Physiological and biochemical analysis of the nitrogen metabolism and lead treatment in hybrid poplar clone NM6 (Populus nigra L. x P. maximowiczii A. Henry)

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PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF THE NITROGEN METABOLISM AND LEAD TREATMENT IN HYBRID POPLAR CLONE NM6

(POPULUS NIGRA L. X P. MAXIMOWICZII A. HENRY)

By

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DISSERTATION

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Doctor of Philosophy

In

Biological Sciences: Integrative and Organismal Biology

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On August 31,
Approval signatures are on file with the University of New Hampshire Graduate School
DEDICATION

I dedicate my dissertation work to my parents. To my mom, Chandra Weerakoon and dad, Vimal Weerasinghe, you have both sacrificed so much to help me achieve my dreams and goals and gave me unconditional support and love. Thank you for your constant support and words of encouragement, they have emboldened me to achieve this major milestone. I hope I have made you proud.

This work is also dedicated to my sister, Thushantha Harshi, who has been a constant source of support and encouragement during the challenges of graduate school and life. You have helped shape me into the person I am, I love you and hope I have made you and my little nephew, Pevin, proud.
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# TABLE OF CONTENTS

Dedication ........................................................................................................ iii
Acknowledgements ......................................................................................... iv
List of Figures ................................................................................................. vii
List of Tables ................................................................................................ x
Abbreviations ............................................................................................... xi
Abstract .......................................................................................................... xiii

Introduction ..................................................................................................... 1

*Chapter 1*: Efficacy of foliar application of different forms of nitrogen and study its physiological and biochemical effects of Poplar clone NM6 (*Populus nigra l. X p. Maximowiczii* A. Henry) ................................................................. 18

Abstract .......................................................................................................... 18

Introduction ..................................................................................................... 19

Methods .......................................................................................................... 23
  - Plant material ............................................................................................. 23
  - Laboratory media preparation ................................................................ 26
  - Tissue preparation ...................................................................................... 33
  - Total soluble protein ............................................................................... 33
  - Chlorophyll content .................................................................................. 34
  - Leaf gas exchange measurements .......................................................... 34
  - Plant growth parameters and moisture content ..................................... 35
  - Soluble sugars .......................................................................................... 35
  - Total carbon and nitrogen ....................................................................... 36
  - Polyamines and amino acids separation by HPLC ................................. 36
  - Statistical analyses .................................................................................. 38

Results ............................................................................................................. 39
  - Morphological differences of the plants between the 3 experiments .......... 39
  - Experiment 2 ............................................................................................ 42
    - Total soluble protein content ............................................................... 42
    - Leaf chlorophyll and carotenoids ......................................................... 44
    - Gas exchange ....................................................................................... 44
Biomass and height ................................................................. 48
The surface leaf area ............................................................ 48
Moisture content ................................................................. 48
C/N ratio in response to different N forms ............................ 48
Foliar soluble sugars ............................................................. 52
Soluble foliar amino acids .................................................... 54
Amino acids derived from α-ketoglutarate - (the glutamate family) .................................................... 54
Amino acids derived from 3-phosphoglycerate ............................ 56
Aromatic amino acids ............................................................. 56
Alanine and branched chain AAs .......................................... 57
Aspartate family AAs ............................................................. 57
Foliar polyamines ................................................................. 57
Experiment 3 ............................................................................. 67
Total soluble protein .............................................................. 68
Leaf chlorophyll and carotenoids ........................................ 70
Gas exchange ........................................................................ 70
Moisture content ................................................................. 70
C and N percentage in response to different N forms ............... 70
Foliar soluble sugars ............................................................. 70
Soluble foliar amino acids .................................................... 73
Amino acids derived from α-ketoglutarate - (the glutamate family) .................................................... 73
Amino acids derived from 3-phosphoglycerate ............................ 74
Aromatic amino acids ............................................................. 74
Alanine and branched chain AAs .......................................... 75
Aspartate family AAs ............................................................. 75
Foliar polyamines ................................................................. 76
Soluble amino acids in roots .................................................. 76
Discussion ............................................................................. 83
Conclusions ........................................................................... 96

Chapter 2: To study the physiological and biochemical responses of poplar NM6 plants to lead stress and its reversal by putrescine spray .................................................... 97

Abstract .............................................................................. 97

Introduction ........................................................................... 98

Methods .............................................................................. 103

Derivatization of thiol compounds ......................................... 103

Results .............................................................................. 105

Total soluble protein content .............................................. 105
Leaf chlorophyll and carotenoids ........................................ 106
Gas exchange ........................................................................ 106
Foliar soluble sugars ............................................................. 110
Soluble foliar amino acids .................................................... 112
Discussion ..................................................................................................................128
Conclusions ...............................................................................................................135

Chapter 3: To produce and metabolically characterize transgenic plants of hybrid poplar clone nm6 with the mode gene that regulates polyamine biosynthesis ........................136

Abstract .....................................................................................................................136
Introduction ...............................................................................................................137
Methods .....................................................................................................................142
  Genetic transformation ............................................................................................142
  Plant material and growth conditions ..................................................................142
  Agrobacterium culture and transformation procedure ........................................143
  Genomic DNA isolation .........................................................................................146
  Polymerase chain reaction (PCR) ..........................................................................147
  Agarose gel electrophoresis ...................................................................................147
Results .......................................................................................................................148
Discussion .................................................................................................................150
Conclusions and current status ...............................................................................153
References ...............................................................................................................154
LIST OF FIGURES

Figure 1. Nitrogen cycle .................................................................5
Figure 2. Structures of polyamines ..................................................13
Figure 3: Interactive pathways for biosynthesis of polyamines ...............16
Figure 4. Plants at the time of transfer to the larger pots .........................25
Figure 5. Arrangement of pots on the bench during the experiment(s) .........25
Figure 6. Fertilizer used in the Greenhouse for maintenance ......................26
Figure 7. Experimental setup to measure the wastage from spray ..............29
Figure 8. The position from where the leaves were sampled .......................30
Figure 9. Picture of a punched leaf (leaves were punched, avoiding mid veins) 31
Figure 10. The effect of foliar spray after 13 days on the morphological traits in experiment 2 ..40
Figure 11. The effect of foliar spray after 14 days on the morphological traits in experiment 3 ..41
Figure 12. The effect of foliar spray on the cellular concentrations of total soluble protein in experiment 2 .................................................................43
Figure 13. The effect of foliar spray on the cellular concentrations of total chlorophyll and carotenoids in experiment 2 .................................................................45
Figure 14. The effect of foliar spray on the cellular concentrations of total chlorophyll and carotenoids on the day of harvest in experiment 2 .................................................................46
Figure 15. The effect of foliar spray on the gas exchange rate on the day of harvest post treatment in experiment 2 .................................................................47
Figure 16. The effect of foliar spray on the plant biomass on the day of harvest, post-treatment in experiment 2 .................................................................49
Figure 17. The effect of foliar spray on the plant leaf surface area on the day of harvest, post-treatment in experiment 2 .................................................................50
Figure 18. The effect of foliar spray on the sugar content on different days, post-treatment in experiment 2 .................................................................53
Figure 19. The effect of foliar spray on the amino acid content on different days, post-treatment in experiment 2 .................................................................59
Figure 20. The effect of foliar spray on the Serine content ..........................60
Figure 21. The effect of foliar spray on the Leucine, Isoleucine, Valine and Alanine content.....61
Figure 22. The effect of foliar spray on the Aspartic acid, Methionine and Lysine..............62
Figure 23. The effect of foliar spray on the Putrescine, Spermidine and Spermine.............63
Figure 24. The effect of foliar spray on the total soluble protein content..........................64
Figure 25. The effect of foliar spray on the total chlorophyll and carotenoids..................65
Figure 26. The effect of foliar spray on the gas exchange rate ......................................66
Figure 27. The appearance of the randomly chosen plants at time zero.............................69
Figure 28. The effect of foliar spray on the sugar content ..............................................72
Figure 29. The effect of foliar spray on Glutamic acid, Glutamine, Proline, GABA...........77
Figure 30. The effect of foliar spray on Serine and Cystine...........................................78
Figure 31. The effect of foliar spray on the Phenylalanine.............................................78
Figure 32. The effect of foliar spray on Leucine, Isoleucine, Valine, Alanine.....................79
Figure 33. The effect of foliar spray on Aspartic acid, Methionine, Lysine.......................80
Figure 34. The effect of foliar spray on Putrescine, Spermidine, Spermine......................81
Figure 35. Representation of the effect of foliar spray on the root amino acid content......82

Figure 36. Poplar NM6 plants at the beginning of the experiment before spray ...............105
Figure 37. Healthy Poplar NM6 plants post-exposure to PbCl₂ treated with and/or without Put spray.................................................................106

Figure 38. Effect of root application of two different concentrations of on the total soluble protein of leaves .................................................................108
Figure 39. Effect of root application of two different concentrations of PbCl₂ on the total chlorophyll content and carotenoids..............................................109
Figure 40. Effect of root application of two different concentrations of PbCl₂ on the gas exchange of leaves .................................................................109
Figure 41. Effect of root application of two different concentrations of PbCl₂ on sugar contents of leaves .................................................................111
Figure 42. Effect of root application of two different concentrations of PbCl₂ Aspartic acid, Glutamic acid, Glutamine.................................................................115
Figure 43. Effect of root application of two different concentrations of PbCl₂ Alanine, Proline, GABA.................................................................116
Figure 44. Effect of root application of two different concentrations of PbCl₂ Valine, Isoleucine, Leucine........................................................................................................117

Figure 45. Effect of root application of two different concentrations of PbCl₂ on Cysteine, Methionine, Ornithine. ........................................................................................................118

Figure 46. Effect of root application of two different concentrations of PbCl₂ on day 22 on the amino acid contents of roots..................................................................................119

Figure 47. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on day 22 on the polyamine contents of leaves.......................................................................122

Figure 48. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on day 22 on the polyamine contents of roots ..................................................................123

Figure 49. Effect of root application of two different concentrations of PbCl₂ on the thiol compound contents of leaves ....................................................................................125

Figure 50. Effect of root application of two different concentrations of PbCl₂ on different days on the PC content of leaves ..................................................................................126

Figure 51. Effect of root application of two different concentrations of PbCl₂ on different days on the thiol compound contents of roots ..................................................................127

Figure 52. Vectors used for inducible expression (pMDC7) and constitutive expression (pMDC32) of mODC 2x ...........................................................................................................144

Figure 53. Different steps in the transformation system for poplar NM6 .........................................................148

Figure 54. Confirmation of constitutive mODC transgene integration using PCR ........................................149

Figure 55. Confirmation of inducible mODC transgene integration using PCR ............................................149
LIST OF TABLES

Table 1. Nutrient mix (without N) used for fertilization during the duration of the study …….27

Table 2. Types and Concentrations of N Fertilizers used in experiment 1 and experiment 2 conducted in 2019, water without N was sprayed on the control plants ……………………………..28

Table 3. Nitrogen spray treatments, concentration, and different forms used for experiment 3 conducted in 2021 ………………………………………………………….32

Table 4. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the plant moisture content of the hybrid poplar NM6 on different days, post-treatment ……………………51

Table 5. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the plant N% and C% of the hybrid poplar NM6 on different days, post-treatment ……………………51

Table 6. Height of the tallest plant of each treatment measured on day 17………………….69

Table 7. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the plant moisture content of the hybrid poplar NM6 on different days, post-treatment …………………….71

Table 8. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the plant N% and C% of the hybrid poplar NM6 on different days, post-treatment …………………….71

Table 9. Composition of media for cultivation, transformation, selection, and regeneration of hybrid poplar clone NM6 …………………………………………………………142

Table 10. Primers used for PCR…………………………………………………………………………147
ABBREVIATIONS

ABA= Absciscic 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-difluorophenacyl)-L-alanyl]-S-phenylglycine t-butyl ester; DFMO= Difluoro methyl ornithine; DNA= Deoxyribonucleic acid; ddH$_2$O= Double distilled water; DTPA= Diethylenetriamine pentaacetic acid; DW= Dry weight; E= Transpiration rate; EDTA= Ethylene diamine tetraacetic acid; GABA= y-aminobutyric acid; Gln= Glutamine; Glu= Glutamate, Gly= Glycine; y-EC= Gamma-glutamylycysteine; 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

The first objective of my Ph.D. research was focused on understanding the reasoning for changes observed in the physiology and metabolism of the poplar plants in response to foliar application of nitrogen. Increased use of nitrogen in the soil in agriculture, in the form of nitrogen fertilizers, has helped to promote high-yield agriculture over the past decades. However, a large fraction of nitrogen applied to croplands in the form of fertilizer and manure ends up entering the freshwater system causing degradation of the water quality and eutrophication of groundwater, rivers, lakes, and coastal and marine ecosystems. Chemical fertilization is also associated with a number of problems, such as low fertilizer utilization rates, soil acidification, and soil salinization. Foliar fertilizer application is an effective method to increase the contents of trace elements in crops and crop yield and to improve the soil environment. While many studies have focused on the effects of foliar application of nutrients, understanding of the metabolic effects of foliar application of nitrogen is scarce.

The second objective of my Ph.D. research was focused on evaluating and understanding the effects of soil application of lead the physiology and metabolism of the NM6 poplar plants in response to soil application of lead in order to determine if this species has tolerance to lead toxicity. Soil pollution and contamination due to non-degradable heavy metals such as lead, has become a worldwide concern. Lead in soil can enter the food chain and eventually accumulate in the human body. Lead directly or indirectly affects photosynthesis, plant’s nutrient uptake, seedling growth, enzyme activities, water imbalance, and membrane permeability. Phytoremediation techniques exploit natural hyperaccumulators or transgenic plants that can uptake and accumulate toxic heavy metals such as lead. Phytoremediation is a plant-based
technology employed on either wild-type or genetically modified plant species including poplar for restoring contaminated land and water sources.

The third objective of my Ph.D. research aimed at understanding the effectiveness of enhanced production of Put via genetic manipulation using the mouse *ornithine decarboxylase* (mODC) gene in plants on stress tolerance in this species. This gene has been very effective in enhancing putrescine production in cell cultures of poplar NM6, tobacco, carrot, and *Arabidopsis thaliana* plants. The goal was to produce transgenic plants of poplar NM6 that could be grown in pots in the greenhouse and tested for stress tolerance.

The first introduction gives an overview of the economical values of poplar plants and a general introduction to the nitrogen cycle. My first chapter investigates the efficacy of foliar application of different forms of nitrogen on the growth and nitrogen metabolism in young poplar plants. I found that different forms of nitrogen are metabolized differently in plants. Additionally, the level of nitrogen starvation achieved did affect the outcome significantly. My second chapter investigates the changes in the metabolism in response to lead stress in poplar plants. Here we concluded that single application of lead to large plants was not sufficient to induce a strong metabolic response. I found that foliar putrescine could alleviate some of the mild effects of lead observed in the present study. Further investigations will be needed to understand the tolerance of poplar NM6 to lead stress. My third chapter aimed to produce and metabolically characterize transgenic plants of hybrid poplar NM6 expressing a mODC gene that regulates polyamine biosynthesis constitutively and inducibly. Altogether, this research provides a better understanding of the changes in the plant nitrogen metabolism in response to different stress conditions.
INTRODUCTION

Poplars, also known as cottonwoods and aspens, are a group of plants belonging to the genus *Populus* in the Salicaceae family. Properties of woody perennial plants such as poplar include extended life spans, increased generation times, ability to adapt to more harsh environments of a wide range, and growth behavior (Dillen et al. 2011, Douglas 2017).

Poplars are mostly dioecious plants, and numerous interspecific hybrids produced from them do not reproduce sexually. They are easily propagated by cuttings (Slavov and Zhelev 2010, Guleria et al. 2021, Sanderson et al. 2023). Their bark and other parts (leaves, buds, branches, and exudates) are a rich source of bioactive compounds with essential restorative properties (Hamad et al. 2019). The black poplar, also known as *Populus nigra* is dispersed throughout the Northern Hemisphere and in Africa, generally in riparian habitats along river drainages and flood plains (Rennenberg et al. 2010, Slavov and Zhelev 2010, De Rigo et al. 2016). The use of the poplar buds as a healing ointment with beneficial effects against inflammations was first mentioned in John Gerard’s book (1597) in Europe (Gerard 1974, Kis et al. 2020). Poplar plants are known for their ethnomedicinal uses in different parts of the world to treat fever, cold, stomach pain, wounds, etc. (Guleria et al. 2021). They also have properties of antimicrobial, anti-inflammatory, anti-oxidative, anti-rheumatic, antiseptic, astringent, and diuretic. Some poplar species (e.g., *P. balsamifera*, *P. tremuloides*, and *P. fremontii*) were used in treating skin disorders, wounds, and respiratory disorders by several North American Indian tribes. The phenolic compounds that are present in poplar are responsible for some of these properties because phenolic compounds are known for their antimicrobial activity by inhibiting cytoplasmic membrane function, nucleic acid biosynthesis, and antioxidant activity (Tyśkiewicz et al. 2019, Guleria et al. 2021, Zhang et al. 2022b).
The poplar plants are often tall with either a branched or a straight stem (Cervera et al. 2005), and most of them are faster-growing compared to most other tree species. Their leaves vary from being narrow or lanceolate to oblong, deltoid, heart-shaped, longer than wide, wider than long, or equal in both dimensions (Eckenwalder 1996, Tebbi and Debbache-Benaida 2022). The large dense root system of the plant ensures efficient uptake of groundwater and nutrients (Guleria et al. 2021). Poplar is often propagated from cuttings, which quickly initiate adventitious roots (De Almeida et al. 2017).

Poplars are also known for their commercially useful properties, such as their vegetative propagation capability, fast growth, multiple short rotations, and high biomass yield (Douglas 2017). These characteristics of poplar species such as *P. trichocarpa, P. balsamifera, P. deltoids, P. nigra*, or suckering roots of aspens such as *P. tremula* allow them to be suitable for experimental or commercial applications or experiments in growth chambers, greenhouses or in the field (Guleria et al. 2021).

Poplar plants are extensively cultivated around the world for the pulp and paper industry, veneer, wood products, lumber, energy, reforestation of lowlands, etc. (Balatinecz et al. 2001, Rishi et al. 2001, Stolarski et al. 2020). The faster-growing habit, a widespread root system, and relatively high transpiration rates make poplars suitable for phytoremediation as well as bio-indicators for ozone pollution in the environment (Rugh et al. 1998, Pietrini et al. 2009, Bryant et al. 2020, Liang et al. 2023).

Given all the properties of poplars, they are highly suitable for phytoextraction from deeper parts of the soil. Their above-ground biomass/canopy facilitates higher heavy metal (HM) accumulation than herbs and shrubs. Their dense, deep root system prevents soil erosion (Yadav et al. 2010). Some poplar species' root system also acts as a barrier preventing HM contamination
from spreading to the surrounding environment. Studies have shown that poplars are suitable for the uptake or binding of several HMs, including lead (Pb), cadmium (Cd), mercury (Hg), nickel (Ni), copper (Cu), and zinc (Zn) \cite{Algreen2014, Ashraf2019}. Furthermore, genetic manipulation approaches with heavy metal-binding protein genes can further enhance their remediation capability and increase clean-up efficiency \cite{Yadav2010, Algreen2014, Aghaalikhani2017, Liang2023}.

Nitrogen (N) is one of the most abundant elements on Earth. Dinitrogen gas (N\(_2\)) makes up approximately 78\% of the atmosphere and exists primarily in the gaseous form \cite{Chanway2014, Sethi2023}. Atmospheric N must be reduced to ammonia (NH\(_3\)) to become biologically available, which can then be assimilated into various essential biochemicals. Since N\(_2\) possesses a highly stable triple bond (N≡N) that needs immense amounts of energy to break, it is largely inaccessible to living creatures, except to nitrogen-fixing bacteria like \textit{Rhizobium}, \textit{Klebsiella}, \textit{Frankia}, and Cyanobacteria \cite{Bloom2015, Sethi2023}.

As one of the essential mineral nutrients for plant growth and biomass production, N is involved in the synthesis of amino acids (AAs), proteins, nucleic acids, lipids, chlorophyll, and various other N-containing metabolites in all living organisms \cite{Kusano2011, Lee2023}. The element that is required by organisms in significant amounts, except for carbon (C), oxygen (O), and hydrogen (H), is N; it constitutes 1-5\% of the dry matter in plants \cite{Epstein1853, Bloom1853, Hawkesford2012, Kirkby2023}. The overall amount and types of N available to plants by roots throughout their life cycle impact their growth and environmental interactions \cite{Andrews2013, Rehman2023}. As plants require N in more significant quantity N deficiency is a limiting element for plant growth and development \cite{Cameron2013, Canovas2018, Goncalves2020}.
The N cycle (Fig. 1) is a repeating cycle of processes during which N moves through both living and non-living things: the atmosphere, soil, water, plants, animals, and bacteria. In the atmosphere, N exists as N₂, but in the ground, it exists in its oxidized forms, e.g., as nitrogen oxide (NO), nitrogen dioxide (NO₂), and nitrate (NO₃⁻). Nitrogen fixation is the process of converting atmospheric N₂ into various biochemical forms through energy-releasing abiotic processes such as lightning, forest fires, and volcanic activity. The N oxides produced in the atmosphere dissolve in the rain and descend to the ground as NO₃⁻ or NH₃ molecules (Bezdicek and Kennedy 1998).

Most plants can use organic N, NH₄⁺, and NO₃⁻ as N sources, and their uptake is determined by the availability of these compounds in the soil solution (Bloom 2015). Some plant species can also use atmospheric N through a symbiotic relationship with specific bacteria of the genus Rhizobium (Shridhar 2012, Lindström and Mousavi 2020). The various forms of N present in the soil, their complexity and diversity, their chemical and physical properties, and the different ways plants adapt to absorb them all influence the intricate nature of plant N nutrition (Chanway et al. 2014, Bloom 2015). During the N assimilation process, NO₃⁻ must first be reduced to NH₄⁺ by nitrate reductase. Conversion of NO₃⁻ to NH₄⁺ takes place within the chloroplast (O'Leary and Plaxton 2015). The preferred source of N for plants is NH₄⁺, given that its uptake is less energetically demanding than that of NO₃⁻ because it does not require reduction before incorporation into AAs and proteins (Xu et al. 2012, Zou et al. 2012, Wu et al. 2019). The activity of microbial communities and the soil redox potential is significantly influenced by the release of oxygen (O₂) and root exudates in the rhizosphere (Näsholm et al. 2009, Muratore et al. 2021). Evidence shows that the absorption of NH₄⁺ or NO₃⁻ by roots frequently results in acidification or alkalization of the rhizosphere (Marschner 1995). Rice roots in paddy soils, for example, release
O₂ via their aerenchyma and create fast nitrification on their surface, allowing them to take up N as NO₃⁻ at a pace equivalent to NH₄⁺ absorption (Kirk and Kronzucker 2005, Li et al. 2008). Soil NO₃⁻ and NH₄⁺ concentrations could range from <100 µM to >10 mM (Miller et al. 2007). To cope with this range of variations in soil NO₃⁻ and NH₄⁺, plant roots contain absorption mechanisms for both NO₃⁻ and NH₄⁺, which are made up of constitutive and NO₃⁻-inducible components and are influenced by root architecture as well as the activities of NO₃⁻ and NH₄⁺ transporters (Miller et al. 2007). Many membrane proteins aid in NO₃⁻ absorption,
compartmentation, translocation, and remobilization (Glass 2003, Garnett et al. 2009). Two \( \text{NO}_3^- \) transport systems, the NRT1 and NRT2 families, have been identified to act coordinately to uptake \( \text{NO}_3^- \) from the soil and transport it throughout the entire plant (Wang and Tsay 2011, Wang et al. 2020).

More than 98% of N found in soil exists within organic matter and is typically not directly available to plants. Only a small portion of this total N is in inorganic forms (Näsholm et al. 2009). These inorganic forms are inter-convertible via biological processes involving soil microorganisms (Dechorgnat et al. 2011, Cameron et al. 2013). Inorganic N plays a prominent role in many cultivable soils, and many crop plants depend on inorganic N forms. The availability of N is a critical issue in crop productivity in cultivated soils (Khmelevtsova et al. 2022).

Because of the significance of inorganic N to the growth and development of plants, N fertilizers are widely used worldwide, despite their negative impacts on ecosystems and significant social and economic costs. Excessive use of N fertilizers to improve crop yield results in N pollution of ground and surface waters. The loss of N to the environment is a significant concern associated with increasing N fertilization. Many studies have demonstrated that up to 50% of the fertilizer applied to a plant through soil reaches that plant; the remaining N is lost to the environment in groundwater, surface water, and the atmosphere via microbial activity and volatilization (Celikkol Erbas and Guven Solakoglu 2017). Loss of \( \text{NO}_3^- \) by leaching and surface runoff is considered the dominant form of N that is lost. Under normal agricultural practices, about 6.7% to 19.0% of applied N is lost in different cropping years in cultivated lands, followed by \( \text{NH}_3 \) volatilization, another important N loss pathway in calcareous soils (Zhang et al. 2015).

Ammonium fertilizers and urea widely used worldwide can be easily transformed into \( \text{NO}_3^- \), the final oxidized form of inorganic N, especially under aerobic soil conditions such as
in dryland soils (Li et al. 2009). In agricultural soils, NO$_3^-$ is one of the most critical pollutants due to excess production and rapid movement in the field (Wang and Li 2019). Generally, NO$_3^-$ predominates in aerated soils, and when it is not absorbed by plant roots or utilized by microorganisms, it is available for leaching. Nitrate leaching occurs because NO$_3^-$ has a very weak affinity to form surface complexes with soil minerals (Strahm and Harrison 2006). In soil, NO$_3^-$ concentrations vary from 100 $\mu$m to approximately 20 mM, even reaching 70 mM leading to quick diffusion of NO$_3^-$ (Dechorgnat et al. 2011). Consequently, it can quickly disseminate to areas that have lower concentrations, such as the rhizosphere. Among the applied N fertilizers, NO$_3^-$ is the most common form of N, most readily lost to groundwater (Wang et al. 2018). Nitrate leaching threatens water quality as it can lead to NO$_3^-$ contamination, potentially causing eutrophication and compromising groundwater supplies (Powlson and Addiscott 2005, Barton and Colmer 2006, Romanelli et al. 2020).

More recently, there have been attempts to use foliar N applications as supplementary or sole source of fertilizer, which has various advantages, including reducing N losses due to denitrification and leaching, quicker nutrient utilization, and increased N availability when root activity is limited (Ishfaq et al. 2022). Proof of the physiological impact of nutrient absorption via leaves was demonstrated in the 19th century. For example, Gray’s work in 1843 showed how applying the nutrient solution to grapevines through their leaves could serve as an alternative method of fertilizer use (Fernández and Eichert 2009, Fernández and Brown 2013). Alongside this research, advancements were made in understanding the structure of leaf surfaces (Brongniart 1834, de Moura et al. 2023). During the second half of the 19th century, studies on gas exchange, transpiration, leaf anatomy, and physiology were published by several authors (Boussingault 1868, Merget 1873, de Moura et al. 2023). During the 20th century, scientists used a combination of
radioactive isotopes and electron microscopy techniques to lay the groundwork for foliar fertilization research (Mocellin 2004, Fernández and Eichert 2009).

One way to promote optimal plant growth and development is by spraying a solution containing one or more nutrient elements directly onto their leaves (White et al. 2015, Ruiz-Navarro et al. 2019). This allows for the distribution of nutrients throughout the rest of the plant (Mocellin 2004, Fernández and Brown 2013). This fast, efficient, and effective approach to combat plant malnutrition and nutrient loss; provides nutrients to plants more readily than traditional soil application via the roots (Fageria et al. 2009, Malhotra et al. 2020, de Moura et al. 2023). Frioni et al. (2021) evaluated the effect of foliar and soil application of an extract of Ascophyllum nodosum, a brown alga, on vines subjected to continuing water stress. These authors found that the two forms of treatment had contrasting results, with foliar spraying being more effective than soil application, protecting the integrity of the photosynthetic apparatus and rapidly restoring the physiological function of the leaf during the rehydration period. Similarly, Zhou et al. (2021) studied the effect of foliar and soil applications of selenium (Se) and silicon (Si) to reduce cadmium (Cd) toxicity in wheat varieties (Triticum turgidum L.). Soil application of Si and Se effectively controlled Cd concentrations in both types, while the foliar method succeeded for only one variety. To reduce the impact on the environment caused by soil N fertilization, it is vital to have a clear understanding of the impact of foliar N fertilization and how the foliar applied N is metabolized in the plant.

Although foliar fertilization has benefits, it can take time to predict how plants respond due to various factors. These factors include the specific plant species, composition of the leaf cuticle, application timing, phenological aspects, and environmental conditions (Portu et al. 2015). For foliar uptake to be effective, it is crucial that the stomata remain open, and that the temperature is
not above room temperature, as this can lead to damage such as leaf burns. Furthermore, foliar applications are ineffective on windy and rainy days (Fageria et al. 2009). Del Amor and Cuadra-Crespo (2011) reported that in pepper plants (Capsicum annuum L., cv. Herminio), temperature influences the antioxidant response of the plant after foliar application of urea. The success of this fertilization technique may also depend on the timing of the spray (de Moura et al. 2023). A study on cucumber plants discovered that applying manganese (Mn) to the leaves can increase resistance against powdery mildew, a fungal disease caused by Podosphaera fuliginea. Eskandari and Sharifnabi (2020) working with cucumber discovered that applying manganese (Mn) to the leaves can increase resistance against powdery mildew, a fungal disease caused by Podosphaera fuliginea. They showed the most effect occurred when the interval between nutrient spraying and pathogen inoculation was the shortest.

It is important to consider that the rate at which nutrients are delivered depends on the type of N source. The main N type used as fertilizer in soil and foliar applications in agriculture worldwide is urea. Besides acquisition from the environment, urea can also accumulate in plant cells as a consequence of secondary N metabolism (Mérigout et al. 2008).

It has been established that in soils that have been cultivated or disturbed and are well aerated, NO$_3^-$ is the foremost form of N that is available to and taken up by most vascular plants (Dong et al. 2002, Hachiya et al. 2012, Cánovas et al. 2018). The majority of the NO$_3^-$ is translocated to the shoots, where it is first reduced to NO$_2^-$ by nitrate reductase in the cytoplasm and then to NH$_4^+$ by nitrite reductase (NiR) in the plastids and glutamine synthetase (GS) in both the plastids and cytoplasm. The GS/GOGAT (glutamine-2-oxoglutarate aminotransferase) cycle converts NH$_4^+$ generated from NO$_3^-$ or directly from NH$_4^+$ absorption into AAs.
Ammonium fertilizers deliver nutrients quicker than urea, but NO$_3^-$ is the most efficient and speedy option (Shi-Wei et al. 2007). In addition, when it comes to leaf burn, there is a limit to the amount of N that can be applied without causing harm, especially in moderate climates with elevated temperatures during application (Woolfolk et al. 2002, Blandino et al. 2020). The burning of leaves due to foliar applications of urea N is primarily linked to the presence of biuret (a compound made from the condensation of two urea molecules), a toxic substance for crops (Mikkelsen 1990). The accumulation of NH$_4^+$ leading to NH$_4^+$ toxicity compared with NO$_3^-$-fed plants was observed in peas, lettuce, spinach, lupine, tomato, wheat, A. thaliana, and also for M. truncatula plants which were subjected to spray with high NH$_4^+$ concentrations (Cruz et al. 2006, Ariz et al. 2011, Sarasketa et al. 2016, Vega-Mas et al. 2019).

Plant nutrition primarily relies on NH$_4^+$ as the most abundant source of available N, despite its lower soil concentration than NO$_3^-$ (Marschner 2011). Toxicity symptoms typically develop in plants grown in soils with high levels of NH$_4^+$ but without NO$_3^-$, except for plant species that thrive on NH$_4^+$ nutrition, such as rice, blueberry, cranberry, onion, and leek (Britto and Kronzucker 2002). Phenotypic symptoms of NH$_4^+$ toxicity include reduced plant growth, changes in root architecture, decreased root/shoot ratio, and leaf chlorosis (Li et al. 2014, Liu and von Wirén 2017, Xiao et al. 2023). Other effects of excess NH$_4^+$ include inhibition of the uptake of cations, e.g., K$^+$, Mg$^{2+}$, or Ca$^{2+}$, which cause alteration in plant ion imbalance, intra-cellular alkalinization, and extracellular acidification, inhibition of root respiration and stimulation of photorespiration, interference with photosynthetic activity, increased oxidative stress, altered expression/activity of NH$_4^+$ assimilating enzymes, disruption of hormonal homeostasis, and high energy cost to maintain low levels of cytosolic NH$_4^+$ content (Kronzucker et al. 1996, Britto and Kronzucker 2002, Cruz et
The symptoms displayed by the plants are collectively known as ‘ammonium syndrome’ (Xiao et al. 2023).

To prevent toxicity, plants must balance the absorption and utilization of NH$_4^+$ (Bittsánszky et al. 2015). The plant's initial reaction to elevated NH$_4^+$ varies depending on the plant type. In NH$_4^+$-sensitive plants, such as *Arabidopsis*, shoots tend to be the most sensitive part of the plant to NH$_4^+$ (Domínguez-Valdivia et al. 2008, Li et al. 2014). Since roots are the first NH$_4^+$ sensor (Drath et al. 2008), alteration of the root system architecture may be the initial signal of NH$_4^+$ toxicity (Domínguez-Valdivia et al. 2008, Pan et al. 2016). In roots, common symptoms include shorter primary root, inhibition of root elongation, embracing primary and lateral roots, the stimulation of lateral root branching, changes in the insertion of lateral roots in the main root (Domínguez-Valdivia et al. 2008, Li et al. 2010, Rogato et al. 2010, Pan et al. 2016), and a loss of gravitropism (Zou et al. 2012). Research on *Lotus japonicus* using split-root systems revealed that NH$_4^+$-induced changes specific to roots are facilitated by NH$_4^+$ transport (Rogato et al. 2010).

It is widely known that for plants to absorb N, they require both ATP and C skeletons. This is because the metabolic pathways of C and N are closely linked at a fundamental biochemical level. The partitioning of assimilated C between the synthesis of organic acids, starch, and sucrose is noticeably affected by N availability (Gutiérrez et al. 2007, Nunes-Nesi et al. 2010, Hennion et al. 2019, He et al. 2022). In plants, starch has been found to correlate with protein content as an integrator of overall biomass production (Sulpice et al. 2009). The assimilation of N is vital for not only photosynthesis but also photorespiration and respiration.

Photosynthesis metabolism plays a crucial role in N assimilation. A significant quantity of N is required for photosynthesis, especially in the 1,5-bisphosphate carboxylase-oxygenase (Rubisco) and light-harvesting complexes (Zhu et al. 2008, Evans and Clarke 2019). Alternatively,
the assimilation of inorganic N requires energy, ATP, and C skeletons from photosynthesis products and intermediates (Stitt and Krapp 1999, Kelly 2018). Carbon fixed in the leaves is used directly and indirectly (after transfer to roots) for AA biosynthesis (Oaks and Hirel 1985, Tränkner et al. 2018). There is a direct positive correlation between carbohydrate levels and the ability of leaf systems to reduce NO$_3^−$. When plants experience N deficiency, there is a notable increase in carbohydrate levels (Oaks and Hirel 1985), and leaf N assimilation rates are much higher in the presence of light than in the dark (Matt et al. 2001). To summarize, leaves use photosynthesis to convert CO$_2$ into carbohydrates, influenced by stomatal conductance, mesophyll conductance, and photosynthetic metabolism. (Gago et al. 2019). After fixation, as much as 50 – 80% of the carbohydrates are exported from the leaves to roots via phloem as sucrose (Ainsworth and Bush 2011, Braun et al. 2014).

The interaction between photosynthetic C and N metabolism in leaves is also impacted by photorespiration due to the dual affinity of Rubisco for O$_2$ and CO$_2$ in C3 plants (Bauwe et al. 2010). Elevated CO$_2$ concentrations were induced in photorespiration, reducing NADH levels available for NO$_3^−$ reduction and NH$_4^+$ usage (Dutilleul et al. 2005). Furthermore, higher atmospheric CO$_2$ competes with the reductant (e.g., NADPH) in the chloroplast stroma, affecting NO$_3^−$ assimilation. As the released CO$_2$ and NH$_4^+$ are reassimilated by Rubisco and the GS/Fd-GOGAT system, respectively, photorespiration represses photosynthesis efficiency (Nunes-Nesi et al. 2010).
Polyamines (PAs) are a group of low molecular weight, polycationic, aliphatic amines involved in numerous metabolic processes in all living organisms (Nahar et al. 2016, Wuddineh et al. 2018, Paschalidis et al. 2019, Parrotta et al. 2023). These include putrescine (Put, a diamine), spermine (Spm, a tetramine), and spermidine (Spd, a triamine), (Fig. 2). Besides Spm, Spd, and Put; there are other PAs such as cadaverine, nor-spermidine, nor-spermine, caldo-pentamine, and thermospermine (Ali et al. 2020). Polyamines play multiple roles in the growth and development of all living organisms. Polyamines are found in all cells and have a variety of essential roles due to their simple structure and involvement in a multitude of physiological activities (Smirnova et al. 2018). Since PAs are rich in N, they are a promising group to investigate for their biological significance (Minocha et al. 2014, Ali et al. 2020).

It is typical for plants to adjust PA cellular contents in response to various forms of abiotic and biotic stressors, e.g., salinity, drought, HMs, and high- and low-temperature stresses (Alcázar et al. 2020). Extensive research has been conducted on the PA biosynthetic pathway in plants,
which differs from the pathways in animals (Talaat et al. 2015). Amino acids like arginine (Arg), ornithine (Orn), and methionine (Met) are the precursors of PA biosynthesis (Majumdar et al. 2013). In plants, Arg and Orn are converted to Put through Arg decarboxylase (ADC; EC 4.1.1.19) and ornithine decarboxylase (ODC; EC 4.1.1.17), respectively. Putrescine is converted to Spd and Spm by sequential additions of aminopropyl groups from decarboxylated S-adenosyl-Met (dcSAM – which is the product of SAM using SAM decarboxylase; EC 4.1.1.50) by the enzymes spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.16), respectively (Majumdar et al. 2013, Pál et al. 2021) (Figure 3). It is known that the Arabidopsis genome lacks the gene for encoding ODC and thus make all Put via ADC (Hanfrey et al. 2001, Bhardwaj et al. 2023). Lastly, PA synthesis may vary between tissues and organs. For example, the shoot apical meristem of tobacco (Nicotiana tabacum) is the predominant site of Spd and Spm biosynthesis, while Put is primarily synthesized in roots (Moschou et al. 2008, Moschou et al. 2012). Apart from their universal role in cell development and growth, PAs carry a net positive charge; hence, they can interact with DNA, RNA, ATP, specific proteins, and phospholipid heads of cellular membranes (Ghosh et al. 2017). These interactions allow them to play crucial roles at the molecular level, like regulation of transcription and translation, thus regulating cell division, elongation, and differentiation (Kuznetsov et al. 2006, Handa and Mattoo 2010, Lenis et al. 2017).

As N-rich compounds, PAs and AAs have been observed to increase in abundance under N. The excess of N is stored in organic forms such as AAs and PAs as metabolism changes. In this way, NH$_3^+$/NH$_4^+$ toxicity will be reduced. Furthermore, under low N availability, it is possible to use the N from PAs for other critical uses (de Oliveira et al. 2018, Hou and Wu 2018, Jian et al. 2018). Perennial plants like poplars use external N and internal metabolic processes to recycle N and maintain N homeostasis via PAs (Rennenberg et al. 2010, Cánovas et al. 2018).
Over the years, there have been various studies done to understand the relationship between PAs and abiotic stress in plants, suggesting that PAs function in adaptive responses to various environmental stresses (Galston and Sawhney 1990, Alcázar et al. 2010, Mattoo et al. 2010, Alcázar et al. 2011, Tiburcio et al. 2014, Alcázar et al. 2020). The first report on Put accumulation under K deficiency was reported decades ago (Richards and Coleman 1952). Sometimes, all three common PAs (Put, Spd, and Spm) significantly increase in abundance in response to abiotic stress (Yang et al. 2007); however, in most cases, only one of the three PAs shows a significant increase. An instance of this is the response of apple callus, where Put was elevated, while Spd and Spm levels only showed minimal alteration (Liu et al. 2006). Under salt and cold stress, the Spd content of sweet orange callus increased significantly (Liu et al. 2006), and following salt stress, there were a substantial accumulation of Spd and Spm in grape (Vitis vinifera) plants (Ikbal et al. 2014).

Recent studies indicate that PAs are an integral part of signaling systems in plants (Nahar et al. 2016). Numerous studies have shown that transgenic overexpression of the PA biosynthetic genes is a highly effective method for increasing the naturally occurring PA levels and enhancing the plant’s ability to withstand stress (Liu et al. 2015). Higher PA production also increases cell N and C utilization, thus increasing the total biomass (Majumdar et al. 2016). It appears that PAs may boost the production or stimulation of endogenous hormones and promote protein synthesis (Handa and Mattoo 2010). According to Chen et al. (2011), the intake of N (as NO$_3^-$ or NH$_4^+$) by plants positively impacts the accumulation of all AAs. Studies suggest that supplying crops with PAs exogenously can also enhance their ability to withstand abiotic stress, e.g., drought (Kahlaoui et al. 2018), cold (Nayyar and Chander 2004), salinity (Ndayiragije and Lutts 2006), and HMs (Wang et al. 2007). Furthermore, the transformation with PA-biosynthetic genes such as ODC, ADC, SPDS, or SAMDC is associated with improved tolerance of plants to stress (Liu et al. 2007).
The current findings demonstrate that stress response often involves the upregulation of the PA biosynthetic genes - *ADC*, *SPDS*, *SPMS*, and *SAMDC*; however, there are differences in the timing and degree of their induction ([Liu et al. 2006], [Mohapatra et al. 2009], [Liu et al. 2015], [Majumdar et al. 2015]). The *ADC* and *ODC* gene expression has been extensively studied in various plants and is known to play a significant role in responding to stress ([Urano et al. 2004], [Liu et al. 2006], [Wang et al. 2007], [Wang et al. 2011b], [Liu et al. 2015]). Based on the research conducted on wheat, maize, rice, and *Arabidopsis*, it seems that Spd plays the most significant role as a PA in leaves, followed by Spm and Put, while in the roots, the order is: Spd ≥ Put > Spm ([Pál et al. 2014], [Bányai et al. 2017], [Tajti et al. 2019], [Pál et al. 2021]).
Because AAs play a central role in cellular metabolism, stress (abiotic and biotic), and N balance between organs of organisms, multiple AAs sensing mechanisms have been reported to exist that monitor the levels of AAs suggesting that changes in amino acid levels via altered AAs metabolism or transport might trigger pleiotropic responses within cells (Besnard et al. 2021 and references therein). In Arabidopsis stress caused the degradation of highly abundant proteins such as subunits of photosystems and ribosomes causing the accumulation of free AAs Batista-Silva et al. (2019). In addition, low abundant AAs (branched-chain AAs (Lysine (Lys), Methionine (Met), Histidine (His)) and aromatic AAs (phenylalanine (Phe), tyrosine (Try), and tryptophan (Trp)) were further broken down during this process which indicated their usage as an alternative respiratory substrate to compensate for the decreased photosynthesis. Using the metabolomics approach, Xu et al. (2022) identified shared and unique metabolic signatures for various types of stress factors (e.g. cold, heat, drought, and highlight) that included changes in AAs metabolism, sugar metabolism, glycolysis, TCA cycle, GABA shunt, glutathione metabolism, purine metabolism, and the urea cycle in Arabidopsis. Increases in cysteine accompanied by a reduction in glutathione under different stress treatments pointed to the management of oxidative stress as a general phenomenon in abiotic stress in this study. A non-protein AAs, citrulline, has also been shown to act as an osmolyte under abiotic stress conditions in watermelon and many other plant species as described in Song et al. (2020). Another uncommon AAs, Orn has also been assigned a regulatory role in PA metabolism (Majumdar et al. 2013, Majumdar et al. 2015)
CHAPTER 1: EFFICACY OF FOLIAR APPLICATION OF DIFFERENT FORMS OF NITROGEN AND STUDY ITS PHYSIOLOGICAL AND BIOCHEMICAL EFFECTS OF POPLAR CLONE NM6 (*POPLUS NIGRA* L. X *P. MAXIMOWICZII* A. HENRY)

Abstract

We examined the effects of foliar supplementation with three nitrogen sources (urea, NH₄NO₃, and CoRoN) at two different concentrations each to evaluate their efficacy as spray fertilizers for the growth of hybrid poplar cultivar NM6 (*Populus nigra* L. *x P. maximowiczii* A. Henry;) that were nitrogen starved to achieve two levels of initial nitrogen status. Our goal is to reduce nitrogen fertilizer use and loss of nitrogen to the environment making it more economical and environment-friendly for cultivated forestry practices. Our objectives were to determine if: (1) Foliar nitrogen application is an effective method of fertilization for poplar cultivars; (2) Different nitrogen sources are metabolized similarly by the plants; and (3) If initial nitrogen status makes a significant difference in the outcome of these efforts. The analyses involved free polyamines, amino acids, soluble protein, chlorophyll content, gas exchange in the foliage, and overall plant biomass. The plants were started from vegetative cuttings grown in the UNH greenhouse facility. The data showed that (a) the effects of foliar nitrogen application on leaf chemistry, biomass, and foliar nitrogen content varied according to the form and concentration of nitrogen source used, (b) different nitrogen sources are metabolized differently by poplar plants, (c) the initial nitrogen status of plants did affect the outcome of foliar nitrogen application.
Introduction

During the past decades, the negative impact of unsustainable agricultural practices on climate change via increased greenhouse gas emissions and excess fertilizer usage has become evident (Robertson and Vitousek 2009, Popp et al. 2014, Malhi et al. 2021, Yan et al. 2022). As the global population (currently 7.6 billion) is expected to increase to approximately 9.8 billion by 2050 and to 11.2 billion by 2100 (UN Department of Economics and Social Affairs - https://www.un.org/development/desa/pd/content/World-Population-Prospects-2022/, the global fertilizer demand, particularly for nitrogenous fertilizers, is expected to increase to feed the increasing population. The main N fertilizer being used today is based on industrially produced synthetic ammonia (NH$_3$) by the Haber-Bach process. Between 1950 and 2008 the amount of synthetic N fertilizer applied in the soil increased from approximately 10 billion kgs of N year$^{-1}$ to approximately 100 billion kgs of N year$^{-1}$ (Robertson and Vitousek 2009). However, half or more of the reactive N in the fertilizers is lost to the surrounding environment causing unintended adverse environmental and health impacts as well as economic loss (Li et al. 2018). Inappropriate excess N application to increase crop production leads to loss of N from agricultural soils causing a decline in soil quality, groundwater pollution, and eutrophication of freshwater and marine ecosystems (Cameron et al. 2013, Celikkol Erbas and Guven Solakoglu 2017, Henryson et al. 2020, Menegat et al. 2022). Furthermore, the release of atmospheric greenhouse gases such as nitrous oxide (N$_2$O) and ammonia (NH$_3$) leads to pollution of the atmosphere (Zhang et al. 2015, Liu et al. 2023). Hence, there is a strong urgency to improve nitrogen-use efficiency (NUE) to decrease environmental degradation while increasing crop productivity for sustainable agricultural practices.
Compared to soil application of fertilizer, foliar application of fertilizer can deliver nutrients directly to the aerial plant parts, thereby reducing soil N exposure (Pantoja-Benavides et al. 2021a). Additionally, through foliar fertilization plants can efficiently receive a targeted amount of nutrients to reduce the negative impact on the environment, and enhancing human health will be an added benefit (Fernández and Eichert 2009). Many factors influence the effectiveness of the absorption rate of the nutrients applied via foliar spray. Some of these factors are uptake and availability rate, reduced phytotoxicity, deficiency correction capability, rate of metabolism in the leaves, and effect on yield and quality parameters (Hu et al. 2023 and references therein).

Studies using $^{15}$N tagged N-fertilizer revealed (1) that the quantity of $^{15}$N was higher in foliar-treated grape seedlings compared to soil-fertilized plants (Sun et al. 2017); and (2) foliar application increased the uptake rates of soil-applied nutrients (Niu et al. 2021 and references therein). A review by Mahil and Kumar (2019 and references therein) described studies in which fertilizers were applied to foliage as nanoparticles in wheat and watermelon and that the nanoparticles were taken up in the phloem tissue of the leaves. These authors also mentioned that in some other studies foliar application of nanoformulations of N, phosphorous (P), potassium (K), and micronutrients increased the plant height, number of branches, and overall yield in black gram and chickpeas as well. Foliar application of various concentrations of urea induced an increase in net photosynthetic rate and stomatal conductance in bean plants (Hassan et al. 2015). Because the nutrients are utilized faster, foliar fertilization also provides a rapid method to cure nutrient deficiencies other than N. However, so far, most of the studies using foliar N spray are limited to crop plants.

Because of their capacity for fast growth and high biomass yield, poplar plants have high nutrient requirements (Dillen et al. 2011, Zhu et al. 2023). Nitrogen is generally considered the
most limiting nutrient in poplar plantations (Rennenberg et al. 2010). Thus to obtain poplar plants with more biomass, N fertilization of the poplar plantations is necessary (Dong et al. 2004, Yan et al. 2023). A study on the foliar application of urea has shown that foliar application in autumn at the end of canopy growth, increased the N content in poplar stock plants, independent of N supplied during the growing season (Dong et al. 2004). This study also showed that N obtained by the plant from foliar urea application was more easily mobilized than N taken up from the roots at the beginning of the experiment. Not many attempts have been made with foliar application of N to poplars since this study.

Nitrate (NO$_3^-$) and ammonium (NH$_4^+$) are the most common forms of N used by plants, with NO$_3^-$ being the preferred source in poplar (Nunes-Nesi et al. 2010, Gagne et al. 2019). Although N is an important element for plants, carbon (C) is the major component of plant biomass (Nunes-Nesi et al. 2010). As with N, C also plays a key role in photosynthesis whose products such as glucose, ADP, NADP$^+$ are critical for N assimilation (Cui et al. 2019, Kasemsap and Bloom 2023). Furthermore, the splitting up of C between organic acids, starch, cellulose, lignin, sugars, and proteins is also affected by the availability of N as well as C to the plant (Royer et al. 2013).

Although the N assimilation pathways from the roots have been well characterized, not much progress has been made to understand the metabolic pathway of plants following the agrochemical spraying to the aerial parts of crop plants (Fernández and Brown 2013). Generally, N acquired by the plant through foliar use a similar pathway where NH$_4^+$ is assimilated through Glutamine (Gln) (Castro-Rodríguez et al. 2011, Luo et al. 2013) or Aspartate (Asp)-Asparagine (Asn) (Cánovas et al. 2018) as initial metabolites. The predominant AA in poplar plants that is
involved in N transport and remobilization from leaves is Gln (Sauter and van Cleve 1992, Babst and Coleman 2018).

A suite of select metabolic markers is a useful tool for profiling and monitoring the health of various plants including trees by detecting physiological and biochemical changes induced by biotic and abiotic stress including nutrient deficiencies and surplus (Mashabela et al. 2023). This tool focuses on the use of building block primary metabolites that are required for growth and/or signaling metabolites such as soluble polyamines (PAs), soluble AAs, carbohydrates, and small organic acids.

The major goal of our current study was to evaluate whether foliar spray of N is an effective method of N supply for hybrid poplar by evaluating changes in concentrations of PAs, AAs, chlorophyll, total soluble proteins, carbon (C) and N assimilation, and growth (biomass) in poplar plants upon N fertilization. The expected outcomes of the study were to: (i) demonstrate the ability of the plants to assimilate N via leaves, (ii) to compare the effectiveness of four different forms of N being used as foliar spray. This approach, if successful, could reduce the application of large amounts of N in the soil to reduce the cost as well as environmental pollution. We hypothesized that foliar application of N fertilizer is an effective method to apply N fertilizer, and different chemical forms of N will be utilized differently by the plants.
Methods

To understand the biochemical responses of the hybrid poplar (*Populus nigra* L. *x P. maximowiczii* A. Henry) to foliar application of three forms of N, three experiments (experiment 1, experiment 2, and experiment 3) were conducted over two years. The results and discussion for experiment 2 and experiment 3 are presented in chapter 1. Experiments 1 & 2 were conducted in the summer of 2019, whereas experiment 3 was conducted in the summer of 2021. Modifications to the experimental design were made in experiments 2 and 3, following what we learned from experiment 1.

To study the biochemical responses of the hybrid poplar (*Populus nigra* L. *x P. maximowiczii* A. Henry) to Pb heavy metal exposure and foliar application of Put, an experiment was conducted in 2021. The results and discussion of this experiment are presented in chapter 2.

Most of the analyses performed were similar in both experiments. Hence, this section includes the methodology for the analyses for both chapters.

Plant material

The dormant stem cuttings of hybrid poplar (*Populus nigra* L. *x P. maximowiczii* A. Henry; (Clone NM 6) were grown at the University of New Hampshire McFarlane research greenhouse facility. This study used young cuttings from a 5-year-old healthy NM6 tree at 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 two weeks in grow tubes (17.78 cm long) containing PRO-MIX soil by Mycorrhizae (Premier Tech Horticulture, Quakertown, PA, USA). The grow tubes were placed in a tray to hold the extra water and moisten the soil. After two weeks, the plants were
transferred to pots (19 cm diameter x 15 cm height) and filled with vermiculite and perlite (1:1) (Whittemore Company, Lawrence, MA, USA), with each pot having only one plant (Fig. 4). The cuttings were planted in soil to a 5 – 7 cm depth. The pots were spaced about 5 cm apart on a bench in a staggered fashion and were grown for 45 days (Fig. 5). All plants chosen for this study had a single stem showing no axillary branches.

The experiments were conducted from June to September 2019 in the greenhouse. The greenhouse had natural sunlight with a 16-hour photoperiod. The average temperature ranged between 22 ºC to 24 ºC, and relative humidity was ~64% in the greenhouse till the end of the experiment. In this study, the plants once transferred to larger pots 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) for about six weeks (Fig. 6). The plants were regularly irrigated with 200 mL water at 8 AM and again at 2 PM during the starvation period. Experiments 1 and 2 started in the third week of August. To summarize, these plants were kept on the maintenance media for over a month after the transfer to larger pots and before two-week starvation period. Thus, they were much larger and greener compared to plants in experiment 3 described below.

Fully grown plants were deprived of fertilizer for two weeks prior to the treatments. During the experiment, the plants were fertilized with an N-free fertilizer mix (Table 1). It was given once a week in two doses of 200 ml each in the morning (8 AM) and afternoon (2 PM) via roots. The drip lines were closed at 2 PM the day before applying fertilizer and opened at 2 PM the same day after applying fertilizer. The volume was decided based on having no leakage out of the pots. The plants were selected randomly for treatments, with six replicates for each treatment. Throughout the treatments, the water controls received only water without fertilizer.
Figure 4. Plants at the time of transfer to the larger pots

Figure 5. Arrangement of pots on the bench during the experiment(s). The plants shown in this picture are larger and greener and were used in experiments 1 and 2.
Laboratory media preparation

Three forms of N fertilizers were used as foliar spray. They were NH₄NO₃ (Easy Peasy Plants, Inc, Alvin, IL, USA), urea (46-0-0) (Easy Peasy Plants, Inc, Alvin, IL, USA), and CoRoN (28-0-0 – Helena Professional Products, Collierville, TN). CoRoN is a unique foliar fertilizer that contains polymethylene urea solution [https://www.helenaagri.com/products/coron-category/coron-2/]. Each was used at different concentrations, as shown in Table 2. Some scorching on the leaves was noticed after the 1st spray with the higher concentration (1%) in experiment 1. Hence it was decided to use the lower concentrations (0.5%, 0.25%, and 0.125%) for experiment 2. The foliar N spray solutions included 0.05% (v/v) Silwet L-77 surfactant (Phytotechnology Laboratories, Lenexa, Kansas City, USA). After spraying on the leaves, they were gently tapped with a hand manually to get rid of the extra spray solution sitting on the leaves. Equal quantity of water containing Silwet was sprayed to the control plants.
Table 1. Nutrient mix (without N) used for fertilization during the duration of the study.

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Molecular weight (gmol⁻¹)</th>
<th>Element</th>
<th>Atomic weight (gmol⁻¹)</th>
<th>ml of 1 M stock for 100 plants/week (given daily) at 10X concentration given as part of maintenance medium without N</th>
<th>ml of 1 M stock for 100 plants/week at 10X concentration (given once a week at 0.5X of normal concentration) during the one-month long duration of the study</th>
<th>Final amounts prepared for 100 plants (400 ml) given to plants/week</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>74.55</td>
<td>K</td>
<td>39.10</td>
<td>148.00</td>
<td>74.00</td>
<td>740.00</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>147.01</td>
<td>Ca</td>
<td>40.08</td>
<td>186.86</td>
<td>93.43</td>
<td>934.32</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>120.37</td>
<td>Mg</td>
<td>24.31</td>
<td>154.07</td>
<td>77.03</td>
<td>770.33</td>
</tr>
<tr>
<td>KH₂PO₄* for K calc</td>
<td>136.09</td>
<td>K</td>
<td>39.10</td>
<td>936.10</td>
<td>468.05</td>
<td>4680.51</td>
</tr>
<tr>
<td>FeEDTA</td>
<td>367.05</td>
<td>Fe</td>
<td>55.85</td>
<td>3.80</td>
<td>1.90</td>
<td>18.98</td>
</tr>
<tr>
<td>KH₂PO₄* for P calc</td>
<td>136.09</td>
<td>P</td>
<td>95.00</td>
<td>105.26</td>
<td>52.63</td>
<td>526.32</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>197.91</td>
<td>Mn</td>
<td>54.94</td>
<td>1.93</td>
<td>0.97</td>
<td>9.66</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>61.83</td>
<td>B</td>
<td>10.81</td>
<td>3.93</td>
<td>1.96</td>
<td>19.63</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>287.50</td>
<td>Zn</td>
<td>65.38</td>
<td>1.62</td>
<td>0.81</td>
<td>8.11</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>159.60</td>
<td>Cu</td>
<td>63.55</td>
<td>0.33</td>
<td>0.17</td>
<td>1.67</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>241.90</td>
<td>Mo</td>
<td>95.95</td>
<td>0.22</td>
<td>0.11</td>
<td>1.11</td>
</tr>
<tr>
<td><strong>Sum of all nutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>1542.13</strong></td>
<td><strong>771.06</strong></td>
<td><strong>7710.63</strong></td>
</tr>
<tr>
<td><strong>Water</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>2457.87</strong></td>
<td><strong>3228.94</strong></td>
<td><strong>32289.37</strong></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>4000.00</strong></td>
<td><strong>4000.00</strong></td>
<td><strong>40000.00</strong></td>
</tr>
</tbody>
</table>
Table 2. Types and Concentrations of N Fertilizers used in experiment 1 and experiment 2 conducted in 2019, water without N was sprayed on the control plants.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fertilizer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>Urea</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>NH₄NO₃</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>CoRoN</td>
<td>0.5%</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Urea</td>
<td>0.25%</td>
</tr>
<tr>
<td></td>
<td>NH₄NO₃</td>
<td>0.125%</td>
</tr>
<tr>
<td></td>
<td>CoRoN</td>
<td>0.25%</td>
</tr>
</tbody>
</table>

Before the fertilizer application, the pot surface was covered with a plastic bag to prevent the fertilizer solution from reaching the soil. Each pot was carried to a separate spot away from the rest of the plants for the treatments. The foliar treatments were given using 500 ml plastic spray bottles (Zep commercial sales and service, Atlanta, GA, USA) with a spray spout, open halfway to control the pressure of the spray until the leaves appeared saturated. In a separate trial, the approximate volume of solution lost during the spray was measured using paper towels (Fig. 7) around the plant. The paper towels were spread around the area on the floor and the corner walls behind where the spray treatment was done and weighed before and after treatment. The amount wasted was calculated at an average of 60%, reducing the net use to 20 mL per plant. However, the amount lost to the air above the plant could not be estimated.
Figure 7. Experimental setup to measure the wastage from spray.

The foliar N-fertilizer spray was given twice during the experiment; once at the beginning, on day 1, and then on day 14. Experiment 1 and experiment 2 were conducted with the same number of plants and replications, only three days apart. The only difference in setup was the concentrations of N fertilizers described in Table 2. Each plant was sprayed ~50mL of the solution. Samples were collected for various analyses on days 0, 3, 8, 13, 17, 22, and 27 after the start of the N applications. On day 28, harvesting of the plants was done for each experiment.
In experiment 3, unlike experiment 2, the plants were left for N starvation (without any N fertilizing after transfer to larger pots) for ten days in the pots when only water was added. Thus, these plants were much smaller than the ones used in experiments 1 and 2 and were never fed with regular maintenance media containing N after transfer to larger pots. The experiment began in mid-July. Six plants were given 200 ml of regular fertilizer with N used in the greenhouse using the drip lines at 8 AM and 2 PM daily. These plants were excluded from root application of the lab-made nutrient mix without N. The experiment had nine treatments and six replicates per treatment (Table 3). The control plants were treated with a water spray and N-free lab-made fertilizer (Table 1), which was administered through the roots. The plants were given the spray treatment weekly until the end of the experiment (every 7th day from day 0). The spray treatment of 0.5% NH$_4$NO$_3$ was discontinued after two weeks due to severe scorching. Hence data are not available on day 17 of sample collection. After three weeks, the 0.125% and 0.25% NH$_4$NO$_3$
treatments were also stopped altogether. Samples were collected for various analyses on days 3, 6, 10, 17, 24, and 31 after the start of the N applications.

Since the cuttings were shorter and less mature than the ones used in the 2019 study, less volume of N needed to be sprayed. For this, a separate experiment was carried out using extra plants to determine the amount of N fertilizer that each plant should be sprayed with. These plants were sprayed with water containing 0.05% (v/V) Silwet L-77 surfactant. This experiment revealed that a total of 27 ml was needed to saturate the leaves. The experiment had nine treatments and six replicates per treatment (Table 3). The rest of the details for this experiment were similar to the ones described above for experiment 1.

![Punched Leaf](image)

**Figure 9. Picture of a punched leaf (leaves were punched, avoiding mid veins).**

Plant leaf samples were collected at zero time and after the treatments for biochemical analyses in experiments 2 and 3. In the experiment 2 leaves were collected, one each from near the top and bottom of the plant (Fig. 8). After punching (Fig. 9, see details below under tissue preparation), and the leaf discs were mixed thoroughly from top and bottom leaves before weighing for analyses. But in experiment 3, the leaves were only collected from the top 1/3 of the plant. As much as possible, the fully expanded and ‘similar in texture’ leaves were selected for the
first collection. The following fully expanded leaves closest to the top were chosen for the second, and the next were collected for the third collection. We had postulated here that the young leaves, which are very close to the apical shoot meristem, would grow in three weeks bearing the same structure as the 5th leaf from the apical meristem, and that did happen. In the experiment 3 root tissues were collected on day 31. 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 blotted on filter papers or paper towels were immediately put into 2 mL microfuge tubes containing respective solutions for further analysis.

Table 3. Nitrogen spray treatments, concentration, and different forms used for experiment 3 conducted in 2021.

<table>
<thead>
<tr>
<th>Different fertilizer treatments applied</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control with GH fertilizer with N</td>
<td></td>
</tr>
<tr>
<td>Control with pre-lab-made mix no N</td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>0.25%</td>
</tr>
<tr>
<td></td>
<td>0.125%</td>
</tr>
<tr>
<td>urea</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>0.25%</td>
</tr>
<tr>
<td>CoRoN</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>0.25%</td>
</tr>
</tbody>
</table>
**Tissue preparation**

The tissue sample collection was done in the morning and finished within two-to-three hours. All sample collections were made in the greenhouse. The leaf surface was cleaned with dry paper towels to remove any dust particles, or the spray solution used for the treatment. The leaves were punched using a paper puncher in a plastic weigh boat, avoiding the midvein and leaf edges (Fig. 8). Punched discs were thoroughly mixed before sample collections from the two leaves (top and bottom) of the same plant. At the end of the experiment, on day 27 (experiment 2), the plants were harvested for biomass analysis. Tissue was collected from above- and below-ground parts for biomass in experiment 2. Above ground parts of each plant were divided into three parts: top, middle, and bottom. Leaves and stems of all treatments were manually separated from the top, middle, and bottom and weighed for biomass. From each section, the number of leaf scars and petiole average weight were recorded. Using the average weight of these leaves, the weight of the lost leaves for sampling was also added for total biomass. In experiment 3, on day 31 when the experiment was terminated, a small piece of the root was collected and thoroughly cleaned for C and N analysis. The root samples were collected from three replicates only for each treatment for AAs and polyamines analyses. Gas exchange measurements for the leaves were also taken on different days. No samples for biomass calculations were collected at the time of harvest for experiment 3.

**Total soluble protein**

For total soluble protein quantification, approx. 50+2 mg FW of leaf sample was extracted in 1 mL of Tris buffer (100 mM Tris-HCl, 20 mM MgCl₂, 10 mM NaHCO₃, 1 mM NaEDTA, 5 mM DTT, and 10% (v/v) glycerol; pH 8.0) by three freeze-thaw cycles (Bauer et al. 2004). The supernatant (centrifugation 13,000 x g for 5 min) was analyzed for total soluble protein content as
per Bradford (1976) using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA). Absorbances were recorded at 595 nm with a Spectronic™ 200 spectrophotometer (ThermoFisher Scientific, Waltham, MA; spectral bandwidth <4 nm, wavelength accuracy of ±2 nm, and wavelength setting reproducibility of ±1 nm). Protein contents were determined using a 5-point standard curve (0-13 μg Bovine Serum Albumin).

**Chlorophyll content**

Using 10 mg of frozen leaf tissue, chlorophyll and carotenoids were extracted in 95% EtOH and detected as per Minocha et al. (2009). Total chlorophyll, chlorophyll a, chlorophyll b, and total carotenoids were analyzed using the Spectronic™ 200 spectrophotometer. Absorbances were recorded at 470, 649, and 664 nm. Equations from Lichtenthaler (1987) were used to calculate total chlorophyll, chlorophyll a, chlorophyll b, and total carotenoids separately.

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

**Leaf gas exchange measurements**

Gas exchange parameters were typically measured from 9 AM – 12 noon with a portable photosynthesis system (LI-6400/XT, Li-Cor, Lincoln, NE USA). Six replicate measurements per treatment group were used. Net photosynthesis and stomatal conductance were measured under a photosynthetic photon flux density (PPFD) of 1000 μmol m\(^{-2}\) s\(^{-1}\) from a red-blue LED chamber (6 cm\(^2\)); airflow was set to 700 μmol s\(^{-1}\); reference CO\(_2\) concentrations were kept at 400 μmol
mol\(^{-1}\); and block temperature was set to 25\(^0\)C. Leaf humidity was not controlled but ranged between 50-60%.

**Plant growth parameters and moisture content**

In experiment 2, the plant biomass was calculated based on height and stem diameter differences and the weight of the leaves and petioles. Readings were taken on the day of harvest. To measure the stem basal diameter, a digital caliper was used. In experiment 3, the height of the plants was taken on day 0 from six randomized untreated plants and again on day 17 from the same plants.

To measure the leaf surface area in experiment 2, an image of the leaf was taken on the day of harvest, and the leaf area was measured using ImageJ 1.49v software (Schneider et al. 2012).

To determine the moisture content of leaf tissue, the fresh weight (FW) ~200\pm10 mg of the leaf was measured right after it was brought to the lab. For dry weight (DW) determination, the leaf samples were dried in an oven at 70 °C for 48 hours and weighed. Relative water content was calculated using the following formula -

\[
\text{Moisture content (\%) = } \left(\frac{\text{FW} - \text{DW}}{\text{FW}}\right) \times 100
\]

**Soluble sugars**

To quantify various soluble sugars, 50±2 mg FW of leaf tissues was incubated at 65 °C for 30 min in 1 mL 80\% ethanol (method 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 \(\mu\)m 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 used to quantify sugars using external standard curves. For our experiment, we were interested in 11 sugars (xylose, arabinose, fructose, mannose, glucose, galactose, sucrose, trehalose, rhamnose monohydrate, maltose monohydrate, and raffinose pentahydrate) which were quantified with a Shimadzu RID-10A refractive index detector (RID) maintained at 30 ºC (Shimadzu Scientific Instruments Inc., Columbia, MD). The data were analyzed using Perkin Elmer TotalChrom software (version 6.3.2.1). 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.

**Total carbon and nitrogen**

Four replicates from the control, 0.25% NH₄NO₃, 0.5% Urea and 0.5% CoRoN treated plants for both experiments were selected to determine the total C and N in the samples. The leaf samples were oven-dried at 70 ºC for 24 hours, and 2±10 mg of tissue were weighed. Total C and N were determined by elemental analysis using a Thermo Flash EA Series 1112 analyzer (ThermoFisher Scientific Inc., Waltham, Mass.) as per EPA method 440.0 (https://cfpub.epa.gov/si/si_public_record_report.cfm).

**Polyamines and amino acids separation by HPLC**

Free PAs and AAs were dansylated and quantified via Reverse-Phase HPLC as per ([Minocha and Long 2004](#)) with recent modifications as described here. Samples were incubated at 60 ºC for 30 minutes, cooled for 3 minutes, and then centrifuged at 13,000 rpm for 30 seconds. Forty-five µl of glacial acetic acid was added to terminate the reaction. Sample tubes were then kept open for 3 minutes to allow the resulting CO₂ to evolve. A SpeedVac (Savant, Farmingdale, NY, USA) was used to evaporate the excess acetone. Finally, 1.735 µl of filtered HPLC grade methanol was added to the remaining 265 µl of derivatized sample in each tube, for
a total volume of 2 ml. Samples were analyzed using a Series 200 Perkin Elmer HPLC system (Waltham, MA, USA), fitted with a fluorescence detector. The analytical column used was a Phenomenex (Torrance, CA, USA) Synergy Hydro-RP™, 4 µm, 150 x 4.6 mm I.D., which was fitted with a Phenomenex C18 Security Guard™, 5 µm, 4 x 3 mm I.D cartridge guard column and the system was equipped with a Perkin Elmer C18, 10 µm, 33 x 4.6 mm I.D scavenger column. Excitation and emission wavelengths in the detector were set at 340 and 515 nm, respectively. Raw data were collected and processed using TotalChrom HPLC software (Perkin Elmer, v 6.2.1) as nmolesmL⁻¹ methanol and were converted to nmolesg⁻¹ FW. HPLC analysis followed guidance within EPA SW-846 Test Method 8000D.

Note that in the AAs data, many peaks could not be quantified at all tested times. This happened sometimes on different days within the same experiment and between different experiments conducted with plants of the same poplar clone. There are many reasons why this occurred; 1) the peak area in the sample was zero or very low and thus could not be quantified, indicating that this AA(s) was absent or below the detection level of our system, 2) the quantification of AAs, Asp and Glu is very sensitive to the pH of the sodium acetate buffer used in our reverse phase HPLC method. Because these two AAs are most polar, slight variations in the pH of the buffer made a huge impact on the resolution of these two peaks, and thus at times, even though both AAs were present, peak resolution was poor and only one of the two could be accurately quantified, 3) Glutamine in this method comes on the right shoulder of the wide dansyl-hydroxide peak (see chromatograph of AAs profile for the standards) and thus its detection limit is much higher than most other AAs; if the width of the dansyl-OH peak increases (for some unknown reason), it engulfs the base of the Glu peak. Therefore, Glu can be detected only above this threshold area, and on that day the system is not able to detect low concentrations even if
samples do contain Glu, 4) and finally the most important factor is the age and condition of the analytical column. As the columns age, the performance wanes and it affects the separation and resolution of certain peaks. At any point, the profile of AA standards still looks very good because they do not have the many unknown amine peaks that the samples do have and those interfere with either the separation or the resolution of the AAs’ peaks. Due to economic reasons, it is not always feasible to repeat the whole analysis (though we did repeat several runs) or to keep replacing expensive HPLC columns after only a few runs. Foliage extracts of many tree species are known to have high concentrations of many different metabolites that can foul a column decreasing its performance and lifespan.

**Statistical analyses**

The effects of treatment on all the analyses done were tested using a one-way ANOVA followed by Tukey’s HSD post hoc test with JMP Pro 15 (https://www.jmp.com/en_us/home.html) to determine the significant differences ($P \leq 0.05$) between control and treatment groups on a given day (The numbers of each replicate for each data set are shown in the figure and table legends).
Results

Morphological differences of the plants between the 3 experiments

A preliminary experiment (experiment 1) was conducted to determine the appropriate concentrations of the three forms of N fertilizer that were to be used for detailed experiments and analyses. It was observed that the application of foliar 1% NH₄NO₃ caused severe leaf scorching and necrotic leaf margins in plants (Supplementary Fig. S1). Moderate leaf marginal scorching was also observed in plants treated with 1% urea and 1% CoRoN. In experiments 2 and 3, the plants sprayed with 0.5% urea showed minimal marginal scorching in the leaves (Figs. 10B and 11B). Leaf scorching was not observed in plants that were sprayed with 0.25% urea and 0.5% and 0.25% CoRoN in either of these two experiments. However, considerable leaf necrosis and scorching were seen in plants that received 0.5% NH₄NO₃ in experiment 3 as well (Fig. 11A). Because of the leaf scorching observed in experiment 1 (conducted in 2019), the experiment was discontinued, and the detailed data are presented for experiments 2 (conducted in 2019) and 3 (conducted in 2021) only. On day 17 of experiment 3, the leaves of plants sprayed with 0.5% NH₄NO₃ were also severely scorched and showed necrosis symptoms. Hence samples were not collected from these plants on day 17 onwards. It was decided to keep on collecting samples from this treatment 0.5% NH₄NO₃ weekly spray treatment was stopped after week two. For the same reasons, after three weeks all NH₄NO₃ treatments were stopped while urea and CoRoN spray continued for the 4th week. Data are presented separately for experiments 2 and 3 because the treatments and the days of sample collections were not identical.
Figure 10. The effect of foliar spray of (A) 0.5% \( \text{NH}_4\text{NO}_3 \), 0.25% \( \text{NH}_4\text{NO}_3 \), 0.125% \( \text{NH}_4\text{NO}_3 \), (B) 0.5% and 0.25% urea, (C) 0.5% and 0.25% CoRoN after 13 days on the morphological traits of the hybrid poplar NM6 in experiment 2.
Figure 11. The effect of foliar spray of (A) 0.5% NH$_4$NO$_3$, 0.25% NH$_4$NO$_3$, 0.125% NH$_4$NO$_3$, (B) 0.5% and 0.25% urea, (C) 0.5% and 0.25% CoRoN after 14 days on the morphological traits of the hybrid poplar NM6 in experiment 3.
Experiment 2

This experiment involved two concentrations of each of the three fertilizers and two rounds of spray – once on day zero and again on day 14. Leaf samples were collected on days 0, 3, 8, 13, 17, 22, and 27. On the final day, the plants were harvested, and samples were collected not only for biochemical analyses but also for total FW and DW of different parts of the plant, including roots.

Total soluble protein content

Overall, in response to NH$_4$NO$_3$, urea, and CoRoN treatments, the total soluble protein contents varied somewhat on different days, but no major statistically significant differences were seen on most days in response to different treatments. For NH$_4$NO$_3$, total soluble proteins were slightly higher 3 days after the spray treatment (Fig. 12A). Total soluble proteins on the day of harvest (Fig. 12B) were similar in leaves collected from different heights along the stem (lower, middle, and bottom 1/3s) for all treatments.
Figure 12. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the cellular concentrations of total soluble protein of the hybrid poplar NM6 leaves in experiment 2. (A) On different days of sample collection and (B) On the day of harvest, post-treatment. The data presented are mean ± SE (n = 6).
Leaf chlorophyll and carotenoids

In experiment 2, the three forms of N treatments at two different concentrations each had no significant impact on the content of total chlorophyll, or chlorophyll a and chlorophyll b (Figs. 13A-C). The carotenoids likewise showed no significant change with either time of collection after the treatment or with the treatment itself, except for a small but significant decrease on day 3 with 0.25 % urea and 0.5 % CoRoN (Fig. 13D). At harvest also, no differences were seen in leaves collected from different heights along the stem (lower, middle, and bottom 1/3s) (Fig. 14).

Gas exchange

Gas exchange properties of the plants were measured only on the final day of the experiment before harvest. Leaves from three parts of each plant (top, middle and bottom) were tested for their transpiration rates, stomatal conductance, and photosynthetic rates (Fig. 15). All three parameters were somewhat lower in the lowest 1/3 of the plants. Plants treated with the higher concentration of NH₄NO₃ also had lower values (though insignificant) for transpiration (Fig. 15A), stomatal conductance (Fig. 15B), and photosynthetic rate (Fig. 15C) for the top and the bottom parts of the plant. In comparison with the control plants, different fertilizers typically showed no significant impact on transpiration rates on any given day (Fig. 15A). Foliar applications of 0.5% urea showed a significant increase in stomatal conductance in leaves from the middle parts of the treated plants (Fig. 15B). On the day of harvest, the leaves at the lowest 1/3 of the plants exhibited lower photosynthetic rates than the leaves at the top and middle positions (Fig. 15C). No significant difference in photosynthetic rates was observed between the control and the treated plants for any location of the leaves on the stem.
Figure 13. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the cellular concentrations of (A) total chlorophyll, (B) chlorophyll a, (C) chlorophyll b, and (D) carotenoids of the hybrid poplar NM6 leaves on different days post-treatment. The data presented are mean ± SE (n = 6). The letters above each bar represent differences (P ≤ 0.05) between the control and other treatments on a given day.
Figure 14. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the cellular concentrations of (A) total chlorophyll (B) chlorophyll a (C) chlorophyll b, and (D) carotenoids of the hybrid poplar NM6 leaves on the day of harvest post-treatment. The data presented are mean ± SE (n = 6).
Figure 15. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the gas exchange rate of the hybrid poplar NM6 leaves on the day of harvest post treatment. (A) transpiration (B) stomatal conductance (C) photosynthetic rate. The data presented are mean ± SE (n = 6). The letters above each bar represent differences (P ≤ 0.05) between the control and other
**Biomass and height**

Overall, the total biomass and height of all plants in experiment 2 showed no significant treatment-related differences when measured on the day of harvest (Fig. 16A). As shown in Figure 16B, the shoot-to-root ratio was lower for all treatments relative to the control; the plants treated with 0.5% CoRoN showing the lowest value. However, this was not a significant decrease. The height of the plants and stem basal areas were similar for all treatments (Figs. 16C, D).

**The surface leaf area**

The combined surface area of the leaves collected from the top and bottom positions of the plants in experiment 2 showed minimal changes (if any) with any treatment when compared to the control plants (Fig. 17).

**Moisture content**

A significant difference in the moisture content of the leaves collected on different days was not observed in the treated vs. control plants. The moisture content ranged between 62%-72% (table 4).

**C/N ratio in response to different N forms**

The total C and N on the final day were measured for all plants; however, for days 3 and 17, only on the collections from plants treated with 0.25% NH₄NO₃ and 0.5% of urea and CoRoN were measured. No significant differences were observed for unfertilized controls on different days or between the various treatments on any day (Table 5). This resulted in a rather comparable C:N ratios in all samples.
Figure 16. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the plant biomass of the hybrid poplar NM6 on the day of harvest, post-treatment. (A) weight (B) shoot to root ratio (C) height and (D) stem basal area. The data presented are mean ± SE (n = 3).
Figure 17. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the plant surface leaf area of the hybrid poplar NM6 on the day of harvest, post-treatment. The data presented are mean ± SE (n = 6).
Table 4. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the plant moisture content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 6).

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>0.125% NH$_4$NO$_3$</th>
<th>0.25% NH$_4$NO$_3$</th>
<th>0.25% Urea</th>
<th>0.5% Urea</th>
<th>0.25% CoRoN</th>
<th>0.5% CoRoN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>64.42 ± 0.01%</td>
<td>65.71 ± 0.01%</td>
<td>69.65 ± 0.04%</td>
<td>66.23 ± 0.01%</td>
<td>62.48 ± 0.02%</td>
<td>63.68 ± 0.03%</td>
<td>65.40 ± 0.00%</td>
</tr>
<tr>
<td>3</td>
<td>65.11 ± 0.01%</td>
<td>65.79 ± 0.01%</td>
<td>66.09 ± 0.01%</td>
<td>66.95 ± 0.01%</td>
<td>65.85 ± 0.01%</td>
<td>66.51 ± 0.01%</td>
<td>64.07 ± 0.02%</td>
</tr>
<tr>
<td>8</td>
<td>65.99 ± 0.00%</td>
<td>65.35 ± 0.01%</td>
<td>65.89 ± 0.01%</td>
<td>67.50 ± 0.01%</td>
<td>66.07 ± 0.01%</td>
<td>66.15 ± 0.00%</td>
<td>65.97 ± 0.00%</td>
</tr>
<tr>
<td>13</td>
<td>42.23 ± 0.02%</td>
<td>65.85 ± 0.01%</td>
<td>66.93 ± 0.00%</td>
<td>67.67 ± 0.00%</td>
<td>67.01 ± 0.01%</td>
<td>65.98 ± 0.01%</td>
<td>66.68 ± 0.01%</td>
</tr>
<tr>
<td>17</td>
<td>76.09 ± 0.02%</td>
<td>67.89 ± 0.01%</td>
<td>67.71 ± 0.01%</td>
<td>71.31 ± 0.05%</td>
<td>68.48 ± 0.06%</td>
<td>70.65 ± 0.03%</td>
<td>66.82 ± 0.05%</td>
</tr>
<tr>
<td>22</td>
<td>67.44 ± 0.00%</td>
<td>66.98 ± 0.02%</td>
<td>68.94 ± 0.02%</td>
<td>66.05 ± 0.02%</td>
<td>65.89 ± 0.02%</td>
<td>68.28 ± 0.02%</td>
<td>64.34 ± 0.01%</td>
</tr>
<tr>
<td>27</td>
<td>63.61 ± 0.01%</td>
<td>63.76 ± 0.01%</td>
<td>65.18 ± 0.01%</td>
<td>65.32 ± 0.01%</td>
<td>62.01 ± 0.02%</td>
<td>63.80 ± 0.01%</td>
<td>65.68 ± 0.02%</td>
</tr>
</tbody>
</table>

Table 5. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the plant N% and C% of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3 days</th>
<th>17 days</th>
<th>27 days</th>
<th>3 days</th>
<th>17 days</th>
<th>27 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.78 ± 0.07</td>
<td>2.58 ± 0.1</td>
<td>2.27 ± 0.1</td>
<td>44.52 ± 1.19</td>
<td>45.72 ± 0.4</td>
<td>44.78 ± 0.43</td>
</tr>
<tr>
<td>0.125% NH$_4$NO$_3$</td>
<td>na</td>
<td>na</td>
<td>2.18 ± 0.06</td>
<td>na</td>
<td>na</td>
<td>44.57 ± 0.67</td>
</tr>
<tr>
<td>0.25% NH$_4$NO$_3$</td>
<td>2.4 ± 0.05</td>
<td>2.42 ± 0.13</td>
<td>2.16 ± 0.02</td>
<td>45.96 ± 0.27</td>
<td>44.92 ± 0.34</td>
<td>44.39 ± 0.27</td>
</tr>
<tr>
<td>0.25% Urea</td>
<td>na</td>
<td>na</td>
<td>2.26 ± 0.14</td>
<td>na</td>
<td>na</td>
<td>44.7 ± 0.42</td>
</tr>
<tr>
<td>0.5% Urea</td>
<td>2.85 ± 0.24</td>
<td>2.67 ± 0.07</td>
<td>2.36 ± 0.04</td>
<td>43.93 ± 2.46</td>
<td>45.26 ± 0.1</td>
<td>44.72 ± 0.5</td>
</tr>
<tr>
<td>0.25% CoRoN</td>
<td>na</td>
<td>na</td>
<td>1.88 ± 0.08</td>
<td>na</td>
<td>na</td>
<td>43.24 ± 0.92</td>
</tr>
<tr>
<td>0.5% CoRoN</td>
<td>2.78 ± 0.13</td>
<td>2.56 ± 0.07</td>
<td>2.15 ± 0.09</td>
<td>45.95 ± 0.29</td>
<td>45.17 ± 0.16</td>
<td>44.13 ± 0.66</td>
</tr>
</tbody>
</table>
**Foliar soluble sugars**

The leaf samples collected on several days post-treatment with different forms of N were analyzed for a group of common soluble sugars by HPLC using 11 sugars as standards: namely rhamnose, xylose, arabinose, fructose, mannose, trehalose, glucose, galactose, sucrose, maltose, and raffinose. However, xylose + arabinose, and maltose + trehalose peaks were not detected for most of the days in the samples that were collected. All of the collected plant samples showed sugar levels within a range of 0-35 mg g\(^{-1}\) DW, with the content of sucrose being higher than other sugars on most days and most treatments. In this experiment, a significant change in the sugars was not observed (Fig. 18).
Figure 18. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the sugar content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 4). (A) Sucrose (B) Fructose (C) Rhamnose. On a given day * indicates data not detected during the analysis.
**Soluble foliar amino acids**

The soluble AAs in the leaves were quantified to ascertain the effect of N spray on AA contents in the foliage. The HPLC system used here was able to quantify 18 out of 20 common proteinogenic AAs and two common non-proteinogenic AAs (GABA and Orn). However, for poplar leaves, depending upon their age (as judged by their location on the stem), the chromatographic profiles of several AAs were not separated clearly and could not be quantified individually. For example, the peaks of Arg, Thr, and Gly did not separate well in the HPLC run (supplementary data-S8). Thus, the data on clearly identified peaks are presented here. The contents of AAs followed the following trend in order from higher to lower related to abundance: Ala>Glu>GABA>Asp>Ser>Cys>Lys>Val>Pro>Ile>Met>His>Orn>Trp. The combined peak of Arg, Thr, and Gly showed the highest total AA content.

Note that in the AAs data, many peaks could not be quantified at all tested times. This happened sometimes on different days within the same experiment and between different experiments conducted with plants of the same poplar clone. There are many reasons why this occurred, and these are described in detail under the methods section.

**Amino acids derived from α-ketoglutarate - (the glutamate family)**

Glutamic acid is a key intermediate in cellular N metabolism, being involved as the first step in N assimilation, and serving as a substrate for the biosynthesis of key AAs and numerous other metabolites, including the biosynthesis of PAs. In experiment 2, all N-treated plants showed a significant decrease in Glu levels after the first spray (day 3) compared to the control plants (Fig. 19A). The plants sprayed with 0.5% urea and CoRoN showed a full recovery of Glu content on day 13, but a decline followed again after the 2\textsuperscript{nd} spray as was the case for the 1\textsuperscript{st} spray. The content of Glu in plants sprayed with CoRoN was the lowest compared to all other treatments on day 17.
(below the detection limit). On the day of harvest, Glu significantly decreased in the NH$_4$NO$_3$, 0.5% urea and 0.25% CoRoN treated plants.

Ornithine, a substrate used by ODC, was always present in minimal quantities. In experiment 2, Orn was not detected in the treated samples. However, this is more likely due to poor HPLC separation than the treatment effect.

Similar to Glu, cellular Pro decreased significantly on day 3 and stayed low until day 27 (Fig. 19B). It is worth noting that there was a significant increase in Pro on day 27 after being low between days 3, 8, and 22 especially in the N-treated plants. However, the Pro content increased 8 days after the first treatment in the leaves sprayed with 0.125% NH$_4$NO$_3$. Cellular Pro almost doubled in the untreated control plants on day 17. Proline concentration was lower than Glu in control cells at all times. Interestingly, Gln was not detected in control or treated plants in experiment 2. Therefore, data are not presented here. γ-Aminobutyric acid (GABA) is a non-protein AA that can be produced directly from Glu by GAD as well as from the catabolism of Put by diamine oxidase. The reactions of the GABA shunt serve as an intermediate in recycling C of Glu and Put. Similar to Glu and Pro, GABA also declined significantly 3 days after the 1$^{st}$ and 3$^{rd}$ days after the 2$^{nd}$ spray treatments indicating a short-term spray effect on the plants (Fig. 19C). Similar to Glu and Pro, a sharp decline in GABA content was seen in plants sprayed with CoRoN, which was also the lowest GABA content compared to all other treatments on day 17. However, the GABA content increased 8 days after the first treatment in the leaves sprayed with 0.125% NH$_4$NO$_3$ but decreased somewhat in urea-treated plants (Fig. 19C). Furthermore, towards the end of the experiment (day 27), GABA increased again in all treatments, including controls (Fig. 19C).

Finally, His, the most basic of this group of AAs, showed a significant decrease almost to undetectable levels on most of the days after time zero (Supplementary Fig. S8B).
Amino acids derived from 3-phosphoglycerate

Serine and Gly, which are readily interconvertible, are mainly derived from 3-phosphoglycerate (Fig. 2), although Gly can also be produced by direct transamination of glyoxalate (Bourguignon et al. 1999). Both pathways use Glu as the donor of the amide group. We were unable to determine the concentration range of Gly as it did not separate from Arg and Thr peaks in the HPLC run. As with most other AAs, Ser also decreased with all N treatments on days 3 and 17 (Fig. 20). On other days, Ser showed significant fluctuations among the various treatments without a clear pattern of changes with time. The content of Ser increased transiently (vs. the controls) in plants sprayed with 0.5% CoRoN on day 8 and with NH₄NO₃ on days 8 and 27. Cysteine, derived from Ser, was not present in the leaf samples.

Aromatic amino acids

Of the three AAs in this group, two (Tyr and Phe) use Glu as the amide group donor, while Trp takes its amino group from Ser (Siehl 1998). Their carbon skeleton comes from PEP via chorismate (Fig. 3). In this experiment, it was difficult to calculate the content of Trp as it did not separate well from Phe (supplemental data-S8C). The combined contents of Trp+Phe were quite high and remained mostly unchanged during the experiment except for 17 days after CoRoN treatment.
Alanine and branched chain AAs

Leucine, Val, and Ile constitute a group of branched-chain AAs, the former two being derived from pyruvate (Pyr) and the third from oxaloacetate (OAA). All three of these AAs were lower than the controls on 3 and 17 days after the N spray; on all other days, with a few exceptions, their contents were comparable to the control plants on most other days (Figs. 21A-C). In contrast, Ala did not show any significant change among the plants sprayed different forms of N, not even on days 3 and 17 where N treatments reduced the content of most other AAs (Fig. 21D).

Aspartate family AAs

Six AAs, including Ile (discussed above under branched-chain AAs) that constitute the Asp family, utilize Oxaloacetic acid (OAA) as the carbon skeleton (Fig. 2); four of them, i.e. Thr, Asp, Met, and Lys, were quantified here. While Asp and Lys (Fig. 22A-B) showed similar changes to most other AAs on days 3 and 17, Met was closer to the detection limit of the instrument and revealed no significant changes in its content with any of the N treatments (Fig. 22C). From day 13 to 27, Asp showed a steady decrease with time (Fig. 22A). Lysine, on the other hand, showed much smaller changes with time as well as with treatments on given days of analyses (Fig. 22B). The highest contents of Lys were seen on the harvest day for 0.25% urea and 0.5% CoRoN treatment.

Foliar polyamines

Following the treatments, foliar PAs showed varied outcomes. Similar to most AAs, Put content also decreased with all N treatments on days 3 and 17 with the exception of CoRoN on day 17 (Fig. 23A). Putrescine content showed an overall decreasing trend from day 0 to day 27, whereas a statistically significant increase in Spd was observed in plants treated with 0.125%
NH₄NO₃ and 0.25% urea on days 8 and with 0.125% NH₄NO₃ and 0.5% urea on day 22 of sampling (Fig. 23B). Spermidine declined significantly in the CoRoN treated plants on day 17, which is also three days after the 2nd spray treatment. Overall Spd content did not fluctuate much with time. The Spm content decreased significantly (vs. the controls) in plants treated with CoRoN on day 8 (Fig. 23C). However, the Spm content was significantly high compared to the control plant on day 17 in plants treated with 0.25% urea and on day 22 in plants treated with 0.125% NH₄NO₃; Spm content increased with time in most cases (Fig. 23C).
Figure 19. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the amino acid content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). (A) Glutamic acid (B) Proline and (C) GABA. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05).
Figure 20. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the Serine content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05).
Figure 21. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRon on the amino acid content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). (A) Leucine (B) Isoleucine (C) Valine and (D) Alanine. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05).
Figure 22. The effect of foliar spray of NH₄NO₃, urea, and CoRon on the amino acid content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). (A) Aspartic acid (B) Methionine and (C) Lysine. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05). On a given day * indicates data not detected during the analysis.
Figure 23. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the polyamine content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). (A) Putrescine (B) Spermidine and (C) Spermine. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05).
Experiment 3

Figure 24. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the cellular concentrations of total soluble protein of the hybrid poplar NM6 leaves on different days of sample collection post-treatment. The data presented are mean ± SE (n = 6).
Figure 25. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the cellular concentrations of (A) total chlorophyll (B) chlorophyll a (C) chlorophyll b, and (D) carotenoids of the hybrid poplar NM6 leaves on different days post-treatment. The data presented are mean ± SE (n = 6). The letters above each bar represent differences (P ≤ 0.05) between the control and other treatments on a given day.
Figure 26. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the gas exchange rate of the hybrid poplar NM6 leaves on different days post-treatment. (A) transpiration (B) stomatal conductance (C) photosynthetic rate. The data presented are mean ± SE (n = 5). The letters above each bar represent differences (P ≤ 0.05) between the control and other
Experiment 3

As described under methods, under the objective (1) of analyzing the effects of three different forms of N fertilizer spray on physiological and biochemical parameters of young poplar NM6 plants, experiment 3 setup involved the following major differences from experiment 1 (preliminary study) and experiment 2: (i) the plants were much younger than those used in experiments 1 and 2; (ii) all plants from stem cuttings in small tubes were transferred to larger pots which were not given any greenhouse maintenance fertilizer (GHM +N) that was given in the other two experiments for about two months, and they were starved right away before N spray; (iii) N spray was given every week for five weeks instead of once in two weeks in experiments 1 and 2, (iv) there was an additional control in which normal amount of N was provided via roots as GHF+ N; and (v) only top leaves were sampled on different days. Nitrogen spray treatments and sample collections were done the same way on several days as for experiment 2. All analytical procedures were also the same. Control plants mentioned in the figures below are the GHF-N plants where the fertilizer without N was given once a week by hand at 3.5.X (not 7X to avoid toxicity to plants by too much nutrition at any one-time concentration of daily dose of GHF+N. This was similar to the previous experiments.

Total soluble protein

Figure 24 shows that the total soluble protein content in the plants ranged between 0.02-0.06 mg g-1 FW. A significant change or a trend was not observed in the total soluble content in the different groups of plants compared to the control plants.
Leaf chlorophyll and carotenoids

The foliar contents of total chlorophyll, and chlorophyll a and b remained mostly unchanged during the entire 4.5-week study for all treatments except on day 10, where total chlorophyll, as well as chlorophyll a and b contents were significantly higher in plants treated with 0.5% NH$_4$NO$_3$ (Fig. 25A-C). The total chlorophyll content in this experiment ranged between 0.5 to 1.5 mg g$^{-1}$ FW vs. <1 mg g$^{-1}$ FW in experiment 2. Even though small changes in the carotenoids content were seen on some days, a significant trend in the carotenoid contents was not observed in the treated plants vs. the control (no N) plants (Fig. 25D). The carotenoid contents of the leaves were also less in this experiment vs. those in experiment 2.

Gas exchange

In experiment 3, transpiration rates were significantly higher for plants sprayed with 0.25% and 0.5% NH$_4$NO$_3$ on day 17 as well as with 0.5% NH$_4$NO$_3$ and 0.25% or 0.5% urea on day 31 (Fig. 26A); and so were the stomatal conductance measurements for treatments with 0.125% NH$_4$NO$_3$ (Fig. 26B). An intriguing observation was that experiment 3 had a transpiration rate ranging from <3 to about 6 mol.m$^{-2}$.s$^{-1}$ (Fig. 26A), whereas experiment 2 had a much lower range of 0.001-0.005 mol.m$^{-2}$.s$^{-1}$ (Fig. 15A). However, it should also be noted that in experiment 2, the analyses were done only on the final day of the experiment (on three different leaves), when the plants were much older. Although no differences were observed in the photosynthetic rates on most days, except for a small but significantly higher values in the 0.5% urea and 0.5% CoRoN-treated plants (Fig. 26C). Overall photosynthetic rates were also lower in these plants vs. those in experiment 2 on the final day of collection.
Figure 27. The appearance of the randomly chosen plants at time zero

Table 6: Height of the tallest plant of each treatment measured on day 17

<table>
<thead>
<tr>
<th>Foliar fertilizer treatments</th>
<th>Height of the tallest plant (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99.06</td>
</tr>
<tr>
<td>0.5% NH$_4$NO$_3$</td>
<td>101.6</td>
</tr>
<tr>
<td>0.25% NH$_4$NO$_3$</td>
<td>91.44</td>
</tr>
<tr>
<td>0.125% NH$_4$NO$_3$</td>
<td>101.6</td>
</tr>
<tr>
<td>0.5% Urea</td>
<td>93.98</td>
</tr>
<tr>
<td>0.25% Urea</td>
<td>106.68</td>
</tr>
<tr>
<td>0.5% CoRoN</td>
<td>96.52</td>
</tr>
<tr>
<td>0.25% CoRoN</td>
<td>104.14</td>
</tr>
</tbody>
</table>
**Moisture content**

Experiment 3 plants did not show any significant differences in the moisture content among different treatments, except for three sets of samples collected on day 24; the treatments being the two treatments with urea and 0.5% CoRoN. The moisture content mostly ranged between 61%-72% (Table 8). Although not significant, a lower moisture content was observed on day 24 in the plants sprayed with 0.5% urea and CoRoN. Furthermore, the highest moisture content was seen in the plants sprayed with 0.25% and 0.5% NH₄NO₃ on day 31.

**C and N percentage in response to different N forms**

Similar to experiment 2, the C and N were analyzed only in plants treated with 0.25% NH₄NO₃, 0.5% urea and 0.5% CoRoN except for at the time of harvest, day 31 when plants from all treatments were quantified for %N and % C (Table 9). The %N varied between the highest of 2.34±0.12%, and the lowest of 1.18±0.05% on days; both these numbers were for 0.5% CoRoN on days 3 and 24, respectively. Following N treatments, there was no significant difference between the control (unfertilized) and a treatment on a given day for any of the N forms (Table 9). The % of C in the foliage was between 41.77% to 49.42%. This resulted in rather comparable C:N ratios in all samples that had been deprived of N for two weeks.

**Foliar soluble sugars**

Although significant differences were not observed in the sugar content in experiment 3, the fructose showed an overall decreasing trend by day 24 (Fig. 28).
Table 7. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the plant moisture content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 6).

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>0.125% NH$_4$NO$_3$</th>
<th>0.25% NH$_4$NO$_3$</th>
<th>0.5% NH$_4$NO$_3$</th>
<th>0.25% Urea</th>
<th>0.5% Urea</th>
<th>0.25% CoRoN</th>
<th>0.5% CoRoN</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>71% ± 0.03%</td>
<td>67% ± 0.03%</td>
<td>67% ± 0.01%</td>
<td>68% ± 0.01%</td>
<td>68% ± 0.01%</td>
<td>65% ± 0.01%</td>
<td>66% ± 0.01%</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>67% ± 0.01%</td>
<td>67% ± 0.01%</td>
<td>70% ± 0%</td>
<td>68% ± 0.01%</td>
<td>66% ± 0%</td>
<td>67% ± 0.01%</td>
<td>67% ± 0.01%</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>64% ± 0.02%</td>
<td>63% ± 0%</td>
<td>66% ± 0.01%</td>
<td>67% ± 0.02%</td>
<td>65% ± 0.01%</td>
<td>65% ± 0.01%</td>
<td>64% ± 0.01%</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>59% ± 0.02%</td>
<td>61% ± 0.01%</td>
<td>63% ± 0.01%</td>
<td>na</td>
<td>63% ± 0.01%</td>
<td>63% ± 0.01%</td>
<td>61% ± 0.02%</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>70% ± 0.02%</td>
<td>70% ± 0.02%</td>
<td>70% ± 0.03%</td>
<td>67% ± 0.01%</td>
<td>56% ± 0.07%</td>
<td>64% ± 0%</td>
<td>64% ± 0.05%</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>66% ± 0.01%</td>
<td>66% ± 0.03%</td>
<td>71% ± 0.06%</td>
<td>79% ± 0.07%</td>
<td>69% ± 0.02%</td>
<td>68% ± 0.01%</td>
<td>65% ± 0.02%</td>
<td></td>
</tr>
</tbody>
</table>

Table 8. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the plant N% and C% of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Nitrogen</th>
<th>% Carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 days</td>
<td>10 days</td>
</tr>
<tr>
<td>Control</td>
<td>1.84% ± 0.16%</td>
<td>1.24% ± 0.07%</td>
</tr>
<tr>
<td>0.125% NH$_4$NO$_3$</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>0.25% NH$_4$NO$_3$</td>
<td>1.82% ± 0.02%</td>
<td>1.49% ± 0.15%</td>
</tr>
<tr>
<td>0.5% NH$_4$NO$_3$</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>0.25% Urea</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>0.5% Urea</td>
<td>2.28% ± 0.14%</td>
<td>1.66% ± 0.09%</td>
</tr>
<tr>
<td>0.25% CoRoN</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>0.5% CoRoN</td>
<td>2.34% ± 0.12%</td>
<td>1.62% ± 0.15%</td>
</tr>
</tbody>
</table>
Figure 28. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the sugar content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 4). (A) Sucrose (B) Fructose (C) Rhamnose. On a given day * indicates data not detected during the analysis.
**Soluble foliar amino acids**

Similar to experiment 2 only low quantities of His and Orn were detected in experiment 3. However, in contrast to experiment 2, Gln showed high quantities in the plant samples collected in experiment 3 (Fig. 29B). As described for experiment 2, several AAs were not accurately quantified due to HPLC issues; thus, the data are not presented here.

**Amino acids derived from α-ketoglutarate**

*(The glutamate family)*

The treated samples had very little amount of Orn. This is more likely due to the presence of Orn below the detection limits of HPLC in all samples except for GH+N controls where Orn was detected.

In experiment 3, the spray treatments did not show any unique responses on the Gln concentrations either on different days or for different treatments (Fig. 29B). It is interesting to note that, in comparison to other AAs, the levels of Gln remained consistently high in the samples collected in experiment 3.

Compared to the control plants, with two exceptions, the Pro content decreased significantly on days 3 and 6 in plants sprayed with all three forms of N. As observed with several other AAs in both experiments, Pro levels varied with time as well (Fig. 29C). Proline is a minor AA in these samples showing overall low content except on day 24 with CoRoN spray, when it was the highest in 0.25% CoRoN-treated leaves. This increase was x3 fold higher compared to the control plants.

The amounts of GABA also showed some unique responses in experiment 3 (Fig. 29D). A significant increase in GABA was observed in plants treated with 0.5% NH₄NO₃ and 0.25%
CoRoN on day 6. Also, higher GABA levels were seen consistently with CoRoN on days 10, 24 and 31 and with urea on days 10 and 31; its content remained the highest on most days of analyses thereafter. The only exception was day 17 when the lowest GABA content was observed. Histidine could not be separated well to be quantified in most N-treated samples; therefore, data are not presented here.

**Amino acids derived from 3-phosphoglycerate**

In experiment 3, no notable trend was observed in the Ser content of the leaves with any treatment, except that its contents were the lowest on day 24 and highest on day 31 in all samples. As shown in Fig. 30B, Cys was also not properly resolved by HPLC on days 6, 10, and 24. An increase in Cys with one or more concentrations of NH$_4$NO$_3$ and/or CoRoN was seen on days 3, 17 and 31 when separation was reasonable.

**Aromatic amino acids**

Phenylalanine content also could not be resolved for two of the six days for all samples due to poor HPLC separations for the reasons described under methods section (Fig. 31); but its content for all treatments was lower on days 6 and 24 relative to days 3 and 31. Although there were a few significant increases in Phe with NH$_4$NO$_3$ (day 24) and CoRoN (day 31), no consistent pattern of changes overtime with N treatments were observed for this AA. Tyrosine was not resolved by the HPLC method used in this experiment; therefore, data for this AA is not available.
**Alanine and branched chain AAs**

In experiment 3, Leu and Ile contents varied on different days; the overall contents of the two were quite similar on most days these amino acids were properly quantifiable in the chromatograms. As with some other AAs, both or one of these AAs was not well separated or it was below the detection limit. The highest amounts for both were observed for day 31, with only minor differences among the treatments (Figs. 32A, B). There were significant increases in Leu on day 10 and Ile on days 10 and 24 with most N treatments. On day 31, the contents of both these AAs were significantly lower in the plants treated with 0.5% CoRoN. In contrast, the Val content was significantly higher in plants treated with 0.5% CoRoN (Fig. 32C); otherwise, its contents varied within a narrow range during the entire experiment, with a few one-time changes with specific treatments. Alanine, which was a dominant AA in the leaves showed only a small change with time and no significant change with treatment on any day (Fig. 32D).

**Aspartate family AAs**

The contents of Asp were quite variable on different days (being lowest on day 24); it was also not quantifiable on days 3 and 17 due to HPLC issues (Fig. 33A). No significant changes in Asp were observed with N treatments. The content of Met was similar among most treatments on all days (Fig. 33B). Once again, no significant changes in Met were observed with N treatments. Lysine content was similar on most days; except that in the plants treated with 0.5% urea, Lys content was the highest on day 24. However, it was significantly lower (~40% lower than control) for 0.25% and 0.5% NH$_4$NO$_3$ (Fig. 33C).
**Foliar polyamines**

For this experiment, Put was the lowest of the three PAs and was not detected on some days (Fig. 34A). In the plants treated with 0.25% urea and CoRoN, the Put content decreased significantly on days 6. Both Put and Spm content were significantly higher with 0.5% urea on day 24 (Fig. 33A,B). During the first 10 days, Spd was the dominant PA (Fig. 34B); however, by day 31, Spm became the most abundant PA in the leaves, especially in plants treated with 0.5% urea (Fig. 34C). Spermine content decreased on day 24 with both concentrations of CoRoN.

**Soluble amino acids in roots**

Root AA contents were only quantified for samples collected on day 31 (day of harvest). In contrast to leaf samples, Cys was the most abundant AA in the roots, followed by Glu, Ser, and Ala (Fig. 35). Whereas Glu and Ser contents exhibited differences among some treatments, others were within similar range among all treatments. As with the leaf samples, His contents were the lowest in all root samples.
Figure 29. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the amino acid content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). (A) Glutamic acid (B) Glutamine (C) Proline and (D) GABA. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05). On a given day * indicates data not detected during the analysis.
Figure 30. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the amino acid content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). (A) Serine and (B) Cystine. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05). On a given day * indicates data not detected during the analysis.

Figure 31. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the Phenylalanine content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05). On a given day * indicates data not detected during the analysis.
Figure 32. The effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the amino acid content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). (A) Leucine (B) Isoleucine (C) Valine and (D) Alanine. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05). On a given day * indicates data not detected during the analysis.
Figure 33. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the amino acid content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). (A) Aspartic acid (B) Methionine and (C) Lysine. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05). On a given day * indicates data not detected during the analysis.
Figure 34. The effect of foliar spray of NH₄NO₃, urea, and CoRoN on the polyamine content of the hybrid poplar NM6 on different days, post-treatment. The data presented are mean ± SE (n = 5). (A) Putrescine (B) Spermidine and (C) Spermine. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05).
Figure 35. Representation of the effect of foliar spray of NH$_4$NO$_3$, urea, and CoRoN on the root amino acid content of the hybrid poplar NM6 on different days, post-treatment. (A) Control (B) 0.125% NH$_4$NO$_3$ (C) 0.25% NH$_4$NO$_3$ (D) 0.5% NH$_4$NO$_3$ (E) 0.25% Urea (F) 0.5% Urea (G) 0.25% CoRoN (H) 0.5% CoRoN.
Discussion

The primary objective of this part of the study was to test the efficacy of foliar application of different forms of N on NM6 poplar plants (Wildtype). The effects of foliar spray of three forms of N (NH₄NO₃, urea, and CoRoN) at various concentrations were tested on the physiological and metabolic changes including water content, total soluble proteins, chlorophyll, carotenoids, gas exchange (transpiration, stomatal conductance, photosynthesis), soluble sugars, soluble AAs and PAs. We observed that the metabolism of poplar leaves changes in response to various foliar N treatments, particularly in the CoRoN-treated plants. A preliminary experiment was first performed to select the range of N concentration that caused the least visible damage to the plants. Based on the outcomes, detailed biochemical results of experiment 2 and its repeat (with some modifications) in experiment 3 are presented here. The preliminary results showed that higher concentrations of N spray often caused visible damage to the leaves within a few days. Based on these results, two concentrations of each form of N were selected for experiment 2, whose results are presented here in detail. Experiment 3 was a repeat of the 2nd experiment with a few small changes. The assimilation of different forms of N applied to the leaves resulted in varied responses. The changes observed in biomass, total protein, chlorophyll, C/N ratio, and moisture content were similar in the two (named here as 2nd and 3rd experiment) experiments. However, the AAs and PAs were not consistent, and showed differences between the two experiments.

Several studies have recently been published on using foliar spray as a way of plant fertilization. The argument is that N fertilizers are often used in large quantities, a significant portion of which are either converted back to N₂ gas by microbial activity or are washed out of the soil and cause N pollution (Wang et al. 2018, Ciani et al. 2021, Liu et al. 2022, Britannica 2023). Poplars are fast-growing tree species used world-wide as a major source of lumber, biomass, and
for phytoremediation of metallic as well as organic pollutants (Pietrini et al. 2009, Aghaalikhani et al. 2017, Ashraf et al. 2019, Liang et al. 2023). In order to reduce genetic variation in selected plants, a number of inter-specific hybrids have been produced that do not produce viable pollen or seeds (Costa et al. 2017). The result is that millions of plants are produced by cuttings and sold worldwide. Obviously, large amounts of different forms of N fertilizers are used on a large scale.

In attempts to reduce N pollution in the environment, studies have been conducted to use foliar spray of N and analyze its effectiveness to support plant growth, especially during the early stages in nurseries (Bi and Scagel 2007). The most common chemical forms of N used are NH₄NO₃, urea, and a special form called CoRoN, which is highly suitable for foliar spray (Bohner et al. 2015, Oosterhuis et al. 2015, Castro et al. 2022, Murillo-Peña et al. 2023).

In our study, we studied a number of physiological and metabolic aspects of foliar N spray by measuring total soluble protein, chlorophyll, moisture content, total N and C, gas exchange rate, PAs and AAs contents, and total biomass in response to foliar N applications. These variables help us understand how poplar plants metabolize N when exposed to N fertilizers via foliar spray (Gagne et al. 2019, Pantoja-Benavides et al. 2021b). This knowledge can further help develop agronomic strategies for optimizing plant growth and yield; especially in the nurseries and minimize N loss and environmental damage.

Whereas roots are the primary plant part that absorbs N from the soil, most of its conversion to usable forms (first NH₃ and then AAs) occurs in the leaves (REFERENCES). This of course involves the transport of N from roots to leaves. For foliar N fertilization to be successful, it must be able to penetrate the outer protective layers of the leaf and be absorbed through the cuticle, stomata or other epidermal structures to reach the internal cells of the leaf (Fernández and Brown 2013, Eichert and Fernández 2023). The texture of the plant leaf surface, which leads to
hydrophobicity, also factors in the ability of plants to absorb nutrients (Ishfaq et al. 2022). However, the advantage of foliar application is that it can be directly assimilated in the leaves.

In this study, N solutions were sprayed on the adaxial side of the leaf. Only a few studies have been reported to understand how the surface roughness of poplar plants contributes to absorbing nutrients. Thus, it is difficult to draw any conclusions as to what extent the surface poplar leaves acted as a barrier, impeding foliar nutrient uptake. The adaxial leaf surface of hybrid poplar has few stomata (Hu et al. 2014); hence in this study, solutes sprayed on the leaf surface most likely enter the leaf through the cuticle (Schönherr 2006).

The leaves sprayed with higher concentrations of NH$_4$NO$_3$ showed severe scorching within a short period, whereas the leaves that received urea treatments showed mild scorching on the leaves, limited to the margins. The leaf scorching by NH$_4$NO$_3$ could be attributed to NH$_4^+$ accumulation in the leaves. A recent study on wheat revealed that the leaves sprayed with urea-NH$_4$NO$_3$ (UAN) solution displayed visible signs of damage in leaf tissue. This damage was noticeable in the form of chlorosis at the tip of the leaves; it also resulted in the deterioration of the chloroplasts and decreasing GS2 activity in the leaves (Gonçalves et al. 2020). It is also known that sodium nitrate, ammonium sulfate, and UAN can cause more severe leaf scorching compared to urea as N source (Trivelin et al. 1988, Clapp and Parham 1991), suggesting that NH$_4^+$ toxicity is promoted by the lack of balance between N uptake and assimilation (Bittsánszky et al. 2015, Esteban et al. 2016, Jian et al. 2018). This was confirmed by our experiment, where the leaves sprayed with (NH$_4$)$_2$SO$_4$ showed more scorching than those sprayed with CoRoN or urea. CoRoN is a urea-based fertilizer (https://www.helenaprofessional.com/products/details/coron/), which is specially formulated for foliar spray.
Urea is often utilized as a supplementary source of N for foliar fertilization due to its quick absorption, which results in a high recovery rate (within a few hours). This has been demonstrated in various crops such as apple (Dong et al. 2002), grass (Stiegler et al. 2011), sugarcane (Trivelin et al. 1988), tea (Ruan and Gerendás 2015), tomato (Tan et al. 1999), and wheat (Smith et al. 1991). Being a small and neutral molecule with high solubility, urea rapidly penetrates the leaf cuticle by lipophilic (Witte 2011, Fernández and Brown 2013) and stomatal pathways, the latter facilitated by aquaporins (Wang et al. 2008, Heinen et al. 2014, Dawson and Goldsmith 2018). Its hydrolysis occurs within the leaf by urease producing NH₃ and CO₂ (Bohner et al. 2015). These properties could also potentially lead to leaf scorching (Castro et al. 2022) because of its repaid accumulation in the cells. Adding a urease inhibitor to the solution can lessen this reaction, although it may raise the total urea concentration in the leaf and worsen the leaf tip scorching (Krogmeier et al. 1989). Hence, in our experiment, we opted to increase the frequency of urea spray at relatively lower concentrations; and found it to be less harmful.

As the treatments were sprayed with the surfactant, for all the treatments, the drops of fertilizer were adhering to the leaves, including the control treatments, which contributed slightly to leaf scorching. The high scorching at the edges of the plant leaves could be due to the droplets that dripped off from the leaves accumulated at the edge. The scorching leaf edge was also noticed in both experiments 2 and 3; however, this was minimal to none for the 0.125% and 0.25% urea. The scorching in the leaves of experiment 3 was not observed in the 0.25% concentrations of urea and CoRoN. The level of scorching in urea- and CoRoN-sprayed plants suggests that these two forms of N are much better choices for foliar fertilization when compared to NH₄NO₃. Oosterhuis and Steger (1999) also came to the same conclusion with foliar fertilization of cotton plants with urea and CoRoN, as well as Kotz-Gurgacz et al. (2018) with common bean (Phaseolus vulgaris).
CoRoN is a slow-release N liquid fertilizer. It is a combination of 25% control release N (long chain polymethylene urea) coupled with 75% fast release low biuret urea (Oosterhuis and Steger 1999). This combination increased leaf absorption and improved yield potential (Oosterhuis and Steger 1999, Oosterhuis et al. 2015). CoRoN is also a slow-drying foliar fertilizer that retains moisture while reducing crystallization on the leaf. This ultimately lowers the risk of NH₃ volatilization and allows for a stronger adherence to plant leaves, leading to increased N uptake. Increased uptake of CoRoN and growth enhancement were reported in foliar-sprayed cotton plants with minimal phytotoxicity (Oosterhuis and Steger 1999, Wilborn et al. 2006, Oosterhuis et al. 2015). With the observed decrease in Glu content, it could be argued that at the “entry point of N,” CoRoN is being used up by the plants faster than the other forms of N.

Understanding how plants regulate their PA and AA metabolism is crucial due to their critical role in responding to both biotic and abiotic stress, as well as their contribution to development and interaction with other macromolecules and pathways (Minocha et al. 2014, de Oliveira et al. 2018, Decouard et al. 2022, Murillo-Peña et al. 2023). Furthermore, AA metabolism is the cornerstone of plant N management since it is actively involved in efficiently using N for protein biosynthesis (Dellero 2020). In most plants, AAs constitute the most abundant chemical forms in which N is transported. The activity of GS and Glu dehydrogenase (GDH) was significantly improved in plants treated with different N fertilizers via the roots of wheat (Effah et al. 2023). Duan et al. (2023) showed that in the blackberry cultivar ‘Ningzhi 4’ (Kiowa × Hull) treated with NH₄⁺, the AAs, Gln, and Asn increased vs. the plants that did not receive N fertilizer.

In synthesis of other AAs (analyzed in this study), Glu plays a major role. It is the precursor of the Glu family AAs, that include Pro, GABA, Orn, and Gln (Majumdar et al. 2016). Other AAs, like citrulline and Arg, are derived from Orn, and His from Gln. (Mohapatra et al. 2009) have
shown that a common signal transduction mechanism may trigger all sub-pathways, coordinating the direction of Glu into the 3 interacting pathways. Biochemically, these AAs (Gln, Glu, GABA, Pro, Arg) are close to the “entry point” of inorganic N (i.e., GS/GOGAT] cycle) into the organic N metabolism. Furthermore, the C skeleton of Glu and Gln is directly related to primary energy metabolism, i.e. the TCA cycle (Trovato et al. 2021). As stated widely in the literature, there is little doubt that Glu is a central molecule in AA metabolism in higher plants. In addition, Glu serves as the precursor for synthesizing chlorophyll in developing leaves (Yaronskaya et al. 2006).

Our results show that the Glu content decreased in experiment 2 in all treatments particularly after 3 days of spray. While a decrease in Glu was also observed in experiment 3, it happened only after the 3rd foliar spray. In experiment 3, there was no significant change in the content of Gln, which is directly synthesized from Glu. In experiment 2, the Gln content was undetectable, thus no conclusion is drawn. This observation contrasts with the results of a previous study in our lab with two clonally propagated shrub willow cultivars, namely, ‘Fish Creek’ (Salix purpurea L.) and ‘Preble’ (S. viminalis L. × S. sachalinensis F. Schmidt × S. miyabeana Seemen) (Gagne et al. 2019). In these willow cultivars, increases in Glu and Gln were observed, indicating a rapid assimilation of these two AAs in plants sprayed with NH₄NO₃. One possible explanation for the reduction of these two AAs in our experiment 2 could be the plant age. Another explanation could be altered metabolism of N assimilation in the leaves. A study with foliar-sprayed urea or UAN on wheat showed that foliar N-fertilization not only increased the free NH₄⁺ in the leaves but also decreased the proportion of Glu in the total pool of free AAs (Castro et al. 2022). In another study conducted with the hybrid poplar (P. alba × P. berolinensis), Hu et al. (2019) reported increased Glu and Gln in the plants treated with the mixed N solution.
Among the AAs involved in stress defense, Pro is essential because it accumulates in most plant species in response to different stresses and is believed to contribute to stress tolerance (Szekely et al. 2008, Trovato et al. 2021). Accumulation of Pro has also been reported in plants treated with high concentrations of N in sugar beet (Monreal et al. 2007). Proline and GABA are both derived from Glu, whereas Pro can be synthesized from Orn (Mohapatra et al. 2009). Monreal et al. (2007) suggested that excess N increases Pro synthesis from Orn. Our data show that in experiment 2, the Pro and GABA decreased in all the treated plants at 3 days after N spray. In contrast, an earlier study with transgenic poplar N6 cell cultures showed higher cellular Pro levels on the first two days, which dropped to the level of control plants from day 3 onwards (Mohapatra et al. 2009). Of course, it is very likely that the fast growth of cell cultures may be responsible for this rapid metabolic change. Brugière et al. (1999) proposed that plants may synthesize Pro to assimilate excess NH$_4^+$, thus reducing NH$_4^+$ toxicity. It can be suggested that in our plants, by day 27, the plant has stored enough N and has a high N reservoir, leading to Pro accumulation. Murillo-Peña et al. (2023) recently reported that Pro levels of grapevines sprayed with urea increased at the end of fruit ripening stage, where grapevine would face osmotic stress due to the accumulation of sugar. This accumulation was not seen in our experiment 3, where only young leaves were sampled. Hence it could be speculated that increase in Pro in experiment 2 was seen because leaf samples were collected and pooled from both the top and bottom part of the plant.

Past studies from our lab have shown that Orn plays a crucial role in signal transduction, acting as both a sensory and a regulatory molecule, although the exact mechanism remains unknown (Majumdar et al. 2013, Majumdar et al. 2015, Majumdar et al. 2016). Orn also serves as the intermediate in the formation of Arg and Pro (Roosens et al. 2002, Couchet et al. 2021). In experiment 3, Orn was not detected for most days in the young leaves that were sampled. Similar
observations were made in the willow cultivars treated with N fertilizers, where Orn was the least abundant AA, always remaining below 5 nmol g\(^{-1}\)FW (Maegan Gagne, Master’s thesis, 2014). It has been noted that Arg has the highest N/C ratio, making it the most effective storage form of N (Titus and Kang 1982). When urea is the only source of N, the transport of AAs in the plant and N storage are unbalanced (Cao et al. 2010).

When N is abundant, plants often store it as Arg (Otálora et al. 2018). Furthermore, Arg is a terminal product in plants for protein synthesis and PA biosynthesis (Morris 2007). Unfortunately, Arg did not separate as a clear peak from Thr and Gly in the current HPLC procedure. However, the combined AA peaks show low levels after the first treatment in experiment 3 and higher peaks on day 31. It could be argued that Arg was accumulated in the young leaves by the end of 4 weeks of N spray after demand for the synthesis of other metabolites was met. It is likely that the decrease in Orn in poplar leaves is due to increased production of Arg.

Interestingly, the other AAs showed varied concentrations in response to N treatments with the time of sample collection. Similar to Glu, Pro and GABA, Lys and Asp contents were lower within 3 days after N spray in experiment 2. A similar pattern of decline was seen in Ile, Leu, Val, and Ser. Often in plants, Lys is derived from Asp, Thr and Met (Varisi et al. 2008), and is present in low levels (Trovato et al. 2021). A close connection between the metabolism of Ser and Lys was shown by (Kishor et al. 2020). By expressing the phas::DHPS in the AtLKR/SDH knockout mutant of Arabidopsis, Zhu and Galili (2003) showed the metabolic relationships between Lys and Thr, Lys and Met, and Lys, Glu and Asp. The reduction of the AA levels can be explained by the fact that the metabolism and catabolism of these AAs are interconnected, and the reduction in one AA affects the other AAs of the chain.
If AAs like Cys or Lys are accumulated in high concentrations, they may be toxic to the plants; hence, they must be degraded (Zhu and Galili 2004). For example, transgenic rice plants overexpressing the bacterial Lys-biosynthetic pathway genes showed reduced nutritional qualities and a dark-brown endosperm (Yang et al. 2016). It is possible that the decrease in Cys and Lys levels, observed just 3 days after foliar spray of N on poplar leaves, is a precautionary measure to prevent toxicity that may occur due to over accumulation of these two AAs. Serine and Lys were accumulated in the leaves in experiment 2 in plants treated with NH$_4$NO$_3$. Similar observations were made by Sun et al. (2019), where the accumulation of Lys was observed in response to NH$_4^+$ supply. Serine content can also be high under conditions of increased photorespiration (Fernie and Bauwe 2020). Fu et al. (2023) exposed tobacco (Nicotiana tabacum) leaves to environments containing three different levels of O$_2$ (2%, 21% and 40%) and found that the rates of export of Ser and Gly to alternative sinks were high. Increased photorespiration rate is the leading cause of Ser accumulation, which can also provide NH$_3$ during N deficiency (Shi and Bloom 2021).

In experiment 2, the accumulation of Ser on day 8 and day 22 in the NH$_4$NO$_3$-treated plants can be explained by the buildup of toxic quantities of NH$_4^+$ as a result of photorespiration. Although other processes such as AA catabolism, phenylpropanoid metabolism, and NO$_3^-$ reduction could result in the accumulation of high levels of NH$_4^+$, accumulation of Ser was only seen due to photorespiration (Bauwe et al. 2012).

Methionine is an essential AA that is often found in low concentrations, and is the substrate for Cys (Kishor et al. 2020). The reduced levels of Met were observed in experiment 2 in all treated plants. The only exception was on day 27, where the plants treated with 0.125% NH$_4$NO$_3$ showed an increase of Met. Based on experiment 3, it was found that the young leaves from the top of the plants had higher levels of Met accumulation compared to experiment 2 after the 3$^{rd}$ foliar N
treatment. Girija et al. (2020) reported similar results with Arabidopsis. The authors concluded that the abundance of Met residues in storage proteins is likely the main factor limiting Met accumulation in Arabidopsis.

Branched-chain AAs are often accumulated in response to various stresses (Joshi et al. 2010, Hildebrandt et al. 2015). Lysine was present in relatively small amounts in poplar cells; and a reduction in Lys was seen on most days of experiment 2, except for day 22 for urea and NH$_4$NO$_3$ treatments. Lys accumulation is also an indicator of abiotic stress as shown by Obata and Fernie (2012) in Arabidopsis exposed to abiotic stresses. In the Moroccan variety of Barley (Hordeum vulgare L.), Lys accumulated in leaves when the plants were exposed to both low and high N (Decouard et al. 2022). Both these AAs also serve as substrates in the biosynthesis of lipids and glucosinolates (Binder et al. 2007).

Further studies to understand how total AA contents differ with in response to different N levels (NO$_3^-$ and NH$_4^+$). Xu et al. (2023) showed that with NO$_3^-$-N, total AA concentrations increased with the increasing total N concentration, while NH$_4^+$-N concentrations showed a trend of first increasing and then decreasing with increasing N.

Polyamines in plants are crucial in the regulation of cell division, protein synthesis, and many other cellular processes in leaves (Minocha et al. 1999, Minocha et al. 2004, Minocha et al. 2014, Minocha et al. 2015, Nahar et al. 2016). It is known that in most plants both Arg and Orn serve as precursors for Put (Bhatnagar et al. 2001, Bhatnagar et al. 2002). The availability of higher N often influences cellular PA biosynthesis in plants. For example, a rapid increase in Put was seen in willow leaves sprayed with urea compared to the control plants (Gagne et al. 2019). The current study shows a similar trend in experiment 3, where spraying 0.5% urea increased Put and Spd in plants 3 days after treatment. In experiment 2, following the same trend of AAs such as
Glu, the Put contents at 3 days after the first treatment remained low in all plants throughout the experimental period (Fig. 23A). It is interesting to note that Put accumulated after the second foliar treatment with CoRoN in the same plants on day 17. In the controls, Put accumulation was higher than most treatments, perhaps due to the low N stress. Spermidine and Spm contents did not fluctuate much in response to different treatments, with controls being within the same range on most days. All three PAs were present in relatively low amounts during most days of analyses.

Putrescine and other PAs are intricately connected to AAs, organic acids, and hormonal communications in plants (Hu et al. 2020). Putrescine conversion to higher PAs (e.g., Spd and Spm) is considered an adaptive strategy for the plants to environmental alterations (Li et al. 2015). The Spd/Put ratio is also a biomarker of plant regeneration and growth (Silveira et al. 2006). For example, in Rice plants, Spd/Put ratio (<2.3) showed an increase in embryo growth (Shoeb et al. 2001). Increased Spd/Put ratio was also observed during somatic and zygotic embryo development of Pinus radiata (Minocha et al. 1999) and during somatic embryo development in Picea rubens Sarg. (Minocha et al. 2004). The present study did not show significant changes in the Spd/Put ratio. Based on this observation, it appears that these plants did not experience substantial growth during the experimental period. The data from the plant height, biomass, and stem basal area measurements support this conclusion.

Plants can adapt to their new environment when the rate of N application is adjusted (Kiba and Krapp 2016). An increase in the concentration of N in regions where photosynthesis occurs can enhance the process of N assimilation; however, this can lead to competition with C assimilation for resources like ATP, NADPH, and the C produced by photosynthesis. Variables such as chlorophyll content, stomatal conductance, and photochemical parameters such as Fv/Fm ratio are often used to understand the relative N status of plants and yield level. Our results
experiment 2 show very little difference among various treatments vs. the control for these parameters. A study to explore the responses of N fertilizer on chlorophyll content by Hokmalipour and Darbandi (2011) showed that in 3 cultivars of maize, a significant increase in N was correlated with chlorophyll content in the leaves. Photosynthetic capacity is also closely linked to leaf N content, as chlorophyll and proteins in the thylakoids and Calvin-Benson cycle contain most N (Evans 1989, Perchlik and Tegeder 2018). As stated earlier, N metabolism in plants is also tightly coupled with C metabolism (Coruzzi and Zhou 2001). In this context, regulating AA content and transport through the plant is critical for plant adaptation to C and N status, development, and defense. Varied but insignificant changes observed in the protein content suggest that the rapidly synthesized AAs were not utilized for protein synthesis in our study.

On the other hand, it is known that variations in the supply or metabolic status of one element (C or N) may substantially impact the metabolism of the other. In transgenic A. thaliana plants that expressed cyanobacterial fructose-1,6-bisphosphase and sedoheptulose-1,7-bisphosphatase in the chloroplasts, an increase in photosynthetic intermediates led to deficits of free AAs and a transient imbalance in the ratio of C to N in the leaves, which caused significant changes in the expression of the genes involved in the Calvin cycle and AA biosynthesis (Otori et al. 2017). In experiment 2, total N percentages declined in all treated plants, with the plants treated with 0.5% CoRoN by the day of harvest. This further supports the earlier observations made with other metabolites that CoRoN provides more C and N than other metabolites in the plant.

Increasing leaf N effectively improves C fixation and photosynthetic ability (Perchlik and Tegeder 2018). However, the competition for ATP, NADPH, and C between photorespiration and photosynthesis can limit plant growth (Xu et al. 2023). In the younger leaves of experiment 3, a trend of declining sugar levels was observed for fructose. Although the same observations were
made in glucose and galactose a definite conclusion cannot be made as the two sugars did not separate during the HPLC run. Similar results were reported in Tomato (*Solanum lycopersicum* L.) plants supplied with different N levels, showing that with the increase of N concentration, the contents of glucose and fructose decreased or the carbohydrate accumulation in the leaves was inhibited (Xu et al. 2023).

Foliar applications of NO$_3^-$ and increased leaf temperature with decreased leaf hydraulic performance (water evaporation and conductance), can cause changes in biomass, physiological processes, and leaf morphoanatomical traits (Monteiro et al. 2016). There is a correlation between leaf area and chlorophyll content for some poplar genotypes with rapid growth rates. With *Populus × euroamericana*, a positive correlation of leaf area with chlorophyll content and photosynthetic was reported by addition of N (Ripullone et al. 2003). Another study (Hu et al. 2014) reported that in poplar clones XH and BL3 treated with N, leaf area increased with concomitant photosynthetic rate and chlorophyll content compared to the controls. In experiment 2, significant changes in the plant biomass, transpiration, and photosynthetic rate were not visible. Also, no substantial changes in the leaf area were observed. However, the total biomass and leaf area data were collected on the day of harvest only. Therefore, an overall conclusion cannot be made as to how the different forms of N affected plant growth. Significant changes were observed in the stomatal conductance rate in the plants sprayed with NH$_4$NO$_3$ and urea.
Conclusions

Altogether, this study suggests that the level of N starvation achieved does affect the outcome significantly. Based on the data obtained from analyzing AAs, PAs, C/N ratios, total protein, total chlorophyll and carotenoids, moisture content, growth parameters it can be concluded that different forms of N fertilizers are metabolized differently in plant. Out of the three fertilizers used foliar application of CoRoN, a form of urea, was more effective as a fertilizer than the other two forms used in this study. By increasing the duration of the experiment, more impact on the growth parameters could be observed.
CHAPTER 2: TO STUDY THE PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF POPLAR NM6 PLANTS TO LEAD STRESS AND ITS REVERSAL BY PUTRESCINE SPRAY

Abstract

We examined the effects of one-time soil application of two different concentrations (50 and 150 µM) of lead (PbCl₂) accompanied by 1 mM putrescine (Put) foliar spray on 9-months-old hybrid poplar NM6 (Populus nigra L. x P. maximowiczii A. Henry) plants. Our objectives were to analyze: (1) metabolic changes in leaf and root tissues in response to lead; and (2) to test the effects of foliar Put spray to ameliorate the effects of lead in the leaves. These plants were produced by tissue culture of leaves from a 3-year-old tree grown at the UNH Kingman Farm. The tissue-culture plants were rooted and grown in pots at the UNH Greenhouse facility for two years. Leaf samples were analyzed: (1) on days 3, 7, 14, and 21 after lead application for free polyamines, AAs, phytochelatins, gas exchange, total soluble proteins, and chlorophyll contents; (2) roots collected on the day of harvest (22 days) were analyzed for free polyamines and phytochelatins. Results show that: (a) although lead caused changes in leaf metabolism (AAs, phytochelatins, chlorophyll, and carotenoids), the effects were not always dose dependent.; and (b) amelioration of lead effects by Put was minor and visible only on certain days of analyses for some metabolites. The effects of lead in leaves on glutamic acid, glutamine, alanine, and phytochelatin (gamma-glutamyl-cysteine) were significant on some days. In roots, only proline was significantly increased at 22 days post-treatment. Though many poplar species can be used for phytoremediation, the lack of significant responses in several physiological and metabolites (gas exchange, total soluble proteins, soluble sugars, polyamines) in the present study with poplar NM6 could be either because the level and frequency of lead treatment may not have been sufficient for the size of the plants or NM6 is not an efficient phytoremediator as some other poplar species.
Introduction

Lead (Pb) is a major HMs that has gained considerable attention as a potent environmental pollutant and a strong plant growth inhibitor. Contamination from Pb has become a severe problem worldwide. Based on the frequency of occurrence, Pb was reported as the second most hazardous substance, after arsenic (As), by the Agency for Toxic Substances and Disease Registry because of its toxic effects on living organisms (ATSDR 2023). Properties such as high density, lower melting point, acid resistance, and easy moulding have made Pb an industry-friendly HM (Mitra et al. 2020). Thus, different anthropogenic practices like electroplating, mining, smelting, burning of fossil fuels, steel industry, atmospheric deposition, and use of inorganic fertilizers and pesticides have raised the Pb concentration in the environment (Tchounwou et al. 2012, Lenart-Boroń and Boroń 2014, Weldeslassie et al. 2018). In 2022, the production of recoverable lead from mining operations was 266,000 in the United States alone (USGS 2023).

In plants, Pb is known to induce a broad range of toxic effects, such as inhibiting plant growth, root elongation, seed germination, seedling development, transpiration, chlorophyll production, lamellar organization in the chloroplast, and cell division (Chandrasekhar and Ray 2019, Mitra et al. 2020). Moreover, Pb can adversely affect various physiological plant processes, leading to dwarfed plants and ultimately impacting crop yield (Zulfiqar et al. 2019). However, the extent of these effects varies and depends on the Pb concentration tested, the duration of exposure, the intensity of plant stress, the stage of plant development, and the particular organs studied (Pourrut et al. 2011, Al-Jobori and Kadhim 2019). Although Pb is not an essential nutrient for plants, it is quickly taken up by plants from the soil and accumulated in the root, while only a small fraction is translocated into the shoots (Patra et al. 2004, Cenkci et al. 2010).
There is a significant interest in using transgenic poplars tolerant to organic pollutants or HMs for phytoremediation. The hybrid *P. tremula × P. alba* plants overexpressing γ-glutamylcysteine synthetase from *E. coli* produced higher levels of glutathione, exhibited enhanced tolerance to chloroacetanilide herbicides, and accumulated more Cd in the root tissue than wild type (WT) plants (Koprivova et al. 2002, He et al. 2015, Samuilov et al. 2016). Transgenic *P. deltoides* plants expressing a modified mercuric ion reductase gene (*merA9* or *merA18*) could grow in the soil containing up to 400 mg/L Hg^{2+}, whereas the WT shoots died (Che et al. 2003). Also, these transgenic plants were able to detoxify phenylmercury acetate (Shah and Pathak 2019). Furthermore, transgenic *P. × canescens* expressing heat shock transcription factor *SpHsfA4c* from *Sedum plumbizincicola* showed increased levels of five enzymes ascorbate peroxidase (APX), catalases (CAT), superoxide dismutase (SOD), peroxidase (POD) and glutathione S-transferase (GST) as compared to the wild-type plants (Yu et al. 2023).

Root uptake is plants’ main pathway for Pb accumulation (Al-Jobori and Kadhim 2019). In soil, Pb may occur as a free metal ion, bound to components like HCO$_3^-$, CO$_3^{2-}$, SO$_4^{2-}$, and Cl$^-$, or it may interact with organic ligands such as humic acids, fulvic acids, and AAs (Uzu et al. 2009, Sammut et al. 2010). Factors determining the speciation of Pb within soil and availability of Pb to plants depend on soil pH, particle size, soil type, organic matters, cation exchange capacity, iron oxides, plant’s root structure, root mycorrhizae, exudates and transpiration rate (Amundson et al. 2015, Tian et al. 2018). Blaylock et al. (1997) observed that at the pH range of 5.5–7.5, phosphate or carbonate precipitates control Pb solubility and availability to plants. Heavy metal mobility decreases with increasing soil pH due to the precipitation of hydroxides, carbonates, or the formation of insoluble organic complexes (Balkhair and Ashraf 2016). The presence of other HMs such as Zn, Cu, Ni, Cd, and Cr has an antagonistic effect on the Pb availability in soil (Orroño et
and root exudation (Leyval and Berthelin 1991) by modifying soil pH and by the forming of soluble organometallic compounds (Mitra et al. 2020).

The “transfer factor,”; the ratio between the concentration of Pb in the plant vs. the concentration of Pb in the soil, can be used to measure the amount of Pb that moves into the plant from the soil (Arshad et al. 2008, Liu et al. 2010). Generally, plants having a transfer factor (TF) greater than one are categorized as hyperaccumulators. In contrast, those with less than one transfer factor are termed non-accumulators of Pb (Arshad et al. 2008). The TF value for Pb is less than one as a higher proportion (95%) of Pb gets accumulated in the roots (Chandra et al. 2018), and a small fraction (5%) is translocated to shoots or leaves (Zhou et al. 2016). In roots, ion-exchangeable sites in the cell walls help to bind Pb extracellularly. Therefore, following uptake, Pb localization is expected within the root due to the strong binding of the carboxyl group of glucuronic acid and galacturonic acid of the cell wall, which restricts the apoplastic transport to aerial parts (Poleć-Pawlak et al. 2007). Following Pb treatment, root cells in Arabidopsis thaliana and Pb-galacturonic acid complexes have been reported (Inoue et al. 2013). Another reason for the lack of transport of Pb from roots to aerial plants is controlled by negatively charged pectins within the cell wall, precipitation of insoluble Pb salts in intercellular spaces, accumulation in plasma membranes, and sequestration in the vacuoles of rhizodermal and cortical cells (Pourrut et al. 2011, Zhou et al. 2016). In Salix spp. It was shown that the majority of Hg is accumulated and retained in the cell wall of the roots, and only a small part is transferred to the shoots. In poplar plants treatment with four different HMs, As, Zn, Cd, and Pb showed that Cd and Zn have a higher accumulation compared to As and Pb in the above-ground biomass (Stoltz and Greger 2002, Vyslouzilova et al. 2003), hence it’s used in HM phytoremediation.
Adverse effects on germination and growth can occur when plants are exposed to Pb, even at micromolar levels (Kopittke et al. 2007). Lead-induced inhibition of seed germination has been reported in *Hordeum vulgare*, *Elsholtzia argyi*, *Spartina alterniflora*, *Pinus halepensis*, *Oryza sativa*, and *Z. mays* (Sengar et al. 2008). Apart from inhibiting the development and sprouting of the seedlings, Pb can also inhibit plant growth. Studies have shown that plants displayed apparent symptoms of growth inhibition, with fewer, smaller, and more brittle leaves having dark purplish abaxial surfaces (Gupta et al. 2010, Shakoor et al. 2014, Dai et al. 2023). This slow progress in plant growth from Pb exposure may be attributed to nutrient metabolic and photosynthetic downward flux (Jatav et al. 2021).

High concentrations of Pb decrease the protein pool via interactions with cytoplasmic proteins and the generation of ROS that lead to oxidative stress (Chatterjee et al. 2004, Mishra et al. 2006, Gupta et al. 2009). Plants exposed to Pb ions show a decline in photosynthetic rate which results from changes in chloroplast ultrastructure, restrained synthesis of chlorophyll, plastoquinone, and carotenoids, obstructed electron transport, inhibited activities of Calvin cycle enzymes, as well as deficiency of CO_{2} as a result of stomatal closure (Stefanov et al. 1995, Zulfiqar et al. 2019).

Unlike photosynthetic activity, the effect of Pb on sugars and gas exchange has been little studied (Seregin and Ivanov 2001). Stomatal conductance of Pb-stressed plants was reported to be reduced by 40–50% as compared to control in plants grown in Pb (500 and 2000 mg kg^{-1}) contaminated soil (Romanowska et al. 2006, Zulfiqar et al. 2019).

Plants possess a sophisticated and interrelated network of defense strategies to avoid or tolerate HM intoxication. Physical barriers are the first line of defense in plants against metals. Some morphological structures like thick cuticles, biologically active tissues like trichomes and
cell walls, and mycorrhizal association can act as barriers when plants face HM stress (Harada et al. 2010). Trichomes, for instance, can either serve as HM storage sites for detoxification purposes or secrete various secondary metabolites to negate the hazardous effects of metals (Wong et al. 2004, Hauser 2014, Emamverdian et al. 2015).

When plants are exposed to HM, a variety of metabolites are produced which accumulate in millimolar concentrations, particularly AAs such as proline (Pro) and histidine (His), peptides such as glutathione (GSH) and phytochelatins (PCs), PAs, nicotinamide, and mugineic acid that act as the scavengers of free radicals (Rabêlo et al. 2018, Nowicka 2022). Nitrogen metabolism, therefore, plays a crucial role in plants' response to HMs. Synthesis of PCs, \((\gamma\text{-Glu-Cys})_n\text{-Gly}, n = 2–11\), and its homologues is a constitutive mechanism to cope with toxic metals in various plants, algae, and fungi. Amino acids such as Gly and Glu are precursors of GSH and phytochelatins (PCs), which are important for metal binding. Furthermore, Arg is important for synthesizing PAs and acts as signaling molecules (Sharma and Dietz 2006).

Cell walls, plasma membrane, vacuoles, anions in the extracellular space, and cytoplasm are the main components of the defense barriers where the Pb ions can be precipitated, excluded by the channels and transporters in plasma membranes, and compartmentalized by the vacuoles (Fahr et al. 2013, Kumar and Prasad 2018). The roots and soil particles interact at the rhizosphere, where water or nutrients absorbed from the soil are transported to the other plant parts through mass flow and diffusion along with other ions and molecules (Lynch and Whipps 1990, Robinson 1991).
Methods

Wild-type poplar (NM6) plants were tissue culture generated and transferred to the Macfarland greenhouse at the University of New Hampshire in November 2020. These plants were grown in pots (17 cm x 15 cm) containing Pro-Mix® MP Mycorrhizae™ Organic™ (Premier Horticulture Inc., Quakertown, PA, USA). All plants were grown in the greenhouse for about a year (mid-August 2021). All treatments consisted of six replicates. The heavy metal treatment was given at time zero and at 7 and 14 days. With minor modifications, pots were saturated with 200 ml of a solution of lead chloride (PbCl₂) at two different concentrations (50 μM and 150 μM) which were decided based on literature review. Twelve plants that received two different concentrations of PbCl₂ were also sprayed with 1 mM Put containing 0.05% (v/v) Silwet L-77 surfactant (Phytotechnology Laboratories, Lenexa, KS, USA). The control plants received an equal amount of water with Silwet. The Put spray was applied by hand using a 900 mL plastic mist spray bottle early morning.

The total soluble protein content, chlorophyll content, leaf gas exchange measurements, soluble sugars, polyamines, and AAs were measured according to the methods as described in chapter 1.

Derivatization of thiol compounds

The derivatization of thiol compounds with monobromobimane (mBBr) was based on the methods of Minocha et al. (2008). About 100 mg of tissue sample was placed in 1 ml of extraction buffer (6.3 mM diethylenetriamine-penta-acetic acid (DTPA) containing 0.1% (v/v) Trifluoroacetic acid (TFA)). Samples were stored at −20°C until the time of analysis. Samples were freeze-thawed three times before analyses to release cellular contents. The supernatant was collected by centrifugation at 13,000 × g for 10 min and was used for subsequent analyses. Briefly, 615 μL of
200 mM 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), buffer (6.3 mM DTPA, pH 8.2) was mixed with 25 μL of 20 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP). A 250 μL mix of standards or sample extract was added to this mixture. Ten microliters of 0.5 mM N-acetyl-l-cysteine (NAC) were added as an internal standard. This reaction mix was incubated at 45°C for 10 min. The derivatization was carried out in the dark for 30 min at 45°C after adding 10 μl of 50 mM mBBr. The reaction was terminated by adding 100 μl of 1 M methanesulfonic acid (MSA). The derivatized samples were filtered with a 0.45 μm nylon syringe filter. Thiol compounds were analyzed using the liquid chromatographic system consisting of a PerkinElmer (Wellesley, MA, USA) Series 200 pump. Data were integrated using TotalChrom HPLC software (PerkinElmer, Version 6.3.2).
Results

A greenhouse experiment was conducted in pots to determine the effects of foliar application of Put on poplar NM6 plants treated with two different concentrations (decided based on literature) of PbCl₂ (via roots) (Fig. 36).

The experiment did not detect visual symptoms of metal toxicity, such as necrotic spots on leaves, leaf chlorosis, browning of roots, and decreased leaf area (Fig 37). At the given doses of PbCl₂, all plants survived the Pb-supplemented soil, and growth inhibition was not measurable.

Figure 36. Poplar NM6 plants at the beginning of the experiment before spray

Figure 37. Healthy Poplar NM6 plants post-exposure to PbCl₂ treated with and/or without Put spray.
Overall, in response to the PbCl$_2$ treatment, the total soluble protein content varied only slightly among different days of sample collection (Fig. 38). The application of 150 µM PbCl$_2$ resulted in a slight increase in soluble proteins on day 7, but this response was not sustained on other days of collection. However, these differences were not statistically significant.

**Leaf chlorophyll and carotenoids**

With the exception of small changes in total chlorophyll on a couple of days (days 3 and 14) of collection, the overall contents of total chlorophyll remained within a narrow limit (about 4-6 mg$^{-1}$g FW) over the experimental period (Fig. 39A). A small but significant decrease in the total chlorophyll was observed in the plants treated with 50 µM PbCl$_2$ and sprayed with Put 14 days after the treatment. Chlorophyll content in all treated plants had recovered to levels similar to the control plants by the last sampling day.

No significant change was observed in either chlorophyll a:b ratio or the total carotenoids in the treated plant samples (Fig. 39B and 39C).
Gas exchange

Irrespective of the treatment, there was a general decline in stomatal conductance, transpiration rate, and photosynthetic rate from day 6 to day 21 in all treatments (Fig. 40A, 40B, and 40C). There was a lack of statistically significant changes among treatment groups in all three gas exchange measurements. However, a small increase in stomatal conductance was observed on day 6 in the Put-sprayed plants compared to the controls.
Figure 39. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on different days on the chlorophyll contents of leaves of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 6). (A) Total chlorophyll (B) Chlorophyll a: b and (C) Total carotenoids. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05).
Figure 40. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on different days on the gas exchange of leaves of the hybrid poplar NM6 plants. The data presented and mean ± SE (n = 6). (A) Stomatal conductance (B) Transpiration, and (C) Photosynthetic rate. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05).
**Foliar soluble sugars**

The leaf samples collected on several days after treatment with Pb and sprayed with Put were analyzed for a group of common soluble sugars by HPLC using 11 sugars as standards, namely rhamnose, xylose, arabinose, fructose, mannose, glucose, galactose, sucrose, trehalose, maltose, and raffinose. However, rhamnose, mannose, trehalose, maltose, and raffinose were not detected on any day in the leaf tissue samples. Also, the peaks for xylose, arabinose, glucose, and galactose were not separated during the analysis. Therefore, the data are presented for the combined peaks (Fig. 41B and 41C). All of the collected plant samples showed sugar levels within a narrow range of 0-25 nmol g⁻¹ FW, with the content of glucose + galactose being higher than other sugars on most days and for most treatments.

In response to Put spray, plants treated with 150 µM PbCl₂, there was a significant increase in fructose content 21 days post-treatment compared to the 150 µM PbCl₂ treatment alone (Fig. 41A). Likewise, on day 7, xylose + arabinose contents were higher in all plants treated with Pb with or without Put spray (Fig. 41B). The sugars, xylose, and arabinose were not detected in the plant samples on days 3 and 21. Foliar, glucose + galactose content in response to PbCl₂ treatment fluctuated somewhat on different days, with the lowest amounts being on day 21 (Fig. 41C). The changes in sucrose content were not notable for any day of analyses, as the statistical analysis showed several outliers on all days the samples were collected (Fig. 41D).
Figure 41. Effect of root application of two different concentrations of PbCl$_2$ (± Put spray) on different days on sugar contents of leaves of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 5). (A) Fructose (B) Xylose+Arabinose (C) Glucose+Galactose and (D) Sucrose. On a given day, * indicates data not available during the analysis.
Soluble foliar amino acids

The individual soluble AAs were quantified by HPLC to study the effects of Pb treatment via soil on AA content in the foliage. Eighteen out of 22 common proteinogenic AAs and two common non-proteinogenic AAs (GABA and Orn) were quantified for all days the samples were collected (Figs. 42-45). However, depending upon the age of the leaves (as judged by their location on the stem), the chromatographic profiles of certain AAs were not well separated; therefore, these AAs could not be quantified individually. For example, the peaks of Arg, Thr, and Gly and those of Ser and Met were not fully separated. Thus, the data on clearly identified peaks are presented here. The contents of AAs showed the following trend in order from higher to lower levels on a FW basis; Ala > Gln > GABA > Asp > Glu > Cys > Lys > Val > Phe > Ile > Pro > Met > His > Orn > Trp. The combined peaks of Arg, Thr, and Gly showed the highest total content (supplemental data). A trend of lower concentration of all AAs on day seven was observed in all samples, irrespective of treatment. In plants treated with 150 µM of PbCl₂, a significant increase in Asp content was observed in the leaves compared to the control plants at 3 days after treatment. Whereas there was a decrease in Asp on day 7, the Asp content increased twofold after 7 days in all plants (Fig. 42A). Similar trends were observed for Glu as well (Figure 42B). Glutamine, one of the most abundant AAs, produced some unique responses, especially two weeks from the PbCl₂ treatment (Fig. 42C).

Irrespective of the treatment, Gln increased significantly on day 7 compared to the control treatment (Fig. 42C). Putrescine-sprayed plants treated with the highest concentration of PbCl₂ exhibited a ~2-fold increase in the Gln content on day 14 compared to the control group. This was also the highest level of Gln observed out of all the sample collection days; the 21-day-old plants showed > 50% decrease in all samples. However, on day 21, all treatments with
150 µM of PbCl₂ (sprayed as well as unsprayed plants) had significantly higher Gln vs. the control plants.

In plants sprayed with Put, Ala increased significantly on days 7 and 14 at 150 µM of PbCl₂ compared to the control plants (Fig. 43A). The increase of Ala in the other treated plants was significant compared to the control plants on the 7th day, even though the amounts of Ala were the lowest in all plants on this day. Proline, predominantly synthesized from Glu, is usually present at higher levels in plants undergoing stress, including HM stress. However, no significant changes in Pro contents were observed in these samples (Fig. 43B). A higher Pro content was observed at 150 µM of PbCl₂ on day 21, but statistical analyses showed outliers which could explain the relatively high error bar.

As shown in Fig. 43C, GABA (which was almost 10-fold higher than Pro) also did not show much effect of either Pb or Put spray, except for the 14th day collection, when an increase was seen with Put spray on 150 µM of PbCl₂-treated plants.

Amino acids Val, Ile, and Leu all showed the lowest foliar concentrations on day 7 and little variation among the treatments on a given day (Fig. 44A, 44B, and 44C). As with Pro, there were outliers in statistical analysis for the 21-day samples.

Cysteine content of the leaf samples was higher on day 14 for most treatments, but no significant effect of treatment was observed (Fig. 45A). Similarly Met did not show any significant changes on all treated days (Fig. 45B).

Ornithine, which was among the lowest in quantity in all collections, was lower than the control plants 3 days after PbCl₂ treatment with no effect of Put spray (Fig. 45C). The lowest Orn content was observed in the highest concentration of PbCl₂ sprayed with Put on day 7. At
the lowest PbCl₂ concentration, Orn decreased on day 21 compared to the control plants. However, this cannot be attributed to a treatment effect, as Orn was detected in only one of the replicates out of the five.

**Soluble amino acids in roots**

As mentioned earlier, the cellular soluble AA contents in the roots were analyzed only for samples collected 22 days after the PbCl₂ treatment, with Pb treatment only. As with the leaf samples, Asp, Glu, Gln, and Cys contents were among the highest in the roots (Fig. 46A). On the other hand, Pro, Val, Ile, Leu, Met, Orn, and Lys were among the lowest concentration in the root tissue (Figs. 46B, 46C, and 46D). Very little effect of Pb treatment on AAs was seen in the root samples.
Figure 42. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 5). (A) Aspartic acid, (B) Glutamic acid (C) Glutamine. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05).
Figure 43. Effect of root application of two different concentrations of PbCl$_2$ (± Put spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 5). (A) Alanine (B) Proline (C) GABA. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05).
Figure 44. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 5). (A) Valine (B) Isoleucine (C) Leucine.
Figure 45. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on different days on the amino acid contents of leaves of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 5). (A) Cysteine (B) Methionine (C) Ornithine.
Figure 46. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on day 22 on the amino acid contents of roots of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 4). (A) Aspartic acid, Glutamic acid Glutamine, Cysteine (B) Proline, GABA, Valine, Alanine (C) Serine, Isoleucine, Leucine, Methionine, and (D) Ornithine, Lysine, Histidine.
**Foliar polyamines**

Following the treatment of PbCl$_2$, the foliar PAs showed varied outcomes. No statistically significant differences among various treatment groups were found in any of the three major PAs (Put, Spd, and Spm). On most days of sampling, the most abundant of the three PAs was Spd followed by Put and Spm. The Spd/Put ratios were the lowest on day 7 but were similar among the treatments on a given day of sample collection. The Put and Spd concentrations in the leaves were the lowest on day 21 in contrast to Spm which did not show much change over time in any sample.

The leaf Put content for 50 µM PbCl$_2$ treatment resulted in an increase (not statistically significant) in both sprayed and unsprayed plants compared to the control group on day 3 (Fig. 47A). However, the effects on plants exposed to 150 µM of PbCl$_2$ varied on different days. The Put spray led to lower Put content in plants treated with the higher concentration of PbCl$_2$ on day 7.

Three days after the exposure to 50 µM PbCl$_2$ there was a small increase in the leaf Spd content compared to the control plants (Fig. 47B); on other days, this effect was not seen. The lowest Spd amounts were seen on samples collected on day 21.

Foliar Spm of the treated plants did not show treatment effects on any of the days the samples were collected (Fig. 47C). A small decrease in the Spm content was observed on 14 days in response to Put spray treatment at 150 µM PbCl$_2$. The lowest Put/Spd ratio was observed on day 7 of PbCl$_2$ treatment (Fig. 47D).
**Root polyamines**

In roots, as with other collections, PAs were analyzed on the day of harvest and only for the unsprayed plants (control, 50 µM, and 150 µM PbCl₂). There was a decrease in Put with time of treatment compared to the control plants for 150 µM PbCl₂ on the 22nd day (Fig. 48A). Also, there were no differences in the absolute amounts of the three PAs with time or treatment (Fig. 48A, 48B, and 48C).

**Foliar thiol levels**

The study involved pre-column derivatization of thiols using mBBr. In this analysis, three types of PCs namely PC₂, PC₃, and PC₄, with their sulfur-containing metabolic precursors, GSH, γ-EC, and Cys, were quantified using the HPLC method. Overall, the highest thiol content was observed on day 7 (Figs. 49A, 49B, and 49C).

The contents of GSH (g⁻¹FW) showed no significant change in response to Pb treatment (Fig. 49A). By day 14, all plants had lower amounts of GSH. On day 7, all plants had higher Cys and a sharp decrease in Cys content was observed on day 14 vs. day 7 in all treatment groups (Fig. 49B).

As shown in Figs. 50A and B, PC₂ and PC₃ were not detected in samples collected on the 14th day post-Pb treatment, interestingly even in the control plants (could be an analytical error). On the same day, PC₄ was not seen in the control plants (Fig. 50C). On the 14th day of sampling, the PC₄ decreased by about two-fold compared to day 7. With regard to PC-4, the overall trends were similar to those for PC-2 in that the highest amounts were observed on day 7 and the lowest on day 14.
Figure 47. Effect of root application of two different concentrations of PbCl$_2$ (± Put spray) on day 22 on the polyamine contents of leaves of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 5). (A) Putrescine (B) Spermidine (C) Spermine and (D) Spermidine/Putrescine.
Figure 48. Effect of root application of two different concentrations of PbCl$_2$ (± Put spray) on day 22 on the polyamine contents of roots of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 4). (A) Putrescine (B) Spermidine and (C) Spermine.
**Root thiols**

The content of thiol compounds was measured in the roots on the day of harvest only (22 days post-treatment with PbCl₂), and only four replicates of treated plants were used for analyses. Furthermore, samples were only collected from two treatment groups, namely, 50 μM and 150 μM Pb treatment and the control group. Only 4 PCs were detected in the roots – GSH, Cys, PC₂, and PC₄. Interestingly γ-EC and PC₃ were not detected in the roots. Compared to Cys and PCs, GSH seems to be higher (10-12-fold) in the root samples (Fig. 51A and 51B). Otherwise, no significant effects of treatments were seen in any sample.
Figure 49. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on different days on the thiol compound contents of leaves of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 5). (A) Glutathione, (B) Cysteine (C) Gamma-EC. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05). On a given day * indicates data not detected during the analysis. Note that there are two vertical scaling for gamma-EC.
Figure 50. Effect of root application of two different concentrations of PbCl₂ (± Put spray) on different days on the PC content of leaves of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 5). (A) PC-2, (B) PC-3 (C) PC-4. Letters above the bar represent differences between the control and the specific treatment on a given day (P ≤ 0.05). On a given day, * indicates data not detected during the analysis.
Figure 51. Effect of root application of two different concentrations of PbCl$_2$ (± Put spray) on different days on the thiol compound contents of roots of the hybrid poplar NM6 plants. The data presented are mean ± SE (n = 5). (A) Glutathione, (B) Cysteine (C) PC-2 (D) PC-4.
Discussion

As described earlier (Introduction), environmental pollution of HMs due to a combination of natural and human activities is a serious problem worldwide. Among the HMs, Pb has drawn significant attention due to its heavy industrial use and being a highly toxic contaminant in our soils. Also, it is not an essential or a useful micronutrient for living organisms as some other heavy metals like Cu, Co, Fe, Ni, and Zn are (Asati et al. 2016, Redovniković et al. 2017, Amareh et al. 2023). Essential and non-essential HMs in higher concentrations have similar harmful metabolic effects on plants, e.g., inhibition of growth and photosynthesis, altered C and N metabolism, water balance, nutrient assimilation and senescence (Emamverdian et al. 2015, Atabaye et al. 2020). While high concentrations of all HMs can be toxic and inhibit plant growth (Hall 2002, Gawryluk et al. 2020, Fasae and Abolaji 2022); low concentrations can still cause multiple biochemical changes to affect metabolism, growth and development, thus reducing the productivity of the plant. This would be especially important for fast-growing plants like poplars which are of great commercial value. Plants, on the other hand have also developed a variety of mechanisms to counteract these harmful effects, including but not limited to reducing uptake, binding HMs to cell walls, secretion of metabolites to increase metal binding to soil, and production of biochemicals that can hold the HM within the cells and prevent its metabolic damage.

Many poplars are known to have HM tolerance and are used for phytoremediation of contaminated soil (Shim et al. 2013, Ovečka and Takáč 2014, Čudić et al. 2016, Shi et al. 2021). Several studies have reported increased production and accumulation of PAs when plants are exposed to abiotic stress conditions, including HMs. Also, enhanced abiotic stress tolerance in general has been reported in response to experimental manipulation or external applications of PAs (Ghosh et al. 2012, Parvin et al. 2014). There are reports showing that foliar application of a
PA like Put or Spd can alleviate abiotic stress by modulating water status, membrane stability and overall metabolism (Wasaya et al. 2023). Foliar spray of both Spd and Spm was shown to reverse lipid peroxidation caused by CdCl₂ in *Malus hupehensis* var. *pinyiensis* (Zhao and Yang 2008). Hence using poplar hybrid clone NM6, we studied the effects of foliar spray of Put on several physiological responses of young plants in response to Pb.

While we observed several changes in C and N metabolism, and some changes in other metabolites, especially some thiols; the effects were rather small in most cases. Thiols are a group of sulfur-containing compounds that can interact with HMs to reduce their toxicity. Thiols like GSH, Cys and gamma-Glu-Cys (γ-EC) showed significant increases in response to Pb treatment (Fig. 49) on certain days; and so, did some PCs (Fig. 50). On the other hand, due to the short duration of the experiment and the lower concentrations of Pb used, no effects were seen for other thiols. Consistent with the earlier work on poplars, typical morphological symptoms like browning of leaves, chlorosis, stunted shoot growth, and overall decrease in plant growth were not observed in our short-term experiments. It is known that even though roots can take up Pb, its transfer to the shoots is rather limited because of its high affinity for binding to pectins in the roots and precipitation in the root cells (Zhou et al. 2016, Chandrasekhar and Ray 2019). These observations were recently confirmed by (Gawryluk et al. 2020), who compared the uptake of three HMs (Cu, Zn, and Pb) by several species of grasses, and found that Pb was accumulated in the leaves of several grasses to a much lower level than Cu and Zn. In our study, the accumulation of Pb in the stem and leaf tissues was not analyzed.

Likewise, many of the metabolic effects of Pb like major changes in AAs, soluble sugars or chlorophyll contents were also not seen. Thus, there are two potential explanations for our results. First, the limited metabolic responses in the leaves in the present situation with poplar NM-
6 are due to the indirect effects of Pb on the roots, which may have reduced the uptake of inorganic nutrients and the uptake of water. Alternatively, the clone NM-6 of the hybrid poplar used in our study is genetically tolerant to HMs; the tolerance coming from one of the parents of this hybrid; i.e. *P. nigra*, which is known to be a HM tolerant species (*Baldantoni et al. 2014, Redovniković et al. 2017*). No direct work on HM tolerance of NM6 has been published yet. Accumulation of various PCs were also not affected by the presence of Pb in the soil (Fig. 51).

Regardless of the above arguments, similar to other HMs, Pb has been found to harm plant growth and biomass in both herbaceous and perennial plants. For example, *Hattab et al. (2016)* reported that increasing concentrations of Pb (100 to 500 mg L\(^{-1}\)) caused a reduction in the growth tolerance index of the *Acalypha indica* plant and reduced shoot length up to 49%, FW up to 68%, and DW up to 45%. *Stobrawa and Lorenc-Plucińska (2008)* reported that cuttings of black poplar (*Populus nigra* L.) when grown in Pb polluted soil, showed poor rooting in the cuttings, stunted shoots, small leaves, chlorosis, and eventual death of the plants. There are examples, however, which show that Pb has minimal or no significant effects on plant biomass or morphology, e.g., in *Lactuca sativa* (*Silva et al. 2017*) and *P. nigra* plants (*Redovniković et al. 2017*).

Our results are consistent with the above reports in that there were no visual symptoms of metal toxicity like leaf necrosis, chlorosis, decrease in growth, or leaf drop in our Pb-treated plants; there was only limited visible impact on the roots as well. Throughout the experiment, the plants appeared visually healthy, *i.e.*, the foliage was free of chlorotic spotting and scorching. However, the visual observations contradict some of the biochemical data (*e.g.*, foliar chlorophyll content), suggesting these plants were impacted by the Pb treatments. This reduction in foliar chlorophyll content might be due to the oxidation of photosynthetic pigments due to ROS over-production and lipid peroxidation, which can damage chloroplast structure. Furthermore, changes in pigment
protein complexes, an increase of chlorophyllase activity, degradation, and inhibition of photosynthetic pigments synthesis could also be responsible for the decline in chlorophyll content as suggested by several studies in the past (García-Sánchez et al. 2002, Ma et al. 2015, Chandra and Kang 2016, Jahani et al. 2019). Photosynthetic rates, stomatal conductance, and transpiration rates also declined to some extent as the experiment progressed, although this decline was not statistically significant compared to the control plants.

It can also be argued that the lack of physical damage observed could be because the poplar roots act as a barrier to Pb uptake and slow the transport to the shoots. This was shown by Shi et al. (2021), where *P. nigra* had lower Pb translocation from the roots to aerial tissues than that of *P. × canescens*. The roots have multiple defense mechanisms to block the transport of Pb ions to leaves (Fahr et al. 2013, Shahid et al. 2017).

Studies have shown that soil pH can also affect HM uptake (Adamczyk-Szabela and Wolf 2022). Low soil pH (< 4) can increase Pb mobility and result in higher uptake (Gorlach et al. 1990, Ernst 2000). Our soil pH was about 5.8-5.9, which could have negatively affected the uptake of Pb. Moreover, the synthetic soil mix used in the present study did not contain any significant microbe populations, that could aid in the uptake of Pb. Translocation of Pb differs from species to species and is extremely restrained (Yang and Ye 2015, Yongpisanphop et al. 2017, Xu et al. 2019) including that in poplar (*P. nigra* vs. *P. canescens* for Pb (Shi et al. 2021).

Phytochelatins (PCs) are a group of small peptides that play an important role in reducing the adverse effects of heavy metals in plants (Thangavel et al. 2007, Zhang et al. 2010, Zhang et al. 2022a). The synthesis of PCs and their sulfur-containing metabolic precursors, GSH, γ-EC, Cys, and sulfide, are common detoxification responses to Pb stress in yeast, algae, and higher plants (Maitani et al. 1996, Mendoza-Cózatl et al. 2005, Shi et al. 2021). High concentrations of
PCs increase the plant’s capacity to detoxify and sequester metals, and therefore, the major site of PC accumulation is generally in the roots where uptake occurs (Liang Zhu et al. 1999, Bisht et al. 2023). Overall, we found low concentrations of PCs in both the shoots and roots of poplar NM6 suggesting that the Pb ions may have been immobilized in the soil and unavailable for root uptake. This was unexpected as poplar has been documented as a plant used for phytoremediation of various pollutants, including HMs.

In our study, the most abundant thiol compound in the leaves and the roots were GSH suggesting that not all plants may be capable of inducing PCs upon exposure to metal/metalloids (Inouhe 2005, Gupta et al. 2010, Gupta et al. 2013a). For instance, high levels of PCs have been found in several species when exposed to Cd, including red spruce (Thangavel et al. 2007), O. sativa (Pál et al. 2017), Lolium perenne L. (Shi et al. 2021). High PCs were detected in the leaves of P. x canadensis Mönch., clone A4A, and in the P. nigra L., clone Poli treated with Cd (Pietrini et al. 2010). A study from our lab using the NM6 clone showed increased PCs to Cd exposure (Kundu 2023).

Amino Acids are important metabolites with different functions (in addition to being the substrate for protein biosynthesis), such as cell osmotic regulation, absorption of mineral nutrients, detoxification of HMs, and signaling (Kumar et al. 2016, Kocaman 2023). Synthesis and accumulation of AAs is an important mechanism for stress amelioration in plants. This accumulation can result from stress-induced protein breakdown (Di Martino et al. 2003, Planchet and Limami 2015). They play a more active role in the stabilization of enzymes and/or membranes, in addition to functioning as C and energy storage during growth and photosynthesis (Hildebrandt et al. 2015, Jander et al. 2020). Several AAs, such as Ile and Trp combination with other organic acids in plants, help in the chelation of HMs (Kocaman 2023). Thus, the
accumulation of excess AAs in response to Pb can be considered an important adaptive response to mitigate Pb toxicity (Sharma and Dubey 2005, Zhang et al. 2020), which may explain the increase of several AA content in our Pb-treated plants. The accumulation of free AAs in response to stress has been well documented e.g., wheat (Bassi and Sharma 1993), rice (Yang et al. 2000), spinach (Di Martino et al. 2003), Austrian pine (Sherwood et al. 2015), and tomato (Okunev 2019, Živanović et al. 2020).

Proline, an AA known to aid in osmoregulation, tends to accumulate in response to environmental stressors such as HM (Sharma and Dietz 2006, Shin et al. 2016, Feng et al. 2023). Glutamate is the primary precursor of Pro; however, Pro is also generated from Orn by ornithine-d-aminotransferase. However, during environmental stress conditions, plants follow the Glu pathway rather than the Orn pathway (Zhen and Ma 2009). Our findings are consistent with the above reports, where Pro content was higher in the shoots towards the end of the study compared to the roots (Figs. 43B, 46B). The non-protein AA, GABA, also acts as an important compatible solute in response to HM stress (Sharma and Dietz 2006). While the GABA content was much higher in the leaves than the roots (Figs. 43B vs. 46 C); the effects of Pb-treated plants on the accumulation of these AAs was minimal on different days of analyses. In response to Cd treatment Pro accumulated in high concentrations in the shoots of hybrid poplar plants has been reported (Nikolić et al. 2008).

Accumulation of AAs such as Asp and Glu in response to Pb exposure has been reported in many studies (Zafari et al. 2016). In our study, the contents of both Asp and Glu increased in response to Pb on some days but not all (Fig. 42). Aspartate also feeds into the synthesis of Lys, Met, Thr, and Ile (Angelovici et al. 2009); therefore, a significant increase in Asp only on day 3 may contribute to Asp complexation of Pb in the leaves or its conversion into other AAs. The
accumulation of these AAs might be associated with the storage of precursors for protein synthesis to prepare for rapid recovery of plant metabolism following HM stress.

The PA content in the roots and leaves had variable response to the exposure of plants to Pb. In general studies have shown that PA content could fluctuate depending on the HM the plants are exposed to (Sharma and Dietz 2006, Gupta et al. 2013b). Weinstein et al. (1986) observed up to 10-fold increment in Put content in Cd-treated oat seedlings and detached oat leaves with a marginal rise in Spd and Spm content. The lack of increase in Put and other PAs as well (Fig. 47) in the leaves in response to exogenous application of Put in the present study indicates that the plants are metabolizing the PAs in the cells as fast as it’s absorption. It has been reported that Put could be sufficient to minimize the oxidative damages indirectly induced by HMs (Mandal et al. 2013, Mandal et al. 2014). Putrescine being a diamine with a lower molecular mass, has a composition that allows it to quickly translocate and bind with the negatively charged domains of cell membranes. This could potentially prevent proteins from being oxidized by ROS (Rady and Hemida 2015).

The accumulation of PAs is known to increase tolerance to a variety of stressors (Hasanuzzaman et al. 2019) but the relationship between PAs and stress tolerance is complicated due to the production of H₂O₂ via their catabolism, which may have a toxic effect over long periods of time (Mohapatra et al. 2009, Minocha et al. 2014). Polyamines like Spd also enhance the GSH pool subsequently balancing redox homeostasis (Hasanuzzaman et al. 2019). For example, Spd protects *R. sativus* from the negative impacts of Cr (Yang et al. 2010). In *Potamogeton crispus* plants, upon Cd exposure, free PAs content increased along with GSH contents, which contributed to ROS detoxification (Yang et al. 2010). Although not demonstrated in this study, it is widely known that exogenous application of PAs increases HM tolerance in many plants. For example,
after the application of PAs in European pear (transgenic line), the chelation of metals (Zn, Cd, and Pb) increased, thus enhancing metal tolerance (Wen et al. 2010). However, the levels of Put, Spd and Spm decreased significantly in seedlings of *Nymphoides peltatum* under Pb stress (Qiao et al. 2014).

**Conclusions**

The effects of 50 mM and 150 mM of lead on several physiological and biochemical aspects of poplar (*Populus nigra x tremuloides*) clone NM-6 leaves as well as roots were quite modest perhaps due to the relatively lower concentrations of PbCl₂ that were used in this study and the limited duration of the study. For the same reasons, foliar spray of putrescine did not show major changes in the metabolites that were studied. The other hypothesis to explain these results is the fact that this hybrid clone is derived from a parent (*P. nigra*); which (like many other poplars) is known to be tolerant to heavy metals and is used for phytoremediation.
CHAPTER 3: TO PRODUCE AND METABOLICALLY CHARACTERIZE
TRANSGENIC PLANTS OF HYBRID POPLAR CLONE NM6 WITH THE mODC GENE THAT REGULATES POLYAMINE BIOSYNTHESIS

Abstract

Polyamines (PAs) are aliphatic amines that are present in all living organisms and are an obligatory requirement for cell survival. In higher plants, the most ubiquitous PAs are spermidine (Spd), and spermine (Spm), and their diamine precursor, putrescine (Put). A variety of roles have been proposed for PAs in plant growth, development, and stress response. Past studies in the Minocha laboratory have shown that the PA metabolic pathway is intrinsically connected with the metabolism of several AAs. Ornithine and Arg are direct precursors of Put and are synthesized from Glu. Suspension cultures of poplar (Populus nigra x maximowiczii) clone NM6, transformed with a constitutively expressing a mouse ornithine decarboxylase (mODC) gene, were used to study the effect of up-regulation of putrescine biosynthesis (and concomitantly its enhanced catabolism) on cellular contents of various protein and non-protein AAs. An overall increase in percent cellular N and C content was also observed in high putrescine metabolizing cells compared to control cells. Genetic manipulation of putrescine biosynthesis was shown to increase ornithine consumption due to a major change in the entire ornithine biosynthetic pathway and had pleiotropic effects on other AAs and total cellular carbon and nitrogen as well. My research focused on producing transgenic poplar NM6 plants expressing a mouse ODC genes that regulate the PA biosynthesis with the expectation that they would increase N and C assimilation, leading to increased growth and biomass accumulation and stress tolerance. Their biochemical characterization has not been completed.
Introduction

Plant transformation and regeneration are difficult and time-consuming in many economically important species, especially in forest tree species with a long lifespan (Han et al. 2000, Yevtushenko and Misra 2010, Wen et al. 2022). Genetic engineering in plants allows researchers to understand the function of specific genes in different biological processes, thereby enabling the production of new, improved trees with desirable properties. Techniques such as gene-specific overexpression (sense) and suppression (antisense and RNAi) under the control of constitutive, inducible and organ/tissue-specific promoters are often used (Movahedi et al. 2014, Aggarwal et al. 2022, Wen et al. 2022). The modification of one gene sometimes confers multiple new traits. Poplar species have been genetically engineered for several useful traits using genes from other plants, animals, or microorganisms (Song et al. 2019, Zheng et al. 2021).

The most commonly used transformation methods for poplar are Agrobacterium-mediated transformation and biolistic particle bombardment (Ozyigit and Yucebilgili Kurtoglu 2020, Zheng et al. 2021). Agrobacterium-mediated transformation is the most preferred method because of its simplicity, less laboriousness, and ability to generate events with low copy numbers and intact transgene inserts (Azizi-Dargahlou and Pouresmaeil 2023).

Poplars were among the first woody plants to be successfully transformed. The hybrid poplar $P.\ trichocarpa \times P.\ deltoides$ stems, when infected with $A.\ tumefaciens$, showed tumors containing T-DNA sequence and Agrobacterium strain-specific opines (Parsons et al. 1986). Transformation with $A.\ tumefaciens$ has been achieved for many $Populus$ species and hybrids; e.g. $P.\ nigra$ (Wang et al. 1996), $P.\ tomentosa$ ($P.\ alba \times P.\ adenopoda$) (Shan-ping et al. 1990), $P.\ tremula \times P.\ tremuloides$ (Nilsson et al. 1992), Poplar NM6 (Yevtushenko and Misra 2010), $P.\ davidiana$ Dode x $P.\ bollena$ Lauche, $P.\ angustifolia$ and $P.\ balsamifera$ (Maheshwari and
Kovalchuk 2016) and Poplar 84K (P. alba × P. glandulosa) (Wen et al. 2022). These studies have used different types of explants: leaves, petioles, hypocotyls, stem internodes, stem segments, roots, cell suspension cultures, and sprouts.

Several antibiotic resistance markers have been used for transformation and selection, such as the genes for hygromycin resistance (hygromycin phosphotransferase - HPT) and kanamycin resistance (neomycin phosphotransferase II – NPTII) (Yevtushenko and Misra 2010, Majumdar et al. 2013, Song et al. 2019). Other marker systems have also been developed in plant transformation over the years. Yuan et al. (2023) reported a split selectable marker system using protein splicing elements, “inteins,” for Agrobacterium-mediated co-transformation in plants. The authors successfully demonstrated the utility of these selectable marker systems by stacking two reporters eYGFPuv and RUBY genes, using split kanamycin or hygromycin resistance markers enabling robust plant transformation. However, many factors affect the efficiency of Agrobacterium-mediated transformation. Factors such as the optical density of Agrobacterium culture, antibiotic-mediated Agrobacterium death, concentration of acetosyringone, inoculation duration, and cocultivation duration affect the efficiency of Agrobacterium-mediated transformation (Niazian 2019, Niedbała et al. 2021). In a study with Himalayan poplar (Populus ciliata Wall. ex Royle), the concentration of antibiotic cefotaxime was found to be quite important for shoot regeneration (Aggarwal et al. 2022).

Over the past 38 years, our lab has worked extensively on PA metabolism and its regulation via transgenic approaches and understanding their role in growth and development. A major focus of research has been on the regulation of Glu➔Orn➔Arg➔Glu➔Pro, and Glu/PAs➔GABA pathways. The research has involved several plant species, including tobacco (Nicotiana tabacum), carrot (Daucus carota), A. thaliana, hybrid poplar (Populus nigra x maximowiczii - clone NM6),

The relationship between polyamine and ethylene biosynthesis in plants and its significance for morphogenesis in cell cultures were explored in Minocha (1988). More recently, the studies have involved the effects of genetic manipulation of PAs on the entire transcriptome and the metabolome (Page et al. 2007, Page et al. 2012, Majumdar et al. 2013). Studies with transgenic poplar cell cultures and A. thaliana plants for high Put production suggested that the reduction in cellular Orn (by high-efficiency conversion of Orn→Put) causes an increased flux of Glu to Orn. This would decrease cellular Glu and increase in net uptake of N (Majumdar et al. 2015, Wuddineh et al. 2018). The Spd levels in the cells decreased when Put levels increased due to the lower expression of SAMDC (Page et al. 2007, Shao et al. 2012, Majumdar et al. 2013). It is known that Orn is a precursor to PAs and some AAs (Figure 3), thus the biosynthesis of Orn is directly dependent on the demand for its use in the production of Arg or Pro (other AAs of the Glu-Orn-Pro-Arg-GABA pathway) and also could respond to environmental or intracellular conditions (Majumdar et al. 2013, Shao et al. 2014, Majumdar et al. 2015). A study involving the use of two Araucaria angustifolia cell lines showed that exogenous application of Arg and Orn altered the amount of cellular AAs as well as changed the expression of genes related to PA biosynthesis and catabolism (de Oliveira et al. 2018).

By genetically manipulating plants, important information about regulating metabolism can be revealed. This information can be used to create desired metabolites with optimal levels. Furthermore, according to Page et al. (2007), feedback inhibition was not observed for the native ODC or ADC genes or their enzyme activities due to high Put in the cells. Another study done in
the lab looked at the expression of the genes involved in the Orn/Arg biosynthetic pathway in response to the upregulation of PA biosynthesis in transgenic NM6 cell cultures expressing the mODC gene. Here it was shown that the expression of the genes involved in the Glu-Orn-Arg pathway is constitutively coordinated. This work done with 7-day-old poplar cell cultures showed that increased flow rate through the Glu-Orn-Arg pathway because of increased utilization of Orn by mODC did not affect the expression of genes involved in the Glu-Orn-Arg pathway (Page et al. 2012). A recent study done in the lab looked at the transcriptome of the transgenic A. thaliana plants expressing the mODC gene under the control of a constitutive and an estradiol-inducible promoter using RNA-seq (Eric English MS thesis). This study identified several genes that were differentially expressed in the PA metabolism.

Nitrogen levels influence plant productivity and quality due to association with various growth substances involved in plant stress responses (Macky et al. 2014, Ninou et al. 2017, Paschalidis et al. 2019). The PA/N interaction in plants is of major interest because it connects N metabolism, C fixation, and secondary metabolism pathways. An increase in PAs is often seen as a result of N application. Increased levels of PAs usually result from N-induced higher concentrations of their precursor AAs, such as Orn and Arg (Serapiglia et al. 2008, Tsaniklidis et al. 2016). This interaction between PAs and N plays a major role in plant stress responses (Paschalidis et al. 2019). The first synthesized AA, Glu, participates in N recycling/remobilization into other nitrogenous molecules, ensuring N homeostasis in plants. As a central N molecule, Glu leads to the biosynthesis of proline (Pro), Orn, Arg, and PAs, constituting a crucial pathway for C and N assimilation (Majumdar et al. 2016). These metabolites (Pro, Arg, and Put) are not only important indicators for both biotic and abiotic stress response but also help in the alleviation of the stress responses (Paschalidis et al. 2001, Paschalidis et al. 2009, Ghosh et al. 2022, Bhardwaj
et al. 2023). Furthermore, two important precursor molecules in PA synthesis are SAM, which is produced from Met and also involved in ethylene biosynthesis, and Orn, which is involved in the urea cycle (Wuddineh et al. 2018). Furthermore, PAs play a central role in N/C assimilation and remobilization by regulating C and N recycling via PAO catabolism (Paschalidis and Roubelakis-Angelakis 2005, Kusano et al. 2015, Wang et al. 2019). End products of PA catabolism, H_2O_2, and GABA also help in maintaining N/C homeostasis in plant tissues (Wu et al. 2018).

Stress conditions, such as salinity and drought, increase protease activity, causing the accumulation of NH_4^+ ions inside cells. This NH_4^+ is converted into Gln and Glu by GS/GOGAT. Transgenic mODC plants with depleted Orn (due to overexpression of mODC) exhibited higher Glu conversion into Put, leading to an N shortage in the cell (Majumdar et al. 2016). Consequently, Glu to Orn conversion was increased; and consequently, there was enhanced biosynthesis of Glu from additional N assimilation (Majumdar et al. 2016). The result was faster-growing plants with higher uptake of both N and C.

Plants have the ability to react to stressful conditions by converting N and C into useful signaling molecules, including PAs, Pro, GABA, glycine betaine, and β-Ala. These molecules play crucial roles in protecting plants against stress and reducing the toxicity of NH_3 in cells (Majumdar et al. 2016). A Glu-Pro-Arg-PA-GABA coordinated path is therefore of importance to accomplish an equilibrium among assimilated N/C in plant cells (Skopelitis et al. 2006, Majumdar et al. 2016, Paschalidis et al. 2019). Thus it is expected that genetic manipulation of Put production via mODC should lead to the development of poplar NM6 plants that can respond positively for additional uptake and enhanced metabolism of both N and C. The short-term objective was to produce such plants for detailed analysis of N and carbon and the accumulation of biomass.
Methods

Genetic transformation

Plant material and growth conditions

Leaf explants were collected from young plants produced from cuttings of hybrid poplar

*Populus nigra* L. *x Populus maximowiczii* A. Henry (clone NM6) plant growing in the greenhouse. These explants were washed with tap water and sterilized with 30% (v/v) Clorox (Oakland, CA) for 10 min and rinsed 3X with autoclaved water before using for the transformation experiment.

Table 9. Composition of media for cultivation, transformation, selection, and regeneration of hybrid poplar clone NM6

<table>
<thead>
<tr>
<th>Components&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P1 Plant propagation</th>
<th>P2 Co-cultivation</th>
<th>P3 Callus induction</th>
<th>P4 Shoot induction</th>
<th>P5 Rooting</th>
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<tr>
<td>Macro- and micronutrients</td>
<td>WPM&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Hygromycin</td>
<td>10</td>
<td>10</td>
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<tr>
<td>pH 5.7-5.8</td>
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</table>

<sup>a</sup> All concentrations, except for growth regulators and acetylsyringone, are given in mg/l; thermolabile compounds were filter-sterilized and added to the autoclaved media cooled to 40–50°C

<sup>b</sup>McCown’s Woody Plant Medium salts (*Lloyd and McCown 1980*)
**Agrobacterium culture and transformation procedure**

Poplar plants (clone NM6) were transformed with *Agrobacterium tumefaciens* GV3101 with the vector pMDC32 containing mODC gene controlled by a constitutive (2×35 S) promoter and the vector pMDC7 containing the mODC gene under the control of an estradiol-inducible promoter (Fig. 52) (Majumdar et al. 2013) and using the protocol described by (Yevtushenko and Misra 2010). A single colony of *Agrobacterium tumefaciens* from plates started with cultures stored at -80°C was grown in 50 ml of liquid YEB medium (PhytoTech Lab, Lenexa KS) containing appropriate antibiotics (40 μg mL⁻¹ gentamycin or 20 μg mL⁻¹ rifampicin or spectinomycin 40 μg mL⁻¹) for 2 days at 28°C on a rotary shaker at 225 rpm to mid-logarithmic phase (OD₆₀₀ = ~1). Prior to the resuspension of bacteria, 100 μM acetosyringone was added to the liquid media. The bacterial cells were collected by centrifugation at 2,000g for 15 min and re-suspended in liquid P2 medium (Table 9) to a final optical density of 0.4–0.5 (OD₆₀₀) before inoculation of explants.
Figure 52. Vectors used for (A) inducible expression (pMDC7) and (B) constitutive expression (pMDC32) of mODC. 2x35S and G10-90 are constitutive promoters; RB, right border; LB, left border; attB1 and attB2, recombination sites after LR clonase; Hygr, hygromycin resistance gene; XVE, estradiol-responsive transcription factor; OlexA-4, XVE-responsive promoter; T3A and nos T, terminators (Majumdar et al. 2013)
Healthy 4–6-week-old poplar plants (grown in vitro) were cut into 2–3 cm² leaf pieces, full-length petioles, and internodal stem segments. Petioles and stem segments were cut close to the axillary buds to include the maximum amount of adjacent tissues. All cuttings were done while explants were submerged in a liquid P1 medium (Table 9) containing the bacteria (prepared as described above). The leaf explants were pre-cultured in P2 medium (Table 9), where the leaves were placed with the adaxial side up. All cultures were maintained at 24°C with a 16-h light period (80 µmolm⁻²s⁻¹) for 2 days. The explants were then incubated with a fresh *Agrobacterium* culture in P1 medium for 1 h with slow shaking, blotted with sterile filter paper to remove the excess bacteria, washed twice for 1 h with liquid P2 medium supplemented with 1 g/l timentin, and placed (6–12 explants per 9-cm plate) on P3 medium containing antibiotics for selection of transgenic cells/plants (Table 9). The explants were cultivated on P3 medium at 24°C with a 16-h light period for 2–3 weeks until the callus appearance and then transferred to P4 medium containing zeatin riboside (ZR) to induce shooting. The explants were sub-cultured in fresh antibiotic containing P4 medium every 2 weeks. Regenerated shoots (0.5–1.5 cm high) were excised from the explant and placed on NAA (1-Naphthaleneacetic Acid) containing P5 medium (Table 9) to induce root development. The roots were formed in 3–4 weeks from transferring to the P5 media (Table 9).
Genomic DNA isolation

Genomic DNA was isolated from plant tissues (Putative transformants and control) using the protocol described in Križman et al. (2006). Approximately 50 mg of plant tissue was ground with 1.5 ml of extraction buffer; 100 mM Tris-HCl (pH 8), 2.0 M NaCl, 20 mM EDTA (pH 8), 2 % (w/v) CTAB Hexadecyl-trimethylammonium bromide (Sigma, H6269), 1 % (w/v) PVP (Polyvinylpyrrolidone K10, MW 10,000), and 0.5 % (w/v) activated charcoal (added just before use) in a microcentrifuge tube. The ground tissue was incubated for 30 min at 55°C with gentle agitation. The sample was centrifuged for 10 min at 16,000 xg, the upper aqueous phase removed into a new tube, followed by addition of equal volume of chloroform: isoamyl alcohol (Fisher, A 393-500) (24:1). The samples were centrifuged for 10 min at 16,000 xg, the upper aqueous phase removed into a new tube, followed by addition of equal volume of cold isopropanol. The tubes were incubated for 1 h at 25°C. The DNA was pelleted by centrifugation for 15 min at 14,000 xg at 4°C. The pellet was washed with 50% (v/v) ethanol containing 15 mM ammonium acetate (Sigma, A1542) by centrifugation. The pellet was vacuum-dried in a Speed-Vac and resuspended in 25 µL of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Nucleic acid was quantified spectrophotometrically (Nano-drop), and its quality was assessed by $A_{260}/A_{280}$ ratio ($\geq 1.8$).

Polymerase chain reaction (PCR)

A typical PCR was performed using Eco-Taq PCR MasterMix (Midwest Scientific Inc., #ECO-TAQ1) following the manufacturer’s guidelines with 0.2 µM final concentration each of the forward and the reverse primers (Table 10), 100-150 ng of genomic DNA. Reactions were run in a PTC™ 100 Programmable Thermal Controller (MJ Research Inc., Waltham, MA). The PCR conditions were activation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C
for 30 sec, 57°C annealing temperature for 1 min, and elongation at 68°C for 1.5 min, followed by a final extension at 72°C for 2 min. The reaction tubes were stored at 4°C.

Table 10. Primers used for PCR

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Td (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S-mODC</td>
<td>5’-GAACCATGGGCAGCTTAC-3’</td>
<td>5’-CTACTACATGGCTCTGGA-3’</td>
<td>60.0</td>
</tr>
<tr>
<td>Inducible-mODC</td>
<td>5’-CACCATGAGCACCTTTACTAAGGA-3’</td>
<td>5’-CTACTACATGGCTCTGGA-3’</td>
<td>56.0</td>
</tr>
</tbody>
</table>

**Agarose gel electrophoresis**

Agarose gel electrophoresis was performed to analyze DNA using a 1% Seakem agarose (Lonna, Allendale, NJ, Cat # 50000) dissolved in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), acetate, 1 mM EDTA). Prior to loading, samples were mixed with 6x gel loading dye (NEB, Cat # B7021S) containing EDTA and then electrophoresed at 100V for 40 min along with appropriate DNA ladder (Invitrogen 1 Kb plus DNA ladder, Cat # 10488-085) loaded in the gel. The gel was stained in 0.5 μg mL⁻¹ of ethidium bromide for 15 min and subsequently de-stained for 5 min in distilled water. The gel was visualized and photographed using the Fotodyne gel-documentation system (Fotodyne Incorporated, Hartland, WI). The bands of DNA were identified with reference to the appropriate DNA ladder.
Results

Figure 53. Different steps in the transformation system for *Populus nigra* x *maximowiczii* - NM6. (A) Pre-culture of leaf explants on P1. (B) Callus induction of leaf explants on P3 after co-cultivated with *Agrobacterium tumefaciens*. (C) Shoot induction of leaf explants on P4. (D) Shoots cultured on P4 for shoot induction. (E) Shoot induction on P4 from stem segments (F) Shoots cultured on P5 for root induction

The callus growth was observed from the leaf explants placed on P3 media (Table 9) 2-3 weeks after the initial transformation (Fig. 53B). Shoots regenerated via indirect organogenesis through callus after 2-3 weeks of culture (Fig. 53C). The shoots were light green to dark green in color during regeneration on Hyg-containing medium (Fig. 53C). After several weeks of culture, the leaves appeared to have developed partially red in color (Fig. 53D). Multiple shoots were observed from each callus clump (Fig. 53D). In the stem explants, shoots were observed with the majority of shoots around the cut surface.
Several independent lines (4-6) of putative transgenic plants for the two types of promoter::gene combination plasmids and non-transgenic wild type (WT) plants were produced from tissue culture. The presence of the target gene in many of these plants was confirmed by PCR (Fig. 54 and Fig. 55) using mODC specific primers (Table 10).
Discussion

Genetic transformation is a powerful tool in plant molecular biology research to generate plant varieties with favorable traits and understand and control plant gene expression. Transgenic plants are useful for analyzing the function of genes and the functional characterization of gene products (Wen et al. 2022). Agrobacterium tumefaciens-mediated transformation can be utilized for stable and transient expression of foreign genes in plants. Successful A. tumefaciens-mediated transformation has been reported in numerous plant species, with many of them being cultivated across the world (Krügel et al. 2002, Ishida et al. 2007, Zeng et al. 2019, Holmes and Punja 2023, Zhou et al. 2023). Poplars are deciduous trees of the Salicaceae family. With a small genome size, the availability of extensive genomic sequences, and the feasibility of genetic transformation have allowed poplar to become a model tree species for molecular studies (Maheshwari and Kovalchuk 2016). New transgenic poplars that are resistant to pests (Klocko et al. 2014), stress (Harfouche et al. 2014), and modifications to wood properties (Coleman et al. 2009) have been developed from different poplar species with various transformation systems. Although efficient and reproducible Agrobacterium-mediated transformation and regeneration systems have been obtained using poplar, these have been reported only for a limited number of model genotypes, and the process is still difficult in many poplar species (Song et al. 2006, Yevtushenko and Misra 2010, Song et al. 2019).

Although various methods such as viral transformation, electroporation of protoplasts, and particle bombardment method have been used (Taylor and Fauquet 2002, Miao and Jiang 2007, Vainstein et al. 2011), the Agrobacterium-mediated transformation is the most used method for poplar (Busov et al. 2005, Song et al. 2019). The first successful poplar transformation was reported with hybrid Populus trichocarpa × deltoides (Parsons et al. 1986). A series of

The in vitro tissue-to-plant regeneration system is integral to genetic transformation procedures. Regeneration through organogenesis is an efficient method for in vitro production of whole plants (Shin et al. 2020), but not as well suited for transformation. In transformation, the efficiency of DNA delivery into the targeted cells and the regeneration of transgenic plants largely depends on factors such as explant source, genotype, phytohormone type, and combination (Wang et al. 2011a). For in vitro regeneration of poplar through organogenesis, different explants have been used, including leaves (Yevtushenko and Misra 2010, Wang et al. 2011a, Han et al. 2013, Movahedi et al. 2014), petiole (Thakur et al. 2005), stems (Yadav et al. 2009, Yevtushenko and Misra 2010), roots (Yadav et al. 2009), and shoot tips (Kang et al. 2009). In this study, we used young leaves/explants (4-6 weeks old), tissues that showed faster regeneration, as opposed to more matured leaves (7-8 weeks old). In a study done with the hybrid poplar *Populus davidiana* Dode × *Populus bollena* Lauche, it was observed that by using younger leaf explants, the transformation and regeneration frequency increased (Han et al. 2013). Song et al. (2019) reported that genetic transformation and regeneration frequency were higher in the poplar hybrid *P. alba × P. glandulosa* when younger leaves were used. Changes in the cell wall composition at different physiological and developmental stages could also be a possible reason; with older tissues, the *Agrobacterium*-binding to the cells changes because of changes in the cell wall composition (Verma et al. 2008, Han et al. 2013).
The type and concentration of plant growth regulators (PGRs) affect the results of the regeneration of plants (Wen et al. 2022). Organogenesis requires different PGRs for shoot and root induction (Niazian 2019). In this study, using ZR as the shooting-inducing PGR and NAA as the rooting-inducing PGR showed the highest regeneration of transgenic plants. Similarly, the transformation frequency was higher in the poplar hybrid *Populus nigra* L. *× P. maximowiczii* A. Henry when grown in ZR shoot-inducing media (Yevtushenko and Misra 2010). In our studies also, this method was the most effective.

By genetically manipulating plants, important information related to regulating the metabolism can be revealed. This information can be used to create desired metabolites with optimal levels. Furthermore, genetic manipulation of plants can be used to improve the nutritional values of crop plants. In contrast to the mutants and the constitutive transgenic expression systems for genetic manipulation, the inducible transgenic expression systems permit us to mimic gene expression for shorter periods, as it might occur in nature.

As mentioned earlier in the introduction, our lab has extensively studied the regulation of PA metabolism in tobacco, carrot, poplar, and red spruce using both inhibitors and genetic manipulation. The past work of the lab has shown that the reduction in Orn due to its conversion to Put by transgenic mODC causes increased flux of Glu to Orn, and the ensuing decrease in cellular Glu is met by its increased biosynthesis via net uptake and metabolism of N (Page et al. 2007, Page et al. 2012, Majumdar et al. 2013, Majumdar et al. 2016). As a result of higher Put production and accumulation in transgenic cells, several AAs, sugars, sugar alcohols, and organic acids are also positively affected (Minocha et al. 2014). Consequently, the cells and plants draw more N and C from the outside because their metabolism is inter-twined. Using biochemical approaches, past studies have shown that, like the poplar cells in culture and in transgenic
*Arabidopsis* plants expressing the m*ODC* gene, the induction of m*ODC* resulted in up-and down-regulation of several AAs in the transgenic plants and overall improvement of growth and biomass (Majumdar et al. 2013, Majumdar et al. 2016).

**Conclusions and current status**

The primary goal of this study was to use the approach of genetic engineering to increase Put production (and, in turn, biomass production) in the hybrid poplar (*P. nigra x maximowiczii* - clone NM6), the exact clone whose cell cultures have been used in the lab for several studies in the past. In addition, we also expected to study whether foliar spray of N is an effective method of N supply by studying PA and AA contents, C and N assimilation, and growth (biomass) in genetically modified poplar plants and wild-type plants. However, after generating the transgenic plants in 2020, the experiment could not be carried out with the transgenic plants further due to unforeseen circumstances we faced during Covid-19. Due to the lab shut down during Covid-19, more than >70% of these plants were lost and was able to transfer to the greenhouse to do further research. Due to severe restrictions on working in the lab for most of last year, neither the molecular work nor the biochemical analyses of the transgenic plants (using radioisotopes and hazardous chemicals) could be accomplished, and the loss of plants resulted in a significant loss of my effort of two years.


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