essential nutrient in plant growth and development. It is a key component of many biomolecules, including PAs, AAs, proteins, nucleic acids, and chlorophyll (Lea and Ireland 1999). Nitrogen is taken up by plants as inorganic ions, such as ammonium (NH_4^+) or NO_3^-, or as organic compounds, such as urea, by a tightly regulated process called N assimilation (Wang et al. 2014a, Ashraf et al. 2018). Nitrogen assimilation begins with converting NH_4^+ or NO_3^- into ammonia (NH_3) by enzymes such as nitrate reductase and glutamine synthetase (Fig. 1). Ammonia can then be incorporated into AAs and further used to synthesize proteins and other N-containing compounds, including PAs. In plants, PAs are involved in N metabolism by modulating the uptake, assimilation, and utilization of all N sources. In plants, N metabolism is regulated by genes, hormones, and environmental factors. It is closely linked with other metabolic pathways, such as C, sulfur, and phosphorus (Wang et al. 2014a).

Carbon metabolism in plants refers to the process by which plants acquire and use C for growth and development. This process begins with the absorption of CO_2 from the atmosphere through small openings in the leaves called stomata. The absorbed CO_2 is then converted into glucose through photosynthesis. This glucose is used to fuel plant growth and development and to produce other macro-molecules, such as cellulose and starch, which are used to build the plant cell walls and store energy (Geiger and Servaites 1994, Rosa et al. 2009). Additionally, some glucose produced through photosynthesis is converted into other sugars like fructose and sucrose and transported throughout the plant for use as a source of energy. Polyamines are also known to play a role in plant C metabolism (and *vice versa*) by direct increase in their concentration and affecting the activities of enzymes involved in photosynthesis, such as Rubisco. Polyamines modulate the activity of Rubisco by binding to its regulatory subunit, which helps to stabilize the enzyme and

increase its activity. This can increase the rate of photosynthesis, further increasing plant growth and productivity (Shu et al. 2012).

Effect of abiotic stress on the nitrogen and carbon metabolism

Abiotic stress like drought, high salinity, extreme temperatures, and heavy metal toxicity significantly impact N and C metabolism in plants. When plants are exposed to abiotic stress, they often experience a dearth of water availability because the stomata close to prevent water loss, and therefore a decrease in plant growth and productivity. High salinity can affect N metabolism by reducing the uptake and assimilation of NH_4^+ and NO_3^-, and by altering the activity of enzymes involved in N assimilation (Ashraf et al. 2018). High salinity can directly affect C metabolism by reducing the activity of Rubisco and by altering the balance between C fixation and photorespiration. Another common abiotic stress factor in both cultivated and wild plants that negatively impacts both N and C metabolism is heavy metals in the soil. Heavy metals such as lead (Pb) and Cd can inhibit the activity of enzymes involved in N and C assimilation and damage the chloroplasts (Yang et al. 2019, Yang et al. 2020a, Zulfiqar et al. 2020). Cellular PAs have been shown to interact very positively in regulating heavy metal responses in plants.

Polyamines

Polyamines are a group of low molecular-weight polycationic amines involved in numerous metabolic processes in all living organisms (Majumdar et al. 2013, Gu et al. 2020, Pál et al. 2021, Cruz-Pulido and Mounce 2023). The most common PAs are putrescine (Put), spermidine (Spd), and spermine (Spm), thermospermine, cadaverine, and 1,3- diamino propane (1,3-DAP) (Minocha et al. 2014, Michael 2016). Amino acids like arginine (Arg), ornithine (Orn), and methionine (Met) are the most common precursors of the PA biosynthesis cycle (Majumdar et al. 2013, Pál et al.

2021). For example, Orn is converted to Put through ornithine decarboxylase (ODC; EC 4.1.1.17). Putrescine is then converted to Spd and Spm through a series of reactions by sequential additions of aminopropyl groups derived from decarboxylated S-adenosyl-Met (dcSAM – itself produced from SAM by the enzyme called SAM decarboxylase; EC 4.1.1.50) and involving specific enzymes called spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.16) (Majumdar et al. 2013). So, one way to think about the connection between PAs and AAs is that the former is derived from the latter, specifically Orn, through a series of enzymatic reactions and plays a vital role in plant physiology (Figure 1). In contrast, AAs play a vital role in protein synthesis and other cellular processes.

Polyamines are located all over in the cell including vacuoles, mitochondria, cytoplasm, nucleus, nucleoplasm, chloroplast, cell wall, thylakoid membrane, photosystem complex II and light-harvesting complex (Takahashi 2020, Pál et al. 2021). They are also found in the cell membrane and the apoplast, a compartment outside the plasma membrane but inside the cell wall, where solutes can diffuse and interact with the cell wall. The abundance of PAs in the cell can vary depending on its function and the environment. In plant cells, PAs can be free as well as bound to phenolic acids and biomacromolecules (Majumdar et al. 2016, Michael 2016, Takahashi 2020, Pál et al. 2021). Based on the location of the PAs and their role in the normal developmental processes of plants, a little disturbance in the level of PAs can change the metabolic system (Majumdar et al. 2016, Pál et al. 2021).

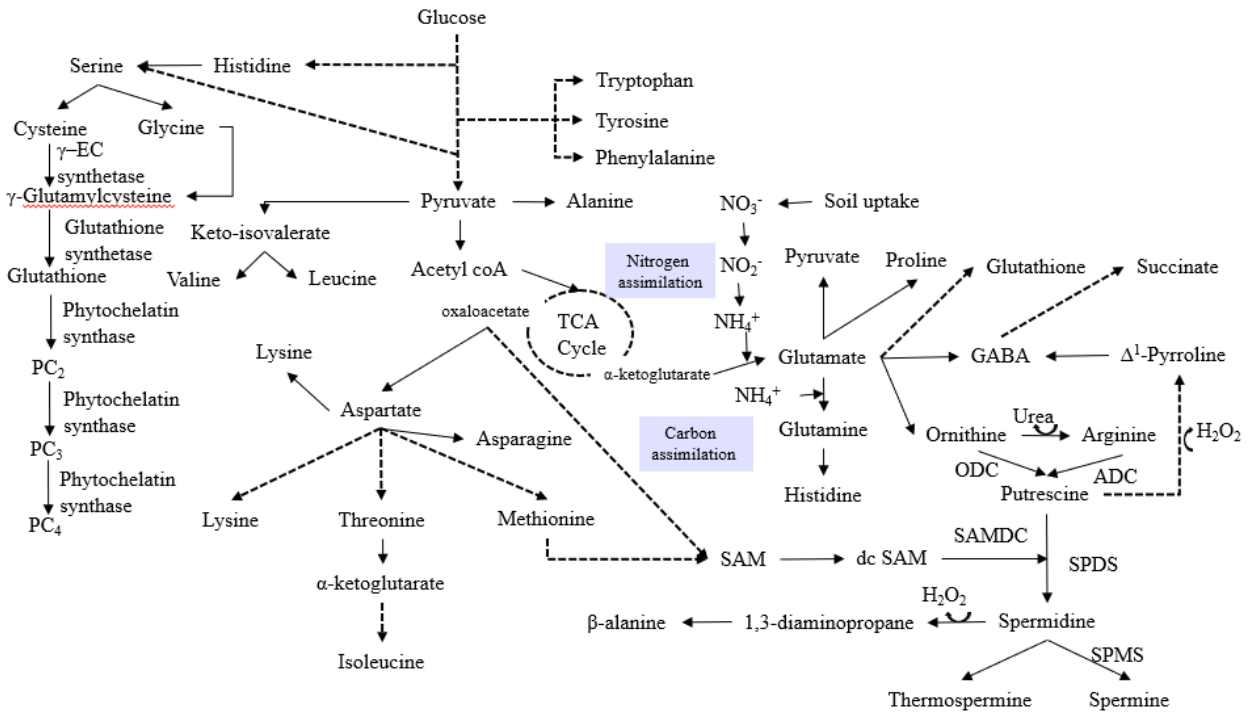

Figure 1: Pathway for biosynthesis of polyamines (PAs), thiol compounds, and phytochelatins (PCs) from amino acids (AAs) (adapted from Cobbett 2000 and Majumdar et al. 2016). Abbreviations: ADC = arginine decarboxylase; ODC = ornithine decarboxylase; GABA = γ-aminobutyric acid; SAMDC = S-adenosylmethionine decarboxylase; SPDS = spermidine synthase; SPMS = spermine synthase. Dashed lines = multiple steps.

Role of polyamines

Polyamines carry a net positive charge; hence, they can interact with numerous cellular components like DNA, RNA, ATP, specific proteins, and phospholipid heads of cellular membranes (Minocha et al. 2014, Nandy et al. 2022). Apart from their universal role in cell development and growth, PAs interact with negatively charged molecules in the cell (Alcázar et al. 2010, Bano et al. 2020, González-Hernández et al. 2022). These interactions allow them to play crucial roles at the molecular level, like regulation of transcription and translation, and gene expression (Abd Elbar et al. 2019), thus regulating cell division as well as cell elongation and differentiation. Due to the interaction with various cell organelles, PAs play critical roles in seed germination, root and shoot growth, floral bud formation, chlorophyll synthesis, gas exchange, ion

transport, and membrane stability, among others. Polyamines are an essential source of N due to their high cellular concentrations in the mM range. Polyamines have also been shown to play a role in plant stress tolerance and have been found to have antioxidant properties (Alcázar et al. 2010, Tamang et al. 2021, Malik et al. 2022). Such properties of PAs help to protect cells from damage caused by reactive oxygen species (ROS) generated under stress conditions. It has been shown that PAs modulate the stress hormones like abscisic acid (ABA) and influence the expression of stress-responsive genes (Tamang et al. 2021). Because of their ubiquitous presence in plants, a slight disturbance in PAs can significantly affect growth and development, including their responses to abiotic and biotic stresses (Minocha et al. 2014, Pál et al. 2018, Takahashi 2020).

Polyamines as a defense system in abiotic stress

Damaging effects on plants due to abiotic stress include reduced plant height, leaf number, stem diameter, fresh weight and dry weight, photosynthesis parameters, chlorophyll, carotenoid anthocyanin, vitamin E, tocopherol, proteins, and total soluble sugars (Paul et al. 2018, Malik et al. 2022). When under stress, plants over-accumulate ROS and carbohydrates in their leaves (Rejšková et al. 2007). Paul et al. (2018) and Malik et al. (2022) have also shown that excess generation of ROS results in oxidative damage to the cellular organelles and cell membranes.

The role of PAs in abiotic stress has been studied for a long time (Galston et al. 1997, Groppa and Benavides 2008, Gill and Tuteja 2010a, Minocha et al. 2014, Nandy et al. 2022). Alcázar et al. (2010) showed that PAs are involved in direct interactions with different metabolic routes and hormonal cross-talks in abiotic stress. When plants are exposed to environmental stress, PAs have been shown to have several protective mechanisms against abiotic stresses like salt stress, heavy metals, and drought in plants (Demetriou et al. 2007, Malik et al. 2022). Hence, timely activation of biochemical pathways for signaling PAs is important for the survival of the plants.

Plant growth

By reducing the oxidative stress caused by excessive salt concentrations, PAs can reduce the detrimental effects of salt on plant growth. Polyamines can help to neutralize ROS and scavenge free radicals, thus protecting the cells from damage and boosting the antioxidant defense system (Paul et al. 2018, Xiong et al. 2018, Malik et al. 2022). It has been shown that treating tea plants with Put enhanced their antioxidant defense mechanism and decreased oxidative damage brought on by salt stress (Xiong et al. 2018). It has also been studied that PAs can reduce ion toxicity under heavy metal stress by chelating divalent metal ions and stopping them from impairing the regular cell activities of sunflower leaves (Groppa et al. 2001).

Photosynthesis

Under abiotic stress, PAs (endogenously produced and exogenously applied) can affect photosynthesis by reducing oxidative stress damage (Demetriou et al. 2007, Duan et al. 2008). As PAs can scavenge free radicals and neutralize ROS, they can protect the photosynthetic machinery and boost the antioxidant defense system of the plants. Additionally, it has been demonstrated that PAs improve photosynthesis by activation of the enzymes involved in the process, like ribulose bisphosphate carboxylase (Rubisco), which is essential for fixing carbon. Duan et al. (2008) showed that administering Spd to cucumber plants enhanced their ability to produce oxygen and protect themselves from free radicals when exposed to salt stress. Polyamines can also increase photosynthetic efficiency by reducing ion toxicity caused by high salt concentrations by chelating divalent metal ions, which can prevent the ions from disrupting the normal functions of the photosynthetic apparatus. It has been argued by Malik et al. (2022) that exogenous applied PAs increased photosynthetic effectiveness, decreased ion toxicity, and increased tolerance under heavy metal stress.

Total soluble carbohydrates

Sugars serve as the primary source of metabolic energy in plants. They are also the signaling molecules that regulate the growth and development of plants (Van Dingenen et al. 2016). Metabolic influx due to endogenous or exogenous PA accumulation affects the carbon (C) metabolism in plants. In leaves, C accumulation is triggered by several metabolites, including NO_3^- and sugars. In response to abiotic stress, sugars like trehalose are one of the primary solutes involved in the osmotic adjustment of plants (Kosar et al. 2019). Exogenous applications of Put also played a prominent role in the Krebs cycle by inhibiting the over-accumulation of carbohydrates in the leaves from reducing salt damage (Yuan et al. 2015, Zhong et al. 2016).

Furthermore, PAs have been shown to protect the plant cells by inhibiting the Cd induced damage to the cell membrane, which in turn can prevent the leakage of sugars, and thus the total sugars in the plant will remain high (Groppa et al. 2001). Additionally, it has been shown by Nahar et al. (2016a) that PAs protect the prominent energy-producing organelles (mitochondria and chloroplasts) in plants, from Cd induced damage. In conclusion, PAs play a crucial role in regulating the accumulation of sugars in plants under Cd stress by protecting the cell membrane, mitochondria, and chloroplasts and stimulating the enzyme activity involved in sugar metabolism.

Polyamines and salt stress

Polyamines affect the metabolite profile in plants under salt stress. Studies have demonstrated that PAs can modify primary metabolites like AAs and organic acids in response to salt stress. Additionally, PAs can promote the accumulation of soluble compounds, such as proline (Pro), to protect the plant cells from the damaging effects of high salt concentrations. It has been studied by Sadak et al. (2012) that sunflower seeds pre-treated with Put resulted in Pro accumulation and improved plant development under salt stress. A study discovered that PAs could increase the

concentrations of AAs, such as Pro, γ-aminobutyric acid (GABA), alanine (Ala), and aspartic acid (Asp), in cucumber plants under salt stress and help the plant to survive under salt stress (Yuan et al. 2016). However Rizhsky et al. (2004) reported that *Arabidopsis thaliana* plants subjected to a combination of drought and heat stress did not accumulate Pro, although they accumulated high levels of sucrose and other sugars. Roychoudhury et al. (2011) demonstrated that Spd treatment improved the gene expression associated with stress response in rice seedlings under salt stress. One of the reasons for salt tolerance in *A. thaliana* is the accumulation of free Put by the induction of *AtADC2* gene (Urano et al. 2004).

Polyamines and cadmium stress

In response to Cd stress, PAs can also modify the levels of several metabolites (Nahar et al. 2016a). According to their research, PAs can modify the levels of AA like Pro, as well as organic acids like citrate and malate in mung bean seedlings under Cd toxicity. Similar to salt stress, excessive Cd concentrations cause oxidative stress, which PAs can lessen by scavenging free radicals. They can also chelate metal ions, which can lessen the toxicity of Cd.

Several authors have reported that PAs control the expression of genes associated with Cd stress and detoxification, which can aid in reducing the toxic effects of the heavy metal on the plant. Exogenous PA therapy for plants has been found to improve plant growth and yield by regulating the expression of genes related to germination, flower development, fruit growth and ripening, under abiotic stress (Gill and Tuteja 2010a). It is important to remember that Spd and Spm have reportedly been shown to be more potent at lowering the toxicity of Cd stress.

Phytochelatins

Plants produce small peptides called phytochelatins (PCs) as strong antioxidants in response to heavy metal stress (Cobbett 2000, Thangavel et al. 2007, Hasanuzzaman et al. 2017). Phytochelatins like glutathione (GSH) help to chelate heavy metals and aid in their sequestration in the vacuoles, thus preventing oxidative denaturation of proteins in plant cells in the presence of heavy metal (Grill et al. 1989, Liu et al. 2015). Recent research has demonstrated that PAs can affect plants under Cd stress in terms of PC production and accumulation. According to a study by Pál et al. (2017) adding PAs to rice seedlings under Cd stress boosted PC synthesis and accumulation. The authors hypothesize that this is due to the capacity of PA to control the expression of genes essential for PC production. However, it was also reported that overexpression of γ-glutamyl-cysteine synthetase in *Arabidopsis thaliana* (Xiang et al. 2001) and *Solanum lycopersicum* (Goldsbrough 1998) did not enhance Cd tolerance. Thus it appears that PAs offer a broad spectrum resistance to a range of stresses in plants (Alcázar et al. 2010, Minocha et al. 2014, Pál et al. 2021). The enhanced tolerance to abiotic stress, which will be covered in greater detail later, is accompanied by an increased level of PAs.

Organic acids

In presence of abiotic stress, plants tend to maintain homeostasis by regulating the complex metabolic system. Several metabolites like the organic acids (aconitic acid, citric acid, fumaric acid, and malic acid) in the tricarboxylic acid (TCA) cycle, as well as mannitol, glucaric acid, pentonic acid, and 4-hydroxybenzoic acid gets accumulated on the plant roots mainly due to the nutrient deficiencies arising in presence of abiotic stress (Zhao et al. 2021a). It has been reported by Li et al. (2020b) that PAs play an important role in the regulation of the levels of organic metabolites. It has been hypothesized by Mattoo and Handa (2008) that stress-regulation in PA

accumulating transgenic tomato is conferred by biosynthesis of extra choline and its conversion to glycine betaine. Regulation on the citrate and malate levels in *Arabidopsis thaliana* conferred salt tolerance (Khan et al. 2020). Moreover, PAs have been shown to play a role in regulating the activity of enzymes involved in the synthesis and degradation of organic metabolites, including those involved in the biosynthesis of secondary metabolites, such as flavonoids and phenylpropanoids (Mattoo and Handa 2008, Nambeesan et al. 2010, Minocha et al. 2014, Khan et al. 2020). In summary, the role of PAs in regulating organic metabolites in plants is a complex and multifaceted one, with implications for plant growth, stress tolerance, and their overall performance.

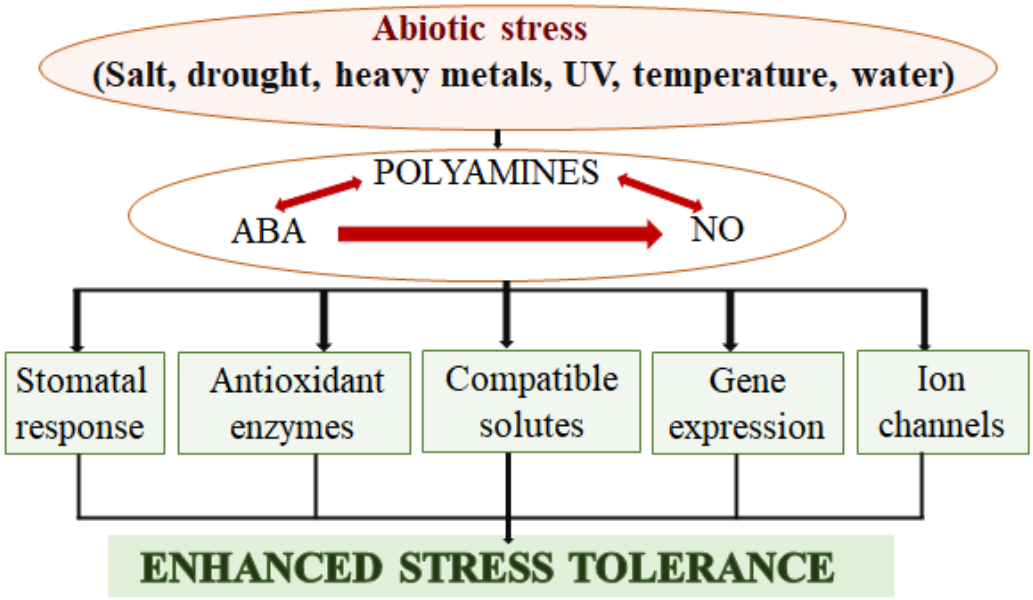

Figure 2: Role of polyamines under abiotic stress (Modified from Shi and Chan. 2013).

Methods of polyamine modification in plants

Two types of experimental approaches have been used to experimentally alter PA metabolism: mutations and genetic engineering (transgene expression). The transgene can be constitutively

expressed or at specific time when plants face environmental hazards, via regulated promoters. Currently, several types of promoters are available, e.g. constitutive, cell and tissue and organ specific, inducible promoters. Our lab has published several papers on the biochemical and cellular effects of inducible and constitutive promoters in regulating growth and development of several plants (Page et al. 2007, Mohapatra et al. 2010a, Majumdar et al. 2013, Shao et al. 2014, Majumdar et al. 2017).

Manipulation of polyamine metabolism against abiotic stress

There are several ways in which PA metabolism can be experimentally manipulated to protect plants against abiotic stress; including: (a) exogenous application of PAs; (b) genetic engineering of PA metabolism; (c) Chemical inhibitors; (d) bio-stimulants.

Manipulation of polyamine metabolism in plants via exogenous application

The exogenous application of PAs can alter the PA and AA metabolism in plants in several ways (Minocha et al. 2014, Ebeed et al. 2017). The application of PAs directly to the plant or the use of biotic or abiotic elicitors that promote PA production are two methods of altering PA accumulation in plant tissue (Ebeed et al. 2017). According to Alcázar et al. (2006b) and Rossi et al. (2021), treating plants with hormones like melatonin, methyl jasmonate (MJ), salicylic acid (SA), or ABA increased PA and antioxidant enzymes under abiotic stresses (Naz et al. 2021, Li et al. 2022). Direct exogenous application of PAs (via roots or leaves) can also alter PA content of the cells by activating antioxidant enzymes involved in PA biosynthesis and degradation (Gill and Tuteja 2010a, Paul et al. 2018, Li et al. 2020a).

In addition, the exogenous administration of PAs can change the ratio of PA production to breakdown, which can impact cellular PA metabolism. S-adenosylmethionine decarboxylase

(SAMDC) and cadaverine dehydrogenase are two enzymes involved in PA breakdown that can be inhibited by the administration of large concentrations of exogenous PAs (El Hadrami and D'Auzac 1992). The effect of exogenous PA administration on PA metabolism can change depending on the plant species, age, and type and concentration of PAs utilized. The action of exogenous PAs may also be modified by other environmental elements such as light, temperature, and water availability, as demonstrated by Ebeed et al. (2017).

Plant materials for my research

Poplars:

Members of the genus *Populus* (poplars) and its related genus Salix (willows) have the properties of faster growth with an usability of their wood and entire biomass. Poplars are among the most rapidly growing and biomass-accumulating forest trees and are considered a model forest tree in sustainable fast-rotation forestry (Dickmann 2001, Hawkins et al. 2003, Plomion et al. 2016, Zalesny Jr et al. 2019). The poplars are a significant source of energy, pulp, and lumber and are used in the phytoremediation of heavy metals (Yi et al. 2022). Most importantly, with the availability of the poplar genome sequences in 2004 and the development of high throughput 'omics' technologies (Ma et al. 2019), one can combine all the potential tools and designed bioengineered plant systems in a short period. Many species and hybrids of poplars and are known to be easy to propagate asexually by cuttings (Navarro et al. (2018) making them highly suitable for short-rotation woody forest crops. Poplars and willows are one of the finest renewable energy resources, and short-rotation forestry gives an advantage. Due to this property, they have been used across the world in agriculture and forestry. Scientists have studied wood physiology and biochemical properties (Bhatnagar et al. 2002, Minocha et al. 2004, Larisch et al. 2012), biofuel

production (Pari et al. 2015), and breeding and selection (Riemenschneider et al. 2001) in poplars. Recent studies have shown that many transgenic poplars can tolerate long-term abiotic stress (Ke et al. 2015, Cheng et al. 2019, Huan et al. 2023).

Poplars and abiotic stress

Woody plants like poplars have an additional stress tolerance system and are widely used in phytoremediation of harmful metals and other pollutants (Robinson et al. 2000, Guerra et al. 2011, Hu et al. 2013). A study published by Hu et al. (2013) found that poplar trees can accumulate high levels of Cd and Zn in the shoots without affecting growth and nutrient uptake. It has been reported by Schützendübel et al. (2002) that the poplar trees responded to Cd stress by increasing the accumulation of PCs, organic acids, and metallothionein in the leaves. Another study published by Lin et al. (2023) found that poplar trees can affect the soil microbial community and enzyme activities in Cd-contaminated soils. It was found that the poplar trees can increase the abundance of Cd-resistant bacteria and the activity of enzymes involved in the cycling of N, C, and P in the soil. Several studies have investigated the mechanisms by which poplar tolerate salt stress (Chen and Polle 2010, Mirck and Zalesny 2015, Zhang et al. 2019a). It has been shown by Zhang et al. (2019a) that overexpression of *NAC13* gene in a poplar hybrid 84 K (*Populus alba* × *P. glandulosa)* enhanced salt tolerance. Apparently, poplars alters their root growth, ionic balance, proteome and metabolomic responses to survive under saline conditions (Zhang et al. 2019b) and heavy metal stress (Shen et al. 2021). The authors found that the poplar trees responded to salt stress by increasing the accumulation of compatible solutes, such as sugars and AAs, and by reducing the accumulation of Na^+ ions in the leaves. A recent study published by He et al. (2023) has shown 84K poplar could accumulate high levels of Cd by regulating the *phytochelatin synthase1* (*PCS1*) gene which further catalyzed antioxidants like GSH. These trees responded to

Cd stress by reducing transpiration, stomatal conductance, and photosynthesis, and concurrently, increasing the accumulation of osmolytes in the leaves (Guerra et al. 2011, Lin et al. 2023).

A hybrid variety of poplar *Populus nigra* X *maximowiczii* (NM6 clone) is widely grown in North America and many other countries for the production of economically important biomass as well as a strong phytoremediation property. This clone has characteristic property of asexual mode of reproduction, high biomass, and genetic stability with no risk of cross-pollination among different species and clones of poplars in the vicinity (Labrecque and Teodorescu 2005, Guerra et al. 2011). Zalesny Jr et al. (2019) also reported that NM6 clone was more salt-tolerant than other willow and poplar species.

Importance of poplars in abiotic stress tolerance

Poplar can be used in a variety of ways to improve abiotic stress tolerance in other plants. Scientists have been able to identify genes involved in the stress response of poplar trees, and these genes can be used to improve the stress tolerance of other plants through genetic engineering (Zhang et al. 2019a, Zhang et al. 2019b). It has been shown that downregulation of genes like *PagSAP1* gene and upregulation of *NAC13* gene in *Populus alba* × *P. glandulosa* conferred tolerance to salt stress (Zhang et al. 2019b). Poplar trees have been shown to produce a variety of root exudates that can help improve the stress tolerance of other plants by altering the soil microbial community and enzyme activities (Qin et al., 2007; Yu et al., 2022). The tolerance of poplars to abiotic stress can also be improved through breeding programs. However, there have been no reports on the physiological and biochemical effect due to exogenous treatment of PAs to ameliorate abiotic stress effects on NM6.

Given the wide use of exogenous application of PAs for amelioration of salt and Cd stress, and the knowledge gap on the effect of exogenous PAs in NM6 in response to salt and Cd stress,

I focused on the physiological and biochemical effects of exogenous PAs in NM6 plants in response to salt and Cd stress.

Related background studies from the Minocha Lab and others

One of the earliest studies from the Minocha lab was to understand the role of PAs on the growth and development of tobacco plants by genetically manipulating them. A human *SAMDC* gene was transferred to tobacco (*Nicotiana tabacum* cv. Xanthi) under the control of 35S promoter of Cauliflower mosaic virus (CaMV) with a *neomycin phosphotransferase* gene (*NPTII* for selection of transgenic plants) via *Agrobacterium tumefaciens.* Whereas Put level was reduced significantly in the transgenic plants, a 2-to-4-fold increase in SAMDC activity and a 2-to-3-fold increase in Spd levels were observed in them *vs.* the non-transformed (NT) plants. At the same time, Spm content remained unchanged or increased *vs.* the NT plants (Noh and Minocha, 1994). This result was similar to the data published earlier by Hamill et al. (1990), where a yeast *ODC* gene was transferred to tobacco roots by *Agrobacterium. rhizogenes.* DeScenzo and Minocha (1993) developed transgenic tobacco plants with mouse *ODC*, also under the control of 35S promoter, which showed a 3-to-20-fold increase in *ODC* activity, and 4-to-12-fold increase in cellular Put, but no increases were observed for Spd and Spm. They suggested that a lack of increase in Spd might be due to insufficient supplies of dcSAM or the SPDS activity.

Several researchers have reported that PAs are able to retard the leaf senescence, whereas ethylene promotes senescence (Altman 1982, Sood and Nagar 2003, Xu et al. 2011, Shu et al. 2012, Koyama et al. 2013, Shao et al. 2014, Qu et al. 2020, Altaf et al. 2022). Polyamines also inhibit ethylene biosynthesis in leaves and flower petals, so there is a competition between ethylene biosynthesis and accumulation of PAs (Mattoo and Handa 2008, Nambeesan et al. 2010, Gao et al. 2021). Nambeesan et al. (2010) had reported Spd delayed post-harvest senescence in transgenic

tomato plants overexpressing yeast *SPDS* gene under CaMV35S and fruit-ripening specific (E8) promoters. The relation between PA and ethylene was studied by Quan et al. (2002) in NM6 poplar cells in our lab by over expressing m*ODC* and a Datura *SAMDC* gene under the control of 35S CaMV promoter. Their results showed significantly higher Put and Spd in the transgenic cells compared to the NT poplar cells but had a constantly higher level of ethylene in the transformed cells *vs*. the NT cells. It was concluded that there was no apparent competition between PAs and ethylene biosynthesis pathways.

The protective role of PAs under biotic and abiotic stress can be attributed to both increased biosynthesis and decreased catabolism (Shao et al. 2012). A study was conducted by Shao et al. (2014) to understand the catabolism of Spd and Spm under the same physiological conditions where PAs were manipulated in the poplar NM6 cells. The studies were conducted using ^{14}C-labeled precursors of Put and Spd. Overexpression of the m*ODC* gene exhibited 8-to-10-fold increase (in poplar cells) and as much as 40-fold increase in Put production in *Arabidopsis thaliana*. Almost 50% increase was observed in Spd, but the Spm content was lower in high putrescine (HP) cells than in NT cells of poplar. The half-life of Spd was found to be 22h in NT and 32h in HP poplar cells, and 52h and 56h in NT and transgenic Arabidopsis plants. It was hypothesized that apart from increased biosynthesis, a lower catabolic rate may be one of the reasons for high PAs during the abiotic and pathogen stress response. High Put content did not increase the uptake of Spd and Spm in Arabidopsis seedlings, which agreed with the previous results reported by Majumdar et al. (2013).

The presence of all the PAs is crucial for normal growth and development, and mutation of one of the enzymes in the PA biosynthesis pathway can halt the growth of plants entirely. It has been shown that a mutation of the SPDS gene prevents the production of Spd from Put and

inactivates the eukaryotic translation initiation factor 5A (eIF5A). *Aspergillus flavus*, an opportunistic pathogen, is responsible for causing mycotoxin contamination in crop plants (Schuster et al. 2002, Yu et al. 2005). Maize seed kernels, when infected with the *spds* mutant gene of *Aspergillus. flavus*, resulted in a massive reduction of fungal growth, sporulation, and aflatoxin accumulation than the control kernels, which was anticipated due to the downregulation of aflatoxin biosynthetic genes (Majumdar et al. 2018). This hypothesis was confirmed with gene expression studies by quantitative polymerase chain reaction (PCR) of aflatoxin biosynthesis genes. Due to interaction between *Aspergillus. flavus* - maize kernels there was increased uptake of PA transporters and an increase in expression of arginine decarboxylase (*ADC)* and *SAMDC* genes in the maize host (Majumdar et al. 2018) which corroborates increased PA synthesis during biotic stress. It is known that the *SAMDC* gene family in maize has multiple members, which code for the same enzyme in the same species and hence has a diverse range of functions. An in-silico study was done by Majumdar et al. (2017) to understand the specific role of the *SAMDC* gene in Arabidopsis development life cycle. Five paralogs of *SAMDC* genes in Arabidopsis have shown similar divergence patterns of promoter and coding sequences in their studies, but the divergence of 5'UTR (untranslated region) and 3'UTR was independent of the promoters, and coding sequence (CDS) was more during the active stages of development. Similar results were obtained while studying somatic embryogenesis in *Daucus carota*, where the expression of SAMDC was higher in embryonic stages of development than in later stages.

Manipulating a single step in the PA biosynthesis process to increase Put production leads to a series of changes in the activities of key enzymes involved in this pathway. Studies by Page et al. (2012) suggested Orn to be the critical regulator of the PA biosynthesis pathway. Upregulation of PA biosynthesis in poplar by transgenic expression of the *mODC* gene in poplar NM6 cells

resulted in insignificant changes in gene expression for enzymes in the Orn/Arg biosynthetic pathway *vs.* the NT poplar cells. Transgenic overexpression of the *mODC* gene also resulted in several fold increase in Put production from Orn and concentration of Orn to be extremely low relative to the amount of Put produced from it, which was in accordance with the previous results from the lab (Bhatnagar et al. 2001, Bhatnagar et al. 2002, Mohapatra et al. 2009, 2010b, Page et al. 2012). High Put production due to overexpression of *mODC* genes is suggested to protect the poplar plants against ROS, but their enhanced turnover makes the plant cells vulnerable to oxidative damage (Mohapatra et al. 2009).

Minocha et al. (2004) studied the effect of variation in the N concentration of the medium in transgenic and non-transgenic NM6 cell cultures. High Put in transgenic NM6 cells affected physiological parameters like protein accumulation, membrane permeability, mitochondrial respiratory activity, and their overall growth rates. The transgenic cell cultures were less tolerant to NH4NO3 while higher tolerance to KNO_3. Quantitative RT-PCR studies on the expression of three members of the *pSAMDC* gene family in transgenic NM6 (overexpressed with *mODC* gene) showed upregulation of pSAMDC1 and pSAMDC3 and downregulation of pSAMDC2 compared to transgenic NM6 overexpressed with *GUS* gene. The overall activity of SAMDC was lower in HP cells *vs.* the control cells (Page et al. 2007).

It was hypothesized by Mohapatra et al. (2009) that higher Put accumulation would have a protective role against ROS in plants. They compared the activities of various antioxidant enzymes and cellular metabolites of the ROS scavenging pathway in HP NM6 cell lines overexpressed with the *mODC* gene. Their studies have shown HP cells had greater membrane damage, high glutathione reductase, monodehydroascorbate reductase, and proline and significantly lower glutathione and glutamate *vs.* control cells. It was concluded that high Put confers protection

against ROS but increases oxidative damage on the NM6 cells. Our lab also studied the effect of aluminum and calcium stress on transgenic NM6 cells (Mohapatra et al. 2010a). In response to Ca, there was reduced accumulation of Put with low mitochondrial activity and cell biomass. The HP cells had a higher accumulation of thiol compounds and PCs under Al stress. Their studies concluded HP cells are at an advantage under Al stress due to the reduced Al uptake from the medium. Transgenic manipulation of a single PA (overexpressed with CONST. *mODC* gene) in poplar cells increased accumulation of Ala, Thr, Val, Ile, and GABA and decreased accumulation of Glu, Gln, Orn, Arg, His, Ser, Gly, Cystine, Phe, Trp, Asp, Lys, Leu and Met. Compared to the control cells, HP cells had higher N and C content suggesting the *ODC* gene played an important role in manipulating the Orn biosynthesis pathway (Mohapatra et al. 2010b).

Polyamines and AAs like Pro, Arg, Orn are a significant link to N-metabolizing pathways in all plants (Galston and Sawhney 1990, Moschou et al. 2012, de Oliveira et al. 2018, Paschalidis et al. 2019, Feng et al. 2023). By producing intermediates like nitric acid (Yamasaki and Sakihama 2000, Astier et al. 2018) and GABA (Bouche and Fromm 2004, Li et al. 2021a), their accumulation depends on N assimilation, but the metabolic regulatory process is yet to be unraveled. Majumdar et al. (2016) studied increase in PA biosynthesis resulted in increased N and C assimilation in transgenic Arabidopsis (containing *mODC* gene). They concluded that there was increased utilization of Orn which enhanced the overall conversion of glutamine (Gln) to Arg and PA, whereas Pro and Arg are regulated independently. The general expression of 28 genes studied here did not show any change even though the biosynthesis of Orn increased by several folds.

To understand how N affects the growth and development of forest plants under abiotic stress, it is crucial to understand as to how plants can utilize N under various stresses. Several commercially important clones of poplars (the genus *Populus*) have been produced and cultivated

on a large scale over the years (Zalesny Jr et al. 2019). Poplar clones like NM6 (*Populus nigra x maximowiczii*) plants are one of the dominant tree species in North America with a faster growth rate that have been used commercially all over the world for the production of wood, biomass for bioenergy and phytoremediation of heavy metals (Guerra et al. 2011, Lin et al. 2023). This clone has been the focus of study in our lab for a long time.

Goal and Objectives of the study

Taking advantage of the >30-year-long history of studies conducted by our lab, broadly on the PAs and other related metabolites in poplar cell cultures (Mohapatra et al. 2009, Mohapatra et al. 2010a, Mohapatra et al. 2010b), *Arabidopsis thaliana* and other plants (Majumdar et al. 2013, Shao et al. 2014), and the knowledge gap in poplar NM6 under abiotic stress, I studied the role of PAs in NM6 plants under abiotic stress using young greenhouse-grown plants.

The goal of the study was to understand the physiological and biochemical effects of salt and Cadmium, and their interactions with PAs sprayed on the leaves in poplar NM6 young plants.

The two specific objectives of the study were: (1) to analyze the effects of two concentrations of NaCl on the physiology and biochemistry of poplar NM6 plants and potential mitigation of these effects by spray with putrescine; and (2) to analyze the effects of two concentrations of Cadmium chloride on the physiology and biochemistry of poplar NM6 plants and potential mitigation of these effects by spray with spermidine.

Initial attempts were also made to develop transgenic NM6 plants overexpressing genes for the manipulation of PAs using a constitutive and an inducible *SPDS* gene with the aim of testing the growth response and physiological responses of the plants to different forms of nitrogen. However, due to unique situations raised by COVID-19 pandemic during which period certain

types of experiments were not possible, the focus of the study was shifted to wild-type plants only. Hence, wild-type NM6 plants were used throughout the research, and experiments were designed accordingly. Two research questions were addressed to explore the importance of PAs in abiotic stresses - (1) What effects does abiotic stress have on young poplar plants grown in the greenhouse? (2) How do exogenous PA applications reduce the detrimental effects of abiotic stress on these young poplar plants? To explore these questions, two different greenhouse experiments were set up on salt stress (NaCl) and heavy metal (Cd) stress and studying their interactions with foliar spray of Put and Spd, respectively.

The greenhouse experiment on salt stress hypothesizes that foliar spray of Put will confer tolerance to salt in poplar NM6 plants. The experiment on Cd stress hypothesizes that repeated Spd application on leaves will reduce the toxic effects of Cd on the young poplar plants. The study involved the analyses of FW, DW, chlorophyll contents, photosynthesis, total soluble proteins and sugars, AAs, and PAs, for both NaCl and Cd. Additionally, phytochelatin biosynthetic pathway members were analyzed for Cd treatment and a few samples for salt treatment were analyzed for major components of the metabolome. Measurements for increase in the plant stem growth and diameter, and real-time chlorophyll contents on the leaves were taken on several days. At the end of the 3-week treatments, the cellular contents of PAs, AAs, and PCs (in Cd stress only) in the roots were also done.

Table 1: Effect of exogenous putrescine in plants under salt stress

Plant	Concentration of NaCl	Concentration of Put	Effects on plant in presence of Put over salt	Reference
Hordeum vulgare	9.3 and 14 dS m^{-1}	100 and 200 ppm	1. Increment in vegetative characters and yield 2. Improved leaf and stem anatomy 3. Decrease in antioxidant enzymes and glycine betaine 4. Increase in proline and total carbohydrates	Seleem et al. 2021
Panas ginseng	0, 150 mM	0.3, 0.6. 0.9 mM	1. Enhancement of salt tolerance 2. Enhancement of ginsenoside content	Islam et al. 2021
Cucumis sativus L.	90 mM	8 mM	1. Alleviation of photoinhibition due to salt stress	Wu et al. 2019
Camellia sinensis	50 mM and 100 mM	5 mM	1. Reduced polyphenol levels 2. Increase in Put and Spm level 3. Decrease in Spd level	Xiong et al. 2018
Cucumis sativus L., cv.Jinyou No. 4	75 mM	1 mM	1. Promotion of chlorophyll and xanthophyll cycle 2. Conversion of uroporphyrinogen III to protoporphyrin IX 3. Higher photosynthetic rate 4. Reduced accumulation of sucrose and starch 5. Regulation of hormone level, leaf structure, carbohydrate metabolism 6. Increment in plant growth	Yuan et al. 2018 Zhong et al. 2016 Yuan et al. 2015 Yuan et al. 2014
Cucumis sativus L. cv.Jinyou No. 4	75 mM	1 mM	1. Regulation of photosynthetic efficiency 2. Reduced fatty acid contents in salt stress 3. Regulation if protein expression at transcription and translation level	Shu et al. 2015
Gossypium barbadense L. ev. Giza 90	3000 ppm, 6000 ppm, and 9000 ppm	1 ppm and 2 ppm	1. Increase plant growth and yield characters 2. Increase in chemical constituents of salt tolerance 3. Increase in free amino acids, sugars, soluble phenols	Darwish et al. 2013

			4. Increase in chlorophyll level	
Cucumis sativus L. cv.ChangChun mici	65 mM	10 mM	1. Increase in net photosynthetic rate 2. Promotion of salt tolerance	Zhang et al. 2009
Cicer arietinm	100 mM	0.5 mM	1. Decrease in the antioxidant enzymes level	Sheokand et al. 2008
Hordeum vulgare L	200 mM	0.5 mM	1. Stabilization of root tonoplast integrity 2. Regulation of endogenous PAs on the root	Zhao and Qin 2004

CHAPTER 2: MATERIALS AND METHODS

Plant material and growth conditions

This study used young cuttings of a hybrid poplar (*Populus nigra x maximowiczii* - NM6), which were collected from a 5-year-old healthy NM6 tree at the UNH Kingman Farm. The cuttings were rooted and maintained at the UNH MacFarlane greenhouse for one growing season of 2.5 months (from Mid of April to the end of June) before starting the experiment. During this growing period, the cuttings (~ 15 cm height, 0.6 cm stem diameter) were maintained under mist for the first 2 weeks in grow tubes (17.78 cm) containing PRO-MIX soil by Mycorrhizae. Semi-opaque plastic tubes were used, which allowed light inside while preserving moisture, which fostered the environment for faster growth. The grow tubes were placed in a tray to hold the extra water to keep the soil moist and grown for 2 months, and the grow tubes were watered regularly. After 2 months, plants which were approximately 30-38 cm height with 9-10 leaves, were selected and transferred to 33 cm pots. The pots were filled with mixtures of vermiculite and perlite (1:1).

The experiments were conducted from July 2021 to September 2021. The experiments were conducted in an environmental-controlled greenhouse to maintain a standard environment. The greenhouse had natural sunlight with a 16 hours photoperiod. The average temperature ranged between 22 ºC to 24 ºC, and relative humidity was 70% in the greenhouse till the end of the experiment. All plants were irrigated and fertilized twice daily in automated drip-line irrigation using Jack's Pure water LX - Professional (https://www.jrpeters.com/17-4-17-pure-water-lx). The plants were irrigated with 200 mL water at 8 AM and again at 2 PM regularly. Plants were watered again in the evening on days when the temperature was above 32 ºC.

Design of experiment

This study was done with two different types of stress (salt and heavy metal) treatments. This section describes how each of these stress experiments were designed.

Objective 1: Salt stress

To conduct the experiment on salt stress, salt treatment (with NaCl) was initiated 21 days after transferring the plugs with plants to 33 cm pots. The plants were 3 months and 1 week old from the day of collection of cuttings. For this experiment, 36 plants were divided into 6 groups. The treatments were: treatment A (control (water)), treatment B (100 mM NaCl), treatment C (200 mM NaCl), treatments D (1 mM Put spray of control), treatment E (100 mM NaCl + 1 mM Put spray), and treatment F (200 mM NaCl + 1 mM Put spray). Putrescine was purchased from Sigma Aldrich in the form of Put dihydrochloride, ≥98% (molecular weight= 161.07) and molecular weight of NaCl was 58.44 g/mol. 0.5% Silwet was added as a surfactant to 1 mM Put solution. Each treatment had 6 replicates. The drip lines were closed at 2 PM the day before applying the salt treatment. The salt treatment was applied via roots by pouring 200 mL of NaCl solution into the pots at 8 AM and again at 2 PM on day 0. The drip lines were opened at 2 PM on the same day after salt treatment via roots. Saucers were placed at the bottom of the pots to prevent water leakage from the plants. The plants were sprayed with 1 mM Put immediately after the salt treatment for groups D, E, and F. During the experiment, each plant received 30 mL Put on average. The Put was only applied on the leaves while keeping the soil covered carefully with plastic sheets to prevent spraying of Put on the soil. After spraying on the leaves, leaves were tapped with hands manually to get rid of the extra spray solution sitting on the leaves. The spray treatment was given on days 0, 3, 6, and 13. After the treatments, leaf samples (~6^{th} leaf from top) were collected for various analyses on days 3, 6, 13, and 20.

Objective 2: Cadmium stress

For the heavy metal stress experiment, heavy metal (with Cd in the form of cadmium chloride- $CdCl_2$) treatment was initiated 42 days after transferring the plugs to 33 cm pots. The plants were 4 months old from the day of collection of cuttings. Similar to the salt experiment, a separate set of 36 plants was used and divided into 6 groups; three were controls (no Spd treatment), and the other 3 were sprayed with Spd. The treatments were: treatment A (water), treatment B (50 µM $CdCl_2$), treatment C (150 µM $CdCl_2$), treatment D (1 mM Spd spray), treatment E (50 µM $CdCl_2$ + 1 mM Spd spray), and treatment F (150 µM $CdCl_2$ + 1 mM Spd spray). Each treatment had 6 replicates. Spermidine was purchased from Sigma Aldrich in the form of Spd trihydrochloride, ≥98% (molecular weight= 254.63), and $CdCl_2$ was in the form of $CdCl_2.2.5\ H_2O$ (formula weight=228.34). The drip lines were closed at 2 PM the day before applying the heavy metal treatment. The heavy metal treatment was applied only once via roots by pouring 200 mL of $CdCl_2$ solution into the pots at 8 AM. The drip lines were opened on the same day of the heavy metal treatment application at 2 PM. Saucers were placed at the bottom of the pots to prevent water leakage from the plants. To 1mM Spd solution, 0.5% Silwet was added as a surfactant. During the experiment, each plant received about ~40 mL Spd, and spray was done only on the leaves while keeping the soil covered carefully with plastic sheets to prevent spraying of Spd on the soil. Similar to the salt experiment, after the leaves were sprayed, they were manually tapped with hands to remove any extra Spd solution sitting on the leaves. The spray treatment was given on days 0, 3, 8, and 15. Sample collection was done on days 0, 4, 7, 14, and 21.

Sample collection

Samples from the plants were collected in experiment 1 and 2 for analysis before and after the treatments were applied. This includes leaf and root tissue collection. This section describes how and when the leaves and roots were collected and preserved for analyses which were done in the lab.

Leaves:

Leaf discs were collected a day before applying stress treatments and were recorded as zero-time. Sampling was done from one plant of each treatment group. The leaves were washed with fresh water and pat dried by placing them between 2 layers of paper towels before collecting with specific extraction solutions/buffers (Table 3).

On the other days of sample collection after the treatment, a fully expanded 6^{th} leaf from the top was collected and punched (using a paper punch, 6.35 mm diameter), and leaf discs were collected in respective solvents/buffers similar to day 0. Physiological traits such as relative water content (RWC) and chlorophyll content were measured with leaf disc on various days post-treatment. Gas exchange measurements were taken with LICOR-6400. For various biochemical analyses of the plant, leaf discs were collected for soluble sugars, PAs and AAs, total protein, and chlorophyll contents. All samples (except biomass samples) were stored at -20 ºC until further analyses.

Roots:

Root tissues were collected on the day of harvesting. The roots were thoroughly washed in running tap water to remove vermiculite and perlite. Fine secondary roots (2-3 mm in size) floating on the water were collected and chopped with scissors from the main root. Approximately 100 mg of the root tissues were immediately put into 2 mL microfuge tubes containing respective solutions

for further analysis (Table 2). The root tissues were collected for PAs and AAs analyses. Additionally, the root tissues of plants treated with Cd were also collected for PC analyses.

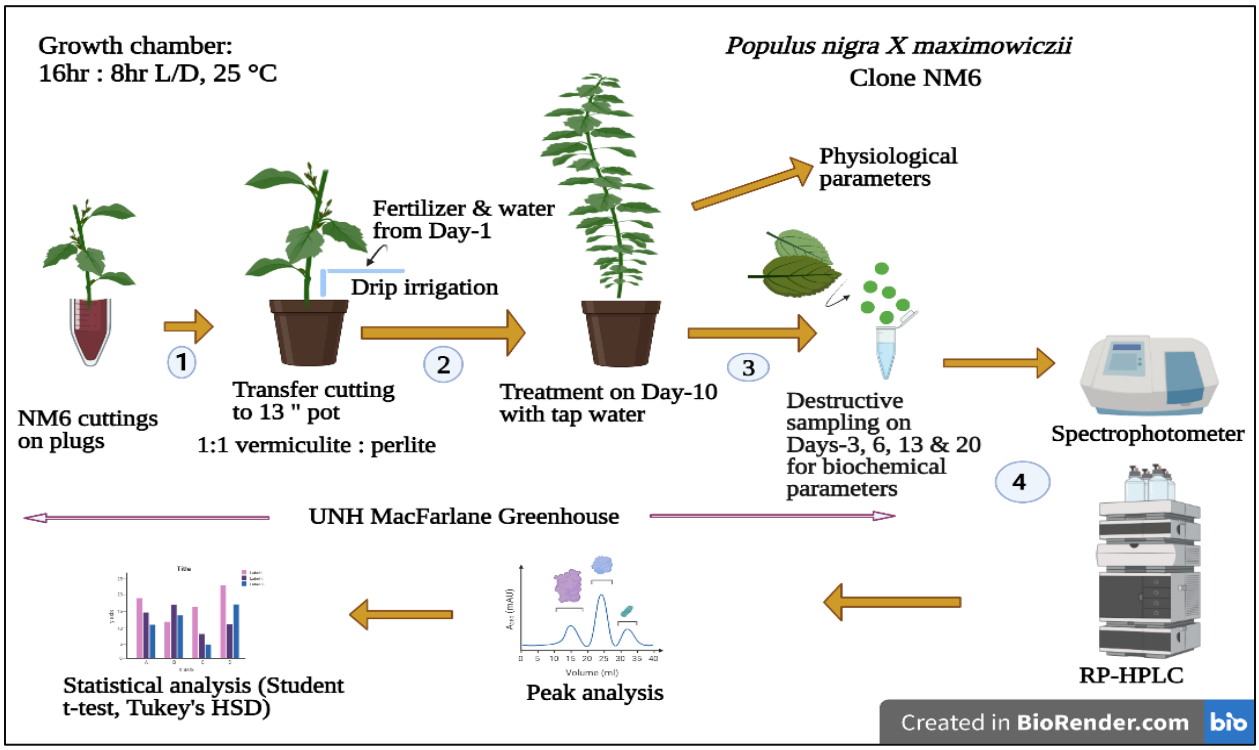

Figure 3: Flowchart for the abiotic stress experiment.

Table 2: Tissue samples collection in various extraction solutions/buffers.

Amount of tissues (mg)	Analysis	Solutions	Amount of solutions (mL)
40±2 (leaf) 100±2 (root)	Polyamine and Amino acids by HPLC	5% perchloric acid	1.00
50±2 (leaf)	Total protein	100 mM Tris buffer	0.25
50±2 (leaf)	Sugars	80% ethanol	1.00
200±2 (leaf)	Relative water content	Direct freezing	NA
100±2 (leaf and root)	Phytochelatins	6.3 mM diethylenetriamine pentaacetic acid (DTPA) + 0.1 % trifluoroacetic acid (TFA)	1.00
100±2 (leaf)	Metabolomics by GC-MS	2:1 chloroform: methanol	0.50

Soluble sugars

To quantify various soluble sugars, 50±2 mg FW of leaf tissues was incubated at 65 ºC for 30 min in 1 mL 80% ethanol modified from Blagden et al. (2022). This extract was kept at room temperature for 5 min and vortexed at medium speed for 2 min. This was followed by centrifugation at 13,000 g for 8 min. The supernatant was filtered into an autosampler vial using a 0.45 µm nylon syringe filter (Pall Corp., Port Washington, NY) fitted onto a 3 mL syringe (Becton, Dickinson and Company, Franklin lakes, NJ). Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) was employed to quantify sugars using external standard curves. The mobile phase was 80% acetonitrile (ACN) + 20% double distilled water (ddH_2O), at a flow rate of 2 mL min^{-1}. The injection volume of the extract was 40 µL into a 200 µL loop, with a 25 cm analytical column (Phenomenex Luna-NH_2, 5 µm, 100 Å 250 x 4.6 mm #00G-4378-EO). For our experiment, we were interested in 11 sugars (xylose, arabinose, fructose, mannose, glucose, galactose, sucrose, trehalose, rhamnose monohydrate, maltose monohydrate, raffinose pentahydrate) which were quantified with a Shimadzu RID-10A refractive index detector (RID)

set at 30 ºC (Shimadzu Scientific Instruments Inc., Columbia, MD). The total run time was 15 min, including washing and stabilizing the column before the next sample was injected. The column temperature was maintained at 25 ºC. A Perkin Elmer TotalChrom software (version 6.2.1) was used to process the data and analyze the chromatograms. Peaks were identified by using retention times of the known standards. An 8-point external standard curve (3 mg mL^{-1}) was created to identify and quantify each sugar (Table 3). The area and concentration of each sugar were added to create a combined curve for sugars that did not separate for quantification of approximate amounts of the unresolved peaks. The unseparated sugars were xylose + arabinose, glucose + galactose, and trehalose + maltose.

Table 3: Preparation of standard solutions from a mixed working stock solution of 11 sugars (xylose, arabinose, fructose, mannose, glucose, galactose, sucrose, trehalose, rhamnose monohydrate, maltose monohydrate, raffinose pentahydrate) in 80% EtOH.

Standards	Stock solution (µL)	80% EtOH (µL)	Total volume (µL)	Final concentration of stock (mg mL^{-1})
S0	0	1000	1000	0
S1	12.5	987.5	1000	0.062
S2	25	975	1000	0.125
S3	50	950	1000	0.25
S4	100	900	1000	0.5
S5	200	800	1000	1.0
S6	300	700	1000	1.5
S7	400	600	1000	2.0
S8	600	400	1000	3.0

Polyamines and amino acids

To quantify different PAs and AAs, approximately 40±2 mg fresh leaf discs were collected in 5% perchloric acid ($HCLO_4$) in a ratio of 1: 25 (w: v) in 2 mL microfuge tubes. Next, the samples were freeze-thawed three times and processed for dansylation which was modified from Minocha et al. (1994), (Minocha and Long 2004). The samples were vortexed for 2 min at high speed after

thawing for the final time and centrifuged at 14,000 g for 8 min. For each sample and the external standards, 20 µL internal standards mix (0.05 mM heptane diamine (for PAs) + 0.05 mM α-methyl-DL phenylalanine (for AAs) dissolved in 5% $HCLO_4$) was added to each tube, followed by 100 µL of the freeze-thawed extract. Then 100 µL 2.691 M Na_2CO_3 and 100 µL freshly prepared dansyl chloride (20 mg/mL in acetone). The mixture was vortexed and incubated at 60 ºC for 30 min. After incubation, the microfuge tubes were cooled at room temperature for 3 min and 45 µL glacial acetic acid was added to each tube to terminate the reaction before evaporating the acetone in a speed-vac for 10 min. Finally, 1735 µL methanol was added to the mixture. The methanol extract was filtered with 0.45 µm nylon syringe filter fitted onto a 3 mL syringe before transferring the solution to autosampler vials. A 15 cm column (Phenomenex Synergi Hydro-RP 80 Å, LC Column 150 x 4.6 mm, 4 µm) was used to separate AAs and PAs. The AAs and PAs were quantified using a fluorescence detector (Series 200 PerkinElmer) set at 340 nm for excitation and 510 nm for emission. An external standard was used for the relative quantification of the PAs and AAs using a standard curve (Table 4). The chromatograms were analyzed using Perkin Elmer TotalChrom software (version 6.2.1), where a multiplication factor was incorporated into the software to obtain the quantities of each component in nmol g^{-1} FW of tissue.

Table 4: Preparation of PA standard solutions from a mixed working stock solution (0.04 mM Put, 0.02 mM Spd and Spm) of three PAs in 5% $HCLO_4$.

Stock Solution (µL)	5% $HCLO_4$ (µL)	Total Volume (µL)	Final Conc. Put	Final Conc Spd and Spm (mM)
0	500	500	0.000	0.000
25	475	500	0.002	0.001
50	450	500	0.004	0.002
125	375	500	0.010	0.005
250	250	500	0.020	0.010
125	0	125	0.040	0.020

Total soluble proteins

To perform this analysis, 50 ± 2 mg fresh leaf discs were extracted in a freshly prepared Tris buffer (100 mM, pH 8.0) containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM ethylene diamine tetra-acetic acid (EDTA), and 10% glycerol (v/v) by 3X freezing and thawing as per (Minocha et al. 2019). The extract was centrifuged at 13,000 g for 5 min and the supernatant was used for analyzing total soluble protein content as per Bradford (1976) using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as a standard. Absorbance was recorded at 595 nm with a spectrophotometer. A Standard Curve with a range of concentrations (e.g. 0.1-0.5 mg/ml) was used to quantify the protein content of the samples (Fig. 8A).

Relative water content

To calculate relative water content (RWC), the fresh weight (FW) of the leaf tissue was measured immediately after bringing the leaf tissue to the lab. For dry weight (DW), the tubes were incubated in an oven at 70 ºC for 48 hours. The leaf samples were weighed again after 24 hours to ensure there was no more water left. Relative water content was calculated using the following formula -

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

Chlorophyll content

Two leaf discs (8-13 mg FW) were transferred into 2 ml microfuge tubes containing 1 mL of 95% ethanol, and chlorophyll content was quantified according to the method described by Minocha et al. (2009). The tubes were incubated for 16 hours) in a water bath at 60 ºC. The tubes were wrapped with aluminum foil to prevent chlorophyll degradation. The samples were vortexed for 1 min, centrifuged at 13000 g for 5 min, and the absorbance was taken at A_{664}, A_{649}, and A_{470} nm. To calculate the total chlorophyll, chlorophyll a, chlorophyll b, and carotenoids, the following equations were used as described by (Lichtenthaler 1987):

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

Thiol compounds and phytochelatins analysis

To quantify the thiol compounds and the phytochelatins (PC_2, PC_3, PC_4), 100 ± 2 mg (FW) leaf discs were collected. Immediately after collection, the leaf discs were placed in pre-weighed microfuge tubes and 1 mL extraction buffer (6.3 mM DTPA with 0.1 %, v/v TFA). The samples were kept on ice while transporting from the greenhouse to the laboratory and stored at -20 ºC until analysis. The samples were freeze-thawed 3X before analysis, as per the procedure of Minocha et al. (1994). On the analysis day, the samples were centrifuged at 13,000 g for 10 min, and 250 µL

of the supernatant was used for subsequent analyses. A total of 6 external standards (Cys, GSH, γ-EC, PC_2, PC_3, PC_4) and 1 internal standard [N-acetyl-L-cysteine (NAC)] solutions, were used. The standards were prepared in an extraction buffer, and a series of working standards (S1-S8) were made, as described in Table 5.

Table 5: Preparation of thiol and PCs standard solutions

Standards	Stock Solution (µL)	Extraction buffer (µL)	Total Volume (µL)	Cys, GSH, and γ-EC Conc. of standard		PC_2, PC_3, and PC_4 Conc. of standard	
				before tagging (mM/L)	after tagging (pM/µL)	before tagging (mM/L)	after tagging (pM/µL)
S0	0.0	300.0	300	0.000	0.000	0.000	0.00
S1	7.5	292.5	300	0.001	0.25	0.0004	0.10
S2	15.0	285.0	300	0.002	0.50	0.0008	0.20
S3	22.5	277.5	300	0.003	0.75	0.0012	0.30
S4	30.0	270.5	300	0.004	1.00	0.0016	0.40
S5	37.5	262.5	300	0.005	1.25	0.0020	0.50
S6	75.0	225.0	300	0.010	2.50	0.0040	1.00
S7	150.0	150.0	300	0.020	5.00	0.0080	2.00
S8	300.0	0.0	300	0.040	10.00	0.0160	4.00

The derivatization of the thiol compounds (cysteine (Cys), glutathione (GSH), gamma-glutamylcysteine (γ-EC)) and PCs was performed according to the protocol described in Minocha et al. (2008). In brief, 615 µL of 200 mM 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS) buffer (6.3 mM DTPA, pH 8.2) was added to 25 µL of 20 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) that was used as a disulfur reductant. A mix of 250 µL standards or sample extract was added to the above mixture. An internal standard of 10 µL of 0.5 mM NAC and the external standard curve was used for the relative quantification of the thiol compounds. The final reaction mix was pre-incubated at 45 ºC for 10 min in a water bath to completely convert the disulfide bonds to sulfhydryls and change the thiols to a reduced state. The reduced thiols were derivatized by adding 10 µL of freshly prepared 50 mM monobromobimane (mBBR), followed by an incubation of the samples in a water bath at 45 ºC for 30 min in the dark. Finally, the reaction

was terminated by adding 100 µL of 1 M methanesulfonic acid (MSA) and vortexed for 30 secs to a homogenous mixture. Next, the derivatized extract was filtered into an autosampler vial using a 0.45 µm nylon syringe filter. A blank reaction mix was included with an extraction buffer in place of the sample to identify peaks arising from the reagents. A 15 cm column (Phenomenex Synergi Hydro-RP 80 Å, LC Column 150 x 4.6 mm, 4 µm) was used to separate individual thiol compounds. For every sample, 10 µL of the mix was injected into a 200 µL loop for quantifying the thiol compounds with a fluorescence detector with wavelengths 380 nm for excitation and 470 nm for emission. The thiol compounds were separated by using solvents (1) 99.9% ACN and (2) 89.9% water + 10% ACN, and both the solvents had 0.1 % TFA (v/v). The total run time was 33.8 min, including the column cleaning and stabilization before the next sample was injected. The chromatograms were analyzed using Perkin Elmer TotalChrom software (version 6.2.1).

Sample preparation for metabolomics analysis

To study the entire metabolome in the leaf tissue, samples were collected on day-14 post-treatment from the plants that were treated with Cd for all treatments. Leaf discs 100 ± 2 mg (FW) were transferred to 2 ml microfuge tubes containing 500 µL of 2:1 mix of pre-cooled chloroform: methanol and stored in ice until they were brought to the lab. All samples were stored at -80 ºC until further analysis before shipping to the Roy J. Carver Biotechnology Center at the University of Illinois Urbana Champaign (https://biotech.illinois.edu/metabolomics). For analysis, only 3 replicates from 4 treatments (control, 50 µM Cd, 150 µM Cd, and 150 µM Cd + 1.0 mM Spd) were sent for metabolomics analysis based on the results obtained by PA quantification with RP-HPLC. To perform the metabolomics analysis, the samples were freeze-dried and quantified using GC-MS. The quantification of entire metabolites and some data results were done by the Carver Biotechnology Staff.

Determination of plant growth parameters

Plant growth rate was calculated based on height and stem diameter differences. Readings were taken on days 0, 15, 30 and 45 post treatment with salt. To measure the stem basal diameter, a digital caliper was used. A region near the base of the stem was marked for measurement from the same location for each collection. Increase in height and diameter of the stem is expressed as % using the following formula:

Increase in height (%) = [(Height on next day - height on previous day)/ Height of day 1] * 100

Increase in diameter (%) = [Diameter on next day – diameter on previous day)/ Diameter of day 1] * 100

Leaf gas exchange

Gas exchange parameters like photosynthetic rate (P_n), transpiration rate (E), and stomatal conductance (g_s) were quantified on fully expanded 6th leaf from the apex for each plant (3-4 plants/replicates per treatment) with a portable photosynthesis system (LI-6400/XT, Li-COR Biosciences, Lincoln, NE USA). Gas exchange was measured from 9 AM-12 Noon under a PPFD of 1000 μmol m^{-2} s^{-1} from a red–blue LED chamber (6 cm^2); air flow was set to 500 μmol s^{-1}; reference CO_2 concentrations were kept at 400 μmol mol^{-1} and the block temperature was set to 25 ºC. Leaf humidity was not controlled but ranged between 50-60%.

SPAD reading

To estimate the chlorophyll content (“greenness” in the top, middle, and bottom leaves of the Cd-treated plants, SPAD 502 Plus Chlorophyll Meter (Item # 2900P, Spectrum Technologies, Inc., Aurora, Illinois), was used. Prior to taking the readings, leaf surface was cleaned with Kimwipes to remove the extra dust. All the readings were taken after 9 days post-treatment

between 10 AM - 12 Noon. The meter was set to zero before taking measurements of leaves and 2 readings were taken from each side of the midrib while carefully avoiding the veins as much as possible while the third reading was taken at the tip of the leaf. These 3 readings were averaged automatically by the SPAD meter to generate one final heterogenous chlorophyll distribution in each leaf.

Data analysis

All statistical analyses were done with JMP Pro 15 (www.jmp.com/en_us/home.html). Data were subjected to one-way analysis of variance (ANOVA) to determine statistically significant differences between control and treatment groups within each day. When comparisons were made between control and treatment, significant differences were represented with letters (a-c). Another comparison was made between with and without PA spray between respective treatment, the statistical differences were expressed with asterisks (*-***). Significant differences were determined at $p < 0.05$ using Tukey's Test between control and stress treatment and the t-test between without and with spray.

The metabolomics dataset was analyzed by multivariate statistical analysis using Metaboanalyst 5.0 online analysis software (https://www.metaboanalyst.ca/). The concentration values of the metabolites identified in the metabolome of poplar plants treated with Cd and control treatment were set to remove features with more than 50% missing values, and the remaining missing values were replaced by $1/5^{th}$ of the minimum positive value of each variable. The samples were normalized by square root transformation and pareto scaled for an appropriate result which was used to perform Principal component analysis (PCA), pattern correlation analysis, hierarchical clustering analysis, variable importance in projection (VIP) scores, and heatmap analysis. The specific measurements used for each plot will be individually described in the results section.

CHAPTER 3: RESULTS

Effect of salt and its interaction with putrescine

As described under Materials and Methods, 6 weeks-old plants were treated with two different concentrations of NaCl (100 mM and 200 mM), which was given via roots only once. A group of these plants were sprayed with 1 mM Put on several days. The various physiological and biochemical parameters studied on several days after treatment are described here.

Plant growth and morphological symptoms

Changes in height and stem diameter was measured for various days till 45 days after salt treatment. The increase in stem length and diameter was maximum in Put-sprayed plants treated with 100 mM NaCl. A similar growth rate was seen in 100 mM NaCl-treated plants and Put-sprayed plants under 200 mM NaCl. Plants under 200 mM NaCl had the lowest stem length and diameter growth compared to all other treatments (Fig. 4). Morphological symptoms in the salt-treated plants started to show after 13 days of salt exposure. Interestingly, the leaves of the plants exposed to 100 mM NaCl had higher chlorosis and leaf burn symptoms than those treated with 200 mM NaCl. After 20 days, it was seen that the leaves of the Put-sprayed plants had lower chlorosis symptoms *vs.* unsprayed under salt treatment (Fig 5).

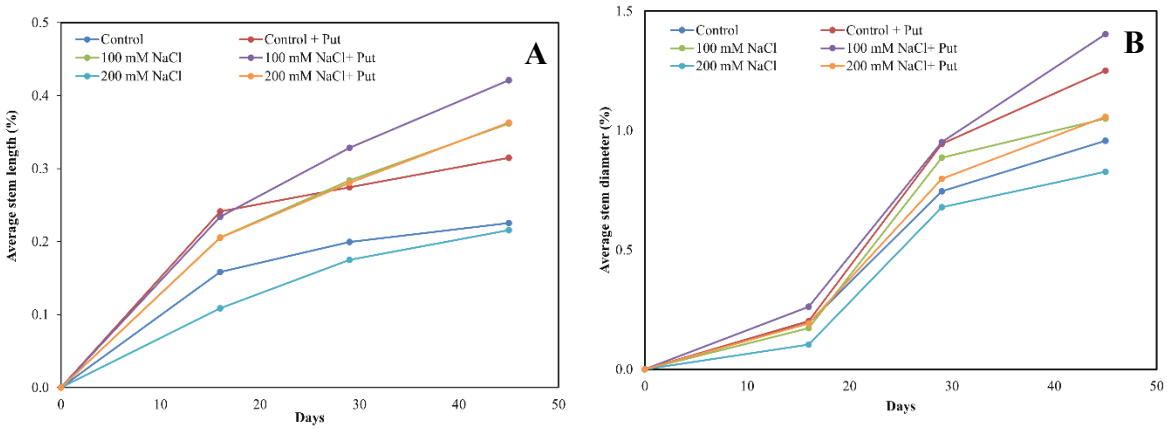

Figure 4: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the (A) stem length and (B) stem diameter of the hybrid poplar NM6 plants. Data are mean of 6 replicates.

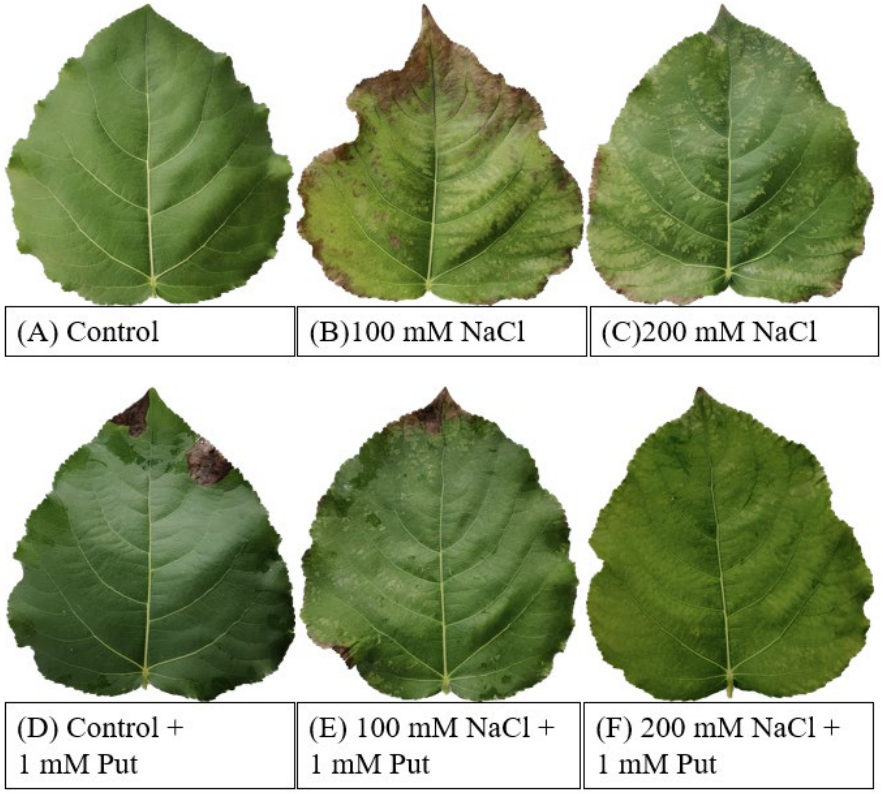

Figure 5: The effect of two different concentrations of NaCl (± Putrescine spray) after 20 days on the morphological traits of the hybrid poplar NM6 plants.

Chlorophyll contents

Treatment with 200 mM NaCl significantly increased chlorophyll a, b, and carotenoids compared to the control on the 3rd day as measured by spectrophotometry (Fig. 6). Compared to day 3, there was a trend in the decrease of all three pigments with time by day 13. In Put-sprayed plants, chlorophyll was often higher *vs.* unsprayed plants for 100 mM NaCl until the 13th day; although the results were not statistically significant. Treatment with Put did not significantly affect chlorophyll or carotenoids at any time. However, salt treatment did not affect total chlorophyll on any day.

Gas exchange

Leaf gas exchange was measured in the 6th fully matured leaf at 7 and 35 days after NaCl treatment (Fig. 7). On the 7th day, NaCl treated plants had lower P_n and g_s compared to the control plants. However, on the 35th day, gas exchange was consistently higher (but statistically insignificant) on the NaCl-treated plants compared to the control. The P_n, g_s, and E of the plants sprayed with Put compared to those not sprayed under control treatment on several days showed no significant increase. In Put-sprayed plants, gas exchange was always higher *vs.* unsprayed for 100 mM NaCl, and on the 35th day, with a significant increase in the g_s and E.

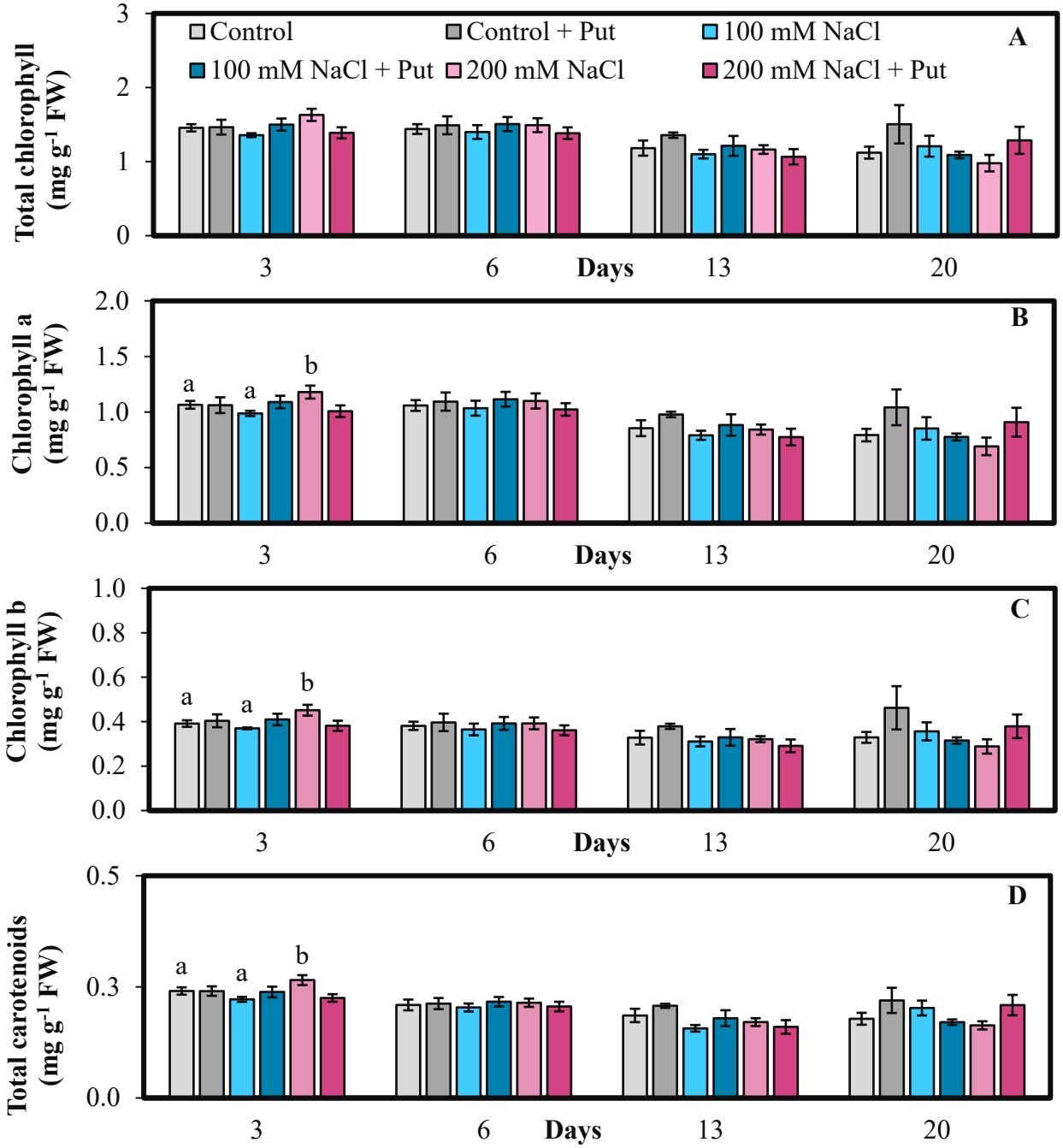

Figure 6: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the chlorophyll contents (spectrophotometric analysis) of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 6 replicates. (A) Total chlorophyll, (B) Chlorophyll a, (C) Chlorophyll b, and (D) Total carotenoids. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 100 mM NaCl, and 200 mM NaCl.

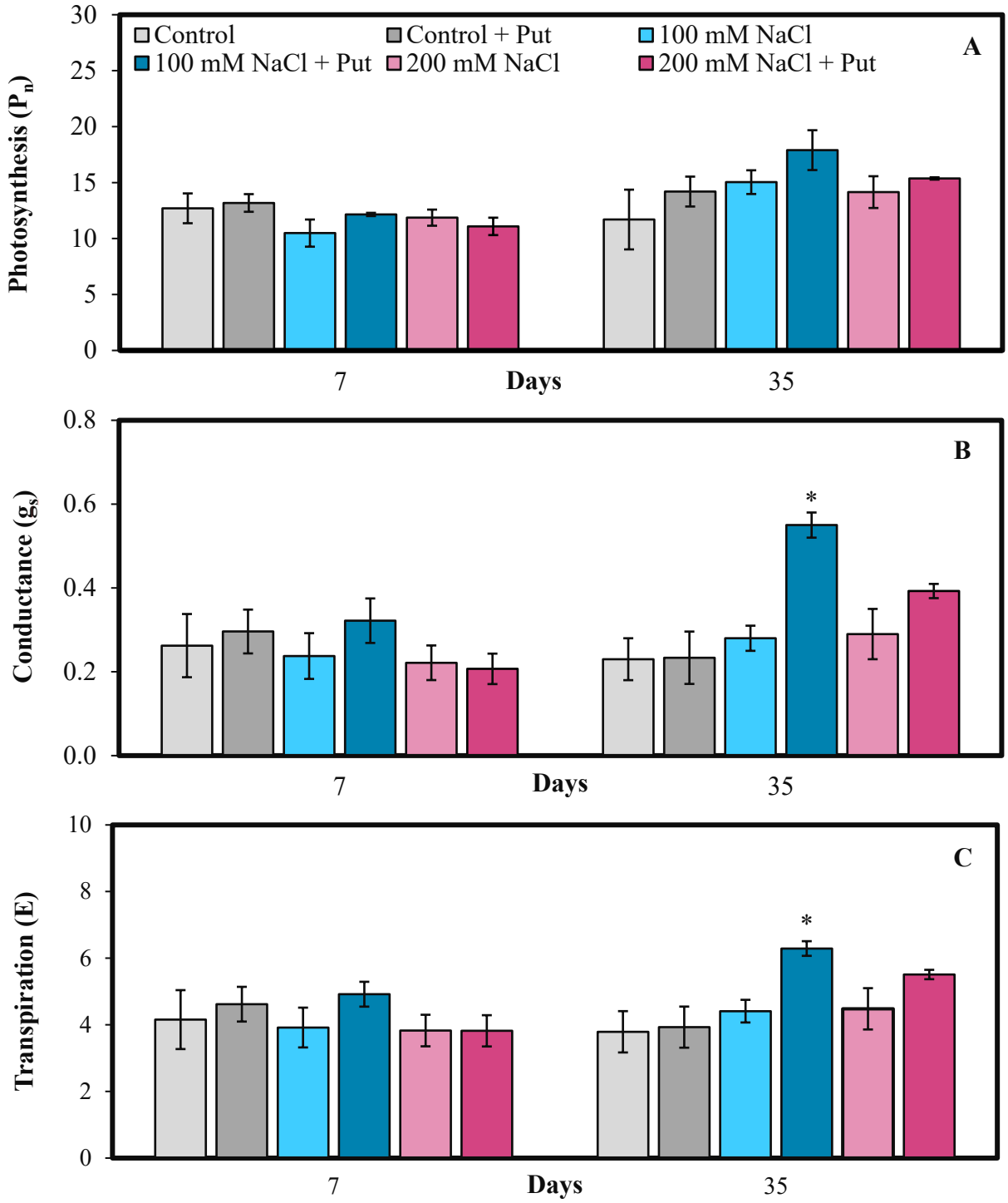

Figure 7: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the chlorophyll contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 4 replicates. (A) Photosynthesis-P_n, (B) conductance-g_s, and (C) transpiration-E. On a given day, an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

Soluble protein contents

In response to 100 mM NaCl treatment, there was a significant decrease in the total soluble protein contents on the 13th day (Fig. 8B). Soluble protein content was higher (but statistically insignificant) in Put-sprayed plants *vs.* unsprayed for 100 mM NaCl on the 3rd and 6th day, while sprayed plants treated with 200 mM NaCl had significantly lower soluble protein content compared to unsprayed plants on the 13th day.

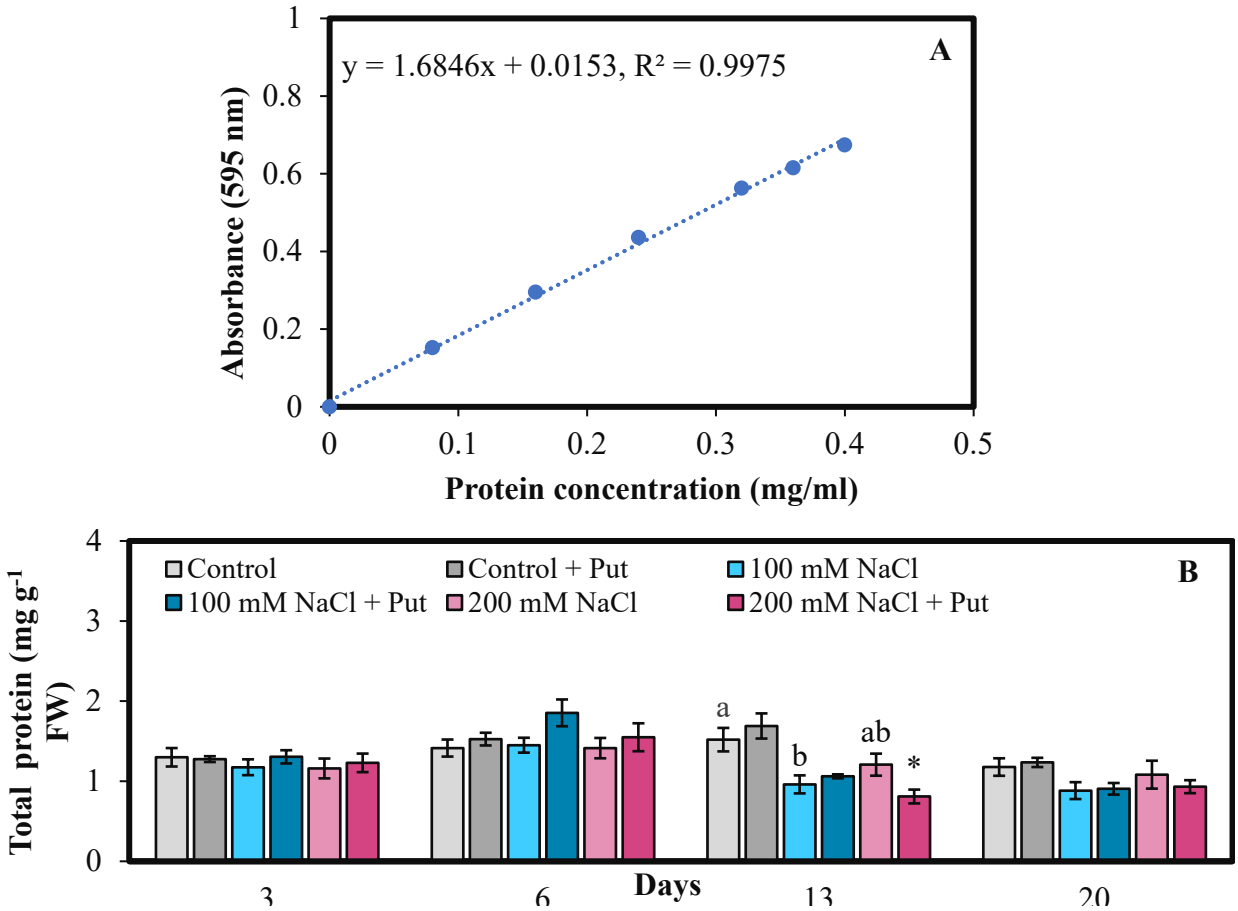

Figure 8: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the total protein content of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 6 replicates. (A) Standard curve and (B) Total protein. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 100 mM NaCl, and 200 mM NaCl, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

Relative water content

There was a significant increase in the RWC on the 13th and 20th day in the plants given NaCl treatment as compared to the control (Fig. 9A). However, significant increase was only seen on the 13th day in 200 mM NaCl-treated plants. Similarly, FW/DW ratio was significantly higher in 100 mM NaCl-treated plants on the 13th and 20th day, while 200 mM NaCl-treated plants had significantly higher FW/DW ratio on the 13th day (Fig. 9B).

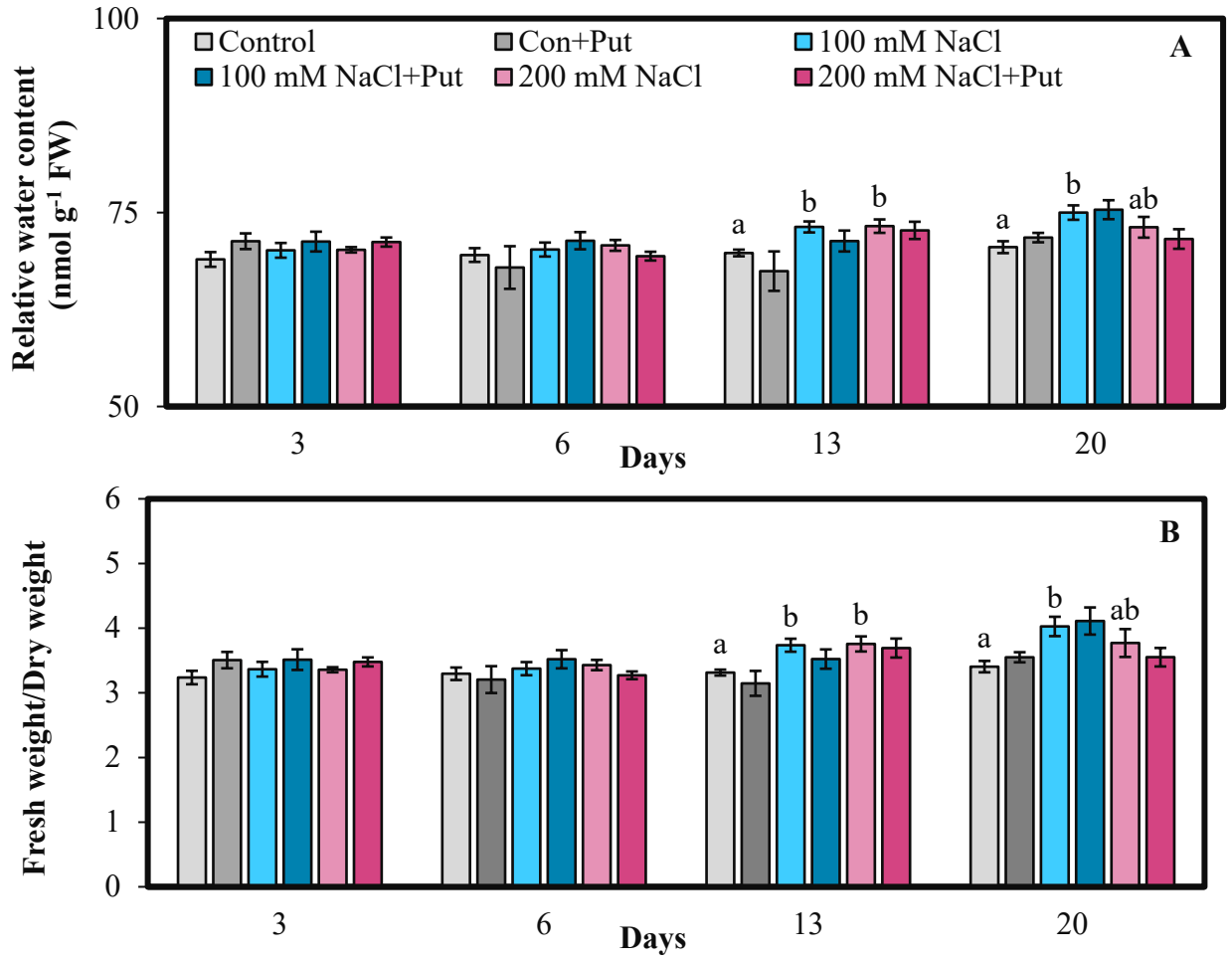

Figure 9: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the (A) Relative water content (B) Fresh weight/dry ratio weight of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 6 replicates. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 100 mM NaCl, and 200 mM NaCl.

Foliar polyamines content

Cellular Put in the leaves showed no significant changes in response to 100 mM NaCl until the 20th day (Fig. 10A). However, for treatment with 200 mM NaCl there was an increased trend in cellular Put content compared to the control on the 3rd, 6th, and 13th day. Unlike 200 mM NaCl, plants under 100 mM NaCl have shown inconsistent trend compared to the control (decrease on the 3rd, increased on the 6th, and similar to the control on the 13th day). On the 20th day, a significant increase under 100 mM NaCl and a decrease under 200 mM NaCl treatment was seen in Put compared to the control plants. In Put-sprayed plants, there was a significant change in the cellular Put content *vs.* unsprayed under control treatment on the 3rd, 6th, and 20th day. There was no significant effect on the Put sprayed salt-treated plant on any days.

There was a decrease in Spd content in the leaves with the progress of time with salt treatment (Fig. 10B). But, on the 20th day, Spd content was higher than that on the 13th day in response to 100 mM NaCl. On the 13th and 20th day, Spd significantly decreased under 200 mM NaCl compared to the control plants. Exogenous Put spray increased (but statistically insignificant) Spd content in the leaves *vs.* unsprayed in NaCl-treated plants until the 13th day. On the 20th day, Put-sprayed plants had lower (but not significant) Spd *vs.* unsprayed for 200 mM NaCl.

Fig. 10C shows that an increase in NaCl concentration increased Spm content on the plants on the 3rd, 6th, and 20th days compared to the control. However, on the 13th day, the Spm was lower on the plants treated with NaCl compared to the control. On the 3rd and the 20th day, plants sprayed with Put showed a significant increase in the cellular Spm compared to those not sprayed in control plants. From day 6th onwards, Put spray led to similar or higher Spm content in the leaves *vs.* unsprayed NaCl-treated plants. On the 13th day, there was a significant Spm increase *vs.* unsprayed in 200 mM NaCl-treated plants.

Root polyamines content

In roots, PA contents were analyzed (with HPLC) only at 21 days after NaCl treatment. Putrescine was the most abundant PA in the roots, and its contents were higher (almost 2X) in response to growth in the presence of salt (Fig. 11). On the other hand, salt caused a major decrease in Spd (>4-fold) and Spm; the effect on Spd being the greatest (Table 6).

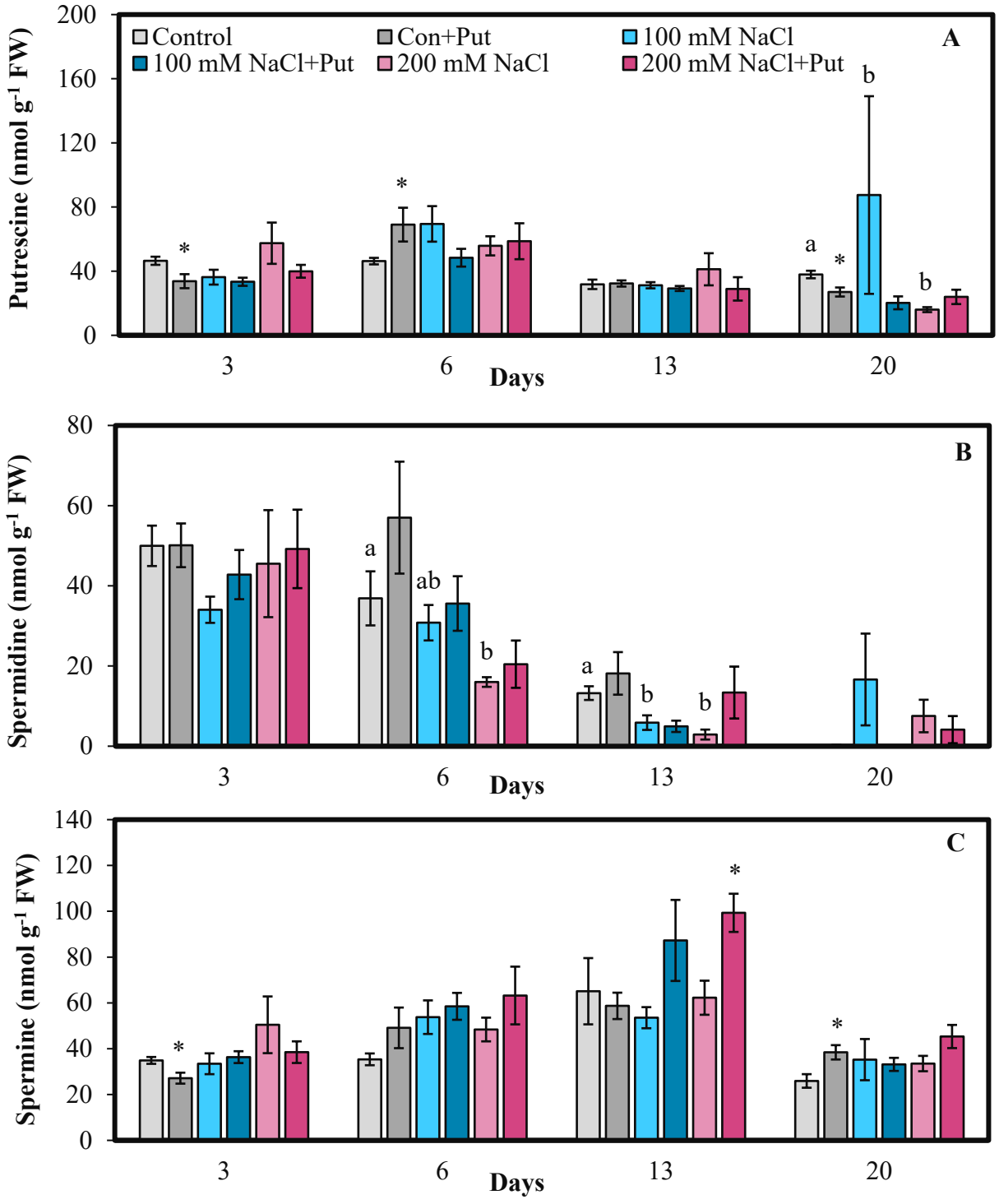

Figure 10: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the polyamines content of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Putrescine, (B) Spermidine, and (C) Spermine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 100 mM NaCl, and 200 mM NaCl, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

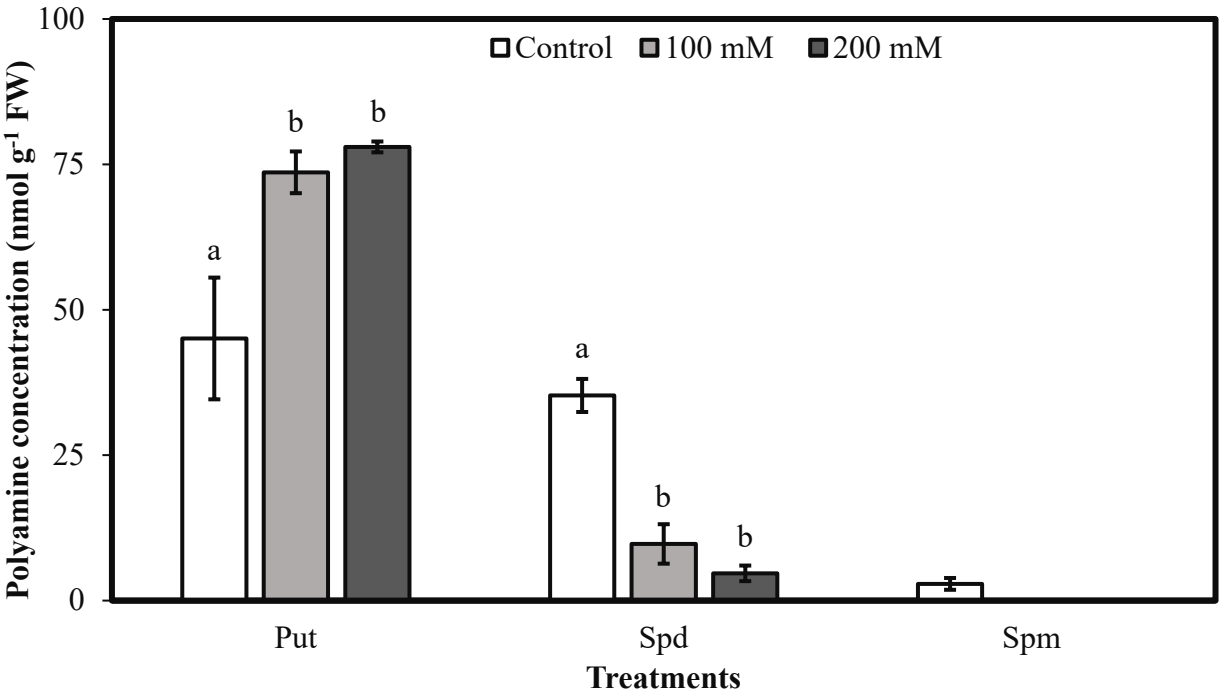

Figure 11: The effect of two different concentrations of NaCl on day 21 on the polyamines (Putrescine, Spermidine, Spermine) content of roots of the hybrid poplar NM6 plants. Data are mean of ± SE of 3 replicates. A different letter indicates the treatment effect was significantly different ($p \leq 0.05$) between control, 100 mM NaCl, and 200 mM NaCl.

Foliar amino acids content

Arginine, Gln, and Glu (all precursors of PA biosynthesis and other AA biosynthetic pathways) were the most abundant AAs in the poplar leaves on most days, followed by GABA, Phe+Cystine, and Ser. Leucine, Met, Leu, Ile, Ala and His are the least abundant, some of them being below the detection limits on many days of analysis.

On the 3rd day, Arg+Thr+Gly in the leaves increased in the presence of 200 mM NaCl compared to the control (Fig. 12A). After NaCl treatment for 6 days, there was a 1.5-fold increase in the Arg+Thr+Gly content on the NaCl-treated plants. On other days, Arg+Thr+Gly content was either less or similar in NaCl-treated plants compared to the control. In Put-sprayed plants, Arg+Thr+Gly content significantly decreased *vs.* unsprayed in control plants only on the 3rd day, while there was an increase by 169% ($p=0.08$) on the 6th day. In Put-sprayed plants under NaCl, Arg+Thr+Gly was either similar or higher by several folds compared to the control on all days. However, Arg+Thr+Gly was always lower on Put-sprayed plants *vs.* unsprayed under 200 mM NaCl on all days.

On the 3rd day, Gln increased in 200 mM NaCl-treated plants compared to the control as shown in Fig. 12B. From the 6th day, Gln content was always higher in salt-treated plants *vs.* control. There was a significant increase by 2-fold in the Gln content under 100 mM NaCl on the 6th day while 200 mM NaCl-treated plants showed a significant increase by 2-fold on the 6th and 13th days compared to the control. There was a significant increase in the Gln content by almost 2-fold in Put-sprayed plants *vs.* unsprayed for the control on the 6th and 20th day while there was a significant decrease *vs.* unsprayed for 200 mM NaCl on the 13th day. In Put-sprayed plants, there was a significant increase in the Gln content *vs.* unsprayed for control plants.

Serine content was always higher under salt treatments than in control (Fig. 12C). Salt treated plants showed 5-fold and 2-4-fold increases in the Ser content compared to the control on the 6th and the 13th day, respectively. In Put-sprayed plants, Ser was higher (p=0.07) *vs.* unsprayed for 100 mM NaCl on the 3rd day while there was a significant increase in the Put-sprayed plants *vs.* unsprayed for control plants on the 6th day. Similarly, Ser increased significantly by ~ fig9-10-fold and 2-fold in Put-sprayed plants *vs.* unsprayed for 100 mM NaCl on the 6th and 13th day. Plants sprayed had significant increase in Ser content *vs.* unsprayed for 200 mM NaCl on the 20th day.

After 3 days of salt treatment, Orn was higher by 2-fold, especially in the salt-treated plants compared to the controls (Fig. 13A). In response to NaCl, a 2-3-fold increase was seen in the Orn content compared to the control on the 3rd and 6th day. Ornithine was almost 2-fold higher in plants sprayed *vs.* unsprayed for 200 mM NaCl on the 6th day. On the 13th day, sprayed plants showed only a small increase in Orn content by several folds *vs.* unsprayed irrespective of any treatment; and a significant increase of 1.5-fold was seen on the 20th day. A 3-fold increase was seen in Put-sprayed plants in the Orn content *vs.* unsprayed for 200 mM NaCl on the 13th day as well.

After 3 days of salt treatment, Ala decreased under 100 mM and increased under 200 mM NaCl as shown in Fig. 13B. On the 6th day, a significant increase by more than 2-fold was seen in the Ala content on the 100 mM NaCl-treated plants compared to the control. There was no effect of salt on the plants compared to the controls on other days of sampling. Alanine content was always lower in Put-sprayed plants irrespective of treatment *vs.* control on the 3rd day. On the 6th day, Ala significantly decreased in Put-sprayed plants *vs.* unsprayed under 200 mM NaCl, while Ala was somewhat higher in concentration (p= 0.0532) in sprayed plants *vs.* unsprayed for 200 mM NaCl on the 20th day. On all days when plants were sprayed with Put under NaCl, Ala was either similar or higher by several folds in concentration compared to the controls.

Compared to the control, His increased by at least 2-fold in response to 200 mM NaCl on all days except the 13th (Fig. 13C). On the 13th day, His was the lowest in these plants. In Put-sprayed plants, the response was quite variable with Put sometime increasing His and at other times lowering its content, depending on the day of analysis. For example, His was lower in unsprayed plants for NaCl treatment on the 3rd day, but higher by as much as 4-5-fold on day 6.

Glutamic acid was not detected under our HPLC condition on the 3rd day (Fig. 14A). On other days, salt treated plants showed significantly higher Glu content by 2-3-folds *vs.* the control on the 6th day. Glutamic acid increased at least by 1.5-fold in Put-sprayed control plants *vs.* unsprayed plants ($p=0.059$) on day 3 and for 200 mM NaCl ($p=0.0529$) on day 20.

Like Glu, Asp was also not detected on the 3rd day as shown in Fig. 14B. Salt made no significant changes in Asp compared to the control on any day. Unlike the control, Asp was consistently higher in the salt-treated and Put-sprayed plants *vs.* those unsprayed, irrespective of the treatment. There was a significant increase in the Asp content on the salt-treated plants sprayed with Put than those not sprayed for 100 mM NaCl on the 6th day. On the 13th day, Asp decreased with the increase in NaCl concentrations compared to the control plants. However, in Put-sprayed plants, Asp increased *vs.* those unsprayed for 200 mM NaCl treatment.

As with Glu and Asp, Phe+Cystine peak was also not detectable; these AAs were however detected on days 3 and 20 (Fig. 14C). Three days after the 200 mM NaCl treatment, Phe+Cystine increased by almost 2-fold compared to the control. In Put-sprayed plants, Phe+Cystine was higher by 1.5-fold ($p=0.0553$) *vs.* unsprayed for the 200 mM NaCl on the 20th day.

At 3 days of 200 mM NaCl treatment, GABA increased by almost 2-fold compared to the control (Fig. 15A); at this time, Put prevented this increase. Almost a 3-fold increase in GABA in the control plants sprayed with Put was seen on the 6th day, while there was no difference between

the two concentrations of NaCl; with or without Put spray. On 20th day again, Put spray was ineffective both in the presence or absence of salt.

An increase in Val content was observed in the plants treated with NaCl for 20 days *vs.* most other days (Fig. 15B). Whereas, Val was very low in the control plants on day 6 with or without salt; put spray significantly increased the Val content on days 3 and 13.

Isoleucine, whose amounts were in the same range as Val increased by 2-fold in control on day 13 and decreased on day 20 in the 100 mM NaCl treated plants (Fig. 15C). At other times, very small changes occurred this amino acid on other days, with or without salt and/or Put spray. For 200 mM salt treatment, Val content increased, Put reversed this effect.

Leucine was consistently low till the 13th day of collection, irrespective of any treatment as shown in Fig. 16A. Putrescine treatment changed the Pro content on days 3 (control plants, and 6 and 20 in salt-treated plants (Fig. 16B), with the highest amounts being present on day 20. Methionine, like some of the other minor amino acids, was detected only on the 20th day (Fig. 16C). The presence of 200 mM salt had no effect on Met, except the Put spray significantly (4-5-fold) lowered the content of this amino acid on day 20. Like Met, Trp was detected only on the 13th day (Fig. 17A) and exhibited no effect of salt but enhancement by Put spray.

The presence of 100 mM salt caused a decrease in Lys content on day 3, on other days it was higher than the control plants (Fig. 17B). The effects of Put were positive in enhancing the Lys content in most days, except on day 3.

Root amino acids content

Amino acid contents of the roots were analyzed (with HPLC) only at 21 days after NaCl treatment. While Gln was the most abundant AA in the control roots, Arg+Thr+Gly was the most

abundant under salt treatment (Fig. 18). Salt significantly increased Gln accumulation (in both concentrations of NaCl) compared to the control (Fig. 19A). After 21 days of salt exposure, Arg+Thr+Gly significantly increased by 8-9-fold *vs.* control plants (Fig. 19A). Plants treated with 200 mM NaCl had significantly higher Phe+Cystine content than the control and 100 mM NaCl (Fig. 19B). Plants treated with 200 mM NaCl had a significantly higher (6-9-fold) accumulation of Orn (Fig. 19B). Histidine increased by 1.7-fold (100 mM NaCl) and 2-fold (200 mM NaCl) compared to the control (Fig. 19B). Serine increased by 2-fold (significant) in 100 mM NaCl and 1.6-fold in 200 mM NaCl (not significant) than control (Fig. 19B).

Alanine, Leu, and Lys content decreased in the roots under salt treatment (Fig. 20A). Interestingly, GABA, one of the important signaling molecules under salt stress, increased in response to 100 mM NaCl and decreased in response to 200 mM NaCl (Fig. 20A). Similarly, Pro decreased with the increase in NaCl concentration, although the Pro content was always higher under NaCl compared to the control (Fig. 20B). Compared to the control, a decrease in Ile content was seen in plants under 100 mM NaCl (Fig. 20B). Similarly, a decrease in Ile content was seen in plants under 200 mM NaCl. There was no difference in Val in 100 mM NaCl-treated plants; however, Val was undetected under 200 mM NaCl. Tryptophan was not detected in the roots of control plants (Fig. 20B). However, the accumulation of Trp was lower in 200 mM NaCl when compared to 100 mM NaCl (Fig. 20B). In our results, Met, Asp, and Glu were absent in any plants, irrespective of the treatments (Table 6).

The PCA 2d score plot showed a distinct cluster for the root AAs and PAs under control and 200 mM NaCl treatment (Fig. 21A). There was also a distinct cluster for 100 mM NaCl and 200 mM NaCl because of the difference in AA content. The AAs and PAs that mostly distinguished the cluster of the PCA and PS-LDA model are represented by VIP sore plot. Fig. 21B shows the

VIP score of the most essential AAs and PAs in the roots under salt treatment. The 5 most important AAs differentiating the control and the Cd treatment were Arg+Thr+Gly, Lys, Phe+Cystine, Spd, and Orn. The root AAs and PAs were correlated with each other (Fig. 22A), and further analyses show that Gln was differentially correlated with other AAs (Fig. 22B).

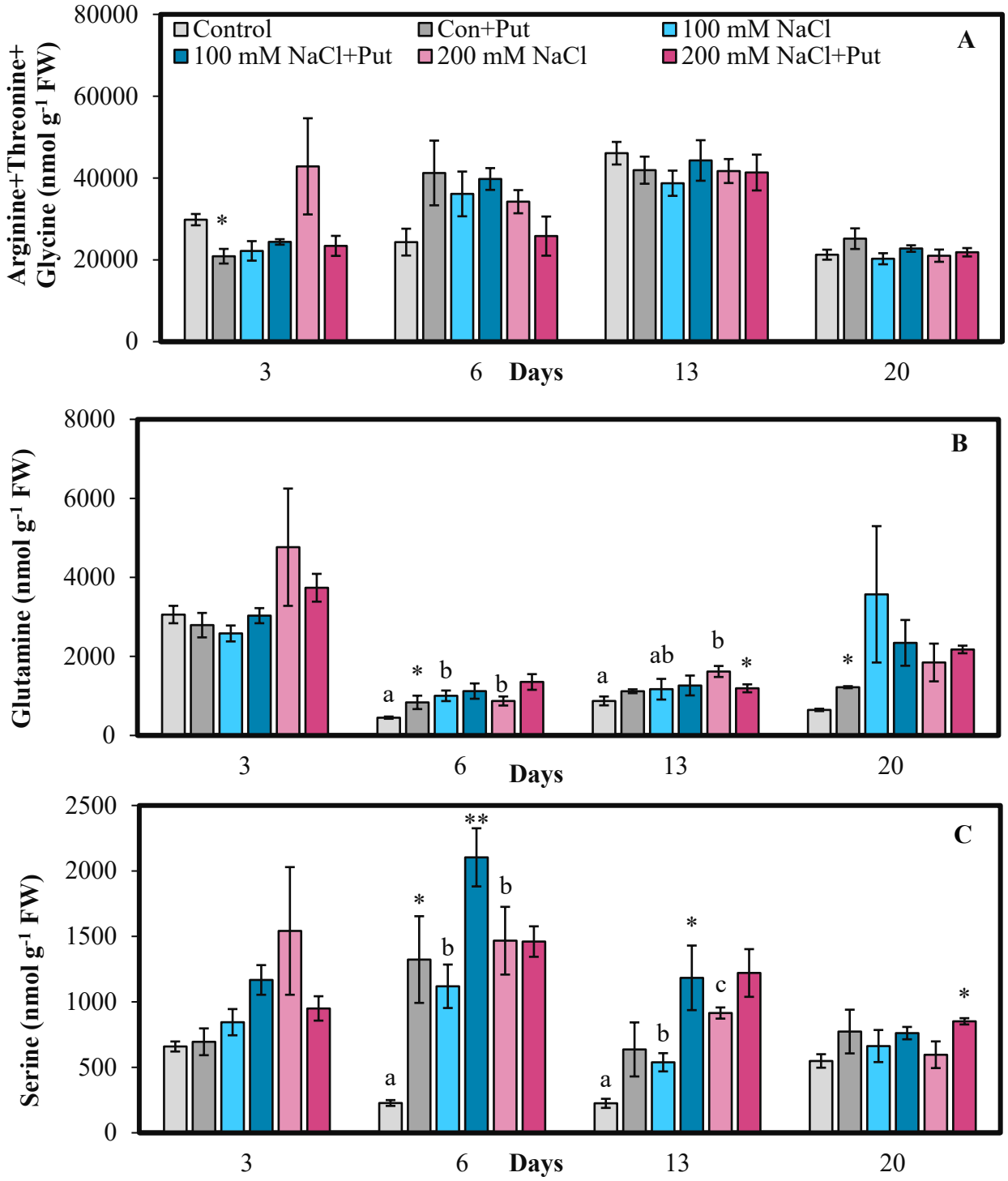

Figure 12: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the amino acids content of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Arginine+Threonine+Glycine, (B) Glutamine, and (C) Serine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 100 mM NaCl, and 200 mM NaCl, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

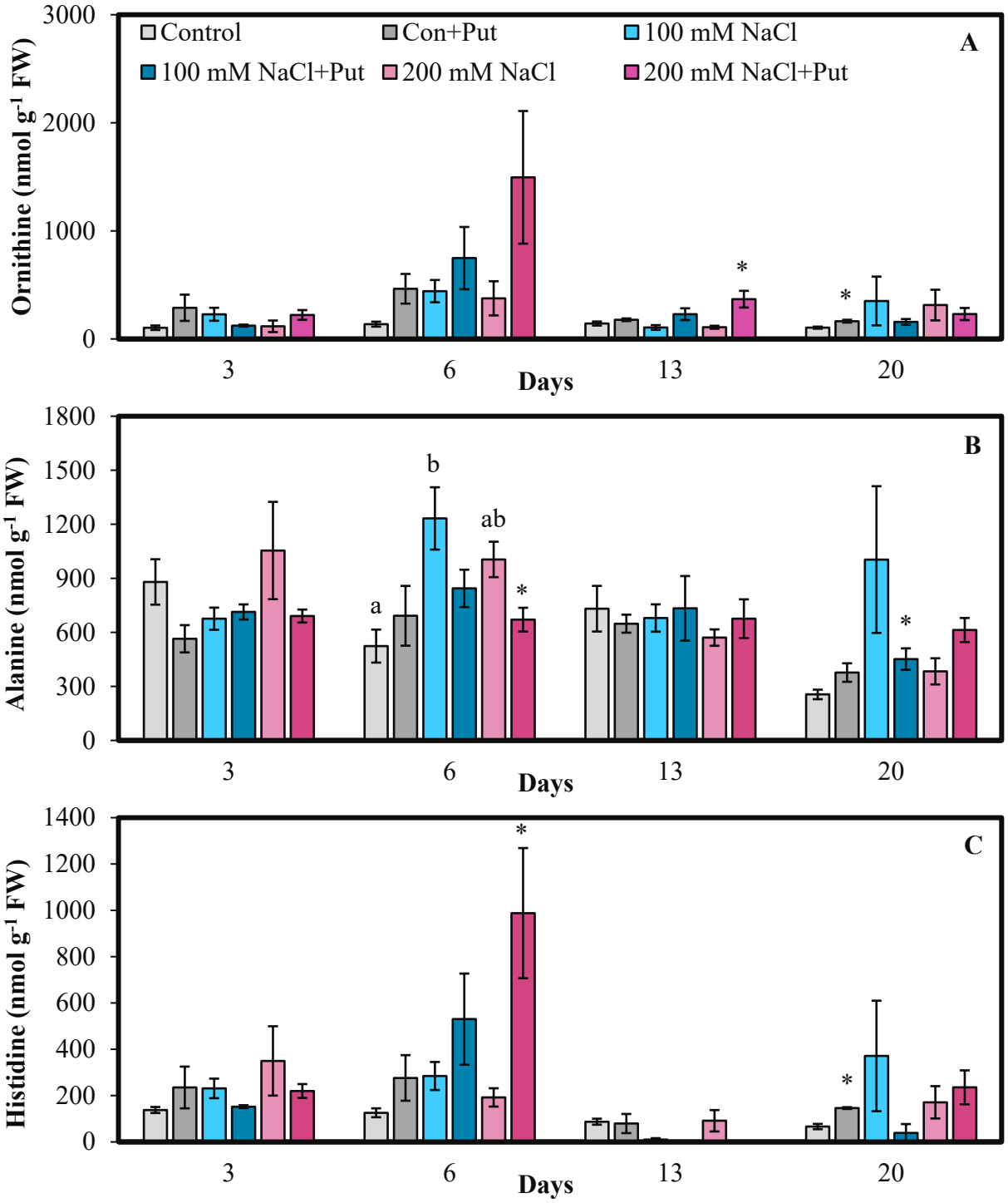

Figure 13: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the amino acids content of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Ornithine, (B) Alanine, and (C) Histidine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 100 mM NaCl, and 200 mM NaCl, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

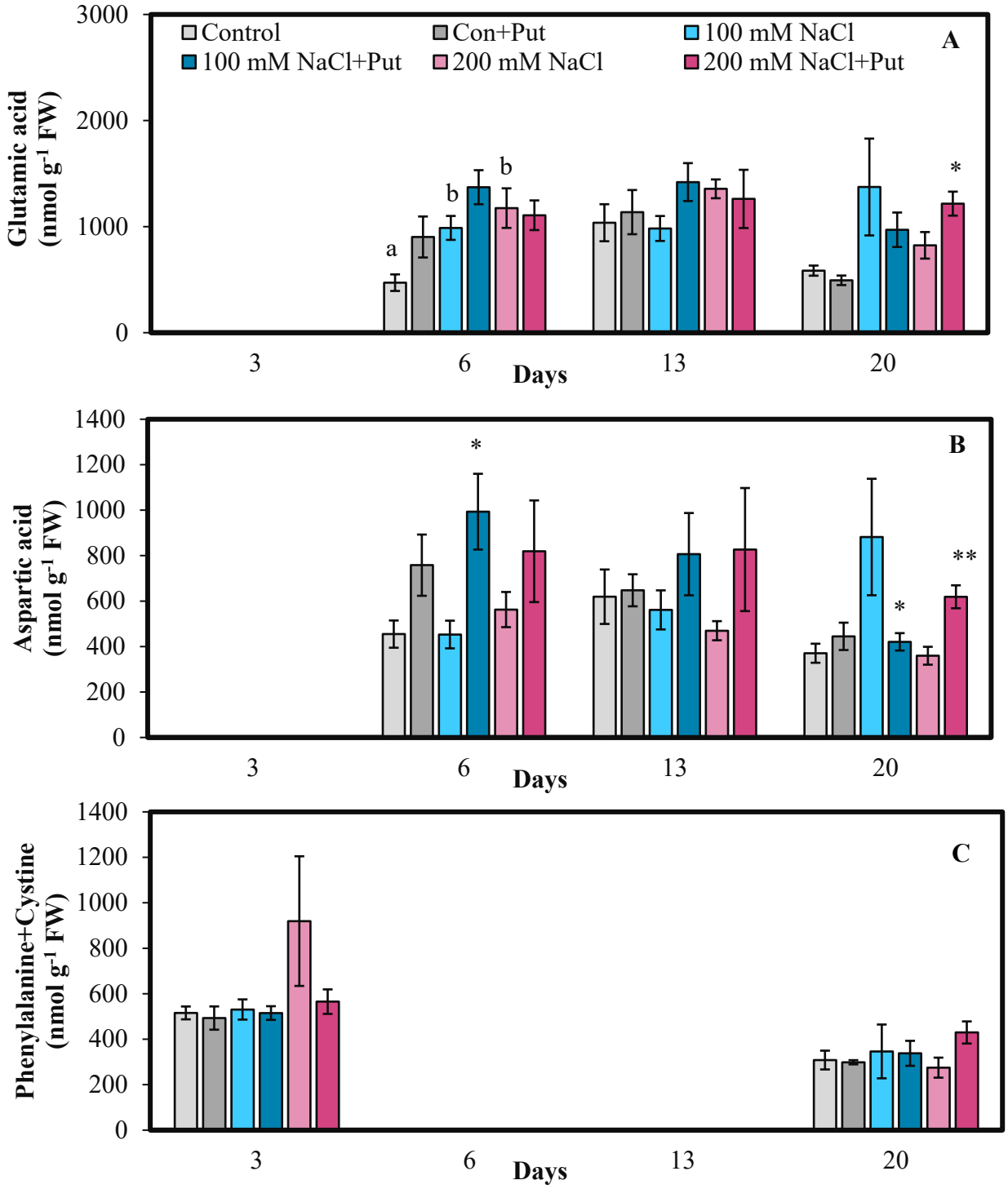

Figure 14: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the amino acids content of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Glutamic acid, (B) Aspartic acid, and (C) Phenylalanine+Cystine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 100 mM NaCl, and 200 mM NaCl, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

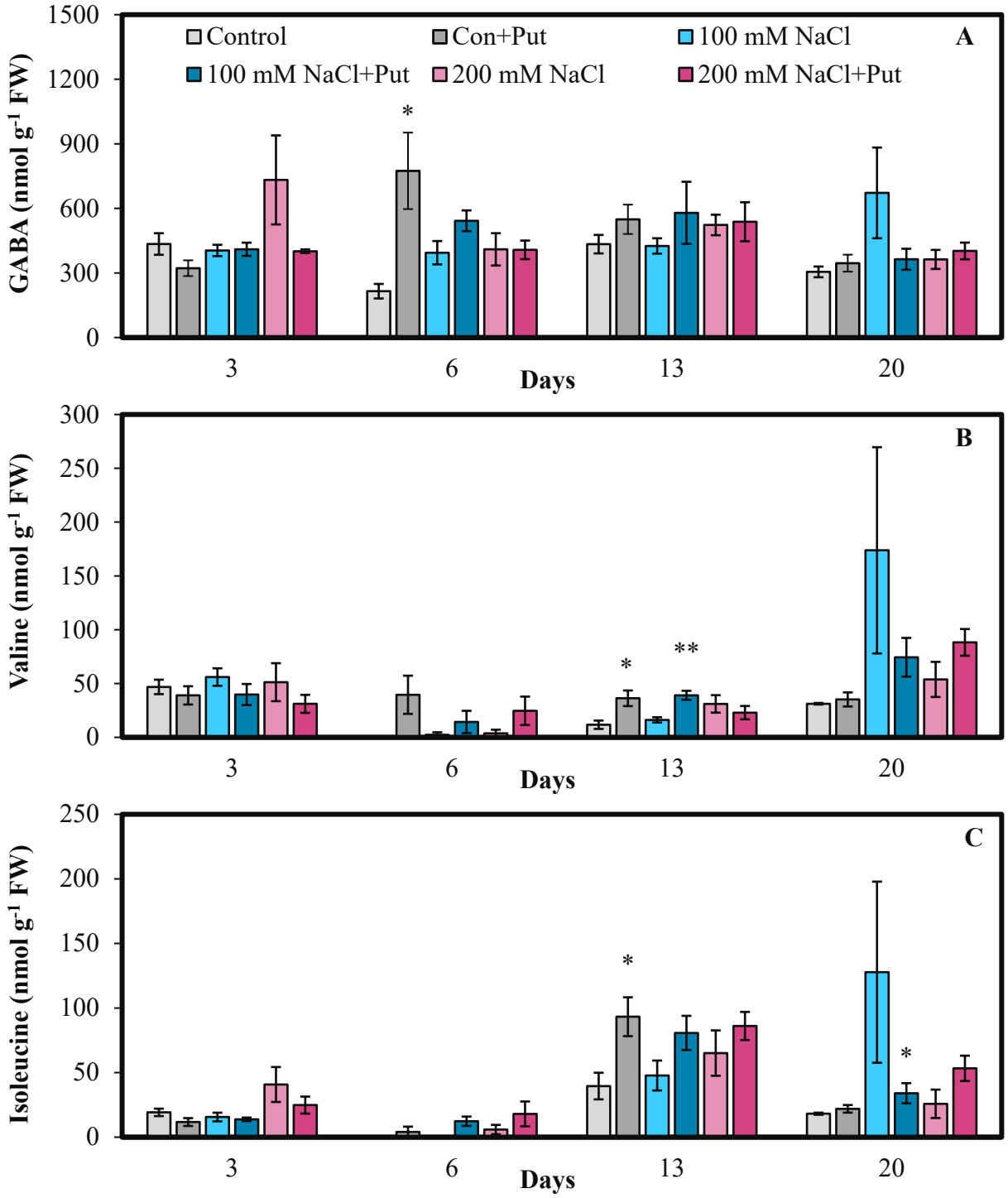

Figure 15: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the amino acids content of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) GABA, (B) Valine, and (C) Isoleucine. On a given day, an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

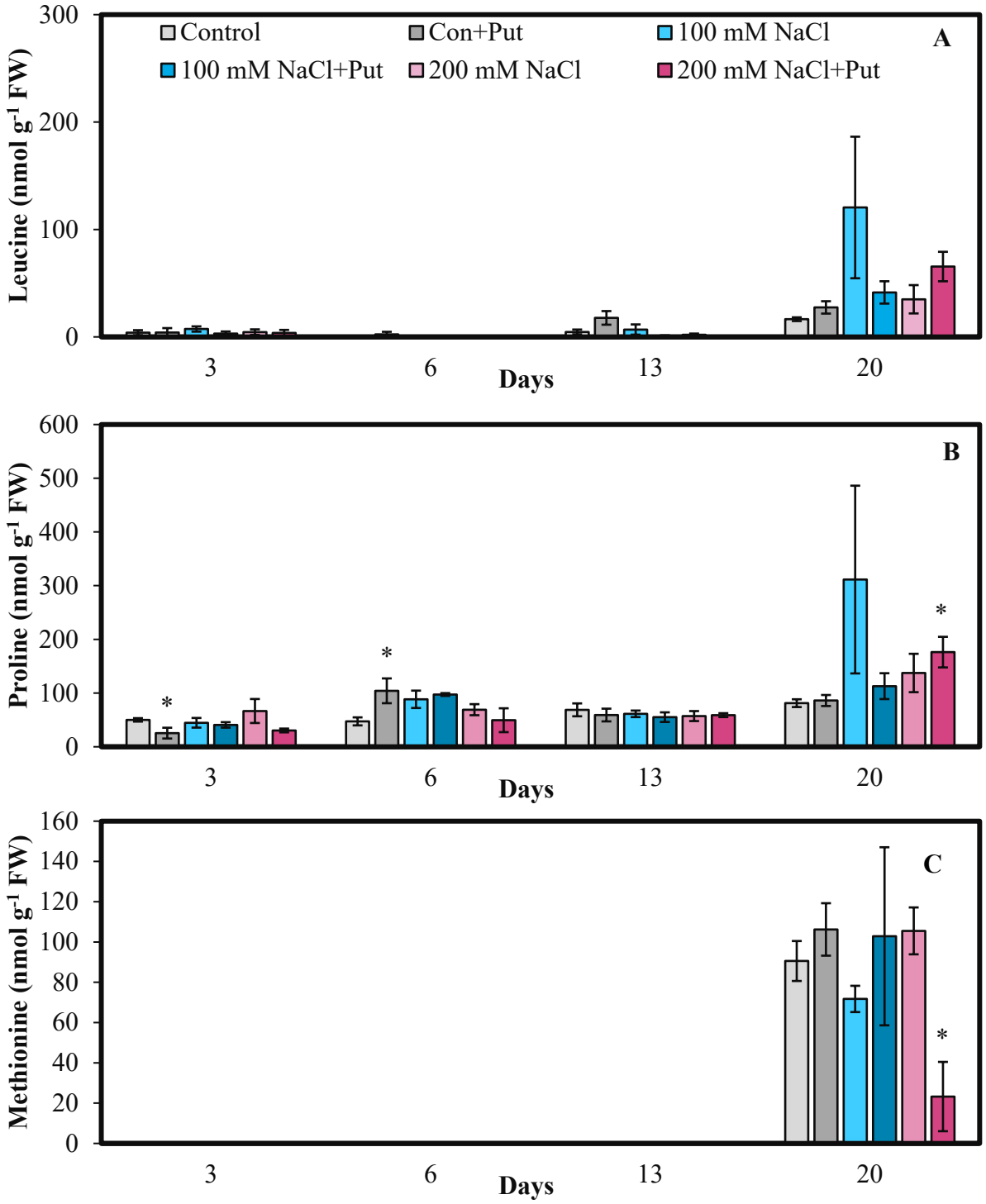

Figure 16: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the amino acids content of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Leucine, (B) Proline, and (C) Methionine. On a given day, an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

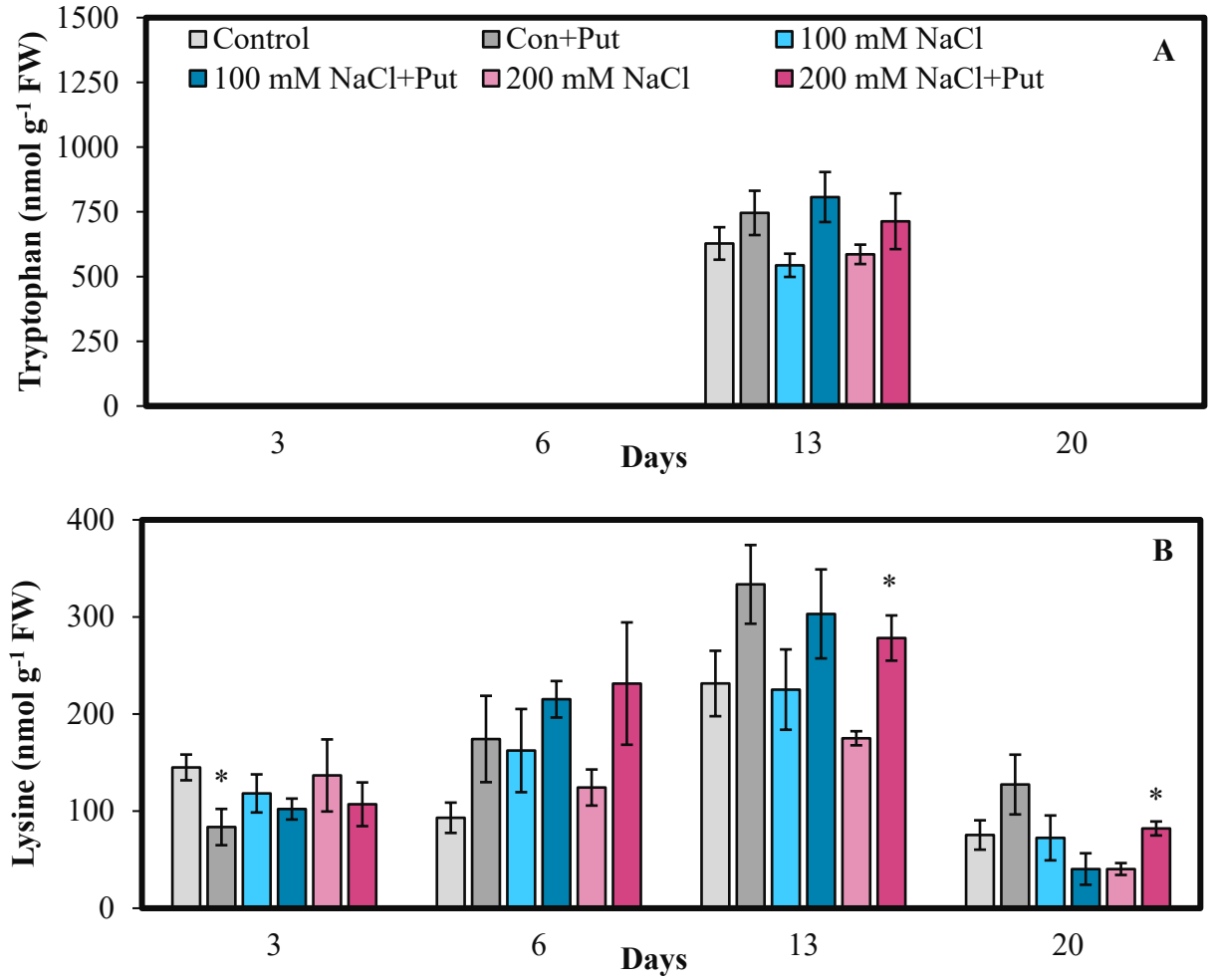

Figure 17: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the amino acids content of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Tryptophan and (B) Lysine. On a given day, an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

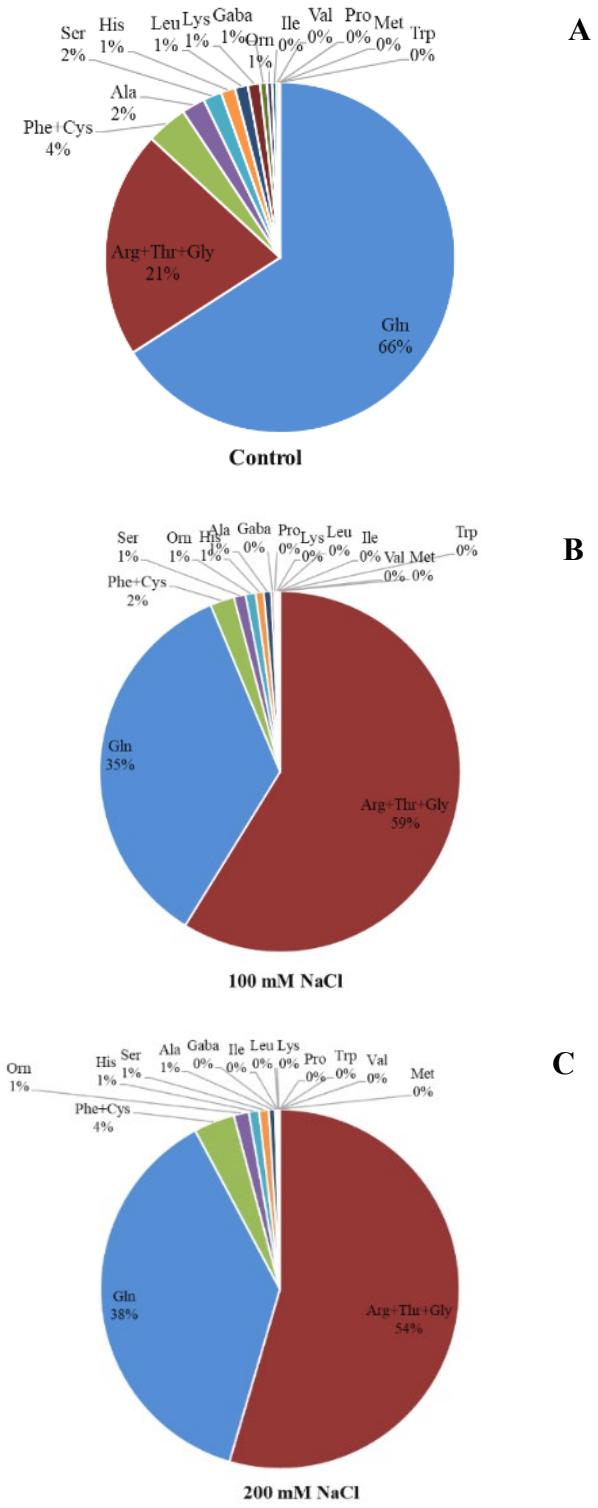

Figure 18: Pie chart representation of amino acids in the roots of the hybrid poplar NM6 plants under (A) control, (B) 100 mM NaCl, and (C) 200 mM NaCl on day 21.

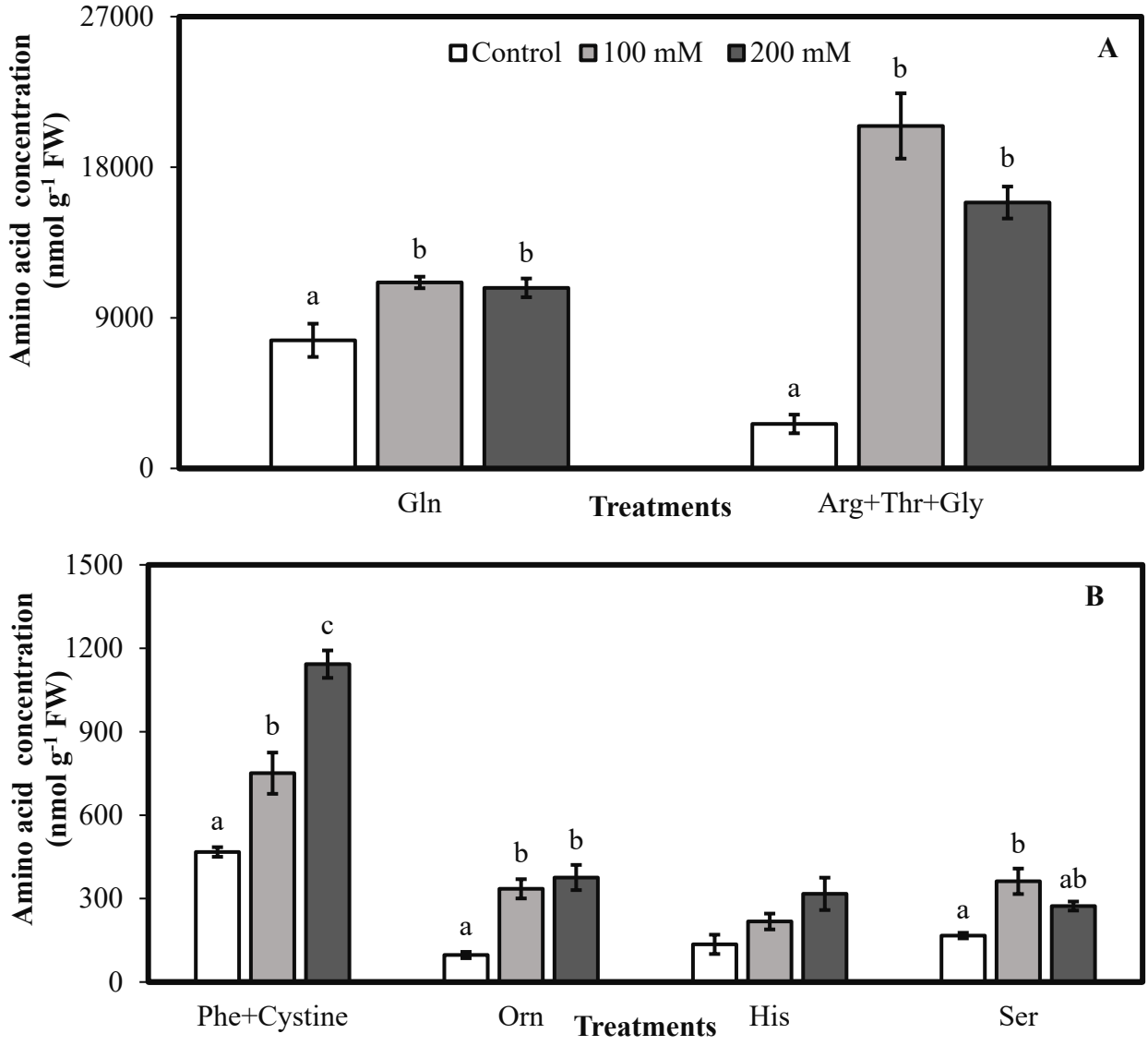

Figure 19: The effect of two different concentrations of NaCl on day 21 on the amino acids content of roots of the hybrid poplar NM6 plants. Data are mean of ± SE of 4 replicates. (A) Glutamine, Arginine+Threonine+Glycine and (B) Phenylalanine+Cystine, Ornithine, Histidine, Serine. A different letter indicates the treatment effect was significantly different ($p \leq 0.05$) between control,

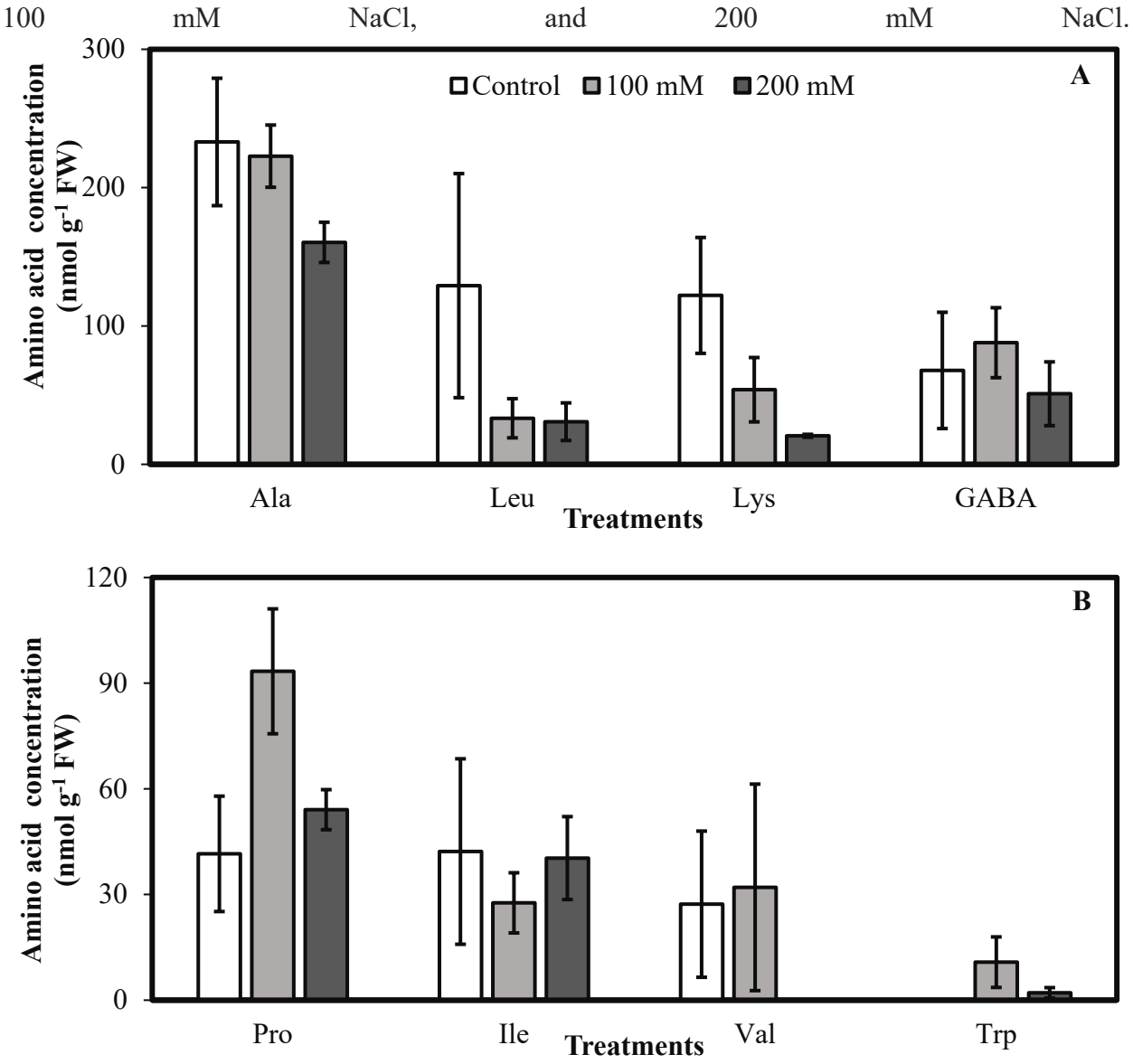

Figure 20: The effect of two different concentrations of NaCl on day 21 on the amino acids content of roots of the hybrid poplar NM6 plants. Data are mean of ± SE of 4 replicates. (A) Alanine, Leucine, Lysine, GABA and (B) Proline, Isoleucine, Valine, Tryptophan.

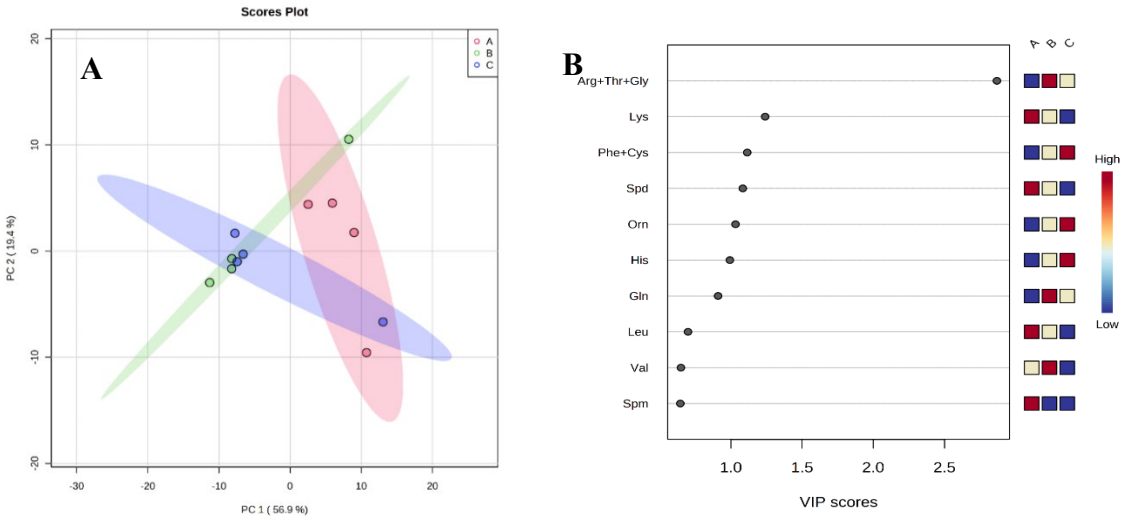

Figure 21: (A) Principal component analysis of roots. A-Control, B-100 mM NaCl, C- 200 mM NaCl. (B) Metabolomic features identified according to the Variable Importance in Projection score. Top 10 primary metabolites (amino acids and polyamines) with values of VIP scores > 1.0 are reported. The intensity of the colored boxes denotes the relative metabolite abundance in each treatment group.

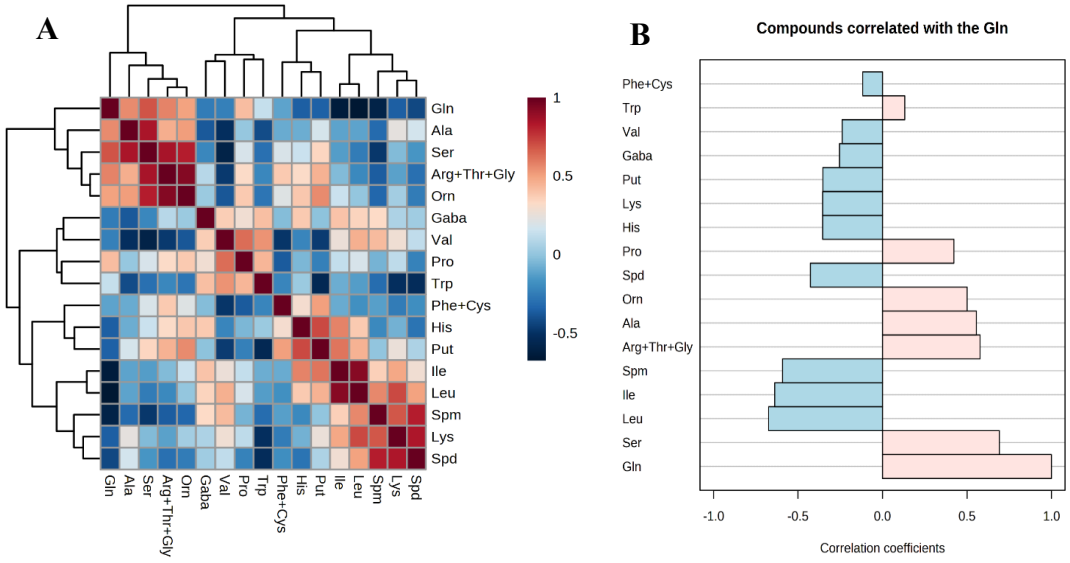

Figure 22: (A) Correlation coefficients of amino acids and polyamines analyzed in the poplar roots treated with two different concentrations of salt. (B) Pattern correlation analysis. The graph reports the significant features detected and ordered according to their correlation coefficient when correlated with Gln. The red color represents a positive correlation, and the blue color represents a negative correlation. Correlation distance- Pearson r.

Table 6: Amino acids and polyamines (nmol g^{-1} FW) content in the leaves and roots of poplar plants exposed to two concentrations of NaCl (100 mM, 200 mM) with or without 1 mM Put spray (data are mean ± SE, n=5 for leaves, n=3 for roots).

Days	Treatment	Asp	Glu	Gln	Ser	Arg+Thr+Gly	Ala
Leaves							
3	Control	ND	ND	3060.01 ± 220.5	659.18 ± 38.27	29829.63 ± 1378	879.9 ± 126.01
	Control +Put	ND	ND	2791.18 ± 309.46	695.06 ± 102.32	**20889.58 ± 1789.62** *	564.41 ± 75.72
	100 mM NaCl	ND	ND	2581.06 ± 201.67	844.9 ± 100.18	22191.44 ± 2384.77	675.82 ± 61.54
	100 mM NaCl + Put	ND	ND	3031.23 ± 191.16	1167.45 ± 113.29	24383.26 ± 669.55	713.34 ± 41.87
	200 mM NaCl	ND	ND	4763.96 ± 1484.23	1541.83 ± 487.54	42850.23 ± 11752.66	1054.38 ± 269.99
	200 mM NaCl + Put	ND	ND	3738.02 ± 352.01	949.9 ± 93.15	23416.83 ± 2446.33	690.72 ± 35.97
6	Control	454.72 ± 59.89	**471.68 ± 78.22** a	**450.57 ± 27.7** a	**227.61 ± 22.19** a	24337.87 ± 3276.96	**523.8 ± 91.64** a
	Control + Put	758 ± 134.69	902.39 ± 193.25	**837.24 ± 169.72** *	**1323.17 ± 331.01** *	41237.47 ± 7908.59	691.92 ± 165.95
	100 mM NaCl	452.96 ± 60.91	**987.9 ± 112.78** b	**1003.15 ± 133.73** b	**1119.11 ± 165.91** b	36114.55 ± 5466.55	**1232.51 ± 173.01** b
	100 mM NaCl + Put	**993.34 ± 166.61** *	1371.48 ± 160.58	1122.66 ± 194.15	**2103.81 ± 222.03** **	39763.5 ± 2660.57	844.06 ± 103.81
	200 mM NaCl	562.68 ± 77.39	**1174.44 ± 186.58** b	**871.91 ± 114.43** b	**1467.18 ± 258.64** b	34218.09 ± 2831.74	**1004.78 ± 98.43** ab
	200 mM NaCl + Put	819.2 ± 223.62	1107.2 ± 139.82	1353.37 ± 198.23	1460.62 ± 116.37	25807.88 ± 4785.39	**670.58 ± 65.97** *
13	Control	619.18 ± 119.74	1036.72 ± 174.45	**873.88 ± 112.16** a	**225.03 ± 34.69** a	46063.37 ± 2761.68	731.33 ± 126.73
	Control + Put	647.47 ± 70.43	1136.69 ± 208.28	1118.34 ± 48.73	636.71 ± 206.35	41919.5 ± 3310.19	648.19 ± 50
	100 mM NaCl	561.19 ± 85.96	982.58 ± 117.56	**1170.17 ± 261.79** ab	**538.62 ± 69.6** b	38726.51 ± 3084.19	679.55 ± 75.89
	100 mM NaCl + Put	806.44 ± 180.97	1419.85 ± 178.78	1264.57 ± 251.38	**1183.61 ± 246.57** *	44288.86 ± 4953.06	733.48 ± 179.33
	200 mM NaCl	469.62 ± 42.06	1356.12 ± 88.73	**1619.11 ± 139.47** b	**914.79 ± 42.62** c	41707.49 ± 2929.1	570.98 ± 45.44
	200 mM NaCl + Put	826.66 ± 270.67	1261.17 ± 274.46	**1192.84 ± 100.95** *	1220.66 ± 182.13	41338.89 ± 4378.27	675.79 ± 107.64
20	Control	370.53 ± 41.9	585.11 ± 47.74	646.34 ± 29.87	548.73 ± 51.53	21263.76 ± 1221.09	255.45 ± 26.5
	Control + Put	444.86 ± 60.18	493.96 ± 44.94	**1221.34 ± 25.62** *	773.38 ± 166.89	25176 ± 2521.15	376.91 ± 51.4
	100 mM NaCl	881.81 ± 56.29	1373.4 ± 44.38	3571.47 ± 151.59	662.68 ± 72.5	20269 ± 1604.38	1004.17 ± 26.24
	100 mM NaCl + Put	**420.69 ± 38.32** *	970.43 ± 162.5	2343.01 ± 578.95	761.36 ± 47.28	22766.57 ± 795.08	**451.64 ± 59.62** *
	200 mM NaCl	359.54 ± 39.37	823.66 ± 125.21	1846.26 ± 477.79	596.25 ± 101.89	21020.16 ± 1496.68	383.52 ± 72.63
	200 mM NaCl + Put	**618.89 ± 50.52** **	**1216.64 ± 113.08** *	2176.25 ± 96.44	**851.45 ± 24.01** *	21858.65 ± 1022.11	613.05 ± 66.88
Roots							
21	Control	ND	ND	**7656.76 ± 993.89** a	**166.99 ± 10.05** a	**2658.09 ± 557.42** a	233.02 ± 46.05
	100 mM NaCl	ND	ND	**11113.98 ± 344.48** b	**362.16 ± 45.8** b	**20464.67 ± 1951.02** b	222.76 ± 22.5
	200 mM NaCl	ND	ND	**10790.9 ± 559.6** b	**273.25 ± 16.18** ab	**15889.44 ± 954.48** b	160.43 ± 14.54

Table 6: continued.

Days	Treatment	GABA	Val	Met	Ile	Leu	Trp	Phe+Cystine
Leaves								
3	Control	434.62 ± 50.15	46.86 ± 6.72	ND	19.19 ± 2.87	3.93 ± 2.35	ND	515.64 ± 28.26
	Control + Put	322.15 ± 36.64	39.01 ± 8.49	ND	11.71 ± 3.04	4.1 ± 4.1	ND	493.04 ± 51.14
	100 mM NaCl	404.58 ± 26.41	56.05 ± 8.12	ND	15.6 ± 3.35	7.34 ± 2.46	ND	530.66 ± 44.41
	100 mM NaCl + Put	409.97 ± 30.81	39.77 ± 9.82	ND	13.83 ± 1.35	3.07 ± 1.96	ND	515.1 ± 30.06
	200 mM NaCl	732.24 ± 206.79	51.26 ± 17.66	ND	40.77 ± 13.53	4.44 ± 2.57	ND	919.47 ± 285.18
	200 mM NaCl + Put	400.93 ± 8.66	31.12 ± 8.38	ND	24.85 ± 6.58	3.74 ± 2.79	ND	565.19 ± 53.91
6	Control	215.25 ± 33.72	ND	ND	ND	ND	ND	ND
	Control + Put	**774.51 ± 177.68** *	39.56 ± 17.75	ND	4.07 ± 4.07	2.38 ± 2.38	ND	ND
	100 mM NaCl	394.07 ± 54.27	2.36 ± 2.36	ND	ND	ND	ND	ND
	100 mM NaCl + Put	542.47 ± 48.28	14.31 ± 10.35	ND	12.33 ± 3.61	ND	ND	ND
	200 mM NaCl	409.67 ± 75.08	3.68 ± 3.44	ND	5.89 ± 3.61	ND	ND	ND
	200 mM NaCl + Put	407.29 ± 43.18	24.67 ± 13.19	ND	18 ± 9.63	ND	ND	ND
13	Control	433.78 ± 42.87	11.72 ± 3.88	ND	39.59 ± 10.3	4.62 ± 2.22	627.91 ± 62.68	ND
	Control + Put	549.24 ± 68.19	**36.29 ± 7.26** *	ND	**93.27 ± 15.05** *	17.7 ± 6.34	745.88 ± 85.57	ND
	100 mM NaCl	425.57 ± 35.67	16.21 ± 2.47	ND	47.74 ± 11.58	6.85 ± 4.76	543.52 ± 44.87	ND
	100 mM NaCl + Put	579.45 ± 144.17	**39.04 ± 4.2** **	ND	80.72 ± 13.23	0.73 ± 0.73	807.2 ± 96.62	ND
	200 mM NaCl	523.02 ± 47.76	31.08 ± 8.12	ND	65.1 ± 17.56	1.96 ± 1.14	585.87 ± 37.5	ND
	200 mM NaCl + Put	537.85 ± 90.68	22.96 ± 6.17	ND	86.09 ± 10.9	ND	713.63 ± 107.61	ND
20	Control	305.04 ± 24.93	31.24 ± 0.72	90.57 ± 9.92	18.17 ± 0.81	16.48 ± 1.73	ND	308.26 ± 41.34
	Control + Put	345.5 ± 39.46	35.23 ± 6.54	106.24 ± 13.02	21.93 ± 2.94	27.45 ± 5.75	ND	298.48 ± 9.24
	100 mM NaCl	671.95 ± 38.42	173.78 ± 0.81	71.74 ± 8.34	127.74 ± 2.73	120.48 ± 1.61	ND	346.24 ± 44
	100 mM NaCl + Put	363.71 ± 48.8	74.43 ± 17.97	102.84 ± 44.21	**34.02 ± 7.81** *	41.42 ± 10.39	ND	337.89 ± 54.99
	200 mM NaCl	362.88 ± 44.27	53.82 ± 16.32	105.53 ± 11.64	25.83 ± 11	35.03 ± 13.18	ND	274.81 ± 44.09
	200 mM NaCl + Put	402.27 ± 38.84	88.26 ± 12.4	**23.27 ± 17.2** *	53.28 ± 9.82	65.51 ± 13.71	ND	429.59 ± 48.66
Roots								
21	Control	67.88 ± 42.03	27.25 ± 20.75	ND	42.19 ± 26.33	129.16 ± 80.98	ND	**467.57 ± 17.34** [a]
	100 mM NaCl	87.91 ± 25.29	32.03 ± 29.32	ND	27.63 ± 8.54	33.3 ± 14.15	10.79 ± 7.17	**750.88 ± 74.2** [b]
	200 mM NaCl	51.01 ± 23.05	ND	ND	40.34 ± 11.77	30.81 ± 13.58	2.08 ± 1.49	**1142.88 ± 49.36** [c]

Table 6: continued.

Days	Treatment	Pro	Orn	Lys	His	Put	Spd	Spm
Leaves								
3	Control	50.1 ± 3.17	103.95 ± 20.71	145.03 ± 13.24	137.89 ± 12.96	46.47 ± 2.53	49.97 ± 5.04	34.9 ± 1.49
	Control + Put	**25.41 ± 10.03** *	288.5 ± 121.48	83.6 ± 18.66 *	235.08 ± 90.15	33.74 ± 4.37 *	50.1 ± 5.45	**27.15 ± 2.39** *
	100 mM NaCl	44.68 ± 9	228.22 ± 60.26	118.24 ± 19.61	231.03 ± 42.26	36.24 ± 4.63	34.03 ± 3.27	33.44 ± 4.55
	100 mM NaCl + Put	40.74 ± 5.09	123.23 ± 9.56	102.14 ± 10.8	152.23 ± 6.25	33.38 ± 2.54	42.8 ± 6.14	36.33 ± 2.58
	200 mM NaCl	66.62 ± 22.33	116.91 ± 53.57	136.75 ± 37.12	349.68 ± 149.64	57.43 ± 12.87	45.53 ± 13.35	50.44 ± 12.38
	200 mM NaCl + Put	30.38 ± 3.51	222.01 ± 45.28	107.06 ± 22.51	219.83 ± 29.93	39.93 ± 4	49.21 ± 9.79	38.5 ± 4.73
6	Control	47.29 ± 7.45	136.9 ± 22.71	93.16 ± 15.61	125.77 ± 19.21	46.25 ± 2.04	**36.87 ± 6.74** a	35.37 ± 2.57
	Control + Put	**104.19 ± 23.09** *	464.17 ± 137.34	174.3 ± 44.47	276.25 ± 98.43	69.01 ± 10.54 *	57.01 ± 13.96	49.11 ± 8.87
	100 mM NaCl	88.38 ± 16.15	442.31 ± 102.99	162.35 ± 42.83	284.62 ± 60.5	69.47 ± 11.08	**30.8 ± 4.42** ab	53.77 ± 7.33
	100 mM NaCl + Put	97.33 ± 2.59	748.06 ± 288.29	215.18 ± 18.8	530.23 ± 196.72	48.38 ± 5.55	35.59 ± 6.8	58.5 ± 5.87
	200 mM NaCl	69.07 ± 10.31	375.84 ± 158.11	124.31 ± 18.59	192.11 ± 39.61	55.77 ± 5.96	**16.01 ± 1.2** b	48.39 ± 5.17
	200 mM NaCl + Put	49.45 ± 22.22	1495.4 ± 613.62	231.38 ± 63.01	**987.86 ± 280.98** *	58.65 ± 11.21	20.46 ± 5.9	63.23 ± 12.6
13	Control	68.77 ± 11.99	142.72 ± 18.88	231.45 ± 33.69	87.29 ± 12.92	31.77 ± 2.95	**13.24 ± 1.71** a	65.1 ± 14.46
	Control + Put	59.11 ± 11.83	177.56 ± 12.37	333.53 ± 40.53	79.38 ± 41.37	32.3 ± 1.92	18.16 ± 5.31	58.7 ± 5.76
	100 mM NaCl	61.36 ± 6.1	106.12 ± 21.69	225.19 ± 41.37	9.92 ± 6.13	31.21 ± 1.93	**5.88 ± 1.82** b	53.55 ± 4.61
	100 mM NaCl + Put	55.15 ± 8.96	229.03 ± 54.61	303.12 ± 45.83	ND	29.19 ± 1.56	4.96 ± 1.41	87.27 ± 17.69
	200 mM NaCl	57.17 ± 9.29	108.37 ± 13.8	175.06 ± 7.26	91.37 ± 46.16	41.17 ± 10.02	**2.9 ± 1.24** b	62.26 ± 7.44
	200 mM NaCl + Put	58.91 ± 3.61	**368.03 ± 77.31** *	**278.27 ± 23.29** *	ND	28.94 ± 7.29	13.38 ± 6.48	**99.32 ± 8.34** *
20	Control	81.2 ± 7.24	104.73 ± 9.52	75.45 ± 15.12	66.39 ± 11.51	37.91 ± 2.35 a	ND	25.93 ± 2.96
	Control + Put	86.14 ± 10.27	**164.38 ± 13.14** *	127.39 ± 30.77	146.18 ± 3.91 *	**27 ± 2.82** *	0.18 ± 0.18	**38.43 ± 3.13** *
	100 mM NaCl	311.33 ± 1.67	351.26 ± 29.24	72.43 ± 19.29	371.2 ± 40.74	**87.46 ± 61.63** b	16.64 ± 11.45	35.24 ± 8.99
	100 mM NaCl + Put	112.88 ± 24.07	157.37 ± 26.88	40.4 ± 16.22	38.53 ± 38.53	20.24 ± 4.02	ND	33.16 ± 2.86
	200 mM NaCl	137.39 ± 35.73	313.58 ± 141.97	40.4 ± 6.12	171.06 ± 69.72	**16 ± 1.55** b	7.53 ± 4.06	33.53 ± 3.36
	200 mM NaCl + Put	**176.21 ± 28.5** *	230.42 ± 56.19	**82.16 ± 7.2** *	235.64 ± 73.33	23.95 ± 4.48	4.12 ± 3.39	45.33 ± 5.06
Roots								
21	Control	41.54 ± 16.38	**97.07 ± 11.36** a	122.09 ± 41.88	135.48 ± 34.73	**54.76 ± 12.19** a	**40.67 ± 5.76** a	7.09 ± 4.28
	100 mM NaCl	93.36 ± 17.73	**335.17 ± 34.64** b	53.94 ± 23.26	217.62 ± 28.46	**73.64 ± 3.59** b	**9.74 ± 3.39** b	ND
	200 mM NaCl	54.08 ± 5.69	**375.86 ± 45.27** b	20.63 ± 1.05	317.05 ± 58.23	**81.63 ± 3.68** b	**4.33 ± 1.01** b	ND

Foliar soluble sugars

In response to NaCl treatment, fructose content significantly increased on the 6th day. In Put-sprayed plants, fructose was significantly higher *vs.* unsprayed under control treatment on the 6th and 13th day (Fig. 23A). In Put-sprayed plants, fructose was significantly higher *vs.* unsprayed for 200 mM NaCl on the 3rd day (Fig. 23A). Fructose was significantly higher in Put-sprayed plants *vs.* unsprayed for 100 mM NaCl on the 6th day. On other days, there was no significant difference between the control and NaCl treatment.

On the 13th day, glucose+galactose contents were significantly lower in response to NaCl treatment (Fig. 23B). Glucose+galactose contents were significantly higher in Put-sprayed plants *vs.* unsprayed for 200 mM NaCl on the 6th day.

On the 13th day, sucrose was significantly higher for 200 mM NaCl (Fig. 23C). Sucrose was significantly lower in Put-sprayed plants than those unsprayed for 200 mM NaCl on the 3rd day. On the 6th day, sucrose significantly decreased in Put-sprayed plants *vs.* unsprayed for 100 mM NaCl. However, there was a significant increase in Put-sprayed plants *vs.* unsprayed for 100 mM NaCl on the 13th day.

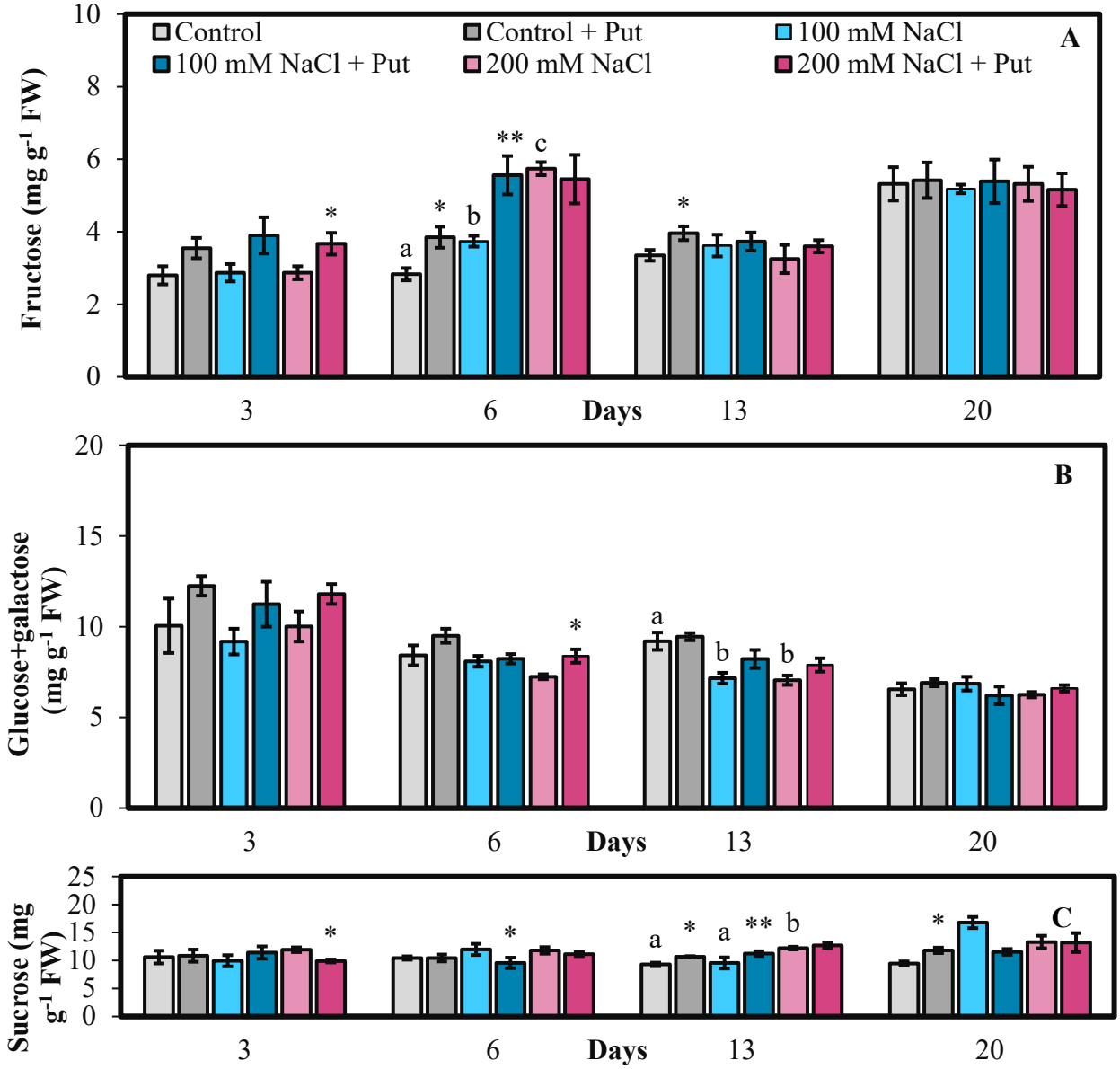

Figure 23: The effect of two different concentrations of NaCl (± Putrescine spray) on different days on the soluble sugars content of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 6 replicates. (A) Fructose, (B) Glucose+galactose, and (C) Sucrose. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 100 mM NaCl, and 200 mM NaCl, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

Effect of cadmium and its interaction with spermidine

Morphological symptoms

Morphological symptoms in the Cd-treated plants started to show after 7 days of Cd exposure. The first signs of Cd damage started to appear in the middle leaves. The top leaves of the plants turned yellowish at first, and by the 21st day, the leaf tips showed browning under 50 µM Cd. Interestingly, the top leaves of the plants exposed to 150 µM Cd had darker pigmentation compared to those of 50 µM Cd-treated plants on the 21st day (Fig. 24). Also, the 50 µM Cd-treated plants sprayed with Spd had browning symptoms, unlike the leaves of Spd-sprayed 150 µM Cd treated plants. On day 21, the middle leaves of the plants sprayed with Spd under Cd treatment had browning symptoms (Fig. 25).

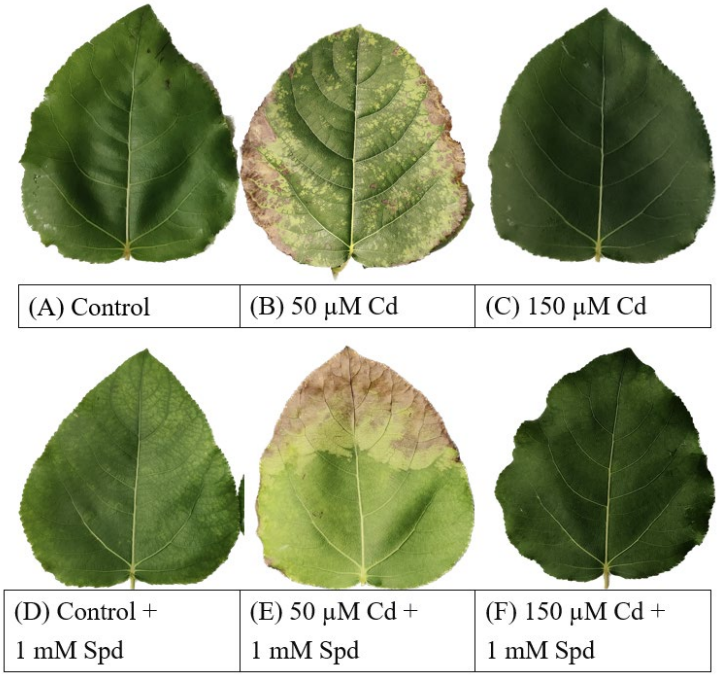

Figure 24: The effect of two different concentrations of $CdCl_2$ (±Spermidine spray) after 21 days on the leaf morphological traits of the hybrid poplar NM6 plants.

Figure 25: The effect of two different concentrations of $CdCl_2$ (±Spermidine spray) after 21 days on the morphological traits of the hybrid poplar NM6 plants.

Chlorophyll content

The leaves near the top 1/3rd of the plant had significantly lower SPAD chlorophyll index than the middle and bottom leaves for all treatments (Table 7). The SPAD chlorophyll index in leaves near the top of the plant was highest on day 9 in response to 150 µM Cd treatment (Fig. 26). The bottom leaves of the Spd-sprayed plants had significantly higher chlorophyll index *vs.* unsprayed control and 50 µM Cd-treated plants.

Treatment with 50 µM Cd significantly reduced the leaf chlorophyll contents as measured by spectrophotometry (Fig. 27A). In Spd-sprayed plants, there was a 2-fold increase in the chlorophyll contents *vs.* unsprayed Cd treatment. Total chlorophyll content was significantly higher than the control 7-day-old plants after the exposure to 150 µM Cd. In Spd-sprayed plants treated with 150 µM Cd, total chlorophyll content was always higher than the respective controls. Chlorophyll a significantly changed in response to Cd treatment compared to the control (Fig. 27B). After 50 µM

Cd treatment for 7 days, chlorophyll a was always less than the control plants on most days, while in response to 150 µM Cd, chlorophyll a was higher. Plants sprayed with Spd had significantly higher chlorophyll a *vs.* unsprayed for 50 µM C treatment. Although somewhat higher, the Spd-sprayed plants did not have significant increase *vs.* those unsprayed for 150 µM Cd treatment. Chlorophyll b decreased significantly for 50 µM Cd treatment as compared to the control on various days (Fig. 27C). Fourteen days after 150 µM Cd treatment, there was a significant increase in the chlorophyll b content as compared to 50 µM Cd-treated plants. In response to Spd spray, there was a significant increment of chlorophyll b *vs.* no Spd spray for 50 µM Cd treatment. Thus, the chlorophyll a: b ratio was not consistent on all days of sampling. For 50 µM Cd, carotenoids significantly increased compared to the control on the 3rd and the 14th day (Fig. 27D). On most days, Spd-sprayed plants had significantly less carotenoids *vs.* unsprayed for 50 µM Cd.

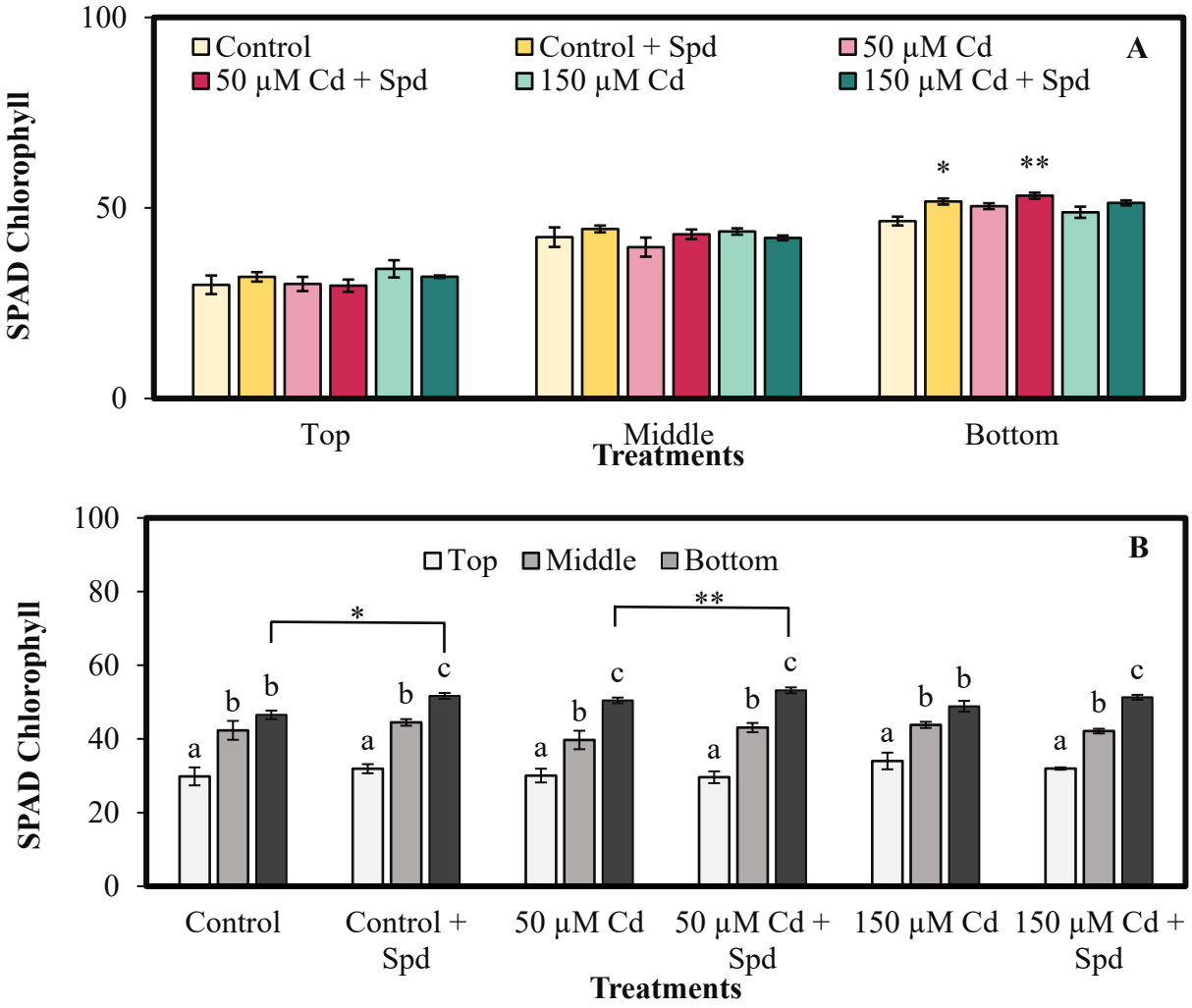

Figure 26: The effects of two different concentrations of $CdCl_2$ (+ Spermidine spray) on the SPAD chlorophyll content of leaves of the hybrid poplar NM6 plants 9 days after treatment. (A) Treatment effect on three different leaf positions of plants. (B) Leaf position effect on the treatment. Data are mean ± SE of 6 replicates. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

Table 7: SPAD meter readings for the top, middle, and bottom leaves in poplar plants exposed to two concentrations of Cd (50 µM, 150 µM) with or without 1 mM Spd spray (data are mean ± SE, n=6).

SL No.	Treatment	Top	Middle	Bottom
1	Control	**29.83 ± 2.44** [a]	**42.33 ± 2.57** [b]	**46.55 ± 1.15** [b]
2	Control + Spd	**31.92 ± 1.24** [a]	**44.48 ± 0.90** [b]	**51.68 ± 0.80** [c*]
3	50 µM Cd	**30.05 ± 1.87** [a]	**39.72 ± 2.51** [b]	**50.47 ± 0.76** [c]
4	50 µM Cd + Spd	**29.60 ± 1.60** [a]	**43.08 ± 1.26** [b]	**53.2 ± 0.80** [c**]
5	150 µM Cd	**34.02 ± 2.26** [a]	**43.82 ± 0.83** [b]	**48.87 ± 1.47** [b]
6	150 µM Cd + Spd	**31.97 ± 0.32** [a]	**42.13 ± 0.63** [b]	**51.32 ± 0.66** [c]

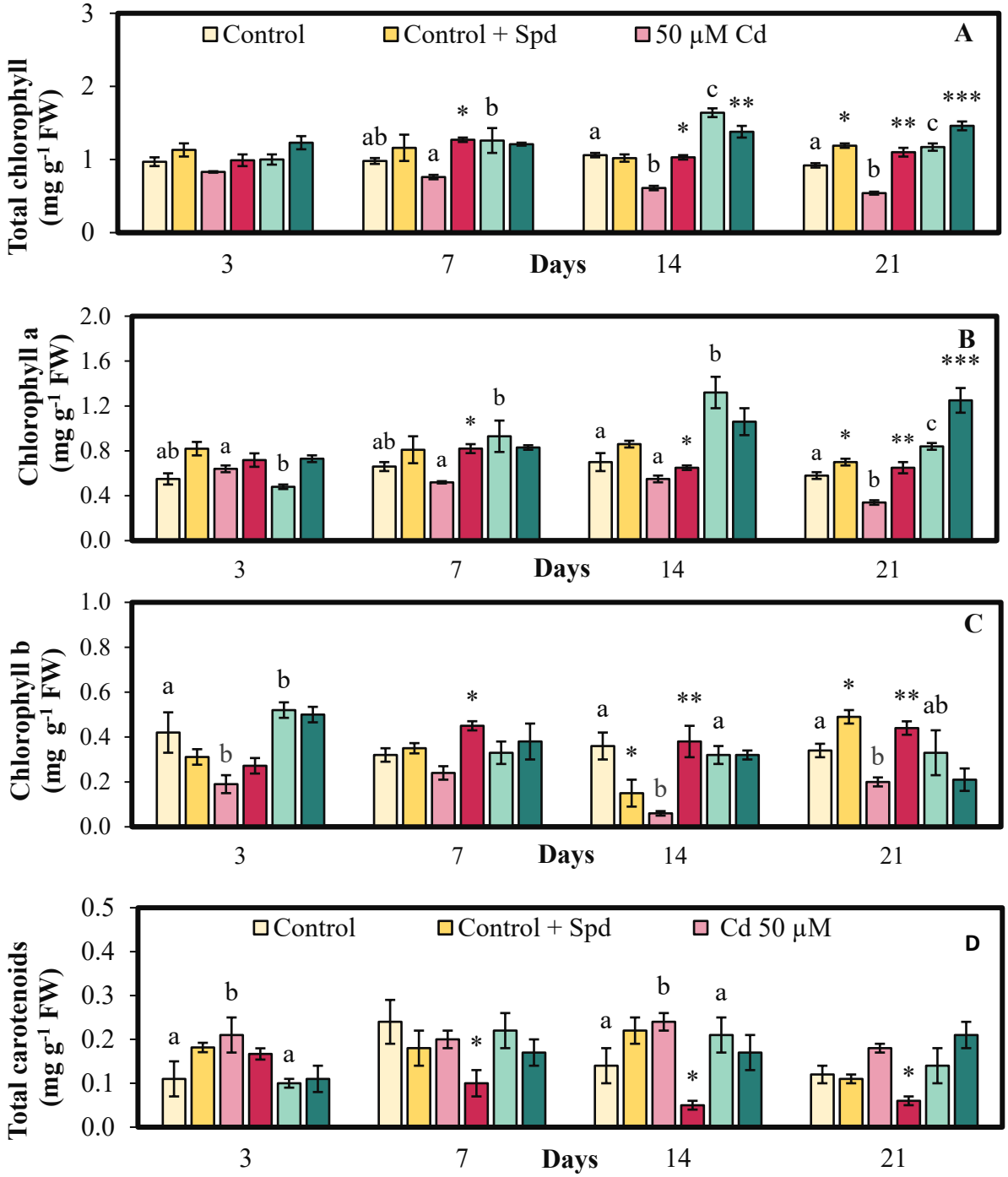

Figure 27: The effect of two different concentrations of $CdCl_2$ (+ Spermidine spray) on different days on the chlorophyll contents (spectrophotometric analysis) of leaves of the hybrid poplar NM6 plants. Data are mean ± SE of 6 replicates. (A) Total chlorophyll, (B) Chlorophyll a, (C) Chlorophyll b, (D) Total carotenoids. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

Gas exchange

Leaf gas exchange was measured in the 4th fully mature leaf 6 days after Cd treatment. Irrespective of the treatment, photosynthesis (P_n), stomatal conductance (g_s), and transpiration (E) values increased with the progress of the days of treatment. A significant increase (~2-fold) was seen in the P_n and E in the leaves of Cd-treated plants compared to the control on the 6th day (Fig. 28). Although not significant, P_n, g_s, and E were consistently lower on the Spd-sprayed plants *vs.* unsprayed plants under Cd treatment. After 6 days, almost a 2-fold increment was seen in the P_n, g_s, and E in response to 150 µM Cd. The lowest P_n was in the leaves of the plants treated with 50 µM Cd, which was similar to the trends in chlorophyll content trends on the 14th day. Compared to the controls, g_s and E content increased by more than 2-fold after 6 days in plants in response to 150 µM Cd, but after 14 days, they decreased significantly. On the 21st day, P_n, g_s, E was lower in sprayed plants *vs.* unsprayed in response to Cd treatment.

Total soluble proteins content

In response to Cd treatment, there was a significant decrease in the total soluble protein contents on the 7th and 14th day (Fig. 29A). On the 21st day, soluble protein content was still lower (statistically insignificant) in these plants. In Spd-sprayed plants, there was a significant increase in the soluble protein content *vs.* unsprayed treated with 50 µM Cd on the 3rd and 7th day, while sprayed plants *vs.* unsprayed treated with 150 µM Cd had significantly higher soluble protein content only on the 7th day.

Relative water content

Relative water content was not affected under Cd exposure for 14 days. In response to 50 µM Cd treatment, RWC significantly decreased on the 21st day (Fig. 29B). At the same time, RWC

was significantly higher in response to 150 µM Cd in comparison to 50 µM Cd on the same day. In Spd-sprayed plants, there was a significant decrease in the RWC *vs.* unsprayed under Cd treatment on the 14th day.

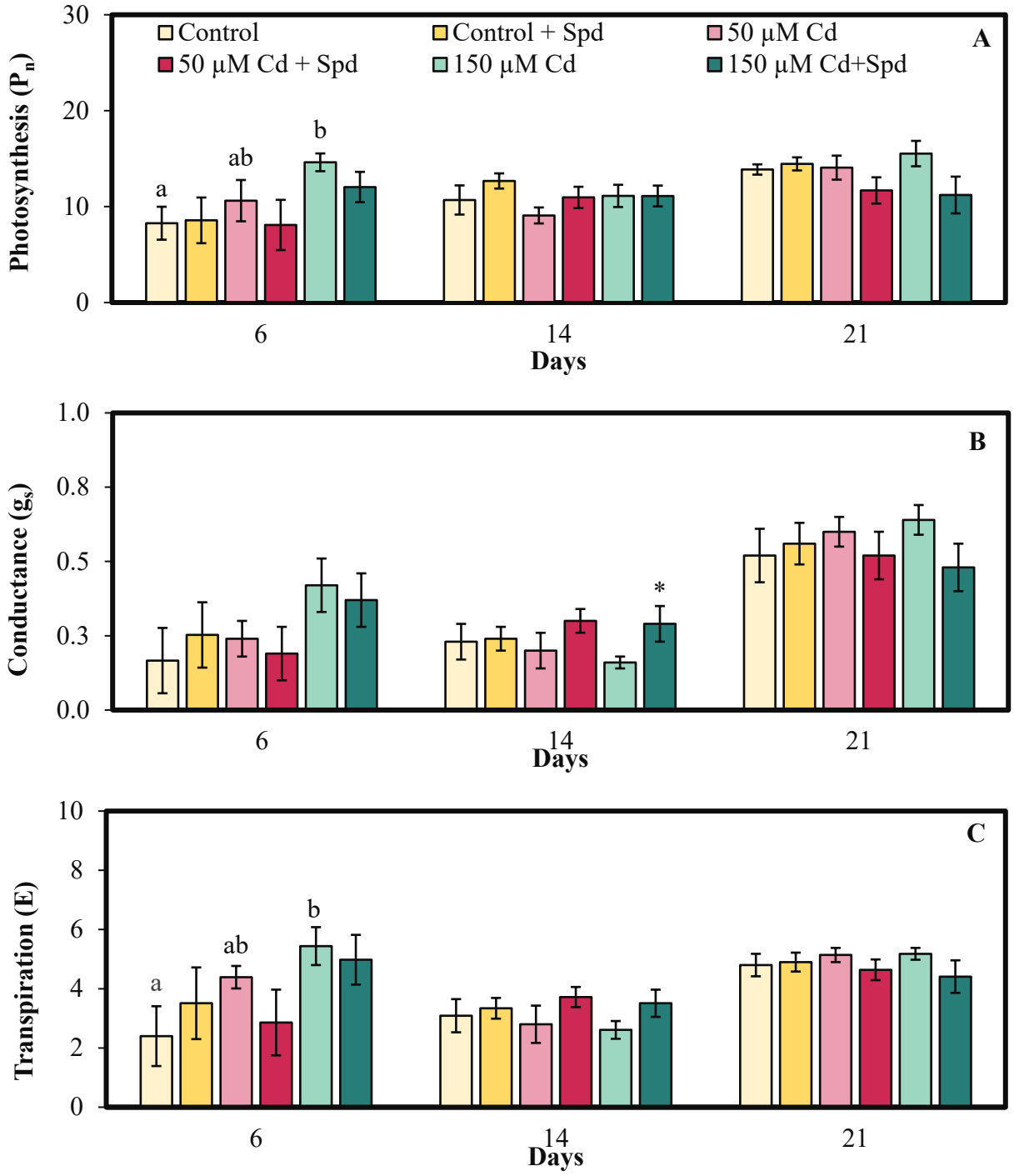

Figure 28: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the gas exchange in the leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 6 replicates. (A) Photosynthesis-Pn, (B) conductance-gs, and (C) transpiration-E as measured by LICOR-6400. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

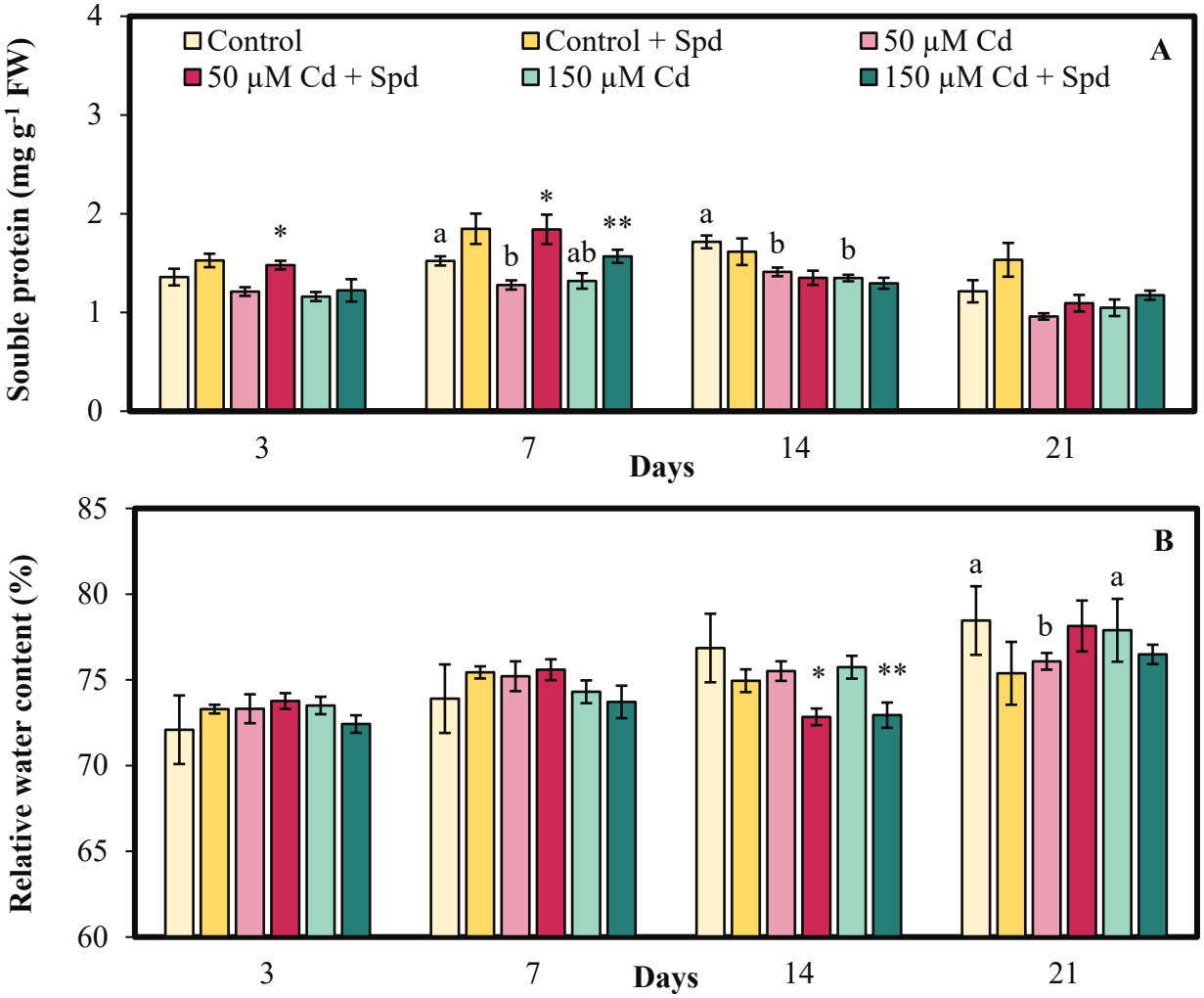

Figure 29: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the (A) soluble protein and (B) relative water content (%) of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 6 replicates. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

Foliar thiols content

Significant increases in the leaf Cys and γ-EC contents were seen in response to 150 µM Cd but not for 50 µM Cd on the 3rd day (Fig. 30A, B). On day 7 of Cd treatment, Cys content was higher (but not significant) under Cd treatment than the control plants. The Cys content increased from the 3rd to the 7th day, irrespective of treatment, and decreased afterwards. On the 14th day, Cys content was significantly higher in Spd-sprayed plants *vs.* unsprayed plants for 50 µM Cd. Spermidine also caused a small (but statistically insignificant) increase in Cys content for plants treated with 150 µM Cd. Unlike Cys, there was no significant effect of any treatment on the γ-EC content on any day.

Similar to Cys, GSH content significantly increased after 3 days in response to Cd treatment compared to the control. There was a sharp decrease in GSH content after the 3rd day in all other treatments. On the 7th day, for 150 µM Cd, GSH was significantly higher compared to the control, and there was a further increase in Spd-sprayed plants *vs.* unsprayed plants. On the 14th day, Spd spray showed significant increases in GSH content in the plants under Cd treatment (Fig. 30C).

As shown in Fig. 31A, PC_2 was detected only on the 7th day after Cd treatment and its content was lower than the control plants. In Spd-sprayed 50 µM Cd-treated plants, PC_2 was higher than those not sprayed. However, PC_2 was lower in sprayed plants *vs.* unsprayed for the 150 µM Cd treatment.

With an increment of Cd concentration, PC_4 showed a small decrease as compared to control plants on the 3rd day. Irrespective of the treatment, PC_3 and PC_4 increased on the 7th day compared to the 3rd day and decreased on the 14th day (Fig. 31B, C). Also, on the 14th day, PC_4 was significantly higher in Spd-sprayed plants *vs.* unsprayed plants for 50 µM Cd treatment.

Roots thiols content

The content of PCs was measured in the roots on the day of harvesting (21 days post-treatment with Cd) and only 3 groups of plants were tested (control, 50 μM, and 150 Cd μM). Only 5 of the 6 PCs were detected - Cys, GSH, PC_2, PC_3, and PC_4 in most plants. Cysteine, GSH, and PC_2 contents were higher in the roots in response to Cd treatment (Fig. 32A-C). However, for PC_3, it was detected only in Cd-treated plants as shown in Fig. 32D. In the presence of Cd, PC_4 increased significantly compared to the control plants (Table 8, Fig 32E).

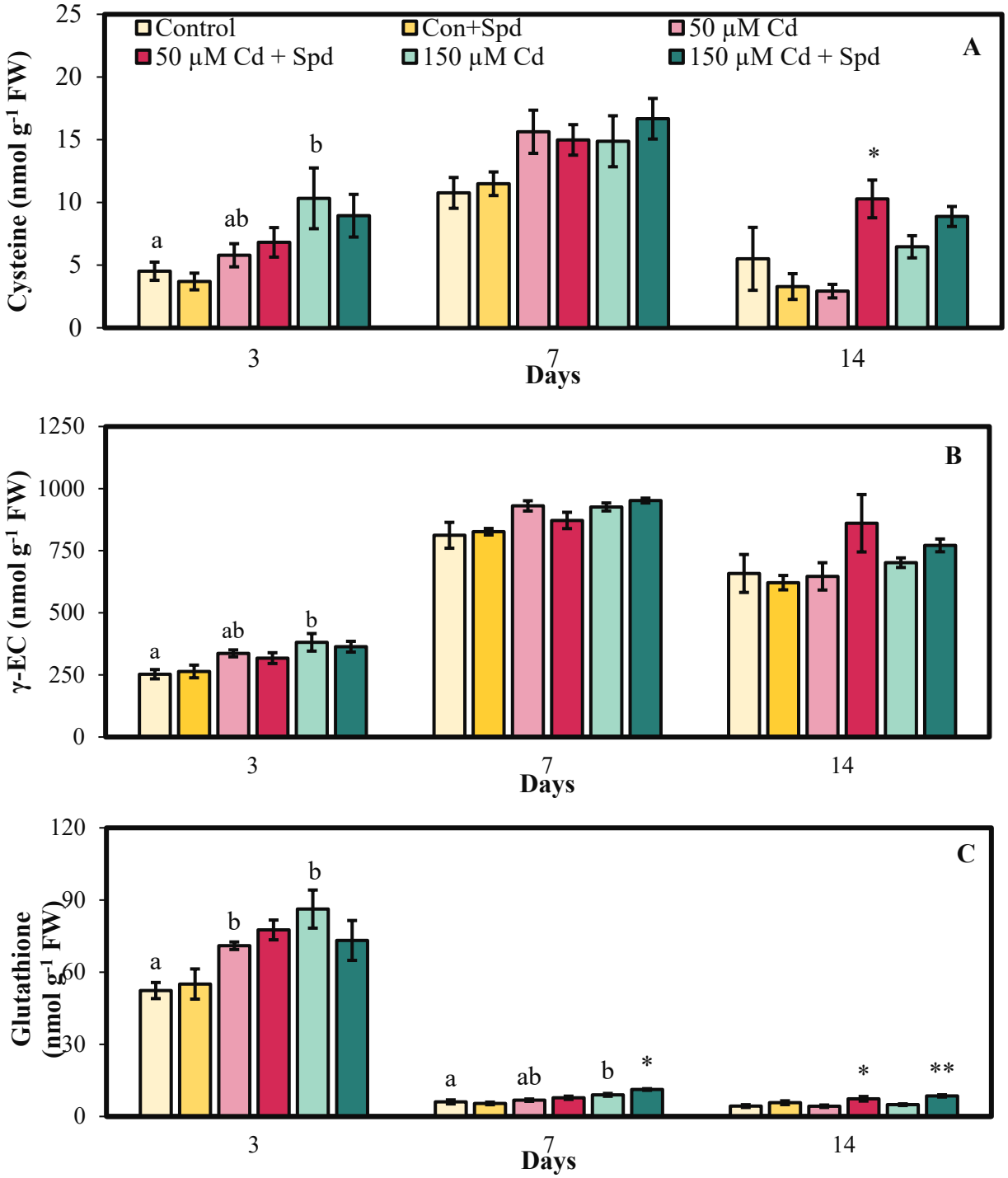

Figure 30: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the thiol compound contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Cysteine, (B) γ-EC, and (C) Glutathione. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

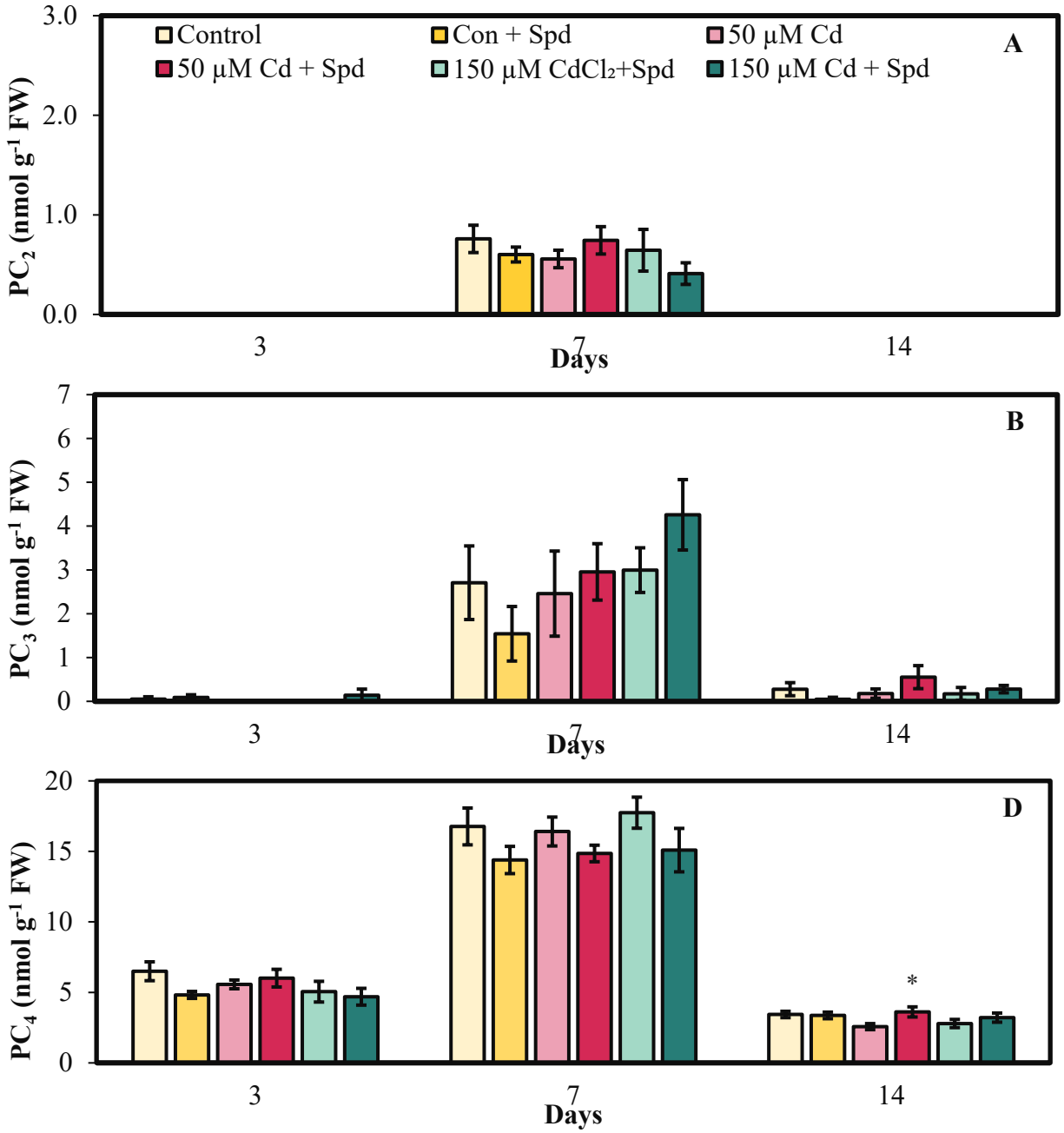

Figure 31: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the PC contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) PC_2, (B) PC_3, and (C) PC_4. On a given day, an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

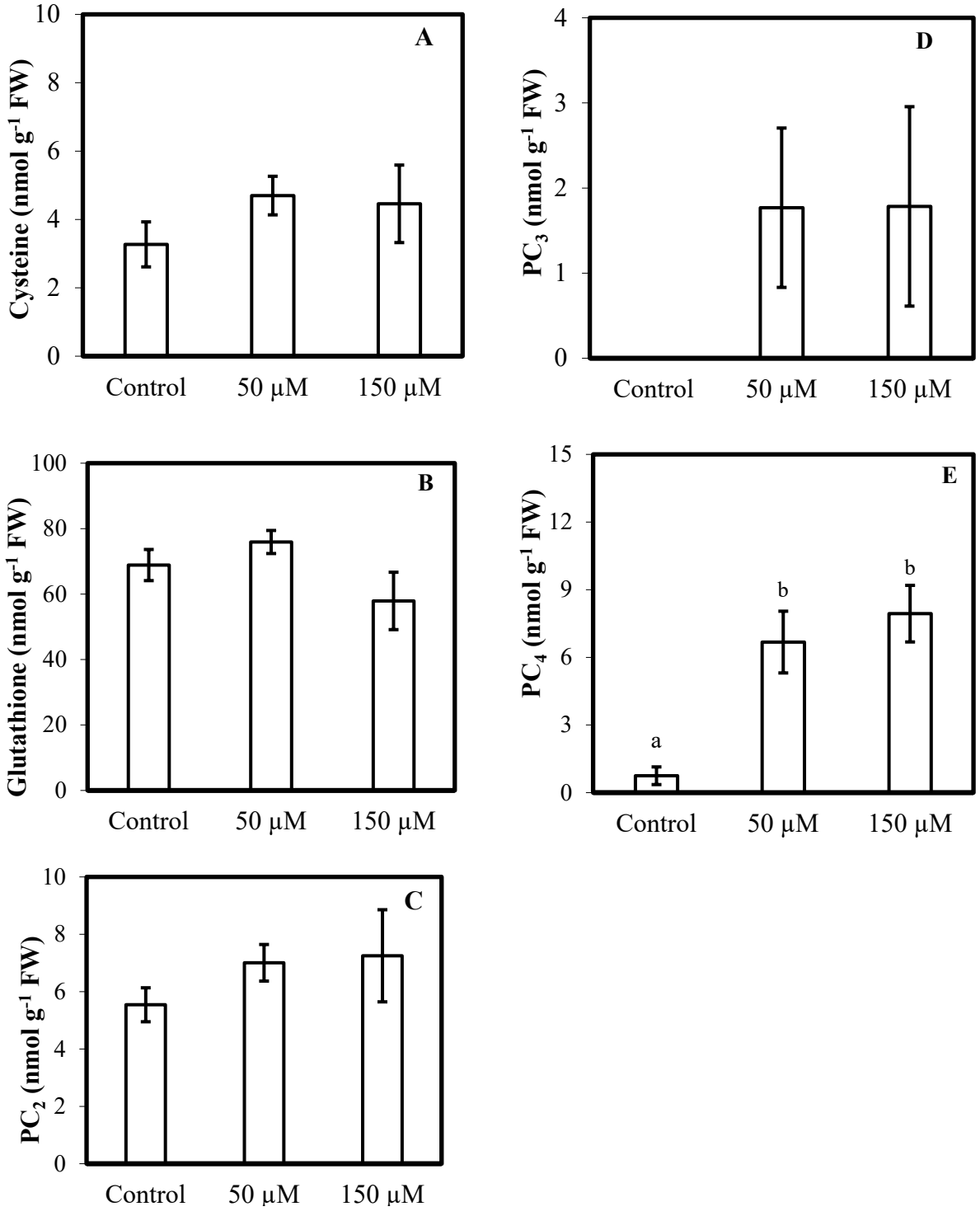

Figure 32: The effect of two different concentrations of $CdCl_2$ on the thiol compound and PC contents of roots of the hybrid poplar NM6 plants. Data are mean ± SE of 5 replicates. (A) Cysteine, (B) Glutathione (C) PC_2, (D) PC_3 and (E) PC_4. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd.

Table 8: Phytochelatin (nmol g^{-1} FW) content in the leaves and roots of poplar plants exposed to two concentrations of Cd (50 µM, 150 µM) with or without 1 mM Spd spray (data are mean ± SE, n=5).

Day	Treatment	Cysteine	Glutathione	γ-EC	PC_2	PC_3	PC_4
Leaves							
3	Control	**4.51 ± 0.73** [a]	**52.36 ± 3.35** [a]	**252.91 ± 18.69** [a]	ND	0.05 ± 0.05	6.50 ± 0.67
	Control + Spd	3.69 ± 0.67	55.06 ± 6.27	263.96 ± 25.55	ND	0.09 ± 0.06	4.82 ± 0.25
	50 µM Cd	**5.78 ± 0.92** [ab]	**70.98 ± 1.57** [b]	**336.75 ± 14.00** [ab]	ND	ND	5.57 ± 0.31
	50 µM Cd + Spd	6.81 ± 1.18	77.56 ± 4.12	317.64 ± 21.71	ND	ND	6.01 ± 0.63
	150 µM Cd	**10.32 ± 2.42** [b]	**86.23 ± 7.92** [b]	**381.25 ± 35.37** [b]	ND	ND	5.05 ± 0.73
	150 µM Cd + Spd	8.92 ± 1.70	73.19 ± 8.29	363.60 ± 21.66	ND	0.14 ± 0.14	4.69 ± 0.59
7	Control	10.76 ± 1.23	**6.06 ± 0.83** [a]	812.15 ± 52.10	0.76 ± 0.14	2.71 ± 0.84	16.77 ± 1.31
	Control + Spd	11.49 ± 0.94	5.43 ± 0.54	826.56 ± 13.11	0.60 ± 0.07	1.54 ± 0.62	14.39 ± 0.97
	50 µM Cd	15.63 ± 1.72	**6.78 ± 0.49** [ab]	930.54 ± 20.67	0.56 ± 0.09	2.46 ± 0.97	16.41 ± 1.02
	50 µM Cd + Spd	14.98 ± 1.22	7.78 ± 0.67	871.83 ± 32.90	0.74 ± 0.14	2.95 ± 0.64	14.85 ± 0.59
	150 µM Cd	14.87 ± 2.03	**9.00 ± 0.58** [b]	926.19 ± 16.03	0.64 ± 0.21	2.99 ± 0.51	17.75 ± 1.10
	150 µM Cd + Spd	16.67 ± 1.62	**11.22 ± 0.33** *	952.05 ± 9.67	0.41 ± 0.11	4.26 ± 0.80	15.09 ± 1.54
14	Control	5.50 ± 2.51	4.32 ± 0.57	658.45 ± 76.37	ND	0.28 ± 0.15	3.43 ± 0.23
	Control + Spd	3.29 ± 1.03	5.72 ± 0.79	621.43 ± 28.97	ND	0.05 ± 0.05	3.37 ± 0.23
	50 µM Cd	2.92 ± 0.54	4.26 ± 0.50	646.73 ± 54.96	ND	0.18 ± 0.11	2.57 ± 0.21
	50 µM Cd + Spd	**10.28 ± 1.51** *	**7.36 ± 0.96** *	860.60 ± 115.58	ND	0.55 ± 0.26	**3.62 ± 0.36** *
	150 µM Cd	6.46 ± 0.88	4.90 ± 0.35	701.85 ± 19.34	ND	0.17 ± 0.15	2.79 ± 0.29
	150 µM Cd + Spd	8.88 ± 0.80	**8.56 ± 0.46** **	771.44 ± 25.78	ND	0.28 ± 0.08	3.21 ± 0.32
Roots							
21	Control	3.27 ± 0.66	68.88 ± 4.76	ND	5.54 ± 0.59	ND	**0.75 ± 0.39** [a]
	50 µM Cd	4.70 ± 0.56	75.92 ± 3.52	ND	7.01 ± 0.64	1.77 ± 0.94	**6.68 ± 1.37** [b]
	150 µM Cd	4.46 ± 1.13	57.91 ± 8.77	ND	7.25 ± 1.61	1.78 ± 1.17	**7.94 ± 1.26** [b]

Foliar polyamines content

On all days of analyses, exposure to 50 µM Cd led to an increase in leaf Put content *vs.* the control, whereas plants exposed to 150 µM Cd showed variable effects on different days. In Spd-sprayed plants, Put decreased on all days except for day 14 (Table 9, Fig. 33A). A significant decrease in Put content was seen in sprayed plants *vs.* unsprayed control treatment on the 3rd day. The Spd spray led to higher Put content in the leaves *vs.* unsprayed, irrespective of the treatment at 14 days after Cd treatment.

The exposure to Cd treatment caused an increase in the leaf Spd compared to the control 3 day after Cd treatment (Fig. 33B). However, under 150 µM Cd, Spd content was less compared to 50 µM Cd. On other days, Cd exposure resulted in less Spd than the control. Leaf Spd content was not affected by Spd spray for any Cd treatment on any day.

With respect to Spm, on the 3rd day, the plants sprayed with Spd had significantly less Spm *vs.* unsprayed plants treated with 50 µM Cd (Fig. 33C). There was a significant decrease in the Spm content at 14 days in response to Cd treatment. Spermine was always low by several folds in Spd-sprayed plants *vs.* unsprayed plants treated with Cd except on the 14th day, when Spm was higher in sprayed plants than those unsprayed and treated with 150 µM Cd.

Root polyamines content

In roots, PA content was measured on the day of harvesting and only the unsprayed plants were studied (control, 50 µM, and 150 Cd µM). There was an increase in all three PAs in the roots on the 21st day after Cd exposure but was significant only under 150 µM Cd (Fig. 34). The magnitude of increase was highest for Spm for 150 µM Cd treatment with respect to control. The

relative abundance of PAs was reported to be 38% for Spd, 34% for Put, and 28% for Spm under control. However, the results also show that the relative abundance under 150 µM Cd was 48% for Spm, 29% for Spd, and 23% for Put respectively.

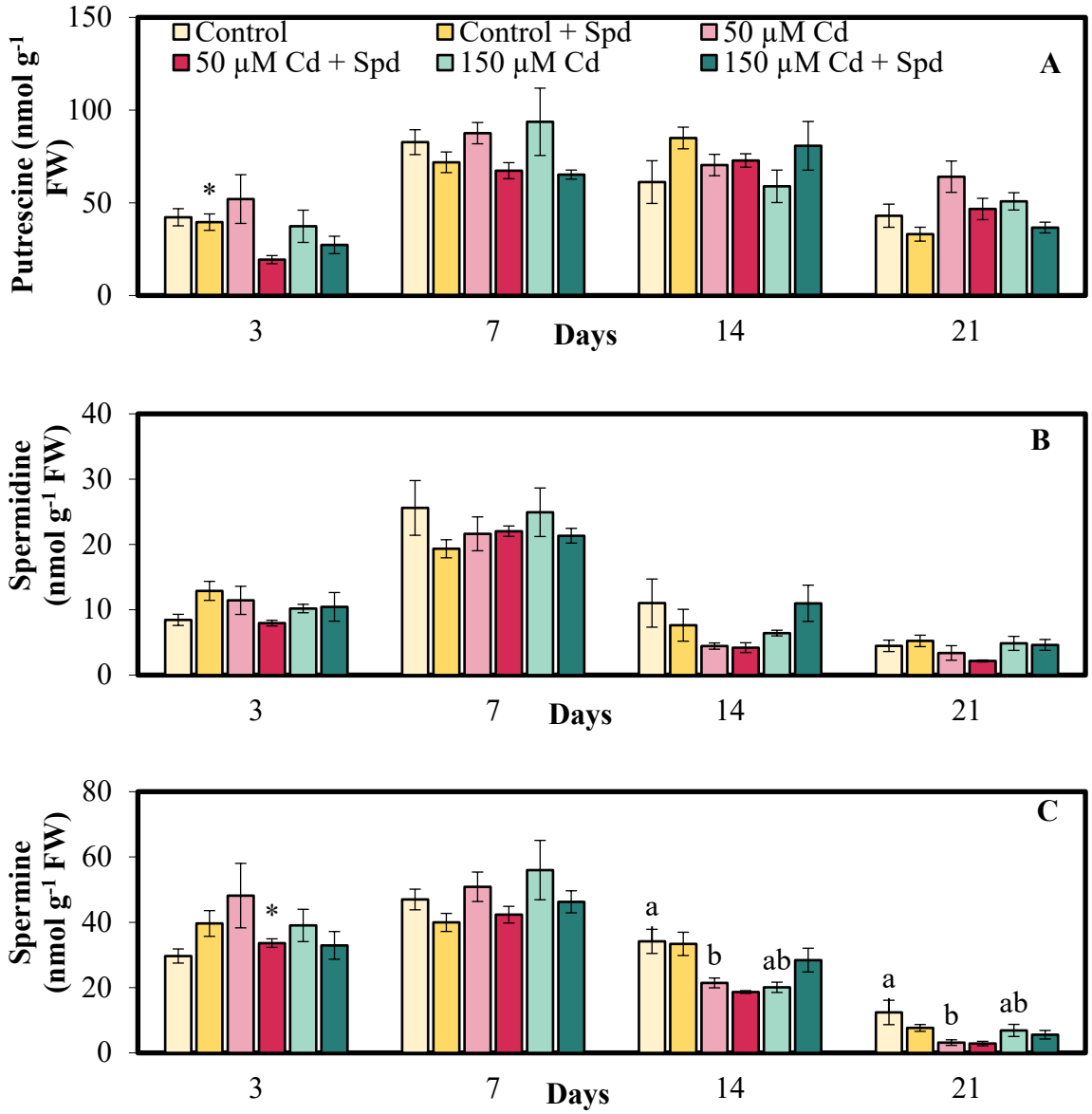

Figure 33: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on polyamine contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Putrescine, (B) Spermidine, and (C) Spermine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

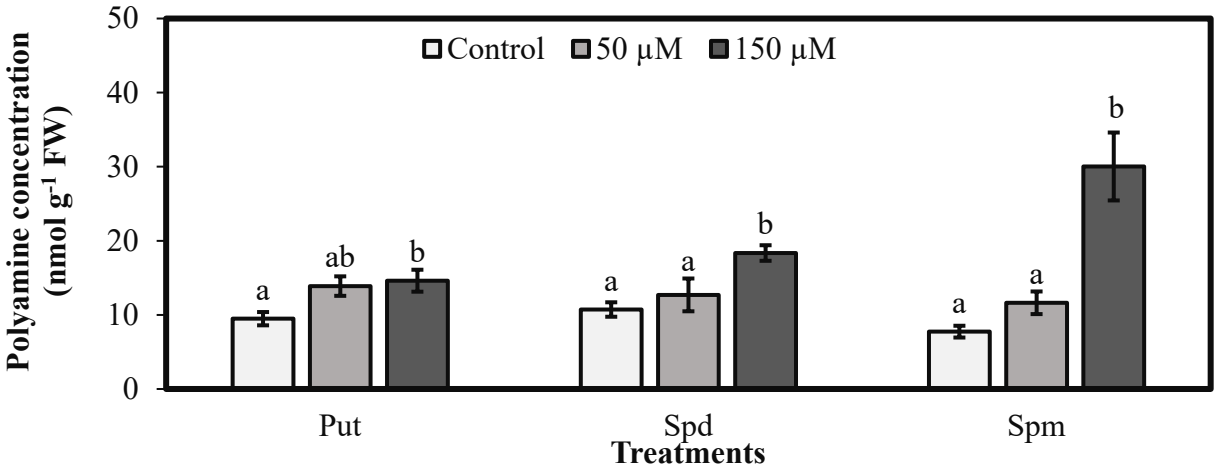

Figure 34: The effect of two different concentrations of $CdCl_2$on day 21 on the polyamines (Putrescine, Spermidine, Spermine) content of roots of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. A different letter indicates the treatment effect was significantly different ($p \leq 0.05$) between control, 50 µM Cd, and 150 µM Cd.

Foliar amino acids content

The individual AAs were analyzed to identify the effect of Cd exposure on AA metabolism in the leaves. Three AAs- Arg+Thr+Gly were not separated from each other by the HPLC method used here. Our results show that Arg+Thr+Gly increased almost a 2-fold in the leaves in response to Cd treatment, but the effect was not concentration-dependent (Fig. 35A). In the presence of 50 µM Cd, these 3 AAs significantly increased compared to the control on the 21st day. Spray treatment significantly decreased the Arg+Thr+Gly content under 50 µM Cd after 21 days. In plants under Cd stress and sprayed with Spd, these 3 AAs were always higher or similar in concentration compared to the control.

Histidine content was only detected at 14 days after exposure to Cd stress (Fig. 35B). On this day, His content increased significantly on the plants sprayed with Spd compared to those not sprayed. On the 21st day, His content was lower than that on the 14th day. However, a significant increase was seen in the His content in plants under Cd treatment compared to the control. The His content was higher in150 µM Cd treated plants sprayed with Spd *vs.* those not sprayed on the 21st day.

With the progress of days, the Glu content increased by almost 2-3-fold irrespective of any treatment (Fig. 35C). On the 7th and the 14th day, Glu changed significantly on the plants sprayed with Spd under Cd stress. Three days after the Cd treatment, Glu increased by almost 2-folds for 50 µM and 1.5-folds for 150 µM in comparison to control plants. On day 7, the Glu contents doubled with Cd and decreased following the application of Spd under Cd. However, on the 14th day, the Glu content was reversed in Cd-treated plants following Spd application. Glutamine decreased in plants treated with Cd only and increased following Spd application ± Cd treatment.

Three days after the Cd treatment, Asp increased by 180% (50 µM) and 161% (150 µM) in comparison to the control (Fig. 36A). There was a decrease in the Asp in the plants with the Spd application compared to those without the Spd application. On the 7th day, a decrease was observed on the Asp content in the Cd-treated plants sprayed with Spd compared to those not sprayed. On the 14th day, there was a decrease in the Asp under Cd treatment compared to the control. However, Asp was significantly higher in the Cd-treated plants sprayed with Spd compared to those not sprayed.

On the 3rd day, Gln increased under Cd treatment (Fig. 36B). Plants sprayed with Spd exhibited an increase in the Gln content on the 3rd day compared to those not sprayed, irrespective of any treatment. On the 7th day, Gln content increased significantly under 150 µM Cd. Application of Spd itself increased the Gln content by ~2-fold after 14 days. The maximum Gln content was seen on plants given Spd spray under 150 µM Cd on the 14th day and 21st day.

Similar to His, Cystine was not detected during the first two weeks of the experiment (Fig. 36C). Cystine was similar in content under Cd-treated and control plants but was significantly increased in the Spd-sprayed plants, irrespective of the treatment. The application of Spd caused an increase in Cystine content in the absence of Cd. Similarly, under 150 µM Cd, Cystine was increased compared to the control. Cystine was always higher in Spd-sprayed plants *vs.* control plants.

Three days after the Cd treatment, Ser was higher than the control (Fig. 37A). It was significantly lower on the 7th day, but they were higher on the 14th day in plants sprayed with Spd than those not sprayed under Cd treatment. The increase in Ser was highest under 150 µM Cd upon application of Spd on the 14th day. When plants were sprayed with Spd under Cd, Ser was either similar or higher by several folds in concentration compared to the control.

Alanine, Phe, and Met significantly decreased in Spd-sprayed plants *vs.* unsprayed under Cd treatment (Fig. 37B, C, 38C). On various days, plants that were sprayed with Spd under Cd stress, Phe were almost restored to the same value as control (Fig. 38C), and Met was consistently decreased (Fig. 38C) compared to the control.

As shown in Fig. 38A, GABA increased in concentration under Cd treatment compared to the control on the 3rd and 7th days. Compared to the control, GABA decreased on the 14th day in the absence of spray application in plants under Cd treatment. However, when plants were sprayed with Spd under Cd, GABA was higher in concentration than the control on the 14th day.

Three days after treatment, Pro increased under Cd treatment compared to the control, although they were not significantly different. However, in the presence of Spd spray, the Pro content was lower in concentration compared to those not sprayed under 50 μM Cd treatment and was significantly less only on the 7th and the 14th day (Fig. 38B). In the presence of Spd spray, Pro content was lower in concentration compared to those not sprayed under 150 μM Cd treatment till the 7th day, and it was higher in concentration on the 14th and the 21st day.

Amino acids like Ile and Leu kept decreasing with the passage of days, irrespective of the treatment. Sprayed plants have shown less Ile content *vs.* unsprayed under 50 μM Cd (Fig. 39A). Leucine decreased significantly on the 7th day after the plants were treated with Cd compared to the control (Fig. 39B).

Ornithine was significantly altered in the plants under Cd treatment 3 days after treatment (Fig. 39C). After exposure to 50 μM Cd for 14 days, Orn increased by 148%, and when spray was applied under 150 μM Cd, Orn increased more than 2-fold compared to the control. When plants were sprayed with Spd under Cd stress, Orn was almost restored to the same value in control on

several occasions. Similar to Pro, Orn was consistently lower in concentration in the presence of Spd spray compared to those not sprayed under 50 µM Cd treatment on all days.

Lysine was significantly high on the 21st day in 50 µM Cd-treated plants (Fig. 40A). However, when plants were sprayed with Spd under 150 µM Cd, Ile, Leu, Lys, and Val was similar or increased in concentration to the control. Similar to Pro and Orn, Val was consistently lower in concentration in the presence of Spd spray than those not sprayed under 50 µM Cd treatment on all days (Fig. 40B). When plants were sprayed, Val significantly decreased *vs.* unsprayed under 50 µM Cd on the 14th day. In Spd-sprayed plants, Val content was lower in concentration compared to those not sprayed under 150 µM Cd treatment till the 7th day, and it was higher in concentration on the 14th and the 21st day. However, the Val was significantly higher in plants sprayed on the 21st day *vs.* unsprayed under 150 µM Cd treatment.

Root amino acids content

To analyze the AA content in the roots, they were collected after treating the plants with Cd for 21 days. An increase was seen in the Arg+Thr+Gly, Gln, Glu, Asp, Cystine, Orn, GABA, Lys, Ser, and Ala contents of roots in response to Cd exposure (Fig. 41). Amino acids like Cystine significantly increased under Cd exposure (Fig. 41C), while Orn (Fig. 41C) and GABA (Fig. 42A) significantly increased in response to 150 µM Cd compared to control. However, AAs like Trp + Phe, His, and Leu decreased under Cd. Other AAs like Ile, Met, Val, and Pro, have shown variable effect under Cd exposure compared to the control (Table 9, Fig. 42B).

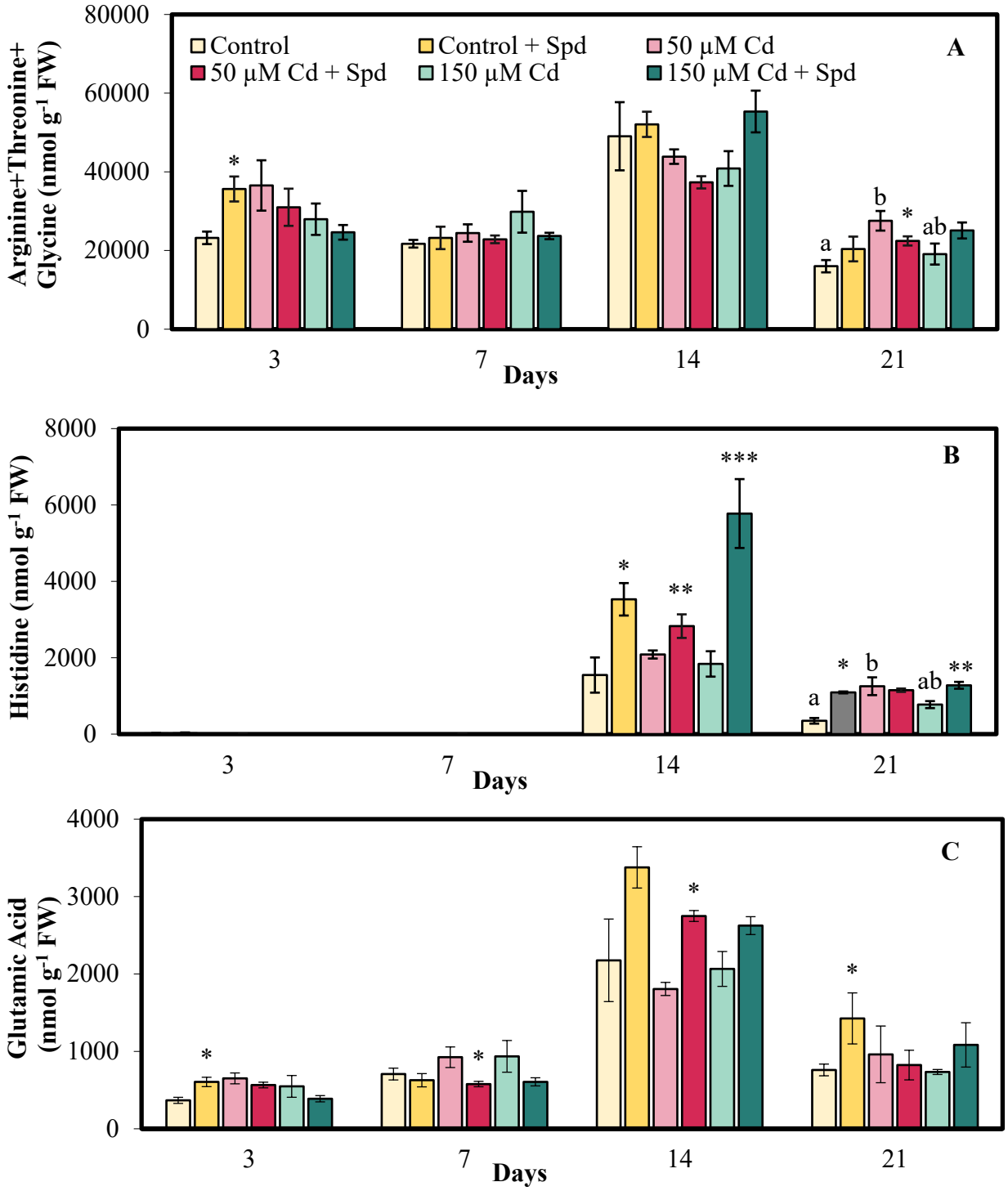

Figure 35: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Arginine+Threonine+Glycine, (B) Histidine, and (C) Glutamic acid. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

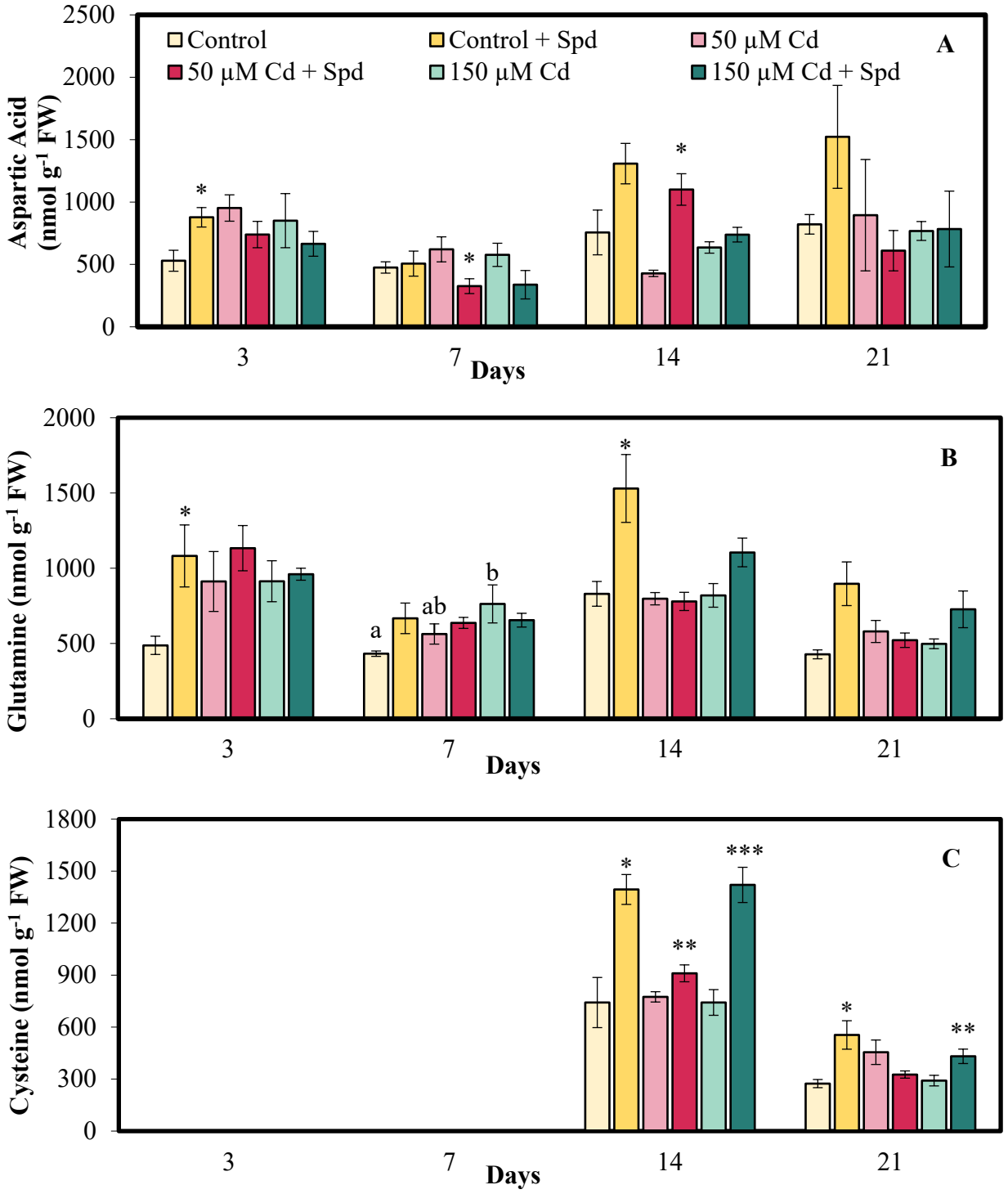

Figure 36: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Aspartic acid, (B) Glutamine, and (C) Cystine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

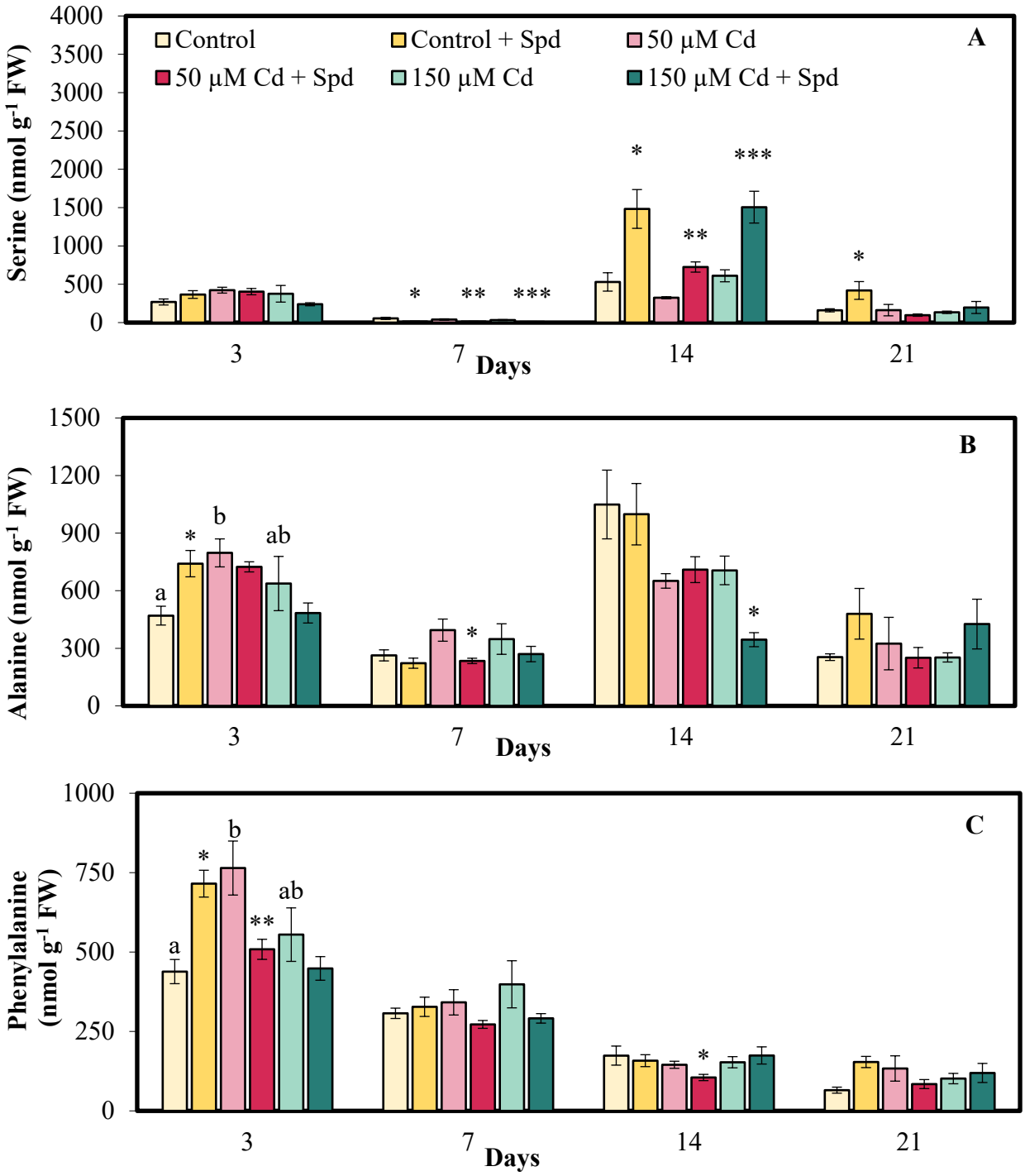

Figure 37: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Serine, (B) Alanine, and (C) Phenylalanine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

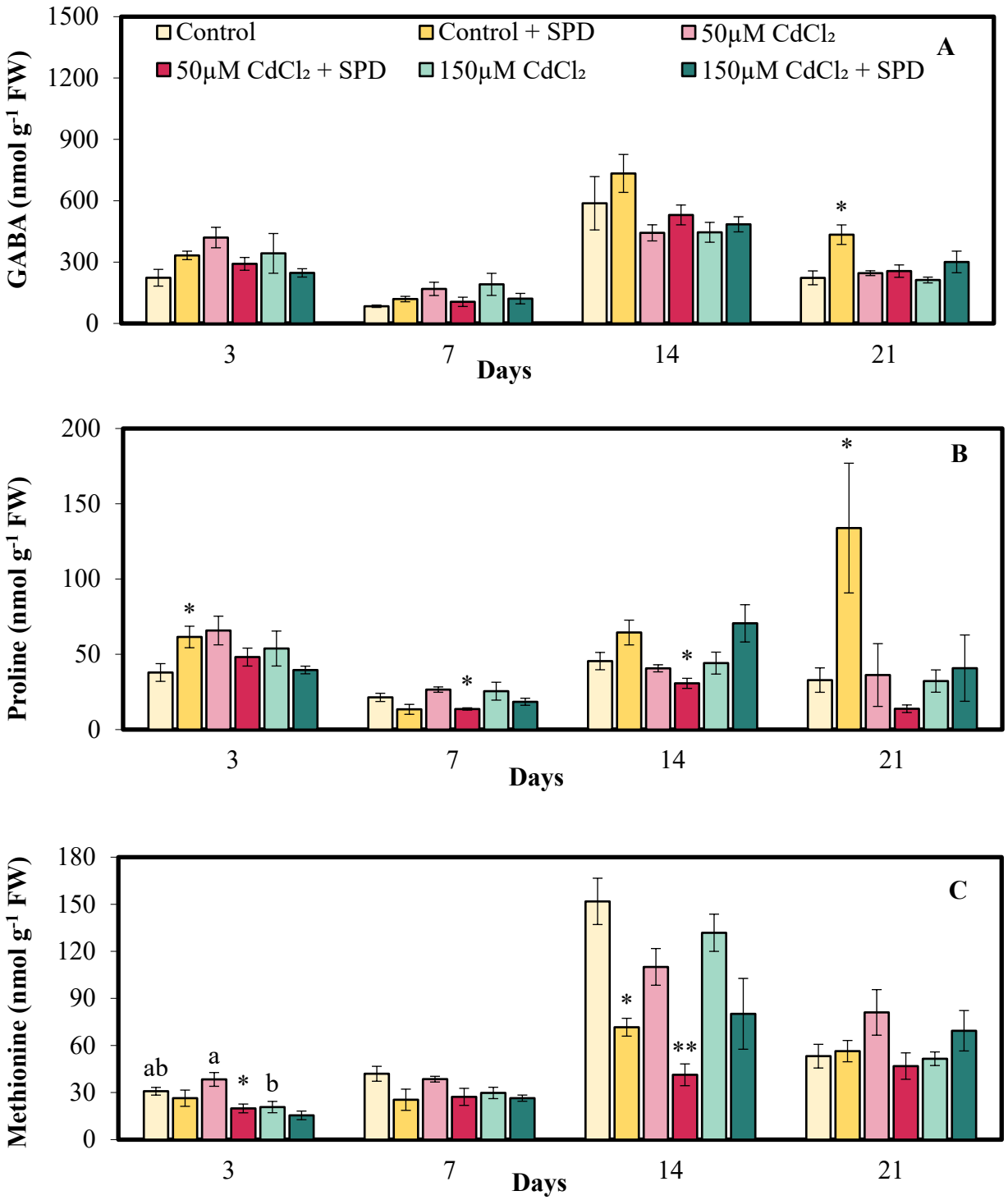

Figure 38: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) GABA, (B) Proline, and (C) Methionine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

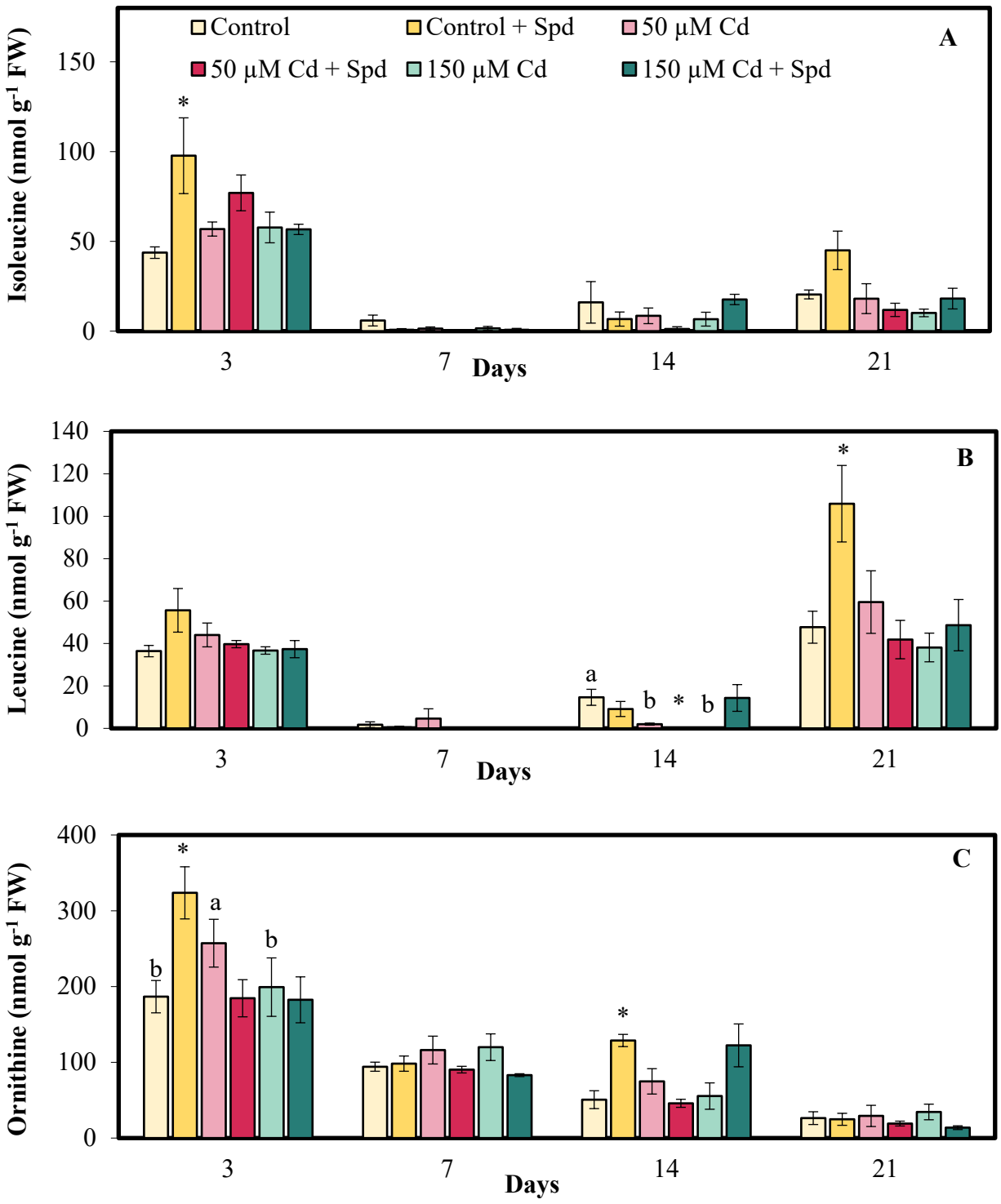

Figure 39: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Isoleucine, (B) Leucine, and (C) Ornithine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

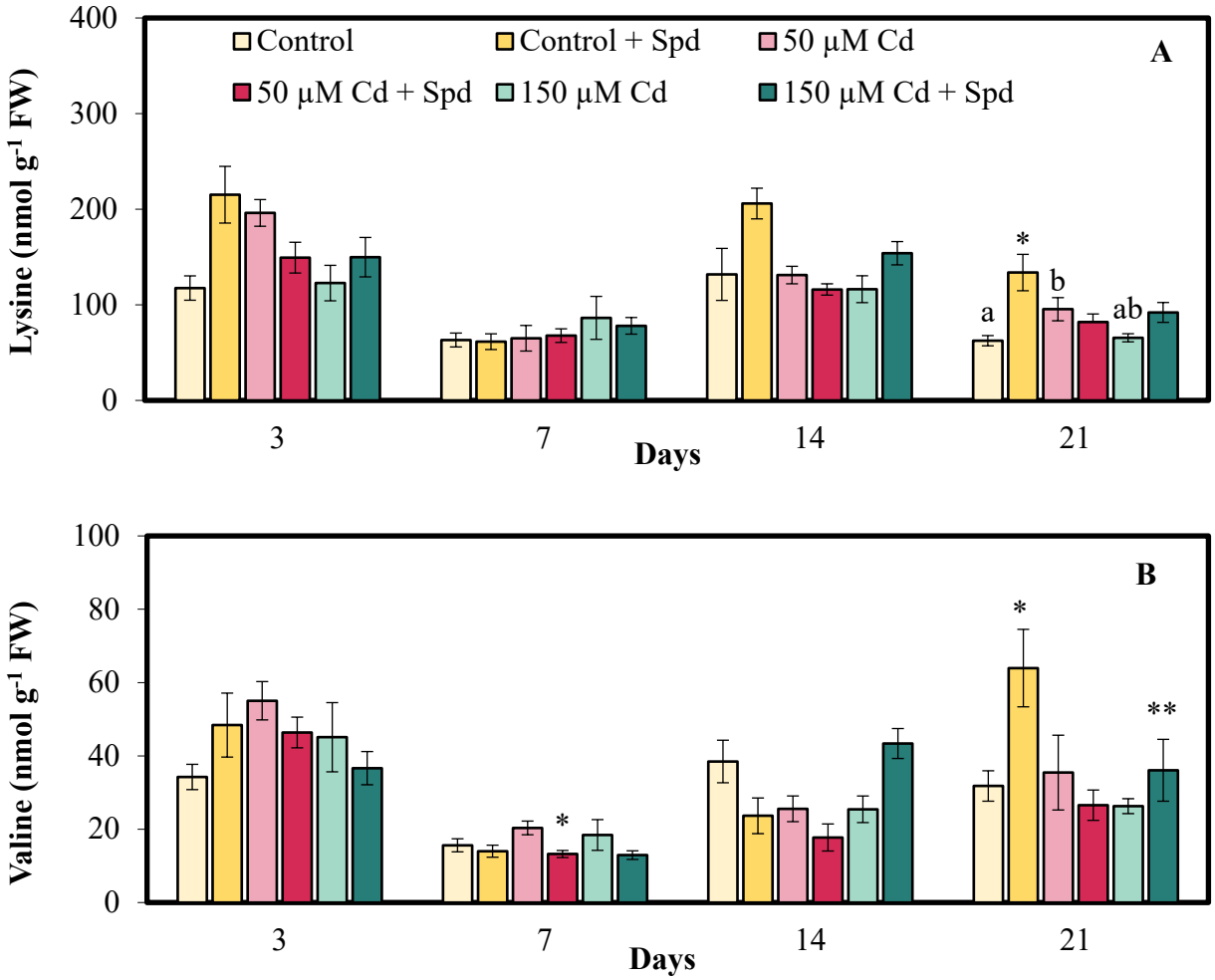

Figure 40: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Lysine and (B) Valine. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

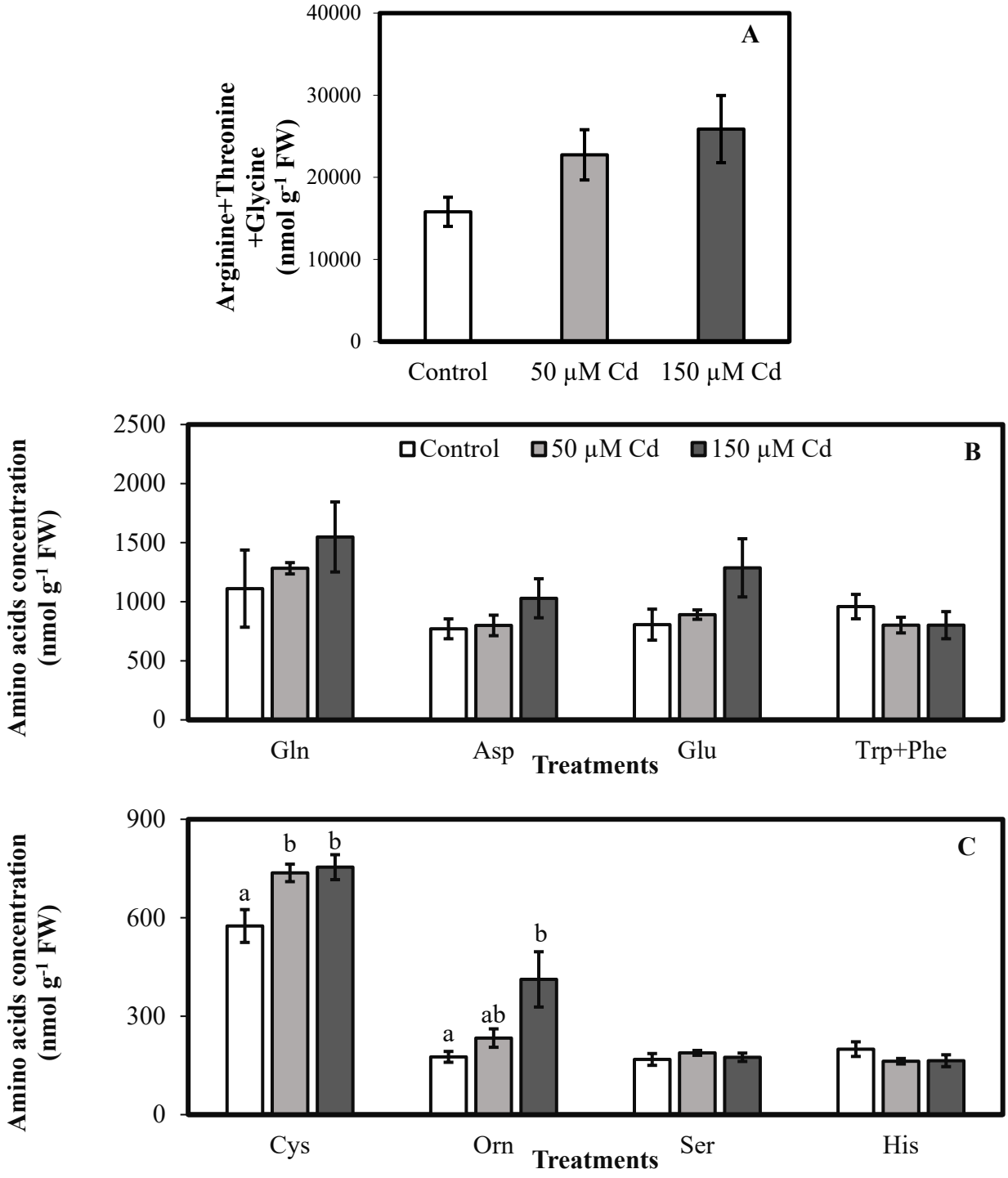

Figure 41: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on day 21 on the amino acid contents of roots of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) Arginine+Threonine+Glycine, (B) Glutamine, Aspartic acid, Glutamic acid, Tryptophan+Phenylalanine, and (C) Cystine, Ornithine, Serine, Histidine. A different letter indicates the treatment effect was significantly different ($p \leq 0.05$) between control, 50 µM Cd, and 150 µM Cd.

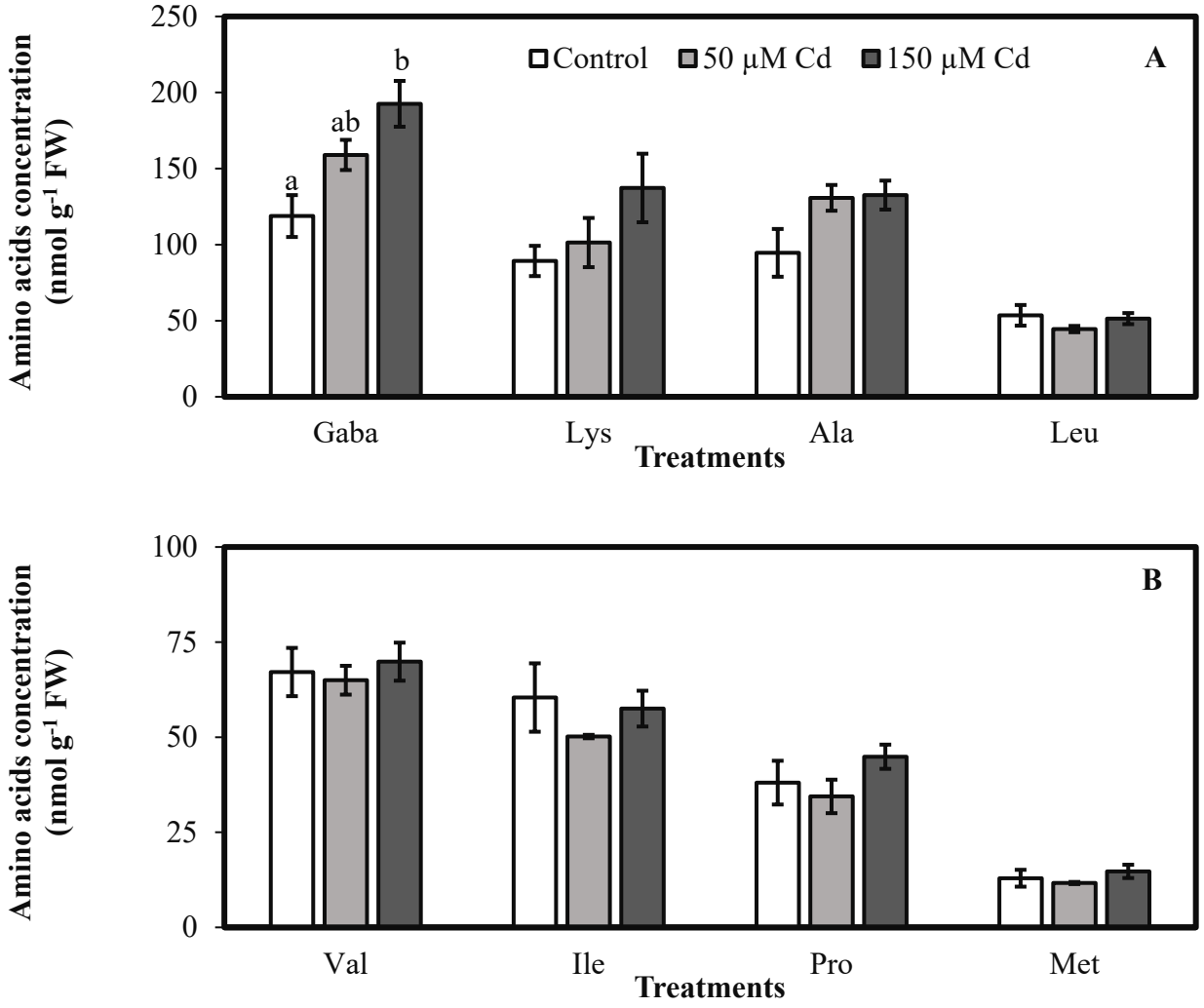

Figure 42: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on day 21 on the amino acid contents of roots of the hybrid poplar NM6 plants. Data are mean of ± SE of 5 replicates. (A) GABA, Lysine, Alanine, Leucine and (B) Valine, Isoleucine, Proline, Methionine. A different letter indicates the treatment effect was significantly different ($p \leq 0.05$) between control, 50 µM Cd, and 150 µM Cd.

Foliar soluble sugars

The individual soluble sugars were analyzed to identify the effect of Cd exposure on sugar metabolism in the leaves. There was an effect of Cd concentration on the fructose content (Fig. 43A). In response to 150 µM Cd treatment, a significant decrease in fructose content was seen on the 3rd compared to the 50 µM Cd treated plants. However, there was no significant difference between the control and 50 µM Cd treatment. On the 7th day, fructose was significantly high in response to 50 µM Cd. Compared to the control, there was a decrease (insignificant) in the fructose content after exposure to 50 µM Cd for 14 days. There was no effect on the glucose+galactose content in response to Cd treatment (Fig. 43B).

The sucrose content was the same on the 7th day, and on the 14th and 21st day, sucrose was higher (not significant) on the Cd-treated plants compared to the control (Fig. 43C). However, sucrose content was significantly increased in Spd-sprayed plants *vs.* unsprayed under Cd treatment on the 14th day.

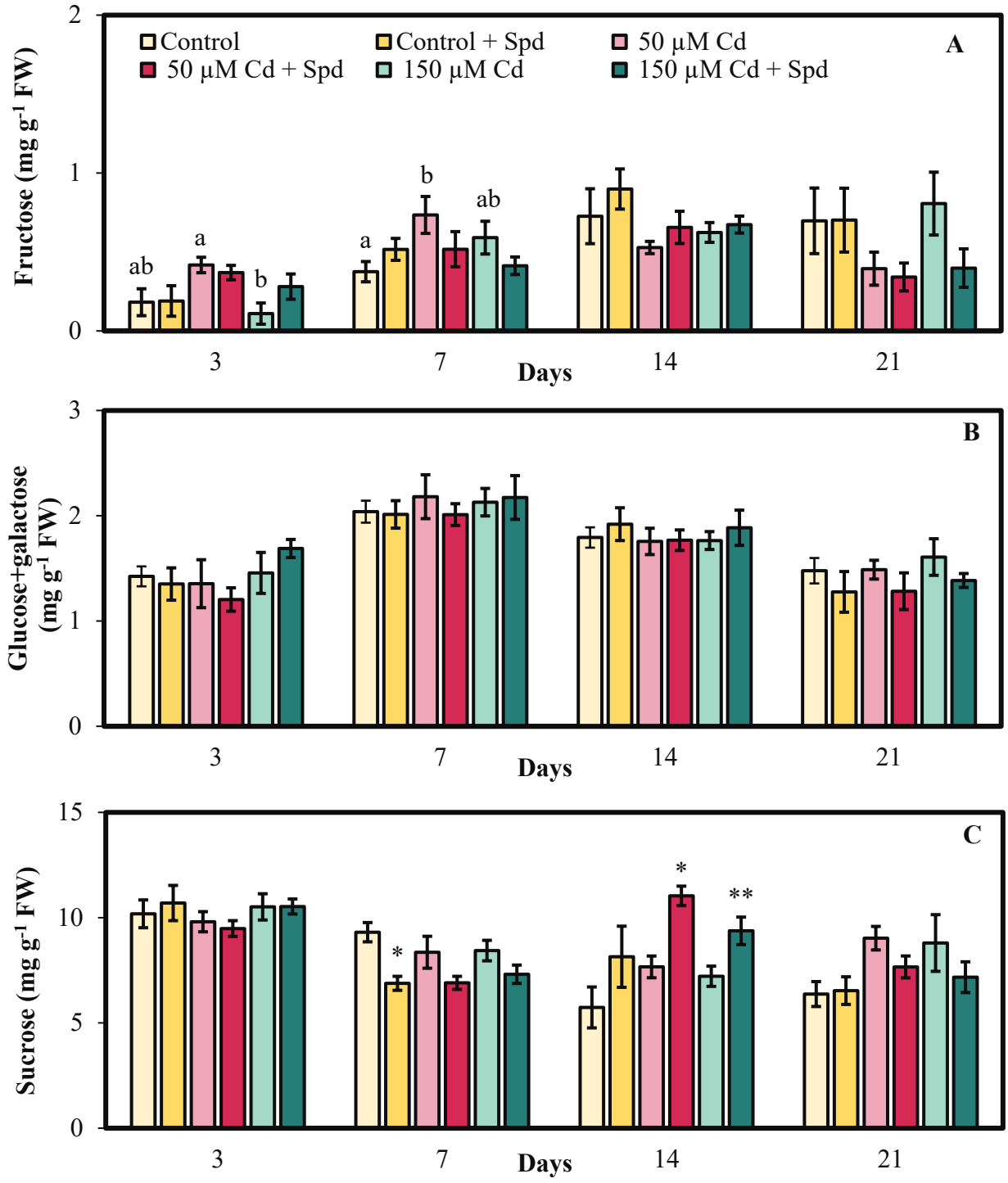

Figure 43: The effect of two different concentrations of $CdCl_2$ (± Spermidine spray) on different days on the soluble sugar contents of leaves of the hybrid poplar NM6 plants. Data are mean of ± SE of 6 replicates. (A) Fructose, (B) Glucose+galactose, and (C) Sucrose. On a given day, a different letter on the bar indicates statistical significance ($p < 0.05$) between control, 50 µM Cd, and 150 µM Cd, while an * on the bar indicates statistical significance ($p < 0.05$) between sprayed and unsprayed plants for each treatment.

Table 9: Amino acids and polyamines (nmol g^{-1} FW) content in the leaves and roots of poplar plants exposed to two concentrations of Cd (50 µM, 150 µM) with or without 1 mM Spd spray (data are mean ± SE, n=5).

Days	Treatment	Asp	Glu	Gln	Ser	Arg+Thr+Gly	Ala
Leaves							
3	Control	529.6 ± 83.96	367.31 ± 39.25	487.77 ± 60.55	268.75 ± 39.2	23220.75 ± 1593.13	**470.25 ± 49.04** [a]
	Control + Spd	**877.78 ± 77.93** *	**605.25 ± 60.51** *	**1081.69 ± 205.89** *	366.22 ± 50.11	35639.1 ± 3174.82 *	**741.08 ± 68.58** *
	50 µM Cd	952.11 ± 105.54	651.23 ± 70.16	912.02 ± 199.06	422.68 ± 38.17	36537.34 ± 6398.86	**797.1 ± 72.89** [b]
	50 µM Cd + Spd	739.6 ± 105.71	565.5 ± 36.52	1133.46 ± 150.33	403.59 ± 41.11	30995.77 ± 4723.86	724.33 ± 26.32
	150 µM Cd	850.99 ± 216.87	547.73 ± 140.63	913.68 ± 136.14	375.72 ± 108.65	27955.86 ± 3988.1	**637.39 ± 141.26** [ab]
	150 µM Cd + Spd	665.28 ± 99.61	388.39 ± 40.43	960.34 ± 39.83	238.95 ± 19.57	24623.27 ± 1858.78	484.00 ± 52.19
7	Control	475.71 ± 45.41	707.2 ± 76.26	**432.36 ± 17.87** [a]	55.07 ± 13.59	21720.92 ± 972.86	263.22 ± 28.72
	Control + Spd	506.56 ± 100.3	627.31 ± 85.62	667.19 ± 101.51	**12.99 ± 5.55** *	23206.14 ± 2852.55	222.49 ± 26.42
	50 µM Cd	620.95 ± 100.22	924.46 ± 133.02	**563.23 ± 67.24** [ab]	39.41 ± 9.73	24443.85 ± 2211.96	394.64 ± 57.73
	50 µM Cd + Spd	325.98 ± 59.38	577.84 ± 34.8	636.98 ± 36.56	**13.29 ± 3.29** **	22841.66 ± 979.23	**234.31 ± 14.01** *
	150 µM Cd	576.94 ± 92.88	935.69 ± 204.72	**762.97 ± 126.32** [b]	32.72 ± 6.54	29850.19 ± 5310.08	348.25 ± 79.48
	150 µM Cd + Spd	337.46 ± 113.92	606.64 ± 52.61	654.91 ± 45.65	**10.34 ± 4.86** ***	23692.66 ± 814.71	270.14 ± 40.05
14	Control	756.4 ± 179.33	2176.43 ± 533.18	829.93 ± 82.27	530.27 ± 119.87	49046.13 ± 8663.46	1049.24 ± 179.02
	Control + Spd	1307.99 ± 162.14	3377.05 ± 266.91	**1530.06 ± 225.43** *	**1482.24 ± 252.86** *	52077.2 ± 3207.01	998.61 ± 160
	50 µM Cd	428.18 ± 25.48	1806.41 ± 84.57	797.56 ± 40.84	323.53 ± 13.36	43865.75 ± 1838.43	651.09 ± 37.85
	50 µM Cd + Spd	**1100.68 ± 126.27** *	**2749.15 ± 70.48** *	779.64 ± 60.4	**724.83 ± 66.66** **	37331.08 ± 1554.02	709.75 ± 67.3
	150 µM Cd	635.96 ± 45.58	2065.14 ± 225.15	819.67 ± 78.52	610.12 ± 77.93	40848.49 ± 4410.36	705.83 ± 74.45
	150 µM Cd + Spd	738.7 ± 58.80	2624.78 ± 115.36	1104.98 ± 95.24	**1505.73 ± 207.61** ***	55335.76 ± 5301.63	**344.99 ± 36.54** *
21	Control	821.49 ± 78.15	760.44 ± 75.46	427.97 ± 29.65	159.99 ± 20.06	**16008.46 ± 1560.72** [a]	253.94 ± 17.33
	Control + Spd	1522.6 ± 412.56	**1425.88 ± 329.63** *	897.02 ± 145.11	**418.88 ± 116.01** *	20392.09 ± 3144.41	479.94 ± 132.04
	50 µM Cd	894.82 ± 446.34	961.58 ± 366.31	579.95 ± 73.29	162.49 ± 74.29	**27558.62 ± 2496.15** [b]	324.68 ± 136.72
	50 µM Cd + Spd	610.18 ± 161.72	823.3 ± 191.48	521.79 ± 47.95	97.14 ± 14.80	**22438.87 ± 1164.88** *	250.91 ± 53.37
	150 µM Cd	768.22 ± 75.65	733.9 ± 32.21	498.16 ± 32.18	133.87 ± 15.63	**19099.52 ± 2670.02** [ab]	252.48 ± 23.88
	150 µM Cd + Spd	783.85 ± 303.72	1083.56 ± 286.22	726.97 ± 121.95	197.13 ± 78.35	25095.34 ± 2035.4	426.29 ± 129.57
Roots							
21	Control	770.57 ± 84.09	805.98 ± 131.18	1110.98 ± 326.74	168.24 ± 17.97	15800.03 ± 1772.4	94.62 ± 15.7
	50 µM Cd	799.44 ± 86.94	890.89 ± 39.81	1283.1 ± 47.92	188.24 ± 7.42	22740.04 ± 3060.31	130.78 ± 8.46
	150 µM Cd	1029.28 ± 165.35	1286.73 ± 246.25	1548.21 ± 296.39	174.85 ± 12.7	25876.17 ± 4092.01	132.59 ± 9.51

Table 9: (continued)

Days	Treatment	GABA	Val	Met	Ile	Leu	Phe	Cystine
Leaves								
3	Control	223.43 ± 40.91	34.25 ± 3.45	**30.83 ± 2.46** [ab]	43.72 ± 3.21	36.43 ± 2.67	**438.52 ± 38.07** [a]	ND
	Control + Spd	332.74 ± 20.76	48.4 ± 8.73	26.40 ± 5.17	**97.69 ± 21.1** *	55.64 ± 10.28	**715.29 ± 42.22** *	ND
	50 µM Cd	419.91 ± 49.97	55.04 ± 5.23	**38.37 ± 4.38** [a]	56.86 ± 3.92	44.04 ± 5.61	**764.43 ± 85.00** [b]	ND
	50 µM Cd + Spd	291.37 ± 31.32	46.39 ± 4.19	**19.88 ± 2.79** *	77.00 ± 9.96	39.72 ± 1.7	**508.61 ± 31.55** **	ND
	150 µM Cd	342.59 ± 97.13	45.09 ± 9.44	20.75 ± 3.61 [b]	57.73 ± 8.53	36.71 ± 1.74	**554.84 ± 84.22** [ab]	ND
	150 µM Cd + Spd	247.18 ± 20.35	36.65 ± 4.53	15.46 ± 2.75	56.67 ± 2.87	37.35 ± 4.05	448.47 ± 37.18	ND
7	Control	83.99 ± 5.76	15.61 ± 1.78	41.97 ± 4.75	5.92 ± 3.02	1.74 ± 1.34	307.15 ± 16.27	ND
	Control + Spd	119.22 ± 13.25	14.01 ± 1.64	25.42 ± 6.75	0.73 ± 0.67	0.52 ± 0.52	327.63 ± 30.36	ND
	50 µM Cd	169.02 ± 32.46	20.35 ± 1.86	38.54 ± 1.82	1.45 ± 0.89	4.61 ± 4.61	341.65 ± 39.83	ND
	50 µM Cd + Spd	105.63 ± 22.68	**13.24 ± 0.96** *	27.26 ± 5.43	0.35 ± 0.35	ND	272.20 ± 12.34	ND
	150 µM Cd	191.08 ± 53.97	18.43 ± 4.19	29.76 ± 3.62	1.59 ± 1.03	ND	398.50 ± 74.14	ND
	150 µM Cd + Spd	121.35 ± 25.58	12.94 ± 1.18	26.43 ± 1.97	0.79 ± 0.74	ND	291.26 ± 14.89	ND
14	Control	587.66 ± 130.42	38.48 ± 5.8	151.84 ± 14.76	16.03 ± 11.56	**14.66 ± 3.76** [a]	173.93 ± 30.01	741.72 ± 144.64
	Control + Spd	733.54 ± 92.77	23.66 ± 4.86	**71.64 ± 5.69** *	6.72 ± 3.94	9.15 ± 3.55	158.06 ± 18.76	**1394.16 ± 86.19** *
	50 µM Cd	442.87 ± 39.35	25.57 ± 3.51	110.08 ± 11.69	8.53 ± 4.35	**2.0 ± 0.48** [b]	145.13 ± 11.13	774.46 ± 29.72
	50 µM Cd + Spd	530.59 ± 48.46	17.75 ± 3.69	**41.31 ± 6.94** **	1.23 ± 1.23	ND	**105.09 ± 9.84** *	**910.24 ± 48.74** **
	150 µM Cd	445.66 ± 48.56	25.44 ± 3.63	131.84 ± 11.86	6.67 ± 3.85	**0.28 ± 0.28** [b]	153.15 ± 17.6	742.09 ± 74.08
	150 µM Cd + Spd	484.71 ± 37.02	43.38 ± 4.09	80.18 ± 22.58	17.64 ± 2.88	**14.34 ± 6.30** *	174.46 ± 26.98	**1420.42 ± 101.65** ***
21	Control	223.00 ± 34.00	31.79 ± 4.15	53.19 ± 7.55	20.43 ± 2.47	47.69 ± 7.54	65.24 ± 9.50	274.03 ± 23.53
	Control + Spd	**434.05 ± 47.76** *	**63.95 ± 10.56** *	56.4 ± 6.78	44.97 ± 10.72	**105.91 ± 18.03** *	153.83 ± 17.66	**554.58 ± 82.07** *
	50 µM Cd	245.9 ± 11.82	35.44 ± 10.2	81.09 ± 14.52	18.10 ± 8.32	59.53 ± 14.74	133.46 ± 39.84	454.86 ± 70.55
	50 µM Cd + Spd	255.84 ± 30.28	26.56 ± 4.13	46.88 ± 8.42	11.85 ± 3.69	41.87 ± 9.04	84.37 ± 14.19	326.28 ± 20.82
	150 µM Cd	212.37 ± 14.2	26.28 ± 2.03	51.55 ± 4.33	10.15 ± 2.12	38.14 ± 6.76	101.69 ± 16.33	291.27 ± 30.62
	150 µM Cd + Spd	300.71 ± 52.91	**36.08 ± 8.44** **	69.39 ± 12.88	18.13 ± 5.76	48.65 ± 12.09	119.38 ± 30.05	**431.32 ± 41.62** **
Roots								
21	Control	**118.81 ± 13.77** [a]	67.13 ± 6.35	12.92 ± 2.22	60.43 ± 8.99	53.57 ± 6.78	958.87 ± 103.24	**574.86 ± 50.05** [a]
	50 µM Cd	**158.97 ± 9.92** [ab]	64.98 ± 3.79	11.66 ± 0.28	50.17 ± 0.45	44.53 ± 2.07	802.17 ± 66.39	**736.62 ± 26.62** [b]
	150 µM Cd	**192.58 ± 15.06** [b]	69.86 ± 5.00	14.72 ± 1.75	57.51 ± 4.71	51.41 ± 3.64	801.45 ± 114.81	**754.09 ± 38.04** [b]

Table 9: (continued)

Days	Treatment	Pro	Orn	Lys	His	Put	Spd	Spm
Leaves								
3	Control	37.92 ± 5.89	**186.63 ± 21.34** [b]	117.53 ± 12.73	17.71 ± 9.86	42.21 ± 4.63	8.45 ± 0.85	29.67 ± 2.16
	Control + Spd	**61.51 ± 7.15** *	**323.67 ± 34.41** *	215.14 ± 29.65	21.54 ± 21.54	**39.58 ± 4.43** *	12.89 ± 1.46	39.63 ± 3.94
	50 µM Cd	65.81 ± 9.53	**257.19 ± 31.54** [a]	196.11 ± 13.95	6.81 ± 6.81	52.02 ± 13.14	11.44 ± 2.15	48.17 ± 9.87
	50 µM Cd + Spd	48.16 ± 5.96	184.53 ± 24.52	149.34 ± 16.09	ND	19.35 ± 2.19	7.95 ± 0.43	**33.63 ± 1.31** *
	150 µM Cd	53.84 ± 11.61	**199.22 ± 38.58** [b]	122.74 ± 18.55	1.93 ± 1.93	37.33 ± 8.70	10.19 ± 0.65	39.03 ± 4.95
	150 µM Cd + Spd	39.59 ± 2.54	182.47 ± 30.3	149.84 ± 20.62	ND	27.27 ± 4.70	10.44 ± 2.19	32.92 ± 4.25
7	Control	21.39 ± 2.73	94.19 ± 6.00	63.19 ± 7.27	ND	82.71 ± 6.71	25.6 ± 4.19	46.98 ± 3.16
	Control + Spd	13.45 ± 3.3	98.24 ± 10.05	61.44 ± 8.22	ND	71.86 ± 5.57	19.34 ± 1.38	39.96 ± 2.76
	50 µM Cd	26.56 ± 1.77	116.16 ± 18.35	65.03 ± 13.39	ND	87.57 ± 5.73	21.63 ± 2.59	50.88 ± 4.51
	50 µM Cd + Spd	13.69 ± 0.75	90.33 ± 4.38	67.77 ± 7.11	ND	67.33 ± 4.34	22.03 ± 0.8	42.33 ± 2.54
	150 µM Cd	25.5 ± 5.94	119.95 ± 17.6	86.35 ± 22.46	ND	93.69 ± 18.16	24.93 ± 3.72	55.99 ± 9.09
	150 µM Cd + Spd	**18.49 ± 2.34** *	83.04 ± 1.88	78.03 ± 8.69	ND	65.21 ± 2.45	21.33 ± 1.12	46.27 ± 3.37
14	Control	45.5 ± 5.75	50.67 ± 11.81	131.81 ± 27.2	1546.3 ± 460.69	61.17 ± 11.53	11.02 ± 3.68	**34.15 ± 3.70** [a]
	Control + Spd	64.47 ± 8.23	**128.79 ± 8.07** *	205.91 ± 16.03	**3529.9 ± 424.92** *	84.97 ± 5.84	7.63 ± 2.44	33.39 ± 3.56
	50 µM Cd	40.69 ± 2.35	74.84 ± 16.74	131.13 ± 9.14	2084.55 ± 105.5	70.39 ± 5.77	4.44 ± 0.48	**21.43 ± 1.50** [b]
	50 µM Cd + Spd	**30.71 ± 3.35** *	45.89 ± 5.31	116.05 ± 5.89	**2827.12 ± 308.65** **	72.85 ± 3.63	4.20 ± 0.75	18.64 ± 0.43
	150 µM Cd	44.16 ± 7.28	55.41 ± 17.41	116.34 ± 14.05	1837.31 ± 332.41	58.89 ± 8.76	6.43 ± 0.43	**20.08 ± 1.57** [ab]
	150 µM Cd + Spd	70.57 ± 12.37	122.33 ± 28.3	153.96 ± 12.19	**5775.56 ± 901.86** ***	80.74 ± 13.14	10.99 ± 2.78	28.42 ± 3.63
21	Control	32.87 ± 8.10	26.31 ± 8.42	**62.47 ± 5.35** [a]	**347.85 ± 73.67** [a]	43.05 ± 6.24	4.48 ± 0.87	**12.39 ± 3.74** [a]
	Control + Spd	**133.85 ± 43.09** *	24.74 ± 7.91	**133.75 ± 19.03** *	**1091.52 ± 24.91** *	33.08 ± 3.75	5.23 ± 0.87	7.65 ± 1.05
	50 µM Cd	36.23 ± 20.84	29.23 ± 14.00	**95.4 ± 12.08** [b]	**1253.54 ± 233.01** [b]	64.10 ± 8.45	3.39 ± 1.12	**3.16 ± 0.85** [b]
	50 µM Cd + Spd	13.84 ± 2.53	19.14 ± 3.20	81.98 ± 8.44	1149.53 ± 44.18	46.67 ± 5.75	2.16 ± 0.15	2.86 ± 0.68
	150 µM Cd	32.23 ± 7.41	34.45 ± 10.31	**65.52 ± 4.24** [ab]	**772.57 ± 91.99** [ab]	50.79 ± 4.67	4.85 ± 1.07	**6.88 ± 1.85** [ab]
	150 µM Cd + Spd	40.82 ± 22.02	13.71 ± 2.46	92.04 ± 10.39	**1278.24 ± 89.67** **	36.66 ± 2.89	4.62 ± 0.82	5.56 ± 1.31
Roots								
21	Control	38.05 ± 5.75	**176.07 ± 16.55** [a]	89.29 ± 9.95	199.63 ± 22.32	**9.49 ± 0.90** [a]	**10.73 ± 0.98** [a]	**7.74 ± 0.80** [a]
	50 µM Cd	34.43 ± 4.40	**233.34 ± 28.08** [ab]	101.39 ± 16.17	162.78 ± 8.35	**13.89 ± 1.32** [ab]	**12.70 ± 2.21** [a]	**11.64 ± 1.53** [a]
	150 µM Cd	44.86 ± 3.18	**412.14 ± 84.21** [b]	137.26 ± 22.52	164.28 ± 18.1	**14.61 ± 1.48** [b]	**18.35 ± 1.05** [b]	**30.03 ± 4.58** [b]

Foliar metabolomic analysis

The primary metabolites in the Cd-treated leaves were quantified with Gas Chromatography-Mass Spectrometry (GC-MS) metabolome analysis at the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana, IL. A total of 129 metabolites were identified; of which, 99 were quantified. The results of the heat map and hierarchical cluster analyses of the top 50 primary metabolites are shown here (Fig. 44, 45). The identified metabolites could be mainly classified into 8 groups, including AAs, amines, organic acids, sugars/polyols, organic acids, vitamins, lipids, and phytohormones (Table 10).

It is noteworthy that 1 metabolite from phenylpropanoid biosynthesis pathway (chlorogenic acid), 3 from pentose phosphate pathway (erythritol, glucose-6-p, ribose), mannose, n-acetyl glutamic acid, ornithine, propane-1,2-diol, salicylic acid-glucopyranoside were absent under 50 µM Cd and pyrrole-2-carboxylic acid was absent under control and 150 µM Cd treatment. Again, erythrose, an intermediate of pentose phosphate pathway, was present in Spd-sprayed plants under 150 µM Cd. Surprisingly, among the AAs, Asn, β-Ala, Pro, Val and among the PAs, Put and Spd were not detected by GC-MS in sample, although they were easily quantified by HPLC.

Heatmap and cluster analysis of steady state metabolite concentrations

The heatmap analysis (Fig. 44) showed that amino acids like Gly, Ser, and Ala were high under 150 µM Cd compared to the control in all replicates. Under 150 µM Cd, Orn and Glu were reduced in all plants compared to the control; Asp decreased in some of the replicates in the 150 µM Cd-treated plants (Fig. 44A). However, a combined result of all the plants indicates that Gln, Orn, Phe, and Trp were lower in the 150 µM Cd *vs.* 50 µM Cd (Fig. 44B). However, Spd-sprayed

plants showed that Gly, Trp, GABA, Orn, and Ser were higher, and Asp was reduced *vs.* unsprayed for 150 µM Cd. Several sugars were identified, which included 1, 6-anhydroglucose, sucrose, fructose, maltose, arabitol, glucose, trehalose, mannose, erythritol, and glycerol in the form of galactosyl glycerol, 1-monohexadecanoylglycerol, glycerol 3-p, and digalactosylglycerol. Differential metabolic changes were also observed in flavonoids like caffeic acid and coumaric acid and fatty acids like oleic acid and linoleic acid under Cd stress. Sugars like trehalose were only identified in plants under 50 µM Cd treatment. Mannose was present only under 150 µM Cd + 1 mM Spd (Table 10). When Cd concentration was increased, sugars like mannose, erythritol, mannitol, maltose, and sucrose decreased, and glycerol increased compared to the control. Glucose, mannose, erythritol, sucrose, arabinose, glycerol 3-p increased, whereas glycerol and arabitol decreased in Spd-sprayed plants for 150 µM Cd (Fig. 45). Another metabolite that increased in Spd sprayed plants under 150 µM Cd was tocopherol-a.

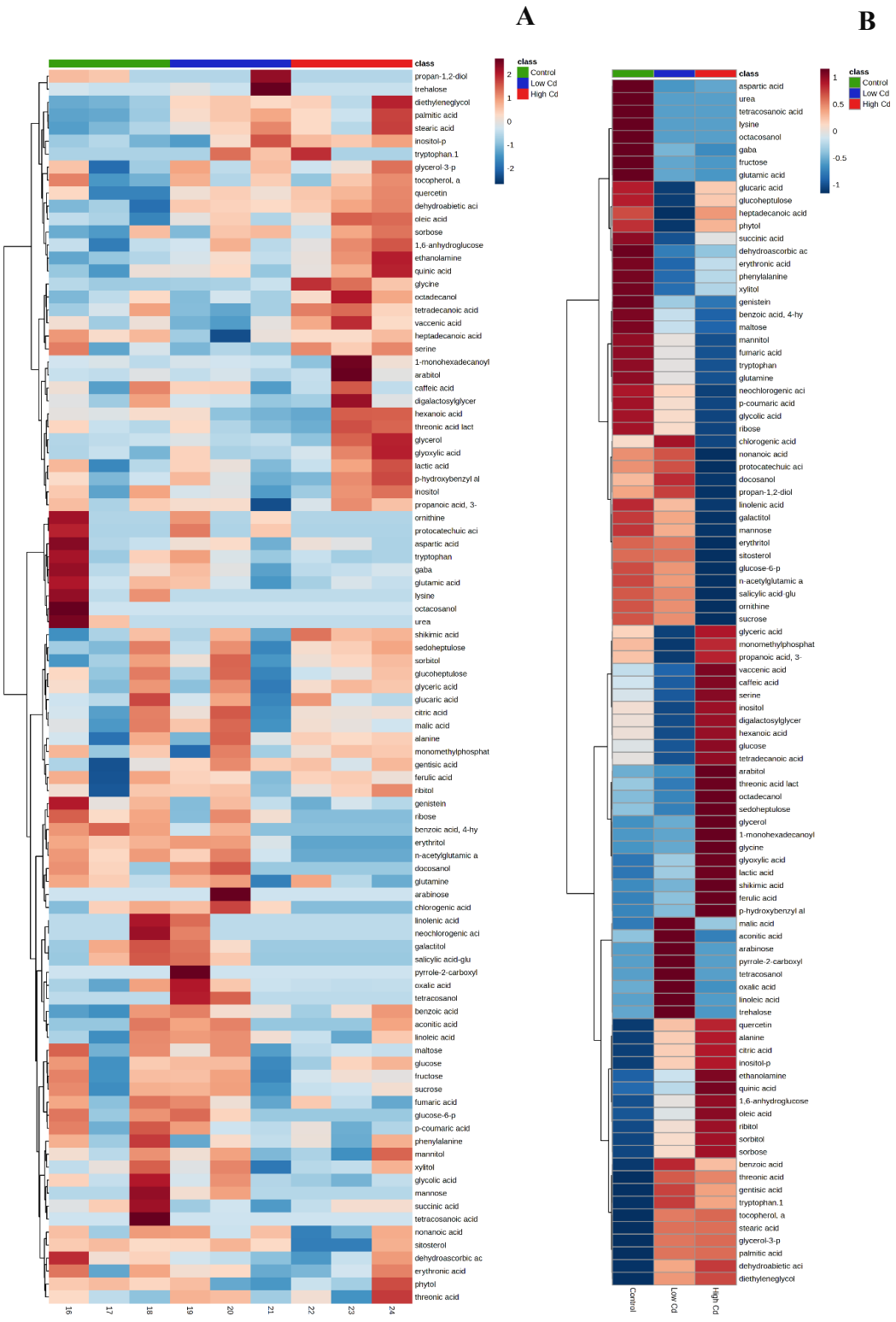

Figure 44: The effect of two different concentrations of $CdCl_2$ on the differentially expressed metabolites in the leaves of the hybrid poplar NM6 plants on day 14. Heat map and hierarchical cluster analysis of (A) all samples and (B) average. The metabolite levels were clustered by compounds identified (rows) and biological replicates (columns) using 3 replicates per treatment. Heatmap generated with parameters set to Euclidian distance and ward linking clustering method.

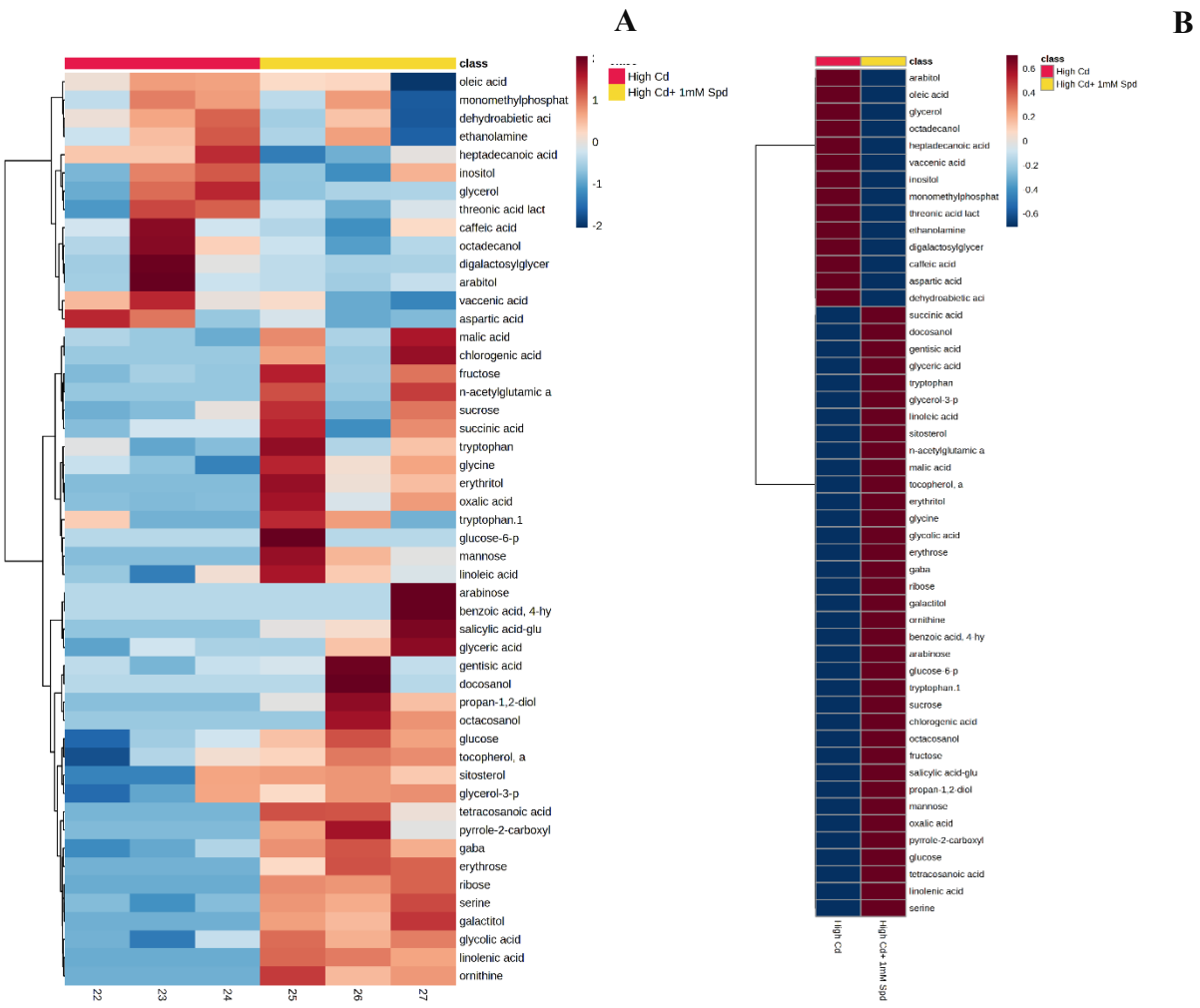

Figure 45: The effect of 150 µM $CdCl_2$ (± Spermidine) on the differentially expressed metabolites in the leaves of the hybrid poplar NM6 plants on day 14. Heat map and hierarchical cluster analysis of (A) all samples and (B) average. The metabolite levels were clustered by compounds identified (rows) and biological replicates (columns) using 3 replicates per treatment. Heatmap generated with parameters set to Euclidian distance and ward linking clustering method.

Multivariate unsupervised data analysis under cadmium treatment

To reduce the dimensionality of the data and visualize sample grouping, an unsupervised multivariate data analysis method, principal component analysis was performed on the GC-MS data generated from the plants treated with Cd. The 2d score plot of principal component analysis did not reveal a distinct cluster between the control and Cd-treated plants (Fig. 46A), but all of them separated from the Cd-treated samples. Similar results were seen between samples treated with only 150 µM Cd and 150 µM Cd with Spd spray (Fig. 46C). As PC1 on the X-axis captures

the greatest variation and can be seen in Fig. 47B, it accounted for 37.2% of the total variance around the metabolites, while PC2 on the Y-axis captures the second greatest variation and accounts for 25.2% of the total variance around the metabolites (Fig. 46C, D).

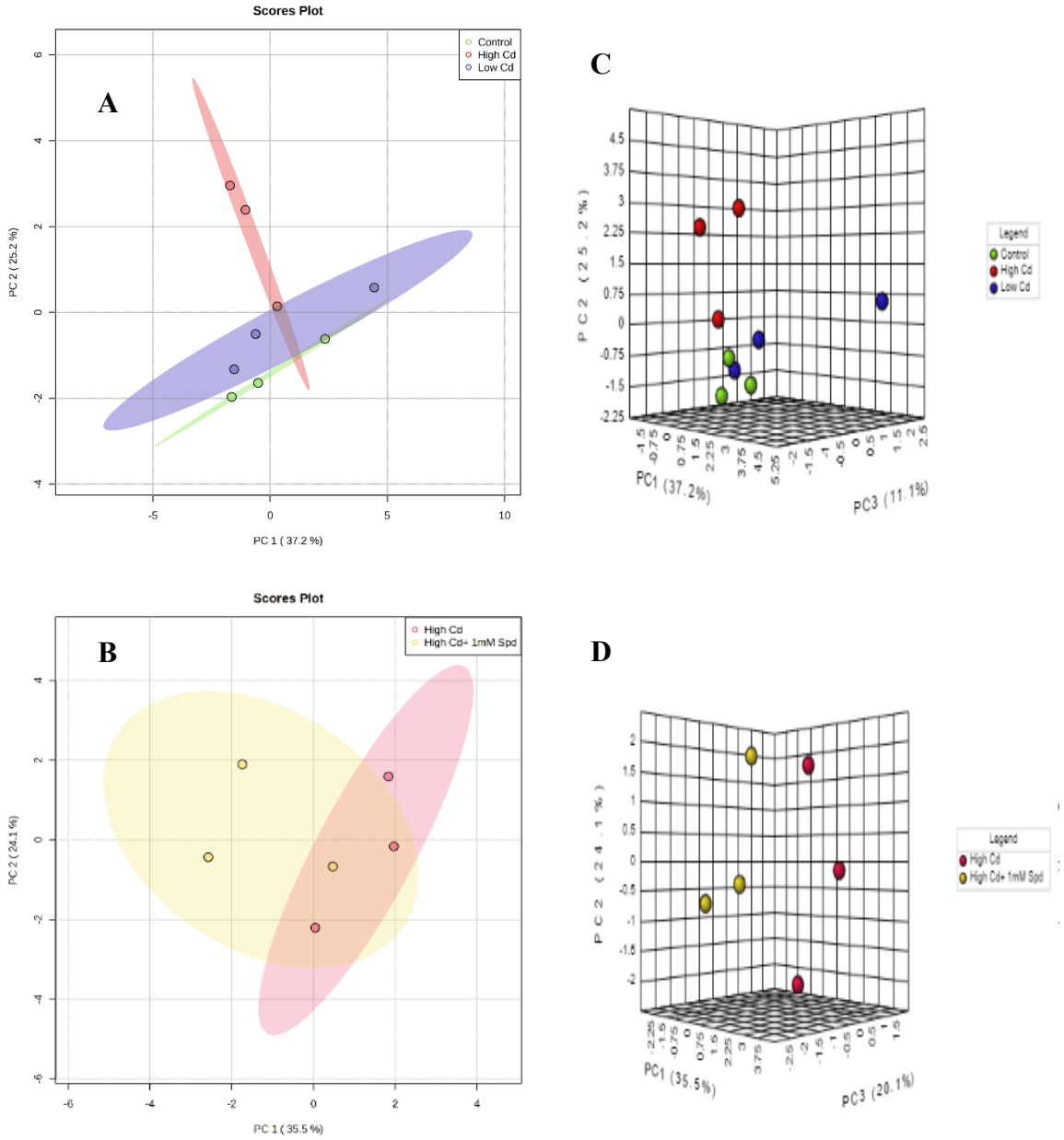

Figure 46: The effect of two different concentrations of $CdCl_2$ on the metabolic profiles in the leaves of the hybrid poplar NM6 plants on day 14. (A) PCA 2d- score plot for control, Low Cd and High Cd-treated samples, (B) PCA 3d- score plot for control, Low Cd and High Cd, (C) PCA 2d-score plot for High Cd and Cd + 1mM Spd, (D) PCA 3d-score plot for High Cd and Cd + 1mM Spd. Control- green, Low Cd- blue, High Cd- red, High Cd+1 mM Spd- yellow.

Multivariate supervised data analysis

As there was no visual separation shown in between treatments by principal component analysis, partial least squares discriminant analysis (PLS-DA) was performed. PLS-DA is a supervised multivariate method that classifies the observation into the group from giving the largest predicted indicator variable. The 2d score plot of the PLS-DA revealed clear discrimination in the treatments between all the samples (Fig. 47A, C). The 3d score plots of PCA and PLS-DA showed distinct separation between control and 150 µM Cd treated samples and Spd-sprayed plants *vs.* unsprayed for 150 µM Cd. PLS-DA is a useful model that also ranks variables based on their importance which can be generated by the Variable importance for projection (VIP) score.

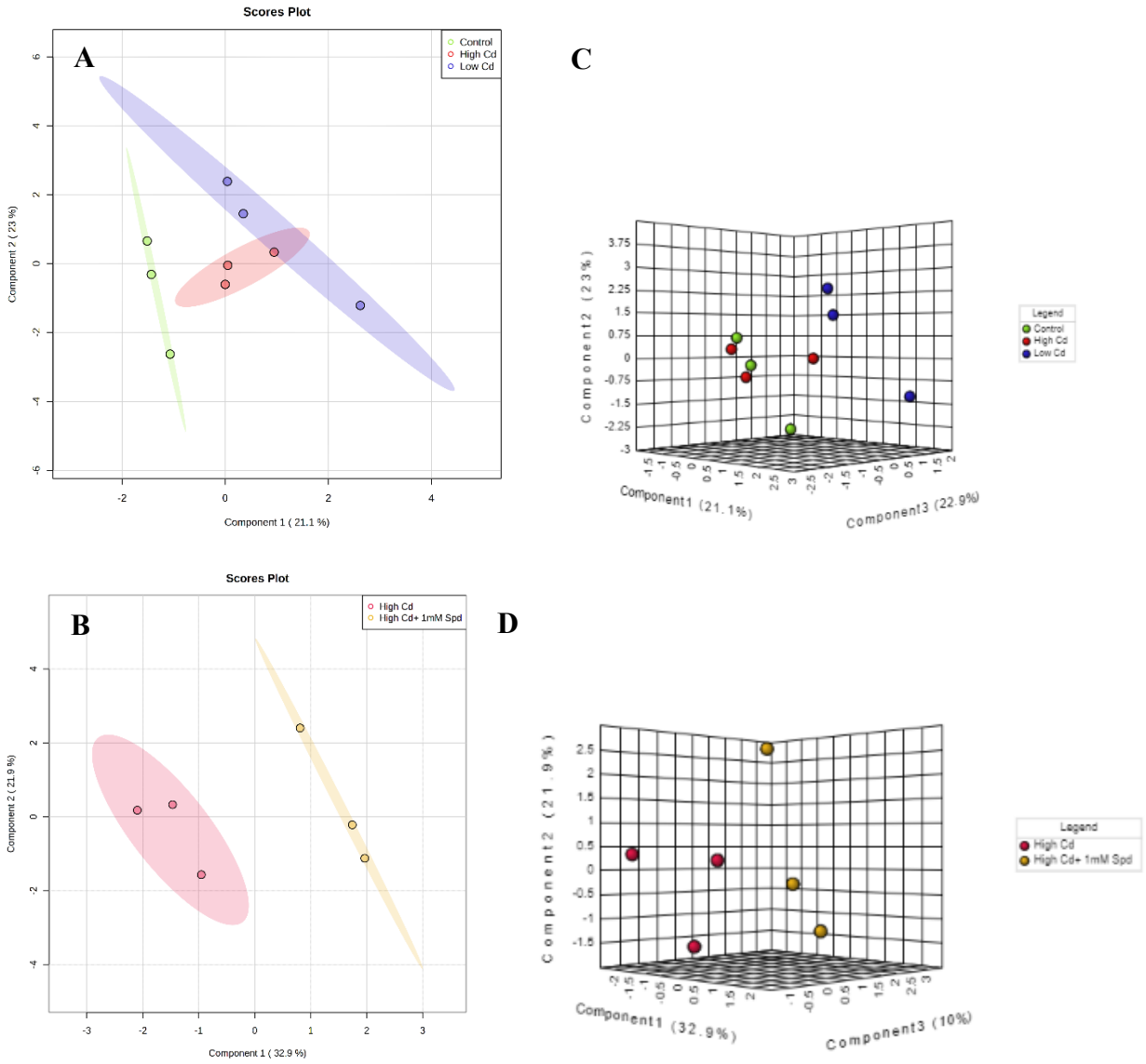

Figure 47: The effect of two different concentrations of $CdCl_2$ on the metabolic profiles in the leaves of the hybrid poplar NM6 plants on day 14. (A) PLS-DA 2d- score plot for control, Low Cd and High Cd-treated samples, (B) PLS-DA 3d- score plot for control, Low Cd and High Cd-treated samples, C) PLS-DA 2d-score plot for High Cd and Cd + 1mM Spd -treated samples, (D) PLS-DA 3d-score plot for High Cd and Cd + 1mM Spd -treated samples. Control- green, Low Cd- blue, High Cd- red, High Cd+1 mM Spd- yellow.The most important variables (metabolites) with a score greater than 1 can be used to visualize separation in the treatment group based on the PLS-DA model. Fig. 48 shows the VIP score plot of 30 most important metabolites identified in the experiment when different treatments were applied. The most important metabolites in distinguishing the control and the Cd treatment were glyoxylic acid, quinic acid, sitosterol,

erythritol, and oleic acid. Among the metabolites identified, 4 AAs were the most important metabolites. Among these 3 AAs (Trp, Orn, and Glu) the concentration decreased, and for 1 AA (Gly) the concentration increased under increasing Cd concentrations. The analysis also reveals that sugars like glycerol, arabitol, and glycerol-3-p increased, and erythritol decreased under 150 µM Cd.

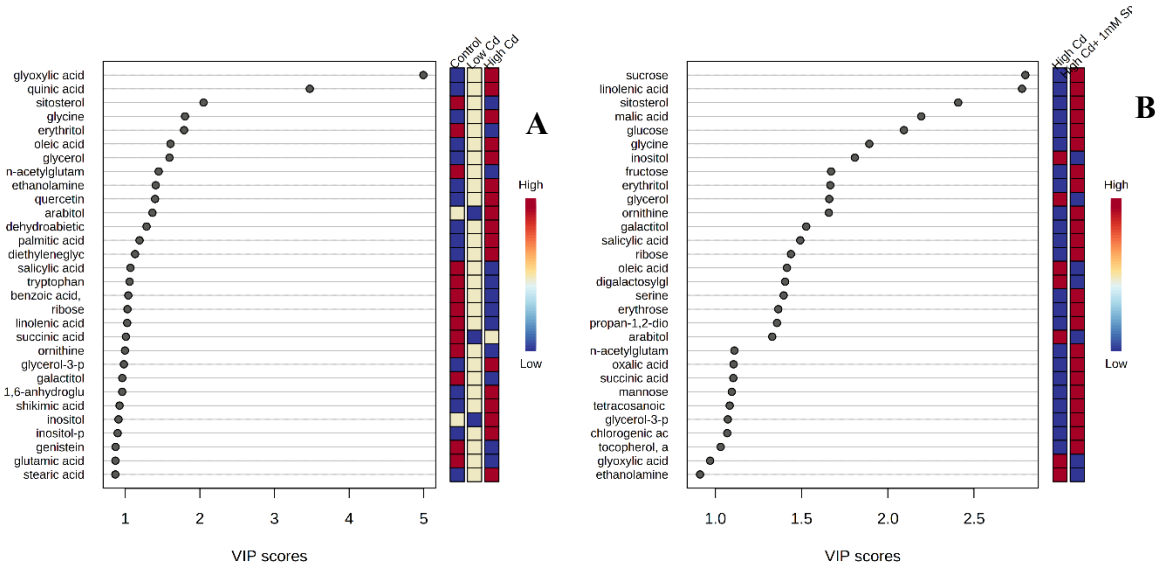

Figure 48: The effect of two different concentrations of $CdCl_2$ (± Spermidine) on the top 30 most important metabolites (VIP score plots) in the leaves of the hybrid poplar NM6 plants on day 14. (A) Control, Low Cd- 150 µM Cd, and High Cd-150 µM Cd, (B) High Cd-150 µM Cd and High Cd+1 mM Spd. The relative abundance of metabolites is indicated by a colored scale from blue to red representing the low and high, respectively.

Changes in metabolites

A volcanic plot was created to combine the results from fold change (FC) analysis and T-test and represented under one single graph which shows the metabolites which are significantly different based on their statistical significance. There were no significant differences in the metabolite distribution between control and 50 µM Cd after 14 days of treatment (Fig. 49A). When comparison was made between control and 150 µM Cd, 6 metabolites (glycine, inositol-p, erythritol, 4-hydroxy benzoic acid, n-acetylglutamic acid, and ribose) were significantly different

(Fig. 49B). Volcano plot between 50 µM Cd- and 150 µM Cd-treated plants revealed that metabolites like serine, glycine, octadecanol, erythritol, chlorogenic acid, and n-acetylglutamic acid were significantly different on the 14th day after Cd treatment (Fig. 49C). Plants sprayed with Spd under 150 µM Cd show that 12 metabolites were significantly different compared to plants only under 150 µM Cd. These metabolites were serine, ornithine, glycine, mannose, erythritol, erythrose, pyrrole-2-carboxylic acid, tetracosanoic acid, galactitol, ribose, linoleic acid, oxalic acid (Fig. 49D).

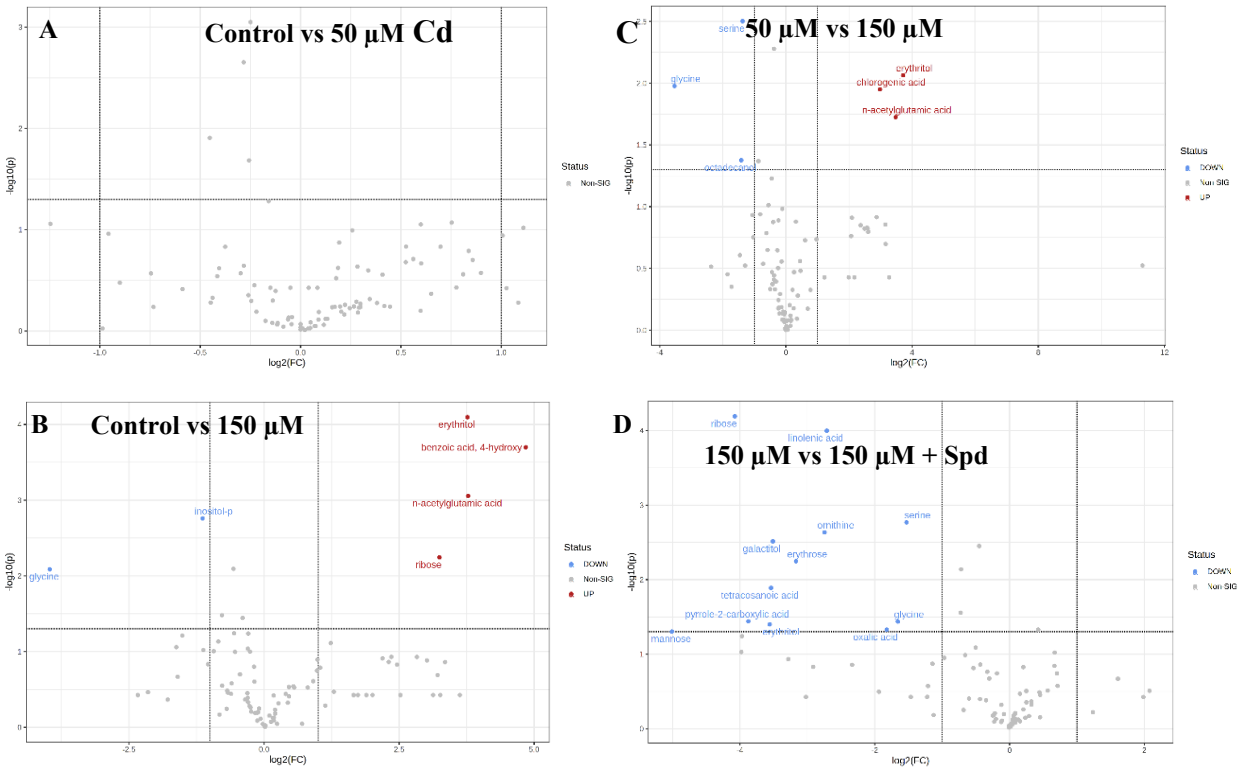

Figure 49: The effect of two different concentrations of $CdCl_2$ on the significantly different metabolites in the leaves of the hybrid poplar NM6 plants on day 14. (A) Control vs 50 µM Cd, (B) Control vs 150 µM Cd, (C) 50 µM Cd vs 150 µM Cd, (D) 150 µM Cd vs 150 µM Cd +1 mM Spd.

Table 10: Metabolite content (relative conc. mg^{-1} FW) in the leaves of the poplar plants exposed to two concentrations of Cd (50 µM, 150 µM) with or without 1 mM Spd spray (data are mean ± SE, n=3).

Metabolites	Treatments			
	Control	50 µM Cd	150 µM Cd	150 µM Cd + 1 mM Spd
Amino acids				
Tryptophan	0.28 ± 0.08	0.20 ± 0.04	0.15 ± 0.01	0.18 ± 0.02
Phenylalanine	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Glutamine	1.01 ± 0.18	0.95 ± 0.32	0.74 ± 0.26	0.75 ± 0.38
Serine	0.02 ± 0.01	0.01 ± 0.00	0.03 ± 0.00	0.07 ± 0.01
GABA	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.00
Glutamic acid	0.08 ± 0.02	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.01
Aspartic acid	0.05 ± 0.03	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
Ornithine	0.27 ± 0.00	0.09 ± 0.02	ND	0.07 ± 0.01
Alanine	0.48 ± 0.09	0.56 ± 0.10	0.60 ± 0.01	0.63 ± 0.05
Glycine	0.01 ± 0.00	0.01 ± 0.00	0.09 ± 0.02	0.29 ± 0.05
Amine				
Ethanolamine	0.88 ± 0.02	0.98 ± 0.02	1.15 ± 0.07	0.97 ± 0.11
Sugars				
Trehalose	0.08 ± 0.00	8.03 ± 6.42	ND	ND
Mannose	0.01 ± 0.00	ND	ND	0.01 ± 0.01
1, 6- anhydroglucose	0.11 ± 0.01	0.13 ± 0.01	0.16 ± 0.01	0.15 ± 0.00
Sucrose	51.87 ± 9.56	51.12 ± 8.68	46.25 ± 2.03	56.99 ± 5.45
Fructose	0.36 ± 0.07	0.34 ± 0.07	0.33 ± 0.01	0.65 ± 0.13
Maltose	0.08 ± 0.03	0.05 ± 0.02	0.03 ± 0.00	0.06 ± 0.01
Mannitol	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.06 ± 0.01
Inositol	6.24 ± 1.11	4.91 ± 0.60	7.35 ± 1.18	5.37 ± 0.91
1-monohexadecanoylglycerol	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.02	0.02 ± 0.00
Arabitol	0.08 ± 0.01	0.07 ± 0.01	0.37 ± 0.22	0.09 ± 0.01
Digalactosylglycerol	0.17 ± 0.07	0.08 ± 0.02	0.30 ± 0.17	0.07 ± 0.00
Glucose	0.92 ± 0.18	0.89 ± 0.17	0.94 ± 0.11	1.55 ± 0.08
Erythritol	0.09 ± 0.01	0.09 ± 0.02	ND	0.08 ± 0.03
Erythrose	ND	ND	ND	0.03 ± .01
Galactitol	0.04 ± 0.01	0.03 ± 0.01	ND	0.04 ± 0.01

Metabolites	Treatments			
	Control	50 µM Cd	150 µM Cd	150 µM Cd + 1 mM Spd
Organic acids				
Citric acid	18.66 ± 3.67	19.80 ± 4.46	19.52 ± 0.13	19.20 ± 3.56
Caffeic acid	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
Glucaric acid	0.21 ± 0.06	0.16 ± 0.05	0.19 ± 0.04	0.22 ± 0.05
Lactic acid	0.20 ± 0.01	0.20 ± 0.01	0.22 ± 0.01	0.22 ± 0.02
Malic acid	2.24 ± 0.49	2.47 ± 0.55	2.20 ± 0.09	3.48 ± 0.50
Oleic acid	0.34 ± 0.03	0.44 ± 0.03	0.58 ± 0.05	0.35 ± 0.12
p-coumaric acid	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
Quinic acid	1.65 ± 0.54	2.47 ± 0.48	5.07 ± 1.27	4.31 ± 1.69
Succinic acid	0.90 ± 0.20	0.42 ± 0.06	0.61 ± 0.04	0.92 ± 0.23
Shikimic acid	0.04 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
Tocopherol a	0.13 ± 0.03	0.15 ± 0.01	0.16 ± 0.02	0.22 ± 0.01
Xylitol	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Vitamin				
Dehydroascorbic acid	0.05 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.06 ± 0.02
Phytohormones				
Sitosterol	1.28 ± 0.06	1.27 ± 0.08	1.48 ± 0.00	1.43 ± 0.09
Lipids				
Glycerol	0.16 ± 0.01	0.18 ± 0.02	0.49 ± 0.16	0.16 ± 0.01
Gycerol-3-p	0.11 ± 0.04	0.19 ± 0.03	0.19 ± 0.04	0.28 ± 0.01
Monohexadecanoylglycerol	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.02	0.02 ± 0.00
Phytol	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00

CHAPTER 4: DISCUSSION

Effect of putrescine on poplar salt tolerance

Salt stress adversely affects plants by limiting nutrient uptake, toxic ion accumulation, and increasing oxidative stress (Hasanuzzaman et al. 2013, Safdar et al. 2019, Rady et al. 2023), which hampers plant growth and productivity (Liu et al. 2023, Rodríguez Coca et al. 2023). 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 (Parida and Das 2005, de Oliveira et al. 2013, Hasanuzzaman et al. 2013). 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 (Ding et al. 2010, Minocha et al. 2014, Nguyen et al. 2018, Zhao et al. 2021b). 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. Also, 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, Srivastava et al. 2021, Zafar et al. 2022, Eisa et al. 2023). Putrescine often enhances plant growth and development under salt stress (Nahar et al. 2016b, Xiong et al. 2018, Alcázar et al. 2020, Ma et al. 2022). High tolerance to salt stress due to foliar Put application has been reported in many plant species (Table 1).

In this study, the effect of foliar Put spray was compared under two different salt concentrations for effects on growth, chlorophyll content, photosynthesis, FW/DW ratio, and biochemical responses of *P. nigra X maximowiczii* (NM6 hybrid poplar clone). Prolonged (up to 3 months) exposure to salt, led to leaf chlorosis and browning symptoms in *A. thaliana*, *P. alba*, and *P. russkii* (Nguyen et al. 2019, Ma et al. 2023). In this study, leaf tips were burned, and chlorosis and browning symptoms started to appear in the middle part of the plant after 13 days of exposure to 100 mM NaCl; surprisingly, 200 mM NaCl-treated plants had less leaf burning and chlorosis symptoms. Plants sprayed with Put had brown spots towards the tip, but the leaves had darker pigmentation, while Put-sprayed salt-treated plants had a scorching effect/salt-burn in the middle part of the plant. The salt-treated plants had slower growth, and leaves withered gradually similar to the reports published by Cao et al. (2020) in salt-treated cotton plants. This study also showed, plants that were treated 100 mM NaCl and sprayed with Put had fastest growth than all other treatments. It has been shown that prolonged exposure to abiotic stress alters gene expression and helps to form a molecular memory (Wang et al. 2014b, Georgii et al. 2019). Wang et al. (2014b) also showed that drought-stressed *Z. mays* had higher photosynthesis rates in the later stages of stress periods. Similarly, after 35 days of salt exposure, NM6 plants had a significantly higher photosynthesis rate than after 7 days of exposure. After 35 days of salt exposure, Put-sprayed vs. unsprayed plants had higher gas exchange rates which was similar to the reports published by Xiong et al. (2018) and Shen et al. (2019) in *C. sinensis* and *C. sativus*.

Polyamines are small nitrogenous compounds that play an important roles in reducing the adverse effects of salt stress in plants (Alcázar et al. 2010, Saha et al. 2015, Choudhary et al. 2023). It has been shown that under salt stress, there is often excessive accumulation of PAs which helps to inhibit lipid peroxidation, stabilizes cell membranes and ionic balance, and regulate genes under

salt stress (Minocha et al. 2014, Alcázar et al. 2020). In response to salt stress, enzymes like ADC and ODC increase the biosynthesis of PAs in plant cells (Tailor et al. 2019, Attia et al. 2022). This increase in PAs is often accompanied by many enzymes. After synthesis from Met, S-adenosylmethionine (SAM) enters into the PA biosynthetic pathway as decarboxylated SAM (dcSAM) and is used to produce Spd by SPDS. The enzyme SPMS converts Spd to Spm and tSpm (Groppa and Benavides 2008). Spermidine is back converted to Put while releasing H_2O_2 (Moschou et al. 2012). Marco et al. (2019) have shown that Spm synthase mutant Arabidopsis plants were less tolerant to salt stress, again suggesting the importance of PAs under stress. Polyamine response is also plant-specific under salt stress (Shu et al. 2015, Baniasadi et al. 2018, Ghalati et al. 2020, Huo et al. 2020, Zhang et al. 2020b). Similar to Xiong et al. (2018) in *C. sinensis*, our studies have shown increased Put and Spm content in the leaves in response to 100 mM NaCl, although they were day specific. It was also seen that Spd significantly decreased in the leaves over time in response to salt. Although there was an increased accumulation of Put in the roots of salt-treated plants, Spd decreased, and Spm was not detected in response to salt treatment.

Salt tolerance is induced by an increased accumulation of several amino acids (Gupta et al. 2013, Singh and Roychoudhury 2020, Srivastava et al. 2021). However, the accumulation of AAs for increased salt tolerance is plant specific (Hijaz et al. 2018). As a byproduct of PA catabolism, H_2O_2 regulates GABA, which plays an essential role in physiological functions under salt stress (Gupta et al. 2013). Polyamines interact with various metabolic pathways as stress messengers, including the accumulation of AAs (GABA, Ala, Pro) and sugars (Marco et al. 2011, Gupta et al. 2013, Singh and Roychoudhury 2020, Srivastava et al. 2021). In our study, Glu, Ser, Ala, His, Arg+Thr+Gly, Gln, GABA, and Pro increased in the leaves in response to salt. Surprisingly, the

increase in most of these AAs was most prevalent after 6 days of salt exposure, suggesting a time-specific effect on AAs. This study also showed increased Gln, Arg+Thr+Gly, Phe+Cys, Orn, His, Ser, and Pro in the salt-treated roots.

Catabolism of PAs is related to increased accumulation of GABA which further promotes salt tolerance (Gupta et al. 2013). In our study also, GABA, Glu, Asp, Trp, and Lys accumulation increased when plants were sprayed with Put under salt treatment. However, Ala content decreased in Put-sprayed plants *vs.* unsprayed plants under salt treatment corroborating the statement that changes in AAs under stress are specific to plant species. However, a decrease in the Ala and GABA contents in the roots under 200 mM NaCl suggested the potential translocation of these AAs to the leaves, similar to the reports published by Hijaz et al. (2018) in *C. sinensis*. Glutamine was negatively correlated with Pro, Orn, and Ala in the roots suggesting increased Gln production resulted in reduced production of the other AAs. We also found that total soluble protein content was significantly reduced under salt stress; thus, being responsible for the accumulation of amino acids. With Put spray, total soluble protein content under 100 mM NaCl stress increased significantly; however, under 200 mM NaCl Put sprayed plants had lower protein content than those unsprayed under the same NaCl concentration. Interestingly, Put spray on non-stressed plants did not change the protein content. This study showed that there was a significant increase in RWC after 13 days of salt exposure. Again, with Put spray, there was no change in the RWC under salt stress suggesting that the effects on soluble protein were most likely due to synthesis or degradation of cellular proteins. Similar to RWC, FW/DW ratio increased after 13 days of salt exposure. The metabolic connection of AA catabolism with the TCA cycle is also related with increased salt tolerance (Ali et al. 2019).

One of the most common adaptive responses of plants to abiotic stress is an accumulation of soluble sugars, as studied in *A. thaliana*, . *euphratica, Beta vulgaris, and Morus multicaulis* (Watanabe et al. 2000, Liu et al. 2008, Liang et al. 2018, Liu et al. 2022). In response to salt stress, plants enhance the production/accumulation of osmoprotectants or osmolytes for cellular osmotic adjustment and mitigate the damaging risk caused by ROS (Liu et al. 2022, Azeem et al. 2023). Carbohydrates including fructose, trehalose, glucose, and sucrose are essential osmolytes, which provide carbon and energy for the normal functioning of cellular metabolism. Increased accumulation of these osmolytes is also known to help maintain high K^+ and low Na^+/K^+ ratios, prevent membrane injury, reduce protein damage and dehydration, and stabilize the normal metabolic processes in response to salt stress. The current studies have shown increased accumulation of fructose and sucrose while glucose+galactose significantly decreased after 6 days of salt exposure. Exogenous Put significantly increased sugar accumulation in NM6 plants under salt stress suggesting Put spray increased sugar accumulation via interconnection between PA and C metabolism pathway under salt stress similar to the reports published by Yuan et al. (2015) in *C. sativus*.

Effect of spermidine on poplar tolerance to cadmium stress

Heavy metals like Pb, Cr, Cd, Zn, and Hg interfere with various physiological and biochemical processes in plants (Cenkci et al. 2010, Ali et al. 2011, Paunov et al. 2018, Hananingtyas et al. 2022, Chen et al. 2023). The primary symptoms of heavy metal toxicity are necrotic spots, chlorosis, reduced leaf size, lower photosynthesis and transpiration, reduced water and nutrient uptake, and alteration of N metabolism (Atabayeva et al. 2020, Riyazuddin et al. 2022, Sun et al. 2022). Additionally, heavy metals generate oxidative stress by overproduction of ROS, resulting in lipid peroxidation, protein damage, enzyme inhibition, damage to nucleic acids, and cell death

(Atabayeva et al. 2020, Sun et al. 2022). Some plants have one or more defense systems for detoxifying heavy metals by accumulating them in the root system and sequestration in the vacuole (Hasanuzzaman et al. 2019, Atabayeva et al. 2020, Li et al. 2023). Poplars are fast growing, deep rooted, easily propagated and some species and hybrid clones of poplars have shown to have high bioaccumulation coefficients, suggesting these plants could be used in phytoremediation strategies (Robinson et al. 2000, Zalesny Jr et al. 2019, Ancona et al. 2020).

Similar to the studies reported by Han et al. (2020) and Teng et al. (2022), this study also showed a variety of morphological and biochemical changes in response to Cd exposure. Brown spots and chlorosis symptoms started to appear after 7 days of exposure to 50 μM Cd via roots; surprisingly, the plants treated with 150 μM Cd were healthier, and showed no signs of chlorosis. Plants sprayed with Spd under 150 μM Cd had dark pigmentation than the Spd-sprayed control plants; while Spd-sprayed 50 μM Cd plants had chlorosis and necrosis symptoms. It has been shown that prolonged exposure to heavy metals alters normal physiological activities, including reduced chlorophyll content and photosynthesis (Nagajyoti et al. 2010, Mourato et al. 2015). In this study, chlorophyll significantly decreased in response to 50 μM Cd; however, there was a significant increase under 150 μM Cd. Similar result were reported by Chandra and Kang (2016) and Yang et al. (2020b) in poplar hybrids and *Davidia involucrata* under mixed heavy metal stress. It has been reported earlier that exogenous treatment of plants with salicylic acid (Wang et al. 2021a), melatonin (Li et al. 2022), and Spd (Gu et al. 2022) alleviated Cd toxicity in them. Our results show that there was a variable effect of Spd spray on plants exposed to different Cd concentrations on chlorophyll because its content was always higher in Spd sprayed *vs.* non sprayed 50 μM Cd-treated plants. However, chlorophyll increased in the Spd-sprayed plants *vs.*

non-sprayed plants treated with 150 μM Cd after 21 days. Similar to chlorophyll, an increase in photosynthetic rate, conductance, and transpiration was seen in response to 150 μM Cd.

Phytochelatins (PCs) are another group of small peptides that play an important role in reducing the adverse effects of heavy metals in plants (Hasan et al. 2017, Baker et al. 2020, Raychaudhuri et al. 2021, Li et al. 2023). A common detoxification response to Cd toxicity in higher plants is synthesizing PCs and their sulfur-containing metabolic precursors like GSH, γ-EC, and Cys (Liu et al. 2015, Hasanuzzaman et al. 2017, Raychaudhuri et al. 2021) which bind to the heavy metal ions within the cell. However, the type and the amount of PCs depend on the concentration of Cd, plant species and organ, and duration of exposure (Thangavel et al. 2007, Raychaudhuri et al. 2021, Zare et al. 2022). In our study, the most abundant thiol compound in the leaves was γ-EC, and in the roots it was GSH, whose concentration significantly increased in response to Cd treatment. Similar results have been reported in red spruce cell suspension cultures (Thangavel et al. 2007), *Z. mays* (Szalai et al. 2013), *O. sativa* (Pál et al. 2017), *Lolium perenne* L. (Shi et al. 2021), and transgenic *Populus X canescens* (Yu et al. 2023) when exposed to Cd. Apart from the thiol compounds, a higher level of PCs was noticed with the increase in Cd concentration in the roots indicating the plant is actively responding to the metals by synthesizing these peptides. Unlike leaves, γ-EC was absent in the roots in our study. In our previous studies with the NM6 poplar cell cultures subjected to Al stress similar results were obtained (Mohapatra et al. 2010a). However, there was a difference in their range which can be attributed to the type of heavy (toxic) metal and plant cells. Similar to the reports published by Gao et al. (2022), the leaf PCs were at peak level by the 7^{th} day of Cd exposure and decreased afterward. The higher levels of PC_4 accumulation (8-10-fold) in Cd-exposed poplar roots compared to control suggests that tolerance to higher Cd toxicity is correlated with elevated PC synthesis.

Several recent studies have shown that PAs are essential signaling compounds in heavy metal stress responses (Szalai et al. 2020, Spormann et al. 2021), which could act as metal chelators (Raychaudhuri et al. 2021), and reduce the Cd-induced oxidative damage by ROS. Spermidine spray significantly increased Cys and GSH content in the poplar leaves under Cd stress, which is consistent with the results of Nahar et al. (2016c) and Li et al. (2020a).

Accumulation of PAs often confers additional protection to the plants in response to abiotic stress of other types as well (Minocha et al. 2014, Bano et al. 2020, Raychaudhuri et al. 2021). In the current study, Put and Spd increased, and Spm significantly decreased in the leaves of Cd-treated plants. In the roots also, PAs significantly increased under Cd stress, and the abundance of each PA varied under different Cd concentrations. Whereas Put was most abundant, followed by Spd and Spm under 50 µM Cd; Spm was the most abundant PA, followed by Spd and Put under 150 µM Cd. The results from an earlier report with the cell cultures of the same hybrid (Mohapatra et al. 2010a) suggest that changes in different PAs depend on the plant parts and type of heavy metal. The results of the current study further show that the effect of Spd spray on the PA content of the leaves also varies with the Cd concentration given to the roots. These results are similar to those published earlier on the effects of exogenous PAs in *Glycine max* (Chmielowska-Bąk et al. 2013), *Arabidopsis thaliana* (Han et al. 2014), *Oryza sativa* (Pál et al. 2017), and *Triticum aestivum* (Tajti et al. 2018) when treated with Cd stress.

Tolerance to heavy metals is also conferred with the increased accumulation of several AAs in the plant cells (Mohapatra et al. 2010a, Zanganeh et al. 2019, Alsherif et al. 2023). High Glu (a precursor for synthesizing Pro, PAs, and GSH) content has been reported in response to heavy metal treatment (Jozefczak et al. 2012, Sharma et al. 2016); and so is an increase in Arg and His, which reduce NH_4^+ toxicity (Esteban et al. 2016). Our results showed that Cd affected AAs like

Arg+Thr+Gly, Asp, Gln, and Orn in the shoots as well as roots. An increase of His, Leu, and Lys in the shoots and Cys, GABA, and Glu in the roots suggests that they were specifically secreted to cope with a high concentration of Cd which is similar to the results shown by Alsherif et al. (2023). In response to Spd spray on Cd-treated plants, there was a significant increase in the leaf Asp, Cys, His, Glu, and Ser content under Cd stress. Most AAs were also affected in the control plants sprayed with Spd. The increase in Cys under Cd could be related to the increased production of GSH and PCs as shown by Guo et al. (2020) and Ni et al. (2023). An increase in Arg in response to Cd could also contribute to its protective role since it is a precursor of PAs and Pro biosynthesis. Increase in Phe as aromatic amino acid in response to Cd could also contribute to biosynthesis of secondary metabolites (e.g. flavonoids) which play important role in abiotic stress tolerance (Zhao et al. 2021a).

Various forms of environmental stress often induce or promote the generation of ROS in plants, which causes severe damage to DNA, enzymes, and proteins. (Xu et al. 2008, Gill and Tuteja 2010b, Balfagón et al. 2020, Gu et al. 2022). This study showed that total soluble protein content was significantly reduced under Cd stress. With Spd spray, however, the total soluble protein content under Cd stress increased significantly. Similar results have been reported by Tang et al. (2019) and Gu et al. (2022) in Salix and *O. sativa* under Pb and Cd stress which corroborates our hypothesis that exogenous Spd helps alleviate the adverse effects of Cd stress in plants.

Organic acids are an inherent part of plant metabolism due to their involvement in fatty acid and carbohydrate biosynthesis (Osmolovskaya et al. 2018). Organic acids also regulate the cellular pH, bind to metal ions and compartmentalize them in the vacuoles, further reducing their accumulation in the cytoplasm (Osmolovskaya et al. 2018, Kocaman 2022). Chelation of toxic ions is a commonly effective mechanism that is enhanced by the organic acids of the TCA cycle.

Our study also looked at various organic acids, lipids, sugars, and sugar alcohols via the analysis of the entire metabolome of the leaves. The results show that in response to Cd, several-fold increases in oleic acid, linoleic acid, succinic acid, glycerol, inositol, malic acid, quinic acid, shikimic acid, succinic acid, and tocopherol a were seen similar to the reports published by Zhao et al. (2021a). With Spd spray, there was a higher accumulation of glucaric acid, malic acid, succinic acid, and tocopherol a under Cd stress. These results indicate that TCA cycle-related metabolites like malic acid, succinic acid, and others were associated with ameliorating the adverse effects of Cd stress in the case of poplar (Wang et al. 2021b).

Fructose, glucose, trehalose, and sucrose are essential in glycolysis and the TCA cycle in plants (Misra and Mall 2021). It is also known that fructose also serves as a precursor of fatty acids and proteins, trehalose prevents dehydration, and sucrose helps cell growth (Khan et al. 2020, Afzal et al. 2021). In the present study, sucrose, fructose, maltose, mannitol, and galactitol accumulation decreased under Cd treatment. At the same time, the contents of 1, 6-anhydroglucose, inositol, 1-monohexadecanoylglycerol, arabitol, digalactosylglycerol, trehalose, and glucose were enhanced under Cd treatment, suggesting these soluble sugars played an important role under Cd stress (Zhao et al. 2021a). In addition, some other sugars, like erythrose, that were not involved in the primary metabolism of Cd-treated plants were accumulated in plants when sprayed with Spd under Cd stress. Our HPLC analysis generated a combined result for Arg+Thr+Gly, but GC-MS metabolomic analysis could separate these AAs. GC-MS analysis showed increased accumulation of Gly under Cd stress, but in Spd-sprayed plants under Cd stress, Gly was highest. This result also corroborates our hypothesis that Spd helped to alleviate the adverse effects of Cd stress with increased accumulation of osmoprotectants like Gly. Overall Cd-stress-induced metabolomic responses by GC-MS analysis for poplar leaves showed different but

overlapping metabolomic alterations, mainly on sugars, AAs, PAs, and organic acids. Metabolic profile analysis demonstrates that Spd sprays in poplar plants under Cd stress generated profound biochemical changes.

Conclusions

Plants develop various strategies to cope with stress and altered soluble protein content, RWC, FW/DW ratio, photosynthesis, and metabolic responses in Put-sprayed *P. nigra X maximowiczii* (clone NM6) plants confirming their potential roles in alleviating salt stress, thereby contributing to higher biomass under salt stress. Poplar plants can reduce surface runoff, and their deep root system can preserve soil and water. Hence, they are an excellent resource for silviculture. Due to their unique role, genetic manipulation of *Populus nigra x maximowiczii* (clone NM6) for salt tolerance can be an important way of using these plants for silviculture and sound economic value with the high salt-tolerant property.

Likewise for Cd stress, exogenous Spd application increased the levels of proteins and several AAs and promoted chlorophyll and GSH synthesis. The overall results suggest a close relationship between AAs, PAs, and PCs and their involvement in the Cd detoxification mechanisms. Due to the unique role of PCs as metal chelators, they could serve as an early indicator for Cd stress. Considering all parameters, it can be suggested that timely applications of exogenous Spd via foliar spray could ameliorate the adverse effect of heavy metal stress in poplar plants. The already known use of several species and hybrids of poplars for phytoremediation is consistent with the tolerance of *P. nigra x maximowiczii* (clone NM6) plants to high concentrations of Cd.

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